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Post-transcriptional processes regulate gene expression and immune evasion of African trypanosomes

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Vanessa Luzak

aus

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Marie Curie

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List of Abbreviations

4-SU	4-thio**u**ridine
4-TU	4-**t**hio**u**racil
AAT	**A**nimal **A**frican **t**rypanosomiasis
ADP	**A**denosine **di**phosphate
ALPH1	**A**paH-like **ph**osphatase 1
ATP	**A**denosine **t**ri**p**hosphate
BaseJ	ß-d-glucosyl-hydroxymethyluracil
BDF1-6	**B**romo**d**omain **f**actor 1-6
bp	**B**ase **p**airs
BSF	Trypanosoma infective **b**lood**s**tream **f**orm
Caf1	**C**hromatin **a**ssembly **f**actor 1
Ccr4-Not	**C**arbon **C**atabolite **R**epression 4—**N**egative **O**n **T**ATA-less complex
CTD	**C**-**t**erminal **d**omain
Dcp1/2	mRNA **d**e**c**a**p**ping enzyme ½
DNA	**D**eoxyribo**n**ucleic **a**cid
Dot1B	**D**isruptor **o**f **t**elomeric silencing 1B
eRF1/3	**E**TS domain-containing t**r**anscription **f**actor 1/3
ES	(Antigen) **E**xpression **s**ite
ESAG	**E**xpression **s**ite **a**ssociated **g**enes
ESB	**E**xpression **s**ite **b**ody
ESB1	**ESB**-specific protein 1
FACT	**FA**cilitates **c**hromatin **t**ranscription remodeler
GO-term	**G**ene **o**ntology **term**
H1	**H**istone 1
H2A	**H**istone 2A
H2A.X	**H**istone 2A variant X
H2A.Z	**H**istone 2A variant Z
H2B	**H**istone 2B

H2B.V	**H**istone **2B v**ariant
H3	**H**istone **3**
H3.V	**H**istone **3 v**ariant
H4	**H**istone **4**
H4.V	**H**istone **4 v**ariant
HAT	**H**istone **a**cetyl**t**ransferase
HAT	**H**uman **A**frican **t**rypanosomiasis
HDAC	**H**istone **dea**cetylases
IDR	**I**ntrinsically **d**isordered **r**egion
ISWI	**I**mitation **swi**tch remodeler
LiP-MS	**Li**mited **p**roteolysis coupled to **m**ass **s**pectrometry
m^{6}A	N**6**-**M**ethyl**a**denosin
m^{7}G	**7**-**m**ethyl**g**uanylate
Mb	**M**ega **b**ases
MCM-BP	**M**inichromosome **m**aintenance **c**omplex **b**inding **p**rotein
mTOR	**m**ammalian **t**arget **o**f **r**apamycin
mRNA	**m**essenger **RNA**
miRNA	**mi**cro **RNA**
NGD	**N**o-go **d**ecay
NMD	**N**onsense-**m**ediated **d**ecay
NSD	**N**on-**s**top **d**ecay
Nup-1/2	**Nu**clear **p**ore complex subunit **1/2**
OR	**O**lfactory **r**eceptor
Orc1/CDC6	**O**rigin **r**ecognition **c**omplex/**c**ell **d**ivision **c**ycle **6**
P body	**P**rocessing **bodies**
PABP	**p**oly(A)-**b**inding **p**rotein
Pan2-Pan3	**p**oly(A)-**n**uclease **2/3** deadenylation complex
PCA	**P**rincipal **c**omponent **a**nalysis
PCF	Trypanosoma **pro**cyclic lifecycle **f**orm
PIP5K	**P**hosphatidyl**i**nositol-4-**p**hosphate **5**-**k**inase
PIP5Pase	**P**hosphatidyl**i**nositol **5**-**P**hosphat**ase**

PLC	**P**hospho**l**ipase **C**
poly-A	**Poly**a**a**denylic acid
pre-mRNA	Unprocessed **m**essenger **RNA**
PTM	**Post-trans**lational **m**odification
PTU	**P**olycistronic **t**ranscription **u**nit
RAP1	**R**epressor **a**ctivator **p**rotein 1
RBP	**R**NA **b**inding **p**rotein
rDNA	**R**ibosomal **DNA**
RNA	**R**ibo**n**ucleic **a**cid
RNAi	**RNA i**nterference
RNA pol I	**RNA p**olymerase **I**
RNA pol II	**RNA p**olymerase **II**
SET27	**Set** domain containing **27**
SIZ1/PIAS1	SIZ1/**p**rotein **i**nhibitor of **a**ctivated **S**TAT E3 SUMO ligase
SLAM-seq	Thiol (**S**H)-**Li**nked **A**lkylation for the **M**etabolic **seq**uencing of RNA
SL array	**s**pliced **l**eader **array**
SL-RNA	**s**pliced **l**eader **RNA**
Sm	**Sm**ith proteins
snRNP	**s**mall **n**uclear **r**ibo**n**ucleo**p**roteins
SPARC	**S**et27 **p**romoter-**a**ssociated **r**egulatory **c**omplex
SUMO	**S**mall **u**biquitin-like **mo**difier
TAD	**T**opologically **a**ssociated **d**omain
TF	**T**ranscription **f**actor
TIF2	**T**RF-**i**nteracting **f**actor **2**
TRF	**T**elomere **r**epeat binding **f**actor
TSS	**T**ranscription **s**tart **s**ite
TTS	**T**ranscription **t**ermination **s**ite
TT-seq	**T**ransient **t**ranscriptome **seq**uencing
UMPS	**U**ridine **m**ono**p**hosphate **s**ynthase
UPF1	**Up-f**rameshift protein 1
VEX1	**V**SG **ex**clusion protein 1

VEX2	**V**SG **ex**clusion protein **2**
WHO	**W**orld **H**ealth **O**rganization
VSG	**V**ariant **s**urface **g**lycoprotein
Xrn	E**x**o**r**ibo**n**uclease

List of publications

Luzak, V., Osses, E., Danese, A., Odendaal, C., Cosentino, R.O., Stricker, S.H., Haanstra, J.R., Erhard, F., Siegel, T.N., 2024. SLAM-seq reveals independent contributions of RNA processing and stability to gene expression in African trypanosomes. *Nucleic Acids Research*, Volume 53, Issue 3, gkae1203, https://doi.org/10.1093/nar/gkae1203.

Luzak, V., 2022. Nuclear Condensates: New Targets to Combat Parasite Immune Evasion? *Frontiers in Cellular and Infection Microbiology*, Volume 12, https://doi.org/10.3389/fcimb.2022.942200.

Luzak, V., López-Escobar, L., Siegel, T.N., Figueiredo, L.M., 2021. Cell-to-Cell Heterogeneity in Trypanosomes. *Annual Reviews Microbiology*, 8:75:107-128.

Faria, J.*, **Luzak, V.***, Müller, L.S.M., Brink, G.B., Hutchinson, S., Glover, L., Horn, D., Siegel, T.N., 2021. Spatial integration of transcription and splicing in a dedicated compartment sustains monogenic antigen expression in African trypanosomes. *Nature Microbiology*, 6, 289-300.

*Contributed equally, co-first authorship

1. Contributions to Publications

1.1 Publication I

Luzak, V., Osses, E., Danese, A., Odendaal, C., Cosentino, R.O., Stricker, S.H., Haanstra, J.R., Erhard, F., Siegel, T.N., 2024. SLAM-seq reveals independent contributions of RNA processing and stability to gene expression in African trypanosomes. *Nucleic Acids Research*, Volume 53, Issue 3, gkae1203, https://doi.org/10.1093/nar/gkae1203.

This study in *Nucleic Acid Research* comprises a main part of my PhD project. I conceptualized all experiments with input from T.N. Siegel. I performed and analyzed the metabolic labeling experiments in Fig. 1B, 1C, 1D, 2B, 2C, 2D, 3B, 3C, 3D, 3E, 4A, 4B, 4C, 4D, S2A, S2B, S3A, S3B, S5A, S5B, S5C, S5D. Further, I performed and analyzed the Western Blot in Fig. S1A, and the RNA-seq experiments in S1B, S1C and S1D. I have received experimental support with RNA-seq experiments, library preparations and the growth curves in Fig. 1E from E. Osses. F. Erhard has supported me with data analysis of SLAM-seq experiments. C. Odendaal and J.R. Haanstra have performed the modeling analysis in Fig. 4E, 4F, S4A and S4B. I conceptualized the analysis of a published single-cell RNA-seq dataset in Fig. 5, which was performed by A. Danese, with input from R. Cosentino and S.H. Stricker.

For publication, I wrote the manuscript together with T.N. Siegel. I prepared all figures, including schematic Fig. 1A, 2A, 3A, with input from T.N. Siegel. I handled the revision requests to finalize the publication, with input from T.N. Siegel.

1.2 Publication II

Faria, J.*, **Luzak, V.***, Müller, L.S.M., Brink, G.B., Hutchinson, S., Glover, L., Horn, D., Siegel, T.N., 2021. Spatial integration of transcription and splicing in a dedicated compartment sustains monogenic antigen expression in African trypanosomes. *Nature Microbiology*, 6, 289-300.

*Contributed equally, co-first authorship

This study in *Nature Microbiology* comprises the second main part of my PhD project. I designed, performed and analyzed all Hi-C experiments (Fig. 1A, 1B, 2A, 2B, 2C, 4E, 4F, 4G, S1A, S1B, S3A, S3B, S3C, S8A, S8B, S8C, S9A). For Hi-C experiments shown in Fig. 1A, 1B, 2A, 2B, 2C, I received experimental & conceptual support from L.S.M. Müller. Further, I received support with informatic analysis of Hi-C data from B.G. Brink. To strengthen my findings, we needed to validate the Hi-C

results with an orthogonal experimental approach. Therefore, we collaborated with J. Faria, a super-resolution imaging expert in D. Horn's lab, who carried out and analyzed the super-resolution imaging experiments shown in the paper. The imaging results were highly complementary with the Hi-C findings, and since J. Faria and I had provided a similar amount of input to the publication at the timepoint of submission, we agreed on a shared first authorship.

For publication, I have prepared Figures 1A, 1B, 2A, 2B, 2C, 4E, 4F, 4G, S1A, S1B, S3A, S3B, S3C, S8A, S8B, S8C, S9A, as well as the summary Fig. 4H, and edited the remaining figure panels. I wrote the manuscript text together with J. Faria and input from T.N. Siegel and D. Horn. Together with J. Faria, I handled the revision requests to finalize the publication, with input from T.N. Siegel and D. Horn.

1.3 Publication III (Appendix)

Luzak, V., 2022. Nuclear Condensates: New Targets to Combat Parasite Immune Evasion? *Frontiers in Cellular and Infection Microbiology*, Volume 12, https://doi.org/10.3389/fcimb.2022.942200.

I was invited to contribute with an *Opinion* article to the special issue "Rising stars in Parasite and Host 2022". For this article, I have researched the role of biomolecular condensates in nuclear biology, and discussed a potential role of condensates in parasite immune evasion. I wrote the manuscript and prepared the figure, receiving feedback from T.N. Siegel.

1.4 Publication IV (Appendix)

Luzak, V., López-Escobar, L., Siegel, T.N., Figueiredo, L.M., 2021. Cell-to-Cell Heterogeneity in Trypanosomes. *Annual Reviews Microbiology*, 8:75:107-128.

This review article summarizes research from bacteria and more complex eukaryotes, describing the important role of cell-to-cell heterogeneity in biological processes. Few is known about the role of cell-to-cell heterogeneity during parasite infections. Therefore, we have highlighted where heterogeneity has been described throughout the life cycle of African trypanosomes, and what role it could play for parasite survival and infectivity, based on the examples from bacteria and more complex eukaryotes.

I researched and wrote the Introduction, Antigenic variation and Conclusion chapter, with input from T.N. Siegel, L. López-Escobar and L.M. Figueiredo. L. López-

Escobar and L.M. Figueiredo researched and wrote the Parasite Life Cycle and Tissue Colonization chapters, with input from me and T.N. Siegel. I prepared Figure 1, and edited Figure 2 and 3, which were drafted by L. López-Escobar.

2. Summary

Gene expression, the process by which genetic information is translated into proteins, is a multi-step process consisting of transcription, RNA processing and degradation. Transcription is thought to be the most important regulatory step, determining whether or not a protein is expressed in a cell and at what levels. The regulation of gene activity by upstream promoter sequences, as well as by *cis*-regulatory enhancer sequences that interact with their target genes in 3D, has been extensively studied in eukaryotes. RNA processing and degradation have also been shown to influence gene expression levels. However, disentangling the individual roles of RNA processing and degradation on gene expression remains technically challenging, and therefore the regulatory roles of post-transcriptional processes have received less attention than transcription.

In contrast to most eukaryotes, transcription is thought to be a largely unregulated process in African trypanosomes, which are single-celled parasites that cause fatal diseases in humans, livestock and wildlife in sub-Saharan Africa, although tight regulation of gene expression is required for parasite survival and infectivity. Interestingly, African trypanosomes have evolved two gene expression systems: (1) Most protein-coding genes are located on chromosome cores and are transcribed by RNA polymerase II from large polycistronic transcription units, most likely in an unregulated manner. However, for African trypanosomes to undergo their complex life cycle and survive in different environments, expression regulation of these genes is essential, and it has therefore been hypothesized that regulation occurs post-transcriptionally. However, the individual roles of RNA processing and degradation have remained elusive. (2) A large antigen gene family consisting of >2000 variant surface glycoproteins is located separately within the large subtelomeric arrays. While most of the arrays remain silent, a single antigen gene is actively expressed in the mammalian host and transcribed by RNA polymerase I. Selective expression of only one antigen and frequent switching to another active antigen is essential for African trypanosomes to undergo antigenic variation and evade the immune system. How selective antigen expression is controlled by the parasite has remained largely elusive.

The aim of this thesis was to elucidate the regulatory role of post-transcriptional processes on gene expression in African trypanosomes, where transcriptional regulation appears to be absent. Using metabolic RNA labelling, I was able to show that both post-transcriptional processes, RNA processing and degradation, independently regulate expression of RNA polymerase II transcribed genes and further regulate transcript variability within a parasite population. Furthermore, using chromosome conformation capture analysis, I was able to show that three-dimensional genome folding plays an important role in selective antigen expression. African trypanosomes have developed a potential post-transcriptional enhancer, an mRNA processing hotspot that could control selective RNA processing of the active antigen

gene through physical interaction. These data indicate that post-transcriptional processes play a critical role for African trypanosome survival and infectivity, opening up new avenues for potential treatment strategies and establishing African trypanosomes as potent model organisms to study post-transcriptional control mechanisms.

3. Zusammenfassung

Die Umsetzung der genetischen Information in Proteine wird als Genexpression bezeichnet und ist ein mehrstufiger Prozess, der unter anderem aus Transkription, RNA-Prozessierung und RNA-Abbau besteht. Man geht davon aus, dass die Transkription hierbei der wichtigste regulatorische Schritt ist, der bestimmt, ob und in welchem Umfang ein Protein in einer Zelle exprimiert wird. Die Regulation der Genaktivität durch vorgeschaltete Promotorsequenzen sowie durch *cis*-regulierende Enhancer-Sequenzen, die mit ihren Zielgenen in 3D interagieren, wurde in Eukaryonten eingehend untersucht. Weiterhin konnte gezeigt werden, dass auch die RNA-Prozessierung und der RNA-Abbau die Expression eines Gens beeinflussen. Die genaue Rolle von RNA-Prozessierung und RNA-Degradation für die Genexpression zu verstehen ist jedoch nach wie vor eine technische Herausforderung, weshalb den beiden posttranskriptionellen Prozessen bisher weniger Aufmerksamkeit geschenkt wird als der Transkription.

Im Gegensatz zu den meisten Eukaryonten gilt die Transkription bei afrikanischen Trypanosomen als weitgehend unregulierter Prozess, obwohl eine strikte Kontrolle der Genexpression für das Überleben und die Infektiosität dieser Parasiten erforderlich ist. Afrikanische Trypanosomen sind einzelligen Parasiten, die in Gebieten südlich der Sahara tödliche Krankheiten bei Menschen, Nutztieren und Wildtieren verursachen. Interessanterweise haben Trypanosomen zwei Genexpressionssysteme entwickelt: (1) Die meisten proteinkodierenden Gene befinden sich auf den Hauptchromosomen, sind in langen polycistronischen Transkriptionseinheiten organisiert und werden von der RNA-Polymerase II transkribiert. Damit Trypanosomen ihren komplexen Lebenszyklus durchlaufen und in verschiedenen Umgebungen überleben können, ist die Regulation der Expression dieser Gene von entscheidender Bedeutung. Man geht davon aus, dass die Transkription der RNA Polymerase II transkribierten Gene unreguliert abläuft, und konnte bisher die regulatorischen Rollen der beiden posttrankriptionellen RNA-Prozessierung und RNA-Degradation nicht entschlüsseln. (2) Eine große Genfamilie, die mehr als 2000 verschiedenen Oberflächenglykoproteinen kodiert, ist gesondert in langen Arrays an den Chromosomenden lokalisiert. Während die meisten Arrays nicht exprimiert werden, wird ein einzelnes Antigen im Säugerwirt aktiv von der RNA-Polymerase I transkribiert. Die selektive Expression nur eines Antigens und der häufige Wechsel zu einem anderen aktiven Antigen ist für Trypanosomen entscheidend, um durch Antigenvariation dem Immunsystem zu entgehen. Wie genau diese selektive Antigenexpression durch den Parasiten gesteuert wird, ist weitgehend ungeklärt.

Ziel dieser Arbeit war es, die regulatorische Rolle posttranskriptioneller Prozesse bei der Genexpression in Trypanosomen aufzuklären, in denen eine transkriptionelle Regulation nicht vorhanden zu sein scheint. Mit Hilfe von metabolischer RNA-Mar-

kierung konnte ich zeigen, dass beide posttranskriptionellen Prozesse, RNA-Prozessierung und RNA-Degradation, unabhängig voneinander die Expression von RNA Polymerase II transkribierten Genen regulieren und darüber hinaus die Transkriptvariabilität innerhalb einer Parasitenpopulation kontrollieren. Weiterhin konnte ich mit Hilfe der Chromosomenkonformationsanalyse zeigen, dass die dreidimensionale Genomfaltung eine wichtige Rolle bei der selektiven Antigenexpression spielt, und afrikanische Trypanosomen einen potentiellen posttranskriptionalen Enhancer entwickelt haben, der die selektive RNA-Prozessierung des aktiven Antigen-Gens durch physikalische Interaktion steuern könnte. Diese Daten deuten darauf hin, dass posttranskriptionelle Prozesse eine entscheidende Rolle für das Überleben und die Infektiosität von Trypanosomen spielen, was neue Wege für mögliche Behandlungsstrategien eröffnet und Trypanosomen als geeignete Modellorganismen für die Untersuchung posttranskriptioneller Kontrollmechanismen etabliert.

