REGULATION OF ALC1'S SNF2 ATPASE: INTERPLAY BETWEEN THE SNF2 DOMAIN, THE MACRODOMAIN AND OTHER CONSERVED ELEMENTS

BY

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ABSTRACT

ALC1, also known as CHD1L, was originally identified as a gene present on a human chromosome 1q21 region amplified in ~50% of human hepatocellular carcinomas (HCC). ALC1 overexpressing cells form tumors in nude mice, and transgenic mice overexpressing ALC1 develop several types of spontaneous tumors. ALC1 has also been proposed as a novel candidate gene for congenital anomalies of the kidneys and urinary tract (CAKUT).

ALC1, a member of the SNF2 family of ATPases, has an N-terminal SNF2-like ATPase that is most closely related to that of ISWI, and a C-terminal macrodomain that binds selectively to poly(ADP-ribose) (PAR). Between the ATPase and the macrodomain is an evolutionarily conserved region with no clear homology to any known domains. This "linker" region can be further divided into three sub-regions of greatest conservation. It was previously shown that wild type ALC1 possesses DNA-dependent ATPase and ATP-dependent nucleosome remodeling activities that are strongly dependent on the presence of poly(ADP-ribose) polymerase PARP1 (or the closely related PARP2) and its substrate NAD^+. Importantly, a point mutation in the ALC1 macrodomain that interferes with PAR binding prevents PARP1- and NAD-dependent ALC1 activation.

In this work, we dissected the mechanism by which PARP1 and NAD^+ activate ALC1 nucleosome remodeling. We demonstrate that ALC1 activation depends on the formation of a stable ALC1·PARylated PARP1·nucleosome intermediate. In addition, by exploiting a novel PAR footprinting assay, we obtained evidence that the ALC1 macrodomain remains stably associated with PAR on autoPARylated PARP1 during the course of nucleosome remodeling reactions. Results of biochemical experiments described here argue (i) that stable binding of the ALC1 macrodomain to autoPARylated PARP1 is critical for ALC1 activation and (ii) that

activation of ALC1 depends on formation of a stable ALC1-autoPARylated PARP1-nucleosome intermediate.

In the course of this study, we also find that apart from being regulated by PARP and NAD^+, ALC1 possesses and additional mode of intradomain control via conserved domains in the linker region and the macrodomain. First, we identified a region, NMAC (N-terminal to Macrodomain ATPase Coupling) domain needed to couple ATP hydrolysis to nucleosome remodeling. Deleting NMAC led to a robust PARP and NAD^+-dependent ATPase, which lacked appreciable remodeling activity. In addition, we identified an additional mode of control via the macrodomain when we replaced ALC1's PAR binding macrodomain with another macrodomain from the variant histone macroH2A1.1 and discovered this chimeric protein was constitutively active independent of PARP and NAD^+. Taken together our findings suggest a model of positive control of the SNF2 ATPase via the macrodomain and an additional level of control over its remodeling activities via the NMAC and other conserved linker elements.

ACKNOWLEDGEMENTS

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and made sure I was progressing in a timely manner towards graduation. I would like to thank all of the members of the Conaway Lab including: Nawel Mahrour, Aaron Gottschalk, Hidehisa Takahashi, Chieri Sato, Shigeo Sato, Vijay Kantharia, Shawn Hall and Margaret Banks. The members of the lab provided me with tremendous support and advice all while promoting a wonderful work environment that was full of fun times and much laughter. I will forever treasure the long hours spent with my lab mates, inside the lab and outside as well. I would like to particularly thank the core facilities here at Stowers Institute including Julie Conkwright in Screening; Tari Parmely, Maria Katt, and Valerie Neubauer in Tissue Culture; and all the members of the molecular biology core facility who have through their collaborative services allowed my research to flourish. My friends at SIMR and KUMC made the last few years of my life immensely enjoyable.

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To Shachi & Rhea

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CHAPTER I

INTRODUCTION

Chromatin Structure and Nucleosome Architecture

Packaging of information and its appropriate retrieval is an evolving process in the physical world. If we take a look at the contemporary data management systems, we see that the first data collection systems were mere notches on a fine surface (punch cards/vinyl). As the need to store more data in a compacted format arose, the discs evolved and became laser discs and eventually DVD's and Blu-ray's. With the evolution of the data storage systems, the data reading systems had to grow in parallel in order to adapt to the compacted reading formats. The storage of data in biological systems is performed by chromosomes. In the search for transferable genetic materials in eukaryotic cells, histones, along with DNA were candidate transferable genetic material as they had mass very similar to that of DNA and were components of chromatin. Their universal presence with eukaryotic chromosomes further strengthened this notion. However, after the discovery of the structure of DNA and complementary base pairing, histones were soon regarded as inert packaging materials for DNA [1].

Electron microscopy by Joseph Gall in the early 1960's helped describe the structure of chromatin in nucleated newt erythrocytes as highly flexible fibers of uniform diameter [2]. During this time, it was thought that eukaryotic DNA was composed of a linear strand of DNA coated with an evenly repeating rearrangement of 5 histone proteins forming 100Å fibers. The 5 types of histones, namely, H3, H4, H2A, H2B, and H1, were thought to bind DNA and each other to serve as an insulation for DNA. However, in the next 15 years, DNAase digestion studies by Hewish and Burgoyne and electron microscopy on interphase nuclei by Olins and Olins in 1974, and Woodstock and Stanchenfield in 1976 suggested a periodic particulate structure of chromatin and pointed at the existence of multiple forms of chromatin, including

both 70 Å and 15 Å strands resembling "beads on a string" [3-5]. The above studies helped establish the nature of chromatin as a dynamic structure that changes with biological need. It can be envisioned as a polymeric fiber with a monomeric repeating unit.

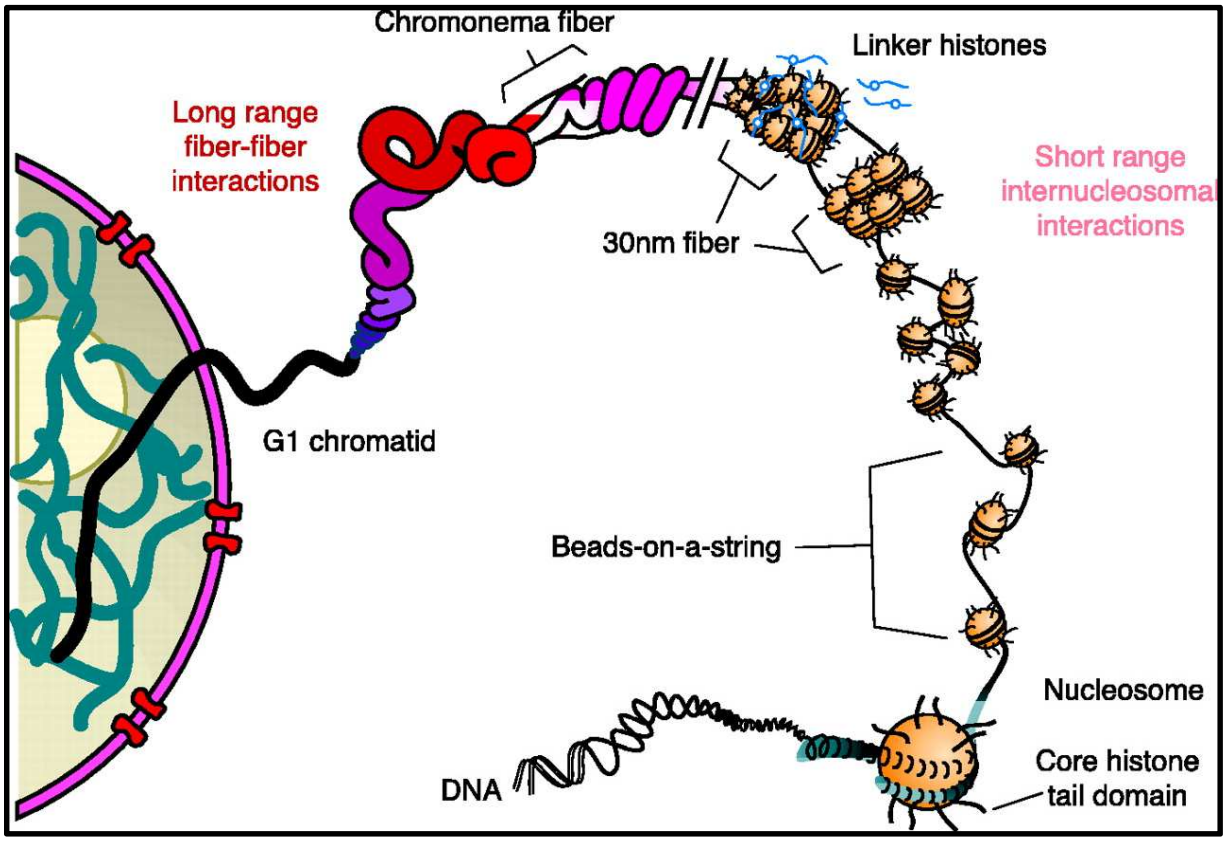


Figure 1: Multiple levels of chromatin folding. DNA compaction within the interphase nucleus (left) occurs through a hierarchy of histone dependent interactions that can be subdivided into primary, secondary, and tertiary levels of structure. Strings of nucleosomes compose the primary structural unit. Formation of 30nm fibers through histone tail-mediated nucleosome-nucleosome interactions provides a secondary level of compaction, whereas tail-mediated association of individual fibers produces tertiary structures (such as chromonema fibers). Figure adapted from Horn, PJ & Peterson, CL. *Science* 297(5588):1824 (2002) [6].

However, the monomeric unit of this fiber was not fully understood yet. It was thought to be some combination of DNA and histone proteins, but its precise composition was of great

interest in the 1970's. In 1974, Kornberg and Thomas published a landmark paper that helped understand this monomeric unit and its organization in chromatin [7]. The histone proteins under mild extraction conditions, consisted of a tetramer of histone proteins H3 and H4, and two copies of a dimer formed between histone H2A and H2B [8]. They observed that a periodic pattern of 205 bp of DNA repeats was obtained by nuclease digestion of rat liver chromatin and concluded that approximated 1 copy of each subunit per 100 base pairs of DNA given the observed ratio of histones [9, 10]. This along with chemical cross-linking of proteins followed by sedimentation analysis allowed Kornberg and Thomas to deduce that the fundamental unit of chromatin is composed of an octamer of histones, an H3/H4 tetramer and two H2A/H2B dimers, wrapped by about 200bp of DNA. This DNA thread wrapped around a spool of histone octamer was called the nucleosome, the basic fundamental repeating subunit of chromatin. This discovery of the nucleosome revolutionized our understanding of chromatin structure and opened doors for understanding gene regulation (Fig. 1).

In 1997, Karolyn Luger and Tim Richmond obtained a high-resolution structure of the nucleosome particle using X-ray Crystallography [11]. Their 2.8 Å structure validated the nucleosome model proposed by Kornberg and Thomas. This model illustrated the globular nature of histone octamers and detailed interactions between histones and DNA. They discovered that 146 base pair of DNA are wrapped around an octamer of histones, making 14 histone-DNA contacts through the phosphodiester backbone of the inner surface of the DNA superhelix (Fig. 2). Most importantly lack of any contact of the DNA bases with the histone suggested sequence independent binding of histones to DNA and explained the ability of histones to pack any piece of DNA. The stoichiometry of histones was the same as predicted by Kornberg's and Thomas's,

as it was found that a tetramer of histones H3 and H4 were flanked by two sets of dimers of histones H2A and H2B. Interestingly, they found that a characteristic histone fold mediates both histone/histone and histone/DNA interactions. Perhaps the most important observation was that the highly basic and flexible histone N-terminal tails pass over and between the gyres of DNA to contact neighboring particles.

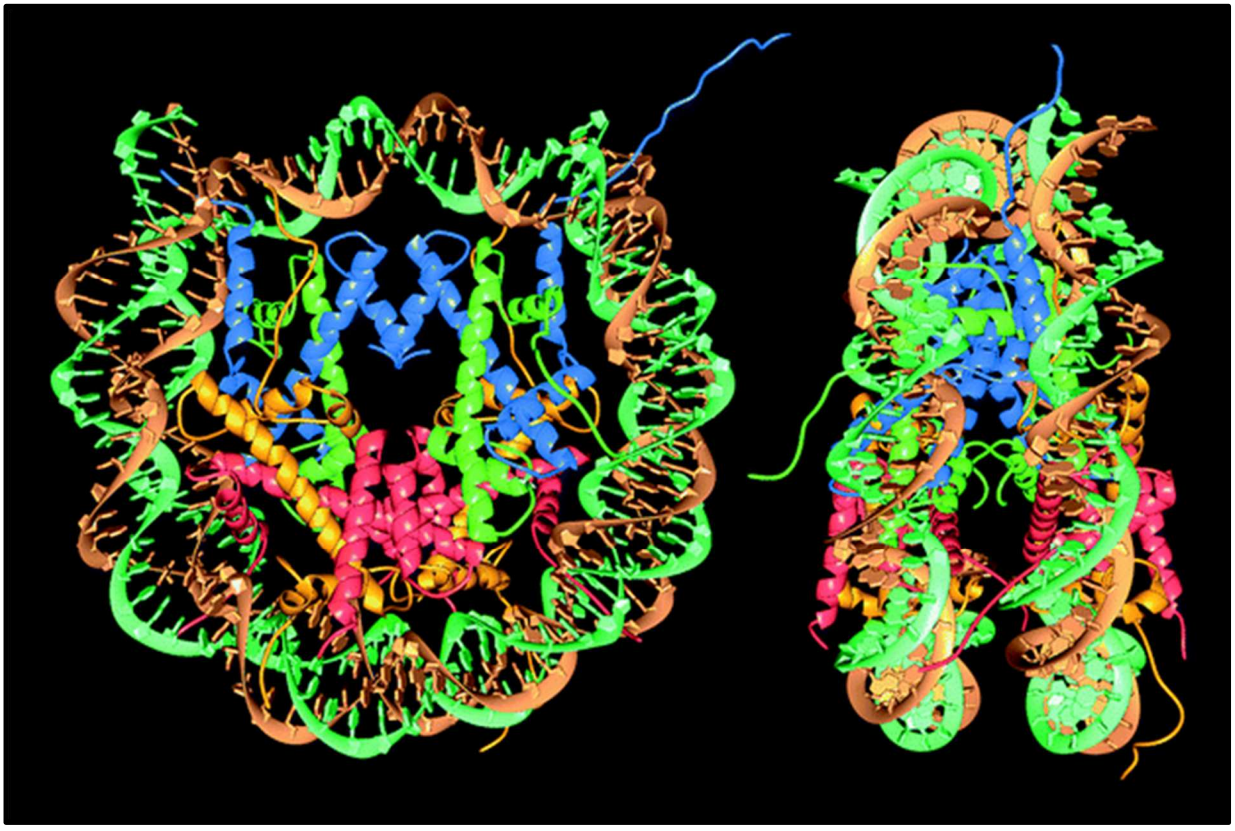


Figure 2: Nucleosome core particle structure. Nucleosome core particle: ribbon traces for the 146-bp DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains (blue: H3; green: H4; yellow: H2A; red: H2B. Image adapted from Luger, K et al. *Nature* 389(6648):231 (1997) [11].

These unstructured histone tails are the perfect candidates for reversible chemical modifications allowing them to carry information beyond the DNA. The highly flexible nature of these tails also suggest some of the N-terminal histone tails protrude from the nucleosomes and

can contribute to interactions with other nucleosomes and presumably lend a role in the formation of higher order structures. Various permutations and combinations of post-translational modifications on these tails gave rise to the "histone code" that creates various nucleosomal interfaces, downstream interaction with various chromatin proteins including neighboring histones and other regulatory enzymes. Many studies throughout the recent decades have supported this histone code hypothesis and suggested that these N-terminal tails play an important role in regulating a variety of nuclear processes.

Packaging of DNA by histones still does not explain the extent to which DNA is packaged in the eukaryotic cell. In mammalian cells, roughly 2 meters of linear DNA is packed into a nucleus of approximately 10 μm in diameter. Thus, packaging by histones alone does not satisfy the degree of compaction observed in cells. To achieve this, a system of higher level packaging of the nucleosomes into chromatin is necessary. Chromatin exists as an intricate assembly of protein and nucleic acids. The basic architectural unit of chromatin is the nucleosome, composed of roughly two turns of DNA wrapped around an octamer of histones. Nucleosomes are further folded into a series of higher order structures that make up chromosomes. This tight packaging not only compresses DNA but also occludes the DNA adding a layer of regulatory control for gene expression. To explain the hierarchical nature of chromatin compaction, we need to look at the organization of the nucleosome structure. The most simple chromatin structure consists of nucleosomes evenly spaced across DNA with roughly 50 base pairs of linker DNA separating them. This structure has been shown to appear as 11nM "beads on a string" structure by electron microscopy under conditions of low ionic strength. This 11nM fiber, under high salt conditions and with the help of linker histones, such as histone H1 [12] and other non-histone proteins, becomes more compact as nucleosomes are

folded into higher order arrays to form chromatin fibers of roughly 30nm in diameter [13]. The structure of this 30 nM fiber is not known in detail, but it is thought to be a two start helix as concluded from the crystal structure of the tetra nucleosome. This 30 nm chromatin fiber constitutes the second level of chromatin compaction. These chromatin fibers woven together form chromosomes (Fig. 1). A further level of chromatin and DNA compaction can be attained in the metaphase chromosome. The most important contributor to this second level of compaction is Histone H1, also called the linker histone, as it promotes coiling or folding of the 30nM fiber into a helical assembly of zigzagging nucleosomes [14, 15]. In addition, histone tails can contact neighboring nucleosomes and lead to further compaction as suggested by the Luger crystal structure where H4 tails may contact a patch on the H2A-H2B dimer of the neighboring nucleosome. This opens the opportunity to reversibly control the level of compaction and accessibility of chromatin by modifying the histone tails of the nucleosomes.

Nucleosomal Packaging

From the above discussion, we gather that chromatin can be envisioned as a highly efficient compaction system that organizes DNA. While this packaging of nucleosomes offers a great opportunity to compact and protect DNA, the wrapping of DNA around the nucleosome and subsequent superstructures of the octamer occlude the sequence of DNA to any external proteins. The wrapping of DNA around histone octamers occlude one face of the DNA double helix and was initially hypothesized to interfere with many nuclear transactions, including transcription, replication, DNA damage repair and recombination. Nucleosomes packaging promoter sequences inhibit transcription initiation events by both bacterial and eukaryotic RNA polymerases *in vitro* [16]. Transcription activities *in vivo* are also inhibited by nucleosomes in a similar manner as concluded from genetic experiments in yeast [17]. It may appear that nucleosomes are needlessly preventing transcription as if there is a cost to pay for efficient packaging and compaction. However, the cell utilizes this property of the nucleosome based inhibition. The packaging of eukaryotic DNA into chromatin provides a means to partition the genome into transcriptionally active and transcriptionally repressed regions. Different patterns of partitioning allow diverse transcriptional programs to arise from a single genetic blueprint. Transcriptional repression is achieved at the level of the chromatin fiber by histone tail modifications. Histone tail interactions and their specific modifications allow for different levels of compaction and access to the DNA. For example, increase in histone acetylation levels catalyzed by histone acetyltransferases (HATs) relieves condensation of the chromatin fiber. This can be easily reversed by histone deacetyltransferases (HDACs) (Fig. 3). Additionally, at

the level of a single nucleosome, contacts between DNA and the histone fold pose a significant hindrance for transcription machinery to access the DNA.

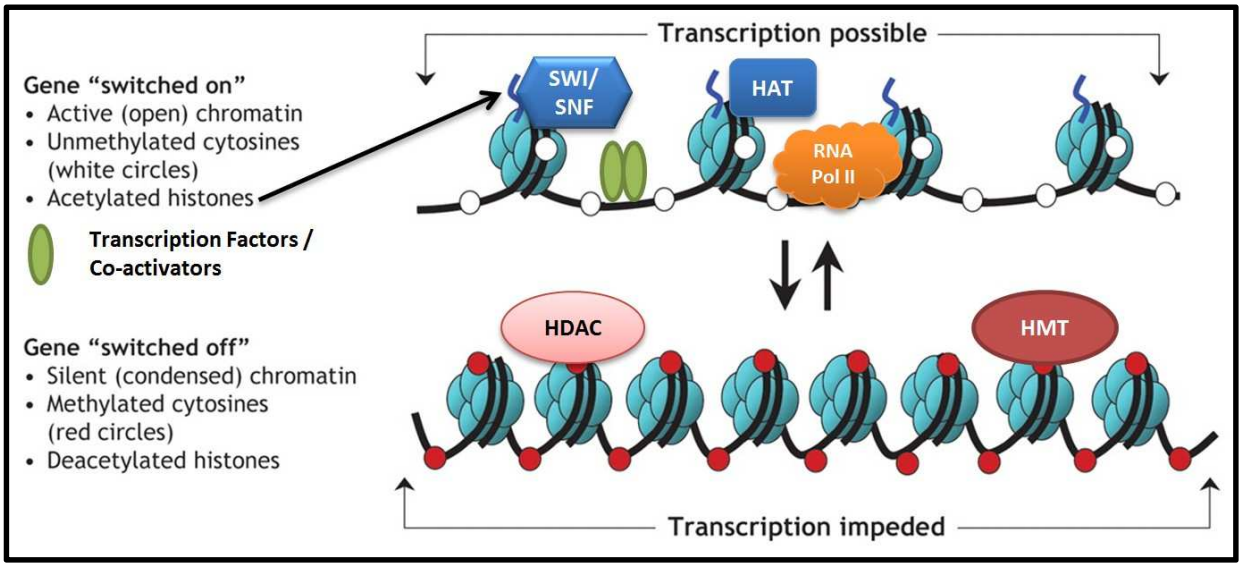


Figure 3: Chromatin remodeling complexes in the dynamic regulation of transcription: In the presence of acetylated histones (HAT mediated) and absence of methylase (HMT) activity, chromatin is loosely packaged. Additional nucleosome repositioning by chromatin remodeler complex, SWI/SNF opens up DNA region where transcription machinery proteins, like RNA Pol II, transcription factors and co-activators bind to turn on gene transcription. In the absence of SWI/SNF, nucleosomes cannot move farther and remain tightly aligned to one another. Additional methylation by HMT and deacetylation by HDAC proteins condenses DNA around histones and thus, make DNA unavailable for binding by RNA Pol II and other activators, leading to gene silencing. Figure adapted from [18]. Davis P.K., Brackmann, R.K. Chromatin remodeling and cancer (2003).

Information in this form is inaccessible and of no potential benefit to the cell. Highly compact chromatin structure presents a structural barrier for cellular proteins to engage any DNA on nucleosomes in biochemical processes. DNA wound on a nucleosome is inaccessible to transcription or gene regulatory machinery as well as enzymes that function in replication (needed for cell proliferation) and repair (needed to maintain genome integrity). In order to read

this compacted information, eukaryotes have evolved a highly sophisticated system of proteins that include histone modifiers, histone chaperones and chromatin remodelers, that work together to alter the chromatin landscape and allow or inhibit access to the DNA as the need may arise. The establishment of specific chromatin states during the course of development as well as their maintenance through the disruptive events of transcription, DNA replication, and DNA repair require rapid rearrangements of chromatin structure. ATP-dependent chromatin-remodeling enzymes – one of which (Amplified in Liver Cancer 1 (Alc1)) is the main focus of this thesis, provide a means of generating such changes in chromatin structure.

Histone modifying enzymes and their roles

Nucleosome packaging presents a significant barrier for cellular processes. The fact that access to DNA wrapped around a nucleosome is essential to life processes, suggested that the nucleosomes had to be more dynamic in nature than thought. Nucleosomes by themselves are stable and show very limited mobility on their own, appearing to be ideal spools for wrapping DNA, but have function outside of packaging DNA by the virtue of the histone tails. Kornberg and collegues were the first to show in 1987 that the promoters assembled with nucleosomes were refractory to transcriptional initiation by both SP6 polymerase and the mammalian RNA polymerase II complex *in vitro*. However, both the polymerases were able to read through the nucleosome once transcription initiation was permitted [16]. Additionally there was a concomitant loss of nucleosomes as the polymerase transcribed through, suggesting a hitherto unknown displacement mechanism. In further support, Han and Grunstein showed loss of nucleosomes at select promoters upon H4 depletion [17]. This resulted in transcriptional activation of multiple genes. These findings suggested that nucleosome occupancy was a key regulator of transcription machinery and many other downstream biological processes.

The next obvious question was to probe the precise nature of regulation exercised by nucleosomes. The mechanism by which packaging and compaction work in tandem to provide a precise level of control was a major question in the field. Grunstein and collegues made a major breakthrough in this field when they found that deletions within the hydrophobic core of the histone H4 were lethal and blocked chromosomal segregation [17]. The N-terminus was found to be dispensable for growth, but essential for repressing the silent mating loci in *S. cerevesiae*. In a later study in the same organism, they found that loss of a region in the H4 N-terminus

encompassing a number of highly conserved lysine residues resulted in drastic reduction of *GAL1* and *PHO5* [19]. Taken together these findings clearly indicated the unique biological functions of histones and their highly conserved tails that had specific biological roles. These findings also established the major role of conserved residues on histone tails that play a major role in regulation.

Research over the past two decades has revealed that covalent modifications of histone proteins and DNA can fundamentally alter the organization and function of chromatin, and that they have a crucial role in the regulation of all DNA-based processes, such as transcription, DNA repair and replication. These modifications are dynamically laid down and removed by chromatin-modifying enzymes in a highly regulated manner. N-terminal tail of histones along with defined positions of the globular domain can carry several post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP ribosylation (Fig. 4) [16, 20-26]. All the 4 canonical histones have been reported to be reversibly acetylated, each acetylated location bearing a different biological function. Acetylation of histones leads to a more "relaxed" form of chromatin that is permissible to transcription machinery, and deacetylation of chromatin leads to a more "packed" and transcriptionally inactive region of chromatin [27]. This is supported by the finding that histone deacetylation caused by HDACs (Histone DeACtylase) can repress transcription. HDAC activity is a common feature of several corepressor complexes such as *RPD3*, and *HDAC1*. Yeast and mammalian *RPD3*s can be directly fused with a heterologous DNA-binding domain and are sufficient to mediate transcription repression, sensitive to HDAC inhibitors [28].

