Allosteric activation mechanism of a human oncogenic chromatin remodeler ALC1

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By

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Affidavit

I hereby declare that my dissertation entitled "**Allosteric activation mechanism of a human oncogenic chromatin remodeler ALC1**" is an original work. All the experimental and other works included in this dissertation has not been submitted elsewhere for any other dissertation or diploma thesis. The contribution of different authors has been quoted in the text. I wrote this dissertation independently with no other sources and aids than mentioned in the text, declaration of contributions and the acknowledgements.

Hari Raj Singh

München

Date: 20th November 2017

Table of contents

Preface

My dissertation is designed in such a way that every section either is already a published unit (it has been mentioned in the thesis wherever that is the case) or which can ultimately be used towards publishable articles. The core of my dissertation research work as entitled, comprises studies on the activation mechanism of ALC1 remodeler, however the document also includes a few other published studies. I have not included all of the so far unpublished work that I have performed during my dissertation period rather there is a bit of mention in the discussion and the future outlook section.

Introduction: This part can be potentially extended into one review/perspective article. This will introduce and describe the chromatin structure, state of the art in the chromatin remodelling mechanisms. In addition, this section also includes a previously written review article entitled "poly-ADP-ribosylation in DNA damage response". Further, previously published preview article and a news and views article are also included in the introduction section.

Methods: In this part, I am including the materials and methods section from the published first author manuscript.

Results: This part of my thesis comprises of in total three published research articles including my first author research article. All three publications are connected with each other due to their involvement in chromatin/nucleus biology.

Discussion and Outlook: Here, I bring back the introduction in perspective aiming to analyze the results and discussion section in a bigger picture of chromatin remodelling mechanisms and their biological significance using ALC1 remodeler as a model system of choice. This part is being prepared to contribute towards perspective article focused on the ALC1 chromatin remodeler along with a part from the introduction. Most of the results that are not included in my thesis are also mentioned. However, that is kept on the bare minimum since I am not including the data. This part of the thesis also includes outlook and future perspectives based on my thesis work on ALC1 chromatin remodeler. In addition, I will be including an Epigenome engineering essay article as well in this part of the thesis. The essay articles bring together my perspective on the field at the end of my thesis and serve well as unifying document for the entire thesis.

List of Publications included in this Dissertation

1. Structures of drosophila cryptochrome and mouse cryptochrome1 provide insight into circadian function.
Anna Czarna, Alex Berndt, **Hari R. Singh**, Astrid Grudziecki, Andreas G. Ladurner, Gyula Timinszky, Achim Kramer, Eva Wolf, **Cell,** 2013; **153**, 1394 –1405. DOI:10.1016/j.cell.2013.05.011

2. ACF takes the driver's seat.
Hari R. Singh and Andreas Ladurner, **Molecular Cell**, 2014; **55**, 345–346. DOI:10.1016/j.molcel.2014.07.014

3. ADP-ribosylation signaling during DNA damage repair.
Barbara Golia, **Hari R. Singh**, Gyula Timinszky, Frontiers in Bioscience (Landmark Ed.), 2015; 1; **20**: 440–57. PMID: 25553460

4. The histone chaperone sNASP binds a conserved peptide motif within the globular core of histone H3 through its TPR repeats.
Andrew Bowman, Lukas Lercher, **Hari R. Singh**, Daria Zinne, Gyula Timinszky, Teresa Carlomagno, Andreas Ladurner, Nucleic Acids Research, 2016; **44** (7): 3105-3117. DOI:10.1093/nar/gkv1372

5. Remodelers tap into nucleosome plasticity.
Hari R. Singh, Magdalena Murawska and Andreas G Ladurner, Nature Structural & Molecular Biology, 2017; **24** (4), 441-443. DOI:10.1038/nsmb.3394

6. A poly-ADP-ribose trigger releases the auto-inhibition of a chromatin remodelling oncogene.
Hari R. Singh, Aurelio P. Nardozza, Ingvar R. Möller, Gunnar Knobloch, Hans A.V. Kistemaker, Markus Hassler, Nadine Harrer, Charlotte Blessing, Sebastian Eustermann, Christiane Kotthoff, Sébastien Huet, Felix Müller-Planitz, Dmitri V. Filippov, Gyula Timinszky, Kasper D. Rand, and Andreas G. Ladurner
Molecular Cell, December 2017; **68**(5), 860-871. DOI:10.1016/j.molcel.2017.11.019

Unpublished manuscript included/to be prepared from this dissertation

7. The Epigenome Joins the Club of Engineers.
Hari R. Singh & Andreas G. Ladurner, Essay manuscript
(Prepared for submission included at the end of the dissertation)

8. How Modular allostery regulates ALC1 chromatin remodeler
Hari R. Singh & Andreas G. Ladurner Perspective article
(To be prepared from the Introduction and discussion section of the dissertation)

Declaration of contributions for the published documents

1. Declaration of Contribution to "Structures of Drosophila Cryptochrome and Mouse Cryptochrome1 Provide Insight into Circadian Function"

Anna Czarna performed protein expression and purification, crystallization, ITC, Blue light illumination experiments, generation of mutants, UV/VIS spectroscopy. Alex Berndt performed cloning, expression, purification of full-length dCRY, crystallization and data collection of full-length dCry; prepared Figure 6B and 6C. Hari Raj Singh did cloning, plasmid preparation, transfections in mammalian cells and performed microscopic experiments, data generation and figure preparation for Figure 6A. Astrid Gridziecki performed experiments for Figure 6B and 6C. Andreas Ladurner discussed the data, provided resources and handled or advised correspondence with the journal. Gyula Timinszky generated the image analysis pipeline for Figure 6A and interpreted the data in 6A. Achim Kramer designed and analyzed experiments in Figure 6B and 6C and contributed to the paper writing.

Eva wolf analyzed and interpreted data except for data shown in Figure 6, wrote the manuscript

2. Declaration of Contribution to "ACF Takes the Driver's Seat"

Hari Raj Singh made the first draft, prepared the Figure, co-wrote and corrected the article with Andreas Ladurner; Andreas Ladurner communicated the article with the journal editors.

3. Declaration of Contribution to "Poly-ADP-ribosylation signaling during DNA damage repair"

Hari Raj Singh co-wrote this review with Barbara Golia and Gyula Timinszky. In particular, I wrote the section 4 of the review article, which deals with "**The effects of poly-ADP-ribosylation on chromatin structure upon DNA damage**" and was additionally involved in writing and corrections of the rest of the document while Barbara Golia and Gyula Timinszky wrote most of the rest of the review article. Gyula Timinszky prepared the figure and communicated the final draft to the editors.

4. Declaration of Contribution to "The histone chaperone sNASP binds a conserved peptide motif within the globular core of histone H3 through its TPR repeats"

Andrew Bowman designed and carried out the experiments, conceived of the project in discussion with Andreas Ladurner, prepared the manuscript for publication. Lukas Lercher designed and carried out NMR experiments, analyzed the data. Hari Raj Singh discussed and design of F2H assays with AB performed all the F2H assays. Daria Zinne expressed and purified labeled proteins for NMR studies. Gyula Timinszky discussed and designed the project, corrected the manuscript. Teresa Carlomagno supervised the NMR work. Andreas Ladurner discussed the project, provided resources, co-wrote the paper and handled the publication process.

5. Declaration of Contribution to "Remodelers tap into nucleosome plasticity"

Hari Raj Singh wrote the first draft, Magdalena Murawska prepared the Figure 1, co-wrote and corrected the article, Andreas Ladurner wrote and corrected the article and communicated with the editors.

6. Declaration of Contribution to "A Poly-ADP-ribose Trigger Releases the Auto-inhibition of a Chromatin Remodeling Oncogene"

Conceptualization, H.R.S., M.H., G.T. and A.G.L.; Methodology, H.R.S., A.P.N., I.R.M., G.K., F.M.P., G.T., K.D.R. and A.G.L.; Investigation, H.R.S., A.P.N., I.R.M., G.K., M.H., N.H., C.B., C.K. and S.H.; Formal Analysis, H.R.S., A.P.N., I.R.M., G.K., M.H., N.H., C.B., F.M.P., G.T., K.D.R. and A.G.L.; Writing – Original Draft, H.R.S. and A.G.L.; Writing – Review & Editing, H.R.S., G.K. and A.G.L.; Funding Acquisition, H.A.V.K., D.V.F., F.M.P. and A.G.L.; Resources, H.A.V.K., D.V.F., S.E., S.H. and C.K.; Supervision, S.H., F.M.P., G.T., K.D.R. and A.G.L.

7. Declaration of Contribution to "The Epigenome Joins the Club of Engineers"

Hari Raj Singh prepared the first present draft. Andreas G. Ladurner and Hari Raj Singh will correct, re-purpose and communicate with the editors.

8. Declaration of Contribution to "How Modular allostery regulates ALC1 chromatin remodeler"

Hari Raj Singh will prepare the first draft from the introduction and the discussion section of the manuscript. Andreas Ladurner and Hari Raj Singh will correct, re-purpose and communicate with the editors.

Summary

The packaging of the genetic material in the form of chromatin is the fundamental level of regulation for genome-templated processes. Chromatin folding acts as a crucial platform for nuclear processes by regulating the spatio-temporal access to the underlying DNA sequence, thereby regulating DNA transcription, replication, recombination, repair and genome maintenance. Many mechanisms exist to establish this regulation, one of which is via the regulated recruitment and activation of ATP-dependent chromatin-remodelling enzymes. These remodelers use the energy of ATP to remodel, space and/or disrupt nucleosomes or other DNA–protein complexes.

One such previously described chromatin remodeler is ALC1 (Amplified in Liver Cancer 1), which is implicated in human cancers, and requires the activity of NAD^+-dependent enzyme poly-ADP-ribose (PAR) polymerase 1 (PARP1) for its remodelling activity. ALC1 has a C-terminal poly-ADPr binding macrodomain and an N-terminal Snf2-like ATPase motor domain separated by a linker. This modular architecture provides a way to couple DNA damage induced PARP1-mediated poly-ADP-ribosylation with ATP-dependent remodelling. ALC1's ATPase activity is strictly dependent on its intact ADPr-binding pocket of the macrodomain, suggesting the existence of a currently unique, post-translationally regulated allosteric activation mechanisms for this chromatin remodeler. However, how PAR regulates ALC1 structure and function was not known.

In my core PhD project, I was able to establish that the macrodomain interacts with the ATPase domain and mediates auto-inhibition. DNA damage-induced PARP1 activation suppresses the inhibitory interaction. Poly-ADPr binding to the macrodomain releases auto-inhibition. We identified tri-ADPr as the minimal ligand acting as a potent allosteric effector, capable of disrupting ATPase-macrodomain interaction. The loss of interaction triggers an ungated, active conformation. Consistently, ALC1 fragments lacking the macrodomain decompact chromatin without requiring PARP1 activation. Further, the ATPase restricts the macrodomain's interaction with PARP1 unless DNA damage is induced. In addition, I found that somatic cancer mutants disrupt ALC1's auto-inhibition and promote chromatin remodeling. Our data show that the NAD^+-metabolite PAR induces a conformational

switch in the ALC1 that releases auto-inhibition to drive chromatin relaxation. Modular allostery in this chromatin remodeling oncogene triggers its robust, DNA-damage-dependent activation. My research may catalyze the development of small molecule therapeutics using ALC1 as potential target of clinical relevance.

During my PhD, I also worked on many other projects out of which two are part of published results and therefore are also included in this cumulative dissertation;

1. The NASP histone chaperone - histone H3 interactions and the histone chaperoning mechanism thereof, 2. Circadian rhythm protein-protein interaction i.e Cry 1 interaction surface with the FBXL3 and PER2 and insights into the circadian function thereof.

Zusammenfassung

Die Verpackung des genetischen Materials in Form von Chromatin ist ein grundlegender Mechanismus für die Regulierung genomgestützter Prozesse. Die Chromatinstruktur dient hierbei als wichtige Plattform für nukleäre Vorgänge. Durch die spezifische Steuerung des räumlichen und zeitlichen Zugangs der zugrundeliegenden DNA-Sequenz werden genomgestützte Prozesse wie Transkription, Replikation, Rekombination, Reparatur und Genom-Engineering reguliert. Es existieren unterschiedliche Mechanismen, um diese Regulation zu gewährleisten. Einer dieser Mechanismen erfolgt über die Rekrutierung und Aktivierung ATP-abhängiger Chromatin-Remodeling-Enzyme. Diese sogenannten Remodeler nutzen die Energie von ATP, um Nukleosomen oder andere DNA-Protein-Komplexe zu verschieben, umzugestalten, deren Strukturen aufzulockern oder ganz aufzulösen, und hierdurch die genomassoziierten Prozesse zu steuern. Ein solcher kürzlich beschriebener Chromatin-Remodeler ist ALC1 (Amplified in Liver Cancer 1), von dem vielfach gezeigt werden konnte, dass er bei der Entstehung verschiedener Krebsarten beteiligt ist. ALC1 benötigt für seine Remodeling-Aktivität das NAD^+-abhängige Enzym Poly-ADP-Ribose-Polymerase 1 (PARP1). ALC1 besteht aus einer C-terminalen ADP-Ribose-bindende Makrodomäne und einer N-terminale Snf2-ähnlichen ATPase-Motordomäne, die über eine Linkerregion miteinander verbunden sind. Diese modulare Struktur ermöglicht es, die durch DNA-Schäden induzierte PARP1-vermittelte Poly-ADP-Ribosylierung mit ATP-abhängigem Chromatin-Remodeling zu koppeln. Es konnte weiterhin gezeigt werden, dass die ATPase-Aktivität von ALC1 strikt von seiner intakten ADP-Ribose-Bindungstasche der Makrodomäne abhängig ist. Dies weist auf eine einzigartige allosterische Regulation dieses Chromatin-Remodelers durch post-translationale Modifikationen hin. Allerdings ist der detaillierte Struktur-Funktionsmechanismus noch nicht bekannt.

Im Hauptteil meiner Doktorarbeit zeige ich, dass die Makrodomäne mit der ATPase-Domäne interagiert und Autoinhibition vermittelt. Die durch DNA-Schädigung induzierte PARP1-Aktivierung und die Bindung von Poly-ADP-Ribose (PAR) an die Makrodomäne beendet diese inhibitorische Interaktion. Wir konnten Tri-ADP-

Ribose als den minimalen Liganden identifizieren, der als ein potenter allosterischer Effektor die ATPase-Makrodomänen-Wechselwirkung aufbricht, aus der eine Konformationsänderung in eine offene, aktive Form resultiert. Hiermit übereinstimmend dekomprimieren ALC1-Fragmente, denen die Makrodomäne fehlt, Chromatin, ohne dass eine PARP1-Aktivierung erforderlich ist. Des Weiteren unterbindet die ATPase-Domäne die Interaktion der Makrodomäne mit PARP1, sofern keine DNA-Schädigung induziert wird. Ich konnte außerdem zeigen, dass somatische Krebsmutationen dieses Chromatin-Remodelers die Auto-Inhibition unterbrechen und die Chromatin-Remodellierung aktivieren. Unsere Daten zeigen, dass der NAD^+-Metabolit PAR einen Konformationswechsel in ALC1 induziert, hierdurch die Autoinhibition aufgehoben wird und letztendlich Chromatinrelaxation resultiert. Eine modulare Allosterie in diesem onkogenen Chromatin-Remodeler löst eine stabile DNA-Schadens-abhängige Aktivierung aus. Diese Arbeit könnte die Entwicklung von niedermolekularen Therapeutika unterstützen, die ALC1 als potentielles Wirkstoffziel für eine klinischen Anwendung haben.

Während meiner Doktorarbeit habe ich an zwei weiteren Projekten gearbeitet, von denen Teile meiner Ergebnisse in Publikationen eingeflossen sind:

1. Die Histonchaperon NASP-Histon H3 Interaktion und der zugrundeliegende Histonchaperon-Mechanismus, 2. Protein-Protein Interaktionen des circadianen Rythmus, u.a. die Interaktion von Cry 1 mit FBXL3 und PER2, und der Einfluss dieser Interaktion auf den circadianen Rythmus.

1. Introduction

1.1 The problem of DNA packaging

The large size of the eukaryotic DNA (total length of human genome is about 2 meters) and relatively smaller size of the nucleus (about 10 uM in diameter) poses an interesting problem of the packaging of the genetic material within the limited confines of the nucleus, while also requiring access to the packaged DNA whenever and wherever needed for the genome templated processes to occur in a regulated manner. Therefore, nature devised nucleosome.

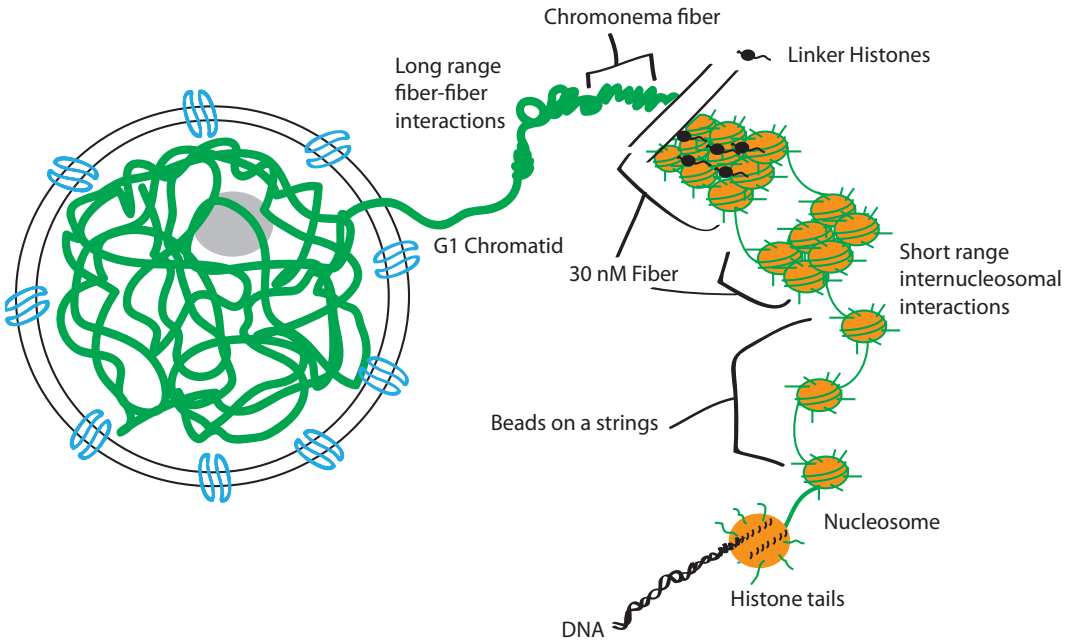

Figure 1: An overview of the packing of the DNA in the form of chromatin. DNA is packaged in the form of chromatin as depicted in the figure within the interphase nucleus (shown at left). This occurs through a series of hierarchical histone-dependent interactions that are subdivided into primary, secondary, and tertiary levels of structure. Bead on the strings made up of nucleosomes makes the primary structural unit. Secondary level of compaction is 30-nm fibers showing the higher level of organization above bead on the strings structure. This is mediated by histone tail–mediated nucleosome-nucleosome interactions as well as linker histones mediated compaction, these fibers further fold to make higher order chromatin structure giving rise to the tertiary structures (such as chromonema fibers). (Figure adapted from [1])

Nucleosome is a cylindrical structure that wraps 147 bp of DNA (in 1.67 turns) around a basic core of histone proteins called octamer consists of two of each H2A, H2B, H3 and H4[2]. This structure helps package the genome (i.e. structural organization)[2] as well as act as a signal integration platform for genome function (i.e. signal interpretation)[3]. Indeed now we know that nucleosome is the basic repeating unit of chromatin that dictates genome structure-function regulation. Nucleosome is a rather rigid cylindrical structure which has histone tails coming out of it[4]. While rigidity contributes well towards packaging and genome protection from genotoxic insults, the histone tails can provide a platform for context dependent structure-function regulation via acting as signalling platform[5].

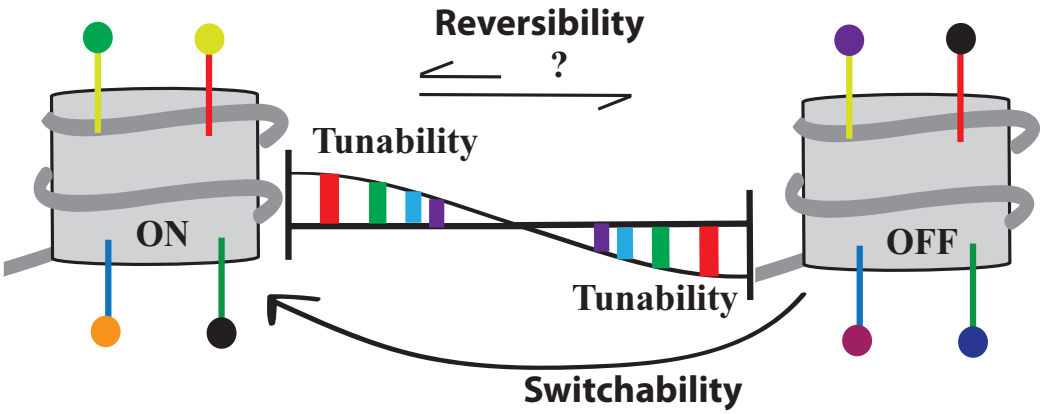

Figure 2: Nucleosome, epigenetics and chromatin signalling. Nucleosome can be seen as monomeric unit within a fiber of chromatin polymer (see also figure 1), which can provide specific structure-function context towards different chromatin states. As shown in the Figure 2 the tails emanating from the nucleosome can be post-translationally modified depending on the input signal. Therefore, a particular combination of the modified states can in principle provide a context dependent regulation of the genome-templated processes for e.g. Transcription. However, as the ability to package genome information in different context increases; the ability to read the underlying information must also concomitantly increase. There are hundreds of proteins with the reader modules that can recognize these PTMs [6]. Different colours represent different modifications and also represent their potential impact on the nuclear processes for eg Transcriptional tunability (depicted as arbitrary range in the figure) as shown in the figure. In some way, place of nucleosome in chromatin can be thought as a metaphor of a particular book in a big university library that has a set of coded information (a set of chromatin modifications) on it defining its position in space and time thereby allowing regulation on the accessibility of its content to the reader.