4. Introduction I – Eukaryotic gene expression is a highly regulated process

Gene expression refers to the process by which the information encoded in a gene is converted into a functional gene product, such as a protein or non-coding RNA, that in turn fulfills a cellular function (Jacob & Monod, 1961; Orphanides & Reinberg, 2002). Most eukaryotic genomes contain ten-thousands of different genes, and the respective gene products are not all required at the same level or at the same time. Instead, regulating the expression of individual genes is essential for cells, enabling functional specialization and an immediate reaction to changing environments. Before we will focus on the importance of regulating gene expression for the eukaryotic pathogen *Trypanosoma brucei* in chapter 5, chapter 4 introduces regulation of gene expression in more complex eukaryotes, such as mammals. The well-characterized mammalian process will serve as a reference for the discoveries we and others have made in African trypanosomes.

We can find many examples of regulated gene expression in the human body: all cells in a body share the same set of genes, but many of those genes are only expressed in certain tissues or cell types to enable their specific function (Fagerberg et al., 2014; Uhlén et al., 2015): Rhodopsin and opsin proteins are preferentially expressed in retinal cells, where both proteins are responsible to convert light into nerve signals that are subsequently reported to the brain, forming the molecular basis of vision (Boll, 1877; Costanzi et al., 2009; Hofmann & Lamb, 2023). Similarly, kidney cells express a specific subset of membrane transporter proteins that establish the sophisticated filtration system to regulate blood homeostasis (Bowman, 1842; Drozdzik et al., 2021), and skin cells express a multitude of keratin proteins to create a protective barrier between our body and the environment (Breinl & Baudisch, 1907; Fuchs, 1995). In summary, regulation of gene expression is a core process for living organisms: it allows tissue-specific functions in multi-cellular organisms, and further, it is essential for unicellular organisms to survive in different environments.

4.1 Gene expression is a multi-step process

The expression of a gene into a protein is a multi-step process (Orphanides & Reinberg, 2002) (**Figure 1**), and each step in the process can regulate the final output of the process, which are the resulting protein levels: (1) Transcription is the first step in the cascade, and describes the process by which a gene is transcribed into pre-messenger RNA (pre-mRNA) by the enzyme complex RNA polymerase II (Jacob & Monod, 1961; Roeder & Rutter, 1969). The rate of transcription can be regulated at the stage of initiation, elongation and termination, and determines how often a

gene is transcribed into unprocessed pre-mRNA (Cramer, 2019). (2) Co-transcriptionally, nascent pre-mRNA is processed. During *cis*-splicing, the spliceosome complex excises sequences of the pre-mRNA called introns, and joins exon sequences (Berget et al., 1977; Chow et al., 1977; Marasco & Kornblihtt, 2023; Wilkinson et al., 2020). In addition, co-transcriptional capping of the 5´mRNA end and polyadenylation of the 3´mRNA end confer stability to the mature mRNA and initiate nuclear export (Drummond et al., 1985; Edmonds & Abrams, 1960; Furuichi et al., 1977). The efficiency of the three processing reactions, *cis*-splicing, capping and polyadenylation, varies between transcripts and determines whether a pre-mRNA becomes mature mRNA and is exported to the cytoplasm, or whether it is prematurely degraded in the nucleus (Bhat et al., 2024). (3) Finally, the rate of cytoplasmic mRNA degradation is regulated by specific enzyme complexes such as deadenylases and exonucleases, and determines how long particular mRNAs are present in the cell (Houseley & Tollervey, 2009; Łabno et al., 2016). In conclusion, these three steps (transcription, RNA processing and stability) together shape the pool of total mRNA present in a cell. Regulation of transcription by chromatin and by three-dimensional genome folding is explained in more detail in chapters 4.2 and 4.2.1, respectively, while RNA processing and RNA degradation are explained in chapter 4.3.

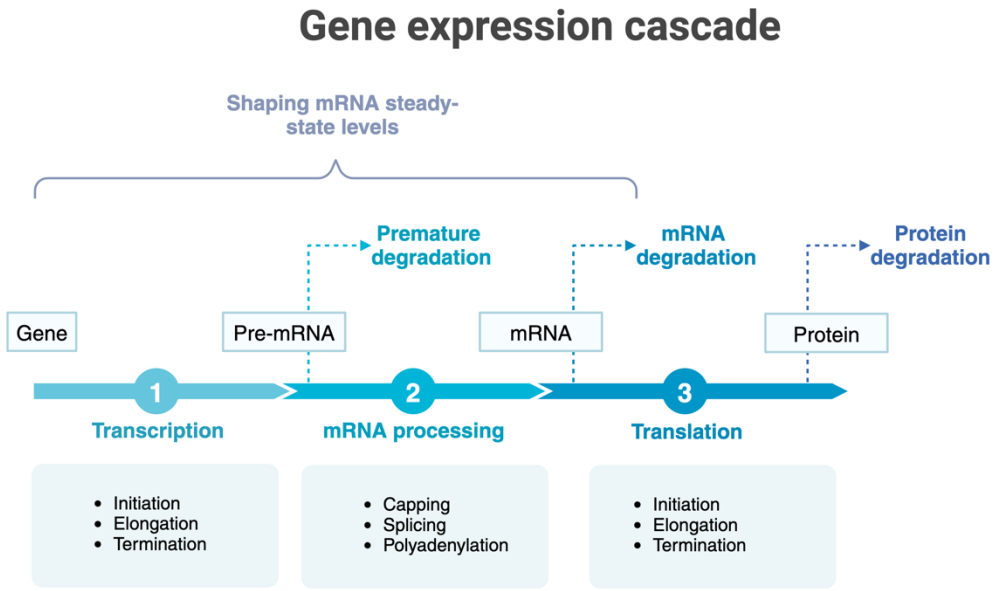

Figure 1. The expression of a gene into a protein is a multi-step process comprising transcription, RNA processing and translation. Transcription is the in initial step in the gene expression cascade, regulated on the level of initiation, elongation, and termination, generating pre-messenger RNA (mRNA) as output. Pre-mRNA is either prematurely degraded, or further processed into mature mRNA by 5'end capping, *cis*-splicing, and 3' end polyadenylation. Mature mRNA is exported from the nucleus to the cyto-

plasm, where it is either translated into protein, stored or degraded. Similar to transcription, the process of translation is further subdivided into initiation, elongation, and termination. Created in BioRender. Luzak, V. (2024) BioRender.com/s84s826.

Further, there is regulation from mRNA to protein: (4) Translation is the process by which the information encoded in an mRNA is translated into a protein, catalyzed by ribosomes (Palade, 1955). The process of translation is highly regulated, and its efficiency greatly varies between mRNAs. Thereby it determines how often an mRNA is translated into protein (Ingolia et al., 2009). (5) In addition, protein stability is a regulated process, and determines how long a given protein is present in the cell before degradation (Christiano et al., 2014). Taken together, it was illustrated that gene expression is a complex process composed of transcription, RNA processing and RNA stability which control total mRNA levels, as well as translation efficiency and protein stability, which further regulate final protein levels in the cell.

Due to its complexity, gene expression is often quantified in a simplified manner by measuring total mRNA levels, for example by RNA-sequencing (RNA-seq), a high-throughput sequencing method that captures total RNA levels in a genome-wide manner (Hrdlickova et al., 2017). Measuring total mRNA levels indicates whether genes are in general expressed at high or low levels, or whether they are up- or downregulated upon a stimulus. However, in order to understand the contribution of transcription, RNA processing or stability to a detected change in total RNA levels, more specialized methods are required that enable the measurement of these processes individually. Several experimental approaches have been established within the last decade, relying on metabolic labeling of newly synthesized RNA (Rädle et al., 2013; Windhager et al., 2012): for example, transcription dynamics can be measured by transient transcriptome sequencing (TT-seq) (Schwalb et al., 2016), RNA processing efficiency by nanopore analysis of co-transcriptional processing (nanoCOP) (Drexler et al., 2020) and RNA stability by Thiol (SH)-Linked Alkylation for the Metabolic sequencing of RNA (SLAM-seq) (Herzog et al., 2020). These techniques have paved the way to uncover the diverse pathways within the gene expression cascade that a cell can utilize to regulate the expression of a protein: transcriptional regulation, regulation of RNA processing rates, RNA stability, or translation rates. With such a multitude of potential regulatory nodes, the question arises which are the main regulatory steps that most strongly determine final protein levels in a cell. Further, it has remained elusive which functional difference it makes for a cell to regulate protein levels, e.g. on the level of transcription or on the level of translation.

4.2 Transcription is regulated on the level of chromatin

Transcriptional regulation has been extensively studied in more complex eukaryotes, and is assumed to be a main regulatory step during gene expression. Gene activity is largely controlled on the level of chromatin (Clark-Adams et al., 1988; Han & Grunstein, 1988), a complex of DNA and specific regulatory and structural proteins such as histones (Van Hoide et al., 1974). DNA contained in the nucleus of a human cell, for example, would be around two meters long when fully unwrapped, and yet has to fit into a nucleus of a few micrometers in diameter. Therefore, DNA requires packaging (**Figure 2a**): 146 bp of DNA sequence is repeatedly wrapped around histone octamers, consisting of two copies of histone proteins H2A, H2B, H3 and H4, to form nucleosomes and organize DNA as "beads on a string" (Kornberg & Thomas, 1974; Luger et al., 1997; Olins & Olins, 1974). To obtain a higher level of compaction, nucleosomes can be further organized into higher order structures, supported by the linker histone H1 (Bednar et al., 1998; Hansen, 2012; Nishino et al., 2012; Tremethick, 2007).

Packaging in the form of chromatin enables the organization of otherwise large and unstructured DNA molecules, e.g. during cell division, and was shown to protect DNA from damage (Kouzarides, 2007). Further, regulation of transcription occurs on the level of chromatin (Dupont & Wickström, 2022; B. Li et al., 2007): transcription of a gene is facilitated by opening up the surrounding chromatin. Open chromatin is commonly referred to as euchromatin (Heitz, 1928)(**Figure 2b**), and describes areas of the cell nucleus where mostly active genes are located. On the other hand, transcription of a gene can be impaired by tight chromatin compaction, as it is found in heterochromatin, the state in which most inactive genes are found (Cremer & Cremer, 2001). In order to control the density of chromatin, the unstructured tails of histone proteins can be chemically modified (Allfrey et al., 1964; Brownell et al., 1996; Fischle et al., 2003), or specific histone variants, such as H2A.X or H3.3, can be introduced (Henikoff & Smith, 2015).

To date, numerous protein complexes have been identified that influence the state of chromatin and thereby regulate gene expression (B. Li et al., 2007; Morrison & Thakur, 2021): (1) Chromatin writers are protein complexes that place specific post-translation modifications (PTMs) at certain amino acid residues of histones, especially at the accessible histone tails (Kouzarides, 2007; Talbert & Henikoff, 2021)(**Figure 2c**). For example, acetylation of lysine residues is mediated by writer proteins from the histone acetyl transferase (HAT) families, and was described to open up chromatin and facilitate transcription due to the modification's negative charge (Roth et al., 2001). In addition to opening up chromatin, histone acetylation further recruits reader proteins, such as bromodomain-containing transcription factors, that activate gene transcription (Jacobson et al., 2000; Ladurner et al., 2003). On the other hand, eraser complexes enable reversible regulation, such as histone

deacetylases (HDAC) that deacetylate histone tails and thereby reduce or prevent transcription (Marks et al., 2003). Besides acetylation, many more histone modification types have been described and functionally characterized, such as mono-, di- and tri-methylation, phosphorylation, ubiquitination, ADP-ribosylation and SUMOylation (Jenuwein & Allis, 2001; Kouzarides, 2007; Talbert & Henikoff, 2021; Tan et al., 2011). Understanding the complexity of the combinatorial histone code on gene activity is currently at the heart of epigenetics research.

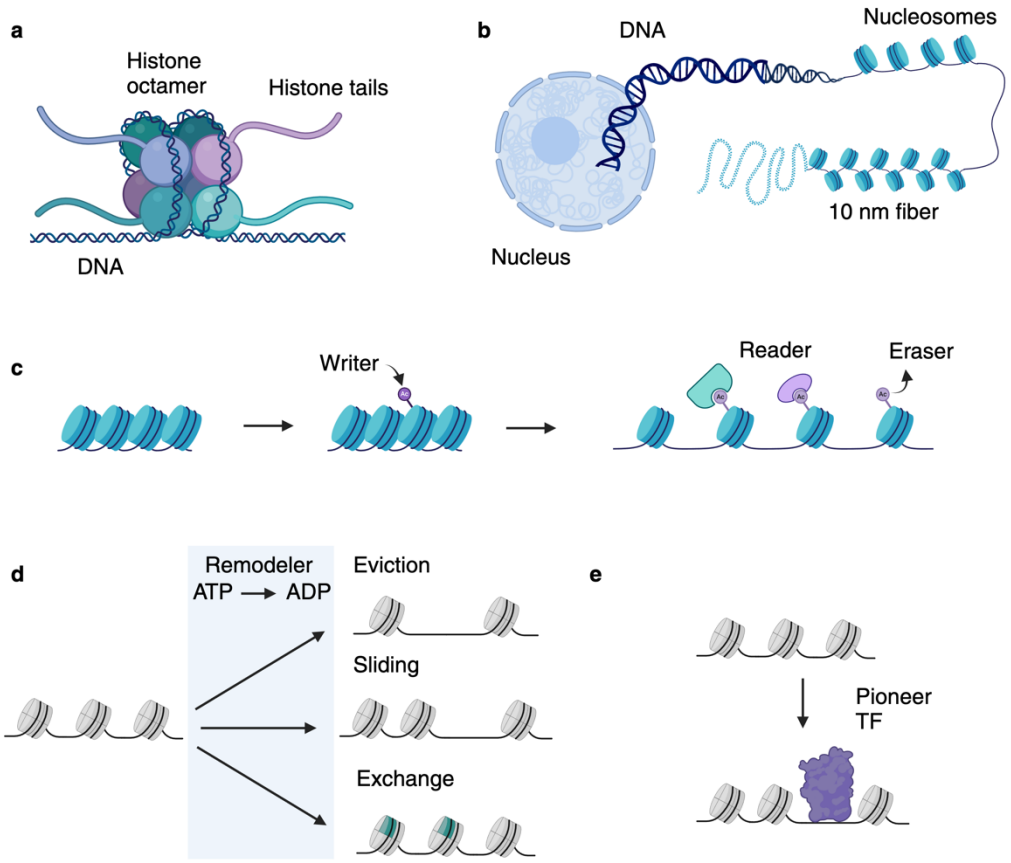

Figure 2. Chromatin is a complex of DNA and proteins that facilitates gene regulation. (a) The smallest unit of chromatin is the nucleosome. It is comprised of 146 bp DNA wrapped around a histone octamer, that is formed by two dimers of histone H2A, H2B, H3 and H4. Histone tails are unstructured protein regions, especially accessible for post-translational modifications. (b) In the nucleus, DNA is wrapped around nucleosomes. Nucleosomes can form higher order structures for more compaction. Euchromatin is accessible for transcription and contains active genes, whereas for heterochromatin denser nucleosome packaging is described, which is less accessible and represses transcription. (c) The unstructured tails of histones are commonly modified by so called "writer" proteins, e.g., histone acetyl transferases. These post-translational modifications such as acetylation modify local chromatin density and further recruit so called "reader" proteins, thereby regulating chromatin activity. "Eraser" proteins remove post-translational modifications, allowing for dynamic chromatin regulation. (d) Chromatin remodelers are protein complexes that can e.g. evict, slide or exchange nucleosomes in an ATP-dependent manner, thereby regulating local chromatin structure. (e) Pioneer transcription factors recognize certain DNA

sequences, and can open up chromatin upon binding. Created in BioRender. Luzak, V. (2025) https://BioRender.com/u82r589.

In addition to histone modifying enzymes, (2) protein complexes from different chromatin remodeler families restructure chromatin and thereby influence gene transcription (**Figure 2d**). Remodelers are protein complexes that remove, exchange or slide histone octamers in an ATP-dependent manner (P. B. Becker & Hörz, 2002; Tyagi et al., 2016). By sliding or removing nucleosomes, chromatin remodelers can influence the local chromatin density, while by nucleosome exchange, remodelers can influence the composition of chromatin, e.g. by incorporating histone variants or by changing histone PTMs. Finally, (3) pioneer transcription factors (TFs) represent the third group of proteins that regulate gene transcription by influencing chromatin. Pioneer TFs are recruited by DNA sequence motifs and can open up the local chromatin to activate gene transcription (Gualdi et al., 1996; Zaret & Carroll, 2011)(**Figure 2e**). In summary, a complex network of protein complexes and histone PTMs ensures correct gene activity. It is essential for a cell to preserve this network, since its disruption or malfunction is involved in several devastating diseases, including different types of cancer (Dawson & Kouzarides, 2012; Kadoch & Crabtree, 2015; Parreno et al., 2024).

Chromatin modifications that affect gene activity are well characterized to occur at the upstream promoter of a gene, where they mainly influence transcription initiation, and along gene body, where they affect transcription elongation and termination (Cramer, 2019; Veloso et al., 2014). However, many of the histone PTMs and chromatin regulators introduced above do also localize to specific intergenic regions, that do not encode proteins and are located away from promoters and genes on the linear genome. These intergenic regions are referred to as enhancers, and the next chapter will explain how such enhancers regulate gene activity via interaction with gene promoters in the three-dimensional space.

4.2.1 Selective transcription can be regulated by three-dimensional genome organization

Enhancer sequences are *cis*-regulatory elements that enhance gene transcription by physically interacting with a gene´s promoter (Schoenfelder & Fraser, 2019)(**Figure 3a**). Enhancers are usually composed of a <1500 bp long DNA sequence that exhibits an open chromatin structure and activating histone modifications, such as H3K27 acetylation and H3K4 mono-methylation (Barral & Déjardin, 2023). A combination of transcription factors and active polymerases are recruited to enhancer sequences, which are transcribed into enhancer RNAs (eRNA) (L. Liu et al., 2021; Michida et al., 2020; Spitz & Furlong, 2012). Three-dimensional genome folding is supported by several factors, such as the mediator complex (Allen & Taatjes, 2015; Y.-J. Kim et al., 1994), in order to induce enhancer interaction with the respective gene promoter.

Thereby, the local concentration of transcription-promoting factors increases around the target gene, and positively influences the rate of gene transcription. Taken together, enhancers serve as an additional layer of gene regulation beyond local chromatin modifications at the gene itself. The frequency and amplitude of enhancer-promoter interactions allow dynamic gene transcription control (Fukaya et al., 2016; Larsson et al., 2019).