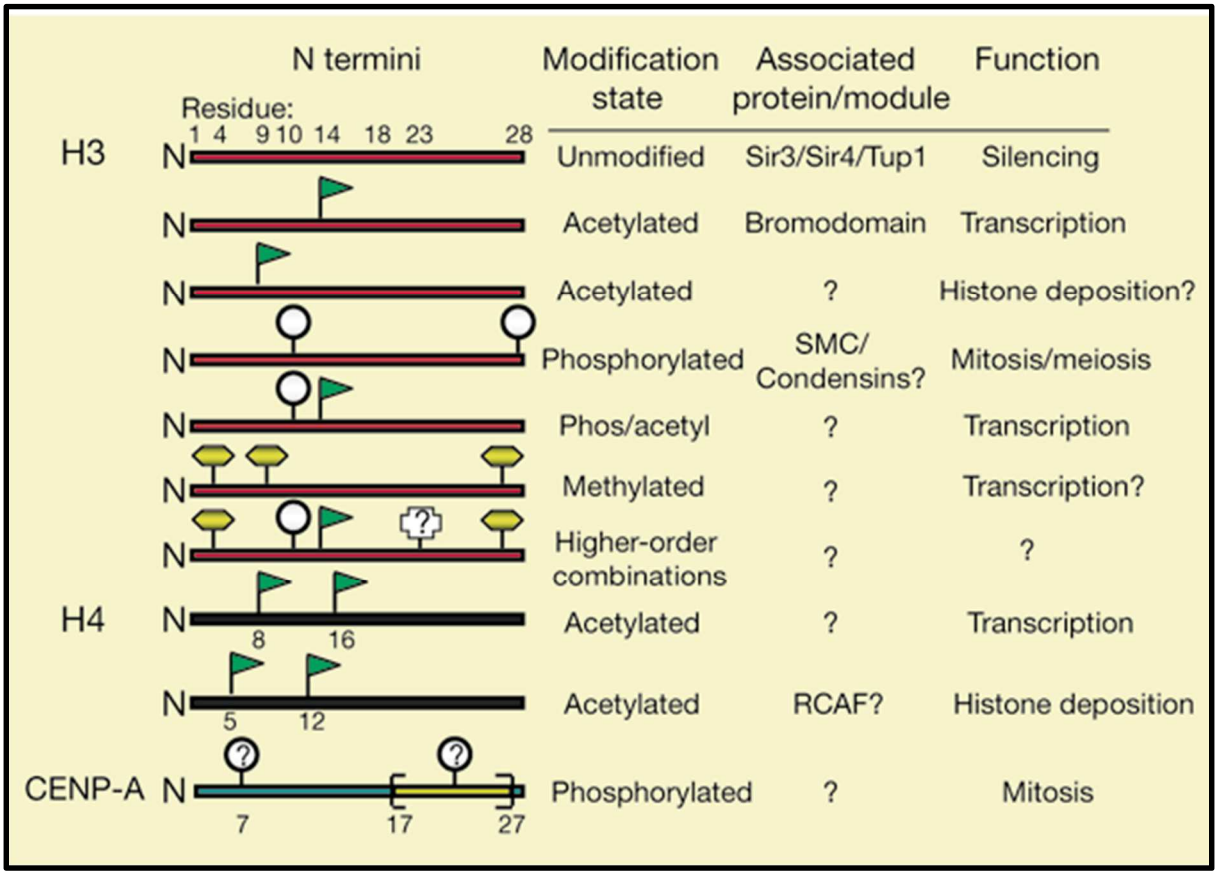


Figure 4: Histone modifications involved in chromatin reorganization. Histone N-tails are post-translationally modified, and certain combinations of histone modifications appear to generate a "histone code" defining the chromatin state. Histone modifications occur at selected residues and some of the patterns shown have been closely linked to a biological event (for example, acetylation and transcription). Emerging evidence suggests that distinct H3 (red) and H4 (black) tail modifications act sequentially or in combination to regulate unique biological outcomes. How this hierarchy of multiple modifications extends (depicted as 'higher-order combinations') or how distinct combinatorial sets are established or maintained in localized regions of the chromatin fiber is not known. Relevant proteins or protein domains that are known to interact or associate with distinct modifications are indicated. The CENP-A tail domain (blue) might also be subjected to mitosis-related marks such as phosphorylation; the yellow bracket depicts a motif in which serines and threonines alternate with proline residues. Figure adapted from C. David Allis et al *Nature* 403: 41-45 (2000) [29].

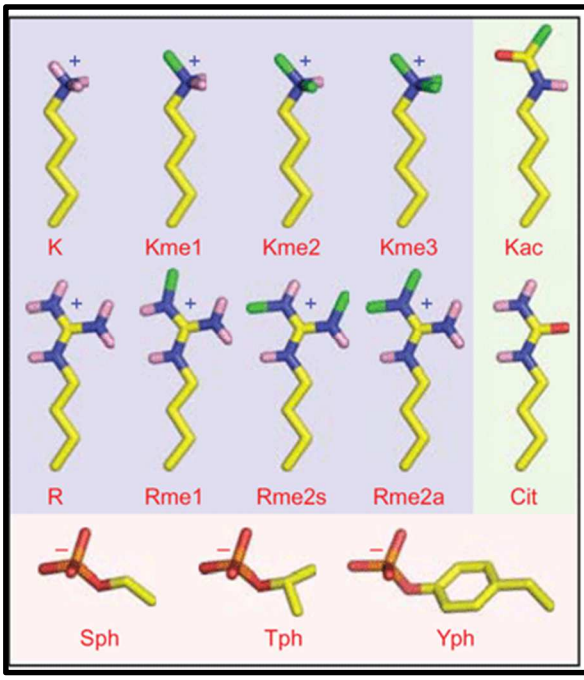
Active transcription is found to be usually associated with acetylation of multiple residues in H3 and H4, and with di- or tri-methylation of H3 at lysine 4 (K4) position.

Condensed heterochromatin and intergenic regions of the genome are often transcriptionally inactive and are commonly associated with H3 K9 methylation and H3 K27 methylation [24].

The histone modifications on individual histone tails and their combinations seemed to hold encoded information for transcriptional regulation, but it was still unclear how these effects were imparted. It was speculated that charge-neutralization of highly basic histone N-terminal tails *via* modifications would change local chromatin structure ultimately leading to disruption of histone-DNA contacts. This explains the effects of acetylation, but methylation of lysine groups has no effect on overall charge, indicating that there is a more complex mechanism at play than simple charge-charge interactions [30].

The more complex mechanism was supported by the discovery of many transcription regulatory proteins possessing functional domains that bind to specific histone modifications. Grunstein and Gasser labs in 1995 discovered that silent information regulator 3 (Sir3) and Sir 4, previously known to be required for repression of yeast silent mating-type loci, associated with methylated histone tails through a chromodomain [31]. This binding event was required for targeted recruitment of SIRs within the genome. Importantly, when the histone tails were acetylated, Sir3 and Sir 4 failed to bind, suggesting the regulatory nature of histone modifications. Zhou and collegues solved the crystal structure of a bromodomain, a domain commonly found in transcriptional activators known to bind acetylated histone residues [32]. Identification of these binding domains fueled the search for additional histone reader molecules (Fig. 5). Biophysical and biochemical methods have uncovered a vast number of histone reader domains with two-fold recognition – residue specific and chemical modification specific. These domains bind to modified histones and serve as multi-purpose platforms for integrating various

chromatin modifying complexes based on the modification imparted on the histone tail. These chromatin modifiers and chromatin remodelers influence import processes such as transcription, replication, DNA repair and cell cycle progression [33].



	Reader module	PTM mark
	Bromodomain	Many histone Kac, (Kac)
Royal	Chromodomain	H3K9me2/3, H3K27me2/3
Royal	Double chromodomain	H3K4me1/2/3
Royal	Chromo barrel	H3K36me2/3
Royal	Tudor	(Rme2s)
Royal	Double/tandem tudor	H3K4me3, H4K20me3 H4K20me1/2, (Kme2)
Royal	MBT	H4K20me1/2, H1K26me1/2 H3K4me1, H3K9me1/2
	PHD finger	H3K4me3, H3K4me0 H3K9me3, H3K36me3
	WD40 repeat	H3R2/K4me2, (R, Sph, Tph)
	14-3-3	H3S10ph, H3S28ph, (Sph, Tph)
	BRCT	H2AX-S139ph, (Sph, Tph)

Figure 5: Histone tail modifications and their binding partners. Left: Stick models of different classes of post-translationally modified amino acid residues, highlighting small chemical group side chains on histone tails. Yellow, carbon; blue, nitrogen; pink, polar hydrogen; red, oxygen; orange, phosphorus; green, methyl groups of post-translational modifications. Background is shaded by charge of side chains at physiological pH: light blue, positive; pink, negative; light green, uncharged. Right: Reading the histone (protein) code. Shown grouped by domain family are known chromatin-associated modules and the histone marks they have been reported to bind. Parentheses denote examples where structural information is known about related family members and their interactions with non-histone PTMs. Figure adapted from [34].

Chromatin remodeling – How and Why?

All DNA-dependent processes require selective and controlled access to DNA sequences. With the evolution of highly compact and efficient packaging of DNA around nucleosomes and their modified histone tails, the need for selective and regulated exposure of DNA to nuclear factors is necessary. Regulatory sequences, such as promoters and enhancers, of actively transcribing genes are shown to be hypersensitive to nuclease digestion. One interpretation of this observation could be that these *cis* regulatory DNA sequences have unrestricted access to transcription machinery and are to be devoid of nucleosomes. If indeed this is the case, then it is highly suggestive of an active mechanism to regulate nucleosome occupancy in these key regions of the eukaryotic genome. A mechanism to regulate nucleosome occupancy also provides an opportunity to regulate gene expression *via* chromatin structure. In addition to that, this mechanism and its effectors need to be functioning in a reversible or complimentary fashion, as an essential feature of regulating the DNA sequences *via* nucleosome occupancy involves deposition and packaging of nucleosomes on exposed DNA sequences (Fig. 6).

Histone tail modifications provide a potential marker for regions that are targeted to be rendered nucleosome free for greater access or packaged tightly to limit accessibility. Although histone tails play an important role in higher order packaging, they have a very minor impact on nucleosome core structure and do not influence any histone core-DNA interactions – key interactions that need to be disrupted for unrestricted access to DNA.

The solution to this problem of restricted DNA access is a broad class of proteins and their assemblies that function as chromatin remodelers. Remodelers are DNA-translocating motors that utilize the energy derived from ATP hydrolysis to change the contacts between

histones and DNA. Remodelers function to fully package the genome, specialize chromatin regions by allowing histone modifying enzymes access to histone tails, and provide regulated access to DNA sequences. [35-41]. Remodelers can 1) mediate nucleosome sliding, in which the position of a nucleosome on the DNA changes, 2) induce the creation of a remodeled state, in which the DNA becomes more accessible but histones remain bound, 3)cause the complete dissociation of DNA and histones, or 4) histone replacement with a variant histone. ATP-dependent remodelers work in conjunction with other factors, most notably histone chaperones and histone modifying enzymes. Remodelers play very important roles in essential cellular processes such as gene expression and its regulation, DNA repair, replication and recombination, and nucleosome deposition.

Two functions that are common among all protein remodeling complexes are 1) a functional ATPase, and 2) the ability to manipulate DNA as a helicase. The first point is quite intuitive as energy is needed for these activities and ATP provides an ideal energy source for these reactions. The second point relates to the diverse functionality the helicase domain has achieved by surrounding itself with a) accessory proteins to guide its functions and/or b) an accessory domain(s) often present within the same protein sequence that can target and manipulate the helicase functionality.

In order to be exhaustive, five basic common properties of remodelers include: (a) an affinity for the nucleosome, beyond DNA itself; (b) domains that recognize covalent histone modifications; (c) a similar DNA dependent ATPase domain required for remodeling and to serve as a DNA-translocating motor to break histone-DNA contacts; (d) domains and/or proteins that regulate the ATPase domain; and (e) domains and/or proteins for interaction with other

chromatin or transcription factors. Together, these shared properties allow nucleosome engagement, selection, and remodeling. However, distinctive targeting and tasks require remodeler specialization.

Currently, four different classes of remodeling complexes are recognized: SWI/SNF, ISWI, CHD or Mi-2 and Ino80. Each class is defined by a unique subunit composition that often works in distinct assemblies. Common to all the classes is the presence of a distinct ATPase that is discussed in the following section [35, 36, 42].

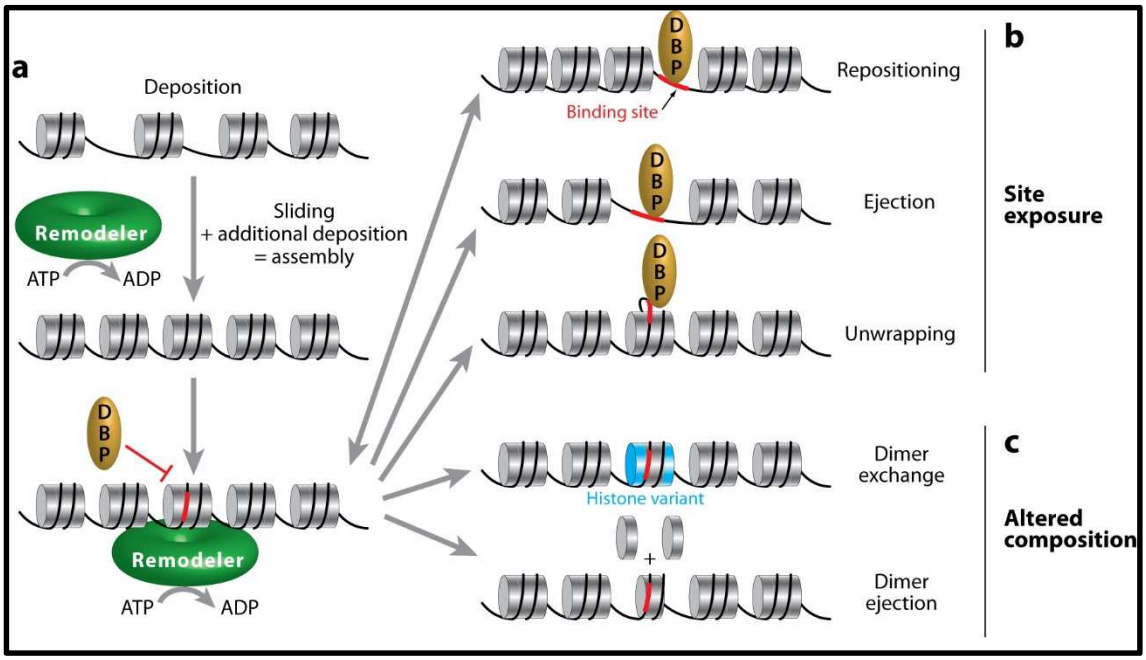


Figure 6: The different outcomes of chromatin remodeling. Remodelers (*green*) can assist in chromatin assembly by moving already deposited histone octamers, generating room for additional deposition (*a*). Remodeler action on a nucleosome array results in various products that can be classified in two categories: (*b*) site exposure, in which a site (*red*) for a DNA-binding protein (DBP), initially occluded by the histone octamer, becomes accessible by nucleosomal sliding (repositioning), or nucleosomal eviction (ejection), or localized unwrapping, and (*c*) altered composition, in which the nucleosome content is modified by dimer replacement [exchange of H2A-H2B dimer with an alternative dimer containing a histone variant (*blue*)] or through dimer ejection. Figure adapted from [43].

The RecA-like ATPase of SNF2 remodelers

In 1988, Gorbalenya and Koonin discovered a large group of proteins that shared a series of short ordered motifs [44, 45]. The majority of members with known function were nucleic acid strand separating helicases. As a result these sequences became known as helicase motifs and were labelled sequentially I, Ia, II, III, IV, V and VI (Fig. 7). A number of additional conserved blocks with broad distributions within these helicase-like proteins were identified, such as the TxGx and Q motifs [46, 47] (Fig 8).

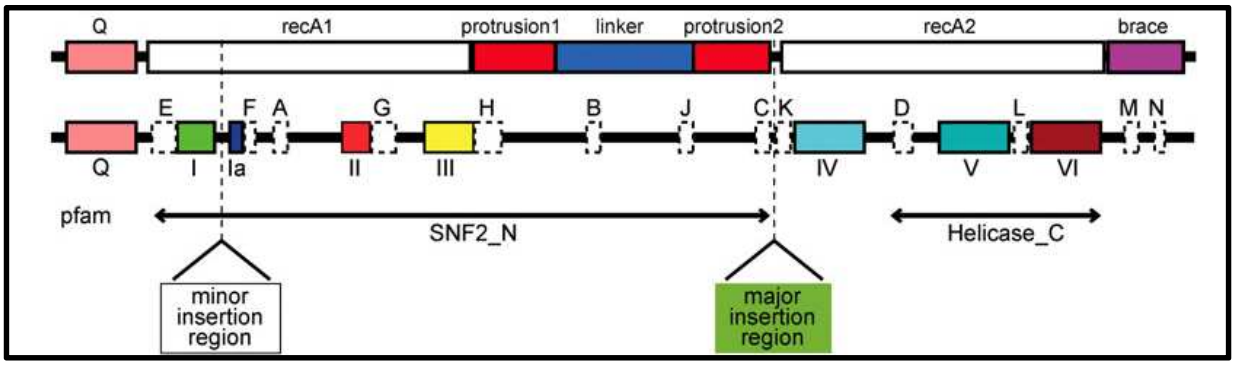


Figure 7: Conserved blocks contribute to distinctive structural feature of Snf2 family proteins. Schematic diagram showing location of structural elements and helicase motifs. Conserver blocks are shown as white boxes. Figure adapted from [48].

The latest bioinformatics approaches have subdivided proteins containing helicase motifs into several superfamilies based on the primary sequence similarity [48]. Overall, helicases can be grouped into six helicase-like superfamilies SuperFamily 1-6 (SF1-SF6). Among these superfamilies, SF1 and SF2 are closely related by sequence similarity in their common core of highly conserved two RecA-like domains [49]. SF3-SF6 are ring-forming helicases. Proteins

with a helicase-like region of similar primary sequence to *Saccharomyces cerevisiae* Snf2p make up the Snf2 family within SF2 [48] (Fig. 8).

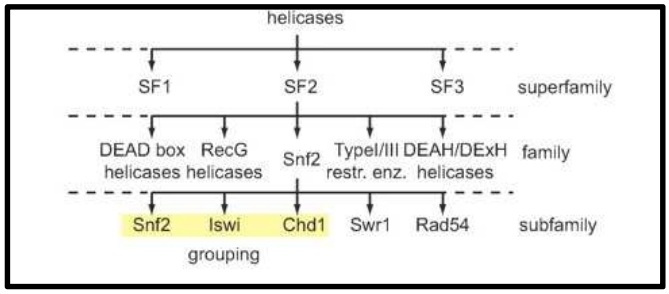


Figure 8: Schematic diagram illustrating hierarchical classification of superfamily, family and subfamily levels. Figure adapted from [48].

The mechanical motion required to fulfil the general function of a helicase is achieved by a change in the relative orientation of the highly conserved two recA-like domains. These helicase-like enzymes hydrolyze ATP in an active site cleft located between the two recA-like domains to derive the energy for this change in orientation [50]. Structural and mutagenesis studies have shown that each of the conserved helicase motifs in the active site cleft between the recA-like domains plays a role in the transformation of chemical energy from ATP hydrolysis to mechanical motion. This enzymatic process has been suggested to represent one application of a more general mechanism used in many proteins containing a recA-like domain [51] (Fig. 9).

The two recA-like domains adopt a bi-lobular structural fold with a central opening cleft as its active site (Fig. 9). It is known that Motif I contains the "Walker A" motif, responsible for binding the triphosphate tail of ATP [47]. Being a helicase, binding to DNA is thought to be mediated *via* Motif Ia that forms the edge of a shallow groove across the surface of the protein and may be involved in binding to DNA. Motif II contains part of the "Walker B" motif and is

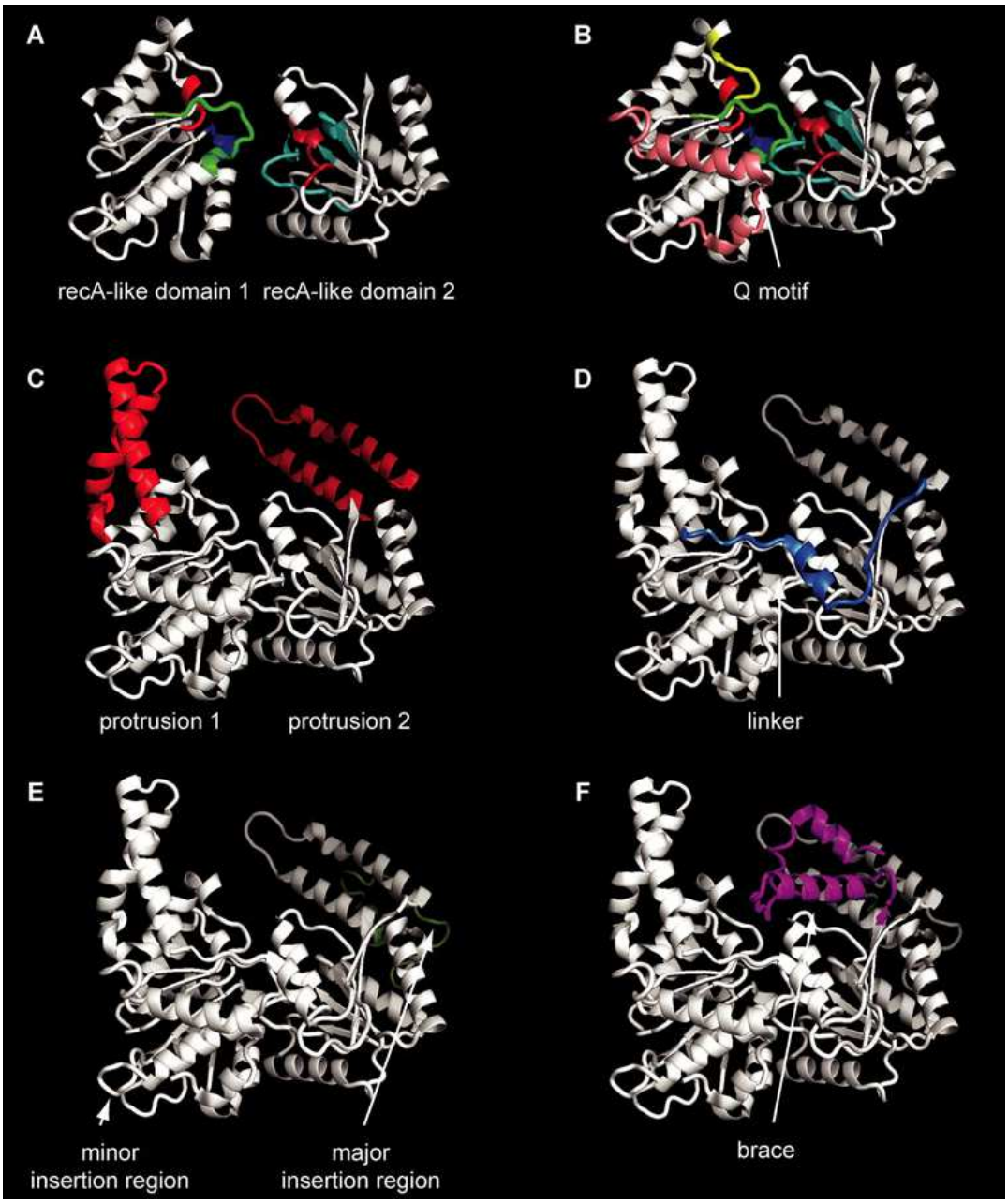


Figure 9: Conserved blocks contribute to distinctive structural features of Snf2 family proteins. Structural components of Snf2 family proteins relevant to the conservation are illustrated on the zebrafish Rad54A structure [pdb 1Z3I [52]]. (**A**) core recA-like domains 1 and 2 including colouring of helicase motifs (I in green, Ia in blue, II in bright red, III in yellow, IV in cyan, V in teal and VI in dark red). (**B**) Q motif (pink). (**C**) antiparallel alpha helical protrusions 1 and 2 (red) projecting from recA-like domains 1 and 2, respectively. (**D**) Linker spanning from protrusion 1 to protrusion 2 (middle blue). (**E**) Major insertion region behind protrusion 2 (light green). (**F**) triangular brace (magenta). Figure adapted from [48].

the DEAD box motif, implicated in binding Mg^{2+} which is a necessary requirement for ATP hydrolysis by both DNA and RNA helicases [53, 54]; Motif III is thought to be involved in the coupling of ATP hydrolysis to helicase activity [47], Motif IV forms the linkage between the two recA like domains by running underneath and then becoming a part of the ATP binding site. Motifs V and VI flank both sides of the ATP binding site and provide important interfaces suspected to be regulating the helicase activity of the protein [49].

As stated earlier, within the helicase-like 2 superfamily (SF2), there are a group of proteins with high primary sequence similarity to *S. cerevisiae* SNF2p. These proteins are classified into the SNF2-like subfamily (Fig. 10). As with SNF2p, they bear two recA-like helicase domains at their core, and many of the members of this family were first identified as ATPases residing within chromatin remodeling complexes. Today, it is widely recognized that SNF2-like proteins serve as a core subunit of chromatin remodeling multi-subunit complexes and are the engine that is required for ATP-dependent chromatin remodeling processes [41]. The SNF2-like family is ubiquitous in eukaryotes and is also present to a lesser extent in both eu- and archaeabacteria. Comprehensive bioinformatic analysis of completed genomes has allowed researchers to catalogue SNF2-like members [55]. Guided by primary sequence similarity in the helicase-like regions, 24 distinct SNF2-like subfamilies can be recognized. Many of the subfamilies correlate with known biological function. Despite the similarity of the core ATPase motor of these enzymes, there is profound diversity within the SNF2-like family with each family having its own set of distinct functions. The human SNF2-like proteins are genetically non-redundant *in vivo*, suggesting that the SNF2-like family helicase–like region has specifically evolved to execute distinct functions unique to each subfamily [56].