This layer of information over and above the DNA sequence is also known as the epigenetic information[7,8]. Figure shows my hypothetical model of this particular aspect of the chromatin phenomenon.

In the coming sections, I will provide a brief history and the introduction to the problem and rationales for the chromatin remodelling. Finally, I will introduce a unified framework for the chromatin structure-function regulation as well as a unified framework for chromatin remodeler activation mechanisms.

1.2 Chromatin to nucleosome: a brief history

From the early discovery in 19th century to high-resolution structure-function characterization of the chromatin associated phenomenon. Chromatin field has a very rich history involving many renowned scientific figures of the second half of 20th century's biological research after the discovery of the DNA double helix structure.

19th century biologist and founder of cytogenetics Walther Flemming using light microscopy first observed the ribbon like structures in the nucleus of eukaryotic cells which strongly absorbed basophilic dyes (aniline), he thus named that structure chromatin (Latin: chroma for colour; taena for ribbon) [9,10]. In 1884, Albrecht Kossel described the presence of basic proteins i.e. histones in the nucleus by acid extraction[11,12]. After more than half a century later, Maurice Wilkins and others using X-ray diffraction experiments on intact nuclei demonstrated the possible presence of a repeating structure larger than the DNA double helix. Later on, Aaron Klug and colleagues using X-ray diffraction on natural chromatin showed the presence of repeating units of about 100 Angström. This was what later on would be known as nucleosome with a diameter of about 110 Angström (discussed in[13]).

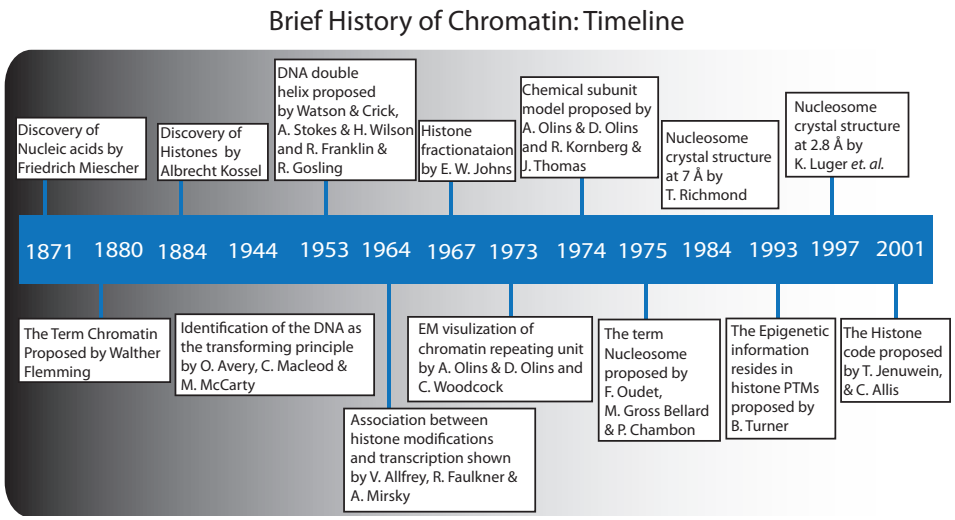

Figure 3: A brief history of chromatin. Figure 3 shows a brief history showing the major milestones in the history of chromatin (The figure adapted from[13])

Based on the biochemical characterization, Roger Kornberg showed that each of these repeating nucleosome particles contain two of each core histones H2A, H2B, H3 and H4; one linker histone H1 and about 200 bp of DNA. He found that Histone H2A crosslinks with H2B and H3 crosslinks with H4 in solution and H3 and H4 exist as tetramer. Further, the mass of DNA in nucleosome was as much as the mass of the histones and the histone H1 were half as much as any core histone. His reconstituted nucleosome using core histones (H2A, H2B, H3 and H4) and DNA reproduced the same x-ray diffraction pattern as was observed in case of natural chromatin[14]. Gary Felsenfeld and colleagues had already shown the size of the repeating unit to be 200 bp DNA using nucleases to cut the chromatin. Putting all of these data together Kornberg proposed his idea of nucleosome with 200bp DNA in complex with one linker histone and two of each core histones [14,15].

Later on Pierre Chambon and colleagues isolated chromatin from chicken red blood cells and removed linker histones using trypsin digestion. Using electron microscope they observed the basic repeating unit in a "bead on a string" kind of structure. Chambon coined the terms "nucleosome" for the bead in this repeating structure of chromatin[16].

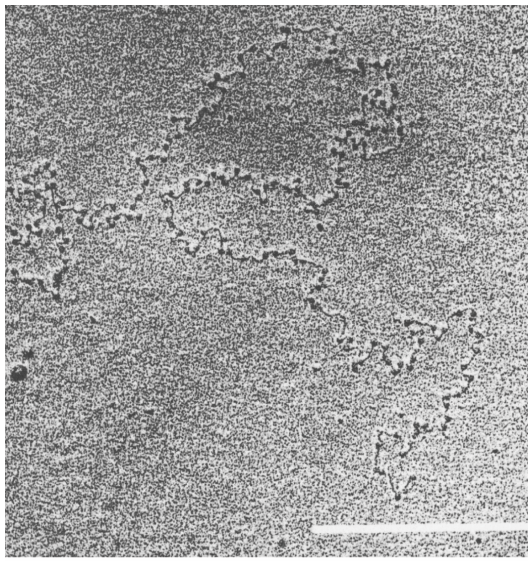

Figure 4: Nucleosome is the basic structural unit of chromatin. Figure shows the early electron micrographs of histone H1 depleted chromatin from chicken red blood cells from Chambon and colleagues showing a bead on the strings structure (bar 500 nM) (Image adopted from[16]).

1.3 Higher order chromatin structure

The core nucleosome structure showed that DNA winds about the 1.66 left handed helical turns around the core histone octamer [2], condensing the length of DNA by a factor of about 5 to 6. This amount of condensation is insufficient for packaging of the DNA in the eukaryotic nucleus. Therefore higher order packaging must occur.

1.3.1 Linker histones are needed for further chromatin compaction

In spite of a highly basic octamer around which the negatively charged DNA is wrapped the overall nucleosome structure remains negatively charged. Linker histones that bind with linker DNA (thus the name) via is C-terminal domain (CTD) between two nucleosomes not only are useful to further neutralize the negative charge but also they can promote the higher order chromatin structure folding. Typically, linker histones dock at the nucleosome dyad axis via its globular winged helix domain and highly basic CTD interacts with the linker DNA possibly bending it and promoting higher order structural organization of 11 nM beads on the strings fiber. In this section I will discuss the higher order chromatin structure organization with more focus on the linker histones and the nucleosome interactions. Partly because I believe that this one question continue to remain a key that once understood might unlock the mechanism of higher chromatin structure folding.[17]

1.3.2. Linker histone and nucleosome together form the chromatosome particle

Though when Roger Kornberg first conceived the idea of nucleosome he had included linker histone H1 as inherent part of it [14]. However, later on Pierre Chambon in his beads on the string model did not include histone H1 as part of the nucleosome as such [16]. Since then Chambon's description of the nucleosome has been the accepted norm, instead nucleosome with bound linker histone H1 is called chromatosome [17]. Nucleosomal organization overall has the negative charge, so some other basic proteins like linker histones would be needed to neutralize it as well as further package the DNA, as nucleosomal organization is in not enough. chromatosome is thought to be involved

in the higher level of chromatin structure organization via promoting further decompaction and the formation of 30nM fiber[18,19].

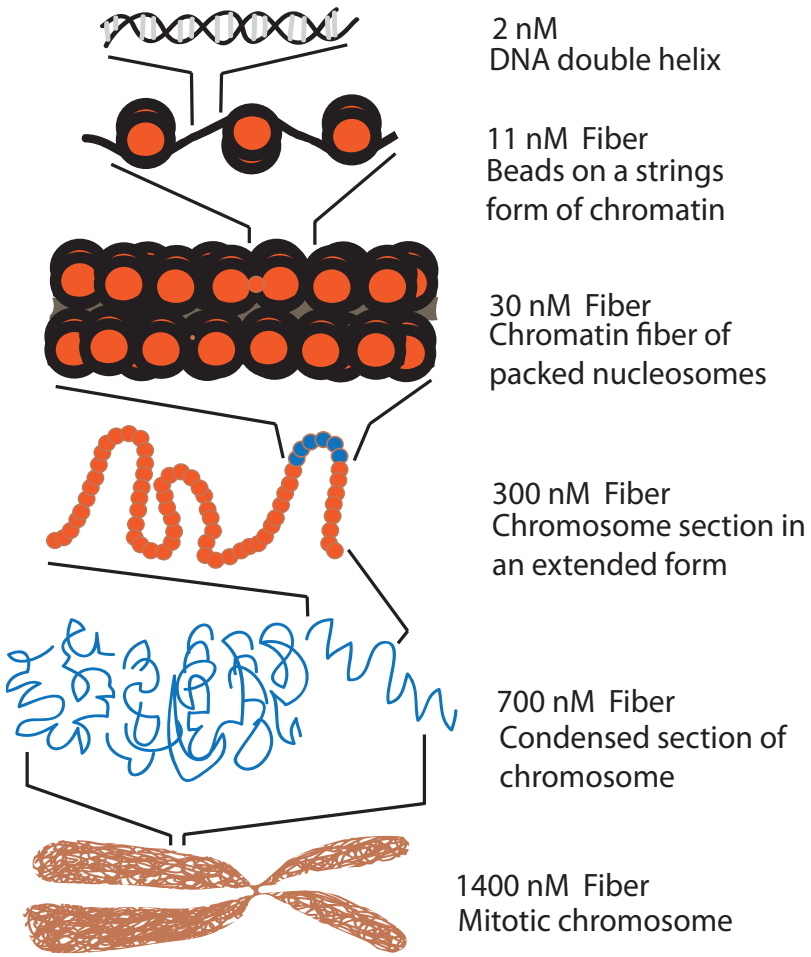

Figure 5: Higher order chromatin structure. A model of the organization of DNA in the form of chromatin is shown. DNA, which has the diameter of 2nM, is wrapped around a core of basic histone octamer forming an 11nM beads on a strings structure. The bead on a string structure upon binding with linker histones can form a sort of more condensed often referred to as 30nM fiber, existence of which is disputed. Nonetheless, the 30nM fiber (on otherwise more compact forms) goes through higher order chromatin looping (as in the interphase nuclei). These loops are further compacted and organized by proteins like condensins and cohesins in the most compacted structure that is mitotic chromosome in somatic cells. The mechanisms and structural information over and above the nucleosome even at the level of linker histones is limited and is an active area of research. Please note that the most compacted form of chromatin packaging happens in the nucleus of the sperm cells with the help of basic proteins called protamines (Figure adapted from[20]).

Linker histones of most organisms have tripartite domain architecture involving a short extended unstructured N-terminal nose, conserved structured globular domain and a

large basic unstructured C-terminal tail domain [17]. Although, the structures of the globular domain of H1/H5 and the nucleosome core particle as well as the chromatosome particle (nucleosome with the globular domain of H5) are available [21]. There is still a little understanding of the structures of H1 terminal domains, however it is known that CTD and NTD get structured upon DNA binding [17]. Location of the Histone H1's globular domain on the nucleosome and thereby the location of the N- and C-terminal domains within a chromatosome can shed light on the folding of Chromatosome in a higher order chromatin fiber by revealing the way in which the linker DNA would bend. In addition, the differential ways in which globular domain of different H1 variants interact with the nucleosome might be responsible for the linker histone variant specific structure-function variations within the chromatin. This information is very crucial in order to delineate the mechanistic details of the linker histone mediated dynamic modulation of chromatin structure and functional implications to the chromatin associated processes [17]. Recently a crystal structure of the chromatosome particle with the globular domain of the H5 linker histone was reported [21] (Reviewed in [22]).

1.3.3 Linker histone's globular domains have a conserved winged-helix domain

The crystal structures of the globular domains of the Chicken linker histones H5 in the absence of DNA has been determined a long time back. These structural studies have revealed the overall structural similarity with a well-known DNA binding winged-helix domain, a three-helix bundle distantly related to the helix-turn-helix superfamily of proteins. In general H5 and H1 globular domain involves three alpha helices followed by a beta hairpin whereas, the canonical winged helix domain consists of two beta wings, three alpha helices and three beta strands in which the second and third helices form helix-turn-helix DNA binding motif.

1.3.4 Docking of the globular domain on the nucleosome determines higher order chromatin structure

Recently the crystal structure of the chromatosome particle was solved. Authors determined the co-crystal structure of the H5 globular domain bound to the nucleosome. Briefly the structure shows that linker histone globular domain binds to the dyad axis and interacts with both the linker DNAs. The manner in which the globular domain binds to the nucleosome dyad determines the higher order chromatin structure. If the binding happens on the dyad axis (thereby symmetric interaction with the linker DNA) then the resulting chromatin structure is more condensed whereas if the binding happens off the dyad axis (thereby asymmetric interaction with the linker DNA) then the resulting chromatin structure is less condensed. Two other studies show off-dyad (asymmetric) mode of globular domain binding to the nucleosome [23,24]. The different linker histone variant's globular domain can bind with the nucleosome in different binding modes and therefore can direct the formation of a different kind of higher order chromatin structure [17].

1.3.5 Linker histone variants can contribute to functional diversification

The interaction and location of globular domain in the nucleosome and thereby the location of N- and C-terminal domains within a chromatosome has direct implications for the folding of chromatosome in a higher order chromatin fiber. In addition, although in the linker histone family the globular domain is a highly conserved independent module, especially designed for the specific binding at the nucleosome dyad and with entering and exiting DNA. The differential ways in which globular domain of different H1 variants interact with the nucleosome might be a contributor to the variant specific function which is attributable to the minor sequence differences among linker histone globular domain. Probably the minor sequence difference leads to the difference in the binding affinity of a linker histone globular domain to the nucleosome and this difference is involved in directing the variant specific chromatin structure-function modulation [17,22].

1.3.6 Linker histone tails promote chromatin condensation

Further, the previous reports suggest that globular domain closes the nucleosome gate by simultaneously binding with the entering and exiting DNA. The C-terminal tail of the linker histones then can interact with the linker DNA and induce the stem like structure via electrostatic interaction, in particular the interaction between SPKK motifs with minor grooves of the linker DNA region that is thought to induce the bend in the DNA promoting the higher order structure formation and condensation. The role of N-terminal tail in chromatin condensation is marginal, if any [17,22].

1.3.7 The 30 nM fiber is the next layer of chromatin organization

There is well defined and very well characterized structures of the nucleosome and now also of the chromatosome lacking the linker histone tails (the so called NTD and CTD). However the structure above and over the 11 nM fiber is not yet well defined. What is however clear is that the compaction over and above the 11nM fiber must occur considering that 11nM fiber alone can not be accommodated in the small nucleus. Next level of compaction is the 30 nM fiber. Initially electron microscopic studies by Aaron Klug described the formation of 30 nM diameter fibers like structure from isolated nucleosomes (Finch and Klug, 1976). There are two main helical models for the 30 nM fiber structure first the one start helix (Solenoid) and two-start helix (Zig-zag) as shown in the figure 6. However, the presence of any 30 nM within the interphase nuclei remains controversial and alternative models suggest that there is no regular 30 nM fiber in the nucleus. A recent Cryo-EM study on the 30 nM fiber with linker histones support Zig-zag model [23].

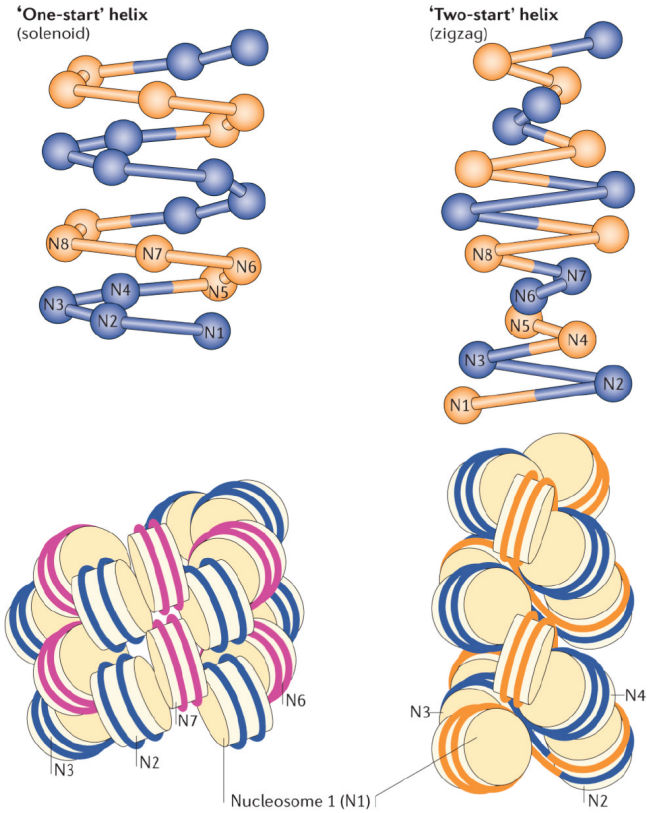

Figure 6: The two models for the 30 nM fiber. Solenoid also known as the one start helix model involves the interactions between the two consecutive nucleosomes on the other hand zig-zag also known as the two start helix model involves interactions between the two alternative nucleosomes. Further, the solenoid model involves the bending of linker DNA inside of the helix whereas in the zig-zag model the straight linker DNA criss-crosses between the two helices (Figure is adopted from [23,25]).

1.4 Nucleosome is the basic unit of chromatin structure-function regulation

Nucleosome structure in particular histone tails provide a signal integration platform that involves PTMs as flags for recruitment of regulatory factors, reader module dependent recruitment of functional effectors and chromatin remodeler mediated nucleosome structure modulation.

The crystal structure of a nucleosome core particle at 2.8 Å resolution[2] revealed in unprecedented details the organization of 147 bp of the DNA around the octamer of each core histones. The negatively charged DNA is wrapped around the basic histone octamer. Octamer contains the two dimers of H2A-H2B histones and a tetramer (dimer of a dimer) of H3-H4 histones. Both H2A-H2B and H3-H4 forms the dimers via a conserved and alpha-helical histone fold motif (three alpha helices connected by two short loops). Interestingly, many other genome regulatory proteins also harbor the histone fold motifs. The structure of the nucleosome did not show the N and C-terminal histone tails, which are unstructured, flexible and floppy in nature. With one exception being the Histone H4 N-terminal tail that was shown to have made contacts with another nucleosome's H2A-H2B acidic patch. This interaction between H4 tail and the H2A-H2B acidic patch would later on turn out to be one of the major regulatory points in chromatin structure-function regulation (Please see also the figure 7).

1.4.1 Histone modification regulate genome templated processes

Gcn5 was the first histone acetyl transferase enzyme that was shown to add acetyl groups to the histone tails indicating a signalling role for the nucleosome [26]. In the signalling role of nucleosome different post-translational modifications (PTMs) like acetylation, phosphorylation, ADP-ribosylation *etc* can act as flags marking a distinct chromatin territory for a context dependent spatio-temporal regulation of chromatin structure and function. These discoveries lead to an explosion of new PTMs and new enzymes that can bring about these modifications[27]. It turns out that most of these

enzymes are part of previously described transcription complexes like co-activators and co-repressors. This suggest that a combination of different modifications will mark chromatin for a specific structural state that will encode for a particular genome function.[7,8,28,29]

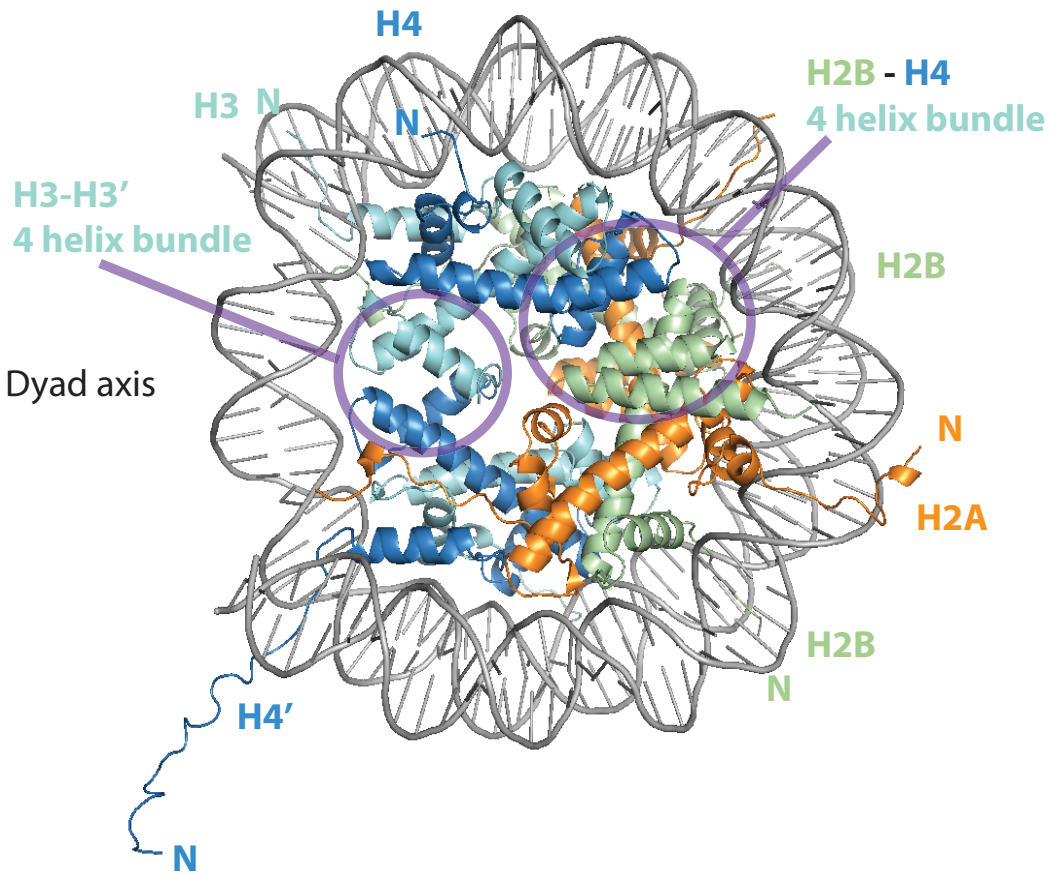

Figure 7: The crystal structure of the nucleosome core particle: Figure shows the structure of the nucleosome core particle consisting of 146 bp DNA (ribbon traces for the 146-bp DNA phosphodiester backbones in grey) wrapped around the core octamer of two of each histones H2A, H2B, H3 and H4 (Cyan: H3; Sky blue: H4; Orange: H2A; Light green: H2B. (Figure adapted from[2] PDB id: 1AOI).