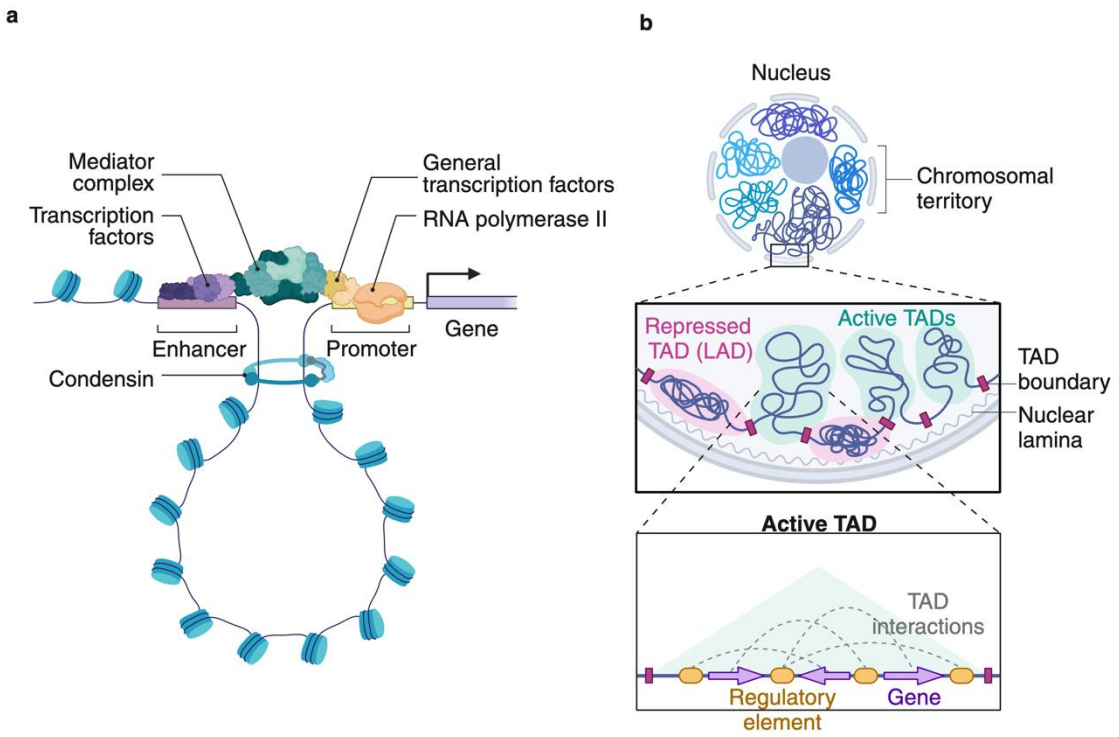

Figure 3. Gene activity is regulated by three-dimensional folding of interphase chromosomes within the nucleus. (a) Gene activity is regulated by *cis*-regulatory enhancer elements, that are located distant on the linear chromosome, and are brought into spatial proximity of the target gene by establishing three-dimensional contacts, mediated by specific proteins such as the mediator complex. Enhancer sequences recruit activating transcription factors, as well as active RNA polymerase II, and thereby provide the machinery to boost transcription of the target gene. (b) Within the nucleus, interphase chromosomes are organized into so called topologically associated domains (TADs). 3D contacts between enhancers and promoters are enhanced within TADs and restricted between TADs. Further, transcriptionally active TADs tend to cluster and build A compartments, while transcriptionally silent TADs form B compartments. Transcriptionally silent TADs are often found to be lamina associated domains (LADs). Created in BioRender. Luzak, V. (2025) https://BioRender.com/m22j975.

The first enhancer structure was described for the mammalian immunoglobulin heavy chain gene (Banerji et al., 1983; Gillies et al., 1983; Mercola et al., 1983). Subsequently, an ever increasing number of enhancers for individual genes were discovered (Blackwood & Kadonaga, 1998), which were mostly located up to 1 Mb away from their target gene on the linear genome. Today, it is assumed that the human genome contains thousands of enhancers, and that the majority of genes is regulated by at least one enhancer (Javierre et al., 2016; Larsson et al., 2019; Pennacchio et

al., 2013; Rubin et al., 2017; Schoenfelder et al., 2018). Further, super-enhancers represent a special group of regulatory DNA elements that exceed the 1 Mb distance limit by far. Each super-enhancer is composed of several individual enhancer sequences, that can be located even on different chromosomes, and come together in three-dimensional space (Blayney et al., 2023; Hnisz et al., 2013). Super-enhancer structures have been described to regulate the expression of cell-lineage specific genes, that require robust expression at relatively high levels (Hnisz et al., 2013; Jia et al., 2019; F. Tang et al., 2020). The multitude of individual enhancers within a super-enhancer is thought to provide robustness to target gene transcription, ensuring cell-lineage specific genes to be transcribed at consistently high rates, and with low cell-to-cell variation. Such a multi-enhancer hub, referred to as the Greek islands, regulates one of the most selective expression processes in the mammalian genome (Monahan et al., 2017): in each olfactory neuron, only one of more than a thousand olfactory receptor genes interacts with this enhancer hub and is expressed, while the remaining olfactory receptor genes are transcriptionally silenced.

The systematic analysis of enhancer-promoter interactions has been facilitated by the advent of chromosome conformation capture (3C) techniques coupled to next generation sequencing, such as Hi-C, which enable the mapping of three-dimensional DNA-DNA interaction frequencies in a genome-wide manner (de Wit & de Laat, 2012; Dekker et al., 2002; Denker & de Laat, 2016). For Hi-C analysis, the three-dimensional conformation of the genome is fixed *in situ* using formaldehyde (Belaghzal et al., 2017; Belton et al., 2012; Lieberman-Aiden et al., 2009). Subsequently, the genome is digested by a restriction enzyme, and then DNA is re-ligated *in situ*. During the re-ligation event, any two DNA fragments that were in close spatial proximity at the timepoint of fixation can be ligated to each other. The resulting hybrid sequences resemble interaction pairs of two genomic regions that were in close contact in the nucleus, and paired end Illumina sequencing of these hybrid sequences enables the mapping of interaction pairs throughout the genome. Beyond enhancer-promoter interactions, 3C methods have revealed overarching three-dimensional structures for interphase chromosomes, such as topologically associated domains (TADs)(**Figure 3b**). TADs direct and restrict enhancer-promoter interactions (Dixon et al., 2012; Nora et al., 2012), and form A and B compartments by interacting transcriptionally active and inactive TADs, respectively (Harris et al., 2023; Lieberman-Aiden et al., 2009). In this chapter, we have learned that the folding of the interphase genome is not random, but rather highly organized; from enhancer-promoter loops, that mostly take place within TADs, to A and B compartments on a larger scale. Interestingly, not only transcription, but also other nuclear processes are influenced by 3D genome folding, such as DNA repair and replication (Pope et al., 2014; Zagelbaum et al., 2023).

4.3 Post-transcriptional processes affect steady state mRNA levels

After transcriptional regulation has determined whether and how much pre-mRNA is made from each gene, there are several post-transcriptional processes that influence the level of mature mRNA for each gene as an additional layer of regulation (**Figure 4**). mRNA processing determines how much pre-mRNA is made into mature mRNA and exported to the cytoplasm. Typical mRNA processing in mammals consists of at least three biochemical reactions: *cis*-splicing, capping and polyadenylation (Berget et al., 1977; Chow et al., 1977; Edmonds & Abrams, 1960; Furuichi et al., 1977), which occur either co-transcriptionally or shortly after transcription termination. While properly processed mature mRNA is exported to the cytoplasm for storage and translation, unprocessed pre-mRNA is pre-maturely degraded (Bhat et al., 2024; Davidson et al., 2012; L. Zhang et al., 2021). Further, the stability of mature mRNA transcripts is actively regulated by the cell. Several partially redundant RNA degradation pathways have been characterized (Bai et al., 1999; Decker & Parker, 1993; Łabno et al., 2016; Larimer et al., 1992; Mitchell et al., 1997), and RNA stability is further influenced by nuclear export, cytoplasmatic transport and storage of a transcript, by its translation efficiency, and by covalent RNA modifications (Barbieri & Kouzarides, 2020; Denes et al., 2021; Fan et al., 2018; Presnyak et al., 2015; Riggs et al., 2020). In this chapter, the biochemical pathways are summarized that enable mRNA processing and mRNA stability in mammalian cells, and thereby function as potential regulatory nodes on the post-transcriptional level.

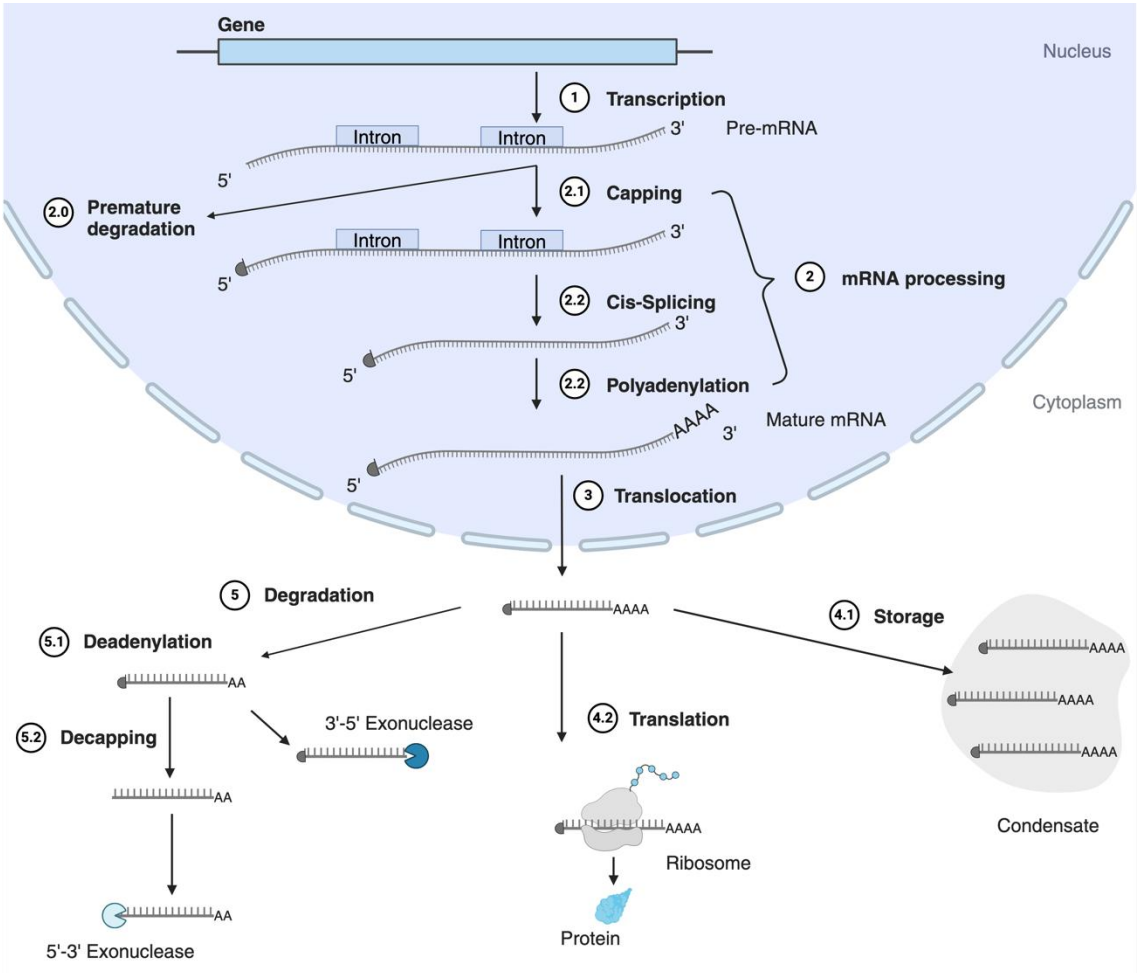

Figure 4. Post-transcriptional processes affect the fate of an mRNA. (1) Directly after transcription of a gene has started in the nucleus of a cell, post-transcriptional processes begin to shape the lifecycle of an RNA: (2) RNA processing by capping, cis-splicing and polyadenylation occurs co-transcriptionally, as well as premature RNA degradation, a process that competes with processing. (3) Mature mRNA is then translocated from the nucleus to the cytoplasm of a cell, where it is either stored in membrane-less condensates (4.1), or used as translation template for protein biogenesis (4.2) by ribosome complexes. Finally, mature mRNA is degraded by distinct cytoplasmic pathways, initiated by deadenylation of the 3' end, and performed by exonucleases. Created in BioRender. Luzak, V. (2024) BioRender.com/g10s442.

4.3.1 Pre-mRNA is processed by capping, *cis*-splicing and polyadenylation

mRNA processing consists of three main biochemical processes, that occur in the nucleus either co-transcriptionally or shortly after transcription termination. The first process is referred to as capping, and describes the co-transcriptional addition of a 7-methylguanylate (m^7G) cap to the 5´mRNA end (Furuichi et al., 1977). The capping process is composed of multiple steps catalyzed by several enzymes, that add guanosine triphosphate by an untypical 5´-5´ linkage to the 5´end of each mRNA, before modifying it by methylation (Ensinger et al., 1975). The unusual 5´-5´ linkage and methylation protect the 5´ end of capped mRNAs from conventional RNA degra-

dation by exonucleases. The second process is *cis*-splicing, which acts co-transcriptionally on the protein coding sequence (CDS) of pre-mRNAs (Berget et al., 1977; Chow et al., 1977). The coding sequence of most eukaryotic genes is composed of so called intron and exon sequences. Intron sequences are not part of the final protein coding sequence and are therefore removed by splicing. Further, the splicing process allows for alternative exon combinations while generating the final coding sequence, which serves as a mechanism to expand the coding repertoire (Marasco & Kornblihtt, 2023). The third process is polyadenylation of the 3´end (Edmonds & Abrams, 1960; Edmonds & Caramela, 1969), which is initiated shortly before transcription termination and protects the 3´end of the mRNA from exonuclease degradation. A polyadenylic acid (poly-A) tail is added to the mRNA that can be up to several hundred bases long and serves as a recruitment platform for regulatory proteins such as poly(A)-binding protein (PABP) that further stabilize the polyadenylated mRNA and initiate nuclear export of the mature mRNA.

While properly processed mature mRNA is exported from the nucleus to the cytoplasm for further storage and translation, pre-mRNA that is not properly processed remains unprotected in the nucleus and has high propensity for pre-mature degradation (Bhat et al., 2024; Siddiqui et al., 2007; Vinciguerra & Stutz, 2004). Nuclear exonucleases that coordinate degradation of pre-mRNA have been described, but the concrete pathways have remained largely unexplored (H. Liu et al., 2014; Moore, 2002; Rambout & Maquat, 2024). Instead, the focus of RNA degradation research has been on mature mRNA turnover in the cytoplasm, a complex network of pathways that is explained in the following chapter.

4.3.2 Mature mRNA stability is regulated by several pathways

mRNA stability is highly regulated in eukaryotes and determines how long a certain transcript is present in the cell before degradation, which strongly influences steady state levels of the respective transcript. The responsible degradation pathways are complex and partly redundant, in order to guarantee efficient regulation of mRNA stability. Functional mRNA transcripts are mostly degraded by exonucleases, which are enzymes that can digest RNA either from the 5´or the 3´end. The 5´end of an mRNA is protected from degradation by the cap structure, while the 3´end is protected by the poly-A tail. In order for exonucleases to digest mRNA, the protective structures at the 5´or 3´end have to be removed. RNA degradation is mostly initiated by shortening of the poly-A tail by the Ccr4-Not, Pan2-Pan3 or other deadenylase complexes (Bai et al., 1999; Dupressoir et al., 2001; Wahle & Winkler, 2013), or alternatively by uridinylation of the poly-A tail (Łabno et al., 2016). The shortened poly-A tail results in decapping of the 5´end by the Dcp1/2 complex (Decker & Parker, 1993) and subsequent digest by 5´-3´ Xrn endonucleases (Larimer et al., 1992). Alternatively, mRNAs with a shortened poly-A tail can by digested by the exosome

complex and other 3´-5´exonucleases (Malecki et al., 2013; Mitchell et al., 1997). Also, decapping and 5´-3´degradation by Xrn can occur independently of poly-A shortening. These complex pathways are coordinated by cis-regulatory sequences in the mRNA itself, as well as by trans-acting proteins and non-coding RNAs that are recruited by these sequences (Łabno et al., 2016).

Additional RNA degradation pathways exist, which rely on endonucleases, that can cleave RNA molecules within the sequence. For example, the degradation of non-functional mRNAs is coupled to ribosome activity during translation and mediated by endonucleases (Shoemaker & Green, 2012): transcripts with a premature stop codon are degraded by the nonsense-mediated decay (NMD) and no-go decay (NGD) pathways, while transcripts lacking a stop codon are degraded by the non-stop decay (NSD) pathway (Shoemaker & Green, 2012). Also, endonuclease activity is involved in RNA interference (RNAi), a pathway that controls RNA levels via reverse complementary miRNAs (Fire et al., 1998).

The accessibility of mRNAs for degradation is strongly influenced by the proteins that bind to a transcript, as well as the respective biological process the mRNA is involved in. For example, it was shown that nuclear export (Fan et al., 2018), cytoplasmatic transport (Denes et al., 2021) and cytoplasmatic storage in P bodies or stress granules (Riggs et al., 2020) influence mRNA half-life. Further, translation efficiency affects mRNA stability (Presnyak et al., 2015), with highly translated transcripts being more stable than transcripts with low translation efficiency. Recently, covalent RNA modifications such as m^6A have been discovered in mRNAs (Arzumanian et al., 2022; Barbieri & Kouzarides, 2020), that affect RNA stability. Taken together, cells undertake a tremendous effort and invest in complex, robust post-transcriptional regulation mechanisms that strongly shape the output of gene expression beyond transcriptional control. These post-transcriptional processes might have been slightly understudied in comparison to transcriptional regulation, but need to be integrated when studying how cells control the conversion of a gene into protein.

In this first chapter, the complexity of the gene expression cascade and involved cellular pathways were described for well characterized more complex eukaryotes. However, this thesis focuses on gene expression in African trypanosomes – which are less complex eukaryotes that act as unicellular pathogens and rely on tight gene expression regulation in order to infect humans and livestock. The following chapter will therefore introduce the importance of gene expression regulation in African trypanosomes – and the open questions that I have addressed with my research.

5. Introduction II – Regulation of gene expression is essential for trypanosome survival and infectivity

Trypanosomes are unicellular eukaryotes from the early branching supergroup of Excavata (Adl et al., 2012). While they share many biological processes with other eukaryotes, early branching has allowed the emergence of divergent molecular processes in some cases, as it will be described in the following chapters. Trypanosomes rely on a parasitic life style, infecting humans and other mammalian species, such as livestock and wildlife (Lukeš et al., 2014). Bloodsucking insects serve as vectors for transmission: African trypanosomes are transmitted by the tsetse fly, causing debilitating diseases, such as sleeping sickness in humans and nagana in live-stock and wildlife, in sub-Saharan Africa (Bruce et al., 1895; Cox, 2004; Kennedy, 2013), whereas American trypanosomes are transmitted by kissing bugs, causing Chagas disease in Central and South America (Álvarez-Hernández et al., 2021; Chagas, 1909; Sousa et al., 2024). African and American trypanosomes are closely related; however, their cell biology, immune evasion strategy and disease phenotype partially differ. In this thesis, I have worked with a species of African trypanosomes (*Trypanosoma brucei brucei*), and therefore the term "trypanosome" will refer to African trypanosomes throughout this thesis, if not stated otherwise.

