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Untersuchung von RNA-Modifikationen in Bakterien und Nematoden

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aus

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- „NAIL-MS reveals the repair of 2-methylthiocytidine by AlkB in *E. coli* ", **Valentin F. Reichle**, Dimitar P. Petrov, Verena Weber, Kirsten Jung, Stefanie Kellner, *Nat. Commun*. **2019**, 10, 5600.
- „Surpassing limits of static RNA modification analysis with dynamic NAIL-MS.", **Valentin F. Reichle**, Steffen Kaiser, Matthias Heiss, Felix Hagelskamp, Kayla Borland, Stefanie Kellner, *Methods (San Diego, Calif.)* **2019**, *156*, 91-101.
- „NAIL-MS in E. coli Determines the Source and Fate of Methylation in tRNA. ", **Valentin F. Reichle**, Verena Weber, Stefanie Kellner, *ChemBioChem* **2018**, *19*, 2575-2583.
- „Observing the fate of tRNA and its modifications by nucleic acid isotope labeling mass spectrometry: NAIL-MS. ", Matthias Heiss, **Valentin F. Reichle**, Stefanie Kellner, *RNA Biology* **2017**, 14, 1260-1268.

Konferenzbeiträge

- „New tRNA modification in *E.coli*: Discovery and characterization by NAIL-MS. ", Gordon Research Conference 2019 RNA editing (Barga, Italy, März **2019**)
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- „Biosynthetic isotope labeling reveals dynamics of the epitranscriptome and novel modified nucleosides", XVIIth Symposium on Nucleic Acid Components (Cesky Krumlov, Tschechische Republik, Juni **2017**)

Trendberichte

- „Biochemie 2016: Der Einfluss des Epitranskriptoms auf Krankheiten und dessen Analytik.", **Valentin F. Reichle**, Stefanie Kellner, *Nachrichten aus der Chemie* **2017**, 65, 316-319.

Weitere Publikationen

- „Synthetic quinolone signal analogues inhibiting the virulence factor elastase of *Pseudomonas aeruginosa*.“, David Szamosvari, **Valentin F. Reichle**, Monika Jureschi, Thomas Böttcher, *Chemical Communications* **2016**, *52*, 13440-13443.

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Abkürzungen

A	Adenosin
Å	Ångström
A. suum	*Ascaris suum* (lat.)
AGT	O^6-Alkylguanin DNA-Alkyltransferase
amol	Attomol
ASL	*anticodon stem loop* (engl.)
Asp	Asparagin
BER	Basenexzisionsreparatur
bTGT	tRNA-Guanin Transglykosylase
bzw.	beziehungsweise
C	Cytidin
C. elegans	*Caenorhabditis elegans* (lat.)
C.e.	*Caenorhabditis elegans* (lat.)
CGA	Cytidin, Guanosin, Adenosin
CID	*collision induced dissociation* (engl.)
CIP	*calf intestinal phosphatase* (engl.)
Cys	Cystein
D	Dihydrouridin
Da	Dalton
DMRM	*dynamic multiple reaction monitoring* (engl.)
DNA	*deoxyribonucleic acid* (engl.)
E. coli	*Escherichia coli* (lat.)
E. c.	*Escherichia coli* (lat.)
Elp	Elongator Komplex
engl.	englisch
ESI	*electrospray ionization* (engl.)
fmol	Femtomol
FTO	*fat mass and obesity-associated protein* (engl.)
G	Guanosin
g/mol	Gramm pro Mol
GCH1	GTP-Cyclohydrolase 1
GTP	Guanosintriphosphat
H. polygyrus	*Heligmosomoides polygyrus* (lat.)
H.p.	*Heligmosomoides polygyrus* (lat.)
His	Histidin
HPLC	*high performance liquid chromatography* (engl.)
HRMS	*high resolution mass spectrometry* (engl.)
I	Inosin
IAA	Iodoacetamid
IFN-α	Interferon-α
IL-7	Interleukin 7

IL-8	Interleukin 8
IS	Interner Standard
IUPAC	*International Union of Pure and Applied Chemistry* (engl.)
L. sigmondontis	*Litomosoides sigmondontis* (lat.)
L.s.	*Litomosoides sigmondontis* (lat.)
lat.	lateinisch
LC	*liquid chromatography* (engl.)
LOD	*limit of detection* (engl.)
lrRNA	*large ribosomal RNA* (engl.)
MALDI	*matrix-assisted laser desorption/ionization* (engl.)
mAU	*milli absorbance units* (engl.)
max.	maximal
Min.	Minuten
miRNA	mikro RNA
mL	Milliliter
MMS	Methylmethansulfonat
Mod.	Modifikation
MoTTs	*modification tunable transcripts* (engl.)
MRM	*multiple reaction monitoring* (engl.)
mRNA	*messenger RNA* (engl.)
ms	Millisekunde
MS/MS	*tandem mass spectrometry* (engl.)
mtRNA	mitochondriale RNA
m/z	Masse pro Ladung
n	Stoffmenge
NADPH	Nicotinamidadenindinukleotidphosphat
NAIL-MS	*nucleic acid isotope labeling coupled mass spectrometry*
NER	Nukleotidexzisionsreparatur
ng	Nanogramm
NH_4OAc	Ammoniumacetat
NHEJ	*non-homologous end joining* (engl.)
NLS	*neutral loss scan* (engl.)
nm	Nanometer
NMR	*nuclear magnetic resonance* (engl.)
O. ochengi	*Onchocerca ochengi* (lat.)
O. volvulus	*Onchocerca volvulus* (lat.)
P. aeruginosa	*Pseudomonas aeruginosa* (lat.)
P.a.	*Pseudomonas aeruginosa* (lat.)
PBC	4-Bromomethyl-7-propargyloxycoumarin
PCR	*polymerase chain reaction* (engl.)
piRNA	*piwi interacting RNA* (engl.)
Q	Queuosin
QQQ	Triple Quadrupol Massenspektrometer
qPCR	quantitative Echtzeit PCR

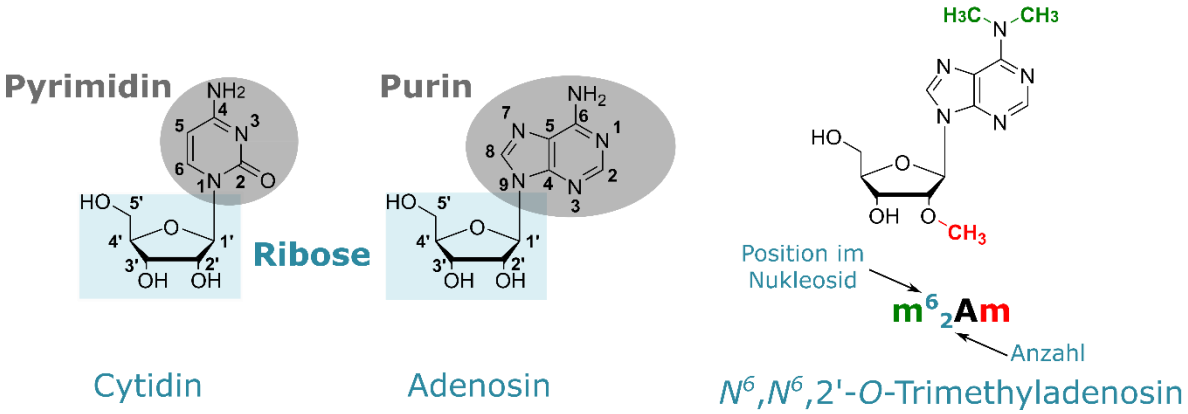
RISC	*RNA-induced silencing complex* (engl.)
RNA	*ribonucleic acid* (engl.)
ROS	*reactive oxygen species* (engl.)
rRFN	Relativer Responsivitätsfaktor für Nukleoside
rRNA	*ribosomal RNA* (engl.)
Rt	Retentionszeit
S. cerevisiae	*Saccharomyces cerevisiae* (lat.)
SAM	*S*-Adenosyl-Methionin
SEC	*size exclusion chromatography* (engl.)
Sek.	Sekunde
sfGFP	*superfolder green fluorescent protein* (engl.)
SIL-CARD	*stable isotope labeling comparative analysis of RNA digests* (engl.)
SILAC	*stable isotope labeling by amino acids in cell culture* (engl.)
SIL-IS	*Stable Isotope Labeled Internal Standard* (engl.)
siRNA	*short interfering RNA* (engl.)
sncRNA	*small non-coding RNA* (engl.)
SPD	*snake venom phosphodiesterase* (engl.)
srRNA	*small ribosomal RNA* (engl.)
T	Ribothymidin
TIC	*total ion current* (engl.)
tiRNA	*tRNA-derived stress induced RNA* (engl.)
TLR	*Toll-like receptor* (engl.)
TrmU	2-Thiouridylase I
tRF	*tRNA-derived fragment* (engl.)
tRNA	*transfer RNA* (engl.)
tsRNA	*tRNA-derived small RNA* (engl.)
U	Uridin
µg	Mikrogramm
UV	Ultraviolett
uvm.	und viele mehr
Vgl.	Vergleich
yW	Wybutosin
z.B.	zum Beispiel
z.T.	Zum Teil

Abkürzungsverzeichnis für RNA-Modifikationen

Ψ	Pseudouridin
ac^4C	*N*^{4}-Acetylcytidin
Am	2‘-*O*-Methyladenosin
Cm	2‘-*O*-Methylcytidin
$cmnm^5ges^2U$	5-Carboxymethylaminomethyl-2-geranylthiouridin
cmo^5U	Uridin-5-Oxyessigsäure
ct^6A	zyklisches *N*^{6}-Threonylcarbamoyladenosin
D	Dihydrouridin
f^5C	5-Formylcytidin
galQtRNA	Galactosyl-queuosin
Gm	2‘-*O*-Methylguanosin
hm^5Cm	2’-*O*-Methyl-5-hydroxymethylcytidin
ht^6A	Hydroxy-*N*^{6}-threonylcarbamoyladenosin
I	Inosin
i^6A	*N*^{6}-Isopentenyladenosin
inm^5U	5-(Isopentenylaminomethyl)uridin
io^6A	*N*^{6}-(Cis-hydroxypentenyl)adenosin
k^2C	2-Lysidin
m^1A	1-Methyladenosin
m^1G	1-Methylguanosin
m^1I	1-Methylinosin
$m^1s^4Ψ$	1-Methyl-4-thiopseudouridin
$m^2{}_2G$	*N*^{2},*N*^{2}-Dimethylguanosin
$m^2{}_2{}^7G$	*N*^{2},*N*^{2},*7*-Trimethylguanosin
m^2A	2-Methyladenosin
m^2G	*N*^{2}-Methylguanosin
m^3C	3-Methylcytidin
m^3U	3-Methyluridin
$m^4{}_2C$	*N*^{4},*N*^{4}-Dimethylcytidin
m^4Cm	*N*^{4},2′-*O*-Dimethylcytidin
m^5C	5-Methylcytidin
m^5s^2U	5-Methyl-2-thiouridin
m^5U	5-Methyluridin
m^5Um	5,2‘-*O*-Dimethyluridin
m^6A	*N*^{6}-Methyladenosin
$m^6{}_2A$	*N*^{6},*N*^{6}-Dimethyladenosin
m^7G	7-Methylguanosin
malonylCoA	5′ (3′ -Dephosphomalonyl-Coenzym A)

mcm^5s^2U	5-Methoxycarbonylmethyl-2-thiouridin
mcm^5U	5-Methoxycarbonylmethyluridin
$mcmo^5Um$	2'-O-Methyluridin 5-Oxyessigsäuremethylester
mnm^5s^2U	5-Methylaminomethyl-2-thiouridin
mnm^5U	5-Methylaminomethyluridin
mO^6G	O^6-Methylguanosin
ms^2A	2-Methylthioadenosin
ms^2C	2-Methylthiocytidin
ms^2ct^6A	2-Methylthio-zyklisches-N^6-Threonylcarbamoyladenosin
ms^2i^6A	2-Methylthio-N^6-isopentenyladenosin
ms^2io^6A	2-Methylthio-N^6-(cis-hydroxyisopentenyl)adenosin
ms^2t^6A	2-Methylthio-N^6-threonylcarbamoyladenosin
ms^4U	4-Methylthiouridin
$msms^2i^6A$	2-Methylthiomethylenthio-N^6-isopentenyladenosin
NAD	Nikotinamid Adenin Dinukleotid
ncm^5U	5-Carbamoylmethyluridin
oQ-tRNA	Epoxyqueuosin
preQ1-tRNA	7-Aminomethyl-7-deazaguanosin
Q	Queuosin
s^2C	2-Thiocytidin
s^2U	2-Thiouridin
s^4U	4-Thiouridin
T	Ribothymidin
t^6A	N^6-Threonylcarbamoyladenosin
tm^5s^2U	5-Taurinomethyl-2-thiouridine
Um	2'-*O*-Methyluridin
yW	Wybutosin

Die Nomenklatur von RNA-Modifikationen



Bei RNA-Modifikationen liegt in der Regel meist eine Purinbase (Adenin, Guanin) oder eine Pyrimidinbase (Cytosin, Uracil) zugrunde. Bei einem Nukleosid mit Pyrimidinbase beginnt die Zählweise am Stickstoff der glykosidischen Bindung und wird in die Richtung fortgesetzt, dass die im Ring befindlichen Heteroatome möglichst kleine Nummerierungen erhalten. Die Nummerierung der Ringatome bei Purinen wird dagegen nicht nach den internationalen IUPAC-Regeln durchgeführt und bildet daher eine Ausnahme. Die Nummerierung erfolgt traditionsbedingt nach der oben dargestellten Zählweise und beginnt daher am von der glykosidischen Bindung entferntesten Ringstickstoff und endet am Stickstoff der glykosidischen Bindung. Die Nummerierung der Ribose orientiert sich an der Fischer-Projektion. Die Ziffern erhalten zur besseren Unterscheidung zusätzlich zu den eigentlichen Nummern einen Apostroph. Werden die Pyrimidin- oder die Purinbasen modifiziert, so wird die Abkürzung für den chemischen Rest (m für Methylgruppe, s für Schwefelgruppe, n für Amingruppe, o für Sauerstoffgruppe, c für Carboxylgruppe, uvm.) mit einer hochgestellten Zahl für deren Ringposition vor das Kürzel des ursprünglichen, nicht modifizierten Nukleosids (A für Adenosin, G für Guanosin, C für Cytidin, U für Uridin) gestellt. Kommt die gleiche Modifikation öfter als einmal an derselben Ringposition vor, so folgt für deren Anzahl stehend eine tiefgestellte Ziffer. Chemische Modifikationen des Riboserests werden erst nach dem Kürzel des ursprünglichen Nukleosids genannt (z.B. Am, Gm, Cm, Um). Besteht der modifizierende Rest aus einer Kette verschiedener chemischer Gruppen, so werden auch die Kürzel entsprechend der Reihe nach genannt (z.B. mcmo^{5}U). Modifikationen mit komplexeren Strukturen, für welche diese Art der Nomenklatur ungeeignet erscheint, können auch durch neue Buchstaben abgekürzt werden (z.B. Q für Queuosin oder yW für Wybutosin).

Zusammenfassung

In verschiedenen Lebensprozessen werden dem aufkommenden Gebiet der Epitranskriptomik und den einhergehenden RNA-Modifikationen eine zunehmende Bedeutung beigemessen. Diese erfordert eine intensivere Untersuchung und ein besseres Verständnis für die vielen unterschiedlichen chemischen Veränderungen in den RNA-Molekülen. Kenntnisse über deren Biosynthese, zelluläre Funktionen oder deren Abbauprozesse sind dabei fundamental. In dieser Arbeit habe ich einen neuen Ansatz mit der Bezeichnung NAIL-MS (**N**ucleic **A**cid **I**sotope **L**abeling coupled **M**ass **S**pectrometry) verwendet, um neue RNA-Nukleosid-Strukturen in Bakterien zu identifizieren oder RNA-Modifikationen, die unter verschiedenen Bedingungen isoliert wurden, zu quantifizieren. Mit Hilfe eines NAIL-MS-basierten Pulse-Chase-Experiments konnte ich zudem RNA-Modifikationen über die Zeit verfolgen. Mit Hilfe dieser Methoden identifizierte ich 43 unbekannte Nukleosidstrukturen, von denen ich zwei Strukturen intensiver charakterisiert habe. Eines der Derivate weist eine auf Queousin (Q) basierende Struktur auf und zeigt einen Massenunterschied von 2 Da im Vergleich zu Q. Bei einer weiteren Struktur handelt es sich um ein Methylierungsprodukt der bereits bekannten Modifikation 2-Thiocytidin (s^2C). Die Methylierung findet an der 2-Thiocarbonylgruppe statt, wodurch 2-Methylthiocytidin (ms^2C) entsteht. Durch meine Arbeit konnte festgestellt werden, dass ms^2C eher ein Methylierungsschaden als eine zielgerichtete RNA-Modifikation ist. Es war daher interessant zu sehen, wie das Bakterium mit dem RNA-Schaden umgeht. In einem Pulse-Chase-Experiment, unter Verwendung des Methylierungsreagenz Methyl-Methansulfonat (MMS), wurden ms^2C und andere Methylierungsschäden wie m^1A oder m^3C über die Zeit verfolgt, wodurch deren potenzielle Reparatur beobachtet werden konnte. Ich konnte durch meine Experimente zeigen, dass einige tRNA-Methylierungsschäden direkt durch die oxidative Demethylase AlkB repariert werden, keiner jedoch so effizient wie ms^2C. Es wurde damit erstmals gezeigt, dass auch eine schwefelgebundene Methylgruppe als Substrat für das Enzym AlkB in Frage kommt. Ein weiterer hier identifizierter, an einem Schwefelatom stattfindender Methylierungsschaden (ms^4U) wird jedoch nicht durch AlkB repariert. Sowohl für ms^2C als auch für ms^4U wurde hingegen ein alternativer Reparaturpfad unter Verwendung einer ^{34}S-Isotopenmarkierung aufgedeckt. Es wurde festgestellt, dass die gesamte Thiomethylgruppe von den Nukleosiden entfernt werden kann und das gebildete

kanonische Nukleosid durch die ursprünglichen Thiotransferasen TtcA (s^2C) oder Thil (s^4U) erneut thioliert wird. Nach der Bildung von ms^2C und ms^4U ist eine dramatische Abnahme der Translationseffizienz und tRNA-Stabilität wahrscheinlich, daher ist es für den bakteriellen Organismus essenziell, den Schaden in den langlebigen tRNA-Molekülen direkt zu reparieren.

In einem zweiten Projekt habe ich die RNA-Modifikationsprofile verschiedener Nematodenarten untersucht. Neben der Dokumentation mehrerer unbekannter Nukleosidstrukturen wurden außerdem mehrere bekannte tRNA-Modifikationen absolut quantifiziert. Darüber hinaus wurde isolierte miRNA aus dem Urin von nicht infizierten Rindern mit miRNA von Rindern verglichen, die mit dem pathogenen Nematoden *O. ochengi* infiziert waren. Die miRNA enthielt eine Vielzahl von tRNA-Modifikationen, von denen 5-Methoxycarbonylmethyluridin (mcm^5U) und 5-Methoxycarbonylmethyl-2-thiouridin (mcm^5s^2U) die stärksten Unterschiede aufwiesen. Interessanterweise korrelierten bei fast allen untersuchten Proben von infizierten Rindern die Werte der Abnahme an mcm^5s^2U mit den Werten der Zunahme an mcm^5U. Bemerkenswert ist, dass die Auswirkungen umso stärker werden, je stärker die Infektion den Organismus des Wirts beeinträchtigt. Diese Ergebnisse sind sehr gewinnbringend, denkt man über eine neue Klasse von auf RNA-Modifikationen basierenden Biomarkern nach.

Abstract

The arising field of epitranscriptomics and the importance of RNA-modifications in different processes of life requires the need of better investigation and understanding of the huge number of different chemical alterations in the RNA-molecules as well as knowledge about their biosynthesis, cellular function or degradation processes. In this work, I used a new approach named NAIL-MS (**N**ucleic **A**cid **I**sotope **L**abeling coupled **M**ass **S**pectrometry) to identify new RNA-nucleoside structures in bacteria, to quantify RNA-modifications isolated during different conditions, or follow RNA-modifications over time applying a NAIL-MS based Pulse-Chase experiment. With this tools in hand, I identified 43 unknown nucleoside structures, from which 2 were characterized more intensively. One derivative is a Queousine (Q) based structure with 2 Da difference compared to Q. Another structure is a methylation product of the already known modification 2-Thiocytidine (s^2C). The methylation takes place at the 2-thiocarbonylgroup resulting in 2-Methylthiocytidine (ms^2C). I found that ms^2C is rather a methylation damage than a cell directed RNA-modification. Therefore, it was interesting to see how the bacteria handles the RNA-damage. In a Pulse-Chase experiment under the use of the methylating reagent methyl-methanesulfonate (MMS), ms^2C and other methylation damages like m^1A or m^3C were tracked over time and their potential repair could be observed. I found that some tRNA methylation damages are directly repaired by the oxidative demethylase AlkB, but none was repaired as efficient as ms^2C. It is the first time that a sulfur-connected methylgroup was observed to be substrate of the enzyme AlkB. Nevertheless, another here identified sulfur-methylation damage (ms^4U) is not repaired by AlkB. For both ms^2C and ms^4U, however, an alternative repair pathway was revealed by using ^{34}S-isotope labeling. I found that the whole thiomethyl-group can be removed from the nucleosides and the formed canonical nucleoside is re-thiolated by the original thiotransferases TtcA (s^2C) or Thil (s^4U). A dramatic decrease of translation efficiency and tRNA-stability is expected after ms^2C and ms^4U formation, therefore it is essential for the bacterial organism to directly repair the damage in the long living tRNA-molecules.

In a second project I investigated RNA-modification profiles of different nematodes. Multiple unknown nucleoside structures were documented and in addition, absolute quantification was done for several known tRNA-modifications. Furthermore, isolated miRNA from urine of uninfected cattle was compared with miRNA isolated from cattle

infected with the pathogenic nematode *O. ochengi*. The miRNA contained a lot of tRNA-modifications, from which 5-methoxycarbonylmethyluridine (mcm^5U) and 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) showed the strongest differences. Intriguingly, a decrease of mcm^5s^2U was correlated with an increase of mcm^5U in almost all the investigated samples of infected cattle. It is noteworthy that the effects become stronger the worse the infection impacts the host's organism. These results could become very important thinking about a new class of biomarkers, based on RNA-modifications.

1. Einleitung und Theorie

1.1. RNA – ein vielfältiges Molekül

1.1.1. Verschiedene RNA-Funktionen

Die Ribonukleinsäure oder RNA (von *engl. Ribonucleic acid*), als Vermittler zwischen genetischem Code (DNA) und den Proteinen, nimmt in vielen Prozessen des Lebens zentrale und vielfältige Funktionen ein. Neben der Translation von Proteinen sind RNA-Moleküle in vielerlei regulatorischen Zellprozessen involviert. Verschiedenste RNA-Typen formen dabei ein ineinandergreifendes System innerhalb der Zelle. Die verschiedenen RNA-Stränge werden an der DNA synthetisiert (Transkription). Je nach Zell- und RNA-Typ werden die RNA-Stränge anschließend in ihre jeweiligen Zellbereiche geleitet, wo sie ihre eigentlichen Funktionen ausüben. Diese Funktionen können vielfältig sein. So besitzen manche RNA-Spezies katalytische Fähigkeiten, populärstes Beispiel hierfür ist sicherlich die ribosomale RNA (rRNA). Diese übersetzt (translatiert) im Zusammenspiel mit der *transfer RNA* (tRNA) und der *messenger RNA* (mRNA) den genetischen Code in die jeweiligen Proteine. Des Weiteren gibt es kleine nichtcodierende RNA-Moleküle in den Zellen (sncRNAs), wie beispielsweise die regulatorischen *short interfering RNAs* (siRNAs) oder *piwi interacting RNAs* (piRNAs), welche durch Bildung eines *RNA-induced silencing complex* (RISC) die Translation eines bestimmten mRNA Stranges verhindern.[1] Es gibt außerdem kurze nichtcodierende RNA-Stränge, sogenannte mikro RNAs (miRNAs), mit ähnlicher zellregulierender Wirkung. Diese spielen beispielsweise auch in der Virulenz von verschiedensten Parasiten eine Rolle.[2] Kleine, nichtcodierende RNA-Fragmente, die aus der Spaltung bestimmter tRNA-Moleküle resultieren (tRFs oder tiRNAs), treten gehäuft bei unterschiedlichsten Stressreaktionen auf.[3] Sie können als direkte Stressantwort in der Zelle entstehen, andererseits jedoch auch von parasitären Organismen in den Wirt eingeschleust werden und dort offenbar die Virulenz verstärken.[3-4]

1.1.2. Der strukturelle Aufbau der RNA

Ein typisches RNA-Molekül besteht aus einem Einzelstrang von verschiedenen aneinandergereihten Nukleotiden. Die vier Grundbasen sind Cytosin, Guanin, Adenin und Uracil. Das RNA Polymer (siehe **Abbildung 1a**) wird beginnend vom 5‘ Ende in

den Zellen synthetisiert (transkribiert) und faltet sich dann als Einzelstrang in zum Teil komplexe Strukturen mit teils spezifischen inter- und intramolekularen Bindungseigenschaften. Die tRNA hat eine Länge von 74 – 93 Nukleotiden (*E. coli*)[5] und besteht aus insgesamt 3 Armen, einer Schleife und einem Aminosäure-Akzeptorstamm.[6] Vom 5'-Ende beginnend kommt zuerst der D-Arm, gefolgt vom *Anticodon Stem Loop* (ASL), welcher auch als Anticodonakzeptorstamm bezeichnet wird. Die Variable Schleife schließt sich dem ASL an und geht schließlich zum TΨC-Arm, oder kurz T-Arm, über. Das 3'-Ende der tRNA wird schließlich vom Aminosäure-Akzeptorstamm gebildet. Der D-Arm und der T-Arm sind nach den in der jeweiligen Schleife vorkommenden chemisch veränderten Bausteinen (RNA-Modifikationen) benannt. D steht für Dihydrouridin und T für Ribothymidin, wobei letzteres der Struktur von 5-Methyluridin (m^5U) entspricht. In der zweidimensionalen Sekundärstruktur, oder im Fall von tRNA auch als Kleeblattstruktur (e*ngl. cloverleaf structure*) bezeichnet (**Abbildung 1b**), scheinen der D- und T-Arm maximal voneinander entfernt zu sein.[7] In der dreidimensionalen tertiären Struktur der tRNA in **Abbildung 1c** sieht man jedoch, dass die beiden Arme miteinander interagieren und dem Molekül damit die charakteristische L-Struktur geben.[6]

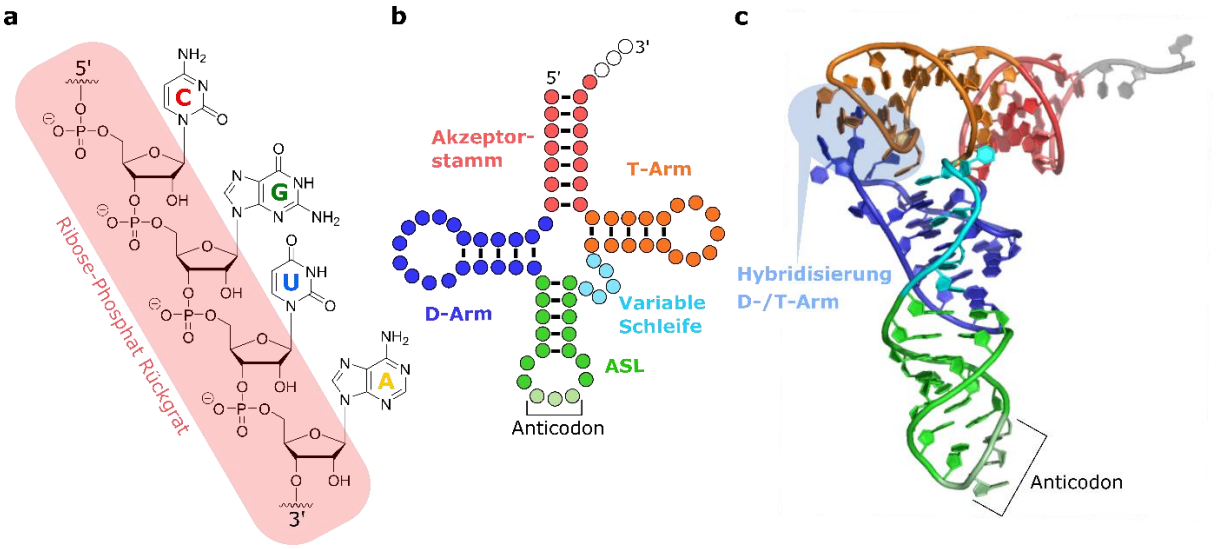


Abbildung 1: Struktur der RNA. **a** Primärstruktur der RNA mit markiertem Ribose-Phosphatgerüst. Die Basen sind mit dem jeweiligen Anfangsbuchstaben gekennzeichnet. C=Cytosin, G=Guanin, U=Uracil, A=Adenin. **b** Sekundärstruktur einer tRNA. Die verschiedenen Teile sind farblich unterschieden. Jeder Punkt entspricht einem Nukleotid mit variabler Base. Striche deuten Watson-Crick Hybridisierungen an. **c** Tertiärstruktur einer tRNA mit gleicher Farbcodierung wie in b. Zusätzlich ist der Bereich markiert, in welchem T-und D-Arm hybridisieren.

Allerdings gibt es bekanntlich nicht nur eine tRNA, sondern für jede Aminosäure mindestens eine spezifische tRNA (Isoakzeptoren), die wiederum in unterschiedlichen Organismen unterschiedliche Sequenzen und Modifikationen tragen können. Es handelt sich also um eine ungeheure Vielzahl an sich voneinander unterscheidenden tRNA-Molekülen auf der Erde, wovon die allermeisten in der charakteristischen L-Form vorliegen. Neben generellen Sequenzunterschieden gibt es Unterschiede in der Variablen Schleife, welche sich stark in der Anzahl der Nukleotide unterscheiden kann. Große strukturelle Unterschiede sind außerdem im Anticodonstamm zu finden. Nicht nur die Anticodon-Sequenzen unterscheiden sich hier, sondern auch eine Vielzahl an unterschiedlichsten Modifikationen sind in diesem Bereich der tRNA zu finden, insbesondere an der sogenannten Wobble-Position, welche im Verlauf dieser Arbeit noch genauer thematisiert werden wird. Diese spielen oftmals eine wichtige Rolle in der direkten Codon-Erkennung oder auch als Stabilisator für die ASL-Struktur insgesamt.[8] Keine andere RNA-Spezies in keiner untersuchten Lebensform trägt prozentual mehr RNA-Modifikationen als die tRNA.[5]

1.1.3. Modifizierte RNA – vielfältige posttranskriptionale Veränderungen

Die RNA-Modifikationen können sich strukturell dramatisch voneinander unterscheiden (siehe **Abbildung 2**). Es können mehrere Modifikationen innerhalb desselben Nukleotids vorkommen und kleine Strukturerweiterungen können zu großen funktionellen Veränderungen führen.[9] Viele Modifikationen beruhen auf einfachen Methylierungen der Basen oder der 2'-Hydroxygruppe der Ribose. Sie kommen neben der tRNA auch in der rRNA, der mRNA und einigen weiteren RNA-Spezies vor.[10] Die am häufigsten auftretende Modifikation ist Pseudouridin (Ψ), welches durch eine Isomerisierung des Uridins entsteht.[11] Weitaus komplexere Strukturen sind oftmals am Anticodonstamm der tRNA (cmnm^{5}ges^{2}U, Wy, galQtRNA) oder auch an den Endstrukturen bestimmter mRNAs anzutreffen (NAD-cap, malonylCoA-cap).[5, 12] Generell können die Basen an fast jeder Position modifiziert werden. Alkylierungen, Oxidationen, Aminierungen und Deaminierungen, Thiolierungen oder gar Acetylierungen sind möglich, Kombinationen dieser Modifikationen eingeschlossen. Erwähnenswert sind auch Aminosäure-Modifikationen wie t^{6}A oder k^{2}C, die ebenfalls erweitert (ht^{6}A) oder kombiniert werden können (ms^{2}t^{6}A). Der Natur scheinen bei der Generierung von RNA-Modifikationen keine Schranken gesetzt, dennoch haben alle

Modifikationen einen der vier Grundbausteine Cytidin, Uridin, Guanosin oder Adenosin als Grundstruktur.[5]

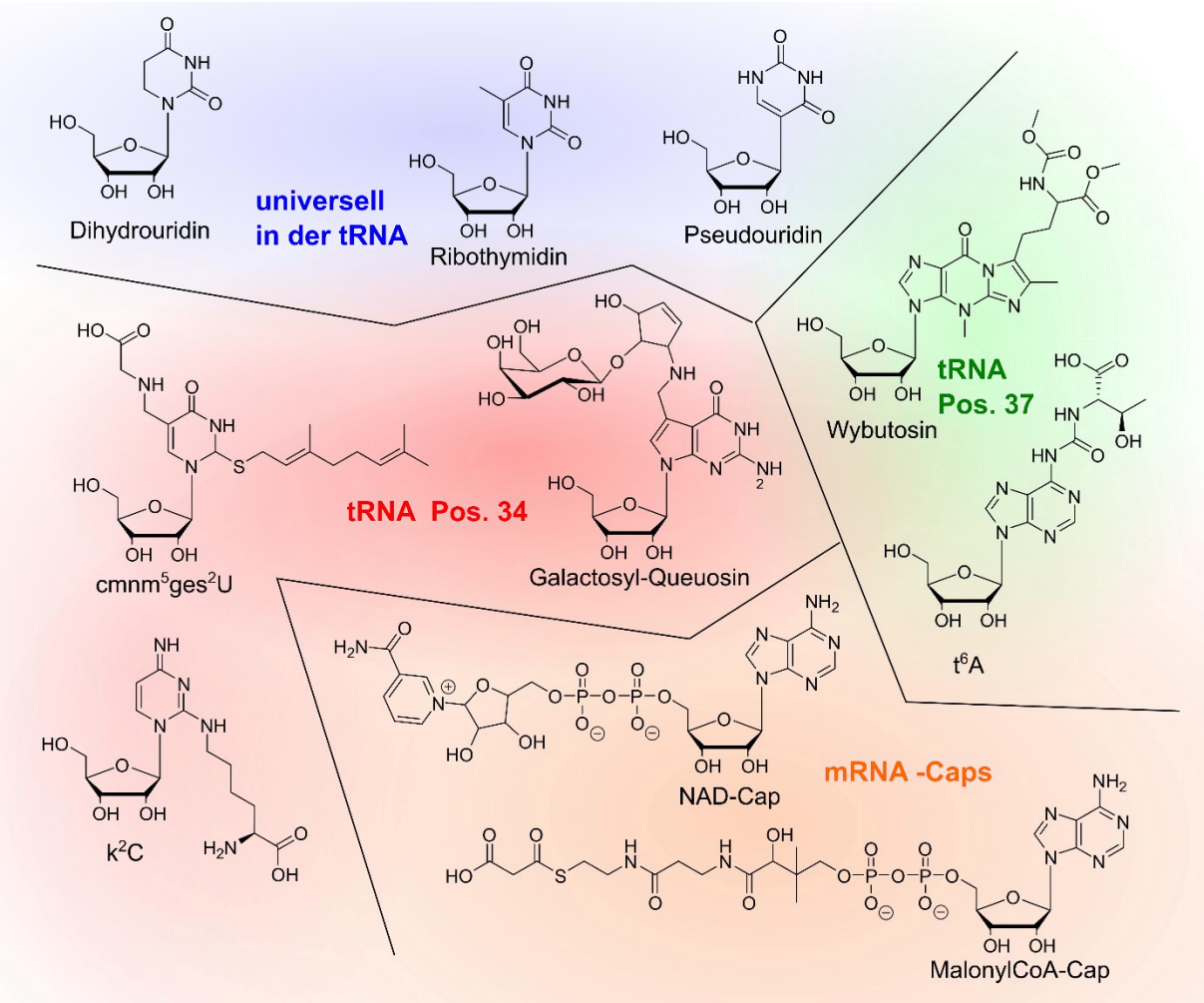


Abbildung 2: Strukturformeln verschiedener RNA-Modifikationen.[5] Die Strukturen sind unterteilt in die verschiedenen RNA-Bereiche oder Sequenzpositionen in denen sie vorkommen.

1.1.4. Die Dynamik der RNA-Modifikationen

Diese Vielfalt der tRNA-Moleküle wird verstärkt durch eine dynamische Erweiterung der RNA-Modifikationen oder einer Entfernung von Modifikationen. Es gibt derzeit etwa 170[5] verschiedene bekannte RNA-Modifikationen mit einer Vielzahl an unterschiedlichsten biochemischen Funktionen. Zahlreiche Enzyme modifizieren den synthetisierten RNA-Strang an spezifischen Stellen innerhalb der Sequenz. Dadurch verändert sich laufend das Modifikationsprofil der RNA. Es wird zwischen Writer-Enzymen und Eraser-Enzymen unterschieden. Die Writer-Enzyme bringen Modifikationen an der RNA an oder erweitern Modifikationen. Eraser-Enzyme dagegen entfernen RNA-Modifikationen.

1.1.5. Regulatorische Möglichkeiten der tRNA außerhalb des Ribosoms

Eine regulatorische Funktion der hochmodifizierten tRNA-Moleküle wird durch die spezifische enzymatische Spaltung der tRNA-Struktur eingeleitet. Dabei entstehen tRNA-Fragmente (tRFs) oder durch Stress induzierte tiRNAs, welche unter dem Sammelbegriff *tRNA-derived small RNAs* (tsRNAs) zusammengefasst werden können (**Abbildung 3**).[13] Diese Art der Fragmentbildung konnte in eukaryotischen, als auch in prokaryotischen Zellen nachgewiesen werden.[14] tRFs entstehen durch die RNA-Spaltung von Dicer-Enzymen, einer Klasse von Typ III Endoribonukleasen.[15] Bei der Fragmentierung der tRNA in tiRNAs spielt in höheren eukaryotischen Organismen dagegen das Enzym Angiogenin eine wichtige Rolle. Dieses schneidet abhängig von den am Anticodon platzierten Modifikationen[16] die verschiedenen tRNA-Isoakzeptoren zwischen Position 31 und 32 am ASL in zwei Hälften.[15] Diese so entstandenen, sehr spezifischen, kleinen RNAs mit einer Länge von 29 bis 50 Nukleotiden können in Zellen verschiedene stressbedingte Prozesse induzieren.[3, 17] So spielen tiRNAs eine Rolle bei verschiedenen neurodegenerativen Krankheiten wie ALS[18] oder Parkinson[19], aber auch in der Entwicklung von verschiedenen Krebsarten.[17b]

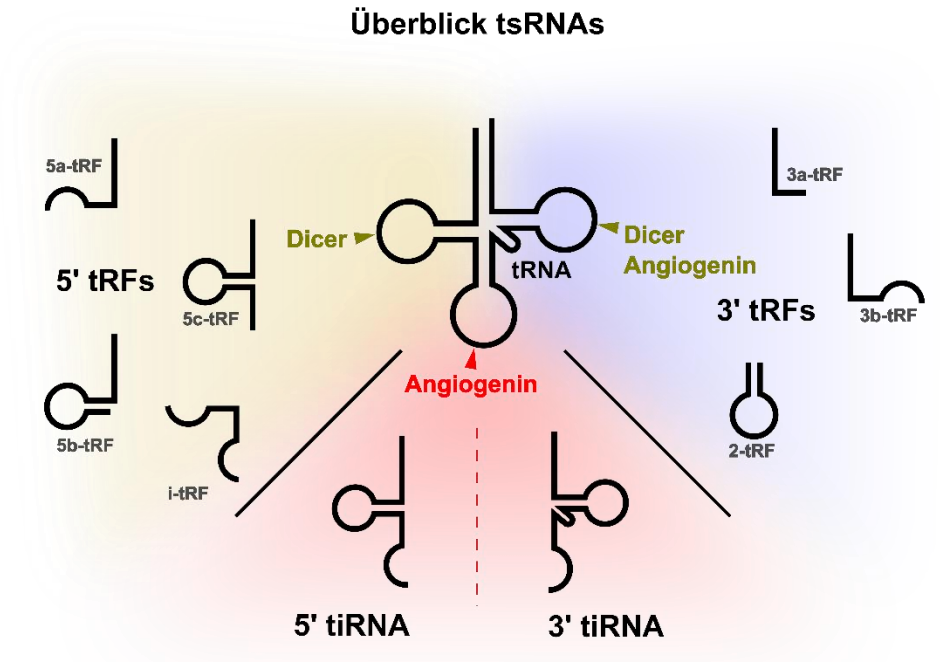


Abbildung 3: Schnittstellen von Dicer Enzymen und dem Enzym Angiogenin in der tRNA. Die entstandenen *tRNA-derived small RNAs* (tsRNAs) werden in tiRNAs und in tRFs unterteilt. In den Unterklassen können jeweils mehrere Varianten gebildet werden.[13]

1.1.6. Der Einsatz von sncRNAs als Virulenzfaktor

Ein besonders interessanter Aspekt ist auch die Verbreitung von sncRNAs in verschiedenen Parasiten. So wurde beispielsweise beobachtet, dass in extrazelluläre Vesikel verpackte miRNA des Malaria-Erregers *Plasmodium falciparum* in Endothelzellen des Wirts eindringen können und damit einen regulatorischen Vorgang in Gang setzen könnten. Es wird spekuliert, dass die Endothelwände dadurch durchlässiger werden und der Erreger sich leichteren Zugang in den Wirt verschafft.[2d] Was für eine Rolle die tsRNAs bei der jeweiligen Virulenz im Wirt spielen wird derzeit untersucht. Das Humanpathogen *P. aeruginosa*, oft Verursacher von Lungeninfektionen oder Blutvergiftungen, scheidet exosomal einen sncRNA-Mix in die Umgebung aus. Ein darunter identifiziertes tRNA Fragment inhibiert nach Eindringen in die Lungenendothelwand des Wirts die Ausschüttung von Interleukin-8 (IL-8) und hemmt somit die Immunantwort.[4] Neben den Einzellern gibt es auch mehrzellige Parasiten wie etwa *Onchocerca volvulus* (*O. volvulus*) oder *Litomosoides sigmondontis* (*L. sigmondontis*) aus dem Stamm der Fadenwürmer (Nematoden). Diese geben über Exozytose ebenfalls kleine tRNA-Fragmente in die Umgebung ab.[2a, 20] Bislang konnte noch nicht endgültig geklärt werden, welche Funktionen die tRNA-Fragmente nach Ausscheidung in den Wirtsorganismus übernehmen, jedoch wird vermutet, dass die Zell-Zell Kommunikation oder die Immunantwort gestört werden.

1.1.7. sncRNA als Biomarkermolekül

Unabhängig davon, inwiefern die parasitären tsRNAs tatsächlich zur Virulenz beitragen, könnten sich solche tsRNAs oder miRNAs im Blutserum oder im Urin des Wirts anreichern. Sie stellen damit ein für die Diagnostik interessantes Biomarkermolekül dar. Aktuelle Untersuchungen identifizierten bereits zahlreiche miRNAs und tiRNAs mit potentiellen Biomarker-Eigenschaften zur Diagnose oder zur Früherkennung verschiedener Krankheiten. Dies trifft für bestimmte Krebsarten[21], neurodegenerative Krankheiten[22] oder auch im Falle von Diabetes[23] oder kardiovaskulären Krankheiten[24] zu. Dabei wird die miRNA oder tiRNA meist anhand von qPCR quantifiziert.

1.2. RNA-Modifikationen in Bakterien und Eukaryoten

1.2.1. Beeinflussung der Bakterienvirulenz durch RNA-Modifikationen

Im ersten Kapitel wurden verschiedene Funktionen von RNA-Molekülen beschrieben. In diesem Abschnitt soll näher auf die einzelnen Funktionen der RNA-Modifikationen eingegangen werden. In virulenten Bakterien können RNA-Modifikationen auf unterschiedlichste Art und Weise dazu beitragen in den Wirt einzudringen und dessen Infektion begünstigen. Für einen Tyrosin-codierenden tRNA Isoakzeptor in *E. coli* konnte beispielsweise die RNA-Modifikation Gm (2‘-O-Methylguanosin) an Sequenzposition 18 der tRNA, als Teil einer immunsupressiven Struktur, identifiziert werden. Im Wirt wirkt die Struktur antagonistisch an den Toll-like Rezeptoren 7 und 8 (TLR-7/8) und verhindert damit die Ausschüttung von Interferon-α (IFN-α).[25] Weitere Virulenz verstärkende Mechanismen in Bakterien konnten im Zusammenhang mit den Modifikationen cmo^5U[26], Queuosin[27], m^4Cm[28], Cm, Um[29] und mnm^5s^2U[30] identifiziert werden.

Eine wichtige Rolle spielen RNA-Modifikationen außerdem in der Bildung von Antibiotikaresistenzen.[31] Viele Antibiotika stören oder inhibieren die bakterielle Translation durch Bindung des bakteriellen Ribosoms. Bei der Resistenzbildung gegen solche Antibiotika stehen unter anderem RNA-Modifikationen in den bakteriellen ribosomalen Untereinheiten im Fokus.[32] Hinsichtlich verstärkt auftretender Resistenzen gegenüber herkömmlichen Antibiotika[33] und der seit Jahrzehnten währenden Problematik bezüglich multiresistenter Krankenhauskeime[34], müssen weitere Untersuchungen angestellt werden, um die komplexen Eigenschaften und Strukturen des bakteriellen Epitranskriptoms vollständig aufzuklären. Dadurch sollen die Erforschung neuer Antibiotika-Targets ermöglicht, sowie neue Strategien zur Umgehung von Resistenzen gefunden werden.

1.2.2. Thiolierte RNA-Modifikationen in Bakterien

Die einzelnen Basen der RNA können neben Methylierungen (z.B. m^1A, m^5U, m^6A, m^7G, m^3C, etc.) Deaminierungen (z.B Inosin), Zyklisierungen (z.B. Q oder Wy), Isomerisierungen (z.B. Pseudouridin) und vielen weiteren chemischen Veränderungen auch thioliert werden. In Bakterien finden Thiolierungen an verschiedenen Positionen in der tRNA statt (siehe **Abbildung 4**). Die Einflüsse der einzelnen schwefelhaltigen

Modifikationen auf verschiedene biochemische Vorgänge können dabei ganz unterschiedlich sein.

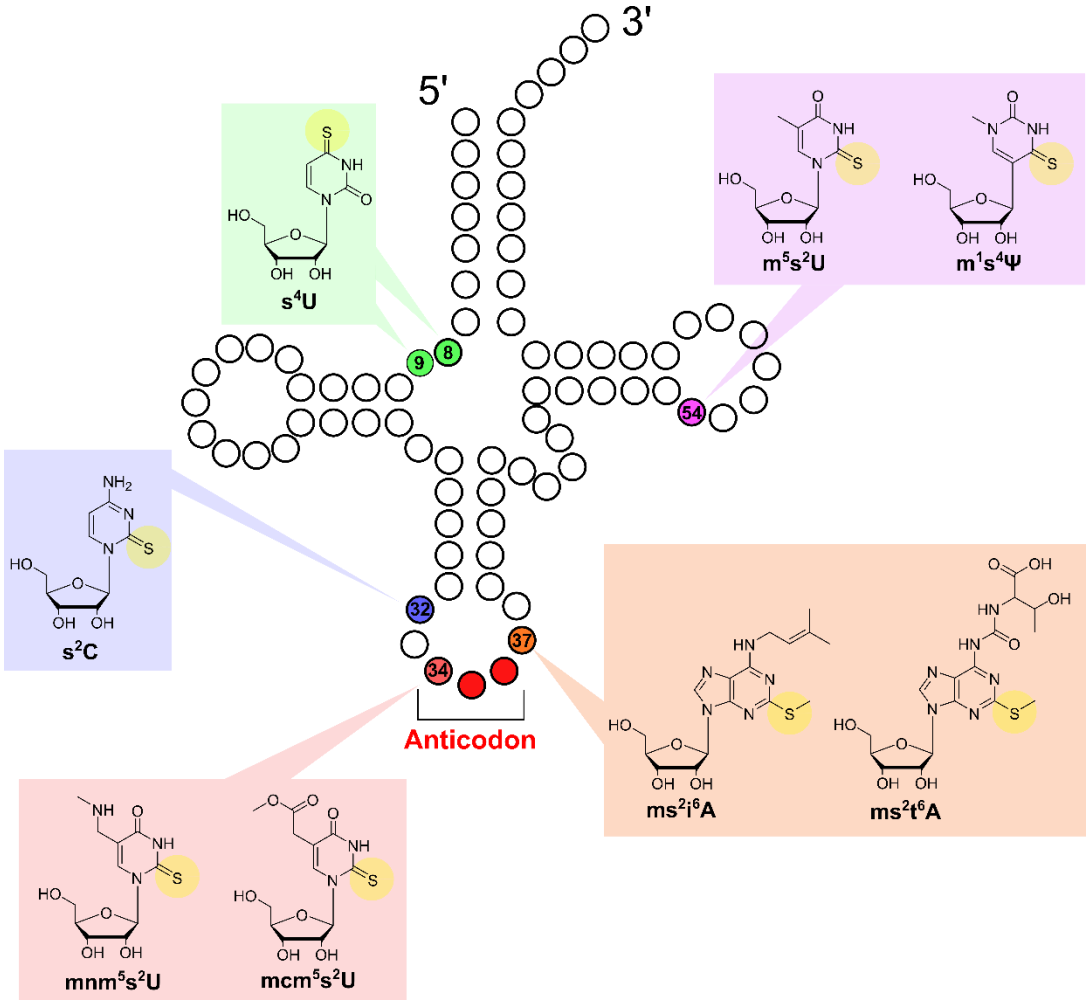


Abbildung 4: Thiolierte RNA-Modifikationen. Alle Positionen in denen thiolierte Modifikationen vorkommen sind in der dargestellten tRNA-Kleeblattstruktur farblich markiert. In den daneben stehenden Nukleosidstrukturen sind die Schwefelgruppen gelb markiert. Die bei der Wobble-Position 34 und an Position 37 abgebildeten Strukturen können zusätzlich in weiter abgewandelten Formen vorkommen. (Abbildung inspiriert von Čavužić *et al.*[35])

Die Thiolierung von Cytidin an Ringposition 2 (s^2C) in der tRNA von mehreren bakteriellen Isoakzeptoren (in *E. coli* und *Salmonella enterica*: $tRNA^{Ser}_{GCU}$, $tRNA^{Arg}_{ICG}$, $tRNA^{Arg}_{UCU}$ und $tRNA^{Arg}_{CCG}$) wird durch die Thiotransferase TtcA durchgeführt.[36] Die Modifikation s^2C wird ausschließlich an der Sequenzposition 32, am ASL der tRNA eingeführt. Das unmodifizierte Cytidin an Position 32 interagiert normalerweise über Wasserstoffbrückenbindungen mit dem Adenosin an Position 38 im ASL.[37] Durch den Austausch des Sauerstoffs mit dem Schwefel werden die elektronegativen Eigenschaften der Position verringert und die Interaktion mit A38 wird stark

eingeschränkt.[38] Außerdem wird wegen der eingeschränkten Flexibilität zwischen der sterisch anspruchsvollen Thiocarbonylgruppe und der 2‘-Hydroxygruppe die 3‘-*endo* Konformation der Ribose bevorzugt. Durch eine Verstärkung der Polarisierbarkeit ist zudem eine Veränderung der Stapeleigenschaften mit den benachbarten Basen wahrscheinlich (siehe Vgl. s^2U, **Seite 11**).[38-39] Diese Strukturänderungen haben eine Erhöhung der Flexibilität der Anticodon-Schleife zur Folge, was bei der Translation dazu führt, dass etwa $tRNA^{Arg}{}_{ICG}$ vermehrt die Codone CGU oder CGC, jedoch nicht mehr das Codon CGA translatiert. s^2C bewirkt in diesem Fall also eine Verschiebung der Codon-Anticodon Affinität zugunsten der Codone CGC und CGU.[38] Interessanterweise weist $tRNA^{Ser}{}_{GCU}$ ebenfalls eine Affinität für verschiedene Codone auf. Auch hier könnte s^2C eine Codon-Präferenz bewirken. Transkripte mit derartigen Eigenschaften werden als modifikationsabgestimmte Transkripte (MoTTs; von engl. *modification tunable transcripts*) bezeichnet und spielen eine wichtige Rolle in verschiedenen Stressantworten und Überlebensstrategien der Zelle.[40] Zudem wurde gezeigt, dass in Abwesenheit von s^2C gehäuft Frameshift-Mutationen während der Translation auftreten können.[41]

Wir sehen wie der Austausch eines einzigen Atoms innerhalb der Struktur von Cytidin bemerkenswerte Veränderungen mit weitreichenden Folgen verursacht. Solch ein Austausch ist nicht nur bei s^2C zu finden, sondern wurde auch für die beiden Ketogruppen der Uridine beobachtet. Demnach wurden, in einigen Bakterien und Archaeen an Position 8 und in manchen tRNAs auch an Position 9[42] in der Basenabfolge, ein Uridin mit einer C4-Thioketogruppe (s^4U) gefunden. Das am C2-Kohlenstoff thiolierte Uridin (s^2U) kommt dagegen fast ausschließlich im Anticodon an der Position 34 der tRNA vor und ist primär in Lysin, Glutamin und Glutaminsäure Isoakzeptoren zu finden.[43] Beiden Modifikationen werden verschiedene Eigenschaften zugeordnet. Dementsprechend ist s^4U wichtig für die Strukturstabilität des tRNA Gerüsts und ist dadurch in einen zellulären tRNA-Qualitätskontrollprozess eingebunden, durch welchen hypomodifizierte tRNAs schneller degradiert werden.[44] Außerdem fungiert s^4U als Sensor bei UV-Schäden durch die Formierung von intramolekularen Kreuzverknüpfungen (*Crosslinking*).[45] Diese Eigenschaft kann genutzt werden um mRNA-rRNA oder Protein-RNA Interaktionen zu untersuchen.[46] Außerdem kann s^4U dank der starken nukleophilen Eigenschaften mit 4-bromomethyl-7-propargyloxycoumarin (PBC) selektiv verknüpft und über Click-Chemie weiter

derivatisiert werden, beispielsweise durch die Anbringung eines Fluorophors.[47] Die künstliche Markierung des Transkriptoms von Säugetierzellen mit s^4U, ermöglicht anschließende Alkylierung durch Iodoacetamid (IAA), oder eine Immobilisierung über die Bildung von Disulfidbrücken an Methylthiosulfonatresten. Durch solche RNA-Markierung ist es möglich Dynamik- und Stabilitätsstudien in totaler RNA *in vivo* durchzuführen.[48] Interessant bleibt die Frage, ob in der Zelle vorkommende Elektrophile durch s^4U angegriffen werden können, und ob dies einen Einfluss auf die tRNA hat. Während s^4U ausschließlich in der bakteriellen und archaealen tRNA vorkommt, ist s^2U zudem in allen eukaryotischen Organismen zu finden. Wie bereits erwähnt, ist s^2U hauptsächlich Bestandteil der Modifikationen im Anticodon an Position 34 der tRNA, dennoch wurde in thermophilen Bakterien- und Archaeenarten (*Thermus thermophilus, Nanoarchaeum equitans*) an tRNA Position 54 die Modifikation m^5s^2U entdeckt.[49] Es wurde herausgefunden, dass diese die Schmelztemperatur der tRNA erhöht und somit für die Hitzebeständigkeit der Mikroorganismen wichtig ist.[50] Selbiges gilt für die erst kürzlich in der Archaeeenart *Ignicoccus hospitalis* entdeckte Modifikation $m^1s^4\Psi$, welche ebenfalls an Position 54 der tRNA vorkommt.[49b]

Auch in Adenosin können Schwefelatome eingebaut werden. So ist es für Prokaryoten, als auch in eukaryotischen Organismen bekannt, dass das Adenosin 37 am ASL thiomethyliert werden kann. Beobachtungen zufolge wird für die Übertragung der Thiomethylgruppe durch die jeweilige Methylthiotransferase (MiaB in Bakterien) in der Regel zuvor die Modifikation der exozyklischen N^6-Aminogruppe angebracht.[51] Dadurch entstehen mehrfach modifizierte Basen, wie ms^2i^6A oder ms^2t^6A, sowie erweiterte Formen davon.[51-52] Die thiomethylierten Adenosine sind, wie die s^2U modifizierten Uridine am Anticodon, in die direkte Anticodon-Codon Interaktion während der Translation eingebunden und können diese dramatisch beeinflussen.[39]

1.2.3. Einfluss der Wobble-Uridine und ms^2A Modifikationen auf die Translation

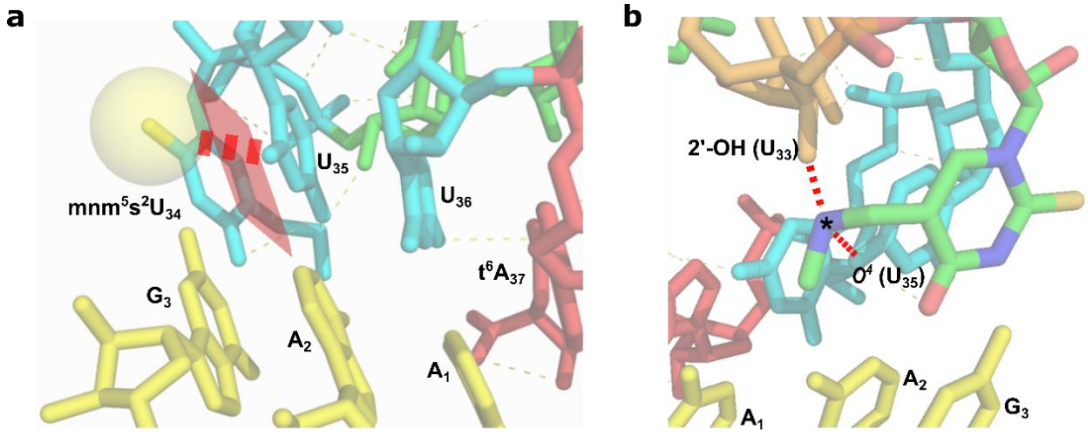
In der sogenannten Wobble-Hypothese von Francis H. C. Crick[53] wurde bereits 1966 postuliert, dass durch eine modifizierte Base im Anticodon einer tRNA, diese dadurch verschiedene Codone decodieren kann. Die Codone können sich demnach an Position 3 unterscheiden. Deshalb wird die dritte Position im Codon als wackelig oder flatterig (*engl. wobbly*) beschrieben.[53] Mit der Zeit rückten insbesondere modifizierte Uridine in den Vordergrund, welche die beschriebenen Effekte maßgeblich beeinflussen.[54] Diese sogenannten Wobble-Uridine, an Position 34 der tRNA, tragen vielfältige

Modifikationen am C5-Kohlenstoff der Uracil-Ringstruktur, und können, wie beschrieben, auch an Position 2 eine Thiocarbonylgruppe tragen. Diese kann in Sonderfällen sogar geranyliert werden[55] oder durch ein Selen ersetzt werden.[56]

Ein häufiges und vergleichsweise gut untersuchtes 2-thiomodifiziertes Wobble-Uridin in Bakterien und mitochondrialer tRNA ist 5-methylaminomethyl-2-thiouridin (mnm^5s^2U). Das eukaryotische Synonym zu dieser Modifikation ist 5-methoxycarbonylmethyl-2-thiouridin (mcm^5s^2U). mnm^5s^2U ist primär wichtig für die Stabilisierung des Codon-Gerüsts und damit für eine funktionierende Translation generell. Das eukaryotische mcm^5s^2U dagegen hat primär die Funktion eine bessere Codon-Erkennung zu gewährleisten. Dies kann im Falle eines mcm^5s^2U Defizits jedoch durch eine Überexpression des nicht modifizierten Isoakzeptors ausgeglichen werden, während der Verlust von mnm^5s^2U in Bakterien letal wirkt.[57]

Die Thiolierung bedingt eine parallele Stapelung der Anticodon-Basen (**Abbildung 5a**) und erzeugt eine Präferenz für eine 3'-*endo*-Konformation (Vgl. s^2C, **Seite 8-9**) bei den Anticodon-Uridinen. Dadurch erfolgt eine schnellere und effizientere Codonerkennung, welche die Translation begünstigt.[58] Es werden glutamin-, glutaminsäure- und lysinhaltige Proteine schneller und folglich häufiger prozessiert. Bei Aminosäuremangel kann das Wobble-Uridin nicht mehr thioliert werden, wodurch die Produktion von Aminosäure generierenden Proteinen verstärkt wird. Die s^2U Modifikation nimmt somit eine regulatorische Funktion im Aminosäure-Haushalt der Zellen ein.[59] Sie stärkt außerdem den Codon-Anticodon Duplex und weist eine Präferenz für Adenosin Paarungen auf. Codone mit einem G oder U an dritter Stelle werden durch die s^2U Modifikation schwächer translatiert.[60] Deshalb wird das Codon GAA in Anwesenheit der 2-Thiolierung vom tRNA Isoakzeptor $tRNA^{Glu}_{UUC}$ bevorzugt gebunden, während die mnm^5-Modifikation wiederum eine Präferenz für das Wobble-Codon GAG zeigt.[61] Je nach Codoneinsatz und tRNA-Modifikationsgrad werden mRNA Transkripte unter diesen Umständen unterschiedlich schnell und häufig translatiert, was zu einer Regulierung des Proteoms führt. Es ist jedoch nicht bekannt, dass durch Wobble-Präferenzen unterschiedliche Aminosäuren unter Einsatz des gleichen Codons translatiert werden können. So translatiert $tRNA^{Ser}_{GCU}$ die Codone AGC und AGU, während AGA und AGG aus derselben Codon Box von $tRNA^{Arg}_{CCU}$ und $tRNA^{Arg}_{UCU}$ translatiert werden.[62] Es ist jedoch nicht bekannt, dass eines dieser Codone sowohl von einer $tRNA^{Ser}$ als auch von einer $tRNA^{Arg}$ gelesen werden kann.

Interessanterweise kann es in der Natur aber Unterschiede bei der Interpretation des genetischen Codes geben. Dies wurde erstmals 1979 von Barrell *et al*.[63] für die Translation in humanen Mitochondrien beschrieben. Demnach codiert AUA in mitochondrialer RNA (mtRNA) für Methionin anstatt für Isoleucin und UGA für Tryptophan anstatt eines Stopp-Codons.[63] Derartige, durch RNA-Modifikationen bedingte Änderungen stellen die Theorie des universellen genetischen Codes stark in Frage.[64] Für mnm^5s^2U konnte zudem beobachtet werden, dass durch die An- oder Abwesenheit der Modifikation ein Unterschied in der Bevorzugung verschiedener Stopp-Codone stattfindet.[65] Beide Modifikationen, mnm^5s^2U als auch mcm^5s^2U, beeinflussen durch unterschiedliche elektrochemische Eigenschaften die Stabilität der Anticodon-Schleife und der Duplex Formation zwischen Codon und Anticodon (**Abbildung 5b**).[39] Zusätzlich sorgt das modifizierte Adenosin an Position 37 (siehe **Abbildung 4**) im ASL für weitere Flexibilität der ASL-Schleife, da eine Interaktion mit U33 verhindert wird (**Abbildung 5c**). Im Gegenzug ergibt sich eine stabilere Stapelung des Codon-Anticodon Duplex. Der t^6A- bzw. i^6A-Rest interagiert dazu mit der ersten Codonbase, einem Adenosin bzw. Uridin, und stabilisiert so die Codon-Anticodon Bindung an tRNA-Position 36 (**Abbildung 5c**).[39, 66]



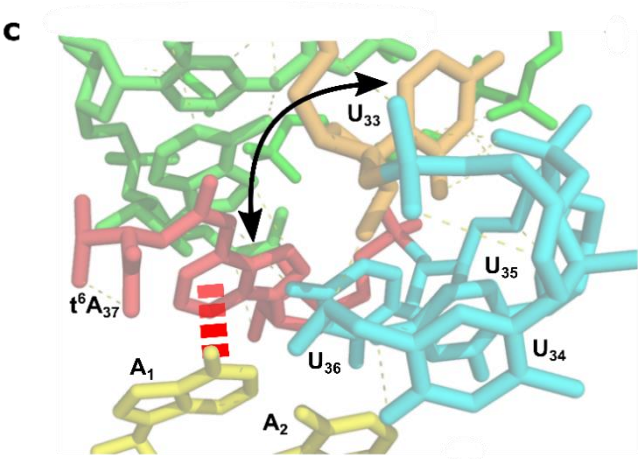


Abbildung 5: Strukturelle Einflüsse durch Modifikationen an der Wobble-Position und Position 37 der tRNA am Beispiel der $tRNA^{Lys}_{UUU}$. **a** Schichtung der Anticodon-Uridine U_{34} und U_{35} durch die s^2U Modifikation (gelbe Kugel). Die eingezogene Ebene (rote Fläche) soll die Schichtung der Basen verdeutlichen. Die gestrichelte Linie induziert die Interaktion des Schwefels mit U_{35}. **b** Interaktion des Stickstoffs des mnm^5-Rests mit der 2'-OH Gruppe der Ribose von U_{33} und der Ketogruppe an Position 4 von U_{35}. * Stickstoff des mnm^5-Rests. Farblegende für mnm^5s^2U: Grün = Kohlenstoff; dunkelblau = Stickstoff, rot = Sauerstoffe, gelb = Schwefel. **c** Einfluss von t^6A_{37} auf die Struktur des ASL und die Codonerkennung. Die Schichtung des an Position 1 im Codon befindlichen Adenosins A_1 mit t^6A_{37} der tRNA, wird anhand einer roten, breit gestrichelten Linie zwischen den geschichteten Basen verdeutlicht. Eine Interaktion zwischen den tRNA-Basen U_{33} und A_{37} ist durch die veränderte Lage des t^6A-Rests und den dadurch resultierenden Abstand (Doppelpfeil) nicht mehr gegeben. Gelb = Codon mRNA; dunkelrot = t^6A37; hellblau = Anticodon, bei **b** nur U_{35} und U_{36}; orange = U_{33}, grün = restliches tRNA-Gerüst. Die Grafiken a bis c wurden mit der Software Pymol™ (2006, DeLano Scientific LLC) erstellt und anschließend mit einer Grafiksoftware bearbeitet. Die verwendete PDB-Datei (*Protein Data Bank*) entstammt der Publikation um Rozov *et al*.[65]

In Eukaryoten stabilisiert der ms^2A Rest im Falle von ms^2t^6A die Stapeleigenschaften im ASL als Ausgleich für die Destabilisierung der Stapeleigenschaften durch den mcm^5-Rest am Wobble-Uridin. Daher ist $ms^2t^6A_{37}$ in Eukaryoten auch fast ausschließlich nur in Isoakzeptoren die $mcm^5s^2U_{34}$ tragen zu finden.[39] Neben t^6A und ms^2t^6A können sich auch die Modifikationen i^6A oder ms^2i^6A an Position 37 der tRNA befinden. In Bakterien wirkt ms^2i^6A regulatorisch auf den Eisenhaushalt der Zelle. So wird bei Eisenmangel i^6A nicht mehr zu ms^2i^6A modifiziert, was eine Umstellung in der Proteinsynthese, zugunsten Eisen-bindender Stoffe, bewirkt.[58b, 67]. Erst kürzlich wurde ein erweitertes Derivat von ms^2i^6A mit der Bezeichnung $msms^2i^6A$ in *E. coli* entdeckt.[68] Dessen Funktion ist zum jetzigen Zeitpunkt jedoch noch unklar.

Die hier beschriebenen Zusammenhänge spielen auch bei für den Menschen relevanten Krankheiten eine Rolle. Demnach gibt es einen Zusammenhang zwischen Veränderungen der mcm^5s^2U Mengen in der tRNA und der Entwicklung

neurodegenerativen Erkrankungen.[69] Außerdem stehen einige Krebsarten in Verbindung mit den mcm^5/ncm^5-Modifikationen generierenden Enzym Elongator Komplex (Elp).[69] Der Mangel an ms^2t^6A verhindert die korrekte Translation von Insulin und trägt zur Entwicklung von Diabetes Typ II bei.[66b, 70] Der zentrale Mechanismus der Translation und dessen molekulare Zusammenhänge sind demnach eng korreliert mit Krankheiten, welche die Menschheit heute vor große Herausforderungen stellen. Mögliche Modifikationsunterschiede innerhalb einer tRNA Population im Zusammenhang mit Infektionen oder Krankheiten sind daher von großem Interesse.

1.2.4. RNA-Modifikationen in Nematoden

Bei den Fadenwürmern (Nematoden) gibt es Arten, die für mehrere Infektionskrankheiten in Mensch und Tier verantwortlich sind. Die Flussblindheit (Onchozerkose) wird durch die Nematodenart *O. volvulus* verursacht und ist vor Allem in Afrika und in Teilen Südamerikas verbreitet.[71] Im Jahr 2017 waren etwa 20,9 Mio. Menschen weltweit infiziert, wovon 14,6 Mio. Fälle in Kombination mit Hauterkrankungen auftraten und 1,15 Mio. Fälle sogar zur Erblindung führten.[72] Der von der Kriebelmücke übertragene Erreger (*O. volvulus* Larve, Stadium L3) befällt ausschließlich Menschen, ist aber auch in nicht-humanen Primaten überlebensfähig.[73] Zudem konnten Maus-Modelle mit immundefizienten Mäusen generiert werden.[74] Ein artverwandter und in den Tropengebieten Afrikas heimischer Nematode mit dem Namen *Onchocerca ochengi* (*O. ochengi*) befällt dagegen Rinder. Mit *O. ochengi* befallene Rinder weisen beulenartige Hautwucherungen auf, die von den bis zu 30 cm langen weiblichen Exemplaren hervorgerufen werden.[75] *O. ochengi* wird inzwischen seit fast 30 Jahren allgemein als Modellorganismus für *Onchocerca*-Spezies eingesetzt[76], dennoch wurden die RNA-Modifikationen von parasitären Nematoden bisher kaum untersucht. Wenige Informationen über Vorkommen und Dynamik von RNA-Modifikationen wurden bisher lediglich für die Nematodenarten *Caenorhabditis elegans* (*C. elegans*) und *Ascaris suum* (*A. suum*) veröffentlicht. In **Tabelle 1** sind die bisher in der tRNA identifizierten Modifikationen dieser beiden Arten aufgeführt.

Tabelle 1: Alle bisher in Nematoden beschriebenen RNA-Modifikationen, inklusive Unterscheidung der Nematodenart und der jeweiligen Literaturquellenangaben.

tRNA Modifikation	*C. elegans*	*A. suum*	Literaturquellen
m^1A	+	+	[77], [78], [79]
m^6A	+		[80], [79]
f^5C		+	[78]
m^5C	+		[79]
hm^5Cm	+		[81]
m^1G	+	+	[78], [79]
m^2G	+	+	[77], [80], [79]
$m^2{}_2G$	+		[82], [80], [79]
m^7G	+		[79]
t^6A	+	+	[78], [79]
i^6A	+		[80]
ms^2t^6A	+		[80]
ms^2i^6A		+	[77], [78]
m^5U	+		[79]
ncm^5U	+		[83],
mcm^5U	+		[80]
mcm^5s^2U	+		[83], [84], [80]
mnm^5U	+		[80]
Cm	+		[80], [79]
Ψ	+	+	[78], [79]
Queuosin	+	+	[85], [78]
Inosin	+		[80]

Es wurde außerdem herausgefunden, dass der Mangel an mcm^5s^2U zur verlangsamten Translation bestimmter Codone und schließlich zu stressinduzierten Proteinaggregaten in *C. elegans* führt.[86] Des Weiteren konnte bei Hitzestress, aber insbesondere bei Nahrungsknappheit, in *C. elegans* ein Rückgang an mcm^5s^2U beobachtet werden.[80] Weitere Untersuchungen, gerade im Zusammenhang mit den genannten *Onchocerca* Vertretern und RNA-Modifikationsveränderungen während einer Infektion, stehen weiter aus.

1.3. Der Einfluss von Methylierungsstress auf die Nukleinsäuren

1.3.1. Zellstress im Überblick

Durch vielfältige Umwelteinflüsse können Zellen auf unterschiedliche Art und Weise geschädigt werden. Im Menschen kann Zellstress durch physischen und psychischen Stress[87], Infektionen[88] oder auch durch energiereiche Strahlung[89] ausgelöst werden. Auf molekularer Ebene bedeutet das, je nach Stressart, eine chemische Modifizierung verschiedener in der Zelle befindlicher (Makro)moleküle. Durch Anwesenheit verschiedener reaktiver Stressoren, wie beispielsweise reaktive Sauerstoffspezies (ROS) oder auch alkylierende Reagenzien, können so Proteine, Membranen, die DNA und die RNA geschädigt werden.[90] Dadurch entstehende Mutationen im Genom können verschiedene Krebsarten auslösen[91], die Zellen zur Apoptose (programmierter Zelltod) zwingen[92] oder Immunreaktionen auslösen[93]. In bakteriellen Organismen kann häufig Methylierungsstress auftreten, beispielsweise ausgelöst durch akuten Nährstoffmangel, wodurch in *E. coli* Guanosin-O^6 methylierende, nitrosylierte Glycin Derivate gebildet werden.[94] Ein weiterer endogener und häufig in der Zelle vorkommender Methylgruppen-Donor ist das Coenzym *S*-Adenosyl-Methionin (SAM).[95] Als exogene Methylierungsreagenzien kommen halogenierte Methylgruppen in Frage, wie das von Pflanzen und Pilzen ausgeschiedene Methylchlorid oder die in maritimen Umgebungen vorkommenden Methylhalogene Methyliodid, beziehungsweise Methylbromid.[90a] Eine Methylierung kann auch durch das natürliche Antibiotikum Streptozotocin oder durch Chemotherapeutika wie Temozolomid erzeugt werden.[90a]

1.3.2. Schädigung des Erbguts und der RNA durch Methylierungsstress

Die Methylierungen können an verschiedenen Stellen der DNA oder RNA Struktur stattfinden. Besonders betroffen sind die Ringstickstoffe der Nukleinsäurebasen. Aber auch exozyklische Amine oder Ketogruppen sind für Alkylierungen anfällig (**Abbildung 6a**).[90a] Neben der Base werden außerdem die 2‘-OH Gruppe im Riboserest der RNA sowie die Sauerstoffgruppen am Phosphodiester-Rückgrat methyliert (**Abbildung 6b**).[90a] Einige der Methylierungsprodukte wie Cm, Am, Um oder Gm sind dabei enzymatische Produkte mit funktionellen Eigenschaften.[96] Manche Stickstoffmethylierungen in den Basen (z.B. m^7G oder m^6A) können dagegen sowohl enzymatisch als auch durch ein methylierendes Stressor-Reagenz direkt erfolgen.[90a,

97] Andere Modifikationen wie m^1A oder m^3C wiederum kommen in der bakteriellen RNA ausschließlich als Methylierungsschaden vor.[98] In **Abbildung 6c** sind einige stressbedingte Methylierungsprodukte der RNA- oder DNA-Basen dargestellt.

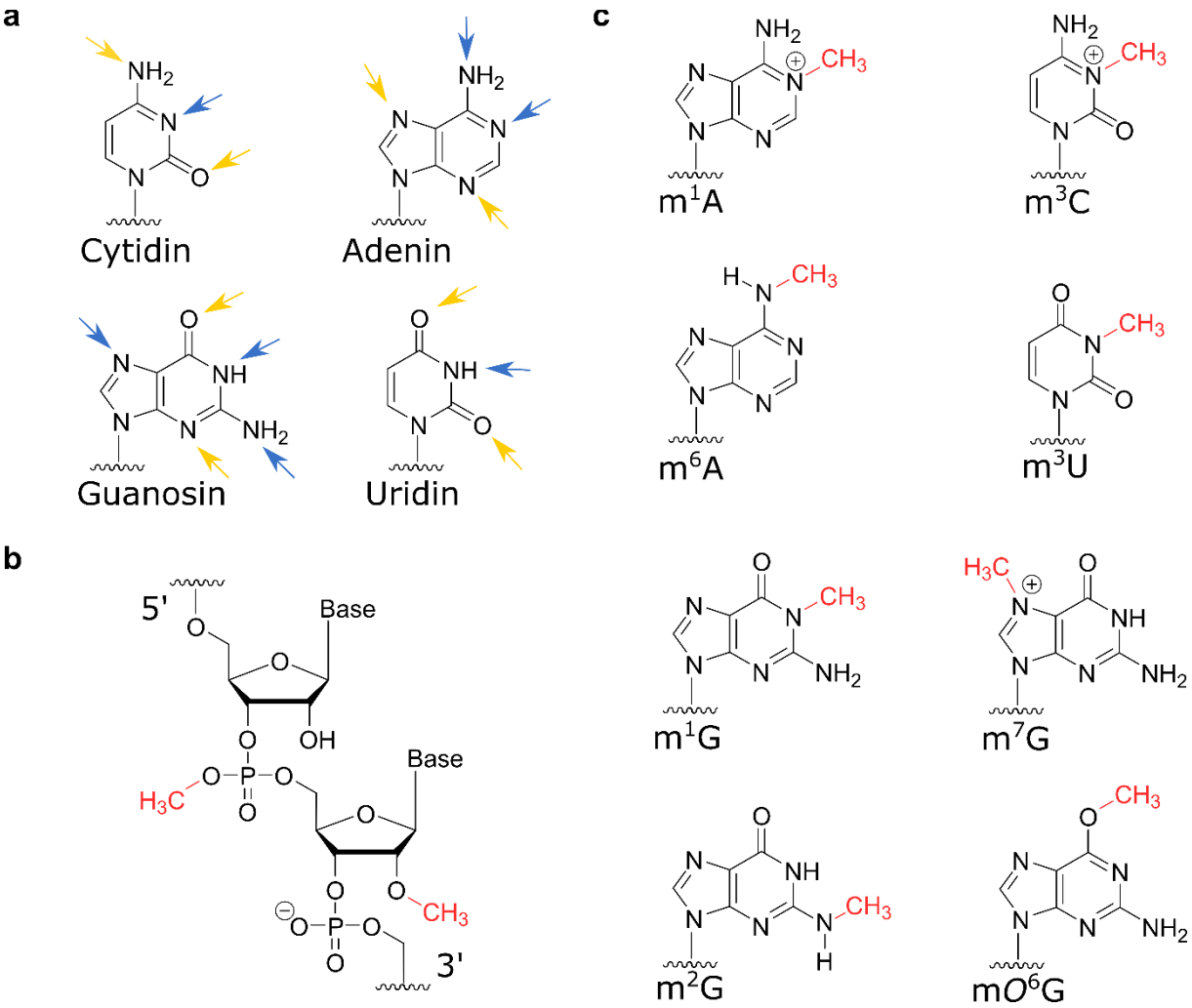


Abbildung 6: Methylierungen in der RNA und der DNA. **a** Alkylierungspositionen in den vier RNA-Basen. Blaue Pfeile markieren Positionen mit einer höheren Methylierungsrate. Gelbe Pfeile deuten auf Positionen, die weniger häufig methyliert werden. **b** Methylierungen im Ribose-Phosphatgerüst der RNA. **c** Verschiedene und häufiger auftretende Methylierungsprodukte in RNA und DNA. (Abbildung inspiriert von Yan *et al.*[90a])

Eine Methode zur Diskriminierung zwischen enzymatisch angebrachten und direkt durch die Stressor-Reagenzien übertragenen Methylgruppen stand bisher noch nicht zur Verfügung und wird im Rahmen dieser Arbeit in Anwendung auf die RNA erstmals beschrieben (Reichle *et al.*, *ChemBioChem*, **2018**, *19*, 2575-2583; siehe auch **Kapitel 3.4.** dieser Arbeit). Methylierungen sind im Allgemeinen wichtiger Bestandteil der Epigenetik[99] und von funktionalen RNA-Modifikationen.[9] Bei unspezifischer Anbringung von Methylgruppen im Genom oder in der RNA kann dies jedoch zu

ungewollten Strukturänderungen führen. Durch die Methylierung bestimmter Positionen kann keine Watson-Crick Basenpaarung mehr stattfinden und die Sequenz kann von den Polymerasen oder Ribosomen nicht mehr gelesen werden. Dies führt zu einer veränderten Genexpression bei DNA-Schäden oder zu einer verringerten Translationsrate bei RNA-Schäden.[90a] Es ist bekannt, dass die ungezielte Formierung von m^7G in der RNA auch zur Inhibierung von RNA-Protein Interaktionen führen kann.[89] Zudem sind vermutlich RNA-Moleküle von Alkylierungsreaktionen stärker betroffen als DNA-Stränge, da als Einzelstränge vorliegende Nukleinsäuren zugänglicher für die Alkylierungsreagenzien sind.[100] Aufgrund der häufigen und vielfältigen RNA-Modifikationen, ist es durchaus möglich, dass bereits modifizierte RNA-Basen durch Stressor-Moleküle weiter modifiziert werden können. Eine besonders reaktive Seitengruppe stellen die Schwefelgruppen in thiolierten Modifikationen dar. Wie in Proteinen die Cysteine[101], so können in der tRNA die Thiouridine oder die Thiocytidine ein hohes Alkylierungspotential aufweisen (mehr dazu in Reichle *et al.*, *Nat. Commun*. **2019**, 10, 5600 und Reichle *et al*., *ChemBioChem* **2020**, *21,* 1-5*;* **Kapitel 3.2.** und **Kapitel 3.3.**).

1.3.3. Stressantworten der Zellen

Zellschädigungen anhand der beschriebenen Stressarten finden täglich millionenfach statt, weshalb die Zellen mehrere Wege gefunden haben mögliche RNA- und DNA-Schäden zu reparieren. Bekannte Mechanismen zur Reparatur der DNA sind beispielsweise die homologe Rekombination oder die Nicht-homologe Endverknüpfung (NHEJ) bei DNA Doppelstrangbrüchen, die Nukleotid-exzisionsreparatur (NER) bei strukturellen Veränderungen der DNA oder etwa die Basenexzisionsreparatur (BER) mit Hilfe Basen entfernender Glykosylasen.[102] Des Weiteren gibt es direkt am Schaden angreifende Reparaturmechanismen, wobei zusätzliche chemische Seitengruppen, wie die Methylierungen nach einem entsprechenden Stress, wieder enzymatisch entfernt werden. Zu diesen Enzymen gehören die O^6-Alkylguanin DNA-Alkyltransferasen (AGTs) oder die Vertreter der AlkB Dioxygenasen.[102] Das multifunktionale Protein Ada agiert dabei als Initiator der Alkylierungsstress-Antwort. Die C-terminale Einheit dieses Proteins (C-Ada) fungiert als AGT und repariert neben O^6-Methylguanosin auch ethylierte oder benzylierte O^6-Derivate.[102-103] Die N-terminale Ada-Einheit (N-Ada) weist dagegen eine Zinkfinger-Proteinstruktur auf, welche in der Lage ist, mit Hilfe eines reaktiven Cysteins im aktiven

Zentrum, Methylierungen vom DNA-Phosphatgerüst auf das Protein Ada zu transferieren (siehe **Abbildung 7a**).[103] Durch die irreversible Methylgruppenübertragung am N-Ada, wird Ada als Transkriptionsfaktor aktiviert und aktiviert wiederum das ada Operon, welches die Gene *ada*, *alkA*, *alkB* und *aidB* beinhaltet.[102, 104] Die Expression dieser Gene induziert im weiteren Verlauf der Stressantwort die direkte Reparatur der alkylierten DNA. AlkA ist eine in der Evolution in allen Organismen konservierte DNA-Glykosylase, die bei der Basenexzisionsreparatur wichtig ist, während über die Funktion von AidB noch keine stressrelevanten Erkenntnisse vorliegen.[102] Das AlkB Enzym ist eine oxidative Demethylase und entfernt laut Literatur vorzugsweise Methylgruppen, die an Ring-Stickstoffen der Nukleinsäurebasen gebunden sind (z.B. m^1A oder m^3C).[105] Im Menschen existieren 9 AlkB Homologe (AlkBH1-AlkBH8, FTO) deren Substrate sich jeweils unterscheiden können.[102] Das bakterielle AlkB und das Humanhomolog AlkBH3 überraschten 2003 durch die Erkenntnis, dass beide auch in RNA-Einzelsträngen eine effiziente Demethylierung durchführen können, was später auch für das AlkB Homolog FTO bestätigt wurde.[106] AlkB ist außerdem in der Lage das künstlich erweiterte Substrat 1,N^6-Ethenoadenin umzusetzen (siehe **Abbildung 7b**)[107] und es scheint möglich, dass sich weitere natürliche AlkB-Substrate in der RNA befinden. Die Demethylierung durch AlkB findet mit Hilfe des Cofaktors Fe(II), des Coenzyms α-Ketoglutarat und unter Einbezug eines Sauerstoffmoleküls (O_2) statt. In Folge einer Hydroxylierung der Methylgruppe, spaltet sich diese im wässrigen Milieu als Formaldehyd ab, wodurch die Base in ihren ursprünglichen Zustand zurückversetzt wird (siehe **Abbildung 7c**).[107]

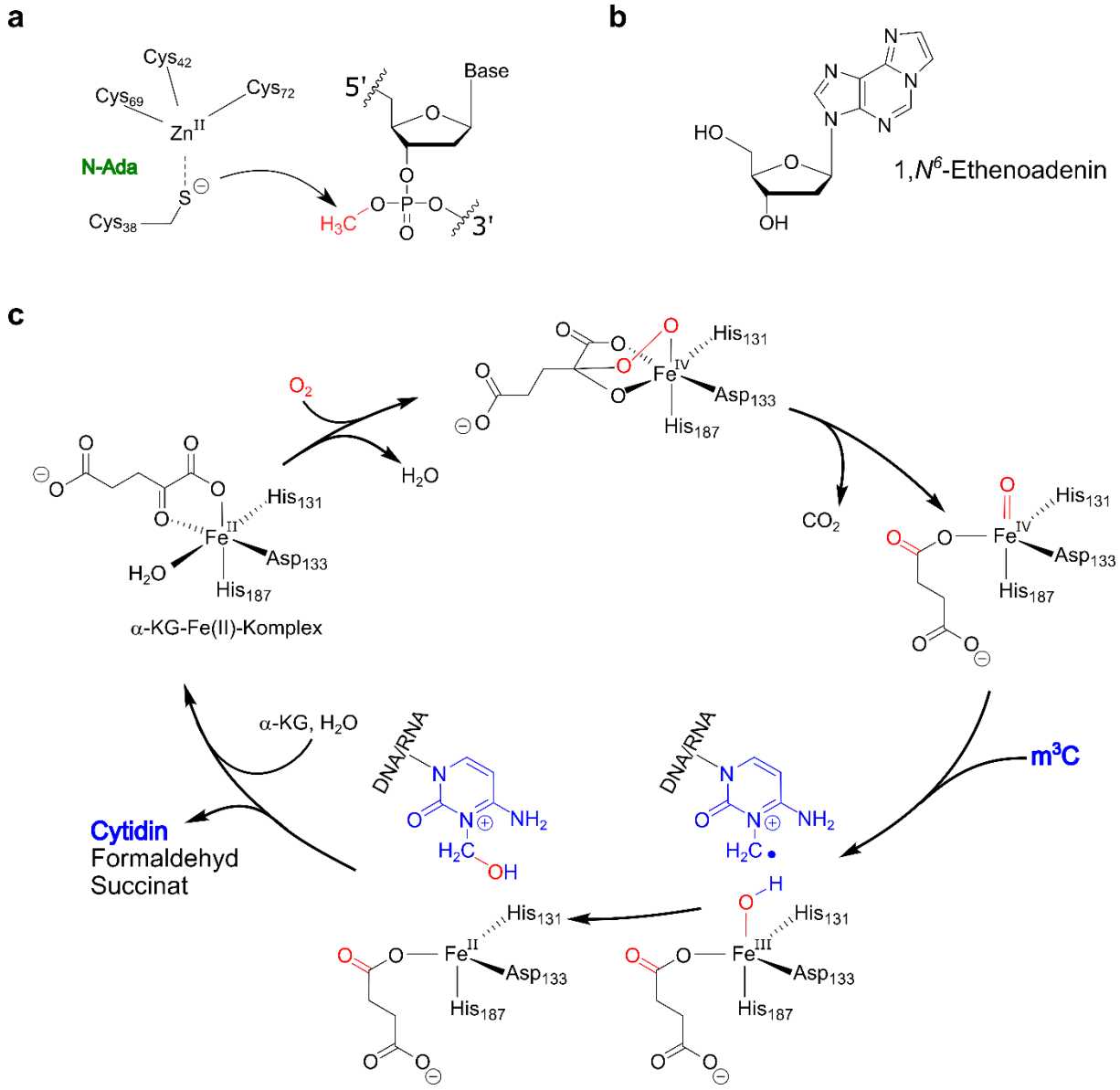


Abbildung 7: Dealkylierung von Nukleinsäurebasen. **a** Zinkfinger-Domäne des N-Ada Enzyms und Mechanismus der Demethylierung des Phosphat-Rückgrats. **b** Struktur des AlkB Substrats 1,N^6-Ethenoadenin. **c** Mechanismus einer Eisen(II)/α-Ketoglutarat abhängigen Dioxygenase, adaptiert auf den Demethylierungsprozess von m^3C durch AlkB.[103, 108]

Weitere enzymatische oder nicht-enzymatische Demethylierungsvorgänge in der RNA oder DNA wurden bisher nicht beschrieben, können jedoch nicht ausgeschlossen werden. Eine vollständige Aufklärung der DNA und RNA-Reparaturmechanismen in den verschiedenen Organismen und Zelllinien muss weiterhin Bestandteil der aktuellen Forschung bleiben. Zellstress bedingte Krankheiten und deren Therapiemöglichkeiten können dadurch zukünftig besser erforscht und evaluiert werden.

1.4. Methoden zur Detektion von Nukleinsäuren

1.4.1. Massenspektrometrie zur Detektion von Nukleinsäuren

Ende der 80er Jahre des letzten Jahrhunderts führte die Entwicklung neuer Ionisierungstechniken zu einer Revolution in der MS-Analytik von großen Biopolymeren, so auch in der Nukleinsäure-Analytik. Mit der Einführung der Elektrospray-Ionisierung (ESI)[109] und der *Matrix-Assisted Laser Desorption/Ionization* (MALDI)[110] Technik wurden neue Sequenzierungsmethoden entwickelt.[111] Jede der beiden Ionisierungstechniken weist je nach Anwendung Vor- und Nachteile auf. Durch den Einsatz von MALDI können zwar größere Polymere leichter Ionisiert werden, jedoch tragen die induzierten Ionen meist nur eine Ladung. Dies macht die Signale einfacher auswertbar, aber große Moleküle als Ionen gleichzeitig auch instabiler.[112] MALDI-MS wird oft für die Analyse von RNA-Oligomeren verwendet[113] und ist vergleichsweise kostenintensiv.[114] Ein Hauptargument für den Einsatz der weniger kostenintensiveren Anwendung einer ESI-Quelle ist die Kompatibilität mit einer vorgeschalteten Flüssigchromatographie (LC).[111] Eine Methoden-Kombination aus LC und MS zur Analyse von Nukleosiden wurde erstmals von S. C. Pomerantz und J. A. Mc Closkey[115] im Jahr 1990 eingesetzt. Die ESI bietet gegenüber der damals in der LC-MS häufiger eingesetzten Thermospray-Ionisierung und gegenüber der MALDI eine begünstigte Ionen-Bildung. Außerdem wird durch die Möglichkeit der schnellen Regulierung des Spannungsfelds in der Quelle eine an die Moleküle angepasste Fragmentierung möglich.[111-112, 115] Die massenspezifische Filterung und spezifische Analyse von Fragmenten kann durch den Einsatz von *Tandem Mass Spectrometry* (MS/MS), erreicht werden. Für die Aufnahme von MS/MS Spektren wird bei der ESI-MS/MS eine Kollisionszelle zwischengeschaltet (CID, von engl. *Collision Induced Dissociation*). Insbesondere für die quantitative Bestimmung von kleinen Molekülen bietet die LC-ESI-MS/MS Methode eine geeignete Plattform.[116]

1.4.2. Aktuelle Methoden zur Entdeckung neuer RNA-Modifikationen

Ein sehr spannender Bereich im Gebiet der Epitranscriptomics ist weiterhin die Entdeckung und Erforschung noch unbekannter RNA-Modifikationen. Trotz der Tatsache, dass bereits über 170[5] verschiedene RNA-Modifikationen in unterschiedlichsten Organismen beschrieben wurden, werden jährlich weitere Modifikationen entdeckt. Die Art und Weise, sowie die Techniken und Strategien, derer

sich die Wissenschaftler dabei bedienen, können sich sehr unterscheiden. Einige neu entdeckte Strukturen sind in **Abbildung 8** abgebildet. Sakai *et al*. 2016[117] konnte durch tRNA-Isoakzeptoren Aufreinigung, RNase T1-Verdau und anschließender LC-MS/MS Analyse das bis dato unbekannte Wobble-Uridin mcmo^{5}Um identifizieren. Die bis dahin nicht bekannte 2‘-O-Methylierung konnte anhand der gebildeten Fragmente im hochauflösenden Massenspektrometer (HRMS) zugeordnet werden. Die Modifikation macht in *E. coli* etwa 4,2 % der Wobble-Uridine in allen tRNA$^{Ser}_{UGA}$ Isoakzeptoren aus. Über dieselbe Strategie und über Bestätigung der Struktur anhand eines synthetischen Standards konnten in der gleichen Forschungsgruppe im Jahr 2017 die Modifikationen ht^{6}A und ms^{2}ct^{6}A an Position 37 der tRNA identifiziert werden.[64, 118] Die synthetischen Nukleosid-Standards zeigen dieselben Retentionszeiten und dieselben molekularen Massen wie die aus dem Naturprodukt isolierten Nukleoside. ht^{6}A wurde aus der tRNA^{Lys} der Seegurkenart *Mesocentrotus nudus* isoliert, wo es die Codon-Anticodon Interaktion zum Codon AAA blockiert.[64] ms^{2}ct^{6}A wurde dagegen in der tRNA^{Lys} des grampositiven Bakteriums *Bacillus subtilis* und des einzelligen Parasiten *Trypanosoma brucei* entdeckt, wo es zusammen mit cmnm^{5}s^{2}U für die Proteinintegrität verantwortlich ist.[118]

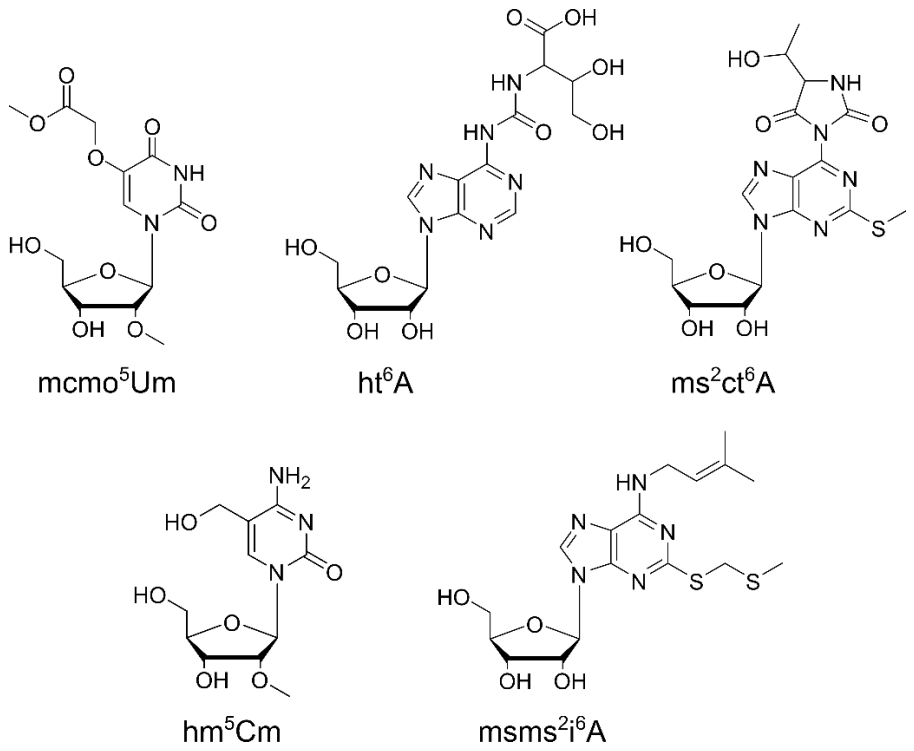


Abbildung 8: Neu entdeckte RNA-Modifikationen der letzten Jahre.

Eine weitere Möglichkeit um auf die Struktur einer neuartigen RNA-Modifikation zu schließen ist die Markierung der RNA durch zugefütterte Isotope. So wurde ebenfalls im Jahr 2017 von Huber *et al.*[81] während eines auf Isotopenmarkierung aufgebauten Dynamik-Experiments in HEK-Zellen die Modifikation hm^5Cm entdeckt. Die zusätzliche 2'-O-Methylgruppe wurde, wie bei $mcmo^5Um$, anhand der Fragmentierung im HRMS identifiziert. Die zusätzliche Detektion der ^{15}N- und CD_3-Isotopologe der neuen Modifikation ergaben weitere Hinweise auf die Summenformel, bevor dann über einen synthetischen Standard die chemische Struktur eindeutig verifiziert werden konnte. Die Anwendung von Isotopen fand auch Eingang in der Studie von Dal Magro *et al*.[68], wodurch die Modifikation $msms^2i^6A$ identifiziert wurde. Hierbei kamen Isotopenmarkierungen gleich mehrmals zum Einsatz. Zuerst wurde die RNA von *E. coli* mit ^{13}C-, ^{15}N- oder ^{34}S-Isotopen markiert, um nach einem MS-Screening Hinweise auf die Summenformel potentieller RNA-Modifikationen zu bekommen. Die erhobenen Daten lieferten 37 unbekannte Signale. Bei der Strukturanalyse der potentiellen Modifikation mit Summenformel $C_{17}O_4N_5H_{25}S_2$ ($msms^2i^6A$) wurden Deuterium-Wasserstoff Austauschexperimente in D_2O durchgeführt und durch die Zugabe von CD_3-*S*-Methionin ins Nährmedium konnte die Thioacetal-Gruppe an Position 2 bestätigt werden. Bei der Entdeckung und Charakterisierung neuer RNA-Modifikationen führte die innovative Anwendung von stabilen Isotopenmarkierungen somit zu bemerkenswerten Ergebnissen.

1.4.3. Isotopenmarkierung als innovatives Werkzeug in der RNA-Analytik

Ein vollständiger Austausch von ^{12}C, ^{14}N oder ^{32}S Atomen durch ^{13}C, ^{15}N oder ^{34}S Isotope in *E. coli* kann durch Verwendung eines Minimalmediums M9 mit $^{13}C_6$-Glukose, ^{15}N-NH_4Cl oder ^{34}S Na_2SO_4 erfolgen. Der Einsatz von Isotopenmarkierungen zur Aufklärung von biochemischen Abläufen ist nicht erst seit Dal Magro *et al*.[68] in den Biowissenschaften als innovative Methode etabliert. Bereits Melvin Calvin wendete Isotopenmarkierung in Zellen an, um die biochemischen Abläufe des Calvin-Zyklus nachzuvollziehen.[119] In der Welt der Nukleinsäuren wurde die Isotopenmarkierung beispielsweise bei der Aufklärung der semikonservativen Replikation der DNA im Meselson-Stahl Versuch von 1958 eingesetzt.[120] Eine neuere Methode mit Isotopenanwendung ist die Markierung von Aminosäuren zur Verfolgung von Proteinen in Zellkultur (SILAC).[121] Zudem führten Isotopenmarkierte Derivate in Verfolgungsstudien von DNA-Modifikationen bereits zu beeindruckenden Ergebnissen

in der Epigenetik.[122] Bei der Untersuchung von RNA-Strukturen und -Dynamiken über Kernspinresonanzspektroskopie (NMR) kommt bereits synthetisierte isotopenmarkierte RNA zum Einsatz.[123] Aber auch in der massenspektrometrischen Analytik findet die Isotopenmarkierung von RNA-Molekülen zunehmend Eingang. Eine zusätzliche Erweiterung für die biosynthetische Isotopenmarkierung stellt dabei die Markierung von Methyl-Modifikationen durch CD_3 Gruppen dar. Die Zugabe der deuterierten Aminosäure CD_3-*S*-Methionin ins Medium erweitert damit das Spektrum der Markierungsmöglichkeiten im Epitranskriptom verschiedener Organismen.

Im Jahr 2011 wurde eine MS-analytische Methode vorgestellt, in welcher biosynthetisch ^{15}N-markierte RNA-Oligomere sowohl die Identifizierung co-eluierender Kompositionsisomere, als auch die Detektion posttranskriptionaler Modifikationen in RNA-Oligomeren ermöglichte.[116a] Eine von Patrick A. Limbach dagegen stetig weiterentwickelte isotopenbasierte Strategie ist die CARD oder SIL-CARD (von engl. *Stable Isotope Labeling Comparative Analysis of RNA Digests*) Technik.[124] Die CARD/SIL-CARD Methode dient insbesondere zur gezielteren Analyse von Veränderungen im Modifikationsprofil der RNA. Eine Methode zur relativen Quantifizierung von rRNA-Modifikationen, basierend auf ^{15}N-Isotopenmarkierungen, wurde 2014 von Popova *et al*.[125] vorgestellt. Diese Methode ist ausschließlich auf RNA-Moleküle mit bekanntem Modifikationsprofil und bekannter Sequenz anwendbar und muss für jedes RNA-Molekül neu berechnet und programmiert werden, was die Anwendbarkeit der Methode sehr begrenzt.