1.4.2 Histone PTM recognition module connect PTMs with functional response

The discovery of a bromodomain binding to the acetylated histone tails answered this long-standing question [30]. Interestingly TAF_{250} bromodomains were part of the largest subunit of transcription initiation complex TFIID's. This generated new possibilities wherein one can imagine histone modifying enzymes, which are also part of the complexes associated with genome function like transcription can mark chromatin towards a context dependent regulation in response to a signal for e.g. transcriptionally active and repressed state[27]. These modifications can then act as flags towards functional interpretation of the underlying modified states via recruiting the specific functional protein complexes for e.g. TFIID for transcription initiation[30]. In addition, many of these proteins and complexes contain both the modifying enzyme and the binding modules immediately suggesting a propagation (via positive feedback mechanisms) and/or auto-regulation mechanism (via an auto-inhibition mechanisms) [7,8]. While the proteins that can de-modify the PTMs can switch one chromatin state to another chromatin state for e.g histone deacetylases/demethylases, DNA demethylases. Indeed, hundreds of histone and other nuclear protein modifying enzymes - their substrates and target sites (the so called writers), their binding modules (the so called interpreters), de-modifying enzymes - their substrates and target sites (the so called erasers) and effector proteins (which combines binding modules with the modification/functional modules) have been identified[4].

The structure of nucleosome immediately conveys that it's a very stable structure stabilized by the multiple histone-DNA contacts and therefore needs to be unwrapped so as to provide access to the underlying DNA sequence[2].

1.4.3 Chromatin remodeler remodel nucleosomes using the energy of ATP

There are ATP dependent chromatin remodelling enzymes that can pump the ds DNA around the nucleosome therefore can perform nucleosome remodelling. Snf2 domain containing protein BRG1 (related to SNF2 family nucleic acid helicases) can create multiple remodelling states of the nucleosome and can provide access to an underlying

restriction site embedded in the nucleosome[31]. On the other hand, ISWI remodeler was shown to create regularly spaced arrays of nucleosomes promoting chromatin assembly[32].

These discoveries not only established nucleosome as the basic repeating unit of the chromatin structure but also led to an explosion of research activity focusing on the histone PTMs, their functional interpretation and chromatin remodelling - establishing nucleosome as a signalling platform for chromatin templated processes as well as a the basic structural repeating unit. This ultimately culminated into histone code hypothesis followed by histone PTM cross talk and then to the proposal of the epigenetic language[3,28,29,33].

1.5 ATP dependent chromatin remodelling

Just by looking at the nucleosome structure one can not escape but immediately and intuitively grasp the underlying fact that the structure is very well stabilized by the histone-DNA interactions and therefore is well-suited for the packaging task. However, on the other hand it poses a great barrier for the DNA templated processes, which needs to have access to the underlying DNA sequences. Fortunately, there are ATP dependent chromatin remodelling machine that can use the energy of ATP to remodel this otherwise very stable structure.

1.5.1 ATP dependent nucleosome remodelling is a complex task

Common to all of these chromatin-remodelling machines is a core protein with snf2 domain module (a member of SF2 superfamily) that specializes in the task of pumping double stranded DNA around. The auxiliary subunits (in case of multi-subunit complexes) and other domains within the snf2 domain containing proteins (in case of single subunit remodelers) direct the remodelling task towards a specific and spatio-temporally regulated outcome[34].

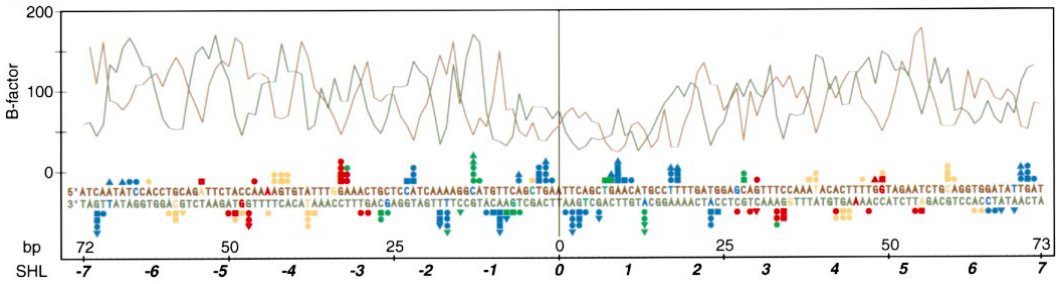

Figure 8: The extensive histone-DNA interactions in the nucleosome core particle. The figure below shows the DNA phosphate B-factors (a measure of spread in the electron density showing the mobility of particular atom) versus base pair location along the dyad axis (also represented as Super Helix Location Zero) in the nucleosome structure. In addition, the extensive interactions between the phosphodiester chains of DNA and histones are indicated on a base pair resolution – squares, circles and triangles represent main-chain hydrogen bonds; the side-chain hydrogen bonds, and the hydrophobic bonds respectively. The bases that are shown in blue, green, red, and yellow indicate close proximity to an arginine side chain finger inserted into the corresponding DNA minor grooves. Further the figure shows higher mobility of the phosphate groups when the DNA is not contacted and stabilized by the interaction with the histones (Figure adopted from[2]).

Now, that nucleosome structure is stabilized by so many interactions is a formidable task to disrupt even with the energy of ATP and DNA translocation activity of the snf2 domain. This is because of the fact that every time you disrupt the interaction between the histones and DNA the tendency of the DNA would be to always fall back on to the histone octamer and this thermodynamic energy barrier represents a herculean task that is nucleosome remodelling. (Please also see the Figure 9)

1.5.2 Different chromatin remodeler families remodel the nucleosomes towards multiple different but defined outcomes

The core catalytic subunit of the chromatin remodelling enzymes is part of the SF2 superfamily of ATP dependent RNA and DNA helicases. This domain in chromatin remodelling enzymes is also called the snf2 ATPase domain. The snf2 ATPase domain like the SF2 helicase family contains the two RecA like lobes connected by a structured linker segment. In addition, snf2 domain also contains minor and major insertions in the RecA like lobe 1 and RecA like lobe 2 respectively. Further there are alpha helical extensions protruding from each lobe. Each family of remodelers contains a common snf2 ATPase subunit. Further, the same catalytic subunit i.e. the snf2 domain can associate with different accessory subunits therefore giving rise to multiple complexes as in case of developmental stage specific expression of different complexes[35]. There are a total of 29 genes encoding snf2 domain containing proteins in human genome. Based on unique domains residing within, or adjacent to, the ATPase domains, chromatin-remodeling enzymes are divided into four families: SWI/SNF, ISWI, CHD and INO80. ATRX is the orphan chromatin remodeler. In humans CHD1 family has total of 10 members CHD1 to CHD9 and ALC1 (Amplified in liver cancer 1) also known as CHD1L (CHD1 like). All CHD1 family snf2 domain protein except ALC1 have chromodomains, which bind to, methylated histone tails whereas ALC1 has macrodomain that binds to the Poly-ADPr. ALC1 is the only chromatin remodeler that has a globular Poly-ADPribose recognition module Macrodomain.

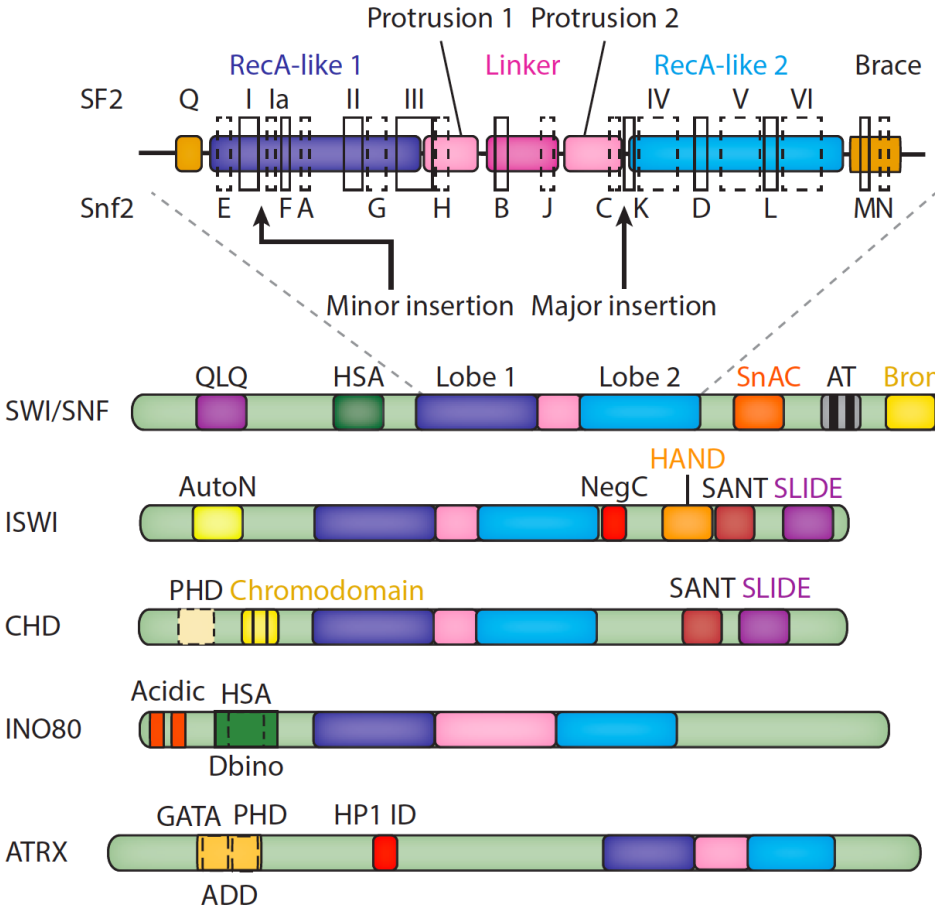

Figure 9: The chromatin remodeler families. There are 5 major chromatin remodelling enzyme families. RecA like lobes are shown in the purple and light blue. Protrusion 1 and 2 are shown in light magenta and the linker is shown in the dark magenta. There are other unique domains in each family adjacent to the catalytic snf2 domain. For SWI/SNF family these are N-terminal HSA (Helicase-SANT-Associated) and C-terminal Bromodomain; for ISWI family a C-terminal HSS (HAND-SANT-SLIDE); for CHD family it is the presence of chromodomains except for ALC1. Ino80 family is characterized by a split ATPase domain with a long insertion between two lobes and an HSA domain. Other domains and motifs abbreviated are SNaC: Snf2 ATPase coupling, PHD: Plant homeodomain, ADD: ADD domain is a fusion of GATA like zinc finger and PHD domain. (Figure adopted from [36])

It should be noted that snf2 domain containing proteins can not only remodel nucleosomes but have also been shown to remodel other DNA-protein complexes such as the Mot1-mediated remodeling of TATA box binding protein (TBP) [37,38], transcription-coupled repair factor Cockayne syndrome protein B (CSB)- mediated remodeling of repair fators at the site of stalled RNA polymerase[39]. Although so far underappreciated, remodelling of DNA-protein complexes other than nucleosomes may be a widespread phenomenon.

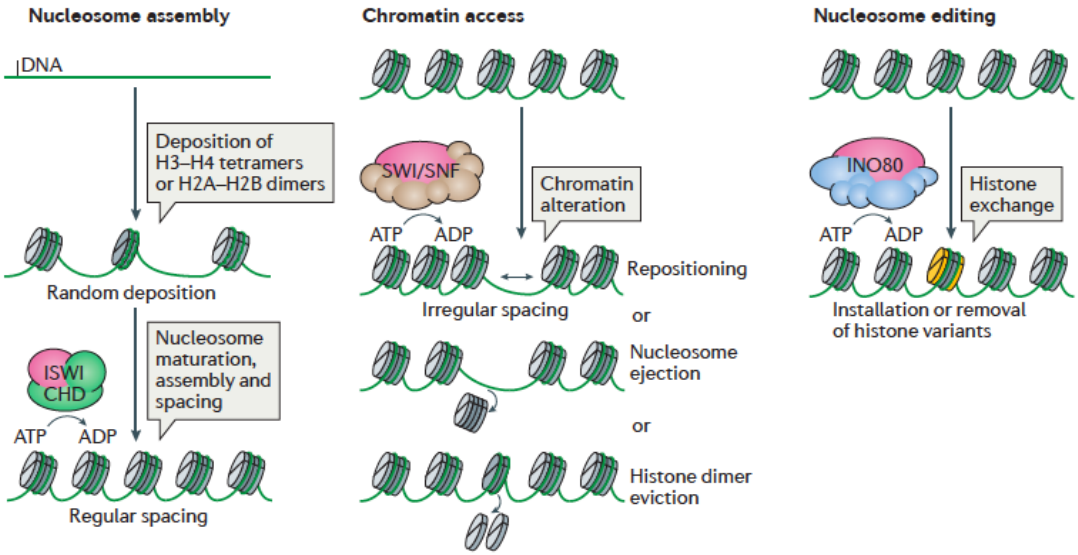

Figure 10: Functional outcomes of chromatin remodelling activities. At the level of nucleosome ATP dependent chromatin remodelling can serve three main purposes – First, nucleosome assembly via acting in concert with the histone chaperones; Second, chromatin access via sliding of the histone octamer along DNA (also helps nucleosome spacing) or via eviction of the histone octamer/dimer and Third, nucleosome editing via histone exchange (Figure adopted from[34]).

1.6 Regulation of chromatin remodelling enzymes

Considering the fact that the substrate for chromatin remodelers is more or less of common nature i.e. nucleosome/other stable DNA-protein complexes and the fact that a common catalytic snf2 domain ATPase subunit has to deal with it towards different remodelling outcomes indicate that there must be a framework for the chromatin remodelling activity regulation utilizing allostery.

Allostery is the fundamental level of meaningful information encoding in the biological systems. In fact, chromatin remodelers are regulated via very sophisticated allosteric activation mechanisms. Common to all snf2 domain proteins is the DNA translocation that works through an Inchworm like mechanism. Snf2 domain containing proteins usually contain other regulatory accessory domains and motifs as well as in case of multi-subunit complexes other accessory subunits. It is these accessory domains, motifs and accessory subunits that specialize the general task of DNA translocation towards a defined outcome whether it is nucleosome spacing or octamer eviction or histone variant exchange. In the case of the remodeler CHD1, chromo-domains act as a targeting module via an interaction with histone tails at the same time the linker region between chromodomains acts as an inhibitory lock for ATPase stimulation. Upon chromodomain binding with histone tails this lock is removed. These steps in the remodeling reaction provide an interesting mechanism for nucleosome targeting, substrate specificity and processivity at the same time. Previously it was shown that the enzymatic activity of the chromatin remodeler

In fact there must be remodelling strategies that are employed by these enzymes, which can deal with this feature of the nucleosome structure in order to achieve efficient remodelling. In addition, the naturally condensed state of chromatin requires the nucleosomes to be evenly spaced so as to promote the higher order chromatin organization, a phenomenon once again requiring chromatin remodelers but with a highly controlled activity so as to not disrupt but just evenly space the nucleosome structure. These special challenges of chromatin structure require special ways through which nucleosome remodelers should act. Indeed, we are now beginning to understand these mechanisms and the nitty-gritty of the nucleosome remodelling and indeed these

remodelers have very sophisticated ways to handle the nucleosome remodelling as well as any other relevant DNA-Protein complex remodelling[40,41].

1.6.1 Histone H4 tails directly regulates remodeler's activity

Histone H4 tail bind with DNA at the SHL2 of the nucleosome[42]. Interestingly, ATPase motor of the chromatin-remodelling enzyme is thought to act primarily on the SHL2 disrupting histone-DNA contacts through torsional strain[34]. H4 tail was first shown to activate the ISWI remodeler's activity[43]. This H4 tail's basic patch mediated ISWI activity stimulation is a general feature of many ISWI family remodelers[44-50].

1.6.2 CHD1 remodeler's allostery ensures substrate specificity and processivity by coupling the recruitment with de-repression

Yeast CHD1 chromatin remodeler is kept in a Pre-remodelling auto-inhibited conformation wherein chromodomains fold back on to the ATPase domain and the acidic helix between the chromodomains is directly interacting with the DNA binding surface at the lobe 2 gating its DNA binding. Only when the remodeler's chromodomains are engaged with the histone tails this negative regulatory acidic helix is displaced releasing the auto-inhibition[51].CHD1 mechanism couples remodeler recruitment with de-repression while also making sure substrate specificity and processivity through anchoring. In fact it appears to be a general theme
In the regulation of remodelers [52,53].

1.6.3 ISWI remodeler uses clutches and brakes to regulate its activity

Drosophila ISWI has an inherently active ATPase domain, which has two auto-inhibitory modules an N-terminal AutoN (H4 tail like sequence) motif and a C-terminal NegC motif. AutoN can act like a brake inhibiting ATP hydrolysis while NegC acts like a clutch inhibiting the conversion of ATP hydrolysis into a productive remodelling event. Only when the remodeler is present in the context of the nucleosome and is presented with H4 tail on the nucleosome. H4 tail inhibits these two (AutoN and NegC modules) thereby activating the remodeler. HSS domain provides further positive regulation upon binding with DNA[46]. Multiple electrostatic interaction surfaces and their exchange is likely to be a general theme in nucleosome remodelling.

CHD1 remodeler also contains an ISWI like NegC module at its ATPase doiman's C-Terminus named as the bridge segment[51]. Another very interesting example of the allostery mediated regulation of the ACF remodeler is discussed later in detail as published Preview manuscript[47,54].

1.6.4 Mot1p combines chaperoning and remodelling to ensure the productive remodelling

Mot1 chromatin remodeler remodels TBP-DNA complex using a Bottle opener kind of mechanism. TBP binds to the DNA with high affinity and induces an 80-degree kink upon binding. Mot1 remodeler engages with the back of TBP via its HEAT repeats and the snf2 domain mediated DNA translocation removes the TBP from DNA. Further, an acidic latch domain re-engages with the TBP's DNA binding pocket preventing re-binding to DNA. In that Chaperoning and remodelling co-operation is also likely general theme considering that many multi-subunit chromatin remodelers also have histone chaperone subunits[38,40].

1.6.5 RSC4 uses post-remodelling auto-inhibition to regulate its activity

RSC4 remodeler's bromodomains recruits it to the sites of GCN5 mediated histone acetylation. After remodelling the GCN5 acetylate the RSC4 protein which makes the bromodomains to re-engage with the RSC4 itself ending its recruitment to nucleosome and thereby its activity. This post-remodelling auto-regulation mechanism could also be a conceptual theme regulating the remodeler's activities[55].

1.6.6 ISWI scans the chromatin with low binding events using a continuous sampling mechanism

Fluorescence microscopic and spectroscopic approaches in the living cells have shown that ISWI continuously sample the chromatin via low affinity binding events and only 1-3 % of the total enzyme is in an active remodelling competent state. The transient binding events test the propensity of the nucleosome translocation and are largely unproductive. Only where the translocation is required a high affinity-binding event happens resulting in the efficient remodelling[56-60]. Scanning of chromatin by the remodelers is particularly relevant when there is no apparent reader module however

this again is a likely general concept for not only the remodeler activity regulation but also for many other chromatin factors including transcription factors and Cas9[61-63].