When African trypanosomes are transmitted from the tsetse fly vector to the mammalian bloodstream and vice versa, they undergo a complex lifecycle of at least seven stages (**Figure 5**), in order to navigate the diverse environments they encounter (Luzak et al., 2021; Matthews, 2005; Plimmer & Bradford, 1900). (1) Metacyclic trypanosomes reside in the salivary gland of the tsetse fly, and are optimally adapted to invade the mammalian bloodstream upon a bite. In the bloodstream, metacyclic cells differentiate into (2) slender bloodstream form cells, the main infective form of the parasite that is optimally adapted to evade the immune system. While most slender cells actively replicate and thereby maintain infection, a subset differentiates into (3) non-replicative stumpy forms when the population reaches a certain density. Stumpy forms are optimally adapted for transmission to the tsetse fly vector, and are taken up by the next tsetse fly bite. Upon uptake, stumpy forms differentiate into (4) procyclic forms that are optimally adapted to reside in the midgut of the fly, and to migrate to the proventriculus, where they become (5) mesocyclic and (6) long epimastigote cells. Entering the salivary glands, long epimastigotes become (7) short epimastigote cells and subsequently (1) metacyclics, which completes the life cycle.

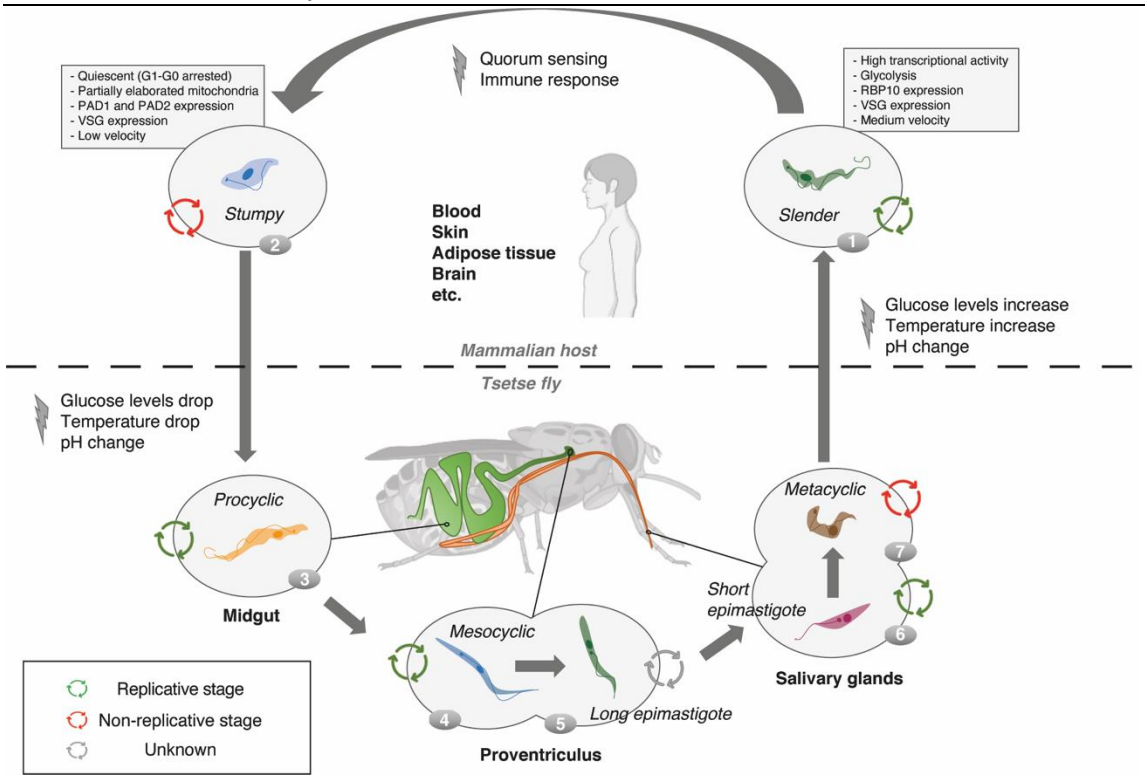

Figure 5. African trypanosomes undergo a complex lifecycle, with seven life cycle stages optimally adapted to the respective environment. (1) Metacyclic trypanosomes reside in the salivary gland of the tsetse fly, and invade the mammalian bloodstream upon a bite by the transmitting tsetse fly. Once in the bloodstream of the mammalian host, metacyclic cells differentiate into (2) slender bloodstream form cells, the main infective form of the parasite that is optimally adapted to evade the immune system via antigenic variation. While most slender cells maintain infection, a subset differentiates into (3) non-replicative stumpy forms, which are taken up by the bite of next tsetse fly. Upon uptake, stumpy forms differentiate into (4) procyclic forms that reside in the midgut of the fly, and migrate to the proventriculus, where they become (5) mesocyclic and (6) long epimastigote cells. Entering the salivary glands, long epimastigotes become (7) short epimastigote cells and subsequently (1) metacyclics, which completes the life cycle. This figure was generated for the review from Luzak et al. 2021 and is used with permission from Annual Reviews.

Each life cycle stage is well adapted to the respective temperature, pH and nutrients in each environment. These physical and biochemical parameters can change dramatically between the different environments. For example, procyclic cells in the fly midgut are exposed to temperatures from 20 (night time) – 27 °C (day time) and an alkaline environment, where they mostly feed on proline in the absence of glucose (Wargnies et al., 2018; Weiss et al., 2019). Interestingly, the migration behavior of procyclic cells in the midgut is regulated by the alkaline environment (Shaw et al., 2022). This pH-mediated migration behavior is required for infection of the fly, illustrating one of many examples how well adapted and how tightly connected trypanosomes are with their diverse environments. In contrast, slender bloodstream form cells have to adapt their cell biology to 37°C -42 °C in the host, a rather neutral blood pH around 7 and mainly feed on glucose. In addition, they are constantly challenged by the mammalian immune system in the bloodstream, which they have to actively evade.

In summary, trypanosomes regularly undergo tremendous changes of their immediate environment while completing the life cycle. They even utilize changes in environmental parameters, e.g. to control migration in the fly midgut or to induce differentiation (Quintana et al., 2021; Shaw et al., 2022). In response to such drastic environmental changes, trypanosomes need to adapt their cellular functions by changing the expressed set of proteins under each condition (Siegel et al., 2010; Urbaniak et al., 2012). Therefore, tight mechanisms to regulate gene expression are required by the parasite. A special case of extreme gene regulation occurs during infection in bloodstream form cells: in order to evade the host immune system, the infective cells express large amounts of a single surface antigen, while >2000 other surface protein genes remain silent at any time. The regular exchange of the expressed antigen is an extremely successful immune evasion strategy referred to as antigenic variation and will be explained in the next chapter.

5.1 Selective antigen expression enables immune evasion

5.1.1 Antigenic variation is an efficient immune evasion strategy

Antigenic variation refers to the ability of pathogens to periodically exchange the antigen proteins displayed on their surface, in order to evade the host immune system (Deitsch et al., 2009). It has proven a very successful immune evasion strategy and pathogens from diverse phyla, such as bacteria, fungi and protozoan parasites, employ antigenic variation to survive and establish infections. African trypanosomes serve as a model organism to study antigenic variation, since they have evolved a particularly large repertoire of more than 2000 antigen genes (Horn, 2014).

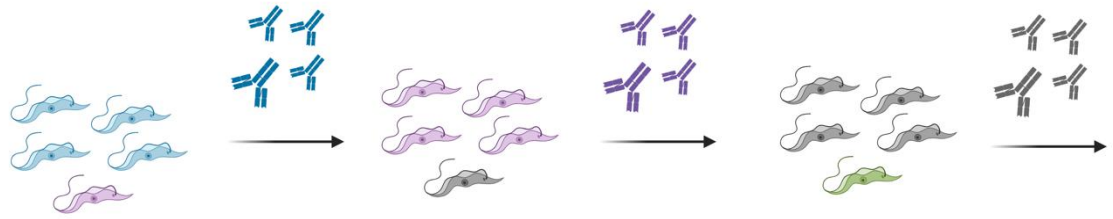

Figure 6. Antigenic variation allows African trypanosomes to efficiently evade the host immune system. Infective trypanosomes reside in the bloodstream of the mammalian host, and express a dense antigenic coat on their surface, which is comprised of millions of copies of the same antigen, namely a variant surface glycoprotein (VSG). The host immune system detects the dense antigenic layer and mounts an immune response. However, in the meantime few trypanosomes have switch the expressed VSG antigen, and exchanged the layer on their surface. Thereby, they can evade the immune response and maintain infection. Created in BioRender. Luzak, V. (2024) BioRender.com/i50u865.

After transmission, slender bloodstream form trypanosomes reside extracellularly in the mammalian bloodstream, where they are constantly confronted with the host immune system. Each bloodstream form parasite expresses a single antigen gene, referred to as variant surface glycoprotein (VSG), at high levels by RNA polymerase I, and millions of copies of this particular VSG protein cover the parasite surface and its invariant membrane proteins (Cross, 1975). The host immune system mounts an efficient response to the displayed VSG protein (**Figure 6**). However, by then, some parasites in the population have switched and express another VSG protein, thereby evading recognition by the immune response and sustaining the parasite population. African trypanosomes have a large repertoire of > 2000 VSG genes, alongside with molecular mechanisms that allow the generation of new, mosaic antigen genes during infection (Kamper & Barbet, 1992). Due to such inexhaustible repertoire, trypanosome infections can last for years and often end detrimental for the host organism (Horn, 2014; Kamper & Barbet, 1992; Kennedy, 2013).

5.1.2 The trypanosome genome harbors specialized regions for antigen expression

The genome of *T. brucei* is mainly composed of 11 megabase chromosomes, in addition to intermediate and mini-chromosomes, and with its size of around 30 Mb, the parasite genome is around 100 times smaller than the human genome (Berriman et al., 2005; Cosentino et al., 2021; Müller et al., 2018). The 11 megabase chromosomes can be functionally divided into (**Figure 7** a): (1) The diploid chromosome core regions that harbor around 8000 RNA polymerase II transcribed genes, tRNA and rRNA genes (Cosentino et al., 2021). (2) A rather unconventional subtelomeric genome portion, which is solely committed to immune evasion in the mammalian host: the large repertoire of > 2000 antigen genes is located on the heterozygous subtelomeric regions of chromosomes, as well as on intermediate chromosomes (Cosentino et al., 2021). While the diploid core regions are actively transcribed by RNA polymerase II, the large subtelomeric antigen repertoire remains transcriptionally silent most of the time. The fact that antigen genes comprise >20% of total genes underlines the huge investment of trypanosomes in immune evasion and its importance for parasite survival.

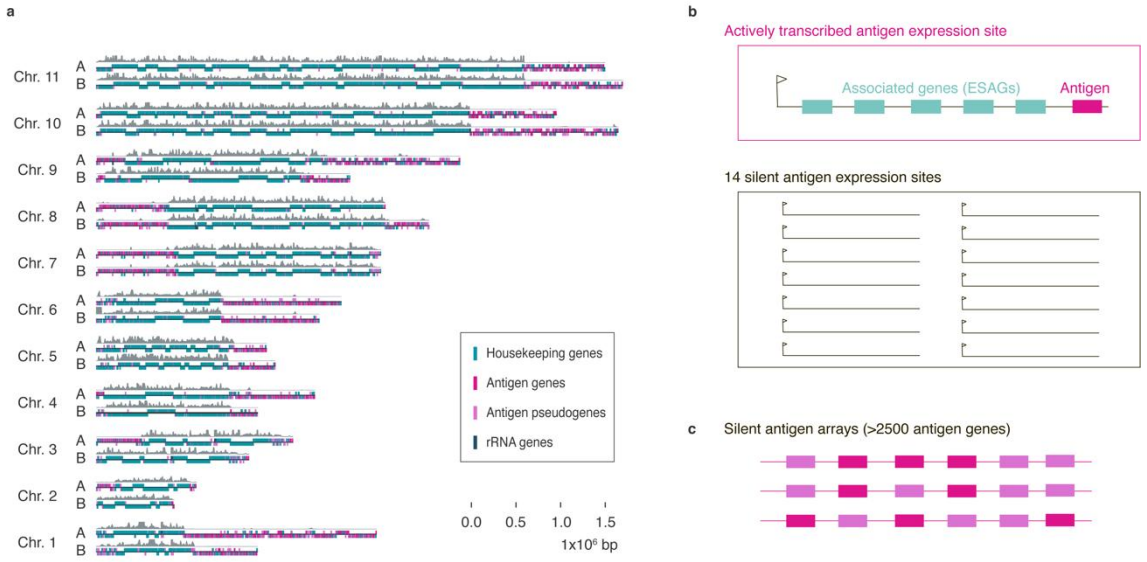

Figure 7. The genome of African trypanosomes is subdivided into actively transcribed chromosome cores and long subtelomeric arrays harboring the transcriptionally silent antigen gene repertoire. (a) Trypanosomes harbor 11 megabase chromosomes. The diploid chromosome cores harbor RNA polymerase II transcribed genes (shown in green), while the haploid subtelomeric arrays harbor antigen genes (shown in magenta). RNA-seq is displyed in grey on top of the chromosomes. Antigen genes are either located in a downsream location of 15 antigen expression sites (b), or in long subtelomeric arrays (c). While subtelomeric arrays and 14 antigen expression sites remain silent, one antigen expression site is expressed in infective bloodstream form cells.

Only in bloodstream form cells during infection, one of 15 specific antigen expression sites becomes active (**Figure 7** b), leading to one VSG antigen being expressed per cell (Cosentino et al., 2021; Horn, 2014; Müller et al., 2018). The 15 antigen expression sites are specialized polycistronic transcription units located in the subtelomeric regions or on intermediate chromosomes (Barcons-Simon et al., 2023; Hertz-Fowler et al., 2008; Müller et al., 2018). Each expression site (ES) harbors an upstream promoter for RNA polymerase I, which is distinct from rDNA-associated promoters. Several expression-site-associated genes (ESAGs) are localized downstream of the promoter, and a single VSG gene resides at the telomeric end of each expression site, flanked upstream by 70 bp repeats and downstream by the telomeric repeats (Hertz-Fowler et al., 2008). The expression of only one of these 15 expression sites ensures that a single VSG protein covers the surface of the parasite. How trypanosomes regulate highly selective VSG expression has been a long-standing question in infection biology. Within recent years, regulatory factors involved in selective VSG expression have been identified. However, regulatory mechanisms and pathways have largely remained elusive to date, which is described in the following chapter.

5.1.3 Selective antigen expression is assumed to be tightly regulated in trypanosomes

Highly selective and robust antigen expression is essential for trypanosome survival in the mammalian host. On the one hand, if selective antigen expression is lost, several antigens are expressed on the surface of each parasite and a coordinated switch from one antigen to another becomes impossible. As a result, the immune system was shown to quickly clear the parasite infection upon loss of mutually exclusive expression (Aresta-Branco, Sanches-Vaz, et al., 2019). On the other hand, if robust expression of the single active antigen would be lost, there would be insufficient amounts of VSG protein to tightly cover the parasite surface. As a result, the immune system could be exposed to invariant surface proteins normally covered by VSG proteins and would kill the parasite population quickly. Due to its importance for parasite survival in the mammalian host, antigen expression is assumed to be highly regulated. So far, multiple proteins have been identified that affect antigen expression, which will be reviewed briefly in this chapter. However, molecular mechanisms or concrete cellular pathways that coordinate activity of the individual factors have remained largely elusive.

The single active antigen expression site is located in an extra-nucleolar RNA polymerase I focus, referred to as the expression site body (ESB)(Navarro & Gull, 2001)(**Figure 8**), where it associates with specific regulatory proteins: The VSG exclusion factor (VEX) complex composed of VEX1 and VEX2 protein was the first described ESB-specific protein complex (J. Faria et al., 2019; J. R. C. Faria et al., 2023; Glover et al., 2016). VEX2 is a UPF1-like helicase, a helicase family which is involved in non-sense mediated decay and in telomeric heterochromatin formation in more complex eukaryotes (Azzalin et al., 2007; J. R. C. Faria et al., 2023). In a complex with VEX1, both proteins ensure that only one expression site is active at any time. More recently, the expression site body associated protein ESB1 was identified as an activator specific for antigen expression (López-Escobar et al., 2022). ESB1 associates with the active expression site close to the promoter, where it promotes Pol I recruitment and high antigen expression levels. The only classical transcription factor identified at the ESB so far was the basal class 1 transcription factor A (CIFTA) (Nguyen et al., 2014). In addition, the SUMO E3 ligase TbSIZ1/PIAS1 was shown to place a strong SUMOylation signal at expression site body proteins, which is required for high levels of VSG transcription (López-Farfán et al., 2014; Saura et al., 2019). Interestingly, SUMOylation is a well-characterized signal that removes rDNA genes from the nucleolus for repair (Capella et al., 2021), and might therefore play a crucial role in extra-nucleolar ESB formation.

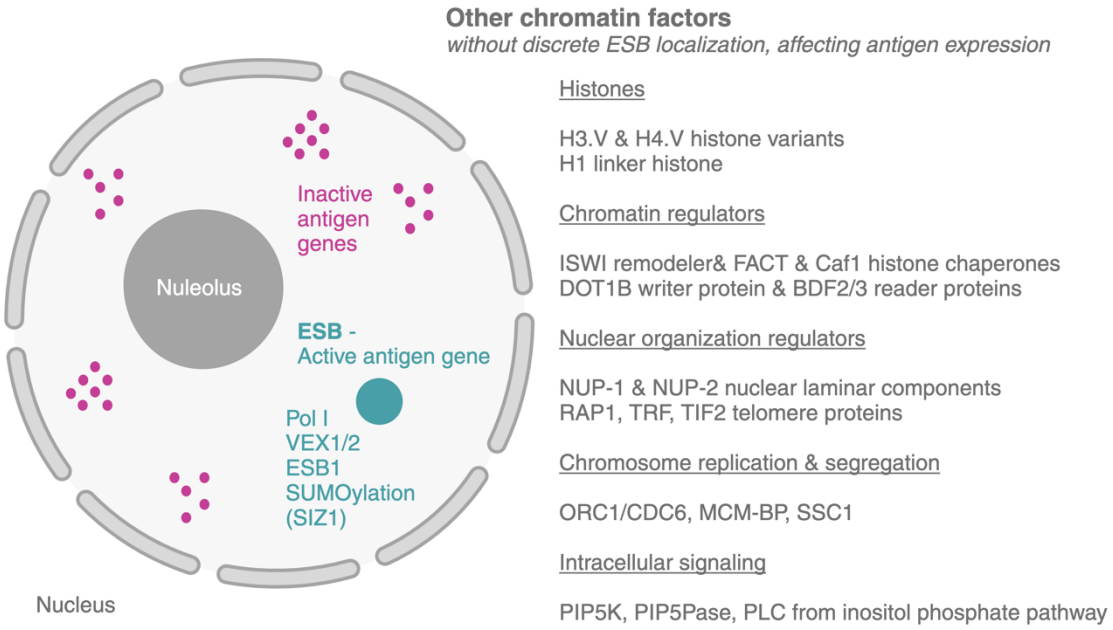

Figure 8. Multiple chromatin factors are involved in regulating mutually exclusive antigen expression in infective African trypanosomes. The active antigen gene is expressed by RNA polymerase I (Pol I) in an extranucleolar focus referred to as expression site body (ESB). Inactive antigen genes were described to form several clusters within the trypanosome nucleus, located distant to the ESB. So far, few specific proteins were shown to co-localize with the ESB: VEX1, VEX2, ESB1 and a SUMOylation focus conferred by SIZ1. Upon depletion of these factors, mutually exclusive antigen expression was impaired. Further, a set of chromatin-related proteins with less distinct localization was described to regulate mutually exclusive antigen expression: upon depletion or deletion of several histone variants and histone H1, chromatin writers, readers and remodelers, laminar and telomere proteins, as well as replication and signaling proteins, mutually exclusive antigen expression was impaired. Created in BioRender. Luzak, V. (2025) https://BioRender.com/l28f846.