Im Gegensatz zu den vorangegangenen Methoden, ermöglicht der Einsatz eines synthetischen[126] oder biosynthetisch hergestellten SILIS (engl. *Stable Isotope Labeled Internal Standard*)[127] neben der relativen Quantifizierung auch die absolute Quantifizierung von RNA-Modifikationen. Bei der Quantifizierung mit einem biosynthetisch hergestellten SILIS wurde von Kellner *et al*.[127] der Verdau einer isotopenmarkierten RNA, welche alle kanonischen Nukleoside aber auch alle natürlichen Modifikationen beinhaltet, 1:10 mit dem zu quantifizierenden RNA-Verdau gemischt und über eine DMRM (engl. *Dynamic Multiple Reaction Monitoring*) Methode in einem Triple Quadrupole Massenspektrometer analysiert (siehe **Abbildung 9**). Die Retentionszeiten, der aus dem IS (Interner Standard) stammenden Isotopologe, stimmen mit den Retentionszeiten der nicht isotopenmarkierten Nukleoside aus der Probe überein. Dies ist den gleichbleibenden physikochemischen Eigenschaften zu

verdanken. Es kann demnach für jedes Nukleosid, unabhängig ob die Struktur bekannt ist oder nicht, auf einen strukturgleichen Standard referenziert werden. Dadurch werden Einflüsse durch Ionensuppression oder durch strukturabhängige Ionisierungseigenschaften stets ausgeglichen. Eine Sättigung des Detektors wirkt sich durch die identische Retentionszeit gleichermaßen auf das IS-Nukleosid und auf das Probenmolekül aus. Die für die absolute Quantifizierung nötige externe Kalibrierungsgerade wird dadurch auch bei hohen Konzentrationen kaum in Ihrer Linearität beeinträchtigt.

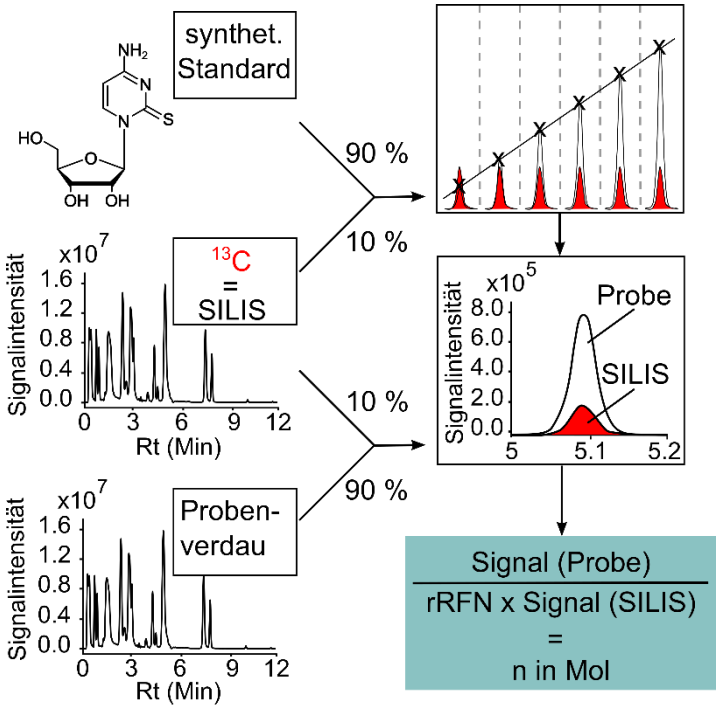


Abbildung 9: Schema zur Anwendung des SILIS. Das isotopenmarkierte Verdauprodukt wird zur Kalibrierung mit verschiedenen Konzentrationen des synthetischen Standards (hier am Beispiel s^2C) jeweils 1:10 verdünnt und gemessen. Derselbe Verdau wird dann 1:10 mit dem Probenverdau verdünnt und gemessen. Über das Verhältnis des Signals des synthetischen Standards zum SILIS Signal wird dann der relative Responsivitätsfaktor für Nukleoside (rRFN) berechnet. Dieser wird mit den MS-Signalen aus der Probenmessung verrechnet um auf die in der Probe vorhandene Stoffmenge der jeweiligen RNA-Modifikation schließen zu können.[127] Rt (Min) = Retentionszeit in Minuten.

In einer Veröffentlichung aus demselben Jahr beschrieben Kellner *et al.*[128] aus der Forschungsgruppe um Mark Helm eine Methode zur Identifizierung unbekannter RNA-

Modifikationen. In dieser Methode werden Kulturen des Bakteriums *E. coli* und des Hefestamms *Saccharomyces cerevisiae* unterschiedlich isotopenmarkiert. Die daraufhin isolierte RNA ist dann entweder gänzlich ^{15}N markiert, ^{13}C markiert oder enthält keine schweren Isotope. Die RNA aus den drei Kulturen wurde jeweils enzymatisch zu Nukleosiden verdaut und mit einer extra entwickelten *Neutral Loss Scan* (NLS) Methode gemessen. Dadurch geht zwar jede Sequenzinformation verloren, jedoch wird die Sensitivität der Messung durch die Minderung der Hintergrundsignale erhöht. Nukleosidstrukturen mit geringer Ionisierbarkeit oder geringer Konzentration in der Probe können dadurch besser erfasst werden. Die Signale des Totalionenstroms (TIC, von engl. *Total Ion Current*) der verschieden isotopenmarkierten Nukleoside wurden anhand der Retentionszeit verglichen. Über den Massenunterschied konnte für jedes, zu einem bestimmten Zeitpunkt eluierendes Nukleosid, die Anzahl der Stickstoff- und Kohlenstoffatome bestimmt werden. Derartige Informationen über die elementare Zusammensetzung wären ohne Isotopenmarkierung nur über die Messung der RNA im HRMS möglich, wodurch die erhöhte Sensitivität wieder eingebüßt werden würde. Über die angewendete Methode konnten letztlich insgesamt 52 Modifikationen den verschiedenen Signalen zugeordnet werden, wovon 10 Signale eine bisher unbekannte Struktur aufwiesen. Ein ähnliches Protokoll zur Messung von Nukleosiden wurde etwa zeitgleich in *Nature Protocols* von Dan Su *et al.*[116b] veröffentlicht.

2. Ziel der Arbeit

Eine Kombination der in 2014 entwickelten Methoden zur Nukleosid-Analyse[116b, 127-128] (siehe **Kapitel 1.4.3.**) führte zur Entwicklung einer neuen Strategie zur absoluten Quantifizierung von RNA-Modifikationen, NAIL-MS (*Nucleic Acid Isotope Labeling coupled Mass Spectrometry*). Diese innovative Technik ist für die Untersuchung verschiedenster RNA-Spezies geeignet und soll für unterschiedliche Strategien angepasst werden. Die vorliegende Arbeit wurde mit dem Ziel angefertigt, RNA-Modifikationen von Bakterien und kleinen eukaryotischen Organismen unter Anwendung verschiedener NAIL-MS Strategien auf unterschiedliche Fragestellungen hin zu untersuchen.

Ein Teil der Arbeit beschäftigt sich mit der Entdeckung und Charakterisierung neuartiger RNA-Modifikationen. Die Signale bisher unbekannter Nukleoside sollten über einen angepassten *Neutral Loss Scan* (NLS) unter Verwendung einer *Multiple Reaction Monitoring* (MRM) Methode für unterschiedlich isotopenmarkierte RNA-Isolate in einem definierten Messbereich identifiziert werden. Zudem sollten die zugehörigen Strukturen anhand der Fragmentierungen im Massenspektrometer und anhand der Herstellung eines synthetischen Standards bestätigt werden.

Eine Verfolgung der RNA-Modifikationen *in vivo* sollte nach Einführung eines auf NAIL-MS basierenden Pulsmarkierungs-Experiments für Hefezellen auch für bakterielle Untersuchungen ermöglicht werden. In diesem Zusammenhang wurden nach Methylierungsstress verschiedene Methylierungsprodukte in der *E. coli* RNA verfolgt. Durch den Einsatz bestimmter Knockout Mutanten sollten potentielle Reparaturenzyme auf bekannte und neue Substrate überprüft werden. Eine Strategie unter Verwendung von CD_3-*S*-Methionin zur Unterscheidung zwischen enzymatisch gewollten Methylierungen und direkten Methylierungen durch das Methylierungsreagenz MMS sollten außerdem Bestandteil dieser Arbeit sein.

Ein anderer Teil der Arbeit umfasst die Quantifizierung von RNA-Modifikationen in Nematoden-RNA und von Wirtsorganismen ausgeschiedener miRNA. Es sollte erstmals über den Vergleich der RNA-Modifikationsprofile ein potentieller Biomarker für die diagnostische Verfolgung der Infektionskrankheit Onchozerkose ermittelt werden. Die RNA-Modifikationen sollen dabei über LC-MS/MS basierte Methoden quantitativ analysiert werden.

3. Ergebnisse und Diskussion

3.1. Methodik zur Identifizierung unbekannter RNA Modifikationen

Im Folgenden wird beschrieben, wie aus dem RNA Gemisch einer Zelle die verschiedenen RNA Typen isoliert werden konnten, welche Techniken dafür verwendet wurden und schließlich wie aus den Enzym-verdauten RNA Fraktionen mit Hilfe von Isotopen Markierung auf die Summenformeln unbekannter Nukleoside geschlossen werden konnte.

Um RNA-Modifikationen genauer untersuchen zu können, ist es wichtig die RNA Stränge zuvor nach Länge und Typ zu trennen. Für die Untersuchung der totalen RNA des pathogenen Organismus *P. aeruginosa PA14* wurde die bereits isolierte RNA über Größenausschlusschromatographie (SEC, von *engl. Size Exclusion Chromatography*) aufgereinigt. Wie der Name bereits induziert, wird bei der SEC nach Größe aufgetrennt, was dazu führt, dass größere Moleküle schneller eluieren als kleinere. Kleinere Moleküle durchlaufen die Säulenmatrix langsamer, da sie durch viele kleine Poren diffundieren müssen. Dadurch wird der Weg der kleinen Moleküle künstlich verlängert. Große Moleküle diffundieren an den Porengängen vorbei und gelangen somit direkter zum Säulenende. In der hier verwendeten Trennmethode wurde eine SEC Säule mit einer Porengröße von 300 Å und eine Flussrate von 1 mL pro Min. verwendet. Die Eluate der entsprechenden Fraktionen wurden über einen Fraktionssammler zeitgesteuert gesammelt (siehe Chromatogramm **Abbildung 10**). Alle aufgefangenen RNA Fraktionen wurden im Anschluss separat zu Nukleosiden verdaut und über eine gestaffelte MRM Methode (von *engl. Multiple Reaction Monitoring*) im Triple Quadrupole Massenspektrometer (QQQ) gemessen und später analysiert.

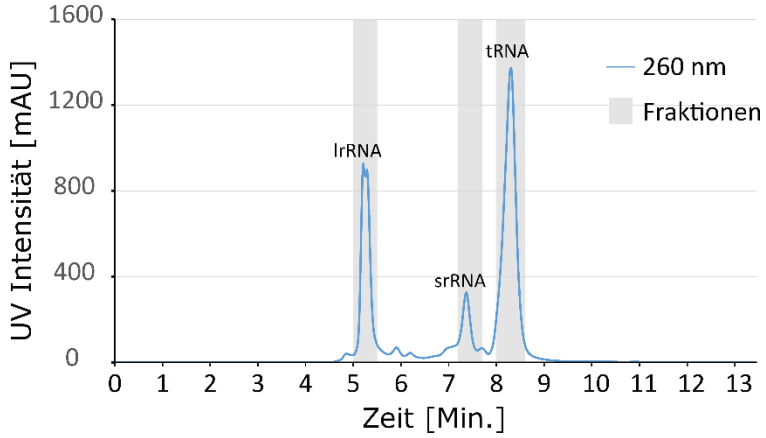


Abbildung 10: SEC-Chromatogramm nach Aufreinigung der totalen RNA von *P. aeruginosa PA14*. Die Signalspur wurde bei 260 nm im DAD gemessen (blau). Über einen Fraktionssammler wurden zeitgesteuert die Fraktionen gesammelt (grauer Bereich). lrRNA = *large ribosomal RNA*, srRNA = *small ribosomal RNA*.

Die angewandte LC-MS/MS Technik mit zusätzlicher MRM Funktion ermöglicht es mehrere verschiedene Massenübergänge in sehr kurzer Zeit hintereinander zu messen. Für die Entdeckung neuer RNA-Modifikationen wurden alle Massen von 240 Da bis 420 Da mit einem jeweiligen Massenübergang von -132 Da gemessen (Schema **Abbildung 11**). Es kann vermutet werden, dass sich in den zu Nukleosiden verdauten RNA Fraktionen neben den eigentlichen Nukleosiden noch eine Vielzahl weiterer Moleküle befinden, welche eine Molekularmasse von im oben genannten Detektionsbereich aufweisen. Um die Nukleosid-Strukturen herausfiltern zu können, bedarf es einem für Nukleoside spezifischen Massenübergang. Dieser wurde bei -132 Da festgelegt, was exakt dem Verlust der Ribose entspricht. Die Fragmentierungen in der Kollisionszelle finden demnach meist an der N-glykosidischen Bindung der Nukleoside statt. Dadurch werden die unspezifischen Hintergrundsignale minimiert, was wiederum zu einer enormen Erhöhung der Sensitivität im Massenspektrometer führt.

Des Weiteren ist die Sensitivität von der in der Methode verwendeten Zyklen-Zeit (*engl. Cycle time*) abhängig. Die Zyklen-Zeit entspricht der Zeitspanne, welche das Massenspektrometer benötigt, um jeden eingestellten Massenübergang einmal zu messen. Bei der in dieser Methode verwendeten Zyklen-Zeit von 0,81 Sek. und einer Anzahl von 60 Massenübergängen lässt sich eine Verweilzeit (engl. *dwell time*) von 13,5 ms pro Massenübergang berechnen. Würde man für ein bestimmtes Nukleosid annehmen, dass es 8 Sek. lang von der HPLC Säule eluiert, so könnten für dieses

Signal knapp 10 Datenpunkte gemessen werden. Um ein Signal quantifizieren zu können sind aber >20 Datenpunkte erforderlich[129], weshalb die gemessenen Daten ausschließlich qualitativ ausgewertet wurden.

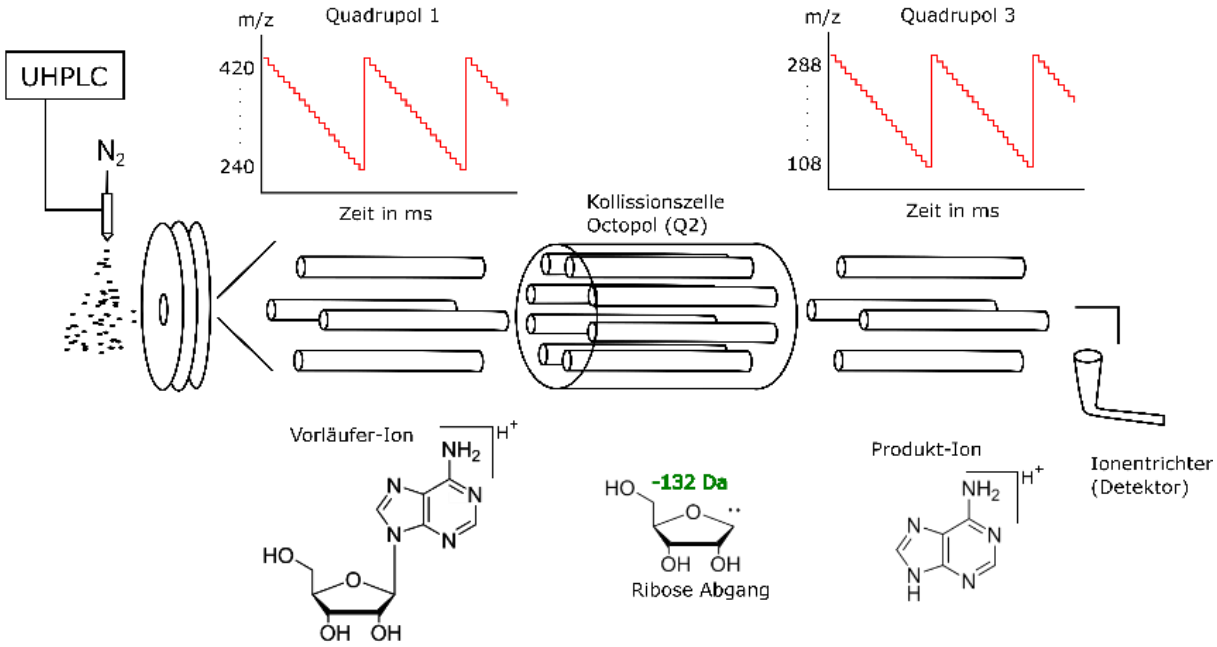


Abbildung 11: Skizze der gestaffelten MRM Methode und eines QQQ Massenspektrometers. Die Graphen oberhalb der einzelnen Kompartimente stellen schematisch die zur Ionen-Filterung nötigen Spannungsänderungen in den Quadrupolen dar. Unterhalb sind die Strukturen der in den Quadrupolen isolierten Moleküle am Beispiel von Adenosin dargestellt. Im Falle der Kollisionszelle ist stattdessen der abgetrennte Ribose-Rest abgebildet.

In **Anhang Tabelle 1** finden sich alle identifizierten Signale der RNA Fraktionen mit Retentionszeit und Massenübergang. Diese große Anzahl an Signalen enthält trotz Vorfilterung einen nicht unerheblichen Anteil an falsch-positiven Nukleosid Signalen. Um das Risiko von Artefakt-Signalen zu minimieren wurde deshalb die RNA von *P. aeruginosa* Zellen mit ^{13}C-Kohlenstoff, ^{15}N Stickstoff oder ^{34}S Schwefel Isotopen markiert, wie zuvor die unmarkierte RNA über SEC aufgetrennt und dann mit der gestaffelten MRM Methode massenspektrometrisch gemessen. Die Datensätze wurden der Reihe nach fraktionsgeordnet (tRNA, srRNA, lrRNA) ausgewertet. Dies bedeutet, dass beispielsweise erst alle tRNA Signale der ^{13}C markierten RNA analysiert und mit den Signalen von **Anhang Tabelle 1** verglichen wurden. Ausschließlich die Signale, welche einen realistischen Massen-Unterschied zwischen der nicht Isotopen markierten und der ^{13}C markierten Probe aufwiesen und sich im MRM-Chromatogramm exakt übereinander legen ließen, wurden weiterhin auf der

Favoriten-Liste geführt. Im nächsten Schritt wurden dann die Signale der ^{15}N markierten tRNA analysiert und diese mit den verbliebenen Signalen der Favoriten-Liste abgeglichen. Die wiederum verkürzte Liste wurde dann schließlich noch mit den tRNA-Signalen der ^{34}S markierten tRNA abgeglichen. Das Verfahren zur Datenauswertung wurde gleichermaßen auf die beiden rRNA Fraktionen angewandt.

Von den übrig gebliebenen Signalen konnte anhand der Massenunterschiede zwischen den Isotopologen auf die Anzahl der Kohlenstoffe, Stickstoffe und Schwefel Atome der zum Signal gehörenden Summenformel geschlossen werden. Diese Strukturrelevanten Informationen wurden im Folgenden mit der MODOMICS[5] Datenbank abgeglichen, wodurch die in **Tabelle 2** aufgeführten bereits bekannten RNA-Modifikationen den einzelnen Signalen zugewiesen werden konnten.

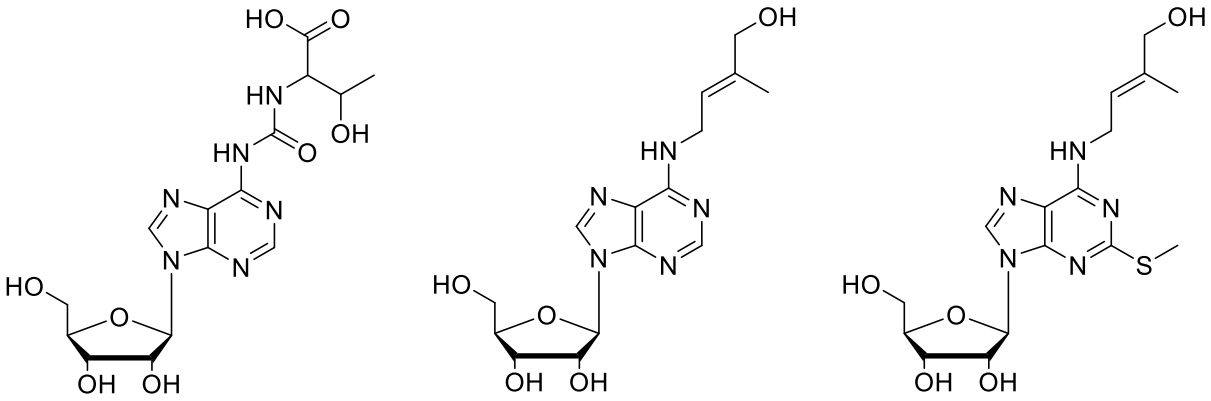
Unerwarteter Weise wurden selbst nach zweifacher Aufreinigung (Chromatogramme siehe **Anhang Abbildung 1**) der RNA Fraktionen über SEC bestimmte RNA Modifikationen in RNA Fraktionen identifiziert, welche bis zum heutigen Tage ausschließlich in anderen Fraktionen bekannt waren.

Tabelle 2: Liste der in den einzelnen RNA Fraktionen identifizierter Nukleoside oder deren Modifikationen. In Rot hervorgehoben sind die RNA-Modifikationen, die in der eingeteilten Fraktion bisher noch nicht beschrieben wurden (MODOMICS, Stand 2020)[5]. Abkürzungen: PA lrRNA = *P. aeruginosa large ribosomal RNA*; PA srRNA = *P. aeruginosa small ribosomal RNA*; PA tRNA = *P. aeruginosa transfer RNA*; *Kein synthetischer Standard zur Bestätigung der Retentionszeit verfügbar.

PA lrRNA	PA srRNA	PA tRNA
C	C	C
U	U	U
m^5C	m^5C	m^5C
m^5U	m^5U	m^5U
m^3U	-	m^3U
A	A	A
m^2A	m^2A	m^2A
m^6A	m^6A	m^6A
G	G	G
m^7G	m^7G	m^7G
m^1G	m^1G	m^1G
-	-	i^6A
io^6A*	io^6A*	io^6A*
t^6A	t^6A	t^6A
m^2G	m^2G	-
-	-	s^2C
-	-	s^4U
-	I	I
-	-	ms^2A

-	-	mnm^5s^2U
-	acp^3U	acp^3U
-	-	k^2C*
-	-	ct^6A*
-	ms^2io^6A*	ms^2io^6A*

Sehr außergewöhnlich war die Tatsache, dass Signale, die ausschließlich den in tRNA bekannten Modifikationen t^6A, io^6A und ms^2io^6A zuzuordnen waren (Strukturformeln siehe **Abbildung 12**), mit absteigenden Intensitäten in tRNA, srRNA und lrRNA detektiert werden konnten (siehe auch **Anhang Tabelle 1**). Als Erklärung hierfür könnte in Frage kommen, dass die strukturell verwandten Nukleoside extrem gut ionisieren und deshalb als kleinste Verunreinigungen detektierbar sind. Für t^6A beispielsweise konnte ein LOD (engl. *Limit of Detection*) von weniger als 10 amol bestätigt werden (**Anhang Abbildung 2**), was vergleichsweise sehr gering ist (Vgl. Cytidine, 25 fmol). Anhand fehlender synthetischer Standards konnte weder für io^6A noch für ms^2io^6A bestätigt werden, ob es sich wirklich um die vermuteten Modifikationen handelt, noch ob diese in besonders geringen Mengen detektierbar sind.



t^6A io^6A ms^2io^6A

Abbildung 12: Strukturformeln der Adenosin-basierten und an Position 6 modifizierten Modifikation t^6A, io^6A und ms^2io^6A. Die zu diesen Strukturen gehörenden Signale sind in fast jeder Fraktion zu finden.

Im Falle von Inosin (Kürzel: I) ist interessant zu sehen, dass es nicht nur in tRNA zu finden ist sondern auch in der lrRNA Fraktion, welche vermutlich mitunter auch mRNA Verunreinigungen mit ähnlicher Größe beinhaltet. Erst 2018 wurde erstmals von Bar-

Yaacov *et al*.[130] A zu I Editierung in bakterieller mRNA beschrieben, was womöglich darauf schließen lässt, dass Inosin nicht zwangsläufig in der rRNA vorkommt, dafür aber in ausreichenden Mengen in der mRNA, verursacht durch A zu I Editierung.

Nach erfolgreicher Feststellung, dass die Methode zur Identifizierung modifizierter Nukleoside geeignet ist, wendet sich dieser Abschnitt im weiteren Verlauf den Signalen zu, welche potentielle neue Nukleosid Strukturen beinhalten könnten. In **Tabelle 3** sind alle Signale aufgelistet, die nach Messung der isotopenmarkierten RNA zur weiteren Charakterisierung noch interessant geblieben sind. Signale wurden erst ab einer Intensität von $>10^3$ gelistet. Signale mit niedrigerer Intensität hoben sich zu wenig vom Hintergrundrauschen ab oder zeigten nur unförmige Signale. Aus **Tabelle 3** ist außerdem herauszulesen in welchen RNA Fraktionen die jeweiligen Signale detektiert werden konnten. Die Signale wurden anhand von Form und Symmetrie, sowie anhand der Vergleichbarkeit über die unterschiedlich isotopenmarkierten Proben hinweg bewertet und in unterschiedliche Klassen eingeteilt. Signale mit einem Kreis (o) oder gar einem Minus (-) zeigen entweder sehr unterschiedliche Signalstärken oder kleine aber auffällige Retentionszeit-Verschiebungen über die verschiedenen Isotopologe hinweg auf. Sie zeigen oftmals keine scharfen, symmetrischen sondern stattdessen breite, zackige Signale. Sind die Signale in allen drei RNA Fraktionen zu sehen und zeigen dabei hohe, ähnlich starke Signalintensitäten sind dies wahrscheinliche Anzeichen für ein systematisch auftretendes Artefakt Signal.

Die interessantesten Signale sind mit einem Plus (+) gekennzeichnet. Kommen diese zusätzlich nur in einer RNA Fraktion vor und zeigen ähnlich hohe Signalintensitäten über die verschiedenen Isotopologe hinweg auf sind dies höchstinteressante Moleküle, die es weiter zu untersuchen gilt. Im Übrigen wurden alle in **Tabelle 3** gelisteten Signale auch in vergleichbarer Intensität im Bakterium *Escherichia coli* nachgewiesen.

Tabelle 3: LC-MS/MS Signale, die in allen drei Isotopen markierten und in der nicht Isotopen markierten RNA gefunden wurden. Neben Retentionszeit (Rt.) sind der zum Signal gehörende Massenübergang (MS^1 = Vorläufer-Ion, MS^2 = Produkt-Ion), sowie Anzahl der jeweiligen Isotopen und Zugehörigkeit der RNA Spezies aufgelistet. Plus (+) = Symmetrische und deutliche Signale mit vergleichbarer Intensität (Isotopologen übergreifend). Kreis (o) = mindere Signalqualität, große Intensitätsunterschiede zwischen den Isotopologen, z.T. Retentionszeit leicht verschoben. Minus (-): Signale klein und unsymmetrisch, Unsicherheit bezüglich der Zusammengehörigkeit der überlagerten Signale.

Rt. [min.]	m/z MS^1	m/z MS^2	^{13}C	^{15}N	^{34}S	lrRNA	srRNA	tRNA
5.32	272	140	9	3	0	+	+	+
8.31	273	141	9	2	0		o	
5.53	274	142	10	3	1			+
10.57	279	147	9	2	1	-		
3.59	296	164	11	5	0			o
12.05	296	164	12	5	0	+	+	+
4.02	299	167	11	6	0	-	-	-
5.10	299	167	11	6	0			-
3.75	306	174	10	5	0		+	-
1.74	312	180	9	3	0		o	
4.57	312	180	10	5	0			o
3.30	312	180	10	5	0	+	-	+
5.55	313	181	12	6	0	+		
4.21	316	184	11	3	0		+	o
2.00	318	186	9	3	0		-	-
4.04	319	187	9	2	0		+	o
9.07	322	190	10	5	0	+	o	+
1.74	326	194	9	3	0	o	o	o
6.68	330	198	11	3	0			+
3.08	338	206	10	5	0	o	+	o
6.79	342	210	14	5	0		+	+
1.84	343	211	9/13	2	0	o	o	-
1.85	343	211	9	2	2	o	o	o
10.71	349	217	9	2	1			+
5.76	350	218	10	5	0	-	-	-
8.34	354	222	10	5	0	o	-	-
5.26	356	224	10	5	0	o	+	o
10.99	356	224	10	5	0	-	+	-
3.69	366	234	10	5	0	-	o	-
3.94	372	240	9	3	0	+	o	
5.65	372	240	9	3	0		o	o
2.23	374	242	9/11	5	1			-

1.74	377	245	13	6	0	o	o	o
5.50	380	248	11	5	0	+		
1.74	381	249	15/20	2	1/0	-	-/o	-
5.78	382	250	10	5	0	-	-	-
7.22	394	262	9	3	1			o
8.19	395	263	12	6	0			-
3.81	398	266	10	5	0	-	o	-
4.24	408	276	17	5	0			+
5.50	412	280	11	5	0	+		
5.13	412	280	11	5	0			+
5.46	413	281	15	6	0			+

Im Folgenden sollen zwei Signale, welche ausschließlich in tRNA zu finden waren, herausgegriffen werden, um deren mögliche Strukturen näher zu bestimmen, beziehungsweise Anhaltspunkte für bestimmte Struktureigenschaften zu finden.

Für den Massenübergang von m/z 408→276, gefunden bei 4,24 Min. und einem m/z Unterschied von +17/+5/+0 zwischen nicht Isotopen markierter und $^{13}C/^{15}N/^{34}S$ markierter tRNA, lässt sich eine Summenformel herleiten, die aus 17 Kohlenstoffatomen, 5 Stickstoffatomen und keinem Schwefelatom besteht (Signale siehe **Anhang Abbildung 3**). Zudem müssen Sauerstoff Atome und Wasserstoff Atome dazu gerechnet werden um auf die Molekülmasse von 407 Da zu gelangen. Sind keine weiteren Elemente in der Molekülstruktur vorhanden, so kann folgende Summenformel für das neuartige Nukleosid bestimmt werden:

$$\mathbf{C_{17}H_{21}N_5O_7}$$

Die Anzahl an Sauerstoffen und Wasserstoffen wurde in einem logischen Verhältnis so berechnet, dass daraus eine wahrscheinliche nukleosidische Molekülstruktur abgeleitet werden konnte. Vergleicht man des Weiteren die Summenformel und die Molekülmasse mit den bereits bekannten Modifikationen aus der Datenbank[5], so fällt auf, dass in unmittelbarer Nähe der neuen Modifikation bereits die Struktur der RNA Modifikation Queuosin (M=409,16 g/mol, $C_{17}H_{23}N_5O_7$) zu finden ist. Außerdem beträgt die Retentionszeit für Q unter den gleichen Bedingungen 4,10 Min., eine Differenz von 0.12 Min. zum zu identifizierenden Molekülsignal. Bekanntlich fragmentiert Queuosin wie die meisten anderen RNA-Modifikationen an der N-glykosidischen Bindung zwischen Ribose und Base, jedoch zeigen Queuosin und die mit Queuosin verwandten

Moleküle eine weitere, mit der ersteren Fragmentierung konkurrierende Fragmentierung auf, welche an der Amin-Bindung zum Cyclopenten-2,3-diol Rest stattfindet. Zur Überprüfung der strukturellen Verwandtschaft zu Queuosin wurden bei einer nachträglichen Messung der tRNA Fraktion die angepassten Massenübergänge für die alternative Fragmentierung mit in die Methode aufgenommen (m/z 408 → 163 falls die Ribose mit abgeht und 408 → 295 falls die Ribose am Ion gebunden bleibt). Die tRNA-Fraktionen des ^{15}N und des ^{13}C isotopenmarkierten RNA-Isolats wurden mit den jeweils angepassten Massenübergängen ebenfalls auf die entsprechenden Fragmente hin überprüft. Das Resultat der Messung ist in **Abbildung 13a** dargestellt und zeigt ein vergleichsweise schwaches Signal für beide Fragmentierungen zur angegebenen Retentionszeit. Dennoch wurden die Signale in allen unterschiedlich isotopenmarkierten RNA-Isolaten detektiert und sie heben sich deutlich vom Hintergrundrauschen ab. Wird anhand der vorliegenden Daten davon ausgegangen, dass es sich bei dem unbekannten Signal um eine oxidierte Form der Queuosin Base handelt, so ist es wahrscheinlich, dass eine mögliche Oxidation an einer der beiden Hydroxygruppen stattfindet. In **Abbildung 13b** sind neben der Queuosin-Struktur insgesamt acht Strukturen abgebildet, wovon **Derivat 1** oder **Derivat 2** vermutlich am wahrscheinlichsten gebildet werden.

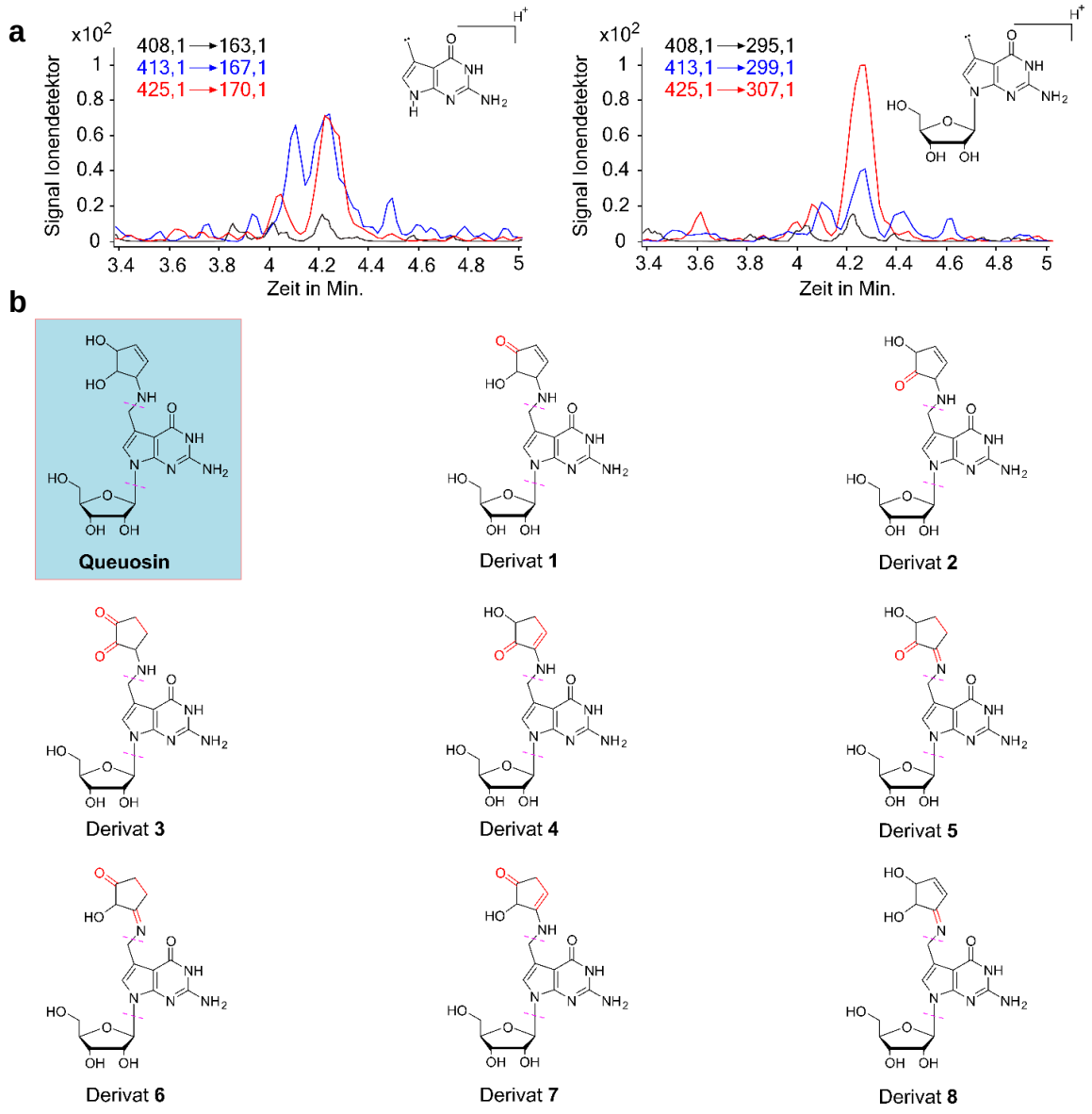


Abbildung 13: Fragmentierung der potentiellen Queuosin-Derivate. **a** Chromatogramm mit überlagerten Signalen aus den unterschiedlich isotopenmarkierten tRNA-Fraktionen beider konkurrierender Produkt-Ionen sowie deren Massenübergänge (schwarz = nicht isotopenmarkiert, rot = ^{13}C-isotopenmarkiert, blau = ^{15}N-isotopenmarkiert). **b** Originalstruktur von Queuosin und mögliche Strukturformeln des gesuchten Queuosin-Derivats. Die von der Originalstruktur abweichenden Bereiche sind rot gekennzeichnet.

Betrachtet man die vollständig aufgeklärte Biosynthese des Queuosins[131] (**Abbildung 14**) so wird ersichtlich, dass die oxidierte Form vermutlich nicht während der Biosynthese auftritt. Vielmehr ist es wahrscheinlich, dass Queuosin entweder enzymatisch oder durch eine ungewollte Oxidation, durch oxidativen Stress verursacht, zu **Derivat 1** oder **2** reagiert.

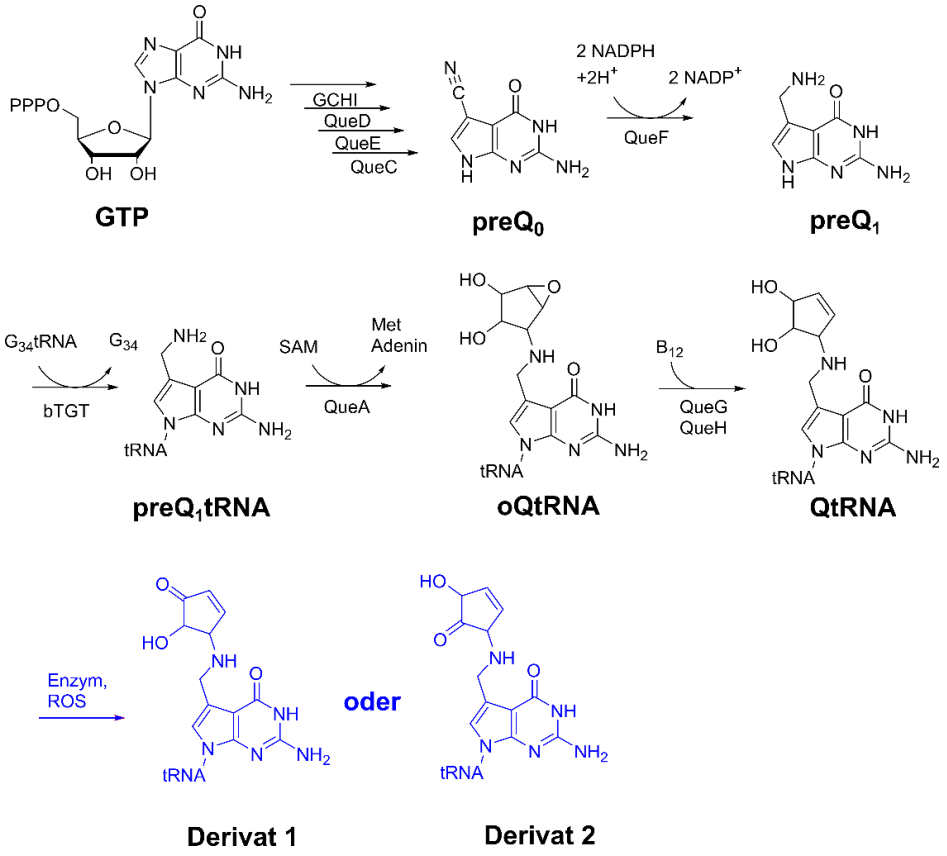


Abbildung 14: Biosynthetischer Aufbau von Queuosin aus GTP. In Blau markiert sind die potenziellen erweiterten oder geschädigten Strukturen dargestellt. Abkürzungen: GCH1 = GTP-Cyclohydrolase 1; bTGT = tRNA-Guanin Transglykosylase. Biosyntheseweg adaptiert von Fergus *et al*.[132]

Für weitere Analysen und zur Überprüfung wäre eine Synthese der vorgeschlagenen Strukturen, sowie eine MS^3 Analyse des Isolats, verglichen mit dem Fragmentierungsmuster des synthetischen Standards, sinnvoll. Des Weiteren wäre interessant zu sehen, ob die Modifikation auch in einem Queuosin Knockout Stamm vorkommt. Stress-Studien unter Anwendung von oxidativem Stress könnten die Frage klären, ob es sich bei dem identifizierten Signal um eine stressinduzierte Modifikation handelt oder ob die Modifikation davon unbeeinflusst bleibt.

Das zweite unbekannte Signal, das in dieser Arbeit herausgegriffen werden soll wurde bei einem Massenübergang von m/z 274→142 und in der angewendeten LC-MS/MS Methode bei einer Retentionszeit von 5,53 Min. gefunden. Die Isotopomere zeigen Signale bei m/z 274 +10/+3/+2 für die $^{13}C/^{15}N/^{34}S$ markierten tRNA Fraktionen. Alle weiteren Untersuchungen und Experimente zur vollständigen Charakterisierung dieses Signals werden im nachfolgenden Kapitel 3.2. und den beinhaltenden Publikationen abgehandelt.

3.2. Charakterisierung neuartiger tRNA-Modifikation in *P. aeruginosa* und *E. coli*

Prolog

Das bei m/z = 275 entdeckte Signal konnte durch Synthese und massenspektrometrische Analyse als 2-Methylthiocytidin (ms^2C) identifiziert werden. Über *in vivo* Experimente mit *E. coli* und unter Anwendung unserer innovativen NAIL-MS Strategien konnte eine verstärkte Bildung der neuartigen Struktur durch direkte Methylierung der RNA-Modifikation 2-Thiocytidin (s^2C) während Stress-Studien mit Methylmethansulfonat (MMS) beobachtet werden. Die Quantifizierung von ms^2C nach Stresszugabe ergab, dass ms^2C einer der am häufigsten auftretenden bis dato bekannten Methylierungsschäden in bakterieller tRNA ist. Unter Anwendung einer neuen NAIL-MS Pulse Chase Strategie konnte ein sehr schneller Abbau von ms^2C in den Zellen beobachtet werden, welcher hauptsächlich auf die oxidative Demethylase AlkB zurückzuführen ist. Unsere NAIL-MS Methode deckte außerdem einen alternativen Reparaturmechanismus auf. Durch Einführung eines sfGFP Reportergens in *E. coli* und Fluoreszenzmessungen konnte zudem eine verminderte Translationsrate beobachtet werden, welche vermutlich auf die Schädigung der tRNA-Struktur durch ms^2C zurückzuführen ist.

„NAIL-MS reveals the repair of 2-methylthiocytidine by AlkB in *E. coli* “, **Valentin F. Reichle**, Dimitar P. Petrov, Verena Weber, Kirsten Jung, Stefanie Kellner, *Nat. Commun*. **2019**, 10, 5600.

Autorenbeitrag

Alle LC-MS/MS basierten Messungen, die Synthese von ms^2C und alle NAIL-MS basierten Experimente wurden von mir durchgeführt. In enger Zusammenarbeit mit *Verena Weber* wurden die Knockout-Screenings und das Screening der Überexpressionsstämme sowie die *in vitro* Studien durchgeführt. Für die Planung und Durchführung des Fluoreszenz-Reporterassays zur Messung der Translationseffizienz waren *Dimitar P. Petrov* und *Kirsten Jung* verantwortlich. Das Manuskript wurde in Gemeinschaftsarbeit von *Stefanie Kellner*, *Kirsten Jung* und mir angefertigt.

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NAIL-MS reveals the repair of 2-methylthiocytidine by AlkB in *E. coli*

Valentin F. Reichle[1], Dimitar P. Petrov[2], Verena Weber[1], Kirsten Jung[2] & Stefanie Kellner[1*]

RNAs contain post-transcriptional modifications, which fulfill a variety of functions in translation, secondary structure stabilization and cellular stress survival. Here, 2-methylthiocytidine (ms^2C) is identified in tRNA of *E. coli* and *P. aeruginosa* using NAIL-MS (nucleic acid isotope labeling coupled mass spectrometry) in combination with genetic screening experiments. ms^2C is only found in 2-thiocytidine (s^2C) containing tRNAs, namely $tRNA^{Arg}_{CCG}$, $tRNA^{Arg}_{ICG}$, $tRNA^{Arg}_{UCU}$ and $tRNA^{Ser}_{GCU}$ at low abundances. ms^2C is not formed by commonly known tRNA methyltransferases. Instead, we observe its formation in vitro and in vivo during exposure to methylating agents. More than half of the s^2C containing tRNA can be methylated to carry ms^2C. With a pulse-chase NAIL-MS experiment, the repair mechanism by AlkB dependent sulfur demethylation is demonstrated in vivo. Overall, we describe ms^2C as a bacterial tRNA modification and damage product. Its repair by AlkB and other pathways is demonstrated in vivo by our powerful NAIL-MS approach.

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Each Nucleic acid is composed of four canonical nucleobases, which are connected through ribose (RNA) or 2′-O-deoxyribose (DNA) and phosphate. In addition, both DNA and RNA are target of modifying enzymes which introduce methylations or other functionalities. To this day, over 160 modified nucleosides have been identified in RNA from organisms in all domains of life[1].

Due to the nucleophilic character of the canonical nucleobases, both RNA and DNA are prone to direct methylation. Inside cells these electrophiles comprise molecules such as S-adenosylmethionine (SAM), which is the common methyl-donor for enzymatic methylation reactions but it is also known to directly alkylate nucleic acids[2]. In addition, bacteria use various electrophiles as chemical warheads (e.g., streptozotocin, azaserine or methylenchloride) to harm other bacteria by methylation and alkylation of their macromolecules[3–6]. Thus, bacteria are constantly forced to overcome these alkylating reagents and various adaptive mechanisms can be found. To study the impact of direct methylation, electrophiles such as methyl-methanesulfonate (MMS) or dimethyl sulfate (DMS) are used. Methylation damage of both RNA and DNA is repaired by direct demethylation through the alpha-ketoglutarate dependent dioxygenase AlkB in bacteria[7,8]. So far the methylated aromatic nitrogens of 3-methylcytidine (m^3C), 1-methyladenosine (m^1A) and 1-methylguanosine (m^1G) are reported substrates of AlkB. In addition, the exocyclic amines of N2,N2-dimethylguanosine ($m^{2,2}G$) can be demethylated by AlkB in vitro, which finds application in the RNA modification sequencing technique ARM-Seq[9]. So far, no non-nitrogen methylations have been described as substrates of AlkB in RNA.

In addition to the nucleobase methylations introduced by direct alkylation, cells in all domains of life use SAM for the enzymatic methylation of RNA. The occurrence of these natural methylated nucleosides makes the detection of damage-derived methylated nucleosides challenging. With the application of CD_3-labeled methionine in a nucleic acid isotope labeling coupled mass spectrometry (NAIL-MS) assay, this discrimination can be achieved and, e.g., enzymatic 7-methylguanosine (m^7G) and MMS-damage derived m^7G can be quantified independently[10]. With NAIL-MS, the analysis of alkylation damage of canonical nucleosides was possible in the presence of natural methylated nucleosides. A key finding in this study was that m^7G damage is as common as m^1A, which was believed to be the only dominant RNA damage product upon direct methylation in vivo[11]. In addition, NAIL-MS was used in a pulse-chase setup to follow the in vivo demethylation kinetics of m^1A and m^3C during MMS recovery[10]. Especially m^3C is quickly and efficiently (~80%) repaired within nine hours of recovery whereas only 20% of m^1A damage sites are repaired within the same timeframe[12]. For m^3C, the demethylation by AlkB is reported in vitro, but so far not in vivo[13].

While methylation is a major modification of RNA, especially tRNA contains additional and more complex modifications. One group of unique tRNA modifications is the enzymatic thiolation. For example, bacteria thiolate cytidine at position 32 of the tRNA to form 2-thiocytidine (s^2C). The thiolated nucleosides at position 32 and 37 of tRNA flank the anticodon (position 34–36) allowing for a more flexible anticodon loop and thus improve the binding of the codon during translation. s^2C is incorporated into bacterial $tRNA^{Arg}_{CCG}$, $tRNA^{Arg}_{ICG}$, $tRNA^{Arg}_{UCU}$ and $tRNA^{Ser}_{GCU}$ by the thiotransferase TtcA at position 32. Its effects on tRNA structure and bacterial translation has been addressed in several studies[14,15].

Here, through the use of genetic and analytical tools, we identify and confirm the structure of 2-methylthiocytidine as an endogenous modification in bacterial tRNA. In accordance with the nomenclature provided by the RNA community[1] it is abbreviated as ms^2C. The new modification is present in the same tRNA isoacceptors as its precursor modification s^2C, but only 1% of all s^2C modified tRNAs are methylated to ms^2C. Due to the low abundance of ms^2C in vivo and its formation by SAM in vitro, ms^2C might present an endogenous tRNA lesion. During exposure of bacteria to the natural antibiotic streptozotocin and the alkylating reagent MMS, up to 50 % of the s^2C modified tRNAs become methylated and ms^2C is formed. Thus, ms^2C is an additional damage product of bacterial RNA and of comparable abundance to the main methylation products m^1A and m^7G in tRNA[10,11]. With our unique NAIL-MS approach, we observe a fast and efficient repair of ms^2C and regeneration of s^2C in the damaged tRNAs. With NAIL-MS, we identify high demethylation activity of AlkB towards ms^2C and m^3C, and only slow demethylation of the described substrate m^1A. In addition, we observe s^2C from rethiolated cytidine after dethiomethylation by an AlkB independent mechanism. Overall, NAIL-MS is a tool which is useful for the identification of nucleoside structures and allows deeper insights into RNA demethylation mechanisms in vivo.

Results

2-methylthiocytidine is a bacterial tRNA modification. With the goal of discovering novel modified nucleosides in bacterial tRNA, *Escherichia coli* and *Pseudomonas aeruginosa* were grown in differentially stable isotope labeled growth media as recently reported[16,17]. The total tRNA were purified and analyzed by mass spectrometry. After data evaluation we found a promising candidate eluting at 5.2 min with an *m/z* of 274 from unlabeled tRNA, an *m/z* of 284 from [^{13}C]-labeled tRNA, an *m/z* of 277 from [^{15}N]-labeled tRNA and an *m/z* of 276 from [^{34}S]-labeled tRNA (Fig. 1a, Supplementary Table 1). The sum formula of the nucleobase is $C_5H_xN_3O_yS_1$ (Supplementary Fig. 1) and the nucleoside is thus $C_{10}H_XN_3O_XS_1$. Through the addition of the heavy labeled amino acid CD_3-methionine to the growth medium, we predicted the presence of a single methyl-group in the nucleoside candidate. Aiming at clarification of the analyte's structure, we screened *E. coli* knockouts for its absence. TtcA is responsible for 2-thiolation of cytidine and in the Δ*ttcA* strain, the *m/z* 274 signal is lost alongside 2-thiocytidine (s^2C) (Supplementary Fig. 2a, Supplementary Tables 2 and 3)[18]. The mass difference of the novel nucleoside (*m/z* 274) and s^2C (*m/z* 260) is 14 Dalton, which is a strong indicator of methylation of s^2C.

Due to the high nucleophilicity of thiols, we predicted that both the chemical synthesis and biosynthesis of 2-methylthiocytidine (ms^2C) is feasible, and thus the methylation reaction at position 2 of s^2C was performed. Interestingly, the synthetic route was already described in 2010, when the Suzuki lab[19] used 2-methylthiocytidine as an intermediate of agmatidine synthesis. We followed their synthetic route under milder conditions and received pure ms^2C in 28% yield (Supplementary Figs. 2b and 3). We mixed the synthesized ms^2C with the heavy isotope labeled tRNA digests and observed perfect co-elution for the synthesized compound and its native isotopologues (Fig. 1a). Additional high resolution MS, MS^2 and MS^3 spectra of the synthesized product and of native tRNA confirmed the structure of the novel nucleoside to be ms^2C in *E. coli* (Fig. 1b) and *P. aeruginosa* (Supplementary Fig. 4).

With the synthetic standard of ms^2C and an internal standard produced by metabolic isotope labeling[20], we could now quantify the absolute abundance of ms^2C in specific tRNAs from the unstressed *E. coli* BW25113 wild-type strain (WT). The precursor of ms^2C, s^2C is reported in three of the five $tRNA^{Arg}$ isoacceptors and one of the five $tRNA^{Ser}$ isoacceptors[1,14,21]. Therefore, we designed DNA probes (Supplementary Table 4) against tRNAs

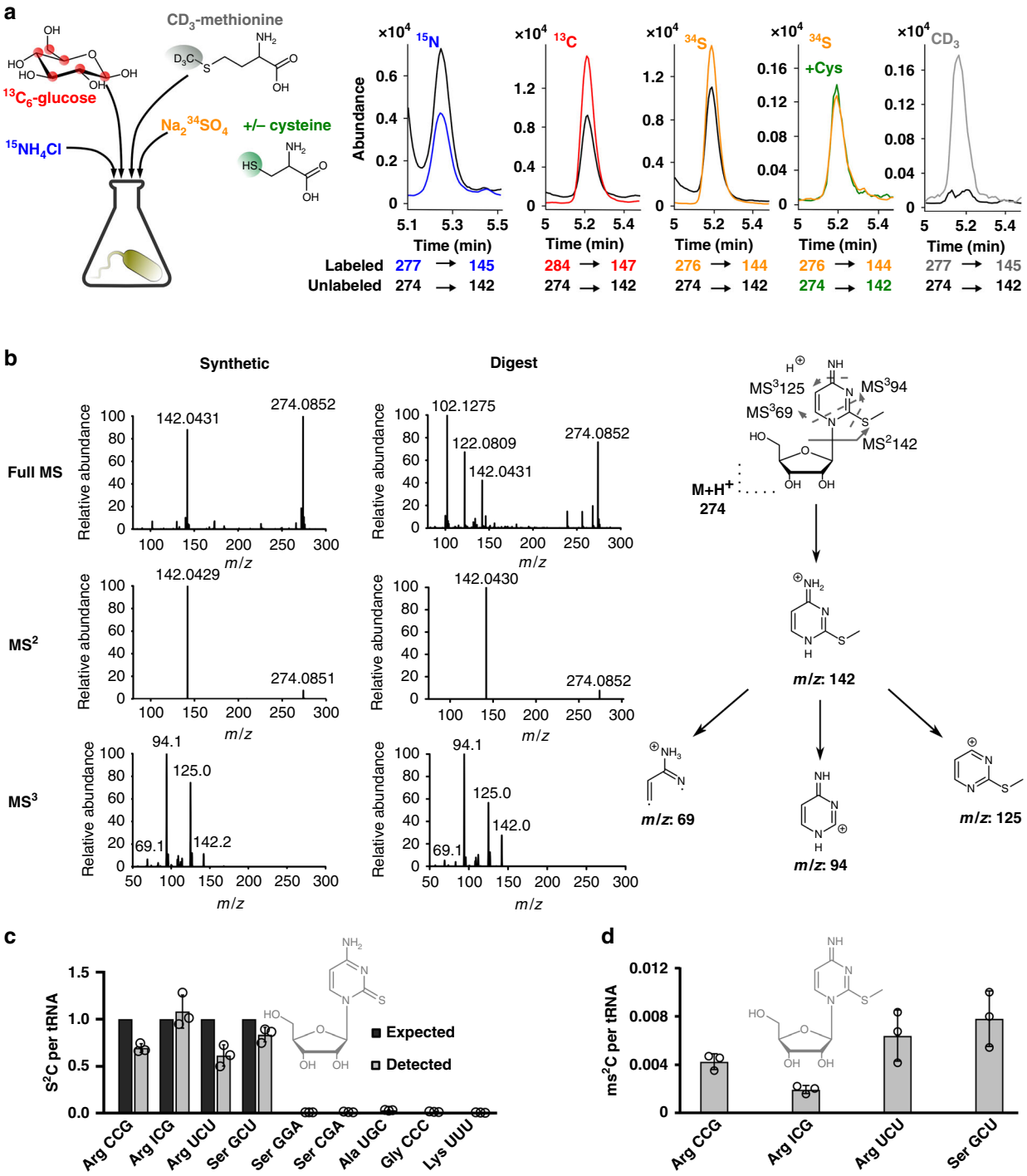

Fig. 1 Verification of 2-methylthiocytidine (ms^2C) structure and distribution in bacterial tRNA isoacceptors. a Different isotope labeling in bacteria. Co-elution of the synthesized ms^2C nucleoside (black) and ms^2C from [^{15}N], [^{13}C] or [^{34}S] labeled bacteria and products of metabolic labeling with [^{32}S]-cysteine in [^{34}S] medium and CD_3-methionine labeling. The respective isotope is indicated above each chromatogram. **b** High resolution mass spectrometry and fragmentation of ms^2C by direct injection of the synthetic standard or the natural bacterial digest and collision induced dissociation. **c** Expected (black)[1] and experimentally detected (gray) abundance of s^2C per respective tRNA isoacceptor. **d** Experimentally detected abundance of ms^2C per respective s^2C containing tRNA isoacceptor. All experiments are from $n = 3$ biol. replicates, error bars reflect standard deviation. Source data of (**c**) and (**d**) are provided as a Source Data file.

with a reported s^2C, namely $tRNA^{Arg}_{CCG}$, $tRNA^{Arg}_{ICG}$, $tRNA^{Arg}_{UCU}$ and $tRNA^{Ser}_{GCU}$, and purified these isoacceptor tRNAs from total tRNA[22]. As a control we decided to isolate tRNAs with C32 ($tRNA^{Ser}_{GGA}$, $tRNA^{Lys}_{UUU}$), a tRNA with 2'-O-methylcytidine at position 32 (Cm32, $tRNA^{Ser}_{UGA}$) and tRNAs without a C32, $tRNA^{Gly}_{CCC}$ and $tRNA^{Ala}_{UGC}$. As expected, we found s^2C in $tRNA^{Arg}_{CCG}$, $tRNA^{Arg}_{ICG}$, $tRNA^{Arg}_{UCU}$ and $tRNA^{Ser}_{GCU}$ (Fig. 1c). The complete modification profile of these tRNAs is shown in Supplementary Table 5 and Supplementary Fig. 5. ms^2C was also found in these s^2C-containing tRNA isoacceptors, however according to our data only 0.2–1.0% of actually s^2C-modified tRNAs contain ms^2C.

For exploration of the biosynthetic pathway, we quantified the abundance of ms^2C in total tRNA from the *E. coli* WT and various isogenic knockout strains (Supplementary Table 2) in glucose-containing M9 minimal medium (Supplementary Fig. 6) and LB medium (Supplementary Fig. 7). In total tRNA from WT *E. coli* grown in M9 we found 0.08 s^2C per average tRNA, and only ~0.00032 ms^2C. In the Δ*ttcA* mutant no s^2C and ms^2C were detectable (corresponding to less than 0.00005 modifications per tRNA). We observed decreased ms^2C formation in various knockout strains grown in M9 medium but not in LB medium. To exclude an involvement of these enzymes in ms^2C formation, we analyzed *E. coli* strains that overexpress tRNA modifying enzymes (Supplementary Fig. 7 and Supplementary Table 6). This confirmed that none of the investigated enzymes is involved in ms^2C biosynthesis. The overall low abundance and high fluctuation of ms^2C in unstressed bacteria hints towards its nature as an endogenous tRNA lesion. Indeed, in vitro experiments showed the formation of ms^2C from s^2C in the presence of SAM and MMS (Supplementary Fig. 8).

2-thiocytidine is a main substrate of MMS alkylation in vivo. Intrigued by the possibility that s^2C is methylated in vivo by naturally occurring methyl donors like SAM, we were wondering about its methylation during alkylation stress. We exposed WT *E. coli* to the LD_{50} dose of MMS (20 mM), non-lethal MMS doses (3 mM and 0.5 mM) and the LD_{50} dose of the natural antibiotic streptozotocin (STZ, 200 µM) in M9 medium (Supplementary Fig. 9). The total tRNA was isolated for absolute quantification by LC-MS/MS (Supplementary Table 7). The results for the main RNA damage product m^1A and ms^2C are shown in Fig. 2a. We observed ~0.1 m^1A per tRNA and ~0.05 ms^2C per tRNA in the 20 mM MMS exposed bacteria. With the non-lethal dose of 3 mM MMS, the formation of damage is reduced to 0.014 m^1A and 0.018 ms^2C per tRNA. With low doses of alkylating agent, the s^2C damage was more prominent in total tRNA than the adenosine damage. The absolute abundance of other modified nucleosides is shown in Supplementary Figs. 10 and 11. STZ methylated both adenosine (0.03 m^1A/tRNA) and s^2C (0.008 ms^2C/tRNA). Overall, ms^2C formed readily during alkylation stress and in comparable extent to m^1A.

While m^1A is known to be a direct methylation product, the increase in ms^2C might be caused by either direct methylation or SAM-derived enzymatic methylation. To elucidate the origin of the methylation in ms^2C under MMS stress, we used nucleic acid isotope labeling coupled mass spectrometry (NAIL-MS) to discriminate direct and SAM-derived methylation during MMS stress in vivo. Adapted from our recently established assay[10], we cultured *E. coli* WT bacteria in the presence of CD_3-methionine to allow complete CD_3-labeling of all SAM dependent methylated nucleosides. One aliquot of these bacteria was exposed to the LD_{50} dose of MMS, while the other remained unstressed. After 1 h of MMS exposure the tRNA was purified and analyzed by LC-MS/MS. Enzymatically methylated nucleosides carry a + 3 label whereas the directly MMS methylated nucleosides are unlabeled and thus the two species are distinguishable by mass spectrometry. Absolute quantification of the enzymatically and directly methylated nucleosides in the unstressed and stressed cells revealed the abundance of methylated nucleosides in the samples. The fold changes between the stressed and unstressed samples were calculated and plotted in Fig. 2b. We observed only little adaptation of enzymatically introduced tRNA modifications under these growth conditions. The strongest increase was 1.7 fold, observed for Um (2′-O-methyluridine) from total tRNA. In both the stressed and unstressed samples, ms^2C was again found to be labeled through CD_3-methionine dependent pathways, with a slightly higher abundance in the stressed samples. This small increase in CD_3-methylated ms^2C does not reflect its substantial abundance in Fig. 2a. In contrast to the SAM dependent methylations, we found a high increase for the known products of direct methylation, namely m^3U (5 fold), m^6A (47 fold), m^7G (64 fold) m^1A (64 fold) and m^3C (74 fold)[10]. However, the highest increase was for the product of direct s^2C methylation, ms^2C, which was over 500 fold increased in the stressed bacteria compared to the unstressed (absolute quantities in Supplementary Fig. 12). Thus, we prove that the ms^2C observed in Fig. 2a originated from direct s^2C methylation by MMS.

The previous experiments were done with total tRNA, but only four tRNA isoacceptors carry s^2C. To study the impact of direct methylation on these s^2C-containing tRNAs, we performed a comparative NAIL-MS experiment. For this purpose, the unstressed *E. coli* are grown in $^{13}C_6$-glucose labeled medium and the MMS exposed bacteria (LD_{50}) in unlabeled medium. After cell harvesting, the unstressed and stressed bacteria were mixed to avoid purification biases, as recently suggested[12]. From the resulting co-purified total tRNA, the tRNA isoacceptors Ser^{GCU}, Arg^{CCG}, Arg^{ICG} and Arg^{UCU} were isolated, digested and subjected to LC-MS/MS analysis. The mass spectrometer was set to distinguish the ^{13}C-labeled nucleosides (unstressed bacteria) from the unlabeled nucleosides (stressed bacteria) and thus the abundance of each nucleoside can be determined (Supplementary Figs. 13 and 14, Supplementary Tables 8 and 9). On average, we found 0.8 s^2C per respective unstressed tRNA, but after MMS exposure, the abundance of s^2C drops substantially (Fig. 2c, left). For tRNA Ser^{GCU}, Arg^{CCG} and Arg^{UCU} we found a 4-fold decrease to <0.2 s^2C per tRNA. $tRNA^{Arg}_{ICG}$ was the only exception with a ~2-fold decrease to around 0.4 s^2C per tRNA. As expected, the abundance of ms^2C was increased in the stressed samples, which indicated that the lost s^2C was directly methylated to ms^2C in these tRNAs (Fig. 2c, middle). In all purified tRNAs, the ms^2C abundance was higher than the m^1A abundance (Fig. 2c, right).

This data demonstrates that s^2C is a better substrate of the methylation agent MMS than adenosine.

2-methylthiocytidine may influence translation. The s^2C containing tRNAs are major targets of direct methylation by MMS. s^2C is located at position 32 where it fulfils important roles in translation of the serine codons AGU and AGC by $tRNA^{Ser}_{GCU}$ and the arginine codons CGG ($tRNA^{Arg}_{CCG}$), AGA ($tRNA^{Arg}_{UCU}$) and CGC, CGU and CGA ($tRNA^{Arg}_{ICG}$)[14]. The methylation of s^2C and formation of ms^2C might result in electronic and electrostatic shifts of the nucleobase, which potentially changes the properties of the whole anticodon loop of the affected tRNAs. Thus translation of the respective serine and arginine codons might be influenced by the methylation of s^2C. To address this question, the effect of MMS on the synthesis of superfolder green fluorescent protein (sfGFP) in *E. coli* WT was determined. We decided to study sfGFP (for sequence and used primers see Supplementary Table 10), which contains one AGC (at position 2) and one AGT codon for serine, five GCU, two CGC and one CGG codon for arginine. 10 codons are translated by s^2C modified tRNAs, which will be damaged to carry ms^2C under MMS stress. Thus the translation of sfGFP could be effected by MMS due to the s^2C methylation. Expression of *sfgfp* was under control of the P_{BAD} promoter, and unstressed or stressed (exposures to 20 mM MMS for 1 h) cells of the mid-log growth phase were induced by arabinose. Fluorescence intensity of single *E. coli* cells of strain BW25113 was determined after 1 and 5 h (Supplementary Fig. 15). In the presence of 3 mM MMS, fluorescence was significantly lower under acute stress (1 h), but cells

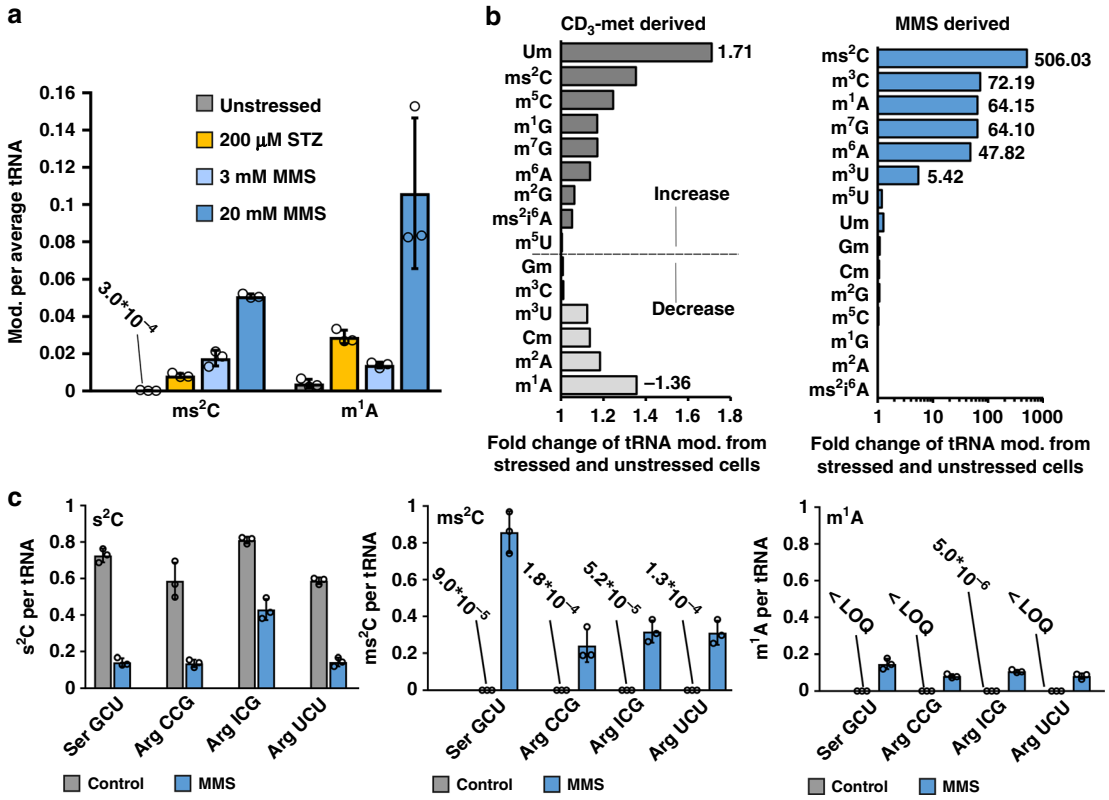

Fig. 2 Absolute abundance of modified nucleosides in total tRNA and tRNA isoacceptors after methylation stress. a Abundance of mod. (ms^2C and m^1A) per average tRNA after 0 mM (unstressed, gray), 200 µM Streptozotocin (STZ, natural methylating agent, 50% lethality, orange), 3 mM MMS (no lethality, light blue) and 20 mM MMS (50% lethality, dark blue). **b** Impact of MMS exposure (unstressed vs. 20 mM stressed) on *E. coli* total tRNA modifications (mod.). The origin of the methylated tRNA modifications is displayed in separate plots. Left: impact of MMS exposure on CD_3-met (met = methionine) derived methylation displayed as the fold change ratio of stressed-to-unstressed. Right: impact of MMS exposure on directly methylated nucleosides (MMS derived methylation) displayed as the fold change of stressed-to-unstressed. **c** Absolute abundance of s^2C (left), ms^2C (middle) and m^1A (right) in various tRNA isoacceptors without (gray) and with (blue) 20 mM MMS exposure. In the negative control (without MMS) *E. coli* were grown in ^{13}C medium (gray bars, control) and the MMS exposed bacteria in non-labeled (blue bars, MMS) media. Co-purification of tRNA isoacceptors was done in a comparative NAIL-MS experiment as detailed in the text. All experiments are from $n = 3$ biol. replicates and error bars reflect standard deviation. Source data are provided as a Source Data file.

recovered after 5 h indicated by the increase in fluorescence. Similar results are seen for cells after exposure to 20 mM MMS (Fig. 3a). We mutated a single codon in the *sfgfp* gene to replace the AGC codon for serine with AGT and TCC (Fig. 3b). While AGC and AGT are read by the same s^2C modified $tRNA^{Ser}_{GCU}$, the TCC codon is read by $tRNA^{Ser}_{GGA}$, which is not s^2C modified and thus the anticodon is not affected by the MMS stress (see Fig. 1c). We tested the influence of these codons (AGC, AGT, TTC) at position 2 in sfGFP on the fluorescence in the WT strain. Fluorescence of stressed cells producing the AGT- and AGC-variants of sfGFP is more affected in comparison to the TCC construct (Fig. 3c). At the used MMS doses, we previously observed about 80% ms^2C modified tRNA Ser^{GCU} while only 20% were s^2C modified (Fig. 2c). Thus we see a direct correlation of ms^2C abundance and decreased translational efficiency for the respective codons. Moreover, translation of the AGT-containing sfGFP was slightly more affected by the methylating agent than the AGC-containing construct. These results indicate a negative effect of ms^2C on translation.

2-methylthiocytidine is efficiently repaired by AlkB. The ms^2C damage has a potential effect on the translation of *E. coli*. It is highly probable that the methylation damage is recognized and repaired by the bacteria to adapt to the stress. This repair is either possible by direct demethylation by AlkB as shown for m^1A[23] or by tRNA degradation. To address the question whether ms^2C is repaired and which mechanism dominates, we designed a pulse-chase assay involving stable isotope labeling. Figure 4a shows the principle of the assay. The goal of this assay is to discriminate the damaged tRNAs from the tRNAs transcribed during the recovery. Thus, we can follow the fate of the damaged tRNAs and their nucleosides independently from dilution by transcription. For this purpose, cells were grown in media containing only [^{14}N]- and [^{32}S]-nutrients. Consequently, the RNA was completely labeled with [^{14}N] and all s^2C have a [^{32}S] label (original s^2C), *e.g.* *m/z* (s^2C) 260. All methylated nucleosides had a CH_3 mark (e.g., original m^1A *m/z* 282). In this medium, the cells were exposed to MMS (LD_{50}) and s^2C was converted to ms^2C and, e.g., A to m^1A. After exposure, MMS was removed by exchanging the media with heavy-isotope media. During the following recovery period, newly transcribed RNA were [^{15}N] labeled, newly methylated nucleosides were $[CD]_3$ labeled and new s^2C had a [^{34}S] label (new s^2C, *m/z* 265 and new m^1A, *m/z* 290).

The dashed line in Fig. 4b, represents the decrease of m^1A if referenced to the sum of all tRNAs (original + new transcripts). The fast decrease is caused by dilution due to ongoing tRNA transcription. In the past, this decrease could be misinterpreted as active tRNA demethylation, but with NAIL-MS we can follow the fate of modified nucleosides inside living cells by excluding

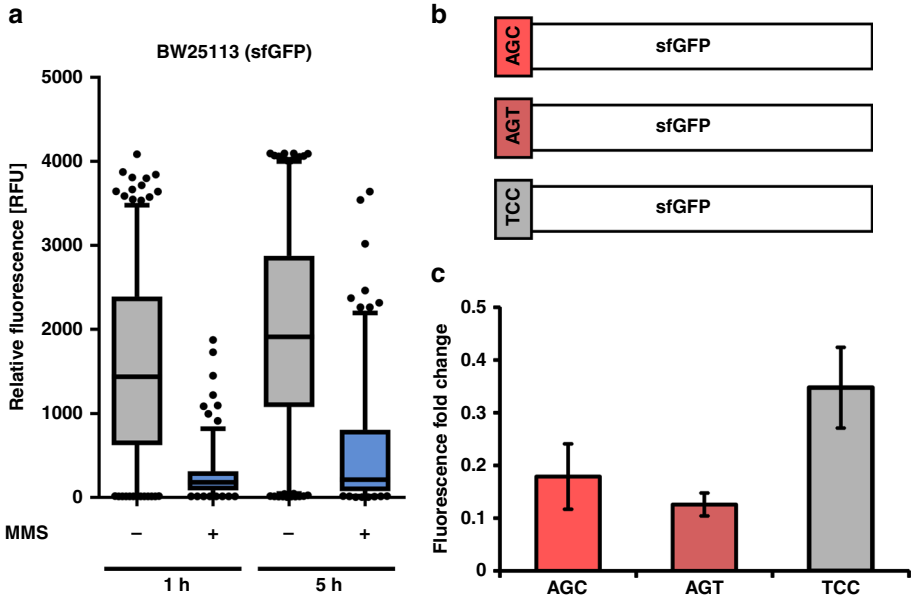

Fig. 3 Impact of s^2C methylation on translation. a Long-term effects of 20 mM MMS stress on sfGFP synthesis in *E. coli* WT cells. Single cell fluorescence was determined for a minimum of 300 *E. coli* cells producing sfGFP under control of the L-arabinose inducible promoter (pBAD24). Fluorescence was measured 1 and 5 h after addition of arabinose to unstressed cells or cells pre-exposed to 20 mM MMS stress for 1 h. (box plots represent the 5-95% percentile) **b** Illustration of the used sfGFP constructs. The serine on amino acid position 2 of the used sfGFP variant was encoded by different codons: AGC, AGT and TCC. **c** Influence of codons on sfGFP synthesis after exposure of cells to MMS stress. Fluorescence was measured after 1 h of induction with arabinose. The fold change was determined by dividing the mean fluorescence of stressed cells (20 mM MMS) by the mean fluorescence of unstressed cells. All experiments are from $n = 3$ biol. replicates and error bars reflect standard deviation. Source data are provided as a Source Data file.

transcription processes. By referencing m^1A abundance to the original tRNAs, we saw a slow but steady decrease of m^1A in the damaged tRNAs over time which reflects true demethylation (Fig. 4b, solid line). For ms^2C, we saw a fast and nearly complete repair over the same observation period (Fig. 4b). This decrease was not caused by increased tRNA degradation as MMS exposed bacteria showed the same original-tRNA dilution pattern as unstressed bacteria (Supplementary Fig. 16). We isolated tRNAs Ser^{GCU} and Arg^{ICG} from the NAIL-MS pulse-chase experiments, and for these damaged tRNAs we saw no increased degradation either (Supplementary Fig. 17). Thus we conclude that the removal of ms^2C from damaged tRNA is not caused by tRNA degradation.

m^1A is known to be demethylated by AlkB[13,23]. To test whether AlkB also demethylates ms^2C to s^2C, we isolated total tRNA from MMS exposed bacteria and tested the AlkB activity in vitro. After addition of AlkB to the damaged tRNA, we could no longer detect ms^2C and m^1A in the tRNA (Fig. 4c). Instead, we could observe an increase of s^2C, which indicates that AlkB demethylates ms^2C to s^2C. This finding is rather surprising as, until now, only nitrogen demethylation was observed for AlkB but never sulfur demethylation[11,23].

To verify that AlkB demethylates ms^2C containing tRNAs in vivo, we decided to repeat the NAIL-MS pulse-chase assay in an *alkB* deficient *E. coli* strain. After exposure to 20 mM MMS, we observed a fast decrease of ms^2C in the WT strain and a slower decrease in the isogenic Δ*alkB* mutant (Fig. 4d). Interestingly, we observed an increase of s^2C in the WT strain but not in the Δ*alkB* strain (Fig. 4e). Our data indicates that a portion of ms^2C is demethylated by AlkB under recovery of the original $[^{32}S]$-s^2C. The other portion of ms^2C seems to be lost by an AlkB-independent mechanism. For m^3C we also observed an AlkB dependent demethylation, but surprisingly not for m^1A (Supplementary Fig. 18). We repeated the experiment in the presence of 3 mM MMS and 0.5 mM MMS to ensure the viability of the cells. 3 mM MMS corresponds to 20% lethality in the Δ*alkB* strain (Supplementary Fig. 19) and no lethality in the WT strain. Directly after 3 mM MMS exposure, the abundance of ms^2C, m^1A and m^3C per original tRNA was comparable in the WT and Δ*alkB* strain (Supplementary Fig. 20). After 2 h of recovery, the abundance of ms^2C, m^1A and m^3C was more reduced in the WT strain compared to the Δ*alkB* mutant. This indicates repair of ms^2C by AlkB in vivo as observed in Fig. 4d. For 0.5 mM MMS, we saw an AlkB independent reduction of m^1A, m^3C, and ms^2C of comparable extent. On tRNAs with a damage abundance below a threshold level, AlkB appeared to be uninvolved in repair.

In summary, we demonstrate that ms^2C damaged tRNAs are yet undescribed substrates of AlkB, both in vitro and in vivo.

Dethiomethylation as an alternative repair pathway. The in vitro as well as the in vivo analyses of AlkB convinced us of the role of AlkB as key player in ms^2C demethylation. Based on our data shown in Fig. 4, we assume direct demethylation by AlkB, which results in the recovery of $[^{32}S]$-s^2C.

Chemically, the thiomethyl group is an acceptable leaving group in the presence of nucleophiles such as water. Dethiomethylation might occur inside the cell and would result in the formation of canonical cytidine. In theory, this cytidine should then be a substrate of TtcA and a rethiolation to s^2C would result (Fig. 5a). Such an s^2C turnover event is observable in vivo with our pulse-chase NAIL-MS experiment. During the design of the experiment, we chose two media with different sulfur isotopes. The stress medium contains the sulfur-32 isotope and thus all s^2C of original tRNAs is $[^{32}S]$-s^2C with an *m/z* of 260. The recovery medium contains no sulfur-32, but sulfur-34 instead. Thus, s^2C in new transcripts is not only three units heavier (nitrogen-15) but five units ($[^{15}N_3/^{34}S]$-s^2C, *m/z* 265). In case of the imagined scenario of spontaneous dethiomethylation of ms^2C into cytidine followed by rethiolation into s^2C, only sulfur-34 is available and

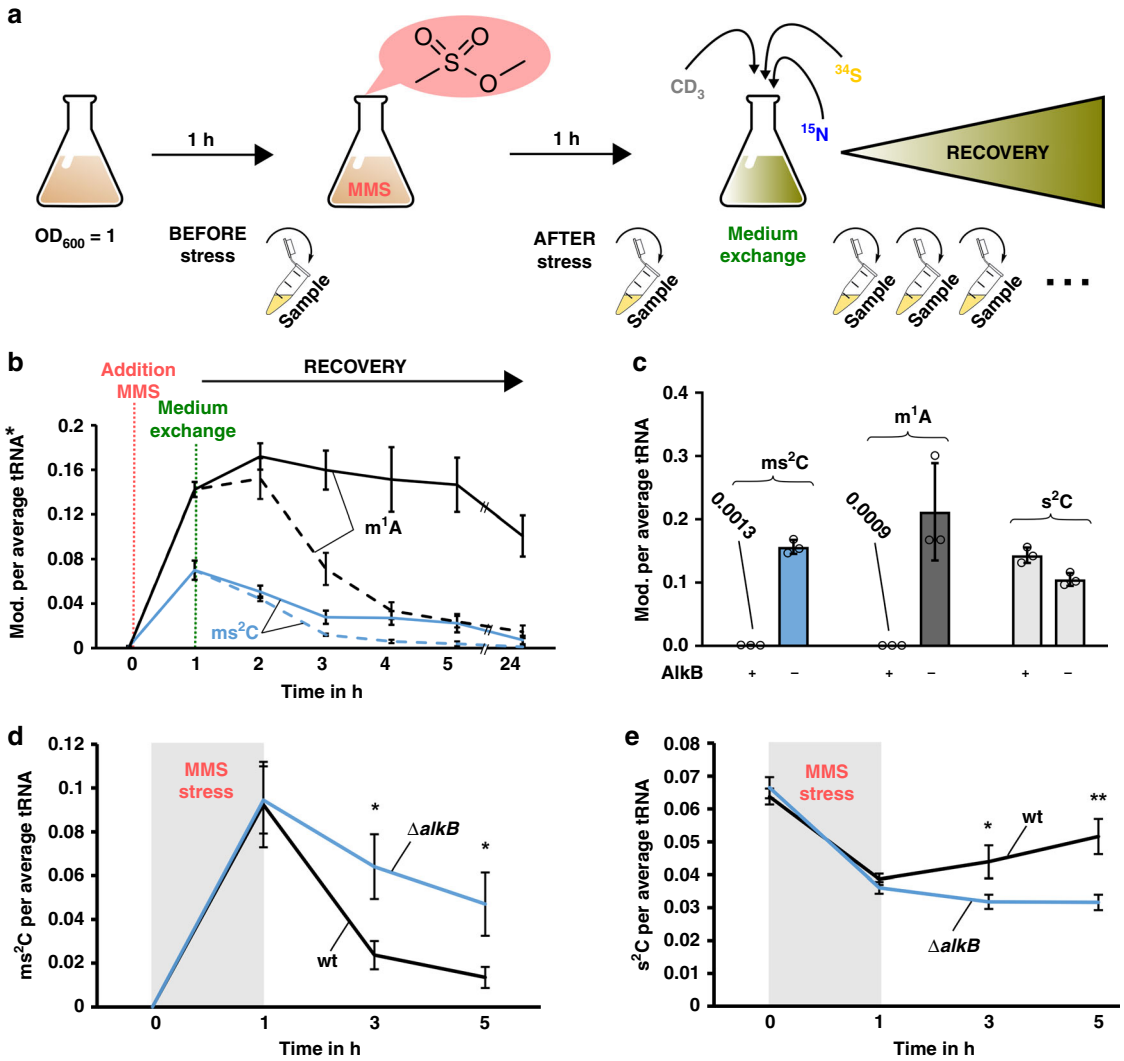

Fig. 4 Principle and results of pulse-chase NAIL-MS experiments to determine the repair of ms^{2}C in vivo. a Principle of a pulse-chase NAIL-MS experiment. The bacteria are grown in unlabeled (n.l.) media before and after exposure to MMS (structure shown). After 1 h MMS exposure, the media is removed and fresh, [^{15}N], [^{34}S] and [CD$_3$]-methionine containing media is added. Samples are drawn during the recovery time for tRNA isolation. **b** Formation of the nucleoside damage product ms^{2}C (blue) and m^{1}A (black) per average tRNA after 20 mM MMS exposure. *Dashed lines: abundance of mod. (ms^{2}C and m^{1}A) per all tRNAs (sum of original, unlabeled and new, [^{15}N]-labeled transcripts). Solid lines: abundance of mod. (ms^{2}C and m^{1}A) per original tRNA. **c** Abundance of mod. (ms^{2}C in blue, m^{1}A in dark gray and s^{2}C in light gray) per tRNA incubated with purified AlkB in vitro. The substrate tRNA is isolated from *E. coli* bacteria exposed to 20 mM MMS. **d** ms^{2}C abundance of *E. coli* WT (black) in comparison to *alkB* deficient *E. coli* (Δ*alkB*, blue) after MMS stress (20 mM, 1 h) and during recovery in a pulse-chase NAIL-MS experiment, as described in a) and b). **e** s^{2}C abundance of *E. coli* WT (black) in comparison to *alkB* deficient *E. coli* (Δ*alkB*, blue) after MMS stress (20 mM, 1 h) and during recovery. All experiments are from n = 3 biol. replicates and error bars reflect standard deviation. *p*-values from student t-test (equal distribution, two-sided): *$p < 0.05$ and **$p < 0.01$. Source data are provided as a Source Data file.

thus the original [^{32}S]-s^{2}C would turn into [^{34}S]-s^{2}C with an *m/z* of 262. The original [^{32}S]-s^{2}C produced by AlkB demethylation and the hypothetical turnover [^{34}S]-s^{2}C formed after dethiomethylation/rethiolation can be distinguished by mass spectrometry (Supplementary Table 11).

The result for the unstressed and 20 mM MMS treated WT strain is shown in Fig. 5b. Immediately after the stress, the number of turnover [^{34}S]-s^{2}C was comparable and rose over time. The increase of [^{34}S]-s^{2}C in unstressed bacteria was most likely caused by regular s^{2}C biosynthesis into original tRNAs, which were not fully mature at the time of medium exchange. The presence of only [^{34}S] at these later time points led to the formation of [^{34}S]-s^{2}C in the control bacteria. In contrast, MMS exposure led to more [^{34}S]-s^{2}C. To test involvement of AlkB in the formation of turnover [^{34}S]-s^{2}C, we plotted the [^{34}S]-s^{2}C abundance observed in the Δ*alkB* strain (Fig. 5c). Here, we saw an increase in turnover s^{2}C, which was comparable to the turnover in the WT strain. We thus conclude that the turnover is not a consequence of the AlkB repair process.

The increase in turnover s^{2}C can be explained by dethiomethylation of ms^{2}C followed by rethiolation of the formed cytidine. Currently it is unclear, whether the dethiomethylation occurs spontaneously after a nucleophilic attack of water or by an enzymatic pathway. From a chemical perspective, we believe that spontaneous dethiomethylation is the most likely.

Discussion

In this study, we identified s^{2}C as a substrate of direct methylation in bacterial tRNAs, and the mechanisms of the subsequent

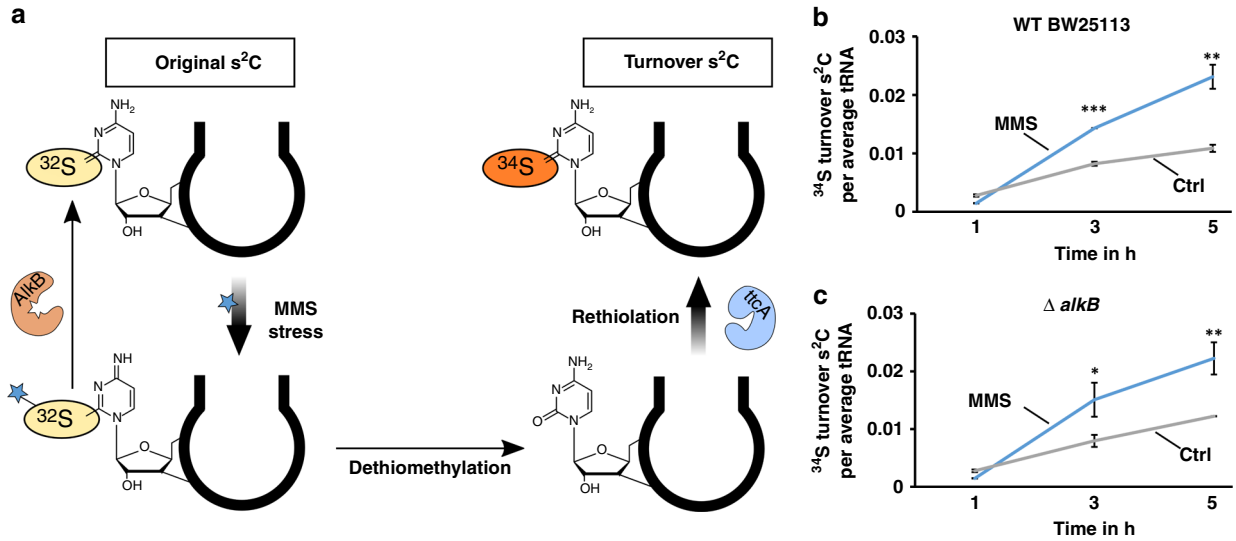

Fig. 5 Pulse-chase NAIL-MS analysis of turnover s^{2}C after 20 mM MMS exposure in WT BW25113 and *E. coli* Δ*alkB* knockout strain. a Concept of turnover s^{2}C generation and its detection by NAIL-MS. **b** Abundance of [^{34}S] turnover s^{2}C in WT BW25113 during recovery after MMS stress (20 mM) and unstressed control. **c** Abundance of [^{34}S] turnover s^{2}C in the Δ*alkB* strain during recovery after MMS stress (20 mM) and unstressed control. All experiments are from $n = 3$ biol. replicates and error bars reflect standard deviation. *p*-values from student t-test (equal distribution, two-sided): $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Source data are provided as a Source Data file.

repair by AlkB. We initially identified the new structure of the methylated nucleoside in total tRNA of *E. coli* and *P. aeruginosa*. The structure of the ms^{2}C (2-methylthiocytidine) nucleoside was confirmed after synthesis by various analytical tools including fragmentation patterns in high resolution mass spectrometry. Methylated derivatives of other thiolated nucleosides like s^{4}U or mnm^{5}s^{2}U might also form endogenously, but were not detected in our nucleoside discovery experiments.

ms^{2}C was found at position 32 of tRNA$^{Arg}_{ICG}$, tRNA$^{Arg}_{CCG}$, tRNA$^{Arg}_{UCU}$ and tRNA$^{Ser}_{GCU}$. Only 1% of the s^{2}C modified tRNA isoacceptors carry ms^{2}C under unstressed growth conditions. The endogenous formation of ms^{2}C was not found to be connected to common tRNA modifying enzymes. At this stage we cannot determine whether the endogenous, but low abundant, ms^{2}C modification of bacterial tRNA is introduced enzymatically or by direct methylation through, e.g., SAM. For low abundant nucleoside modifications it is often an open question whether they are lesions or functional entities[24].

Intrigued by the observation that ms^{2}C forms naturally in the presence of the methyl donor SAM, we were wondering whether ms^{2}C is formed by direct methylation during alkylation stress after MMS exposure. MMS is a direct mono-methylating agent, which is known to methylate RNA nucleosides and DNA nucleosides. From our systematic study, we know that the main damage products of canonical nucleosides are m^{1}A and m^{7}G[10]. In the current study, we also focused on the quantities of ms^{2}C formed during an LD$_{50}$ MMS exposure of *E. coli*. We find 0.1 m^{1}A damage sites per average tRNA and surprisingly 0.05 ms^{2}C per average tRNA. At lower MMS doses (3 mM), the number of m^{1}A damage sites is reduced to 0.014 per average tRNA and 0.018 ms^{2}C per average tRNA. It is now clear that ms^{2}C is an equally prominent damage product as m^{1}A in total tRNA. If one considers that the average tRNA in bacteria is composed of 15 adenosines while only 0.1 s^{2}C are found in the average tRNA, our results are rather intriguing. Although adenosine is 150 fold more abundant than s^{2}C in total tRNA, the methylation products m^{1}A and ms^{2}C are of comparable quantities. From our data, we conclude that s^{2}C is an at least equal or even better substrate of direct methylation than adenosine in the tRNA of the bacterium *E. coli*.

Although the abundance of modified nucleosides is low in comparison to the amount of canonical nucleosides in the total RNA pool, modified nucleosides fulfil a variety of important functions within the cell. The damage of a modified nucleoside will most likely interrupt the modification's function and might be disadvantageous to the organism. We have tested how the methylation of s^{2}C influences the translation in vivo. Serine can be translated by tRNA$^{Ser}_{GGA}$ (no s^{2}C) reading the TCC codon or tRNA$^{Ser}_{GCU}$ reading the AGC or AGU codons. We found that *sfgfp* with the AGC or AGU codon was less efficiently translated than *sfgfp* with the TCC codon under MMS stress. This finding indicates that the translation of the AGC and AGT codon is disturbed after MMS stress, potentially due to the presence of ms^{2}C.

The repair of damaged ribonucleosides might occur by two potential mechanisms. RNA is a transient molecule and constantly transcribed from DNA within the cell. Upon damage, it is possible to remove the damage by controlled degradation of the RNA. Due to the fact that tRNA maturation and modification is energy consuming, the second repair scenario of direct damage repair by, e.g., demethylation, is more attractive. In bacterial RNA, the repair of m^{1}A and m^{3}C by oxidative demethylation by the enzyme AlkB has been shown[7]. In our NAIL-MS studies, we use stable isotopes such as carbon-13, nitrogen-15 or sulfur-34 to distinguish RNA that was present during the stress event (here, MMS exposure) and new RNA transcribed during the recovery period. We can thus distinguish repair by RNA degradation and demethylation. With NAIL-MS, we observe AlkB dependent demethylation of ms^{2}C, m^{3}C and potentially m^{1}A.

The AlkB dependent repair of m^{3}C had been suggested in previous studies but has never been shown in vivo. Surprisingly, m^{1}A repair was similar in the WT and Δ*alkB* mutant. Only for the 3 mM MMS dose, 3 h after stress exposure, the WT strain showed less m^{1}A per tRNA compared to the Δ*alkB* strain. This is in accordance with the only other in vivo study, where m^{1}A repair by AlkB was observed in radioactive tRNA 3 h after stress at a low dose of alkylating agent[23].

In unstressed Δ*alkB* bacteria, we did not observe an accumulation of ms^{2}C (Supplementary Fig. 21). This is to be expected if one considers the low copy number of AlkB in unstressed

wildtype bacteria (1 copy of AlkB in log phase *E. coli* cells[25]). Only after exposure to alkylating agents, *alkB* is induced and six hours after 3 mM MMS exposure 6000 AlkB copies/cell have been found[11].

Concerning ms^2C repair, we found an increase of original [^{32}S]-s^2C in vivo, which reflects direct sulfur demethylation by AlkB. With our sophisticated NAIL-MS approach we could additionally observe an AlkB independent formation of s^2C which originated from dethiomethylation of s^2C to cytidine and subsequent rethiolation. The initial dethiolation step might be enzymatically catalyzed, but due to the electrophilic nature of the C2 in ms^2C, a direct nucleophilic attack by water is also possible.

So far, m^1A was thought to be the main methylation damage product in bacterial RNA and thus to be the main substrate of AlkB. Our data shows that ms^2C is a methylation damage in bacterial tRNAs of comparable extent after MMS exposure. In addition, AlkB is faster during the repair of ms^2C damaged tRNAs compared to m^1A damaged tRNAs.

Since its discovery, AlkB has been an enzyme full of surprises. In this work, we present the next surprise, which is AlkBs extended substrate repertoire and its preference for the sulfur methylated nucleoside ms^2C.

Methods

Salts, reagents, and nucleosides. All salts were obtained from Sigma-Aldrich (Munich, Germany) at molecular biology grade unless stated otherwise. The isotopically labeled compounds $^{15}NH_4Cl$ (>98 atom %) and [D_3]-L-methionine (98 atom %) were obtained from Sigma-Aldrich. Isotopically labeled $^{13}C_6$- glucose (≥99 atom %) and isotopically labeled $Na_2{}^{34}SO_4$ (99.11 atom %) were obtained from Eurisotope (Saarbruecken, Germany). All solutions and buffers were made with water from a Millipore device (Milli-Q, Merck, Darmstadt, Germany). The nucleosides adenosine, cytidine, guanosine, uridine, and N2-methylguanosine (m^2G) were obtained from Sigma-Aldrich. 1-Methyladenosine (m^1A), 2-methyladenosine (m^2A), N3-methylcytidine (m^3C), N6-methyladenosine (m^6A), 7-methylguanosine (m^7G), 5-methylcytidine (m^5C), 5-methyluridine (m^5U), 2'-O-methylcytidine (Cm), 2′-O-methylguanosine (Gm), 1-methylguanosine (m^1G), and 3-methyluridine (m^3U) were obtained from Carbosynth (Newbury, UK).

Synthesis of 2-methylthiocytidine. Twenty milligrams of s^2C were stirred in 1 mL anhydrous ethanol under nitrogen atmosphere.7.4 mg of $NaHCO_3$ and 24 µL of methyl iodide were added and the reaction was stirred overnight at room temperature. The clear yellow solution was evaporated and two times purified by silica gel chromatography in dichloromethane with 10 % methanol. The Rf value (TLC) in DCM/MeOH (10:1) was 0.46.

^1H NMR (400 MHz, D_2O):δ = 8.39 (d, J = 7.7 Hz, 1H, H-6), 6.60 (d, J = 7.7Hz, 1H, H-5), 5.98 (d, J = 3.0Hz, 1H, H-1′), 4.38 (t, J = 3.4 Hz, 1H, H-2′), 4.23-4.18 (m, 2H, H-3′, H-4′), 3.99 (d, J = 12.7Hz, 1H, H-5′a), 3.85 (dd, J = 2.7 Hz, 13.4 Hz, 1H, H-5′b), 2.67 (s, 3H, CH_3). ^{13}C NMR (500 MHz, D_2O):δ = 166.4 (C-2), 162.1 (C-4), 141.5 (C-6), 102.1 (C-5), 92.7 (C-1′), 84.6 (C-3′), 74.9 (C-2′), 68.5 (C-4′), 59.8 (C-5′), 14.4 (C-7).

***E. coli* and *P. aeruginosa* strains**. The used *E. coli* wild-type strain BW25113 and the isogenic knockout strains were purchased from the Keio database[26]. *Pseudomonas aeruginosa* PA14 was a generous gift of Prof. Peter Dedon. Knockout strains with kanamycin resistance were grown on kanamycin LB agar plates (50 µg/mL). The *E. coli* strain AG1 (ME5305) transformed with the indicated pCA24N-based vectors was used for gene overexpression and ordered from the ASKA library database[27]. Strains with chloramphenicol resistance were grown in the presence of chloramphenicol (30 µg/mL). All cultures were grown in a shaking incubator at 37 °C at 250 rpm (Orbit = 10 mm). Overnight cultures were grown in 5 mL of the media used for the respective experiment. The cells were grown starting with an OD_{600} of 0.1, 0.5 or 1 (as specified in the respective section) and grown until reaching stationary phase (OD_{600} ~ 4).

Growth media LB/ M9/ isotope labeled. For LB media, LB Broth (Luria Miller) from Roth (Karlsruhe, Germany) was used. 1.5% (wt/vol) agar plates were prepared with LB-broth and Agar-Agar (Kobe I from Roth) according to manufacturer's protocol.

M9 minimal medium was used with and without the indicated isotopes. Unlabeled M9 was prepared by mixing a 10 × M9 stock solution with glucose, $MgCl_2$, Na_2SO_4, and $CaCl_2$ (as detailed below). For unlabeled 10 × M9 stock solution, Na_2HPO_4 (68 g/L), KH_2PO_4 (30 g/L), NaCl (2.5 g/L), and NH_4Cl (10 g/L) were mixed and autoclaved. For ^{15}N-labeled 10 × M9 stock solution, $^{15}NH_4Cl$ (10 g/L) was used. $MgCl_2$ (0.1 M), $CaCl_2$ (0.1 M), Na_2SO_4 (0.1 M), and 20% (wt%) glucose were prepared by sterile filtration. A 20% (wt%) $^{13}C_6$-labeled glucose solution was prepared for ^{13}C-labeled M9 media. For ^{34}S-labeled M9 media a 0.1 M $Na_2{}^{34}SO_4$ solution was prepared. Final M9 media was prepared by mixing, e.g., 500 µL M9 stock solution with 100 µL glucose, 100 µL $MgCl_2$, 100 µL Na_2SO_4, 5 µL $CaCl_2$ and water to a final volume of 5 mL. For ^{15}N-labeled cultures, the ^{15}N-10 × M9 stock solution was used. For ^{13}C-labeled cultures, the 20% (wt%) $^{13}C_6$-labeled glucose solution and for ^{34}S-labeled cultures, the 0.1 M $Na_2{}^{34}SO_4$ solution was used. For CD_3-methylome labeling, 200 µL CD_3-methionine (stock 5 g/L) was added to 5 mL of culture volume.

***E. coli* knockout/overexpression strain library**. The knockout strains were cultured in LB or M9 media, starting from OD_{600} = 0.5. After 3 h the cells were harvested and the RNA was isolated and purified. The overexpression strains were cultured in LB media starting with an OD_{600} = 0.5 and an IPTG concentration of 0.1 mM. After 1 h incubation at 37 °C and 250 rpm (Orbit = 10 mm) the IPTG concentration was increased to 1 mM to induce full overexpression. The bacteria were incubated for further 2 h before the cells were harvested and RNA was isolated and purified

Survival assay. Unlabeled M9 media was used throughout the survival assay. A 5 mL culture with OD_{600} 1.0 (or 0.1, respectively) were prepared from an *E. coli* overnight culture. After 60 min incubation (30 min with Streptozotocin), 100 µL of the culture were diluted 10^{-5} or 10^{-6} with sterile water. From this dilution 70 µL were plated on a prewarmed LB agar plate. The colony number of this plate represents 100% bacterial survival. MMS (Methyl-methanesulfonate, 99%) or Streptozotocin (STZ, 10 mM stock solution) was added to the remaining bacteria in defined concentrations. After 60 min (30 min) exposure, 100 µL of the culture were diluted and plated. The LB plates were incubated at 37 °C overnight and the colonies counted for determination of the survival at the respective MMS or STZ concentration.