1.6.7 Remodelers engage with the nucleosome during remodelling reaction

Recently there have been unprecedented insights into the mechanisms of the chromatin remodeler's engagement with the nucleosome giving potential mechanics insights into how these remodelers try to deal with a very herculean task of nucleosome remodelling. ISWI and other remodeler can manage the remodelling outcome via managing the nucleosome plasticity. Binding of remodeler to nucleosome and subsequent effect on the histone octamer structure is again likely to be a general theme considering the stability and the energy barrier that nucleosome poses before the remodeler. It makes even more sense considering that remodelling involves constant strain on the nucleosome (Minus 4 bp) that must be stabilized via interaction with the remodeler. In fact this transition state like nucleosome forms are then manageable by the remodeler (possibly with help from accessory domains and subunits) towards a defined outcome. One recent example of remodeler's ability to manage the remodelling outcomes via tapping into the nucleosome plasticity was published and will be discussed in detail later as the published News and Views manuscript[64,65]. Recent structural studies of the remodelers confirmed the motor domains binding with the SHL 2 of the nucleosome and its anchoring with the N-terminal histone H4 tail [66-68].

1.6.8 The hourglass model captures the essentials of the functional diversification among the chromatin remodelers

Functional diversification through allosteric regulation can equip chromatin remodelers with a wide variety of remodelling possibilities and contexts. In a typical ATPase activation cycle of the swi2/snf2 ATPases, protein-DNA complex is recognized via accessory domains. This puts the ATPase domain in the vicinity of DNA. DNA binding induces favorable conformational changes in the ATPase leading to the ATP hydrolysis and subsequent translocation of the ATPase domain along DNA. Repeated cycles of ATP hydrolysis-translocation result in the remodeling of protein-DNA complex.

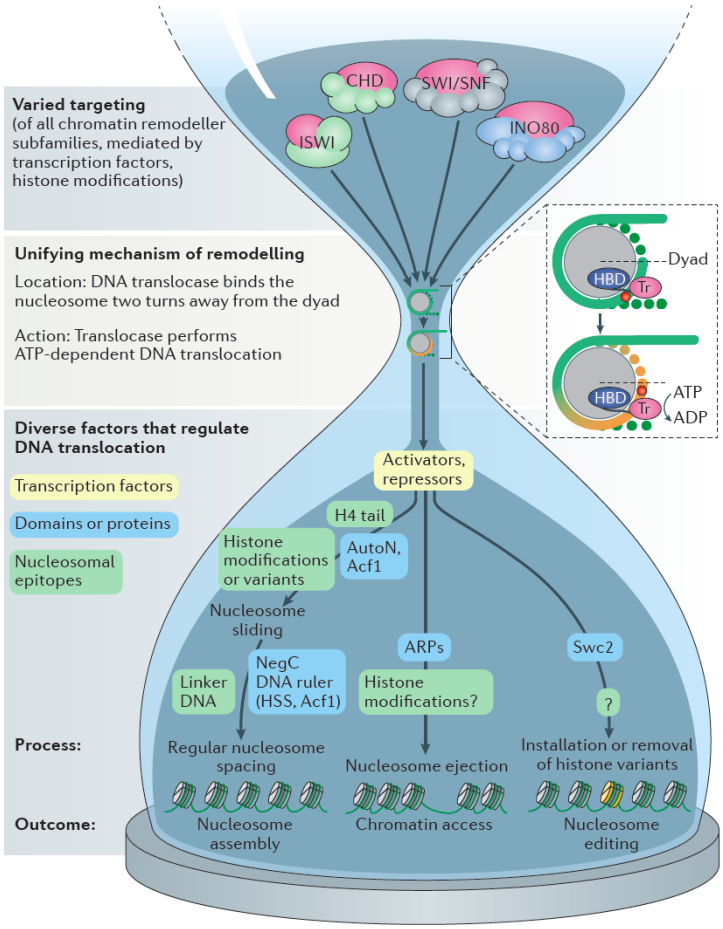

Figure 10: The hourglass model of chromatin remodelling. The hourglass model proposes a unifying mechanism for chromatin remodeler activity that funnel through a common snf2 chromatin remodelling ATPase domain that act as a DNA pump using is DNA dependent translocation activity. This common activity is directed at the nucleosome using a histone binding domain (HBD) and various remodelling outcomes like assembly, access or editing are achieved through smart regulation imparted by regulatory accessory domains/ motifs, accessory subunits and transcription factors responding to various chromatin features like histone PTMs, Linker DNA length and histone variants. Finally the hourglass model shows that chromatin remodelers are not just standard remodelling enzymes preforming the standard task of remodelling once targeted rather they are evolved and selected to deal with a chromatin substrate and are sophisticated smart machines exploiting the second secret of life i.e. allostery towards a specific functional outcome fully utilizing the allostery mediated encoding of the meaningful information at the molecular level. ARPs stand for actin related proteins; AutoN stands for autoinhibitory N-terminal; CHD stands for chromodomain helicase DNA-binding; HSS stands for HAND-SANT-SLIDE; NegC stands for negative regulator of coupling. (Figure adopted from [34])

1.7 Published - Introduction I

Preview

[Molecular Cell 55, August 7, 2014, 345-346]

1.7.1 ACF Takes the Driver's Seat

Hari R. Singh and Andreas G. Ladurner

Department of Physiological Chemistry, Butenandt Institute and LMU Biomedical Center, Ludwig-Maximilians-University of Munich, Butenandtstraße 5, 81377 Munich, Germany

ISWI family chromatin remodeling enzymes generate regularly spaced nucleosome arrays. In a recent Nature report, Hwang et al. (2014) describe how ACF gauges the length of linker DNA when deciding to accelerate nucleosome sliding or to put on the brakes.

Declaration of Contribution to "ACF Takes the Driver's Seat"

Hari Raj Singh made the first draft, prepared the Figure, co-wrote and corrected the article with Andreas Ladurner; Andreas communicated the article with the journal editors.

1.8 Published - Introduction II

News and Views

[Nature Structural & Molecular Biology, 2017; 24 (4), 441-443]

1.8.1 Remodelers tap into nucleosome plasticity

Hari R. Singh, Magdalena Murawska & Andreas G. Ladurner
Department of Physiological Chemistry, Biomedical Center, Ludwig-Maximilians-University of Munich, Planegg-Martinsried, Germany

Chromatin-remodeling enzymes perform the formidable task of reorganizing the structure of a stable macromolecular assembly, the nucleosome. Recently published work demonstrates that the SNF2H chromatin remodeler distorts the histone octamer structure upon binding to the nucleosome, then taps into this induced plasticity to productively achieve nucleosome sliding.

Declaration of Contribution to "Remodelers tap into nucleosome plasticity"
Hari Raj Singh wrote the first draft, Magdalena Murawska prepared the Figure 1, co-wrote and corrected the article, Andreas wrote and corrected the article and communicated with the Editors.

1.9 Published - Introduction III

Review

[Frontiers in Bioscience, Landmark, 20, 440-457, January 1, 2015]

1.9.1 Poly-ADP-ribosylation signaling during DNA damage repair

Barbara Golia, Hari R. Singh and Gyula Timinszky

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Poly-ADP-ribosylation (PARylation) is a post-translational modification generated in high amounts by poly-ADP-ribose polymerases (PARPs) in response to cellular stress, especially genotoxic stimuli. DNA damage-induced PARylation significantly changes local chromatin structure and triggers the accumulation of several DNA damage response (DDR) proteins at the DNA lesions. In this review, we will discuss the regulation of chromatin structure and DNA damage repair machineries by DNA damage induced poly-ADP-ribosylation.

Declaration of Contribution to "Poly-ADP-ribosylation signaling during DNA damage repair"

I co-wrote this review with Barbara Golia and Gyula Timinszky. In particular, I wrote the section 4 of the review article, which deals with "**The effects of poly-ADP-ribosylation on chromatin structure upon DNA damage**" and was additionally involved in writing and corrections of the rest of the document while Barbara Golia and Gyula Timinszky wrote most of the rest of the review article. Gyula Timinszky prepared the figure and communicated the final draft to the editors.

1.10 ALC1 is a paradigm chromatin remodeler

The previous data on the ALC1 remodeler, its domain architecture and presence of various putative regulatory motifs suggested that ALC1 remodeler can capture essentials of all the unified chromatin remodeler activation mechanisms making this single subunit chromatin remodeler a paradigm towards understanding the chromatin remodeler mediated structure function modulation.

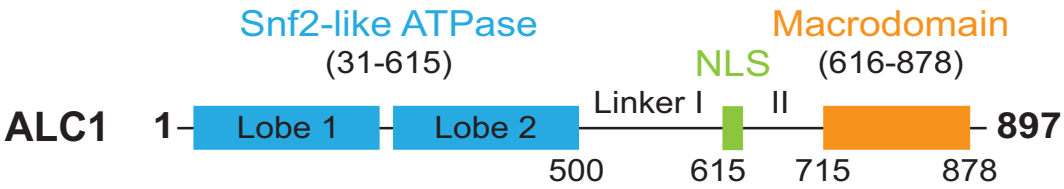

Figure 11: ALC1 is a simple single subunit macrodomain containing chromatin remodeler. ALC1 contains N-terminal ATPase domain (in cyan are shown the two lobes of the snf2 domain) and a C-Terminal macrodomain (in orange) separated by two linker regions in the middle.

ATP-dependent chromatin remodelling enzymes alter nucleosome structure and facilitate transcription, replication and repair[35]. However, little is known about how DNA damage triggers the activity of these chromatin remodelers. ALC1 is a uniquely known example of a chromatin-remodelling enzyme whose activity is strictly regulated by a dynamic posttranslational modification poly-ADP-ribosylation (PARylation). ALC1 harbors a C-terminal macrodomain and N-terminal snf2-like ATPase domain separated by a central linker region. This modular architecture allows PARylation induced by DNA damage to be coupled with Chromatin remodelling. In addition, the macrodomain mediates PARylation-dependent recruitment of ALC1 to sites of DNA damage after which the ATPase domain remodels chromatin at these sites. Crucially, the intact poly-ADPribose-binding pocket is necessary for the ATPase activity, suggesting an allosteric interaction between the macrodomain and the ATPase domain[69-72]. ALC1 is a single protein remodeler as opposed to the multi-protein remodeling complexes such as the Ino80 complex or SWI/SNF complexes[73]. This makes it an ideal system to explore the poorly understood fundamental mechanisms of

chromatin-remodeling using ALC1 as a paradigm chromatin remodeler. Considering the role of ALC1 in cancer and other diseases, there is also a window of potential clinical significance. PARP1 and macrodomain proteins are known targets for the therapeutic intervention in cancer[74-79].

1.10.1 ALC1 contains many previously known linear remodelling activity regulatory motifs

In addition to the globular macrodomain and the snf2 ATPase domain, there are various putative linear sequence motifs within the ALC1 protein outside the core ATPase and macrodomain with similarity with the previously described chromatin remodelling regulatory domains.

A. NegC like motif in ALC1

```
HsALC1          --MDEIDLESILGETKDGQWVSDALPAAEGGSR-
DmISWI          TDITDEDIDVILERGEAKTAEQKAALDSLGESSL
SNF2H           SEITDEDIDGILERGAKKTAEMNEKLSKMGESSL
XlIswI          SEITDEDINAILERGEKKTAEMNEKLSNMGESSL
HsSNF2L1        SELTDEDITTILERGEKKTAEMNERLQKMGESSL
ScIsw2          KVTVDADIDDILKKGEQKTQELNAKYQSLGLDDL
ScIsw1          EKGDDIDLDELLLKSENKTKSLNAKYETLGLDDL
                    : *:  :* .        .      * .
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B. AutoN like motif in ALC1 and its alignment with H4

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H4              MSGRGKGGKGLGKGGAKRHRKVLRDN
HsALC1          -NKGSVLIPGLVEGSTKRKR-VLSP-
                 .  .    ** :*.:**:* **
```

Figure 12: ALC1 and the putative regulatory motifs. Figure shows sequence alignments of the putative NegC like motif and the histone H4 tail like (or AutoN like) motif within the ALC1. **A.** Sequence alignment of ALC1 NegC like motif with other renodeler's similar motif; **B.** ALC1 alignmnet with the H4 tail.

ISWI's AutoN

ALC1 appears to have at least three previously described putative regulatory motifs in its middle linker region. These modules have been shown to regulate either remodelling activity or ATPase activity or both of the remodelers. For e.g. H4 N-terminal like motif

in ISWI (AutoN) inhibits the ATPase activity. It is interesting to note that ALC1 activity has been shown to be regulated by H4 tail, however to what extent this NLS like and H4 tail mimicking fragment within ALC1 (Amino acid 616-635) is playing regulatory role has not been understood.

ISWI's NegC

Another regulatory motif at the end of C-terminus of the ATPase [46] domain named NegC regulates the coupling of ATP hydrolysis with nucleosome remodelling activity. ALC1 also contains a similar motif after its ATPase domain (Amino acid 533-567). However exact mechanisms of their action in the context of ALC1 is not known.

CHD1's Acidic Helix

When we align the chd1 construct for which the crystal structure was solved with the ALC1 sequence until Amino acid 584 aligns very well arguing for a high conservation between the two proteins. Interestingly the region between the Amino acid 689-707 is highly acidic and in that way similar to the acidic helix in the Chd1 remodeler that docks against the DNA binding surface on the ATPase domain.

The sequence between the residues 585-605 remains of an unknown importance and 606-615 is an apparent hinge for the two domains. Interestingly the region between 500-645 has also been shown to have most of the poly-ADP-ribosylation sites.

Presence of these putative regulatory modules generates interesting questions about ALC1 regulation and whether these putative sequences have any role in that. However, What is clear so far is that the region between 616-711 and 533-567 are the most interesting regions within ALC1 protein.

1.10.2 The chromatin remodeler ALC1 is a protein of clinical relevance

ALC1 (amplified in liver cancer1) also known as CHD1L (chromo domain helicase 1 like) was originally identified as a gene encoded in genomic locus 1q21.1 that is amplified in more than 50 % of hepatocellular carcinomas[80-84]. Copy number variants and single nucleotide polymorphisms of this locus have been shown to be associated

with various disease states like congenital anomalies, learning deficiency/ intellectual disability and various cancers[81,83,85-90]. Further ALC1 has been shown to have roles in stem cell renewal and development[91-93]. ALC1-overexpressing cell line showed increased colony formation on soft agar and tumoriginicity in nude mice[81]. ALC1 was shown to promote epithelial-to-mesenchymal transition and thereby metastasis[87,94]. ALC1 was also shown to interact with Nur 77, suppressing its nucleus-to-mitochondrial translocation, thereby sustaining cancer cell proliferation. ALC1 can also act as an inducer of mitotic defects and chromosome mis-segregations, providing another physiological basis for its involvement in cancer manifestation[80,86,88]. In short, it appears that the remodeler ALC1 may be an important protein of clinical significance in cancer.

1.10.3 ALC1 chromatin remodeler connects the Poly-ADPribose signalling with the chromatin remodelling

ALC1 has a C-terminal, ADP-ribose-binding macrodomain and an N-terminal snf2-like ATPase motor domain separated by a central unstructured linker region. This modular architecture allows it to couple DNA damage detection and subsequent PARP1-mediated poly-ADP-ribosylation (PARylation) with chromatin remodeling. This occurs via macrodomain's binding with the poly-ADP-ribose and subsequent remodeling mediated by its activated ATPase domain. ALC1 was shown to have weak ATPase activity, but activity was weakly stimulated upon addition of DNA. It was further stimulated by nucleosomes in a manner dependent on histone H4 N-terminal tail[70]. In addition, it was shown that ALC1's activity is very strongly stimulated upon addition of active PARP1. This stimulation was not observed in the absence of NAD+ or DNA and in the presence of PARP1 inhibitor, indicating the requirement of active PARP1 for its stimulation[69,70]. Incubation of pre-modified PARP1 was able to stimulate its activity showing that stimulation requires auto-modified PARP1 but not the modification of histones or ALC1[70]. But poly-ADPr (PAR) alone was not sufficient to stimulate ATPase activity it was shown to interact with the ALC1 macrodomain. This suggests that ATPase stimulation requires protein-protein interactions in addition to the PAR binding mediated by the macrodomain. These data strongly indicate the existence of molecular communication between the macrodomain

and ATPase domains.

ALC1 was also shown to remodel nucleosomes in a native gel nucleosome-sliding assay in the presence of active PARP1. ALC1 was shown to protect 3-22 nts of ADP-ribose upon binding with the PARylated-PARP1 from PARG digestion[69]. In addition the recruitment behaviour of the ALC1 macrodomain to the UV laser induced DNA damage sites was found to be very different from mono-ADPr binding macrodomains. In a way that the ALC1 macrrodomains stays at the laser cut site for about 40 minutes far far more than other macrodomains. This suggested that ALC1 macrodomain might recognize Oligo-ADPr instead of mono-ADPribose[69,70]

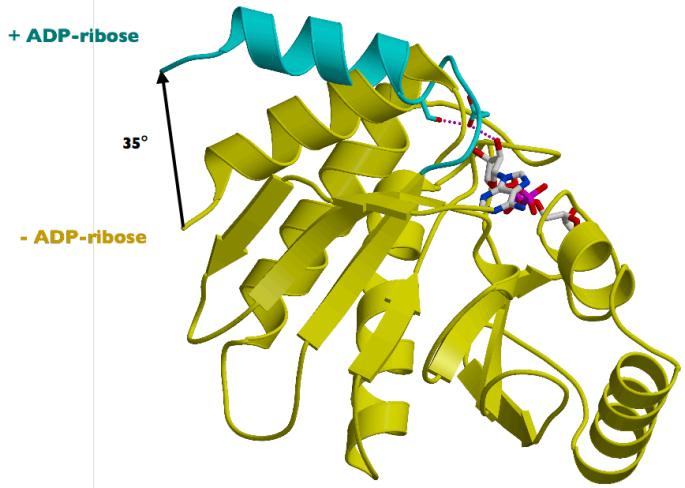

Figure 13: Macrodomain and their ADPr binding and the induced conformational change. Macrodomains are the globular ADPr recognition modules. Figure shows the comparison of the macrodomain of the MacroH2A1.1 structure in ADPr bound and unbound form. The C-terminal helix shown in cyan moves away from the body of the macrodomain's (shown in yellow) by about 35 degree angle upon its binding with the ADPr in the canonical ADPr binding pocket (ADPr is shown in the stick model bound to the pocket). This shows that the macrodomain binding with the ADPr can induce conformational changes (Figures adopted from [95]).

1.10.4 ALC1 remodeler connects the chromatin relaxation with the DNA damage response

ALC1 promotes massive chromatin decompaction at the site of DNA damage[71]. ALC1 has also been shown to interact (most likely bridged through the poly-ADP-ribose) with different DNA Damage Response (DDR)-associated proteins[70]. Its interacting partners, such as PARP1, DNA-PKcs, Ku70-80 and APLF have been described as critical in the balancing of DNA repair pathway choices after DNA damage. Interestingly, PARP1 is known to promote alternative non-homologous end joining (alt-NHEJ) repair pathways, a pathway considered as a backup for the classical NHEJ. PARP1 can also promote homologous recombination (HR) by recruiting ATM at the site of damage[96]. Further, ALC1 affcets the residence time of the TRIM33 at the site of DNA damage[97]. Both ALC1 and PARP1 have been shown to be involved in the NER pathway via DDB2[98]. This is indicative of possible ALC1-mediated modulation of downstream DNA damage signalling and repair pathway choice via either direct interaction with these proteins and/or via its chromatin remodeling activity.

1.11 Aims of this study

1.11.1 Background

Little is known about how DNA damage triggers the activity of chromatin remodelers. In my dissertation, I have studied a unique chromatin-remodeling enzyme whose activity is strictly regulated by a dynamic posttranslational modification, poly-ADP-ribosylation (PARylation). Specifically, I proposed to investigate the molecular basis of the PARylation-induced changes in ALC1 structure leading to the activation of its remodeling activity using cell biological, biochemical, biophysical and hybrid structural approaches. My host lab and others had previously published that the ALC1 chromatin remodeler is strongly activated by active PARP1 and modified PARP1 and ALC1 macrodomain can interact *in vitro* and *in vivo*. Interestingly intact ADP-ribose binding pocket was crucial for ATPase activity. This indicated the requirement of macrodomain-ATPase communication during ALC1 activation. In addition, ALC1 has been also shown to get PARylated (mostly in the linker region) by PARP1 *in vitro*.

1.11.2 Rationale

By understanding the inter and intra domain interactions within the remodeler and other ligands (activator, remodelling substrate etc) involved in the remodelling reaction and by deciphering the consequences of these interactions to the remodelling reaction, we can understand the mechanisms by which remodeler's are regulated.

1.11.3 Aims

The main goal of my PhD is to understand the activation mechanism of the chromatin remodeler ALC1 (Amplified in liver cancer 1). Specifically, how remodeler's recruitment to the site of DNA damage triggers a set of intra-molecular events involving re-shaping of the interaction circuitry and the potential role of the regulatory sequences in it, ultimately driving the ATPase activation using a combination of biochemical, cell biological and structural approaches.

In particular what keeps ALC1 inactive prior to recruitment and how poly-ADP-ribose actives the remodeler upon recruitment. The two related and major goals of my dissertation are:

a) What keeps the remodeler inactive before recruitment?

Previously, ALC1's ATPase activity was shown to be strictly dependent on its intact ADPr binding pocket of the macrodomain indicative of allosteric regulation. However, what keeps the remodeler inactive remains unknown.

b) How is the remodeler activated upon recruitment?

Previously, ALC1 was shown to prevent 3-22 nts from PARG digestion *in vitro* indicative of a potentially extended binding surface for the poly-ADPr. In addition the effect of this ligand binding on the communication between ATPase and the macrodomain as well as the consequences of that interaction on other ligand's like DNA binding with the ALC1 remodeler is not known. In other words how poly-ADP-ribose acticates the remodeler upon recruitment to the site of DNA damage is remains unknown.

For both of these goals, I have combined *in vitro* biochemistry, biophysical assays as well as structural studies (HDX-MS) with live cell imaging/spectroscopy assays.