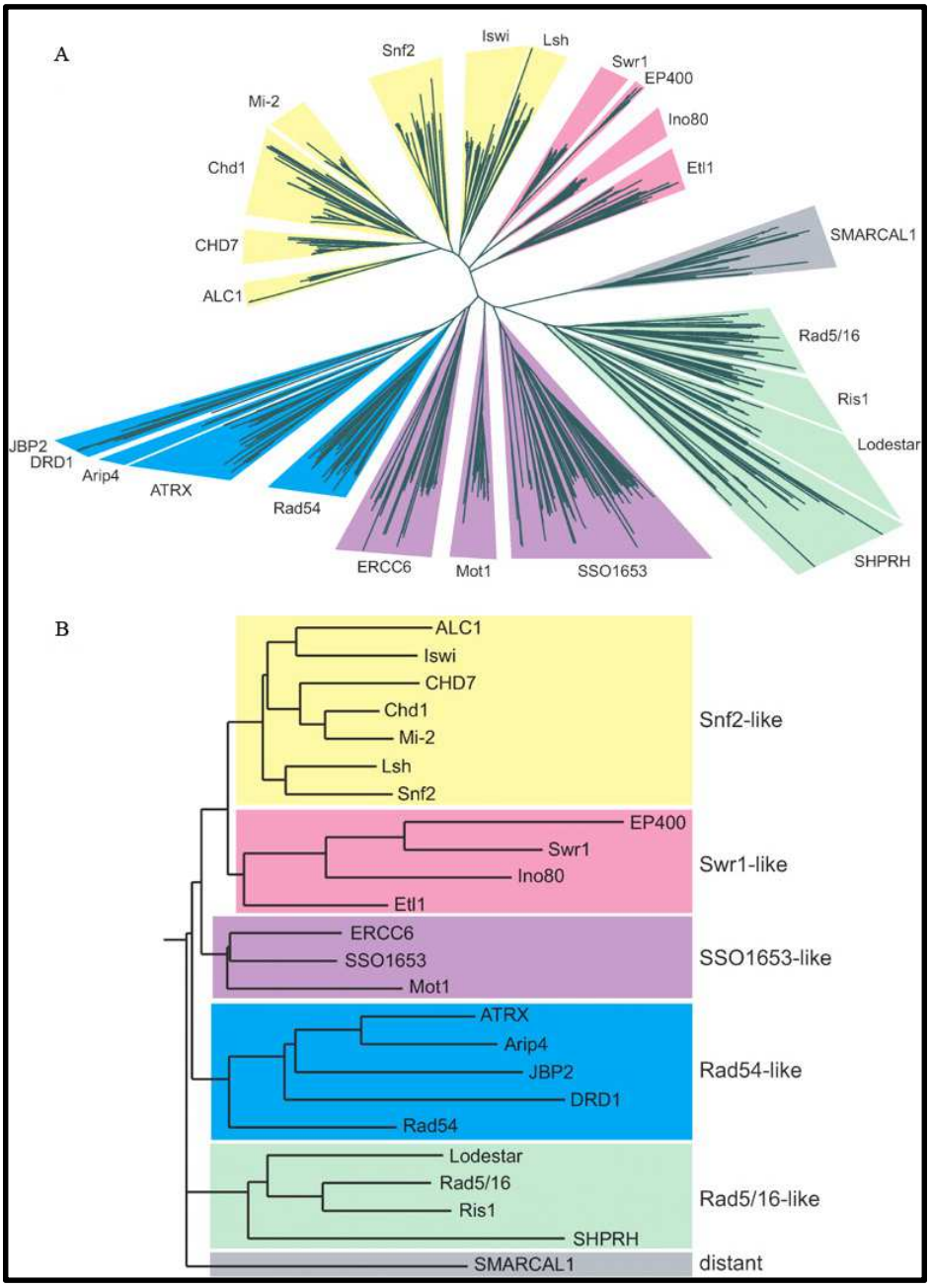


Figure 10: Tree view of SNF2 family. (A) Unrooted radial neighbor-joining tree from a multiple alignment of helicase-like region sequences excluding insertions at the minor and major insertion regions from motifs I to Ia and conserved blocks C–K for 1306 Snf2 proteins identified in the Uniref database. The clear division into subfamilies is illustrated by wedge backgrounds, colored by grouping of subfamilies. Subfamilies DRD1 and JBP2 were not clearly separated, as discussed in text. (B) In order to illustrate the relationship between subfamilies, a rooted tree was calculated using HMM profiles for full-length alignments of the helicase regions. Groupings of subfamilies are indicated by coloring as in (A). Figure adapted from [48].

SNF2 ATPases – a brief history

The ability of nucleosomes to alter chromatin structure *via* their histone tails suggested that proteins capable of influencing nucleosome structure may be important regulators of gene expression. The first chromatin remodeling factor identified was the yeast SWI/SNF protein complex. Genes encoding SWI/SNF subunits were originally identified in two independent screens for mutants affecting either mating type switching or growth on sucrose, hence the names Switching defective (*SWI*) and Sucrose nonfermenting (*SNF*) [57-59]. Further genetic studies revealed that mutations in the histone genes suppressed *swi/snf* phenotypes, providing a direct connections between chromatin and SWI/SNF function. The Winston Lab provided the first evidence to functionally link transcriptional activators and chromatin [60]. Previous reports in literature has been established that Snf5 affected transcription of a large number of genes including the glucose repressible gene *SUC2* that codes for the enzyme invertase. Mutations in Snf5 caused transcriptional repression of *SUC2.* However, these effects could be reversed by mutating the genes encoding for H2A and H2B. Furthermore, localized structural changes in the *SUC2* gene of *snf2* and *snf5* mutant yeast could be rescued by a deletion of one of the two sets of genes encoding histones H2A and H2B, (hta1-htb1) delta, but not by mutating the TATA box to inhibit specific transcription initiation from the *SUC2* promoter. These findings provided the first link between changes in local chromatin structure induced by transcriptional activators Snf2 and Snf5 and its subsequent effect on transcription.

Several proteins encoded by *SWI/SNF* genes (such as SNF2 and SNF5) have been reported to physically associate with each other during immunoprecipitation and chromatographic separation (SWI1/ADR6, SWI2/SNF2, SWI3, SNF5, and SNF6). Multiple

groups independently purified the 10 subunits of the SWI/SNF complex from both yeast and mammalian cells [61-64]. This multisubunit SWI/SNF complex was able to remodel nucleosomes in an ATP-dependent manner *in vitro,* as evidenced by the altered nuclease cleavage pattern and DNA topology. Yeast SWI/SNF complexes were subsequently shown to be required for transcription by DNA-specific activator proteins such as yeast GAL4 and the glucocorticoid receptor expressed in yeast [65, 66]. Workman and Peterson provided a mechanistic explanation by showing that the binding of GAL4 to nucleosomal DNA could be dramatically enhanced by SWI/SNF complexes in the presence of the catalytically active SWI2 subunit and ATP hydrolysis [62]. Additionally, SNF2, SNF5, and SNF6 that were fused with a LexA DNA binding domain were found to be sufficient to activate transcription when tethered to DNA [67]. Thus, the SWI/SNF remodeling complex, was proposed to be a transcriptional coactivator complex that alters structure of promoter chromatin in an ATP-dependent fashion,.

A few years later, a new ATP-dependent chromatin remodeler that exhibited greatly enhanced activity in the presence of GAGA factor was discovered. The new chromatin remodeler was determined to be a complex of 4 proteins was named NURF (Nucleosome Remodeling Factor) and had a 140kDA catalytic subunit named ISWI [68]. The ATPase domain of Iswi had a high degree of homology to SNF2, the catalytic subunit of the Swi/Snf complex. The high degree of homology between the Snf2 and Iswi ATPase domains provided a link between these two complexes and began the hunt for other chromatin remodeling assemblies.

SNF2 sub families

Members of the SNF2 superfamily of ATPases serve as catalytic subunits of ATP-dependent chromatin remodeling enzymes. All SNF2 family ATPases share a conserved ATPase domain. The SNF2 superfamily of ATPases can be further subdivided into subfamilies that have unique collections of functional or structural domains that fall outside of their catalytic domains. However, each family has unique flanking domains that allow further subdivision based on protein motifs and domains outside of the ATPase. These domains and other accessory subunits allow the cell to repurpose this ATPase motor for specific biological processes (Fig. 11).

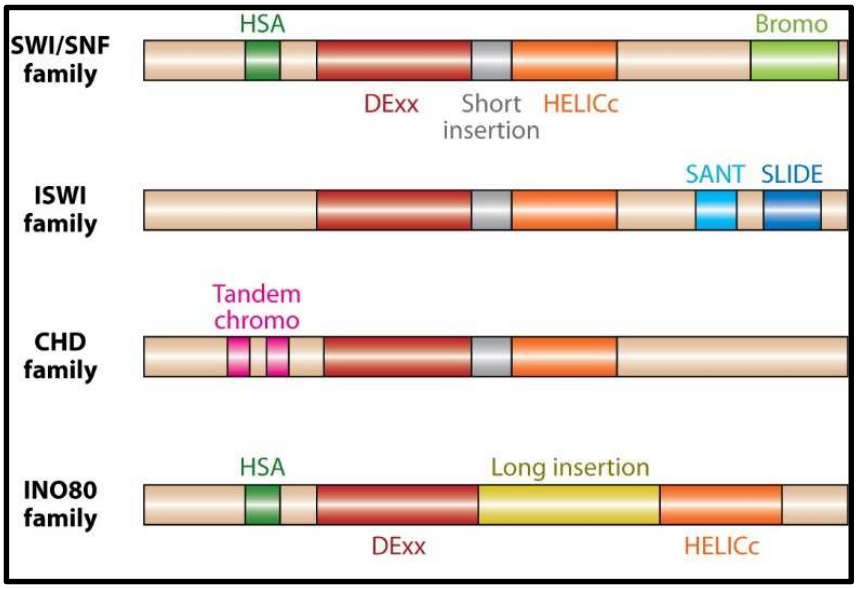


Figure 11: Remodeler Families, defined by their ATPase. All remodeler families contain a SWI2/SNF2-family ATPase subunit characterized by an ATPase domain that is split in two parts: DExx (*red*) and HELICc (*orange*). What distinguishes each family are the unique domains residing within, or adjacent to, the ATPase domain. Remodelers of the SWI/SNF, ISWI, and CHD families each have a distinctive short insertion (*gray*) within the ATPase domain, whereas remodelers of INO80 family contain a long insertion (*yellow*). Each family is further defined by distinct combinations of flanking domains: Bromodomain (*light green*) and HSA (helicase-SANT) domain (*dark green*) for SWI/SNF family, SANT-SLIDE module (*blue*) for ISWI family, tandem chromodomains (*magenta*) for the CHD family, and HSA domain (*dark green*) for the INO80 family. Figure adapted from [43].

SWI/SNF family remodelers

Although first discovered in yeast, the SWI/SNF family of remodelers is evolutionary highly conserved, and homologous complexes have been identified in insects and vertebrates. The budding yeast Snf2 protein is the founding member of the SWI/SNF subfamily and indeed of all SNF2 family remodeling factors. Most eukaryotic cells contain two subclasses of SWI/SNF remodelers, each characterized by the presence of a unique but related catalytic subunit. Human cells contain two distinct SWI2/SNF2-like ATPase subunits, named hBRM (human Brahma) and BRG1 (Brahma-Related Gene 1), which are analogous to yeast SWI2/SNF2 and STH1 [69]. *Drosophila* happens to be an exception to this rule as it contains only a single protein corresponding to yeast SWI2/SNF2 or STH1, called Brahma (BRM) [70, 71]. The evolutionary emergence of multicellularity was accompanied by the loss of some of the subunits that are present in yeast SWI/SNF complexes (SNF6, SNF11, SWP29 and SWP82) and the gain of others. Unlike in yeast, there are two *D. melanogaster* SWI/SNF complexes — the Brahma (BRM)-associated proteins (BAP) complex and the polybromo-containing BAP (PBAP) complex, and these can mediate transcriptional activation and transcription repression. In the transition to vertebrates, there is a large increase in the number of possible complexes as a result of vertebrates gaining the ability to combinatorially assemble several subunits encoded by gene families (Fig. 12) [72].

The catalytic SNF2 ATPase of the SWI/SNF subfamily remodelers contain HSA (helicase-SANT), a post-HSA domain and a C-terminal bromodomain. A pair of actin related proteins (ARP7 and ARP9) is present in yeast SWI/SNF complexes, whereas a dimer of actin and Arp4 (also known as Baf53a or b) are present in higher orthologs [73, 74]. A number of

studies have reported additional subcomplexes in which the SWI/SNF-type remodelers are associated with other factors, such as BRCA1 or components of the histone deacetylating Sin3 complex [75, 76]. Additional conserved domains present in other conserved subunits add another layer of functionality to the already versatile catalytic ATPase. For example, human polybromo subunits have multiple bomodomains, hBAF155/170 has SANT and SWIRM domains and hBAF60 has a SwiB domain. This family has many activities, and is capable of sliding and ejecting nucleosomes at many loci for diverse processes. However, a role of SWI/SNF sub-family in chromatin assembly has yet to be described.

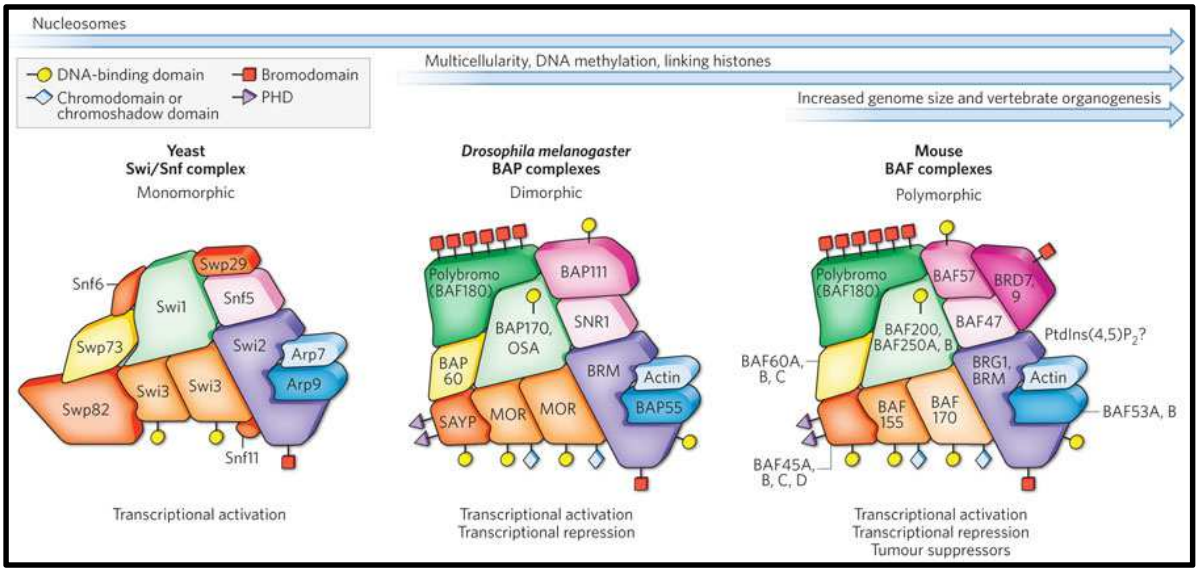


Figure 12: Evolutionary diversification of SWI/SNF complexes. BRD, bromodomain-containing protein; BRG1, brahma-related gene 1; MOR, Moira; PHD, plant homeodomain; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; SAYP, supporter of activation of yellow protein; SNR1, Snf5-related protein 1; Brahma (BRM)-associated proteins (BAP) complex; polybromo-containing BAP (PBAP) complex; brahma-associated factor (BAF). Figure adapted from [72].

ISWI family remodelers

The Iswi (Imitation switch) family of remodelers is comprised of chromatin remodeling complexes containing the ISWI ATPase—including NURF, CHRAC and ACF (2 to 4 subunits) (Fig. 13). They were first identified using *in vitro* assays for nucleosome-remodeling activities in *Drosophila melanogaster* embryo extracts (dNURF, dCHRAC, dACF, hWICH and hNoRC complexes) [68, 77-79]. A characteristic set of domains reside at the C-terminus of ISWI family ATPases: a SANT domain (ySWI3, yADA2, hNCoR, hTFIIIB) adjacent to a SLIDE domain (SANT-like ISWI), which together form a nucleosome recognition module that binds to an unmodified histone tail and DNA [80]. All ISWI remodeling factors require a particular region of the histone H4 tail that is positioned near the DNA surface and presumably functions as an allosteric effector [81, 82].

ISWI family complexes are utilized by most eukaryotes in multiple assemblies, using one or two different catalytic subunits with specialized attendant proteins. Specialized attendant proteins impart many domains, including DNA-binding histone fold motifs (in hCHRAC 15-17), plant homeodomain (PHD), bromodomains (hBPTF and hACF1), and additional DNA-binding motifs [HMGI(Y), for dNURF301]. Many ISWI family complexes (ACF, CHRAC) optimize nucleosome spacing to promote chromatin assembly and the repression of transcription. Attendant proteins can diversify function as seen in NURF, which can randomize spacing and may assist RNAPII activation. All of these complexes function *in vitro* predominantly by relocating histone octamers without disruption. This supports the idea that ISWI complexes reposition, rather than remove, nucleosomes thus modulating the spacing of a nucleosome array. [83, 84]. All three fly complexes are known to have homologs in humans [77-79, 85-88].

Although in mammals two ISWI-homologs exist, SNF2H and SNF2L, so far only SNF2L has been found in the human NURF complex (Barak et al., 2003). ISWI-containing remodeling complexes commonly share subunits related to Acf1, which contain plant homeo (PHD)- and bromodomains [89, 90]. Budding yeast also expresses two forms of ISWI - Isw1p and Isw2p. Yeast CHRAC and ACF resembling complexes contain Isw2p [91, 92]. Isw1p on the other hand resides in two functionally distinct complexes [93]. Functions for ISWI-containing complexes are diverse, ranging from transcriptional regulation of RNA polymerase II and RNA polymerase I to replication [90, 94-99]. It is also well established that Iswi complexes have roles in assembling chromatin and formation of nucleosome arrays with uniform ordered spacing. This ordered spacing of nucleosomes occludes transcription factors and other transcription machinery from promoters, thereby causing repression [99-102].

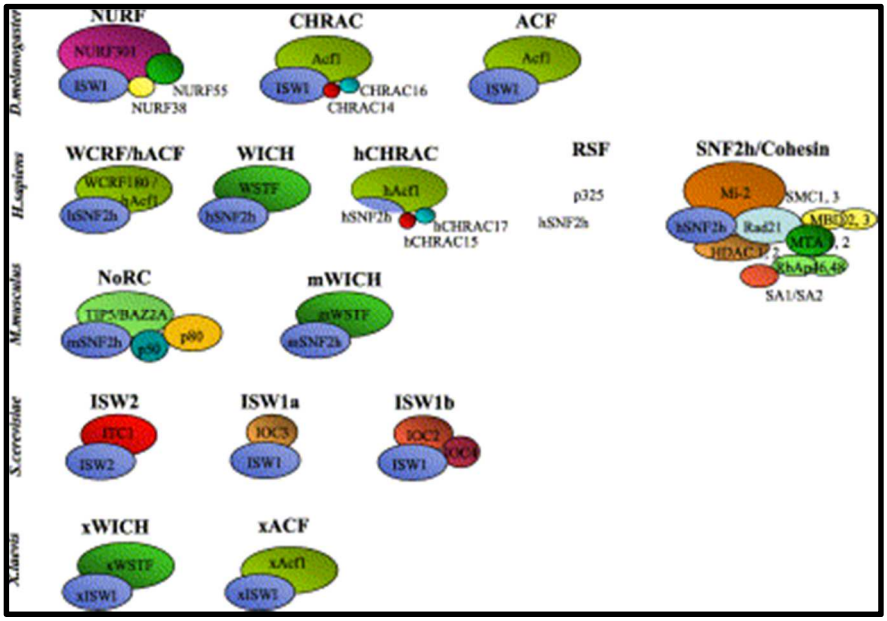


Figure 13: ISWI family of chromatin remodeling complexes. Figure adapted from [101].

CHD family remodelers

A CHD (chromodomain, helicase, DNA binding) family ATPase is the catalytic ATPase core of the CHD family of remodelers. Members of the CHD family are characterized by a tandem arrangement of two chromodomains on the N terminus of the catalytic subunit [103, 104]. The most prominent member of this family is Mi-2, which resides in NuRD complexes that also have histone deacetylase activity [105]. The CHD family ATPase CHD1 is monomeric in lower eukaryotes such as *S. cerevisiae*. However, in vertebrates, CHD1 can be present in large multi-subunit complexes (Fig. 14) [106]. Associated proteins often bear functional domains such as DNA-binding domains and PHD, BRK, CR1-3, and SANT domains. Certain CHD remodelers are known to slide or eject nucleosomes in a chromodomain-dependent fashion to promote transcription, but they can also have repressive roles. Vertebrate Mi-2/NuRD (nucleosome remodeling and deacetylase) complex, which contains histone deacetylases (HDAC1/2) and methyl CpG-binding domain (MBD) proteins, provides a link between histone deacetylation, chromatin remodeling and DNA methylation [107].

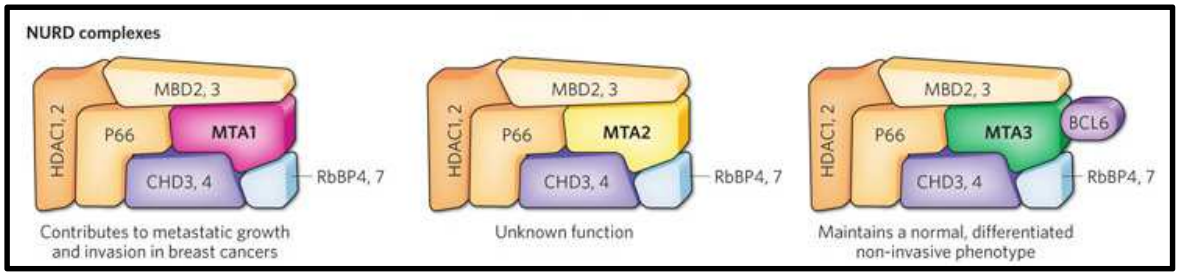


Figure 14: NURD Complexes. NURD complexes (which are members of the CHD family of chromatin-remodelling complexes) incorporate different products of the MTA (metastasis-associated) gene family, and these complexes have distinct, and even opposing, functions in regulating the development and tumorigenesis of mammary tissues. Figure adapted from [72].

INO80 family remodelers

The INO80 subfamily includes the INO80 remodeling complex (INO80.com) and the SWR1 remodeling complex (SWR1.com), which are characterized by split ATPase domains (Ino80 and Swr1 respectively) and the presence of two RuvB-like proteins, Rvb1 and Rvb2 (Fig.15) [37, 108]. The large insertion region is located at the major insertion site of Snf2-like ATPases between helicase motifs III and IV, where the helicase-related (AAA-ATPase) Rvb1/2 proteins (resemble the *E. coli* Holliday junction resolvase) and ARP4 protein bind. Both yeast INO80 and yeast SWR1 complexes contain actin and Arp4. The most well know remodeler of the INO80 subfamily is the Ino80 ATPase from budding yeast, which was identified in a genetic screen for genes involved in transcriptional activation upon inositol starvation, hence the name Ino80 (Inositol requiring 80) [109]. The INO80.com was purified from budding yeast as a 15 subunit complex including the INO80 ATPase [108]. Yeast INO80 has been shown to catalyze ATP-dependent nucleosome sliding activity, DNA-stimulated ATPase activity, and separate DNA strands in a primer-displacement assay, making the INO80 complex the only known Snf2 family remodeling complex to exhibit helicase activity *in vitro*. Higher orthologs include hINO80, hSRCAP (SNF2-related CREB-activator protein), and p400, which also contains HAT activity.

The Conaway lab purified and defined the subunit composition of human INO80-like chromatin remodeling complexes from human cell lines as a 9 subunit complex that is evolutionarily conserved in all eukaryotes [110, 111]. Apart from the 9 conserved subunits, Snf2-like INO80 ATPase, actin, actin-related proteins ARP4, ARP5, and ARP8, AAA^+ ATPases RvB1 and RvB2, IES2, and IES6, there are several species specific subunits for the Ino80 complex. For example, yeast have specific subunits: TATA-binding-protein-associated factor 14 (TAF14),

high mobility group (HMG) domain-containing non-histone protein 10 (NHP10) and four additional IES (INO Eighty Subunits): 1, 3, 4, and 5. In contrast, there are different metazoan-specific subunits, including Gli-Kruppel zinc finger transcription factor Ying-Yang 1 (YY1), nuclear factor related to κB (NFRKB), ubiquitin protease UCH37, forkhead domain associated (FHA) domain-containing MCRS1, pre-B cell acute lymphoblastic leukemia fusion protein TFPT/Amida, and proteins with unknown function FLJ20309 (INO80D), FLJ90652 (INO80E) [112].

INO80 complex is reported to have involvement in multiple processes *in vivo*. INO80 directly occupies several genomic targets in yeast, and mutant strains display transcriptional defects [108, 109]. In human cells, INO80 also contributes to transcriptional regulation of at least some genes regulated by the transcription factor YY1, which is tightly associated with human INO80. Mutation of genes encoding INO80 subunits also interferes with efficient progression of replication forks during DNA synthesis and with maintenance of telomere structure [113-115].

INO80 and Swr-1 have well documented roles in transcription and DNA damage and recombination [37, 108, 109, 111, 116-121]. The inclusion of Ruv-B like helicases as subunits in the complex is highly indicative of the possibility that Ino80 and Swr-1 remodelers evolved to maintain genetic integrity in organisms. This is further supported by the observation that when subunits of Ino80 complex are mutated, it leads to impaired DNA damage response in humans, flies, plants and yeast [108, 122-126]. Ino80 plays an active role in the eviction of nucleosomes proximal to double strand breaks (DSB) and chromatin of the homologous donor locus in an ATP-dependent manner.