***E. coli* incubation with Streptozotocin (STZ)**. From an overnight culture of *E. coli* strain BW25113 (WT) the OD_{600} was brought to 1.0 and the culture was grown for 1 h. To 5 mL bacterial culture 125 µL of a 10 mM STZ solution was added (200 µM final conc.) and the culture was incubated for 30 min. Afterwards, the bacteria were centrifuged and the resulting pellet was used for RNA isolation and purification.

Cell lysis and tRNA purification. The bacteria culture was centrifuged at 1200 × *g* for 5 min. The supernatant was discarded and the cell pellet was resuspended in 1 mL TRI reagent (Sigma-Aldrich) per 5 mL bacteria culture. The total RNA was isolated according to the supplier's manual. tRNA was purified by size exclusion chromatography (SEC) according to published procedures[10]. The tRNA was resuspended in water (30 µL).

Isoacceptor purification. The procedure was adapted from Hauenschild et al.[22]. One microgram of total tRNA was incubated with 100 pmol of the biotinylated DNA probe and purified using Dynabeads T1 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. For tRNA isoacceptor purification, pre-purified total tRNA was used. The sequences of the biotinylated 2′-deoxyoligonucleotide probes are listed in Supplementary Table 4.

Comparative NAIL-MS. One *E. coli* culture was grown overnight in an unlabeled M9 medium and another in a ^{13}C labeled M9 medium. From these cultures, a labeled and unlabeled exposure culture with an OD_{600} of 1.0 and a volume of 5 mL were prepared. After 60 min growth, 8.5 µL MMS (final conc. 20 mM) were added to the unlabeled culture and 8.5 µL of water to the ^{13}C-labeled culture (MOCK). After 60 min of exposure both cultures were mixed and the RNA was purified immediately. Total tRNA and tRNA isoacceptors were isolated as described above. For validation of the comparative NAIL-MS assay, the experiment was repeated by mixing and co-purifying RNA (total tRNA and tRNA isoacceptors) from an unlabeled MOCK treated culture and a ^{13}C-labeled MOCK treated culture.

Methylome discrimination assay. For this assay, CD_3-labeled M9 medium was used at all times. A 5 mL bacterial solution with an OD_{600} of 0.1 was prepared from an overnight culture. After 60 min growth, 8.5 µL MMS (final conc. 20 mM) were added. As a control, 8.5 µL water was added to a second culture. After 60 min of exposure, the RNA was isolated and total tRNA purified by SEC.

sfGFP reporter and single-cell fluorescence microscopy. For in vivo quantification of the effects of tRNA modifications after MMS stress on translation, the *E. coli* strains BW25113, BW25113 Δ*alkB* and BW25113 Δ*ttcA* were transformed with pBAD24 containing a copy of sf*gfp* under the control of the arabinose-inducible P_{BAD} promoter. To test the effects of different serine codons (AGC, AGT, TCC) the only serine codon of the used sf*gfp* sequence (corresponds to amino acid position 2) was exchanged by using the respective primers during the cloning process. To enhance the number of serines, the sf*gfp* sequence was extended at the 5′-end with multiple motifs coding for proline-alanine-serine

(ASPAAPSASAPSAASAAPSAA)-sequence, modified from previous studies[28]. We used the primers PAS-gfp_for/ PAS-AGT_gfp_for / PAS-TCC_gfp_for plus gfp_rev to introduce different types of codons for serine (AGC, AGT or TCC) in the sfGFP variants extended with the proline-alanine-serine (ASPAAPSA-SAPSAASAAPSAA)-sequence or respectively gfp_for / AGT_gfp_for / TCC_gfp_for plus gfp_rev to produce the sfGFP variants with different types of codons for serine (AGC, AGT or TCC). The restrictions sites EcoRI and XbaI were used for integration of the sf*gfp* variants into the pBAD24 plasmid.

For each experiment cells of an overnight culture were inoculated into fresh M9 minimal medium supplemented with 0.5 % (vol/vol) glycerol as a sole carbon source and incubated under vigorous shaking at 37 °C. Cells were grown to mid-log phase, and the culture was divided. One half was stressed by the addition of MMS to final concentrations of 3 and 20 mM, respectively, the other half remained untreated. After 1 h of incubation in presence of MMS the medium was exchanged with fresh, pre-tempered M9 minimal media supplemented with 0.5% (vol/vol) glycerol and 0.2% (vol/vol) arabinose to induce sfGFP expression. As a control, cells were cultivated without addition of arabinose. To measure sfGFP fluorescence, cells were fixed on an agarose pad (1% wt/vol in phosphate-buffered saline) placed on a microscope slide with coverslip. Micrographs were taken on a Leica microscope DMI 6000B equipped with a Leica DFC 365Fx camera (Andor, 12 bit). sfGFP fluorescence was visualized using an excitation wavelength of 460 nm and a 512 nm emission filter with a 75-nm bandwidth. Fluorescence intensities of a minimum of 300 cells per transformant were collected and quantified using Fiji[29].

Pulse-chase NAIL-MS experiment. A single colony of *E. coli* BW25113 or *E. coli* JW2200-KC (Δ*alkB*) was picked and grown in unlabeled M9 medium (5 mL) overnight. From the first overnight culture, a 50 mL culture was prepared in unlabeled M9 medium and grown overnight. From the second overnight culture, 120 mL culture (OD_{600} of 1.0) was prepared in unlabeled M9 medium. After 60 min growth, the first aliquot (7 mL) was taken for RNA isolation. The remaining culture was split into two flasks of 56.5 mL each. One was exposed to MMS (95.7 µL, 20 mM final concentration) the other to water (MOCK) and inverted before both cultures were cultivated for 60 min. An aliquot (7 mL) was drawn from each culture, and the RNA was isolated. The remaining bacteria were centrifuged (1200 × *g*, 5 min), and the MMS/MOCK-containing supernatants were discarded. The bacteria pellets were washed with ^{15}N, ^{34}S and CD_3-methionine labeled M9 medium (5 mL), and each bacterial pellet was suspended in fresh $^{15}N/^{34}S/CD_3$ M9 medium (50 mL). The bacteria were allowed to grow and recover from the MMS/MOCK treatment. Seven millilitres of each bacterial culture were harvested after 1, 2, 3, 4, and 23 h. The RNA was isolated and the tRNA purified by SEC. The experiment was also done with lower volumes and different concentrations of MMS.

SAM and MMS incubation assay in vitro. 4.3 µL of a 93 µM s^2C synthetic standard solution (0.4 nmol) were mixed with 0, 500, 1000, 1500 or 2000 equivalents of SAM (32 mM stock) or MMS (20 mM stock) in a final volume of 100 µL containing 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0). The mixture was incubated at 37 °C for 60 min in a shaking heat block. After incubation, 900 µL water were added (1:10 dilution) and spiked with SILIS to be analyzed by quantitative LC-MS/MS.

AlkB in vitro assay. Five microlitres of purified tRNA (30 ng/µL) from 20 mM MMS exposed *E. coli* was mixed with 45 µL of AlkB. (45 µL AlkB was prepared with 7.5 µL KCl (100 mM), 1.5 µL freshly mixed sodium α-ketoglutarate (10 mM), 2.5 µL Tris buffer (1 M, pH 7.65), 5 µL freshly mixed sodium L-ascorbate (20 mM), 1.5 µL freshly mixed $Fe(II)(NH_4)_2(SO_4)_2$ (10 mM) and 1.5 µL of AlkB enzyme (39.1 µM, Peak Proteins, Cheshire, UK) and water.) The reaction mixture was incubated at 37 °C for 60 min. After incubation, 500 µL of $LiClO_4$ in acetone (2 vol%) was added for tRNA precipitation and mixed thoroughly. After 10 min at room temperature, the sample was centrifuged at 5000 × *g* for 10 min. The RNA pellet was washed with 70 vol% ethanol and resuspended in 20 µL water before digestion for LC-MS/MS analysis.

tRNA digestion for mass spectrometry. tRNA (100 ng) in aqueous digestion mix (30 µL) was digested to single nucleosides by using 0.2 U alkaline phosphatase, 0.02 U phosphodiesterase I (VWR, Radnor, Pennsylvania, USA), and 0.2 U benzonase in Tris (pH 8, 5 mM) and $MgCl_2$ (1 mM) containing buffer. Furthermore, 0.5 µg tetrahydrouridine (Merck, Darmstadt, Germany), 1 µM butylated hydroxytoluene, and 0.1 µg pentostatin were added to avoid deamination and oxidation of the nucleosides[30]. The mixture was incubated for 2 h at 37 °C and then filtered through 96-well filter plates (AcroPrep Advance 350 10 K Omega, PALL Corporation, New York, USA) at 3000 × *g* and 4 °C for 30 min. 1/10 Vol. of SILIS (stable isotope labeled internal standard) as prepared in[31] was added to each filtrate before analysis by QQQ mass spectrometry.

High resolution mass spectrometry. The ribonucleosides were separated using a Dionex Ultimate 3000 HPLC system on an Interchim Uptisphere120-3HDO C18 or a Synergi, 2.5 µm Fusion-RP C_{18}, 100 Å, 100 × 2 mm (Phenomenex®, Torrance, California, USA). Mobile phase A was 2 mM ammonium acetate and mobile phase B was 80% acetonitrile containing 2 mM ammonium acetate. Gradient elution started with 0% B and increased to 12% B after 10 min and to 80% after 12 min. After 4 min elution at 80% B and subsequently regeneration of starting conditions to 100% A after 5 min, the column was equilibrated at 100% A for 8 min. The flow rate was 0.2 mL/min and the column temperature 30 °C. High-resolution mass spectra of precursor and product ions were recorded by a ThermoFinnigan LTQ Orbitrap XL. The parameters of the mass spectrometer were tuned with a freshly mixed solution of adenosine (5 µM). The parameters were sheath gas flow rate, 16 arb; auxiliary gas flow rate, 11 arb; sweep gas flow rate, 4 arb; spray voltage, 5.0 kV; capillary temperature, 200 °C; capillary voltage, 20 V, tube lens 65 V.

QQQ mass spectrometry. For quantitative mass spectrometry an Agilent 1290 Infinity II equipped with a diode-array detector (DAD) combined with an Agilent Technologies G6470A Triple Quad system and electrospray ionization (ESI-MS, Agilent Jetstream) was used. Operating parameters: positive-ion mode, skimmer voltage of 15 V, cell accelerator voltage of 5 V, N_2 gas temperature of 230 °C and N_2 gas flow of 6 L/min, sheath gas (N_2) temperature of 400 °C with a flow of 12 L/min, capillary voltage of 2500 V, nozzle voltage of 0 V, and nebulizer at 40 psi. The instrument was operated in dynamic MRM mode.

For separation a Core-Shell Technology column (Phenomenex, Torrance, CA, USA; Kinetex 1.7 µm EVO C_{18}, 100 Å, 150 × 2.1 mm) at 35 °C and a flow rate of 0.35 mL/min were used in combination with a binary mobile phase of 5 mM NH_4OAc aqueous buffer A, brought to pH 5.6 with glacial acetic acid (65 µL), and an organic buffer B of pure acetonitrile (Roth, LC-MS grade, purity ≥.99.95). The gradient started at 100% solvent A, followed by an increase to 10% over 10 min. From 10 to 15 min, solvent B was increased to 45% and was maintained for 3 min before returning to 10% solvent A and a 3 min re-equilibration period.

Calibration. For calibration, synthetic nucleosides were weighed and dissolved in water to a stock concentration of 1–10 mM. Due to an unknown content of water and salts in the synthesized ms^2C standard, the concentration could not be determined through weighing. Therefore, the concentration of the stock solution was determined by comparison to s^2C containing isoacceptors after MMS exposure. The calibration solutions range from 0.3 to 500 pmol for each canonical nucleoside and from 0.3 to 500 fmol for each modified nucleoside and were spiked with 10% SILIS[20]. The sample data were analyzed by the Quantitative and Qualitative MassHunter Software from Agilent. The areas of the MRM signals were integrated for each modification and their isotope derivatives.

The absolute amounts of the modifications were referenced to the absolute amounts of the respective canonical. In the case of the pulse-chase experiment, the different isotopomers were referenced to their respective labeled canonicals, so that original modifications were referenced to original canonicals and new modifications were referenced to new canonicals. See Eqs. (1) and (2) for s^2C as an example in Supplementary Table 12.

Statistics. All experiments were performed at least three times (biological replicates) to allow student *t*-test analysis. *p*-values of student *t*-test (unpaired, two-tailed, equal distribution) were calculated using Excel or Graphpad Prism.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

A reporting summary for this Article is available as a Supplementary Information file. The source data underlying Figs. 1c, d, 2a–c, 3a, c, 4b–e, 5b, c and Supplementary Figs. 2a, 5–8, 10–18, 20, 21 as well as Supplementary Table 10 are provided as a Source Data file. All relevant data are available from the corresponding author upon reasonable request.

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Author contributions

V.R., D.P., K.J., and S.K. planned the experiments and wrote the manuscript. V.R., D.P. and V.W. conducted the experiments. V.R., D.P., and V.W. performed data analysis.

Competing interests

The authors declare no competing interests.

Additional information

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3.3. Synthese und metabolische Verfolgung von 4-Methylthiouridin in bakterieller tRNA

Prolog

Nachdem ms^2C als sehr prominenter Methylierungsschaden identifiziert werden konnte, drängt sich die Frage auf, ob weitere thiolierte Modifikationen vergleichbare Strukturen aufweisen. Die in Bakterien am häufigsten vorkommende schwefelhaltige Modifikation ist 4-Thiouridin (s^4U). Nach Stress mit MMS wird die Bildung von 4-Methylthiouridin (ms^4U) in der tRNA von *E. coli* mit Hilfe eines synthetischen Standards nachgewiesen. Anhand der relativ niedrigen Mengen an quantifiziertem ms^4U kann diese Modifizierung, entgegen der Häufigkeit an s^4U in der tRNA, eher als seltener Schaden eingeordnet werden. Unter Anwendung unserer NAIL-MS Methoden konnte festgestellt werden, dass nach Bildung von ms^4U unter Methylierungsstress der ms^4U Gehalt schnell wieder abnimmt. Dies erfolgt im Unterschied zu ms^2C nicht durch die Demethylase AlkB, jedoch häufiger über eine Dethiomethylierung mit anschließender Thiolierung des resultierenden Uridins zu s^4U.

„Synthesis and metabolic fate of 4-methylthiouridine in bacterial tRNA.", Christoph Borek[#], **Valentin F. Reichle**[#], Stefanie Kellner, *ChemBioChem* **2020**, *21*, 1-5.

Autorenbeitrag: Alle *in vivo* Experimente, *in vitro* Stabilitätstests und entsprechenden LC-MS/MS Messungen wurden von mir geplant und durchgeführt. Alle chemischen Synthesen wurden von Christoph Borek geplant und durchgeführt. Das Manuskript wurde unter Mitwirkung aller Autoren verfasst.

[#] Die Autoren haben zu gleichen Teilen beigetragen

Synthesis and Metabolic Fate of 4-Methylthiouridine in Bacterial tRNA

Christoph Borek[+],[a] Valentin F. Reichle[+],[a] and Stefanie Kellner*[a]

Ribonucleic acid (RNA) is central to many life processes and, to fulfill its function, it has a substantial chemical variety in its building blocks. Enzymatic thiolation of uridine introduces 4-thiouridine (s^4U) into many bacterial transfer RNAs (tRNAs), which is used as a sensor for UV radiation. A similar modified nucleoside, 2-thiocytidine, was recently found to be sulfur-methylated especially in bacteria exposed to antibiotics and simple methylating reagents. Herein, we report the synthesis of 4-methylthiouridine (ms^4U) and confirm its presence and additional formation under stress in *Escherichia coli*. We used the synthetic ms^4U for isotope dilution mass spectrometry and compared its abundance to other reported tRNA damage products. In addition, we applied sophisticated stable-isotope pulse chase studies (NAIL-MS) and showed its AlkB-independent removal *in vivo*. Our findings reveal the complex nature of bacterial RNA damage repair.

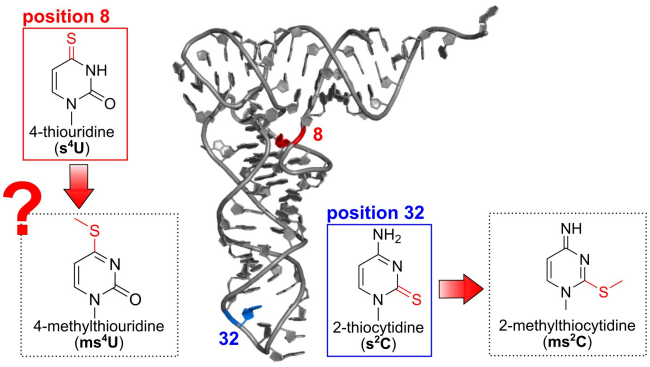

Figure 1. 3D structure of tRNA indicating the positions of enzymatically thiolated nucleobases in bacteria. Red: 4-thiouridine (**3**, s^4U) found at position 8 and the suggested structure of its methylated derivative 4-methylthiouridine (**4**, ms^4U). Blue: 2-thiocytidine at position 32 and its reported derivative 2-methylthiocytidine.

RNA and especially tRNA have complex structures to fulfill their important functions inside the organism. This is possible through the vast chemical variety of building blocks found in RNA. To date over 170 modifications to either ribose or nucleobase have been reported.[1] One group of unique tRNA modifications is enzymatic thiolation. In bacteria, thiolation of uridine (4-thiouridine, s^4U) is commonly found at position 8 of most tRNAs (red in Figure 1). s^4U is a target of ultraviolet light;[2] it leads to a reduced growth of bacteria exposed to UV and, as a consequence, saves bacteria from photomutagenic effects.[3] In addition, s^4U-hypomodified tRNAs were found to be targeted by the RNA degradosome; this leads to a reduced abundance of a subset of bacterial tRNAs.[4] Due to its sulfur decoration, s^4U is a nucleophile, and can be coupled with electrophiles such as bromomethylcoumarin[5] or iodoacetamide.[6] The latter is used to assess RNA transcription and stability after metabolic RNA labeling with exogenous s^4U (SLAM-Seq). Similar to SLAM-Seq, TUC-Seq uses metabolically introduced s^4U, which can be chemically converted to cytidine prior to RNA sequencing[7]. Despite its important function in bacterial tRNA and its broad use as a metabolic label for RNA sequencing, little is known about its chemical reactivity inside cells.

Another sulfur decorated tRNA modification, 2-thiocytidine (s^2C) (blue in Figure 1) has been recently found to be endogenously methylated[8] and efficiently repaired, potentially through its function as a modulator of translation.[9] A direct methylation of s^2C through electrophiles such as *S*-adenosylmethionine, methyl methanesulfonate (MMS) or antibiotics (streptozotocin) was observed. The resulting damage ms^2C (Figure 1) is substrate to the α-ketoglutarate dependent dioxygenase AlkB and repaired both *in vitro* and *in vivo* to restore tRNA function.[8] While s^2C is fully accessible to electrophiles in the anticodon loop of tRNA, s^4U is in tight interaction with nucleosides of the D- and T-loop and might be less accessible to electrophiles. This raises the question of whether s^4U is a target to direct methylation and if so, how much damage forms and how bacteria react to the damage.

To address these questions, we report here the synthesis of the suggested damage product ms^4U (**4**).

The synthesis of ms^4U (**4**) was first attempted *via* the formation of the fully acetylated corresponding 4-triazolic precursor which was meant to react with sodium thiomethanolate[10] to form the desired nucleoside. We encountered several problems in the key step due to partial deprotection of the ribose moiety, which led to further problems with the purification. Therefore, we decided to form ribose-protected 4-thiouridine (**2**) separately with subsequent methylation adopting a procedure for the corresponding 2′-

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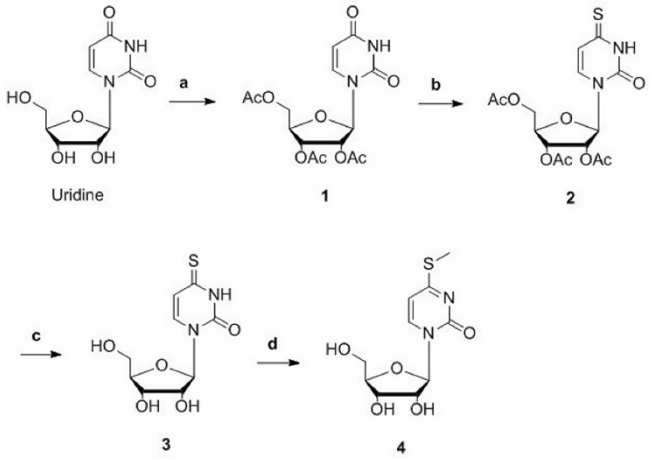
chlorine riboside.[11] The complete reaction is shown in Scheme 1. The initial peracetylation in neat acetic anhydride with catalytic amounts of iodine is a fast and reliable method to protect sugars in general which provided conversion of uridine to compound (**1**) in high yields. The subsequent formation of the 4-thiouridinic compound (**2**) by thiolation with phosphorus pentasulfide yielded 72%. It should be noted, that crystallization from ethanol, as described for the chlorinated compound, could not be observed. The deprotection was conducted by refluxing in concentrated aqueous ammonia solution, and s^4U (**3**) was received presumably in quantitative yield but was used as crude product in the next step. Of note, the more common method under Zemplén conditions[12] was not capable of deprotecting compound (**2**). In a final step, the thio group was selectively methylated by iodomethane to provide ms^4U (**4**) in a moderate overall yield of 40% over four steps.



Scheme 1. Synthesis of the key compound **4**: a) Ac_2O, I_2, RT, 45 min; b) P_2S_5, pyridine, reflux, 4 h; c) NH_4OH conc., reflux, 2 h; d) MeI, EtOH 50%, RT, 1 h.

With the synthetic standard in hands, we developed a sensitive LC-MS/MS method for detection of ms^4U in tRNA from unstressed *Escherichia coli*. With this targeted analysis, we found a peak in native tRNA that corresponds to the synthetic ms^4U in terms of retention time, precursor and product ion mass. In *E. coli* exposed to the LD_{50} dose of methyl methanesulfonate (MMS), the peak increased. A co-injection of the synthesized ms^4U standard and tRNA from MMS exposed *E. coli* grown in stable isotope labeled medium clearly showed 1) perfect co-elution and 2) the expected numbers of carbon, nitrogen, and sulfur atoms in native ms^4U (Figure 2A). In a next step, we confirmed the origin of the methyl group attached to the sulfur following our established methylome discrimination assay.[13] For this purpose, we grew *E. coli* in medium supplemented with $[CD_3]$-S-methionine; this leads to CD_3 labeling of all enzymatically placed methyl groups. After exposing *E. coli* to MMS, we found a high intensity signal for CH_3-methylated ms^4U and only a minor signal for CD_3-methylated ms^4U (Figure 2B). We thus prove the direct methylation of s^4U through the electrophile MMS in bacterial tRNA *in vivo*.

We were next interested to quantify the extent of ms^4U formation in unstressed and MMS-treated tRNA. For this purpose, a stable isotope labeled internal standard (SILIS) of ms^4U was produced by metabolic isotope labeling of *E. coli*. To increase the yield of stable isotope labeled ms^4U, MMS was added to the culture medium for 60 minutes, and the RNA was harvested and processed as previously described.[14] The combination of synthesized ms^4U and metabolically produced ms^4U-SILIS allowed accurate quantification of ms^4U and other modified ribonucleosides in bacterial tRNA (Figure 3). For normalization, we plotted the number of modified nucleosides per 10^6 canonical ribonucleosides (rN).

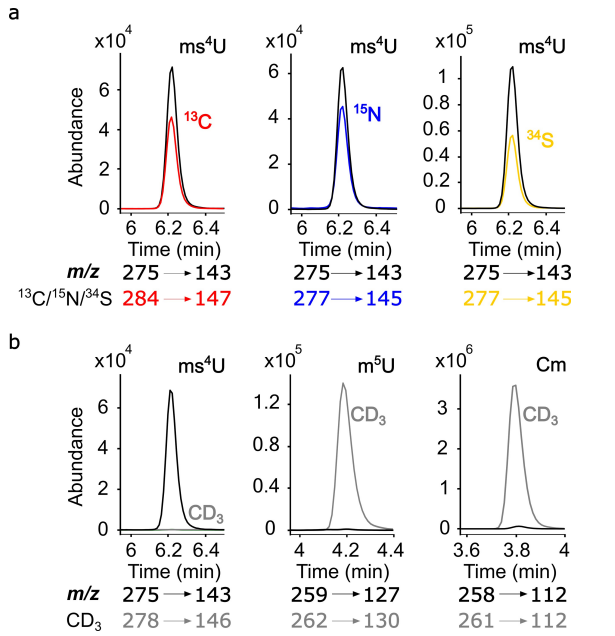


Figure 2. LC-MS/MS analysis of native and synthesized ms^4U. A) Co-injection of synthesized ms^4U (black) and digested tRNA from ^{13}C (red), ^{15}N (blue), and ^{34}S (yellow) metabolically labeled *E. coli* cultures. B) Native tRNA digests screening for enzymatically methylated nucleosides (gray, $[CD_3]$-S-methionine-derived) and damage-derived nucleoside methylation (black). Abbreviations: ms^4U: 4-methylthiouridine (**4**), m^5U: 5-methyluridine, and Cm: 2′-*O*-methylcytidine. The mass transitions (precursor ion→product ion) are given below the respective chromatograms.

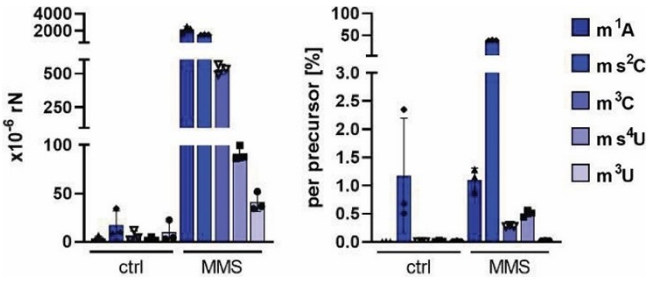


Figure 3. Absolute quantification of damage-derived nucleosides found in tRNA in control *E. coli* and *E. coli* exposed to 20 mM MMS. Left: per 10^6 rN (ribonucleosides) Right: per precursor [%]. Abbreviations: m^1A: 1-methyladenosine, ms^2C: 2-methylthiocytidine, m^3C: 3-methylcytidine, ms^4U: 4-methylthiouridine, and m^3U: 3-methyluridine. From 3 biological replicates. Error bars represent standard deviation.

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In tRNA of unstressed *E. coli*, we found 2.6×10^{-6} ms^4U/rN, which is less compared to the natural abundance of our recently described modification ms^2C (17×10^{-6} ms^2C/rN).[8]

After exposure to MMS, the abundance of the known tRNA damage products is 2147×10^{-6} m^1A/rN, 1543×10^{-6} ms^2C/rN, 2772×10^{-6} m^7G/rN, 530×10^{-6} m^3C/rN, 478×10^{-6} m^6A/rN and 41×10^{-6} m^3U/rN (Figure 3 and Figure S1a in the Supporting Information). ms^4U damage is with 91×10^{-6} ms^4U/rN comparable to m^3U damage in bacterial tRNA. This value appears to be rather low, but if the abundance of damage is normalized to the abundance of its respective precursor nucleoside (*e.g.*, m^1A per A or ms^4U per s^4U) a different conclusion must be drawn. With 0.5% ms^4U/s^4U, ms^4U is of comparable abundance to the known damage product m^1A (1.1% per A; Figures 3 and S1b). S4 in thiouracil is thus similarly reactive towards electrophiles such as MMS as is the N1 in adenine and the N7 in guanine. However, the S2 of thiocytosine is the strongest nucleophile and thus 38% of all s^2C become methylated to ms^2C in tRNA from *E. coli* exposed to MMS. Due to the importance of s^2C during translation, where it negates the wobble inosine binding to codons starting with adenine,[9] its efficient repair by enzymatic demethylation has been reported.[8]

s^4U is found at position 8 in 60%[1] of all bacterial tRNAs and in addition at position 9 in $tRNA^{Tyr}_{QUA}$ from *E. coli*. The chemical properties of sulfur are exploited by the bacteria for oxidative stress sensing through, for example, UV irradiation. Oxidative stress can be triggered by UV irradiation following iron-dependent Fenton chemistry. Therefore, s^4U acts as a sensor for UV irradiation,[15] which leads to delayed growth of bacteria during UV light exposure.[16] Given this important function of s^4U, we were wondering how cells react to tRNAs which have been methylated and carry ms^4U. For this purpose, we designed a pulse chase study based on our NAIL-MS expertise.

The goal of this assay is to discriminate the damaged tRNAs and exclude signals from tRNAs transcribed during recovery from MMS stress. Thus, we can follow the metabolic fate of ms^4U/rN independently from dilution by transcription. For this purpose, cells are grown in medium containing only ^{14}N and ^{32}S. Consequently, the RNA is completely labeled with ^{14}N, and all s^4U have a ^{32}S label (original s^4U), for example, *m/z* (s^4U) 261. In this medium, the bacteria are exposed to MMS (20 mM) and s^4U is converted to ms^4U and, for example, A to m^1A. After exposure, MMS is removed by exchanging the medium with stable isotopes containing medium. During the following recovery period, newly transcribed tRNA will be ^{15}N labeled, enzymatically methylated nucleosides will be CD_3 labeled and new s^4U will have a ^{34}S label (new s^4U, *m/z* 265 and new m^1A, *m/z* 290). The experimental design is shown in Figure 4A. Using LC–MS/MS analysis, we detect the formation of ms^4U during MMS exposure with around 50×10^{-6} ms^4U/original rN. In the subsequent recovery period, we traced the abundance of ms^4U and normalized it to the abundance of original rN. In wild-type *E. coli*, we saw a constant decrease in ms^4U over time (Figure 4B) which is comparable to the decrease found for ms^2C (Figure 4C). For ms^2C, we observed a slower repair in the absence of AlkB. Intriguingly, ms^4U loss is independent of AlkB. We concluded that AlkB is not the demethylase of ms^4U; this

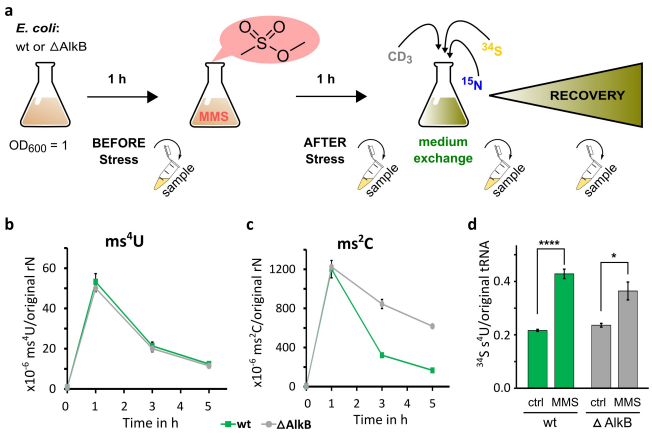

Figure 4. A) Principle of a pulse-chase NAIL-MS experiment. The bacteria are grown in unlabeled medium before and after exposure to MMS. After 1 h of exposure to MMS, the medium is replaced with [^{15}N]-, [^{34}S]- and [CD_3] methionine-containing medium. B) Formation and loss during recovery of ms^4U after exposure to 20 mM MMS in wild-type (wt, green) and AlkB-deficient (ΔAlkB, gray) *E. coli.* C) Formation and loss during recovery of ms^2C after 20 mM MMS exposure in wild-type (wt, green) and AlkB-deficient (ΔAlkB, gray) *E. coli.* D) Abundance of [^{34}S]ms^4U after 5 h in control (ctrl) and MMS-exposed wt and AlkB-deficient bacteria. All data from 3 biological replicates. Error bars represent standard deviation.

opens the way for two hypotheses. The first revolves around a potential, undescribed demethylase or dethiomethylase, which has ms^4U-damaged tRNA as substrate. SelU, a dethiogeranylase might be a potential candidate for this reaction[17]. From a chemical perspective, a direct dethiomethylation through attack of a nucleophile such as water is also theoretically possible. In both scenarios, ms^4U would dethiomethylate to uridine, which is again substrate for enzymatic thiolation. The re-thiolation during the recovery phase can be monitored by analysis of [^{34}S] incorporation into original tRNA. Our NAIL-MS study indeed indicates an increased formation of [^{34}S]-ms^4U in original tRNA from MMS stressed compared to unstressed bacteria (Figure 4D). This *in vivo* data hints at dethiomethylation of damaged tRNA that results in uridine.

While we cannot exclude the involvement of an unknown dethiomethylase, we tested the possibility of spontaneous ms^4U dethiomethylation. For this purpose, we simulated potential cellular environments and exposed synthesized ms^4U as free nucleoside prior to quantitative LC–MS/MS analysis (Figure S2). Dethiomethylation was observed after incubation with dithiothreitol (DTT). No dethiomethylation was observed under acidic/alkaline conditions, in growth medium or in the presence of cysteine or bovine serum albumin (BSA as an example protein).

In summary, we describe the existence of thiomethylated s^4U in bacterial tRNA. The low abundance of ms^4U indicates its formation as a lesion through the constantly present electrophile *S*-adenosylmethionine. During the exposure of bacteria to methylating agents such as MMS, RNA is damaged, and the methylation products of canonical nucleosides (m^1A, m^7G, m^3C, m^3U and m^6A) emerge.

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In addition, modified nucleosides with a pronounced nucleophilic character, such as s^2C and s^4U, become methylated. As evident from Figure 3 (right), s^2C is more prone to direct methylation than s^4U. This can be explained by both the chemical reactivity of the S2 in cytidine compared to the S4 in uridine and its location within the tRNA. Due to the exocyclic amine in cytidine, s^2C has an increased electron density, which improves its nucleophilic character over the S4 in uridine. Furthermore, the uridine S4 is more prone to solvation, which further decreases its nucleophilicity. In addition to the difference in nucleophilicity, s^2C is exposed and accessible in the anticodon loop of the tRNA, whereas s^4U is buried in the D-/T-loop fold.

Our studies reveal a differential reaction of the cells towards these forms of RNA damage. One class of lesions is repaired through enzymatic demethylation using an oxidative demethylation mechanism. Namely, m^1A, m^3C (Figure S3a, b) and ms^2C (Figure 4C) are substrate to enzymatic demethylation through AlkB. The second class comprises lesions that are lost from the RNA over time, but in an AlkB-independent manner (ms^4U and m^6A). The third class of RNA damage comprises m^7G, which is not removed from tRNA (Figure S3d).

Overall, the finding of ms^4U as a natural and stress-induced lesion in bacterial tRNA confirms the importance of tRNA modifications during stress response.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: epitranscriptome · modified nucleoside · NAIL-MS · RNA damage · tRNA

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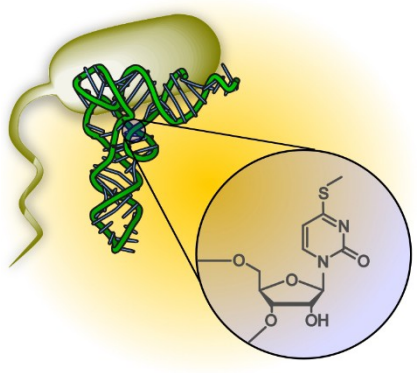
COMMUNICATIONS

Through a combination of synthetic and analytical chemistry, the structure of the modified nucleoside 4-methylthiouridine was confirmed in bacterial tRNA. The modification is of low abundance and forms after bacterial stress induced by methylating agents; this argues for its nature as a natural tRNA lesion. By pulse chase analysis, we show its repair through a dethiomethylation mechanism *in vivo*.



*Dr. C. Borek, V. F. Reichle, Dr. S. Kellner**

1 – 5

Synthesis and Metabolic Fate of 4-Methylthiouridine in Bacterial tRNA

3.4. Verschiedene NAIL-MS Ansätze in verschiedenen Organismen unter Betrachtung von Methylierungsstress

gemeinsamer Prolog

Methylierungsstress führt in der RNA von Bakterien zu ernsthaften, für das Bakterium existenzbedrohenden Schäden. Einige dieser Schäden wurden bisher nicht näher untersucht, da sie dieselbe Struktur wie metabolisch natürlich in die RNA eingebaute RNA-Modifikationen besitzen (m^6A, m^7G). Durch Deuterium-Markierung der Methylgruppen lässt sich in unserer NAIL-MS Methode geschädigte RNA von natürlicher RNA unterscheiden (*ChemBioChem* **2018**). Es wurde dabei auch ersichtlich dass m^3U in Folge von Alkylierungsstress in der tRNA auftreten kann. Dies erklärt auch die m^3U Signale in den Daten aus **Kapitel 3.1**. Die bereits unter **Kapitel 3.2** in *E. coli* angewendete NAIL-MS Pulse Chase Strategie kann indes auch in eukaryotischen Zellen wie Hefe (*RNA Biology* **2017**) oder sogar humanen Zellen angewendet werden (*Methods (San Diego, Calif.)* **2019**). Ausführliche Validierungen bezüglich der Isotopomeren-Reinheit wurden durchgeführt und werden diskutiert. Im Übrigen werden zudem weitere NAIL-MS Ansätze anhand von Beispielen beschrieben und erklärt.

„NAIL-MS in E. coli Determines the Source and Fate of Methylation in tRNA.“, **Valentin F. Reichle**, Verena Weber, Stefanie Kellner, *ChemBioChem* **2018**, *19*, 2575-2583.

Autorenbeitrag: Alle *in vivo* Experimente, die RNA Aufreinigung und Quantifizierung über LC-MS/MS wurden von mir geplant und durchgeführt. Die *in vitro* Experimente wurden von *Verena Weber* geplant und durchgeführt. Die Ausarbeitung des Manuskripts erfolgte durch *Stefanie Kellner* und mich.

„Observing the fate of tRNA and its modifications by nucleic acid isotope labeling mass spectrometry: NAIL-MS. “, Matthias Heiss, **Valentin F. Reichle**, Stefanie Kellner, *RNA Biology* **2017**, 14, 1260-1268.

Autorenbeitrag: Die Planung der NAIL-MS Experimente und die Etablierung der isotopenmarkierten Medien sowie die HRMS-Messungen wurden von *Matthias Heiss* und *Stefanie Kellner* übernommen. Ich habe in enger Zusammenarbeit mit *Matthias Heiss* und *Stefanie Kellner* bei der Durchführung der NAIL-MS Experimente und der

Herstellung des SILIS, bei der Datenauswertung sowie der Erstellung des Manuskripts geholfen.

„Surpassing limits of static RNA modification analysis with dynamic NAIL-MS.“, **Valentin F. Reichle**, Steffen Kaiser, Matthias Heiss, Felix Hagelskamp, Kayla Borland, Stefanie Kellner, *Methods (San Diego, Calif.)* **2019**, *156*, 91-101.

Autorenbeitrag: Die HRMS-Daten der bakteriellen RNA sowie die Daten der Pulse Chase Experimente wurden von mir bereitgestellt. Die Daten zum „Comparative NAIL-Experiment“ aus Abbildung 4 des Manuskripts wurden von *Steffen Kaiser* erhoben und die Daten für die Hefe-Kultivierung stammen von *Matthias Heiss*. Die Methoden und Daten zur Etablierung von NAIL-MS in Zellkultur wurden von *Matthias Heiss*, *Felix Hagelskamp* und *Kayla Borland* bereitgestellt. Bei der Erstellung des Manuskripts trugen alle Autoren gleichermaßen bei.

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Very Important Paper

NAIL-MS in *E. coli* Determines the Source and Fate of Methylation in tRNA

Valentin F. Reichle, Verena Weber, and Stefanie Kellner*[a]

In all domains of life, the nucleobases of tRNA can be methylated. These methylations are introduced either by enzymes or by the reaction of methylating agents with the nucleophilic centers of the nucleobases. Herein, we present a systematic approach to identify the methylation sites within RNA in vitro and in vivo. For discrimination between enzymatic tRNA methylation and tRNA methylation damage in bacteria, we used nucleic acid isotope labeling coupled mass spectrometry (NAIL-MS). With NAIL-MS, we clearly observed the formation of 7-methylguanosine, 3-methyluridine, and 6-methyladenosine during exposure of bacteria to the alkylating agent methyl methanesulfonate (MMS) in vivo. These damage products were not reported to form in tRNA in vivo, as they were masked by the enzymatically formed modified nucleosides in previous studies. In addition, we found formation of the known damage products 1-methyladenosine and 3-methylcytidine in vivo. With a dynamic NAIL-MS setup, we observed tRNA repair by demethylation of these two RNA modifications in vivo. Furthermore, we saw the potential repair of 6-methyladenosine but not 7-methylguanosine in bacterial tRNA.

Introduction

RNA, and especially tRNA, contains a vast variety of modified nucleosides. From the natural RNA modifications known to date (≈160), 70 contain a methylated base or ribose.[1] These methylations are enzymatically incorporated at defined positions of the RNA. In addition, nucleobases display several nucleophilic centers that are prone to reactions with electrophiles, and thus, non-enzymatic methylation and alkylation have been reported.[2] Currently, it is not possible to distinguish enzymatic methylation from direct chemical methylation.

Assessing the nucleophilicity of nucleosides in vitro

The reactivity of each position within the nucleobase is best assessed by exposing the nucleic acid or, even simpler, the nucleoside to the electrophile of interest in vitro. The reaction of DNA with various monoalkylating reagents was summarized in 1983.[2] The conclusion of all analyzed studies was "that all simple, direct-acting alkylating agents react with nucleic acid in vivo on the same sites as in vitro".[2] For cytidine and thymidine/uridine, alkylation of N3 was observed. In adenosine, the N1 position was most reactive, but N3 and N7 alkylation was also found, especially if adenosine was base paired. Guanosine was mainly alkylated at position 7 but also mildly at positions 2, 3, and 6. Figure 1 A shows the common alkylation sites of the canonical nucleosides as defined in the literature. The alkylation of nucleosides has been exploited in vitro for the introduction of functional groups such as coumarin derivatives with[3] and without[4] clickable moieties for further functionalization (Figure 1 A). Here, mainly uridine and thymidine (at position N3) were found to be alkylated under the alkaline reaction conditions.

Beyond the nucleophilic centers of the canonical nucleosides, some modified nucleosides of RNA contain additional nucleophilic sites. These are exploited for the detection of modified ribonucleosides by reaction with various electrophiles, as recently reviewed.[5]

RNA damage and repair

In vivo, alkylation of DNA bases is linked directly to genomic instability, whereas the alkylation of RNA bases is believed to have a less dramatic effect. Interestingly, methylation of adenosine in RNA is as efficiently recognized and repaired as DNA alkylation damage. The key players of ribonucleic acid demethylation in both bacteria and mammals are the α-ketoglutarate-dependent dioxygenases AlkB and AlkBH, respectively.[6] Previously reported in vitro tests showed that methylation of position 1 of adenine and position 3 of cytosine was quickly repaired by AlkB, as these positions were necessary for correct base pairing[7] (Figure 1 B). The authors also showed that MS2 phage RNA, inactivated by methylation, became reactivated upon bacterial expression of AlkB, and thus, the importance of

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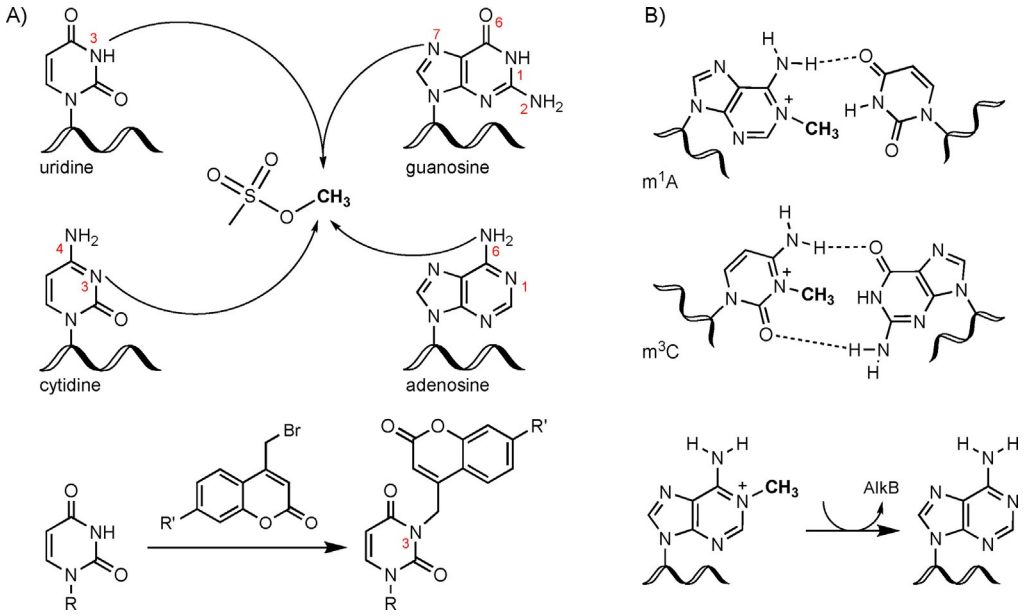

Figure 1. Nucleic acid alkylation sites; the impact on base pairing and repair by AlkB. A) Nucleophilic sites in nucleobases as indicated with the alkylating agent MMS or bromomethylcoumarins (e.g., R′ = azide or propargyl). B) Methylation of N1 in adenine (m^1A) and N3 in cytosine (m^3C) disrupts base pairing and is, thus, repaired by AlkB in bacteria.

RNA methylation repair was presented in vivo. Later studies identified repair of the same methylated bases in mRNA and tRNA. Here, the negative effects on translation and aminoacylation caused by methylation damage were rescued by AlkB.[8] Intriguingly, in this study only 3-methylcytidine (m^3C) and 1-methyladenosine (m^1A) were shown to be repaired in tRNA, but not 7-methylguanosine (m^7G). One reason might be that m^7G is commonly found in many bacterial tRNAs (e.g., m^7G48 in tRNAs Arg, Phe, and Val).[1] It is possible that bacteria cannot distinguish natural m^7G from damage-derived m^7G, and thus, m^7G is not a substrate of demethylation by AlkB. The authors also described a pulse-chase study based on radioactively labeled adenine, which clearly proved m^1A repair by AlkB in vivo after previous induction of AlkB.[8] m^1A and m^3C in bacterial small RNA (mostly tRNA) were identified to account for 99% of the nucleic acid lesions formed upon exposure with methyl methanesulfonate (MMS).[9] As a result of RNA turnover, RNA biosynthesis, and dilution effects, it was not possible to measure the RNA repair in vivo. Nevertheless, the authors demonstrated an accumulation of m^1A in the AlkB-deficient strain, which again hinted towards active repair of the lesion by AlkB.

Stefanie Kellner received a pharmacy degree in 2009 from the University of Heidelberg. She then joined Mark Helm's lab at Johannes Gutenberg University (Mainz) where she received her PhD in 2012. After a one-year postdoc in the Helm lab, she moved to MIT (Boston) to work with Peter Dedon on the DNA phosphorthioate modification of bacteria. In 2016, Stefanie started her own lab at Ludwig Maximilians University (Munich). The main focus of her research is the analytical chemistry of nucleosides, especially modified RNA nucleosides. Since 2017, she has been funded by the Emmy Noether Program of the DFG.

Nucleic acid isotope labeling as a tool to observe nucleic acid repair in vivo

Radioactive labeling of RNA previously allowed observation of m^1A demethylation in vivo.[8] Although the radioisotope labeling approach was ideal to follow the fate of a biological mark, no further studies to explore the demethylation of nucleic acids were conducted. This is most likely due to the regulatory hurdles of running radioisotope laboratories. In 2017, we implemented isotope-labeling techniques that could be done without radioisotopes. The prerequisite for these studies was the complete labeling of the nucleic acid with heavy, non-radioactive isotopes such as carbon-13, nitrogen-15, and sulfur-34 and access to a mass spectrometer. The combination of the different labeling media allowed the creation of a pulse-chase experiment, which was used to observe repair of phosphorothioates in bacterial DNA.[10] In the same year, we adapted the approach to RNA modification analysis in yeast.[11] We followed the modification density of tRNA as a function of the growth phase, and we identified the underlying mechanisms for several modified nucleosides.

Herein, we present a systematic approach to identify the methylation sites within RNA both in vitro and in vivo. For discrimination of enzymatic RNA methylation and RNA methylation damage, we use nucleic acid isotope labeling coupled mass spectrometry (NAIL-MS). With the presented method, we show RNA demethylation in vivo by overcoming the biases

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introduced by RNA turnover, RNA transcription, and dilution. With the strength of NAIL-MS, it is possible to observe the dynamics of RNA modifications and to study RNA repair in vivo.

Results and Discussion

In vitro methylation of nucleosides reveals the most nucleophilic centers

Systematic assessment of nucleoside reactivity requires defined reaction conditions, a reliable way to stop the reaction, and an analytical system for quantification of the reaction products. With the goal to compare the in vitro damage products of the ribonucleosides with the damage products found in tRNA in vivo, we decided to use reaction conditions similar to those of the in vivo environment. The ribonucleosides were incubated in aqueous conditions at pH 7, 100 mM ionic strength, and 37 °C for 60 min. As quenchers, we tested several sulfur-containing nucleophiles for their ability to suppress reaction of canonical nucleosides with the methylating agent MMS. Here, we found dithiothreitol (DTT) at pH 8 in 100-fold excess to be a successful quencher that completely suppressed further methylation after its addition.

An equimolar mixture of the canonical nucleosides cytidine, uridine, guanosine, and adenosine was exposed to increasing doses of MMS at pH 7. After the reaction was quenched by the addition of DTT in alkaline buffer, the stable isotope labeled internal standard from yeast was added,[11] and the number of methylated nucleosides was determined. The quantities of each methylation were normalized to the respective canonical they were derived from and were plotted as damage in percent (Figure 2A for 1-methyladenosine and Figure S1 in the Supporting Information for the other nucleosides). Under equimolar conditions, 0.15 ± 0.09 % of all adenosines were methylated at the N1 position (m^1A). A tenfold excess amount of MMS led to the formation of 0.62 ± 0.02 % m^1A, and a 100-fold excess amount led to nearly 1 % m^1A (0.97 ± 0.2 %). Overall, the N1 of adenosine was one of the most nucleophilic positions and was easily methylated at neutral pH. Figure 2B summarizes the found methylation sites within RNA nucleosides. Position 7 of guanosine was slightly more reactive than position 1 in adenosine. In the same order of magnitude, methylation of position 3 in cytidine and uridine was observed. We also detected the formation of methylation products at positions 1, 2, and 6 in purine nucleosides; however, these sites were only methylated once every 10 000 nucleosides. As expected, no reaction of position 5 in pyrimidines, position 3 in purines, or at the 2′-OH of the ribose was observed in our studies.

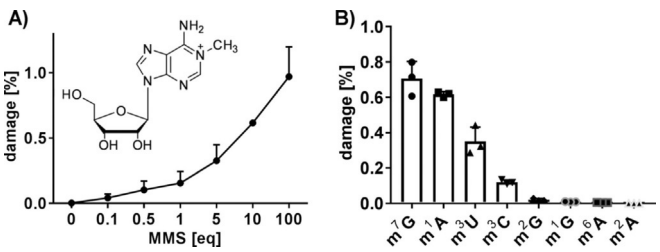

Figure 2. In vitro methylation of nucleosides (error bars represent the standard deviation of three replicate experiments). A) Formation of 1-methyladenosine (m^1A) during exposure of adenosine with increasing amounts of MMS starting from 0.1 equivalents MMS per A (tenfold excess A) up to 100 equivalents MMS per A (100-fold excess MMS). B) Quantities of methylated nucleosides formed after exposing a mixture of canonical nucleosides to a tenfold excess amount of MMS (10 equiv.). Abbreviations: m^7G, 7-methylguanosine; m^1A, 1-methyladenosine; m^3U, 3-methyluridine; m^3C, 3-methylcytidine; m^2G, 2-methylguanosine; m^1G, 1-methylguanosine; m^6A, 6-methyladenosine; m^2A, 2-methyladenosine.

Distinguishing enzymatic methylation marks from RNA damage in vivo

From our in vitro data, the nucleosides 7-methylguanosine (m^7G), 1-methyladenosine (m^1A), 3-methyluridine (m^3U), and 3-methylcytidine (m^3C) seemed to be the most prominent damage products within RNA. To our knowledge, m^1A and m^3C have not been reported as natural modifications in *Escherichia coli* tRNAs,[12] whereas m^7G is a natural tRNA modification and 3-methyluridine is a natural ribosomal RNA modification in *E. coli*.[1] Therefore, it was not yet possible to determine reliably the quantities of, for example, N7 methylation damage of guanosine in vivo.[13] NAIL-MS overcame this limitation. The prerequisite for a discriminatory NAIL-MS experiment was the availability of a heavy isotope-labeling medium, which could label either the damaged or the natural product (e.g., here, methylation or thiolation[10]). Feeding organisms with CD_3-methionine led to the formation of CD_3-SAM and transfer of heavy methyl marks onto the RNA (Figure S2) by the respective enzymes.

With this labeling tool, we could study the methylation damage formation of position 7 in guanosine in the presence of enzymatically formed m^7G by NAIL-MS. For this purpose, the bacteria were cultured in CD_3-methionine-containing M9 medium, which resulted in the formation of heavy, enzymatically methylated nucleosides, for example, CD_3-m^7G (*m/z* 301). Exposure to MMS led to the formation of nucleoside damage products, for example, CH_3-m^7G (*m/z* 298), which is three mass units lighter than the enzymatic CD_3-m^7G. With this NAIL-MS-based RNA-methylome discrimination assay (concept shown in Figure 3A), we could distinguish all enzymatically methylated nucleosides ($m/z + 3$) from the MMS-derived methylation marks (regular CH_3, $m/z \pm 0$) by mass spectrometry.

With the goal to determine the most nucleophilic centers of canonical ribonucleosides in vivo, we first defined the median lethal dose (LD_{50}) of MMS in minimal M9 medium in *E. coli* (BW25113) by colony counting after exposure for 60 min (Figure S3).

The bacteria were grown in the presence of CD_3-methionine and were exposed to the LD_{50} of MMS for 60 min. The total RNA was extracted, and the total tRNA was purified by size-exclusion chromatography[14] and subsequently digested to nucleosides for mass spectrometry analysis. The mass spectrometer was programmed to detect all canonical nucleosides and their methylated derivatives. The enzymatically and MMS-methylated nucleosides were distinguished by their +3 difference in the *m/z* values of the precursor and product ions. Exemplary mass transitions are given for all possible m^7G isotopomers in

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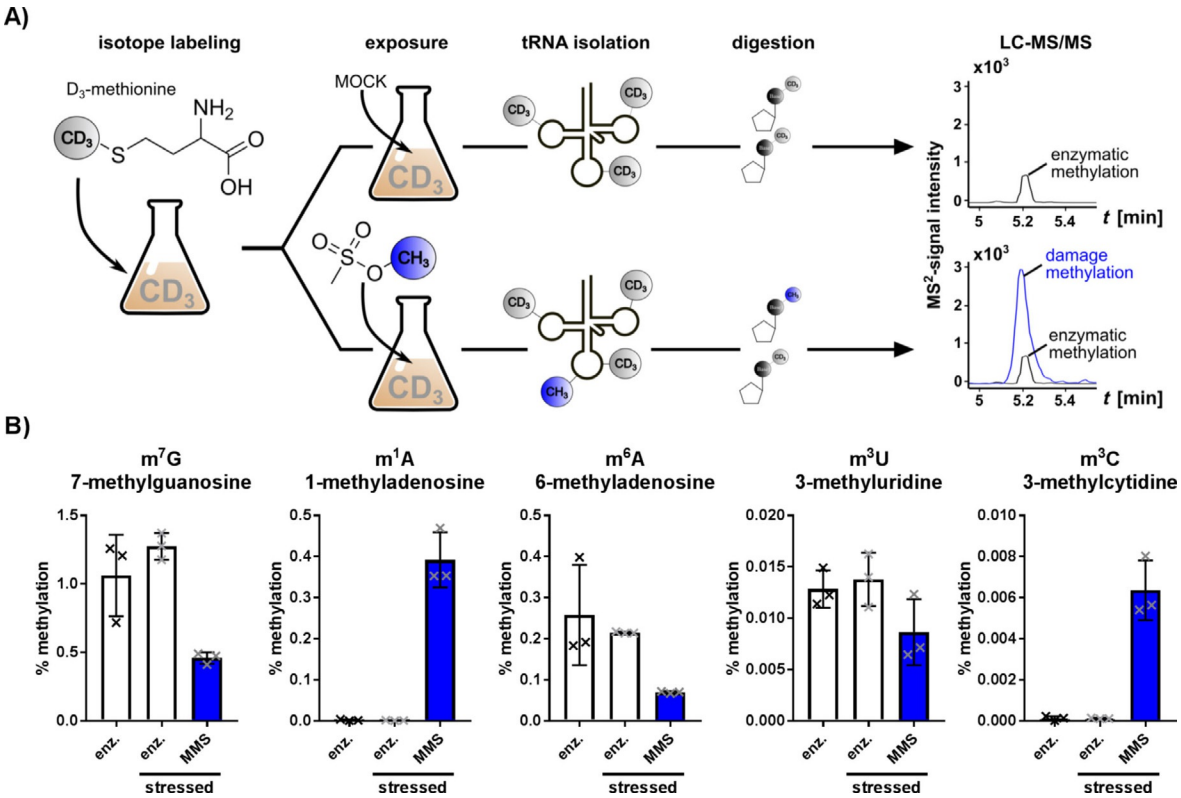

Figure 3. Concept sketch of the RNA methylome discrimination assay. A) CD_3-methionine-containing medium is used for metabolic labeling of native tRNA methylations. Enzymatic methylation (black) and MMS-derived methylation (blue) are distinguished by mass spectrometry. B) Methylation sites in canonical ribonucleosides found in vivo and sorted by abundance (left: high; right: low). The abundance of methylated nucleosides from unstressed cells is compared to that of enzymatically derived nucleosides (white) and MMS-derived nucleosides (blue bars) from MMS-exposed cells. Error bars represent the standard deviation of three biological replicates.

Table 1. Mass transitions of 7-methylguanosine isotopomers in methylation discrimination NAIL-MS.

Nucleoside	Labeling[a]	Precursor[b]	Product[c]	t_R [min]
guanosine	unlabeled	284	152	3.7
	SIL-IS[d]	299	162	3.7
7-methylguanosine	unlabeled[e]	298	166	2.0
	CD_3-label[f]	301	169	2.0
	SIL-IS	314	177	2.0

[a] Used labeling technique. [b] Precursor ion *m/z*. [c] Product ion *m/z*. [d] SIL-IS: stable isotope labeled internal standard prepared from a complete $^{13}C/^{15}N$-labeled *E. coli* culture. [e] From MMS damage. [f] Labeling with CD_3-methionine to detect enzymatic methylation.

Table 1, and the complete list is given in Table S2. The quantities of each methylated nucleoside were normalized to the abundance of its respective canonical nucleoside, and thus, the percentage methylation of a specific site was calculated. The summarized results in Figure 3B show that five methylation sites were found in vivo, namely, m^7G, m^1A, 6-methyladenosine (m^6A), m^3U, and m^3C. No other MMS products could be detected (Figure S4). The highest number of damage was found for guanosine with 0.46 ± 0.034% m^7G per guanosine and adenosine with 0.39 ± 0.05% m^1A per adenosine. With our RNA methylome discrimination, we thus revealed position 7 of guanosine to be the main target of methylation damage in tRNA in vivo. This finding is in accordance with the reported reactivity and our observed reactivity of the N7 position in vitro. However, owing to the natural abundance of m^7G in tRNA,[1] this damage was overlooked in past studies. Another, yet unreported, in vivo damage site is position 6 of adenosine (m^6A). The damage is with 0.07 ± 0.002% per adenosine less prominent than that in the main alkylation sites m^7G and m^1A. Nevertheless, m^6A is more prominent than the in vitro found methylation products m^3U and m^3C, which account for less than 0.008% damage per respective canonical. We also found the formation of m^3U, which is usually a prominent ribosomal RNA modification in *E. coli*. In our hands, m^3U was also formed as a damage product in tRNA. In summary, we found the same major reaction products in the in vitro and in vivo reactions of MMS with nucleobases (m^1A and m^7G). The different reactivity observed for cytidine and uridine was most likely caused by lower accessibility of the N3 position as a result of base pairing in vivo. We also observed base pairing as the main reason for the formation of m^6A in vivo. Although in vitro the N1 of adenosine was found to be the most reactive center, it was blocked because of base pairing in vivo, and thus, the N6 position became an easily accessible nucleophile for reaction with MMS. In our hands, we did not see statistically significant adaptation of enzymatically introduced tRNA modifications after MMS exposure.

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Observing the repair of methylation damage in *E. coli* tRNA by pulse-chase NAIL-MS

The positions within the nucleobases that are damaged by MMS methylation are mainly needed for correct base pairing, and thus, their enzymatic repair is crucial for cell homeostasis and survival. The repair of m^1A was shown by radioisotope labeling in vivo.[8] To our knowledge, there are no reports that present evidence for the in vivo demethylation of the other MMS damaged products. In the past, we used a combination of isotope labeling in bacteria[10] and yeast[11] and pulse-chase experiments to determine the fate of nucleic acid modifications. Here, we adapted these NAIL-MS assays to follow the fate of damaged tRNAs and their damage-derived methylated nucleosides in *E. coli*. The assay was started in unlabeled medium, and thus, all canonical nucleosides and all methylated nucleosides were unlabeled. These unlabeled *E. coli* cultures were exposed to MMS, and thus, canonical nucleosides in the tRNA received an unlabeled methyl group. In this assay, damage methylation and enzymatic methylation could not be distinguished. After 60 minutes' exposure, MMS was removed by medium exchange. The new medium contained nitrogen-15, which resulted in labeling of +5 for purines, +3 for cytidine, and +2 for uridine in the newly transcribed tRNAs. Furthermore, we used CD_3-methionine, which is typically used to study additional methylation of the original tRNAs (mass increase of +3) in case of enzymatic adaptation of the tRNA modification profile. The assay was set up as outlined in Figure 4A. Using mass spectrometry, the MMS-exposed tRNA and the newly transcribed tRNA could be clearly distinguished, and the abundance of modified nucleosides in the damaged, original tRNA could be quantified. An exemplary list for the mass transitions for all observed m^7G isotopomers is given in Table 2 and for the other nucleosides in Table S3.

After MMS exposure for 1 h, the damage products m^1A and m^3C were formed with around 1% damaged adenosines and only 0.08% damaged cytidines (Figure 4B, top row) in tRNA. The abundance of m^1A decreased in the damaged tRNA over the 24 h recovery period. The reason for the decrease in m^1A abundance was most likely active demethylation (potentially by AlkB[8]). With our experimental setup of the NAIL-MS experiment, we could clearly distinguish damaged tRNA from freshly transcribed tRNAs. Thus, the decrease in m^1A was not caused by dilution effects from tRNA transcription but by a reduction in m^1A in the damaged tRNA. We observed even more pronounced removal of m^3C from the damaged tRNAs during the recovery timeframe. With our NAIL-MS assay, we report for the first time, repair of m^3C in vivo. The repair was more efficient for m^3C than for m^1A (Figure 4B), and this was most likely caused by the low abundance of m^3C sites in the tRNAs. Unfortunately, the abundance of m^3U in the samples was too low for quantification. Thus, we could not follow the fate and potential repair of m^3U in vivo. For m^6A and m^7G (Figure 4B, bottom), which occur from enzymatic and MMS methylation in *E. coli* tRNAs, we observed a significant increase after MMS exposure. This increase was most likely due to active methylation by MMS, as shown in the RNA methylome discrimination assay

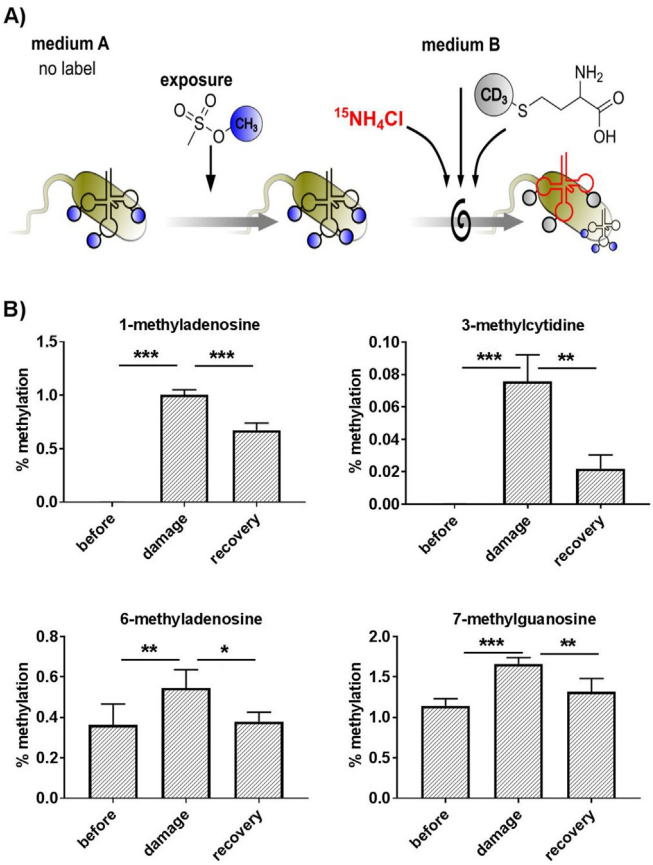

Figure 4. A) Concept of a pulse-chase NAIL-MS assay to study the repair of methylated nucleosides by using isotope labeling to distinguish MMS-damaged tRNAs (black, from medium A) and newly transcribed tRNAs (red, from medium B). B) Abundance of methylated nucleosides before MMS exposure, after 1 h of exposure to MMS, and after 24 h of recovery. Error bars represent the standard deviation of three biological replicates. *P* values of student t-test are indicated as $*p<0.05$, $**p<0.01$, $***p<0.005$.

Table 2. Mass transitions of 7-methylguanosine isotopomers in pulse-chase NAIL-MS for RNA repair observation.

Nucleoside	Labeling[a]	Precursor[b]	Product[c]	t_R [min]
guanosine	unlabeled[d]	284	152	3.7
	^{15}N-label[e]	289	157	3.7
	SIL-IS[f]	299	162	3.7
7-methylguanosine	unlabeled	298	166	2.0
	CD_3-label[g]	301	169	2.0
	$^{15}N/CD_3$-label[e]	306	174	2.0
	SIL-IS	314	177	2.0

[a] Used labeling technique. [b] Precursor ion *m/z*. [c] Product ion *m/z*. [d] No labeled metabolites, original nucleosides. [e] tRNA transcribed in the presence of ^{15}N, de novo nucleosides. [f] SIL-IS: stable isotope labeled internal standard prepared from a complete $^{13}C/^{15}N$-labeled *E. coli* culture. [g] Labeling with CD_3-methionine to detect enzymatic methylation of original nucleoside, methylated after stress.

(Figure 3B). In the discrimination assay, we observed that roughly 30% of all m^7G and m^6A was derived from direct MMS methylation (Figure 3B). In our pulse-chase assay, for which damage and enzymatic methylation are not distinguishable,

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we observed an increase in m^7G and m^6A of again approximately 30% (Figure 4B, bottom).

For both m^7G and m^6A, we detected a decrease during the recovery period, which hinted towards demethylation repair of these damaged nucleosides. Intriguingly, the abundance of m^6A dropped from the elevated damage level to the pre-exposure level. For m^7G, the abundance remained elevated relative to the starting abundance. This indicated that the main methylation damage in bacterial tRNA, m^7G, was repaired more slowly than m^1A, m^3C, and m^6A or, potentially, not at all.

Intrigued by this finding, we wanted to study the behavior of m^6A and m^7G in the original, but damaged tRNAs over time. For comparison, we took unstressed *E. coli* and analyzed the behavior of modifications in the original tRNAs by pulse-chase NAIL-MS as shown in Figure 4A. Figure 5A shows the principle of the analysis. To assess the impact of tRNA degradation and tRNA transcription on the modification content, we plotted the ratio of original canonicals and new canonicals over time. With the presented setup of the NAIL-MS experiment, we observed dilution of the original nucleosides, which was caused by a combination of tRNA degradation and transcription (Figure S5). The assay revealed deeper insight into the differences in the stressed and unstressed cells. The transcription and/or degradation rate of tRNA seemed to be faster in unstressed cells than in stressed cells in the first 3 h after medium exchange. This indicated that there was no massive degradation of tRNAs in the first 3 h of recovery. After that, the ratio flipped, and we found that the original nucleosides were more diluted in the stressed cells than in the unstressed cells. This was potentially caused by faster transcription in the stressed cells or by excessive tRNA degradation of damaged tRNA in the stressed cells after 3 h.

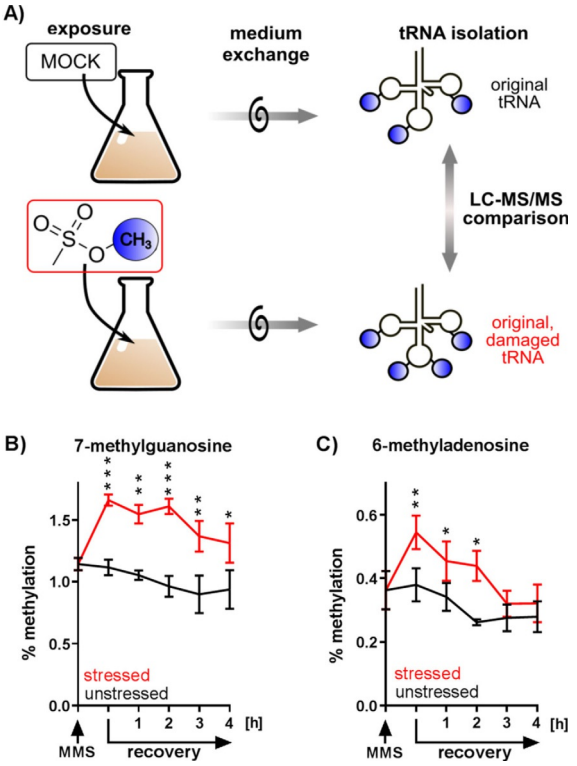

Figure 5. Pulse-chase NAIL-MS study of MOCK- and MMS-incubated bacterial cultures. A) Principle of the study for comparison of the percent methylation in original tRNAs. original tRNAs: tRNAs present during MOCK incubation; original, damaged tRNAs: tRNAs present during the MMS exposure. B) 7-Methylguanosine abundance in stressed (red) and unstressed (black) tRNAs. C) 6-Methyladenosine abundance in stressed (red) and unstressed (black) tRNAs. Error bars represent the standard error of three biological replicates. *P* values of student t-test for significance between stressed and unstressed sample at the same timepoint are indicated as $^*p<0.05$, $^{**}p<0.005$, $^{***}p<0.001$.

We could thus exclude tRNA degradation as the main pathway of tRNA damage repair in the first 3 h after MMS removal. Figure 5B, C shows the results of the methylation content in the original tRNA from stressed (red) and unstressed (black) bacteria.

The abundance of m^7G before MMS exposure was around 1.1%. After MMS exposure, the m^7G abundance rose to 1.6%, whereas the unstressed cells remained at around 1.1% methylation. The difference in m^7G abundance in the stressed and unstressed cells was statistically significant throughout the observed timeframe (Figure 5B). After recovery for 3 h, the abundance of m^7G decreased in the stressed bacteria, but it stayed elevated compared to the unstressed bacteria. This is in stark contrast to the results we saw for m^6A. In Figure 5C, we observed an initial increase in the abundance of m^6A, followed by a rapid decrease in the first 3 h of recovery. In fact, after the 3 h recovery phase, we did not see any statistically significant difference in the abundance of m^6A in the stressed and unstressed bacterial tRNAs. As we excluded tRNA degradation as a tRNA repair mechanism, the fast return of m^6A to the starting abundance hinted towards a demethylation process of m^6A. For m^7G, we did not have any such indication, as the abundance of m^7G stayed elevated in the stressed bacterial tRNAs. Future experiments will deepen our understanding of this intriguing finding.

We also analyzed other modified nucleosides in the bacterial tRNAs. Unlike m^7G and m^6A, we did not observe any difference in the abundance of other modified nucleosides upon comparing the tRNAs from stressed and unstressed cells. This was more proof that the overall compositions of the tRNA pools of the stressed and unstressed bacteria were comparable. We observed a minor decrease in the abundances of m^1G and m^5U in the original tRNAs from both stressed and unstressed bacteria (Figure S6). This decrease was also seen in the abundances of m^7G and m^6A in unstressed cells (Figure 5B,C). This was most likely growth-phase dependent, as already described for *Saccharomyces cerevisiae* by using NAIL-MS.[11]

Conclusion

In the presented work, we systematically defined the main target sites for methylation within ribonucleosides in vitro and in vivo. Using MMS as an electrophile, we found position 7 of guanosine to be the main target in vitro. Position 1 of adenosine was found to be similarly reactive. Furthermore, position 3 of cytidine and uridine were prominent targets for methylation. As expected, we observed no methylation of non-nucleo-

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philic positions such as C5 in cytidine and the 2′-OH of the ribose moiety.

Although 7-methyl-2′-deoxyguanosine was described as a damage product of DNA methylation, m^7G in RNA has not yet been observed as a damage in vivo.[9] In fact, m^1A was described to be the main methylation product of MMS damage in bacterial RNA. The detection of m^7G as a damage product was impossible in previous studies, as m^7G is also formed enzymatically and, thus, damage and enzymatic m^7G could not be distinguished. We developed a NAIL-MS-based methylome discrimination assay that found m^7G to be a major damage product of bacterial tRNAs. After 60 minutes' exposure of *E. coli* to MMS, we found that about 0.5% of all guanosines and adenosines were methylated. The results of our in vivo methylome discrimination assay nicely reflected our in vitro results in terms of these major damage products. For the minor methylation products m^3U and m^3C, we observed less formation in vivo than in the nucleoside-based in vitro assay. We assumed that in vivo base pairing protected the N3 position of these nucleosides from the reaction with MMS. We also found a damage product that we did not expect from our in vitro studies, namely, m^6A. Although in vitro the N1 of adenosine was the most reactive center, it was blocked in vivo because of base pairing, and thus, the N6 position became an easily accessible nucleophile for reaction with MMS. Similar to m^7G, m^6A is a damage product that was previously masked by the abundance of enzymatically produced m^6A in bacterial tRNA.

With the adapted pulse-chase NAIL-MS assay, we followed the fate of the MMS-damaged tRNAs over time. For m^1A, repair by active demethylation was reported in the past.[8] We also found repair of m^1A by demethylation in vivo. However, the decrease that we found in the m^1A abundance was slower than that in the 2004 publication. This discrepancy was explained by the fact that we used regular *E. coli* bacteria, whereas the study in 2004 used bacteria with an induced AlkB demethylase. AlkB is not constitutively expressed, but sublethal doses of MMS lead to its expression within 2 h and a plateau after 4 h of continuous MMS exposure.[9] Thus, they observed immediate repair, whereas we saw no repair in the early recovery phase but only at later time points after MMS induced expression of AlkB.

In addition to the repair of m^1A, we clearly saw the repair of m^3C in vivo. To our knowledge, this is the first report of m^3C repair in vivo upon alkylation stress. Intriguingly, the repair of m^3C was more efficient than the repair of m^1A. One factor might have been the relative low abundance of m^3C in the damaged tRNAs (less than 0.01%) relative to that of m^1A with approximately 0.5% (compare Figures 3B and 4B). It is also possible that m^3C is a better substrate for the demethylase than m^1A. Although we assumed that AlkB, as previously reported, repaired m^1A and m^3C damage, in this study we focused on the chemical fate of the tRNA and not on the biological players of repair. Currently, AlkB is the only known demethylase in *E. coli*, but future studies will reveal its substrate specificity.

Specifically, the results we received for m^6A indicated potential repair of damage-derived m^6A nucleosides in bacterial tRNAs. Here, a systematic screen for potential demethylases in *E. coli* might reveal the biological impact of our observation.

The combination of nucleic acid isotope labeling with mass spectrometry is a powerful tool to study damage and repair of RNA. With NAIL-MS, we uncovered in vivo two MMS damage products that could previously not be detected in RNA. Furthermore, we followed the fate of the damaged RNA and observe its repair mechanisms in vivo.

Experimental Section

Salts, reagents, isotopes, and nucleosides: All salts were obtained from Sigma–Aldrich (Munich, Germany) at molecular biology grade unless stated otherwise. The isotopically labeled compounds $^{15}NH_4Cl$ (> 98 atom%) and [D_3]-L-methionine (98 atom%) were also obtained from Sigma–Aldrich. All solutions and buffers were made with water from a Millipore device (Milli-Q, Merck). The nucleosides adenosine, cytidine, guanosine, uridine, and *N*2-methylguanosine (m^2G) were obtained from Sigma–Aldrich. 1-Methyladenosine (m^1A), 2-methyladenosine (m^2A), *N*3-methylcytidine (m^3C), *N*6-methyladenosine (m^6A), 7-methylguanosine (m^7G), 5-methylcytidine (m^5C), 5-methyluridine (m^5U), 2′-*O*-methylcytidine (Cm), 2′-*O*-methylguanosine (Gm), 1-methylguanosine (m^1G), and 3-methyluridine (m^3U) were obtained from Carbosynth (Newbury, UK).

In vitro methylation of nucleosides: For the in vitro methylation assay, a canonical stock solution was prepared containing 1 mM adenosine, guanosine, cytidine, and uridine, respectively. The stock solution (10 μL) was incubated with potassium phosphate buffer (10 μL, 50 mM, pH 7) and methyl methanesulfonate (MMS; 0.1 mM, diluted in water, 10 μL) at 37 °C for 1 h. Following incubation, potassium phosphate buffer (60 μL, 50 mM, pH 9) was added before the reaction mixture was quenched with freshly prepared dithiothreitol (10 μL, 0.01 M). The same procedure was used to conduct the assay with MMS concentrations of 0.5, 1, 5, 10, 100 mM as well as a water control. The reactions were stopped by the addition of DTT in a hundredfold excess. As part of the sample preparation, the sample solution (10 μL) was diluted with water (990 μL). An aliquot (18 μL) of the dilution was transferred into a vial and was combined with a yeast-derived, stable isotope labeled internal standard (SIL-IS; 2 μL).[5]

In vivo methylation

Strains: A single-colony lysogeny broth (LB) plate of *E. coli* BW25113 was prepared, and for each experiment, a single colony was picked. The LD_{50} of MMS for this strain at OD_{600} values of 0.1 and 1.0 was determined to be 20 mM by colony counting after exposure to various MMS concentrations for 60 min.

Media for isotope labeling: Minimal medium M9 was used with and without the indicated isotopes. Unlabeled M9 was prepared by mixing a 10× M9 stock solution with glucose, $MgCl_2$, Na_2SO_4, and $CaCl_2$. For unlabeled 10× M9 stock solution, Na_2HPO_4 (68 g L^{-1}), KH_2PO_4 (30 g L^{-1}), NaCl (2.5 g L^{-1}), and NH_4Cl (10 g L^{-1}) were mixed and autoclaved. For ^{15}N-labeled 10× M9 stock solution, Na_2HPO_4 (68 g L^{-1}), KH_2PO_4 (30 g L^{-1}), NaCl (2.5 g L^{-1}), and $^{15}NH_4Cl$ (10 g L^{-1}) were mixed and autoclaved. $MgCl_2$ (0.1 M), $CaCl_2$ (0.1 M), Na_2SO_4 (0.1 M), and 20% (*w/w*) glucose were prepared by sterile filtration. For a 5 mL M9 preculture (or 50 mL exposure culture, respectively), 500 μL (5 mL) M9 stock solution was mixed with 100 μL (1 mL) glucose, 100 μL (1 mL) $MgCl_2$, 100 μL (1 mL) Na_2SO_4, and 5 μL (50 μL) $CaCl_2$. For ^{15}N-labeled cultures, the ^{15}N-10× M9 stock

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solution was used. For CD_3-methylome labeling, 200 µL CD_3-methionine (stock 5 g L^{-1}) was added to 5 mL of culture volume.

RNA isolation and tRNA purification: The bacteria culture was centrifuged at 1200 *g* for 5 min. The supernatant was discarded, and the cell pellet was suspended in 1 mL TRI reagent (Sigma–Aldrich) per 5 mL bacteria culture, and the total RNA was isolated according to the supplier's manual.

For purification of tRNA from total RNA, size-exclusion chromatography (SEC)[14] was used with an Agilent 1100 HPLC system with an AdvanceBio column, 300 Å pore size, 2.7 µm particle size, 7.8× 300 mm (Agilent, Waldbronn, Germany) at 40 °C. For elution, an isocratic flow of 1 mL min^{-1} 0.1 M ammonium acetate buffer was used. Eluting RNA was detected at $\lambda = 254$ nm with a diode-array detector. The tRNA fraction was collected, and the solvent was evaporated (GeneVac, EZ-2 PLUS, Ipswich, UK) to a volume of about 100 µL before ethanol precipitation. The tRNA was resuspended in water (30 µL).

Distinguishing enzymatic methylation from damage methylation: A single colony was picked and inoculated in CD_3-labeled M9 medium (5 mL) at 37 °C and 250 rpm shaking overnight. From this culture, a bacterial solution with an OD_{600} of 0.1 was prepared (5 mL, CD_3-labeled M9) by dilution. The culture was allowed to transit from stationary into early log phase by shaking at 37 °C for 60 min with 250 rpm in a shaking incubator. Methyl methanesulfonate (MMS, 99% solution) was added to a final MMS concentration of 20 mM (8.5 µL of MMS stock solution for a 5 mL exposure culture). MMS was distributed evenly in the culture by inverting the tube several times. After 60 min of exposure to MMS at 37 °C and 250 rpm, the RNA was isolated. As a control, water was added instead of MMS.

Pulse-chase NAIL-MS: A single colony was picked and grown in unlabeled M9 medium (5 mL) overnight. From this preculture, a 50 mL culture was prepared in unlabeled M9 medium and grown overnight. Unlabeled bacteria solution (120 mL, $OD_{600} = 1.0$) was prepared by adding the appropriate amount of overnight culture to fresh, unlabeled M9 medium. After 60 min growth (37 °C, 250 rpm), the first aliquot (7 mL) was taken for RNA isolation. The remaining culture was equally split into two flasks of 56.5 mL each. One was exposed to MMS stock solution (95.7 µL) or water and was inverted before both cultures were left to grow for 60 min at 37 °C and with 250 rpm shaking. After 60 min exposure, an aliquot (7 mL) was drawn from each culture, and the RNA was isolated. The remaining bacteria were centrifuged (1200 *g*, 5 min), and the MMS/MOCK-containing supernatants were discarded. The bacteria pellets were washed with ^{15}N CD_3-methionine labeled M9 medium (5 mL), and each was suspended in fresh $CD_3/^{15}N$ M9 medium (50 mL). For recovery, the bacteria were grown at 37 °C, 250 rpm, and after 1, 2, 3, 4, and 10 h, aliquots (7 mL) were drawn for RNA isolation.

tRNA digestion for mass spectrometry: tRNA (100 ng) in aqueous digestion mix (30 µL) was digested to single nucleosides by using alkaline phosphatase (0.2 U, Sigma–Aldrich), phosphodiesterase I (0.02 U, VWR, Radnor, Pennsylvania, USA), and benzonase (0.2 U, Sigma–Aldrich) in Tris (pH 8, 5 mM) and $MgCl_2$ (1 mM) containing buffer. Furthermore, tetrahydrouridine (THU, 0.5 µg from Merck), butylated hydroxytoluene (BHT,1 µM, Sigma–Aldrich), and pentostatin (0.1 µg Sigma–Aldrich) were added to avoid deamination and oxidation of the nucleosides.[15] The mixture was incubated with the RNA for 2 h at 37 °C and was filtered through 96-well filter plates (AcroPrep Advance 350 10 K Omega, PALL Corporation, New York, USA) at 3000 *g* and 4 °C for 30 min. The filtrate was combined with *E. coli* SILIS 10:1 (stable isotope labeled internal standard[16]) and was measured with a triple quadrupole mass spectrometer.

LC-MS instruments and methods

Triple quadrupole instrument no. 1: Agilent 1290 LC equipped with a variable wavelength detector (VWD) combined with an Agilent G6490A Triple Quad system with electrospray ionization (ESI-MS, Agilent Jetstream). The used parameters for this instrument were as follows: 250 V fragmentor voltage, cell accelerator voltage of 5 V, N_2 gas temperature of 150 °C and N_2 gas flow of 15 L min^{-1}, sheath gas (N_2) temperature of 275 °C with a flow of 11 L min^{-1}, capillary voltage of 2500 V, nozzle voltage of 500 V, nebulizer pressure of 30 psi, and positive-ion mode. The instrument was operated in dynamic MRM mode, and the individual mass spectrometric parameters for the nucleosides are given in Table S2.

Triple quadrupole instrument no. 2: Agilent 1290 Infinity II equipped with a diode-array detector (DAD) combined with an Agilent Technologies G6470A Triple Quad system and electrospray ionization (ESI-MS, Agilent Jetstream). Operating parameters: positive-ion mode, skimmer voltage of 15 V, cell accelerator voltage of 5 V, N_2 gas temperature of 230 °C and N_2 gas flow of 6 L min^{-1}, sheath gas (N_2) temperature of 400 °C with a flow of 12 L min^{-1}, capillary voltage of 2500 V, nozzle voltage of 0 V, and nebulizer at 40 psi. The instrument was operated in dynamic MRM mode, and the individual mass spectrometric parameters for the nucleosides are given in Tables S1 and S3.

Chromatography no. 1: For the in vitro assay, we used an RP-18 column (Synergi, 2.5 µm Fusion-RP C18 100 Å, 100×2 mm; Phenomenex) at 35 °C and a flow rate of 0.35 mL min^{-1} in combination with a binary mobile phase of 5 mM NH_4OAc aqueous buffer A, brought to pH 5.6 with glacial acetic acid, and an organic buffer B of pure acetonitrile (Roth, LC-MS grade, purity ≥95.95). The gradient started at 100% solvent A for 1 min, followed by an increase to 10% over 4 min. At minute 5, solvent B was increased to 40% and was maintained for 1 min before returning to 100% solvent A and a 2.5 min re-equilibration period.

Chromatography no. 2: A Core-Shell Technology separation column (Phenomenex, Torrance, CA, USA; Kinetex 1.7 µm EVO C_{18} 100 Å, 150×2.1 mm) at 35 °C and a flow rate of 0.35 mL min^{-1} were used for the in vivo experiments. Solvents A and B were identical to the solvents used for chromatography no. 1. The gradient started at 100% solvent A, followed by an increase to 10% over 10 min. From 10 to 15 min, solvent B was increased to 45% and was maintained for 3 min before returning to 100% solvent A and a 3 min re-equilibration period.

Calibration and equations: For calibration, synthetic nucleosides were weighed and dissolved to a stock concentration of 1–10 mM. In vitro calibration solutions: ranging from 100 fmol to 1 nmol with respect to canonical nucleosides and 100 amol to 1 pmol in terms of modified nucleosides. In vivo calibration solutions: ranging from 0.3 to 500 pmol for each canonical nucleoside and from 0.3 to 500 fmol for each modified nucleoside. The calibration solutions were mixed with the same SILIS as the corresponding samples and were analyzed with the same methods. The value of each integrated peak area of the nucleosides was divided through the respective SILIS area. The linear regression for each nucleoside's normalized signal/concentration plot gave the relative response factor for nucleosides (rRFN).[16] The sample data were analyzed by the Quantitative and Qualitative MassHunter Software from Agilent. The areas of the MRM signals were integrated for each modification and their isotope derivatives. The area value was divided

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through the respective SILIS area value and was divided through the rRFN value from the respective calibration to receive the absolute amount of the modification or canonical. Finally, the absolute amounts of the modifications were referenced to the absolute amounts of the precursor canonical. In the case of the pulse-chase experiment, the different isotopomers were referenced to their respective similarly labeled canonicals, so that original modifications were referenced to original canonicals and new modifications were referenced to new canonicals. See Equations (1) and (2) for m^7G as an example:

$$\frac{m^7G\ (\text{old})}{G\ (\text{old})} = \frac{\text{area } m^7G\ (\text{unlabeled})}{\text{rRFN } m^7G \times \text{area } m^7G\ (\text{SILIS})} \quad (1)$$

$$\frac{m^7G\ (\text{new})}{G\ (\text{new})} = \frac{\text{area } m^7G\ (^{15}\text{N-labeled})}{\text{rRFN } m^7G \times \text{area } m^7G\ (\text{SILIS})} \quad (2)$$

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: isotopic labeling · mass spectrometry · nucleosides · RNA demethylation · tRNA

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Observing the fate of tRNA and its modifications by nucleic acid isotope labeling mass spectrometry: NAIL-MS

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ABSTRACT

RNA in yeast, especially rRNA and tRNA are heavily modified to fulfill their function in protein translation. Using biosynthetic stable isotope labeled internal standards we quantified 12 modified nucleosides in tRNA from *S. cerevisiae* over 24 hours. We observed different quantities of modified nucleosides in dependence of the growth phase. To elucidate the underlying mechanism of the observed tRNA modification profile adaptation, it is necessary to distinguish the pre-existing tRNA pool and its modifications from newly-synthesized tRNAs. By combination of 2 differentially isotope labeled media we developed NAIL-MS, nucleic acid isotope labeling coupled mass spectrometry. During the yeast growth cycle we observe dilution of pre-existing tRNAs by newly-synthesized tRNAs by the growing number of cells. tRNA was found to be highly stable with only little degradation over the observed period. The method was further used to quantify the levels of modified nucleosides in the original and new tRNA pools. By addition of deuterium-labeled methionine, we could observe the incorporation of new methyl marks on pre-existing tRNAs. For 2′-O-methylcytidine (Cm) we observed a global increase in log phase. We identified extensive 2′-OH-cytidine methylation of the pre-existing tRNAs and the new tRNAs which masks an actual decrease of pre-existing Cm. In contrast, global 5-methylcytidine (m^5C) levels decreased during growth due to a drop in m^5C quantities in the original tRNA pool.

The NAIL-MS data suggests different mechanisms for tRNA modification adaptation depending on the individual modification observed. With this new tool it is possible to follow the fate of methylated RNAs during growth and potentially compare the impact of different stress conditions on the epitranscriptome.

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Introduction

The central dogma of molecular biology states that DNA is the storage of the genetic code, which is transcribed into (messenger) RNA, which is translated into proteins by another (transfer) RNA. This fundamental life process is dominated by nucleic acids, which are composed of the 4 canonical nucleosides cytidine, guanosine, adenosine and thymidine in DNA and uridine in RNA, respectively. The sequence of these building blocks defines the genetic code which contains all information necessary for each organism. While DNA is the storage of the genetic code, RNAs can be found as regulators and effectors of all essential life processes. Especially tRNA (tRNA) and rRNA (rRNA) are key players in protein translation and therefore highly abundant. To fulfill their function, both tRNA and rRNA are extensively modified and across all domains of life more than 150 modified RNA nucleosides have been identified.[1] In recent years, the term epitranscriptomics has emerged to describe the modification level and profile of RNAs. The modification is introduced by dedicated RNA modifying enzymes which are referred to as RNA writers. Common RNA writers include methyltransferases, thiotransferases and pseudouridine-synthases and each writer has a specific substrate RNA. While modifications in tRNA and rRNA have been known for decades, modifications in mRNA have only recently been described.[2,3] Alongside mRNA writer enzymes, so-called RNA erasers have been identified which actively demethylate the methylated adenosine in mRNA.

Like all organisms analyzed to date, *S. cerevisiae* tRNAs are heavily modified as recently reviewed.[4] Over 25 different modified nucleosides have been identified in these tRNAs, of which 12 are mostly *S*-adenosyl methionine methylated derivatives of the canonical nucleosides. The quantities of tRNA modifications are known to adapt during cell growth[5] and during stress.[6] Furthermore they are essential for protein homeostasis and cell morphogenesis.[7] The dynamic adaptation of tRNA modifications prompts to ask the question how tRNA modification levels are changed from a mechanistic viewpoint. One possibility is an addition of modifications by tRNA writers to the already existing tRNA pool and the newly synthesized tRNA which results in increased tRNA modification levels. A decrease of tRNA modification levels might be achieved by 2 possible scenarios. The first includes the participation of tRNA erasers that actively remove existing modifications from the tRNA. While RNA erasers are known to exist for mRNAs, only recently ALKBH1 was observed

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in vitro to demethylate 1-methyladenosine to adenosine in certain tRNAs.[8] Another potential way to remove RNA modifications is degradation of the existing, modified RNA and synthesis of new, unmodified RNA. However, current methods cannot distinguish the modification profile of the existing RNA pool and the new RNA pool. Here we present a new technique, NAIL-MS (nucleic acid isotope labeling coupled mass spectrometry) which allows observation of modification dynamics in existing and newly synthesized RNAs from *S. cerevisiae* during growth.

Results

tRNA modification quantities fluctuate during growth

tRNA from yeast was purified from total RNA by size-exclusion chromatography (SEC) as described previously[9] and the tRNA fraction collected. Fig. 1 shows an exemplary chromatogram of total RNA before SEC and purified tRNA after SEC. The large RNA is a mixture of rRNA and other large RNA molecules like mRNA (Fig. S1). The isolated tRNA was digested to nucleosides and subjected to liquid chromatography coupled mass spectrometry (LC-MS). An exemplary chromatogram for detection of the canonical nucleosides and 13 abundant modified nucleosides can be found in Fig. 1. The less common modifications like 2′-O-methyluridine (Um) or wobble-uridines like 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) were not included in the current study due to low detection sensitivity of uridine derivatives. Dihydrouridine was not available as a synthetic standard and could not be included in quantification analysis. Using a biosynthetic stable isotope labeled internal standard (SILIS), produced according to[10] and calibration with synthetic standards, we quantified the absolute level of modification per tRNA in log phase growing cells (calibration curves in Fig. S2). Fig. S3 shows a scheme how RNA modifications are quantified and which calculations and normalization steps are necessary to arrive at the number of a given modification per tRNA.

In total tRNA from log growing cells we find pseudouridine (Ψ) as the most abundant modified nucleoside, followed by the methylated nucleosides 5-methylcytidine (m^5C), 1-methylguanosine (m^1G) and 5-methyluridine (m^5U or rT) as expected (compare Table S1 prepared from[11]). Here, the modification profile of 34 tRNA sequences is listed. For total tRNA the exact number of modified nucleosides depends on the ratio of the isoacceptors and isodecoders. This ratio is dynamic and used as an adaptation mechanism to overcome changes in growth conditions and external stress.[12,13] This adaptation and the unknown modification status of most tRNAs make it difficult to assign a quantitative number of modifications to the total tRNA pool.

During these initial experiments to quantify tRNA modifications in *S. cerevisiae* BY4741 we noticed fluctuations in the quantities of many modifications in dependence of the growth phase (Fig. S4). 2′-O-methylcytidine (Cm), 1-methylguanosine (m^1G) and 7-methylguanosine (m^7G) levels increased in log phase (Fig. 1 and Fig. S4). In contrast, we observed a drop in 5-methylcytidine (m^5C), 5-methyluridine (m^5U) and pseudouridine (Ψ) quantities in log phase. For modified nucleosides 2′-O-methylguanosine (Gm), 2′-O-methyladenosine (Am), 2-methylguanosine (m^2G), 2,2-dimethylguanosine ($m^{22}G$), inosine (I) and 1-methyladenosine (m^1A) the levels stayed constant throughout growth (Fig. S4).

While we cannot explain this phenomenon at the moment we were curious to understand how the levels of modifications are de- or increased mechanistically. This prompts the question whether a change of modification quantity is caused by hyper- or hypomodified new tRNAs while the pre-existing tRNAs stay unaltered or by dynamic adaptation of the pre-existing tRNAs.

Developing media for nucleic acid isotope labeling

Intrigued by these results we wanted to design an isotope based assay which allows discrimination of the original tRNA pool and newly synthesized tRNAs by mass spectrometry. For this

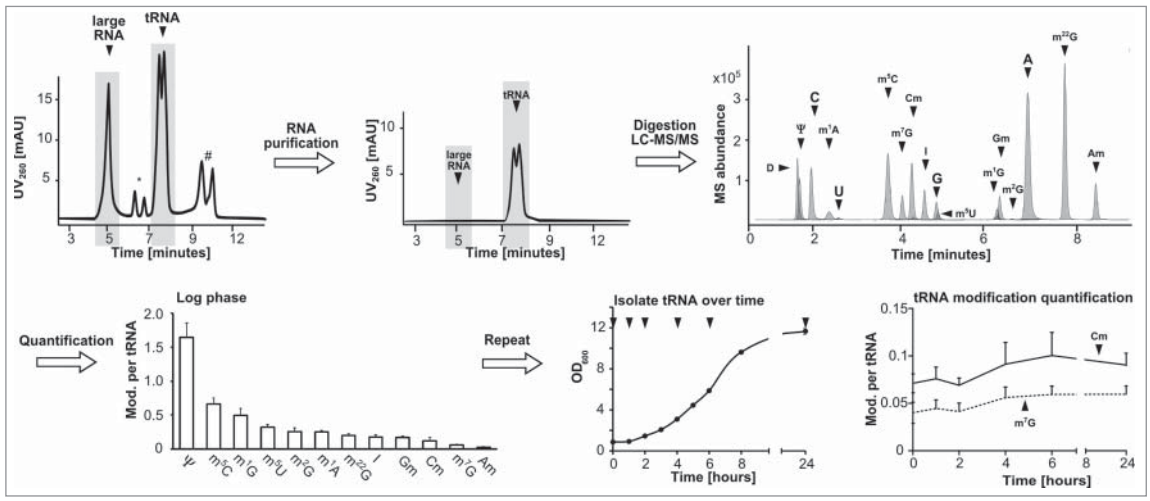

Figure 1. Workflow to determine modification levels in tRNA during yeast growth cycle. The isolated total RNA was purified by size exclusion chromatography (SEC) (*5.8S/5S rRNA peak, #unknown contamination). The tRNA fraction was then digested and the obtained nucleosides separated and quantified over LC-MS to receive the shown chromatogram on the upper right side. The average amounts of the respective modifications per molecule tRNA in log growing cells were calculated and plotted (error bars represent SD of n = 3). The quantification was repeated during growth (arrows indicate sample time points) and the quantities of the modifications Cm and m^7G were plotted over time. Abbreviations: D = dihydrouridine, Ψ = pseudouridine, C = cytidine, m^1A = 1-methyladenosine, U = uridine, m^5C = 5-methylcytidine, m^7G = 7-methylguanosine, Cm = 2′-O-methylcytidine, I = Inosine, G = guanosine, m^5U = 5-methyluridine, m^1G = 1-methylguanosine, Gm = 2′-O-methylguanosine, m^2G = *N*2-methylguanosine, A = adenosine, $m^{22}G$ = *N*2,*N*2-dimethylguanosine, Am = 2′-O-methyladenosine.

type of experiments it is necessary to have 2 growth media that result in differential labeling and therefore different masses of the nucleosides. We have tested commercial rich growth medium (Silantes, Munich, Germany) which allows efficient labeling of nucleobases with either ^{15}Nitrogen or ^{13}Carbon while the ribose remains unlabeled when supplemented with regular ^{12}C-glucose (see Fig. S5). In these media it is possible to introduce additional heavy carbons in the ribose by supplementing with $^{13}C_6$-glucose. Furthermore it is possible to supplement with D_3-methionine which resulted in a 60–70% labeling efficiency of methylated nucleosides with D_3-labeled methyl groups (see Fig. S6). The commercial medium was chosen for preparation of the stable isotope labeled internal standard (SILIS) to receive a max. labeled internal standard. The SILIS nucleosides have a +9 mass shift for uridine and cytidine, and +12 for methylated uridine and cytidine modifications (see Table S2). The purines, guanosine and adenosine, have a +10 while their methylated derivatives have a +13 mass shift (except $m^{22}G$ with a mass shift of +16).

For the time course experiments, the commercial media was not suitable since some modifications like 5-methylcytidine would have formed isotopomers with the same m/z as its corresponding SILIS nucleoside. Therefore, we tested the labeling efficiency in minimal medium (YNB) supplemented with isotope labeled amino acids, glucose and nucleobases. With D_3-methionine, labeling of methylated nucleosides was achieved quickly (increase in deuterated over unlabeled nucleosides shown in Fig. S7). As *S. cerevisiae* BY4741 is a uracil auxotroph strain, we supplemented with $^{15}N_2$-uracil which resulted in a complete labeling of pyrimidines and their modifications. For purine labeling we added the purine biosynthesis precursors ^{15}N-amide labeled glutamine or ^{15}N-aspartic acid. Although these amino acids are the nitrogen source of purines,[14] the incorporation efficiency was very low (Fig. S8). However, a quantitative +1 purine base label could be achieved by growth in $^{13}C_6$-glucose supplemented YNB medium which results in a purine nucleoside mass increase of +6 (+1 from base and +5 from ribose, Fig. 2c and Fig. S9) and +5 for pyrimidines (+5 from ribose). From these results a combination of $^{15}N_2$-uracil with $^{13}C_6$-glucose minimal medium was chosen as the first medium for our experiments. The nucleoside and nucleobase m/z values and their mass increase compared with unlabeled nucleosides for the canonicals are found in Table 1 and Fig. S10.

For the second medium we decided to use YNB minimal medium but supplemented with regular uracil ($^{14}N_2$-uracil) and regular $^{12}C_6$-glucose which results in unlabeled canonicals (Fig. 2a). By combination of these 2 media we can now distinguish the pre-existing nucleosides (heavy $^{15}N/^{13}C$-label) and the newly synthesized tRNA nucleosides (unlabeled). To further allow a detailed analysis of the pre-existing tRNA modifications, we added D_3-methionine to the growth medium which led to the incorporation of heavy methyl groups after medium exchange (Fig. 2b for newly synthesized modifications). In combination with the differential labeling of the nucleoside structure, this step allows to detect newly methylated nucleosides in the pre-existing tRNA pool by a mass increase of +3 (Fig. 2d and Fig. S10) compared with the pre-existing methylated nucleosides. We refer to this event as post-methylation as it happens after experiment initiation.