2. Materials and Methods

A Poly-ADP-ribose Trigger Releases the Auto-inhibition of a Chromatin Remodeling Oncogene

Hari R. Singh,[1] Aurelio P. Nardozza,[1,*11] Ingvar R. Möller,[2,11] Gunnar Knobloch,[1,11] Hans A.V. Kistemaker,[3] Markus Hassler,[1,4] Nadine Harrer,[1] Charlotte Blessing,[1] Sebastian Eustermann,[5] Christiane Kotthoff,[1] Sébastien Huet,[6,7] Felix Müller-Planitz,[1] Dmitri V. Filippov,[3] Gyula Timinszky,[1] Kasper D. Rand,[2,*] and Andreas G. Ladurner,[1,8-10*]

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Declaration of Contribution to "A Poly-ADP-ribose Trigger Releases the Auto-inhibition of a Chromatin Remodeling Oncogene"

AUTHOR CONTRIBUTIONS

Conceptualization, H.R.S., M.H., G.T. and A.G.L.; Methodology, H.R.S., A.P.N., I.R.M., G.K., F.M.P., G.T., K.D.R. and A.G.L.; Investigation, H.R.S., A.P.N., I.R.M., G.K., M.H., N.H., C.B., C.K. and S.H.; Formal Analysis, H.R.S., A.P.N., I.R.M., G.K., M.H., N.H., C.B., F.M.P., G.T., K.D.R. and A.G.L.; Writing – Original Draft, H.R.S. and A.G.L.; Writing – Review & Editing, H.R.S., G.K. and A.G.L.; Funding Acquisition, H.A.V.K., D.V.F., F.M.P. and A.G.L.; Resources, H.A.V.K., D.V.F., S.E., S.H. and C.K.; Supervision, S.H., F.M.P., G.T., K.D.R. and A.G.L.

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3. Results

3.1 Published Results I - Research Article I

3.1.1 A Poly-ADP-ribose Trigger Releases the Auto-inhibition of a Chromatin Remodeling Oncogene

Hari R. Singh,[1] Aurelio P. Nardozza,[1,*11] Ingvar R. Möller,[2,11] Gunnar Knobloch,[1,11] Hans A.V. Kistemaker,[3] Markus Hassler,[1,4] Nadine Harrer,[1] Charlotte Blessing,[1] Sebastian Eustermann,[5] Christiane Kotthoff,[1] Sébastien Huet,[6,7] Felix Müller-Planitz,[1] Dmitri V. Filippov,[3] Gyula Timinszky,[1] Kasper D. Rand,[2,*] and Andreas G. Ladurner,[1,8-10*]

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AUTHOR CONTRIBUTIONS

Conceptualization, H.R.S., M.H., G.T. and A.G.L.; Methodology, H.R.S., A.P.N., I.R.M., G.K., F.M.P., G.T., K.D.R. and A.G.L.; Investigation, H.R.S., A.P.N., I.R.M., G.K., M.H., N.H., C.B., C.K. and S.H.; Formal Analysis, H.R.S., A.P.N., I.R.M., G.K., M.H., N.H., C.B., F.M.P., G.T., K.D.R. and A.G.L.; Writing – Original Draft, H.R.S. and A.G.L.; Writing – Review & Editing, H.R.S., G.K. and A.G.L.; Funding Acquisition, H.A.V.K., D.V.F., F.M.P. and A.G.L.; Resources, H.A.V.K., D.V.F., S.E., S.H. and C.K.; Supervision, S.H., F.M.P., G.T., K.D.R. and A.G.L.

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Background

The genome has to cope with a plethora of environmental changes and stress signals like DNA damage. In addition, it undergoes a number of continuous transitions during transcription, replication and recombination. ATP-dependent chromatin remodelling enzymes alter nucleosome structure and facilitate transcription, replication and DNA repair. However, little is known about how DNA damage triggers the activity of chromatin remodelers.

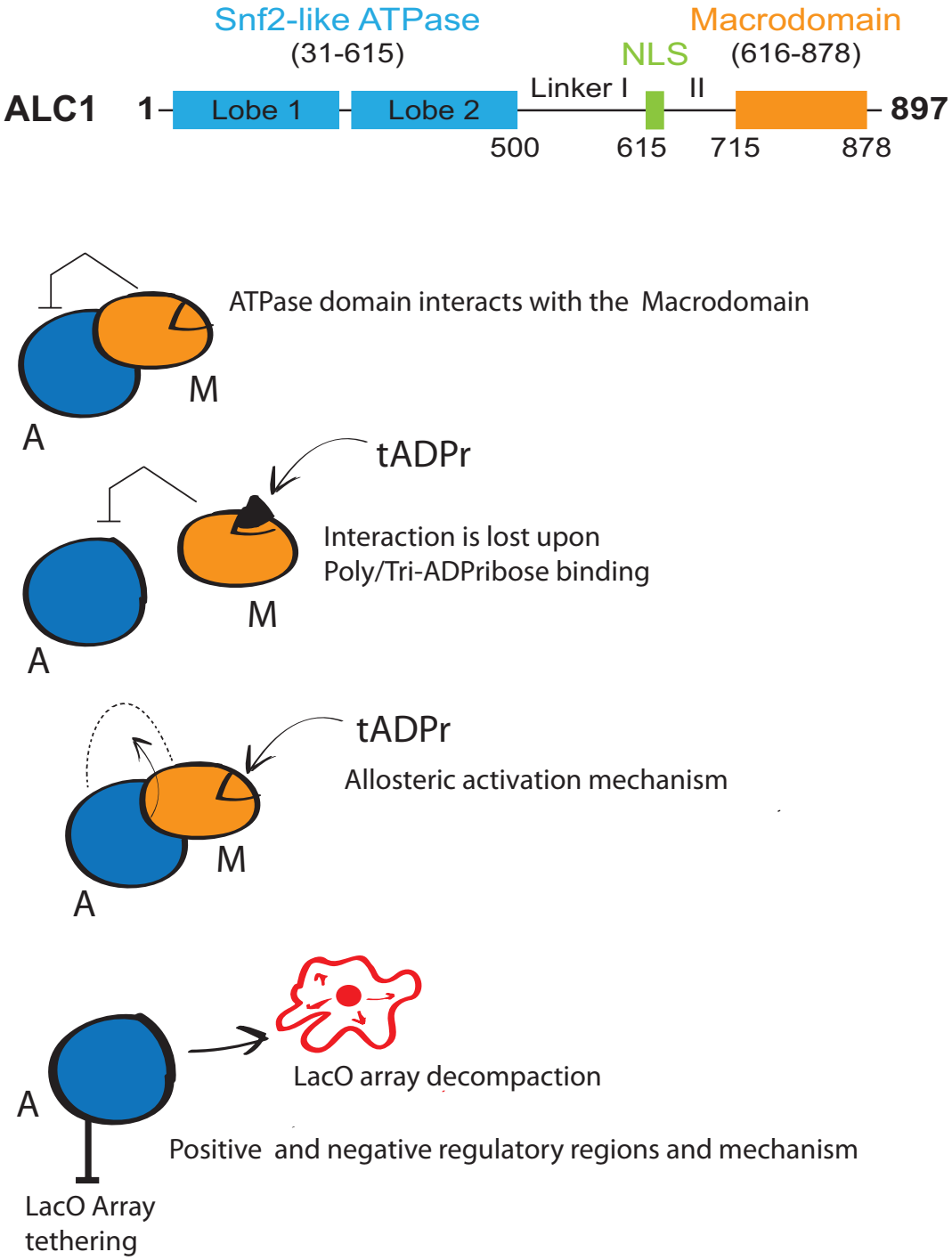
In my PhD, I have studied a unique example of a chromatin-remodelling enzyme whose activity is strictly regulated by a dynamic posttranslational modification (Poly-ADP-ribosylation). Specifically, I have been dissecting out how Poly-ADP-ribose allosterically activates the ALC1 *in vitro* and *in vivo* using biochemical, cell biological and hybrid structural approaches.

The chromatin remodeler ALC1 requires the activity of poly-ADP-ribose polymerase 1 (PARP1), an NAD^+-dependent enzyme for its remodelling activity. ALC1 has a C-terminal ADPr binding macrodomain and N-terminal Snf2 like ATPase motor domain separated by a linker region. This modular architecture provides a way to couple DNA damage induced PARP1-mediated poly-ADP-ribosylation with ATP-dependent chromatin remodelling. ALC1's ATPase activity was shown to be strictly dependent on its intact ADP-ribose binding pocket of the macrodomain indicative of allosteric regulation. However, the in-depth mechanism is not known.

I have been testing the hypothesis that the activation of ALC1 requires communication between the ATPase- and macrodomain. For that I have combined *in vitro* biochemistry, structural studies (HDX-MS and XL-MS) with live cell imaging based assays. In my PhD, I have revealed fundamental new insights into the allosteric activation mechanism of the ALC1.

Note: Above background is not part of the published article

Graphical Summary



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3.2 Published Results II - Research Article II

[Nucleic Acids Research, 2016, Vol. 44, No. 7 3105–3117]

3.2.1 The histone chaperone sNASP binds a conserved peptide motif within the globular core of histone H3 through its TPR repeats

Andrew Bowman[1], Lukas Lercher[2], **Hari R. Singh[1]**, Daria Zinne[1], Gyula Timinszky[1], Teresa Carlomagno[2] and Andreas G. Ladurner[1]

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2. Leibniz University Hannover, BMWZ-Institute of Organic Chemistry, Schneiderberg 38, 30167 Hannover, Germany

Declaration of Contribution to "The histone chaperone sNASP binds a conserved peptide motif within the globular core of histone H3 through its TPR repeats"

Andrew Bowman designed and carried out the experiments, conceived of the project in discussion with AGL, prepared the manuscript for publication. Lukas Lercher designed and carried out NMR experiments, analyzed the data. Hari Raj Singh discussed and design of F2H assays with AB performed all the F2H assays. Daria Zinne expressed and purified labeled proteins for NMR studies. Gyula Timinszky discussed and designed the project, corrected the manuscript. Teresa Carlomagno supervised the NMR work. Andreas Ladurner discussed the project, provided resources, co-wrote the paper and handled the publication process.

3.3 Published Results III - Research Article III

[Cell 153, 1394–1405, June 6, 2013]

3.3.1 Structures of Drosophila Cryptochrome and Mouse Cryptochrome1 Provide Insight into Circadian Function

Anna Czarna,[1,2] Alex Berndt,[3,5] **Hari Raj Singh**,[1] Astrid Grudziecki,[4] Andreas G. Ladurner,[1] Gyula Timinszky,[1] Achim Kramer[4] and Eva Wolf[1]

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Declaration of Contribution to "Structures of Drosophila Cryptochrome and Mouse Cryptochrome1 Provide Insight into Circadian Function"

Anna Czarna performed protein expression and purification, crystallization, ITC, Blue light illumination experiments, generation of mutants, UV/VIS spectroscopy. Alex Berndt performed cloning, expression, purification of full-length dCRY, crystallization and data collection of full-length dCry; prepared Figure 6B and 6C. Hari Raj Singh did cloning, plasmid preparation, transfections in mammalian cells and performed microscopic experiments, data generation and figure preparation for Figure 6A. Astid Gridziecki performed experiments for Figure 6B and 6C. Andreas Ladurner discussed the data, provided resources and handled or advised correspondence with the journal. Gyula Timinszky generated the image analysis pipeline for Figure 6A and interpreted the data in 6A. Achim Kramer designed and analyzed experiments in Figure 6B and 6C and contributed to the paper writing.

Eva wolf analyzed and interpreted data except for data shown in Figure 6, wrote the manuscript.

4. Discussion

4.1 ALC1 is regulated via modular allosteric regulation

In my dissertation I have demonstrated for the first time that the ATPase domain and the macrodomain of ALC1 physically interact and that this interaction ends upon PARylation both *in vivo* and *in vitro*. ALC1 is kept inactive by folding of the macrodomain on to the ATPase domain as an auto-inhibitory module, which interacts with the lobe 2 of the ATPase domain. Upon recruitment to the sites of poly-ADP-ribosylation, the interaction between ATPase domain and the macrodomain is lost. Moreover, the loss of interaction is dependent on the intact canonical ADPr binding pocket of the macrodomain and that poly-ADPr, but not Mono-ADPr, is necessary and sufficient for the loss of interaction. Futher, we identified tri-ADPr as the minimal, necessary and sufficient ligand for the loss of interaction. Further, we have also discovered the positive and negative regulatory regions in the protein for ALC1 remodelling activity using a novel *in vivo* LacO array based remodelling assay. HDX-MS helped in identifying the allosterically relevant regions within the ALC1 protein. In addition, cross-linking mass-spectrometry indicates for the possible re-shaping of the interaction circuitry between ATPase and the macrodomain during its activation. We have identified the molecular underpinnings of recruitment coupled anchoring, and positive regulation,. I think anchoring **will** increase processivity and positive regulation will enhances efficiency of productive remodelling events. More work is needed to further understand the molecular mechanism. Further, PARP1 mediated PARylation of ALC1 could auto-inhibit ALC1 after remodelling reaction, in a way similar to Rsc4 chromatin remodeler[55]; as macrodomain would engage with ALC1's linker region. Activation mechanism also showed certain hotspots for the disease manifestation and relevance of the activation mechanism in the cellular physiology in particular DNA damage response and cancer biology[80].

My results also support the view that the macrodomain of ALC1 not only acts as a recruitment module but is also the repressor, activator and processivity factor acting during different stages of the remodelling reaction. Our present results therefore

provide additional evidence for the emerging concept of remodeler activation through intra-molecular interactions.

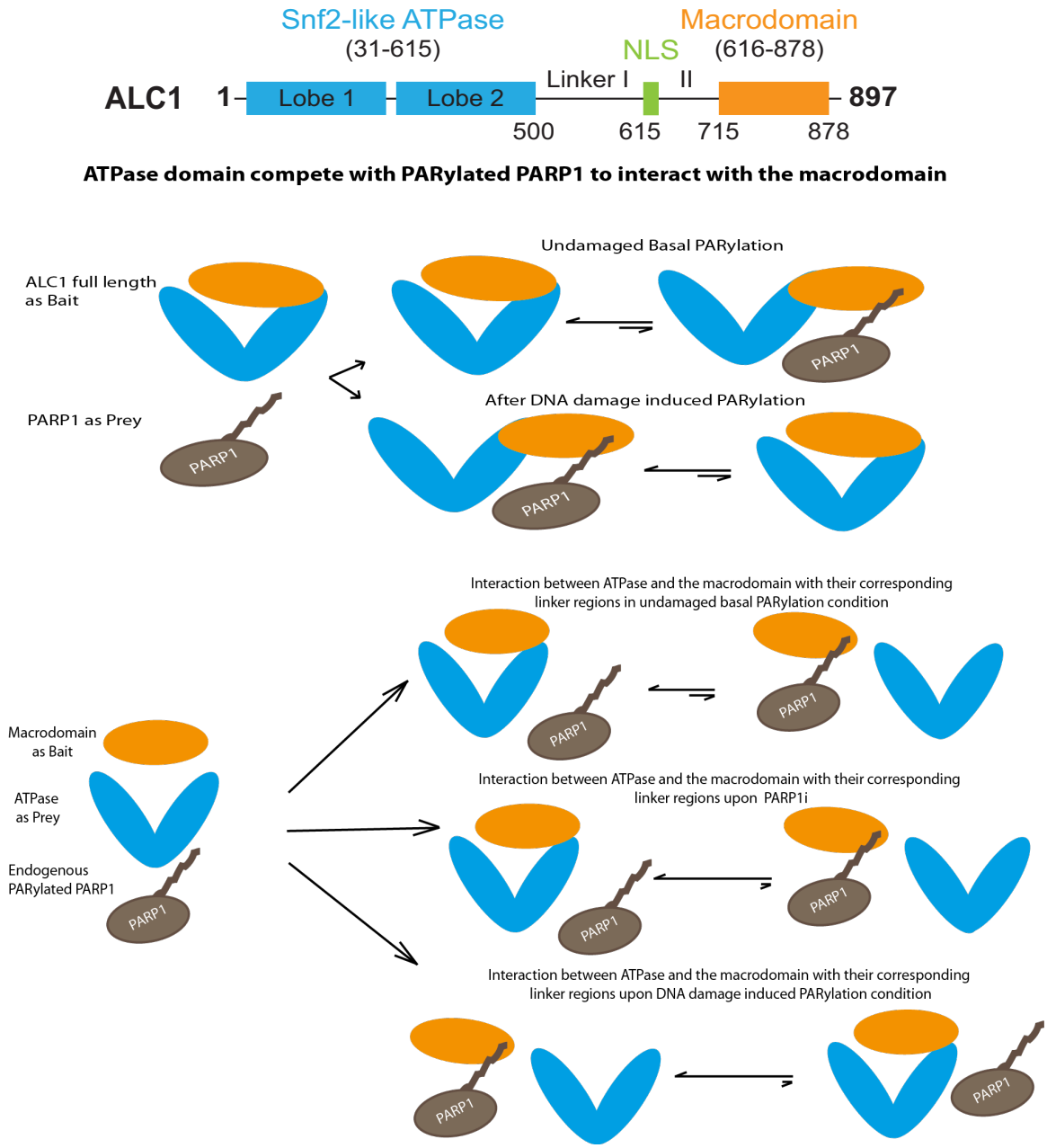

Figure 14: ALC1 modular allosteric regulation. The ATPase domain and the PARylated PARP1 compete to bind with the Macrodomain and exist in equilibrium. In the basal DNA damage/with PARP1inhibitor treatment the ATPase domain dominates the equilibrium whereas upon DNA damage which results in the production of PARylated-PARP1. PARylated-PARP1 will then dominates the binding with the Macrodomain and compete out the ATPase.

Future efforts are directed towards elucidation of the high-resolution structures and more in-depth understanding of the basis of the remodeler function[51,66,68,99].

Specifically, how in atomic details the remodeler is kept in an inactive conformation when not recruited and how the repression is relieved upon its recruitment. To fully grasp the knowledge of the mechanism that underpins the activation mechanism, understanding the structure of the remodeler and its individual domains and complexes including with the nucleosome and PARP1 would be very useful. In addition, high-resolution structures will also give insights into the possible mechanism of the negative and positive regulatory sequence motifs within ALC1 remodeler's sequence.

4.2 Proposal of new domain architecture of the ALC1 chromatin remodeler

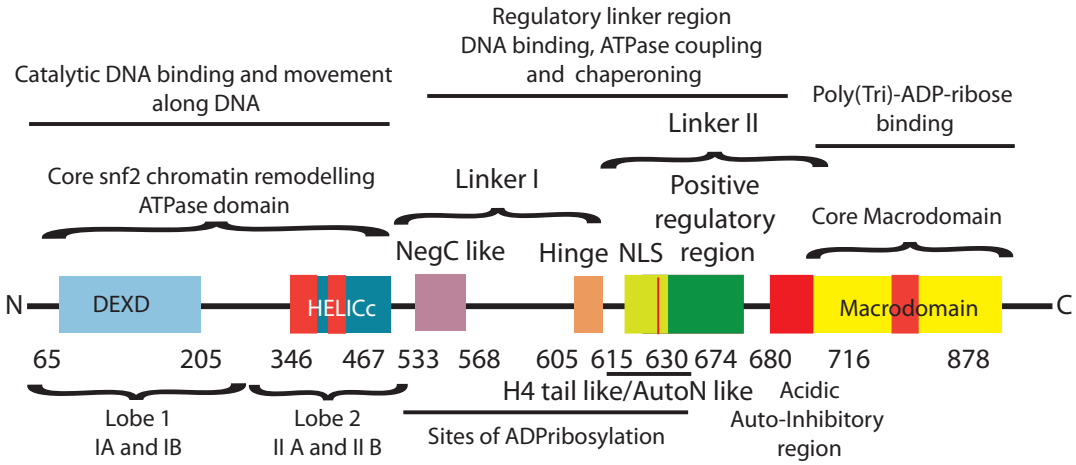

Figure 15: Proposed domain architecture for ALC1 chromatin remodeler. Figure shows the proposed architecture of the ALC1 chromatin remodeler. In future, it would be important to understand the ALC1 remodelling activity and its regulation in the context of different regulatory regions.

Based on my other unpublished data (not included in the thesis) we can now revisit the ALC1 domain architecture which now includes the previously known snf2 core ATPase and core macrodomain, three regions responding to the TriADPr binding in HDX-MS experiments named HDX1, 2 and 3, one NegC like motif, a Hinge between the two linkers, an AutoN like motif (the previously known H4 tail like sequence in ISWI), Positive regulatory domain (in green) and auto-inhibitory negative regulatory region (in red similar to CHD1 acidic helix). While hinge and negC like regions is

more part of ATPase domain and NLS, H4 tail like, basic positive regulatory and acidic negative regulatory sequences is more part of the linker II region associated with the macrodomain (linker I and II association to the respective domains was identified by limited proteolysis and MS). It should be noted that the positive regulatory fragment (in green) is a predicted coiled coil. The linker region that is rich in the regulatory sequences needs more extensive work in particular structural studies in future to dissect out the roles of each motif and define how they work and what happens to them upon tri-ADPr binding with the macrodomain. In particular, *In vitro* FRET/NMR based structure-dynamic studies would be useful.

4.3 ALC1 activation mechanism has parallels with unified remodeler activity regulation

The proposed ALC1 activation mechanism brings together allostery-mediated information encoding with chromatin signaling-mediated regulation of spatio-temporal context towards PARylation dependent chromatin structure-function modulation.