Although highly related to INO80, SWR1 does not affect nucleosome eviction at DSBs (Double Strand Break) and is unique in its ability to restructure the nucleosome by removing canonical H2A-H2B dimers and replacing them with H2A.Z-H2B dimers, thereby inserting the histone H2A variant H2A.Z at sites of DNA damage [127]. Histone H2AZ is known to be responsible for sustained recruitment of DNA repair factors such as Rad51, Mec1, and Ku80, to DSBs. These factors are required for non-homologous end joining (NHEJ) [128, 129].

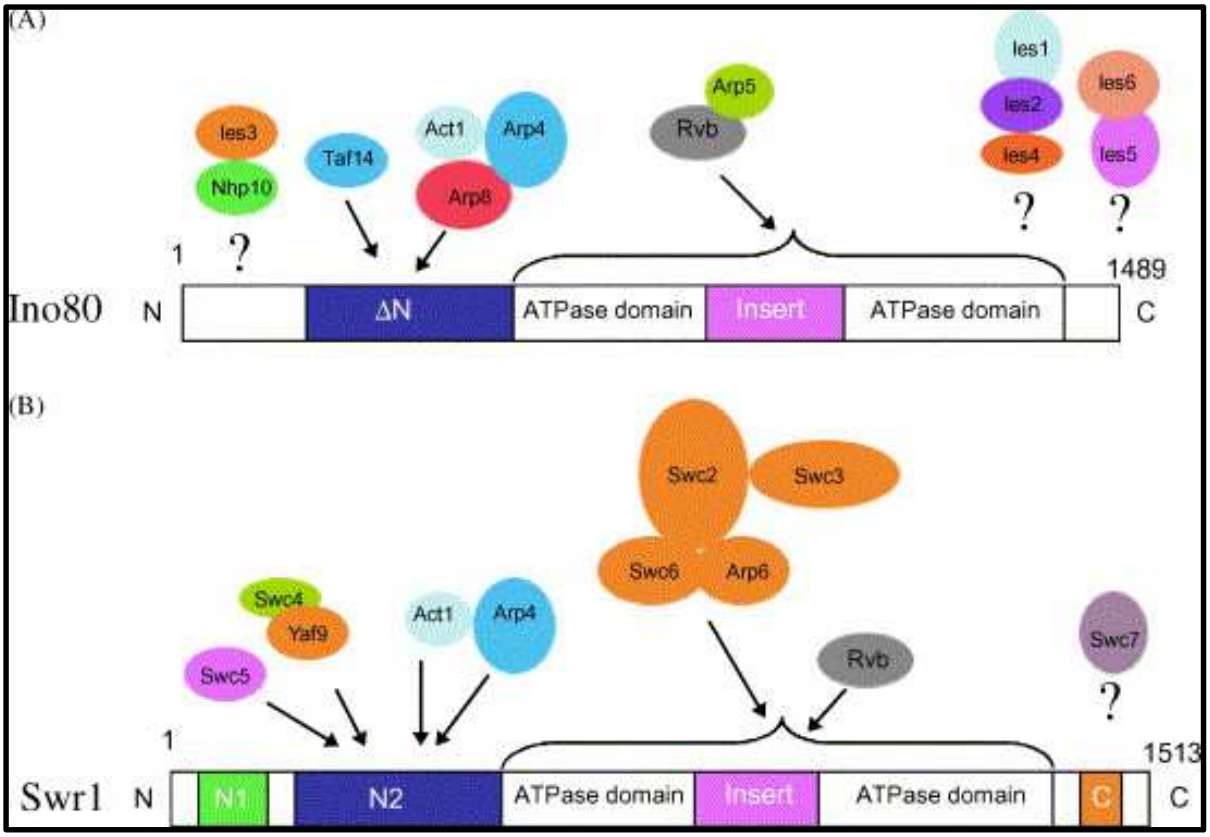


Figure 15: Schematic model of subunit interactions of the INO80 and SWR1 complexes. (A) INO80 subunits and their interactions. (B) SWR1 subunits and their interactions. The known interactions between subunits and ATPases are shown by arrows, while the question marks indicate unknown interactions. Figure adapted from [130]

Accessory domains of SNF2 ATPase

Chromatin modifiers and remodelers change chromatin structure and direct nucleosome dynamics to regulate gene expression. Accessory proteins explain the diverse functionality of the same core ATPase subunit repurposed in several gene regulatory processes. Interestingly, the recognition of which nucleosome needs to be modified and in which direction is dependent on the recognition of the covalent modifications and other epigenetic marks on those nucleosomes. In order to recognize these modifications, the ATPase domain of these catalytic subunits is flanked by binding domains that recognize specific modifications (Fig. 16) [43]. These domains act to target the ATPase and in some cases even regulate the ATPase activity. Whether these domains act in a concerted fashion with other domains and subunits or act as independent modules, is an area of active research.

Binding domains on SNF2 ATPases associate with specific chemical modifications on peptides that are present either on the histone tails or other nuclear proteins (Fig. 17). The bromodomain, a characteristic feature at the C-terminal region of the ATPase of SWI/SNF family of remodelers, binds acetylated lysines in histones and other proteins [131]. The bromodomain is present in both the versions of SWI/SNF complexes present in yeast, flies and humans. The *yeast* RSC contains multiple bromodomains on several proteins, while the *Drosophila* PBAP and the *human* PBAF possess multiple bromodomains on a single protein (polybromo). The existence of multiple bromodomains may suggest to the co-operative ATPase targeting functions of these domains to their substrates.

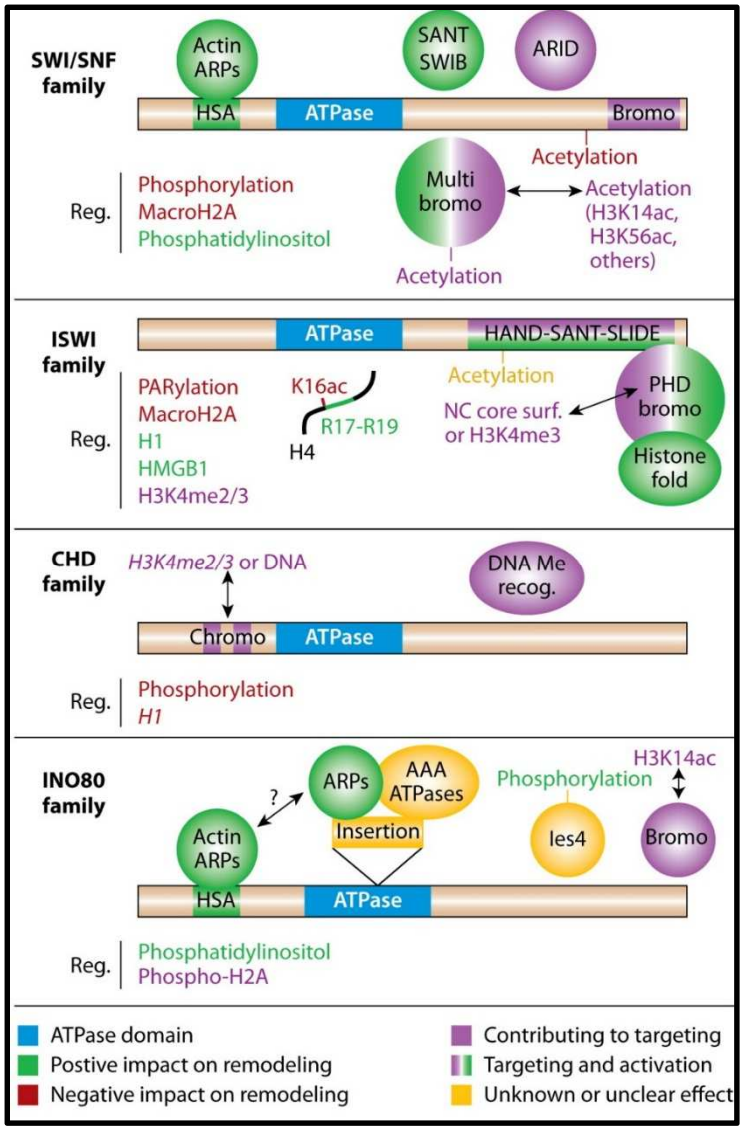


Figure 16: Domains, proteins, and modifications that regulate remodelers. Remodeler regulation is an area of increasing complexity. Depicted here is the ATPase domain itself (*blue*) along with the proteins, domains, and modifications that are involved in remodeler regulation. Regulatory (Reg.) modifications and proteins that positively impact remodeling activities are in green, those negatively affecting remodeling activities are in red, and those contributing to targeting are in purple. Yellow is used for features with unclear or unknown effect. Objects implicated in both activation and targeting are depicted as objects with a green-purple gradient. CHD remodelers can be monomeric or multimeric, with italicized text only for the monomeric form. Figure adapted from [43].

Whether bromodomains are simply involved in targeting or have an impact on remodeler efficiency, or both remains to be understood. Histone acetylation has been linked with increasing remodeling efficiency by SWI/SNF complexes and RNAPII elongation by yRSC [132, 133]. The bromodomains also play a key recognition role, as they can interact with specific acetylated residues. The yRSC4 interacts with the histone tail modification H3K14ac (Lysine14 acetylation on histoneH3) *in vitro* and promotes gene activation *in vivo* [134]. Bromodomains are also present on some ISWI remodelers but have yet to be shown to associate with any particular histone or protein substrate.

The chromodomains, characteristic tandem repeats present on the C-terminal region of the ATPase of CHD family, bind one methylated lysine. The tandem repeat chromodomains function as a single structural unit for binding their substrate. hCHD1 tandem chromodomains bind H3K4me2 or me3, histone tail marks correlated with active chromatin [135, 136]. The tandem chromodomains of Mi-2 are implicated in the recognition of DNA rather than methylated histone tails [137]. Mi-2 mutants lacking the chromodomains fail to bind or remodel nucleosomes. Importantly, to note that the tandem chromodomains of CHD1 are separated by a small patch of highly basic residues known as the “chromo-wedge”. This region of the protein has been shown to directly regulate the ATPase by splaying open the two lobes of the ATPase and competitively inhibiting substrate (DNA) binding of the SNF2 ATPase motor [138]. Another methyl-lysine interacting motif present in several remodelers is the PHD finger or the Plant HomeoDomain. The PHD finger of dNURF stabilizes its interaction with active chromatin that has been marked with H3K4me3 tail modifications [139]. The PHDs have also been implicated in binding the globular domain of core histones and improve the effectiveness of nucleosome mobilization as in the case of dACF1 [140].

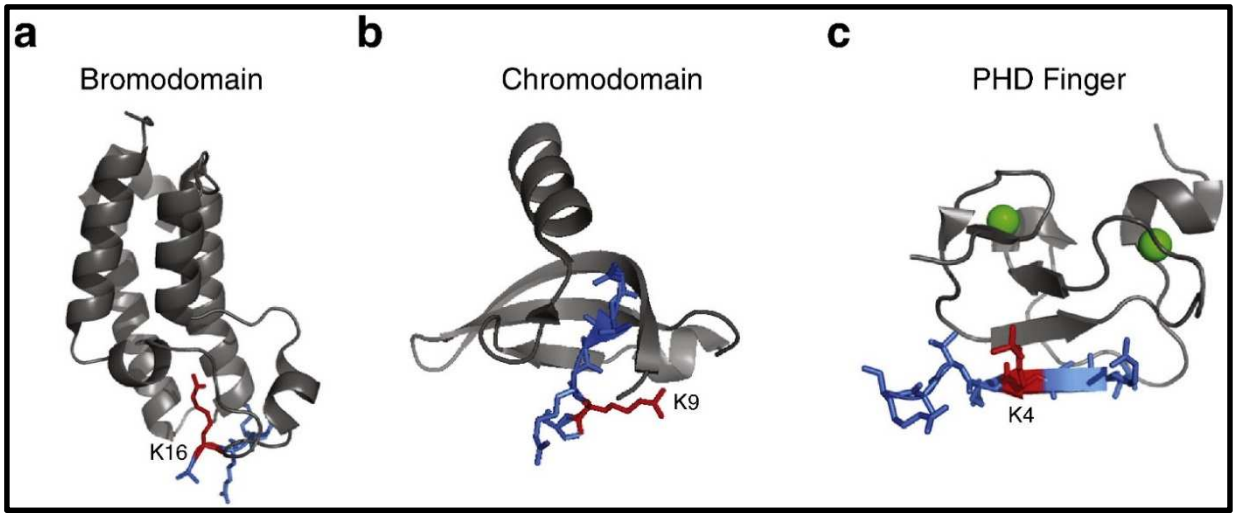


Figure 17: Chromatin remodeling enzymes have specific domains that bind histone modifications. (a) Bromodomains bind acetylated lysines. The bromodomain of Gcn5 bound to an H4 tail peptide acetylated at K16 (PDB: #1E6I). The bromodomain is in gray, the histone peptide in blue, and the modified lysine in red. (b) Chromodomains bind methylated lysines. The chromodomain of HP1 protein Swi6 bound to an H3 tail peptide methylated at K9 (PDB: #1KNA). The chromodomain is in gray, the histone peptide in blue, and the modified lysine in red. (c) PHD fingers bind methylated lysines. The PHD finger domain of ING2 bound to an H3 tail peptide methylated at K4 (PDB: #2G6Q). The PHD finger is in gray, the histone peptide in blue, the modified lysine in red, and the zinc ions are in green. Figure adapted from [131].

The ISWI family is accessorized with three tandem domains, HAND-SANT-SLIDE (HSS), C-terminal to the SNF2 ATPase, that form an extended dumbbell-like structure with two globular domains connected by a spacer helix [141]. The SANT domain (SWI3, ADA2, N-CoR, TFIIIB B") was recognized to be structurally similar to the DNA binding tandem repeats of homeodomain helix-loop-helix motifs of c-myb DNA binding domain (c-myb DBD) [142]. The minimal myb DBD is composed of two repeats and thus a single SANT domain was thought to contribute to general DNA affinity. Recent crystal structure data and SANT's lack of key residues that contact DNA in c-Myb suggested that SANT domains function not as a DNA binding domain but rather as a domains that bind unmodified histone tail motif [80, 141, 143]. In

conjunction with HAND and SLIDE (SANT-like domain), the HSS motif binds extranucleosomal DNA that flanks the nucleosome. The C terminus of Isw2 remodeling complex of the ISWI family, comprising the SLIDE (SANT-like domain) and HAND domains, was found to be associated with extranucleosomal DNA and the entry site of nucleosomes [144]. Together, the HSS domain of Isw2 is involved in anchoring the complex to nucleosomes through their interactions with linker DNA and they facilitate the movement of DNA along the surface of nucleosomes. dISWI ATPase mutants with a SLIDE domain deletion have no appreciable remodeling or ATPase activities, whereas the SANT domain deletion retains low activity [141]. Thus this unique combination of tandem HAND, SANT and SLIDE domains, which is exclusive to the ISWI family of remodelers, cooperatively achieves nucleosome recognition and stimulation of ATPase activity.

In addition to the above, there are other domains which bind non-histone substrates, such as the macrodomain. The SNF2ATPase Alc1 possesses a macrodomain C-terminus to the ATPase. At the time of Alc1's discovery, its substrates were not well characterized. This changed with the recognition of the macrodomain present at the C-terminus of the proteins. The macrodomain has been shown to bind poly-ADP ribose (PAR) and the monomeric ADP (Adenosine diphosphate)-ribose (ADPR) *in vitro* and *in vivo* [145]. This discovery directly connected Alc1, a SNF2 ATPase remodeler, with NAD^+, PAR and effector proteins such as Poly ADP-ribose Polymerase 1 (PARP1) that modify nuclear proteins including histones with ADP ribosylation or Poly (ADP-ribosyl)ation (PARylation) modifications.

PAR – Poly ADP-ribose

PAR is a homopolymer of ADP-ribose (derived from NAD^+ *in vivo*) units linked by glycosidic ribose-ribose 1'-2' bonds [146-149] (Fig. 18). These polymers are synthesized both *in vitro* and *in vivo* and can be extremely heterogenous, reaching lengths of 200-400 units [150]. Long polymers are speculated to form helicoid secondary structures similar to the structures of DNA and RNA, which may be of biological significance [151, 152]. The polymers are not strictly linear and irregularly branch after every 20-50 ADP-ribose units [150, 153-156].

PARylation, in the cell comes in two flavors. First, it can occur as free poly ADP-ribose, which has been suggested to play an important role in stress-dependent signaling processes [157-160]. The large majority of poly-ADP-ribose species are covalently attached to acceptor proteins. Protein ADP-ribosylation can be divided into two subtypes: mono-ADP-ribosylation (MARylation), where only mono-ADP-ribose (MAR) is transferred to an amino acid of a target protein, and poly-ADP-ribosylation (PARylation), which involves the transfer and elongation of the initial ADP-ribose moiety to generate PAR. In both reactions, the acceptor site is initially MARylated, followed by an elongation reaction in the case of PARylation. MARylation is more prevalent in cells than modification by PARylation [161-163].

Schiff base formation with the ε-amine of lysines, and ester formation with carboxyl groups of aspartic acid and glutamic acids is the primary mode of covalent PAR attachment to these acceptor residues. Protein-bound PAR and MAR can be completely hydrolyzed by different enzymes, thus generating free ADP-ribose and free PAR. Most stress-induced free or protein-associated PAR in cells is degraded to ADP-ribose within minutes, accounting for its transient nature in living cells [162]. Of all the PAR found in cells, greater than 90% of it is

found in the nucleus, covalently attached to PARP1 [164]. Lysine, glutamic acid and aspartic acid are the most frequently PARylated residues. However, there is a lack of consensus on the precise residue that is PARylated, making it difficult to predict PARylation patterns among acceptor proteins [165]. This might be largely due to the nature of this modification as there is varibality in the acceptoe residues or the inability to detect it in mass spectrometry due to PAR's heterogeneity; or due to the instability of the ester bond between aspartic and glutamic acid residues under basic pH [166].

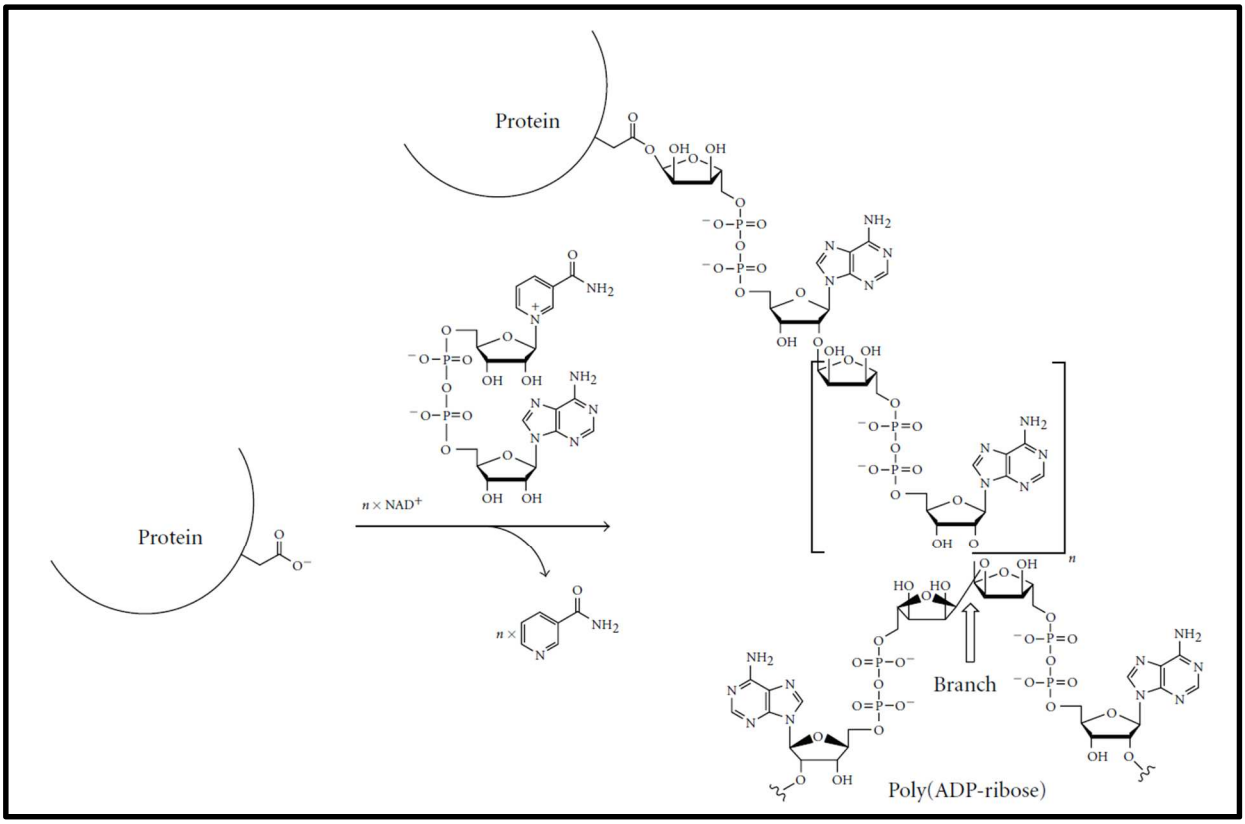


Figure 18: Poly(ADP-ribosyl)ation. PARPs cleave the glycosidic bond of NAD^+ between nicotinamide and ribose followed by the covalent modification of acceptor proteins with an ADP-ribosyl unit. PARPs also catalyze an adduct elongation, giving rise to linear polymers with chain lengths of up to 200 ADP-ribosyl units, characterized by their unique ribose (1″→2′) ribose phosphate-phosphate backbone. At least some of the PARP family members also catalyze a branching reaction by creating ribose (1‴→2″) ribose linkages. Figure adapted from [167]

MARylation or PARylation are the products of the same multi step enzymatic process. The reaction steps are as follows 1) Initiation: Covalent modification of the acceptor protein by Parp. 2) Elongation: Addition of ADPR units to the previously attached mono-ADP-ribose. Whether this elongation is bimolecular, in *trans*, is debatable. 3) Branching: Polymer can be branched due to the nature of the reaction or the enzyme catalyzing the reaction. 4) Release of covalently bound PAR chains from the acceptor protein by the enzymatic activity of Poly-ADP-Ribose Glycohydrolase (PARG), the only known human enzyme to catabolize PAR [168]. Parp1 and Parp2 can facilitate autonomous initiation, elongation and branching of PAR. The strongest stimulation of PARP1 activity is mediated by its binding to DNA strand breaks, which induces its catalytic activation as a monomer or dimer by several hundred-fold [169-172], under these conditions PARP1 accounts for >75% of the overall cellular poly(ADP-ribosyl)ation capacity [173, 174]. Apart from direct DNA damage-dependent PARP1 activation, its activity is also regulated by posttranslational modifications such as phosphorylation, acetylation, and sumoylation and direct protein-protein interactions [175-182]. It has been shown that most of the PAR generated upon genotoxic stimuli is rapidly degraded, with a half-life of 40s to 6 min [169, 183, 184]. This efficient PAR turnover is mediated by poly(ADP-ribose) glycohydrolase (PARG). PARG hydrolytically cleaves PAR chains and releases mono(ADP-ribose) as the main reaction product [183, 185]. Substrates reported to be PARylated include PARP family members (through auto-modification), histones (H1, H2A, H2B, H3, H4), HMG and LMG (High/Low Mobility Group) proteins, p53, PCNA, CTCF, DNA polymerase alpha, telomeric repeat binding factor-1, Centromeric-binding proteins such as CENPA, CENPB, Bub3, MARCK5, F52/GAP43 and Topoisomerases I and II amongst more than 200 nuclear proteins proposed to be covalently modified by PAR *in vitro* [186].

PAR binding Domains

Poly(ADP-ribosyl)ation is involved in cellular signaling *via* specialized protein domains that specifically recognize and bind to different forms of ADP-ribose and/or a part of the PAR polymer. This binding is mediated *via* at least four evolutionary conserved PAR-binding modules that have been discovered within numerous proteins with various function different PAR binding motifs [154, 156, 187-190]. Those include 1) a 20 amino acid PAR binding motif (PBM); 2) distinct macrodomains; 3) WWE domain and 4) a PAR-binding zinc finger (PBZ). The 20-aa motif has been identified in several hundred human protein sequences. This weakly conserved motif consists of 1) a cluster rich in basic amino acids and 2) a pattern of hydrophobic amino acids interspersed with basic residues [187, 190]. The PAR-binding macrodomains simply referred to as the macrodomain and PAR binding zinc fingers are quite rare and have been found on less than fifty human proteins to date.

PAR Binding Motif (PBM)

The PBM was the first discovered protein motif with the ability to recognize and bind PAR and consists of only 8 amino acids with the following sequence: [HKR]xx[AIQVY]-[KR]-[KR]-[AILV]-[FILPV] [191, 192]. Given the loose definition of PBM, it is difficult to predict this motif from genome sequences with a high degree of certainty. Genome-wide scans reveal that the PBM is predicted to be present in more than 800 proteins. Such broad presence of the PBM implies its general role in PAR mediated processes, although the majority of its roles are yet to be confirmed experimentally. The PBM is postulated to form an electro-positive surface

using its basic residues that allows it to bind the negatively charged PAR polymer in a non-specific manner [193].