NAIL-MS (nucleic acid isotope labeling coupled mass spectrometry) reveals dilution of the pre-existing tRNA pool

In a next step we combined the 2 growth media in a single assay. The principle is shown in Fig. 3a. The cells are grown overnight in $^{15}N_2$-uracil/$^{13}C_6$-glucose supplemented YNB medium. To achieve complete labeling of all nucleosides with the heavy label the cells were centrifuged, resuspended in fresh $^{15}N/^{13}C$ YNB medium and grown overnight a second time. At this step high labeling efficiency of all nucleosides is required which was verified by high-resolution mass spectrometry (see Fig. S9 and S10). Minor signals of unlabeled nucleosides are due to impurities of the $^{13}C_6$-glucose and $^{15}N_2$-uracil starting material (99% ATOM ^{13}C and 98% ATOM ^{15}N purity) which causes <5% of unlabeled nucleosides at experiment start. For experiment initiation the cells are briefly centrifuged and

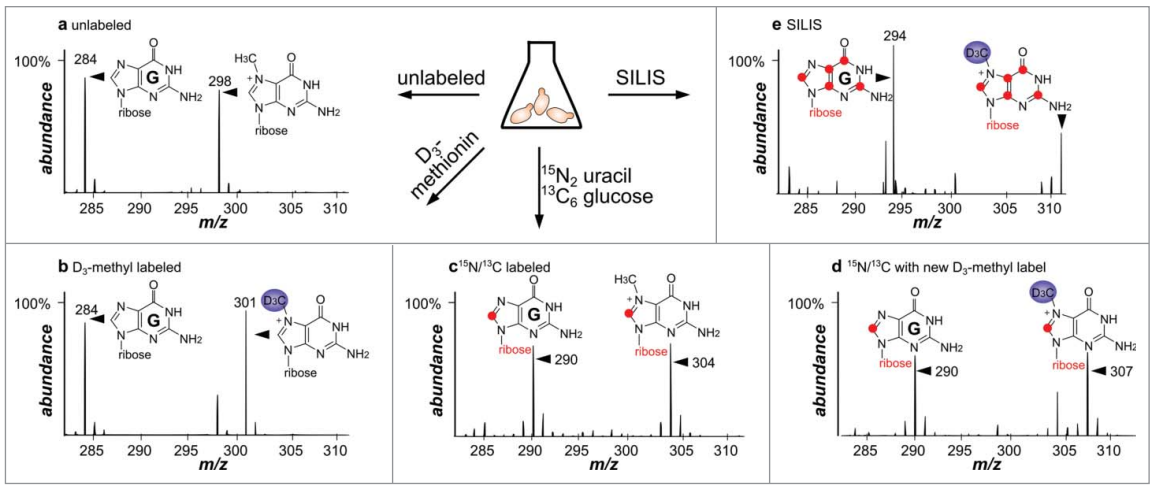

Figure 2. Mass spectra of guanosine (G) and 7-methylguanosine (m^7G) in different growth media. **a** unlabeled medium: $[M+H]^+$ (G) 284, $[M+H]^+$ (m^7G) 298. **b** D_3-methionine containing medium: $[M+H]^+$ (G) 284, $[M+H]^+$ (m^7G) 301. **c** $^{15}N_2$ uracil and $^{13}C_6$ glucose containing medium: $[M+H]^+$ (G) 290, $[M+H]^+$ (m^7G) 304. **d** $^{15}N_2$ uracil, $^{13}C_6$ glucose and D_3 methionine: $[M+H]^+$ (G) 290, $[M+H]^+$ (m^7G) 307. **e** SILIS: $[M+H]^+$ (G) 294, $[M+H]^+$ (m^7G) 311.

Table 1. Mass transition and mass increase compared with non-labeled canonical nucleosides from "heavy" minimal medium with $^{15}N_2$-uracil and $^{13}C_6$ glucose label.

Canonical	$[M+H]^+$ / (mass increase)	$[B+2H]^+$ / (mass increase)
Cytidine	251 / (+7)	114 / (+2)
Uridine	252 / (+7)	115 / (+2)
Guanosine	290 / (+6)	153 / (+1)
Adenosine	274 / (+6)	137 / (+1)

suspended in D_3-methionine supplemented YNB medium. Total RNA isolation and digestion to nucleosides followed by mass spectrometry revealed an increase of newly synthesized canonical nucleosides (light) from the new RNA pool during growth (Fig. S11, black line) while the original RNA was diluted (red). For modified nucleosides we performed mass spectrometry in scan mode after experiment initiation. As expected we could detect the signal of the new, D_3-methylated nucleosides and the H_3-methylated original nucleosides. However, we could also detect the predicted post-methylated nucleoside species with a mass increase of +3 from the original heavy isotope labeled RNA pool (Fig. 2d and Fig. S10). This nucleoside species is the result of additional methylation of canonicals that were not methylated in the pre-existing tRNA pool at experiment initiation. In summary, we can observe 3 different isotopomers of each methylated nucleoside (Fig. 3c) which originate from different RNA pools: 1) methylated nucleosides from the new RNA pool, 2) original nucleosides already methylated at experiment initiation and 3) original nucleosides, methylated AFTER experiment initiation.

With this assay at hand we decided to grow yeast for 24 hours and observe the fate of canonical and modified nucleosides in tRNAs by sensitive triple quadrupole mass spectrometry. The mass transitions of canonical and modified nucleosides were programmed for each observed isotopomer into a multiple reaction monitoring (MRM) method (mass transitions in Table S2 and mass spectra in Fig. S9). The total RNA was isolated from 6 biologic replicates before experiment initiation (T = 0 hours), after 1, 2, 4 and 6 hours (lag phase and log phase) and 24 hours (stationary) as the last time point. Using size-exclusion chromatography we purified total tRNA and subjected all samples to the developed NAIL-MS method after digestion to nucleosides. By injection of tRNA digests we could quantify the amount of tRNA injected and separate the signal of the pre-existing and novel tRNA pool. This quantification revealed an initial degradation of the existing tRNAs in the first 60 to 120 minutes followed by constant levels thereafter (Fig. 3b). We did not observe further tRNA degradation which is consistent with the reported long half-life of tRNA.[15,16] In similar pulse chase studies using tritium labeled uracil and subsequent gel electrophoretic quantification of the radioactively labeled yeast tRNA, no initial drop in tRNA levels was observed.[17] While this difference might be caused by technical errors during our sample collection and RNA isolation, we currently investigate the possibility of stress-induced tRNA degradation caused by the necessary pelleting of yeast cells before experiment initiation.

NAIL-MS reveals differences in the modification profile of the original and new tRNA pool

Fig. 3d shows all signals of 7-methylguanosine (m^7G) which potentially occur in a NAIL-MS experiment after medium exchange. For quantification of newly synthesized m^7G, the signal for the unlabeled and D_3-labeled isotopomers were summed and normalized to the amount of unlabeled tRNA injected (calculated from the signal of unlabeled adenosine). Thereby we

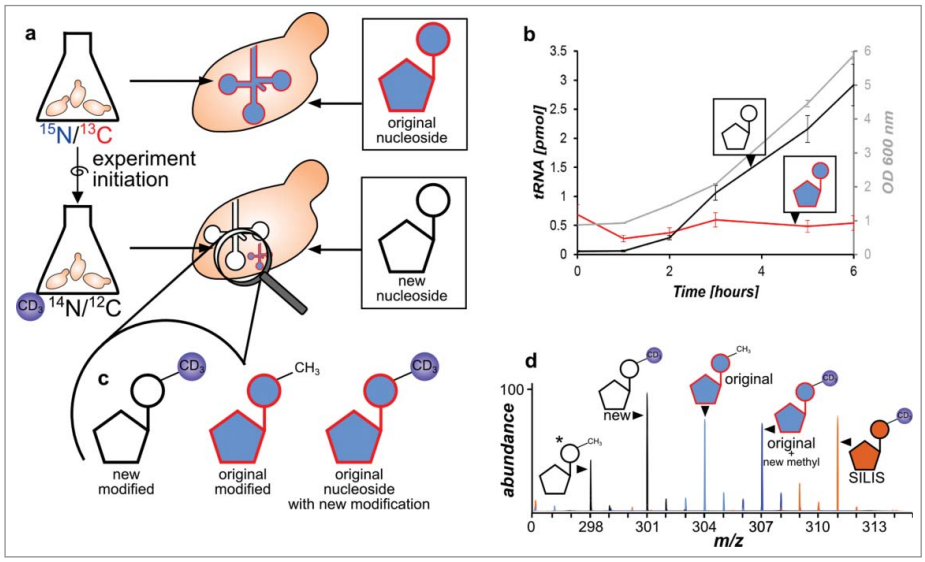

Figure 3. Principle of the developed NAIL-MS assay and RNA species emerging over time. **a** Yeast is grown in heavy isotope medium which results in heavy labeled nucleosides. The experiment is initiated by medium exchange with D_3-methionine labeled medium. New canonicals are unlabeled while pre-existing nucleosides remain $^{15}N/^{13}C$ labeled. **b** Original tRNA quantities stay constant over time (red), while new tRNAs are synthesized (black line) as cells grow (OD 600 gray line) (error bars are SD of n = 3). **c** Different modified nucleosides can be distinguished according to their labeling. New modified nucleosides have an unlabeled canonical building block and a D_3-methyl mark, original modified nucleosides derive from labeled canonicals but carry the unlabeled methylation and original, labeled canonicals which receive a methyl mark after experiment initiation have an additional D_3-methyl group. **d** Mass spectrum of potential isotopomers of 7-methylguanosine (m^7G) in a NAIL-MS experiment: *unlabeled m^7G as a reference (298), from new tRNA (301), from original tRNA with pre-existing methylation (304), from original tRNA but from post-methylation (307) and the signal of the m^7G SILIS (311).

received the number of m^7G per new tRNA. Similarly we added the signal of the original m^7G isotopomer and the post-methylated m^7G (heavy nucleoside + heavy D_3-methyl group) and normalized to the amount of pre-existing tRNA in the sample (calculated from the signal of labeled adenosine).

The plots of Fig. 4 show the number of modifications per original tRNA or new tRNA, respectively, on the y-axis. In the unlabeled experiment (Fig. 1) we observed growth phase dependent fluctuations for 7-methylguanosine (m^7G). By NAIL-MS we identified the reason for the observed increase in a slow and steady increase of m^7G in new tRNAs during log phase (Fig. 4a). The number of m^7G in the original tRNAs appears to stay constant throughout the experiment. However, a detailed analysis of the pre-existing tRNA pool reveals a constant decrease in the number of pre-existing m^7G while new m^7G is incorporated into the original tRNAs.

For 2′-O-methylcytidine (Cm) we observed a growth cycle dependent fluctuation of modification level (Figs. 1 and 4b). By NAIL-MS we observed an initial increase of Cm levels in pre-existing tRNAs while the new tRNA pool forms Cm rather slowly in the first 2 hours. While one explanation for the increase of Cm in the pre-existing tRNAs is degradation of those tRNAs that are not Cm modified during lag phase, the quantification of D_3-labeled pre-existing cytidines revealed a substantial incorporation of new methyl marks in the pre-existing tRNAs (box of Fig. 4b). Curiously we observe a sudden decrease of pre-existing Cm in the original tRNA pool after entering log phase. This could be explained by several scenarios. The most likely explanation is sudden degradation of pre-existing tRNAs with Cm at a certain position (*e.g.* tRNAs with Cm at position 34, see Fig. S13) as these tRNAs might be no longer needed while entering log phase. In parallel, Cm might become necessary at the other positions of pre-existing tRNAs (*e.g.*, at position 4) which leads to increased post-methylation. Another scenario for the decrease in pre-existing Cm is active demethylation of Cm by an unknown RNA eraser. Quantification of specific Cm-modified tRNAs by northern blotting might provide an answer to this open question.

The unmethylated nucleoside modifications pseudouridine and inosine are rapidly introduced into the nascent tRNA as these modifications reach their maximum abundance in the first 60 minutes (Fig. 4c and d). While new inosine levels in new tRNA are immediately comparable to the original tRNA inosine levels, pseudouridine levels are lower in the newly synthesized tRNAs compared with the levels in original tRNA. Furthermore, we observed a slight decrease of pseudouridine modification in the original tRNA pool after 6 hours, which might be linked to degradation of pseudouridine containing tRNAs. As both pseudouridine and inosine are non-methylated tRNA modifications, we cannot further distinguish between pre-existing modifications and those introduced after experiment initiation.

NAIL-MS results of the other methylated nucleosides are shown in Fig. S12. Like Cm, 5-methylcytidine (m^5C) is found at various positions of tRNA (positions 34, 40, 48, 49) and has a pronounced drop in its abundance in the pre-existing tRNAs. This drop can be observed in both the pre-existing and post-methylated m^5C molecules. The other methylated nucleosides Gm, m^1A, $m^{22}G$, m^2G and Am are like inosine rather unresponsive with similar quantities throughout the experiment. Analysis of the incorporation speed into the nascending tRNA pool shows an almost immediate incorporation of m^1G, $m^{22}G$, Gm and Am while Cm, m^7G, m^1A, m^2G, m^5U and m^5C are less abundant during lag phase compared with stationary and log phase.

For all methylated nucleoside modifications we observed incorporation of the heavy D_3-methyl mark into the pre-existing tRNAs. To compare the extent of this delayed post-methylation we formed the ratio of *e.g.*, post-methylated Cm to all Cm in original tRNA and plotted the % of the post-methylated

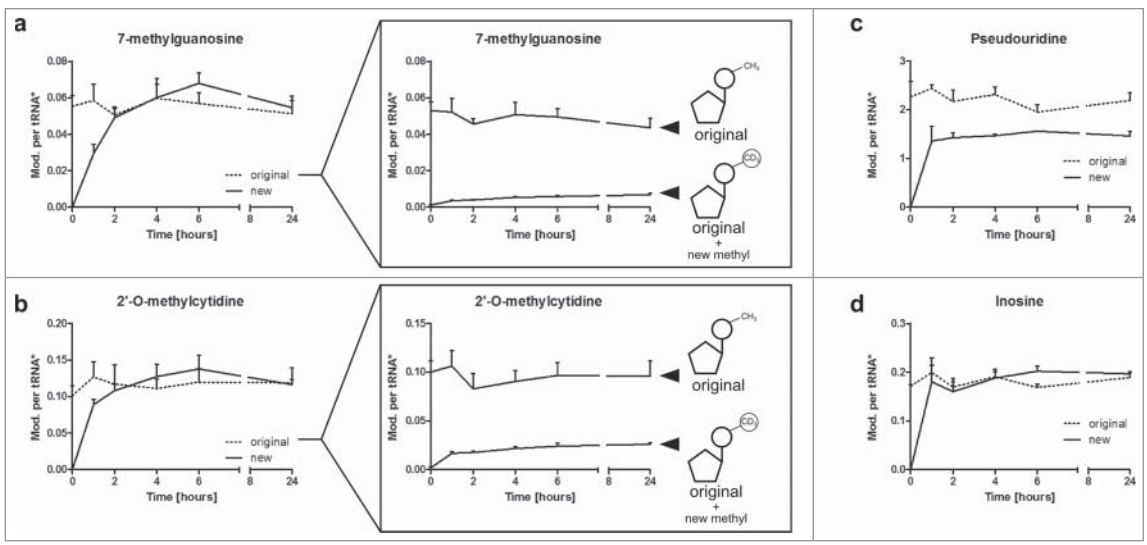

Figure 4. Levels of modifications in tRNA during a time range of 24 h discriminated by the original and newly-synthesized tRNA pool. *The quantity of modification was normalized to its respective origin (*e.g.*, new m^7G was normalized to abundance of new tRNA to receive the number of m^7G per new tRNA while original m^7G was normalized to original tRNA). **a** Level of 7-methylguanosine (m^7G) in new and original tRNAs over time. The constant level of original m^7G is caused by a constant increase in pre-existing m^7G, while post-methylated m^7G increases (box). (error bars reflect the SE of n = 6) **b** 2′-O-methylcytidine (Cm) increases fast in newly synthesized tRNAs, while the level of pre-existing Cm drops (box). This drop is masked by substantial post-methylation of original tRNAs. (error bars reflect the SE of n = 6) c Pseudouridine quantities are lower in newly-synthesized tRNAs while **d** the number of inosine per respective tRNA is constant throughout the experiment (both n = 3)

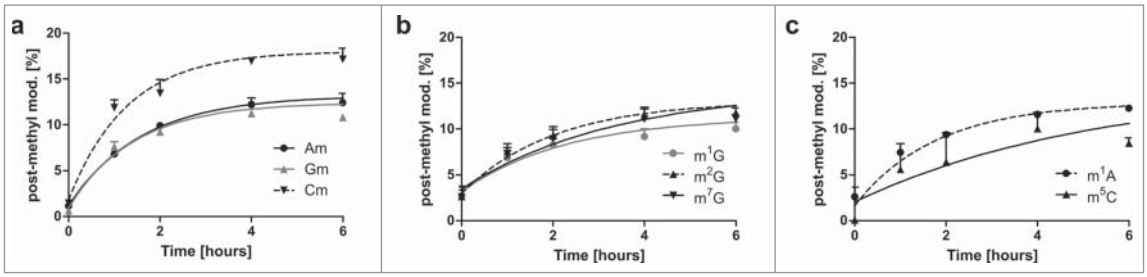

Figure 5. Extent of post-methylation in the original tRNA pool in %. Most modified nucleosides (Am, Gm, m^1G, m^2G, $m^{22}G$, m^1A and m^5C) plateau at 10% which indicates that 10% of all modifications in the original tRNAs were made after experiment initiation (delayed methylation). Cm (graph on the left) is an exception. Here up to 18% of all Cm in original tRNA are incorporated after experiment initiation. (in all graphs the error bars reflect the SE of n = 3)

modifications (Fig. 5). After 6 hours the maximum of post-methylation is reached for all methylated nucleosides. Except Cm, all nucleosides plateau at 10% post-methylation. Cm has a 1.8-fold higher plateau of post-methylation compared with the other methylated nucleosides, indicating the necessity to quickly adapt Cm levels in tRNAs to overcome lag phase.

Discussion

Using stable isotope labeling mass spectrometry with a biosynthetically produced internal standard we quantified 13 modified nucleosides in total tRNA from *S. cerevisiae* grown in minimal medium. The detected quantities from yeast log growing cells were lower compared with the expectation from a database search (Table S1[11]). These differences are partly explained by the low number of tRNA sequences with reported modification profile in the database (only 34 tRNA sequences available) and the general error introduced by absolute quantification approaches. In our quantification we use an "averaged" tRNA generated from the database under the assumption of equimolar contribution of each listed tRNA sequence. Therefore, absolute quantification results might slightly deviate from the true number of modification per tRNA molecule. Another explanation can be found in the dynamic nature of tRNA modifications which adapt depending on the growth phase as observed in Fig. 1 and Fig. S4 or stress.[6] Especially during log phase the quantities of modified nucleosides increased for Cm, m^7G and m^1G, while m^5C, m^5U and Ψ levels decreased and Gm, Am, m^2G, $m^{22}G$, I and m^1A stayed constant. While the biology of these changes remains unclear, we have developed a new tool to separately analyze the original and new transcripts. Using NAIL-MS (nucleic acid isotope labeling coupled mass spectrometry) we could partly identify the mechanism of these modification profile changes for methylated nucleosides (Fig. 4 and Fig. S12).

The mechanism, by which the modification level adapts, depends on the modified nucleoside and potentially the distribution in the RNA. For 7-methylguanosine (m^7G, pos. 46) in tRNA, increased incorporation into newly synthesized tRNAs was observed. We observed a decrease in pre-existing m^7G while post-methylated m^7G increased, resulting in an apparent constant level of m^7G in the pre-existing tRNA.

We made similar observations for 1-methylguanosine (m^1G), 2,2-dimethylguanosine ($m^{22}G$) and 2′O-methylguanosine (Gm) (see Fig. S12). While these methylated nucleosides showed a limited adaptation during growth, 2′O-methylcytidine (Cm, pos 34 and 4 of tRNAs) and 5-methylcytidine (m^5C pos 34, 40, 48 and 49) quantities fluctuated depending on the growth phase. Cm showed an interesting modification profile over time which was partly caused by reduced modification levels in original tRNAs, high modification levels in the new tRNAs and post-methylation of the existing tRNA pool. Especially the reduced modification profile in the original tRNAs after 4 hours is intriguing (compare Fig. 4b and Fig. S12 for m^5C after 6 hours). One potential explanation is degradation of Cm and m^5C modified tRNAs (*e.g.*, $tRNA^{Gly}$, $tRNA^{Phe}$ or $tRNA^{Leu}$) which would result in a reduced number of these modifications in the original tRNAs. It is possible that the observed changes in the tRNA epitranscriptome reflect fluctuations in the ratio of isoaccpetors and isodecoders. Another potential mechanism that was recently described for 1-methyladenosine (m^1A) in human cell culture is active removal of the methylation mark by an RNA eraser. While our current data cannot distinguish these mechanisms, it highlights m^5C and Cm as potential candidates for RNA erasers. Future analysis of single modifications within their respective tRNA subtype will reveal the underlying mechanism.

We interpret methylation of original canonicals as an addition of new methyl marks into existing, intact RNA to adapt the modification profile to the physiologically needed modification status. However, it is possible that the observed signal is caused by salvage of existing heavy-labeled canonicals which are introduced into newly synthesized RNAs followed by subsequent methylation. However, if salvage was the main reason for added methylation to existing canonicals, the quantities of added methylation should be similar among the different modified nucleosides and the level would constantly increase over time as more and more RNA gets degraded and original canonicals get released. Both were not observed in our studies as the % of post-methylated nucleosides in the original tRNAs plateau after 6 hours. Although outside the scope of this manuscript, oligonucleotide mass spectrometry of *e.g.*, a partial RNase T1 digest[18] of NAIL-MS samples would allow to assess the origin of these methylated nucleosides.

We used the NAIL-MS method to analyze 13 modified nucleosides, 2 of those are non-methylated tRNA modifications. For non-methylated nucleosides it is not possible to determine the quantity of added modification to the original RNA pool with the developed assay. However, we could monitor the modification extent in new and original RNAs and compare their quantities of modification.

In principle, it is possible to monitor all methylated nucleosides in original and new RNA and even observe the addition of the methyl mark to original RNA. Especially methylated uridine modifications like 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) or 5-methoxycarbonylmethyluridine (mcm^5U) found at position 34 of tRNA would be interesting targets for such an analysis. These modifications have the strongest influence on protein translation,[7,19] especially during stress.[6,20] Due to their low abundance and low detection efficiency, large sample volumes are necessary to monitor these modifications. With NAIL-MS we now have a tool which allows a new viewpoint on the dynamics of potentially all methylated RNA nucleosides. It can be applied to heavily modified RNAs, as shown here for tRNA, but also to any other purified RNAs species like rRNA, mRNA or small non-coding RNAs.

Materials and methods

Growth medium and conditions for S. cerevisiae BY4741

S. cerevisiae BY4741 was grown in ^{13}C labeled Yeast OD 2 (+ 10 g/L $^{13}C_6$ Glucose (99% ATOM) (Euriso-top, Saclay, France)) rich medium (Silantes, Munich, Germany) for SILIS preparation or in YNB minimal medium for NAIL-MS experiments. 10x YNB (Carl Roth GmbH, Karlsruhe, Germany) was prepared according to manufacturer's manual. 1x YNB medium was supplemented with 10 g/L glucose, 0.02 g/L uracil, a mix of needed unlabeled aminoacids (final concentration: 0.02 g/L arginine, 0.02 g/L histidine, 0.06 g/L leucine, 0.03 g/L lysine, 0.05 g/L phenylalanine, 0.4 g/L serine, 0.2 g/L threonine, 0.04 g/L tryptophane, 0.03 g/L tyrosine and 0.15 g/L valine) and additional needed aminoacids used for labeling (final concentration: 0.1 g/L aspartic acid, 0.1 g/L glutamine and 0.02 g/L methionine). Depending on the desired labeling $^{15}N_2$-uracil, L-aspartic-^{15}N acid, L-glutamin-amide-^{15}N, L-methionine-methyl-D_3 or their unlabeled isotopomers were used. $^{15}N_2$-uracil (98% ATOM ^{15}N) was supplied by Euriso-top. All unlabeled aminoacids and L-aspartic-^{15}N acid (98% ATOM ^{15}N), L-glutamin-amide-^{15}N (98 ATOM% ^{15}N) and L-methionine-methyl-D3 (98% ATOM D_3) were supplied by Sigma-Aldrich, Munich, Germany.

SILIS preparation

S. cerevisiae BY4741 was grown in 200 mL of ^{13}C labeled Yeast OD 2 medium supplemented with 10 g/L ^{13}C-glucose and 0.1 g/L D_3-methionine. The cells were harvested at OD 6 by centrifugation at 3500 xg for 10 minutes. The supernatant was removed and the pellet was resuspended in 20 mL TRI reagent® (Sigma-Aldrich, Munich, Germany). For complete disruption of the cells, an amount of acid-washed beads equivalent to 200 μL (425–600 μm, Roth, Karlsruhe, Germany) were added to the suspension and vortexed for 10 minutes. RNA was isolated as specified in user manual and finally dissolved in 500 μLMilli-Q water. tRNA was isolated from total RNA and digested to nucleosides (see **tRNA isolation via SEC** and **tRNA digestion** below). The tRNA digest from 100 mL OD 6 culture was diluted in 1 mL pure water which resulted in mass spec peak areas 50–100x above the limit of quantification (LOQ) of the modified nucleosides of interest in a 1/10 dilution of the digest. As an external standard 10 mM theophylline was added to a final concentration of 1 mM in the digest solution. The resulting digest/theophylline mixture is referred to as 10x SILIS which was added to a final concentration of 1x to samples and calibration solutions.

Calibration

Synthetic modified nucleosides for preparation of calibration solutions were purchased from: Sigma-Aldrich, Munich, Germany: Nucleoside test mix: inosine (I), pseudouridine (Ψ), 5-methyluridine (m^5U), 2′-O-methylcytidine (Cm), 5-methylcytidine (m^5C); solid nucleosides: adenosine (A), 7-methylguanosine (m^7G), 2′-O-methyladenosine (Am), 1-methyladenosine (m^1A), 1-methylguanosine (m^1G), 2-methylguanosine (m^2G) and *N*2,*N*2-dimethylguanosin ($m^{22}G$); solid nucleosides from Berry&Associates, Dexter, MI, USA: 2′-O-methyluridine (Um) and 2′-O-methylguanosine (Gm). Each nucleoside powder was weighed into a clean tube (5–10 mg per nucleoside) and dissolved in pure water to reach a final concentration of 10 mM. The nucleosides and the test mix were combined to a final concentration of 500 pmol/μL of A, 2000–500 fmol/μL of each modified nucleoside and 1x of the prepared SILIS. The calibration mix was serial diluted 1:5 and 1:10 with 1x SILIS until the lowest calibration concentration of 5 fmol/μL canonical and 5 amol/μL modified nucleoside were reached. 10 μL of each calibration solution was injected onto LC-MS/MS. After LC-MS/MS measurement the value of the integrated MS signals of the unlabeled synthetic nucleosides were set into relation to the integrated MS signals of the heavier SILIS nucleosides. The results were plotted against the nucleoside concentrations and the regression lines from the diagrams (Fig. S2) were used to calculate the respective RFN values, necessary for quantification.[10]

NAIL-MS experiments

Five mL of ^{15}N and ^{13}C-Glucose labeled medium was inoculated with a single *S. cerevisiae* BY4741 culture from an YPD-agar plate in a 50 mL falcon tube. The cells were grown in a shaking incubator over night at 30 °C and 250 rpm. The next day 1 mL of the lysate was centrifuged, the supernatant discarded and the pellet resolved in 5 mL fresh ^{15}N and ^{13}C-Glucose labeld medium to achieve better labeling. After incubation over night at 30°C and 250 rpm again, the OD was determined at 600 nm using an Eppendorf Biophotometer. For experiment initiation part of the culture was centrifuged at 3000 xg for 5 minutes at room temperature. The cell pellet was resuspended in 20 mL of D_3-labeled YNB medium to achieve a starting OD of 1. Using a 100 mL glass flask, the cells were incubated for 24 hours at 30 °C and 250 rpm in a shaking incubator. At set times (T = 0, 1, 2, 4, 6 and 24 hours) the OD was measured and a 2 mL sample was taken for RNA isolation. After centrifugation at 3000 xg for 3 minutes, the pellet was dissolved in 1 mL of TRI reagent®, transferred into a screw-top tube with an amount of acid-washed beads equivalent to 200 μL and vortexed for 10 minutes. Isolation of RNA was done according to user manual. The experiment was repeated with 6 biologic replicates.

tRNA isolation via SEC

Total RNA was loaded on an Agilent/HP 1100 LC system equipped with a binary pump, diode array detector set to

260 nm, autosampler, column thermostat (60 °C) and fraction collector. A size-exclusion column (Agilent Bio SEC-3, 3μm, 300Å, 7.8 × 300 mm, Agilent, Waldbronn, Germany) allowed collection of RNA fractions after isocratic elution with 100 mM ammonium acetate at pH 7. The tRNA fraction was concentrated to about 150μL in a Speed-Vac (Christ, Osterode am Harz, Germany). 5 M NH_4OAc was added to a final concentration of 0.5 M and after addition of 2x Vol. ice-cold ethanol (100%) the RNA was precipitated over night at −20°C. After centrifugation at 12.000 xg for 30 minutes at 4° C the RNA pellet was subjected to an additional ethanol (80 %) wash step to verify complete removal of ammonium acetate and then resuspended in 20 μL pure water. The quality of the thus isolated tRNA was verified by analytical SEC using the same method.

tRNA digestion

tRNA was digested using 0.6 U nuclease P1 (Sigma-Aldrich) in the presence of $ZnCl_2$, ammonium acetate pH 5.3, THU, pentostatine and BHT from[21] at 50°C for one hour followed by overnight incubation at room temperature. The next day, 10 U benzonase (Sigma-Aldrich, Munich, Germany), 0.1 U phosphodiesterase I (VWR, Ismaning, Germany) and $MgCl_2$ in a final concentration of 1 mM were added and incubated at 37°C for one hour. Then 20 U alkaline phosphatase (Sigma-Aldrich, Munich, Germany) and the proper amount to reach a final concentration of 0.1 M Tris-HCl pH 8 were added. After 2 hours at 37 °C the digestion mix was filtered through a 10 kDa MWCO filter (AcroPrep™ Advance, 350 μl, Omega™ 10K MWCO, Pall, Dreieich, Germany) at 3000 xg for 30 minutes. 18 μL of filtrate was mixed with 2 μL of the 10x SILIS and subjected to LC-MS/MS analysis.

High resolution mass spectrometry

For determination of m/z precursor and product values 5 mL cultures of each growth medium was inoculated with a colony of *S. cerevisiae* BY4741. Total RNA was isolated at OD 6 and digested as described for NAIL-MS experiments. The ribonucleosides were separated using a Dionex Ultimate 3000 HPLC system on an Interchim Uptisphere120–3HDO C18. Mobile phase A was 2 mM ammonium acetate and mobile phase B was 80% acetonitrile containing 2 mM ammonium acetate. Gradient elution started with 0% B and increased to 12% B after 10 minutes and to 80% after 12 minutes. After 4 minutes elution at 80% B and subsequently regeneration of starting conditions to 100% A after 5 minutes, the column was equilibrated at 100% A for 8 minutes. The flow rate was 0.2 mL/minute and the column temperature 30°C. High-resolution mass spectra of precursor and product ions were recorded by a ThermoFinnigan LTQ Orbitrap XL.

LC-MS / QQQ

Ribonucleosides were separated using a Synergy Fusion RP, 2.5 μm particle size, 100 Å pore size, 100 mm length, 2 mm inner diameter from Phenomenex (Torrance, CA), on an Agilent 1290 series HPLC system equipped with a diode array detector. Mobile phase A was 5 mM ammonium acetate adjusted to pH 5.3 with glacial acetic acid and mobile phase B was pure acetonitrile. Gradient elution started with 100% A for one minute, increase to 10% B after 10 minutes, 40% after 14 minutes and regeneration of starting conditions with 100% A for 3 additional minutes. The flow rate was 0.35 mL/minute and the column temperature 35°C. For mass spectrometric measurements an Agilent 6490 Triple Quadrupole mass spectrometer set to dynamic multiple reaction monitoring (MRM) mode was used. The MS was operated in positive ion mode with the following parameters: electro-spray ionization (ESI-MS, Agilent Jetstream), Fragmentor Voltage (set in tunefile to) 250 V, Cell Accelerator Voltage 2 V, N2-Gas temperature 150°C, N2-Gas flow 15 L/min, Nebulizer 30 psi, Sheath gas (N2) temperature 275 °C, Sheath gas flow 11 l/min, Capillary 2500 V and Nozzle Voltage 500 V. The mass transition for each modified nucleoside and its isotopomers are found in Table S2.

Data analysis

Using Agilent's Quantitative Data Analysis software, the mass signal areas of the different isotope labeled ribonucleosides were integrated. For methylated ribonucleosides the SILIS isotopmer, the $^{15}N/^{13}C$ labeled isotopomer (original), the $^{15}N/^{13}C/D_3$ labeled isotopomer (original+new methyl mark) and the D_3 labeled isotopomer (new) could be identified. For the canonicals the isotopomers of the SILIS, the original and the unlabeled m/z transitions were analyzed to quantify the amount of injected tRNA. The values of the integrated signals were used for further calculations. The nucleosides were quantified by calculating the nucleoside isotope factor (NIF) and the response factors (RFN) were received by plotting the NIF over the concentration of injected synthetic standard.[10]

$$\mathrm{NIF} = \frac{\mathrm{area}_{\mathrm{MS}}(\mathrm{isotopomer})}{\mathrm{area}_{\mathrm{MS}}(\mathrm{SILIS})}$$

The signal of adenosine in each sample was divided by the response factors and the amount of injected adenosine was received. The value for adenosine was divided by the number of adenosine per tRNA molecule (see also Table S1). Thereby the injected amount of original and new tRNA molecules could be determined.

The modified nucleosides were analyzed by correcting first the original+D value under consideration of the presence of signals with the same m/z introduced by the biosynthetic SILIS (only for 5-methylcytidine and 5-methyluridine). Therefore the integral of the mass signal corresponding to original+D of an independent SILIS measurement was divided by the integrated SILIS mass signal of the same measurement. The resulting correction factor was then multiplied by the SILIS value of the actual sample and subtracted from the sample's original+D mass signal area.

Using the RFN the signal of each modified nucleoside isotopomer to SILIS ratio was converted into the amount of injected modification. The amount of new modified nucleoside was normalized by the amount of new tRNA injected and the number of the modification in new tRNA thus revealed. For original and original/post-methylated modified nucleosides the

same procedure was applied but their amounts related to the amount of original tRNA injected.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Surpassing limits of static RNA modification analysis with dynamic NAIL-MS

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ABSTRACT

Ribonucleic acids (RNA) are extensively modified. These modifications are quantified by mass spectrometry (LC-MS/MS) to determine the abundance of a modification under certain conditions or in various genetic backgrounds. With LC-MS/MS the steady state of modifications is determined, and thus we only have a static view of the dynamics of RNA modifications. With nucleic acid isotope labeling coupled mass spectrometry (NAIL-MS) we overcome this limitation and get access to the dynamics of RNA modifications. We describe labeling techniques for *E. coli*, *S. cerevisiae* and human cell culture and the current instrumental limitations. We present the power of NAIL-MS but we also outline validation experiments, which are necessary for correct data interpretation.

As an example, we apply NAIL-MS to study the demethylation of adenine and cytidine, which are methylated by the damaging agent methyl-methanesulfonate in *E. coli*. With NAIL-MS we exclude the concurrent processes for removal of RNA methylation, namely RNA degradation, turnover and dilution. We use our tool to study the speed and efficiency of 1-methyladenosine and 3-methylcytidine demethylation.

We further outline current limitations of NAIL-MS but also potential future uses for *e.g.* relative quantification of tRNA isoacceptor abundances.

1. Introduction

1.1. State-of-the-art quantification of modified nucleosides

Ribonucleic acids (RNA) are key players in the central dogma of molecular biology. Messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA) participate in protein synthesis, while the group of non-coding RNAs (ncRNAs) are crucial for many processes, including gene regulation as interfering RNAs (miRNAs) and guide RNAs. As RNAs fulfill many important and diverse functions, more than 4 building blocks are needed. Therefore, a large chemical diversity of ribonucleoside modifications can be found [1]. The chemical alterations of the canonical RNA nucleosides are comprised of simple methylations, isomerization, thiolation or even addition of complex groups like amino acids. Modifications that occur on the base are usually indicated by a small letter (*e.g.* m- for methylation or s- for thiolation) before calling the base (C, U, G or A). The position on the base is inserted as superscript between the short abbreviation and the base; *e.g.* 5-methylcytidine is abbreviated as m^5C. Some modifications, like the hypermodification queuosine, are abbreviated with their own capital letter, *e.g.* Q. Specific enzymes insert RNA modifications post-transcriptionally.

Studies of modified RNA rely on sensitive detection of modified nucleosides, which is commonly done with triple quadrupole mass spectrometry (described in various reviews [2–4]). The principle and workflow of such analyses is shown in Fig. 1**a**. After purification of the RNA of interest (here tRNA), the RNA is enzymatically digested into nucleosides and ideally an internal standard is added. The sample is injected on the LC-MS/MS system where the nucleosides are separated chromatographically and their mass transitions are monitored in the mass spectrometer. Using calibration curves, the absolute quantity of each nucleoside can be calculated and the number of modification per tRNA can be plotted.

1.2. Nucleic acid isotope labeling (NAIL)

Key to the quantification of nucleosides by mass spectrometry is the availability of the synthetic nucleoside in weighable quantities (or a known extinction coefficient) and an internal standard. The internal standard is ideally an isotopomer of the nucleoside of interest. While the natural nucleoside contains regular isotopes like hydrogen (H) carbon-12 (^{12}C), nitrogen-14 (^{14}N) its isotopomer will contain one or more heavy isotopes like deuterium (D), carbon-13 (^{13}C) or nitrogen-15 (^{15}N). The isotopomer is therefore heavier than the natural nucleoside

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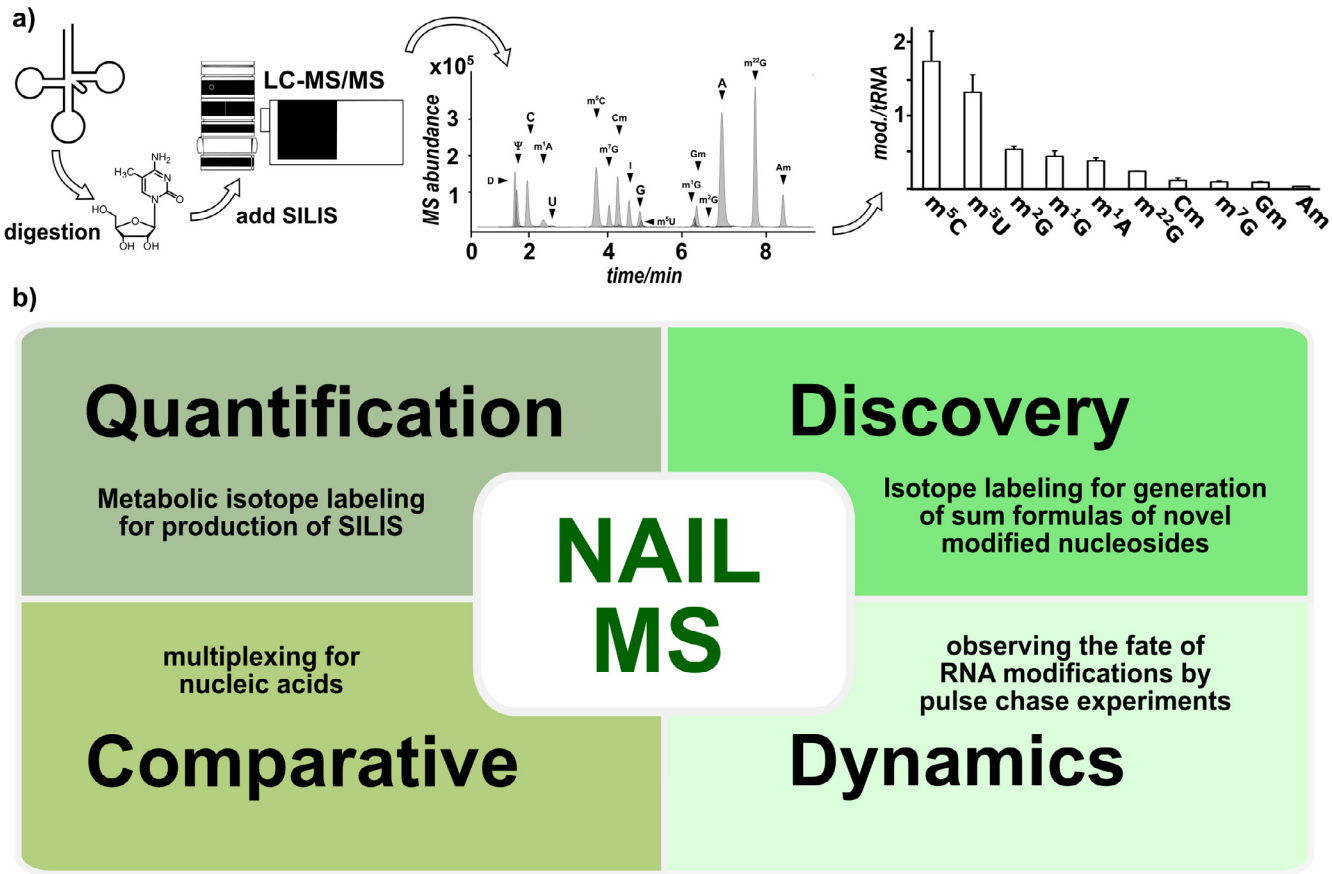

Fig. 1. a General workflow for quantification of modified nucleosides. The RNA is isolated and digested to the nucleoside building blocks (here, 5-methylcytidine). The stable isotope label internal standard (SILIS) is added and the sample is subjected to LC-MS/MS analysis. After chromatographic separation, the nucleosides are detected in the mass spectrometer and the abundance can be calculated and plotted. **b** Current and potential uses of NAIL-MS (nucleic acid isotope labeling coupled mass spectrometry).

and it can be used as an internal standard in mass spectrometry. Such internal standards are referred to as stable isotope labeled internal standards (SILIS) and the technique as isotope dilution mass spectrometry. The generation of these isotopomers can be done either synthetically [5–7] or biosynthetically by metabolic labeling in bacteria, yeast or algae [8–10].

Metabolic labeling relies on the incorporation of heavy isotopes from heavy-labeled growth media into the RNA. The heavy labeled RNA is isolated and processed for preparation of a SILIS for quantification. The usage of SILIS reduces the detection fluctuations of the mass spectrometer and thus reliable quantitative data becomes available to study RNA modifications.

However, nucleic acid isotope labeling (NAIL) should not be limited to quantification purposes. One can envision the use in a variety of experiments, which are or will become valuable tools in the research of modified nucleosides. Fig. 1**b** reviews the current usage of NAIL and potential future uses in RNA modification analysis. In addition to the usage of NAIL in **quantification**, its use to facilitate the **discovery** of novel modified nucleosides is widespread. Here, isotope labeling has become a key tool for sum formula generation and is highly helpful for structure prediction and verification [11–14].

1.2.1. Comparative NAIL

One of the caveats in the area of RNA modification research is the lack of key technologies comparable to those boosting protein research [15,16]. In the field of proteomics, a variety of mass spectrometry tools have emerged and allowed scientists to study proteins and their networks in more detail. Many of these tools focus on the comparison of proteomes (all proteins within a cell) in the context of stress studies or functional analyses by, *e.g.* multiplexing. Multiplexing allows the direct comparison of samples by mixing them within a single tube and performing a single analysis. They can be distinguished by mass spectrometry either by a chemical labeling step prior to mixing or by metabolic isotope labeling during sample generation. Especially metabolic isotope labeling has the advantage of overcoming purification biases. Additionally, mass spectrometric detection fluctuations are of no consequence. Metabolic isotope labeled proteomics (SILAC-Proteomics [17]) allows a direct comparison of proteomes within a single measurement with high accuracy. In principle, SILAC-like multiplexing is possible by NAIL, and we see a significant potential in such a SILAC-like approach in RNA modification research. We refer to this technique as **comparative NAIL**, which is also a useful tool for validation of dynamic NAIL-MS experiments.

1.2.2. Dynamic NAIL

While epigenetics is an intensively studied area, the analogue process in RNA, termed epitranscriptomics, is far less studied. This is mainly caused by our limited number of tools to study the dynamics of RNA modifications and, in addition, the complex process of finding biological consequences to RNA modifications. DNA is the storage of the genetic code. Therefore, modifications of DNA must be removed by an enzymatic process, which leaves the DNA sequence untouched and the DNA intact. Otherwise, mutations would occur and harm the organism. While it should be possible for the cell to use similar removal mechanisms in RNA, RNA has a second option for removal of an unwanted modification – the whole RNA itself is degraded and an unmodified new RNA is transcribed. The potential competition between these two processes makes it difficult to study the dynamics of RNA

modifications. Although many studies described that mRNA is enzymatically demodified, the mechanisms are still reviewed skeptically [18]. These doubts arise from the used techniques for *in vivo* analysis of the demodification process. In these studies, quantitative mass spectrometry of RNA modifications was used to observe an active demodification *in vivo* [19]. However, the absolute number of a modification within an RNA does not reflect the origin of the modification. A decrease in modification density can be explained by enzymatic demodification processes but also by increased degradation of modified RNA or even by increased transcription of unmodified RNA. *Vice versa*, an increase in modification density can be explained by additional modification events in the original RNA or by increased degradation of non-modified RNAs.

Current analyses of nucleic acid modifications are limited in respect to their blindness towards the underlying mechanisms, which lead to changes of modification content. NAIL-MS expands our current analytical toolbox and overcomes limitations by providing insight into the dynamics of nucleic acid modifications.

1.3. Nucleic acid isotope labeling as a tool to observe nucleic acid modification dynamics

From the natural RNA modifications known to date (~160), 70 contain a methylation of the base or the ribose [1]. These methylations are enzymatically incorporated at defined positions of the RNA. Especially m^6A in mRNA has gained major interest since this is the first RNA modification, which is incorporated by a methyltransferase (writer) and can be removed by demethylation *via* FTO or AlkBH5 (erasers).

In yeast, it was found that tRNA modifications are highly dynamic during cellular stress and are used by the cell for efficient stress survival by changing the translational speed of stress response proteins [20]. Although the dynamic nature of tRNA modifications is crucial to cell survival, it remains unclear how the cell achieves the adaptation of the modification profile. Additionally, cells are facing various stressors that can actively methylate the RNA such as methyl-methanesulfonate (MMS). Such methylations are randomly distributed across the accessible sites in RNA. *In vitro* tests showed that methylation of positions 1 of adenine and 3 of cytosine are quickly repaired by AlkB in *E. coli* [21].

Only one study showed the repair of m^1A by AlkB *in vivo*. This early pulse-chase study, based on radioactively labeled adenine, showed removal of m^1A from MMS damaged RNA by AlkB *in vivo*. Other studies that report demethylation processes in RNA did not present clear *in vivo* evidence of demethylation due to the limitations of static LC-MS/MS quantification.

Our first steps to overcome this limitation was in 2017, when we utilized non-radioactive isotope labeling of DNA to observe damage and repair of a DNA modification in bacteria. The combination of different labeling media allowed the creation of a pulse-chase experiment, which was used to observe repair of phosphorothioates in bacterial DNA [22]. The principle is shown in Fig. 2**a**. The bacteria were pre-cultured in heavy labeled media resulting in complete heavy labeling of the DNA nucleosides. In the heavy media, the bacteria were exposed to hypochlorous acid and the DNA present at this moment was damaged. After the exposure the bacteria were placed into unlabeled media and replication produced unlabeled DNA. By this approach it was possible to distinguish DNA present during the exposure from DNA synthesized after the exposure by mass spectrometry. Thus, we could see the loss of original phosphorothioates upon reaction with the chemical *in vivo* and the subsequent repair by the phosphorothiolating dnd enzymes. In the same year, we adapted the approach to RNA modification analysis in yeast [9]. As shown in Fig. 2**b**, we followed the modification density of tRNA in dependence of the growth phase and we identified the underlying mechanisms for several modified nucleosides (here 7-methylguanosine, m^7G, is shown). With NAIL-MS we can assess RNA turnover, RNA biosynthesis and dilution effects and determine the modification content of RNAs in response to *e.g.* growth or stress.

With LC-MS/MS the steady state of modifications is determined and thus is limited to a static view on dynamic RNA modification processes. Here, we present nucleic acid isotope labeling coupled mass spectrometry (NAIL-MS) which overcomes these current limitations and allows dynamic analysis of RNA modifications. We describe labeling techniques for *E. coli*, *S. cerevisiae* and human cell culture and the current instrumental limitations and recommended validation experiments.

We apply NAIL-MS to study the repair of adenine and cytidine, which are methylated by the damaging agent MMS in *E. coli*. With NAIL-MS, we exclude the concurrent processes for removal of RNA methylation, namely RNA degradation, turnover and dilution. The power of NAIL-MS is demonstrated by showing the kinetics of demethylation of damage-derived 1-methyladenosine and 3-methylcytidine in *E. coli in vivo*. We further outline current limitations of NAIL-MS but also potential future uses for *e.g.* relative quantification of RNA abundances.

2. Material and methods

2.1. Salts, reagents, isotopes and nucleosides

All salts were obtained from Sigma Aldrich (Munich, Germany) at molecular biology grade unless stated otherwise. Isotopically labeled compounds: $^{15}N\text{-}NH_4Cl$ (≥98% atom) and L-methionine-methyl-D_3 (98% atom) from Sigma-Aldrich. $^{13}C_6$-glucose (≥99% atom) and $Na_2{}^{34}SO_4$ (≥99.1% atom) from Eurisotop (Saarbruecken, Germany). 1,3-$^{15}N_2$-uracil (98% atom) from Cambridge Isotope Laboratories (Tewksbury, MA, USA). All solutions and buffers were made with water from a Millipore device (Milli-Q, Merck). Nucleosides: adenosine (A), cytidine (C), guanosine (G), uridine (U) and N2-methylguanosine (m^2G) from Sigma Aldrich. 1-Methyladenosine (m^1A), 2-methyladenosine (m^2A), N3-methylcytidine (m^3C), N6-methyladenosine (m^6A), 7-methylguanosine (m^7G), 5-methylcytidine (m^5C), 5-methyluridine (m^5U), 2′-O-methylcytidine (Cm), 2′-O-methylguanosine (Gm), 1-methylguanosine (m^1G) and 3-methyluridine (m^3U) from Carbosynth (Newbury, UK).

2.2. Specific laboratory equipment

Injection vial for HPLC and LC-MS: 0.3 mL PP Snap Ring Micro Vial, 32 × 11.6 mm, transparent, VWR (Radnor, PA, USA), Cat. No. 548-0120.

Injection vial cap: 11 mm Snap Ring Cap, tr., natural rubber/TEF, 60°, 1.0 mm, VWR (Radnor, PA, USA), Cat. No. 548-0014.

Fraction Collector glass vial: 1.5 mL Screw vial, 32 × 11.6 mm clear, VWR (Radnor, PA, USA), Cat. No. VWRI548-0018.

Glass vial cap: 8 mm PP-Screw cap black hole, VWR (Radnor, PA, USA), Cat. No. VWRI548-3322.

Culture tube: Centrifuge tube 50, TPP (Trasadingen, Switzerland), Product No.91050

2.3. Experimental settings

2.3.1. Metabolic isotope labeling

2.3.1.1. Bacteria. For each experiment a single colony from an LB agar plate with *E. coli* BW25113 was picked and used for culture inoculation.

We used minimal media M9 with and without the indicated isotopes for all bacterial cultures.

Unlabeled 10× M9 stock solution: mix 68 g/L Na_2HPO_4, 30 g/L KH_2PO_4, 2.5 g/L NaCl and 10 g/L NH_4Cl and autoclave. Store at room temperature.

Nitrogen-15-labeled 10x M9 stock solution: mix 68 g/L Na_2HPO_4, 30 g/L KH_2PO_4, 2.5 g/L NaCl and (!) **10 g/L $^{15}N\text{-}NH_4Cl$** (!) and autoclave. Store at room temperature.

Other stock solutions: $MgCl_2$ (0.1 M), $CaCl_2$ (0.1 M), Na_2SO_4 (0.1 M) and 20 w/w% glucose (or $^{13}C_6$-glucose) are prepared by sterile

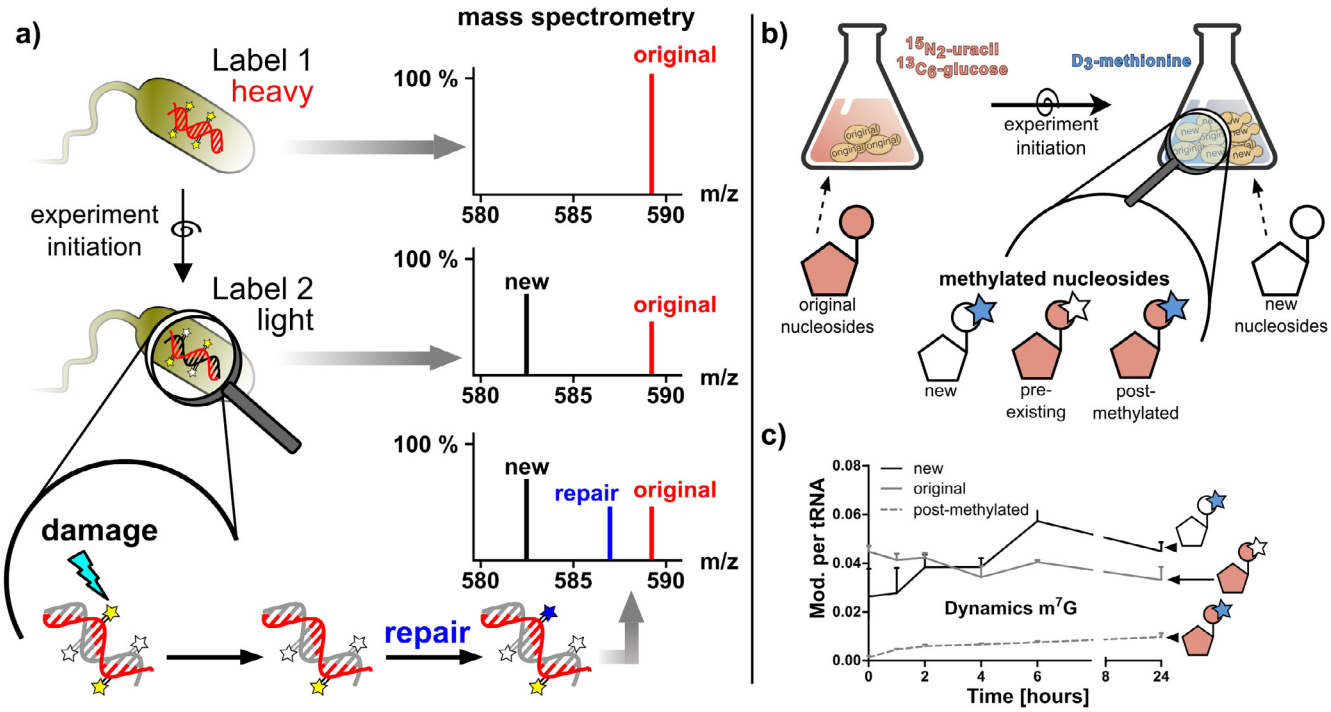

Fig. 2. a Principle of a NAIL-MS assay, which allows the observation of DNA modification damage repair *in vivo*. The bacteria are cultured in heavy isotope containing media, and therefore the DNA is heavy labeled, as observed by mass spectrometry. After exposure to the damage, the media is exchanged to a light media and newly replicated DNA is light labeled. The repair of original, but damaged DNA is observed by the formation of an intermediately labeled species in the mass spectrometer. (Adapted from [22].) **b** Principle of a dynamic NAIL-MS assay of tRNA in *S. cerevisiae* and the dynamics of 7-methylguanosine (m^7G) in tRNAs after experiment initiation. The drop in pre-existing m^7G is masked by an increase of post-methylated m^7G added to the pre-existing tRNAs (from [9]).

filtration.

For an unlabeled 5 mL pre-culture (or 50 mL exposure culture, respectively) mix 500 µL (5 mL) 10x M9 stock solution with 100 µL (1 mL) glucose, 100 µL (1 mL) $MgCl_2$, 100 µL (1 mL) Na_2SO_4 and 5 µL (50 µL) $CaCl_2$.

For ^{13}C-labeled cultures, $^{13}C_6$-glucose was used.

For ^{15}N-labeled cultures, the ^{15}N-10x M9 stock solution was used.

For CD_3-labeling, 200 µL of L-methionine-methyl-D_3 (stock 5 g/L) were added to 5 mL of culture volume.

2.3.1.2. Yeast. A single colony of a *S. cerevisiae* BY4741 YPD agar plate was picked and used for inoculation of 5 mL YNB media. 10x YNB (Carl Roth GmbH, Karlsruhe, Germany) was prepared according to manufacturer's manual. 1x YNB media was supplemented with the following metabolites to a final concentration of: 10 g/L glucose, 0.02 g/L uracil, 0.02 g/L methionine, 0.02 g/L arginine, 0.1 g/L aspartic acid, 0.1 g/L glutamine, 0.02 g/L histidine, 0.06 g/L leucine, 0.03 g/L lysine, 0.05 g/L phenylalanine, 0.4 g/L serine, 0.2 g/L threonine, 0.04 g/L tryptophan, 0.03 g/L tyrosine and 0.15 g/L valine (only L-amino acids were used). Depending on the desired labeling, $^{13}C_6$-glucose and L-methionine-methyl-D_3 were used instead of the unlabeled compounds.

2.3.1.3. Mammalian cells. HEK 293 T cells were cultured in Dulbeccós Modified Eagle Media (DMEM) or RPMI 1640 media (Gibco, Carlsbad, CA, USA). DMEM media was prepared by dissolving 8.4 g DMEM powder D5030 (Sigma Aldrich) in 1 L milli-Q water. Before sterile filtration, carbonate and phenol red were added to a final concentration of 3.7 g/L $NaHCO_3$ and 0.0159 g/L phenol red. Stocks of glucose (200 g/L) and L-glutamine (15 g/L) were prepared and sterile filtered. These were added to the DMEM media before usage to a final concentration of 2 g/L glucose, 0.584 g/L L-glutamine and 10% dialyzed fetal calf serum (Sigma Aldrich, Product No. F0392-500ML). The methionine concentration was 0.15 g/L in the final media. Depending on the desired labeling either $^{13}C_6$-glucose and L-methionine-methyl-D_3 or their unlabeled isotopomers were used. The cells were incubated at 10% CO_2 atmosphere and cultivated in the labeled media for at least 2 days (5 days for complete labeling). For splitting, the cells were treated with TrypLE Express (Gibco, Carlsbad, CA, USA).

2.3.2. RNA isolation and tRNA purification

Bacteria cultures were centrifuged at 1200 × *g* for 5 min, yeast cultures at 3500 × *g* for 5 min. The supernatant was discarded and the cell pellet was suspended in 1 mL TRI-Reagent® (Sigma-Aldrich) per 5 mL culture, respectively. Yeast cells were additionally vortexed for 5 min using acid-washed Glass beads (Sigma-Aldrich) equivalent to ~200 µL. HEK cells were harvested directly in cell culture dishes/flasks using 1 mL TRI-Reagent® per 25 cm^2. After transfer into an Eppendorf tube and incubation at room temperature for 5 min, 200 µL of chloroform (≥99% purity, Roth) were added to 1 mL of the TRI-Reagent® solution of each organism and the mixture was vortexed for at least 10 s. The biphasic solution was allowed to settle at room temperature for 5 min and centrifuged at room temperature for 20 min at 10,000 × *g*. The clear upper phase (~500 µL) was transferred into a new vial, 500 µL of isopropanol (Roth, Karlsruhe, Germany) were added and the solution was mixed thoroughly. The mixture was stored at -20 °C overnight. The precipitated total RNA was pelleted by centrifugation for 40 min at 12,000 × *g* and 4 °C. The RNA pellet was washed two times with 100–200 µL 70% EtOH and finally dissolved in 30 µL water.

For purification of tRNA from total RNA size exclusion chromatography (SEC) [23] was used on an Agilent 1100 HPLC system (Degasser, G1279A; Quat Pump, G1311A; ALS, G1313A; COLCOM, G1316A; VWD, G1314A; Analyt FC, G1364C) with an AdvanceBio column, 300 Å pore size, 2.7 µm particle size, 7.8 × 300 mm (Agilent, Waldbronn, Germany). For elution, a 1 mL/min isocratic flow of 0.1 M ammonium acetate was used. Eluting RNA was detected at 254 nm with a diode array detector. Under these conditions, tRNA elutes at a retention time

between 7 and 8 min. The 1 mL tRNA fraction was collected and evaporated (GeneVac, EZ-2 PLUS, Ipswich, UK) to a volume of ~100 µL before precipitation by addition of 0.1 vol of 5 M ammonium acetate and 2.5 vol of ice-cold ethanol (100%). (!) Ammonium acetate is the precipitation method of choice if mass spectrometric analysis is desired. Sodium ions interfere with mass spectrometric detection of nucleosides and should be avoided if possible (!) After rigorous mixing, the tRNA was allowed to precipitate at −20 °C overnight. The tRNA was pelleted by centrifugation (12,000*g*, 40 min, 4 °C), washed with 70% ethanol and resuspended in 30 µL water.

2.3.3. Pulse-Chase NAIL-MS

A single *E. coli* colony was picked and grown in 5 mL unlabeled M9 media overnight (37 °C, shaking at 250 rpm). From this pre-culture, a second overnight culture was prepared by inoculating 50 mL unlabeled media with the complete 5 mL pre-culture. On the next day, these bacteria were added to 120 mL of unlabeled M9 media to a final OD of 1.0. After 60 min of growth at 37 °C and 250 rpm, the first 7 mL aliquot was taken for RNA isolation. The remaining culture was equally split in 2 Erlenmeyer flasks (100 mL glass size) of 56.5 mL each. One was exposed to 95.7 µL MMS (99% purity), the other to the same amount of water (MOCK) and gently stirred before both cultures were left to grow for 60 min at 37 °C with shaking at 250 rpm. After 60 min exposure, an aliquot of 7 mL was drawn from each culture and the RNA was isolated. The remaining bacteria were centrifuged (1200 × *g*, 5 min) and the MMS/MOCK containing supernatants were discarded. The bacteria pellets were washed with 5 mL $^{15}N/CD_3$-methionine labeled M9 media and each suspended in 50 mL fresh $^{15}N/CD_3$-methionine M9 media. For recovery, the bacteria were grown at 37 °C, 250 rpm and after 1, 2, 3, 4 and 10 h 7 mL aliquots were drawn for RNA isolation.

2.3.4. tRNA digestion for mass spectrometry

100 ng tRNA in 30 µL aqueous digestion mix were digested to single nucleosides by using Alkaline Phosphatase (0.2 U, Sigma-Aldrich, Munich, Germany), Phosphodiesterase I (0.02 U, VWR, Radnor, Pennsylvania, USA) and Benzonase (0.2 U Sigma-Aldrich, Munich, Germany) in Tris (5 mM, pH 8.0) and $MgCl_2$ (1 mM) containing buffer. Furthermore, tetrahydrouridine (THU, 0.5 µg from Merck), butylated hydroxytoluene (BHT, 1 µM, Sigma-Aldrich, Munich, Germany) and Pentostatin (0.1 µg, Sigma-Aldrich, Munich, Germany) were added to avoid deamination and oxidation of nucleosides [2]. All mentioned concentrations/amounts are final concentrations/amounts used in a 30 µL final digestion volume. The mixture was incubated with the RNA for 2 h at 37 °C and filtered through 96 well filterplates (AcroPrep ™Advance 350 10 K Omega™, PALL Corporation, New York, USA) at 4 °C for 30 min at 3000 × *g*, or through single tubes (VWR, 10 kDa MWCO) at room temperature for 7 min at 5000 × *g*. The filtrate was mixed with *E. coli* SILIS 10:1 (stable isotope labeled internal standard [8]) and measured with the triple quadrupole mass spectrometer.

2.4. LC-MS instruments and methods

2.4.1. Triple quadrupole instrument

For quantification an Agilent 1290 Infinity II equipped with a DAD combined with an Agilent Technologies G6470A Triple Quad system and electro-spray ionization (ESI-MS, Agilent Jetstream) was used. Optimized operating parameters: positive ion mode, skimmer voltage 15 V, Cell Accelerator Voltage 5 V, N_2 gas temperature 230 °C and N_2 gas flow 6 L/min, sheath gas (N_2) temperature 400 °C with a flow of 12 L/min, Capillary Voltage of 2500 V, Nozzle Voltage of 0 V and the Nebulizer at 40 psi. The instrument was operated in dynamic MRM mode and the individual mass spectrometric parameters for the nucleosides are given in Supplementary Tables S2 and S3. Mobile phase A was 5 mM NH_4OAc (≥99%, HiPerSolv CHROMANORM®, VWR), brought to pH = 5.3 with glacial acetic acid (≥99%, HiPerSolv CHROMANORM®, VWR). Mobile phase B was pure acetonitrile (Roth, LC-MS grade, purity ≥99.95). A Kinetex EVO column (Phenomenex®, Torrance, California, USA; Kinetex® 1.7 µm EVO C18 100 Å, 150 × 2.1 mm) at a temperature of 35 °C with an eluent flow rate of 0.35 mL/min was used. The gradient started at 100% solvent A, followed by an increase to 10% solvent B over 10 min. From 10 to 15 min solvent B was increased to 45% and maintained for 3 min before returning to 100% solvent A and a 3 min re-equilibration period. Alternatively, a Synergi Fusion-RP column (Phenomenex®, Torrance, California, USA; Synergi® 2.5 µm Fusion-RP 100 Å, 150 × 2.0 mm) at 35 °C and a flow rate of 0.35 mL/min was used for yeast and mammalian cell analysis. Gradient elution started with 100% A for 1 min, increased to 10% B after 5 min, and to 40% after 7 min. The column was flushed with 40% B for 1 min. After regeneration of starting condition for 0.5 min the column was re-equilibrated at 100% A for 3 additional minutes.

2.4.2. High-resolution mass spectrometry

The ribonucleosides were separated using a Dionex Ultimate 3000 HPLC system on an Interchim Uptisphere120-3HDO C18 or an RP-18 column (Synergi, 2.5 µm Fusion-RP C18 100 Å, 100 × 2 mm; Phenomenex®, Torrance, California, USA). Mobile phase A was 2 mM ammonium acetate and mobile phase B was 80% acetonitrile containing 2 mM ammonium acetate. Gradient elution started with 0% B and increased to 12% B after 10 min and to 80% after 12 min. After 4 min elution at 80% B and subsequently regeneration of starting conditions to 100% A after 5 min, the column was equilibrated at 100% A for 8 min. The flow rate was 0.2 mL/min and the column temperature 30 °C. High-resolution mass spectra of precursor and product ions were recorded by a ThermoFinnigan LTQ Orbitrap XL. The parameters of the mass spectrometer were tuned with a freshly mixed solution of adenosine (5 µM). The parameters were sheath gas flow rate, 5 arb; auxiliary gas flow rate, 35 arb; sweep gas flow rate, 0 arb; spray voltage, 5.0 kV; capillary temperature, 200 °C; capillary voltage, 20 V, tube lens 65 V.

2.5. Calibration and equations

For calibration, synthetic nucleosides were weighed and dissolved to a stock concentration of 1–10 mM. Calibration solutions ranging from 0.15 pmol to 500 pmol for each canonical nucleoside and from 0.15 fmol to 500 fmol for each modified nucleoside were prepared in water. The calibration solutions were mixed with the *E. coli* SILIS and analyzed with the appropriate method. The value of each integrated peak area of the nucleoside was divided through the respective SILIS area. The linear regression for each nucleoside's normalized signal/concentration plot gives the relative response factor for nucleosides (rRFN) [8]. The sample data were analyzed by the Quantitative and Qualitative MassHunter Software from Agilent. The areas of the nucleoside signals were integrated for each modification and their isotope derivatives. The area was divided through the respective SILIS area and divided through the rRFN value from the respective calibration to receive the absolute amount of the modification or canonical. Finally, the absolute amounts of the modifications were referenced to the absolute amounts of the precursor canonical. In case of the validation and dynamic NAIL-MS experiment the different isotopomers were referenced to their respective labeled canonicals, so that original modifications are referenced to original canonicals and new modifications were referenced to new canonicals. See the following equations for m^1A as an example:

	m^1A (fmol)	A (fmol)	Normalization
Original Here: unlabeled	$\frac{area\ m1A\ (unlabeled)}{RFN\ m1A \times area\ m1A\ (SILIS)}$	$\frac{area\ A\ (unlabeled)}{RFN\ A \times area\ A\ (SILIS)}$	$\frac{m1A\ (original)}{A\ (original)}$

New	$\frac{area\ m1A\ (labeled)}{RFN\ m1A \times area\ m1A\ (SILIS)}$	$\frac{area\ A\ (labeled)}{RFN\ A \times area\ A\ (SILIS)}$	$\frac{m1A\ (new)}{A\ (new)}$
Here: labeled			

2.6. Other instruments

Orbital Shaker-Incubator ES-20 (BioSan, Riga, Latvia); Rotina 380 R centrifuge (Hettich, Tuttlingen, Germany); Centrifuge 5427 R (Eppendorf, Hamburg, Germany); Perfect Spin 24 R refrigerated micro centrifuge (PeQlab/VWR, Erlangen, Germany); Vortexer (Heathrow Scientific, IL, USA), Product Code: 120212; Nanophotometer N60 Touch (Implen, Munich, Germany), T60966; Speedvac EZ-2^{PLUS} (GeneVac, Ipswich, UK).

3. Important considerations for NAIL-MS studies

As the term NAIL-MS states, there are two key features for setting up such studies. The first is defined isotope labeling of the nucleic acid (NAIL) and the second is the availability of a mass spectrometer (MS). In addition, we recommend rigorous validation of NAIL-MS studies, which will be discussed in detail.

3.1. Labeling techniques

Before starting a NAIL-MS experiment, a labeling technique must be established, which leads to a defined labeling of the nucleic acid. Microorganisms like *E. coli* [8] and *S. cerevisiae* [9] are effortlessly labeled in minimal media or complete media (*e.g.* from Silantes, Munich, Germany). In addition, algae like *Chlamydomonas reinhardtii* or worms such as *Caenorhabditis elegans* can be labeled [10,24]. Fig. 3**a** shows the mass spectra of digested tRNA from an *E. coli* culture in minimal media M9 using $^{13}C_6$-glucose as the carbon source (left) or using CD_3-methionine (right). By feeding $^{13}C_6$-glucose overnight, 83% of carbon atoms in the tRNA are ^{13}C labeled in the exemplary nucleoside guanosine (G). Thus a mass increase of +10 is observed for G compared to unlabeled G (and ~17% of a +9 species). Most enzymatic methylation reactions require S-adenosyl methionine (SAM) as the methyl donor. After the methylation, the resulting S-adenosyl homocysteine is recharged with a methyl group by methionine. Feeding organisms CD_3-methionine leads to the formation of CD_3-SAM and transfer of heavy methyl marks onto nucleic acids [25,26]. This is also observed for 7-methylguanosine (m^7G) from CD_3-methionine supplemented bacterial cultures, which has a mass increase of +3 compared to unlabeled m^7G (note: the +4 is the natural ^{13}C isotope peak of m^7G). We recently shared this labeling technique, which helped Dal Magro et al. to determine the structure of a novel RNA modification in bacteria, namely $msms^2i^6A$ [11].

For purines in *S. cerevisiae*, we mainly observe a +6 mass increase upon culturing in $^{13}C_6$-glucose media overnight and a +3 for m^7G in CD_3-methionine media (Fig. 3**b**). It is noteworthy that both labeling techniques do not lead to a single defined isotopomer. Further validation is necessary to assess the impact of multiple isotopomer formation (see chapter on validation). In addition to the presented $^{13}C_6$-glucose and CD_3-methionine labeling, it is possible to use $^{15}NH_4Cl$ or $^{34}Na_2SO_4$ [22] in *E. coli* or $^{15}N_2$-uracil in yeast for further labeling options. It is also possible to combine the carbon, nitrogen, sulfur and methyl sources into a single media.

While yeast is already less efficiently labeled in minimal media compared to *E. coli* (compare Fig. 3**a** and **b**), labeling in human cell culture is even more difficult. We have tested HEK 293 T and Hela cell lines in both DMEM and RPMI media using $^{13}C_6$-glucose and CD_3-methionine. While labeling of methyl groups with CD_3-methionine is similarly successful in HEK cells (Fig. 3**c**) as it is in yeast cells, $^{13}C_6$-glucose labeling is less promising. We observed for all nucleosides a variety of formed isotopomers, which were found to be independent of the used serum. Culturing for a longer time did not increase labeling efficiency. Fig. 3**c** shows the guanosine signal from tRNA of a 5 day-labeled HEK culture in the presence of 2 g/l$^{13}C_6$-glucose. Here, a mass shift of +5, +6, +7 and +8 is observed for G. The +5 reflects a guanosine with a $^{13}C_5$-labeled ribose but unlabeled base. The +6 to +8 labeled peaks reflect guanosine isotopomers with $^{13}C_5$-labeled ribose and $^{13}C_X$-labeled base. Such a labeling technique is not suitable for comparative NAIL-MS studies (but potentially for dynamic NAIL-MS). $^{13}C_6$-glucose appears not to be the metabolite of choice for successful NAIL-MS experiments in cell culture systems. The determination of a metabolite or even a mixture of metabolites, which can be used to achieve a defined labeling in cell culture, is the major bottleneck in establishing NAIL-MS in cell culture.

When choosing a labeling technique for comparative or pulse-chase NAIL-MS, the abundance of natural isotopes, especially ^{13}C (1.1% rel. abundance) has to be considered. Pyrimidines and purines have 9 and 10 Carbon atoms, respectively. Statistically, ~10% of all nucleosides carry one ^{13}C atom (m/z +1), 1% carry two ^{13}C atoms (m/z +2) and 0.1% carry three ^{13}C atoms (m/z +3). We recommend labeling techniques, which increase the mass by at least 3 Dalton to avoid the detection of the natural ^{13}C-isotopomers of the nucleosides.

The preparation of isotopically labeled media requires the acquisition of isotopically labeled compounds, which are more expensive than the unlabeled compound. We have summarized the cost of 1 Liter NAIL-MS media in an overview in Table 1. Per NAIL-MS experiment around 20–50 mL heavy labeled media is necessary. The least expensive isotope labeling is achieved in bacteria (76 €/L) and cell culture (290 €/L), but here the labeling efficiency is quite low. Yeast minimal media (945 €/L) labeling is affordable, but yeast complete media (2025 €/L) is the most expensive and therefore solely recommended for the production of internal standards.

3.2. Mass spectrometry

For the detection of modified nucleosides, sensitive triple quadrupole instruments are used. The first quadrupole filters for the nucleosides' m/z values (*e.g.* m/z 298 for m^7G). In the collision cell (historically second quadrupole), the nucleoside is fragmented into nucleobase and ribose and the charge remains on the nucleobase. The third quadrupole filters for the nucleobases' m/z values (*e.g.* m/z 166 for m^7G). The detection of product ions from defined precursor ions is called a mass transition. For m^7G, the mass transition is 298 → 166. Commonly, 20–30 modified nucleosides are analyzed in a single run. This is achieved by fast switching from one nucleoside's mass transition to the next (around every 5–10 ms). If ions from the previous nucleoside remain in the mass spectrometer, although the instrument selects for the next nucleoside, false positive signals can be observed. Especially for co-eluting isotopomers with small differences in mass transitions, carry-over in the collision cell falsifies the detected quantities of the compounds. Linear collision cells with hexapoles and octopoles have high carry-over tendencies and should be validated for usability for NAIL-MS methods. The desired method can be tested by injection of single-labeled samples, which should only produce signals for the isotopomers from the used label. Signals from other isotopomer mass transitions are considered artefacts and the method is not usable for NAIL-MS studies. The carry-over error can be reduced by programming only 1–2 nucleosides into the method or by using time-gated selection of mass transitions. The new generation of QQQ instruments use faster collision cells (curved or T-wave), which have less to no carry-over. Note: Carry-over is rarely a problem in common nucleoside quantification as most nucleosides are chromatographically separated and thus do not disturb each other in the mass spectrometer.

3.3. Validation of NAIL-MS experiments

Insufficient labeling and a slow mass spectrometry can result in false positive results or misinterpretation. Thus, NAIL-MS experiments must

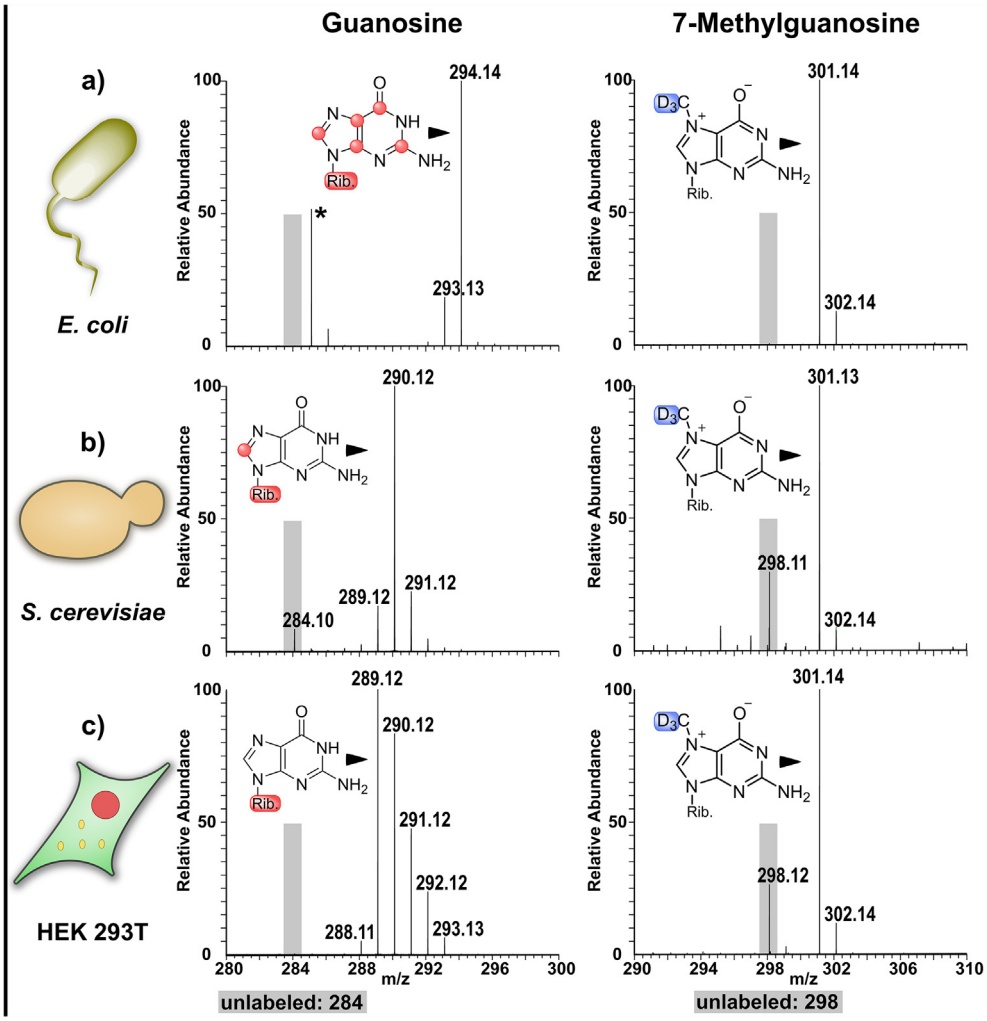

Fig. 3. High resolution mass spectra from ^{13}C labeled guanosine and CD_3-labeled 7-methylguanosine in *E. coli* (a), *S. cerevisiae* (b) and HEK 293 T (c). The grey bars in the spectra point out the expected *m/z* value of the unlabeled compounds. *Coeluting compound.

Table 1
Costs of NAIL-MS suitable media per Liter media in €uro.

Used isotope	CD_3	^{13}C	^{15}N	^{34}S
Bacteria (*E. coli*)	17	360	76	510
Yeast minimal	45	945	/	/
Yeast complete	1125	2025	/	/
Human cell culture	123	290	/	/

be carefully validated before they can be used for biological questions. The first step in validation should be to determine the labeling efficiency. As previously mentioned, it is crucial that the labeling leads to only one labeled isotopomer (*e.g.* 95% *m/z* of +5) and not several (*e.g.* 37% *m/z* +5, 31% *m/z* +6 and 18% *m/z* +7 as seen for guanosine in Fig. 3**c**). We recommend testing each labeling strategy by scanning the nucleoside mass range (*e.g.* *m/z* 240–400) and validate the abundance of all nucleoside isotopomers. If more than one isotopomer is formed under one labeling condition, we recommend optimization of the labeling technique. Sometimes low purity of the isotopically labeled metabolites/salts can also result in labeling inefficiency. In these cases, materials from other suppliers with higher isotope purity should be tested.

For validation of multiplexing and dynamic NAIL-MS we further recommend a control multiplexing experiment as shown in Fig. 4**a**. The media of choice is prepared in the unlabeled and isotopically labeled variant.

At this step it is crucial that both media are identical except for the isotope composition. Small differences in media composition already influence the abundance of modified nucleosides and the validation result. Thus, we recommend parallel preparation of the media using labeled and unlabeled stock solutions (!).

The two media are inoculated with the same number of cells and grown in parallel under identical conditions. After the appropriate amount of time (*e.g.* overnight for *E. coli*, 24 h for yeast and 5 days for HEK cells), the cells are harvested in *e.g.* TRI® reagent and mixed in a single container. From now on all processing steps are performed in parallel to avoid purification biases. From the isolated total RNA, the RNA of interest (*e.g.* tRNA) is isolated and prepared for LC-MS/MS by enzymatic digestion. The sample contains labeled and unlabeled nucleosides, and the amount of each modified nucleoside can be determined, normalized and plotted. Fig. 4**b** shows a successful validation of an *E. coli* $^{13}C_6$-glucose labeling procedure. Here, the quantities of most modified nucleosides from total tRNA are identical in labeled and unlabeled media.

The abundance of D (dihydrouridine) is in general higher in the ^{12}C samples compared to the ^{13}C samples. This is explained by contaminating ^{12}C-dihydrouridine from the used deaminase inhibitor tetrahydrouridine. Other uridine and cytidine derivatives showed a comparable modification profile in the ^{12}C and ^{13}C tRNA. A more than 1.1 fold difference in modification density is observed for m^2A. The

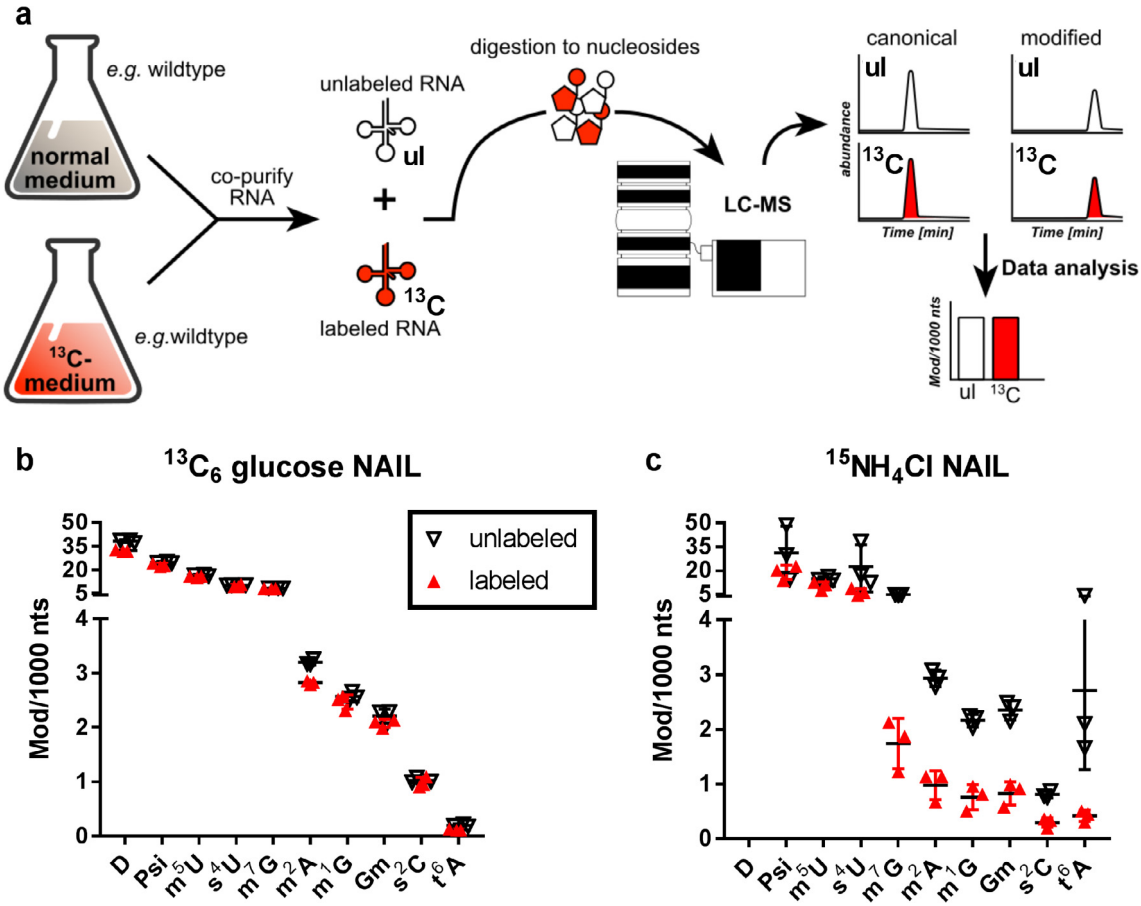

Fig. 4. a The principle of comparative NAIL-MS for validation of labeling. **b** and **c** *E. coli* comparative NAIL validation from a ^{13}C labeled culture mixed with an unlabeled culture (b) and a ^{15}N-labeled culture mixed with an unlabeled culture (c). y-axis labeling: Mod. per 1000 nts (Modification per 1000 nucleotides). Results from labeled RNA are marked in red, from unlabeled in black. (data represents 3 biol. replicates and error bars reflect the standard deviation.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

observed fold difference between labeled and unlabeled tRNA is defined as the limit of precision in comparative NAIL-MS experiments and should be given in a table for any NAIL-MS study (Supplementary Table S1). The $^{13}C_6$-glucose labeling can be used for comparative NAIL studies of organisms with varying genetic background or under various growth conditions. It is also very useful if samples from complex purification procedures are compared as co-purification eliminates potential purification biases. It is also suitable for pulse-chase NAIL-MS studies, which focus on both the original RNA modification content and the new transcript modification content.

Fig. 4**c** shows the validation of a ^{15}N labeling technique in *E. coli*, which is less successful. Here, we observe lower modification content in the ^{15}N labeled bacterial tRNA compared to the unlabeled tRNA. The limit of precision of this validation experiment is larger as shown in Supplementary Table S1. ^{15}N labeling is more error prone compared to ^{13}C labeling in *E. coli* due to the media preparation. M9 media requires the preparation of a M9 salt stock, which also contains the $^{15}NH_4Cl$ salt for ^{15}N labeling. This M9 stock mix has a limited shelf life, and upon aging of the M9 stock mix the modification content in bacteria changes. Thus, ^{15}N labeling can be only used for comparative NAIL when both unlabeled and labeled M9 stock mix are freshly prepared and ideally in parallel. For pulse-chase NAIL-MS ^{15}N labeling is acceptable if, for example, the labeling is only used to distinguish original RNA from newly transcribed RNA. In these studies, modification profiles cannot be compared, but changes in the RNA modification profile of *e.g.* original RNA can be studied and effects of transcription can be excluded.

4. Results

4.1. Observing the repair of methylation damage in E. coli tRNA by pulse-chase NAIL-MS

We recently applied NAIL-MS to discriminate the origin of methylated RNA nucleosides in *E. coli* treated with the methylating agent MMS. We observed direct methylation of all canonical nucleosides and 7-methylguanosine, 1-methyladenosine, 6-methyladenosine, 3-methylcytidine and 3-methyluridine as the main damage products. In a dynamic NAIL-MS assay we followed the fate of these damage products and we observed demethylation of 1-methyladenosin and 3-methylcytidine after 24 h [27]. Here, we want to present the assay in more detail and we performed a NAIL-MS time-course experiment, which allows the determination of the demethylation kinetics. An assay to observe the repair of methylated RNA nucleosides is possible by NAIL-MS under the following conditions: A) A "backbone" labeling technique is needed, which allows discrimination of the damaged RNA from newly transcribed RNA. B) Availability of a labeling technique, which additionally distinguishes the damaged modification from the natural modification (here CD_3-methyl groups) [27]. C) The "damage" label and "backbone" label must be sufficiently resolvable in the mass spectrometer, *i.e.* if the "damage" label is +3, the "backbone label" cannot be +3 but should be ideally larger than +5. D) An internal standard must be available which is clearly distinguishable from all potential heavy isotope combinations from the biological assay.

In this study, we used 15-Nitrogen for the "backbone" labeling which results in a labeling of +5 for purines, +3 for cytidine and +2

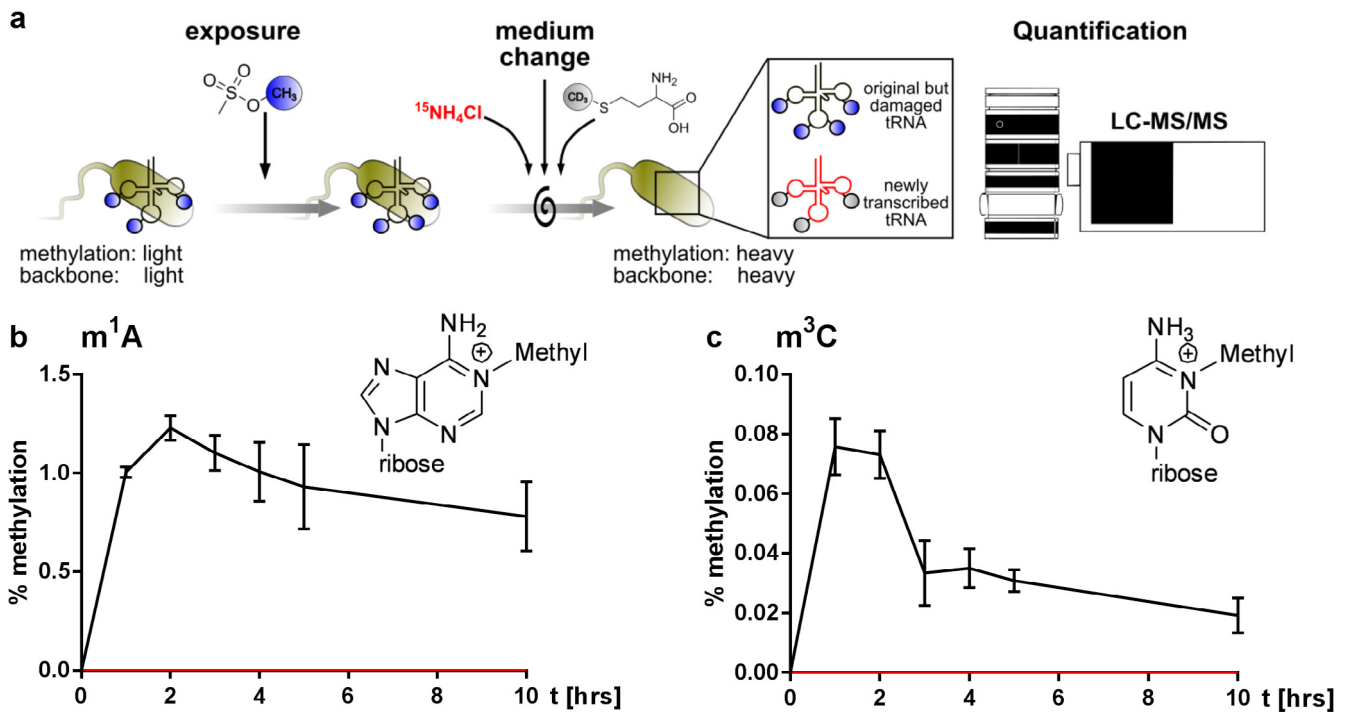

Fig. 5. **a** Concept of a pulse-chase NAIL-MS assay to distinguish the modified nucleosides from damaged tRNAs and newly transcribed tRNAs. (Adapted from [27].) **b** and **c** Pulse-chase NAIL-MS assay for 1-methyladenosine (**b**, m^{1}A) and 3-methylcytidine (**c**, m^{3}C). Color code: Black: damaged tRNAs. Red: newly trtranscribed tRNAs (Error bars represent the standard error of 3 biological replicates.)

Table 2
Mass transitions of 1-methyladenosine isotopomers in pulse-chase NAIL-MS for RNA repair observation.

Compound Group	Compound Name	Precursor Ion	Product Ion	Ret Time (min)
Unlabeled (original)	A	268	136	5.7
	m^{1}A	282	150	1.7
^{15}N and ^{13}C labeled (internal standard)	A SILIS	283	146	5.7
	m^{1}A SILIS	298	161	1.7
CD_3 labeled (post-methylated)	m^{1}A CD_3	285	153	1.7
^{15}N labeled (new)	A ^{15}N	273	141	5.7
^{15}N and CD_3 labeled (new)	m^{1}A ^{15}N_CD_3	290	158	1.7

for uridine. As the "damage/methylation" label we used CD_3-methionine (+3). The assay was set up as outlined in Fig. 5**a** and samples were drawn every hour after removal of the MMS. The bacteria are grown in unlabeled M9 media and exposed to the LD_{50} dose of MMS (Supplementary Fig. S1). The original RNA as well as all methylated nucleosides were unlabeled ($m/z \pm 0$). After 60 min, the MMS is removed by media exchange. The new media contains only ^{15}N as the nitrogen source and CD_3-methionine. Newly transcribed RNA is now labeled with ^{15}N (m/z +5, +3 or +2) and methylated nucleosides have an additional +3 label (m/z is thus +8, +6 or +5). Original tRNA, which is enzymatically methylated after media exchange receives a +3 label from the CD_3-methionine (m/z is thus +3). Using mass spectrometry, the tRNA exposed to the MMS and the newly transcribed tRNA can be clearly distinguished and the abundance of modified nucleosides in the original tRNA can be quantified. An exemplary list for the mass transitions of all m^{1}A isotopomers is given in Table 2 and for the other nucleosides in Supplementary Table S3. Here, and in the recently published study [27], we solely focused on the fate of the damaged tRNA from unlabeled bacteria culture. In such a case, the validation of NAIL-MS can be omitted, as the modification profile of the new transcripts is not of interest.

In *E. coli*, the quantities of the usually unnatural nucleosides m^{1}A and m^{3}C peak after 2 h with around 1% damaged adenosines (Fig. 5**b**) and only 0.08% damaged cytidines (Fig. 5**c**) in tRNA. The abundance of m^{1}A is slowly decreasing in the damaged tRNA over the 10 h recovery period. Two potential scenarios can explain this: The first scenario is degradation of m^{1}A-damaged tRNAs. The sequence of each tRNA contains at least 10 adenines in *E. coli* [1]. 1% of all adenosine methylation damage thus translates to a statistical abundance of m^{1}A in 1 out of 10 tRNAs. Thus 10% of the total tRNA pool needs to be degraded for removal of m^{1}A. If m^{1}A-targeted tRNA degradation was the cause, a faster tRNA dilution rate in comparison to unstressed *E. coli* would be observable. However, we observe the opposite (Supplementary Fig. S2b). The original tRNA pool from MMS stressed bacteria is less quickly diluted by newly transcribed tRNA in comparison to the unstressed bacteria. Thus, we consider the scenario of m^{1}A-targeted tRNA degradation as unlikely.

The second scenario is the proposed repair by demethylation. We see this hypothesis as proven, since we did not observe increased degradation of the original, damaged tRNA pool. Therefore, the only cause for the decrease in m^{1}A abundance is active demethylation of m^{1}A (potentially by AlkB [28]).

With our NAIL-MS assay, we can also follow the speed of m^{3}C repair by demethylation *in vivo*. The repair is even quicker (Fig. 5**c**), which is most likely caused by the comparably low abundance of m^{3}C sites in the tRNAs.

5. Discussion and outlook

LC-MS/MS is the method of choice for quantification of modified nucleosides. However, it is currently limited and only provides data on the quantities of modified nucleosides in an RNA but not about the dynamic changes and the underlying mechanisms. These limitations can be overcome by using nucleic acid isotope labeling coupled mass spectrometry (NAIL-MS). We present currently used approaches of isotope labeling in microorganisms such as *E. coli* and *S. cerevisiae*. Furthermore, we demonstrate the difficulties of adapting these methods

to human cell culture. In our hands, cell culture labeling by addition of $^{13}C_6$-glucose is not sufficient to allow successful application of comparative NAIL-MS experiments. However, for dynamic pulse-chase experiments where *e.g.* only the original RNA is the focus of the analysis, the labeling should allow sufficient resolution to distinguish the original RNA from new transcripts. Thus, we foresee a wide usage of cell culture labeling by addition of $^{13}C_6$-glucose in dynamic NAIL-MS experiments to answer current questions regarding open questions in the field such as: Is the change in the epitranscriptome due to a complete renewal of the transcriptome? Are modifications added to the existing transcriptome to confront *e.g.* stress? And are modifications removed actively *in vivo*? Due to the many isotopomers formed for each nucleoside and its respective modifications, we also foresee that massive validation is necessary to answer these questions by $^{13}C_6$-glucose in dynamic NAIL-MS. Ideally, other metabolites, *e.g.* precursors of nucleoside biosynthesis are found which lead to single isotopomer formation in cell culture. Thus, the usage of comparative NAIL and more elegant pulse-chase experiments would become possible in cell culture.

Regarding comparative NAIL-MS, we also foresee a unique opportunity towards determination of changes in RNA abundance as a consequence of stress or the loss of an RNA modification. Instead of quantifying the abundance of modified nucleosides, it should be possible to focus on the quantities of canonical nucleosides instead. As Fig. 6 shows, the total RNA from a comparative NAIL experiment is analyzed and the ratio of canonical nucleosides from labeled and unlabeled total RNA is determined. The ratio from the total RNA is later used for normalization and set to 100%. From the total RNA mixture, the RNA of interest, here isoacceptor tRNAs, can be purified [29]. The quantities of canonical nucleosides from an isoacceptor tRNA can be determined and subsequently the ratio of labeled and unlabeled is formed. In a control experiment, the abundance of all tRNA isoacceptors is expected to be identical in the unlabeled and labeled samples. The ratio of the pure isoacceptor tRNA can be compared to the total RNA ratio and plotted in %. Indeed, we observe in our $^{13}C_6$-glucose validation experiments from *E. coli* (Fig. 6) the same ratio of labeled and unlabeled canonical nucleosides for total RNA and several purified tRNA isoacceptors.

In a comparative NAIL experiment, it should be possible to detect changes in *e.g.* tRNA isoacceptor abundances because of stress or genetic manipulation of an RNA writer. By mixing an unlabeled control sample with a labeled, but *e.g.* stressed sample, the potential changes of tRNA isoacceptor abundance should be detectable. We expect that the ratio of canonical nucleosides would be different in total RNA and the purified tRNA isoacceptor and thus the impact of the stress on the relative abundance of the tRNA isoacceptor should be revealed. If our assumption is correct, we see a broad usability of comparative NAIL-MS to determine relative abundances of RNAs.

As an example, we show the repair kinetics of 1-methyladenosine (m^1A) and 3-methylcytidine (m^3C) in bacterial tRNA after exposure to MMS *in vivo*. We find that m^1A is slowly removed from original tRNA over the timeframe of observation. This is in accordance with previous work done with radioisotope labeling [28]. In contrast to the published work, we used *E. coli* without previous induction of AlkB which is reflected in the rather slow repair of m^1A observed in our NAIL-MS study. Similarly, we can observe the removal of m^3C from the bacterial tRNA which appears to happen a lot faster. The reason can be the relatively low abundance of m^3C damage compared to m^1A damage. It is also possible that m^3C is the better substrate for the tRNA demethylase.

In this study, we found around 1% of all adenines in tRNA are methylated by the methylating agent MMS after one hour of exposure to the LD_{50} dose. From a chemical point of view, the N1 position of adenine can be considered a good nucleophile which is easily methylated by an electrophile such as MMS. Therefore, we wonder how strongly the N1 position of adenine interacts with natural electrophiles such as S-adenosyl methionine (SAM). SAM is the natural methylating agent of the cell, and the methylation reaction of nucleic acids usually depends on an enzyme which activates the nucleoside first. However, chemically, the N1 position of adenosine is already a good nucleophile, and it should be possible, that some methylation occurs by reaction with SAM inside the cell, especially in those environments, which are rich in SAM and those RNAs that have one or more non base-paired adenosines. Considering the current dispute on the distribution of m^1A in mRNA [30–32], we wonder if some of the 0.02% m^1A (per A) [33] in mRNA is due to non-enzymatic methylation by SAM. In eukaryotic mRNA, the 5′ end is methylated (m^7G of the mRNA cap) and thus the 5′ end and its adenosines are always exposed to high amounts of SAM. Statistically, a sub-stoichiometric methylation of these adenosines is possible and should be taken into account during data interpretation. Of course, the origin of the 5′ UTR methylation, enzymatic or chemical, does not play a major role upon determining the function of the methylated adenosine.

With nucleic acid isotope labeling coupled mass spectrometry (NAIL-MS) we overcome current limitations and assess the dynamics of RNA modifications. We show the repair of m^1A and m^3C *in vivo* by discriminating the concurrent processes for removal of RNA methylation, namely RNA degradation, turnover and dilution. Dynamic NAIL-MS and comparative NAIL-MS are powerful tools which finally allow the observation of dynamic processes of RNA and its modifications.