ALC1 exists in an auto-inhibited state wherein the recruitment module i.e. PAR binding macrodomain (in yellow) folds back on to the functional module i.e. snf2 ATPase domain (two lobes in cyan) the positively charged part of the linker region which is (shown in light red here) associated with the core macrodomain functions as a low affinity chromatin scanning module before recruitment (see figure 15). Upon finding the site of PARylation, the interaction between the ATPase domain and the Macrodomain is disrupted via re-arrangement of the conformations of both ATPase and the macrodomain. Concomitantly the linker region (in light red here) associated with the core macrodomain can now act as a positive regulatory fragment. The reported PARylation of ALC1 by PARP1 immediately suggest an auto-inhibition mechanism post remodelling in a way similar to RSC4 chromatin remodeler[55,70,100].

The proposed mechanism will provide a conceptual framework for the understanding of the activation mechanisms among the chromatin remodelling enzymes. As chromatin remodelers face different challenges than many other allosterically regulated enzymes - this class of enzymes have evolved specific allosteric strategies to deal with these challenges. In particular, common to all chromatin remodelers is an engineering

of a DNA motor domain with many other accessory domains/modules specialized for - context specific targeting, anchoring, processivity, efficiency, chaperoning, hoping mediated molecular gauging *etc* functions in order to achieve the desired remodelling outcome. ALC1 activation mechanism also provides an opportunity to target these specific regulatory hotspots for therapeutic intervention.

4.4 Future Perspective on the ALC1 activation mechanism

4.4.1 Interplay of multiple interaction surfaces in ALC1 activation mechanism

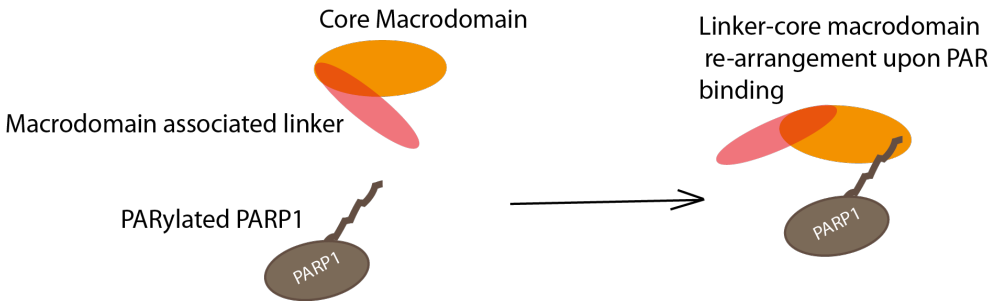

Figure 16: Poly/Tri-ADPr binding may induce structural changes within the ALC1 macrodomain. Potential Allostery within the ALC1 Macrodomain (shown in yellow) and regulatory region containing the linker II (shown in light red)

My other unpublished data (not included in the thesis) show that ALC1 exists in an inactive chromatin scanning competent conformation before recruitment to the sites of DNA damage induced PARylation. Further unpublished data shows that 2nd and 3rd ADPr (1st being the canonical ADPr) interact outside of the canonical ADPr binding pocket and that is crucial to trigger the allosteric activation mechanism. The results also show that the HDX3 region on the core macrodomain (832-858) is also a surface that shows crosslinks with the linker II region for inactive ALC1 (Figure S1 of the research article I). Interestingly the mutations in the corresponding region, which is also, the

positive regulatory fragment (KRRR/AAAA) shows an effect on the LacO array compaction (more decrease in the LacO decompaction than the pseudo wt mutant of the macrodomain when expressed in trans with the constitutively active ATPase) and a subtle increase in the F2H interaction (Figure 5 of the research article I). Further mutations in the HDX3 and this XL-MS region show effect in the F2H assays with the ATPase (no interaction), increase in the chromatin binding (FCS) no change in the compaction of 1-673 mediated decompaction of the LacO array (Figure 4, figure 5, Figure S1, S6 of the research article I). The fact that macrodomain's canonical ADPr pocket binding mutant (G750E) still recruits to the site of laser induced DNA damage, interacts with the hyperactive PARP1-L713F mutant (unpublished data), further support our findings of the tri-ADPr binding to the macrodomain and presence of extra ADPr binding surface in addition to the canonical ADPr binding surface. I think this extra ADPr binding surface on the macrodomain (in addition to the canonical ADPr binding) would induce conformation rearrangement on the XL-MS surface triggering the allosteric activation by concomitantly causing the loss of interaction between the ATPase and the macrodomain while also making sure that the positive regulatory fragment becomes available. This idea is further supported by thermo-fluor assays with the macrodomain (+linker II) alone as in the presence of the tri-ADPr macrodomain shows a huge stabilization indicating for a large conformation re-arrangement within the macrodomain and linker II region (Figure S3 of the research article I). Therefore, I think that the modular ALC1 activation mechanism that we have proposed also involve releasing the positive regulatory fragment from the macrodomain upon binding of poly-ADPr with the macrodomain, concomitantly causing the loss of interaction between the two domains. This suggest that a complex interplay of multiple interaction/competitive surfaces drives the activation of the ALC1 chromatin remodeler through rewiring of the intra and inter-molecular interaction circuitry. High-resolution structural insights from x-ray crystallography and Cryo-EM of the active (tri-ADPr bound), inactive and complexes with other ligands for e.g. nucleosome, PARP1 and ATP analogues will be needed to further understand the nature of these conformational changes and how various regulatory regions fit in the final activation mechanism model.

5. Essay manuscript

Note: Following pages contain my ideas around the future perspective on the field of chromatin biology. In particular how the synthetic biology can help further advance chromatin biology. This is also intended to give a unified perspective on my thesis which not only includes the chromatin remodeler activation mechanism as core thesis as entitled but also histone chaperoning mechanism by NASP as well as circadian rhythm protein cry1 structure and its protein-protein interactions and the previously reported feedback regulations thereof. Considering that circadian rhythm, histone chaperoning and chromatin remodelling can be unified within a wider chromatin biology and transcriptional regulation context. I decided to present my ideas with a discussion on a few recent publications utilizing chromatin phenomenon towards developing synthetic biology tools and applications thereof.

5.1 The Epigenome Joins the Club of Engineers

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Authors contributions: Hari Raj Singh prepared the first draft. Andreas Ladurner and Hari Raj Singh will correct and communicate with the editors.

The Epigenome Joins the Club of Engineers

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The regulation of chromatin plasticity is at the heart of how organisms control the identity and activity of all cells in their body. Recent work shows that using proteins we can target these epigenetic systems and engineer circuits to manipulate the epigenetic landscape. Epigenome engineering provides an innovative framework for the controlled switching of epigenetic states, establishing tools within a wider synthetic biology framework that will prove useful in discovery research and biomedicine.

"*What I cannot create, I do not understand.*" - **Richard Feynman**

INTRODUCTION

Chromatin has the dual task of packaging the eukaryotic genome and acting as a signal integration platform for the regulation of DNA-mediated processes, including gene transcription, gene silencing, DNA replication, recombination and repair [4,8]. Chemical modifications of both histone proteins and DNA, regulate whether and how chromatin binding factors access the DNA template with exquisite spatio-temporal control [27]. These so-called epigenetic modifications of the chromatin substrate act as important drivers of cellular differentiation during development, establishing an "epigenetic memory" that can robustly maintain cellular identity and generally represents a formidable barrier to cellular reprogramming. Often aberrations in nature's tightly engineered epigenetic circuits that maintain the stable inheritance of the modified chromatin states go awry in disease[5,101,102]. Indeed, mutations in epigenetic regulators directly contribute to cancer etiology. This has fuelled an active interest in targeting

epigenetic modifiers using small-molecular regulators in order to derive new therapeutic approaches.

Efforts to identify, characterize and correct epigenetic phenomenon strongly profit from the availability of tools that allow us to manipulate and switch gene function, for example using approaches that make it possible to silence or activate gene expression with exquisite spatio-temporal control. In this perspective, we will summarize some of the more current approaches that have been successfully developed to design and manipulate epigenetic processes in order to alter gene activities and cellular function [103-121]. Taken together, many of these recently developed tools and approaches point to a new era for the field of epigenetics. The ability to engineer the epigenome with a high degree of precision is poised to open up new research avenues and – increasingly – also to herald the development of new applications, both in the context of new therapeutics as well as in the wider arena of biotechnology.

Manipulation and the engineering of the chromatin states

Being able to manipulate a system in its native context and/or synthesizing a phenomenon from scratch greatly improves our ability to understand the underlying principles as has been witnessed in past with the progress in organic chemistry and physics. On the other hand biology has traditionally been done as an observational science. Now, in the new era of synthetic biology we too can think of understanding systems and its components much like an organic chemist and physicist by synthesizing and engineering it. The promise of synthetic biology by and large depends upon our ability to better manipulate the native systems and orthogonally synthesize a particular trait or phenomenon in a spatio-temporally regulated manner. In fact as Richard Feynman puts it in his famous dictum "***What I cannot create, I do not understand***." captures the essence of our article. Since, only when you synthesize a system is when you understand it well enough and therefore can confidently state the underlying principles. Epigenetic phenomenon, chromatin mediated genome function regulation and the underlying principles have been for a long time eluded the biologists since Conrad Waddington's proposal of the idea. This is due to the lack of tools with which we can engineer the epigenetic states and thereby start to understand and dissect-out the underlying principles. Since, chromatin also acts as a wonderfully equipped,

cellular-state-computing system constantly taking cues from the environment and calculating the genome regulation response. This feature makes it an amenable system towards exploring the possibilities of developing an Epigenome engineering framework using the rationale design from synthetic biology approaches and combining those approaches with the diverse range of naturally occurring epigenetic toolbox available to us.

The chromatin engineering metaphor

The emerging approach of Epigenome engineering framework will not only have applications in therapeutics and biotechnology but it will also allow us to better dissect out and develop an abstraction of the chromatin structure-function regulation; that has been eluding us so far. For e.g. we will be able to address the questions like: What makes chromatin plastic enough to adapt in response to a range of environmental conditions while, at the same time robust enough for the epigenetic phenomenon to work as in case of development (for e.g. canalization)? In other words, what are the sources of epigenetic redundancy that give rise to environmental robustness as well as the developmental robustness? What are the usefulness of noise and stochasticity if any? And why these characteristics are selected throughout evolutionary course? How has chromatin contributed to the extremely successful eukaryotic evolution? Can we develop an abstraction of the epigenetic phenomenon and phenotypic emergence there of? How does a complex epigenetic phenomenon emerge out of a set of interactions among very simple modular components? How different layers of chromatin organization cross talk with each other in regulating the genome function? Can we get to the bottom of what allows and equips the chromatin with an ability to sense and compute various environmental inputs and come up with different epigenetic states that delivers the response?

To answer these questions we need to have tools that can manipulate the system in a spatio-temporally controlled manner as well as acquire the ability to engineer the chromatin states in a targetable manner that can lock chromatin in a particular epigenetic state at will.

Now, a few recent reports [108] [104,106,115,122] demonstrate technical breakthroughs, with the development of engineered epigenetic transcriptional effectors that allow the stable

modification of endogenous genes by switching between different transcriptional states via changing their epigenetic profiles. These reports establish new ways, with which we can now rationally dissect out chromatin modification-based epigenetic heritability mechanisms, facilitate inheritable gene expression. Further these reports show us how cell identity can be successfully reprogrammed. These and related recent tools and publications [123] has established exciting new ways and give hope for the design of tailor-made therapeutic interventions based on epigenetic editing. Putting all of these together, our increasing ability to edit the epi-genome [106,108,115,123], being able to use optogenetic/small molecule mediated control [104,109,119,122,124], a rich presence of feedback regulation in the naturally occuring epigenetic inheritance mechanisms[125,126] is leading to an epigenome engineering framework (**Figure 23**).

Among the 5 reports specifically discussed in detail here, three reports [104,106,108] use DNA methylation/de-methylation as a robust epigenetic effector system combined with modularity in the DNA targeting domains towards engineering of a specifically targeted epigenetic loci. While, One report [115] demonstrate the direct conversion of fibroblasts to neuronal cells using targeted epigenetic remodelling via Cas9 based transcriptional activators to the endogenous loci. Finally, Other two report demonstrate the small molecule mediated targeting of the epigenetic probes via Cas9[122].

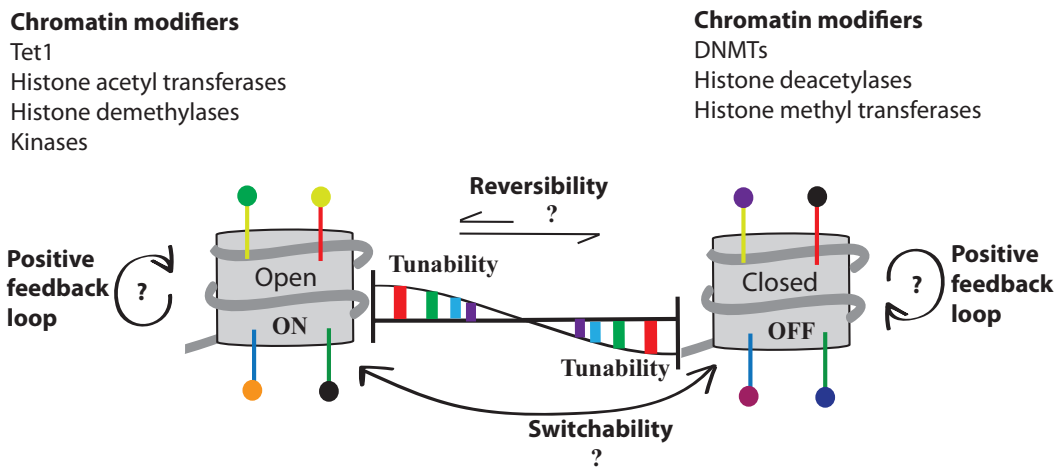

Figure 17: The chromatin engineering metaphor. Nucleosome can be seen as monomeric unit within a fiber of chromatin polymer which can provide specific structure-function context towards different chromatin states. As shown in the Figure 2 the tails emanating from the nucleosome can be post-translationally modified depending on the input signal. Therefore, a particular combination of the modified states can in principle provide a context dependent regulation of the genome-templated processes for e.g. Transcription. However, as the ability to package genome information in different context increases; the ability to read the underlying information must also concomitantly increase. There are hundreds of protein reader modules that can recognize these PTMs. Different colours represent different modifications and also represent their potential Impact on the nuclear processes for eg Transcription and tunability as shown in the figure. In some way place of nucleosome in Chromatin can be thought as a metaphor of a particular book in a big university library that has a set of coded information (a set of chromatin modifications) on it defining its position in space and time thereby allowing its readily access of its content to the reader. This layer of information over and above the DNA sequence is also known as the Epi-genetic information. Figure shows author's hypothetical model of the chromatin phenomenon. Chromatin can store information and inputs in different states, which can correspond to the different states of gene expression for example. The challenge of manipulating and engineering chromatin and the underlying epigenetic phenomenon is really the challenge of engineering a complex system with inherent stochasticity, noise and yet unknown variables. Though, the research in the field has identified multiple mechanisms - through which chromatin might act as a layered information storage and retrieval system. Therefore, some of those can be utilized towards chromatin engineering while also asking the questions related to the yet unknown variables. The epigenome engineering might help us in better understanding the abstract principles of chromatin structure-function regulation and can help us address the why question? For example as the figure 1 shows different modified states can maintain different gene expression states which are in-turn maintained using a set of yet not completely understood positive feedback loops. The strength and involved players in the feedback loops will determine the differences in the strength of the gene expression outputs and stability of the particular state. Being able to engineer these states opens a window of opportunity wherein one can quantitatively and rationally measure the contribution from each of the components. **The local chromatin state hypothesis:** A hypothetical model for the local chromatin states is shown. The figure shows two extreme states of the local chromatin states at the nucleosomal level that dictate the spatio-temporal regulation of the genome templated processes in this case transcription. There lies a spectrum of interchangeable states between these two states (shown here as tunability as different colour representing the spectrum).

Epigenome engineering

DNA methylation is thought to play a crucial role in developmental gene regulatory control systems; via many differentially methylated regions (DMRs), which play crucial roles in different stages of normal development and disease. However, their functional

significance has been lacking due to the lack of tools that allow the targeted manipulation of the DMRs[127-132].

Now, Liu et. al. reported a molecular toolbox that can edit the DNA methylation in a targeted manner. The authors have exploited the modularity of dCas9 as programmable target DNA binding module guided by gRNA and fused that to the key enzymes in the DNA methylation (DNMT3A) and de-methylation (TET1) pathway[108]. This has allowed authors to edit the DNA methylation state on specific genomic loci demonstrating its usefulness for gene expression control, cellular re-programming applications and higher order chromatin structure-function modulation (See Figure 18).

First of all authors have demonstrated the specificity and proof of concept by targeting the Tet1-dCas9 to a hyper methylated reporter DAZL-snrpn-GFP which contains a promoter of an imprinted gene snrpn that is upstream to the GFP encoding sequence and downstream to the germ cell specific gene-Dazl's promoter element DAZL. Dazl is a hypermethylated and silenced in ES cells whereas snrnp promoter reports the methylation status of the neighbouring region and as a consequence is also hypermethylated leading to no GFP expression. Interestingly, Tet1-dCas9 fusion protein targeting of the snrpn region via gRNA results in robust GFP expression. Remarkably, genomic DNA sequencing shows that demethylation was specifically localized to the snrpn promoter region demonstrating the high target specificity.

To check whether the system also works with the de-novo sequences author replaced the hypermethylated DAZL [103,118,133-135] promoter sequences with Gapdh promoter, which is unmethylated and expressed in ES cells. Likewise, this leads to the expression of GFP. Consistently, dCas9-DNMT3A targeting to the snrpn promoter leads to the localized and specific DNA methylation of the snrpn promoter and the resulting silencing of the GFP expression. Moreover, the DNA methylation was specifically localized to the snrpn promoter region only demonstrating the high target specificity.

Further, a comparison between the TALE based targeting with the dCas9 based targeting shows that dCas9-DNMT3A/Tet1 system has higher efficacy and base resolution.

To further demonstrate the applicability of the DNA de-methylation in a replication independent manner authors used post-mitotic neuronal cells and targeted the BDNF

promoter with dCas9-Tet1. As a result de-methylation of the BDNF promoter lead to the induction of the BDNF gene expression. PARP1 inhibition as well as the inhibition of the Tet1 abolishes the BDNF induction establishing a causal relationship between de-methylation of the BDNF promoter and the subsequent gene activation.

DNA methylation has also been known to have roles in establishing barrier between the cell lineages. Using the MyoD regulation by the distal enhancer DMR as a model system of choice authors further demonstrated that de-methylation in the distal enhancer region leads to the MyoD expression in fibroblasts[136]. This synergistically facilitates the muscle cell differentiation and Myotube formation induced by 5-Aza-2'deoxy cytidine treatments.

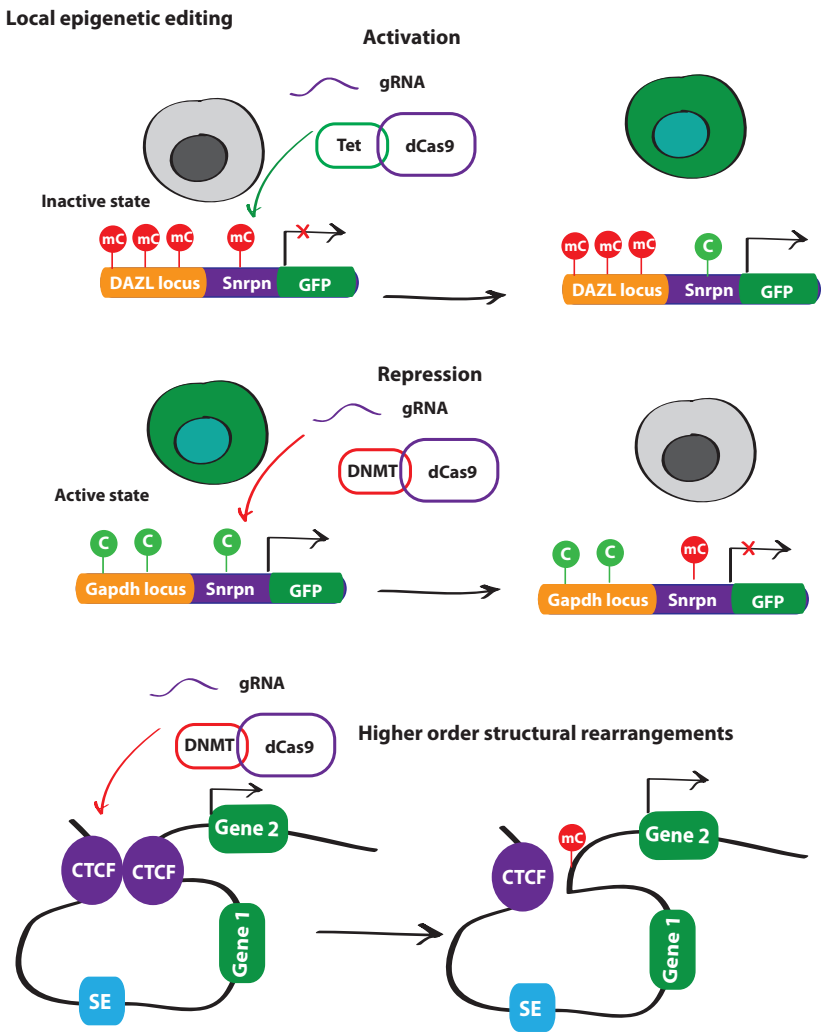

Figure 18: Editing and manipulating the epigenetic landscape. Authors showed the dCas9 mediated targeted manipulation of the DNA methylation states and

resulting transcriptional states upon local and specific epigenetic editing of the targeted loci. Further, the system can be used towards engineering different higher order chromatin looping via (as shown in the figure) targeting of the DNA methylation to the CTCF binding sites. This leads to changes in gene expression in the neighbouring loop. (Figure adapted from[108])

Further, CTCF is a known genome organizer and insulator, which forms higher order chromatin loops to promote gene silencing also known as CTCF mediated insulated neighborhoods[137]. Authors targeted CTCF binding sites with Cas9-DNMT3A bordering the super enhancer containing loops miR290 and Pou5f1, leading to de-novo CpG methylation of the CTCF binding sites. This changes the gene expression in the neighboring regions but not in the same loops showing that methylation of the CTCF binding site interferes with its insulator function. Further, 3C assay (chromosome conformation capture) shows an open conformation of these particular CTCF targeted sites upon DNA methylation and resulting lack of CTCF anchoring as shown by Chip assays. This establishes the Cas9-DNMT3A as a powerful tool for higher order chromatin structure modulation.