Further, transcriptional regulation on the level of chromatin most probably plays a role in selective antigen expression, since several chromatin-related factors were shown to influence selective antigen expression (Cestari & Stuart, 2018): For example, depletion or deletion of (1) the linker histone H1 (A. C. Pena et al., 2014; Povelones et al., 2012) and the histone variants H3.V & H4.V (Müller et al., 2018), (2) the chromatin writer DOT1B which places H3K76me3 marks (Figueiredo et al., 2008), (3) the bromodomain-containing chromatin readers Bdf2/3 (Schulz et al., 2015), (4) the chromatin remodeler ISWI (Hughes et al., 2007), and (5) the histone chaperones FACT (Denninger & Rudenko, 2014) and Caf1 (Alsford & Horn, 2012) resulted in de-repression of inactive antigen genes. Also, bloodstream form trypanosomes exhibit the unconventional DNA modification ß-d-glucosyl-hydroxymethyluracil (BaseJ), which was shown to be involved in antigen silencing (Reynolds et al., 2014).

Since regulation on the level of chromatin seems to play a role in selective antigen expression, comparable to mechanisms described in more complex eukaryotes, would

it be possible that 3D genome conformation plays an important role as well? There are several findings that support the hypothesis that spatial regulation within the nucleus is involved in selective antigen expression: As explained above, the active antigen gene is transcribed in a specified nuclear location, the ESB (Navarro & Gull, 2001), while inactive antigen genes are localized distant from this site in the nucleus. Also, the nuclear lamina proteins Nup-1 and 2 were shown to play a role in antigen repression, further suggesting a functional organization within the trypanosome nucleus (DuBois et al., 2012; Maishman et al., 2016). And finally, it was shown that a general disruption of chromatin structure leads to de-regulation of antigen expression (Müller et al., 2018). Taken together, these findings suggest that three-dimensional genome folding could play a role in antigen expression, leading to the question whether there might be a specific enhancer or super-enhancer structure in trypanosomes involved in antigen selection, as it was described for other highly selective expression mechanisms (Monahan et al., 2017).

5.1.4 Selective transcription is unlikely to regulate mutually exclusive antigen expression

It has been a long-standing question in infection biology how mutually exclusive antigen expression is regulated in trypanosomes (Horn, 2014). Although individual factors that affect mutually exclusive antigen expression have been identified in recent years, it has remained largely unclear which steps of the antigen expression cascade are regulated by them. Interestingly, it has been shown that transcription initiation is detected at all 15 antigen expression sites (Kassem et al., 2014; Vanhamme et al., 2000), suggesting that mutually exclusive expression cannot be regulated by strict transcriptional regulation. Instead, it has been suggested that transcription elongation and/or mRNA processing could be the main regulatory step, resulting in only one expression site being fully expressed. mRNA processing and transcription elongation have been shown to be directly coupled in other organisms (Uriostegui-Arcos et al., 2023). So far, it has remained elusive how selective processing of only one of 15 initiated ESs could be regulated in trypanosomes. Could it be regulated by specific DNA-DNA interactions, as suggested in the previous chapter? If so, would trypanosomes have evolved a divergent type of enhancer structure, which would not primarily affect transcription of its target gene as common enhancers in other mammalian cells do, but rather regulate selective processing of the target gene pre-mRNA, functioning as a post-transcriptional enhancer?

5.2 Regulation of RNA polymerase II transcribed genes is required for trypanosome survival in rapidly changing environments

Tight regulation of antigen expression, which was explained in the previous chapters, is exclusively occurring in the infective bloodstream form life cycle stage, in order to evade the immune system in the mammalian host. In contrast, regulation of RNA polymerase II transcribed genes, which are responsible for essential cellular functions such as metabolism, cell-cell communication or motility, is essential for all life cycle stages, in order to adapt these cellular functions to the changing environments (Briggs et al., 2023; Howick et al., 2022; Matthews, 2005; Siegel et al., 2010). In this thesis, RNA polymerase II transcribed genes in trypanosomes are defined as all protein-coding genes except the antigen gene family. While antigen genes mainly reside in the haploid-like subtelomeric chromosome arms, with most of them being transcriptionally silent, and one being highly expressed in bloodstream form cells by RNA polymerase I, RNA polymerase II transcribed genes are localized in the diploid chromosome core regions, and are actively transcribed by RNA polymerase II (Berriman et al., 2005; Cosentino et al., 2021; Müller et al., 2018)(**Figure 7**). In contrast to most other eukaryotes, RNA polymerase II transcribed genes are not regulated by gene-specific promoters, but are rather organized in long polycistronic transcription units (PTUs), which harbor up to hundreds of genes. Such particular linear genome organization makes distinct regulation of individual genes a challenging task for trypanosomes. To better illustrate the challenge that trypanosomes face, I would like to compare gene expression of individual genes in more complex eukaryotes with gene expression from polycistronic operons in prokaryotes, and place trypanosome gene expression in this context, as a hybrid concept sharing aspects of both systems (**Figure 9**).

In more complex eukaryotes, the initial regulatory step in the gene expression cascade is selective transcription of individual genes, while other genes remain silent. Here, the regulation occurs at the level of individual genes, via a regulatory promoter element (Smale & Kadonaga, 2003), that is located upstream of the respective gene. Gene-specific transcription rates can be further enhanced by the interaction of the promoter with a distal enhancer element, as introduced above. The coordinated expression of multiple genes is controlled by complex transcriptional programs, orchestrated by transcription factors binding to regulatory DNA sequence motifs in promoter sequences (Lambert et al., 2018). After selective transcription has occurred, RNA processing efficiency (Bhat et al., 2024) and RNA half-lives further shape RNA steady state levels of individual transcripts (E. Yang et al., 2003), contributing to a variation in transcript levels between genes of >1000-fold.

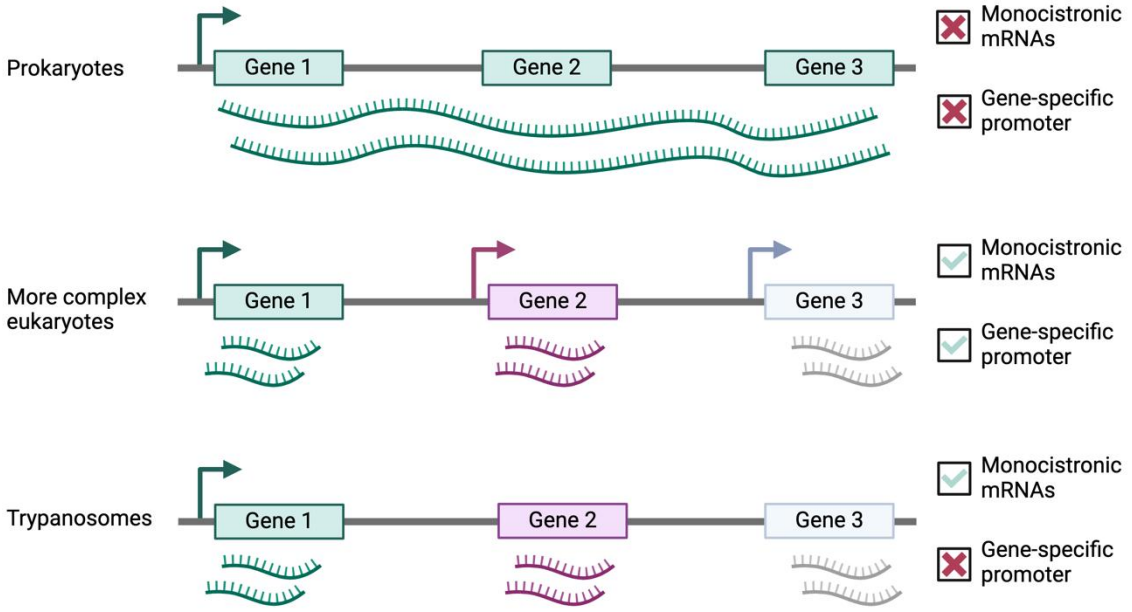

Figure 9. African trypanosomes have evolved a hybrid gene expression process, lacking gene-specific transcriptional regulation, but allowing transcript-specific regulation of monocistronic mRNAs. (1) In most prokaryotes, functionally related genes are transcribed from one polycistronic unit, referred to as operon. Here, all genes within one operon are co-regulated under a single promoter. The polycistronic transcript is directly translated, without RNA processing into individual monocistronic mRNAs. RNA stability can differ within a polycistronic transcript, e.g. due to secondary structures or ribosome binding sites with differing affinities. Regulation of individual genes does not occur at the level of transcription and RNA processing, but rather by RNA stability in prokaryotes. (2) In contrast, most complex eukaryotes harbor individual genes with gene specific promoters, that are transcribed into monocistronic mRNAs. Here, gene-specific regulation is possible at the level of transcription, as well as RNA processing and RNA stability. (3) In trypanosomes, most genes are organized within polycistronic transcription units, which harbor up to hundreds of functionally unrelated genes. Gene-specific regulation is not possible on the level of transcription. However, in contrast to prokaryotes, the polycistronic pre-mRNA is processed into monocistronic mRNAs, facilitating gene-specific regulation on the post-transcriptional level by RNA processing and RNA stability. Created in BioRender. Luzak, V. (2025) https://BioRender.com/h40q462.

In contrast, transcription is not regulated on the level of individual genes in prokaryotes: functionally related genes are organized downstream of a single promoter in polycistronic transcription units (PTUs), referred to as operons (Jacob et al., 1960). Here, selective transcription occurs on the level of operons: Similarly to eukaryotic transcription factors, prokaryotic sigma factors bind to the promoter of operons and thereby activate transcription of the entire unit with all the contained genes upon demand. Bacterial PTUs are transcribed into long polycistronic transcripts that harbor several protein coding sequences, and translation occurs directly on these polycistronic RNAs (Kohler et al., 2017; Miller et al., 1970). RNA processing by capping, splicing and polyadenylation does not occur for individual coding sequences and therefore does not play a major regulatory role in bacteria (Brown,

2002). Bacterial polycistronic transcripts display a certain half-life similar to individual mRNAs in more complex eukaryotes, and one could think they provide a similar half live to functionally related encoded genes. However, RNA stability can greatly vary within a polycistronic transcript, e.g. due to stabilizing secondary structures or ribosome binding sites with differing affinity (Rauhut & Klug, 1999). In summary, regulation of prokaryotic gene transcription occurs in functional gene groups, and it is RNA stability that affects individual transcript levels.

Organization of genes into polycistronic transcription units is rare in eukaryotes, and found for example in in *C. elegans*, *Drosophila* and trypanosomes. PTUs in trypanosomes harbor up to hundreds of genes, co-regulated by a single transcription start site (see chapter 5.2.1). In contrast to bacteria, all PTUs are transcribed at any time, and genes within a PTU do not have any functional relationship. Therefore, co-regulation of genes in one unit is not favored by the parasite. Instead, regulation of individual transcripts is required to obtain adequate levels. Processing of the polycistronic pre-mRNAs into individual mature mRNAs enables of individual transcripts regulation on the post-transcriptional level in trypanosomes: by RNA processing and RNA stability (see chapter 5.2.2). However, it has remained unclear (1) whether transcription contributes to gene expression regulation, e.g. by differential efficiency between or along PTUs, and (2) whether it is RNA processing or RNA stability that is mainly responsible for the observed drastic differences in steady state mRNA levels in trypanosomes.

5.2.1 Transcription by RNA polymerase II is assumed to be constitutive in trypanosomes

With the particular organization of RNA polymerase II transcribed genes into large PTUs, the process of transcription has indeed less regulatory potential than in most eukaryotes, where transcription is regulated on the level of individual genes. However, heterogenous levels of pre-mRNA could arise from PTUs due to differences in transcription elongation along PTUs, and due to differences in initiation frequency between different PTUs.

The transcription start sites (TSSs) of PTUs exhibit striking similarities: (1) Instead of focused promoters composed of a set of distinct regulatory DNA sequences, which are found in most eukaryotes and prokaryotes, transcription start sites in trypanosomes represent dispersed promoters with a less distinct, G-T rich DNA composition (Wedel et al., 2017). (2) It was shown that TSSs are occupied by a specific set of chromatin-related factors, such as histone variants H2A.Z and H2B.V (Siegel et al., 2009), which reduce nucleosome stability and facilitate transcription (**Figure 10**). Also, a specific set of permissive histone modifications, such as H4K10ac, is enriched at TSSs (Kraus et al., 2020; Siegel et al., 2009). (3) Further, a set of chromatin reader, writer and eraser proteins, as well as chromatin remodelers, is found at PTU

TSSs (Staneva et al., 2021): bromodomain factor BDF3 (Siegel et al., 2009), the SET27 promoter-associated regulatory complex (SPARC) complex (Staneva et al., 2022) and the SWR remodeler that deposits H2A.Z in more complex eukaryotes (Staneva et al., 2021). Beyond the transcription start sites, also the transcription termination sites (TTSs) of PTUs exhibit a distinct biochemical composition, characterized by histone variants H3.V and H4.V (Siegel et al., 2009) and other chromatin-related factors (Staneva et al., 2021). In summary, the biochemical and genetic characterization of PTUs and their chromatin environment has revealed high similarity among different transcription start sites. Depletion of several TSS-associated factors had a negative impact on all RNA polymerase II transcribed PTUs to a similar extent (Kraus et al., 2020; Staneva et al., 2022). These data support the long-standing assumption that RNA polymerase II occurs in a constitutive manner in trypanosomes, meaning that there is no strong difference in PTU initiation and elongation rates, and RNA polymerase II transcribed genes are transcribed at similar levels (C. E. Clayton, 2002).

Despite the strong evidence listed above, constitutive transcription has not been experimentally verified in trypanosomes. In order to test the hypothesis of constitutive transcription, an approach is required that can compare nascent transcript levels from different PTUs, in order to measure transcriptional dynamics before RNA processing and degradation affect RNA levels.

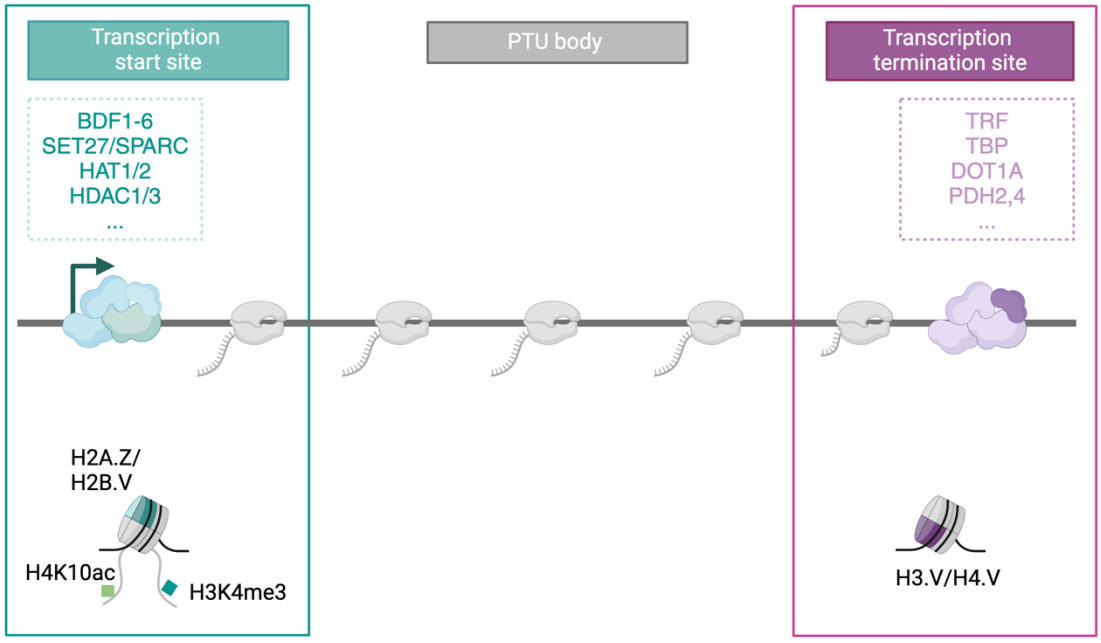

Figure 10. Transcription start sites display a characteristic biochemical composition that facilitates transcription in African trypanosomes. Transcription start sites of trypanosome PTUs are marked by a certain functional biochemical composition, that enables transcription initiation. Histone variants H2A.Z and H2A.V are recruited to these GT-rich DNA sequences, and histone marks H4K10ac and H3K4me3 are deposited by specific writer enzymes, e.g. histone acetyltransferases 1 and 2 (HAT1/2)

and histone methyltransferases. These marks further recruit reader proteins such as bromodomain factors 1-6 (BDF1-6) and the SPARC complex, that facilitate transcription, and eraser proteins such as histone deacetylases 1 and 3 (HDAC1/3). Similarly, also transcription termination sites (TTSs) are marked by a functional biochemical composition. Histone variants H3.V and H4.V are located at TTSs, recruiting chromatin factors that facilitate transcription termination. Created in BioRender. Luzak, V. (2024) BioRender.com/s80d913.

5.2.2 RNA processing and RNA degradation have the potential to regulate RNA polymerase II gene expression in trypanosomes

Unlike genes in bacterial operons, genes located in the same transcription unit in trypanosomes are not functionally related, and therefore co-regulation of a PTU's transcripts is not required or even unfavorable. Instead, tight regulation of individual RNA polymerase II transcribed genes is essential for the survival of trypanosomes: in order to go through different life cycle stages (Siegel et al., 2010), and to survive in very different environments (Matthews, 2005; Trindade et al., 2016). In addition to life cycle specific transcript levels, it has been shown that steady state levels of individual transcripts are regulated in a cell cycle dependent (Archer et al., 2011) and circadian (Rijo-Ferreira et al., 2017) manner. Steady state RNA levels vary up to 25-fold for different genes in trypanosomes (de Freitas Nascimento et al., 2018; Siegel et al., 2010), even when genes are located in the same PTU. This indicates that gene expression is tightly regulated. Since constitutive transcription could not give rise to such strong variation in RNA levels, both post-transcriptional processes, RNA processing and RNA stability, were proposed to play a major role for gene expression regulation in trypanosomes (C. Clayton, 2019).