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The Kellner lab thanks Thomas Carell and his group for instrument

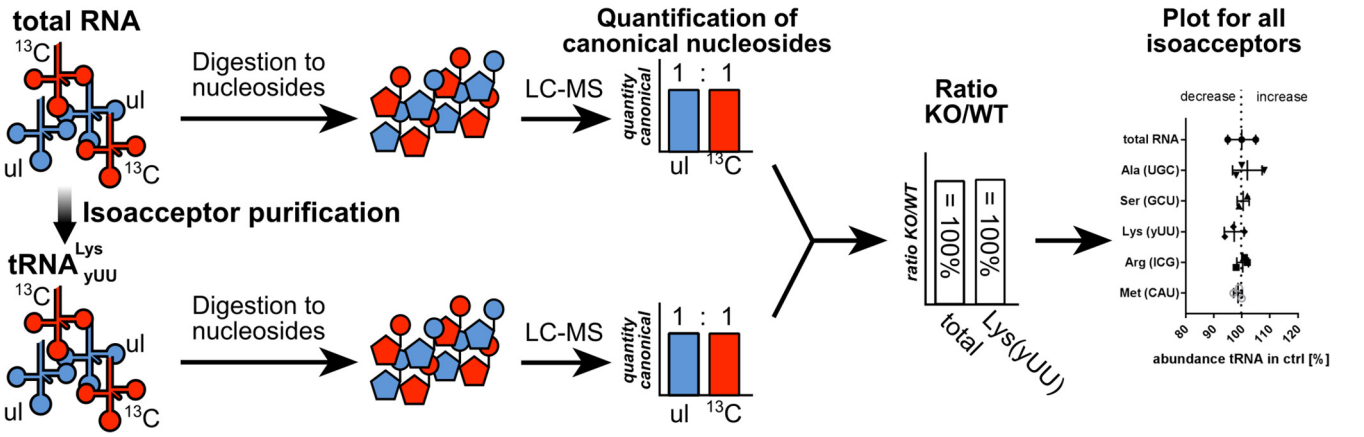

Fig. 6. Concept of relative tRNA isoacceptor abundance determination by NAIL-MS. The ratio of co-purified total RNA from unlabeled (ul) and ^{13}C labeled cells is 1:1 in this example. From the same RNA sample, a tRNA isoacceptor is purified by oligonucleotide hybridization. After digestion the ratio of the canonical nucleosides is determined and compared to the ratio from total RNA. The resulting data of each isoacceptor tRNA is plotted for all isoacceptors (n = 3).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2018.10.025.

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3.5. RNA Modifikationen als Biomarker zur Nematoden-Diagnostik

Prolog: Wie bereits in der Einleitung beschrieben sind zahlreiche regulatorische und krankheitsbedingte Abläufe in unterschiedlichsten Zelltypen durch RNA-Modifikationen beeinflusst. Im Vergleich zu den vorherig untersuchten RNA Schäden in Bakterien werden im Folgenden die RNA-Modifikationen von verschiedenen Fadenwürmern (Nematoden), also von eukaryotischen Parasiten, quantifiziert und verglichen. Neben *C. elegans N2*, *Heligmosomoides polygyrus* (*H. polygyrus*) und *L. sigmondontis* soll auch der besonders für den Menschen relevante Nematodenvertreter *O. volvulus* untersucht werden. Dieser löst die Flussblindheit oder Onchozerkose aus[133], wogegen bisher noch keine zufriedenstellenden Therapien und Diagnostik-Verfahren etabliert sind. Deshalb wird derzeit intensiv an einer verbesserten Therapie der Krankheit und an neuen Diagnosemethoden geforscht.[134] Anstelle des humanen Erregers wird nun seit knapp 30 Jahren auch mit dem Rinder-Parasiten *O. ochengi* geforscht. Dieser ist für den Menschen ungefährlich und wird aufgrund seiner genetischen und physiologischen Nähe in der Forschung als Modell-Organismus für *O. volvulus* verwendet.[76] Mit Hilfe der Analyse von aus dem Urin infizierter Rinder isolierter miRNA Fragmente soll untersucht werden, ob *O. ochengi* und damit vielleicht auch *O. volvulus* im Wirt einen Einfluss auf deren RNA-Modifikationsprofil hat. Die Ergebnisse dieser Untersuchungen könnten zur Identifizierung eines neuen auf RNA-Modifikationen basierenden Biomarkers führen. Dieser könnte einen erheblichen Vorteil zur Verbesserung der Diagnostik liefern.

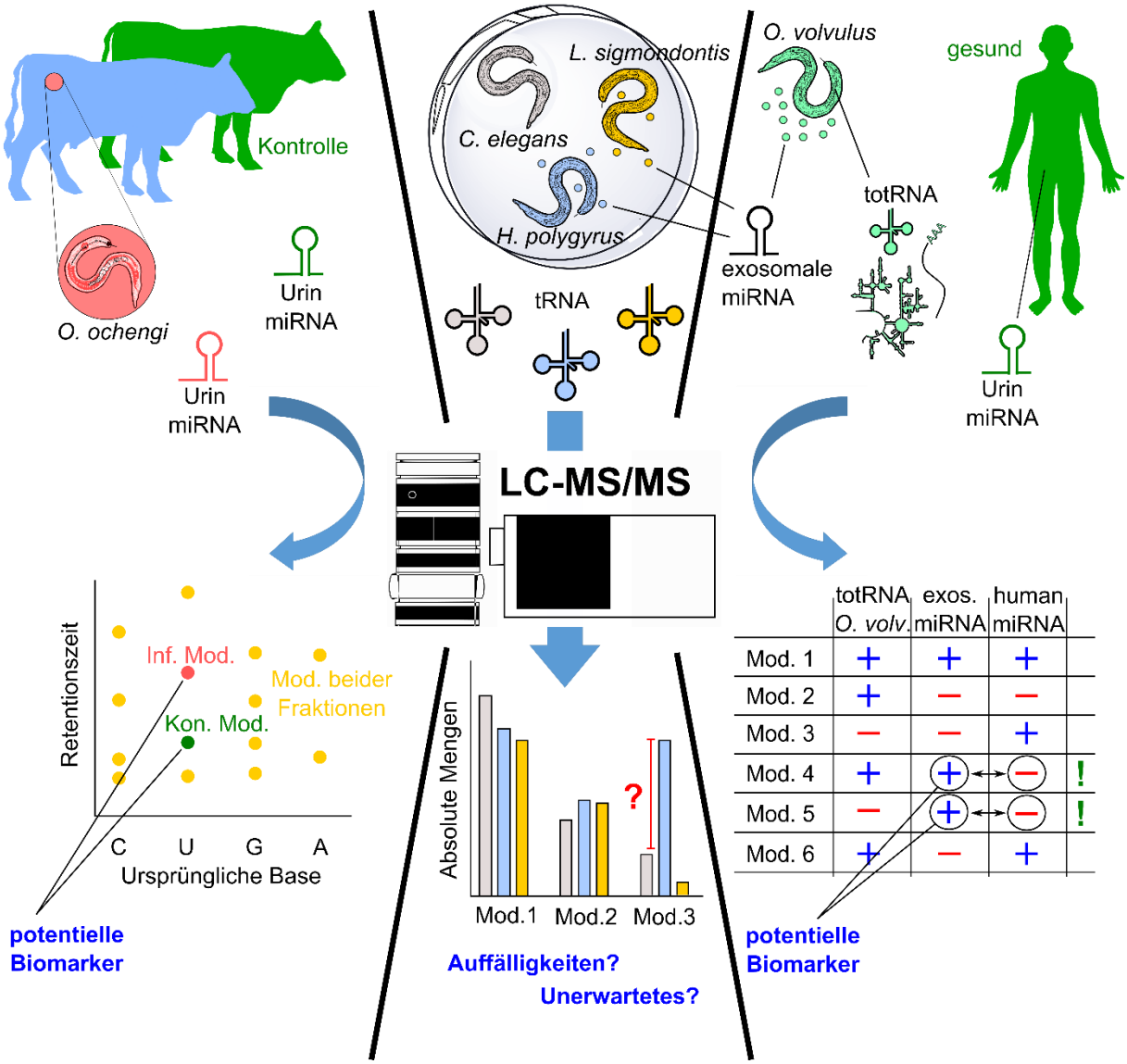


Abbildung 15: Schematische Darstellung der Fragestellungen des Nematoden Projekts, aufgegliedert in drei Hauptteile. Links: Vergleich der Urin miRNA-Modifikationen infizierter und nicht infizierter Rinder. Gibt es quantitative Veränderungen oder verschwinden Modifikationen ganz? Tauchen vielleicht neue Signale auf? Mitte: Quantifizierung des tRNA-Modifikationsprofils verschiedener Nematodenarten. Worin unterscheiden sich die Arten? Rechts: Modifikationsvergleich zwischen Nematoden RNA, exosomaler RNA und humaner miRNA. Gibt es Unterschiede, Modifikationen, welche spezifisch für die Würmer sind und könnten diese dann mit dem Urin des Wirts ausgeschieden und quantifiziert werden? Abkürzungen: Mod. = Modifikation, Kon. Mod. = Modifikation aus Kontrollprobe, Inf. Mod. = Modifikation aus Infektionsprobe, exos. miRNA = exosomale miRNA, *O. volv*. = *O. volvulus*.

3.5.1. RNA-Modifikationsprofil verschiedener Nematoda-Modellorganismen

Der bekannteste und am besten untersuchte Vertreter der Fadenwürmer ist der Modellorganismus *C. elegans N2*. Unter anderem aufgrund seiner definierten Anzahl an Zellen, seines transparenten Körpers sowie der einfachen Kultivierung, wird dieser Organismus bevorzugt für zahlreiche Studien zur Erforschung verschiedenster Fragestellungen herangezogen. *C. elegans N2* ist der erste mehrzellige eukaryotische Organismus, dessen Genom vollständig sequenziert wurde.[135] Des weiteren wird *C. elegans* in der Erforschung der Zelldifferenzierung, bei der Untersuchung neurologischer Fragestellungen[136], im Bereich der RNA Inhibierung (*RNA silencing*)[137] oder etwa zur Lösung von Fragestellungen zu Genom bedingten Alterungsprozessen[138], eingesetzt. Dies sind nur einige wichtige Forschungsgebiete in denen *C. elegans* als Modellorganismus nicht mehr wegzudenken ist. *C. elegans N2* wird üblicherweise auf *E. coli* Platten kultiviert und hat sich bereits genetisch an die künstliche Kultivierung angepasst.[139] Der Organismus wird in dieser Studie aufgrund seiner Modelleigenschaften und aufgrund seiner bereits intensiv erforschten Biologie in die Messungen miteinbezogen.

Weitere in diesem Zusammenhang untersuchte Nematodenarten sind *H. polygyrus* und *L. sigmondontis*, zwei in den Gedärmen von Nagern heimische parasitäre Nematoden-Vertreter, die in Kombination mit Mäusen verschiedene Maus-Modelle zur Untersuchung von humanen Wurminfektionen ermöglichen.[140] Damit spielen diese Würmer ebenfalls eine relevante Rolle in der Forschung und erweitern das Spektrum an nematodischen Modell-Organismen. Dennoch ist derzeit noch wenig über deren RNA-Modifikationsprofile bekannt. Deshalb wurde die Nematoden RNA in meiner Dissertationsarbeit vorerst qualitativ nach bekannten und unbekannten RNA-Modifikationen untersucht, bevor in einem späteren Kapitel verstärkt auf die Quantifizierung der entdeckten RNA-Modifikationen eingegangen werden soll.

Die in dieser Arbeit untersuchten Nematoden wurden in der Arbeitsgruppe um Dr. Amy Buck, *University of Edinburgh*, kultiviert, anschließend die totale RNA aus den adulten Nematoden extrahiert, in Wasser gelöst und tiefgefroren an mich übersendet. Die totale RNA wurde nicht weiter aufgetrennt sondern direkt enzymatisch zu Nukleosiden verdaut und über dieselbe massenspektrometrische MRM Methode gemessen wie die RNA Fraktionen unter **Kapitel 3.1**. Es wurden bei jeder Messung äquivalente Mengen an verdauter totaler RNA injiziert (30 µg). Da die daraus erhaltenen Signale nicht

ausreichend zur Quantifizierung geeignet sind und keine zusätzlichen Artefakt-Signale durch einen internen Standard hervorgerufen werden sollten, wurden die erhaltenen Signale ausschließlich qualitativ betrachtet und nach deren Intensität klassifiziert. Wie aus **Tabelle 4** ersichtlich, konnten einige Signale bereits bekannten RNA-Modifikationen zugeordnet werden. Die Anzahl der Plus-Symbole soll die Signalintensitäten der verschiedenen Modifikationen in den verschiedenen Nematodenarten verdeutlichen. So stehen einfache Plus (+) für eine Intensität kleiner $1x10^4$, doppelte Plus (++) für eine Signalintensität kleiner $5x10^6$ und dreifache Plus (+++) für Signalintensitäten größer $5x10^6$. Hierbei muss stets bedacht werden, dass Signalintensitäten untereinander verglichen nicht zu einer quantitativen Aussage führen, es sei denn es handelt sich um dasselbe Molekül. Somit können Moleküle die selten in der Probe zu finden sind sehr viel höhere Signale produzieren als sich strukturell davon unterscheidende Moleküle, welche in größeren Mengen vorhanden sind.

Überraschenderweise wurden in *C. elegans N2* sowie in *H. polygyrus* neben anderen RNA Modifikationen auch die tRNA Modifikation s^2C und dessen Methylierungsprodukt ms^2C (siehe **Kapitel 3.2.**) detektiert. Bisher wurde ms^2C ausschließlich in Prokaryoten, s^2C zusätzlich in Archaea[141], beide jedoch nie zuvor in eukaryotischer RNA beschrieben.

Tabelle 4: Liste der anhand der Masse pro Ladung (m/z) identifizierten und anhand der Retentionszeiten der synthetischen Standards verifizierten RNA-Modifikationen. Die Signalintensitäten sind in Stufen eingeteilt. (-) Kein Signal; (+) Signalintensität kleiner $1x10^4$; (++) Signalintensität kleiner $5x10^6$; (+++) Signalintensität größer $5x10^6$. Rot eingefärbt sind Modifikationen, deren Intensitäten zwischen den unterschiedlichen Nematodenarten stark schwanken.

			Signalintensitäten			
Vorläufer Ion [m/z]	**Produkt Ion [m/z]**	Rt. [Min.]	*C. elegans N2*	*H. polygyrus*	*L. sigmondontis*	**Mod.**
247	**115**	1.452	++	++	++	**D**
258	**126**	2.574	+++	+++	+++	**m^5C**
258	**126**	2.001	++	++	++	**m^3C**
259	**127**	3.839	++	++	++	**m^5U**
259	**127**	4.609	+	+	++	**m^3U**
260	**128**	2.264	++	+	-	**s^2C**
269	**137**	3.506	++	++	++	**I**
274	**142**	5.255	+	++	-	**ms^2C**
282	**150**	2.096	+++	+++	+++	**m^1A**
282	**150**	8.406	+++	+++	+++	**m^6A**
283	**151**	4.913	++	++	++	**m^1I**
286	**154**	5.487	++	++	++	**ac^4C**

296	**164**	12.015	+++	+++	+++	**$m^6{}_2A$**
298	**166**	2.346	+++	++	++	**m^7G**
298	**166**	5.051	+++	+++	+++	**m^1G**
298	**166**	5.43	+++	+++	+++	**m^2G**
312	**180**	6.78	+++	+++	+++	**$m^2{}_2G$**
317	**185**	5.251	+	+	++	**mcm^5U**
326	**194**	5.754	+++	+++	+++	**$m^2{}_2{}^7G$**
333	**201**	7.711	++	++	++	**mcm^5s^2U**
336	**204**	14.195	++	++	++	**i^6A**
413	**281**	7.446	++	++	++	**t^6A**

Eine mögliche Erklärung für die Anwesenheit von s^2C und ms^2C könnte sein, dass durch die als Nahrung aufgenommenen *E. coli* Bakterien, und durch deren Verdau innerhalb der Würmer, die Bakterien spezifischen RNA Modifikationen in den Nukleotid-Kreislauf der Nematoden gelangen und so in deren eigene RNA eingebaut werden könnten. Des Weiteren ist nicht auszuschließen, dass sich während des RNA Extraktionsvorgangs noch etliche *E. coli* Zellen oder zumindest deren RNA Fragmente innerhalb des Nematodenkörpers befinden, was damit zu einer Co-Extraktion der *E. coli* RNA führen würde (**Abbildung 16**). Um den Ursprung der beiden RNA-Modifikationen überprüfen zu können, würde ich ein weiteres Experiment vorschlagen, in welchem die Nematoden während der Kultivierung anstatt mit Wildtyp-*E. coli* mit Zellen des s^2C-Knockout *E. coli* Stamms ΔTtcA gefüttert würden. Wären nach dieser Behandlung immer noch starke Signale für s^2C und ms^2C in der isolierten Nematoden RNA sichtbar, wäre dies ein Hinweis dafür, dass es in Eukaryoten ein Homolog zur bakteriellen Thiotransferase TtcA gibt und die hier identifizierten Signale nicht durch Co-Aufreinigung von nematodischer und bakterieller RNA entstanden sind.

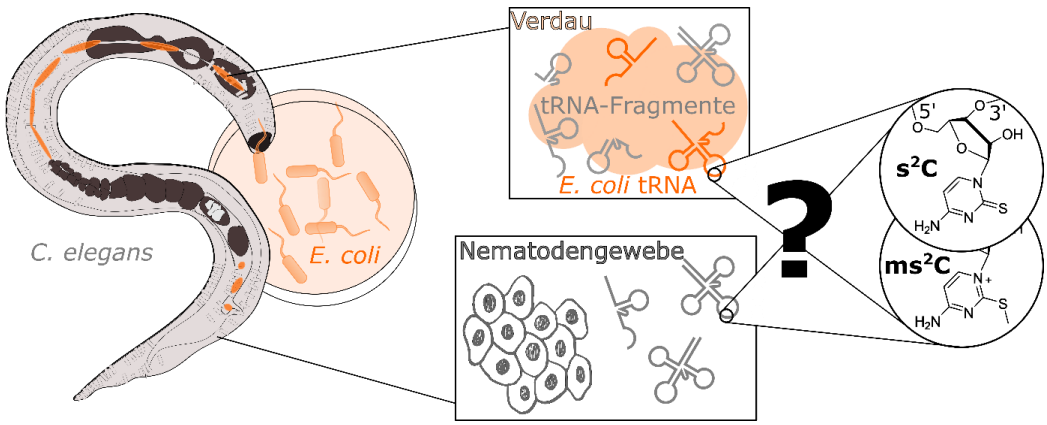


Abbildung 16: Ursprung der Modifikationen s^2C und ms^2C in *C. elegans*. Unter Laborbedingungen werden die Nematoden auf Petrischalen kultiviert, welche *E. coli* Bakterien enthalten. Bei der RNA-Isolierung stellt sich die Frage, ob die nematodeneigene RNA getrennt von der aus dem Verdauungstrakt der Nematoden stammenden RNA gewährleistet ist.

Abgesehen von s^2C und ms^2C wurden alle identifizierten Modifikationen über alle drei untersuchten Arten hinweg mit meist ähnlich starken Signalintensitäten gemessen. Verglichen mit den Messungen für *C. elegans* und *H. polygyrus* konnte eine deutliche Zunahme der Signalintensitäten für m^3U und mcm^5U in *L. sigmondontis* beobachtet werden. Dies kann für *L. sigmondontis* eine Veränderung der Translationseigenschaften bedeuten. So ist mcm^5U, wie in der Einleitung beschrieben, an der Wobble-Position 34 der tRNA zu finden, wodurch die Erkennung der passenden Codons während der tRNA-Bindung beeinflusst wird.[39] Die Modifikation m^3U kommt in der tRNA dagegen ausschließlich als Methylierungsschaden vor, während in der bakteriellen rRNA m^3U an Position 1498 der 16S Untereinheit enzymatisch angebracht wird.[142] Es nimmt dabei vermutlich eine strukturstabilisierende Funktion in der rRNA ein, welche förderlich für die Translation ist.

Neben den bekannten Modifikationen konnten außerdem viele Signale detektiert werden, für die keine Modifikationen bestätigt werden konnten (siehe **Anhang Tabelle 2**). Manche dieser Signale wurden bereits in der tRNA von *Pseudomonas aeruginosa PA14* nachgewiesen (Vgl. **Anhang Tabelle 1**). Andere wiederum stimmen mit dem m/z-Wert von bekannten Modifikationen überein[5], ohne dass die Retentionszeiten der potentiellen Modifikationen bestätigt noch widerlegt werden konnten. Eine Liste von besonders interessanten Signalen mit ungeklärter zugehöriger Molekülstruktur findet sich in **Tabelle 5**. Die hier aufgelisteten Signale deuten aufgrund ihrer großen Intensitätsschwankungen zwischen den einzelnen Nematodenarten nicht auf ein Artefakt, sondern tatsächlich auf unbekannte Strukturen aus den RNA Isolaten hin. Um die Signale dahingehend zu untersuchen, ob es sich um neuartige RNA-Modifikationsstrukturen handelt wäre es durchaus denkbar die Nematoden mit isotopenmarkierten Bakterien zu füttern um schließlich isotopenmarkierte Nematoden, beziehungsweise deren entsprechend markierte RNA zu generieren. Die unterschiedlich markierten RNA-Fraktionen könnten dann genau wie in **Kapitel 3.1** analysiert werden um vermeintliche Artefakt-Signale ausschließen zu können.

Tabelle 5: Liste unbekannter Signale mit großen Intensitätsunterschieden zwischen den einzelnen Nematodenarten. (-) Kein Signal; (+) Signalintensität kleiner $1x10^4$; (++) Signalintensität größer $1x10^4$ aber kleiner $5x10^6$.

			Signalintensitäten		
Vorläufer Ion [m/z]	**Produkt Ion [m/z]**	Rt. [Min.]	*C. elegans N2*	*H. polygyrus*	*L. sigmondontis*
295	**163**	4.038	-	+	+
301	**169**	3.901	+	++	++
301	**169**	6.135	-	++	++
315	**183**	4.826	-	++	++
316	**184**	11.123	++	++	+
317	**185**	4.849	-	+	+
332	**200**	6.843	-	+	+
343	**211**	7.019	-	+	+
360	**228**	6.468	++	-	-
362	**230**	6.467	++	-	-
372	**240**	5.306	+	+	-
396	**264**	8.721	++	++	+
400	**268**	6.565	+	++	+
406	**274**	7.244	+	-	-

3.5.2. Analyse der RNA von *O. volvulus* auf potentielle Biomarker

In Bezug auf humane Parasiten wurde in einer weiteren Versuchsreihe die RNA von *O. volvulus* untersucht. Dieser befällt menschliches Bindegewebe.[133] Er ist somit Verursacher der Onchozerkose, oder auch bekannt als Flussblindheit (siehe Einleitung **Kapitel 1.2.4**).[133] Es ist bekannt, dass *O. volvulus* als auch *H. polygyrus* und *L. sigmondontis* exosomale miRNA sekretieren.[20] *In vitro* Experimente deuten darauf hin, dass diese über Endozytose in Wirtszellen immigrieren kann[143] und die Zell-Zell Kommunikation zwischen den Zellen stört[144]. Andere Studien konnten sogar zeigen, dass die freie miRNA die Immunantwort hemmt und somit für Immunresistenzen verantwortlich ist.[143, 145] Diese exosomale miRNA gelangt nachweislich in den Blutkreislauf des infizierten Wirtsorganismus[20] und wird möglicherweise mit anderen kleinen RNAs über den Urin ausgeschieden. Für verschiedenste Krebsarten[146] und andere Krankheiten wie beispielsweise Diabetes Typ II[147] werden bereits miRNAs aus dem Urin von Patienten isoliert um diese mit Hilfe verschiedener analytischer Methoden als potentielle Biomarkermoleküle zu untersuchen. Auch als Onchozerkose-Biomarker sind miRNA-Isolate aus dem Urin von Wirtsorganismen denkbar und sollen dahingehend untersucht werden. Der Fokus soll hierbei auf die Analyse der miRNA-Modifikationsprofile gerichtet werden.

Es wurden aus Nematoden sekretierte exosomale miRNA (2,7 µg) sowie *O. volvulus* tRNA (3,6 µg) aus adulten Würmern enzymatisch zu Nukleosiden verdaut und über die bereits zuvor erwähnte gestaffelte MRM Methode (**Kapitel 3.1., 3.2.** und **3.5.1.**) gemessen. Des Weiteren wurde aus dem Urin eines gesunden Probanden isolierte miRNA (160 ng) auf die gleiche Art und Weise vorbereitet und gemessen wie die vorherigen Proben und mit in den Signalvergleich einbezogen. Ziel dieses Vergleichs ist es Signale zu entdecken, welche zwar in *O. volvulus* tRNA und/oder auch der exosomalen miRNA anzutreffen sind, jedoch nicht im Urin des gesunden Menschen. Einige Signale, gelistet in **Tabelle 6**, konnten direkt anhand der synthetischen Standards einigen bekannten RNA-Modifikationen zugeordnet werden.

Tabelle 6: Liste mit eindeutig identifizierten RNA-Modifikationen aus den verschiedenen Isolaten. Signalintensitäten: (-) kein Signal; (+) < 10^4; (++) <10^5; (+++) >10^5

			Signalintensitäten		
Modifikation	Vorläufer Ion [m/z]	Rt. [Min.]	Exosomale miRNA	*O. volvulus* totale RNA	humane miRNA (Urin)
m^5C	258	2.616	+	+++	++
m^5U	259	3.765	-	+	-
m^1A	282	1.735	+	+++	++
m^6A	282	8.325	+	+++	++
m^1I	283	4.804	-	+	++
ac^4C	286	5.364	-	++	+
$m^6{}_2A$	296	11.916	+	+++	+
m^7G	298	2.148	+	+++	+
m^1G	298	4.936	+	+++	++
m^2G	298	5.322	+	+++	++
$m^2{}_2G$	312	6.674	++	+++	+++
$m^2{}_2{}^7G$	326	5.688	-	++	+
mcm^5s^2U	333	7.623	-	+	-
i^6A	336	14.109	+	++	+
t^6A	413	7.33	+	++	+

Aufgrund der relativ niedrigen Verfügbarkeit des zu analysierenden RNA-Materials wurden vergleichsweise niedrige Signale gemessen. Diese Tatsache kann dazu geführt haben, dass einige Modifikationen unter die Detektionsgrenze gefallen sind und somit nicht mehr detektiert werden konnten. Insbesondere weniger einfach ionisierende Moleküle wie die Uridine im Allgemeinen (D, m^5U, m^3U, mcm^5U) werden dadurch nur mit kleinen Signalen (m^5U, mcm^5s^2U) oder gar nicht mehr detektiert (D, m^3U, mcm^5U). Dies bedeutet, dass in dieser Messreihe zwar Schlüsse gezogen

werden können, welche RNA-Modifikationen in den verschiedenen RNAs vorkommen, jedoch kann nicht explizit geklärt werden, ob die Liste an gefundenen Signalen vollständig ist. Es muss außerdem berücksichtigt werden, dass durch die unterschiedlichen Mengen an in das LC-MS System injizierter RNA die Signalstärken zwischen den Messungen stark variieren. So wurde in etwa das 23-fache an *O. volvulus* totaler RNA im Vergleich zur Menge an humaner Urin miRNA in das Massenspektrometer injiziert. Die dadurch verzerrten Ergebnisse müssen daher immer in Relation zur verwendeten RNA Menge betrachtet werden.

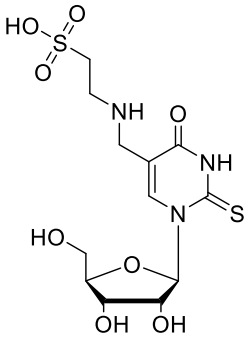
Die in **Tabelle 6** gelisteten Modifikationen geben zudem Aufschluss zu Herkunft und Identität der miRNA-Fragmente, da einige gefundene Modifikationen spezifisch für bestimmte RNA-Spezies sind. $m^6{}_2A$ ist demnach ausschließlich in rRNA bekannt, während i^6A, t^6A, m^1I und mcm^5s^2U erwartungsgemäß in tRNA vorkommen. Es konnte also nachgewiesen werden, dass sich Fragmente beider RNA Typen in den miRNA Fraktionen befinden.

In **Tabelle 7** sind Signale gelistet, welche sich zwar den m/z-Wert mit bekannten RNA-Modifikationen teilen, jedoch Mangels synthetischer Standards nicht eindeutig als diese identifiziert werden konnten.

Tabelle 7: Liste von gefundenen Signalen bei Massenübergängen von bereits bekannten Modifikationen. Die Retentionszeiten können aufgrund eines fehlenden synthetischen Standards nicht bestätigt werden. Die in Spalte 1 gelisteten Modifikationen sind ausschließlich Strukturvorschläge. Signalintensitäten: (-) Kein Signal; (+) Signalintensität kleiner $1x10^4$; (++) Signalintensität größer $1x10^4$ aber kleiner $5x10^6$.

			Signalintensitäten		
Modifikation	Vorläufer Ion [m/z]	Rt. [Min.]	Exosomale miRNA	*O. volvulus* totale RNA	humane miRNA (Urin)
$m^4{}_2C$	272	1.385	+	++	+
preQ0tRNA	308	5.362	-	+	-
preQ0tRNA	308	6.381	-	+	-
inm^5U	342	1.709	-	+	-
io^6A	352	12.412	++	+	++
ms^2i^6A	382	3.626	-	+	-
ct^6A	395	2.822	++	++	++
tm^5s^2U	398	3.626	-	+	-
ms^2io^6A	398	13.471	-	-	+

Es ist daher zweifelhaft, dass zwei Modifikationen wie $m^4{}_2C$ oder preQ0tRNA, welche bisher nur in Bakterien oder Archaea bekannt sind[5], tatsächlich den entdeckten Signalen entsprechen. Auch über die potentielle tRNA Modifikation inm^5U, welche angeblich im Bakterium *Thermodesulfobacterium commune* vorkommt ist sehr wenig bekannt und die Ergebnisse wurden nie in einem wissenschaftlichen Journal publiziert.[5] Starke Signale für die *N*^{6}-Adenosin basierten Modifikationen (io^6A, ms^2i^6A, ct^6A, ms^2io^6A) sind wegen deren guter Ionisierbarkeit auch bei wenig eingesetzter RNA durchaus wahrscheinlich. Dabei ist anzumerken, dass das potentielle Signal für io^6A sehr verstärkt in beiden miRNA Fraktionen vorkommt. Außerdem war das potentielle Signal für ms^2io^6A ausschließlich in der humanen miRNA Fraktion detektierbar. Diese tRNA spezifische RNA-Modifikation liefert einen zusätzlichen Hinweis zu den i^6A und t^6A Signalen, dass auch in der humanen Urin miRNA fragmentierte tRNA vorkommt. Eine weitere interessante eukaryotische tRNA-Modifikation mit der Bezeichnung tm^5s^2U (5-Taurinomethyl-2-thiouridin, Strukturformel **Abbildung 17**), welche in dem mitochondrialen Isoakzeptor $tRNA^{Lys}{}_{UUU}$ von Eukaryoten an Position 34 (Wobble-Position) gebildet wird, könnte sich hinter dem Signal bei m/z = 398 und einer Retentionszeit von 3.626 Min. verbergen. Dieses Signal konnte nur in der *O. volvulus* RNA nachgewiesen werden. Die Tatsache, dass das potentielle Signal für tm^5s^2U weder in der humanen miRNA noch in der exosomale miRNA nachgewiesen werden konnte, liegt vermutlich an der hohen Detektionsgrenze dieser Uridin-basierten Modifikation.



tm^5s^2U

Abbildung 17: Strukturformel von 5-Taurinomethyl-2-thiouridin.

Es fanden sich insgesamt sieben über die Masse nicht zuordenbare Signale in der humanen Urin miRNA (siehe **Tabelle 8**). Drei dieser Signale wurden in dieser Messreihe ausschließlich in der Urin miRNA gefunden, wobei eines davon zusätzlich

schon in *C. elegans*, *H. polygyrus* und *L. sigmondontis* nachgewiesen werden konnte (m/z=420, Rt.=5,875 Min., Vgl. **Anhang Tabelle 2**), ein anderes zusätzlich nur in *H. polygyrus* (m/z=320, Rt.=8,792 Min.) und nur ein Signal wurde bisher noch nicht beschrieben und ist damit als einziges in der humanen Urin miRNA lokalisiert. Dabei handelt es sich um das Signal bei m/z=271 mit einer Retentionszeit von 3,84 Min.

In der exosomalen miRNA konnten trotz vergleichsweise höherer Mengen eingesetzter RNA nur drei unbekannte Signale identifiziert werden, wovon jedoch alle auch in den beiden anderen RNA-Fraktionen zu sehen waren (siehe **Tabelle 8**). Die Molekulare Masse des dem Signal zugehörigen Moleküls mit Massenübergang 258 →126 und einer Retentionszeit von 4,763 Min. beträgt 257 g/mol, was der molekularen Masse von m^3C, m^4C oder m^5C entspricht. Möglicherweise birgt dieses Signal die Struktur eines weiteren methylierten Cytidins. Denkbare Strukturen könnten auch hier über die Nutzung von Isotopenmarkierungen besser aufgeklärt werden. Es wurde im Übrigen kein Signal gefunden, welches in der exosomalen Nematoden-miRNA und in der *O. volvulus* RNA, jedoch nicht in der humanen miRNA vorkommt. Es konnten insgesamt auch keine bekannten RNA-Modifikationen in der exosomalen miRNA identifiziert werden, welche nicht auch in der humanen Urin miRNA detektierbar waren.

Tabelle 8: Signale potentieller Nukleoside mit nicht zuordenbaren Massen bereits bekannter Strukturen. Bei *P.a.* wurden nur Signale der der **Anhang Tabelle 1** berücksichtigt. Signalintensitäten: (+) < 10^4; (++) < $5x10^5$, Abkürzungen: *C.e. = C. elegans N2, H.p. = H. polygyrus, L.s. = L. sigmondontis, P.a. = Pseudomonas aeruginosa,* * Signal leicht verschoben (max. 0.2 Min.)

		Signalintensitäten			
Massenüber-gänge [m/z]	Rt. [Min.]	Exosomale miRNA	*O. volvulus* totale RNA	Humane miRNA (Urin)	Signal bereits detektiert in
258 → 126	4.763	+	+	+	
266 → 134	1.695	+	++	+	*C.e., H.p., L.s.*
271 → 139	3.84	-	-	+	
278 → 146	7.477	-	+	+	
280 → 148	2.611	-	+	-	*C.e.*, H.p.*, L.s.**
306 → 174	3.696	-	+	-	*C.e.*, H.p.*, L.s.*; P.a.*
320 → 188	8.792	-	-	+	*H.p.**
326 → 194	1.701	-	++	-	*C.e.*, H.p.*, L.s.*, P.a.*
366 → 234	3.609	-	+	-	*C.e.*, H.p.*, L.s.*, P.a.*
387 → 255	1.693	-	+	-	*C.e., H.p., L.s.*
388 → 256	1.859	-	+	-	*C.e.*, H.p.*, L.s.*, P.a.**
400 → 268	4.133	+	+	+	

419 → 287	1.769	-	+	-	
420 → 288	1.846	-	+	-	
420 → 288	5.875	-	-	+	*C.e.*, H.p.*, L.s.**

Leider konnten somit aus den hier ermittelten Daten keine potentiellen Biomarker identifiziert werden. Es bedarf erneuter Messungen mit größeren RNA-Mengen um aussagekräftigere Ergebnisse zu erhalten. Die hier gezeigten Ergebnisse können dabei als Vergleich zu Rate gezogen werden. Die Auswahl der Proben sollte neben einer humanen Negativkontrolle auch miRNA eines infizierten Patienten beinhalten. Generell wären biologische Triplikate vorteilhaft.

3.5.3. Unterschiedliche Spezies und deren spezifische Modifikationsprofile

Nachdem in den vorherigen Kapiteln auf die Identifizierung von Signalen eingegangen wurde, beschäftigt sich der folgende Abschnitt mit der Quantifizierung der identifizierten RNA-Modifikationen. Zur Analyse wurde die enzymatisch verdaute tRNA der vier Modell-Nematoden *C. elegans*, *H. polygyrus*, *L. sigmondontis* und *O. ochengi* verwendet. Zum Vergleich wurden außerdem die tRNA von *HEK* 293 Zellen und von *E. coli* BW25113 enzymatisch verdaut und analysiert. Wie in **Abbildung 18a** zu sehen ist, unterscheiden sich die verschiedenen RNA Isolate bereits an der Zusammensetzung der kanonischen Nukleoside. Demnach ist in *HEK* 293 Zellen einiges mehr an Guanosin zu finden als vergleichsweise in allen gezeigten Nematoden-Arten. In der bakteriellen tRNA ist der Guanosin Anteil am kleinsten. Auffällig ist außerdem der große Anteil an Uridin in der tRNA des Parasiten *O. ochengi*. Aufgrund dieser Variabilität erscheint es sinnvoll die zu quantifizierenden RNA-Modifikationen auf die Summe der kanonischen Nukleoside insgesamt zu referenzieren, statt die einzelnen Modifikationen auf einzelne Vorläufermoleküle (beispielsweise m^1G auf G) zu normieren. Die darüber quantifizierten Werte für die einzelnen RNA-Modifikationen aus den verschiedenen Organismen sind den Graphen in **Abbildung 18b** zu entnehmen.

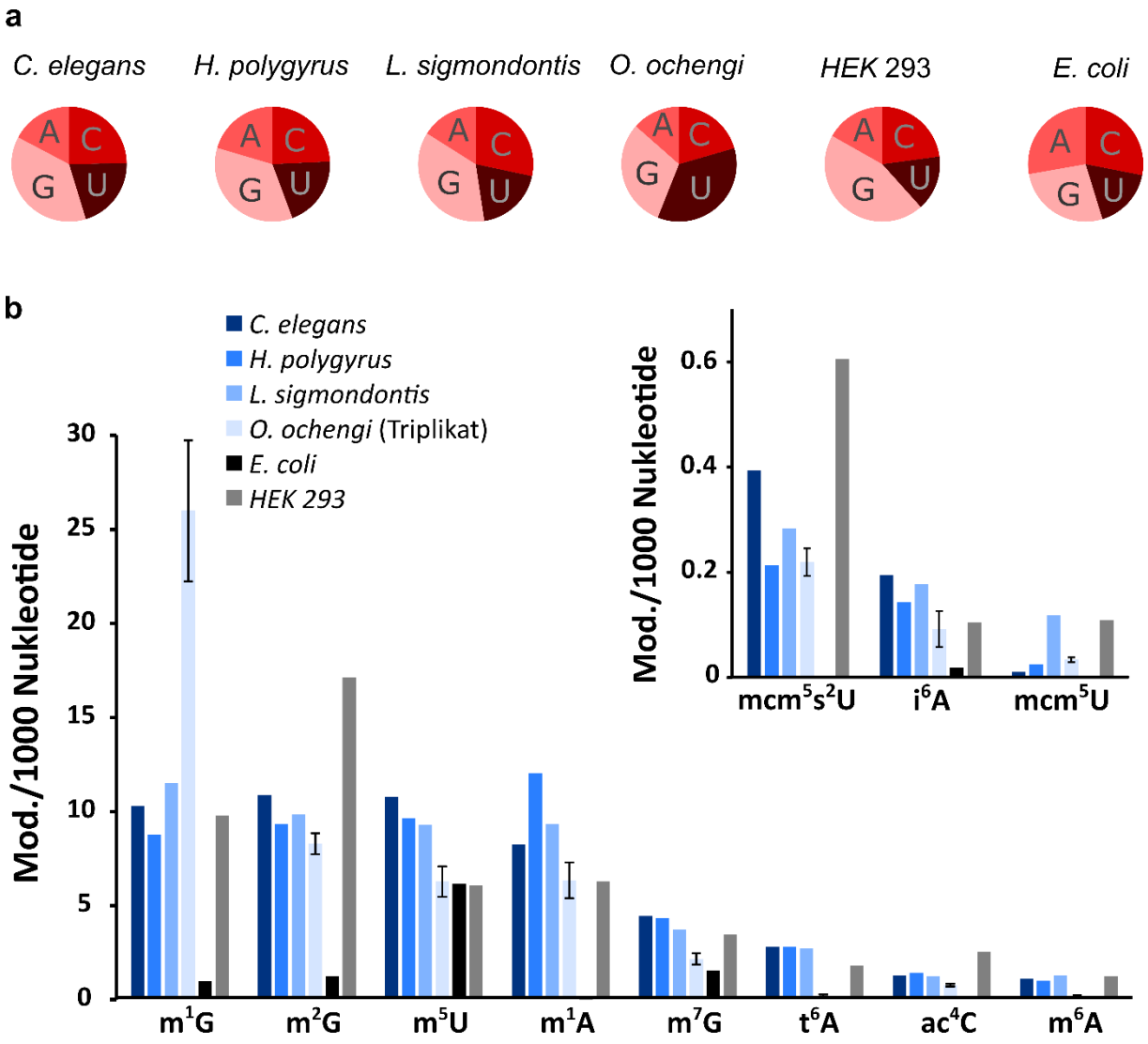


Abbildung 18: tRNA Nukleotid-Zusammensetzung und tRNA-Modifikationsprofile verschiedener Nematodenarten und Organismen. **a** Die dargestellten Tortendiagramme bilden die Anteile der kanonischen Basen in der totalen tRNA der verschiedenen Organismen ab. C = Cytidin, U = Uridin, G = Guanosin, A = Adenosin. **b** In den beiden Balkendiagrammen sind die absoluten Mengen für einige bekannte RNA-Modifikationen aus der totalen tRNA der verschiedenen Organismen gegen die Menge der kanonischen Nukleobasen aufgetragen (Modifikation pro 1000 Nukleotide). Ausschließlich für *O. ochengi* wurden die Messungen für n=3 biologische Replikate durchgeführt. Die Fehlerbalken entsprechen der Standardabweichung vom Mittelwert.

Auffallend sind insbesondere die vergleichsweise niedrigen Werte von *O. ochengi* für t^6A und m^6A, während m^1G in diesem Organismus stark erhöht vorliegt. Interessanterweise sitzen diese drei tRNA-Modifikationen an derselben Position entlang verschiedener tRNA Isoakzeptoren. So sind t^6A und m^1G häufig an Position 37 in tRNA unterschiedlichster Spezies aus allen drei Lebensdomänen anzutreffen, während eine N^6-Methylierung in Adenosin 37 bisher nur für bakterielle oder

mitochondriale tRNA beschrieben ist.[5] Ansonsten weisen die Ergebnisse für *O. ochengi* viele Gemeinsamkeiten mit der tRNA der anderen Nematoden auf (siehe **Abbildung 19a**). Auch die *HEK* 293 tRNA zeigt ein ähnliches Profil, während die prokaryotische tRNA phylogenetisch weiter von den Nematoden entfernt ist und daher auch ein sich stärker unterscheidendes Modifikationsprofil aufweist (siehe **Abbildung 19b**). Erwähnenswert sind außerdem die quantitativen Unterschiede, welche für die in geringer Menge auftretende tRNA-Modifikation mcm^5U beobachtet wurde. Bekanntermaßen ist dieses Molekül die Vorläuferstruktur für mcm^5s^2U an Position 34 der tRNA. Die Funktionen der Seitenketten wurden bereits in der Einleitung diskutiert. Für *C. elegans* wurde demnach bereits ein Zusammenhang zwischen einer geringeren mcm^5s^2U Anteil unter Stressbedingungen festgestellt werden. Dies könnte im Umkehrschluss zu einer Erhöhung des mcm^5U-Levels in den betroffenen tRNAs führen. In *E. coli* konnten diese Modifikationen wie erwartet nicht detektiert werden.

Die über die Quantifizierung gewonnenen Informationen sind wichtige Bestandteile zur Feststellung posttranskriptionaler Unterschiede zwischen dem Transkriptom pathogener und nicht-pathogener Nematoden. Durch die gewonnenen Daten wird die Sichtweise hinsichtlich der phylogenetischen Zusammenhänge der Würmer erweitert. Zusätzlich können die detektierten Modifikationsmengen mit Spezies-spezifischen Modifikationsprofilen verglichen werden. Es kann abgewogen werden, ob beispielsweise die aus dem Urin isolierte miRNA dem nematodischen Parasiten (z.B. von *O. ochengi*), dem Wirt oder sogar dem Mikrobiom des Wirtes als Ursprungsorganismus zuzuordnen ist.

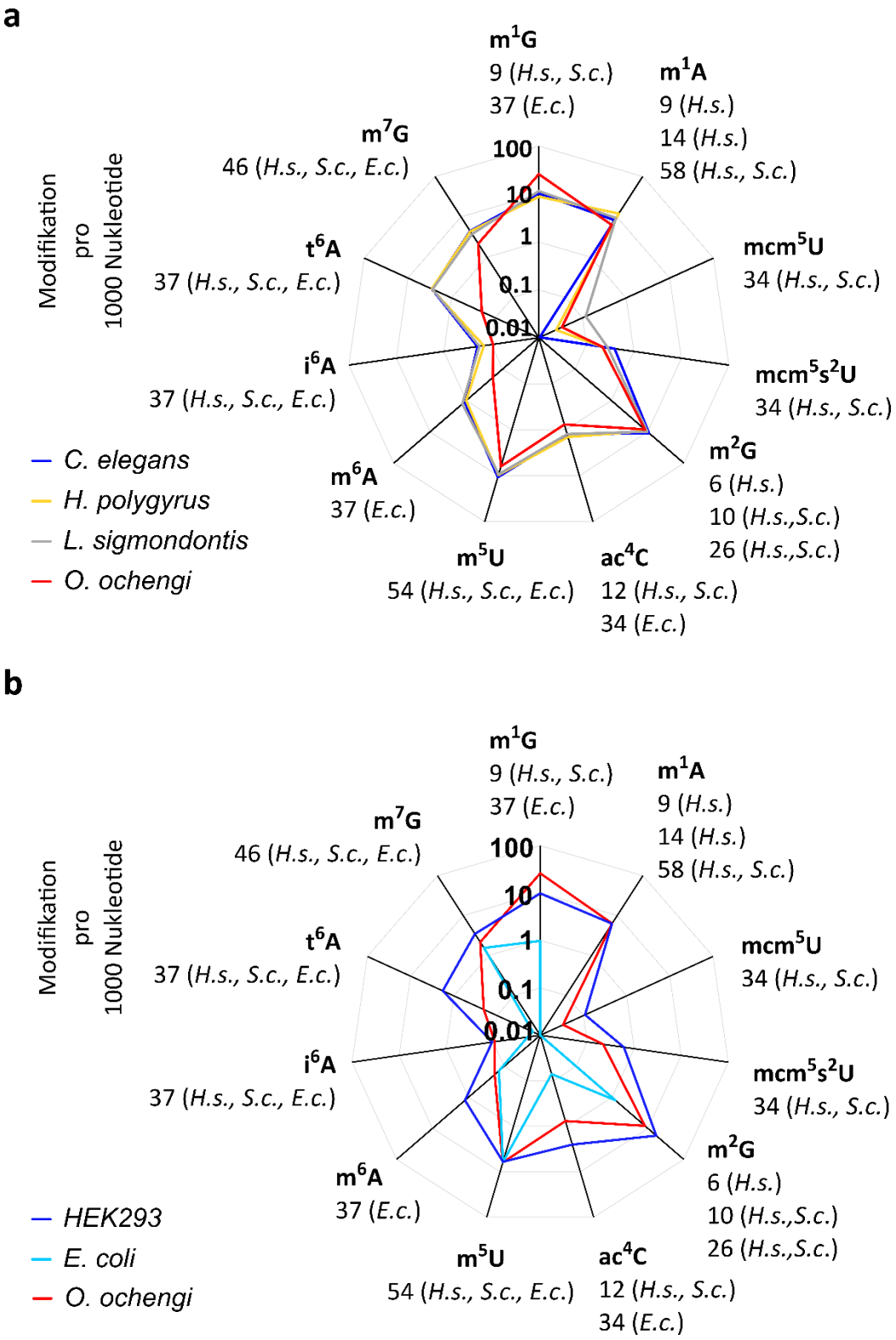


Abbildung 19: tRNA-Modifikationsprofile verschiedener Organismen im Spinnennetz-Diagramm. Die Nummern unter der Modifikationsbezeichnung stehen für die möglichen Positionen in der tRNA mit den jeweiligen Organismen in Klammern dahinter. **a** Vergleich des tRNA-Modifikationsprofils von *O. ochengi* mit deren anderer Nematodenvertreter. **b** Vergleich des tRNA-Modifikationsprofils von *O. ochengi* mit humanen (*HEK* 293) und bakteriellen (*E. coli*) tRNA-Modifikationen. Abkürzungen: *H.s. = Homo sapiens, S.c. = Saccharomyces cerevisiae, E.c. = E. coli.*

3.5.4. Untersuchung von miRNA aus dem Urin gesunder und infizierter Kälber auf Unterschiede im Modifikationsprofil

Mit der gewonnenen Klarheit über die Modifikationsverhältnisse innerhalb der tRNA der unterschiedlichen Nematoden sollen als nächstes die Modifikationsverhältnisse in der über den Urin von infizierten Rindern ausgeschiedenen miRNA untersucht werden. Hierzu wurden 7 Urinproben von *O. ochengi* infizierten Rindern und zur Kontrolle 8 Urinproben von gesunden Rindern entnommen, die miRNA extrahiert und nach dem Enzymverdau massenspektrometrisch auf Modifikationen untersucht (siehe **Abbildung 20a**). Vor der endgültigen Zersetzung der isolierten miRNA in einzelne Nukleoside durch den enzymatischen Verdau wurde eine Qualitätskontrolle bezüglich Länge und Reinheit der zu analysierenden RNA vorgenommen. Dazu wurde jeweils eine kleine Menge der Proben (1 µL) über einen RNA Small Chip oder über einen RNA Pico Chip der Firma Agilent in einem Bioanalyzer 2100 (Agilent, Waldbronn) analysiert (**Anhang Abbildungen 4a und 4b**). Da die RNA-Konzentrationen in den Proben zum Teil sehr niedrig waren, konnte die RNA-Länge mancher Proben nur im sensitiveren RNA Pico Chip identifiziert werden. Dieser zeigt bei allen Proben eine starke und definierte Bande im Bereich einer RNA Länge zwischen 25 bis 200 Nukleotide. Eine Kontamination mit sehr kleinen RNA Fragmenten (< 25 Nukleotide) sowie mit größeren RNA Fragmenten (rRNA, mRNA) kann damit ausgeschlossen werden. Eine spezifischere Längenbestimmung der miRNA konnte für die meisten Proben über den RNA Small Chip erreicht werden. Hierbei wurde über die Banden im Gel eine definierte RNA-Länge von etwa 30 Nukleotiden gemessen. Dies entspräche auch der Länge möglicher enthaltener tsRNAs.

Es wurden durchschnittlich von jeder Probe 52,6 ng (mindestens 10 ng, maximal 120 ng) miRNA enzymatisch zu Nukleosiden verdaut und massenspektrometrisch analysiert. Nach dem Ausschluss zweier Datenpunkte aufgrund des Verdachts einer Verwechslung und der Anwendung des Grubbs-Tests (siehe Material und Methoden) reduzierte sich die Anzahl der für die statistische Auswertung verwendeten Proben in der infizierten als auch in der Kontrollgruppe um jeweils eine Probe. Statistische Auswertungen unter Einbezug der Ausreißer finden sich dennoch im Anhang (**Anhang Abbildung 5, 6 und 8**).

Es wurde festgestellt, dass einige tRNA-Modifikationen (Vgl. t^6A, mcm^5U, mcm^5s^2U) in ähnlich hohen Mengen in der miRNA enthalten sind wie in reinen eukaryotischen

tRNA-Isolaten (Vgl. **Abbildung 19)** Diese Entdeckung gibt einen weiteren deutlichen Hinweis darauf, dass die isolierte miRNA zu einem sehr großen Teil aus 3‘-tRFs oder 3‘-tiRNAs besteht.

Das Diagramm in **Abbildung 20b** zeigt einen signifikanten Abfall der Modifikation mcm^5s^2U ($p < 0,01$) in der miRNA von infizierten Rindern, während die nicht thiolierte Vorläufer-Modifikation mcm^5U einen signifikanten Anstieg ($p < 0,0001$) im Vergleich zu den Kontrollproben verzeichnet. Auch die Modifikationen Am ($p < 0,005$) sowie m^6A und m^7G (beide bei $p < 0,05$) zeigen signifikante Veränderungen nach einer Infektion mit *O. ochengi* (Die angegebene p-Werte wurden mit einem zweiseitigen „Student’s t-Test“ berechnet). Dennoch ist bei mcm^5U und mcm^5s^2U das Verhältnis der nach Infektion gemessenen Mengen zur ursprünglichen Menge im gesunden Tier am beeindruckendsten. So vervielfältigt sich der mcm^5U Gehalt in der Urin miRNA um knapp das Vierfache, während mcm^5s^2U um etwa 40 % absinkt (**Abbildung 20c,** absolute Werte, welche nicht aus **Abbildung 20b** ersichtlich, sind in **Anhang Abbildung 7** aufgeführt). Damit zeigen mcm^5s^2U sowie mcm^5U die massivsten Verluste beziehungswiese Steigerungen innerhalb der gemessenen Modifikationen auf. Betrachtet man zusätzlich deren biosynthetischen Zusammenhang, so erscheinen die Ergebnisse für mcm^5s^2U und mcm^5U außerordentlich bedeutsam und sind richtungsweisend für weitere Analysen und Auswertungen dieser Arbeit.

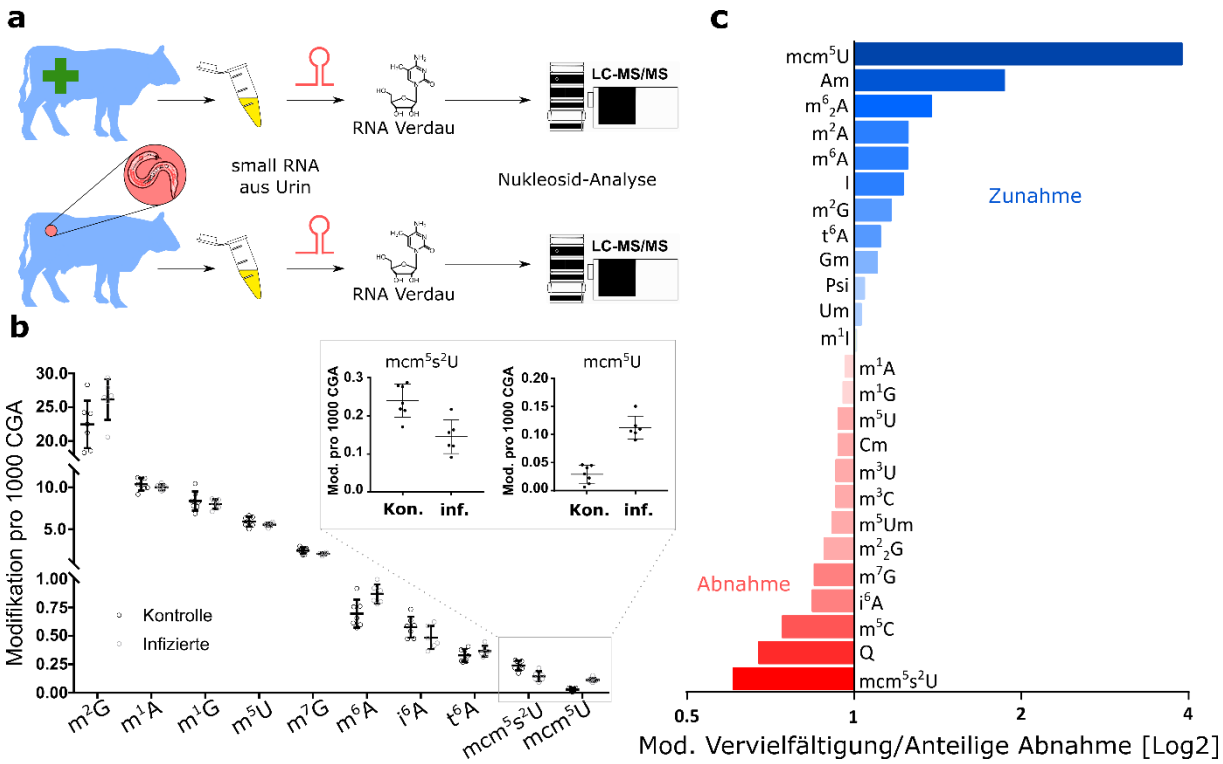


Abbildung 20: Probenvorbereitung und LC-MS/MS Analyse**. a** Schematische Darstellung der Probenentnahmen und -vorbereitung von gesunden (oben) Rindern und mit *O. ochengi* infizierten Rindern (unten). **b** Absolute Mengen an verschiedenen tRNA-Modifikationen, referenziert auf ein Tausendstel des gesamten Anteils an Cytidin, Guanosin und Adenosin (CGA). Es sind die Mittelwerte der infizierten als auch der gesunden Rinder angezeigt. Die Fehlerbaken entsprechen der Standardabweichung. Die Einzelwerte sind ebenfalls im Graph dargestellt. Die Ergebnisse der sich nach Infektion am stärksten verändernden Modifikationen mcm^5s^2U und mcm^5U sind zur einfacheren Betrachtung zusätzlich in einem Vergrößerungsfenster dargestellt. **c** Veränderungen der Modifikationsmengen nach Infektion, dargestellt in einem Balkendiagramm mit logarithmischer Skalierung.

Die beiden tRNA-Modifikationen mcm^5U und mcm^5s^2U gehören in die Gruppe der sogenannten „Wobble-Uridine“, welche in unterschiedlichen tRNA-Isoakzeptoren an Position 34 der Nukleotid-Abfolge enzymatisch angebracht werden. Biosynthetisch sind die beiden Modifikationen nur einen enzymatischen Reaktionsschritt voneinander entfernt, so wird im Menschen die Position 2 am Basengerüst von mcm^5U über die mitochondriale tRNA-spezifische 2-Thiouridylase I (TrmU) thioliert [148], woraus dann die Modifikation mcm^5s^2U resultiert **(Abbildung 21**).

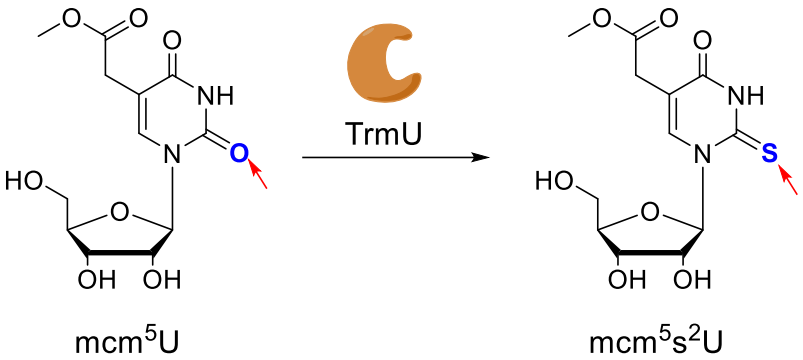


Abbildung 21: Schematische Darstellung der enzymatischen Reaktion von TrmU mit den zugehörigen Strukturformeln des Substrates mcm^5U und des thiolierten Endprodukts mcm^5s^2U. Die ausgetauschten Atome sind blau eingefärbt und mit einem roten Pfeil markiert.

Die Interpretation, der in **Abbildung 20b** und **20c** dargestellten Daten ergibt, dass durch die Infektion mit *O. ochengi* die Thiotransferase für die mcm^5U Thiolierung gehemmt, beziehungsweise deren Translation ausgeschaltet wird, während eine aktive Desulfurierung der mcm^5s^2U Modifikation ebenso vorstellbar wäre. Eine Verdünnung der miRNA mit fremder miRNA (ausgehend vom Parasiten) oder eine Veränderung der Isoakzeptoren Zusammensetzung innerhalb des tRNA Pools in den Zellen des Wirtes, als Reaktion auf die Infektion, könnte ebenfalls eine mögliche Erklärung für die Verschiebung der Modifikationsverhältnisse sein. Es besteht außerdem die Möglichkeit, dass durch den Infektionsbedingten Stress im Wirt der Angiogenin Spiegel steigt und dieses bevorzugt mcm^5U-haltige tRNAs schneidet. Doch auch diese Theorie erklärt nicht die Abnahme der mcm^5s^2U Modifikationen im Urin der infizierten Tiere. Weitere Untersuchungen sollen Aufschluss über den Ursprung der gemessenen Modifikationen geben.

3.5.5. Bestimmung des Nukleosid-Ursprungs der untersuchten Modifikationen

Anhand eines Modifikationsprofil-Vergleichs soll im Folgenden untersucht werden, ob die gemessenen Modifikationen aus der RNA des Parasiten stammen, ob sie aus der RNA des Wirts in den Urin gelangen, oder gar bakteriellen Ursprungs sind. Außerdem soll anhand eines speziell angepassten enzymatischen Verdauansatzes bestätigt werden, dass die Modifikationen nicht frei als Nukleoside oder Nukleotide mit der miRNA co-aufgereinigt wurden, sondern tatsächlich aus den RNA-Oligomeren entstammen (**Abbildung 22a**).

Da aus den bereits genannten Gründen davon ausgegangen werden kann, dass die Zusammensetzung der aus dem Urin isolierten miRNA hauptsächlich aus tRFs besteht, ist es durchaus sinnvoll das Modifikationsprofil der miRNAs mit den tRNA Modifikationsprofilen möglicher Ursprungsorganismen zu vergleichen um daraus auf die ursprüngliche Herkunft der RNA Fragmente schließen zu können.

Zuvor soll dennoch überprüft werden, ob die eukaryotischen Modifikationen mcm^5U und mcm^5s^2U aus der zu untersuchenden miRNA stammen oder ob eine Verschiebung der gefundenen Modifikationslevels auch durch einzelne im Urin vorkommende modifizierte Nukleotide oder Nukleoside entstehen kann. Dazu wurden zwei Kontroll-Proben und drei Proben aus infizierten Rindern stellvertretend für alle Proben untersucht. Es wurden jeweils zwei gleich große Volumenanteile entnommen, wovon jeder nach einem anderen Verdauprotokoll behandelt wurde. Der eine Teil wurde mit einer Alkalischen Phosphatase (CIP) behandelt, welche ausschließlich in der Lage ist potentielle Nukleotide in Nukleoside umzuwandeln oder RNA Stränge am 5‘ Ende zu dephosphorylieren. jedoch könnte das Enzym keine Strangbrüche induzieren. Im Gegensatz dazu wurde die andere Hälfte zusätzlich mit Benzonase und Phosphodiesterase II (SPD) inkubiert, sodass in diesem Verdau auch die RNA-Stränge zu Nukleosiden verdaut werden konnten. In der verwendeten LC-MS/MS Methode wurden die Konzentrationen der in den Verdau-Lösungen vorhandenen nukleosidischen Modifikationen bestimmt und miteinander verglichen. Die Ergebnisse können der **Abbildung 22c** entnommen werden. Es ist zu sehen, dass im CIP-Verdau aller gemessenen Proben vergleichsweise nur geringe Mengen an mcm^5s^2U zu finden waren. Im totalen Verdau konnten jedoch hohe Konzentrationen gefunden werden, die 91,5 bis 99,5 % der detektierten mcm^5s^2U Moleküle insgesamt ausmachen. Für mcm^5U sind die Werte etwas geringer, sodass hier 81,7 bis 97,6 % den Oligo-RNAs zuzuordnen sind. Es wurde somit also bestätigt, dass die untersuchten Modifikationen zu einem Hauptteil aus intakten miRNA-Strängen entstammen.

Im nächsten Schritt wurden die Modifikationsprofile bakterieller tRNA (*E. coli*), der tRNA von *O. ochengi* und der miRNA der gesunden Kontroll-Tiere mit dem Modifikationsprofil der aus den infizierten Tieren isolierten miRNAs verglichen (siehe **Abbildung 22b**). Das Modifikationsprofil der *E. coli* tRNA weist, wie zu erwarten, kaum Gemeinsamkeiten mit den restlichen Modifikationsprofilen auf. Eine viel stärkere Übereinstimmung in vielen tRNA-Modifikationen ist für die tRNA von *O. ochengi* zu

beobachten. Gegen die Annahme, dass die miRNA hauptsächlich aus den Wurmparasiten entstammt, spricht jedoch die hohe Übereinstimmung des Modifikationsprofils der Urin miRNA der infizierten Rinder mit dem Modifikationsprofil der Urin miRNA der Kontrolltiere. Ausschließlich für mcm^5U und mcm^5s^2U schwanken die Werte stark in der gezeigten Profilüberlagerung, was durchaus auch der logarithmischen Skalierung zuzuschreiben ist. Dennoch verdeutlicht diese Darstellung nochmals die Vervielfältigung bzw. extreme Verringerung bezüglich des Eigenwerts der Modifikationsmengen dieser beiden Modifikationen. Obgleich die Profile im Ganzen darauf hinweisen, dass der Hauptanteil der miRNA im Urin vom Wirt selbst stammt, könnte man dagegen argumentieren, dass es gerade anhand der kleinen aber signifikanten Mengenzunahme von mcm^5U sein kann, dass aus *O. ochengi* stammende tRFs mit sehr hoher Wobble-Uridin Konzentration zusätzlich im Urin Eingang finden und unverhältnismäßig zu ihrem eigentlichen Anteil den mcm^5U Anteil erhöhen. Doch auch diese Theorie kann nicht die Verringerung des mcm^5s^2U Gehalts erklären. Eine sehr plausible Erklärung dagegen könnte sein, dass ausgeschiedene Signalstoffe der Würmer oder Reaktionen des Immunsystems des Wirtsorganismus dazu beitragen, dass mcm^5s^2U als Modifikation oder mcm^5s^2U tragende Isoakzeptoren nicht mehr in vollem Umfang in den Wirtszellen gebildet werden. Stattdessen könnten durch die Infektion hervorgerufene tiRNAs die Modifikationszusammensetzung im miRNA-Pool so verändern, dass insgesamt ein größerer Anteil an tRFs mit einem niedrigeren mcm^5s^2U aber dafür höheren mcm^5U-Gehalt vorzufinden ist.

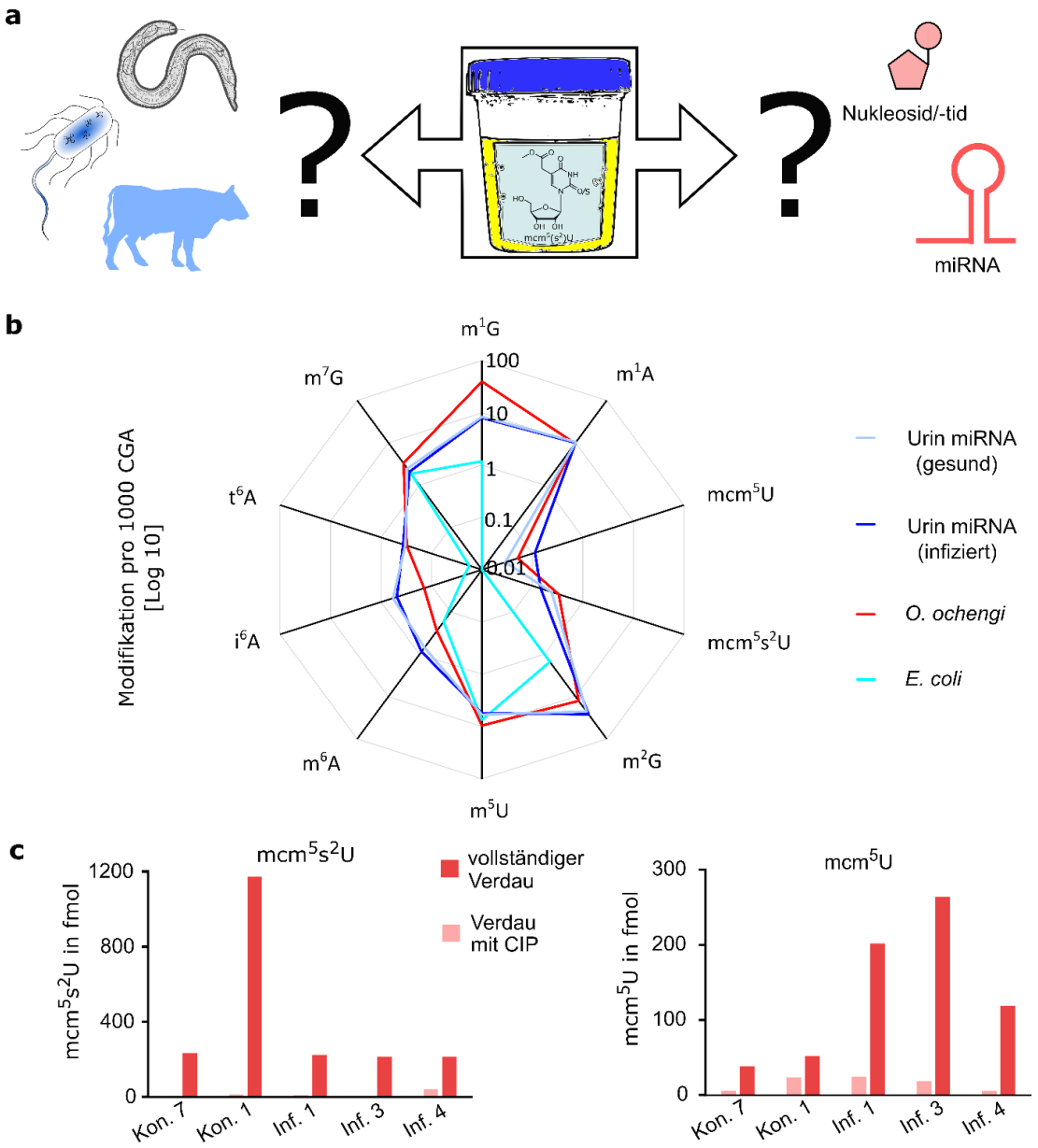


Abbildung 22: Herkunft und ursprünglich vorliegende Form von mcm^{5}s^{2}U und mcm^{5}U. **a** Schema zur Verdeutlichung der Fragestellung. **b** Spinnennetzdiagramm mit den aufgezeichneten Modifikationsprofilen von *E. coli* tRNA, *O. ochengi* tRNA, sowie den RNA Modifikationsprofilen der Urin miRNA der gesunden und infizierten Rinder. **c** Gemessene Stoffmengen in den Proben für mcm^{5}s^{2}U und mcm^{5}U nach den jeweils verschiedenen Verdauschritten.

Es wird zu überprüfen sein, ob ähnliche Verschiebungen der Modifikationslevel für mcm^{5}U und mcm^{5}s^{2}U als Produkt einer Immunantwort auch unter anderen Krankheits- oder Stressbedingungen stattfinden. Nachdem die Frage der Herkunft von mcm^{5}U und mcm^{5}s^{2}U in der miRNA behandelt wurde, soll im nächsten Schritt geklärt werden, ob sich der Effekt der Verschiebung der Modifikationslevel über den Krankheitsverlauf hinweg verändert.

3.5.6. Die Dynamik des Verhältnisses von mcm^5s^2U zu mcm^5U während einer Infektion

Die Dynamiken der Modifikationsveränderungen können anhand der bereits erhobenen Daten von den einzelnen infizierten Tieren, welche sich bei Urinentnahme jeweils in einem unterschiedlichen Infektionsstadium befanden, ausgewertet werden.

Als Messvariable für den Infektionsgrad wurde die Anzahl der Beulen auf der Hautoberfläche der infizierten Rinder herangenommen. Die Beulenanzahl wurde dann gegen die Modifikationsmengen pro 1000 CGA aufgetragen (**Abbildung 23**). Der Ausgangspunkt (0 Beulen) wurde mit dem Mittelwert aller Kontrollproben definiert. Wie aus den Graphen ersichtlich wird, korreliert der Anstieg des mcm^5U Gehalts als auch der Abfall von mcm^5s^2U tatsächlich mit der Anzahl an Beulen.

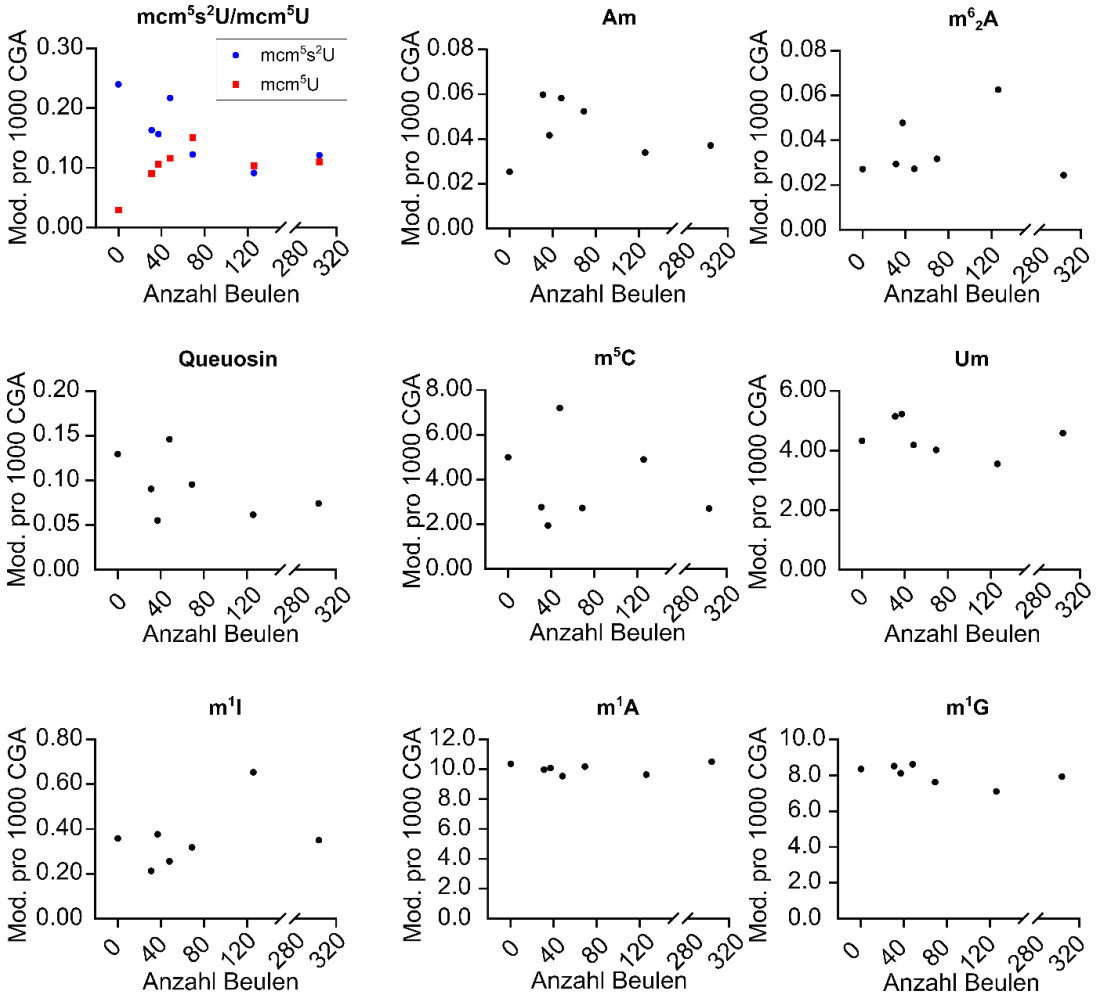


Abbildung 23: Graphische Darstellung der quantitativen RNA-Modifikationsdaten aller Rinder, aufgetragen gegen die Anzahl der krankheitsbedingten Hautwucherungen (Beulen) als Indiz für den Grad der Infektion. Eine Korrelation zwischen den RNA-Modifikationsmengen und dem Infektionsgrad soll damit erkennbar gemacht werden. Eine dynamische Änderung ist am ehesten für mcm^5U (rot) und mcm^5s^2U (blau) zu beobachten.

Eine Korrelation der Modifikationslevel, einhergehend mit dem Grad der Infektion ist ein deutlicher Hinweis auf die direkte Abhängigkeit der Modifikationen mit dem Wurminfekt. Ab einem bestimmten Infektionsgrad (~100 Beulen) scheinen die mcm^5U und mcm^5s^2U Mengen sich nicht weiter anzunähern und stagnieren auf etwa gleichem Level im weiteren Krankheitsverlauf. Es ist bemerkenswert, dass in den meisten Proben genau die Menge an mcm^5U hinzukommt, die an mcm^5s^2U abgenommen hat.

Andere Modifikationen mit ebenfalls hohen Änderungsraten nach einer Infektion (Q, m^5C, Am, $m^6{}_2A$) zeigen keine derartige Abhängigkeit gegenüber der Beulenanzahl. Die Datenpunkte dieser Modifikationen scheinen eher willkürlich verteilt. Zur Kontrolle wurden auch Korrelationsplots von sich wenig verändernden Modifikationen (Um, m^1I, m^1A, m^1G) erstellt, welche auch hier auf keine Abhängigkeit mit dem Krankheitsverlauf schließen lassen, jedoch vergleichsweise konstante Mengenverhältnisse aufweisen.

Obwohl in der humanen Urin miRNA unter **Kapitel 3.5.2.** kein mcm^5s^2U entdeckt werden konnte, müssen weitere Proben von Onchozerkose-Patienten und Kontrollproben mit ausreichender Menge an miRNA analysiert werden. Nur dadurch kann bestätigt werden, ob die beiden „Wobble-Uridine“ auch unter Einfluss von *O. volvulus* veränderte Mengenverhältnisse aufweisen und damit als neuartige Biomarker in der Diagnostik eingesetzt werden könnten.

Unter **Kapitel 3.5.** konnten in verschiedenen Nematodenarten neben vielen bereits bekannten RNA-Modifikationen auch MS-Signale von noch potentiell unbekannten RNA-Modifikationen entdeckt werden. Neben in Eukaryoten üblichen tRNA-Modifikationen wurden interessanterweise aber auch die bakteriellen tRNA-Modifikationen s^2C und ms^2C in *C. elegans N2* und *H. polygyrus* gefunden. Zur Identifizierung potenzieller Onchozerkose-Biomarker wurden des Weiteren verschiedene RNA Fraktionen aus *O. volvulus* und humaner miRNA auf bekannte und unbekannte RNA-Modifikationen untersucht. Keines der detektierten Signale entsprach jedoch den Anforderungen eines potentiellen Biomarkers. Ein Signal welches für in allen hier gemessenen Fraktionen auftrat, weist die Masse eines methylierten Cytidins auf, kann anhand der Retentionszeit jedoch nicht den bekannten Modifikationen m^3C, m^4C oder m^5C zugeordnet werden. Einige bekannte tRNA-Modifikationen der verschiedenen Nematodenarten wurden quantifiziert und die Profile wurden untereinander verglichen, wodurch artspezifische Unterschiede, insbesondere

für *O. ochengi*, festgestellt werden konnten. In der aus Rinderurin isolierten miRNA mit und ohne *O. ochengi* Infektion konnten eindrückliche Daten gewonnen werden. Insbesondere die Veränderungen der beiden RNA-Modifikationen mcm^5s^2U und dessen Vorläufer-Modifikation mcm^5U weisen signifikante Veränderungen während einer Infektion auf. Es war zudem möglich mittels Modifikationsprofil-Vergleichen, sowie unter Anwendung angepasster enzymatischer Verdauprotokolle, auf den Ursprung des Hauptanteils der isolierten RNA zu schließen. Die Auswertung der Daten bezüglich der Dynamik über den Krankheitsverlauf hinweg zeigt des Weiteren eine beeindruckende Verschiebung der Mengenverhältnisse von mcm^5U und mcm^5s^2U zueinander hin an. Beide Werte pendeln sich im Verlauf der Infektion bei etwa 0.1 Modifikationen pro 1000 Nukleotide ein. Bestätigen sich die Ergebnisse auch in Onchozerkose-Patienten jedoch nicht für andere Infektionskrankheiten, würden sich die RNA-Modifikationen mcm^5U und mcm^5s^2U als vielversprechende Biomarkermoleküle im Bereich der Onchozerkose-Diagnostik einsetzen lassen.

4. Ausblick

Es wurde gezeigt, wie durch die neu entwickelten NAIL-MS Methoden neue RNA-Modifikationen entdeckt werden können und wie deren Ursprung und deren Verbleib in der RNA verfolgt werden kann. Insbesondere zur Untersuchung von RNA-Schäden erweist sich die NAIL-MS Technik als außerordentlich geeignet. Hervorzuheben sind auch die hierbei erzielten Erfolge bei der Aufklärung der RNA-Reparaturmechanismen und der Identifikation hierbei beteiligter Enzyme.

Ich bin überzeugt davon, dass sich mit fortschreitender Weiterentwicklung der LC-MS/MS Technik und über die hier vorgestellten NAIL-MS Methoden weitere, bisher unbekannte, RNA-Modifikationen entdecken und untersuchen ließen. Eine Ausweitung der NAIL-MS Methoden ist außerdem auch für menschliche Zellen in Zellkultur vorstellbar und wird bereits angewendet. Die Technik könnte dort bei der Aufdeckung komplexer Zusammenhänge zwischen bestimmten RNA-Modifikationen und bisher unheilbaren Krankheiten eine wichtige Rolle spielen. In pathogenen Bakterien können die entwickelten NAIL-MS Methoden zur Verfolgung von RNA-Modifikationen und deren Einfluss auf die Virulenz-Aktivierung angewendet werden. Die exakte Quantifizierung ermöglicht die Ermittlung der genauen Anteile der Modifikationen innerhalb der RNA und kann unter verschiedenen Bedingungen, auch unter den üblichen Stressbedingungen während des Befalls eines Wirtsorganismus, untersucht werden. Wird die genaue Funktion einzelner RNA-Modifikationen während einer Infektion klarer, besteht die Möglichkeit ein ganzes Feld an neuen Antibiotika Targets zu definieren.

Eine Veränderung des RNA-Modifikationsprofils wurde für Rinder während einer Infektion mit der Nematodenart *O. ochengi* festgestellt. Der deutlichste Unterschied konnte für die Wobble-Uridine mcm^5U und mcm^5s^2U nachgewiesen werden. Derartige Veränderungen erhöhen die Aussicht auf eine neue Generation von Biomarker-Molekülen auf der Basis von Nukleosiden. Die Chance, dass Biomarker auf Nukleosidbasis, in Zukunft auch bei der Diagnose in für den Menschen relevanten Krankheiten, eine größere Rolle spielen könnten, nimmt mit zunehmendem Verständnis für die Funktionen von RNA-Modifikationen zu.

Es wurde gezeigt, wie mcm^5s^2U unter Stresseinwirkung (hier die Infektion durch *O. ochengi*) anteilig in der RNA abnimmt, während mcm^5U anteilig ansteigt. Zu einem

späteren Zeitpunkt im Verlauf der Infektion nähern sich beide Modifikationen einander an und sind in einem späten Stadium anteilig gleichauf. Ein ähnliches Verhalten für mcm^5s^2U wurde bereits unter anderen Stressbedingungen in Nematoden gezeigt und könnte auch in Säugetieren einer generellen Stressreaktion auf RNA-Modifikationsbasis entsprechen. Dies muss jedoch erst durch weitere wissenschaftliche Studien bewiesen werden.

5. Material und Methoden

Die im Folgenden beschriebenen Materialien und Vorgehensweisen beziehen sich auf die unveröffentlichten Ergebnisse. Protokolle zu Materialien und Methoden der veröffentlichten Ergebnisse finden sich direkt in der Publikation unter der Sektion „Materials and Methods“ oder in den jeweiligen Zusatzinformationen. Lediglich interne Hinweise oder detailliertere Beschreibungen zur Durchführung der bereits in den Publikationen beschriebenen Verfahren werden in diesem Abschnitt ergänzt.

Chemikalien und Nukleoside

Alle Salze wurden von Sigma-Aldrich (München, Deutschland) in molekularbiologischer Qualität erhalten, sofern nicht anders angegeben. Chemikalien wie MMS (99 %, Art.-Nr.: 129925-25G) wurden ebenfalls von Sigma-Aldrich erworben. Die isotopenmarkierten Verbindungen $^{15}NH_4Cl$ (> 98 Atom-%, Art.-Nr.: 299251-1G) und [D_3] -L-Methionin (98 Atom-%, Art.-Nr.: 300616-5G) wurden von Sigma-Aldrich erhalten. Isotopenmarkierte $^{13}C_6$-Glucose (≥ 99 Atom-%, Art.-Nr.: CLM-1396-10) und isotopenmarkierte $Na_2{}^{34}SO_4$ (99,11 Atom-%, Art.-Nr.: MS34S) wurden von Eurisotope (Saarbrücken, Deutschland) erhalten. Alle Lösungen und Puffer wurden mit Wasser aus einer Millipore-Anlage (Milli-Q, Merck, Darmstadt, Deutschland) hergestellt. Lösungsmittel in LC-MS Qualität (Acetonitril, Isopropanol, Methanol) wurden von der Firma Roth (Karlsruhe, Deutschland) erworben. Die Nukleoside Adenosin, Cytidin, Guanosin, Uridin und m^2G wurden von Sigma-Aldrich erworben. m^1A, m^2A, m^3C, m^6A, m^7G, m^5C, m^5U, Cm, Gm, m^1G, m^3U, ac^4C und mcm^5s^2U wurden von Carbosynth (Newbury, UK) erworben. Die synthetischen Standards für Queuosin, i^6A und mcm^5U wurde von Peter Dedon M.D., Ph.D. (Massachusetts Institute of Technology, Cambridge, USA) bereitgestellt. t^6A wurde kommerziell von Toronto Research Chemicals (North York, Canada) und m^4C von Berry & Associates, Inc. (Dexter, USA) erworben.

LB-Medium/Agarplatten

Für LB-Medien wurde LB Broth (Luria Miller, Art.-Nr.: X968.1) von Roth (Karlsruhe, Deutschland) verwendet. Agarplatten 1,5% (Gew./Vol.) wurden aus LB und Agar-Agar (Kobe I von Roth, Art.-Nr.: 5210.3) nach Herstellerangaben hergestellt.

Glycerin-Stocks

Einzelkolonie des jeweiligen *E. coli* Stamms gepickt und in 5 mL LB-Medium über Nacht wachsen lassen. Enthielten die Stämme Kanamycin-Resistenzen wurden dem Medium 5 µL Kanamycin (50 mg/mL) zugegeben. Von der Übernachtkultur wurden dann 500 µL in ein Kryo-Tube überführt und anschließend 500 µL steriles 50%iges Glycerol/Wasser (v/v) zugegeben, sehr gut gemischt und sehr zeitnah bei -80°C tiefgefroren.

LC-MS Puffer

In eine saubere 1L Labor Gewindeflasche wurden 0,385 g NH_4OAc (LC-MS Qualität, ≥99%, VWR, Art.-Nr.: 84885.180), gegeben und mit 1000 mL hochreinem Wasser aufgefüllt (Endkonzentration 5 mM). Zur Einstellung des korrekten pH-Werts (5,3) wurden 65 µL Essigsäure (HiPerSolv CHROMANORM for LC/MS, Essigsäure 99%, VWR Chemicals, Art.-Nr.: 84874.180) zugegeben.

SEC-Puffer

In eine saubere 1L Labor Gewindeflasche wurden 7,7 g NH_4OAc (molekular-biologische Qualität, Art.-Nr.: A1542-250G) gegeben und mit 1000 mL hochreinem Wasser aufgefüllt, sodass eine 0,1 M Lösung vorlag.

RNA-Konzentrationsbestimmung

Die RNA-Ausbeuten wurden mit Hilfe eines NanoPhotometer® N60/N50 von Implen (München, Deutschland) bestimmt. Dazu wurde die gefällte RNA in hochreinem Wasser angelöst und 1 µL zur Konzentrationsbestimmung verwendet. Das Ergebnis muss zwischen 5 und 5000 ng/µL liegen, ansonsten ist das Ergebnis ungenau und die Probe muss zur erneuten Messung gegebenenfalls verdünnt werden.

ms^2C Aufreinigung aus RNA-Verdau

Die aufgereinigte tRNA aus 200 mL *E. coli* Kultur wurden über Nacht verdaut und das Verdauprodukt wurde in sechs Läufen über ein Agilent 1100 HPLC System aufgetrennt. Es wurde eine Synergy 4 µm Hydro-RP Säule (80 Å, 250x10 mm, Phenomenex) verwendet, bei 35°C und einer Flussrate von 5 mL/Min. Laufmittel A besteht aus 0,1% Essigsäure und 5 mM NH_4OAc, Laufmittel B aus 100% ACN. Die Methode hat eine Laufzeit 60 Min.

Tabelle 9: Beschreibung des Gradienten der Methode zur Aufreinigung von ms^2C aus einem tRNA-Verdau Gemisch.

Zeit [Min.]	Laufmittel B [%]
0 - 38	0 - 5
38 - 43	5 - 20
43 - 50	20
50 - 53	20 - 0
53 - 60	0

LD_{50} Bestimmung (survival assay)

Aus einer *E. coli* Übernachtkultur wurden 5 ml Kultur mit OD_{600} = 1,0 hergestellt. Nach einer Inkubationszeit von 60 Min. bei 37°C in einem Schüttelinkubator (Orbital Shaker-Incubator ES-20, BioSan, Riga, Lettland) bei 250 rpm wurden 100 µL der Kultur entnommen und 10^{-5} oder 10^{-6} mit sterilem, nicht vorgewärmtem Wasser verdünnt. 5-6 sterile Eppendorff Gefäße wurden dafür **im Voraus mit 900 µL sterilem Wasser befüllt** und die 100 µL aus der Kultur wurden in das erste Gefäß überführt, Das Gemisch wurde **gut gevortext** und es wurden 100 µL daraus ins nächste vorbereitete Gefäß überführt, usw. Aus der letzten Verdünnung wurden 70 µl auf eine **vorgewärmte** LB-Agarplatte pipettiert und mit **8 bis 12** Glaskugeln (4 ± 0,3 mm, Roth, Karlsruhe, Art.-Nr.: HH55.1) pro Platte unter beständigem Schwenken (1-2 Min.) ausplattiert. Die Kolonien Anzahl dieser Platte repräsentiert später 100% bakterielles Überleben. In die verbliebene Kultur wurde direkt nach der 100 µL-Entnahmen eine definierte Menge MMS oder Streptozotozin (STZ, 10 mM Stammlösung) zugegeben, **durch intensives Mischen die zugegebene Substanz gut vermischt** und 60 Min. (30 Min. bei STZ) bei 37°C im Schüttelinkubator inkubiert.

Daraufhin wurden wieder 100 µL der Kultur entnommen, in gleicher Weise verdünnt und ausplattiert wie die Entnahme zuvor und über Nacht im Platteninkubator bei 37 °C inkubiert. Durch das Verhältnis der Anzahl der gezählten Kolonien der zweiten Entnahme und der Anzahl der gezählten Kolonien der ersten Entnahme lässt sich die Überlebensrate der Bakterien beschreiben.

Isoakzeptor Aufreinigung

Es wurde 1 µL 100 µM Biotin-DNA-Oligo für einen bestimmten Isoakzeptor in ein 1,5 mL Eppendorf-Gefäß vorgelegt ($tRNA^{Arg}_{CCG}$ SK65*, $tRNA^{Arg}_{ICG}$ SK66*, $tRNA^{Arg}_{UCU}$ SK67*, $tRNA^{Ser}_{GCU}$ SK63*, $tRNA^{Ser}_{GGA}$ SK64*, $tRNA^{Ser}_{UGA}$ SK57*, $tRNA^{Ala}_{UGC}$ SK33*, $tRNA^{Gly}_{CCC}$ SK53*, $tRNA^{Lys}_{mnm5s2UUU}$ SK55*. Die zugehörigen Sequenzen sind in **Tabelle 4**, Supplementary Information, Reichle *et al.*, *Nat. Commun.* **2019** zu finden, bzw. in **Kapitel 3.2.**). 1 µg in hochreinem Wasser gelöste totale *E. coli* tRNA wurde zugegeben und mit 25 µL 20x SSC Puffer verdünnt. Bis zu einem Gesamtvolumen von 100 µL wurde das restliche Volumen mit hochreinem Wasser aufgefüllt. Das Gemisch wurde zur Denaturierung der RNA für 3 Min. bei 90°C erhitzt (Heizblock A) und direkt im Anschluss zur Hybridisierung mit der DNA für 10 Min. bei 65°C inkubiert (Heizblock B). In einem zweiten 1,5 mL Eppendorf Gefäß wurden 25 µL magnetische Streptavidin-Dynabeads (Dynabeads™ MyOne™ Streptavidin C1, Invitrogen, Thermo Fisher Scientific, Art.-Nr.: 65801D) pro Ansatz gegeben, dabei muss darauf geachtet werden, dass die Beads aus homogener Suspension aus der Stammlösung pipettiert werden, der Bodensatz also durch Mischen gleichmäßig in Lösung gebracht wird. Die Beads werden im Eppendorf Gefäß mit Hilfe der magnetischen Halterung (OZ Biosciences Magnetic Separation Rack) am Gefäßrand konzentriert und die klare Lösung kann abpipettiert werden. Die Beads werden so 3 Mal mit je 25 µL pro Ansatz 1x B&W Puffer gewaschen und dann in 25 µL pro Ansatz 5x SSC Puffer resuspendiert. Die nun äquilibrierten Streptavidin-Dynabeads werden bei Raumtemperatur in den Ansatz pipettiert. Zur Immobilisierung des Biotin-DNA/RNA Hybrids mit den Beads wird der Ansatz 1h lang bei 25°C in einem Mixing Block (MB-102, BIOER, Hangzhou, China) bei 600 rpm inkubiert. Die Entfernung der nicht Hybridisierten RNA erfolgt durch das Entfernen des Überstands von den Beads unter Verwendung der magnetischen Halterung. Die Beads werden mit 50 µL 1x SSC Puffer und dreimal mit 25 µL 0.1x SSC Puffer gewaschen und dann in 30 µL reinem Wasser bei 80 °C (Heizblock C) für 3 Min. denaturiert. Die aufgereinigte tRNA befindet sich nun in Lösung und wird über die magnetische Halterung von den DNA-Beads-Konjugaten getrennt und in ein neues 1,5 mL Eppendorf Gefäß überführt. Bis zur weiteren Verarbeitung wird die RNA bei -20°C gelagert.

*Interne Kennnummer in der Oligo-Datenbank des AK Kellner (Stand 2019)

20x SSC Puffer

Zu 2 mL einer 1,5 M Trinatriumcitrat-Lösung (pH 7,0) wurden 1,75 g NaCl zugegeben. Es wurde mit reinem Wasser auf ein Gesamtvolumen von 10 mL aufgefüllt. Endkonzentrationen: Trinatriumcitrat-Lösung (300 mM), NaCl (3M).

1x B&W Puffer

584 mg NaCl wurden mit 38,5 µL einer 1M EDTA Lösung und 50 µL einer 1M Tris-HCl Lösung (pH 7,5) vereint. Dieses Gemisch wurde mit hochreinem Wasser auf ein Gesamtvolumen von 10 mL gebracht. Endkonzentrationen: NaCl (1M), EDTA (0,5 mM), Tris-HCl (5mM).

Bioanalyzer

Nach Vorbereitung des Agilent RNA 6000 Pico Chips/ des Agilent Small RNA Chips gemäß dem Agilent RNA 6000 Pico Kit Quick Start Guide/ Agilent Small RNA Kit Quick Start Guide wurden jeweils 1 µL der unterschiedlich konzentrierten miRNA-Proben auf den jeweiligen Chip aufgetragen und im Agilent 2100 Bioanalyzer mit den vorinstallierten Methoden „Eukaryote Total RNA Pico Series II.xsy“/ „Small RNA Series II.xsy“ gemessen.

Enzymverdau (Pipettierschema)

Tabelle 10: Beispiel-Protokoll für 10 µg RNA Verdau, 2h bei 37°C.

	15 uL Probe +			15 uL Master-Mix		
Substanz	Stammlsg.		Zielkonz.	1x	5x	50x
$MgCl_2$	10 mM	→	1 mM	3 µL	15 µL	150 µL
Tris pH=8	50 mM	→	5 mM	3 µL	15 µL	150 µL
Benzonase	1 U/µL	→	2 U	2 µL	10 µL	100 µL
CIP (Alk.Phos.)	1 U/µL	→	2 U	2 µL	10 µL	100 µL
SPD (PDE1)	0,1 U/µL	→	0,2 U	2 µL	10 µL	100 µL
Pentostatin	1 mg/mL	→	1 µg	1 µL	5 µL	50 µL
THU	5 mg/mL	→	5 µg	1 µL	5 µL	50 µL
BHT	10 mM	→	10 uM	1 µL	5 µL	50 µL

Alle verwendeten Enzyme, sowie Pentostatin, THU und BHT wurden kommerziell erworben. Benzonase Nuclease (Art.-Nr.: E1014-5KU), CIP (Phosphatase, *Alkaline*

from bovine intestinal mucosa, Art.-Nr.: P0114-10KU), Pentostatin (≥95%, Art.-Nr.: SML0508-5MG) und BHT (Butylated Hhydroxytoluene, Art.-Nr.: PHR1117) wurden von Sigma Aldrich (München, Deutschland), PDE1 (Phosphodiesterase I, *C. adamanteus*, Art.-Nr.: P5263) von Abnova (Taipei City, Taiwan) und THU (Tetrahydrouridin, Art.-Nr.: 584222-10MG) von Merck (Darmstadt, Deutschland) erworben.

CIP-Verdau mi-RNA

Tabelle 11: Beispiel-Protokoll für CIP-Verdau von miRNA, 2h bei 37°C.

	15 uL Probe +			15 uL Master-Mix		
Substanz	Stammlsg.		Zielkonz.	1x	5x	50x
$MgCl_2$	10 mM	→	1 mM	3 µL	15 µL	150 µL
Tris pH=8	50 mM	→	5 mM	3 µL	15 µL	150 µL
CIP (Alk.Phos.)	1 U/µL	→	2 U	2 µL	10 µL	100 µL
Pentostatin	1 mg/mL	→	1 µg	1 µL	5 µL	50 µL
THU	5 mg/mL	→	5 µg	1 µL	5 µL	50 µL
BHT	10 mM	→	10 uM	1 µL	5 µL	50 µL
H_2O				4 µL	20 µL	200 µL

Grubbs-Test

In der statistischen Auswertung gehört der Grubbs-Test, oder auch ESD-Methode (*Extreme Studentized Deviate*) genannt, zu den bekanntesten und oft verwendeten statistischen Methoden, um potentielle Ausreißer mit hoher Wahrscheinlichkeit zu identifizieren. Dazu muss erst das Verhältnis Z berechnet werden. Z ist das Verhältnis des Mittelwerts (MW) aller Proben minus den Wert des potentiellen Ausreißers (V) zur Standardabweichung (SD) aller Werte.

$$Z = \frac{|MW - V|}{SD}$$

Je größer Z, desto weiter liegt der Wert V von den anderen Werten entfernt. Z kann niemals größer werden als

$$Z(Max) = (N - 1)/\sqrt{N},$$

wenn N für die Anzahl aller gemessenen Werte steht. Kritische Werte für Z wurden von Grubbs und anderen Statistikern festgelegt. Liegt Z über dem kritischen Wert, so ist der Wert V mit hoher Wahrscheinlichkeit ein Ausreißer. Die Signifikanz wird über die

Annahme einer Gaußschen Verteilung der Werte berechnet. Ist der Wert < 0.05, so ist der Wert V zu 95 % ein Ausreißer. Die Grenze, ab wann ein Wert ein Ausreißer ist, kann über das Signifikanzniveau α festgelegt werden.[149]

Wird α = 0,05 gesetzt (95% Ausreißerwahrscheinlichkeit), so liegt in Kontrollprobe 5 (**Kon. 5**) der mcm^5U-Wert als Ausreißer vor. Für einen α-Wert von 0,1 (90% Ausreißerwahrscheinlichkeit) wird zusätzlich in Probe 6 (**Inf. 6**) der mcm^5U-Wert als Ausreißer identifiziert. Der mcm^5s^2U-Wert der **Kon. 5** ist zu 80% (α = 0,2) ein Ausreißer.

Anhand dieser Wahrscheinlichkeiten und der Tatsache, dass die Werte sich für mcm^5U als auch für mcm^5s^2U im Bereich der Ausreißer befinden, führten zu der Konsequenz, dass diese in der weiteren Ergebnisauswertung nicht weiter berücksichtigt wurden. Obwohl die Überlagerung der Werte mit der jeweiligen anderen Population die Vermutung auf einen einfachen Vertausch der Proben nahelegt, dies jedoch nicht belegt werden kann, werden die Werte aus Gründen der Datenintegrität nicht zur jeweils anderen Population gerechnet. Die Werte wurden mithilfe der GraphPad Prism Software (Version 7.04) berechnet.

QQQ-Methoden

Alle massenspektrometrischen Messungen wurden an einem Agilent Technologies G6490A Triple Quad System oder einem Agilent Technologies G6470A Triple Quad System durchgeführt. In beiden Fällen war eine LC 1290 von Agilent Technologies vorangekoppelt und es wurde eine ESI Quelle zur Ionisierung verwendet (ESI-MS, Agilent Jetstream). Es wurde jeweils im positiven Ionenmodus gemessen, die Zellbeschleunigungsspannung (*cell accelerator voltage*) lag bei 5 V (oder 2 V in der gestaffelten MRM Methode), die Fragmentorspannung lag bei 380 V (gestaffelte MRM Methode) oder wurde Nukleosid spezifisch optimiert (siehe nachfolgende Tabellen). Die Kollisionsenergie lag bei 10 eV (gestaffelte MRM Methode) oder wurde Nukleosid spezifisch optimiert. Die Gastemperatur (N_2) lag bei konstant 150 °C (G6490) bzw. 230 °C (G6470A) mit einer Flussrate von 15 L/Min. bzw. 6 L/Min. Die Sheath-Gastemperatur lag bei 275 °C (G6490) bzw. 400 °C (G6470A) mit einer Flussrate von 11 L/Min. bzw. 12 L/Min. In beiden Systemen lag die Kapillarspannung (*capillary voltage*) bei 2500 V, die Skimmer-Spannung lag bei 15 V und die Düsenspannung (*nozzle voltage*) bei 500 V (G6490) bzw. 0 V (G6470A). Der Vernebelungsdruck

(*nebulizer pressure*) lag im G6490 System bei 30 Psi und im G6470A System bei 40 Psi. Alle Methoden wurden im DMRM Modus durchgeführt.

Gestaffelte MRM-Methode

Tabelle 12a: LC-Parameter (*binary pump*) Laufmittel A: 5 mM NH_4OAc, pH = 5,3. Laufmittel B: LC-MS grade Acetonitril, 35 °C, Kinetex (1.7 µm EVO C_{18}, 100 Å, 150 x 2.1 mm, Phenomenex, Torrance, CA, USA)

Zeit [Min.]	Laufmittel A [%]	Laufmittel B [%]	Fluss [mL/Min.]
0	100	0	0,350
0	100	0	0,350
10	90	10	0,350
20	20	80	0,350
22	100	0	0,350
25	100	0	0,350

Tabelle 12b: Massenübergänge der gestaffelten MRM-Methode für die nicht isotopenmarkierten Proben, die ^{15}N markierten Proben und die ^{34}S markierten Proben (*P. aeruginosa, E. coli,* Nematodes).