Finally, to show even wider utility of these tools for epigenetic regulation authors demonstrated the activation of the hyper methylated silenced GFP reporter both *ex vivo* and *in vivo* using a transgenic mouse model. Wherein they injected the lentiviral vectors of dCas9-Tet1 with target gRNAs in the dermis and brain of the mice leading to the activation of the methylation sensing GFP reporter.

The above described tools will drive the better understanding of the gene regulatory mechanisms, cellular programming/reprogramming and the higher order chromatin organization. They will also help us better understand the underlying positive feedback loops that maintain these silenced states both in naturally occurring epigenetic circuits as well as engineering of the synthetic epigenetic circuits.

Synthesizing an epigenetic phenomenon

Endogenous retrovirus (ERV) silencing is one of the most robust epigenetic silencing systems. ERVs are silenced in pre-implantation embryos and this state is maintained throughout development and adult life in most of the tissues. DNMTs and KRAB-Zinc Finger Proteins (ZFPs) play crucial roles in the silencing process wherein KRAB-

ZFPs initiate the cascade by recruiting other silencing proteins to the target retroviral DNA site including KAP1, SETDB1, G9a, LSD1, NuRD complex and HP1 to establish a self-reinforcing repressive chromatin state. Finally KAP1 complex recruits the DNMT3A/3B/3L and locks the repressive state by depositing the methyl groups on the CpG sites. Engineered transcriptional repressors (ETRs) have previously been shown to silence the target sites [138] however silencing requires stable ETR expression[103,114].

Recently, Amabile *et.al.*[106] reasoned that combinatorial recruitment of multiple silencing factors may mimic the *in vivo* sequential assembly of the silencing complexes and thereby should be a better approach towards developing more robust ETRs, which can work with transient expression. Using custom DNA binding domains (TetR, TALE and dCas9) and fusing them with a combination of transcriptional repressors (KRAB/K, DNMT3A/D3A DNMT3L/D3L); repurposed the ERVs silencing machinery towards synthetic ETRs. The authors engineered customized, portable, multiplexabale, versatile, transient hit and run ETRs that can synergistically repress the target loci in a highly specific and sharply confined manner (see figure 19).

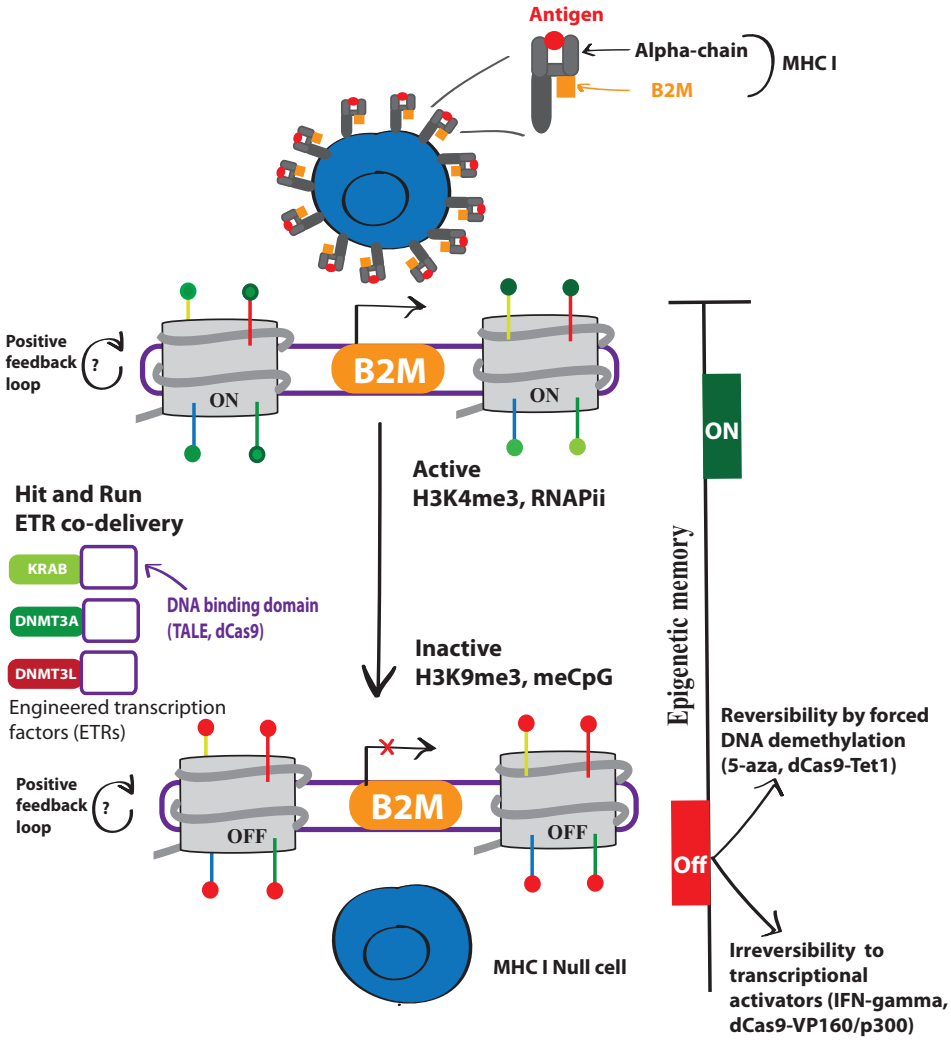

Figure 19: Hit-and-run targeted epigenome engineering. Authors used a strategy wherein they targeted the constitutively expressed B2M gene promoter via a transient silencers or Engineered transcriptional repressors (ETRs) the resulting silencing is resistant to the transcription activators and can only be reversed by DNA demethylation. (Figure adapted from[106])

To demonstrate the hit and run ETR activity authors engineered a cell line where the release of the ETRs from its target site can be controlled by Doxycycline. They inserted an eGFP expression cassette with downstream TetO7 sequences inside ubiquitously transcribed locus AAVS1 and followed the silencing of the eGFP expression in the presence of TetR:K (Fusion ETR of TetR and KRAB) and TetR:D3A(Fusion ETR of TetR and DNMT3A). While TetR:K mediated silencing was fast, spread across the entire locus, and reversible; the TetR:D3A mediated silencing was slow, confined to the target gene and was found to be irreversible.

Authors then tested the double (TetR:K and TetR:D3A) and triple combinations

(TetR:K, TetR:D3A and TetR:D3L) of the ETRs for silencing. The co-delivery of double ETR combination showed fast and irreversible silencing which was further improved by the triple ETR combination co-delivery.

The silencing on the TetO7 site containing reporter showed the proof of principle of the Hit and run silencing process however whether the silencing can be customized to any site on the endogenous genes was lacking. Therefore, the authors engineered dCas9 and TALE based ETRs targeting B2M promoter upstream to a tdTomato gene. Both TALE and dCas9 based ETRs gave rise to the comparable silencing showing the portability to the endogenous genes and customizability towards different DNA binding domains. Next, authors tested whether system was feasible to multiplex gene silencing. They targeted 3 different genes B2M, IFNAR1 and VEGFA either alone or in combination and found a long-term co-repression of the genes.

Authors then asked whether the achieved silencing could be reversed using external transcriptional activators. dCas9-VP160 and dCas9-p300 have been previously shown to activate transcription of endogenous genes. In addition, IFN-gamma is known to activate their endogenous genes of choice. Therefore, they use dCas9-VP160, dCas9-p300 and IFN-gamma as external transcription activators and dCas9-TET1 as de-methylation enzyme to unlock the silenced chromatin state locked-in by the DNA methylation and H3K9me3. Both dCas9-VP160 and dCas9-p300 were able to increase the expression of the control genes but not the gene silenced by the triple ETR combination whereas dCas9-TET1 targeting was able to achieve effective and robust reactivation showing that the Triple ETR induced epigenetic marks maintain the stable silencing and can not be reversed by external transcriptional activators and therefore are resistant to the activation unless directly reversed by de-methylation enzyme TET1.

Finally to test the specificity of their tools authors performed whole genome profiling of the DNA methylation and RNA expression wherein B2M-tdTomato was targeted by both dCas9 and TALE based triple ETRs. Virtually no off-target effects both in the RNA expression as well as in DNA methylation profiles was found showing the very high specificity of the ETR-silencing platform. The big question remains what and how the unknown positive feedback loop, which would ensure the stable epigenetic inheritance, is established during silencing when using combinations of multiple ETRs ensuring stable and irreversible silencing. These tools will further our understanding of

the relative contribution of the different regulatory elements in the gene expression as well as gene and cell therapy applications involving gene silencing without mutagenesis and RNAi mediated targeting.

Cellular reprogramming through epigenome engineering

Epigenetic mechanisms determine and control cell identity through locking of the epigenome in a particular state. The ability to engineer epigenome offers us a window into direct reprogramming of the cell identity. Over expression of the lineage specific transcription factors that can direct the cellular fate has been shown to directly reprogram somatic cells in to different target cell types. However, an approach that can activate multiple lineage specific transcription factors has been lacking. Black et.al. [106] show that the epigenetic reprogramming of the lineage specific endogenous transcription factor genes via dCas9 based transactivators can efficiently reprogram Primary mouse embryonic fibroblasts (PMEFs) to induced neuronal cells - iNs (See figure 20).

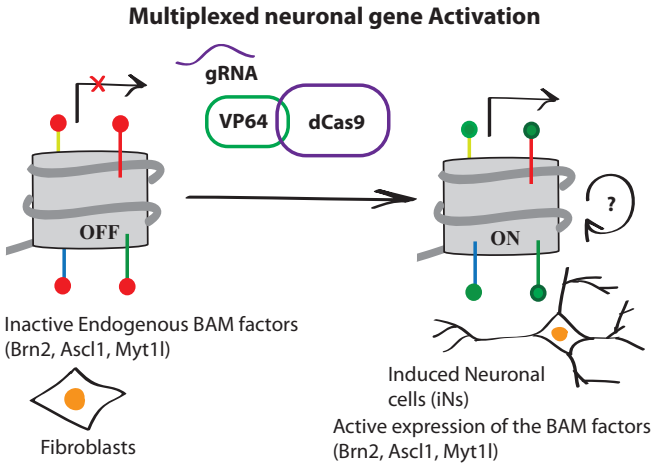

Figure 20: Making neurons directly from fibroblasts. Fibroblast's chromatin state of the BAM factors represents a silenced OFF state, which is a barrier to reprogramming. This state can be re-engineered into ON, reprogramming permissive and transcriptionally active state by recruiting VP64 to these genes via dCas9. Authors showed this was sufficient to convert fibroblasts into neuronal cells[115].

Authors used VP64-dCas9-VP64 triple fusion as a programmable locus specific, transcription activator and achieved multiplex activation of the neurogenic factors Brn2, Ascl1 and Myt1l (BAM factors). This caused rapid remodeling of the epigenetic signatures (increase in H3K27ac and H3K4me3) of the target genes leading to their

high levels of overexpression sustained overtime and resulting cellular reprogramming to iNs.

Achieving spatio-temporal regulation of Epigenome engineering

Dynamic regulation of chromatin enables cellular reprogramming, response to environmental signals and determines cell identity. Approaches that can allow us to better regulate the spatio-temporal control of the gene expression are therefore very useful to further advance our understanding of the underlying mechanisms. Optogenetic tools can provide that necessary control [115]. Recent report by Lo et. al. [104] demonstrate a loci specific alteration of the methylation states at the promoter of Ascl1 pro-neuron gene using targetable optogenetic methylation state effectors (see figure 21).

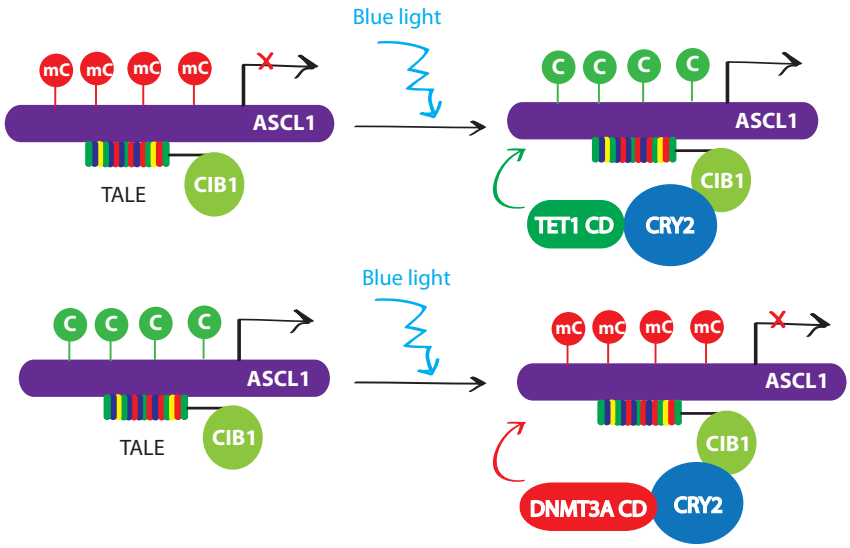

Figure 21: Epigenome engineering meets opto-genetics. Figure shows a description of the optogenetic system used towards targeted manipulation of the DNA methylation states in a light dependent manner. Briefly, CIB1 and CRY2 dimerize upon blue light illumination authors used TALE to anchor the CIB1 on to the target site and recruited the functional DNMT/TET catalytic domains via blue light illumination (figure adapted from[104])

To achieve that, the authors used optogenetic protein pairs CIB1 and CRY2, which can dimerize upon blue light illumination. They fused the CIB1with a TALE DNA binding domain designed to bind the Ascl1 promoter region and used DNMT3A-CD

and TET1-CD fusions with CRY2 to control their recruitment in a light dependent manner.

They targeted the promoter regions and optogenetically induced site-specific methylation and de-methylation in murine striated cells (hypo methylated) and dorsal root ganglion (hyper-methylated), selectively altering the methylation state of the targeted regions leading to changes a decrease and increase in the gene activity respectively. This report along with previous report [104] provides an optogenetic tool for the epigenome manipulation towards better and more precise spatio-temporal control on the gene expression.

Targeted epigenome engineering with chemical probes

The programmable DNA binding modular proteins like ZnFs, TALE, Cas9, LacR, TetR *etc* have greatly enriched our ability of specific epigenome editing using genetically encodable biomolecules. However, delivery of the chemically synthetic small molecular cargo to specific genomic/epigenomic locus remains challenging. Recently a report in PNAS demonstrated the delivery of the synthetic small molecules to a specific locus by exploiting the dCas9 targetability. They exploited the flexibility of the chemical synthesis and the intein-mediated protein trans-splicing (PTS)[122] to site specifically link Cas9-guide RNA complex with the synthetic cargo in-vitro for subsequent delivery of the cargo in the live cells. Interestingly, all the reactions can be performed in cell culture medium owing to the high efficiency and specificity of the inteins (see figure 22).

Using this approach authors show successful targeting of the dCas9 fusions that included either the small-molecule BET inhibitor JQ1 and a peptide- based PRC1 chromodomain ligand UNC3866, separately. JQ1 as well as UNC3866 were capable of recruiting endogenous copies of their cognate-binding partners BRD4 and PRC1 complex to targeted genomic binding sites respectively. This versatile and modular approach offers yet another way of recruiting macromolecular complexes to specific loci without any genetic manipulation and overexpression. This approach further equips us with better ways towards dissecting chromatin based epigenetic mechanisms using this kind of cellular biochemistry.

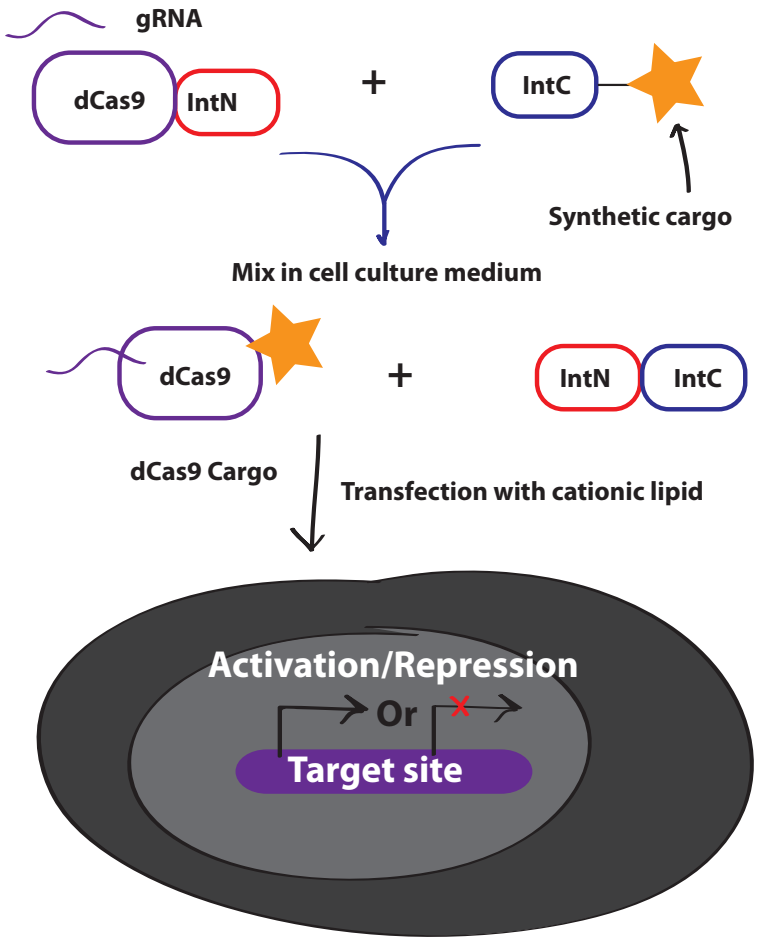

Figure 22: Epigenome engineering meets chemical biology. Shows the one pot assays utilizing the protein trans-splicing to label Cas9-gRNA with the chemical probes of their choice for eg JQ1. By transfecting the Cas9-gRNA-synthetic cargo in the cells authors were able to deliver the small molecule probes on to the Cas9 targeted loci (Figure adapted from[122]).

Towards an epigenome engineering framework

Epigenetic phenomenon can be thought as the nature's own engineered circuits of protein-protein and protein-DNA interactions leading to the heritable states of chromatin modifications. This leads to the epigenetic regulation of DNA templated processes, in particular transcription thereby giving rise to the so called "Epi-(genetic) Memory"[139].

Chromatin is rich in many regulatory elements like feed back systems, readers, writers, recruiters, and remodelers - which is what makes Epigenetic memory possible. Many of them are orthogonal, modular and reversible towards developing them for making customizable and robust chromatin based epigenetic structure-function modulation

devices and synthetic gene circuits. A synthetic biology framework towards sophisticated epigenome engineering using programming, designing, synthesizing and testing of these epigenetic circuits is likely to revolutionize the understanding of the eukaryotic gene regulatory control systems and applications thereof (See Figure 23).
The described reports and the developments in the last few years represent a significant major step towards targeted chromatin based Epigenome engineering. Further these reports brings us a step closer to an engineering solution towards understanding the underlying mechanisms through which the evolution as the tinkerer has been evolving the biological systems[102]. With this we will not only be better understanding the epigenetic language[140] but also we will re-write a new synthetic epigenetic language while also attempting to correct the errors as in case of disease states like cancer and/or cellular reprogramming.

SUMMARY

Chromatin plasticity is at the heart of chromatin-mediated computation of the gene regulatory logic and thereby has the ability to not only sense but also respond to various environmental inputs. At the same time it can also be thought as main source of stochasticity, noise, robustness and evolvability within the naturally occurring eukaryotic gene circuits. Chromatin modifications are the key to the transcriptional activity of the developmental regulators. Decades of research in chromatin field have identified a varied set of molecular mechanisms in unprecedented details but the abstraction of the overall chromatin based DNA templated processes and mechanisms remains unclear. The multi-layered structural and functional organization of chromatin and therefore the inherent difficulty in the abstraction of the underlying mechanisms continue to be the main reason for this knowledge gap. In particular, not being able to separate one layer of regulation from another in naturally evolving systems has been the main source of the practical problems in failing to address these questions. Therefore, we need to approach the problem differently from the traditional chromatin biology approaches.