5.2.2.1 Processing of pre-mRNAs is a two-step process in trypanosomes, mediated by *trans*-splicing and polyadenylation

A polycistronic pre-mRNA results from PTU transcription in trypanosomes, similar to the output of operon transcription in bacteria. However, other than in bacteria, trypanosome pre-mRNA is further co-transcriptionally processed into individual monocistronic mRNAs. However, other than in more complex eukaryotes, RNA processing occurs as a simplified two-step process in trypanosomes (**Figure 11**), composed of (1) *trans*-splicing, which confers the 5´cap structure, and (2) polyadenylation of the 3´end (Sutton & Boothroyd, n.d.; Ullu et al., 1993). The processing machineries have been described, and are largely homologous to more complex eukaryotes, with few divergent factors mediating trypanosome specific functions.

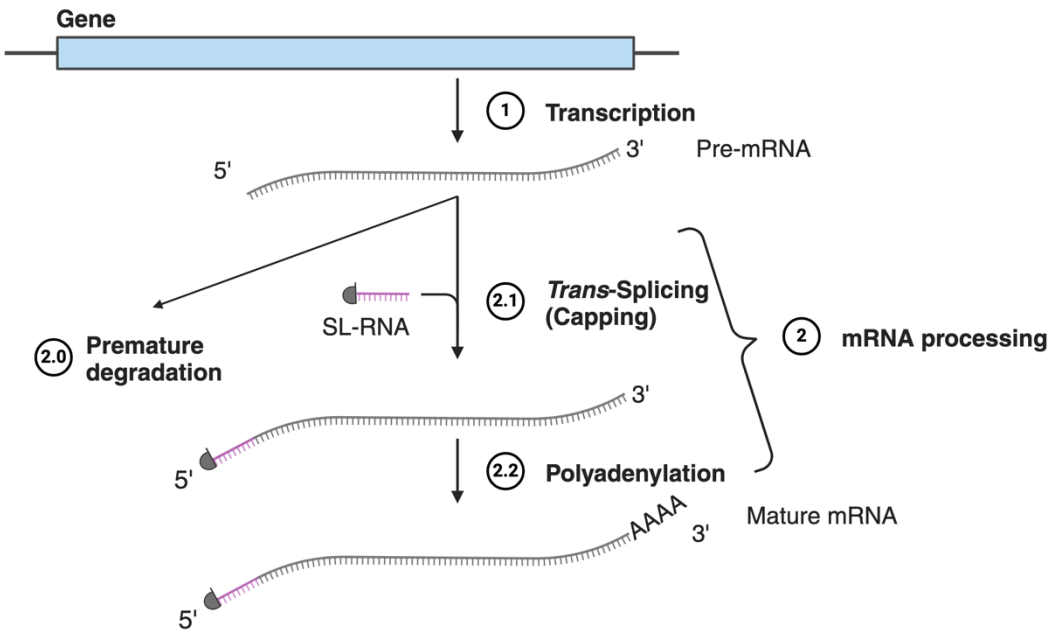

Figure 11. mRNA processing is a simplified two-step mechanism in African trypanosomes. Co-transcriptionally, polycistronic pre-mRNA is processed by (2.1) *trans*-splicing and (2.2) polyadenylation into mature, monocistronic mRNA. During *trans*-splicing, a 39 nt long spliced leader RNA (SL-RNA) is spliced to the 5' end of each mRNA in trypanosomes, conferring the cap structure. Typically, trypanosome genes do not contain introns, besides two genes that are *cis*-spliced. Polyadenylation occurs at the 3' end of an mRNA. mRNA processing presumably competes with premature RNA degradation. Created in BioRender. Luzak, V. (2024) BioRender.com/n00g170.

(1) *trans*-splicing describes a splicing process during which a single mature mRNA transcript is generated by joining two separate pre-mRNAs. In contrast, during conventional *cis*-splicing a mature mRNA is generated from a single pre-mRNA by intron removal. *trans*-splicing was first discovered in trypanosomes (Boothroyd & Cross, 1982; De Lange et al., 1984; Lei et al., 2016), and has subsequently been found in several other lower eukaryotic organisms. More recently, chimeric *trans*-splicing products have also been detected in cancer cells, and their oncogenic potential is currently explored (H. Li et al., 2008). In trypanosomes, *trans*-splicing is responsible for processing of the 5´end of mRNAs: a 39 nucleotide long spliced leader sequence (SL-RNA) carrying an unconventional cap structure is transferred to each pre-mRNA 5´end by *trans*-splicing (Perry et al., 1987). While every mature mRNA in trypanosomes requires processing by *trans*-splicing in order to receive the cap structure, only two genes in trypanosomes were shown to carry intron sequences, and are processed by *cis*-splicing. The trypanosome spliceosome is composed of conventional U2, U4, U5 and U6 snRNPs and Sm core proteins (Tkacz et al., 2010). However, whether U1 is implicated in trans-splicing has remained unclear, since it is thought

to be replaced by the SLRNA snRNP in trypanosomes. In summary, *cis*- and *trans*-splicing seem to be mediated by highly similar molecular machineries. While trypanosomes employ *trans*-splicing to enable the separation of polycistronic pre-mRNAs into individual transcripts, more complex eukaryotes mostly rely on *cis*-splicing to increase the coding repertoire.

(2) The trypanosome polyadenylation complex, which confers stability to the 3´mRNA end by adding the poly-A tail, has a largely conventional composition compared to more complex eukaryotes except from two trypanosome-specific subunits (Koch et al., 2016). In contrast to other eukaryotes, the polyadenylation site is not clearly marked in trypanosomes, resulting in heterogeneity among transcripts. Instead of a clear sequence motif, the polyadenylation site is rather placed within a certain distance from the splice site of the respective downstream transcript (Campos et al., 2008). This "ruler" function suggests that polyadenylation of the upstream and *trans*-splicing of the respective downstream mRNA could occur in a coupled manner, which has been suggested, but remains to be experimentally verified (C. Clayton, 2019).

5.2.2.2 Similar to more complex eukaryotes, multiple pathways control mRNA stability in trypanosomes

RNA stability regulation is complex in most eukaryotes, with multiple components affecting RNA half-life: e.g. cytoplasmic & nuclear RNA degradation pathways, RNA binding proteins, RNA modifications, RNA storage in nuclear condensates and translation efficiency. In this chapter, I will briefly give an overview over the most relevant regulatory processes in trypanosomes, in comparison to more complex eukaryotes.

Conventional cytoplasmatic RNA degradation pathways by exonucleases are present in trypanosomes and were comprehensively characterized (C. Clayton, 2019). mRNA degradation is mostly initiated at the 3´end of an mRNA, by deadenylases shortening the poly-A tail. In more complex eukaryotes, it is mainly Ccr4 or Caf1 deadenylase in complex with Not, that catalyze deadenylation. Similarly, the Caf1-Not complex was identified as the main initiator of mRNA degradation in trypanosomes (E. Erben et al., 2014; Fadda et al., 2013; Färber et al., 2013; Schwede et al., 2008), while no Ccr4 homologue has been identified so far. Also Pan2-depedent deadenylation exists in trypanosomes, but was shown to play a minor role for overall mRNA stability (Fadda et al., 2013; Schwede et al., 2009). Decapping of the 5´mRNA end is initiated by the shortening of the poly-A tail, and conferred by an unconventional decapping complex, lacking a Dcp2 homologue and instead relying on the ApaH-like phosphatase ALPH1 (S. Kramer, 2017b; S. Kramer et al., 2023). After deprotection, mRNA degradation in trypanosomes mainly occurs from the 5´end mediated by the exonuclease XRNA (Xrn1 homologue) (S. Kramer, 2017a; Manful et al.,

2011), supported by the exosome exonuclease which starts at the 3´end. However, the exosome complex was shown to play a minor role in cytoplasmic RNA degradation (Fadda et al., 2013).

Nuclear degradation pathways that affect the stability of pre-mRNAs have been suggested as critical regulators of post-transcriptional gene expression in trypanosomes (C. Clayton, 2019; Fadda et al., 2014). However, similar to more complex eukaryotes, the pathways responsible for nuclear RNA degradation have remained largely unknown. In trypanosomes, the exosome with its nuclear localization and role in rRNA processing is currently discussed as the main machinery of nuclear RNA degradation (Haile et al., 2007; S. Kramer et al., 2016), strongly supported by the finding that exosome depletion resulted in the accumulation of partially unprocessed pre-mRNAs.

Components of endonuclease-mediated RNA degradation pathways are partially present in trypanosomes (C. Clayton, 2019), but play no major role in mRNA degradation. Nonsense mediated decay (NMD) could not be experimentally verified, although homologues for Upf1 and 2 as well as eRf1 and 3 exist in the parasite (Cosentino et al., 2021; Delhi et al., 2011). Components of the RNA interference machinery are functional in trypanosomes and can be leveraged for RNAi knock down of target proteins. However, so far no evidence for endogenous miRNA has been detected, suggesting that this pathway most probably suppresses retroposons and viruses and does not affect endogenous mRNA levels (Lye et al., 2010; Ngô et al., 1998).

In addition to RNA degradation pathways, more than 150 RNA-binding proteins (RBPs) were identified in trypanosomes (C. Clayton, 2019; Lueong et al., 2016b), that bind to cis-regulatory mRNA sequences in a combinatorial manner and thereby regulate RNA stability of specific functional mRNA subsets (E. D. Erben et al., 2014). For example, the RNA binding protein 6 (TbRBP6) regulates the differentiation into infective metacyclic parasites, and RBP10 establishes and promotes infective bloodstream form parasites (Kolev et al., 2012; Mugo & Clayton, 2017). RBP42 regulates the metabolic adaptation of bloodstream form parasites to their environment, and PuREBP1 and 2 regulate nucleobase transporter expression upon purine starvation. Further, the composition of the protein coding sequence was shown to strongly influence RNA stability, with ideal codon usage causing high translation efficiencies and increased RNA half-lives (de Freitas Nascimento et al., 2018; Jeacock et al., 2018). m^6A was the first RNA modification described to increase RNA half-live in trypanosomes (Viegas et al., 2022), and in addition, the incorporation of RNAs in biological condensates, such as stress granules, was shown to regulate RNA stability upon heat shock (S. Kramer, 2014).

5.2.3 The regulatory potential of both post-transcriptional processes has not been studied systematically

Although the pathways and machineries that mediate RNA processing and stability have been largely characterized, the regulatory role of both post-transcriptional processes for gene expression has remained unclear in trypanosomes. mRNA processing rates have been determined for a few individual genes: These initial experiments have demonstrated that processing occurs co-transcriptionally (Haanstra et al., 2008; Ullu et al., 1993), while RNA polymerase II transcribes the polycistronic pre-mRNA. Further, it was shown that splicing efficiency can vary for a subset of example genes, e.g. according to the quality of the poly-pyrimidine tract marking the splice site (Huang & Van der Ploeg, 1991; Siegel et al., 2005). Genome-wide information about processing efficiencies is missing, in order to determine the degree of variation between genes and predict the potential regulatory power of differential RNA processing on gene expression regulation in trypanosomes.

Trypanosome mRNA half-lives have been measured in a genome-wide manner, ranging from few minutes to several hours, with a median half live of 12 minutes in bloodstream forms (Fadda et al., 2014). In this previous study, transcription was blocked using a transcriptional inhibitor and RNA levels were determined at different time points after inhibition by RNA-seq. RNA half-lives from this previous study correlated with steady state RNA levels, but have also shown that half lives alone cannot predict steady state levels in trypanosomes (Antwi et al., 2016; Fadda et al., 2014; Haanstra et al., 2008). However, it has been shown recently that inhibition of an essential cellular process such as transcription can strongly affect RNA half-lives, and therefore interferes with the measurement (Haimovich et al., 2013; Herzog et al., 2020).

In order to understand whether RNA processing or RNA stability is the main process regulating expression levels of RNA polymerase II transcribed genes in trypanosomes, an improved measurement in unperturbed cells would be required, that allows quantification of RNA processing rates as well as RNA stability.

6. Aims of this thesis

The ability to selectively regulate expression of individual genes is essential for trypanosomes to survive, and at the same time, to cause deadly infections in livestock and humans. The regulation of RNA polymerase II transcribed genes enables the parasite to adapt its cell biology, e.g. metabolism and motility, to the diverse environments which it encounters going through its life cycle. In addition, highly selective and mutually exclusive antigen expression by RNA polymerase I is required for infective trypanosomes to survive in the mammalian host.

The strong dependency of trypanosomes on selective gene regulation seems a contradiction to the assumption that transcription is not tightly regulated in this parasite, but rather occurs in a permissive manner. Therefore, it was the aim of this thesis to establish the role of RNA processing and RNA stability, the two post-transcriptional processes that occur after transcription, for selective gene expression regulation in trypanosomes.

6.1 Aim 1 - Does 3D genome folding regulate selective antigen mRNA processing in trypanosomes?

So far, traditional transcriptional enhancers have not been identified in trypanosomes. However, depletion experiments of several chromatin factors (Cestari & Stuart, 2018; Müller et al., 2018) indicate that specific DNA-DNA contacts might play a role during selective antigen expression. Further, it has been shown that all 15 antigen expression sites are transcriptionally initiated (Kassem et al., 2014; Vanhamme et al., 2000), suggesting that selective regulation cannot occur on the level of transcription. Instead, mRNA processing has been suggested as the selective regulatory step.

In this thesis, I aimed to determine whether selective antigen expression is regulated by 3D genome folding, addressing the following questions:

1. Does the active antigen gene engage in specific DNA-DNA contacts with potential regulatory elements?
2. Could such regulatory element mediate mutually exclusive antigen expression by physical interaction with a single antigen gene?
3. Could such regulatory element ensure selective, efficient mRNA processing for the exceptionally high amounts of antigen pre-mRNA levels generated by processive RNA polymerase I?

6.2 Aim 2 - Do post-transcriptional processes regulate expression levels of RNA pol II transcribed genes in trypanosomes?

Despite its central role for gene expression regulation, constitutive transcription by RNA polymerase II has not been experimentally verified in trypanosomes (C. Clayton, 2019). Further, it has remained unclear how expression levels of individual RNA polymerase II transcribed genes are regulated in the context of constitutive RNA polymerase II transcription.

In this thesis, I aimed to systematically measure RNA polymerase II transcription, mRNA processing rates and mRNA stability, in order to address the following questions:

1) Is transcription by RNA polymerase II indeed constitutive in trypanosomes?
2) Is mRNA processing or RNA stability the process that mainly regulates gene expression in trypanosomes?
3) Which consequence does it have for a transcript, whether it is being regulated by mRNA stability or mRNA processing?

7. Publications

7.1 SLAM-seq reveals independent contributions of RNA processing and stability to gene expression in African trypanosomes

The publication can be found here:

Luzak, V., Osses, E., Danese, A., Odendaal, C., Cosentino, R.O., Stricker, S.H., Haanstra, J.R., Erhard, F., Siegel, T.N., 2024. SLAM-seq reveals independent contributions of RNA processing and stability to gene expression in African trypanosomes. *Nucleic Acids Research*, Volume 53, Issue 3, gkae1203, https://doi.org/10.1093/nar/gkae1203

DOI: 10.1093/nar/gkae1203

PMID: 39673807

URL: https://academic.oup.com/nar/advance-article/doi/10.1093/nar/gkae1203/7924192

7.2 Spatial integration of transcription and splicing in a dedicated compartment sustains monogenic antigen expression in African trypanosomes

The publication can be found here:

Faria, J.*, **Luzak, V.***, Müller, L.S.M., Brink, G.B., Hutchinson, S., Glover, L., Horn, D., Siegel, T.N., 2021. Spatial integration of transcription and splicing in a dedicated compartment sustains monogenic antigen expression in African trypanosomes. *Nature Microbiology*, 6, 289-300.

*Contributed equally, co-first authorship

DOI: 10.1038/s41564-020-00833-4

PMID: 33432154

URL: https://www.nature.com/articles/s41564-020-00833-4

8. Discussion

8.1 Selective antigen expression is mediated by three-dimensional genome folding

Highly selective antigen expression by RNA polymerase I is essential for trypanosome survival, enabling evasion of the mammalian host immune response during infection. When parasites lose the ability to selectively express only one antigen gene, and instead express multiple antigens at once, a coordinated exchange of the expressed antigen is impaired, and the immune system can efficiently clear the infection (Aresta-Branco, Sanches-Vaz, et al., 2019). Similar to antigen expression in trypanosomes, highly selective expression of one gene out of a large gene family has been described in other divergent organisms and biological processes. One such example is selective olfactory receptor expression in mammals, a process that was shown to be regulated by three-dimensional folding of the genome (Monahan et al., 2017). The active olfactory receptor gene engages in an exclusive interaction with the Greek island enhancer cluster, that regulates exclusive expression of this particular OR gene, whereas around thousand inactive olfactory receptor genes are located in different, transcriptionally silent nuclear compartments (Bashkirova et al., 2020). Indeed, by applying Hi-C analysis in trypanosomes, we were able to show that the active antigen gene engages in specific DNA-DNA contacts with a potential regulatory element.

8.1.1 The active antigen gene engages in an inter-chromosomal interaction with a post-transcriptional enhancer

So far, transcriptional enhancers have not been identified in trypanosomes, in line with the low level of transcriptional regulation assumed for the parasite. In order to test for any regulatory elements that would interact with the active antigen gene via three-dimensional chromosome folding, I performed Hi-C experiments in trypanosomes that allowed me to map genome-wide DNA-DNA contacts (publication II, chapter 7.2). Genome-wide DNA interactions were mapped in three homogenous cell populations: (1) infective bloodstream form cells expressing antigen 1, (2) infective bloodstream form cells that had switched to express antigen 2, and (3) non-infective procyclic cells. Indeed, we identified a specific DNA element on chromosome 9, which interacted with the active antigen 1 in cell population (1). The interaction with the locus on chromosome 9 was dynamically re-established upon a switch in antigen expression, resulting in antigen 2 interacting with the locus on chromosome 9 in cell population (2). In procyclic cells (3), no VSG antigen is expressed, and in line with this no interaction of the locus on chromosome 9 with a VSG antigen was detected. Instead, procyclin antigens are expressed by RNA polymerase I in procyclic cells,

and we detected an interaction of the locus on chromosome 9 with the procyclin genes, which has not been present in infective bloodstream form cells (1) and (2) where procyclin genes are not expressed. To summarize, in all three cell populations studied, the interaction with the locus on chr. 9 was detected for the respective active antigen genes expressed by RNA polymerase I, whereas inactive antigen genes did not interact to a similar extent, suggesting that the interaction was functional and involved in selective antigen expression.

Interestingly, the Hi-C experiments did not identify a conventional transcriptional enhancer sequence in trypanosomes. Instead, the locus on chr. 9 which interacted with the respective active antigen gene was a well characterized mRNA processing hotspot in trypanosomes: it was the so-called spliced leader (SL) array from which the SL-RNA molecule is transcribed, that is required for *trans*-splicing of each mRNA in the parasite, including antigen mRNAs transcribed by RNA polymerase I. High amounts of SL-RNA and mRNA processing machinery have been detected at this locus (Budzak et al., 2022), suggesting that the mRNA processing hotspot could act as a post-transcriptional enhancer, which increases the efficiency of antigen mRNA processing by bringing the processing machinery in close proximity to the site of antigen transcription.