Vorläufer Ion [m/z]	Produkt Ion [m/z]	Verweilzeit [ms]	Fragmentor [V]	Kollisions-energie [eV]	Zellbeschl. [V]	Polarität
Messung 1						
243	111	10	380	10	2	Positive
244	112	10	380	10	2	Positive
245	113	10	380	10	2	Positive
246	114	10	380	10	2	Positive
247	115	10	380	10	2	Positive
…	…	…	…	…	…	…
…	…	…	…	…	…	…
…	…	…	…	…	…	…
295	163	10	380	10	2	Positive
296	164	10	380	10	2	Positive
297	165	10	380	10	2	Positive
298	166	10	380	10	2	Positive
299	167	10	380	10	2	Positive
Messung 2						
300	168	10	380	10	2	Positive
301	169	10	380	10	2	Positive
302	170	10	380	10	2	Positive
303	171	10	380	10	2	Positive
304	172	10	380	10	2	Positive

…	…	…	…	…	…	…
…	…	…	…	…	…	…
…	…	…	…	…	…	…
356	224	10	380	10	2	Positive
357	225	10	380	10	2	Positive
358	226	10	380	10	2	Positive
359	227	10	380	10	2	Positive
360	228	10	380	10	2	Positive
Messung 3						
360	228	10	380	10	2	Positive
361	229	10	380	10	2	Positive
362	230	10	380	10	2	Positive
363	231	10	380	10	2	Positive
364	232	10	380	10	2	Positive
…	…	…	…	…	…	…
…	…	…	…	…	…	…
…	…	…	…	…	…	…
416	284	10	380	10	2	Positive
417	285	10	380	10	2	Positive
418	286	10	380	10	2	Positive
419	287	10	380	10	2	Positive
420	288	10	380	10	2	Positive

Tabelle 12c: Massenübergänge der gestaffelten MRM-Methode für die ^{13}C markierten Proben (*P. aeruginosa, E. coli*).

Vorläufer Ion [m/z]	Produkt Ion [m/z]	Verweilzeit [ms]	Fragmentor [V]	Kollisions-energie [eV]	Zellbeschl. [V]	Polarität
Messung 1						
240	103	10	380	10	2	Positive
241	104	10	380	10	2	Positive
242	105	10	380	10	2	Positive
243	106	10	380	10	2	Positive
244	107	10	380	10	2	Positive
…	…	…	…	…	…	…
…	…	…	…	…	…	…
…	…	…	…	…	…	…
320	183	10	380	10	2	Positive
321	184	10	380	10	2	Positive
322	185	10	380	10	2	Positive
323	186	10	380	10	2	Positive
324	187	10	380	10	2	Positive
Messung 2						
300	163	10	380	10	2	Positive

301	164	10	380	10	2	Positive
302	165	10	380	10	2	Positive
303	166	10	380	10	2	Positive
304	167	10	380	10	2	Positive
…	…	…	…	…	…	…
…	…	…	…	…	…	…
…	…	…	…	…	…	…
356	219	10	380	10	2	Positive
357	220	10	380	10	2	Positive
358	221	10	380	10	2	Positive
359	222	10	380	10	2	Positive
360	223	10	380	10	2	Positive
Messung 3						
361	224	10	380	10	2	Positive
362	225	10	380	10	2	Positive
363	236	10	380	10	2	Positive
364	237	10	380	10	2	Positive
365	238	10	380	10	2	Positive
…	…	…	…	…	…	…
…	…	…	…	…	…	…
…	…	…	…	…	…	…
442	305	10	380	10	2	Positive
443	306	10	380	10	2	Positive
444	307	10	380	10	2	Positive
445	308	10	380	10	2	Positive
446	309	10	380	10	2	Positive

Methode zur Messung der Rinderurin miRNA

Tabelle 13a: LC-Parameter (*binary pump*) Laufmittel A: 5 mM NH_4OAc, pH = 5,3. Laufmittel B: LC-MS grade Acetonitril, 35 °C, Synergi Fusion-RP 2.5 µm EVO C_{18}, 100 Å, 100 x 2.0 mm.

Zeit [Min.]	Laufmittel A [%]	Laufmittel B [%]	Fluss [mL/Min.]
0	100	0	0,350
0	100	0	0,350
1	100	0	0,350
5	90	10	0,350
7	60	40	0,350
8	60	40	0,350
8,5	100	0	0,350
11	100	0	0,350

Tabelle 13b: Methode zur Messung der Urin miRNA von *O. ochengi* infizierten Rindern und den Kontrollproben. Die Zyklen-Zeit lag bei 700 ms.

Name	Vorläufer Ion [m/z]	Produkt Ion [m/z]	Retentions-zeit [Min.]	Fragmen-tor [V]	Kollisions-energie [eV]	Zell-beschl. [V]	Polarität
Um	259	113	4.3	96	8	5	Positive
U	245	113	2.8	95	5	5	Positive
t^6A	413	281	5.4	130	9	5	Positive
Q	410	295	4.4	115	12	5	Positive
Ψ	245	209	1.7	90	5	5	Positive
mcm^5U	317	185	4.8	95	5	5	Positive
mcm^5s^2U	333	201	6.2	92	8	5	Positive
m^7G	298	166	3.5	105	14	5	Positive
m^6A	282	150	6.5	120	20	5	Positive
$m^6{}_2A$	296	164	7.1	130	21	5	Positive
m^5Um	273	127	5.4	90	13	5	Positive
m^5U	259	127	4.1	145	10	5	Positive
m^5C	258	126	3.5	185	13	5	Positive
m^3U	259	127	4.4	75	9	5	Positive
m^3C	258	126	2.3	88	14	5	Positive
m^2G	298	166	5.0	95	17	5	Positive
m^2A	282	150	6.1	125	21	5	Positive
$m^2{}_2G$	312	180	5.6	102	15	5	Positive
m^1I	283	151	4.0	85	9	5	Positive
m^1G	298	166	4.8	105	13	5	Positive
m^1A	282	150	2.7	110	21	5	Positive
i^6A	336	204	8.1	140	17	5	Positive
I	269	137	3.9	100	9	5	Positive
Gm	298	152	4.8	100	9	5	Positive
G	284	152	4.0	95	17	5	Positive
Cm	258	112	2.8	180	9	5	Positive
C	244	112	2.0	175	13	5	Positive
Am	282	136	5.9	110	17	5	Positive
ac^4C	286	154	4.9	85	9	5	Positive
A	268	136	5.1	110	21	5	Positive
SILIS							
Um SILIS	271	119	4.3	96	8	5	Positive
U SILIS	256	119	2.8	95	5	5	Positive
t^6A SILIS	434	297	5.4	130	9	5	Positive
Q SILIS	432	311	4.4	115	12	5	Positive
Ψ SILIS	256	220	1.7	90	5	5	Positive
mcm^5U SILIS	331	194	4.8	95	5	5	Positive
mcm^5s^2U SILIS	347	210	6.2	92	8	5	Positive
m^7G SILIS	314	177	3.5	105	14	5	Positive
m^6A SILIS	298	161	6.5	120	20	5	Positive

$m^6{}_2A$ SILIS	313	176	7.1	130	21	5	Positive
m^5Um SILIS	286	134	5.4	90	13	5	Positive
m^5U SILIS	271	134	4.1	145	10	5	Positive
m^5C SILIS	271	134	3.5	185	13	5	Positive
m^3U SILIS	271	134	4.4	75	9	5	Positive
m^3C SILIS	271	134	2.3	88	14	5	Positive
m^2G SILIS	314	177	5	95	17	5	Positive
m^2A SILIS	298	161	6.1	125	21	5	Positive
$m^2{}_2G$ SILIS	330	193	5.6	102	15	5	Positive
m^1I SILIS	297	160	4	85	9	5	Positive
m^1G SILIS	314	177	4.8	105	13	5	Positive
m^1A SILIS	298	161	2.7	110	21	5	Positive
i^6A SILIS	356	219	8.1	140	17	5	Positive
I SILIS	283	146	3.9	100	9	5	Positive
Gm SILIS	314	162	4.8	100	9	5	Positive
G SILIS	299	162	4	95	17	5	Positive
Cm SILIS	271	119	2.8	180	9	5	Positive
C SILIS	256	119	2	175	13	5	Positive
Am SILIS	298	146	5.9	110	17	5	Positive
ac^4C SILIS	300	168	4.9	85	9	5	Positive
A SILIS	283	146	5.1	110	21	5	Positive

Methode zur Messung der tRNA verschiedener Nematodenarten

Die tRNA-Nukleoside wurden bei einer Temperatur von 35 °C über eine Synergi Fusion-RP Säule (2.5 µm EVO C_{18}, 100 Å, 100 x 2.0 mm, Phenomenex) gemessen. Laufmittel A: 5 mM NH_4OAc, pH = 5,3. Laufmittel B: LC-MS grade Acetonitril. Der Gradient kann der **Tabelle 13a** entnommen werden.

Tabelle 14: Methode zur absoluten Quantifizierung der tRNA-Modifikationen verschiedener Nematodenarten. Die Zyklen-Zeit lag bei 700 ms.

Name	Vorläufer Ion [m/z]	Produkt Ion [m/z]	Retentions-zeit [Min.]	Fragmen-tor [V]	Kollisions-energie [eV]	Zell-beschl. [V]	Polarität
U	245	113	2.8	95	5	5	Positive
t^6A	413	281	5.4	130	9	5	Positive
mcm^5U	317	185	4.8	95	5	5	Positive
mcm^5s^2U	333	201	6.2	92	8	5	Positive
m^7G	298	166	3.5	105	14	5	Positive
m^6A	282	150	6.5	120	20	5	Positive
m^5U	259	127	4.1	145	10	5	Positive
m^2G	298	166	5.0	95	17	5	Positive
m^1G	298	166	4.8	105	13	5	Positive

i^6A	336	204	8.1	140	17	5	Positive
G	284	152	4.0	95	17	5	Positive
C	244	112	2.0	175	13	5	Positive
ac^4C	286	154	4.9	85	9	5	Positive
A	268	136	5.1	110	21	5	Positive
SILIS							
U SILIS	256	119	2.8	95	5	5	Positive
t^6A SILIS	434	297	5.4	130	9	5	Positive
mcm^5U SILIS	331	194	4.8	95	5	5	Positive
mcm^5s^2U SILIS	347	210	6.2	92	8	5	Positive
m^7G SILIS	314	177	3.5	105	14	5	Positive
m^6A SILIS	298	161	6.5	120	20	5	Positive
m^5U SILIS	271	134	4.1	145	10	5	Positive
m^2G SILIS	314	177	5	95	17	5	Positive
m^1G SILIS	314	177	4.8	105	13	5	Positive
i^6A SILIS	356	219	8.1	140	17	5	Positive
G SILIS	299	162	4	95	17	5	Positive
C SILIS	256	119	2	175	13	5	Positive
ac^4C SILIS	300	168	4.9	85	9	5	Positive
A SILIS	283	146	5.1	110	21	5	Positive

Methode zur Messung der alternativen Fragmentierungen des potentiellen Q-Derivats

Die unterschiedlich isotopenmarkierten tRNA-Nukleoside wurden bei einer Temperatur von 35 °C über eine Kinetex Säule (1.7 µm EVO C18, 100 Å, 150 x 2.1 mm, Phenomenex) gemessen. Laufmittel A: 5 mM NH_4OAc, pH = 5,3. Laufmittel B: LC-MS grade Acetonitril. Der Gradient kann der **Tabelle 12a** entnommen werden.

Tabelle 15: Alternative Fragmentierung des Queuosin-Derivats bei Rt. = 4,24 Min.

Name	Vorläufer Ion [m/z]	Produkt Ion [m/z]	Retentions-zeit [Min.]	Fragmen-tor [V]	Kollisions-energie [eV]	Zell-beschl. [V]	Polarität
nl Qox F1	408.4	295	4.25	115	12	5	Positive
nl Qox F2	408.4	163	4.25	115	12	5	Positive
^{15}N Qox F1	413.4	299	4.25	115	12	5	Positive
^{15}N Qox F2	413.4	167	4.25	115	12	5	Positive
^{13}C Qox F1	425.4	307	4.25	115	12	5	Positive
^{13}C Qox F2	425.4	170	4.25	115	12	5	Positive

6. Anhang und Zusatzinformationen

Anhang Tabelle 1: Liste aller gefundenen Signale nach einem ersten Durchlauf der gestaffelten MRM-Methode mit nicht isotopnemarkierter *P. aeruginosa* tRNA, srRNA und lrRNA. Neben den Massenübergängen und Retentionszeiten werden die Intensitäten der Signale in den jeweiligen RNA Fraktionen gezeigt. Mögliche, den Signalen zuordenbare RNA-Modifikationen sind in der Spalte ganz rechts aufgelistet.

		Signalintensitäten			
Massen-übergänge	Retentionszeit (Min.)	tRNA	srRNA	lrRNA	Vorschläge/ zutreffende Massenübergänge
240 --> 108	14,45	-	6x10^3	6x10^3	-
243 --> 111	1,85	4x10^5	9x10^3	-	-
244 --> 112	1,33	-	-	1x10^7	-
244 --> 112	1,54	-	8x10^6	-	-
244 --> 112	1,75	1x10^7	9x10^6	1x10^7	Cytidin
245 --> 113	1,56	-	-	3x10^6	Pseudouridin
245 --> 113	1,90	9x10^6	4x10^6	4x10^6	Uridin
247 --> 115	1,38 - 1,48	4x10^6	4x10^4	2x10^4	Dihydrouridin
249 --> 117	1,28 - 1,34	1x10^7	1x10^7	1x10^7	-
249 --> 117	1,76	-	3x10^5	-	-
249 --> 117	3,42	-	1x10^5	1x10^5	-
251 --> 119	1,29 - 1,43	1x10^7	7x10^6	4x10^6	-
251 --> 119	1,97	-	-	3x10^5	-
251 --> 119	3,50	3x10^6	2x10^6	9x10^6	-
253 --> 121	18,17	-	-	3x10^3	-
254 --> 122	1,31 - 1,56	6x10^5	6x10^5	5x10^5	-
258 --> 126	2,24	-	-	4x10^5	m^3C, m^4C
258 --> 126	2,46	-	-	4x10^5	m^3C, m^4C
258 --> 126	2,70	1x10^4	2x10^6	5x10^5	m^5C
259 --> 127	3,94	8x10^6	1x10^5	5x10^4	m^5U
259 --> 127	4,73	6x10^5	6x10^3	1x10^5	m^3U
259 --> 127	12,73	-	4x10^3	-	-
260 --> 128	2,25	1x10^6	6x10^3	-	s^2C
261 --> 129	4,77	1x10^6	7x10^3	-	s^4U
261 --> 129	5,96	-	1x10^3	-	-
262 --> 130	1,39	-	3x10^3	-	-
263 --> 131	1,96	-	3x10^4	1x10^4	-
263 --> 131	4,75	-	2x10^3	-	-
263 --> 131	5,08	-	4x10^3	-	-
263 --> 131	10,64 - 11,00	5x10^4	2x10^4	2x10^4	-
263 --> 131	12,72	3x10^3	3x10^3	3x10^3	-
264 --> 132	1,42	-	2x10^3	-	-
264 --> 132	16,94	-	6x10^3	6x10^2	-
265 --> 133	1,29	-	2x10^3	-	-
266 --> 134	1,34	-	-	1x10^5	-

266 --> 134	1,53	-	1x10^5	8x10^4	-
266 --> 134	1,75	7x10^4	1x10^5	1x10^5	Cytidin Na^+
267 --> 135	3,28	1x10^4	1x10^4	6x10^3	-
267 --> 135	4,32	-	3x10^3	-	-
267 --> 135	7,29	-	1x10^3	-	-
268 --> 136	5,78	1x10^7	1x10^7	1x10^7	Adenosin
269 --> 137	3,55	4x10^5	-	4x10^3	Inosin
269 --> 137	3,83	3x10^4	5x10^4	3x10^4	-
271 --> 139	3,13	4x10^3	2x10^3	-	-
271 --> 139	15,95	2x10^3	1x10^3	1x10^3	-
272 --> 140	5,32	2x10^6	2x10^5	4x10^4	$m^{44}C$, f^5C
273 --> 141	5,78	6x10^4	3x10^4	7x10^4	-
273 --> 141	8,34	6x10^4	1x10^4	-	-
274 --> 142	5,57	6x10^4	1x10^3	-	nm^5U, hm^5C
275 --> 143	2,82	2x10^4	-	-	mo^5U
275 --> 143	3,21	-	4x10^3	-	mo^5U
275 --> 143	3,48	-	-	5x10^3	mo^5U
275 --> 143	3,66	3x10^4	4x10^4	-	mo^5U
275 --> 143	6,04	-	3x10^3	-	m^5s^2U
276 --> 144	3,73	5x10^5	4x10^3	-	-
277 --> 145	4,24	2x10^5	3x10^5	2x10^5	-
277 --> 145	4,36	2x10^5	1x10^5	-	-
277 --> 145	6,59	-	6x10^3	6x10^3	-
277 --> 145	7,12	-	6x10^3	8x10^3	-
277 --> 145	12,72	-	2x10^4	2x10^4	-
279 --> 147	10,73	-	1x10^3	2x10^3	-
279 --> 147	11,27	-	1x10^3	1x10^3	-
280 --> 148	1,91	-	3x10^3	-	-
280 --> 148	2,75	-	-	1x10^4	-
280 --> 148	6,22	-	1x10^3	-	-
281 --> 149	10,61	1x10^5	2x10^5	2x10^5	-
282 --> 150	8,13	1x10^7	3x10^6	5x10^6	m^2A
282 --> 150	8,51	1x10^7	3x10^5	6x10^6	m^6A
283 --> 151	3,27	-	2x10^4	2x10^4	-
283 --> 151	3,60	-	2x10^4		-
284 --> 152	3,77	1x10^7	1x10^7	1x10^7	Guanosin
285 --> 153	1,90	5x10^5	3x10^5	-	-
285 --> 153	3,29	1x10^7	1x10^7	1x10^7	-
287 --> 155	8,12	1x10^4	-	-	-
289 --> 157	3,77	3x10^4	2x10^4	3x10^4	-
291 --> 159	1,36	-	2x10^3	-	-
291 --> 159	3,57	8x10^3	-	-	Inosin Na^+
291 --> 159	6,53	-	2x10^3	-	-
292 --> 160	1,79	-	1x10^3	2x10^3	-
293 --> 161	5,50	-	-	1x10^3	-
293 --> 161	8,97	1x10^4	5x10^3	8x10^3	-
294 --> 162	9,09	3x10^3	-	-	-

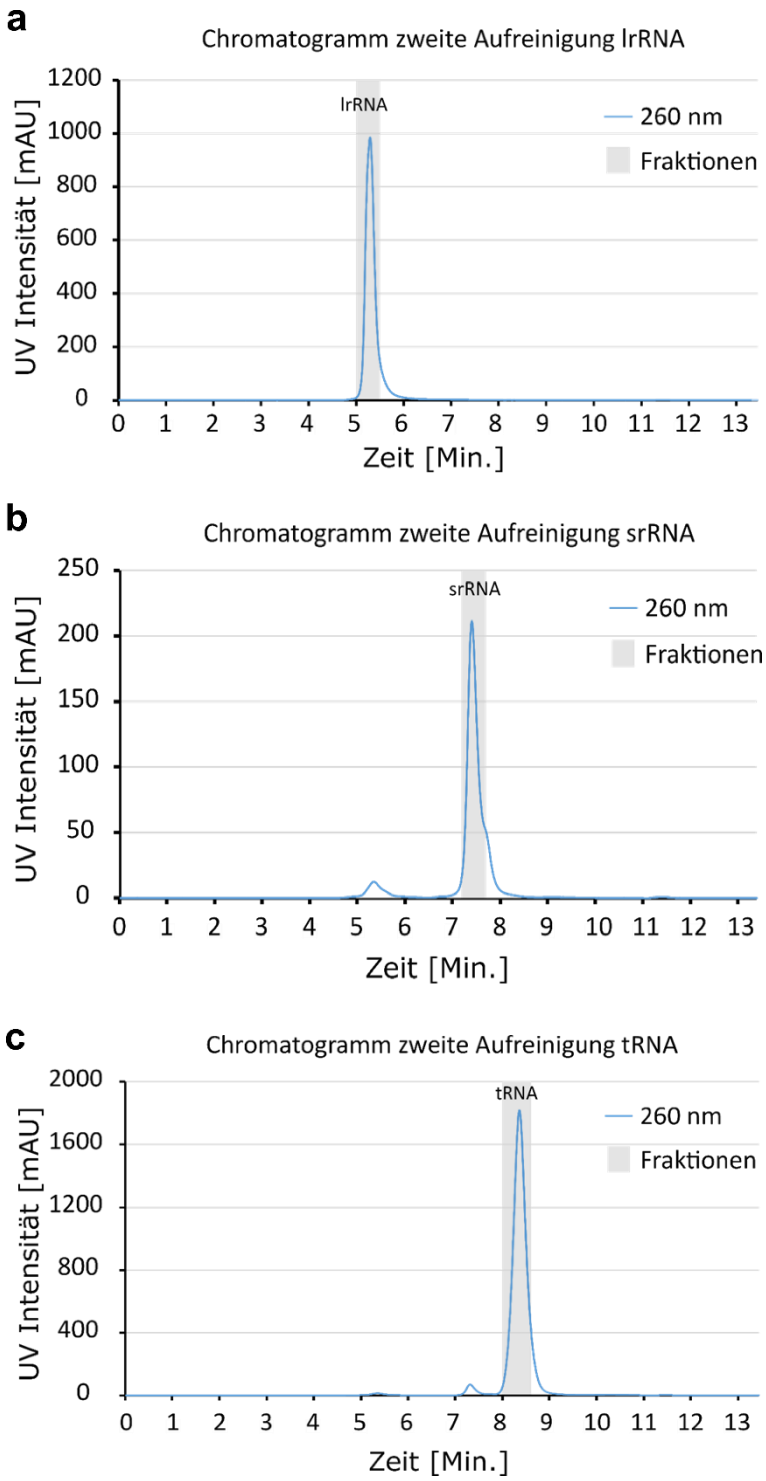
295 --> 163	4,26 - 4,47	4x10^4	3x10^3	-	-
295 --> 163	12,71	1x10^5	2x10^5	2x10^5	-
296 --> 164	3,64	2x10^5	3x10^3	-	f^6A, $m^{28}A$
296 --> 164	12,07	2x10^5	7x10^5	1x10^7	$m^{66}A$
298 --> 166	2,44	1x10^7	1x10^6	2x10^6	m^7G
298 --> 166	5,14	1x10^7	3x10^5	2x10^6	m^1G
298 --> 166	5,54	-	2x10^5	4x10^6	m^2G
299 --> 167	4,03	6x10^5	3x10^5	1x10^5	-
299 --> 167	4,35	7x10^5	7x10^5	4x10^5	*m/z* 277 Na^+
300 --> 168	3,76	-	6x10^3	-	-
300 --> 168	15,97	-	-	4x10^3	-
301 --> 169	3,29	1x10^4	3x10^5	2x10^5	-
301 --> 169	12,09	-	-	6x10^4	-
302 --> 170	5,06	-	1x10^3	-	-
302 --> 170	5,68	-	1x10^4	-	-
303 --> 171	2,50	1x10^4	-	-	-
303 --> 171	5,15	1x10^4	-	-	-
303 --> 171	6,83	1x10^4	8x10^3	7x10^3	-
304 --> 172	2,13	6x10^4	-	-	mnm^5s^2U
305 --> 173	14,34	-	-	1x10^3	-
306 --> 174	3,62	-	-	7x10^4	-
306 --> 174	3,74	5x10^4	-	-	Guanosin Na^+
306 --> 174	3,84	5x10^4	6x10^4	-	-
306 --> 174	3,94	1x10^5	-	-	-
307 --> 175	3,16	-	-	3x10^3	-
307 --> 175	3,31	6x10^3	4x10^3	-	se^2U
309 --> 177	4,24	5x10^3	5x10^3	2x10^3	
310 --> 178	3,32	1x10^3	-	-	ac^6A
311 --> 179	7,75	4x10^3	-	-	-
312 --> 180	1,74	7x10^4	4x10^3	3x10^3	preQ1tRNA
312 --> 180	3,35	1x10^3	2x10^3	1x10^4	preQ1tRNA
312 --> 180	4,63	1x10^4	-	-	preQ1tRNA
312 --> 180	6,91	1x10^3	-	3x10^3	$m^{22}G$
312 --> 180	9,02	-	-	2x10^3	preQ1tRNA
313 --> 181	1,34	-	5x10^3	-	-
313 --> 181	1,85	-	3x10^3	-	-
313 --> 181	5,58	2x10^4	1x10^4	2x10^4	-
314 --> 182	1,80	6x10^3	7x10^3	8x10^3	-
314 --> 182	3,26	5x10^3	-	-	-
314 --> 182	10,56	2x10^4	1x10^3	-	$m^{27}G$
315 --> 183	1,55	-	3x10^3	-	-
315 --> 183	4,06	-	2x10^3	-	-
315 --> 183	4,37	-	4x10^3	2x10^3	-
315 --> 183	6,61	-	3x10^3	3x10^3	-
315 --> 183	15,44	-	3x10^3	2x10^3	-
316 --> 184	1,76	6x10^4	-	-	-
316 --> 184	2,20	6x10^3	-	-	-

316 --> 184	3,75	-	2x10^3	2x10^3	-
316 --> 184	4,24	-	9x10^3	-	-
316 --> 184	11,66	-	1x10^3	1x10^3	-
317 --> 185	3,28	3x10^3	4x10^3	3x10^3	mcm^5U
317 --> 185	7,94	3x10^3	-	-	mcm^5U
317 --> 185	9,23	3x10^3	-	-	mcm^5U
318 --> 186	1,87	8x10^3	5x10^3	-	ncm^5s^2U, $nchm^5U$
318 --> 186	2,03	2x10^4	-	-	ncm^5s^2U, $nchm^5U$
319 --> 187	1,70	4x10^4	-	-	chm^5U,cmo^5U, cm^5s^2U
319 --> 187	3,68	-	1x10^3	-	-
319 --> 187	4,10	1x10^4	2x10^3	-	chm^5U,cmo^5U, cm^5s^2U
320 --> 188	2,52	1x10^5	-	-	m^7G Na^+
320 --> 188	5,15	1x10^5	1x10^3	1x10^4	m^1G Na^+
320 --> 188	5,53	-	3x10^3	4x10^4	m^2G Na^+
320 --> 188	9,05	4x10^5	-	-	-
321 --> 189	13,37	5x10^3	-	-	-
322 --> 190	3,49	-	3x10^3	3x10^3	-
322 --> 190	3,77	-	3x10^3	-	-
322 --> 190	9,12	-	-	1x10^3	-
323 --> 191	6,39	1x10^4	1x10^4	1x10^4	-
325 --> 193	5,46	4x10^3	4x10^3	4x10^3	-
325 --> 193	5,67	-	1x10^3	2x10^3	-
326 --> 194	1,75	1x10^6	1x10^6	8x10^5	-
326 --> 194	3,67	2x10^3	3x10^3	-	-
326 --> 194	6,10	-	1x10^3	-	-
327 --> 195	1,88	1x10^5	2x10^5	-	-
327 --> 195	2,61	-	3x10^3	-	-
329 --> 197	1,48	3x10^4	-	-	-
329 --> 197	3,71	-	-	2x10^3	-
329 --> 197	4,32	3x10^3	-	3x10^3	-
330 --> 198	5,74	2x10^3	-	-	-
330 --> 198	6,74	4x10^3	-	-	-
331 --> 199	1,34	1x10^4	1x10^4	-	-
331 --> 199	1,48	1x10^4	-	-	-
332 --> 200	4,87	3x10^3	-	-	$cmnm^5U$
333 --> 201	1,35	-	4x10^4	2x10^4	mcm^5s^2U, $mchm^5U$, $mcmo^5U$
333 --> 201	2,06	3x10^4	3x10^4	2x10^4	mcm^5s^2U, $mchm^5U$, $mcmo^5U$
333 --> 201	6,30	1x10^5	-	-	mcm^5s^2U, $mchm^5U$, $mcmo^5U$
334 --> 202	6,98	6x10^3	6x10^3	5x10^3	-
335 --> 203	1,39	-	7x10^3	-	-
336 --> 204	2,52	7x10^3	-	-	nm^5se^2U, imG, imG2,
336 --> 204	14,17	3x10^5	2x10^4	7x10^3	i^6A
337 --> 205	5,24	4x10^3	3x10^3	2x10^3	-
337 --> 205	9,35	2x10^3	-	-	-

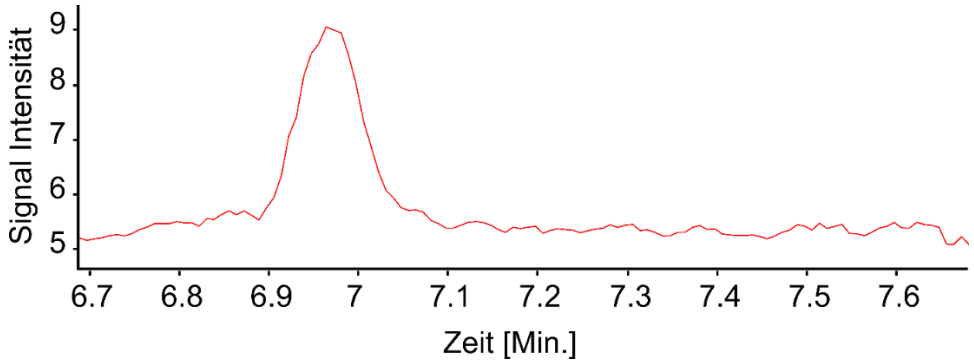
338 --> 206	2,74	-	-	3x10^3	-
338 --> 206	3,17	2x10^4	3x10^3	8x10^3	-
338 --> 206	10,97	-	-	1x10^3	-
338 --> 206	14,17	2x10^3	-	-	-
340 --> 208	10,40	3x10^3	-	-	-
341 --> 209	1,97	-	2x10^3	-	-
341 --> 209	4,23	-	2x10^3	-	-
342 --> 210	1,79	2x10^4	-	-	inm^{5}U
342 --> 210	3,85	4x10^3	-	-	inm^{5}U
342 --> 210	4,72	8x10^3	-	-	inm^{5}U
342 --> 210	6,82	5x10^6	2x10^4	-	inm^{5}U
343 --> 211	1,88	3x10^4	5x10^4	6x10^4	-
343 --> 211	5,06	-	1x10^4	-	-
343 --> 211	5,48	4x10^4	4x10^4	3x10^4	-
344 --> 212	8,15	-	-	1x10^3	-
344 --> 212	8,53	-	-	1x10^3	-
345 --> 213	3,66	4x10^3	4x10^3	2x10^3	-
345 --> 213	12,14	7x10^3	8x10^3	6x10^3	-
346 --> 214	1,75	2x10^5	1x10^4	-	acp^{3}U
347 --> 215	1,86	7x10^4	2x10^4	-	mchm^{5}Um
347 --> 215	2,23	-	3x10^3	-	mchm^{5}Um
347 --> 215	8,87	1x10^4	-	-	mchm^{5}Um
348 --> 216	3,23	8x10^4	2x10^4	-	cmnm^{5}s^{2}U, acp^{3}D
349 --> 217	2,57	4x10^3	3x10^3	-	-
349 --> 217	3,64	4x10^3	3x10^3	-	-
349 --> 217	4,85	-	1x10^3	-	-
349 --> 217	10,77	5x10^3	-	-	-
350 --> 218	5,80	1x10^3	1x10^3	1x10^3	mnm^{5}se^{2}U, mimG
351 --> 219	4,57	3x10^3	2x10^3	3x10^3	-
351 --> 219	14,40	3x10^3	-	-	-
352 --> 220	12,49	2x10^6	1x10^5	2x10^4	io^{6}A
353 --> 221	1,76	-	2x10^3	-	-
354 --> 222	8,38	-	3x10^3	1x10^4	-
354 --> 222	9,13	-	1x10^3	5x10^3	-
355 --> 223	6,28	2x10^5	-	-	mcmo^{5}U Na^+
355 --> 223	12,38	5x10^3	-	2x10^3	-
356 --> 224	5,28	6x10^3	2x10^3	3x10^3	-
356 --> 224	11,04	-	-	1x10^3	-
357 --> 225	2,64	-	1x10^3	-	-
357 --> 225	3,77	-	1x10^3	1x10^3	-
357 --> 225	12,76	-	1x10^3	1x10^3	-
358 --> 226	1,69	-	3x10^3	-	-
358 --> 226	12,07	-	-	1x10^3	-
359 --> 227	1,90	-	4x10^3	-	-
359 --> 227	3,27	-	2x10^3	-	-
359 --> 227	7,34	1x10^5	1x10^5	1x10^5	-
362 --> 230	12,27	1x10^3	-	-	-

363 --> 231	3,62	2x10^4	2x10^4	-	-
363 --> 231	5,16	-	1x10^4	-	-
364 --> 232	1,75	8x10^3	6x10^3	-	-
364 --> 232	4,77	6x10^3	-	-	-
364 --> 232	12,41	4x10^3	-	-	-
364 --> 232	6,01	2x10^3	-	-	-
366 --> 234	3,78	1x10^4	1x10^4	1x10^4	-
369 --> 237	7,26	-	3x10^3	3x10^3	-
369 --> 237	7,43	6x10^3	7x10^3	7x10^3	-
369 --> 237	8,86	5x10^3	-	-	-
371 --> 239	6,27	3x10^3	-	-	-
372 --> 240	3,95	-	-	1x10^3	-
372 --> 240	4,35	1x10^4	-	-	k^2C
372 --> 240	5,66	6x10^3	1x10^3	-	k^2C
373 --> 241	1,40	-	2x10^3	-	-
373 --> 241	5,71	-	2x10^3	2x10^3	-
373 --> 241	12,38	2x10^3	-	2x10^3	-
374 --> 242	2,29	1x10^4	-	-	-
375 --> 243	4,99	4x10^3	-	-	-
377 --> 245	1,75	-	2x10^4	-	-
377 --> 245	6,58	-	2x10^3	2x10^3	-
378 --> 246	1,88	-	2x10^3	-	-
380 --> 248	2,49	1x10^4	-	-	-
380 --> 248	5,00	2x10^5	3x10^4	1x10^3	-
380 --> 248	5,53	-	-	1x10^3	-
381 --> 249	1,75	-	3x10^3	-	-
381 --> 249	11,42	-	2x10^3	-	-
382 --> 250	3,84	1x10^4	1x10^4	-	tm^5U, ms^2i^6A
382 --> 250	3,72	1x10^4	-	1x10^4	tm^5U, ms^2i^6A
382 --> 250	5,79	-	2x10^3	2x10^3	-
383 --> 251	1,47	2x10^4	2x10^4	-	-
384 --> 252	1,78	4x10^4	8x10^4	-	-
384 --> 252	1,66	-	-	7x10^4	-
388 --> 256	1,93	1x10^4	2x10^4	-	-
388 --> 256	5,49	-	-	7x10^3	-
389 --> 257	3,88	5x10^3	5x10^3	4x10^3	-
389 --> 257	4,02	5x10^4	5x10^3	-	-
390 --> 258	1,47	4x10^3	-	-	-
391 --> 259	7,29	5x10^3	5x10^3	4x10^3	-
391 --> 259	8,28	-	-	1x10^3	-
392 --> 260	11,05	6x10^4	6x10^4	7x10^4	-
394 --> 262	1,73	-	4x10^3	-	-
394 --> 262	7,26	2x10^3	-	-	$cmnm^5se^2U$
394 --> 262	8,22	-	-	4x10^4	-
395 --> 263	2,72	-	4x10^4	-	ct^6A
395 --> 263	2,90	5x10^4	-	-	ct^6A
395 --> 263	8,22	4x10^4	3x10^4	-	ct^6A

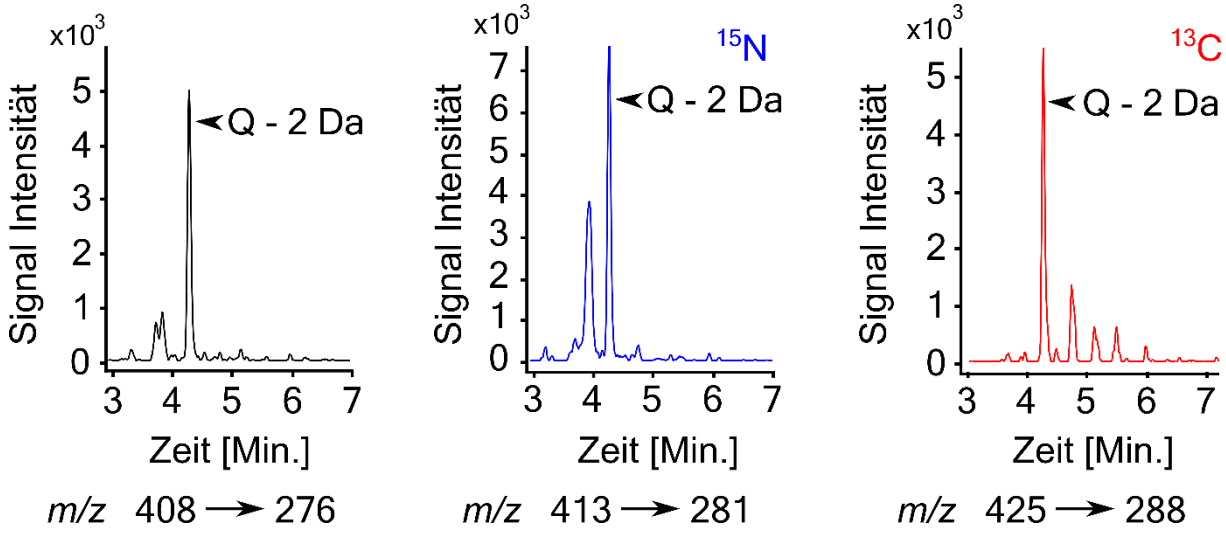
396 --> 264	2,50	1x10^4	-	-	-
397 --> 265	1,88	-	2x10^3	-	-
398 --> 266	3,79	2x10^4	2x10^4	2x10^4	tm^5s^2U, ms^2io^6A
398 --> 266	13,97	4x10^5	4x10^4	-	tm^5s^2U, ms^2io^6A
399 --> 267	3,27	3x10^4	4x10^4	-	-
399 --> 267	13,98	-	-	4x10^4	-
400 --> 268	14,17	-	1x10^3	-	-
401 --> 269	1,70	5x10^3	-	-	-
402 --> 270	4,99	8x10^3	-	-	-
402 --> 270	4,98	-	2x10^3	-	-
404 --> 272	1,87	-	1x10^3	3x10^3	-
404 --> 272	4,70	3x10^4	-	-	-
405 --> 273	14,35	-	1x10^3	5x10^3	-
406 --> 274	3,64 - 3,77	-	3x10^3	2x10^3	-
408 --> 276	1,79	1x10^5	6x10^4	-	-
408 --> 276	4,26	5x10^3	-	-	-
409 --> 277	1,80	-	-	8x10^4	-
409 --> 277	7,10	-	3x10^3	3x10^3	-
410 --> 278	1,74	-	2x10^4	-	-
412 --> 280	5,52	-	-	6x10^3	-
412 --> 280	7,92	5x10^4	-	-	-
412 --> 280	9,09	5x10^5	-	-	-
413 --> 281	1,74	-	9x10^3	-	-
413 --> 281	5.50 - 5,52	2x10^5	2x10^3	-	-
413 --> 281	7,36	5x10^5	1x10^4	1x10^3	t^6A
414 --> 282	12,49	2x10^4	2x10^3	-	-
415 --> 283	1,39	-	2x10^3	-	-
416 --> 284	3,78	7x10^3	3x10^3	4x10^3	-
416 --> 284	12,49	8x10^3	-	-	-
417 --> 285	1,82	-	1x10^3	-	-
417 --> 285	3,99	2x10^3	-	-	-
418 --> 286	4,05	-	1x10^3	1x10^3	-
419 --> 287	1,76	-	2x10^3	-	-
419 --> 287	7,93	2x10^3	-	-	-
420 --> 288	2,52	3x10^3	-	-	-
420 --> 288	13,97	1x10^3	-	-	-
420 --> 288	18,45	1x10^3	-	-	-



Anhang Abbildung 1: Chromatogramme von der zweiten Aufreinigung der verschiedenen RNA-Fraktionen. **a** Zweite Aufreinigung der lrRNA-Fraktion. **b** Zweite Aufreinigung der srRNA-Fraktion. **c** Zweite Aufreinigung der tRNA-Fraktion. In blau ist jeweils die UV-Spur bei 260 nm eingezeichnet. Der grau hinterlegte Bereich entspricht der Zeitspanne über welche die Fraktion gesammelt wurde.



Anhang Abbildung 2: t^6A Signal nach 10 amol Injektion (Massenübergang: 413 →281)



Anhang Abbildung 3: Signale des Q-Derivats mit 2 Da Massenunterschied zur originalen Q-Struktur. Die sind die Signale (von links) des nicht isotopenmarkierten (schwarz), des ^{15}N-markierten (blau) und des ^{13}C-markierten (rot) Q-Derivats des jeweiligen tRNA-Verdaus dargestellt. Die Massenübergänge entsprechen dem für Nukleoside typischen Verlust des Riboserests (-132 Da, bei ^{13}C-Markierung -137 Da).

Anhang Tabelle 2: Liste aller unbekannten Signale der Nematodenarten *C. elegans N2 (C. e. N2), H. polygyrus* (*H. p.*) und *L. sigmondontis* (*L. s.*) zwischen m/z = 240 – 420. Sind die Signale in **Anhang Tabelle 1** ebenfalls zu finden ist dies durch ein blaues Plus (**+**) in der letzten Spalte gekennzeichnet. Bekannte Modifikationen mit unbekannter Retentionszeit und mit dem Signal identischen m/z Werten sind als Vorschläge gelistet.

Vorläufer Ion [m/z]	Produkt Ion [m/]	Rt. [Min.]	*C. e. N2*	*H. p.*	*L. s.*	Vorschläge	Vergleich *P. a. 14*
242	110	6.687	+	+	+		
243	111	1.748	+	+	+		
248	116	10.749	+	+	+		
249	117	1.345	+++	+++	+++		
249	117	1.838	++	++	++		
249	117	1.937	++	++	++		
249	117	3.455	++	++	++		
251	119	1.42	+++	+++	+++		
251	119	3.066	++	++	++		
251	119	3.536	+++	+++	+++		
252	120	2.07	++	++	++		
254	122	1.509	++	++	++		
262	130	12.785	+	+	+		
263	131	2.029	++	++	++		
264	132	9.404	++	++	++		
264	132	10.059	++	++	++		
265	133	10.457	+	+	+		
266	134	1.715	++	++	++		
266	134	1.983	++	++	++		
268	136	5.157	++	++	++		
271	139	15.979	+	+	+		
272	140	1.962	++	++	++	f^5C, $m^4{}_2C$	
272	140	4.078	+	+	+	f^5C, $m^4{}_2C$	
272	140	5.024	+	++	-	f^5C, $m^4{}_2C$	**+**
272	140	11.303	++	++	+	f^5C, $m^4{}_2C$	
275	143	3.185	+	+	+	mo^5U, m^5s^2U	
277	145	4.232	++	++	++		
277	145	4.33	++	++	++		
277	145	6.486	++	++	++		
277	145	7.036	++	++	++		
279	147	18.248	++	+	++		
280	148	2.794	++	++	++		
281	149	17.851	++	++	++		
284	152	1.796	++	++	++	cnm^5U	
285	153	3.272	++	++	++		
289	157	4.297	+	+	+		
291	159	6.313	++	++	++		
291	159	6.448	++	++	++		
292	160	1.925	++	++	++		
292	160	2.081	++	++	++		

293	161	1.65	+	+	+		
293	161	19.906	+	+	+		
294	162	2.004	++	++	++		
295	163	4.038	-	+	+		
295	163	4.39	+	+	+		
296	164	3.314	+	++	++	f^6A, $m^{28}A$	+
297	165	5.052	+	+	+		
297	165	5.437	++	++	+		
297	165	3.314	-	++	++		
301	169	3.901	+	++	++		
301	169	6.135	-	++	++		
302	170	1.78	+	+	+	ncm^5U	
303	171	6.711	+	+	+		
304	172	2.092	++	+	+	mnm^5s^2U	
304	172	5.394	+	+	+	mnm^5s^2U	
306	174	2.073	++	++	++		
306	174	3.118	++	++	++		+
306	174	3.820	++	++	++		+
308	176	5.453	++	++	++	preQ0	
309	177	4.172	++	+	++		
312	180	4.556	++	++	++	preQ1	+
314	182	2.011	++	++	++	$m^{27}G$	
315	183	4.826	-	++	++		
316	184	11.123	++	++	+		
317	185	4.849	-	+	+		
318	186	6.788	+	+	+	ncm^5s^2U, $nchm^5U$	
320	188	2.23	++	++	+	Na^+ Signal m^7G	
320	188	5.017	++	++	++	Na^+ Signal m^1G	
320	188	5.402	++	++	++	Na^+ Signal m^2G	
320	188	8.487	-	++	-	Evtl. K^+ Signal m^6A	
323	191	6.298	++	++	++		
324	192	5.452	+	+	+		
326	194	1.750	++	++	++		+
332	200	6.843	-	+	+		
334	202	6.285	++	++	++		
334	202	6.782	++	++	++		
338	206	7.686	+	+	+	nm^5se^2U	
338	206	11.184	+	-	-	nm^5se^2U	
340	208	2.572	++	++	+		
342	210	5.756	+	+	+	inm^5U	+
343	211	7.019	-	+	+		
344	212	2.096	++	++	++		
345	213	8.333	+	+	+		
346	214	9.61	++	++	++	acp^3Psi	
348	216	2.728	+	+	+	acp^3D	
349	217	4.789	+	+	+		
350	218	5.59	+	+	+	mnm^5se^2U, mimG	+

353	221	13.772	+	+	+		
354	222	8.077	+	+	+		+
355	223	7.716	++	++	++		
356	224	2.486	++	++	++	agmatidine	
356	224	9.252	-	+	+	agmatidine	
356	224	12.368	+	+	+	agmatidine	+
358	226	7.599	++	++	++	inm^5s^2U	
359	227	7.231	++	++	++		
360	228	6.468	++	-	-	m^1acp^3Psi	
362	230	6.467	++	-	-		
363	231	1.807	++	++	++	pG(pN)	
363	231	3.457	++	++	++	pG(pN)	
364	232	2.108	++	++	++		
366	234	3.768	++	++	++		+
368	236	5.457	+	+	+		
369	237	1.972	+	+	+	g^6A	
370	238	1.42	+	+	+		
372	240	5.306	+	+	-	k^2C	+
372	240	4.089	-	-	++	k^2C	
374	242	2.104	++	++	++		+
375	243	8.922	+	+	+		
375	243	13.874	+	+	+		
376	244	1.732	++	++	++		
377	245	6.258	++	++	++		
379	247	4.456	+	+	+		
379	247	5.409	+	+	+		
379	247	9.206	+	+	+		
380	248	2.21	++	++	++		
382	250	3.771	+	+	+	tm^5U	
386	254	12.951	+	+	+		
387	255	1.706	++	++	++		
388	256	1.840	++	++	++		+
393	261	10.942	++	++	++		
394	262	6.769	+	+	+	$cmnm^5se^2U$	+
395	263	2.723	++	++	++	ct^6A	
396	264	2.225	++	++	++		
396	264	7.792	++	++	+		
396	264	8.721	++	++	+		
398	266	3.76	+	+	+	tm^5s^2U	+
398	266	11.438	+	+	+	tm^5s^2U, ms^2io^6A	
398	266	12.237	+	+	+	tm^5s^2U, ms^2io^6A	
399	267	2.069	+	+	++		
399	267	8.774	-	+	+		
400	268	6.565	+	++	+		
401	269	2.625	++	++	++		
401	269	7.006	+	+	+		
401	269	21.364	+	+	+		

403	271	8.233	++	+	+
404	272	2.502	++	++	++
405	273	3.528	+	+	+
406	274	7.244	+	-	-
420	288	5.968	+	+	+
420	288	18.449	+	+	+

Anhang Tabelle 3: Zusammensetzung und Herstellung des M9 Mediums.

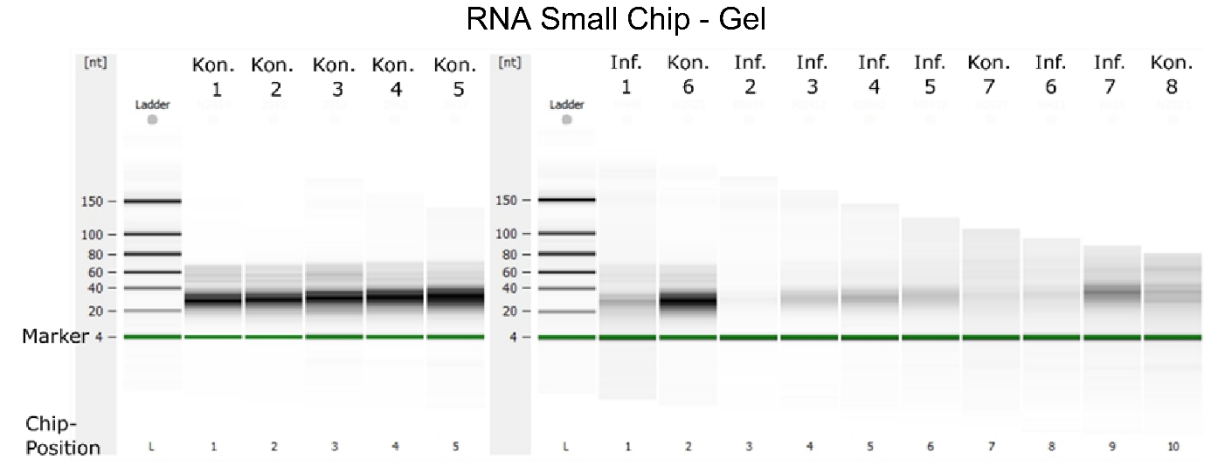
10 x M9 Stammlsg.	**200**	**mL**	**100**	**mL**	**50**	**mL**	**25**	**mL**	**15**	**mL**
Na_2HPO_4	13.6	g	6.8	g	3.4	g	1.7	g	850	mg
KH_2PO_4	6	g	3	g	1.5	g	750	mg	375	mg
NaCl	0.5	g	0.25	g	0.125	g	62.5	mg	31	mg
^{14}N-/^{15}N-NH_4Cl	2	g	1	g	0.5	g	0.25	g	125	mg
hochreines Wasser	200	mL	100	mL	50	mL	25	mL	12.5	mL

M9 Salze (nicht steril)								
$MgCl_2$ *$6H_2O$	203.3	g/mol	0.1	mol/L	20	mL	406.6	mg
$CaCl_2$	110.99	g/mol	0.1	mol/L	20	mL	221.98	mg
^{32}S-/^{34}S-Na_2SO_4	144	g/mol	0.1	mol/L	20	mL	288	mg
^{12}C-/^{13}C-D-Glukose	5	g	zu	20 mL Wasser				
CD_3-S-Methionin	100	mg	zu	20 mL Wasser				

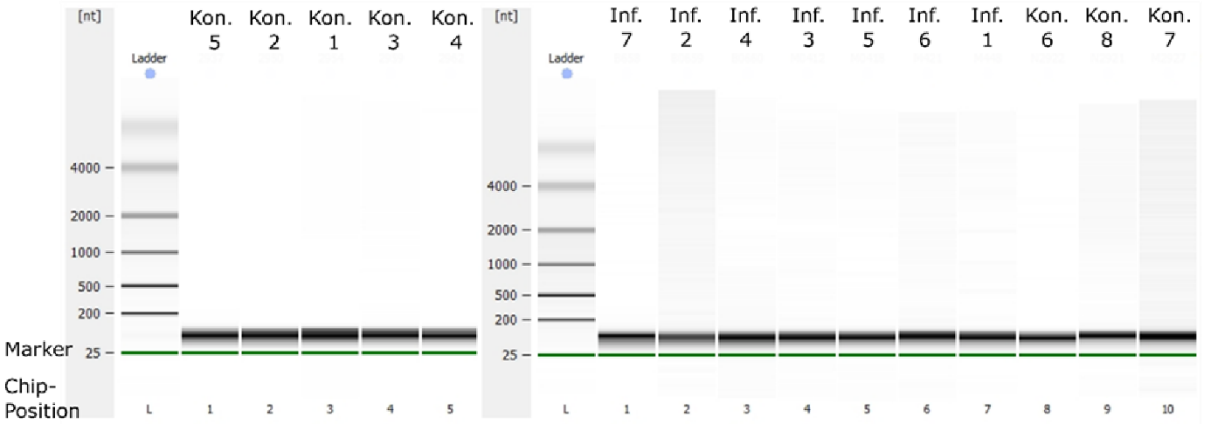
Salze in Wasser lösen und durch Sterilfilterspritze drücken

1 x M9 Medien	**Stammlsg.**		**FINAL**		**5**	**mL**	**10**	**mL**	**100**	**mL**
10 x M9	10	x	1	x	500	µL	1000	µL	10	mL
$CaCl_2$	0.1	M	0.0001	M	5	µL	10	µL	100	µL
$MgCl_2$	0.1	M	0.002	M	100	µL	200	µL	2	mL
Na_2SO_4	0.1	M	0.002	M	100	µL	200	µL	2	mL
D-Glukose	20	%	0.4	%	100	µL	200	µL	2	mL
alternativ:										
CD_3-S-Methionin	0.5	%	0.02	%	200	µL	400	µL	4	mL
Wasser (steril)					4195	µL	8390	µL	83.9	mL
					3995	µL	7990	µL	79.9	mL

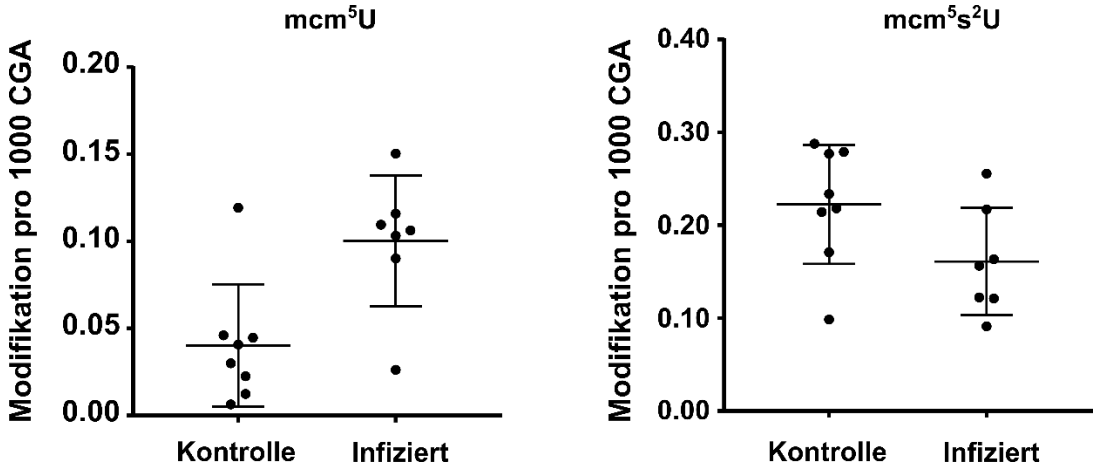
a



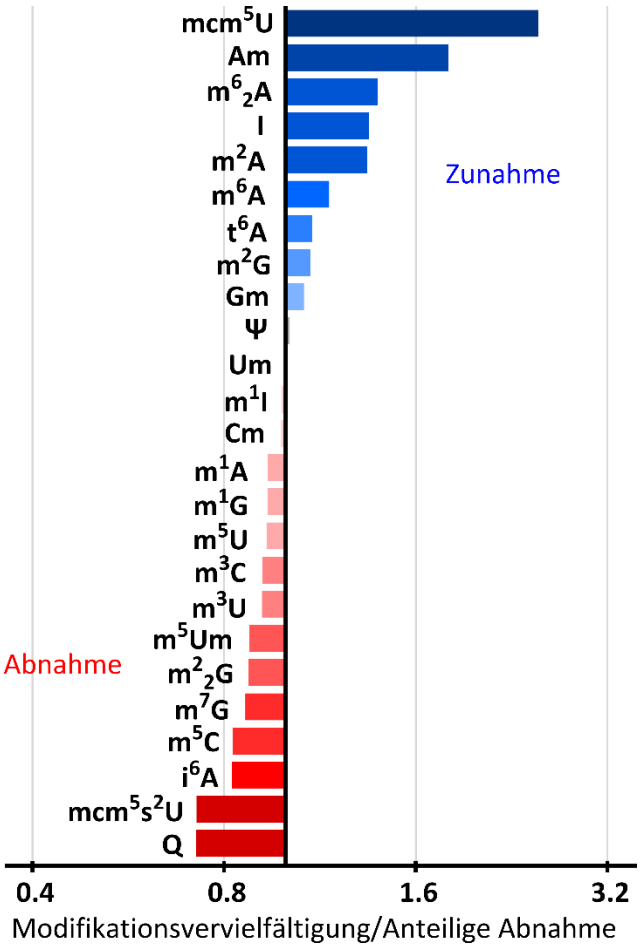
b



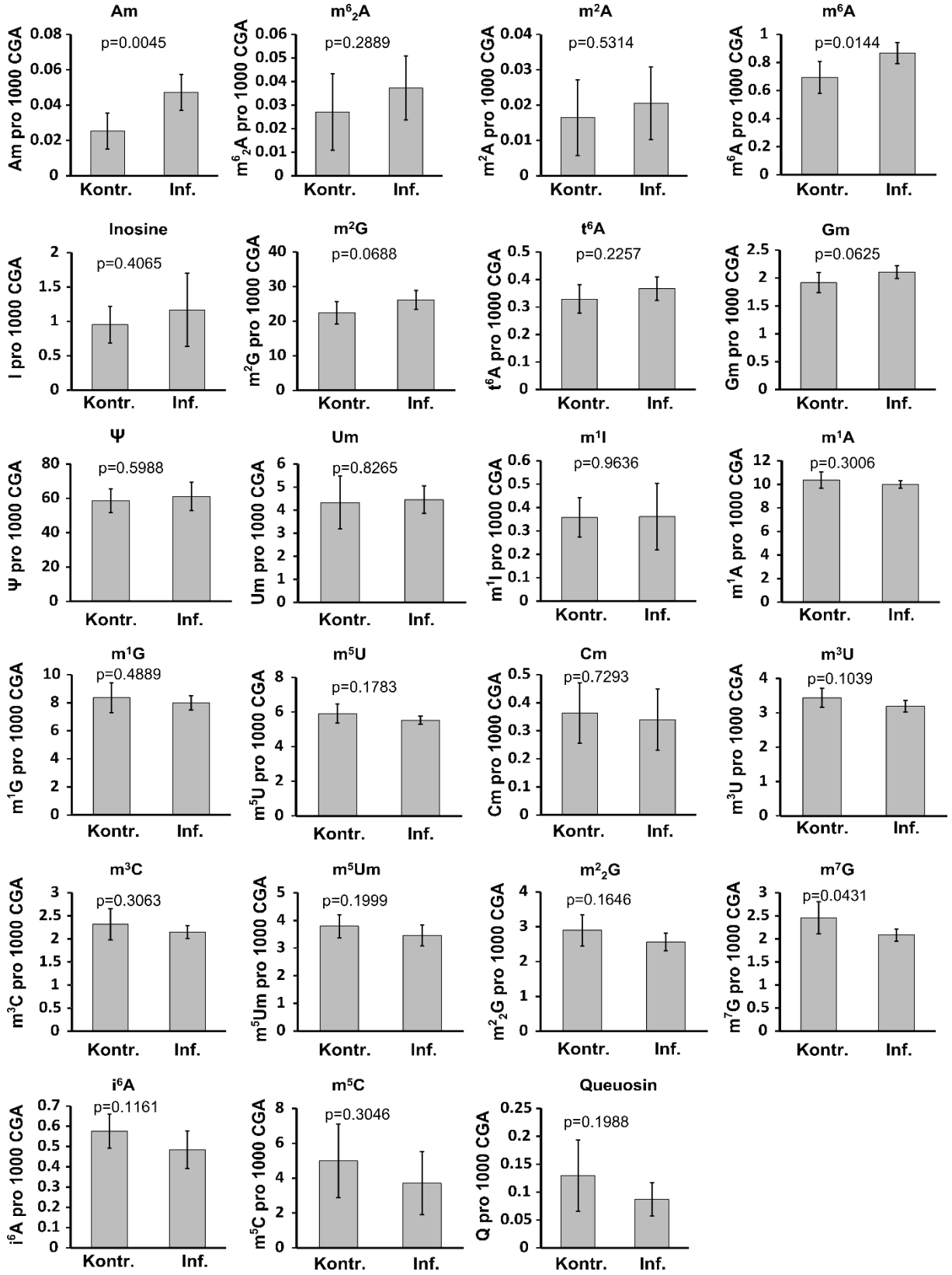
Anhang Abbildung 4: Bioanalyzer-Gele der Urin-miRNA aller *O. ochengi* Proben. **a** RNA Small Chip – Gel mit 1 µL aus jeder Urin miRNA Probe. Die RNA-Leiter (Ladder) bei diesem Chip umfasst RNA-Längen von von 4 bis 150 Nukleotide (nt). **b** RNA Pico Chip – Gel mit 1µL aus jeder Urin miRNA Probe. Die RNA-Leiter (Ladder) bei diesem Chip umfasst RNA-Längen von 25 bis 4000 Nukleotide (nt). Die Sensitivität des RNA Pico Chips ist um ein Vielfaches höher als im RNA Small Chip.



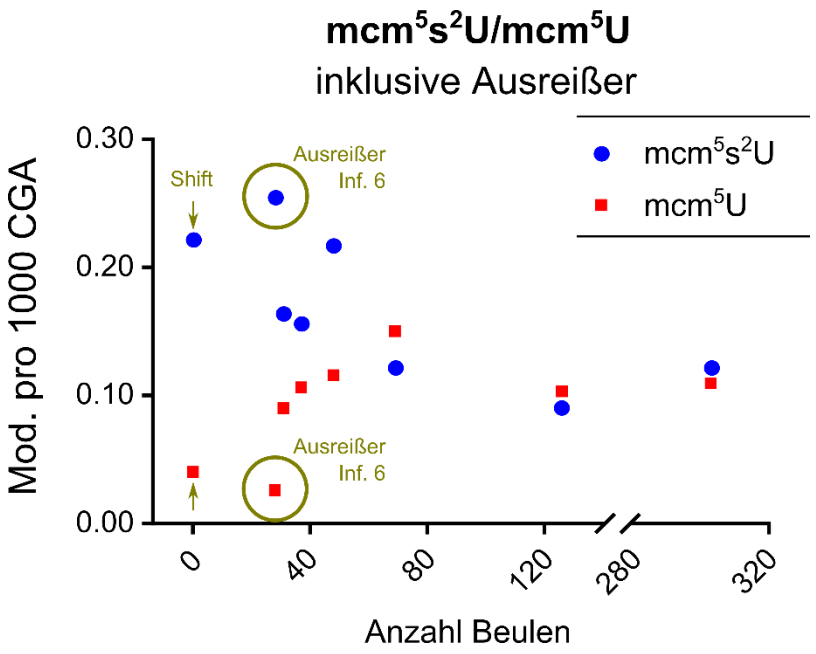
Anhang Abbildung 5: Alle für mcm^{5}U und mcm^{5}s^{2}U gemessenen Werte, inklusive Ausreißer. Die Datenpunkte der liegen in beiden Graphen außerhalb der Fehlerbalken.



Anhang Abbildung 6: Vervielfältigung/anteilige Abnahme der RNA-Modifikationen in der Rinderurin miRNA nach einer Infektion mit *O. ochengi*. Im Gegensatz zu **Abbildung 20** sind in diesem Graphen die Datenpunkte der Ausreißer miteinberechnet.



Anhang Abbildung 7: Absolute Werte der Modifikationen aus der Urin miRNA von infizierten (Inf.) und nicht infizierten (Kontr.) Rindern. Die Fehlerbalken repräsentieren Standardabweichungen und der p-Wert wurde über einen zweiseitigen t-Test berechnet. Die Werte wurden für die **Abbildungen 20b** und **20c** verwendet. n(Kontr.) = 7; n(Inf.) = 6.



Anhang Abbildung 8: Die Menge der mcm^5U und mcm^5s^2U Modifikationen, aufgetragen gegen die Anzahl an Beulen auf der Hautoberfläche der infizierten Rinder. Die Werte mit einer Beulenanzahl von 0 setzen sich aus dem Mittelwert aller Kontrollproben zusammen. Durch die Berücksichtigung der Ausreißer verschieben sich im Vergleich zu dem Korrelationsplot in **Abbildung 23** die Ausgangswerte (Beulenanzahl = 0, grüner Pfeil) zueinander hin. Der Ausreißer aus der Population der infizierten Rinder (Inf. 6) ist für beide Modifikationen grün eingekreist.

Supplement

NAIL-MS reveals the repair of 2-methylthiocytidine by AlkB in *E. coli*

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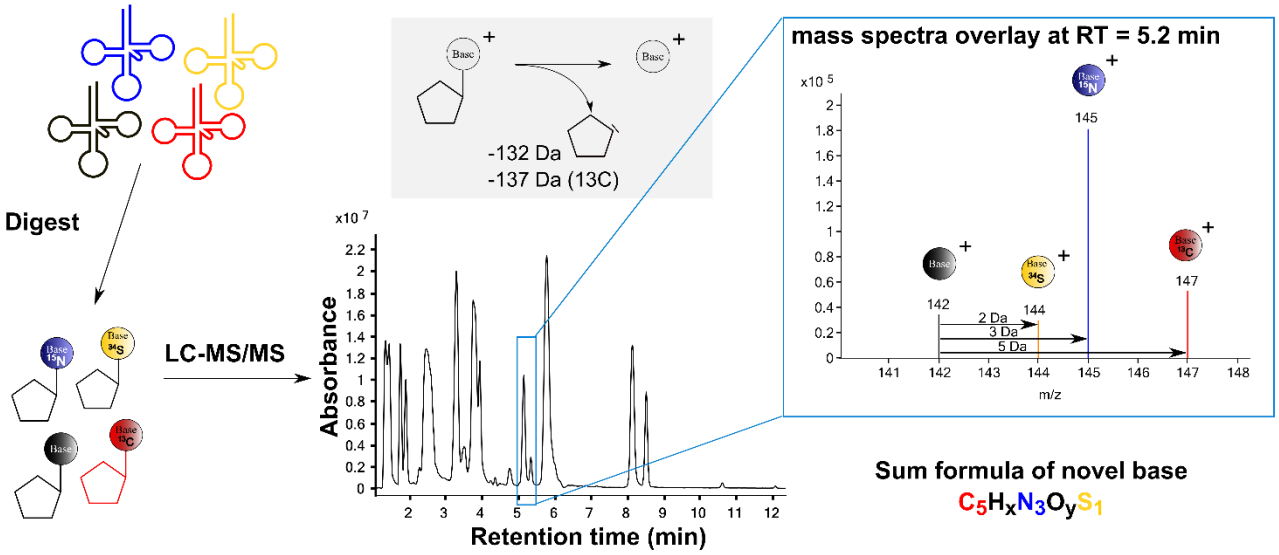


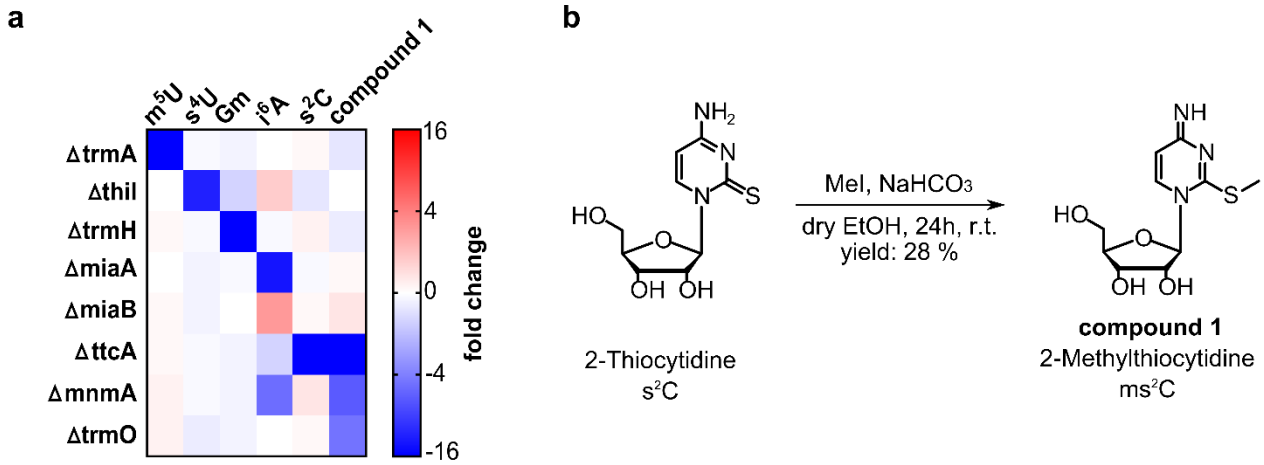
Supplementary Figure 1: Principle concept for the discovery of unknown nucleosides. Bacteria (*E. coli* or *P. aeruginosa*) are fed with stable isotope labeled nutrients in minimal M9 medium supplemented with glucose. The completely carbon-13 (red), nitrogen-15 (blue), sulfur-34 (yellow) or unlabeled (black) tRNAs are isolated and digested to nucleosides. Each RNA digest of isotopically labeled bacteria is analyzed by LC-MS/MS and the total ion count is displayed in the chromatogram. The mass spectra of the chromatographic peaks are isolated and overlayed for the differentially labeled samples. Here, the mass spectra overlay for the new compound ms^2C at retention time (RT) 5.2 min is shown.

Supplementary Table 1a: Parameters of Triple Quadrupole method used for the discovery of ms^2C. The difference between precursor and product ions is 132 units, the common fragmentation of nucleosides (loss of ribose). The table applies only for unlabeled, nitrogen-15 and sulfur-34 labeled RNA samples.

Precursor Ion	Product Ion	Ret. Time	Delta Ret.	Fragmentor	Collision energy	Cell Accelerator Voltage	Polarity
240	108	10	20	250	40	2	positive
241	109	10	20	250	40	2	positive
242	110	10	20	250	40	2	positive
243	111	10	20	250	40	2	positive
..	..	10	20	250	40	2	positive
..	..	10	20	250	40	2	positive
419	287	10	20	250	40	2	positive
420	288	10	20	250	40	2	positive

Supplementary Table 1b: Parameters of Triple Quadrupole method used for the discovery of ms^2C. The difference between precursor and product ions is 137 units, which reflects the loss of a $^{13}C_5$-labeled ribose.

Precursor Ion	Product Ion	Ret. Time	Delta Ret.	Fragmentor	Collision energy	Cell Accelerator Voltage	Polarity
240	103	10	20	250	40	2	positive
241	104	10	20	250	40	2	positive
242	105	10	20	250	40	2	positive
243	106	10	20	250	40	2	positive
..	..	10	20	250	40	2	positive
..	..	10	20	250	40	2	positive
419	282	10	20	250	40	2	positive
420	283	10	20	250	40	2	positive



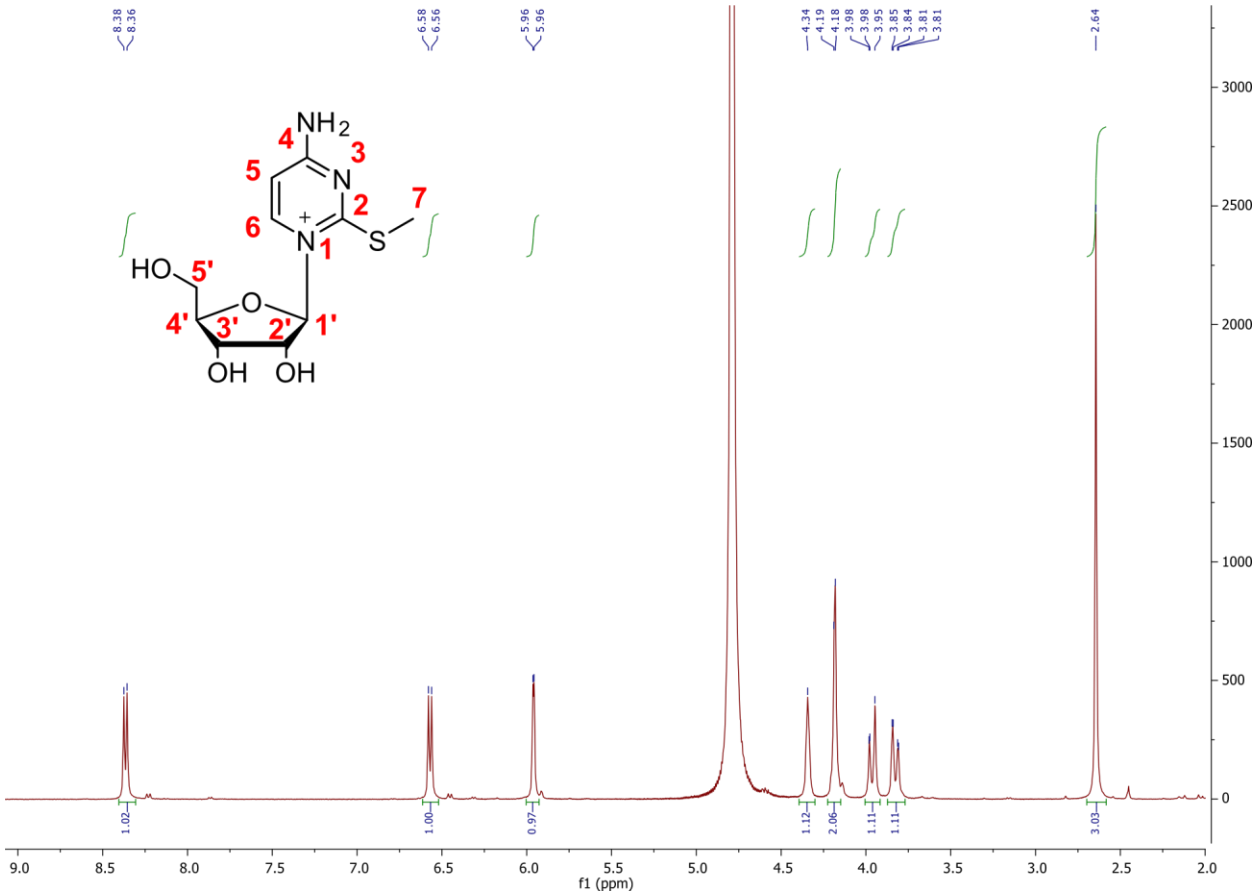
Supplementary Figure 2: Heatmap of *E. coli* knockout screen and synthetic route of ms^2C**. a.** tRNA from the indicated *E. coli* knockout strains was analyzed for the abundance of modified nucleosides. The found abundance is related to the abundance found in wildtype BW25113 strain. Compound 1 is ms^2C. Blue: Lower abundance compared to wildtype. Red: Higher abundance compared to the wildtype. **b.** Synthetic pathway and conditions of the synthesis from s^2C to ms^2C under usage of methyl iodide.

Supplementary Table 2: *E. coli* knockouts used for RNA modification screening in **Supplementary Figure 2** and **6**. Reference: Keio collection.[1]

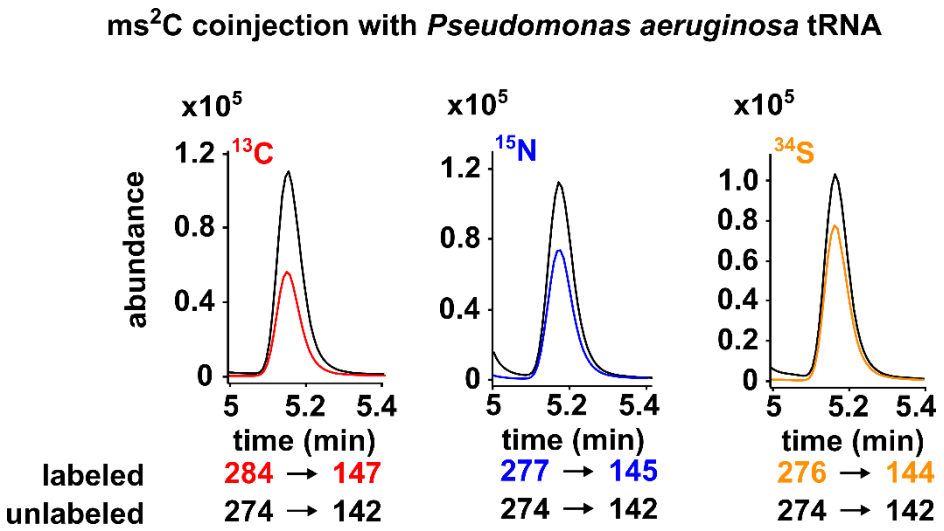
Keio code number	knockout gene	Enzyme	Mod. suppression
BW25113	-	-	-
JW3937-KC	trmA	TrmA	m^5U
JW0413-KC	thiI	ThiI	s^4U
JW3626-KC	trmH	TrmH	Gm
JW4129-KC	miaA	MiaA	i^6A
JW0658-KC	miaB	MiaB	ms^2i^6A
JW1338-KC	ttcA/ydaO	TtcA	s^2C
JW1119-KC	mnmA	MnmA	s^2U
JW0191-KC	trmO/yaeB	TrmO	m^6t^6A
JW2501-KC	yfgB	RlmN	m^2A
JW2762-KC	yqcB	TruC	Psi
JW0396-KC	tgt	TGT	Q
JW2459-KC	tmcA/ypfI	TmcA	ac^4C
JW2559-KC	trmN6/yfiC	TrmN6	m^6A
JW3228-KC	dusB	DusB	D

Supplementary Table 3: MRM parameters for LC-MS/MS analysis of unlabeled tRNA digests (*E.g.* for isoacceptor purification analysis or knockout library analysis of.

Compound Group	Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Ret Time (min)	Delta Ret Time (min)	Fragmentor (V)	Collision Energy (eV)	Cell Accelerator Voltage (V)	Polarity
unlabeled	C	244	112	1.73	1	175	13	5	Positive
	U	245	113	2	1	95	5	5	Positive
	G	284	152	3.718	3	95	17	5	Positive
	A	268	136	5.711	1	110	21	5	Positive
	s2C	260	128	2.5	2	85	13	5	Positive
	ms2C	274	142	5.533	3	85	13	5	Positive
	s4U	261	129	4.753	1	75	17	5	Positive
	Gm	298	152	5.133	1	100	9	5	Positive
	Cm	258	112	3.347	3	180	9	5	Positive
	I	269	137	3.556	1	100	9	5	Positive
	m2A	282	150	8.08	1	125	21	5	Positive
	m1G	298	166	5.08	1	105	13	5	Positive
	t6A	413	281	7.19	1	130	9	5	Positive
	m7G	298	166	2.021	2	105	14	5	Positive
	acp3U	346	214	1.744	2	180	20	5	Positive
	m5U	259	127	3.915	1	145	10	5	Positive
	Psi	245	209	1.49	1	90	5	5	Positive
	i6A	336	204	14.134	1	140	17	5	Positive
SILIS	C SILIS	256	119	1.73	1	175	13	5	Positive
	U SILIS	256	119	2	1	95	5	5	Positive
	G SILIS	299	162	3.718	3	95	17	5	Positive
	A SILIS	283	146	5.711	1	110	21	5	Positive
	s2C SILIS	272	135	2.5	2	85	13	5	Positive
	ms2C SILIS	287	150	5.533	3	85	13	5	Positive
	s4U SILIS	272	135	4.753	1	75	17	5	Positive
	Gm SILIS	314	162	5.133	1	100	9	5	Positive
	Cm SILIS	271	119	3.347	3	180	9	5	Positive
	I SILIS	283	146	3.556	1	100	9	5	Positive
	m2A SILIS	298	161	8.08	1	125	21	5	Positive
	m1G SILIS	314	177	5.08	1	105	13	5	Positive
	t6A SILIS	434	297	7.19	1	130	9	5	Positive
	m7G SILIS	314	177	2.021	2	105	14	5	Positive
	acp3U SILIS	362	225	1.744	2	180	20	5	Positive
	m5U SILIS	271	134	3.915	1	145	10	5	Positive
	Psi SILIS	256	220	1.49	1	90	5	5	Positive
	i6A SILIS	356	219	14.134	1	140	17	5	Positive



Supplementary Figure 3: 400 MHz ^{1}H-NMR spectrum of 2-methylthiocytidine in D_2O.



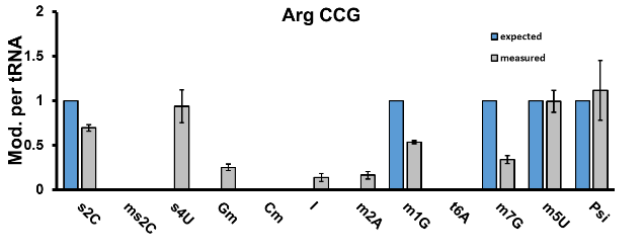
Supplementary Figure 4: Co-injection of synthetic ms^{2}C with digested and isotope labeled *P. aeruginosa* tRNA (grown in the indicated isotope labeled M9 medium). ms^{2}C was co-injected with fully ^{13}C labeled tRNA digest (red), with fully ^{15}N labeled tRNA digest (blue) and with ^{34}S labeled digest (orange). The mass transitions are shown below each chromatogram.

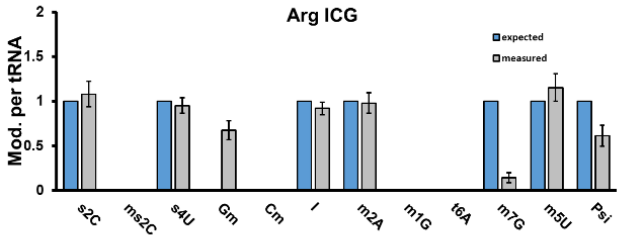
Supplementary Table 4: DNA oligonucleotides for tRNA isoacceptor purification. Every sequence starts with a Biotin [Btn]-tag, followed by three adenosines as a spacer before the actual reverse complementary sequence starts.

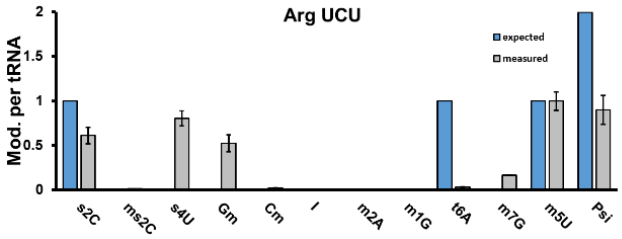
tRNA isoacceptor	Sequence of the probe (5'-3')
$tRNA^{Arg}_{CCG}$:	[Btn]AAAGAGACCTCTGCCTCCGGAGGGCAGCGCTCTATCCAGCTGAGCTA
$tRNA^{Arg}_{ICG}$:	[Btn]AAACCGACCGCTCGGTTCGTAGCCGAGTACTCTACCAGCTGAGCTAC
$tRNA^{Arg}_{UCU}$:	[Btn]AAAGCGGCCCACGACTTAGAAGGTCGTTGCTCTATCCAACTGAGCTA
$tRNA^{Ser}_{GCU}$:	[Btn]AAACCCGGATGCAGCTTTTGACCGCATACTCCCTTAGCAGGGGAGC
$tRNA^{Ser}_{GGA}$:	[Btn]AAACCCCGATACGTTGCCGTATACACACTTTCCAGGCGTGCTCCTT
$tRNA^{Ser}_{UGA}$:	[Btn]AAATGGCGGAAGCGCAGAGATTCGAACTCTGGAACCCTTTCGGGTCGCCGGTTTCAAG
$tRNA^{Ala}_{UGC}$:	[Btn]AAATGGTGGAGCTATGCGGGATCGAACCGCAGACCTCCTGCGTGCA
$tRNA^{Gly}_{CCC}$:	[Btn]AAATGGAGCGGGCGAAGGGAATCGAACCCTCGTATAGAGCTTGGGAAGCTCTCGTTCTACCGAACTACGCCCGC
$tRNA^{Lys}_{mnm5s2UUU}$:	[Btn]AAATGGTGGGTCGTGCAGGATTCGAACCTGCGACCAATTGATTAAA

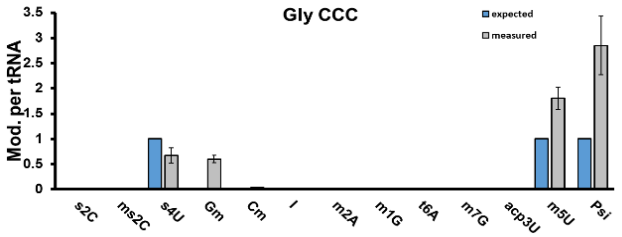
Supplementary Table 5: Modified nucleosides and their positions in the respective tRNA sequence of each isoacceptor related to this work. Reference: MODOMICS [2]

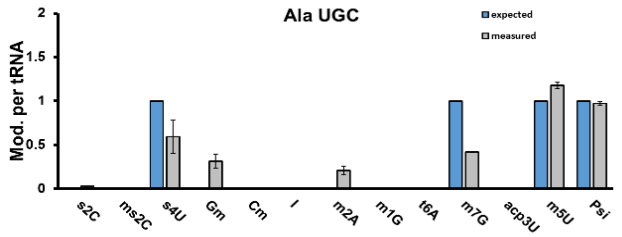
	Modifications and their positions in the respective tRNA sequence																
Modification/ Isoacceptor	**s^4U**	**Gm**	**D**	**s^2C**	**Cm**	**Inosine**	**mnm^5U**	**m^1G**	**mnm^5s^2U**	**cmo^5U**	**m^2A**	**ms^2i^6A**	**m^7G**	**acp^3U**	**ct^6A**	**m^5U**	**Psi**
Arg CCG	x	x	17, 20A	32	x	x	x	37	x	x	x	x	46	x	x	54	55
Arg ICG	8	x	17, 20A	32	x	34	x	x	x	x	37	x	46	47	x	54	55
Arg UCU	x	x	x	32	x	x	34	x	x	x	x	x	x	x	37	54	40, 55
Ser GCU	8	x	20	32	x	x	x	x	x	x	x	x	x	x	37	54	55
Ser GGA	8	18	20, 20A	x	x	x	x	x	x	x	x	x	x	x	x	54	40, 55
Ser UGA	8	18	20, 20A	x	32	x	x	x	x	34	x	37	x	x	x	54	55
Ala UGC	8	x	17	x	x	x	x	x	x	34	x	x	46	x	x	54	55
Gly CCC	8	x	20	x	x	x	x	x	x	x	x	x	x	x	x	54	55
Lys UUU	x	x	16, 17, 20	x	x	x	x	x	34	x	x	x	46	47	37	54	39, 55

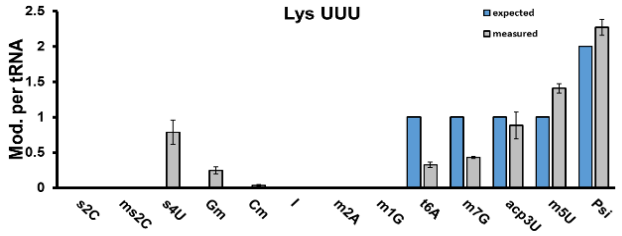


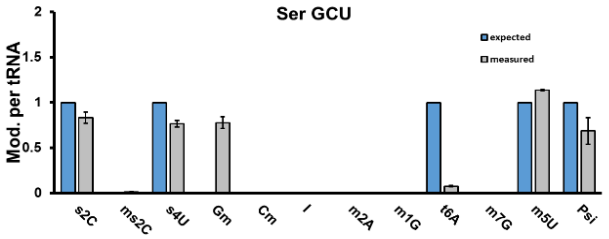


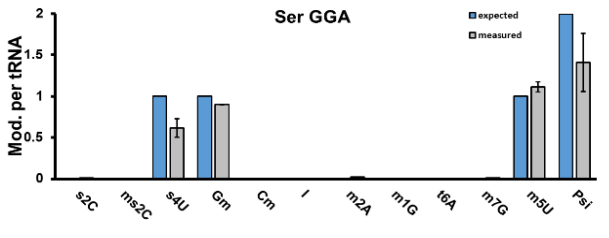


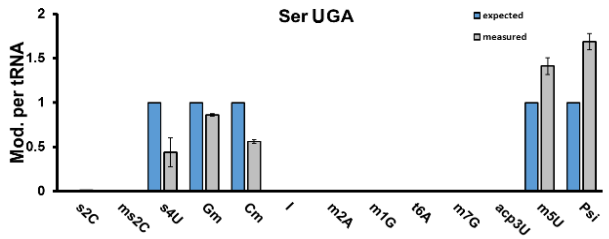




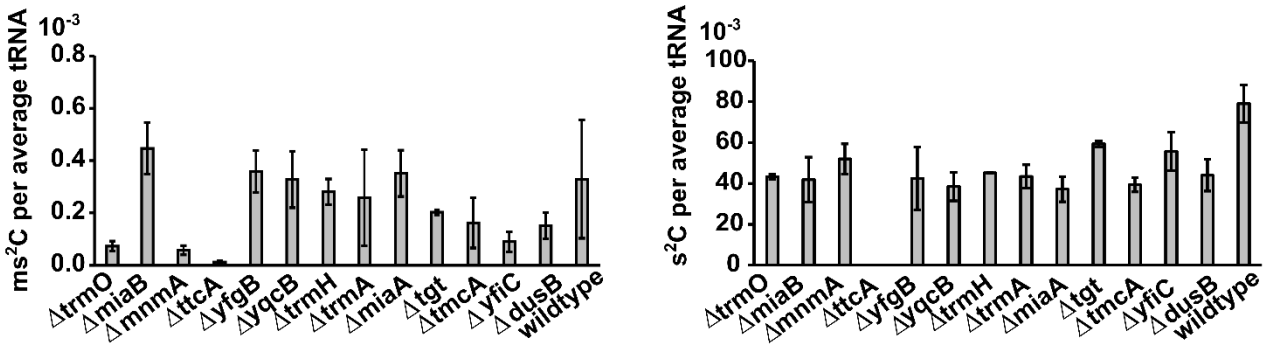








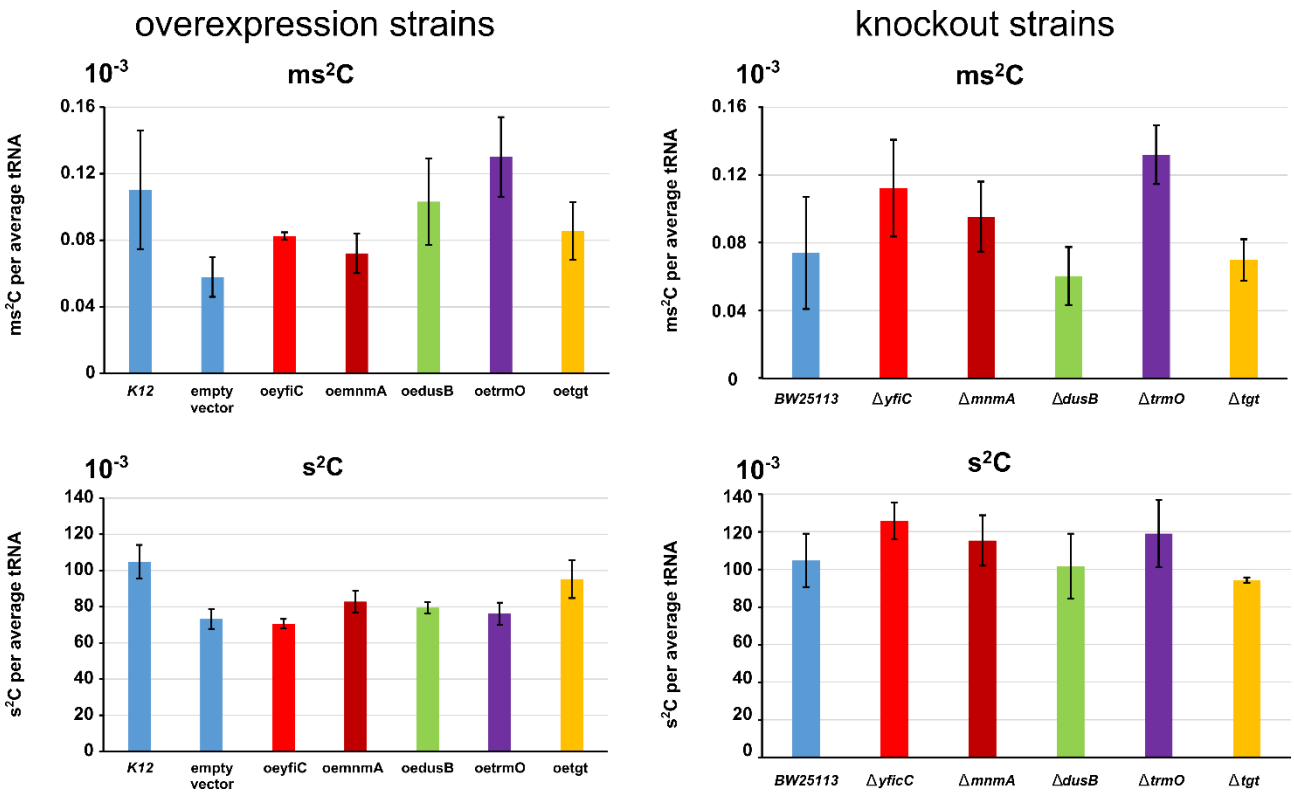
Supplementary Figure 5: Validation of tRNA isoacceptor purification by modified nucleoside analysis. Expected (blue, from MODOMICS) [2] and experimentally determined abundance of modified nucleosides per tRNA. *E.coli* was grown in LB medium. From n=3 biol. replicates, error bars reflect standard deviation. Source data are provided as a Source Data file.



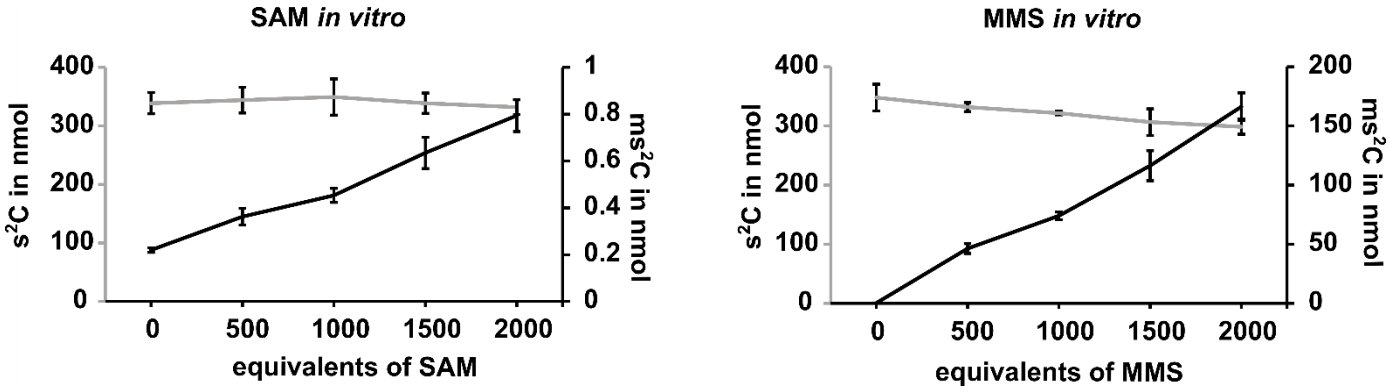
Supplementary Figure 6: *E. coli* knockout studies in M9 medium **Left:** ms^2C abundance in different *E. coli* RNA modification knockouts during stationary growth phase in M9 media. **Right:** s^2C abundance in different *E. coli* RNA modification knockouts during stationary growth phase in M9 media. All experiments are from n=3 biol. replicates and error bars reflect standard deviation. Source data are provided as a Source Data file.

Supplementary Table 6: *E. coli* overexpression strains used for RNA modification screening in **Supplementary Figure 7**. Reference: ASKA library [3]

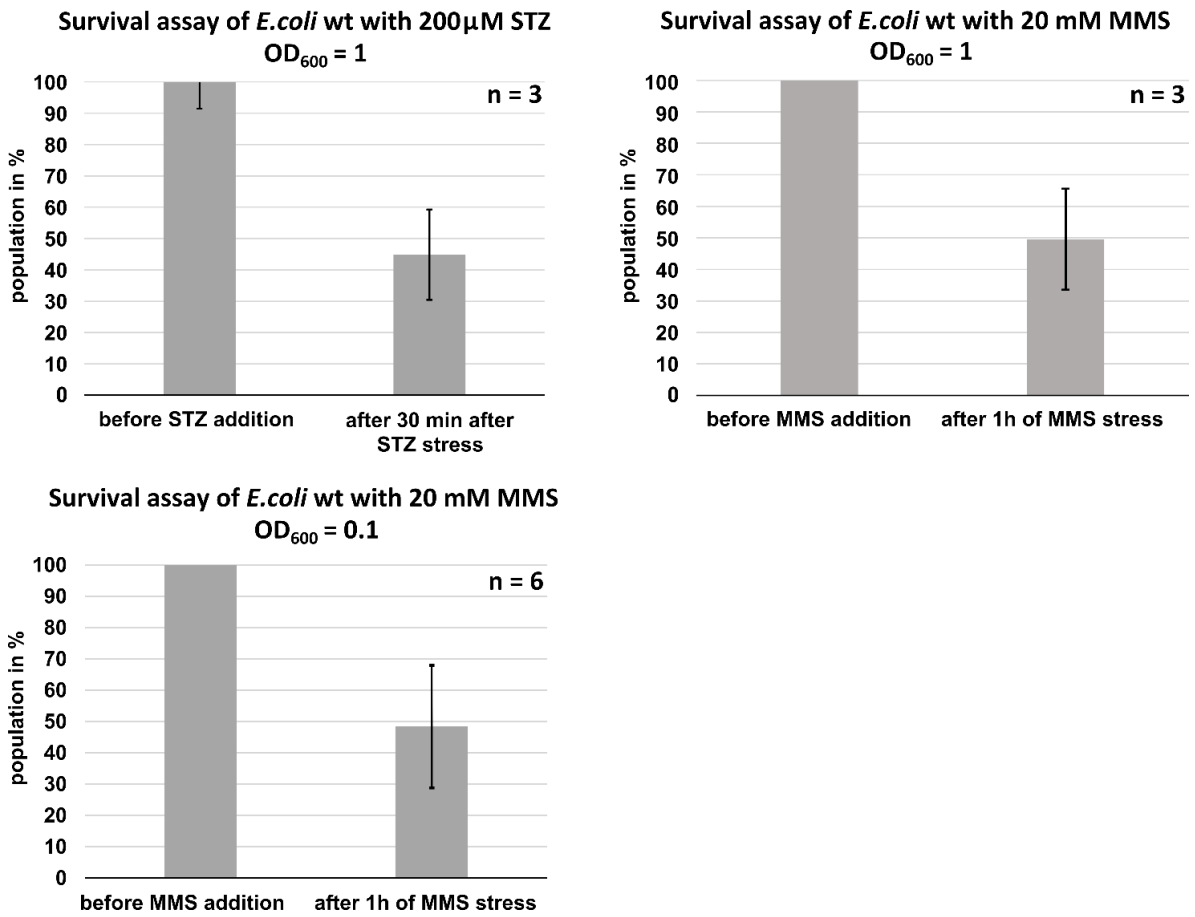
ASKA code number	overexpressed	Enzyme	Modification
AG1 (ME5305)	-	-	-
AG1 (pCA24N-gfp)	-	empty vector	-
JW3228-AP	dusB	DusB	D
JW0396-AP	tgt	Tgt	Q
JW1119-AP	mnmA	MnmA	s^2C
JW0191-AP	tsaA/yaeB	TrmO	m^6t^6A
JW2559-AP	trmN6/yfiC	TrmN6	m^6A



Supplementary Figure 7: ms^2C and s^2C abundance of *E. coli* knockouts and corresponding overexpression strains which showed low ms^2C abundance in M9 media before (Supplementary Figure 6). Here, they were cultured in LB media and harvested in exponential growth phase. As a control, the wildtype strains and the wildtype with the empty overexpressing gene vector are shown as well. The assumption of enzyme dependent ms^2C formation could not be confirmed. All experiments are from n=3 biol. replicates and error bars reflect standard deviation. Source data are provided as a Source Data file.



Supplementary Figure 8: *in vitro* incubation assay of s^2C with SAM and MMS. SAM: S-adenosylmethionine. MMS: Methyl-methanesulfonate. Grey axes and curve: abundance s^2C. Black axes and curve: abundance ms^2C. The experiments were done in triplicates and the error bars reflect standard deviation. Source data are provided as a Source Data file.

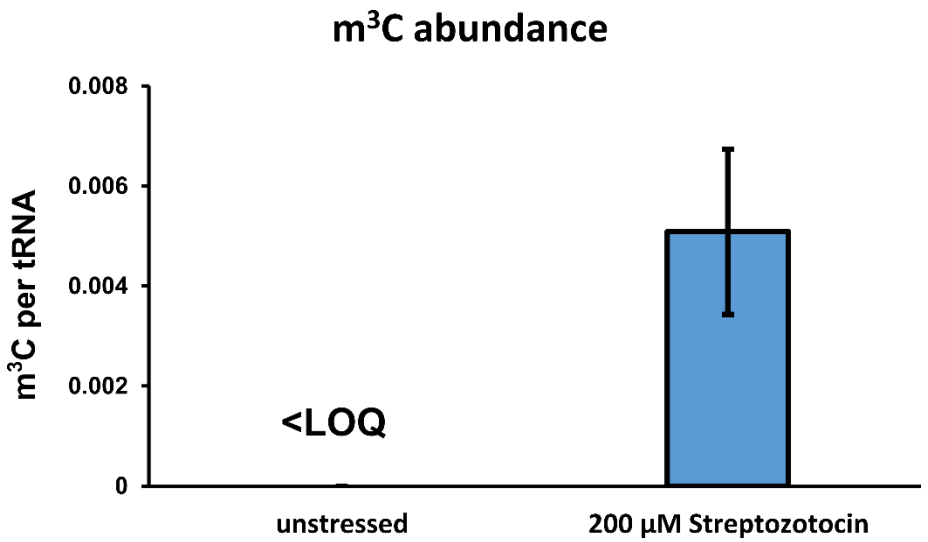


Supplementary Figure 9: Survival assay to determine the LD_{50} of MMS and Streptozotocin (STZ) for the *E.coli* strain BW25113. Cells were brought to either OD_{600} = 1 (top graphs, error bars reflect the standard deviation of n=3 biol. replicates) or OD_{600} = 0.1 (left bottom graph, error bars reflect the standard deviation of n=6 biol. replicates) and exposed to 200 µM STZ for 30 min. (left top graph) or 20 mM MMS for 60 min. (right top graph and left bottom graph).