Poly(ADP-Ribose)-Binding Zinc Finger (PBZ)

The PBZ is a specific and high affinity PAR-binding module using the motif consensus sequence [K/R]xxCx[F/Y]GxxCxbbxxxxHxxx[F/Y]xH [194]. The PBZ is restricted to only eukaryotic proteins (excluding yeast) and only three mammalian proteins have been identified to possess the PBZ domain (APLF, CHFR and SNM1A). In mammals, PAR recognition by the PBZ motifs of CHFR and APLF has been shown to be indispensable for their function in mitosis and DNA repair, respectively [194, 195]. APLF possesses tandem PBZ domains that could independently bind PAR, but the presence of both functional PBZ domains results in more than a 1000 fold increase in PAR binding affinity [196, 197]. The solution structure of the APLF PBZs also revealed that this family of zinc fingers are the most similar in structure to ssRNA-binding C_3H_1 Tandem Zinc Fingers (TZF). Both the PBZ and TZF fingers largely lack secondary structure, and the recognition of the substrate is achieved through main-chain-base hydrogen bonding and the interaction with highly conserved aromatic residues [196] (Fig. 19). The structures of the APLF and CHFR PBZs suggest that a single PBZ module contains two binding sites that can simultaneously recognize adenines in two neighboring ADP-ribose units of the PAR polymer. This recognition makes the PBZ a highly specific PAR binding module [198].

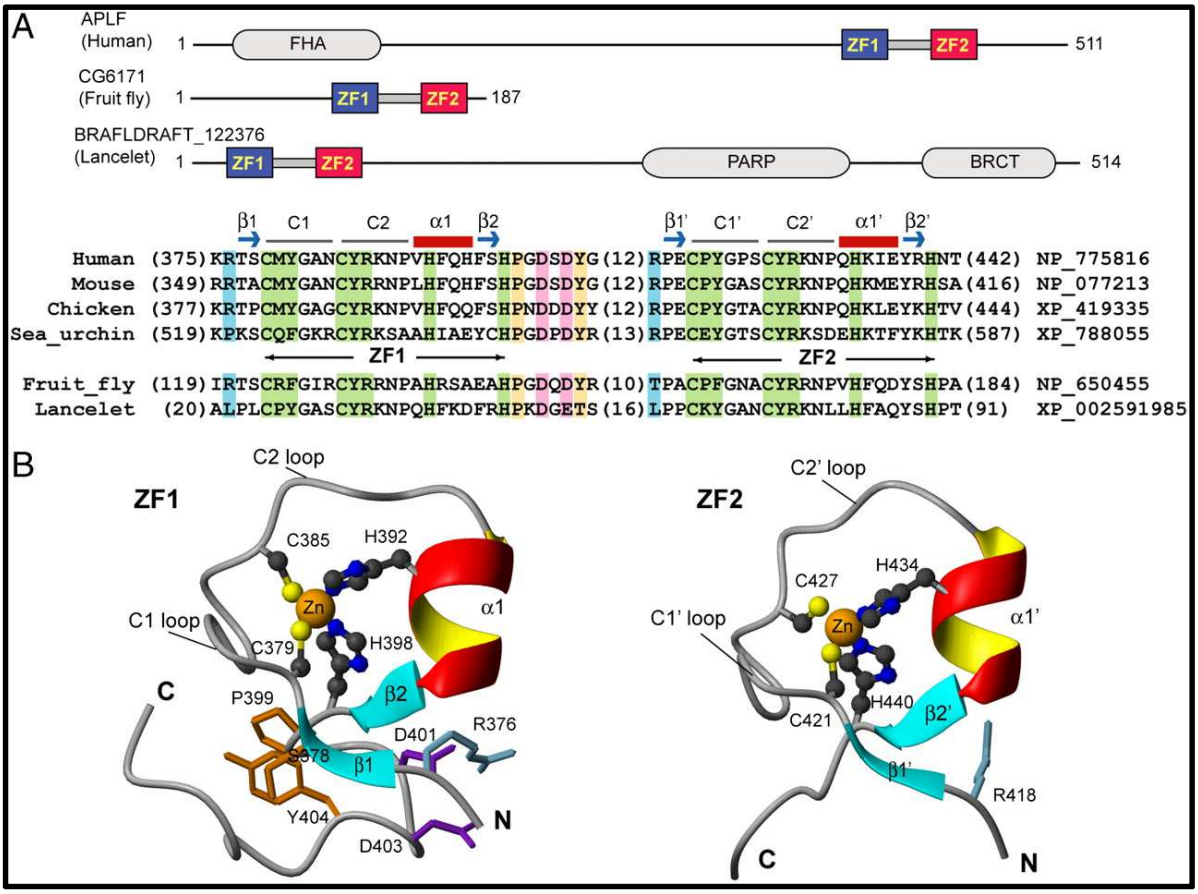


Figure 19 Structure and alignment of APLF PBZ domains. APLF PBZ domains comprise unique zinc finger folds. (*A*) Schematic representation of APLF and all other known tandem PBZ-containing proteins with domain organization, and alignment of PBZ amino acid sequences. (*B*) NMR-derived structure of the APLF PBZ domains (ZF1 and ZF2) and linker region. Figure adapted from [196].

WWE Domain

The WWE domain contains two conserved tryptophan residues and a glutamate residue. This domain was first noted in proteins related to either ubiquitination or poly(ADP-ribosyl)ation [199]. RNF146 ubiquitin ligase E3, or Iduna, contains a functional PAR binding WWE domain that helps binding to proteins targeted for proteosomal degradation through PAR recognition and

their subsequent polyubiquitination by RNF146 [200-202].The interaction between RNF146 and poly(ADP-ribosyl)ated proteins such as PARP1, XRCC1, DNA ligase III, KU70, KU86, histones and axin promotes their degredation [201]. RNF146 does not bind mono-(ADP-ribose), but interacts with iso-ADP-ribose with high affinity. Iso-ADP-ribose represents a minimal PAR fragment that contains a specific 2',1''-*O*-glycosidic ribose-ribose bond unique to the PAR polymer. The phosphate groups on each side of the iso-ADP-ribose molecule interact with the highly positively charged edge of the WWE domain binding site, while the adenine ring is inserted in the binding pocket formed by the half β-barrel and the α-helix. This mode of binding explains the specificity of the WWE domain for iso-ADP-ribose and consequently PAR [200].

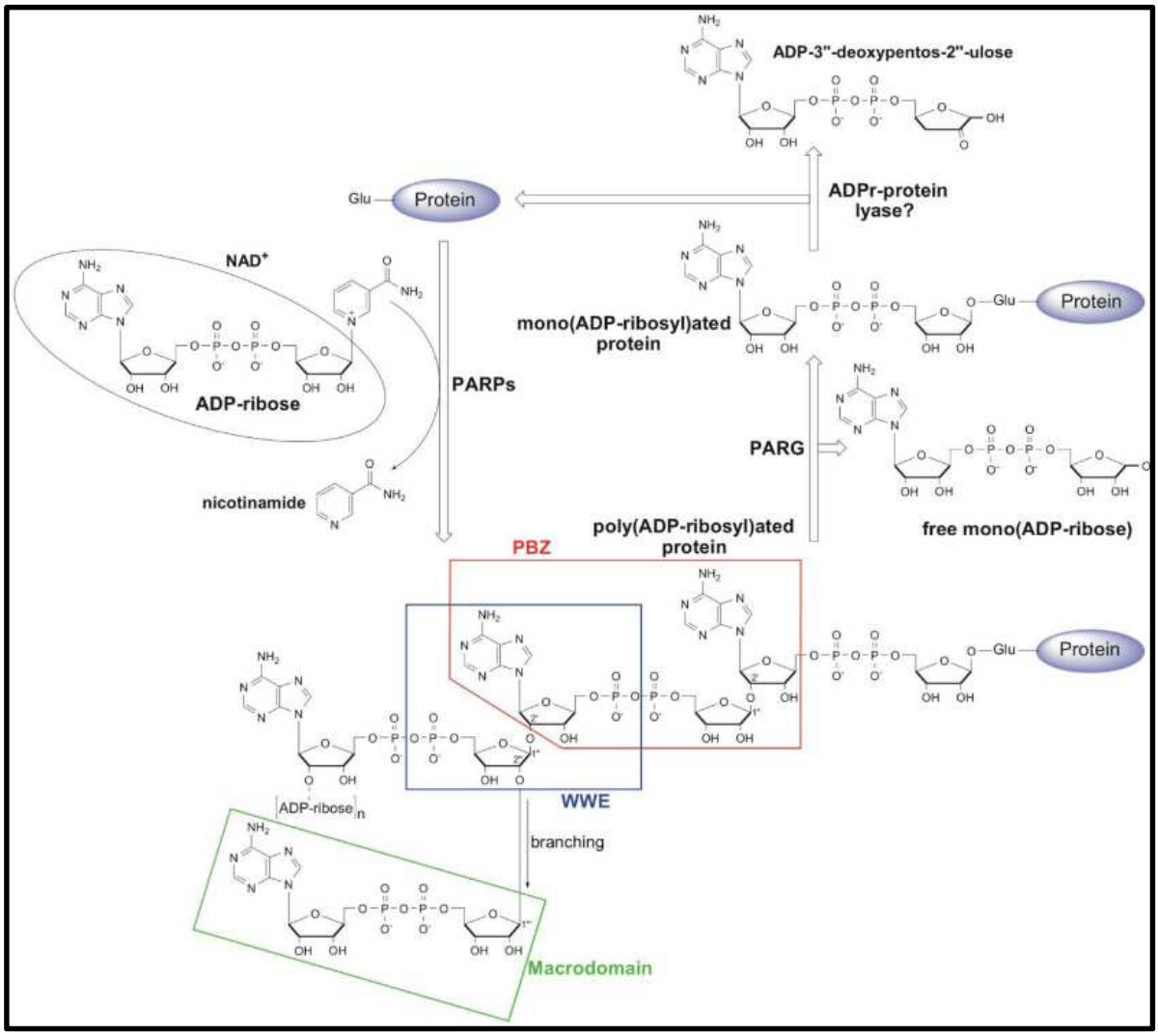


Figure 20: Regions of PAR recognized by different domains. Schematic representation of poly(ADP-ribosyl)ation (PAR) metabolism. The first ADP-ribose is attached *via* an ester linkage to the glutamate on the target protein. The subsequent transfer of additional ADP-ribose molecules through the 2',1"- and 2",1'"-O-glycosidic bond leads to the synthesis of linear and branched PAR polymers, respectively. PARG cleaves PAR chains and releases mono(ADP-ribose), whilst the proximal ADP-ribose is removed by ADP-ribosyl protein lyase. Regions of PAR recognised by Poly(ADP-Ribose)-Binding Zinc Finger (PBZ), WWE and macrodomain PAR-binding modules are boxed. Figure adapted from [203]

Macro domain

The macro domain (also referred to as the macrodomain) is an ancient, highly evolutionarily conserved structural module of 130-190 amino acids capable of binding metabolites of NAD^+ including PAR [204] (Fig. 21). Unlike the PBZ and WWE domains, the presence of macrodomains are widely distributed across all kingdoms of life, including prokaryotes, archaea and some single-stranded RNA viruses that replicate in animal cells. Three are currently >300 proteins in the SMART database that contain a macrodomain [205]. Macrodomains were first discovered in 1992 with the cloning and isolation of macroH2A1, a histone variant highly similar to canonical histone H2A except for the presence of a C-terminal macrodomain [206]. MacroH2A1 has been shown to have alternatively spliced forms that either bind (macroH2A1.1) or do not bind (macroH2A1.2) NAD^+ metabolites [207]. There are 10 genes that encode 11 members of the macrodomain family in humans. These proteins include the macro H2A1.1 and its splice variant macroH2A1.2, MacroD1, MacroD2, C6orf130, MacroD3, Alc1, and macroPARPs Parp9, Parp14 and Parp15 (Fig. 22) [208]. The crystal structure of the macrodomain of Af1521, found in the thermophilic archaebacterium *Archaeoglobus fulgidus,* and macroH2A revealed the general structural fold and the ligand binding pocket of this PAR binding module [145, 207, 209-211] (Fig. 22). The Af1521 macrodomain containing protein, found in the thermophile *Archaeoglobus fulgidus*, exhibits a high affinity for ADP-ribose, as isothermal titration calorimetry suggests an equilibrium dissociation constant (K_D) of 126 nM. The macrodomain affinity for ADP-ribose was nearly 45-fold greater than observed for ADP [145] (Fig. 23).

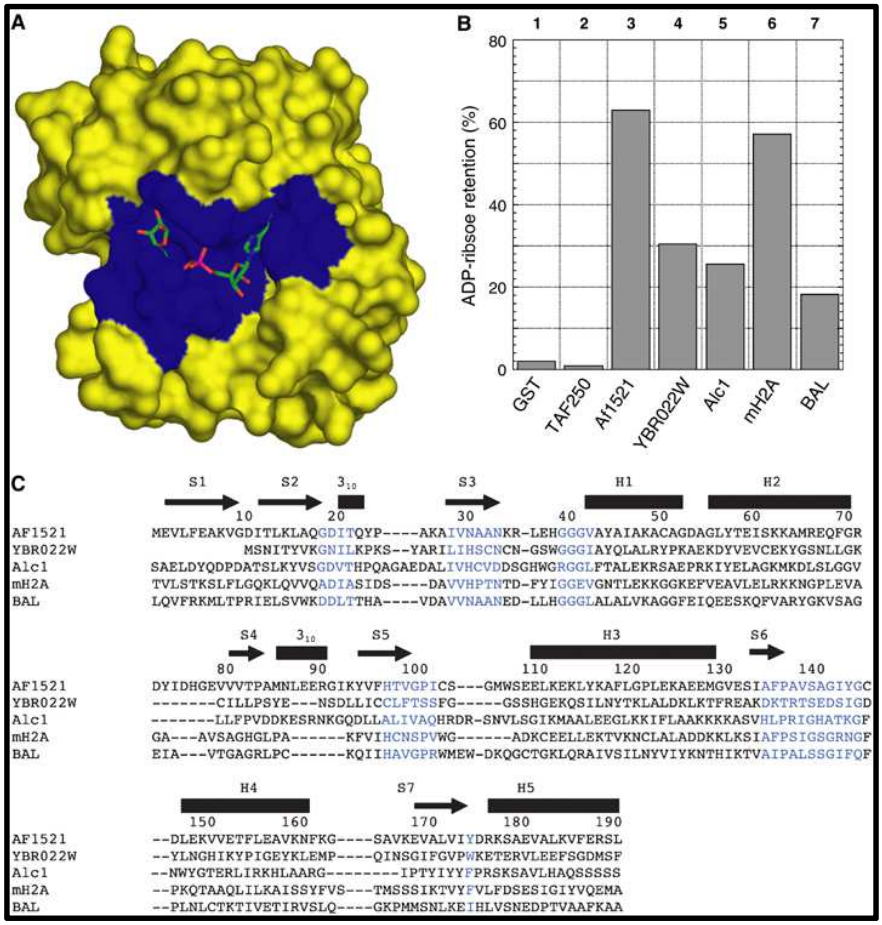


Figure 21 ADP-ribose binding is a conserved feature among *macro* domains. (**A**) Sequence and structure conservation in the *macro* domain family of proteins. Regions with the highest sequence conservation among *macro*domain proteins are shown in blue. These include residues (19–22), (29–34), (40–43), (98–103), (136–146) and 176. (**B**) Structure-guided alignment between select *macro* domain proteins. The alignment was generated using the output of a Blast search, and refined manually on the Af1521–ADP-ribose complex structure. Residues shown in blue correspond to the region colored in blue of panel (A). (**C**) Pulldown assays for the binding of ADP-ribose to yeast and human *macro* domains. Distinct *macro* domain proteins (*A. fulgidus* Af1521, *S. cerevisiae* YBR022W, human Alc1, human macroH2A and human BAL/PARP9) were fused to either GST protein or to a histidine-tag. In all, 30 nmol of fusion proteins was immobilized to a solid support, including two control proteins that should not interact with ADP-ribose (GST and a GST-fusion of the $TAF_{II}250$ (hTAF1) double bromodomain module, and incubated with 30 nmol of ADP-ribose in solution. Following the incubation, the samples were centrifuged and the amount of ADP-ribose that remained in the supernatant was estimated by absorbance measurements. The graphs show the percentage retention on the beads (calculated by subtracting the percentage of ADP-ribose that remained in the supernatant from 100%). The pulldown assay shows that all tested *macro* domain proteins retain some ADP-ribose. Figure adapted from [145].

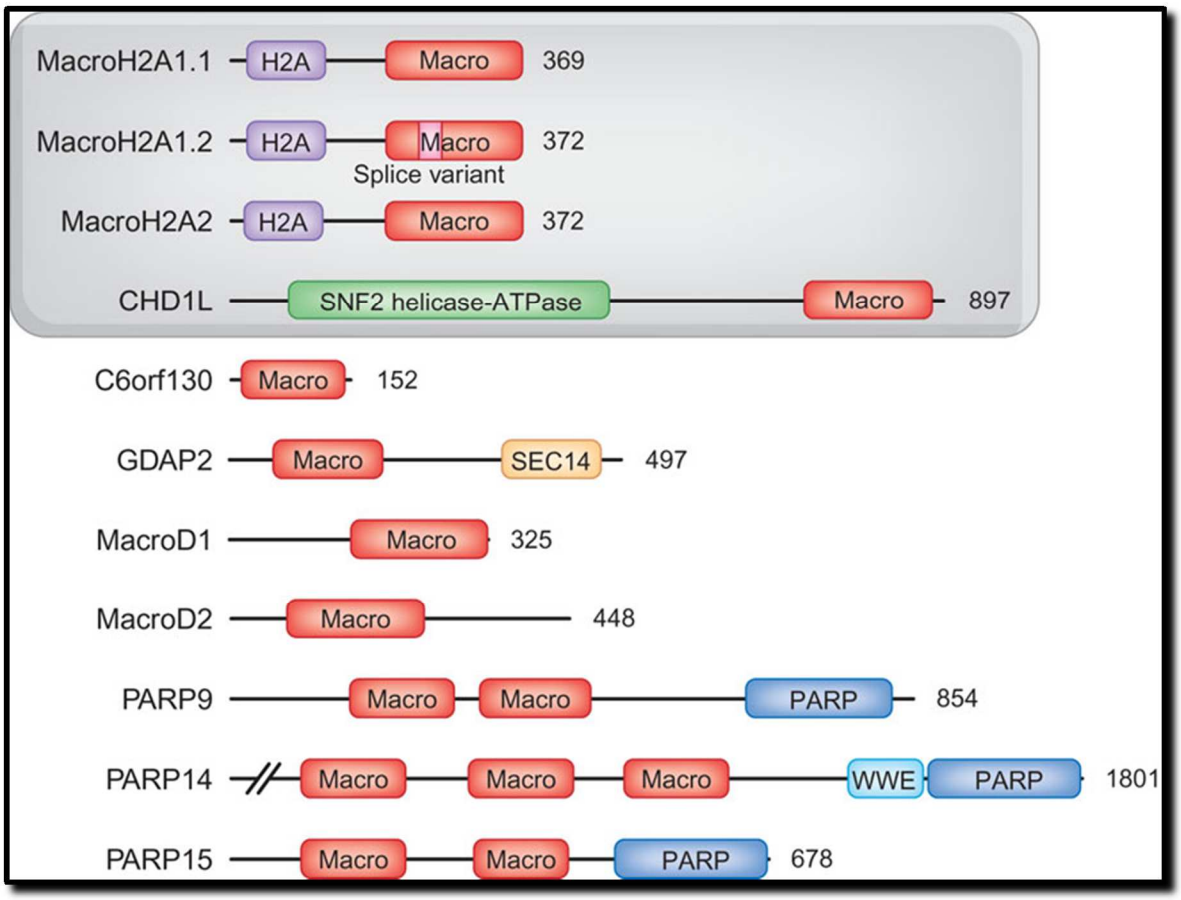


Figure 22 Macrodomain-containing proteins in humans. The human genome has at least 10 genes encoding 11 macrodomain-containing proteins (including the known splice variants of macroH2A1), with a total of 15 distinct macrodomains. Macrodomains are often combined with other functional domains in proteins, including the following: an H2A histone fold domain (H2A) that allows assembly into nucleosomes; an SNF2 helicase-ATPase domain that confers ATP-dependent nucleosome-remodeling activity; an SEC14 domain that may allow lipid binding; a PARP-homology domain that confers PARP enzymatic activity; and a WWE domain that functions as a common protein-protein interaction module in protein-ubiquitylation and ADP-ribosylation reactions. Figure adapted from [208].

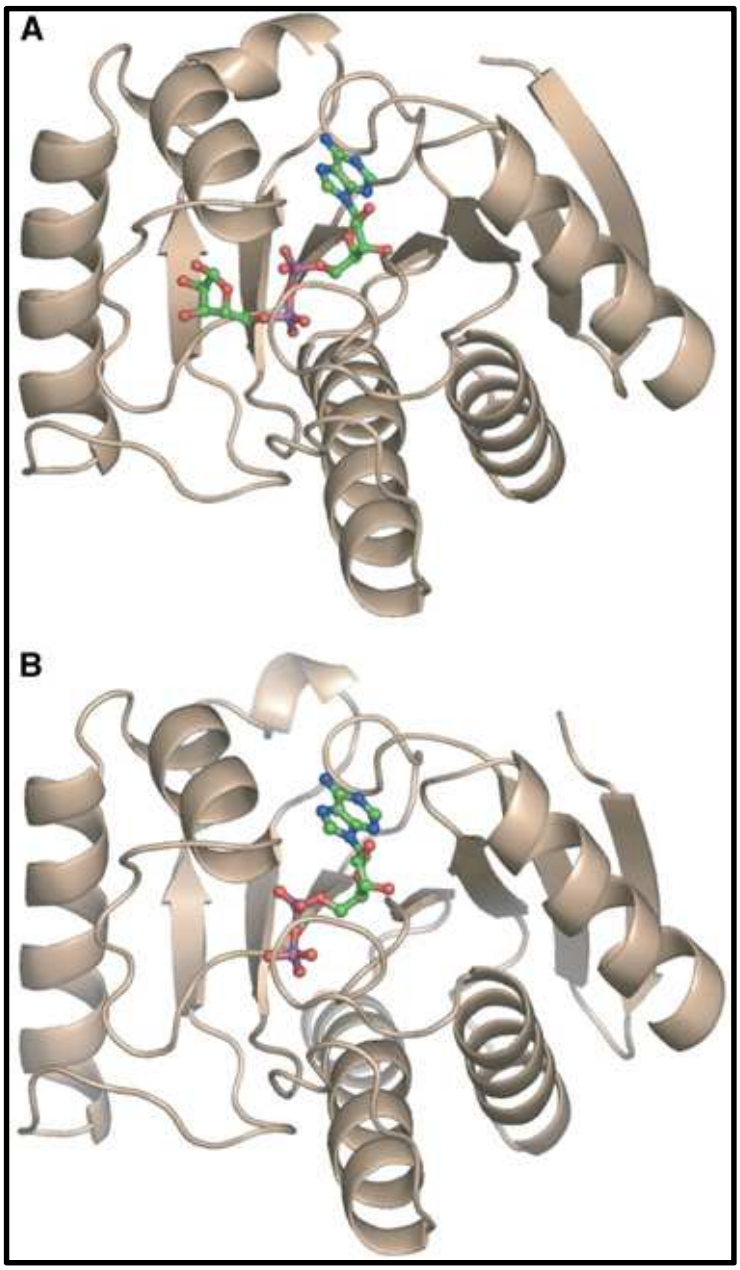


Figure 23: Structure of the complex formed between Af1521 and ADP-ribose. (**A**) The ADP-ribose molecule binds the Af1521 *macro* domain in an L-shaped cleft. The ADP-ribose ligand is shown as a ball-and-stick model. (**B**) Structure of the complex between Af1521 and ADP. The structure is highly similar to that of the complex between Af1521 and ADP-ribose, but a number of interactions that contribute to ADP-ribose specificity and affinity cannot occur. The ADP ligand is shown as a ball-and-stick model. Figure adapted from [145].

The ‘macro fold’ is roughly 25 kDa in size and is composed of a globular mixed α-helix/β-sheet structure, with similarity to the P loop-containing nucleotide triphosphate hydrolases with a deep L-shaped ligand binding pocket [212]. There is a high degree of sequence similarity within this family, particularly for residues that might be involved in catalysis or substrate binding. However, it is likely that the sequence variation that exists among macro domains is responsible for the specificity of function of individual proteins (Fig 24). Despite the high structural conservation, the sequence variation between macrodomains directs their preferences for binding specific forms of ADP-ribose. Histone H2A variant MacroH2A1.1 contains a macrodomain through which it is able to bind PAR, mono(ADP-ribose), as well as *O*-acetyl-ADP-ribose [207, 209]. The structure of histone macroH2A1.1 in complex with ADP-ribose suggests that the macrodomain is only able to bind the terminal ADP-ribose units of the PAR polymer [209]. Selectivity for ADP-ribose binding by macrodomains, determined through crystallographic studies, is in part due to coordinated interactions of aspartic acid residues with the distal ribose group in ADP-ribose [145, 207].

Macrodomains are unique among PAR binding modules, as some have been shown to exhibit catalytic activity and can hydrolyze phosphate groups from nucleotides or ADPR derivatives, suggesting that the domain may be evolving to provide new functions. The first reported catalytically active macrodomains which dephosphorylate ADP-ribose-1’’-phosphate, are viral macrodomains including the severe acute respiratory syndrome coronavirus (SARS-CoV) protein and the yeast homolog Poa1p (YBR022) [213-215]. The eukaryotic MacroD proteins (orthologues of human MacroD1 and MacroD2 proteins and the human C6orf130 protein deacetylate *O*-acetyl-ADP-ribose *in vitro* [210, 211]. Recently crystal structures have

revealed that the PAR hydrolyzing enzyme PARG, has a catalytically active macrodomain fold for the binding and hydrolysis of PAR [216].