RNA polymerase I transcription is highly processive, resulting in large amounts of antigen pre-mRNA that requires processing into mature antigen mRNA in order to avoid premature degradation (Jacobs & Schneider, 2024). Further, RNA polymerase I does not harbor a C-terminal domain (CTD) comparable to RNA polymerase II, which usually provides the physical link to recruit the co-transcriptional mRNA processing machinery (Tafur et al., 2016). High amounts of antigen pre-mRNA and a presumably missing link for co-transcriptional processing might have created the necessity for trypanosomes to evolve an alternative mechanism to couple highly processive antigen transcription with efficient mRNA processing. The spatial integration of the antigen transcription site with an mRNA processing hotspot could serve as such alternative mechanism, which ensures efficient co-transcriptional splicing and prevents pre-mature mRNA degradation for the active antigen gene in close proximity.

The proposed mechanism of a post-transcriptional enhancer differs strongly from the function of conventional transcriptional enhancer structures found in other eukaryotes. Conventional enhancers were shown to increase the rate of target gene transcription, especially on the level of transcriptional initiation, rather than their processing efficiency. Interestingly, a recent study has shown that transcription of the active antigen expression site is dependent on mRNA processing (Budzak et al., 2022). Upon inhibition of *trans*-splicing, especially transcription elongation was inhibited at the active antigen expression site. This suggests that the interaction with the processing hotspot might not only ensure high processing rates of antigen

mRNA, but might also positively affect RNA polymerase I elongation as a secondary effect.

While we were able to show that the active antigen gene selectively interacts with the SL array in an activity-dependent manner, and formulated the hypothesis of a post-transcriptional enhancer in trypanosomes based on this finding, it remains to be proven that antigen mRNA is indeed processed more efficiently in close spatial proximity to the SL array. To address this question, I suggest to induce the physical interaction of an inactive antigen expression site with the SL array. Inducing the interaction with an inactive antigen expression site would answer the following questions: (1) Is mRNA processing efficiency increased upon interaction with the SL array, and (2) is the interaction with the SL array sufficient to fully activate the previously inactive antigen gene? One strategy to induce the physical interaction between two genomic loci is light-activated dynamic looping (LADL) (J. H. Kim et al., 2019), which utilizes inactive Cas9 for sequence-specific recruitment and tethering. This system is currently established in trypanosomes in our department and will yield important insights into the role of the SL array in antigen gene transcription.

In summary, the Hi-C results suggest that dynamic three-dimensional genome folding can not only regulate gene transcription, as it was described for conventional enhancers, but might also regulate the mRNA processing efficiency of respective target genes. The SL array as a potential mRNA processing hotspot is a trypanosome-specific structure, that does not exist in most more complex eukaryotes. However, there is growing evidence found in more complex eukaryotic organisms that RNA processing can indeed be regulated by three-dimensional organization of the genome: Highly transcribed housekeeping genes were found to be located in close proximity to nuclear speckles, which are nuclear condensates highly enriched for RNA processing factors (Chen & Belmont, 2019). Further, heat shock genes were shown to be located distantly from nuclear speckles when inactive, and to move towards nuclear speckles upon activation (Khanna et al., 2014). Finally, a recent study has quantified splicing efficiency and distance to nuclear speckles in a genome-wide manner (Bhat et al., 2024), whereby a strong correlation between a gene's processing efficiency, expression levels and distance to nuclear speckles was revealed. Taken together, our findings support the hypothesis that the role of three-dimensional genome folding extends beyond transcriptional regulation in eukaryotes, and can strongly influence mRNA processing.

8.1.2 The post-transcriptional enhancer is one of multiple mechanisms to ensure selective antigen expression

The interaction between the active antigen gene and the mRNA processing hotspot is part of multiple mechanisms that ensure selective antigen expression at high levels. Antigen expression is affected at all three levels: (1) transcription, (2) RNA processing and (3) RNA stability.

(1) Transcription levels of the active antigen expression site are further boosted by ESB1, which is localized at the active expression site (López-Escobar et al., 2022), and by SUMOylation of RNA polymerase I and other ESB associated factors through the E3 ligase TbSIZ1/PIAS1 (López-Farfán et al., 2014).

(2) While transcription initiation was shown to occur at all 15 antigen expression sites, mRNA processing occurred only at the one active antigen expression site (Vanhamme et al., 2000). In this thesis, I was able to show that such selective mRNA processing could be explained by spatial genome organization. Hi-C has revealed the selective interaction of the active antigen gene with the SL-RNA processing hotspot (publication II, chapter 7.2). The SL-RNA array is a highly repetitive sequence, composed of up to 200 repeats of around 1.5 kb encoding SL-RNA, and much longer than conventional transcriptional enhancers. Such repetitive nature could mimic the function of a so-called super-enhancer that regulates lineage-specific gene expression in more complex eukaryotes: robust expression of the target gene is mediated by multiple regulatory elements. Interestingly, a recent publication has identified additional nuclear condensates with a potential function in RNA processing to associate with the active antigen gene (Budzak et al., 2022)(for review of nuclear condensates potentially involved in antigen expression see publication III, appendix A).

(3) Finally, RNA stability of the active antigen mRNA is extended to several hours, compared to the average half-live of 11 minutes for RNA polymerase II transcribed genes in trypanosomes (publication I, chapter 7.1). It was shown recently that such high stability is mediated by the selective deposition of m^6A RNA modifications in the poly-A tail of the active antigen mRNA. m^6A deposition is dependent on a short conserved 16-mer sequence in the 3´UTR of VSG mRNAs, and occurred only for the dominantly expressed antigen gene, even when a second VSG gene was activated in the same cell (Viegas et al., 2022), raising the possibility that selective RNA modification might be spatially regulated, e.g. with the ESB.

In summary, several specific machineries are put in place in trypanosomes to ensure consistent and high expression of antigen mRNA and in turn sufficient levels of antigen protein to cover the entire cell surface: high transcription levels by RNA polymerase I, subsequently high RNA processing rates to avoid pre-mature degradation, and a strongly increased half-live of several hours of the active antigen mRNA. In addition, there are specific mechanisms to ensure that only one out of the 15 antigen

expression sites is expressed in each parasite, and to coordinate antigen switching (Cestari & Stuart, 2018; J. Faria et al., 2019; Gaurav et al., 2023; Glover et al., 2016). Trypanosomes invest a substantial amount of energy in regulating selective antigen expression, although this process only occurs in one of the seven life cycle stages, representing the important role of this process for trypanosome survival and infection establishment.

8.2 Expression levels of RNA polymerase II transcribed genes are regulated by post-transcriptional processes in trypanosomes

Regulating the expression levels of RNA polymerase II transcribed genes is essential for trypanosome survival, since it enables the parasite to adapt and survive in the diverse environments it encounters throughout its life cycle. Physical parameters, such as temperature, pH and matrix, change drastically during the parasite life cycle, as well as nutrients and immune interactions, when parasites mitigate within the transmitting tsetse fly and the mammalian host. It is regulated gene expression that allows the parasites to adapt essential cellular functions, such as metabolism and motility, to the respective environment (Besteiro et al., 2005; Shaw et al., 2022; Shaw & Roditi, 2023; van Grinsven et al., 2009). Despite its central role for parasite survival, it has remained unclear how gene expression is regulated in this early branching eukaryote. Therefore, I aimed to address the following questions with my thesis, in order to understand gene expression regulation better in trypanosomes: Is transcription indeed a constitutive process, with little regulatory power? And which of the two post-transcriptional processes, RNA processing and RNA stability, has the potential to regulate gene expression levels in the context of permissive transcription?

Genetic engineering of pyrimidine metabolism pathways allowed me to establish efficient metabolic labeling of newly synthesized RNA in trypanosomes (publication I, chapter 7.1). I was able to experimentally verify the long-standing hypothesis that transcription of polycistronic transcription units by RNA polymerase II is a constitutive process in trypanosomes. The TT-seq experiment revealed that transcription initiation and elongation did not strongly vary between and along trypanosome PTUs, respectively. This finding suggests that RNA polymerase II transcribed PTUs are being transcribed with similar rates at any time, and therefore RNA polymerase II mediated transcription does not play a major regulatory role for gene expression in trypanosomes. Instead, applying SLAM-seq has demonstrated that both post-transcriptional processes, RNA processing and stability, strongly vary between individual genes and have the potential to play a regulatory role in gene expression. It

revealed that RNA stability is most likely the dominant regulatory process in trypanosomes, which directly influences RNA steady state levels for a large number of trypanosome genes. On the other hand, RNA processing seems to have a fine-tuning role for most genes, and a strong regulatory role only for a subset of trypanosome genes.

8.2.1 Transcription is indeed constitutive in trypanosomes

Many eukaryotes regulate gene expression on a gene-specific level, via regulatory DNA sequences such as promoters and enhancers that affect transcription of individual genes. It is rather uncommon that all genes are simultaneously transcribed with similar efficiency. However, after showing that transcription is constitutive in trypanosomes, meaning that all RNA polymerase II transcribed genes are transcribed at the same time, the following question arises: How could constitutive transcription be advantageous for trypanosomes, given that it seems a tremendous energy investment to transcribe all RNA polymerase II transcribed genes at the same level, instead of reducing the transcribed gene set to fit the requirements of the present environment?

As one possible advantage, constitutive transcription was proposed to accelerate the cellular response to a sudden change in the environment (Geisel, 2011). Genes can be either transcribed continuously or upon demand in a responsive manner. Responsive transcription allows a cell to be optimally adapted either to state A (before change in environment, without respective protein) or to state B (after change in environment, with respective protein). However, the time to produce the required amount of respective protein takes longer in the case of responsive transcription, since the respective mRNA has to be transcribed and processed prior to translation. In contrast, a protein can be produced more quickly in the case of constitutive transcription, where the mRNA is already present. As a disadvantage of constitutive transcription, the cell is neither optimally adapted to state A or B, since the mRNA and the respective protein might be present to some extent in both states. In conclusion, there are different scenarios that require responsive or constitutive expression of a gene. In the case that a protein is mutually exclusively required in state A, but becomes a burden in state B, selective or responsive transcription is required. This would be the case during antigenic variation in trypanosomes. In contrast, if a protein might not be required but also does not display a burden in both states, constitutive expression allows for a quicker response to a sudden change. Therefore, constitutive transcription of RNA polymerase II transcribed genes in trypanosomes might allow the immediate reaction to sudden and unexpected environmental changes. All RNA polymerase II transcribed genes are transcribed into RNA by the parasite, and upon demand, a specific RNA can be quickly stabilized and translated

into protein. Such immediate response plays an important role for unicellular organisms, since they are constantly confronted with unpredicted changes in their immediate environment and cannot create a stable environment of their own like multicellular organisms can do.

As another possible advantage of constitutive transcription, while it requires more energy for transcription itself, energy can be saved on transcription regulation and allows for a reduced genome size with few regulatory DNA sequences. Indeed, a somewhat "trimmed" linear genome organization is observed for the chromosomal cores of trypanosomes (Berriman et al., 2005). The chromosome cores comprise the largest proportion of the megabase chromosomes, and are mostly composed of protein-coding sequences and very short intergenic regions of up to few hundred bps. The only regulatory sequences are transcription start and termination sites for RNA polymerase II transcribed PTUs, as well as promoters for non-coding RNA transcription by other polymerases. As a result, the gene-rich trypanosome genome is hundred times smaller than the human genome, although it contains only around 2-fold less protein-coding sequences. The smaller size is driven by a reduction of non-coding, potentially regulatory sequences, and shorter protein-coding sequences that do not harbor introns. A small genome size represents a tremendous advantage for fast dividing cells like trypanosomes, since it requires less energy to perform replication. In contrast, a large genome is less problematic for slowly dividing or post-mitotic cells. In addition, a small genome with few regulatory sequences requires a less sophisticated machinery to selectively identify and activate regulatory DNA sequences. In line with this hypothesis, trypanosomes were shown to have a reduced set of transcription factors compared to more complex eukaryotes (C. Clayton, 2019), as well as a degenerated version of the mediator complex (J. H. Lee et al., 2010).

To summarize, there are potential benefits of constitutive transcription for trypanosome fitness, that require experimental validation. Especially the reduction of genome size could be important for this fast-dividing organism. Besides the chromosome cores that harbor RNA polymerase II transcribed genes, the second largest proportion of the trypanosome genome encodes the transcriptionally silent antigen repertoire located in subtelomeres, as well as on mini-chromosomes. Such a large silent repertoire is a tremendous energy investment in terms of replication, and a reduced organization of RNA polymerase II transcribed genes might have been beneficial in order to ensure high replication rates despite the large antigen repertoire.

8.2.2 Post-transcriptional processes regulate gene expression and affect cell-to-cell heterogeneity within trypanosome populations

Variation between individual cells, for example in cell morphology, in transcript or protein levels, or in the amount of post-translational protein modification, has been described in the literature for decades, even for isogenic cells belonging to the same

cell type (Balázsi et al., 2011). With the recent advent of single cell sequencing technologies, the observed variations between cells were verified and can now be quantified properly (B. A. Kramer et al., 2022; Popovic et al., 2018; Vandereyken et al., 2023). Further, it was shown that cell-to-cell variation can fulfill important biological functions, and multiple examples of biological processes exist that rely on cell-to-cell variability (Balázsi et al., 2011). For example, cell-to-cell variability can enable the division of labor within a population of unicellular organisms, so that individual cells can fulfill opposing functions that could not be fulfilled by one cell at the same time (Giri et al., 2019). Further, cell-to-cell variability enables bet hedging, a mechanism describing that diversity within a cell population can enable the population´s survival under unexpected environmental changes (Grimbergen et al., 2015). And finally, an important role of cell-to-cell variability was described during cellular differentiation processes (Balázsi et al., 2011; Bose & Pal, 2017).

In the review article "Cell-to-cell heterogeneity in trypanosomes" (publication IV, appendix B), we have utilized studies that describe functional cell-to-cell variability in bacteria and more complex eukaryotes as examples, and speculated at which stages of their life cycle trypanosome parasites might similarly rely on cell-to-cell variability for survival. We concluded that trypanosome populations would tremendously benefit from cell-to-cell variability during heterogenous differentiation processes, for division of labor and for bet hedging to survive uncertain future events. Further, cell-to-cell heterogeneity has been described for several steps within the trypanosome life cycle: for example, only a small fraction of bloodstream form cells switches the expressed antigen gene during an infection, while most cells remain expressing the previous antigen gene. Another small fraction of infective bloodstream form cells differentiates to stumpy form parasites, a non-replicative life cycle stage that is adapted to be taken up by the tsetse fly vector. It is important to understand how cell-to-cell variation is regulated in trypanosomes, in order to identify mechanisms that regulate essential processes during their life cycle. This study provides a first quantification of cell-to-cell variability in the transcriptome of individual trypanosome parasites (publication I, chapter 7.1), and further starts to explore which transcripts are stably expressed with low variation between individual trypanosomes, and which transcripts are detected less frequently and display more cell-to-cell variation.

Analyzing cell-to-cell variability in single-cell RNA-seq data from trypanosome bloodstream form parasites has revealed that mRNAs with long half-lives show lower cell-to-cell variability than mRNAs with short half-lives (publication I, chapter 7.1). This could be explained by the fact that mRNAs with long half-lives are present for a long time before degradation in individual cells, and therefore display a high likelihood to be detected in many cells at the same time. In this way, mRNA half-live can influence the degree of cell-to-cell variability. By GO-term analysis, mainly

essential cellular pathways such as gene expression, translation and glucose metabolism, which is the main energy source for this life cycle stage, were identified to yield transcripts with low cell-to-cell variability. In contrast, mRNAs with low half-live displayed a higher degree of cell-to-cell variability, most probably due to the fact that they are less long present in individual cells before degradation. Therefore, simultaneous detection in multiple cells becomes less likely, and underlying noise during transcription and mRNA processing cannot be buffered. By GO-term analysis, mainly non-essential cellular pathways that might benefit from variation, e.g. cellular communication, were identified to harbor transcripts with relatively high cell-to-cell variability. Importantly, this finding holds true for transcripts with comparable total RNA levels and either high RNA half live or high RNA processing rate.

The experiments in this thesis represent the first genome-wide quantification of RNA processing rates and an improved measurement of RNA half-lives in trypanosomes. These measurements have further enabled the integration of post-transcriptional parameters with RNA single cell data on cell-to-cell variability. Thereby, it has been revealed that post-transcriptional regulation seems to impact cell-to-cell heterogeneity, and that it makes a difference whether a transcript's total RNA level is mainly regulated via RNA half-live or via RNA processing rate. While a high RNA half-live confers low cell-to-cell variability to a transcript, a transcript with similar total RNA levels that result from high processing rate and low RNA stability exhibits a higher degree of cell-to-cell variability. In order to experimentally verify this observation, perturbation experiments with a single-cell readout will be required: depleting RNA binding proteins and the RNA processing machinery to manipulate RNA processing and RNA stability of target genes will reveal whether this has consequences for cell-to-cell variability.

9. Scientific Outlook – How to translate the findings of this thesis into treatment strategies

African trypanosomes cause debilitating diseases in sub-Saharan Africa, such as human African trypanosomiasis (HAT, or sleeping sickness) in humans and animal African trypanosomiasis (AAT) in livestock such as cattle, pig, and goat, as well as in wild life. So far, there is no prophylactic treatment or vaccine available to prevent African trypanosomiasis infections. However, the number of human African trypanosomiasis (HAT) cases has dropped significantly between 2000 and 2022 by more than 90 %, from around 28.000 to 800 newly reported infections per year (Venturelli et al., 2022). While humans can protect themselves from bites of the transmitting tsetse fly, for example by appropriate clothing and chemical repellents, livestock cannot be efficiently protected by the same means. Therefore, the numbers of animal African trypanosomiases (AAT) cases remain high and present a huge economic burden of more than 4 billion USD per year in the affected rural regions (Venturelli et al., 2022). Besides the economic burden of productive livestock loss, livestock infections form a disease reservoir transmittable to humans, and thereby prevent disease eradication.

If an African trypanosomiasis infection remains untreated, the multiplying parasites enter the central nervous system of the mammalian host, which is fatal for the host organism. Symptoms in livestock can be, for example, weakness, weight loss, dehydration and death. Furthermore, upon infection, livestock becomes unavailable for food and milk production (Büscher et al., 2017). A small set of agents is currently available to treat African trypanosomiasis, most of which are associated with severe side effects, high costs or low efficacy (Chitanga et al., 2011; Venturelli et al., 2022). Most of these agents are used to treat human and animal infections at the same time, and emerging drug resistances threaten to further narrow down the small set of available agents. Due to low commercial interest, the development of new, more effective agents with less side effects is a rather slow process (Venturelli et al., 2022). Most recently, after decades of attempts, immunization of mice against an invariant surface protein of *Trypanosoma vivax* has been achieved (Autheman et al., 2021). Though this is promising development, it remains to be seen whether such vaccine proofs to be effective in larger animals, and further, how widespread immunization of livestock in rural African regions could be organized and financed.