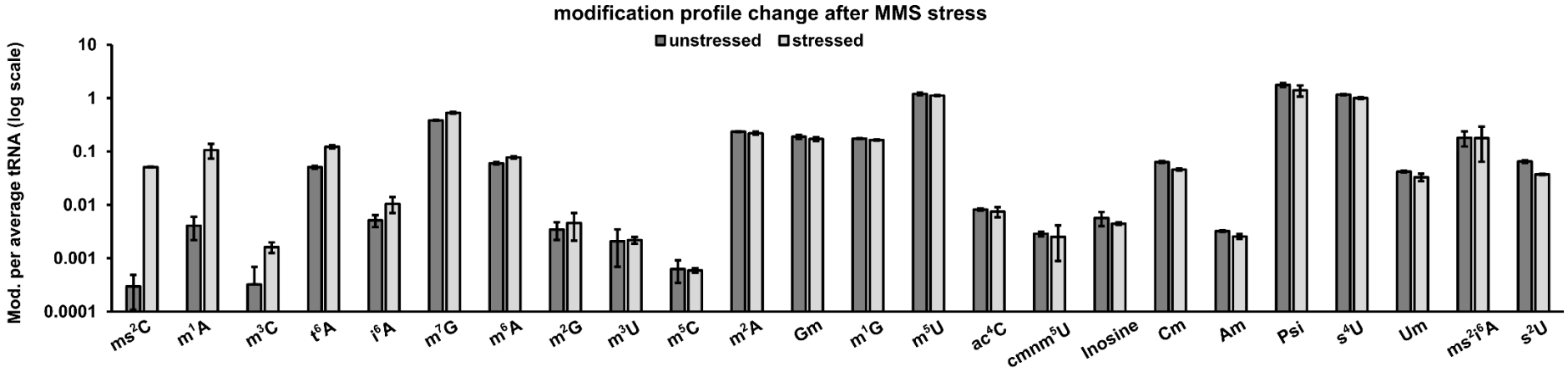
Supplementary Table 7: MRM parameters for RNA methylome discrimination assay.

Compound Group	Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Ret Time (min)	Delta Ret Time (min)	Fragmentor (V)	Collision Energy (eV)	Cell Accelerator Voltage (V)	Polarity
unlabeled	C	244	112	1.73	1	380	40	5	Positive
	U	245	113	1.876	1	380	40	5	Positive
	G	284	152	3.718	3	380	40	5	Positive
	A	268	136	5.711	1	380	40	5	Positive
	ms2C	274	142	5.533	3	380	40	5	Positive
	m1A	282	150	1.745	2	380	10	5	Positive
	m3C	258	126	1.752	1	380	10	5	Positive
	i6A	336	204	14.134	1	380	10	5	Positive
	m7G	298	166	2.021	2	380	10	5	Positive
	m6A	282	150	8.423	1	380	10	5	Positive
	m2G	298	166	5.491	2	380	10	5	Positive
	m3U	259	127	4.696	2	380	10	5	Positive
	m5C	258	126	2.702	2	380	10	5	Positive
	m2A	282	150	8.08	1	380	10	5	Positive
	Gm	298	152	5.133	1	380	10	5	Positive
	m1G	298	166	5.08	1	380	10	5	Positive
	m5U	259	127	3.915	1	380	10	5	Positive
	ac4c	286	154	5.538	2	380	10	5	Positive
	cmnm5U	332	200	1.586	1	380	10	5	Positive
	I	269	137	3.556	1	380	10	5	Positive
	Cm	258	112	3.347	3	380	10	5	Positive
	m5s2U	275	143	6.172	2	380	10	5	Positive
	Am	282	136	7.257	1	380	10	5	Positive
	Psi	245	209	1.49	1	380	10	5	Positive
	s4U	261	129	4.753	1	380	10	5	Positive
	Um	259	113	4.49	1	380	10	5	Positive
	ms2i6A	382	250	10	20	380	10	5	Positive
	s2C	260	128	2.288	1	380	10	5	Positive
	t6A	413	281	7.553	1	380	10	5	Positive
	mnm5U	288	156	1.491	1	380	10	5	Positive
CD3 labeled	ms2C CD3	277	145	5.533	3	380	40	5	Positive
	m1A CD3	285	153	1.745	2	380	10	5	Positive
	m3C CD3	261	129	1.752	1	380	10	5	Positive
	m7G CD3	301	169	2.021	2	380	10	5	Positive
	m6A CD3	285	153	8.423	1	380	10	5	Positive
	m2G CD3	301	169	5.491	2	380	10	5	Positive
	m3U CD3	262	130	4.696	2	380	10	5	Positive
	m5C CD3	261	129	2.702	2	380	10	5	Positive
	m2A CD3	285	153	8.08	1	380	10	5	Positive
	Gm CD3	301	152	5.133	1	380	10	5	Positive
	m1G CD3	301	169	5.08	1	380	10	5	Positive
	m5U CD3	262	130	3.915	1	380	10	5	Positive
	Cm CD3	261	112	3.347	3	380	10	5	Positive
	m5s2U CD3	278	146	6.172	2	380	10	5	Positive
	Am CD3	285	136	7.257	1	380	10	5	Positive
	Um CD3	262	113	4.49	1	380	10	5	Positive
	ms2i6A CD3	385	253	10	20	380	10	5	Positive
	mnm5U CD3	291	159	1.428	1	380	10	5	Positive

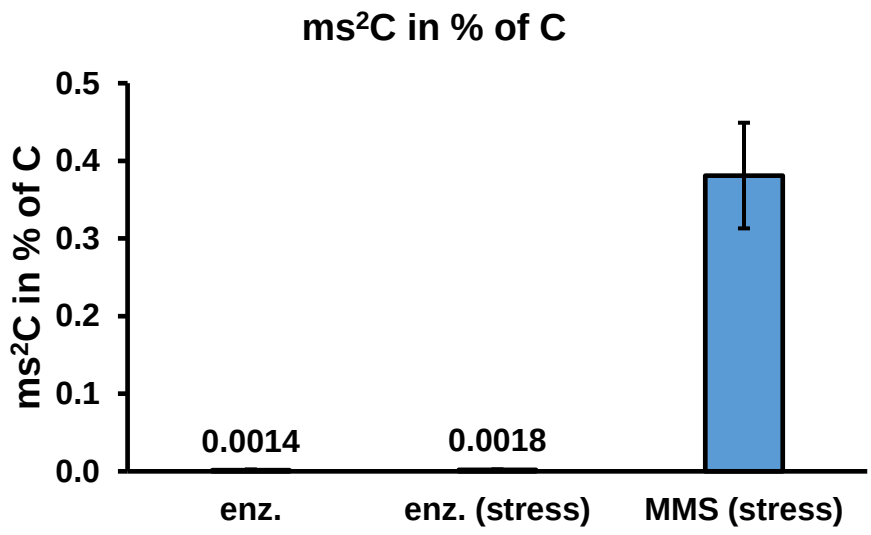
SILIS	C SILIS	256	119	1.73	1	380	40	5	Positive
	U SILIS	256	119	1.876	1	380	40	5	Positive
	G SILIS	299	162	3.718	3	380	40	5	Positive
	A SILIS	283	146	5.711	1	380	40	5	Positive
	ms2C SILIS	287	150	5.533	3	380	40	5	Positive
	m1A SILIS	298	161	1.745	2	380	10	5	Positive
	m3C SILIS	271	134	1.752	1	380	10	5	Positive
	i6A SILIS	356	219	14.134	1	380	10	5	Positive
	m7G SILIS	314	177	2.021	2	380	10	5	Positive
	m6A SILIS	298	161	8.423	1	380	10	5	Positive
	m2G SILIS	312	175	5.491	2	380	10	5	Positive
	m3U SILIS	271	134	4.696	2	380	10	5	Positive
	m5C SILIS	271	134	2.702	2	380	10	5	Positive
	m2A SILIS	298	161	8.08	1	380	10	5	Positive
	Gm SILIS	314	162	5.133	1	380	10	5	Positive
	m1G SILIS	314	177	5.08	1	380	10	5	Positive
	m5U SILIS	271	134	3.915	1	380	10	5	Positive
	ac4c SILIS	300	163	5.538	2	380	10	5	Positive
	cmnm5U SILIS	347	210	1.586	1	380	10	5	Positive
	I SILIS	283	146	3.556	1	380	10	5	Positive
	Cm SILIS	271	119	3.347	3	380	10	5	Positive
	m5s2U SILIS	287	150	6.172	2	380	10	5	Positive
	Am SILIS	298	146	7.257	1	380	10	5	Positive
	Psi SILIS	256	220	1.49	1	380	10	5	Positive
	s4U SILIS	272	135	4.753	1	380	10	5	Positive
	Um SILIS	271	119	4.49	1	380	10	5	Positive
	ms2i6A SILIS	403	266	10	20	380	10	5	Positive
	s2C SILIS	272	135	2.288	1	380	10	5	Positive
	t6A SILIS	434	297	7.553	1	380	10	5	Positive
	mnm5U SILIS	302	165	1.491	1	380	10	5	Positive



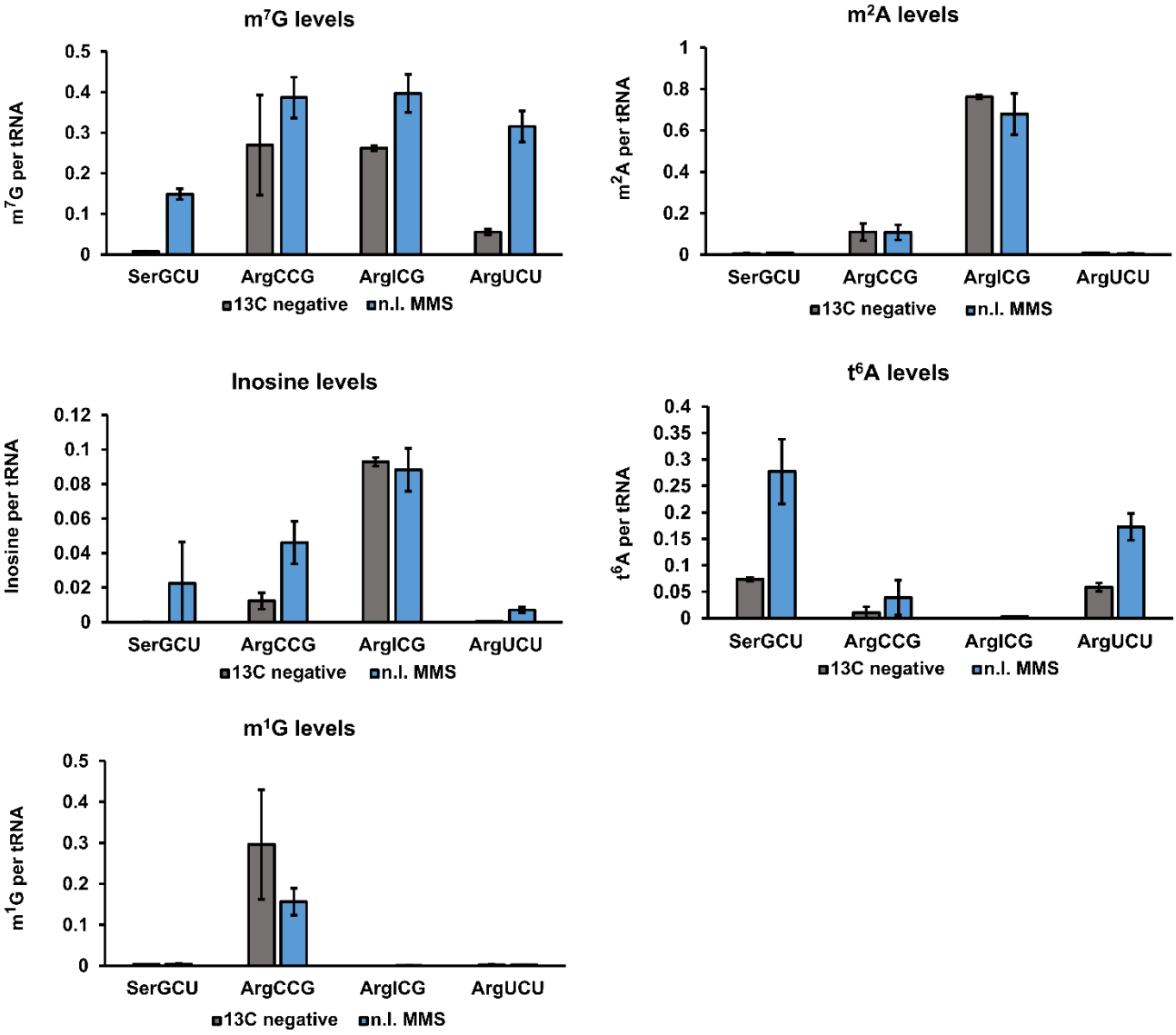
Supplementary Figure 10: m^3C abundance in *E. coli* tRNA after incubation with Streptozotocin (200 µM) for 30 min. The signal for m^3C in the unstressed samples were below the limit of quantification (LOQ). The experiments were done in biological triplicates and the error bars reflect standard deviation. Source data are provided as a Source Data file.



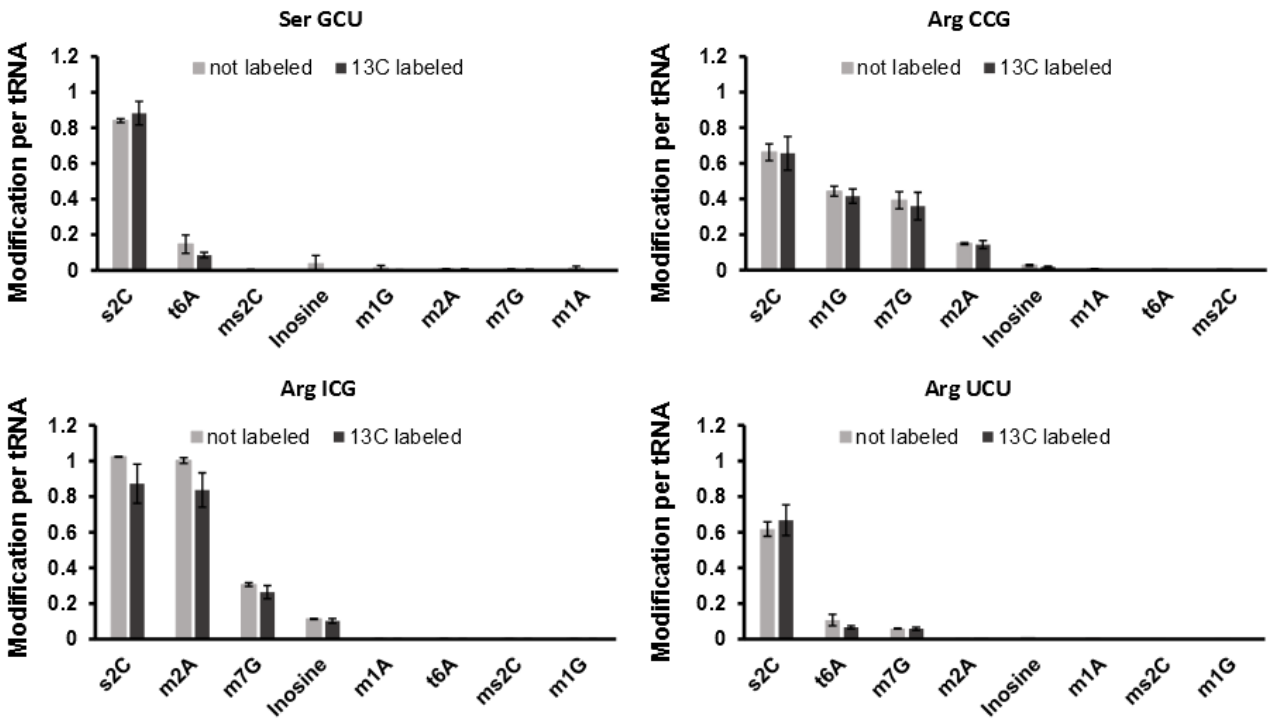
Supplementary Figure 11: Absolute abundance of tRNA modifications in *E. coli* after exposure to 20mM MMS. The dark grey bar shows the modification abundance of an unstressed sample whereas the light grey bar shows the modification abundance after one hour of 20mM MMS stress. From n=3 biol. replicates, error bars reflect standard deviation. Source data are provided as a Source Data file.



Supplementary Figure 12: NAIL-MS discrimination assay to determine origin of ms^2C *in vivo*. SAM dependent ms^2C per C in % of tRNA from unstressed bacteria (enz.) and 20 mM MMS stressed bacteria (enz. stressed). On the right, the amount of directly methylated ms2C per C in % from 20 mM MMS stressed bacteria is shown. From n=3 biol. replicates, error bars reflect standard deviation. Source data are provided as a Source Data file.



Supplementary Figure 13: Modified nucleoside abundance in comparative NAIL-MS experiment for tRNA SerGCU, ArgCCG, ArgICG and ArgUCU. *E. coli* in the negative control (without MMS) were grown in ^{13}C medium (grey bars, ^{13}C negative) and the 20mM MMS exposed bacteria in non-labeled (blue bars, n.l. MMS) media. The different samples were unified and the tRNA isoacceptors were co-purified in a comparative NAIL-MS experiment as detailed in the text. All experiments are from n=3 biol. replicates and error bars reflect standard deviation. Source data are provided as a Source Data file. The validation of the comparative NAIL-MS experiment is shown in **Supplementary Figure 14**.



Supplementary Figure 14: Validation experiment of the comparative NAIL-MS experiment The validation is performed by mixing unstressed *E. coli* from unlabeled media and unstressed *E. coli* from carbon-13 labeled media during harvesting. The tRNA and the tRNA isoacceptors from the two *E. coli* cultures are co-purified. The modified nucleosides per tRNA from the unlabeled bacteria (grey bars) and the labeled bacteria (black bars) are of equal abundance. Thus, the validation is considered a success (Reichle *et al.*, Methods, 2018) [4]. From n=3 biol. replicates, error bars reflect standard deviation. Source data are provided as a Source Data file.

Supplementary Table 8: Limit of precision of the comparative NAIL-MS validation shown in **Supplementary Figure 14**. Abbreviation n.d. = not determined All experiments are from n=3 biol. replicates and error bars reflect standard deviation. Source data are provided as a Source Data file.

	upper fold change				lower fold change				standard deviation			
	Ser GCU	Arg CCG	Arg ICG	Arg UCU	Ser GCU	Arg CCG	Arg ICG	Arg UCU	Ser GCU	Arg CCG	Arg ICG	Arg UCU
s2C	1.04162	1.0275	1.1907	1.05574	0.95838	0.97249	0.8093	0.94426	0.08482	0.14137	0.1429	0.16303
ms2C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
t6A	1.66802	n.d.	n.d.	1.59955	0.33198	n.d.	n.d.	0.40045	0.31097	n.d.	n.d.	0.41107
Inosine	n.d.	n.d.	1.1135	n.d.	n.d.	n.d.	0.8865	n.d.	n.d.	n.d.	0.1089	n.d.
m1G	n.d.	1.0808	n.d.	n.d.	n.d.	0.91916	n.d.	n.d.	n.d.	0.15722	n.d.	n.d.
m2A	n.d.	n.d.	1.2105	n.d.	n.d.	n.d.	0.7895	n.d.	n.d.	n.d.	0.1114	n.d.
m7G	n.d.	1.1128	1.1776	n.d.	n.d.	0.88715	0.8224	n.d.	n.d.	0.12952	0.1311	n.d.
m1A	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Supplementary Table 9: MRM parameters for comparative NAIL-MS analysis.

Compound Group	Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Ret Time (min)	Delta Ret Time (min)	Fragmentor (V)	Collision Energy (eV)	Cell Accelerator Voltage (V)	Polarity
unlabeled	C	244	112	1.73	1	175	13	5	Positive
	U	245	113	2	2	95	5	5	Positive
	G	284	152	3.718	3	95	17	5	Positive
	A	268	136	5.711	1	110	21	5	Positive
	ms2C	274	142	5.533	3	85	13	5	Positive
	s2C	260	128	2.288	2	85	13	5	Positive
	m1A	282	150	1.745	2	110	21	5	Positive
	m7G	298	166	2.021	2	105	14	5	Positive
	m2A	282	150	8.08	1	125	21	5	Positive
	I	269	137	3.556	1	100	9	5	Positive
	t6A	413	281	7	1	130	9	5	Positive
	m1G	298	166	5.08	1	105	13	5	Positive
13C labeled	C 13C	253	116	1.73	1	175	13	5	Positive
	U 13C	254	117	2	2	95	5	5	Positive
	G 13C	294	157	3.718	3	95	17	5	Positive
	A 13C	278	141	5.711	1	110	21	5	Positive
	ms2C 13C	284	147	5.533	3	85	13	5	Positive
	s2C 13C	269	132	2.288	2	85	13	5	Positive
	m1A 13C	293	156	1.745	2	110	21	5	Positive
	m7G 13C	309	172	2.021	2	105	14	5	Positive
	m2A 13C	293	156	8.08	1	125	21	5	Positive
	I 13C	279	142	3.556	1	100	9	5	Positive
	t6A 13C	428	291	7	1	130	9	5	Positive
	m1G 13C	309	172	5.08	1	105	13	5	Positive
SILIS	C SILIS	256	119	1.73	1	175	13	5	Positive
	U SILIS	256	119	2	2	95	5	5	Positive
	G SILIS	299	162	3.718	3	95	17	5	Positive
	A SILIS	283	146	5.711	1	110	21	5	Positive
	ms2C SILIS	287	150	5.533	3	85	13	5	Positive
	s2C SILIS	272	135	2.288	2	85	13	5	Positive
	m1A SILIS	298	161	1.745	2	110	21	5	Positive
	m7G SILIS	314	177	2.021	2	105	14	5	Positive
	m2A SILIS	298	161	8.08	1	125	21	5	Positive
	I SILIS	283	146	3.556	1	100	9	5	Positive
	t6A SILIS	434	297	7	1	130	9	5	Positive
	m1G SILIS	314	177	5.08	1	105	13	5	Positive

Supplementary Table 10: Primers used for the generation of sfGFP constructs used in this study

gfp_for	CGGAATTCCGCATGAGCAAAGGAGAAGAACTTTTCACTGGAG
gfp_for_AGT	CGGAATTCCGCATGAGTAAAGGAGAAGAACTTTTCACTGGAG
PAS-gfp_for	CGGAATTCCGCATGAGCCCAGCTGCACCTAGCGCTAGCGCAGCAAGCGCACCTAGCGCAGCTA GCAAAGGAGAAGAACTTTTCACTGGAG
PAS-ACT_gfp_for	CGGAATTCCGCATGAGTCCAGCTGCACCTAGTGCTAGTGCAGCAAGTGCACCTAGTGCAGCTAG TAAAGGAGAAGAACTTTTCACTGGAG
gfp_for_TCC	CGGAATTCCGCATGTCCAAAGGAGAAGAACTTTTCACTGGAG
PAS-gfp_for_TCC	CGGAATTCCGCATGTCCCCAGCTGCACCTTCCGCTTCCGCAGCATCCGCACCTTCCGCAGCTTCC AAAGGAGAAGAACTTTTCACTGGAG
gfp_rev	GCTCTAGACTTATTTGTAGAGCTCATCCATGCCATGTG

Full sf*gfp* sequence:

5'AGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATT
TTCTGTCCGTGGAGAGGGTGAAGGTGATGCTACAAACGGAAAACTCACCCTTAAATTTATTTGCACTACTGGAAAACT
ACCTGTTCCGTGGCCAACACTTGTCACTACTCTGACCTATGGTGTTCAATGCTTTTCCCGTTATCCGGATCACATGAAAC
GGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGACCT
ACAAGACGCGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAGGGTATTGATTTTAAAG
AAGATGGAAACATTCTTGGACACAAACTCGAGTACAACTTTAACTCACACAATGTATACATCACGGCAGACAAACAAA
AGAATGGAATCAAAGCTAACTTCAAAATTCGCCACAACGTTGAAGATGGTTCCGTTCAACTAGCAGACCATTATCAAC
AAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCGACACAATCTGTCCTTTCGAAAGA
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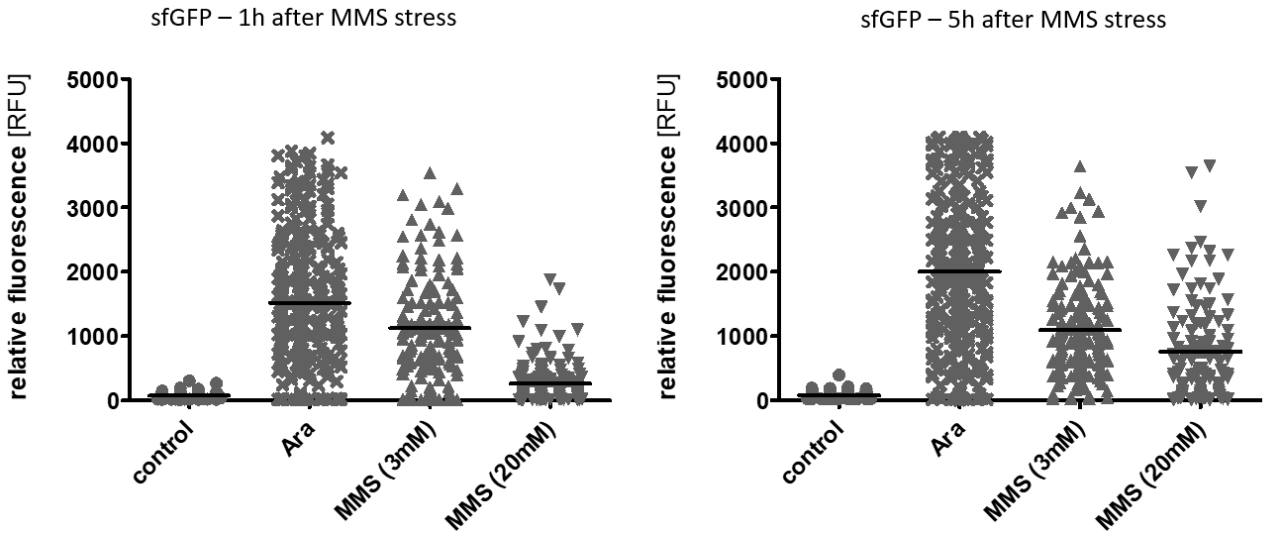
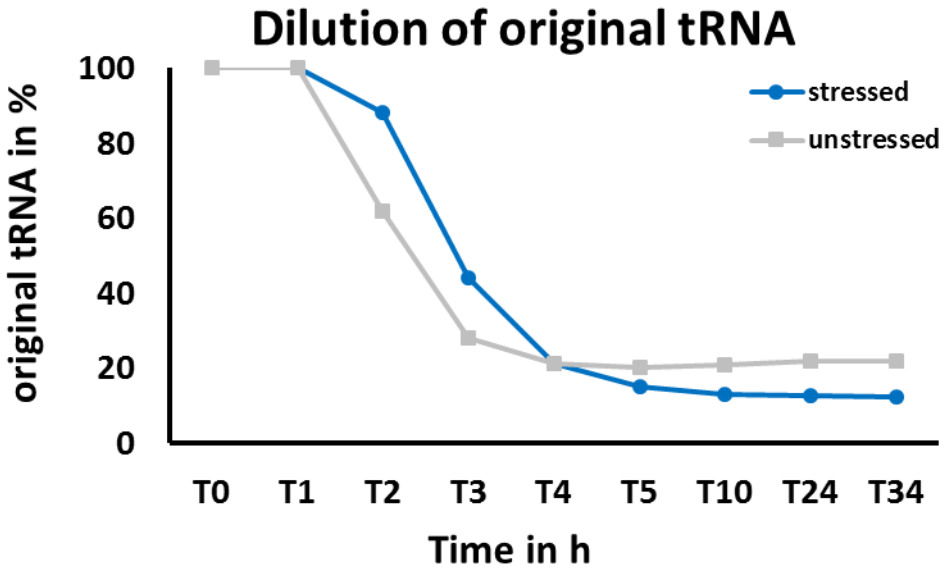
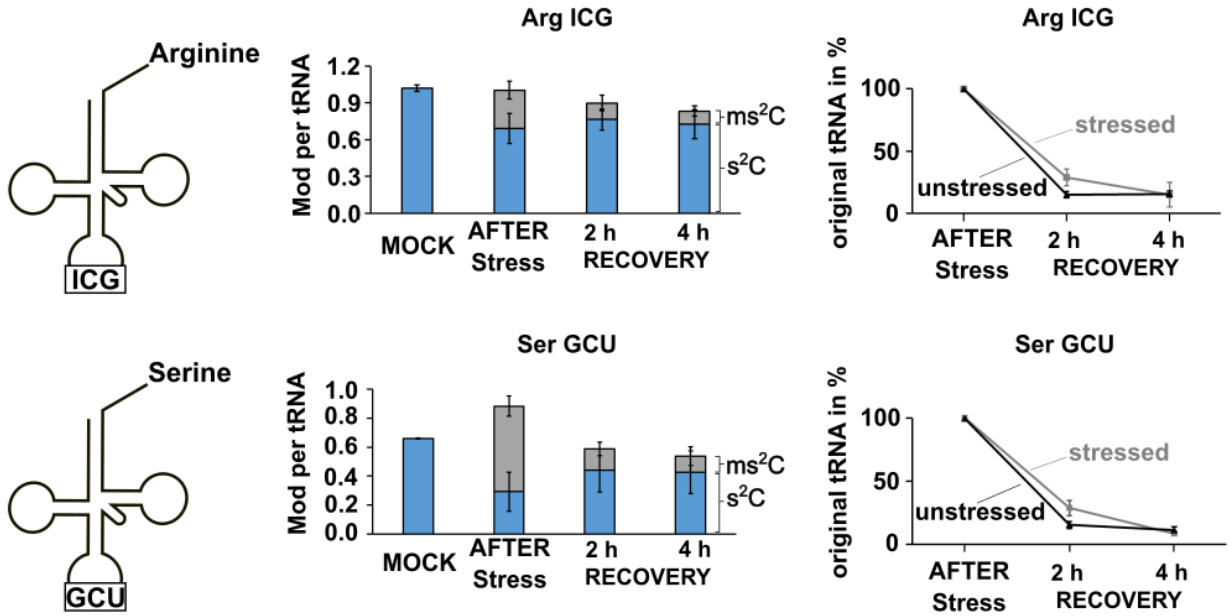
CTACAAATAA-3'



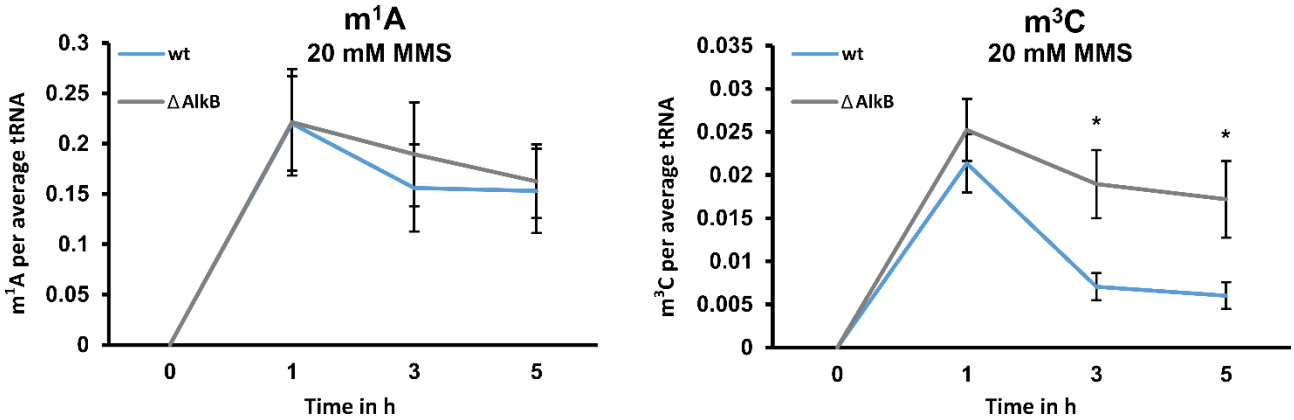
Supplementary Figure 15: Effects of 20 mM or 3 mM MMS stress on sfGFP synthesis in *E. coli* BW25113 cells. Results of single-cell fluorescence microscopy of *E. coli* cells producing sfGFP form the arabinose inducible plasmid pBAD24. Fluorescence was measured 1h or 5h after induction with 0.2% arabinose of non-stressed cells or cells pre-exposed to 20mM or 3 mM MMS stress for 1h. As a control cells were incubated in absence of MMS and arabinose. Black line represents the mean value. Source data are provided as a Source Data file.



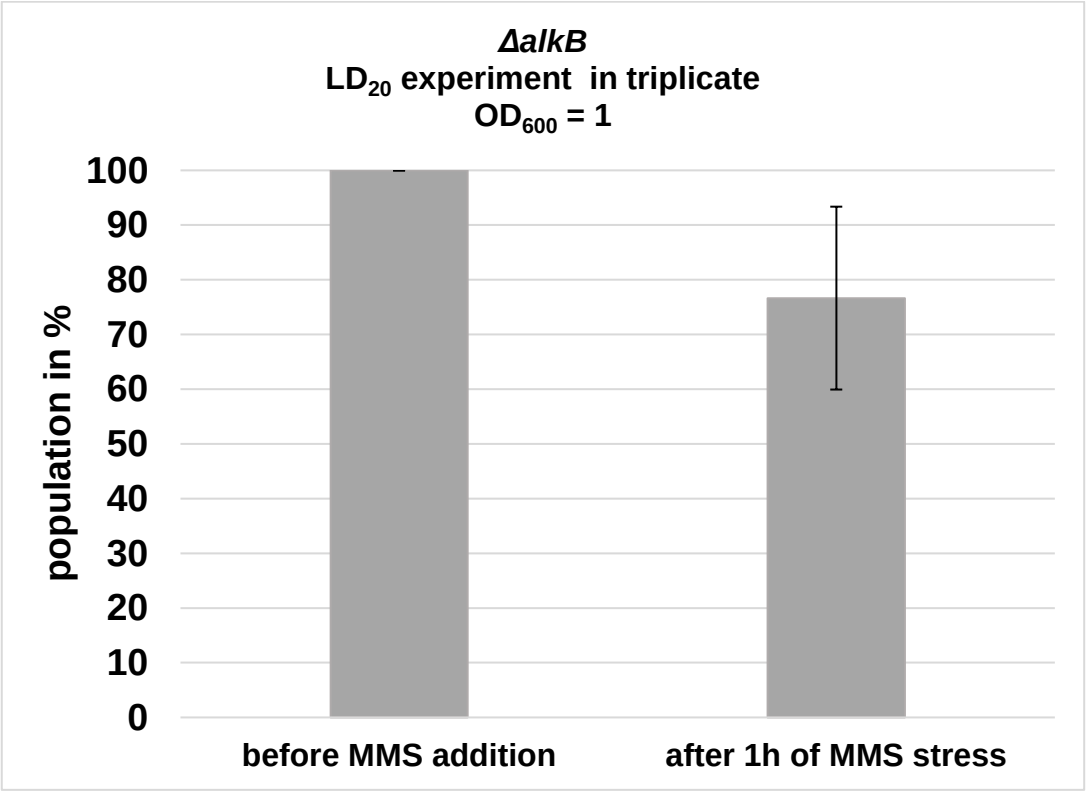
Supplementary Figure 16: RNA dilution curve for total RNA. Comparison of the proportional decrease of original tRNA in comparison to freshly transcribed tRNA during the pulse chase NAIL-MS experiment. The ratio of original (unlabeled canonicals determined by LC-MS) to newly transcribed tRNA (nitrogen-15 labeled canonicals determined by LC-MS) is shown in % original tRNA. blue: from MMS stressed bacteria, grey: from unstressed control bacteria. Mean of n=3 biol. replicates. Source data are provided as a Source Data file.



Supplementary Figure 17: Abundance of s^2C (blue) and ms^2C (grey) in $tRNA^{Arg}_{ICG}$ (top) and $tRNA^{Ser}_{GCU}$ (bottom) present during the 20 mM MMS exposure. The bar diagrams show the abundance of the modified nucleosides in original tRNA from a pulse-chase NAIL-MS experiment after 1 h of MMS exposure, and after 2 and 4 hours of recovery. MOCK indicates tRNA from unstressed bacteria. On the right side, the ratio of original to newly transcribed tRNA is shown in % original tRNA. grey: from MMS stressed bacteria, black: from unstressed control bacteria. The isoacceptors were purified from n=3 biol. replicates and error bars reflect standard deviation. Source data are provided as a Source Data file.



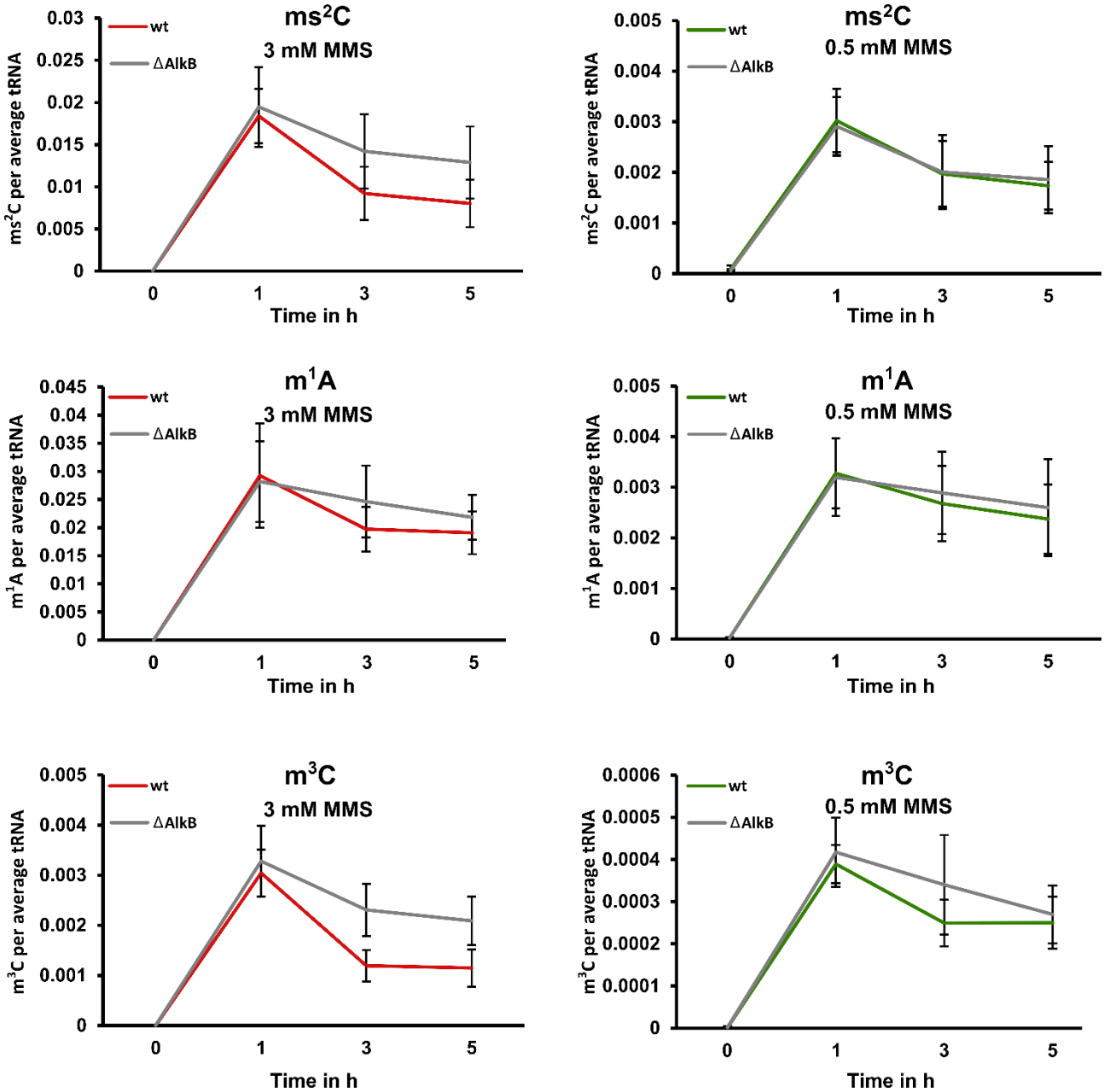
Supplementary Figure 18: Absolute abundance of m^1A and m^3C in wildtype and Δ*alkB E. coli*. Here, the abundance of m^1A and m^3C in original tRNA (distinguished from new transcripts by pulse-chase NAIL-MS as described in **Figure 4a**) in wildtype (wt, light blue) and AlkB deficient *E. coli* (Δ*alkB*, grey) is shown. The bacteria are exposed to 20 mM MMS and harvested directly after the stress (1 h) and after 2 and 4 hours of recovery (3 h, 5 h). All experiments are from n=3 biol. replicates and error bars reflect standard deviation. The statistics were done with student t-test (equal distribution, two-sided): * $p < 0.05$. Source data are provided as a Source Data file.



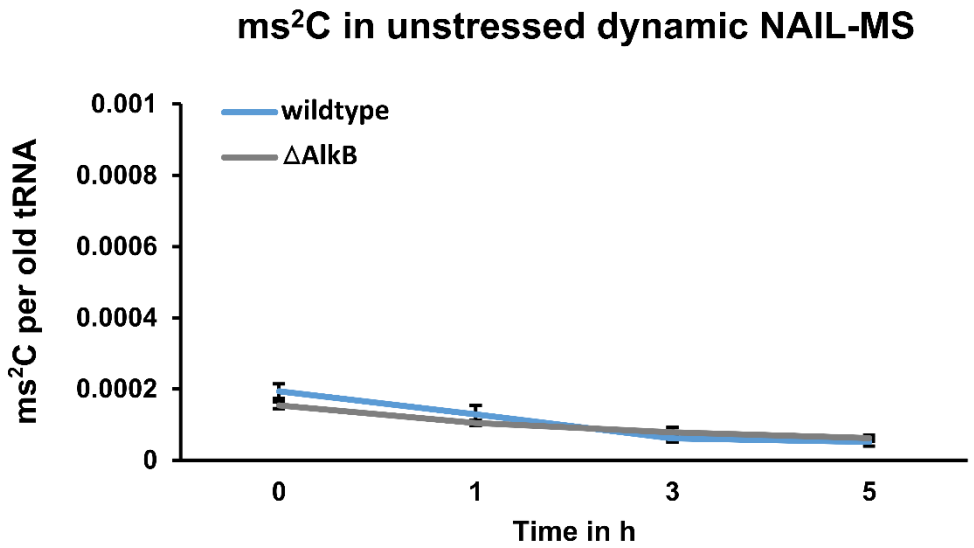
Supplementary Figure 19: Survival assay to determine the lethality of 3mM MMS for the *E.coli ΔalkB* strain Cells were brought to an OD of 1 (error bars reflect the standard deviation of n=3 biol. replicates) and exposed to 3 mM MMS for 60 min.

Supplementary Table 11: MRM parameters for pulse chase NAIL-MS experiments.

Compound Group	Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Ret Time (min)	Delta Ret Time (min)	Fragmentor (V)	Collision Energy (eV)	Cell Accelerator Voltage (V)	Polarity
unlabeled (original)	C	244	112	1.73	1	175	13	5	Positive
	U	245	113	2	2	95	5	5	Positive
	G	284	152	3.718	3	95	17	5	Positive
	A	268	136	5.711	1	110	21	5	Positive
	ms2C	274	142	5.533	3	85	13	5	Positive
	s2C	260	128	2.288	2	85	13	5	Positive
	m1A	282	150	1.745	2	110	21	5	Positive
	m3C	258	126	1.752	2	88	14	5	Positive
	I	269	137	3.272	2	100	9	5	Positive
	m5U	259	127	3.61	2	145	10	5	Positive
	m2A	282	150	7.85	3	120	20	5	Positive
	m7G	298	166	2.7	2	105	14	5	Positive
	t6A	413	281	6.923	2	130	9	5	Positive
15N/CD3/34S labeled (new)	C 15N	247	115	1.73	1	175	13	5	Positive
	U 15N	247	115	1.876	2	95	5	5	Positive
	G 15N	289	157	3.718	3	95	17	5	Positive
	A 15N	273	141	5.711	1	110	21	5	Positive
	ms2C 34S_CD3_15N	282	150	5.533	3	85	13	5	Positive
	s2C 34S_15N	265	133	2.288	2	85	13	5	Positive
	m1A 15N_CD3	290	158	1.745	2	110	21	5	Positive
	m3C 15N_CD3	264	132	1.752	2	88	14	5	Positive
	I 15N	273	141	3.272	2	100	9	5	Positive
	m5U 15N_CD3	264	132	3.61	2	145	10	5	Positive
	m2A 15N_CD3	290	158	7.85	3	120	20	5	Positive
	m7G 15N_CD3	306	174	2.7	2	105	14	5	Positive
	t6A 15N	419	287	6.923	2	130	9	5	Positive
34S labeled (turnover)	ms2C 34S	276	144	5.533	3	85	13	5	Positive
	s2C 34S	262	130	2.288	2	85	13	5	Positive
SILIS	C SILIS	256	119	1.73	1	175	13	5	Positive
	U SILIS	256	119	1.876	2	95	5	5	Positive
	G SILIS	299	162	3.718	3	95	17	5	Positive
	A SILIS	283	146	5.711	1	110	21	5	Positive
	ms2C SILIS	287	150	5.533	3	85	13	5	Positive
	s2C SILIS	272	135	2.288	2	85	13	5	Positive
	m1A SILIS	298	161	1.745	2	110	21	5	Positive
	m3C SILIS	271	134	1.752	2	88	14	5	Positive
	I SILIS	283	146	3.272	2	100	9	5	Positive
	m5U SILIS	271	134	3.61	2	145	10	5	Positive
	m2A SILIS	298	161	7.85	3	120	20	5	Positive
	m7G SILIS	314	177	2.7	2	105	14	5	Positive
	t6A SILIS	434	297	6.923	2	130	9	5	Positive



Supplementary Figure 20: Absolute abundance of ms^2C, m^1A and m^3C at different MMS concentrations in WT and Δ*alkB E. coli*. The experimental set up as described in **Figure 4a** was used to investigate the stress behavior at lower MMS concentrations (3mM and 0.5 mM). The bacteria are exposed to MMS and are harvested directly after the stress (1 h) and after 2 and 4 hours of recovery (3 h, 5 h). All experiments are from n=3 biol. replicates and error bars reflect standard deviation. Source data are provided as a Source Data file.



Supplementary Figure 21: Pulse chase control experiment with unstressed *E. coli* WT compared to unstressed Δ*alkB* knockout cells. ms^2C was not observed to accumulate in the Δ*alkB* knockout. The experimental set up is shown in **Figure 4a** in the manuscript. Experiments were done in biological triplicates and error bars reflect standard deviation. Source data are provided as a Source Data file.

Supplementary Table 12: Formula for absolute quantification of unmodified and modified nucleosides from LC-MS/MS signals (area) of a NAIL-MS Pulse Chase experiment and subsequent reference of the modifications to the respective canonicals. As an example, here we chose 2-thiocytidine which was referenced to its canonical nucleoside, cytidine.

	s^2C (fmol)	C (fmol)	Normalization
(1) original	$\frac{\text{area s2C (unlabeled)}}{\text{rRFN s2C} \times \text{area s2C (SILIS)}}$	$\frac{\text{area C (unlabeled)}}{\text{rRFN C} \times \text{area C (SILIS)}}$	$\frac{\text{s2C (original)}}{\text{C (original)}}$
(2) new	$\frac{\text{area s2C (34S, 15N, CD3)}}{\text{rRFN s2C} \times \text{area s2C (SILIS)}}$	$\frac{\text{area C (15N)}}{\text{rRFN C} \times \text{area C (SILIS)}}$	$\frac{\text{s2C (new)}}{\text{C (new)}}$

Supplementary References

1 Baba, T. *et al.* Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular systems biology* **2**, 2006.0008, doi:10.1038/msb4100050 (2006).

2 Boccaletto, P. *et al.* MODOMICS: a database of RNA modification pathways. 2017 update. *Nucleic acids research* **46**, D303-d307, doi:10.1093/nar/gkx1030 (2018).

3 Kitagawa, M. *et al.* Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research. *DNA research : an international journal for rapid publication of reports on genes and genomes* **12**, 291-299, doi:10.1093/dnares/dsi012 (2005).

4 Reichle, V. F. *et al.* Surpassing limits of static RNA modification analysis with dynamic NAIL-MS. *Methods (San Diego, Calif.)* **156**, 91-101, doi:10.1016/j.ymeth.2018.10.025 (2019).

ChemBioChem

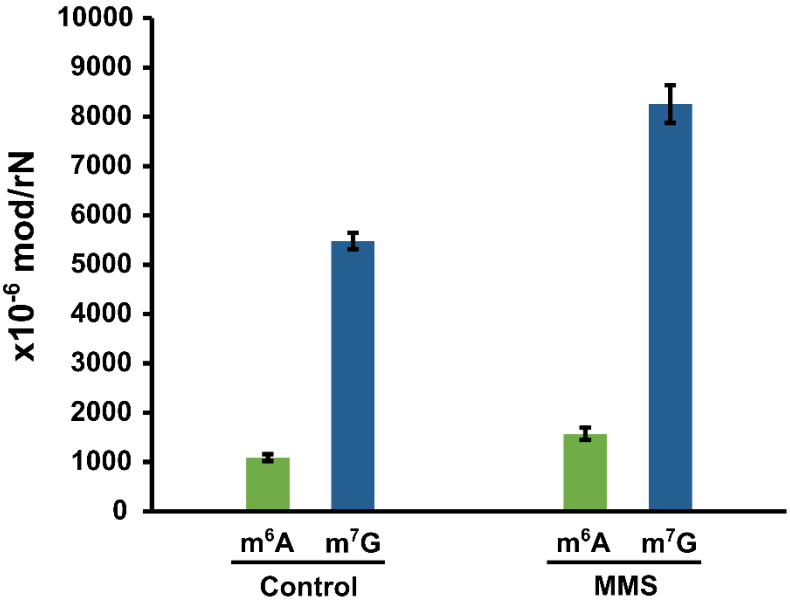
Supporting Information

Synthesis and Metabolic Fate of 4-Methylthiouridine in Bacterial tRNA

Christoph Borek[+], Valentin F. Reichle[+], and Stefanie Kellner*

Nucleoside damage after 20 mM MMS exposure in *E. coli* tRNA 2
Stability of ms^4U in vitro 3
Pulse-chase NAIL-MS study of modified nucleosides 3
Materials & Methods (Microbiology and Anal. Chemistry) 4
Synthetic access to ms^4U 8

a



b

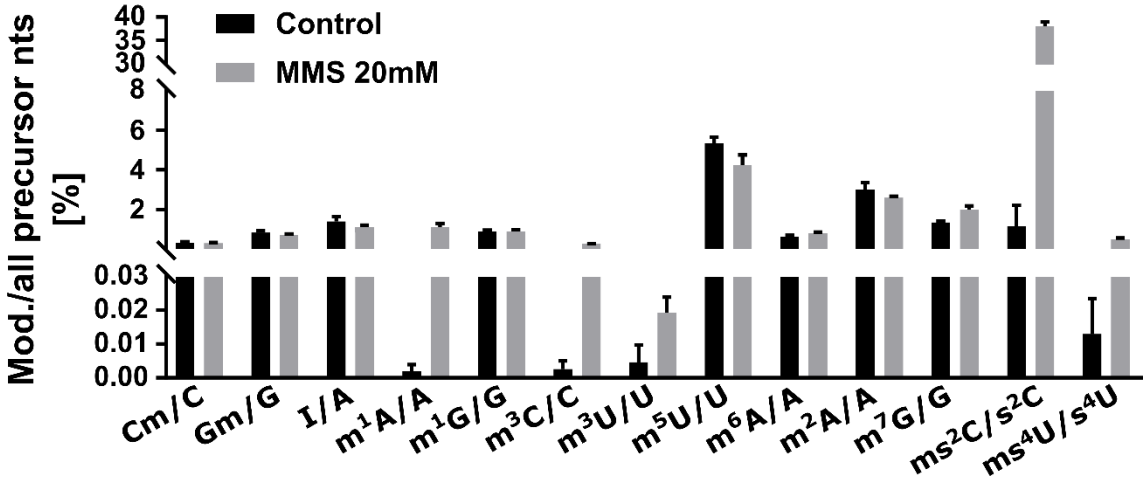


Figure S1: tRNA damage products after 20 mM MMS stress. All experiments are from n=3 biol. replicates and error bars reflect standard deviation. **a** Increase of m^6A (green) and m^7G (blue) after MMS stress in 10^{-6} modifications per ribonucleotide (rN). **b** Percentage of methylated nucleotide in relation to its respective precursor nucleotide (nts).

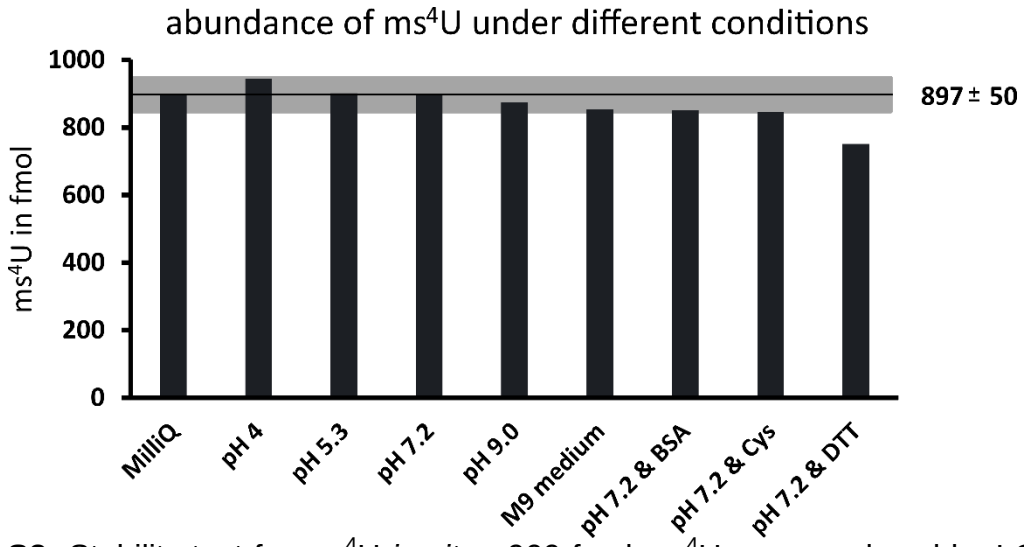


Figure S2: Stability test for ms^{4}U *in vitro*. 900 fmol ms^{4}U were analyzed by LC-MS/MS after 2h at 37°C under different conditions. The grey horizontal bar visualizes a variance of ±50 fmol (±5.6%).

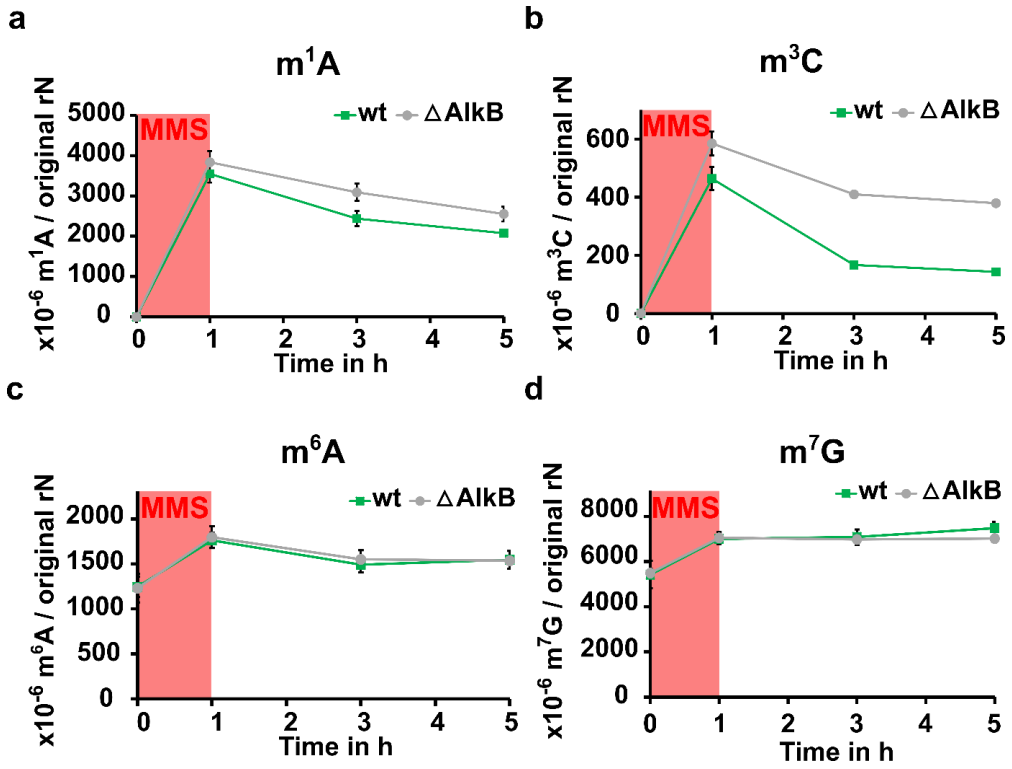


Figure S3: Pulse-chase NAIL-MS study of modified nucleosides not shown in Figure 4. MMS stress (20 mM, 1 h exposure time highlighted in red) in *E. coli* wt (green) and ΔAlkB (grey) strain. The results for m^{1}A (**a**), m^{3}C (**b**), m^{6}A (**c**) and m^{7}G (**d**) are shown. The y-axes scale shows the modification abundance x10^{-6} per original ribonucleotide (rN). All experiments are from n=3 biol. replicates and error bars reflect standard deviation.

Material and Methods

Salts, reagents, and nucleosides

All salts were obtained from Sigma-Aldrich (Munich, Germany) at molecular biology grade unless stated otherwise. The isotopically labeled compounds $^{15}NH_4Cl$ (>98 atom %) and [D_3]-L-methionine (98atom %) were obtained from Sigma-Aldrich. Isotopically labelled $^{13}C_6$-glucose (≥99 atom %) and isotopically labeled $Na_2{}^{34}SO_4$ (99.11 atom %) were obtained from Eurisotope (Saarbruecken, Germany). All solutions and buffers were made with water from a Millipore device (Milli-Q, Merck, Darmstadt, Germany). The nucleosides adenosine, cytidine, guanosine, uridine, and N2-methylguanosine (m^2G) were obtained from Sigma-Aldrich. 1-Methyladenosine (m^1A), N3-methylcytidine (m^3C), N6-methyladenosine (m^6A), 7-methylguanosine (m^7G), 5-methyluridine (m^5U), 2'-O-methylcytidine (Cm), 2′-O-methylguanosine (Gm), 1-methylguanosine (m^1G), and 3-methyluridine (m^3U) were obtained from Carbosynth (Newbury, UK). The nucleoside ms^2C was synthesized as described before.[1]

Strains

E. coli strains. The used *E. coli* wild-type strain BW25113 and the isogenic AlkB knockout strain JW2200-KC were purchased from the Keio database[2] All cultures were grown in a shaking incubator at 37 °C at 250 rpm (Orbit=10 mm). Overnight cultures were grown in 5 mL of the differently labeled M9 media used for the respective experiment. The cells were grown starting with an OD_{600} of 1 and grown until reaching stationary phase (OD_{600}~ 4).

M9 media

M9 minimal medium was used with and without the indicated isotopes. Unlabeled M9 was prepared by mixing a 10 × M9 stock solution with glucose, $MgCl_2$, Na_2SO_4, and $CaCl_2$ (as detailed below). For unlabeled 10 × M9 stock solution, Na_2HPO_4 (68 g/L), KH_2PO_4 (30 g/L), NaCl (2.5 g/L), and NH_4Cl (10 g/L) were mixed and autoclaved. For ^{15}N-labeled 10 × M9 stock solution,$^{15}NH_4Cl$ (10 g/L) was used. $MgCl_2$ (0.1 M), $CaCl_2$ (0.1 M), Na_2SO_4 (0.1 M), and 20% (wt%) glucose were prepared by sterile filtration. A 20% (wt%) $^{13}C_6$-labeled glucose solution was prepared for ^{13}C-labeled M9 media. For ^{34}S-labeled M9 media a 0.1 M $Na_2{}^{34}SO_4$ solution was prepared. Final M9 media was prepared by mixing, *e.g.*, 500µL M9 stock solution with 100µL glucose, 100µL $MgCl_2$, 100µL Na_2SO_4, 5µL $CaCl_2$ and water to a final volume of 5 mL. For ^{15}N-labeled cultures, the ^{15}N-10 ×M9 stock solution was used. For ^{13}C-labeled cultures, the 20% (wt%) $^{13}C_6$-labeled glucose solution and for ^{34}S-labeled cultures, the 0.1 M $Na_2{}^{34}SO_4$ solution was used. For CD_3-methylome labeling, 200µL CD_3-methionine (stock 5 g/L) was added to 5 mL of culture volume.

MMS- stress studies *in vitro*

The pipetting scheme for the different *in vitro* sample mixtures is shown in **Table S1**. The buffer solutions were prepared in MilliQ and adjusted with a pH-meter to the respective pH. M9 medium was not pH adjusted but used in the composition as described before. The BSA solution was prepared from a stock solution (c = 10 µg/µl, in PBS) and diluted with MilliQ. DTT and Cysteine were both solved and diluted with MilliQ.

The prepared sample mixtures were incubated in a heating block at 37°C for 2h. To the sample solutions 900 µL MilliQ were added and the mixtures were vortexed (1:10 dilution). Afterwards 18 µL of each sample solution were combined with 2 µL of our SILIS. The LC-MS/MS injection amount was 10 µL, which means a theoretical ms^4U amount of 900 fmol.

Table S1: Pipetting scheme for *in vitro* ms^4U stability test. Abbreviations: Vol. = Volume;

Sample	Buffer	Vol. ms^4U (0.01 mM)	Vol. Buffer	Add-on	Vol. Add-on	Dil. SILIS
MilliQ	-	10 µL	-	-	-	1:10
pH 4.0	0.1 M Citric Acid	10 µL	90 µL	-	-	1:10
pH 5.3	0.5 mM $NH_4OAc/AcOH$	10 µL	90 µL	-	-	1:10
pH 7.2	0.1 M NH_4HCO_3	10 µL	90 µL	-	-	1:10
pH 9.0	0.1 M $NH_4OAc/NaOH$	10 µL	90 µL	-	-	1:10
M9 medium	0.05 M Na_2HPO_4 0.02 M KH_2PO_4	10 µL	90 µL	-	-	1:10
pH 7.2 & BSA	0.1 M NH_4HCO_3	10 µL	80 µL	0.02 mM BSA	10 µL (2 eq.)	1:10
pH 7.2 & Cys	0.1 M NH_4HCO_3	10 µL	80 µL	0.1 mM Cysteine	10 µL (10 eq.)	1:10
pH 7.2 & DTT	0.1 M NH_4HCO_3	10 µL	80 µL	0.1 mM	10 µL	1:10

Dil. = Dilution; BSA = Bovin serum albumin; Cys = Cysteine; DTT = Dithiothreitol; eq. = Equivalents respective ms^4U.

				DTT	(10 eq.)	

MMS- stress studies *in vivo*

A 5 mL bacterial solution with an OD_{600} of 1 was prepared from an overnight culture. After 60 min growth, 8.5 µL MMS (final conc. 20 mM) were added. As a control, 8.5 µL water was added to a second culture. After 60 min of exposure, the RNA was isolated and total tRNA purified by SEC.

Pulse-chase NAIL-MS experiment

A single colony of *E. coli* BW25113 or *E. coli* JW2200-KC (ΔalkB) was picked and grown in unlabeled M9 medium (5 mL) overnight. From the first overnight culture, a 50 mL culture was prepared in unlabeled M9 medium and grown overnight. From the second overnight culture, 120 mL culture ($OD_{600}$of 1.0) was prepared in unlabeled M9 medium. After 60 min growth, the first aliquot (7 mL) was taken for RNA isolation. The remaining culture was split into two flasks of 56.5 mL each. One was exposed to MMS (95.7µL, 20 mM final concentration) the other to water (MOCK) and inverted before both cultures were cultivated for 60 min. An aliquot (7 mL) was drawn from each culture, and the RNA of the aliquot was isolated. The remaining bacteria were centrifuged (1200 ×g, 5 min), and the MMS/MOCK-containing supernatants were discarded. The bacteria pellets were washed with ^{15}N, ^{34}S and CD_3-methionine labeled M9 medium (5 mL), and each bacterial pellet was suspended in fresh $^{15}N/^{34}S/CD_3$ M9 medium (50 mL). The bacteria were allowed to grow and recover from the MMS/MOCK treatment. Seven mL of each bacterial culture were harvested after 2 and after 4 h. The RNA was isolated and the tRNA purified by SEC.

SILIS preparation

A single colony of *E. coli* BW25113 was picked and grown in $^{15}N/^{13}C$ labeled M9 medium (10 mL) overnight. The overnight culture was diluted with 90 mL freshly prepared $^{15}N/^{13}C$ labeled M9 medium and it was incubated till the OD_{600} reached 1.0. The culture was then exposed to MMS (170 µL, 20 mM final concentration) and after 60 min the cells were harvested and the RNA was isolated and digested as described in the protocols below.

Cell lysis and tRNA purification

The bacteria culture was centrifuged at 1200 ×g for 5 min. The supernatant was discarded and the cell pellet was resuspended in 1 mL TRI reagent (Sigma-Aldrich) per 5 mL bacteria culture. The total RNA was isolated according to the supplier's manual. tRNA was purified by size exclusion chromatography (SEC) according to published procedures.[3] The tRNA was resuspended in water (30µL).

tRNA digestion for mass spectrometry

tRNA (100 ng) in aqueous digestion mix (30µL) was digested to single nucleosides by using 0.2 U alkaline phosphatase, 0.02 U phosphodiesterase I (VWR, Radnor, Pennsylvania, USA), and 0.2 U benzonase in Tris (pH 8,5 mM) and $MgCl_2$ (1 mM) containing buffer. Furthermore, 0.5 µg tetrahydrouridine (Merck, Darmstadt, Germany), 1 µM butylated hydroxytoluene, and 0.1 µg pentostatin were added to avoid deamination and oxidation of the nucleosides.[3] The mixture was incubated for 2 h at 37 °C and 1/10

Vol. of SILIS (stable isotope labeled internal standard) was added to each sample before analysis by QQQ mass spectrometry.

QQQ mass spectrometry

For quantitative mass spectrometry an Agilent 1290 Infinity II equipped with a diode-array detector (DAD) combined with an Agilent Technologies G6470A Triple Quad system and electrospray ionization (ESI-MS, Agilent Jetstream) was used. Operating parameters: positive-ion mode, skimmer voltage of 15 V, cell accelerator voltage of 5 V, N2 gas temperature of 230 °C and N2 gasflow of 6 L/min, sheath gas (N2) temperature of 400 °C with a flow of 12 L/min, capillary voltage of 2500 V, nozzle voltage of 0 V, and nebulizer at 40 psi. The instrument was operated in dynamic MRM mode. We used a flow rate of 0.35 mL/min in combination with a binary mobile phase of 5 mM NH_4OAc aqueous buffer A, brought to pH 5.6 with glacial acetic acid (65µL in 1 L), and an organic buffer B of pure acetonitrile (Roth, LC-MS grade, purity≥.99.95). The *in vitro* experiments, the coinjects and the metabolic CD_3 labeling digest seperation was performed on a Synergi Fusion-RP column (Phenomenex®, Torrance, CA, USA; Synergi® 2.5 µm Fusion-RP 100Å, 150 x 2.0 mm) at 35 °C. The gradient started with 100% A for one minute, increased to 10% B by 5 min, and to 40% B by 7 min. The column was flushed with 40% B for 1 min and returned to starting conditions to 100% A by 8.5 min followed by re-equilibration at 100% A for 2.5 additional minutes. For the pulse-chase experiment and the unlabeled *in vivo* MMS stress study (Figure 3), the digests were separated on a Core-Shell Technology column (Phenomenex, Torrance, CA, USA; Kinetex 1.7µm EVO C18, 100 Å, 150 × 2.1 mm) at 35 °C. The gradient started at 100% solvent A, followed by an increase to 10% over 10 min. From 10 to 15 min, solvent B was increased to 45% and was maintained for 3 min before returning to 10% solvent A and a 3 min re-equilibration period.

Table S2: MRM parameters for co-injection, CD_3-metabolic labeling and MMS *in vitro* experiments.

Comp. group	Comp. Name	Precursor Ion (m/z)	Product Ion (m/z)	Ret Time (min)	Fragmentor (V)	CE (V)	Cell Acc (V)	Polarity
not labeled	C	244	112	2	200	40	5	Positive
	Cm	258	112	3.8	180	9	5	Positive
	m^3C	258	126	1.7	88	14	5	Positive
	m^3U	259	127	4.4	75	9	5	Positive
	m^5U	259	127	4.1	145	10	5	Positive
	ms^2C	274	142	4.5	85	13	5	Positive
	ms^4U	275	143	6.3	70	12	5	Positive
	s^2C	260	128	3.2	85	13	5	Positive
	s^4U	261	129	4.6	75	17	5	Positive
	U	245	113	2.8	95	5	5	Positive
SILIS	C SILIS	256	119	2	200	40	5	Positive
	Cm SILIS	271	119	3.8	180	9	5	Positive
	m^3C SILIS	270	133	1.7	88	14	5	Positive

	m^3U SILIS	271	134	4.4	75	9	5	Positive
	m^5U SILIS	271	134	4.1	145	10	5	Positive
	ms^2C SILIS	286	149	4.5	85	13	5	Positive
	ms^4U SILIS	286	149	6.3	70	12	5	Positive
	s^2C SILIS	272	135	3.2	85	13	5	Positive
	s^4U SILIS	272	135	4.6	75	17	5	Positive
	U SILIS	256	119	2.8	95	5	5	Positive
^{13}C	$ms^4U\ ^{13}C$	284	147	6.3	70	12	5	Positive
^{15}N	$ms^4U\ ^{15}N$	277	145	6.3	70	12	5	Positive
^{34}S	$ms^4U\ ^{34}S$	277	145	6.3	70	12	5	Positive
CD_3 - labeled	$ms^4U\ CD_3$	278	146	6.3	70	12	5	Positive
	Cm CD_3	261	112	3.8	180	9	5	Positive
	$m^5U\ CD_3$	262	130	4.1	145	10	5	Positive

Table S3: MRM parameters for pulse-chase experiments and unlabeled 20 mM MMS *in vivo* stress experiment (Figure 3).

Comp. group	Comp. Name	Ret. Time (min)	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	CE (V)	Cell Acc (V)	Polarity
not labeled (original)	A	5.611	268	136	200	40	5	Positive
	C	1.73	244	112	200	40	5	Positive
	Cm	3.347	258	112	180	9	5	Positive
	G	3.718	284	152	200	40	5	Positive
	Gm	4.78	298	152	100	9	5	Positive
	I	3.272	269	137	100	9	5	Positive
	m^1A	1.745	282	150	110	21	5	Positive
	m^1G	4.85	298	166	100	15	5	Positive
	m^3C	1.752	258	126	88	14	5	Positive
	m^3U	4.7	259	127	75	9	5	Positive
	m^5U	3.61	259	127	145	10	5	Positive
	m^6A	7.85	282	150	120	20	5	Positive
	m^7G	2.7	298	166	105	14	5	Positive
	ms^2C	5.533	274	142	85	13	5	Positive
	ms^4U	7.34	275	143	75	17	5	Positive
	s^2C	2.288	260	128	85	13	5	Positive

	s^4U	4.337	261	129	75	17	5	Positive
	U	1.876	245	113	95	5	5	Positive
$^{34}S/^{15}N/CD_3$ labeled (new)	A ^{15}N	5.611	273	141	200	40	5	Positive
	C ^{15}N	1.73	247	115	200	40	5	Positive
	Cm $^{15}N_CD_3$	3.347	264	115	180	9	5	Positive
	G ^{15}N	3.718	289	157	200	40	5	Positive
	Gm $^{15}N_CD_3$	4.78	306	157	100	9	5	Positive
	I ^{15}N	3.272	273	141	100	9	5	Positive
	m^1A $^{15}N_CD_3$	1.745	290	158	110	21	5	Positive
	m^3C $^{15}N_CD_3$	1.752	264	132	88	14	5	Positive
	m^3U $^{15}N_CD_3$	4.7	264	132	75	9	5	Positive
	m^5U $^{15}N_CD_3$	3.61	264	132	145	10	5	Positive
	m^6A $^{15}N_CD_3$	7.85	290	158	120	20	5	Positive
	m^1G $^{15}N_CD_3$	4.85	306	174	100	15	5	Positive
	m^7G $^{15}N_CD_3$	2.7	306	174	105	14	5	Positive
	ms^2C $^{15}N_CD_3_^{34}S$	5.533	282	150	85	13	5	Positive
	ms^4U $^{15}N_CD_3_^{34}S$	7.34	282	150	75	17	5	Positive
	s^2C $^{15}N_^{34}S$	2.288	265	133	85	13	5	Positive
	s^4U $^{15}N_^{34}S$	4.337	265	133	75	17	5	Positive
	U ^{15}N	1.876	247	115	95	5	5	Positive
$^{15}N/^{13}C/CH_3$ labeled (SILIS)	A SILIS	5.611	283	146	200	40	5	Positive
	C SILIS	1.73	256	119	200	40	5	Positive
	Cm SILIS	3.347	271	119	180	9	5	Positive
	G SILIS	3.718	299	162	200	40	5	Positive
	Gm SILIS	4.78	314	162	100	9	5	Positive
	I SILIS	3.272	283	146	100	9	5	Positive
	m^1A SILIS	1.745	297	160	110	21	5	Positive
	m^1G SILIS	4.85	314	177	100	15	5	Positive
	m^3C SILIS	1.752	270	133	88	14	5	Positive
	m^3U SILIS	4.7	271	134	75	9	5	Positive
	m^5U SILIS	3.61	271	134	145	10	5	Positive
	m^6A SILIS	7.85	298	161	120	20	5	Positive
	m^7G SILIS	2.7	314	177	105	14	5	Positive
	ms^2C SILIS	5.533	286	149	85	13	5	Positive
	ms^4U SILIS	7.34	286	149	75	17	5	Positive
	s^2C SILIS	2.288	272	135	85	13	5	Positive
	s^4U SILIS	4.337	272	135	75	17	5	Positive

	U SILIS	1.876	256	119	95	5	5	Positive
^{34}S labeled (re-thiolation)	s^2C ^{34}S	2.288	262	130	85	13	5	Positive
	s^4U ^{15}N or ^{34}S	4.337	263	131	75	17	5	Positive

Calibrations and data analysis

For calibration, synthetic nucleosides were weighed and dissolved in water to a stock concentration of 1–10 mM. In contrast to the ms^4U standard, due to an unknown content of water and salts in the synthesized ms^2C standard, the concentration could not be determined through weighing. Therefore, the concentration of the stock solution was determined by comparison to s^2C containing isoacceptors after MMS exposure.[1] The calibration solutions range from 0.3 to 500 pmol for each canonical nucleoside and from 0.3 to 500 fmol for each modified nucleoside and were spiked with 10% SILIS. The sample data were analyzed by the Quantitative and Qualitative MassHunter Software from Agilent. The areas of the MRM signals were integrated for each modification and their isotope derivatives. The absolute amounts of the modifications were referenced to the absolute amounts of the respective canonical. In the case of the pulse-chase experiment, the different isotopomers were referenced to their respective labeled canonicals, so that original modifications were referenced to original canonicals and new modifications were referenced to new canonicals. See Eqs. (1) and (2) for s^4U as an example in **Table S4.**

Table S4: Equations for absolute quantification calculations in the pulse-chase experiment. The ms^4U precursor molecule s^4U was chosen here as an example modification.

	s^4U (fmol)	U (fmol)	Normalization
(1) original	$\frac{\text{area s4U (unlabeled)}}{\text{rRFN s4U} \times \text{area s4U (SILIS)}}$	$\frac{\text{area U (unlabeled)}}{\text{rRFN U} \times \text{area U (SILIS)}}$	$\frac{\text{s4U (original)}}{\text{U (original)}}$
(2) new	$\frac{\text{area s4U (34S, 15N, CD3)}}{\text{rRFN s4U} \times \text{area s4U (SILIS)}}$	$\frac{\text{area U (15N)}}{\text{rRFN U} \times \text{area U (SILIS)}}$	$\frac{\text{s4U (new)}}{\text{U (new)}}$

Synthetic access to 4-methylthiouridine

General information

All chemicals were purchased from AlfaAesar, Acros, or Sigma Aldrich in standard purity and used without further purification if not stated otherwise. NMR-Spectra were measured on a *Bruker* Fourier 300 or *Bruker* Avance III HD 300. The chemical shift δ is given ppm, coupling constants J in Hz. As internal standard remaining Protons of the deuterated solvents and their carbons were used with the following shifts:

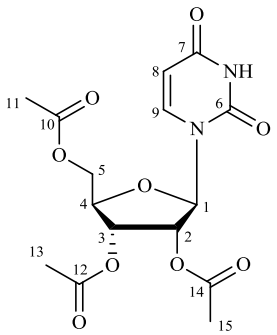
Solvent	δ (ppm) ^{1}H	δ (ppm) ^{13}C
$CDCl_3$	7.260	77.160
d^6-DMSO	2.500	39.520
MeOD	4.870	49.000
D_2O	4.790	

The multiplicities are given with the following abbreviations: s = singlet, s(br) = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet as well as combinations according to the scheme: dt = doublet from triplet. The NMR signals were assigned via DEPT90, DEPT135, 2D correlation spectra (COSY, HSQC, HMBC, NOESY).

TLC were performed on precoated sheets ALUGRAM® Xtra SIL G/UV_{254} by *Macherey-Nagel*. Fluorescence quenching was observed at 254 nm. Composition of mobile phases (eluents) are given in volume fractions (v/v), as standard staining reagent for the sugar compounds anisaldehyde/sulfuric acid was used. Column chromatography was carried out with columns whose dimensions were adapted to the respective separation problem packed with Silica 60 (0,06 - 0,2 mm) from *Roth*.

Syntheses

<u>2,3,5-Tri-*O*-acetyl uridine (1)</u>



To a suspension of 2.15 g Uridine (8.8 mmol, 1 eq.) in 15 ml of acetic anhydride was added a catalytical amount of Iodine. The mixture was stirred at room temperature until all starting material was consumed (about 30 min) and further 15 minutes to complete the reaction (TLC control). Then aqueous sodium thiosulfate solution was added until the solution was completely decolorized. A bath with cold water was applied and three drops of concentrated sulfuric acid was added to decompose the excess of acetic anhydride. The mixture was stirred vigorously for 20 minutes until no further heat development indicated the completion of the reaction. The reaction mixture was portioned between water and chloroform, the water phase was extracted two times with chloroform, and the combined organic layers were thoroughly washed with water and finally with concentrated sodium hydrogen carbonate solution. After drying with anhydrous sodium sulfate, the solvent was removed and the remaining resin was dried in high vacuum to give the peracetylated uridine as a colourless foam.

yield: 3.00 g (7.1 mmol, 92 %)

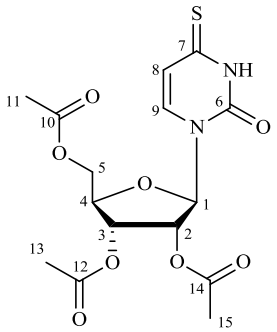
appearence: Colorless foam

R_f : 0.07 (cyclohexane : ethylic acetat [1:1]) / 0.4 (cyclohexane : ethylic acetat [1:4])

^{1}H-NMR: (300 MHz CDCl3) δ 9.95 (s, 1H, -N***H***), 7.37 (d, J = 8.1 Hz, 1H, H9), 5.99 (d, J = 4.6 Hz, 1H, H1), 5.74 (d, J = 8.2 Hz, 1H, H8), 5.35 – 5.24 (m, 2H, H2/H3), 4.29 (s, 3H, H4/H5/H5´), [2.08 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H) 9x H11+13+15] ppm.

^{13}C-NMR: 13C NMR (75 MHz, CDCl3) δ (170.22, 169.66 C10/12/14), 163.26 (C7), 150.45 (C6), 139.50 (C9), 103.35 (C8), 87.43 (C1), 79.83 (C4), (72.67, 70.15 C2/3), 63.17, 20.73 (C5), (20.47, 20.36 C11/13/15) ppm.

2,3,5-Tri-*O*-acetyl thiouridine (2)



2.00 g peracetyleted uridine (5.4 mmol, 1 eq.) and 2.40 g phosphorous pentasulfide (10.8 mmol, 2 eq.) were dissolved in 40 ml of pyridine and heated to reflux. After 30 min 0.1 ml of water was added und the mixture was refluxed for further 3.5 h. Upon cooling, excessing P_2S_5 participated as yellow solid and was removed by filtration. The filtered solids were washed with pyridin two times and the solvent was removed under reduced pressure. To the residue carefully water was added and stirred, until no further H_2S evolution was observed. During this process, the crude product participated as yellow solid which was separated by filtration, dissolved in chloroform and dried with anhydrous sodium sulfate. The amber glass like residue, which form upon solvent removal was dissolved in methanol and subjected to flash column chromatography (very short column, about 10 cm to remove any remaining polar components) with an eluent gradient cyclohexane/ ethylic acetate 1:1 to 1:4.

yield: 3.00 g (7.1 mmol, 92 %)

appearence: Colorless foam

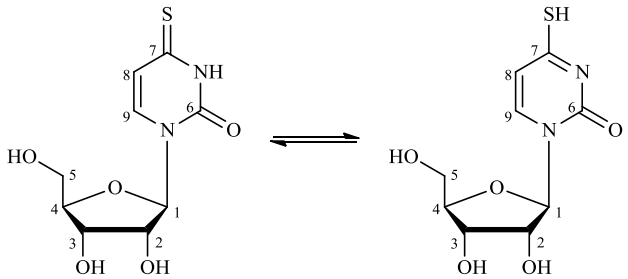
R_f : 0.33 (cyclohexane : ethylic acetat [1:1]) / 0.7 (cyclohexane : ethylic acetat [1:4])

^{1}H-NMR: (300 MHz CDCl3) δ 9.95 (s, 1H, -N***H***), 7.37 (d, J = 8.1 Hz, 1H, H9), 5.99 (d, J = 4.6 Hz, 1H, H1), 5.74 (d, J = 8.2 Hz, 1H, H8), 5.35 – 5.24 (m, 2H, H2/H3), 4.29 (s, 3H, H4/H5/H5´), [2.08 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H) 9x H11+13+15] ppm.

^{13}C-NMR: 13C NMR (75 MHz, CDCl3) δ (170.22, 169.66 C10/12/14), 163.26 (C7), 150.45 (C6), 139.50 (C9), 103.35 (C8), 87.43 (C1), 79.83 (C4), (72.67, 70.15 C2/3), 63.17, 20.73 (C5), (20.47, 20.36

C11/13/15) ppm.

Thiouridine (3)



2.04 g (5.2 mmol) peracetylated thiouridine (**2**) were dissolved in 3 ml methanol and mixed with 30 ml of concentrated NH_4OH and refluxed for 2 h until TLC indicated completion of the conversion. The mixture was then neutralized with diluted acetic acid (25 % in water) to approx. pH 6 and the solvents were evaporated under reduced pressure by co-evaporating with ethanol several times to give an orange oil, which can be directly used for the next step. However, a small amount was dissolved in hot isopropylic alcohol, which was mixed with pentane after cooling, which lead to participation of the product. After filtration and drying in high vacuum, the product is highly hygroscopic and electrostatically chargable. Furthermore it undergoes tautomery very fast (on the timescale of NMR-characterization). A reliable assignment to one of the two tautomeric forms is not possible on the basis of the available spectra alone.

Yield (crude): 1.22 g (4.7 mmol, 90 %)

appearence: Orange resin as crude product, pale yellow solid after recrystallization

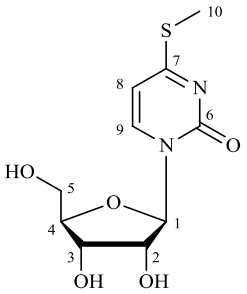
R_f : Not measured

^{1}H-NMR (of crude product): 1H NMR (300 MHz, DMSO-d6) δ 7.83 (d, J = 7.6 Hz, 1H, H9), 6.30 (d, J = 7.5 Hz, 1H, H8), 5.73 (d, J = 4.7 Hz, 1H, H1), 4.03 (t, J = 4.8 Hz, 1H, H2), 3.97 (t, J = 4.7 Hz, 1H, H3), 3.91 – 3.82 (m, 1H, H4), 3.65 (dd, J = 12.1, 3.0 Hz, 1H, H5), 3.56 (dd, J = 12.1, 3.2 Hz, 1H, H5´) ppm. Of note, that measured triplets for H3 and H4 in theory are dd, but splitting was not resolved by the used NMR

spectrometer.

^{13}C-NMR (of crude product): 13C NMR (75 MHz, DMSO) δ 190.35 (C7), 148.13 (C6), 135.95 (C9), 112.64 (C8), 88.57 (C1), 84.97 (C4), 74.00 (C2), 69.57 (C3), 60.53 (C5) ppm

<u>4-Methylsulfanyl-1 β-*D*-ribofuranosyl-1*H*-pyrimidin-2-one MS4U (4)</u>



To 1.22 g (4,7 mmol, 1 eq.) Thiouridine (**3**) in 25 ml 50 % ethanol 1.33 g methyl iodide (9.4 mmol, 584 µl, 2 eq.) and 4.7 ml 1 N NaOH were added and the mixture stirred at room temperature for 1 h. Then, the mixture was neutralized with diluted acetic acid (25 % in water) to approx. pH 6. After a short column (flash column chromatography, 10 cm, eluent chloroform/isopropanol 9:1 → pure isopropanol) the product was obtained as highly hygroscopic yellow foam.

yield: 915 mg (3.4 mmol, 71 %)

appearence: Highly hygroscopic yellow foam

R_f : 0.66 (isopropanol)

^{1}H-NMR: (300 MHz, Methanol-d4) δ 8.35 (d, J = 7.2 Hz, 1H, H9), 6.55 (d, J = 7.2 Hz, 1H, H8), 5.92 (d, J = 2.2 Hz, 1H, H1), 4.87 (s, 7H, H2), 4.24 – 4.19 (m, 2H, H2, H3), 4.13 (ddd, J = 6.4, 2.8 Hz, 1H, H4), 3.97 (dd, J = 12.4, 2.6 Hz, 1H, H5), 3.83 (dd, J = 12.4, 3.0 Hz, 1H, H5´), 2.57 (s, 3H, 3 x H10) ppm.

^{13}C-NMR: (75 MHz, MeOD) δ 180.58 (C7), 156.39 (C6), 142.05 (C9), 105.05

(C8), 92.67 (C1), 85.98 (C4), 76.53 (C2), 70.23 (C3), 61.44 (C5), 13.00 (C10) ppm.

NMR-spectra

Of compound (1) 2,3,5-Tri-*O*-acetyl uridine

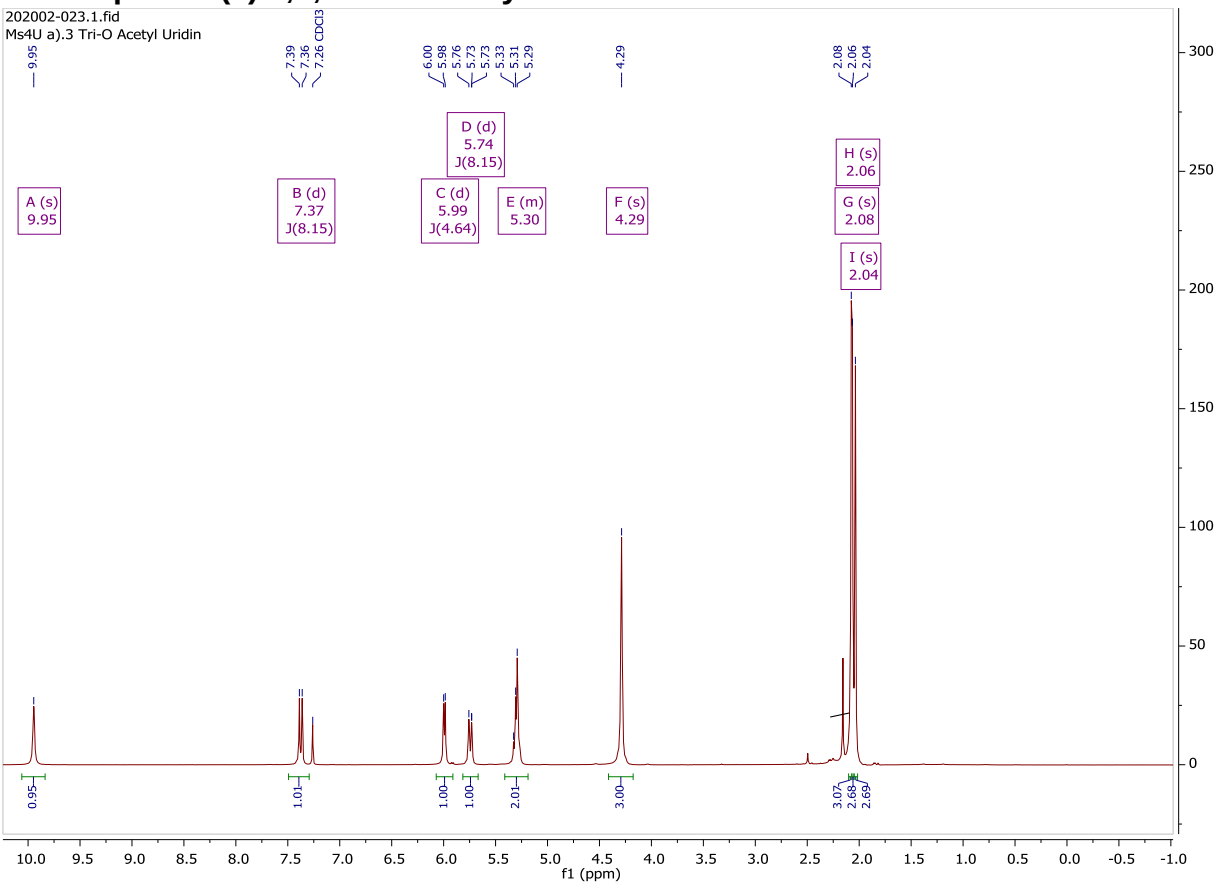


Figure S4 ^{1}H-NMR spectrum of (1)

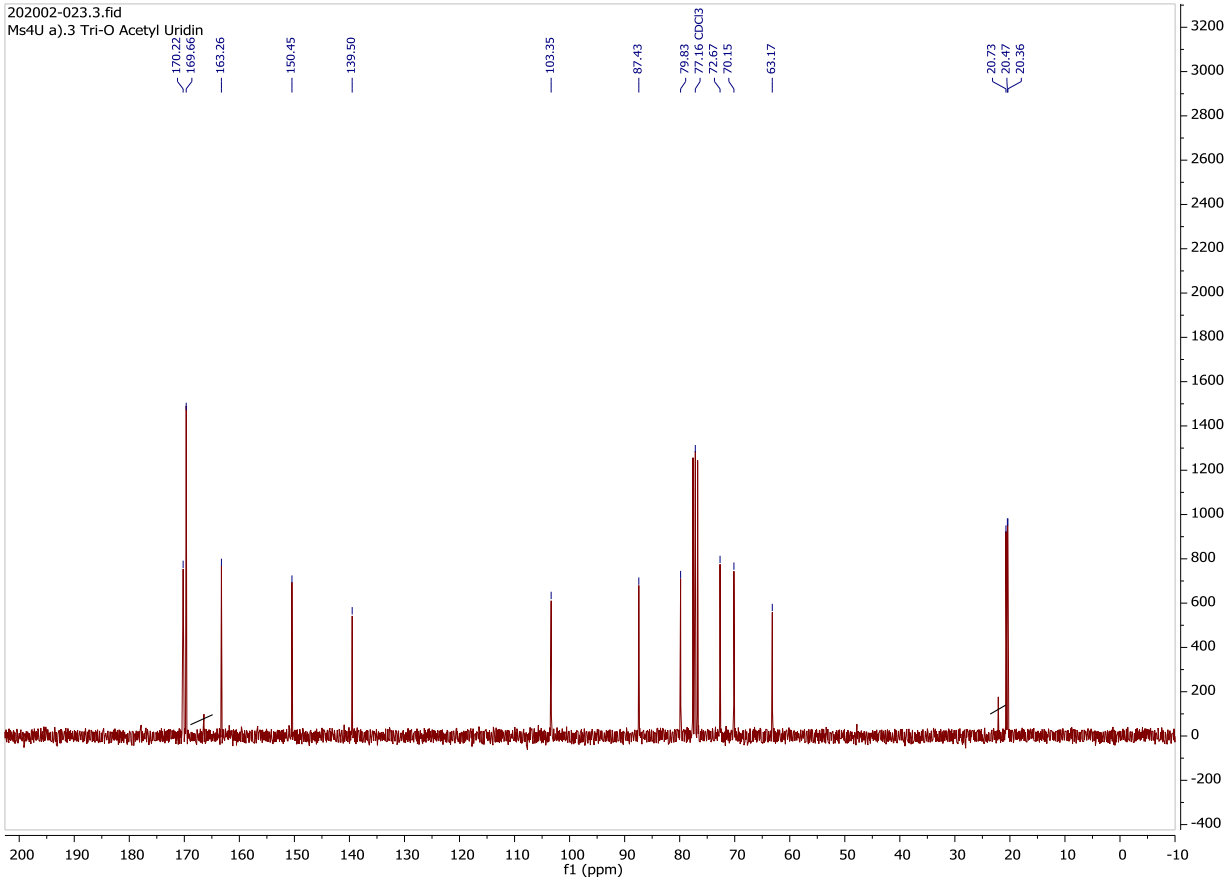


Figure S5 ^{13}C spectrum of (1)

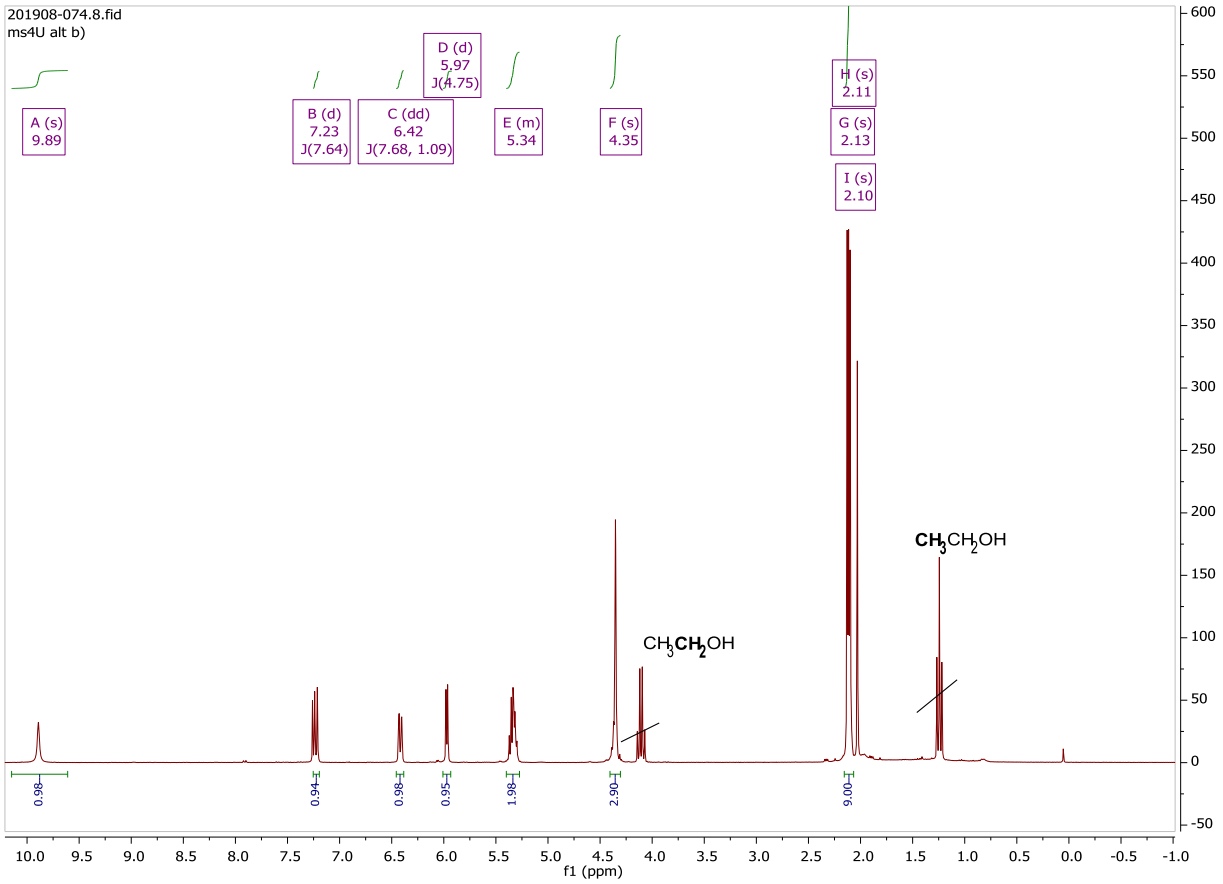


Figure S6 ^{1}H-NMR spectrum of (2)

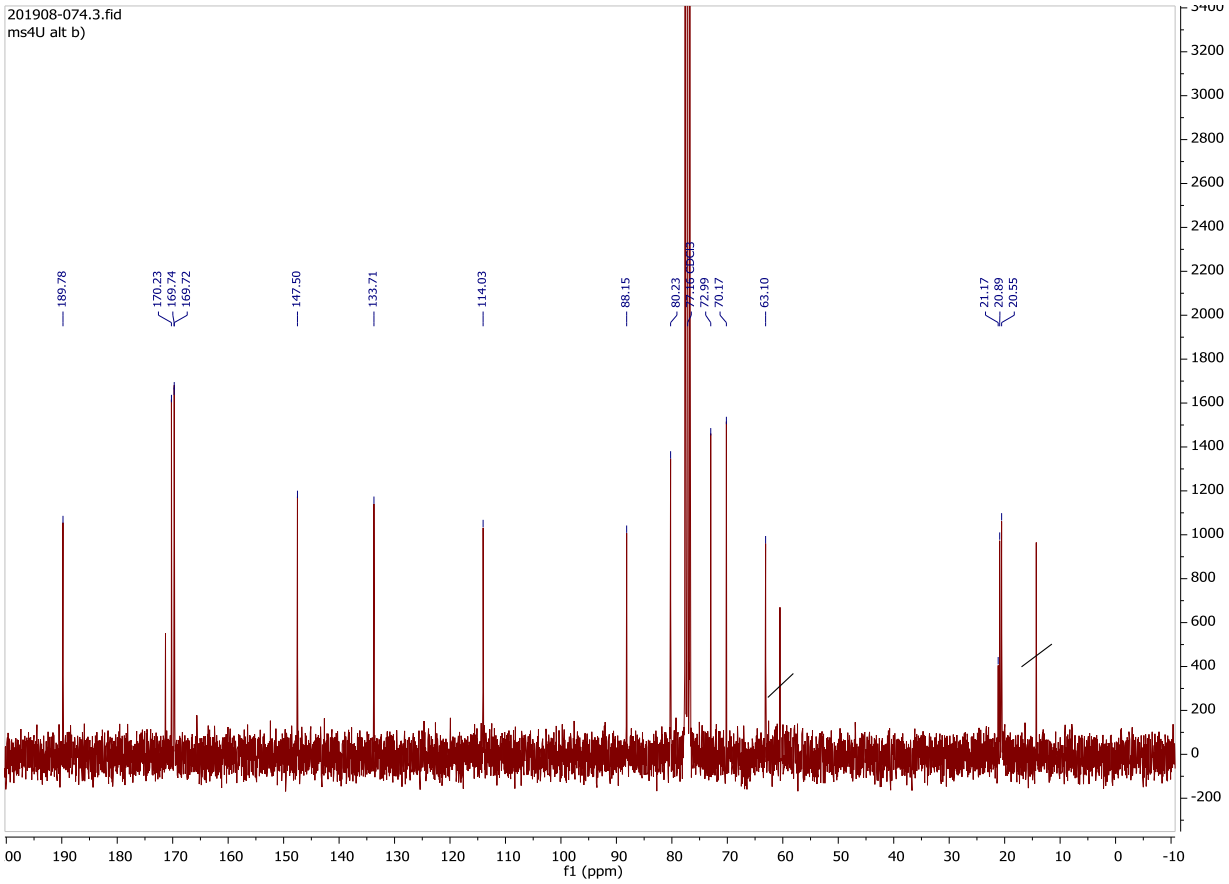


Figure S7 ^{13}C spectrum of (2)

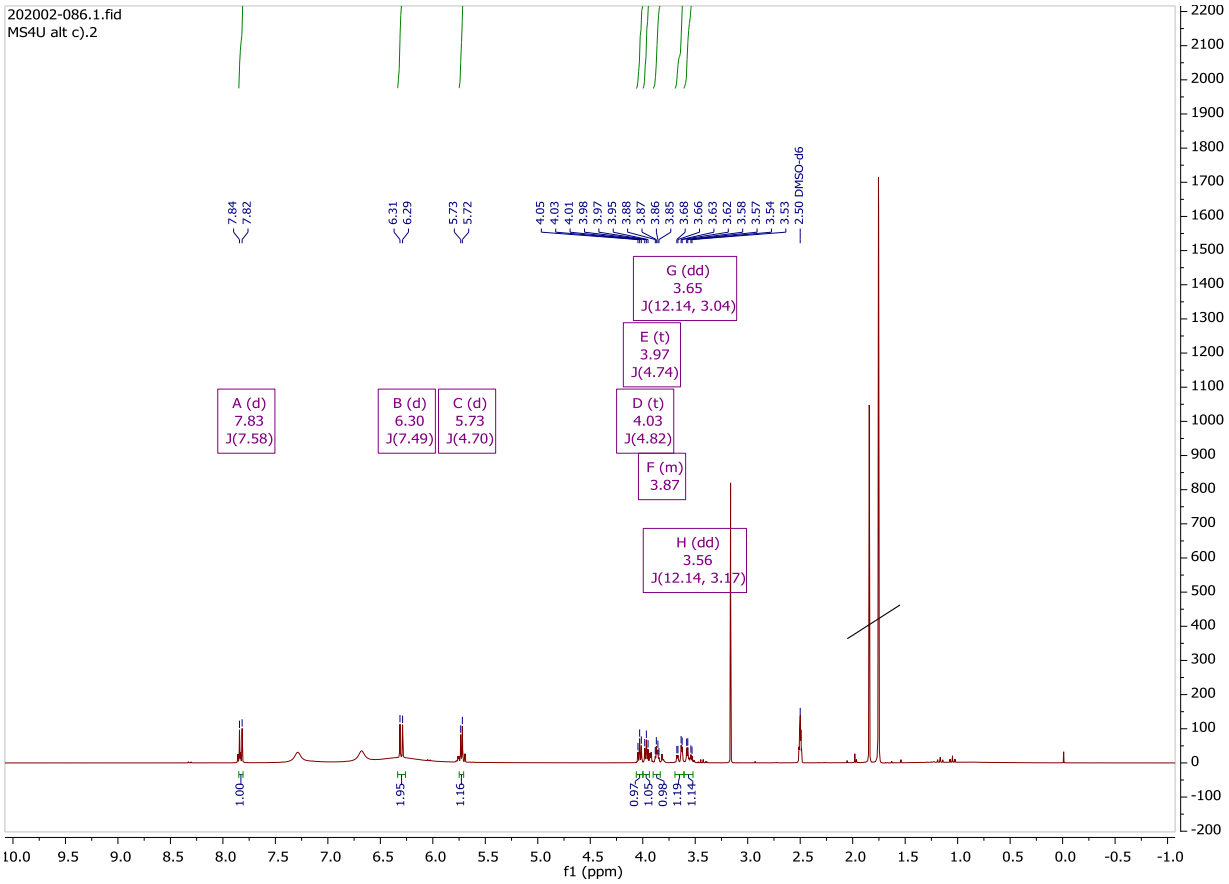


Figure S8 ^{1}H-NMR spectrum of (3)

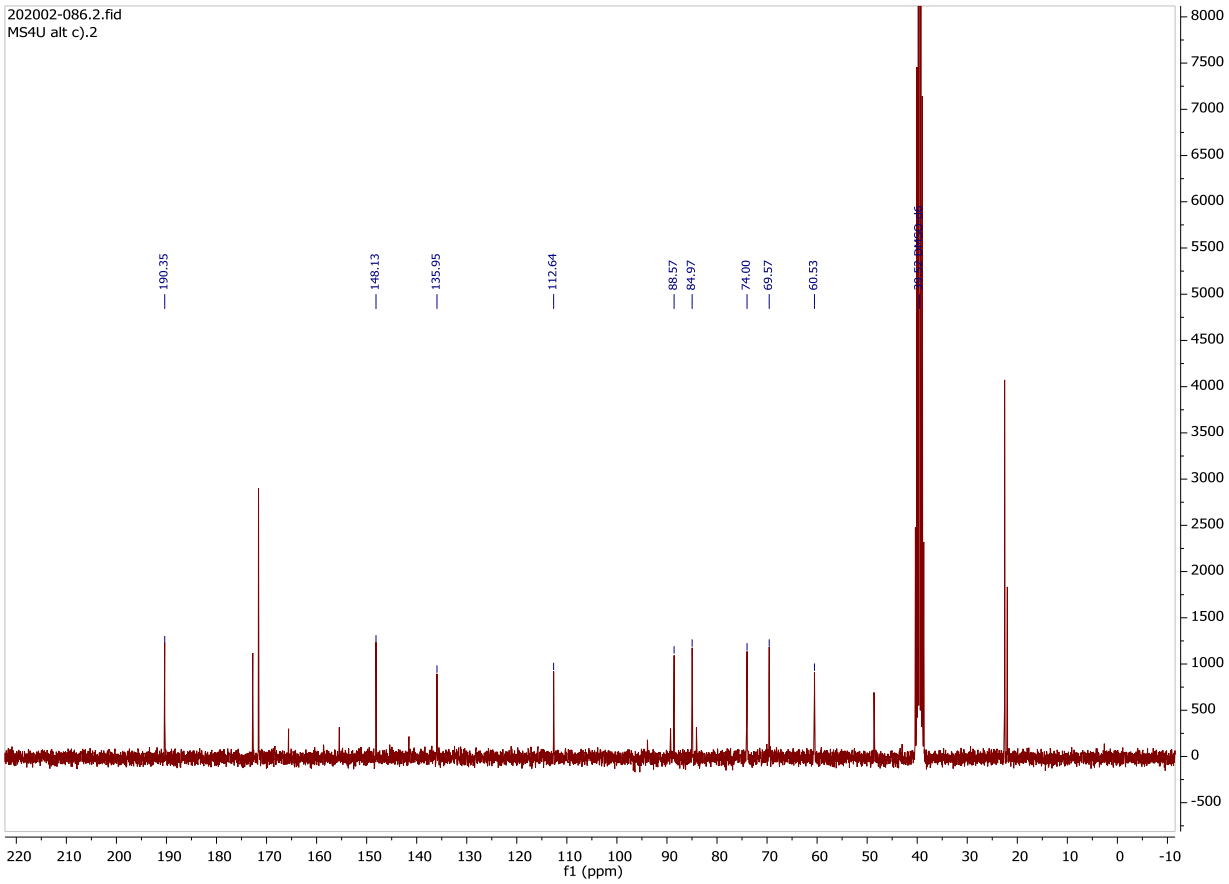


Figure S9 ^{13}C spectrum of (3)

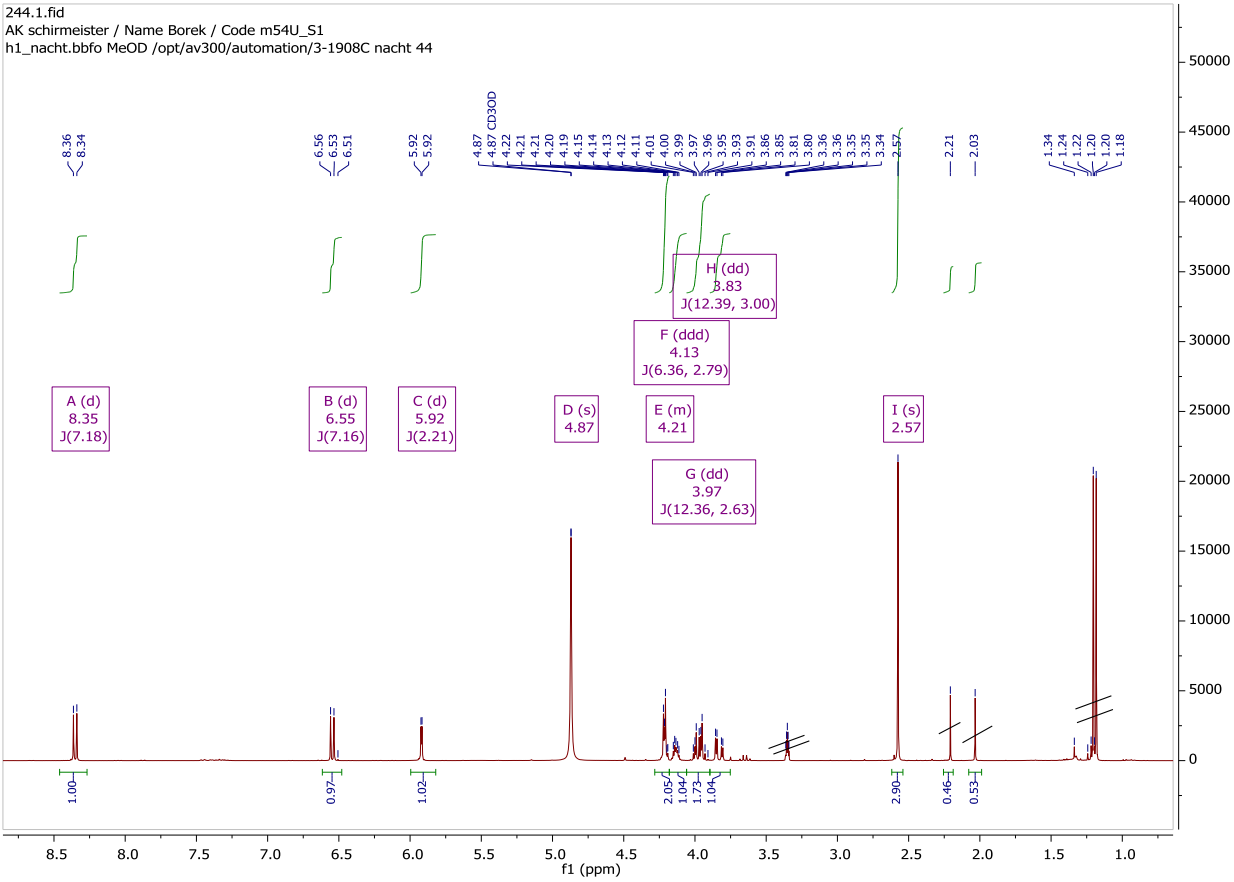


Figure S10 ^{1}H-NMR spectrum of (4)

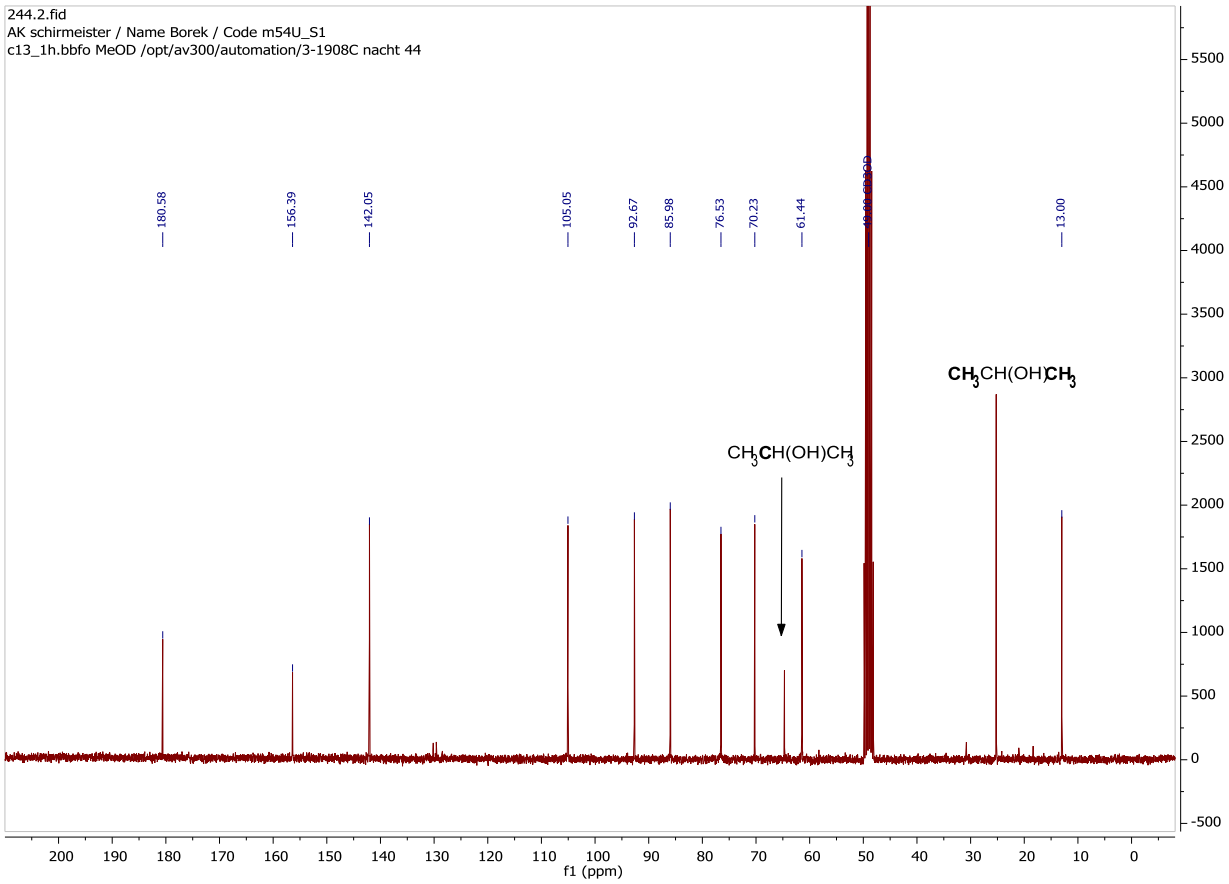


Figure S11 ^{13}C spectrum of (4)

[1] V. F. Reichle, D. P. Petrov, V. Weber, K. Jung, S. Kellner, *Nature communications* **2019**, *10*, 5600.