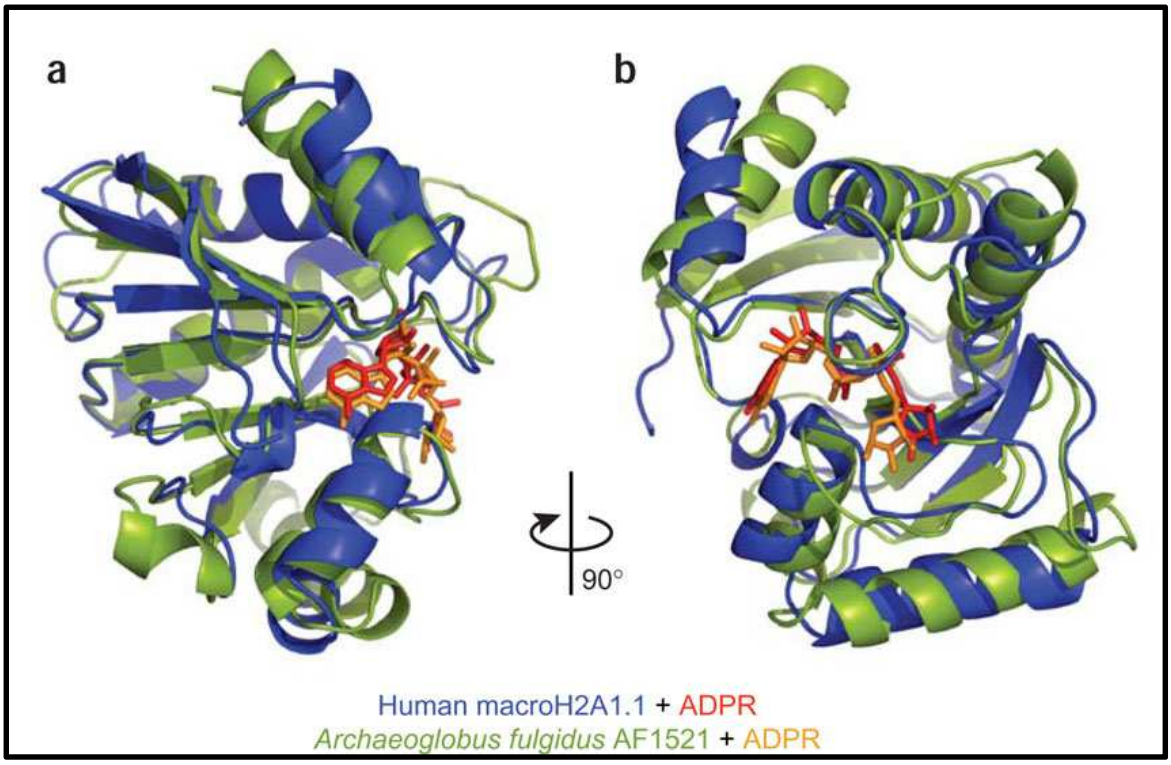


Figure 24 Macrodomain are ancient and highly conserved structural domains that bind metabolites of NAD$^+$. X-ray crystal structures of the macrodomains from *Archaeoglobus fulgidus* (Archaea) AF1521 protein (PDB 2BFQ) and human macroH2A1.1 bound to ADP-ribose (PDB 3IID). The two views are rotated 90° relative to each other. Figure adapted from [208].

The PARP superfamily and its roles

The PARP family of proteins consists of 17 PARPs in humans (PARP-1–17) [217]. PARPs are composed of functionally distinct domains, such as 1) a DNA-binding domain that enables PARPs to bind to RNA and damaged DNA 2) a catalytic domain that is responsible for polymerization of ADP-ribose and 3) accessory domains to support interaction with proteins or NAD^+ metabolites (for detailed overviews see 2, 4 and 5). PARPs are involved in DNA repair processes, transcriptional regulation, cell death in oxidative stress related pathologies, and metabolic and immune regulation [218].

PARP-1 is the founding member of the PARP family and has been the most extensively studied. PARP1 uses NAD^+ as a substrate to catalyze the covalent attachment of ADP-ribose units on the γ-carboxyl group of Glu residues of either acceptor proteins that are usually associated with DNA transactions (heteromodification) or on PARP-1 itself (automodification), in response to DNA-strand breaks. PAR produced by PARP1 can be linear or branched and of variable size that can interact in a selective manner with a number of protein targets that are involved in the cellular response to DNA damage and DNA metabolism. Both Parp1 and its close relative Parp2 play a significant biological role in response to distinct stress response pathways [219, 220]

Crystal structures of the catalytic domains of chicken PARP-1 and mouse PARP-2 show remarkable structural homology with the active site of the ADP-ribosylating *Corynebacterium diphtheria toxin* [221, 222]. The most conserved region in Parp1 orthologues is the β-α-loop-β-α NAD^+ fold, and this fold is considered the PARP signature. This signature was used to search

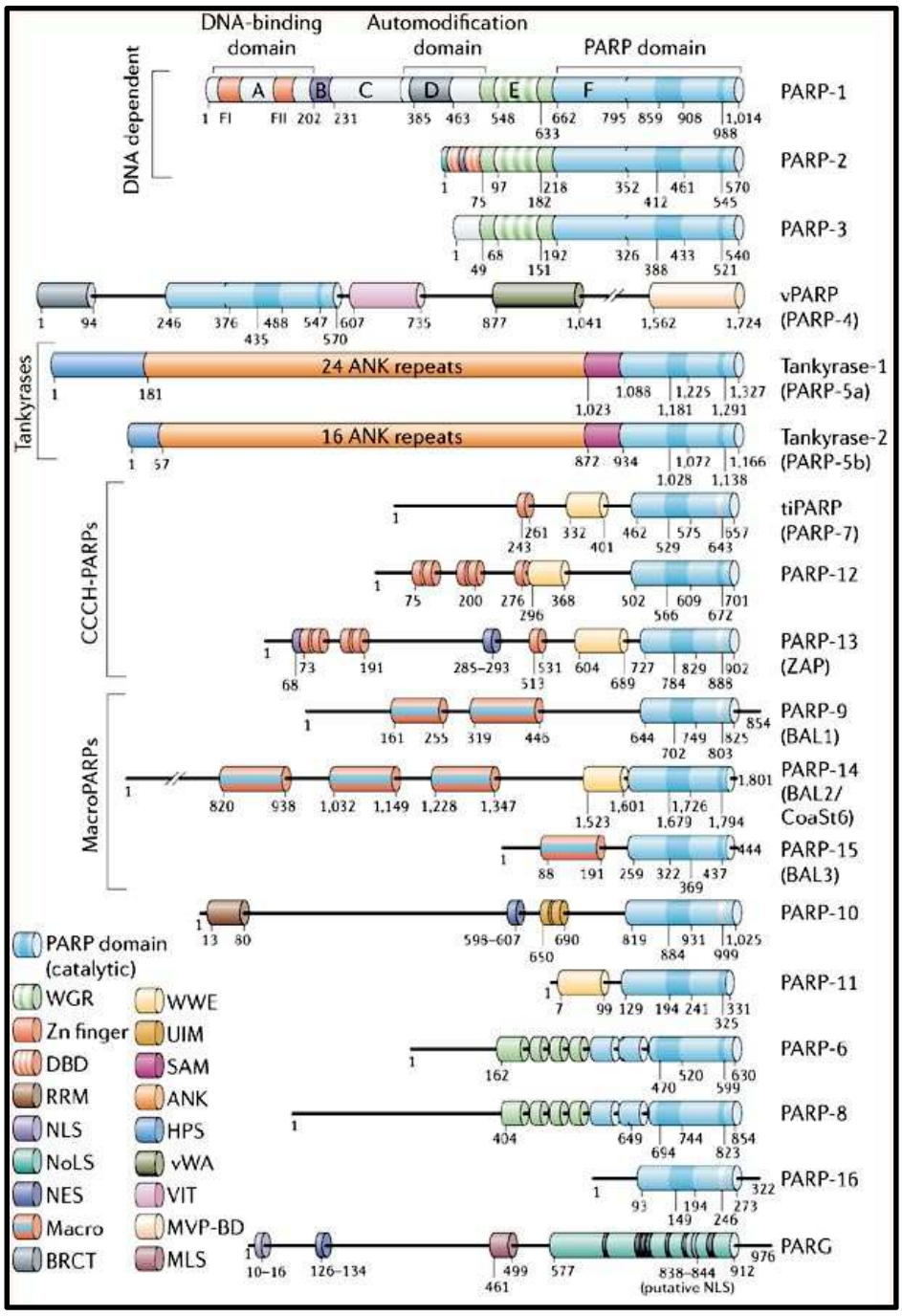


Figure 25: The domain architecture of the 17 members of PARP superfamily and PARG. Protein domains that are illustrated by colored boxes were defined according to the Pfam 19.0 or CCD v2.06 (National Center for Biotechnology Information) databases. Within each putative PARP domain, the region that is homologous to the PARP signature (residues 859–908 of PARP-1) as well as the equivalent of the PARP-1 Glu988 catalytic residue (when present) are darkened. Accessory domains present on PARP are also shown. Figure adapted from [223].

for other human PARP homologues and new putative PARPs were identified, thus increasing the number of PARP-family members to 17 (Fig. 25) [219].

The conservation in the PARP signature varies significantly among the PARP-family members. The catalytic residue, Glu988 of PARP-1, is replaced by a non-conserved residue in some members (PARP7, PARP-9/BAL1, PARP-10, PARP-13 and PARP-16). Whether these proteins have poly- or mono-(ADP-ribosyl)ating activities or may not possess PARylating activity is an area of active research. To date, only PARP-9/BAL1 has been reported to be inactive, whereas poly (ADP-ribosyl)ating activity has been reported for PARP-7 and PARP-10 [224-226].

The PARP domain is located at the C terminus of all the PARP proteins, except in the case of PARP-4 where it is located close to the N-terminus and is adjacent to various domains that are involved in DNA or RNA binding, protein–protein interactions or cell signaling. PARP family members can be divided into subgroups according to their putative functional domains, sequences of their catalytic domains, and their enzymatic activities.

The PARP superfamily can be divided into four groups; 1) DNA-damage-dependent PARP subfamily that comprises the founding member of the family Parp1 and Parp2 [220, 227], 2) the Tankyrase subfamily consisting Parp-5a and Parp-5b, which are characterized by an Ankyrin repeat domain, are involved in telomere homeostasis, and are both reported to be components of the telomeric complex [219, 228, 229], 3) the CCCH-type zinc-finger Parp subfamily consisting of tiPARP, PARP-12 and PARP13 that share similar domain organization comprising $CX_8CX_5CX_3$-like zinc fingers, a WWE domain and a catalytic PARP domain [225,

230-232] 4) the MacroPARP subfamily including PARP-9/BAL1, PARP-14/BAL2/CoaSt6 and PARP-15/BAL3, belonging to the subfamily of macroPARPs, which link 1–3 macro domains to a PARP domain. Macro domains of PARP-9/BAL1 have been shown to bind ADP-ribose and PAR [145].

In addition to the above mentioned classification, a few PARPs are unique enough to have their own classification. One such example is PARP4 or vault-PARP/vPARP, the largest member of the PARP family. PARP-4/vPARP is a catalytic component of vault particles, barrel-shaped ribonucleoprotein complexes that are involved in multidrug resistance of human tumours thought to function in intracellular transport [233]. Parp4 knockout mice display normal vaults but are susceptible to carcinogen-induced colon tumourigenesis, suggesting PARP-4's role in the response to genotoxins [234]. PARP-3 preferentially associates with the daughter centriole within centrosomes and is implicated in the maturation of daughter centriole until the G1-S restriction point [235]. PARP-10 is a partner of the proto-oncoprotein c-Myc, a key transcriptional regulator that controls cell proliferation [224]. PARP-10 PARylates histone H2A, suggesting that it has a role in chromatin regulation [224]. No known domains have been found in PARP-6, PARP-8, PARP-11 and PARP-16 so far (except a WWE domain in PARP-11), which makes it difficult to speculate on their possible functions.

Despite sequence similarity in the catalytic domains between PARP family members, only PARP-1, PARP-2, and tankyrases are known to produce long PAR polymers. Other PARP enzymes are defective in elongation or are inactive [165]. Hence, the majority of both basal and stimulated cellular PARP activity is attributed to PARP-1 (85–90%) and PARP-2 (10–15%). PARP-1 and PARP-2 can inhibit themselves through self-PARylation, also known as

autoPARylation. These two proteins can also modulate the biochemical activity of a plethora of proteins through trans-PARylation [236]. While all members of the PARP family are reported to have autoPARylation activity, PARP1 has the strongest such activity *in vitro* [174, 227-229, 235]. Both PARP1 and PARP2 have been shown to be automodified within their DNA-binding domains, and PARP1 has further been shown to be automodified within its "automodification domain" (AD) [237, 238]. Recently, there has been some disagreement within the field regarding the identity of Parp1 residues targeted for automodification. The earliest reports suggested that 25 to 30 glutamic acid residues within the automodification domain were targeted for poly-ADP-ribosylation. However, it was shown that loss of these glutamic acid residues had no effect on automodification of Parp1 *in vitro* [190]. Lysine to arginine mutations of residues K498, K521, and K524 within the AD of Parp1 strongly reduced the automodification of the enzyme, suggesting these residues may be acceptors for PAR [190]. Interestingly, these residues are in close proximity to the catalytically active site. It has been proposed that the automodification of Parp1 on lysine residues is catalyzed by its NADase activity and results in a glycation linkage to the lysine residue and formation of Lys-ADP-ribose ketamine[186]. It is this Lys-ADP-ribose ketamine intermediate that has been proposed to serve as an acceptor for the elongation reaction catalyzed by glutamic acid residue E998 in human Parp1 [186].

PARP1 and its roles in the nucleus

PARP1 is a versatile enzyme involved in many processes in the cell. PARP1 was first found to play a very active role in DNA damage repair. Using PARP1 inhibitors, researchers were able to establish a firm link between PARP1 activation, NAD^+ depletion and PARylation in cells suffering from DNA damage [239-241]. Inhibition of PARP1 has been shown to increase cytotoxicity through an increase in the half-life of DSBs [242-246]. Inhibition of PARP1 results in accumulation of single and double-strand breaks that are normally repaired through homologous recombination (266-269), or sister chromatid exchange [247-249]. Inhibition of PARP1 has also been shown to promote carcinogenic induced gene amplifications, increased rate of apoptosis and resulting decrease of cellular necrosis [250-254].

PARP1 is actively associated with repair of DNA damage by the base excision repair (BER) pathway and has been shown to interact with multiple components of the BER pathway, including XRCC1, DNA ligase III, and DNA polymerase β [255, 256]. XRCC1 and other proteins such as CHFR and APLF are recruited and can bind directly to PAR, suggesting that PARylation activity itself could contribute to the recruitment of required machinery [194]. Another piece of evidence that indirectly supports the role of PARP1 in the repair of SSBs (single strand break) is that the presence of NAD^+ both *in vivo* and within cell-free DNA BER (base excision repair) assays is necessary for proper repair [257]. *PARP1*$^{-/-}$ mice are known to be extremely sensitive to γ-irradiation. *PARP1*$^{-/-}$ mice are also extremely sensitive to exposure to *N*-methylnitrosurea and cell lines derived from these *PARP1*$^{-/-}$ mice have difficulty proceeding through mitosis after being treated with DNA-damaging agents and rapidly undergo apoptosis [258, 259].

PARP1 is thought to bind electrostatically with DNA ends, SSB lesion and DSB lesions. Binding of PARP1 to DNA ends covers a region of seven nucleotides on each side of the lesion, suggesting that PARP1 binds to DNA strand breaks as a dimer [260, 261]. PARP1 activity is differentially induced by SSBs and DSBs. The amount of PAR synthesized by PARP1 bound to DSBs is far less than SSBs [262, 263]. This indicates that the level of PARylation can directly have an effect on the DNA repair pathway that is subsequently induced. It is important to note that PARylation activity is not required for excision of damaged bases and the subsequent resynthesis of DNA [264-266].

Extensive modification of PARP1 itself and of surrounding chromatin may act as a signal to downstream effectors, such as p53, which are involved in cellular responses to DNA damage but do not participate directly in repair reactions [267]. p53 function is regulated through cellular PARylation as PARP1 inhibition with chemicals significantly suppresses the accumulation of p53 in response to ionizing radiation [268-270]. Moreover, *PARP*$^{-/-}$ cells have considerably lower levels of p53 in the absence of genotoxic stress [271, 272]. Modification of chromatin allows PARP to be involved in gene regulatory processes. Histones are PARylated in response to DNA damage, suggesting PARP1 may play a role in DNA repair through its activity on chromatin [273-275]. Extensive PARylation of chromatin has been shown to yield greater accessibility of DNA to nucleases

Perhaps the most interesting roles of PARP1 and its associated enzymatic activities are their regulation of chromatin structure in both the presence and absence of DNA damage. PAPR1 can covalently modify histones *in vivo* and the subsequent PARylation of histones is thought to contribute to destabilization of nucleosomes and, in turn, to affect higher-order

chromatin structure by decondensation [276-278]. Highly charged PAR moieties have been proposed to strip DNA off basic proteins like histones [277, 279, 280]. It has been reported that all histones can be modified [267] and histones H1 and H2B act as the major acceptors for modification by PARP1 and PARP2 [276, 278].

Recent studies have attempted to characterize the effects of PARP1 on chromatin structure using various techniques. The Kraus lab reported that PARP1 incorporates into chromatin by virtue of its inherent nucleosome binding properties and, in the absence of NAD^+, promotes the formation of compact, transcriptionally repressed chromatin structures highly similar to chromatin structure formed in the presence of histone H1 [281, 282]. However, in the presence of NAD^+, PARP1 automodifies itself and promotes the decondensation of chromatin structures *in vitro*, allowing transcription to occur. These studies show that PARP1 activation can be achieved independent of DNA damage. PARP1 modification of chromatin does not need PARylation of chromatin.

The regulation of chromatin structure by PARP1 has been show to contribute directly to transcriptional regulation [267, 283, 284]. PARP1 is speculated to be recruited to target promoters *via* DNA-binding transcription factors. Once recruited to the target promoter, PARP1 can contribute to transcriptional regulation by acting as a promoter-specific coregulatory factor. Several DNA-binding transcriptional regulators such as NF-κB, nuclear receptors, Hes1, B-Myb, HTLV Tax-1, Sp1, NFAT and Elk1 have been reported to be involved in this process [220, 282, 284-289].

The interplay between PARP1 and histone H2 is very interesting as histone H2 is the only histone with variants that bear a functional PAR binding macrodomain. PARP1 is enriched

at promoters correlated with depletion of histone H2, and those promoters with high PARP1/histone H1 ratio were found to be mostly actively transcribed genes. A PAR binding variant of macroH2A, known as macroH2A1.1 is suppressed in cancer and can interact with PAR generated by PARP1. This macroH2A1.1 protein was recently shown to promote the CBP-mediated acetylation of H2B K12 and K120 that positively or negatively regulate the expression of macroH2A1-target genes in a PARP1 dependent manner. This macroH2A1-regulated H2B acetylation regulation is intact in primary cells, but typically lost in cancer cells [290].

The macroH2A1.1 protein associates with PARP1 through its PAR binding macrodomain *in vivo* and *in vitro* and has been shown to block PARP1 enzymatic activity *in vitro* [291]. Recent studies have demonstrated that macrodomain of macroH2A can promote recruitment of PARP1 to the Xist RNA. The macrodomain of macroH2A1.1, is among the several macrodomain-containing proteins like ALC1 and macroH2A1.2, is recruited to sites of PARP1 activation induced by laser-generated DNA damage (Fig. 26). It has been shown that histone macroH2A1.1 senses PARP1 activation, transiently compacts chromatin, reduces the recruitment of DNA damage factor Ku70–Ku80 and alters γ-H2AX patterns. Whereas the splice variant macroH2A1.2, which is deficient in poly-ADP-ribose binding, does not mediate chromatin rearrangements upon PARP1 activation. This suggests that the chromatin modification achieved by PARP1 and macroH2A1.1 are directly dependent on a catalytically active PARP1 and a functioning PAR binding macrodomain [291].

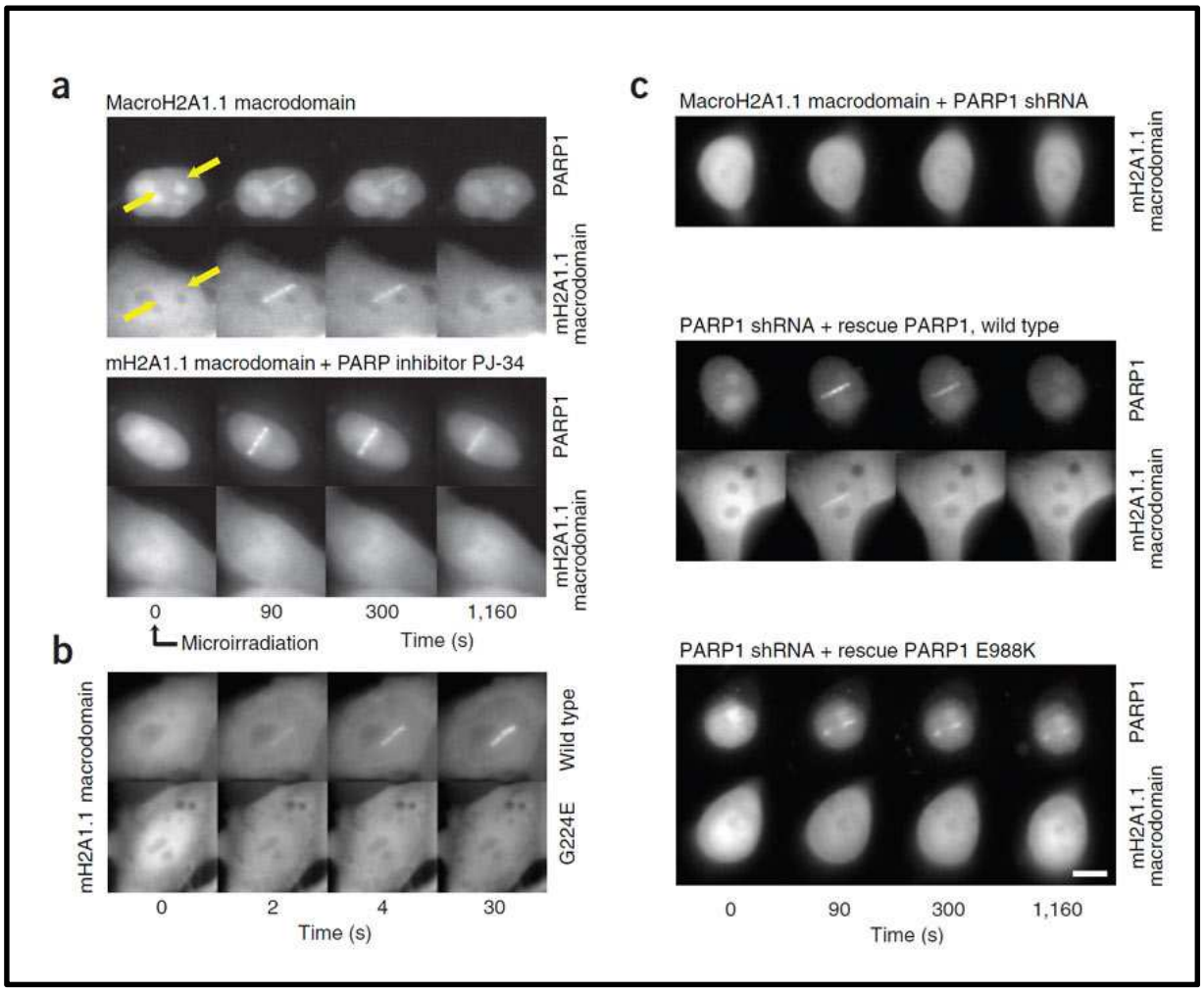


Figure 26 The macroH2A1.1 macrodomain is recruited to in vivo PARylation sites. (a) HeLa cells transfected with enhanced yellow fluorescent protein (EYFP)-tagged macroH2A1.1 macrodomain and mCherry-tagged PARP1 were microirradiated (delimited by two yellow arrows). PARP1 and macrodomain relocalize to PARylated sites (above), with recruitment depending on PARP activity (middle). (b) Mutation of ADPR-binding residues in the macroH2A1.1 macrodomain disrupts recruitment. (c) Catalytically active PARP1 is necessary for macrodomain recruitment. Human AGS cells treated with a PARP1-specific shRNA do not recruit the macroH2A1.1 macrodomain (above). This can be rescued with a wild-type PARP1 construct (middle), but not with the catalytically inactive PARP1 E988K point mutant (below). Scale bar is 5 µm. Figure adapted from [209].

ALC1: A SNF2 ATPase remodeler with a macrodomain

Amplified in Liver Cancer 1 or ALC1; Gene Bank accession number AF537213) has been identified as an oncogene located at Chr1q21 (genomic coordinate: chr 1:146,714,292-146,767,443, strand (+)) [292]. Amplification of 1q21 is one of the most frequent genetic alterations in many solid tumors [246, 293-298]. ALC1 is also known as CHD1L (Chromodomain helicase/ATPase DNA binding protein 1-like) and is encoded by the *CHD1L* gene. Overexpression of CHD1L protein in tumors is considered to be a biomarker of poor prognosis and short tumor-free survival time.

ALC1 exhibits an oncogenic role that is frequently amplified in hepatocellular carcinoma (HCC) and exhibits an oncogenic role during malignant transformation [292] (Fig. 27). ALC1 was found to be amplified in 58%-78% of HCC patients by comparative genomics hybridization. Hepatocellular Carcinoma (HCC) is one of the most frequently diagnosed human cancers affecting more than 1 million individuals annually [299] and has a poor prognosis, with an overall 5-year survival rate of less than 5% [299-301]. Using fluorescent *in situ* hybridization, amplification of *ALC1* was detected in ~50% of HCC cases. *ALC1* mRNA was overexpressed in greater than half of tumor samples taken from HCC patients when compared with their respective non-tumor liver samples. Overexpression of full length *ALC1* cDNA in LO2 human liver cell line promoted an increase in colony formation in soft agar assays [292], demonstrating its tumorigenic abilities. Tumor xenograft experiments in nude mice suggested that *ALC1* could dramatically increase tumorigenicity of immortalized liver cell lines. Further, transgenic *ALC1* expression in mouse induces spontaneous tumors. Nearly 25% of transgenic mice had cancerous lesions while no lesions were found in control mice. These findings strongly suggest that *ALC1*

acts as an oncogene and could be instrumental in the pathogenesis of HCC and other cancers (Fig. 28).