Uncontrolled animal infections, emerging drug resistances, and the potential rise of human infections due to geopolitical instability and to invasion of tsetse fly habitats for commercial reasons, create an unmet need to expand and improve treatment options for African trypanosomiasis. Unraveling the basic biology of African trypanosomes, as done in this thesis, can build the foundation to develop new treatment strategies. In this chapter, I would like to illustrate how the findings of this thesis

shine light on new potential treatment strategies. Further, and where possible, I would like to illustrate how expertise developed for disease treatment with greater commercial interest could be re-purposed to treat trypanosome infections. Finally, I am suggesting experiments that could be performed in a basic research laboratory, in order to explore the potential treatment strategies deduced from my findings.

Importantly, it was not the aim of this thesis to develop a treatment strategy for trypanosome infections. However, in addition to discussing my findings in the context of biological mechanisms to regulate gene expression, I find it important to place my findings in the greater context of African trypanosomiasis eradication, which is a long-standing and currently unmet goal set by the WHO, that should have been completed in 2030.

9.1 Targeting selective antigen expression

While most commonly used agents to treat African trypanosomiasis target essential biological processes, such as energy production by the parasite, with the aim to kill infective trypanosomes (Venturelli et al., 2022), the disruption of the highly efficient antigenic variation mechanism, which enables immune evasion of the parasite, could serve as an alternative target, and has been under investigation for decades. Disrupting selective antigen expression would not immediately kill the parasites, but instead enables the host immune system to efficiently clear the infection (Aresta-Branco, Sanches-Vaz, et al., 2019). Within the last decade, researchers have started to unravel parasite-specific mechanisms that regulate selective antigen expression, and could serve as potential drug targets. In this thesis, a new mechanism was described that illustrates the role of 3D genome folding during selective antigen expression (publication II, chapter 7.2)Further, by performing a control experiment, I showed that pyrimidine starvation can negatively regulate RNA polymerase I mediated antigen expression (publication I, chapter 7.1). In the following, I will explore how both findings could lead to the development of strategies interfering with parasite immune evasion.

9.1.1 Targeting the link between RNA polymerase I activity and nucleotide metabolism

While setting up an approach for efficient metabolic RNA labeling in infective bloodstream form trypanosomes, I found that pyrimidine starvation caused inhibition of RNA polymerase I mediated antigen expression in this life cycle stage (publication I, chapter 7.1). This finding was unexpected, and yet could be an interesting starting point for a treatment strategy. Nucleotide metabolism pathways have been extensively studied in trypanosomes as potential drug targets (de Koning et al., 2005; Hammond & Gutteridge, 1984), with the intention to block nucleotide synthesis and

thereby induce parasite death. Especially purine-related pathways have been characterized, since trypanosomes do not have a functional *de novo* biosynthesis pathway for purine nucleotides. Instead, they fully rely on the salvage of external purine precursors (Berg et al., 2010; Hammond & Gutteridge, 1984), suggesting that the inhibition of the salvage pathway could be an efficient drug target. However, targeting different individual enzymes involved in purine salvage has proven inefficient, due to complex, non-linear salvage pathways circumventing the inhibition of individual metabolic enzymes (Berg et al., 2010). In addition, also pyrimidine-related synthesis pathways were tested as potential drug targets. However, since trypanosomes have a functional *de novo* biosynthesis pathway for pyrimidines in addition to an efficient salvage pathway of external precursors (Hammond & Gutteridge, 1984), the inhibition of individual enzymes in pyrimidine pathways has not yielded promising results to reduce parasitemia in mice (Ali, Creek, et al., 2013; Ali, Tagoe, et al., 2013).

Instead of targeting individual metabolic enzymes to kill trypanosome parasites, the finding that RNA polymerase I activity is linked to pyrimidine nucleotide metabolism could be explored in a different context. It suggests that trypanosome cells have evolved a sensing mechanism that can inactivate RNA polymerase I mediated antigen transcription upon pyrimidine starvation. Interestingly, a similar sensing mechanism for purine starvation was found in cultured mammalian cells (Hoxhaj et al., 2017). It was shown that purine starvation, but not pyrimidine starvation, is sensed by the well-characterized nutrient-sensor mammalian target of rapamycin complex 1 (mTORC1), which in turn negatively affects RNA polymerase I activity (Hoxhaj et al., 2017). Also pyrimidine depletion was sensed by more complex eukaryotic cells and lead to RNA polymerase I inactivation, but by an uncharacterized, mTORC1-indepdendent mechanism (Mullen & Singh, 2023). Beyond the identification of a potential drug target, trypanosomes could therefore serve as an ideal model to study how pyrimidine metabolism is linked to RNA polymerase I activity in general.

In order to identify the molecular sensor that negatively regulates RNA polymerase I activity in the absence of pyrimidines in trypanosomes, I would suggest to employ a screening approach with single cell RNA-seq read-out, a method referred to as Perturb-seq (Replogle et al., 2022). Perturb-seq allows the simultaneous depletion of hundreds of proteins in a cell population, and the characterization of respective transcriptional phenotypes by single cell RNA-seq. The Perturb-seq approach is currently established in trypanosomes by members of the Siegel lab. Once established, Perturb-seq could be used to identify the sensor of pyrimidine starvation in trypanosomes, by introducing a genome-wide depletion library, subjecting cells to 15 minutes of pyrimidine starvation and identifying individual cells in which RNA polymerase I remains active after 15 minutes of starvation by single-cell RNA-seq. Hereby, proteins would be identified that link pyrimidine starvation to RNA polymerase I activity. Alternatively to Perturb-seq, the LiP-MS approach developed by

the Picotti lab in Zurich would allow to map proteins and pathways activated by pyrimidine starvation. In contrast to Perturb-seq, LiP-MS does not require any perturbations and is independent of transcriptional changes as it maps conformational changes in a proteome-wide manner (Malinovska et al., 2023).

After identifying the sensory proteins that mediate inactivation of antigen expression, an approach, such as a small molecule, could be developed to constitutively activate the sensor, in order to interfere with antigen expression during infection. Interestingly, mTOR proteins in trypanosomes are structurally somewhat similar to their mammalian counterparts and can be targeted in trypanosomes (Phan et al., 2020). Ideally, targeting the sensor protein would have a dual effect: a negative effect on parasite fitness in combination with impaired antigen expression.

9.1.2 Targeting 3D genome architecture via nuclear condensates

Chromosome conformation capture approaches, such as Hi-C, have elucidated the highly complex folding of interphase chromosomes in various species, and ongoing research is characterizing the impact of such specific three-dimensional DNA-DNA contacts for nuclear processes. By applying Hi-C in trypanosomes, I was able to describe a frequent inter-chromosomal contact between the active antigen gene and an mRNA processing hotspot (publication II, chapter 7.2). One puzzling question in the field of chromosome folding is how such specific DNA-DNA contacts between different chromosomes could be established in the highly crowded nucleus, and the formation of biomolecular condensates has been suggested as one mechanism to regulate nuclear organization (Banani et al., 2017; Sabari, 2020; Sabari et al., 2020). Biomolecular condensates are membrane-less bodies found in the cytoplasm and nucleoplasm of cells, composed of functionally related proteins and RNAs, and often formed by liquid-liquid phase separation (Boija et al., 2021). Membrane-less bodies can be stable, as it was described for nuclear speckles and the nucleolus, or form and dissolve in a highly dynamic manner, as it was described e.g. for stress granules. Intrinsically disordered protein regions (IDRs) play a crucial role in condensate formation and regulation, as well as post-translational protein modifications and RNA-protein interactions (Mitrea et al., 2022). Miss-regulation of condensate biology has been described in the context of several diseases, and leading experts in the field, such as Anthony Hyman, located at the Max-Planck Institute of Molecular Cell Biology in Germany, and Richard Young, located at the Massachusetts Institute of Technology in the US, have bundled their expertise within Dewpoint Therapeutics, one of several start-up companies with the aim to develop condensate-targeting drugs for commercial use (Mitrea et al., 2022).

Interestingly, both genomic loci involved in selective antigen expression in trypanosomes, the active antigen gene and the SL array, were described to be associated with specific sets of proteins: VEX2, ESB1, and a highly sumoylated focus co-localize

at the active antigen gene with an extra-nucleolar RNA polymerase I focus (publication II, chapter 7.2)(J. Faria et al., 2019; López-Escobar et al., 2022; López-Farfán et al., 2014), while VEX1, several mRNA processing proteins and RNAs co-localize at the SL array (publication II, chapter 7.2)(Budzak et al., 2022). The specific localization of these proteins suggests the formation of membrane-less condensates at both loci, and further suggests that condensate-targeting drugs could potentially be used to combat parasite infections. In the perspective article "Nuclear condensates: new targets to combat parasite immune evasion?", I have described the potential condensates involved in antigen expression in more detail. Further, I have suggested experiments to identify the full repertoire of proteins that localize at the SL array and the active antigen gene, as well as experiments to study the properties of involved intrinsically disordered protein regions and their ability to phase separate (publication III, appendix A). The knowledge about condensate formation and specificity, which is currently established by Dewpoint Therapeutics and multiple research groups around the world, could be transferred to target parasite-specific condensates as a new avenue of treatment in infection biology.

9.2 Targeting expression of RNA pol II transcribed genes

Besides selective antigen expression by RNA polymerase I during immune evasion in the mammalian host, also RNA polymerase II mediated gene expression could be targeted to combat African trypanosomiasis infections. Tight regulation of gene expression is essential for trypanosome parasites, in order to quickly respond to environmental changes, to complete different life cycle stages, and to survive in diverse hostile environments (Lueong et al., 2016a; Siegel et al., 2010; Vickerman, 1985). Interfering with gene expression could lead to maladaptation and a tremendous fitness loss for the parasites.

9.2.1 Targeting global RNA polymerase II transcription at the Achille's heel - transcription start sites

In this thesis, I was able to experimentally confirm the long-standing hypothesis that RNA polymerase II transcription is constitutive in trypanosomes (publication I, chapter 7.1). This finding suggests that the polycistronic transcription units, which harbor RNA polymerase II transcribed genes, seem to be co-activated by the same set of molecular factors, and therefore could be targeted simultaneously by the same intervention strategy, representing an Achille´s heel of trypanosome fitness. The chromatin composition of PTU transcription start sites (TSSs) has been well characterized in recent years: transcription start sites are marked by H2A.Z deposition, a histone variant that opens up chromatin and thereby facilitates transcription initiation (Siegel et al., 2009). Further, 58 histone modifications were detected to be specifically deposited at TSSs (Kraus et al., 2020), and a set of histone reader and writer

proteins, as well as chromatin remodelers, was characterized to specifically localize to TSSs (Staneva et al., 2021, 2022).

This set of regulatory proteins present at TSSs represents promising targets to manipulate genome-wide expression levels of RNA polymerase II transcribed genes. It was shown previously that the depletion of a single regulatory protein, for example the histone acetyl transferase 1 (HAT1), which acetylates the H2A.Z histone variant at TSSs, was sufficient to reduce overall RNA polymerase II transcription to around 50% and resulted in a severe loss of fitness (Kraus et al., 2020). Further, initial tests have shown that epigenetically active compounds that act on histone acetylation, such as histone deacetylase inhibitors, were active in trypanosomatids and did affect cellular fitness (Di Bello et al., 2022; Zuma & de Souza, 2018).

In order to identify which regulatory proteins at TSSs could serve as promising drug targets, and induce the strongest fitness loss upon depletion, I would like to suggest a screening experiment: Trypanosome parasites can be transfected with an RNAi knock down library against regulatory proteins located at TSSs. Samples of the transfected parasite population are taken directly after library transfection, as well as 24 and 48 hours after RNAi induction. By next generation sequencing, it can be determined which factors lead to a strong growth defect and should be considered as drug targets, and which factors introduce a rather mild growth defect after depletion and therefore do not serve as good targets. Here, a special focus should be placed on the protein class of trypanosome chromatin remodelers, since our institute has access to a highly efficient drug screening platform to identify small molecules that target chromatin remodeler activity, established by Prof. Andreas Ladurner and his team at Eisbach Bio. So far, Eisbach Bio mostly aims to target human chromatin remodelers for cancer treatment, as well as virally encoded chromatin remodelers to treat corona virus infections. However, the assay could be adapted to trypanosome remodelers using the tremendous expertise of Eisbach Bio, and a screening of small molecule libraries could identify novel compounds with anti-protozoan activity.

9.2.2 Targeting RNA-protein networks

By applying SLAM-seq, I was able to show that RNA stability is a major regulator of gene expression in trypanosomes (publication I, chapter 7.1). This finding highlights the importance of studying the mechanisms that control RNA stability in trypanosomes: which are the regulatory RNA sequences and respective RNA binding proteins that establish complex regulatory networks? Which roles do RNA modifications play, and the sequestration of RNAs in biomolecular condensates? RNA binding proteins, RNA modifications and biomolecular condensates are in the focus of cancer research (Barbieri & Kouzarides, 2020; Bertoldo et al., 2023; Boija et al., 2021), and will hopefully reveal strategies that could be transferred to combat parasite infections.

In order to identify the RNA sequence motifs that regulate RNA stability of RNA polymerase II transcribed genes in trypanosomes, we are collaborating with the informatician Anna Danese, who has previously helped us analyzing single cell variation in trypanosomes (publication I, chapter 7.1). Anna Danese is currently training the statistical model Saluki on trypanosome transcriptome data, which can identify regulatory RNA sequences (Agarwal & Kelley, 2022) using the RNA half-lives measured by SLAM-seq in this thesis.

Upon identification, major regulatory sequence motifs encoded by endogenous RNAs could be used as short exogenous RNA molecules to interfere with gene expression regulation in trypanosomes. Such short exogenous RNA molecules encoding regulatory sequences would compete for the binding of regulatory RNA binding proteins with endogenous mRNAs and could thereby interfere with proper gene expression regulation. To test the efficacy of such short exogenous sequences as a potential new treatment strategy, they could be overexpressed in trypanosome cell culture, in order to determine whether they are capable of conferring a major fitness loss to the parasites. Interestingly, it has been shown that the regulatory networks of RNA-binding proteins can be manipulated in trypanosomes and affect viability, making this a promising approach (Lueong et al., 2016a). Next, these short regulatory sequences could be added to the culture medium, in order to test whether they would be taken up by parasites and exert their function when added in an extracellular manner.

RNA-based therapeutics are currently under intensive development, mostly in the context of vaccines that immunize against infectious diseases or help targeting cancer cells. RNA production and delivery strategies could be adapted to treat or prevent trypanosome infections. Further, since RNA polymerase II transcribed genes could be targeted in any life cycle stage of the parasite, and do not require treatment of infected humans or livestock, the intervention with short regulatory RNA sequences could occur in a preventive manner, for example when parasites reside in the transmitting tsetse fly. Thereby, side effects for the mammalian host could be avoided.

9.3 Advancing the understanding of eukaryotic gene expression

Above, potential strategies were described how the findings of this thesis could be implemented to develop new anti-trypanosomal treatments in the future. Before following one of the suggested strategies above, one has to consider to following: Which strategy is commercially attractive and applicable in rural regions of sub-Saharan Africa? Ideally, the developed strategy should not be limited to treat *Trypanosoma brucei* infections only, but could have the potential to be adapted to treat infections caused by related parasite species, such as *Trypanosoma cruzi* or *Leishmania*, or infections caused by other agents that employ similar antigenic variation strategies

to evade the immune system (Barcons-Simon et al., 2023; Deitsch et al., 2009; Florini et al., 2022).

The experiments in this thesis represent basic research with the aim to understand trypanosome physiology. Therefore, the most immediate outcome of this thesis is an insight into how an early divergent eukaryote regulates gene expression in order to survive, and in the absence of tight transcriptional regulation. An overwhelming majority of research is performed in a relatively small number of eukaryotic model organisms, such as yeast, *C. elegans*, Drosophila, or mammalian cells. Research in less well characterized eukaryotes, such as protozoan parasites, illustrates that eukaryotic systems are much more diverse. While trypanosomes represent early diverging eukaryotes, and have evolved some biological processes in an alternative manner compared to more complex eukaryotes, studying basic trypanosome biology has previously lead to discoveries of general importance that were transferable to other eukaryotes. For example, trypanosomes were among the first eukaryotic organisms in which RNA interference was described (Ngô et al., 1998), a biological process that has been extensively exploited for basic research in different eukaryotes, and is explored as a treatment strategy for several diseases (Traber & Yu, 2022). Further, the process of *trans*-splicing was first described in trypanosomes (Matthews et al., 1994) and is now under active investigation in the context of cancer (S. J. Tang et al., 2020).

In this context, it will be interesting to explore whether the concept of a post-transcriptional enhancer, as it is described in this thesis, also exists in more complex eukaryotes. Indeed, the SL array, which forms the mRNA processing hotspot in trypanosomes, is a structure unique to trypanosomatids. However, an increasing body of research suggests that nuclear speckles, which are biomolecular condensates in the nuclei of most eukaryotes harboring a large set of mRNA processing factors, could fulfill a similar function (Ilık & Aktaş, 2022). It has been shown that highly expressed genes were located in close proximity to nuclear speckles, and that this association was dependent on gene activity and varied between cell types (L. Zhang et al., 2021). Similarly, heat shock genes were shown to move closer towards nuclear speckles upon activation, and the distance to a nuclear speckle correlated well with the level of gene activation (Khanna et al., 2014). These data suggests that the regulated spatial integration of transcription and mRNA processing machineries by nuclear organization is a concept beyond trypanosomes, that ensures high and selective gene expression. Trypanosomes represent an ideal model organism to study how spatial proximity of a transcribed gene is established with an mRNA processing hotspot.

Further, confirming constitutive transcription and identifying RNA half-lives as a main regulator of gene expression, makes trypanosomes a great model organism to study the complex networks that regulate RNA stability in eukaryotes. Several mechanisms, that play a role in disease phenotypes, have been identified to regulate

RNA stability: sequence-specific RNA binding proteins (Bertoldo et al., 2023), biomolecular condensates such as stress granules (Boija et al., 2021) and RNA modifications (Barbieri & Kouzarides, 2020). *Trypanosoma brucei* expresses >150 RNA binding proteins of different functional classes (Lueong et al., 2016a), exhibits around 15 RNA modifications specific for mRNA, which remain largely unexplored (Viegas et al., 2022), and form biomolecular condensates upon different stressors (S. Kramer, 2014; S. Kramer et al., 2012). The absence of transcriptional regulation paves the way to understand RNA stability regulation with great depth in trypanosomes. Further, metabolic labeling and SLAM-seq analysis established in this thesis represent an ideal genome-wide analysis tool, with the ability to not only monitor how total transcript levels, but also how RNA stability and RNA processing changes upon a perturbation in the regulatory network.

10. References

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Appendix A: Nuclear Condensates: New Targets to Combat Parasite Immune Evasion?

The publication can be found here:

Luzak, V., 2022. Nuclear Condensates: New Targets to Combat Parasite Immune Evasion? *Frontiers in Cellular and Infection Microbiology*, Volume 12:942200, https://doi.org/10.3389/fcimb.2022.942200.

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Appendix B: Cell-to-Cell Heterogeneity in Trypanosomes

The publication can be found here:

Luzak, V., López-Escobar, L., Siegel, T.N., Figueiredo, L.M., 2021. Cell-to-Cell Heterogeneity in Trypanosomes. *Annual Reviews Microbiology*, Volume 75:107-128.

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