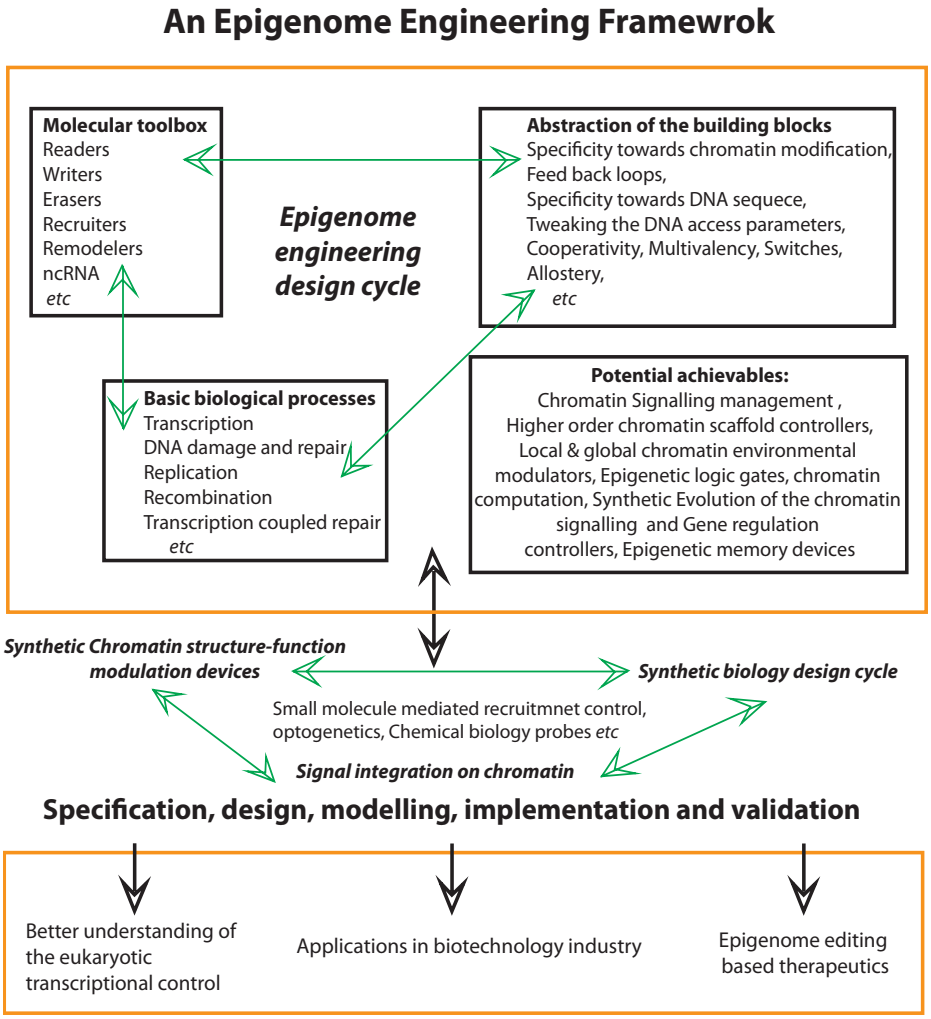

Figure 23. A futuristic vision of an Epigenome engineering framework. A synthetic biology based epigenome engineering framework is poised to drive the better understanding of the Epigenetic phenomenon and tailored Epigenome editing based therapeutics. Briefly, a rationale design of the synthetic devices (the molecular components of the circuit board) for chromatin structure-function modulation will be informed by the knowledge of the availability of the chromatin based molecular toolbox. The presence of diverse epigenetic molecular currency will be utilized; especially the features, which are central to chromatin signalling, like modularity, and reversibility combined with the orthogonality of DNA binding domains as specific recruitmnet platform (ZnF, TALENs and Cas9) are central to the designing process. After a careful design and testing of the engineered circuits, one can translate that to better explore the fundamental mechanisms and/or work towards developing epigenome editing based therapeutics.

PERSPECTIVE

Chromatin based mechanisms however modulatory provide robustness to the

eukaryotic gene regulatory systems and in our view are the main players in the regulatory control systems of the genome templated processes in particular gene expression; thereby has allowed quicker evolution of diverse and complex eukaryotic systems as compared to the prokaryotic evolutionary time scale. Exploiting modular epigenetic switches towards understanding the role of the chromatin in gene expression noise, redundancy among the gene expression controllers thereby robustness and evolvability of the underlying systems and the role of chromatin context in the gene expression. This is set to disrupt the field and has far reaching consequences in basic understanding of the abstract principles of the information processing via signal integration on chromatin.

CONCLUSION

Epigenome engineering in a wider synthetic biology framework is set to disrupt both - the way we try to dissect out and understand biology as well as biomedical applications thereof. In particular the chromatin based epigenetic phenomenon - utilizing the modularity and orthogonality thereof; towards engineering of the desirable epigenetic states is likely to provide the much needed control system engineering for biotechnological and therapeutic applications.

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(For all the unpublished text)

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Abbreviations

ACF: ATP-utilizing chromatin assembly and remodeling factor
ADD: A fusion of GATA like zinc finger and PHD domain
ADPr: ADP-ribose
ALC1: Amplified in liver cancer 1
Alt-NHEJ: Alternative non-homologous end joining
ARPs: Actin Related Proteins
ATM: Ataxia-telangiectasia mutated serine/threonine kinase
ATP: Adenosine tri-phosphate
ATRX: ATRX also known as ATP-dependent helicase ATRX
Auto N: N-terminal autoinhibitory region (AutoN)
Cas9: CRISPR associated protein 9
CHD1: Chromodomain helicase DNA binding protein 1
CHD1L: Chromodomain helicase DNA binding protein 1 like
Cry1: Cryptochrome1
CSB: Cockayne syndrome protein B
CTD: C-terminal domain
CTCF: CCCTC-binding Factor
DDB2: DNA damage-binding protein 2
DDR: DNA damage response
DMSO: Dimethylsulfoxide
DNA: Deoxyribonucleic acid
DNA-PKcs: DNA-dependent protein kinase, catalytic subunit
DNMT: DNA methyltransferase
DTT: D,L-1,4-dithiothreitol
***E. coli*:** Escherichia coli
EDTA: Ethylenediaminetetraacetic acid
F2H: Fluoresce two hybrid
FBXL3: F-box/LRR-repeat protein 3
FCS: Fluorescence correlation spectroscopy
FL: Full length
GCN5: A class of histone acetyltransferase
GFP: Green fluorescent protein
HAT: Histone acetyltransferase
HDAC: Histone deacetylases
HDX-MS: Hydrogen deuterium exchange mass-spectrometry
HEAT repeats: Tandem repeat protein structural motif found in Huntingtin, Elongation factor 3, Protein phosphatase 2A and yeast kinase TOR1.
HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid
HMGs: High mobility group proteins
HP1: Heterochromatin protein 1
HR: homologous recombination
HSA: Helicase-SANT-Associated
INO80: Inositol requiring mutant 80
IPTG: Isopropylthio-b-D-galactoside
ISWI: Imitation switch

ITC: Isothermal Titration Calorimetry
MBD: Methyl binding domain
MeCP2: Methyl CpG binding protein 2
modENCODE: Model organism encyclopedia of DNA elements
Mot1: Modifier of transcription 1
NAD^+: Nicotinamide adenine dinucleotide
NASP: Nuclear autoantigenic sperm protein
ncRNA: non-coding RNA
Neg C: Negative regulator of the coupling of ATP hydrolysis to DNA translocation
NER: Nucleotide excision repair
NuRD: Nucleosome remodeling and deacetylase complex
NTD: N-terminal domain
PAR/poly-ADPr: poly-ADP-ribose
PARPs: Poly-ADP-ribose polymerases
PBS: Phosphate buffered saline
Per2: Period 2
PHD: Plant homeodomain
PMEFs: Primary mouse embryonic fibroblasts
PMSF: Phenylmethylsulfonyl fluoride
PRC: Polycomb repressive complex
PTMs: Post-translational modifications
RNA: Ribonucleic acid
RNA Pol II: RNA polymerase II
RSC4: Remodel the structure of chromatin complex subunit 4
SHL: Super helix location
SNaC: Snf2 ATPase coupling
SWI/SNF: Switch/sucrose non-fermentable
TAF_{250}: Transcription associated factor 250
TALE: Transcription activator-like effector
TBP: TATA box binding protein
TET1: Ten-eleven translocation 1: A methyl cytosine dioxygenase
TFIID: Transcription factor II D
TPR: Tetratricopeptide repeat
TRIM 33: Tripartite motif-containing 33
U2OS: Human bone osteosarcoma epithelial cells
WT: Wild type
XL-MS: Chemical crosslinking followed by mass-spectrometry
ZnF: Zinc Finger

Declaration of contributions

List of Publications included in this Dissertation

1. Structures of drosophila cryptochrome and mouse cryptochrome1 provide insight into circadian function.
Anna Czarna, Alex Berndt, **Hari R. Singh**, Astrid Grudziecki, Andreas G. Ladurner, Gyula Timinszky, Achim Kramer, Eva Wolf, **Cell,** 2013; **153**, 1394 –1405.

2. ACF takes the driver's seat.
Hari R. Singh and Andreas Ladurner, **Molecular Cell**, 2014; **55**, 345–346.

3. ADP-ribosylation signaling during DNA damage repair.
Barbara Golia, **Hari R. Singh**, Gyula Timinszky, Frontiers in Bioscience (Landmark Ed.), 2015; 1; **20**: 440–57.

4. The histone chaperone sNASP binds a conserved peptide motif within the globular core of histone H3 through its TPR repeats.
Andrew Bowman, Lukas Lercher, **Hari R. Singh**, Daria Zinne, Gyula Timinszky, Teresa Carlomagno, Andreas Ladurner, Nucleic Acids Research, 2016; **44** (7): 3105-3117.

5. Remodelers tap into nucleosome plasticity.
Hari R. Singh, Magdalena Murawska and Andreas G Ladurner, Nature Structural & Molecular Biology, 2017; **24** (4), 441-443.

6. A poly-ADP-ribose trigger releases the auto-inhibition of a chromatin remodelling oncogene.
Hari R. Singh, Aurelio P. Nardozza, Ingvar R. Möller, Gunnar Knobloch, Hans A.V. Kistemaker, Markus Hassler, Nadine Harrer, Charlotte Blessing, Sebastian Eustermann, Christiane Kotthoff, Sébastien Huet, Felix Müller-Planitz, Dmitri V. Filippov, Gyula Timinszky, Kasper D. Rand, and Andreas G. Ladurner
Molecular Cell, 2017 (Accepted for publication in December 2017).

Unpublished documents included/to be prepared from this dissertation

7. The Epigenome Joins the Club of Engineers.
Hari R. Singh & Andreas G. Ladurner, Essay manuscript
(Prepared for submission included at the end of the dissertation)

8. How Modular allostery regulates ALC1 chromatin remodeler.
Hari R. Singh & Andreas G. Ladurner Perspective article
(To be prepared from the Introduction and discussion section of the dissertation)

Declaration of contributions for the published documents

1. Declaration of Contribution to "Structures of Drosophila Cryptochrome and Mouse Cryptochrome1 Provide Insight into Circadian Function"

Anna Czarna performed protein expression and purification, crystallization, ITC, Blue light illumination experiments, generation of mutants, UV/VIS spectroscopy. Alex Berndt performed cloning, expression, purification of full-length dCRY, crystallization and data collection of full-length dCry; prepared Figure 6B and 6C. Hari Raj Singh did cloning, plasmid preparation, transfections in mammalian cells and performed microscopic experiments, data generation and figure preparation for Figure 6A. Astid Gridziecki performed experiments for Figure 6B and 6C. Andreas Ladurner discussed the data, provided resources and handled or advised correspondence with the journal. Gyula Timinszky generated the image analysis pipeline for Figure 6A and interpreted the data in 6A. Achim Kramer designed and analyzed experiments in Figure 6B and 6C and contributed to the paper writing.

Eva wolf analyzed and interpreted data except for data shown in Figure 6, wrote the manuscript

2. Declaration of Contribution to "ACF Takes the Driver's Seat"

Hari Raj Singh made the first draft, prepared the Figure, co-wrote and corrected the article with Andreas Ladurner; Andreas communicated the article with the journal editors.

3. Declaration of Contribution to "Poly-ADP-ribosylation signaling during DNA damage repair"

I co-wrote this review with Barbara Golia and Gyula Timinszky. In particular, I wrote the section 4 of the review article, which deals with "**The effects of poly-ADP-ribosylation on chromatin structure upon DNA damage**" and was additionally involved in writing and corrections of the rest of the document while Barbara Golia and Gyula Timinszky wrote most of the rest of the review article. Gyula Timinszky prepared the figure and communicated the final draft to the editors.

4. Declaration of Contribution to "The histone chaperone sNASP binds a conserved peptide motif within the globular core of histone H3 through its TPR repeats"

Andrew Bowman designed and carried out the experiments, conceived of the project in discussion with AGL, prepared the manuscript for publication. Lukas Lercher designed and carried out NMR experiments, analyzed the data. Hari Raj Singh discussed and design of F2H assays with AB performed all the F2H assays. Daria Zinne expressed and purified labeled proteins for NMR studies. Gyula Timinszky discussed and designed the project, corrected the manuscript. Teresa Carlomagno supervised the NMR work. Andreas Ladurner discussed the project, provided resources, co-wrote the paper and handled the publication process.

5. Declaration of Contribution to "Remodelers tap into nucleosome plasticity"

Hari Raj Singh wrote the first draft, Magdalena Murawska prepared the Figure 1, co-wrote and corrected the article, Andreas wrote and corrected the article and communicated with the editors.

6. Declaration of Contribution to "A Poly-ADP-ribose Trigger Releases the Auto-inhibition of a Chromatin Remodeling Oncogene"

Conceptualization, H.R.S., M.H., G.T. and A.G.L.; Methodology, H.R.S., A.P.N., I.R.M., G.K., F.M.P., G.T., K.D.R. and A.G.L.; Investigation, H.R.S., A.P.N., I.R.M., G.K., M.H., N.H., C.B., C.K. and S.H.; Formal Analysis, H.R.S., A.P.N., I.R.M., G.K., M.H., N.H., C.B., F.M.P., G.T., K.D.R. and A.G.L.; Writing – Original Draft, H.R.S. and A.G.L.; Writing – Review & Editing, H.R.S., G.K. and A.G.L.; Funding Acquisition, H.A.V.K., D.V.F., F.M.P. and A.G.L.; Resources, H.A.V.K., D.V.F., S.E., S.H. and C.K.; Supervision, S.H., F.M.P., G.T., K.D.R. and A.G.L.

Declaration of contributions for the unpublished documents

7. Declaration of Contribution to "The Epigenome Joins the Club of Engineers"

Hari Raj Singh prepared the first present draft. Andreas and Hari will correct, re-purpose and communicate with the editors.

8. Declaration of Contribution to "How Modular allostery regulates ALC1 chromatin remodeler"

Hari Raj Singh will prepare the first draft from the introduction and the discussion section of the manuscript. Andreas and Hari will correct, re-purpose and communicate with the editors.

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Curriculum Vitae

Personal details:

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Academic Background:

2007-2009: M.Sc. Department of Biotechnology, University of Pune

2004-2007: B.Sc. Honours Biochemistry, Aligarh Muslim University

2001-2003: Senior Secondary School, St. Paul's School Ajmer

Research Experience:

Since April 2012 to present:

Ph.D. candidate (Dr. rer. nat.) at Biomedical Center - Munich, Physiological Chemistry, Ludwig Maximilian University of Munich

August 2009 to December 2011:

Research scholarship by Council of Scientific and Industrial Research (CSIR), India

January 2009 to May 2009:

Master's thesis reseach at National center for cell science (NCCS), Pune

Merits:

1. Secured all India rank 72 in competetive exam for Master's in biotechnology, competition among tens of thousands, 2007

2. Secured all India rank 43 in graduate aptitude test in engineering, among 13466 candidates, 99.64 percentile, 2009

3. Cleared India's National eligibility test with junior research fellowship (one of the first 200 selected in the country), 2008.

Publications:

1. Structures of drosophila cryptochrome and mouse cryptochrome1 provide insight into circadian function.
Anna Czarna, Alex Berndt, **Hari R. Singh**, Astrid Grudziecki, Andreas G. Ladurner, Gyula Timinszky, Achim Kramer, Eva Wolf, **Cell,** 2013; **153**, 1394 –1405. DOI:10.1016/j.cell.2013.05.011

2. ACF takes the driver's seat.
Hari R. Singh and Andreas Ladurner, **Molecular Cell**, 2014; **55**, 345–346. DOI:10.1016/j.molcel.2014.07.014

3. ADP-ribosylation signaling during DNA damage repair.
Barbara Golia, **Hari R. Singh**, Gyula Timinszky, Frontiers in Bioscience (Landmark Ed.), 2015; 1; **20**: 440–57. PMID: 25553460

4. The histone chaperone sNASP binds a conserved peptide motif within the globular core of histone H3 through its TPR repeats.
Andrew Bowman, Lukas Lercher, **Hari R. Singh**, Daria Zinne, Gyula Timinszky, Teresa Carlomagno, Andreas Ladurner, Nucleic Acids Research, 2016; **44** (7): 3105-3117. DOI:10.1093/nar/gkv1372

5. Remodelers tap into nucleosome plasticity.
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6. A poly-ADP-ribose trigger releases the auto-inhibition of a chromatin remodelling oncogene.
Hari R. Singh, Aurelio P. Nardozza, Ingvar R. Möller, Gunnar Knobloch, Hans A.V. Kistemaker, Markus Hassler, Nadine Harrer, Charlotte Blessing, Sebastian Eustermann, Christiane Kotthoff, Sébastien Huet, Felix Müller-Planitz, Dmitri V. Filippov, Gyula Timinszky, Kasper D. Rand, and Andreas G. Ladurner
Molecular Cell, December 2017; **68**(5), 860-871. DOI:10.1016/j.molcel.2017.11.019

7. The poly(ADP-ribose)-dependent chromatin remodeler Alc1 induces local chromatin relaxation upon DNA damage. Hafida Sellou, Théo Lebeaupin, Catherine Chapuis, Rebecca Smith, Anna Hegele, **Hari R.Singh**, Marek Kozlowski, Andreas G. Ladurner, Gyula Timinszky, and Sébastien Huet, Molecular Biology of Cell, 2016; **27**, 3791–3799. DOI:10.1091/mbc.E16-05-0269

International conferences/meetings:

1. Indo-US workshop on Epigenetic regulation and genome control (emphasis on RNAi and Micro RNA), 16th - 18th December, 2009, CCMB, Hyderabad, India

2. 3rd meeting of the Asian forum of chromosome and chromatin biology: "Chromosome/chromatin dynamics Epigenetics and disease", 4th - 6th December, 2010, JNCASR, Banglore, India

3. Spetses summer school, Chromatin and Systems biology, 30th August - 5th September 2013, Spetses, Greece
Poster: DNA damage activates the chromatin remodeler ALC1 through ADP-ribosylation

4. DNA damage response in physiology and disease, 24th October 2014, Rennes, France
Short talk: DNA damage activates the human chromatin remodeler ALC1 through ADP-ribosylation

5. International synthetic & systems biology summer school, 5th - 9th July, 2015, Taormina, Sicily, Italy
Poster: Poly-ADP ribose binding activates a human oncogenic chromatin remodeler ALC1: a potential synthetic device for chromatin structure-function modulation

6. CAS conference: Synthetic Biology, 27th - 29th July 2015, Biocenter, LMU, Munich
Poster: Poly-ADP ribose binding activates a human oncogenic chromatin remodeler ALC1: a potential synthetic device for chromatin structure-function modulation

7. FASEB meeting, NAD+ Metabolism and Signaling, 9th -14th August, 2015, Timmendorfer Strand, Germany
Poster: Poly-ADP ribose binding activates a human oncogenic chromatin remodeler ALC1

8. Final EpiGeneSys network meeting, 11th - 13th February, 2016, Paris, France
Poster: Poly-ADP ribose binding activates a human oncogenic chromatin remodeler ALC1

9. Annual Chromatin Day: SFB 1064, meeting of munich based chromatin groups every year July Last week
Short talk -2016: A poly-ADP-ribose switch releases the auto-inhibition of the human oncogeneic chromatin remodeler ALC1

10. EMBL Symposium on the complex like of mRNA - 5th-8th October 2016, EMBL, Heidelberg

11. Keystone symposia on "Precision Genome Engineering" January 8 - 12, 2017 Beaver Run Resort, Breckenridge, Colorado, USA
Poster: A poly-ADP-ribose switch releases the auto-inhibition of the human oncogeneic chromatin remodeler ALC1

12. EMBL conference on "The Quantitative principles in Biology" 2 - 4 Nov 2017, EMBL, Heidelberg, Germany

Further training:

1. Scientific writing workshop by International Bioscript, 2012, Munich

2. Managing bibliographies with Endnote by LMU, Graduate center, Munich, 2013

3. Network training work shop on aspects of leadership by Leadership sculptor, 2013, Spetses

4. Introductory R course at LMU, Munich, 2014

5. Designing and presenting poster by International Bioscript, Munich, 2015

6. Workshop on Data analysis using R by Science craft, Munich, 2015

7. Workshop on scientific image processing and analysis Biovoxxel, 2015, Munich

8. Workshop on Scientific writing with Science craft, Munich, 2016

9. Workshop on Proposal writing with Science craft, Munich, 2016

10. Startup Weekend Munich 2017 - Friday, April 28, 2017 - Sunday, April 30, 2017

11. MPI, Munich, Startup community workshop on - "Discover your entrepreneurial opportunities" 1st June - 2nd June 2017

12. Bioentrepreneurship Summit-2017, 10th October - 11th October 2017, BMC, LMU, Munich

13. "Grant Writing "14th november - 15th November 2017 at LMU, Munich