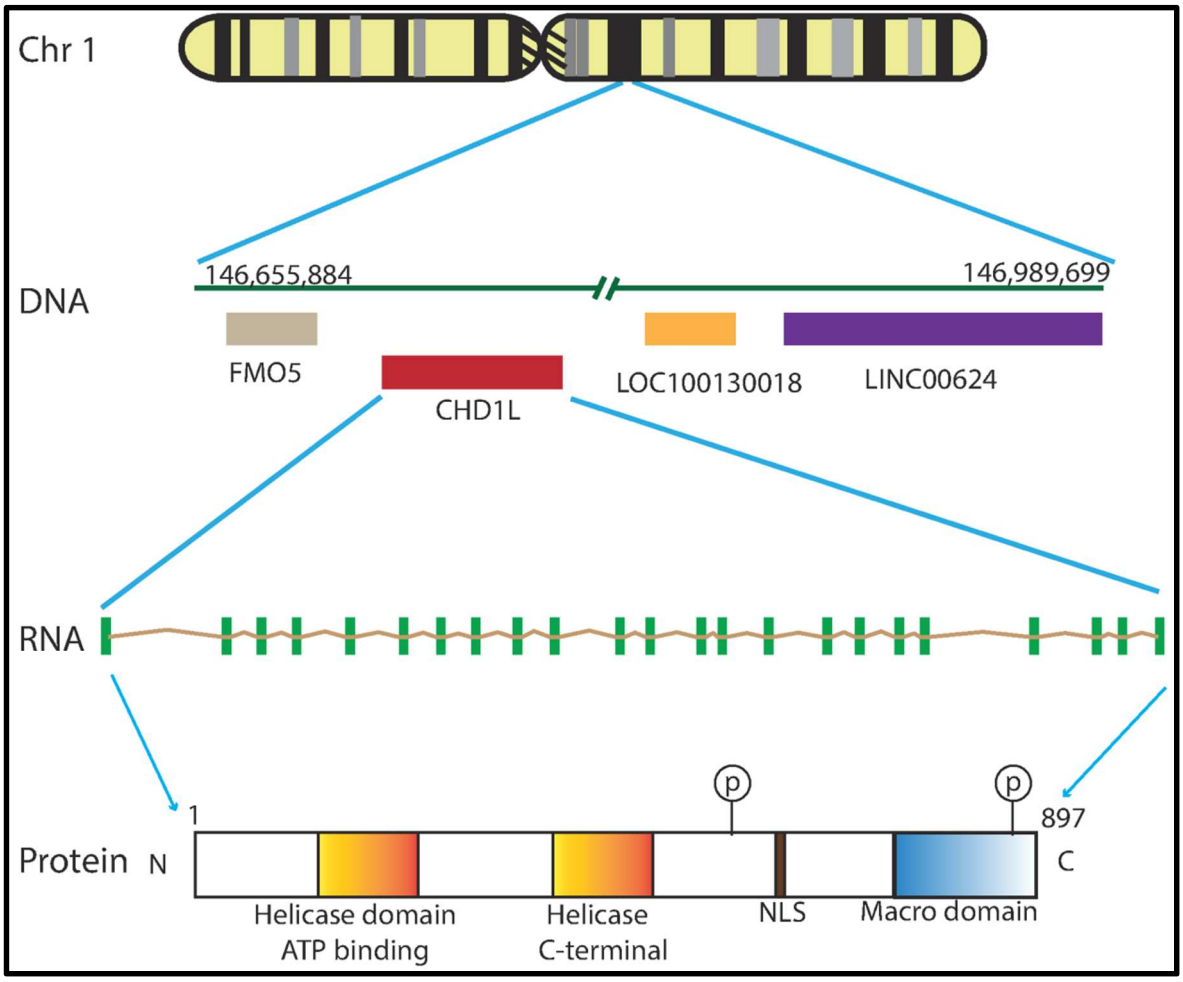


Figure 27 Genomic information of human *CHD1L* gene (chromodomain helicase/ATPase DNA binding protein 1-like gene). The genomic locus of *CHD1L* gene is located on the long arm q21 of Chromosome 1 with 53,152 base pairs in length. It is downstream of *FMO5* gene (flavin containing monooxygenase 5) and upstream of LOC10030018 (prostaglandin reductase 1 pseudo gene) and LINC00624. CHD1L contains 23 exons (green boxes), which may be transcribed with six transcript variants. The full-length transcript (NM_004284.2) and encoded protein structure is illustrated. CHD1L protein is comprised of two helicase domains (yellow color), a C-terminal macro domain (blue color) and nuclear localization sequence (NLS, purple color). There are two putative phosphorylation sites at the relative C-terminus of the protein: phospho-serine at 636 and 891 amino acids, respectively. Figure adapted from [302].

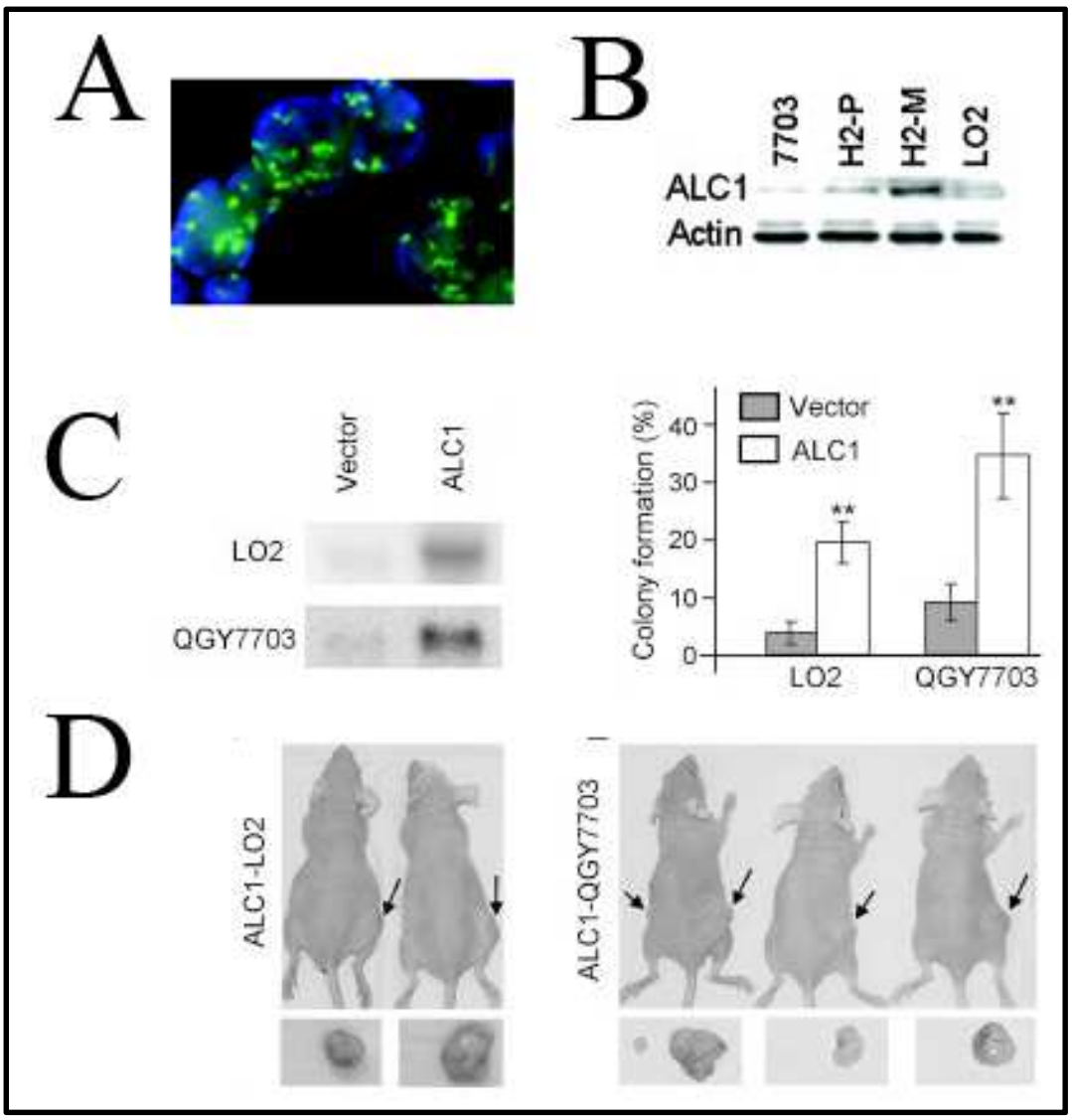


Figure 28 Oncogenic ability of *ALC1* (A) A representative example of *ALC1* amplification in HCC detected by FISH with the BAC clone containing *ALC1*. (B) A 98-kD protein was detected by anti-ALC1 antibody. (C) Expression of ALC1 in *ALC1*-transfected LO2 and QGY-7703 cells detected by northern blot hybridization. Blank vector–transfected cells were used as controls. Left panel shows rates of colony formation in soft agar detected in *ALC1*-transfected and blank vector–transfected LO2 and QGY-7703 cells (**$P < 0.05$). (D) Representative examples of tumors formed in nude mice following injection of *ALC1*-expressing LO2 cells (left) and QGY-7703 cells (right). ALC1-expressing cells and mock cells were injected into the right and left dorsal flanks, respectively. Figure adapted from [292].

Six alternatively spliced transcript variants have been described for the *ALC1* gene, the largest of which, consists of 3036 base pairs with an open reading frame coding an 897aa protein [292]. 64 different mutations on *ALC1* have been reported in the catalogue of somatic mutations in cancer (COSMIC). Among these mutations, the substitution missense mutations that change amino acid residues and may have an effect on function account for 66.67% [302]. Recently, *ALC1* mutations have been detected in congenital anomalies of the kidneys and urinary tract (CAKUT) patient [303]. It is speculated that these mutations have an effect on the functional outcomes of ALC1 in cancer cells. All ALC1 mutations found in CAKUT patients lead to amino acid substitutions in or near the CHD1L macro domain (Fig. 29). Further, these mutants showed decreased interaction with PARP1 by pull-down assay of transfected cell lysates. A recent study that analyzed the coding exons of the 17 known dominant CAKUT-causing genes in a cohort of 749 individuals from 650 families with CAKUT, reported that several mutations previously reported to be disease-causing are most likely benign variants.

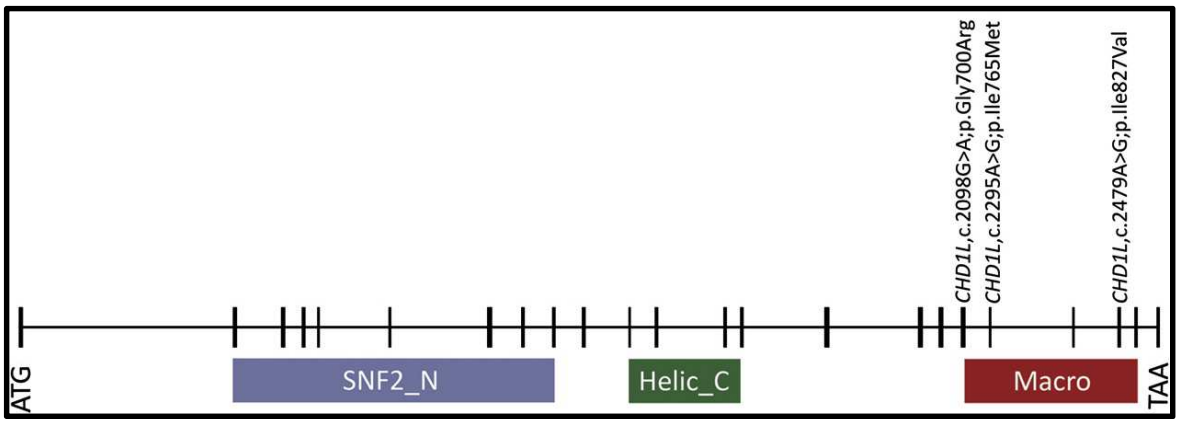


Figure 29 Identification of *ALC1* variants in CAKUT patients. Genomic location of the three missense variants within the *CHD1L* gene: *CHD1L*,c.2098G>A;p.Gly700Arg (exon 18),*CHD1L*,c.2295A>G;p.Ile765Met (exon 19) and *CHD1L*,c.2479A>G;p.Ile827Val (exon 21) relative to the functional Snf2 family N-terminal domain (SNF2_N), the helicase superfamily C-terminal domain (Helic_C) and the macro domain (Macro) of the CHD1L protein. Note that all variants are localized close to or within the macro domain. Figure adapted from [303].

Apart from binding PAR *via* its macrodomain, ALC1 is also able to bind the protein Nur77, a critical member of a p53-independent apoptotic pathway. This binding subsequently inhibits the nucleus-to-mitochondria translocation of Nur77, a key step of Nur77-mediated apoptosis. This binding causes retention of Nur77 protein in the nucleus and results in prevents release of cytochrome c from mitochondria thereby blocking the initiation of apoptosis [304].

There is remarkable sequence homology between the SNF2 domains of ALC1 and its close relative chromodomain helicase DNA binding protein 1 (CHD1). Thus, ALC1 was given the alternative name CHD1L (Chromodomain helicase DNA binding 1-like) [292]. This alternative name is misleading, as unlike CHD1 and all other Chd-type subfamily remodelers, ALC1 is lacking an identifiable chromodomain. Instead of the chromodomain found in CHD-type remodelers, ALC1 has a C-terminal macrodomain. The first preliminary biochemical characterization of ALC1 was achieved simultaneously by the Conaway Lab and the Boulton Lab.

Gottschalk *et al.* showed that ALC1 possesses a PAR-dependent chromatin remodeling and ATPase activity. They showed that the ATPase and chromatin remodeling activities of ALC1 are strongly activated by PARP1 and its substrate NAD^+ through their interaction between an intact macrodomain and PARylated PARP1. They also showed that ALC1 was rapidly recruited to nucleosomes *in vitro* and to chromatin in cells when PARP1 catalyzes PAR synthesis [305].

Ahel *et al.* reported ALC1's ATPase activity was increased markedly in the presence of nucleosomes. This enhanced activity was, however, less pronounced with nucleosomes containing histone H4 mutated at residues 16 to 19 [H4(16-19)A], suggesting that the ALC1

ATPase activity required the histone H4 N-terminal tail. They also showed that the ability of ALC1 to reposition nucleosomes is dependent on the histone H4 tail, as it was not observed with the mutant nucleosome H4 (16-19). To assess the possible consequences of ALC1 overexpression, Ahel *et al* analyzed induction of H2AX phosphorylation (γH2AX, a DNA damage marker) in ALC1-overexpressing and control cells after phleomycin treatment. They found no measurable differences in γH2AX profiles in ALC1-overexpressing cells before phleomycin treatment. However, 80 to 93% of ALC1-overexpressing cells exhibited H2AX phosphorylation upon phleomycin treatment as compared to 44 - 50% in control cells. Phleomycin treatment also showed more damage in alkaline Comet assay in ALC1-overexpressing cells as compared with control cells. This effect was negated by the K77R ALC1 mutation, which renders the enzyme catalytically dead, and was unaffected by PARP inhibitor treatment, suggesting that the phenotype required ALC1 chromatin-remodeling activity but not its recruitment to the sites of DNA damage [306].

The focus of the remaining chapters revolves around ALC1, its interaction with PARP1 and PAR, and domains within ALC1 that can regulate the SNF2 ATPase and associated chromatin remodeling activities. By performing a rigorous biochemical characterization of ALC1, we hoped to gain an insight into the mechanisms that control its activities. Through our work, we find that ALC1 is a unique chromatin remodeler with intrinsic and extrinsic points of control that could functionally repurpose it in different cellular contexts.

CHAPTER II

ACTIVATION OF THE SNF2 FAMILY ATPASE ALC1 BY POLY (ADP-RIBOSE) IN A STABLE ALC1·PARP1·NUCLEOSOME INTERMEDIATE

Abstract:

The human *ALC1*/*CHD1L* oncogene encodes an SNF2 family ATPase with a macrodomain that binds poly(ADP-ribose) (PAR). We and others previously showed that ALC1 possesses a cryptic ATP-dependent nucleosome remodeling activity that is potently activated in the presence of PARP1 and NAD^+, its substrate for PAR synthesis. In this work, we dissected the mechanism by which PARP1 and NAD^+ activate ALC1 nucleosome remodeling. We demonstrate that ALC1 activation depends on the formation of a stable ALC1·PARylated PARP1·nucleosome intermediate. In addition, by exploiting a novel PAR footprinting assay, we obtained evidence that the ALC1 macrodomain remains stably associated with PAR on autoPARylated PARP1 during the course of nucleosome remodeling reactions. Taken together, our findings are consistent with the model that PAR present on PARylated PARP1 acts as an allosteric effector of ALC1 nucleosome remodeling activity.

Introduction

The human *ALC1* (amplified in liver cancer 1; also known as *CHD1L* (chromodomain-helicase-DNA-binding protein 1-like) gene encodes a member of the SNF2 (sucrose non-fermenter 2) superfamily of ATPases. *ALC1* was originally identified as a gene present on a short human chromosome 1q21 region that is amplified in hepatocellular carcinomas [292]. Overexpression of the ALC1 protein was found to transform human cells and to be oncogenic in mice [292, 307]. More recently, the *ALC1* gene was found to be mutated in patients with congenital anomalies of the kidney and urinary tract [303]. Although the mechanism of ALC1 action in these processes is not known, ALC1 has been reported to have roles in DNA repair and in controlling the expression of several genes implicated in tumorigenesis and metastasis [292, 306-308].

ALC1 is unique among SNF2 family members because it includes a macrodomain that is capable of binding selectively to poly(ADP-ribose) (PAR). Previous studies from our laboratory and elsewhere revealed that ALC1 possesses cryptic DNA-dependent ATPase and ATP-dependent nucleosome sliding activities that are strongly activated in the presence of the catalytically active PARP1 and its substrate NAD^+ [305, 306]. Suggesting that binding of the macrodomain to PAR is essential for ALC1 activation, ALC1 macrodomain mutations that prevent PAR binding abolish ALC1 DNA-dependent ATPase and ATP-dependent nucleosome sliding. Nevertheless, we observed that binding of free PAR chains to the macrodomain is not sufficient to activate DNA-dependent ATPase and nucleosome remodeling activities, suggesting that the ALC1 activation process is more complex [305].

In this study, we explored the mechanism by which PARP1 and NAD^+ function together to activate ALC1 nucleosome remodeling and the role of PAR in this process. As described

below, we demonstrate that ALC1 activation proceeds *via* formation of a stable ALC1·PARylated PARP1·nucleosome intermediate. In addition, through development and application of a novel PAR footprinting assay, we obtained evidence that ALC1 activation results from a direct and stable interaction of the ALC1 macrodomain with PAR conjugated to PARylated PARP1. Below, we present these findings, which are consistent with the model that PAR conjugated to PARP1 functions as an allosteric effector that activates ALC1 nucleosome remodeling activity.

The results presented in this chapter result from a collaboration between Dr. Aaron Gottschalk and myself. We each performed reproductions of the experiments performed in Figs 30-32. Dr. Gottschalk and I developed the PARG protection assay used in Fig. 32, and I performed the example shown in the figure.

Results

ALC1 Activation Is Preceded by Rapid Formation of a Benzamide-resistant Intermediate

To begin to investigate the mechanism by which PARP1 and NAD^+ function together to activate ALC1, we sought to isolate and define intermediates in the process. Previous studies using the PARP1 inhibitor benzamide, which prevents PAR synthesis by PARP1, have provided information about the nature of the essential PARP1- and NAD^+-dependent step(s) in ALC1 activation [309]. In these experiments, benzamide was found to block ALC1 DNA-dependent ATPase and ATP-dependent nucleosome remodeling activities when added at the beginning of reactions [305, 306]. This observation, together with evidence that free PAR is not sufficient to activate ALC1, argued that ALC1 activation depends on PARylation of one or more components of the nucleosome remodeling reaction. Indicating that ALC1 PARylation is not essential for ALC1 activation, preincubation of PARP1 and NAD^+ with nucleosomes or with naked DNA prior to addition of benzamide and ALC1 rendered reactions resistant to benzamide [305, 306]. Arguing that histone PARylation does not play an essential role in formation of this benzamide-resistant intermediate, we observed that either nucleosomes or naked DNA can support PARP1- and NAD^+-dependent ALC1 DNA-dependent ATPase activity [305]. Taken together, these findings indicate that a benzamide-resistant intermediate capable of supporting ALC1 activation and containing PARylated PARP1 can form in the absence of ALC1.

To extend our understanding of ALC1 activation, we carried out similar benzamide challenge experiments to explore the kinetics of formation of the benzamide-resistant intermediate. To do so, we preincubated nucleosomes with PARP1 and NAD^+ for varying lengths of time before adding benzamide and ALC1 and assayed for nucleosome remodeling by

monitoring deprotection of a HhaI site located near the nucleosome dyad (diagrammed in Fig. 30A). Consistent with our previous results, addition of benzamide at the beginning of the reaction completely blocked ALC1 activity (Fig. 30B, *lane 3*). Under the conditions of these assays, a benzamide-resistant intermediate that supports ALC1-dependent nucleosome remodeling formed very rapidly in the presence of PARP1 and NAD^+, reaching near maximal levels within 2–4 min (Fig. 30B, compare *lanes 5*, *7*, *9*, *11*, and *13* with *lanes 6*, *8*,*10*, *12*, and *14*, respectively, and Fig. 30C, which shows quantitation of the data from the experiment of Fig. 30B).

To estimate the length of PAR chains synthesized during formation of the benzamide-resistant intermediate in the experiment of Fig. 30B, we performed a parallel experiment in which nucleosomes and PARP1 were incubated for varying lengths of time in the presence of [^{32}P]NAD^+. Reaction mixtures were then treated with proteinase K, and PAR chains were cleaved from the remaining PARP1 protein fragments by base treatment, ethanol-precipitated, and analyzed on denaturing polyacrylamide gels. As shown in Fig. 30D similar amounts of PAR species migrating more rapidly than the position of the *asterisk* (estimated length of 12–15 ADP-ribose units) were synthesized after 0.5 min, a time point at which only a small amount of benzamide-resistant intermediate had formed, and after 4 min, a time sufficient for formation of enough intermediate to support near maximal nucleosome remodeling (compare *lanes 4* and *7*). In contrast, longer species continued to accumulate during this time period (*lanes 4–7*), suggesting that formation of the benzamide-resistant intermediate that supports ALC1 activation requires synthesis by PARP1 of these longer PAR chains.

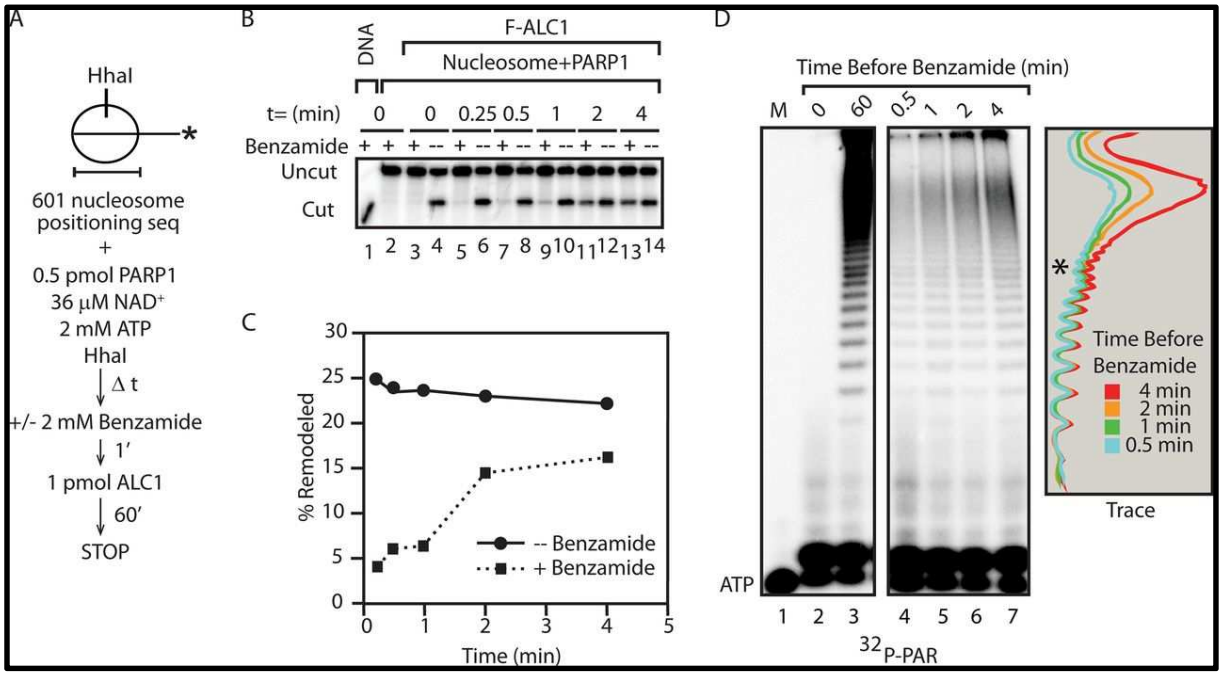


Figure 30 Rapid formation of a benzamide-resistant intermediate in ALC1 activation. **A**, diagram describing the protocol and positioned nucleosome substrate with an HhaI site on 216-bp 601-lat Gal4 DNA fragment used for benzamide challenge nucleosome remodeling assay. The *asterisk* indicates ^{32}P-labeled DNA end. **B**, Naked DNA (*lane 1*) or positioned nucleosomes (*lanes 2–14*) were incubated with HhaI in the absence (*lane 1*) or presence (*lanes 2–14*) of PARP1. Where indicated, PARP1 inhibitor benzamide was added to reactions at the times shown in the figure. One minute later, ALC1 was added, and remodeling reactions proceeded for an additional 60 min, and DNA or nucleosomes were monitored for HhaI restriction enzyme accessibility. **C**, quantitation of experiment shown in **B**. **D**, kinetics of PAR synthesis. Reactions with [^{32}P]NAD$^+$ were performed according to the protocol shown in *A*, except that ALC1 was omitted. PAR synthesized during the reactions was purified, analyzed on denaturing polyacrylamide gels, and visualized on a phosphorimager (left panels). Marker lane (*M*) contains [γ-^{32}P]ATP. The graph on the right shows traces of *lanes 4–7* generated using ImageQuant TL software.