[2] T. Baba, T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, H. Mori, *Molecular systems biology* **2006**, *2*, 2006.0008.

[3] V. F. Reichle, V. Weber, S. Kellner, *Chembiochem : a European journal of chemical biology* **2018**, *19*, 2575-2583.

CHEMBIOCHEM

Supporting Information

NAIL-MS in *E. coli* Determines the Source and Fate of Methylation in tRNA

Valentin F. Reichle, Verena Weber, and Stefanie Kellner*[a]

cbic_201800525_sm_miscellaneous_information.pdf

Supplementary Information

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Figure S5: Dilution of original tRNA with newly built tRNA.

Figure S6: m1G and m5U abundance of MMS stressed and unstressed bacteria over time.

Table S1: Dynamic MRM data from QQQ measurement (in vitro MMS studies)

Table S2: Dynamic MRM data from QQQ measurement (methylome discrimination assay)

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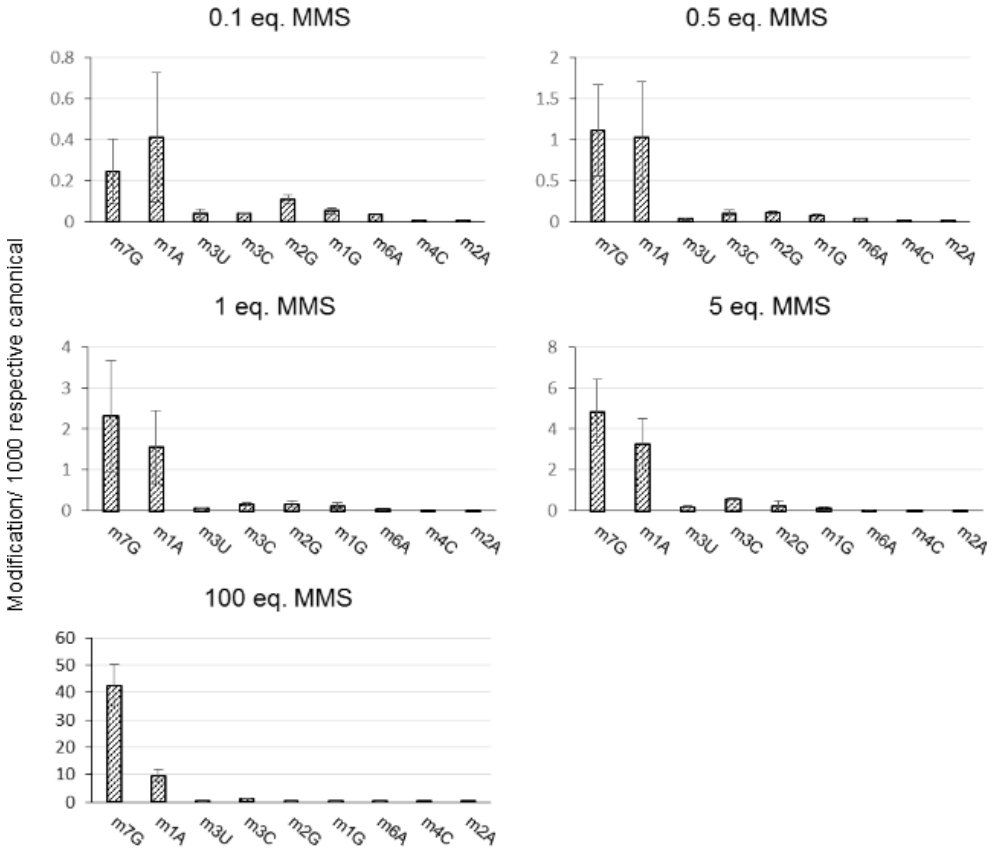

Figure S1 MMS damage in vitro on different nucleosides with different MMS concentrations. The values show the amount of single modification per 1000 respective canonical (precursor canonical of the modification). Abbreviations: m^7G = 7-methylguanosine, m^1A = 1-methyladenosine, m^3U = 3-methyluridine, m^3C = 3-methylcytidine, m^2G = N2-methylguanosine , m^1G = 1-methylguanosine, m^6A = 6-methyladenosine , m^4C = 4-methylcytidine, m^2A = 2-methyladenosine.

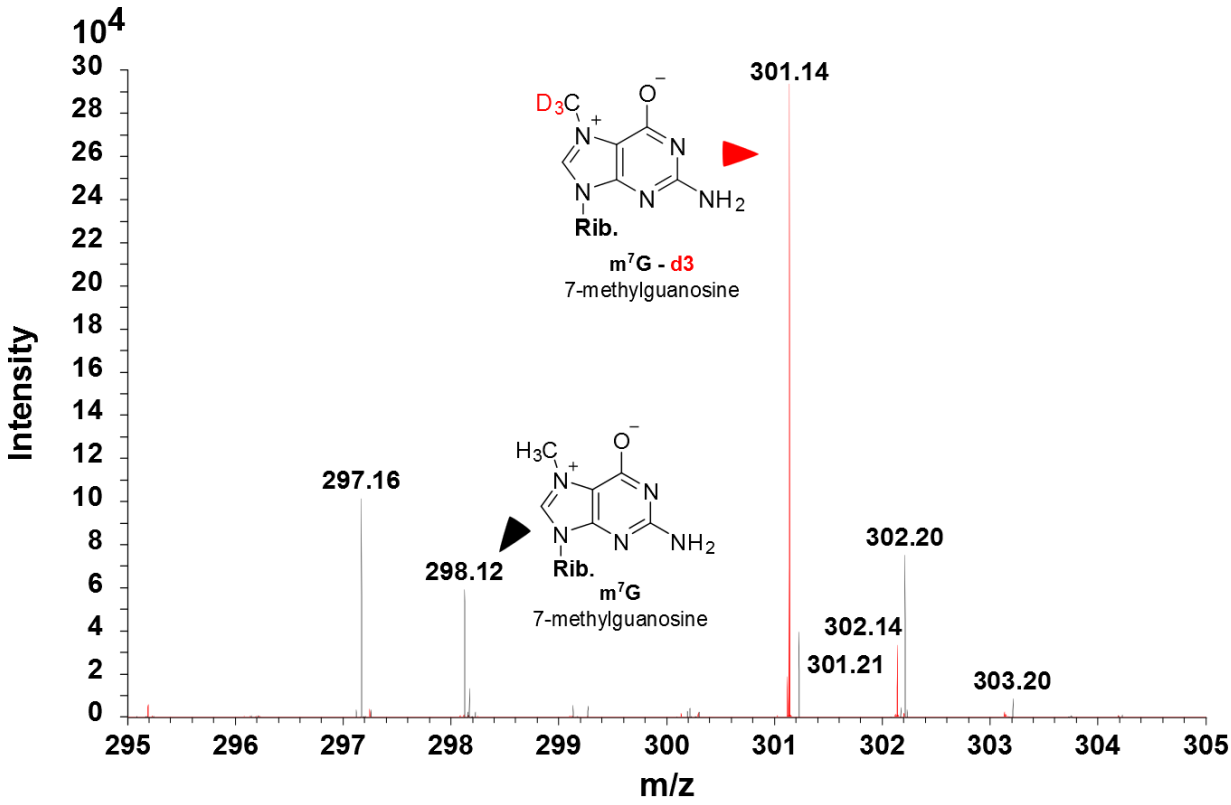

Figure S2 Full MS spectrum of E.coli RNA digest grown in methionine-d3 labeled medium (red) or unlabeled M9 medium (black). The shown spectra were averaged from 7.5 to 8.5 minutes and show a m/z window from 295 to 305. One can see the shift of three Dalton in m^7G because of the deuterated methyl group and there is also a high efficiency in CD3 labeling. Abbreviations: m^7G = 7-methylguanosine

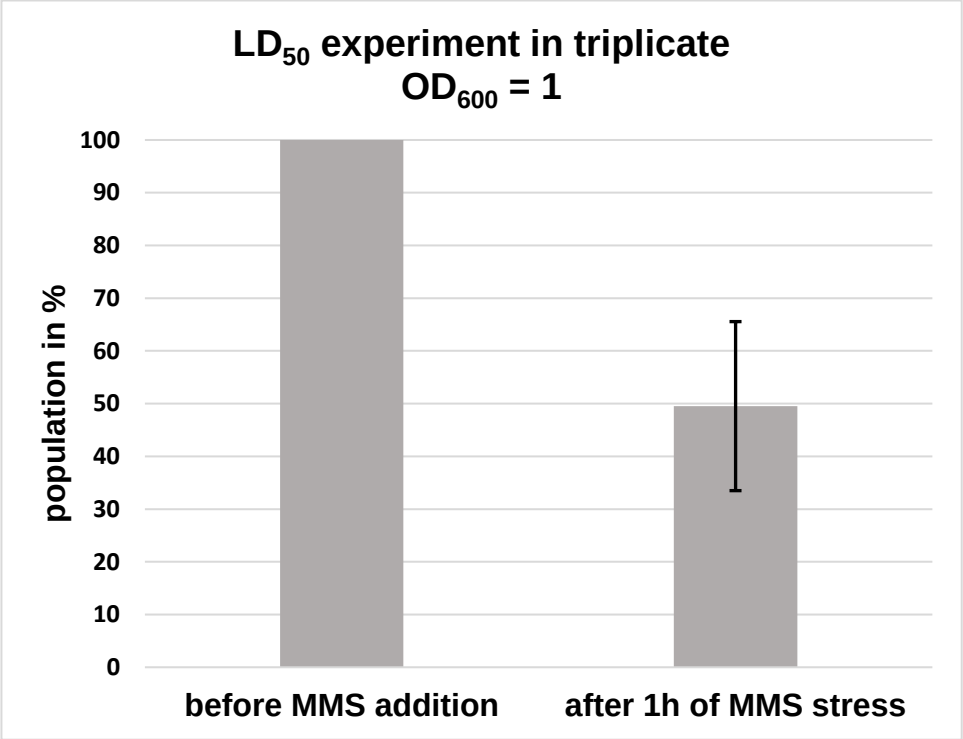

Figure S3 Diagram of LD_{50} assay with E. coli wildtype strain BW25113. The experiment was started with an OD_{600} = 1 and after one hour of growing the bacteria were exposed to 20 mM MMS alkylation stress for one hour. A bacteria dilution of 10^{-4} was plated on LB – agar plates and incubated at 37°C. The next day colony counting showed how many bacteria survived the stress. The experiment was done in triplicate.

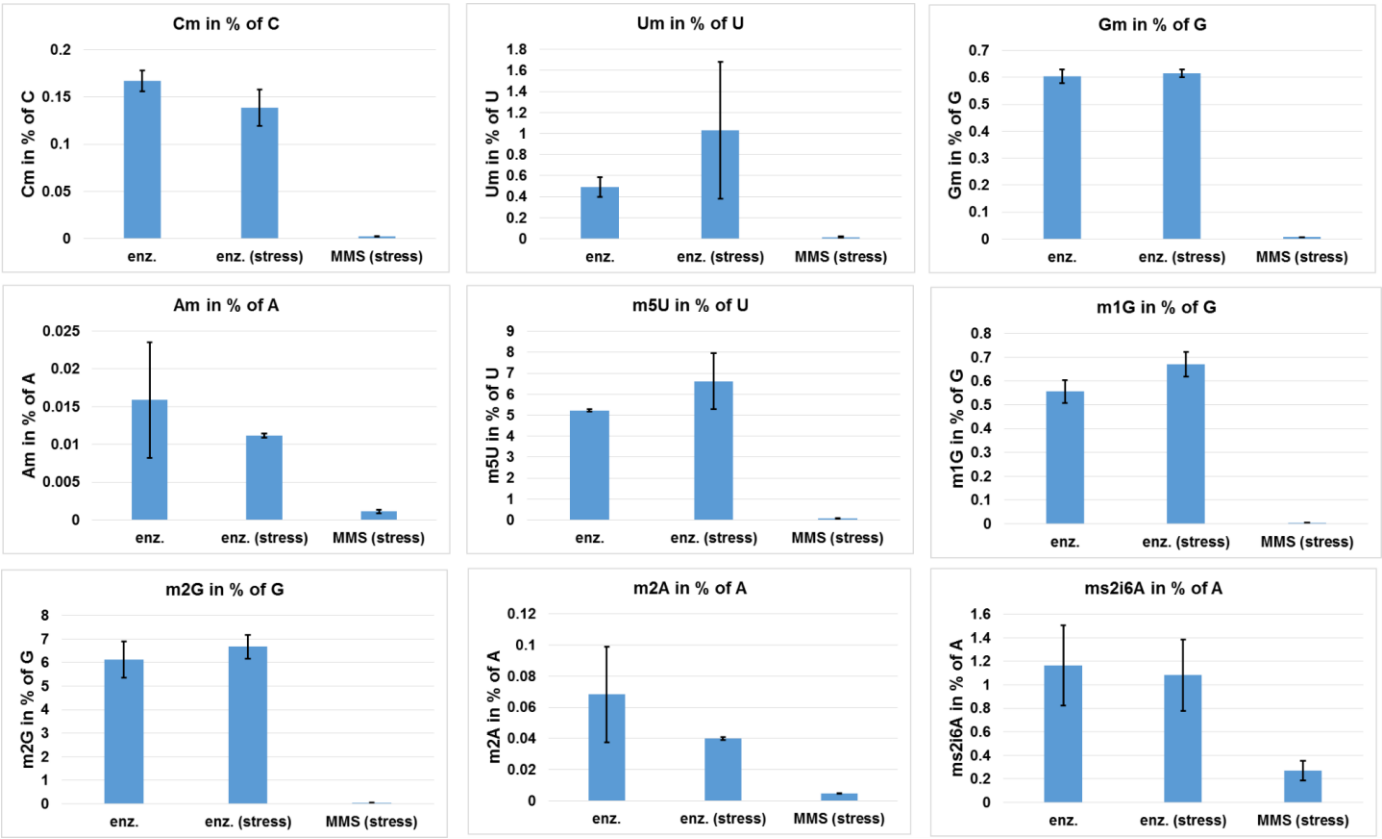

Figure S4: influence of MMS stress on other known tRNA modifications. The LOQ of the respective modifications are for m^7G<0.0008%, m^1A<0.0008%, m^6A<0.0008%, m^3U=0.008%, m^3C=0.0016%, Cm=0.005%, Um=0.08%, Gm=0.013%, Am<0.001%, m^5U=0.08%, m^1G=0.003%, m^2G=0.053%, m^2A=0.002%, ms^2i^6A=0.28%.

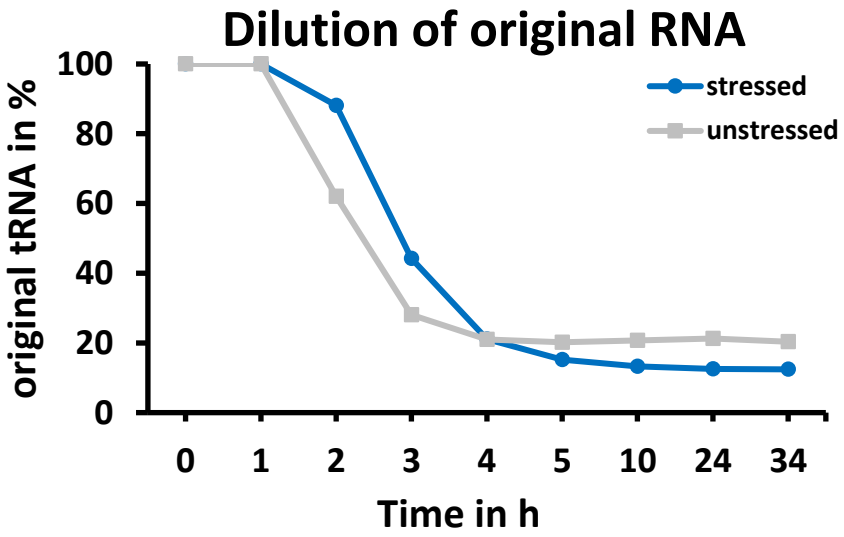

Figure S5: Dilution of original tRNA with newly transcribed tRNA. It seems that in MMS stressed cells (blue) the dilution process is slower than in the unstressed cells (grey). Therefore, it is unlikely that the damaged tRNA degrade more quickly.

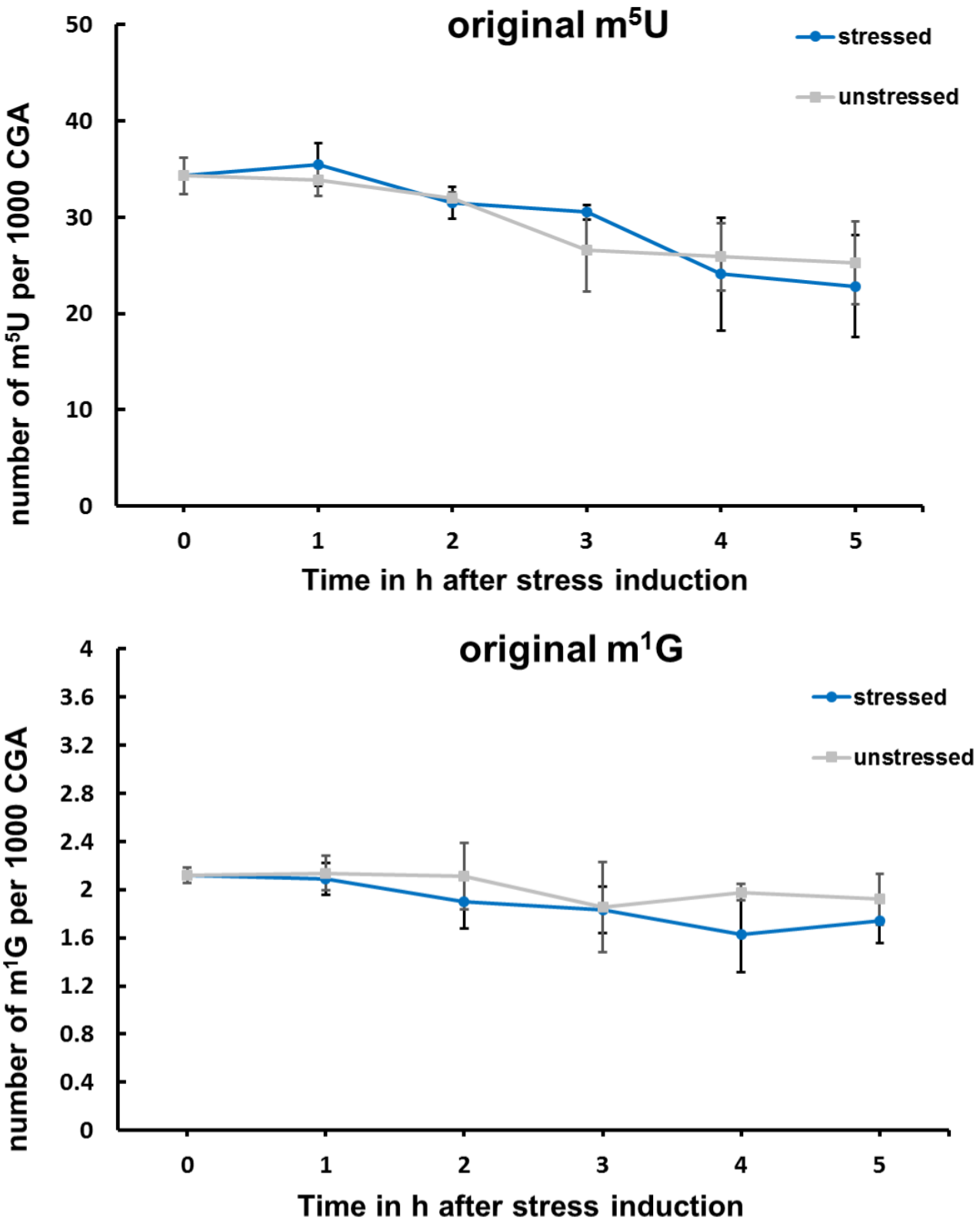

Figure S6: m^5U and m^1G levels of MMS stressed (blue) and unstressed (grey) bacteria over time. After one hour the bacteria were still in exponential growth phase whereas after 5 hours the cells reached stationary phase. One can see the modification levels are decreasing from one state to the other.

Table S1: Dynamic MRM data from QQQ measurement (in vitro MMS studies) . Abbreviations: A = adenosine, C = cytosine, U = uridine, G = guanosine, m^2A = 2-methyladenosine, m^1A = 1-methyladenosine, m^2G = N2-methylguanosine, m^3C = 3-methylcytidine, m^3U = 3-methyluridine, m^1G = 1-methylguanosine, m^6A = 6-methyladenosine, m^7G = 7-methylguanosine, m^5C = 5-methylcytidine, s^4U = 4-thiouridine, m^4C = 4-methylcytidine.

Compound Group	Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
unlabeled	A	268	136	5.3	1	100	40	5	Positive
	C	244	112	1.7	1	100	40	5	Positive
	U	245	113	3	3	100	30	5	Positive
	G	284	152	4.2	1	100	40	5	Positive
	m2A	282	150	6	1	100	10	5	Positive
	m1A	282	150	2.7	3	110	21	5	Positive
	m2G	298	166	4.7	1	95	17	5	Positive
	m3C	258	126	2.4	3	88	14	5	Positive
	m3U	259	127	4.7	1	100	10	5	Positive
	m1G	298	166	4.5	2	105	13	5	Positive
	m6A	282	150	6.2	1	120	20	5	Positive
	m7G	298	166	3.3	1	105	14	5	Positive
	m5C	258	126	3.5	1	185	13	5	Positive
	s4U	261	129	4.5	1	75	17	5	Positive
	m4C	258	126	3.4	1	130	13	5	Positive
SILIS (13C labeled)	A SILIS	283	146	5.3	1	100	40	5	Positive
	C SILIS	253	116	1.7	1	100	40	5	Positive
	U SILIS	254	117	3	2	100	30	5	Positive
	G SILIS	294	157	4.2	1	100	40	5	Positive
	m2A SILIS	295	158	6.4	1	100	10	5	Positive
	m1A SILIS	295	158	2.7	3	110	21	5	Positive
	m2G SILIS	311	174	4.7	1	95	17	5	Positive
	m3C SILIS	270	133	2.4	3	88	14	5	Positive
	m3U SILIS	271	134	4.7	1	100	10	5	Positive
	m1G SILIS	311	174	4.5	1	105	13	5	Positive
	m6A SILIS	295	158	6.2	1	120	20	5	Positive
	m7G SILIS	311	174	3.3	1	105	14	5	Positive
	m5C SILIS	270	133	3.5	1	185	13	5	Positive
	m4C SILIS	270	133	3.4	1	130	13	5	Positive

Table S2: Dynamic MRM data from QQQ measurement (methylome discrimination assay) . Abbreviations: A = adenosine, C = cytosine, Cm = 2'-O-methylcytidine, G = guanosine, Gm = 2'-O-methylguanosine, m^1A = 1-methyladenosine, m^1G = 1-methylguanosine, m^2G = N2-methylguanosine, m^3C = 3-methylcytidine, m^3U = 3-methyluridine, m^5U = 5-methyluridine, m^6A = 6-methyladenosine, m^7G = 7-methylguanosine, U = uridine.

Compound Group	Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
unlabeled	A	268	136	5.711	1	380	40	5	Positive
	C	244	112	1.73	1	380	40	5	Positive
	Cm	258	112	3.347	3	380	10	5	Positive
	G	284	152	3.718	3	380	40	5	Positive
	Gm	298	152	5.133	1	380	10	5	Positive
	m1A	282	150	1.745	2	380	10	5	Positive
	m1G	298	166	5.08	1	380	10	5	Positive
	m2G	298	166	5.491	2	380	10	5	Positive
	m3C	258	126	1.752	1	380	10	5	Positive
	m3U	259	127	4.696	2	380	10	5	Positive
	m5U	259	127	3.915	1	380	10	5	Positive
	m6A	282	150	8.423	1	380	10	5	Positive
	m7G	298	166	2.021	2	380	10	5	Positive
	U	245	113	1.876	1	380	40	5	Positive
SILIS (15N and 13C labeled)	A SILIS	283	146	5.711	1	380	40	5	Positive
	C SILIS	256	119	1.73	1	380	40	5	Positive
	Cm SILIS	271	119	3.347	3	380	10	5	Positive
	G SILIS	299	162	3.718	3	380	40	5	Positive
	Gm SILIS	314	162	5.133	1	380	10	5	Positive
	m1A SILIS	298	161	1.745	2	380	10	5	Positive
	m1G SILIS	314	177	5.08	1	380	10	5	Positive
	m2G SILIS	312	175	5.491	2	380	10	5	Positive
	m3C SILIS	271	134	1.752	1	380	10	5	Positive
	m3U SILIS	271	134	4.696	2	380	10	5	Positive
	m5U SILIS	271	134	3.915	1	380	10	5	Positive
	m6A SILIS	298	161	8.423	1	380	10	5	Positive
	m7G SILIS	314	177	2.021	2	380	10	5	Positive
	U SILIS	256	119	1.876	1	380	40	5	Positive
CD3 (methionine-d3 labeled)	Cm CD3	261	112	3.347	3	380	10	5	Positive
	Gm CD3	301	152	5.133	1	380	10	5	Positive
	m1A CD3	285	153	1.745	2	380	10	5	Positive
	m1G CD3	301	169	5.08	1	380	10	5	Positive
	m2G CD3	301	169	5.491	2	380	10	5	Positive
	m3C CD3	261	129	1.752	1	380	10	5	Positive
	m3U CD3	262	130	4.696	2	380	10	5	Positive
	m5U CD3	262	130	3.915	1	380	10	5	Positive
	m6A CD3	285	153	8.423	1	380	10	5	Positive
	m7G CD3	301	169	2.021	2	380	10	5	Positive

Table S3: Dynamic MRM data from QQQ measurement (Pulse Chase experiment). Modification m1G was analyzed by mass transition of m2G because of the close retention times and the same molecular mass and MS2 fragmentation. Abbreviations: A = adenosine, C = cytosine, Cm = 2'-O-methylcytidine, G = guanosine, Gm = 2'-O-methylguanosine, m^1A = 1-methyladenosine, m^1G = 1-methylguanosine, m^2G = N2-methylguanosine, m^3C = 3-methylcytidine, m^3U = 3-methyluridine, m^5U = 5-methyluridine, m^6A = 6-methyladenosine, m^7G = 7-methylguanosine, U = uridine

Compound Group	Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
unlabeled	A	268	136	5.711	1	110	21	5	Positive
	C	244	112	1.73	1	175	13	5	Positive
	Cm	258	112	3.347	3	180	9	5	Positive
	G	284	152	3.718	3	95	17	5	Positive
	Gm	298	152	4.78	1	100	9	5	Positive
	m1A	282	150	1.745	2	110	21	5	Positive
	m2G	298	166	4.85	3	100	15	5	Positive
	m3C	258	126	1.752	2	88	14	5	Positive
	m3U	259	127	4.2	1	75	9	5	Positive
	m5U	259	127	3.61	2	145	10	5	Positive
	m6A	282	150	7.85	3	120	20	5	Positive
	m7G	298	166	2.7	2	105	14	5	Positive
	U	245	113	1.876	2	95	5	5	Positive
SILIS (15N and 13C labeled)	A SILIS	283	146	5.711	1	110	21	5	Positive
	C SILIS	256	119	1.73	1	175	13	5	Positive
	Cm SILIS	271	119	3.347	3	180	9	5	Positive
	G SILIS	299	162	3.718	3	95	17	5	Positive
	Gm SILIS	314	162	4.78	1	100	9	5	Positive
	m1A SILIS	298	161	1.745	2	110	21	5	Positive
	m2G SILIS	314	177	4.85	3	100	15	5	Positive
	m3C SILIS	271	134	1.752	2	88	14	5	Positive
	m3U SILIS	271	134	4.2	1	75	9	5	Positive
	m5U SILIS	271	134	3.61	2	145	10	5	Positive
	m6A SILIS	298	161	7.85	3	120	20	5	Positive
	m7G SILIS	314	177	2.7	2	105	14	5	Positive
	U SILIS	256	119	1.876	2	95	5	5	Positive
CD3 (methionine-d3 labeled)	Cm CD3	261	112	3.347	3	180	9	5	Positive
	Gm CD3	301	152	4.78	1	100	9	5	Positive
	m1A CD3	285	153	1.745	2	110	21	5	Positive
	m2G CD3	301	169	4.85	3	100	15	5	Positive
	m3C CD3od15N	261	129	1.752	2	88	14	5	Positive
	m3U CD3	262	130	4.2	1	75	9	5	Positive
	m5U CD3	262	130	3.61	2	145	10	5	Positive
	m6A CD3	285	153	7.85	3	120	20	5	Positive
	m7G CD3	301	169	2.7	2	105	14	5	Positive

15N labeled	A 15N	273	141	5.711	1	110	21	5	Positive
	C 15N	247	115	1.73	1	175	13	5	Positive
	Cm 15N	261	115	3.347	3	180	9	5	Positive
	G 15N	289	157	3.718	3	95	17	5	Positive
	Gm 15N	303	157	4.78	1	100	9	5	Positive
	m1A 15N	287	155	1.745	2	110	21	5	Positive
	m2G 15N	303	171	4.85	3	100	15	5	Positive
	m3U 15N	261	129	4.2	1	75	9	5	Positive
	m5U 15N	261	129	3.61	2	145	10	5	Positive
	m6A 15N	287	155	7.85	3	120	20	5	Positive
	m7G 15N	303	171	2.7	2	105	14	5	Positive
	U 15N	247	115	1.876	2	95	5	5	Positive
15N + CD3 labeled	Cm 15N_CD3	264	115	3.347	3	180	9	5	Positive
	Gm 15N_CD3	306	157	4.78	1	100	9	5	Positive
	m1A 15N_CD3	290	158	1.745	2	110	21	5	Positive
	m2G 15N_CD3	306	174	4.85	3	100	15	5	Positive
	m3C 15N_CD3	264	132	1.752	2	88	14	5	Positive
	m3U 15N_CD3	264	132	4.2	1	75	9	5	Positive
	m5U 15N_CD3	264	132	3.61	2	145	10	5	Positive
	m6A 15N_CD3	290	158	7.85	3	120	20	5	Positive
	m7G 15N_CD3	306	174	2.7	2	105	14	5	Positive

Supplement

Observing the fate of tRNA and its modifications by nucleic acid isotope labeling mass spectrometry: NAIL-MS

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Contact corresponding author: stefanie.kellner@cup.lmu.de

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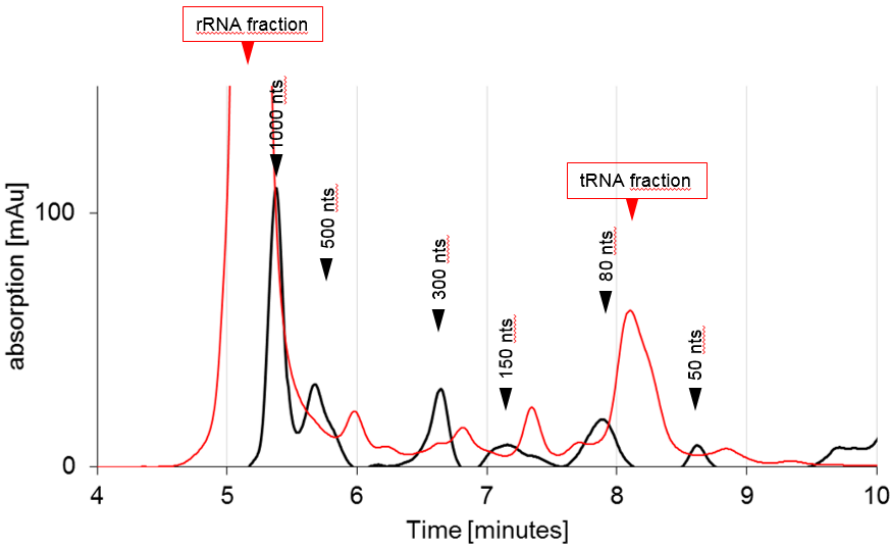
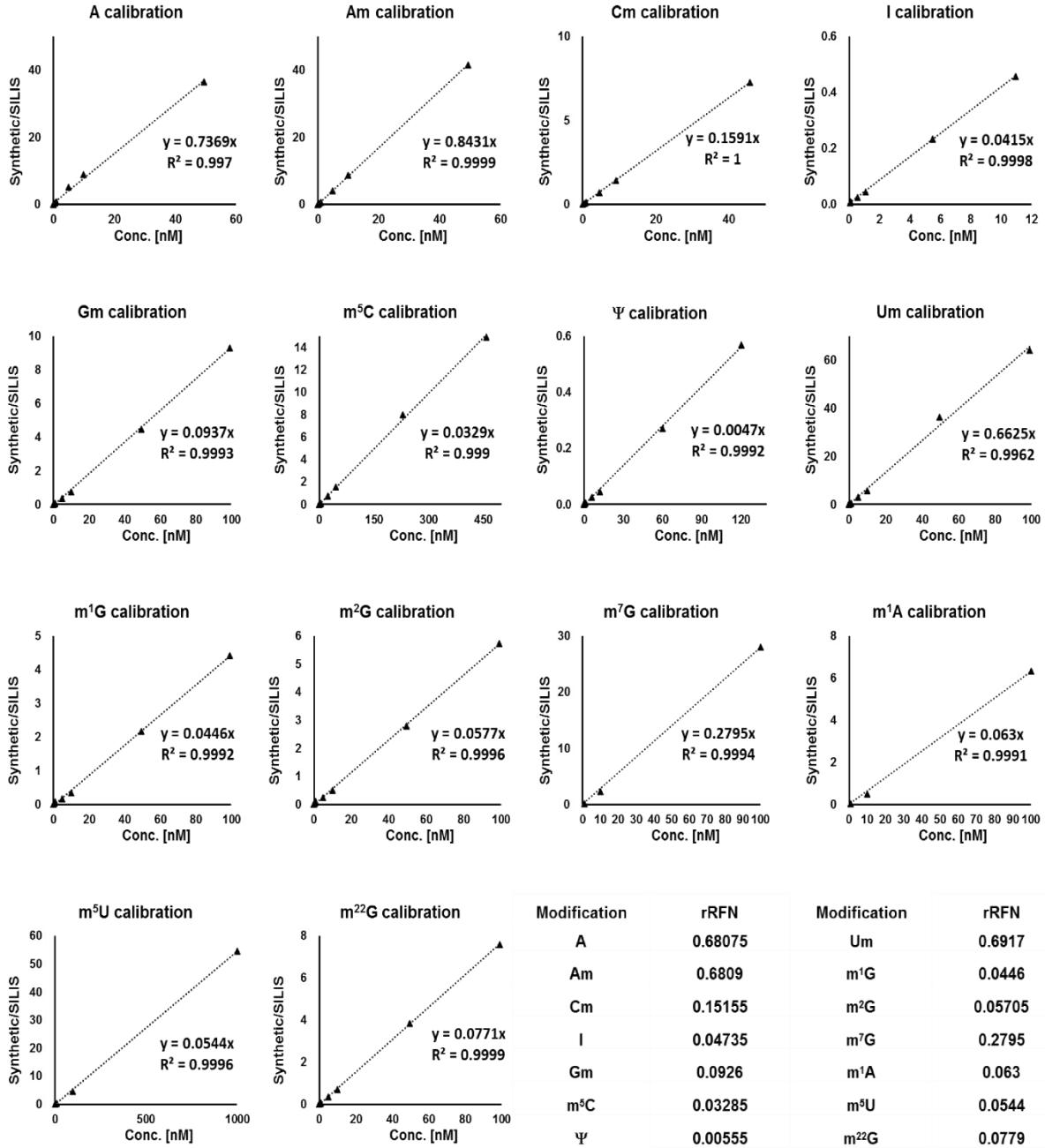


Figure S1 Size-exclusion chromatogram of total RNA from yeast (red) and an oligonucleotide ladder (black). The large RNA fraction (labeled as rRNA fraction) contains all RNAs larger than 1000 nucleotides. tRNA elutes as expected between the 80 and 50 nucleotide marker.



Modification	rRFN	Modification	rRFN
A	0.68075	Um	0.6917
Am	0.6809	m^1G	0.0446
Cm	0.15155	m^2G	0.05705
I	0.04735	m^7G	0.2795
Gm	0.0926	m^1A	0.063
m^5C	0.03285	m^5U	0.0544
Ψ	0.00555	$m^{22}G$	0.0779

Figure S2 Calibration curves of all detected nucleosides. The vertical axis shows the relative value of the signal area of the synthetic nucleosides and the area of the corresponding SILIS signals. On the horizontal axes the concentrations of the synthetic nucleosides are shown. In the table on the bottom the relative response factors for the nucleosides (rRFN) are listed, calculated from the slopes of the regression lines of two replicates. Abbreviations: A = adenosine, Am = 2'-O-methyladenosine, Cm = 2'-O-methylcytidine, I = Inosine, Gm = 2'-O-methylguanosine, m^5C = 5-methylcytidine, Ψ = pseudouridine, Um = 2'-O-methyluridine, m^1G = 1-methylguanosine, m^2G = N2-methylguanosine, m^7G = 7-methylguanosine, m^1A = 1-methyladenosine, m^5U = 5-methyluridine, $m^{22}G$ = N2,N2-dimethylguanosine.

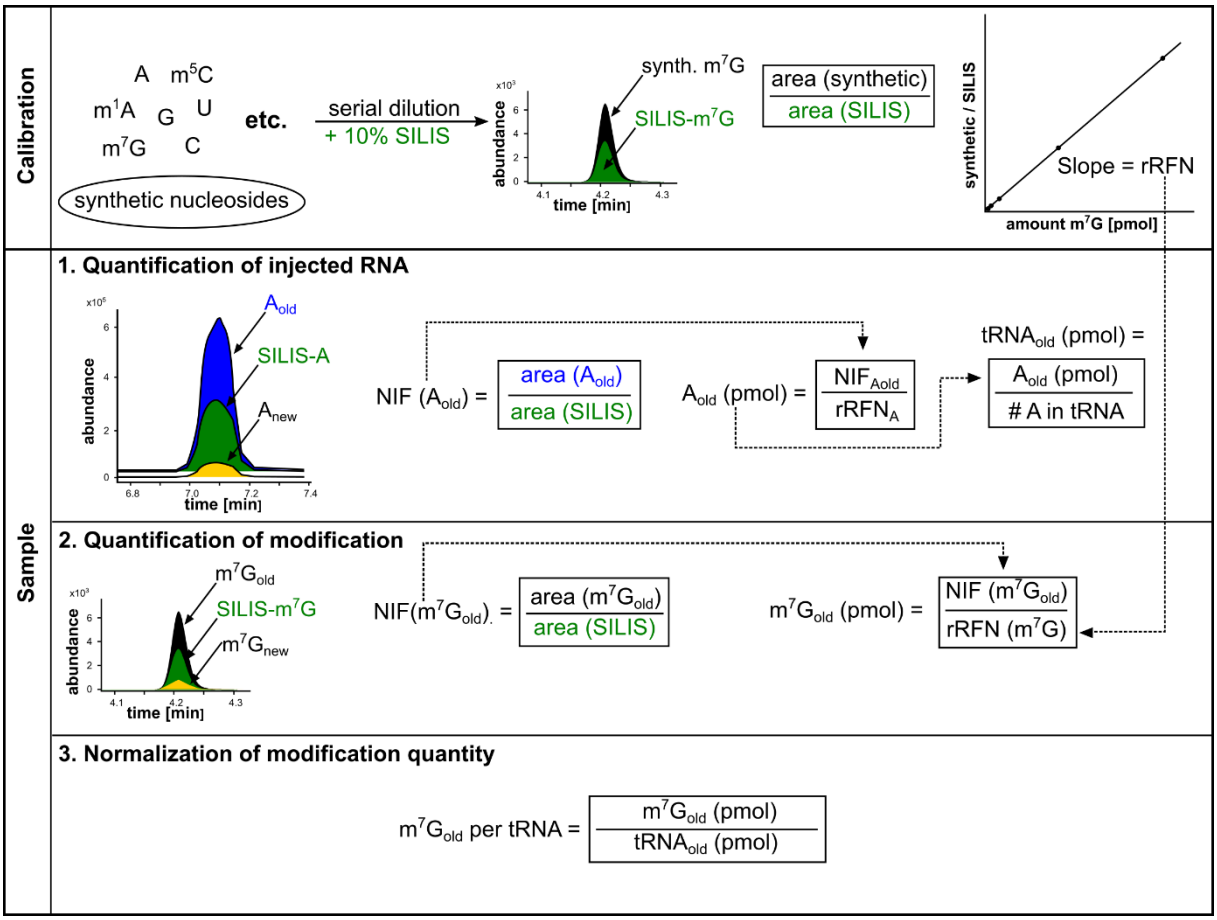


Figure S3 Numeric conversion steps for absolute quantification of tRNA modifications shown for 7-methylguanosine (m^7G). Calibration is performed with synthetic standards. The ratio of synthetic standard to SILIS area is plotted over the amount of injected synthetic standard. The slope of the calibration curve is the relative response factor nucleoside (rRFN) and is done for all calibrated nucleosides (see Figure S2). The amount of injected RNA and modifications is quantified by calculation of the nucleoside-isotope-factor (NIF) which is subsequently devided by the rRFN to reveal the amounts in pmol. The amount of injected modification is normalized to the amount of injected tRNA by dividing through the amount of injected tRNA. The amount of injected tRNA depends on the number of adenosine (A) present in an average tRNA (also see Table S1).

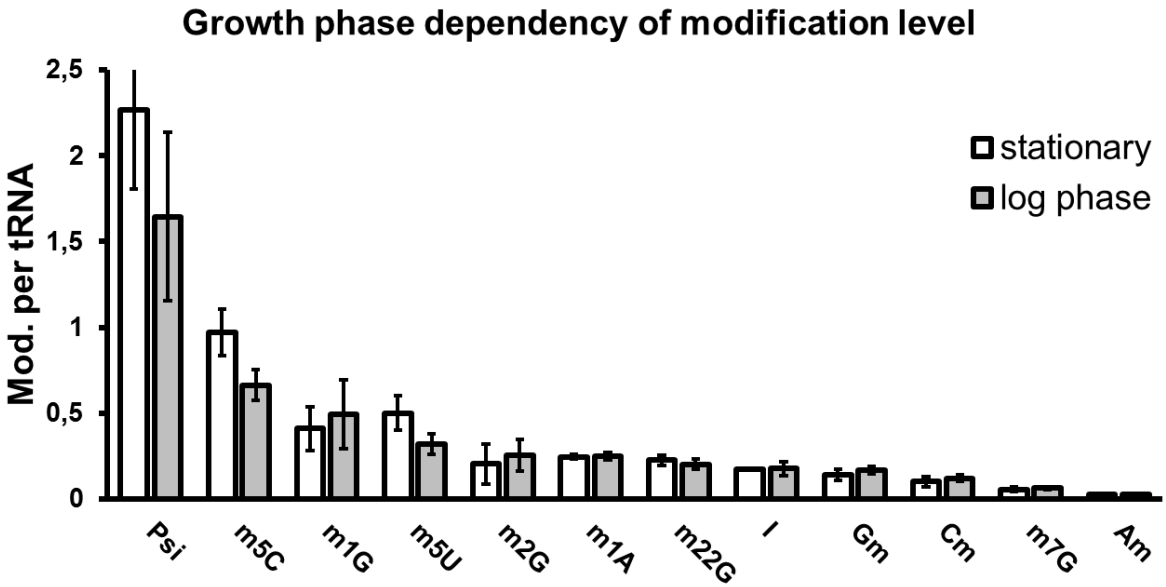


Figure S4 Absolute levels of modified nucleosides in total tRNA during a yeast growth cycle. Abbreviations: Am = 2'-O-methyladenosine, I = Inosine, Gm = 2'-O-methylguanosine, m^5C = 5-methylcytidine, Ψ = pseudouridine, Um = 2'-O-methyluridine, m^1G = 1-methylguanosine, m^2G = N2-methylguanosine, m^1A = 1-methyladenosine, $m^{22}G$ = N2,N2-dimethylguanosine.

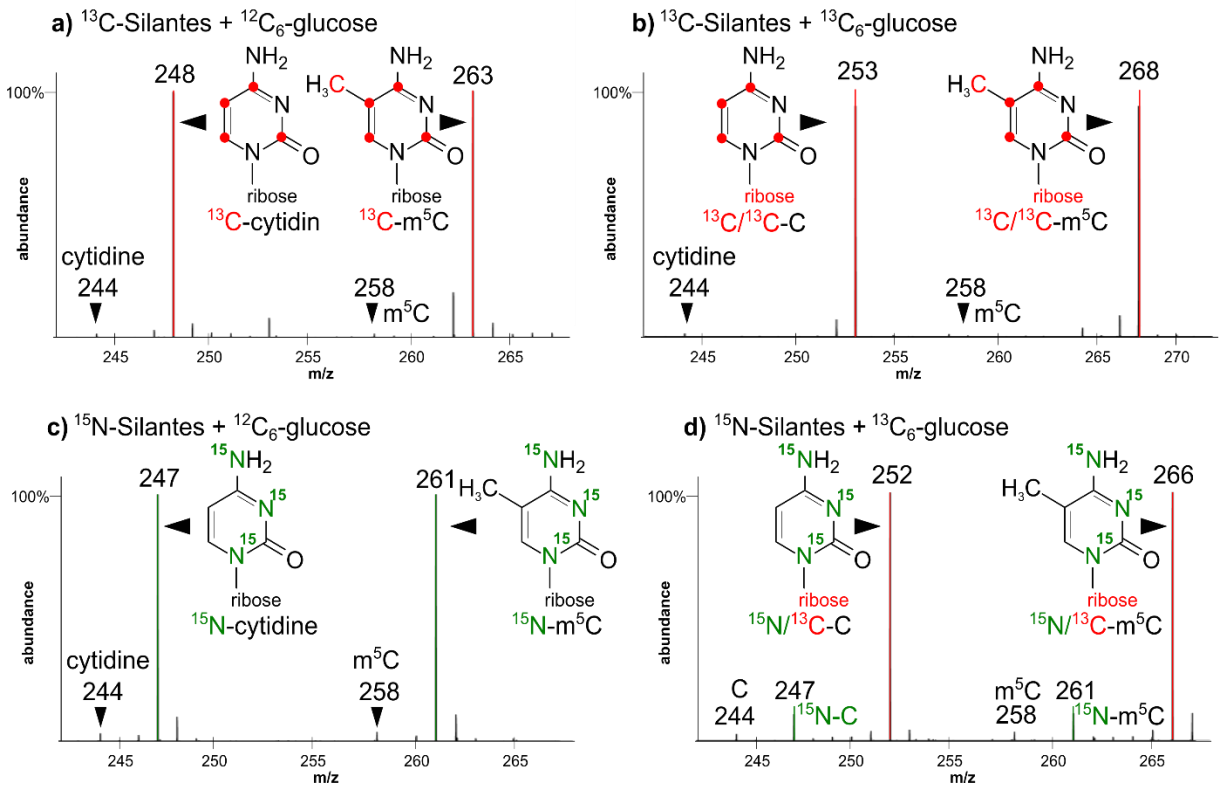


Figure S5 Mass spectra of differently labeled nucleosides. ^{13}C labeled positions in the structure are marked in red and ^{15}N labelled positions are marked in green. The corresponding mass signal in the diagram is marked with the same color.

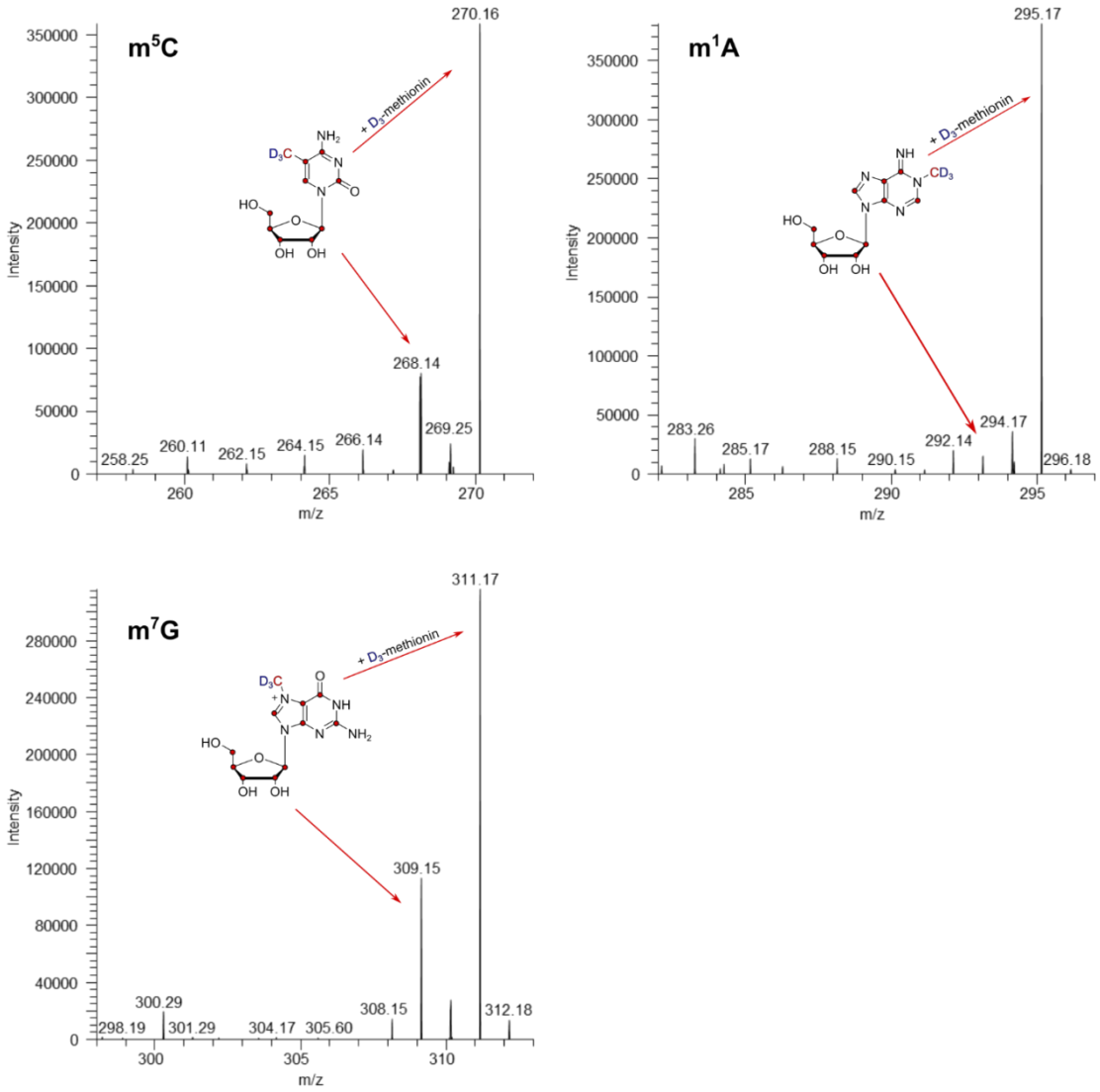


Figure S6 D_3 labeling in ^{13}C Silantes medium. The nucleosides are completely labeled with ^{13}C carbon. The carbon of the methyl group gets removed when additionally labeled with D_3-methionine thus resulting in an increase of +2 m/z. The efficiency of D_3-labeling is about 80% for m^5C and m^1A and about 70% for m^7G.

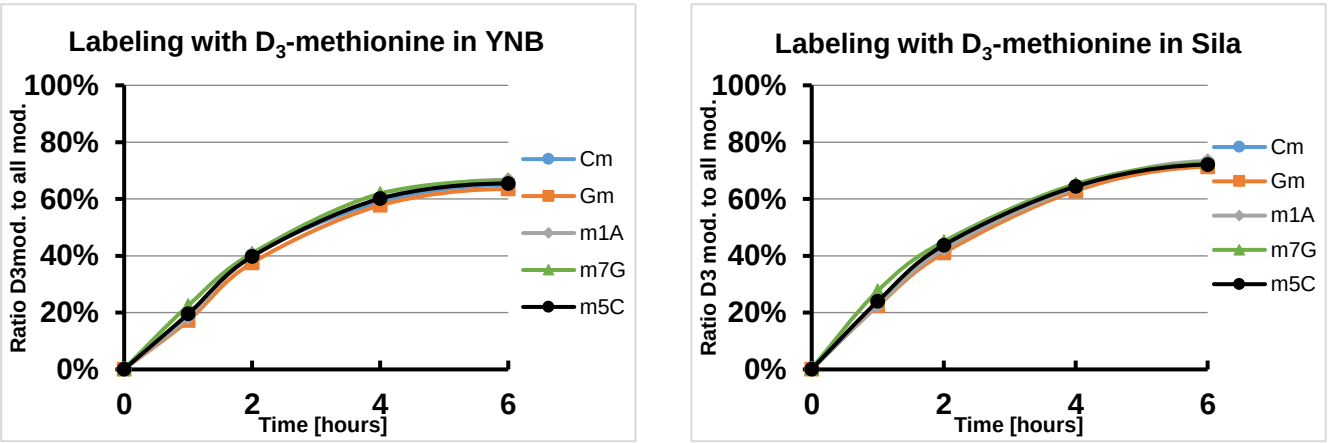


Figure S7 Labeling kinetics of D_3-methionine in YNB and Sila medium. All modified nucleosides are quickly labeled with D_3 by methionine as the ratio of labeled and unlabeled nucleosides shows.

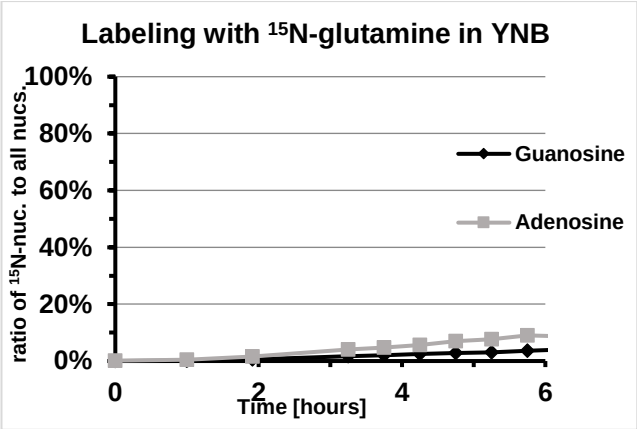


Figure S8 Labeling kinetics of ^{15}N-glutamine in YNB medium. The labeling of purines by ^{15}N-glutamine is very low and not sufficient for the experiment.Here, the ratio of 15N-labeled nucleosides to all nucleosides is plottedfor adenosine and guanosine.

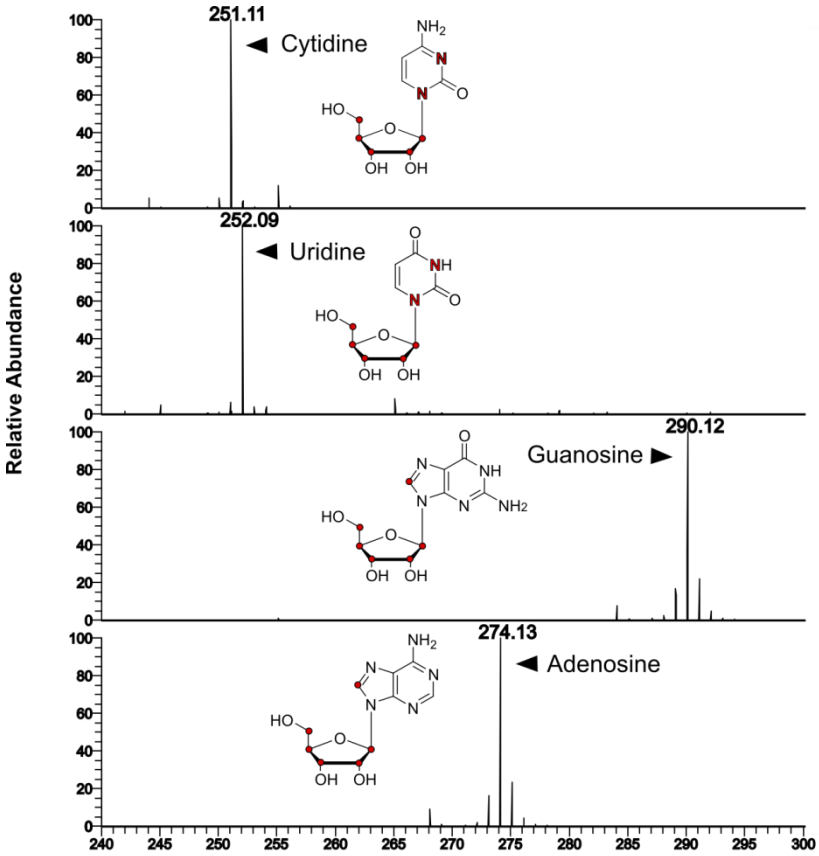


Figure S9 High resolution mass spectra of the four canonicals in ^{13}C/^{15}N YNB medium.Low abundant unlabeled nucleoside mass peaks are from impurities of the isotopically labeled starting materials $^{15}N_2$-uracil and $^{13}C_6$-glucose.

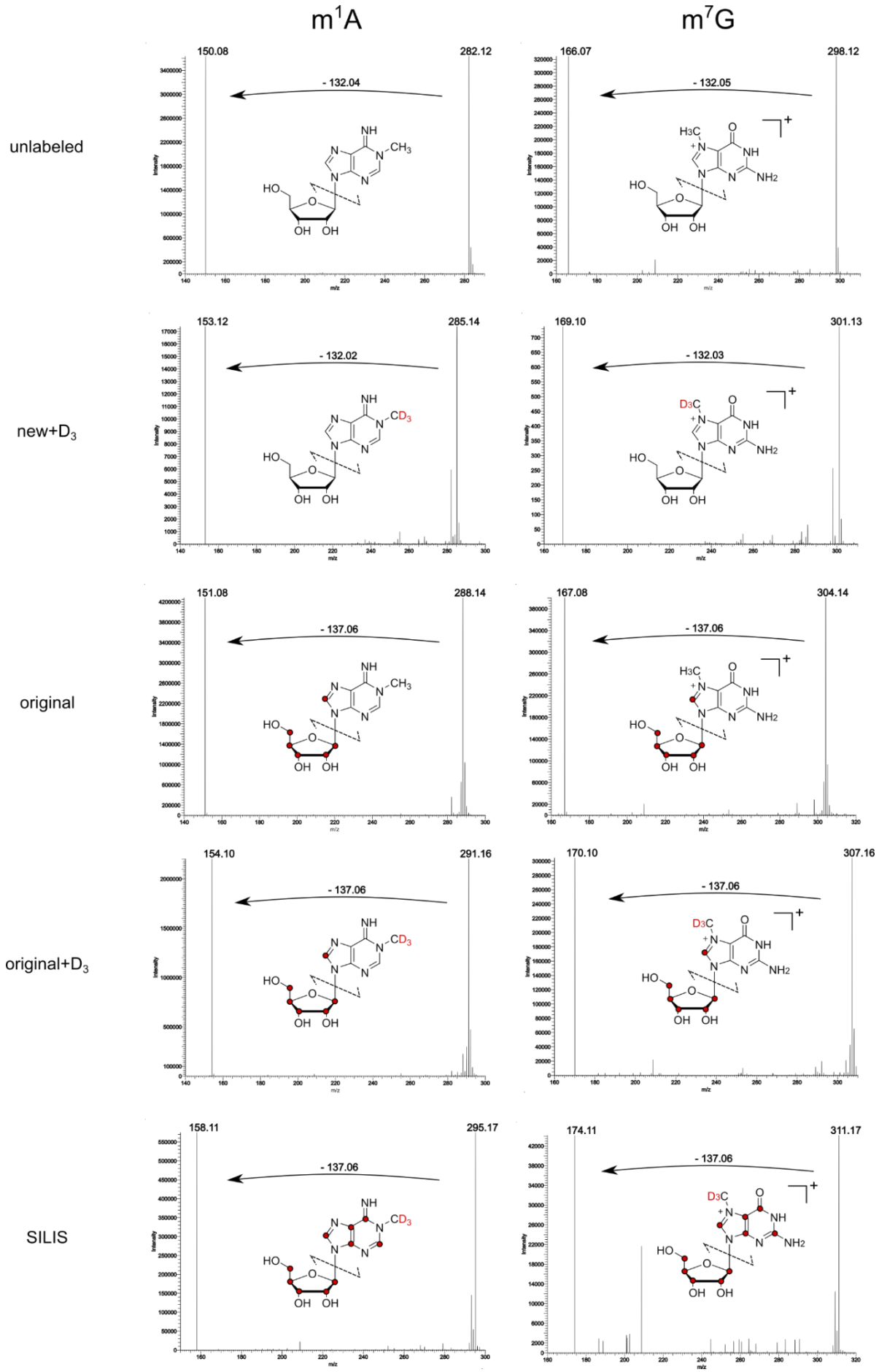


Figure S10 Mass transitions of m¹A (left) and m⁷G (right) for the labeling in the different media.

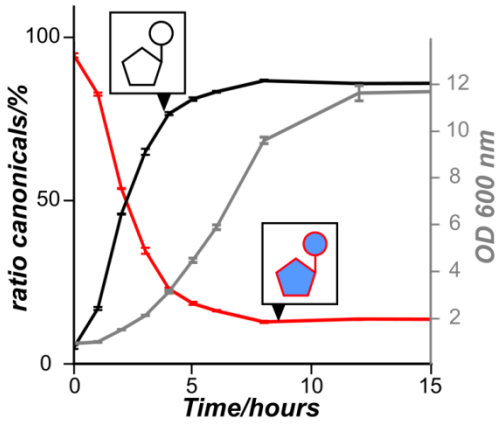


Figure S11 Dilution of pre-existing heavy labeled canonical nucleosides over time displayed as the ratio of heavy-labeled to unlabeled canonicals in %

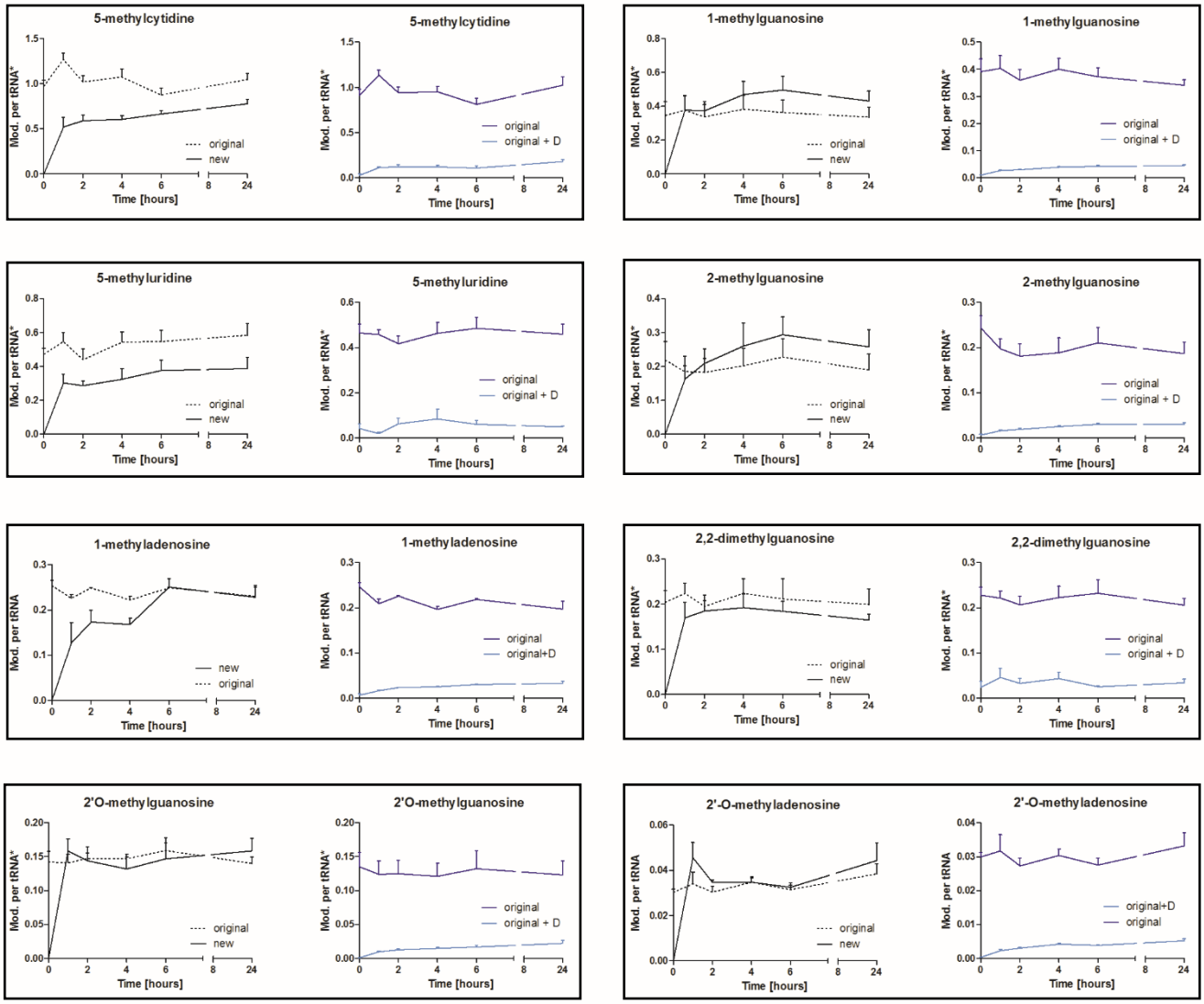


Figure S12 Dynamics of the different modifications in yeast tRNA measured over 24 hours of growth. The diagrams on the left show the level of modifications in new synthesized tRNA (solid black line) and original tRNA (dashed line). The diagrams on the right show the modification levels of original RNA, split into original RNA modifications formed before the medium exchange and original+D RNA modifications which are formed of original canonicals which get methylated after medium exchange (post-methylation). Except m^1A (n=3), the values are the averages of n=6 and SE as standard bars.

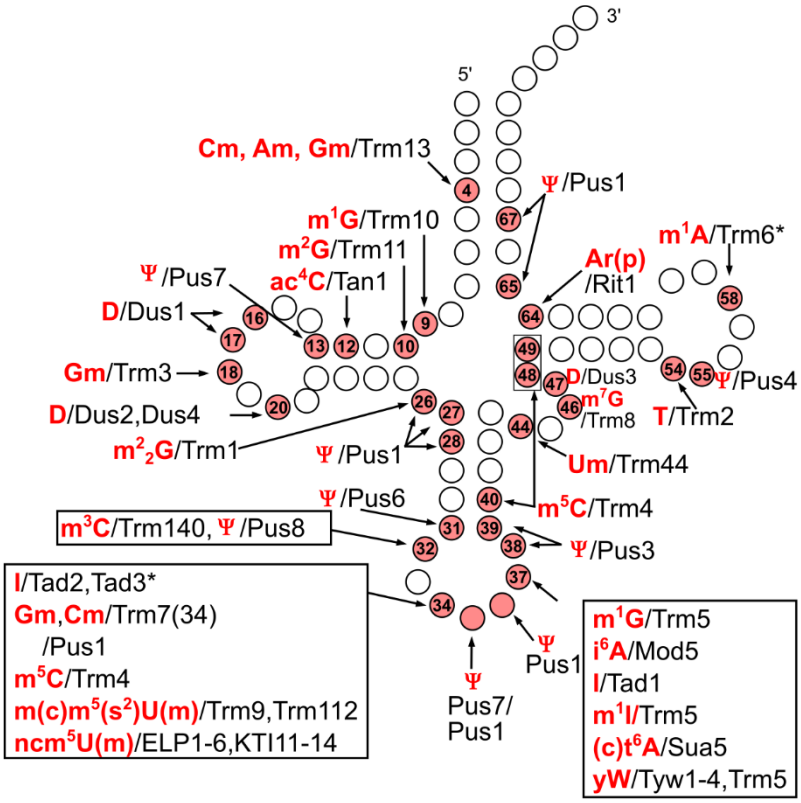


Figure S13 Summary of cytosolic tRNA in S. cerevisiae. Positions with possible modifications are marked in red. Enzymes participating at the modifications biosynthesis are given in black.

Table S1 Expected number of modifications in tRNA of S. cerevisiae. Abbreviations: Nucleosides: C = cytosine, U = uridine, G = guanosine, A = adenosine, Ψ = pseudouridine, D = dihydrouridine, m^5C = 5-methylcytidine, m^5U = 5-methyluridine, m^1G = 1-methylguanosine, m^2G = N2-methylguanosine, m^7G = 7-methylguanosine, $m^{22}G$ = N2,N2-dimethylguanosine, m^1A = 1-methyladenosine, t^6A = N6-threonylcarbamoyladenosine, i^6A = N6-isopentenyladenosine, Cm = 2′-O-methylcytidine, Um = 2′-O-methyluridine, Gm = 2′-O-methylguanosine, Am = 2′-O-methyladenosine, I = Inosine, m^1I = 1-methylinosine, m^3C = 3-methylcytidine, yW = wybutosine, mcm^5U = 5-methoxycarbonylmethyluridine, mcm^5s^2U = 5-methoxycarbonylmethyl-2-thiouridine, ncm^5U = 5-carbamoylmethyluridine, $cmnm^5Um$ = 5-carboxymethylaminomethyl-2′-O-methyluridine, Ar = 2′-O-ribosyladenosine, ac^4C = N4-acetylcytidine. Amino acids (AA): Ala = alanine, Cys = cysteine, His = histidine, Arg = arginine, Asn = asparagine, Asp = aspartic acid, Glu = glutamic acid, Gly = glycine, Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, Phe = phenylalanine, Pro = proline, Ser = serine, Thr = threonine, Trp = tryptophan, Tyr = tyrosine, Val = valine, Ini = ini methionine

		Canonicals				25 modifications																										
		C	U	G	A	Ψ	D	m5C	m5U	m1G	m2G	m7G	m22G	m1A	t6A	i6A	Cm	Um	Gm	Am	I	m1I	m3C	yW	mcm5U	mcm5s2U	ncm5U	cmnm5Um	Ar	ac4C	all Mods in AA	
natural amino acids	Ala	22	11	25	8	2	3		1	1			1								1	1									**10**	Ala
	Cys	17	13	22	12	3	3	1	1			1		1		1															**11**	Cys
	His	14	14	19	17	4	3	1	1	1									1	1											**12**	His
	Arg	20	11	18	14	3	1		1	1	1		1	1	1										1						**11**	Arg
	Asn	19	10	20	15	2	5	1	1		1		1	1	1																**13**	Asn
	Asp	18	13	23	13	3	2		1	1																					**7**	Asp
	Glu	22	11	19	16	3	1	1	1																	1					**7**	Glu
	Gly	21	12	22	13	2	3		1																						**6**	Gly
	Ile	21	9	21	13	5		1	1	1	1			1	1						1										**12**	Ile
	Leu	17	15	19	21	3	2	1	1	1	1		1						1											1	**12**	Leu
	Lys	17	10	19	13	5	4	1	1		1	1	1	1	1													1			**17**	Lys
	Met	18	10	17	18	4	2	1	1			1	1	1	1																**12**	Met
	Phe	15	12	18	17	2	2	2	1		1	1	1	1			1		1					1							**14**	Phe
	Pro	20	12	22	8	4	2		1	2		1		1			1														**12**	Por
	Ser	18	13	23	17	3	3	1	1				1			1		1	1		1									1	**14**	Ser
	Thr	15	11	18	18	2	4	1	1		1		1	1	1						1		1								**14**	Thr
	Trp	16	10	16	16	6	3		1	1	1	1		1			2		1												**17**	Trp
	Tyr	20	7	20	15	3	6	1	1		1		1	1		1			1												**16**	Tyr
	Val	21	8	19	16	3	4	1	1		1		1	1													1				**13**	Val
	Ini	22	8	21	13		2	2		1	1	1	1	1	1														1		**11**	Lui
results	sum	373	220	401	293	62	55	16	19	10	11	7	12	13	7	3	4	1	6	1	4	1	1	1	1	1	1	1	1	2	**241**	
	average per tRNA	18.65	11	20.05	14.65	3.10	2.75	0.80	0.95	0.50	0.55	0.35	0.60	0.65	0.35	0.15	0.20	0.05	0.30	0.05	0.20	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.10	12.05	

Table S2: Dynamic MRM data from QQQ measurement. Abbreviations: C = cytosine, U = uridine, G = guanosine, A = adenosine, Ψ = pseudouridine, D = dihydrouridine, m^5C = 5-methylcytidine, m^5U = 5-methyluridine, m^1G = 1-methylguanosine, m^2G = N2-methylguanosine, m^7G = 7-methylguanosine, $m^{22}G$ = N2,N2-dimethylguanosine, m^1A = 1-methyladenosine, Cm = 2'-O-methylcytidine, Gm = 2'-O-methylguanosine, Am = 2'-O-methyladenosine, Um = 2'-O-methyluridine, I = Inosine.

Compound Group	Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret	Frag-mentor	Collision Energy	Cell Accelerator Voltage	Polarity
new (unlabeled)	A	268	136	7.08	1	250	40	2	Positive
	Am	282	136	8.76	1	250	10	2	Positive
	C	244	112	1.93	1	250	40	2	Positive
	Cm	258	112	4.32	1	250	10	2	Positive
	D	247	115	1.6	1	250	10	2	Positive
	G	284	152	4.91	1	250	40	2	Positive
	Gm	298	152	6.45	1	250	10	2	Positive
	I	269	137	4.62	1	250	10	2	Positive
	m^1A	282	150	3.63	5	250	10	2	Positive
	m^1G	298	166	6.31	1	250	10	2	Positive
	$m^{22}G$	312	180	7.95	1	250	10	2	Positive
	m^2G	298	166	6.7	1	250	10	2	Positive
	m^5C	258	126	3.83	1	250	10	2	Positive
	m^5U	259	127	4.96	1	250	10	2	Positive
	m^7G	298	166	4.21	1	250	10	2	Positive
	Ψ	245	209	1.62	1	250	10	2	Positive
	U	245	113	2.53	1	250	40	2	Positive
	Um	259	113	5.46	1	250	10	2	Positive

new+D (D_3)	Am	285	136	8.76	1	250	10	2	Positive
	Cm	261	112	4.32	1	250	10	2	Positive
	Gm	301	152	6.45	1	250	10	2	Positive
	m^1A	285	153	3.63	5	250	10	2	Positive
	m^1G	301	169	6.31	1	250	10	2	Positive
	$m^{22}G$	318	186	7.95	1	250	10	2	Positive
	m^2G	301	169	6.7	1	250	10	2	Positive
	m^5C	261	129	3.83	1	250	10	2	Positive
	m^5U	262	130	4.96	1	250	10	2	Positive
	m^7G	301	169	4.21	1	250	10	2	Positive
	Um	262	113	5.46	1	250	10	2	Positive
original (^{15}N-Ura/^{13}C-Glc)	A	274	137	7.08	1	250	40	2	Positive
	Am	288	137	8.76	1	250	10	2	Positive
	C	251	114	1.93	1	250	40	2	Positive
	Cm	265	114	4.32	1	250	10	2	Positive
	D	254	117	1.6	1	250	10	2	Positive
	G	290	153	4.91	1	250	40	2	Positive
	Gm	304	153	6.45	1	250	10	2	Positive
	I	275	138	4.62	1	250	10	2	Positive
	m^1A	288	151	3.63	5	250	10	2	Positive
	m^1G	304	167	6.31	1	250	10	2	Positive
	$m^{22}G$	318	181	7.95	1	250	10	2	Positive
	m^2G	304	167	6.7	1	250	10	2	Positive
	m^5C	265	128	3.83	1	250	10	2	Positive
	m^5U	266	129	4.96	1	250	10	2	Positive
	m^7G	304	167	4.21	1	250	10	2	Positive
	Ψ	252	216	1.62	1	250	10	2	Positive
	U	252	115	2.53	1	250	40	2	Positive
	Um	266	115	5.46	1	250	10	2	Positive

original+D (15N-Ura/13C-Glc/D_3-Met)	Am	291	137	8.76	1	250	10	2	Positive
	Cm	268	114	4.32	1	250	10	2	Positive
	Gm	307	153	6.45	1	250	10	2	Positive
	m^1A	291	154	3.63	5	250	10	2	Positive
	m^1G	307	170	6.31	1	250	10	2	Positive
	$m^{22}G$	324	187	7.95	1	250	10	2	Positive
	m^2G	307	170	6.7	1	250	10	2	Positive
	m^5C	268	131	3.83	1	250	10	2	Positive
	m^5U	269	132	4.96	1	250	10	2	Positive
	m^7G	307	170	4.21	1	250	10	2	Positive
	Um	269	115	5.46	1	250	10	2	Positive
SILIS	A	278	141	7.08	1	250	40	2	Positive
	Am	295	141	8.76	1	250	10	2	Positive
	C	253	116	1.93	1	250	40	2	Positive
	Cm	270	116	4.32	1	250	10	2	Positive
	D	256	119	1.6	1	250	10	2	Positive
	G	294	157	4.91	1	250	40	2	Positive
	Gm	311	157	6.45	1	250	10	2	Positive
	I	279	142	4.62	1	250	10	2	Positive
	m^1A	295	158	3.63	5	250	10	2	Positive
	m^1G	311	174	6.31	1	250	10	2	Positive
	$m^{22}G$	328	191	7.95	1	250	10	2	Positive
	m^2G	311	174	6.7	1	250	10	2	Positive
	m^5C	270	133	3.83	1	250	10	2	Positive
	m^5U	271	134	4.96	1	250	10	2	Positive
	m^7G	311	174	4.21	1	250	10	2	Positive
	Ψ	254	218	1.62	1	250	10	2	Positive
	U	254	117	2.53	1	250	40	2	Positive
	Um	271	117	5.46	1	250	10	2	Positive

Supplement

NAIL-MS: A dynamic tool for dynamic RNA modification analysis

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Content

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Figure S2 Pulse-chase NAIL-MS study of mock and MMS incubated bacterial cultures 2

Table S1: Limit of precision of ^{13}C6-glucose and ^{15}N NAIL-MS study in E.coli.. 2

Table S2: *Dynamic MRM data from QQQ measurements for the detection of the limit of precision.* 3

Table S3: Dynamic MRM data from QQQ measurement (Pulse Chase experiment) 4

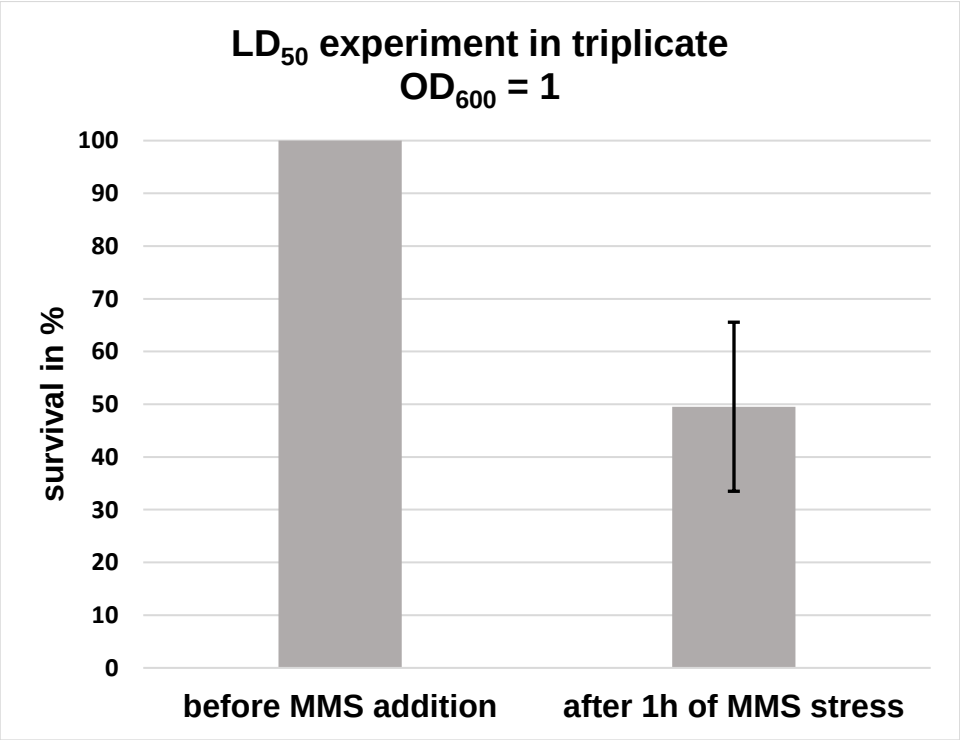

Figure S1: Diagram of LD_{50} assay with E. coli wildtype strain BW25113. The experiment was started with an OD_{600} = 1 and after one hour of growing the bacteria were exposed to 20 mM MMS alkylation stress for one hour. A bacteria dilution of 10^{-4} was plated on LB – agar plates and incubated at 37°C. The next day colony counting showed how many bacteria survived the stress. The experiment was done in triplicate.

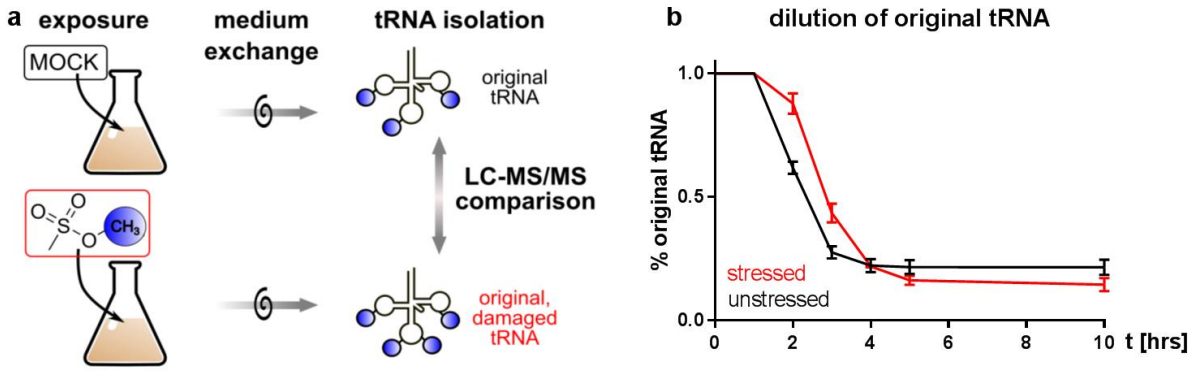

Figure S2: Pulse-chase NAIL-MS study of mock and MMS incubated bacterial cultures. ***a*** *Principle of the study for comparison of % methylation in original tRNAs.* ***b*** *Dilution of original tRNA measured as the ratio of light nucleosides (original) to the sum of all nucleosides (original+new) in %.*

Table S1: Limit of precision of ^{13}C6-glucose and ^{15}N NAIL-MS study in E.coli. Abbreviations: Psi = Pseudouridine, D = Dihydrouridine, acp^{3}U = 3-(3-amino-3-carboxypropyl)uridine, s^{2}C = 2-thiouridine, m^{7}G = 7-methylguanosine, I = inosine, m^{5}U = 5-methyluridine, s^{4}U = 4-thiouridine, m^{1}G = 1-methylguanosine, Gm = 2'-O-methylguanosine, t^{6}A = N6-threonylcarbamoyladenosine, m^{2}A = 2-methyladenosine.

$^{13}C_6$- glucose NAIL-MS study in E. coli			
Modification	Upper fold change	Lower fold change	Standard deviation
Psi	1,07	0,93	0,04
D	1,16	0,84	0,02
s2C	1,04	0,96	0,04
m7G	1,04	0,96	0,03
I	1,19	0,81	0,25
m5U	1,05	0,95	0,00
s4U	1,05	0,95	0,07
m1G	1,04	0,96	0,03
Gm	1,06	0,94	0,02
t6A	1,42	0,58	0,02
m2A	1,12	0,88	0,02

^{15}N NAIL-MS study in E. coli			
Modification	Upper fold change	Lower fold change	Standard deviation
Psi	1.30	0.70	0.20
s2C	1.63	0.37	0.09
m7G	1.64	0.36	0.07
I	1.54	0.46	0.12
m5U	1.31	0.69	0.11
s4U	1.68	0.32	0.06
m1G	1.65	0.35	0.08
Gm	1.64	0.36	0.09
t6A	1.83	0.17	0.04
m2A	1.67	0.33	0.06

Table S2: Dynamic MRM data from QQQ measurements for the detection of the limit of precision.

Compound Group	Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Ret Time (min)	Delta Ret Time (min)	Fragmentor (V)	Collision Energy (eV)	Cell Accelerator Voltage (V)	Polarity
unlabeled	Psi	245	209	1.49	1	90	5	5	Positive
	D	247	115	1.322	1	100	10	5	Positive
	s2C	260	128	2.288	2	85	13	5	Positive
	m7G	298	166	2.7	2	105	14	5	Positive
	I	269	137	3.556	1	100	9	5	Positive
	m5U	259	127	3.61	2	145	10	5	Positive
	s4U	261	129	4.753	1	75	17	5	Positive
	m1G	298	166	5.08	1	105	13	5	Positive
	Gm	298	152	4.78	1	100	9	5	Positive
	t6A	413	281	7	1	130	9	5	Positive
	m2A	282	150	8.08	1	125	21	5	Positive
13C labeled	Psi	254	218	1.49	1	90	5	5	Positive
	D	256	119	1.322	1	100	10	5	Positive
	s2C	269	132	2.288	2	85	13	5	Positive
	m7G	309	172	2.7	2	105	14	5	Positive
	I	279	142	3.556	1	100	9	5	Positive
	m5U	269	132	3.915	2	145	10	5	Positive
	s4U	270	133	4.753	1	75	17	5	Positive
	m1G	309	172	5.08	1	105	13	5	Positive
	Gm	309	157	5.133	1	100	9	5	Positive
	t6A	428	291	7	1	130	9	5	Positive
	m2A	293	156	8.08	1	125	21	5	Positive
SILIS (15N and 13C labeled)	Psi	256	220	1.49	1	90	5	5	Positive
	D	258	121	1.322	1	100	10	5	Positive
	s2C	272	135	2.288	2	85	13	5	Positive
	m7G SILIS	314	177	2.7	2	105	14	5	Positive
	I	283	146	3.556	1	100	9	5	Positive
	m5U SILIS	271	134	3.61	2	145	10	5	Positive
	s4U	272	135	4.753	1	75	17	5	Positive
	m1G	314	177	5.08	1	105	13	5	Positive
	Gm SILIS	314	162	4.78	1	100	9	5	Positive
	t6A	434	297	7	1	130	9	5	Positive
	m2A	298	161	8.08	1	125	21	5	Positive
15N labeled	Psi	247	211	1.49	1	90	5	5	Positive
	D	249	117	1.322	1	100	10	5	Positive
	s2C	263	131	2.288	1	85	13	5	Positive
	m7G	303	171	2.021	2	105	14	5	Positive
	I	273	141	3.556	1	100	9	5	Positive
	m5U	261	129	3.915	2	145	10	5	Positive
	s4U	263	131	4.753	1	75	17	5	Positive
	m1G	303	171	5.08	1	105	13	5	Positive
	Gm	303	157	5.133	1	100	9	5	Positive
	t6A	419	287	7	1	130	9	5	Positive
	m2A	287	155	8.08	1	125	21	5	Positive

Table S3: Dynamic MRM data from QQQ measurement (Pulse Chase experiment). Abbreviations: A = adenosine, C = cytosine, G = guanosine, m^1A = 1-methyladenosine, m^3C = 3-methylcytidine, U = uridine

Compound Group	Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Ret Time (min)	Delta Ret Time (min)	Fragmentor (V)	Collision Energy (eV)	Cell Accelerator Voltage (V)	Polarity
unlabeled	A	268	136	5.711	1	110	21	5	Positive
	C	244	112	1.73	1	175	13	5	Positive
	G	284	152	3.718	3	95	17	5	Positive
	m1A	282	150	1.745	2	110	21	5	Positive
	m3C	258	126	1.752	2	88	14	5	Positive
	U	245	113	1.876	2	95	5	5	Positive
SILIS (15N and 13C labeled)	A SILIS	283	146	5.711	1	110	21	5	Positive
	C SILIS	256	119	1.73	1	175	13	5	Positive
	G SILIS	299	162	3.718	3	95	17	5	Positive
	m1A SILIS	298	161	1.745	2	110	21	5	Positive
	m3C SILIS	271	134	1.752	2	88	14	5	Positive
	U SILIS	256	119	1.876	2	95	5	5	Positive
CD3 (methionine-d3 labeled)	m1A CD3	285	153	1.745	2	110	21	5	Positive
	m3C CD3od15N	261	129	1.752	2	88	14	5	Positive
15N labeled	A 15N	273	141	5.711	1	110	21	5	Positive
	C 15N	247	115	1.73	1	175	13	5	Positive
	G 15N	289	157	3.718	3	95	17	5	Positive
	m1A 15N	287	155	1.745	2	110	21	5	Positive
	U 15N	247	115	1.876	2	95	5	5	Positive
15N + CD3 labeled	m1A 15N_CD3	290	158	1.745	2	110	21	5	Positive
	m3C 15N_CD3	264	132	1.752	2	88	14	5	Positive

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