Cooperative Binding of PARP1 and ALC1 to Nucleosomes

We previously showed that that the binding of ALC1 to immobilized DNA or nucleosomes depends on PARP1 and NAD^+ [305]. These experiments did not, however, address the fate of PARP1 during ALC1 activation. If PARylated PARP1 contributes to both ALC1 activation and subsequent ATP-dependent nucleosome remodeling or DNA-dependent ATP hydrolysis, PARylated PARP1 might be predicted to remain as an integral component of an activated ALC1·nucleosome or ALC1·DNA intermediate throughout reactions. Alternatively, PARylated PARP1 might be required for the initial activation of ALC1, but then be displaced upon the binding of ALC1 to its substrates and/or hydrolysis of ATP.

To address this issue, immobilized nucleosomes or DNA was incubated with PARP1 and NAD^+ with or without ALC1 and ATP. Excess competitor DNA was added as a sink for unbound or disassociated PARP1 and ALC1 after a time sufficient for formation of the benzamide-resistant intermediate. At varying times after addition of competitor, the amounts of PARP1 and ALC1 remaining bound to immobilized nucleosomes and DNA or released were measured by western blotting. As shown in Fig. 31A, PARP1 could be detected in the bound fraction after incubation with immobilized nucleosomes or DNA. Notably, retention of PARP1 on nucleosomes or DNA increased significantly in the presence of ALC1 (compare *lanes 1–3* with *lanes 4–6*), suggesting that the binding of PARP1 and ALC1 to nucleosomes or DNA is cooperative. ALC1·PARP1·nucleosome or ALC1·PARP1·DNA complexes were stable, as they exhibited no apparent dissociation during a 30 minute incubation following addition of competitor. Furthermore, the amount of bound PARP1 was not decreased when binding assays

included ATP (compare *lanes 4–6* with *lanes 7–9*), suggesting that PARP1 is not displaced from ALC1·PARP1·nucleosome or ALC1·PARP1·DNA intermediates upon ATP hydrolysis.

PARylation of PARP1 has been shown to decrease its affinity for DNA or nucleosomes [267, 310]. To investigate the relationship between PAR synthesis and formation of the stable ALC1·PARP1·nucleosome intermediate, we manipulated the extent of PARP1 PARylation by varying the concentration of NAD^+ included in the binding assays. Consistent with previous evidence that the binding of ALC1 to DNA or nucleosomes depends on PARP1 and NAD^+, the amount of ALC1 binding depended on NAD^+ concentration; at the lowest concentration of NAD^+, less ALC1 remained associated with immobilized nucleosomes than when NAD^+ was present at the highest concentrations used (Fig. 31B, *lanes 6–9*). There was little NAD^+-dependent change in the amount of bound PARP1 when the binding reactions included ALC1 (*lanes 6–9*). However, when ALC1 was not included in the binding reactions, substantially less PARP1 remained associated with immobilized nucleosomes at high NAD^+ concentrations, where the extent of [^{32}P]NAD^+ incorporation into PARylated PARP1 was greater (Fig. 31B, *upper panels*, compare *lanes 2–5* with *lanes 6–9*). The NAD^+-dependent decrease in PARP1 bound to nucleosomes depended on PAR synthesis because it was mitigated when benzamide was added to reactions along with competitor DNA (Fig. 31B, *lower panels*). We similarly observed that enhanced binding of PARP1 to DNA in the presence of ALC1 was most pronounced when reactions contained NAD^+ (data not shown). Taken together, our findings are consistent with the model that ALC1 and PARylated PARP1 bind cooperatively to nucleosomes or DNA to form an intermediate that is stable in the presence or absence of ATP.

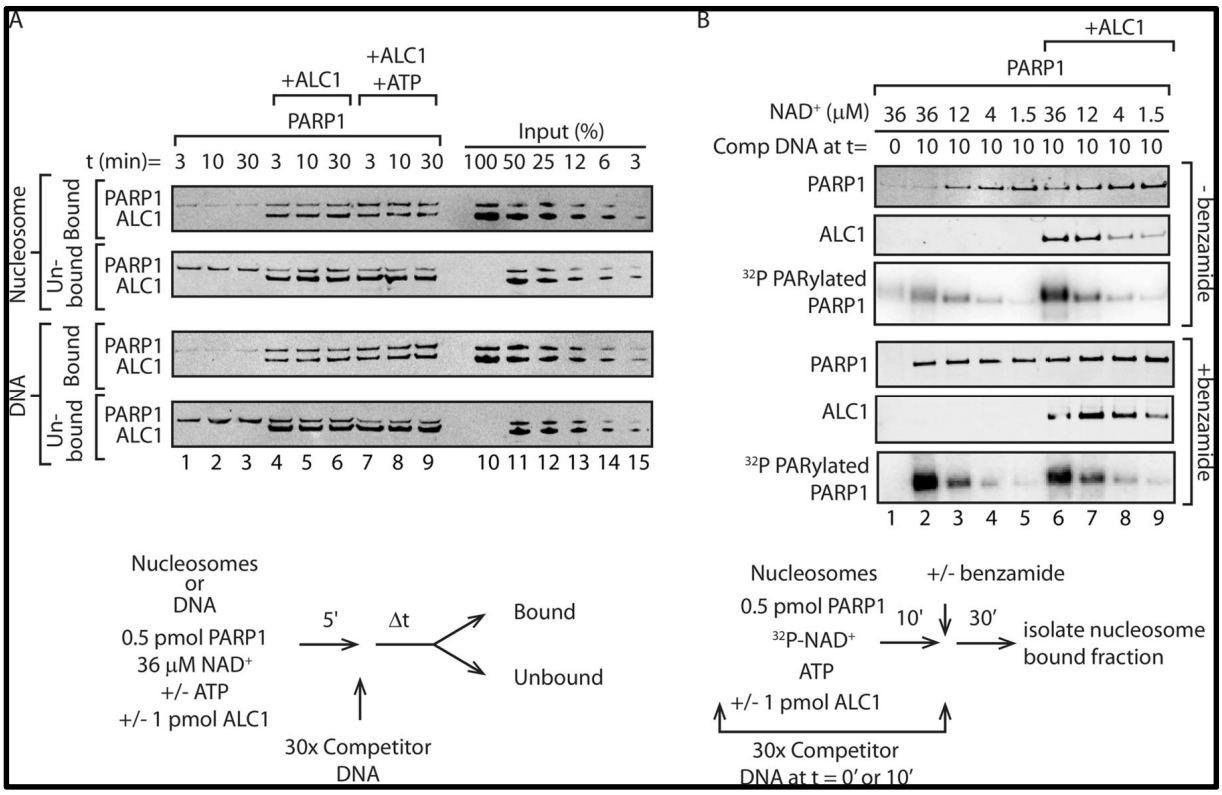


Figure 31 Cooperative binding of PARP1 and ALC1 to nucleosomes. A, biotinylated DNA or mononucleosomes reconstituted with HeLa cell histones were immobilized on streptavidin beads and incubated for 5 min with PARP1 and NAD^+, with or without ALC1 and ATP. After addition of competitor DNA, reactions were incubated for the indicated times. PARP1 and ALC1 in bound and unbound fractions was detected by western blotting. **B**, nucleosome binding reactions were performed with the indicated concentrations of NAD^+ and with or without benzamide as diagrammed in the figure. PARP1 and ALC1 in bound and unbound fractions was detected by Western blotting, and ^{32}P-PARylated PARP1 was detected by phosphorimaging of the same membrane used in the Western blots.

ALC1 protects PAR chains from ~ 3 to more than 20 ADP-ribose units from digestion by poly(ADP-ribose) glycohydrolase (PARG).

We previously observed that the DNA-dependent ATPase and nucleosome remodeling activities of ALC1 were abolished by an ALC1 macrodomain point mutation (D723A) that interferes with its binding to free PAR, suggesting that binding of PAR to the ALC1 macrodomain is likely to play a role in ALC1 activation. To investigate potential ALC1-PAR interactions under nucleosome remodeling conditions, we developed a novel PAR footprinting assay in which we exploited the ability of poly(ADP-ribose) glycohydrolase (PARG) to degrade PAR. PARG catalyzes hydrolysis of α(1″-2′) *O*-glycosidic linkages in PAR and can act as both an endo- and exoglycosidase [311, 312].

PAR footprinting assays were performed as diagrammed in Fig. 32A. Bead-bound nucleosomes were incubated with PARP1 and ^{32}P-NAD^{+} in the presence or absence of ALC1 for a time sufficient to allow formation of stable benzamide-resistant intermediates, and benzamide was added to block further PAR synthesis. After treatment of reaction mixtures with increasing concentrations of PARG, beads were washed, and residual PAR associated with ALC1• PARylated-PARP1•nucleosome or PARylated-PARP1•nucleosome complexes was isolated and analyzed by denaturing gel electrophoresis. As shown in Fig. 32B, ^{32}P-PAR chains in the PARylated-PARP1•nucleosome complex were digested to near completion by PARG. In contrast, a nearly constant fraction of ^{32}P-PAR chains in the ALC1•PARylated-PARP1•nucleosome complex was resistant to digestion at all PARG concentrations used; notably, the size distribution of these protected digestion products (from ~3 to more than 20 ADP-ribose units) was very similar over a 10-fold range of PARG concentrations. Whereas ALC1 carrying a mutation in its ATP binding site behaved very similarly to the wild type

enzyme, PAR was not protected from digestion by ALC1 containing a macrodomain point mutation that prevents it from binding free PAR (Fig. 32C). These results argue that PAR binding by the ALC1 macrodomain is required for the observed PAR protection.

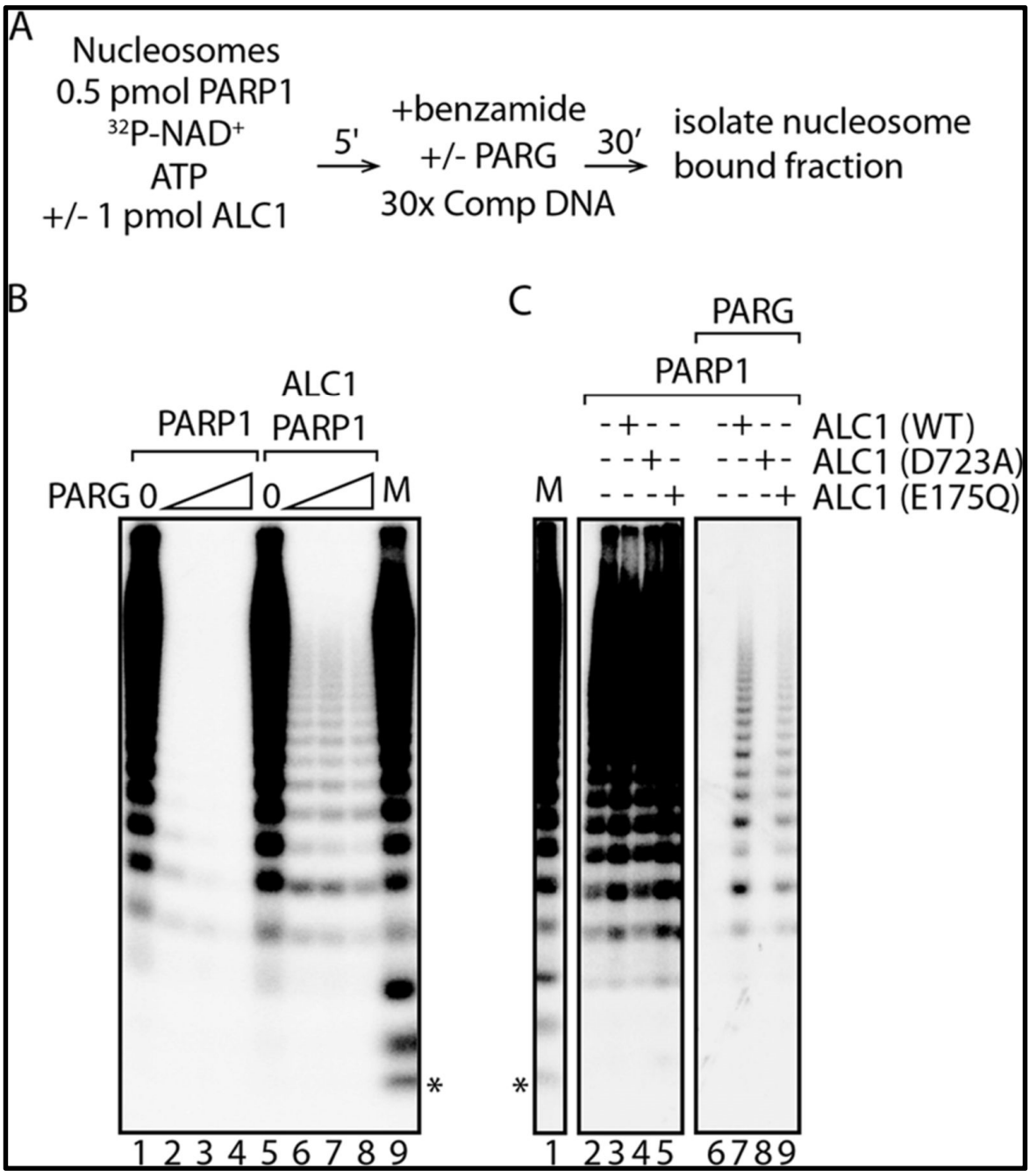


Figure 32 The ALC1 macrodomain protects PAR chains from ~ 3 to more than 20 ADP-ribose units from digestion by PARG. A, diagram showing the protocol used for PAR footprinting assays. **B**, reactions performed as diagrammed in *A* contained no PARG (*lanes 1* and *5*) or 1.5 ng (*lanes 2* and *6*), 5 ng (*lanes 3* and *7*), and 15 ng (*lanes 4* and *8*) PARG. **C**, reactions performed as diagrammed in **A** contained PARP1 and wild-type or mutant ALC1 without or with 5 ng of PARG. Marker lanes (*M*) show the total reaction products synthesized in a reaction containing nucleosomes and PARP1. Free ATP runs at the position indicated by the *asterisk*.

Discussion

ALC1 is an SNF2 family ATPase with a macrodomain. We and others previously demonstrated that ALC1 possesses cryptic DNA-dependent ATPase and ATP-dependent nucleosome remodeling activities that are activated in the presence of PARP1 and NAD^+, its substrate for PAR synthesis [305, 306]. Several lines of evidence argued that autoPARylation of PARP1, rather than PARylation of nucleosomal histones or ALC1, is the essential NAD^+-dependent step in ALC1 activation. First, either nucleosomes or naked DNA can support PARP1- and NAD^+-dependent ALC1 DNA-dependent ATPase activity, indicating that histone PARylation is not essential. Second, an intermediate that activates ALC1 and that is resistant to the PARP inhibitor benzamide can be formed by preincubation of PARP1 and NAD^+ with nucleosomes or with naked DNA in the absence of ALC1 [305, 306].

In this work, we have applied a combination of approaches to dissect in more detail the mechanism by which ALC1 nucleosome remodeling activity is activated by PARP1 and NAD^+. In the course of our investigation, we considered two possible models for the mechanism of ALC1 activation. First, ALC1 activation might occur *via* a direct physical interaction between ALC1 and autoPARylated PARP1, leading to formation of a stable intermediate capable of catalyzing ATP-dependent nucleosome remodeling. Alternatively, given that PARylation of PARP1 has been shown to reduce its affinity for DNA or nucleosomes, it seemed possible that ALC1 might be activated by a different mechanism, perhaps by interacting transiently with autoPARylated PARP1 in a way that renders ALC1 competent to bind and remodel nucleosomes [267, 310, 311].

Our findings are most consistent with the model that activation of ALC1 occurs through direct and stable binding of ALC1 to autoPARylated PARP1. Our biochemical dissection of the mechanism of ALC1 activation argues that it proceeds with the formation of multiple intermediates: an initial complex of PARP1 bound to a nucleosome; a benzamide-resistant intermediate that contains nucleosome-bound PARylated PARP1; and a stable ternary intermediate that is composed of ALC1, autoPARylated PARP1, and nucleosome and that is capable of catalyzing nucleosome remodeling (Fig. 33). Importantly, the results of PAR footprinting assays argue that in the ternary intermediate, the ALC1 macrodomain is bound to PAR. Taken together with our evidence that ALC1 containing a macrodomain point mutation that abolishes its binding to PAR is inactive in nucleosome remodeling, our findings argue that PAR on PARylated PARP1 plays a unique role as an allosteric effector of ALC1 DNA-dependent ATPase and ATP-dependent chromatin remodeling activities.

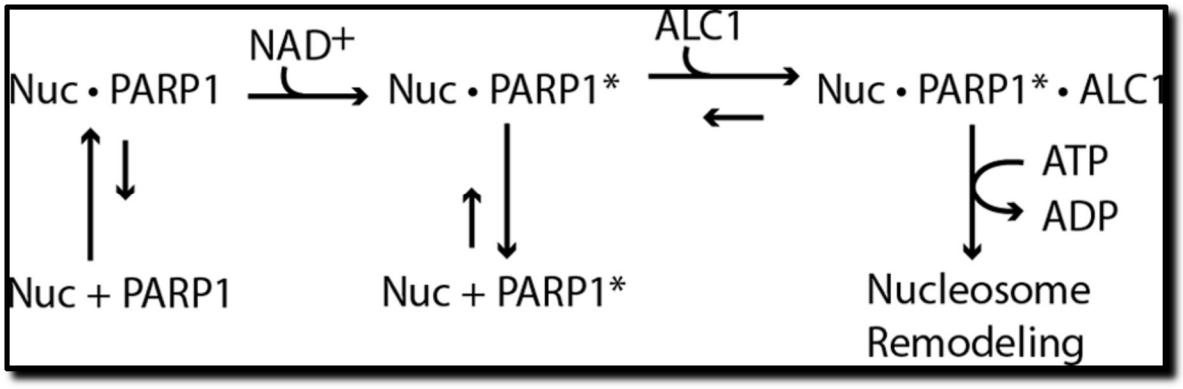


Figure 33 Proposed pathway for ALC1 activation by PARP1 and NAD$^+$. Unmodified PARP1 binds nucleosomes with high affinity. NAD$^+$-dependent PARylation of PARP1 decreases its affinity for nucleosomes or DNA; thus, in the absence of ALC1 the nucleosome-PARylated PARP1 intermediate tends to dissociate. ALC1 and PARylated PARP1 bind cooperatively to nucleosomes, leading to formation of a stable, activated intermediate that can catalyze ATP-dependent nucleosome remodeling. PARP1*, PARylated PARP1; Nuc, nucleosome.

Although a structure of the ALC1 macrodomain is not yet available, the structure of the macroH2A1.1 macrodomain in complex with ADP-ribose predicts that macrodomains bind at the distal ends of PAR chains and could, in principle, bind stably to polymers containing just two or three ADP-ribose units. Our data suggest, however, that such short PAR chains may not be sufficient to recruit ALC1 to PARylated PARP1 and support ALC1 activation, because formation of the benzamide-resistant intermediate correlates with synthesis of PAR chains longer than ~12–15 ADP-ribose units. In the ALC1·PARP1·nucleosome intermediates, PAR chains ranging in size from ~3 to more than 20 ADP-ribose units are protected from endo- and exo-glycolytic digestion by PARG. Although the size of the smallest of these corresponds with what might be expected based on the model for PAR binding by macroH2A1.1, the largest are considerably longer, likely reflecting interactions between PAR and ALC1 that fall outside of the ADP-ribose binding pocket in its macrodomain and that contribute to ALC1 activation.

CHAPTER III

THE MACRODOMAIN OF HISTONE MACROH2A1.1 IS AN ACTIVATOR OF THE ALC1 CHROMATIN REMODELER

Abstract

The human *ALC1* oncogene encodes a SNF2 family DNA-dependent ATPase with a macrodomain that binds poly (ADP-ribose) (PAR). ALC1 possesses cryptic ATP-dependent nucleosomal remodeling and ATPase activity that is potently activated *via* macrodomain dependent interactions with Poly ADP-ribosylated PARP1. In the course of this study, we find that apart from PARP and NAD^+, Alc1 possesses and additional mode of internal control via conserved domains in the linker region and the macrodomain. First, we identified a region, NMAC (N-terminal to Macrodomain ATPase Coupling) domain needed to couple ATP hydrolysis to nucleosome remodeling. Deleting NMAC led to a robust PARP and NAD^+-dependent ATPase, which lacked appreciable remodeling activity. An additional mode of control via the macrodomain was discovered when we replaced ALC1's PAR binding macrodomain with another macrodomain from the macrohistoneH2A1.1. We discovered this chimeric protein was constitutively active independent of PARP and NAD^+. These results suggests a model of positive control of the SNF2 ATPase via the macrodomain and an additional level of control over its remodeling activities via the NMAC and other conserved linker elements.

Introduction

The human ALC1 (Amplified in Liver Cancer 1) protein (also referred to as CHD1L or Chromodomain-Helicase-DNA-binding protein 1-like) is a member of the SNF2 (Sucrose Non-Fermenter 2) superfamily of DNA-dependent ATPases and is capable of catalyzing ATP-dependent nucleosome remodeling. The *ALC1* gene on chromosome 1q21 gene is amplified in a subset of hepatocellular carcinomas, and overexpression of the ALC1 protein was found to transform cultured cells and lead to appearance of spontaneous tumors in mice. More recently, the *ALC1* gene was found to be mutated in individuals suffering the genetic disorder CAKUT (Congenital Anomalies of the Kidney and Urinary Tract). Although it is presently not clear how ALC1 overexpression or mutation leads to these disorders, it has been reported that ALC1 has roles in both DNA repair and the transcription of genes implicated in tumorigenesis and metastasis.

ALC1 is unique among SNF2 family chromatin remodelers because it includes a macrodomain that has been shown to be capable of binding selectively to poly (ADP-ribose) (PAR). Previous studies from our laboratory and elsewhere revealed that ALC1 possesses cryptic DNA-dependent ATPase and ATP-dependent nucleosome sliding activities that are strongly activated in the presence of the poly(ADP-ribose) polymerase PARP1 and its substrate NAD^+. These studies also revealed that ALC1 macrodomain mutations that abolish PAR binding block ALC1 ATPase and nucleosome remodeling, arguing that the ALC1 macrodomain is important for ALC1 activation. In a more recent study, we obtained evidence (i) that activation of ALC1 nucleosome remodeling activity by PARP1 and NAD+ requires assembly of a stable ALC1-PARP1-nucleosome intermediate and (ii) that PAR chains on autoPARylated PARP1 are

the target of the ALC1 macrodomain. Taken together these findings argued that physical interaction of the ALC1 macrodomain with autoPARylated PARP1 is critical for activation of ALC1 DNA-dependent ATPase and nucleosome remodeling activities.

In this report, we explore several possible models for how interaction of the ALC1 macrodomain with autoPARylated PARP1 might lead to activation of the ALC1 SNF2 ATPase. In particular, we present a series of experiments aimed at distinguishing between the possibilities (i) that ALC1 activation occurs via a "de-inhibition" mechanism through release of an intrinsically active ALC1 ATPase from inhibition by sequences outside the SNF2 domain and perhaps including the macrodomain or (ii) that ALC1 activation depends upon a tethering function of the macrodomain or (iii) that ALC1 activation occurs via positive regulation of an intrinsically inactive ATPase. As described below, our findings are consistent with the model that the ALC1 macrodomain functions, in concert with autoPARylated PARP1, as an allosteric activator of an intrinsically inactive ALC1 ATPase. A role for the ALC1 macrodomain as an activator of ALC1 is bolstered by our discovery that replacement of the ALC1 macrodomain with the macrodomain of histone macroH2Afurthermore renders the ALC1 ATPase constitutively active in the absence of PARP1 and NAD^+. Taken together, our findings shed new light on the mechanism of ALC1's allosteric activation, and they reveal that ALC1 can be a target for regulation by more than one cellular macrodomain

Results

Deletion mutants lacking the ALC1 macrodomain are inactive.

ALC1's N-terminal SNF2 ATPase has been classified as a member of the Snf2-like subfamily of SF2 ATPases. The Snf2-like subfamily includes a number of related ATPases, including CHD1. CHD1, ALC1, and other members of this family share sequence similarity throughout the two lobes of their ATPase domains and an additional region of approximately 50 amino acids that extends C-terminal from the second ATPase lobe (shown in green in Fig. 34). The crystal structure of yeast CHD1 reveals that this C-terminal portion of the ATPase bridges ATPase lobes 1 and 2, packing against one face of each lobe in a way that might allow it to sense and/or influence motions of the two ATPase lobes (Fig. 35) [138]. A model of ALC1's SNF2 ATPase domain and C-terminal bridge region generated using Phyre2 (www.sbg.bio.ic.ac.uk/phyre2/) suggests that ALC1 is likely to adopt a very similar structure (Fig. 35).

Hauk *et al.* previously presented evidence that the human CHD1 SNF2 ATPase domain is intrinsically active, but is negatively regulated by amino acid sequences lying outside the SNF2 ATPase domain and the C-terminal Bridge [138]. This inhibitory region, referred to as the chromo-wedge, is located between the tandem N-terminal chromodomains of CHD1. The chromo-wedge appears to bind to the CHD1 SNF2 ATPase domain and sterically interfere with the binding of the SNF2 ATPase to effector DNA. These authors showed that deletion of CHD1 amino acid sequences outside of the CHD1SNF2 ATPase domain led to constitutive activation of the ATPase, and they proposed that binding of the CHD1 chromodomain to methylated nucleosomal histones acts to "de-inhibit" the CHD1 SNF2 ATPase by releasing it from steric

inhibition. Similarly, the ATPase of ISWI, which is also predicted to adopt a structure similar to that of ISWI is an intrinsically active ATPase that is regulated by inhibitory modules that are antagonized by nucleosomal epitopes [313]. Thus, the ATPase activities of both CHD1 and ISWI can be regulated by a combination of intramolecular interactions, in which domains outside of their ATPase domains affect their activities, and by intermolecular interactions with external factors, such as nucleosomal histones.

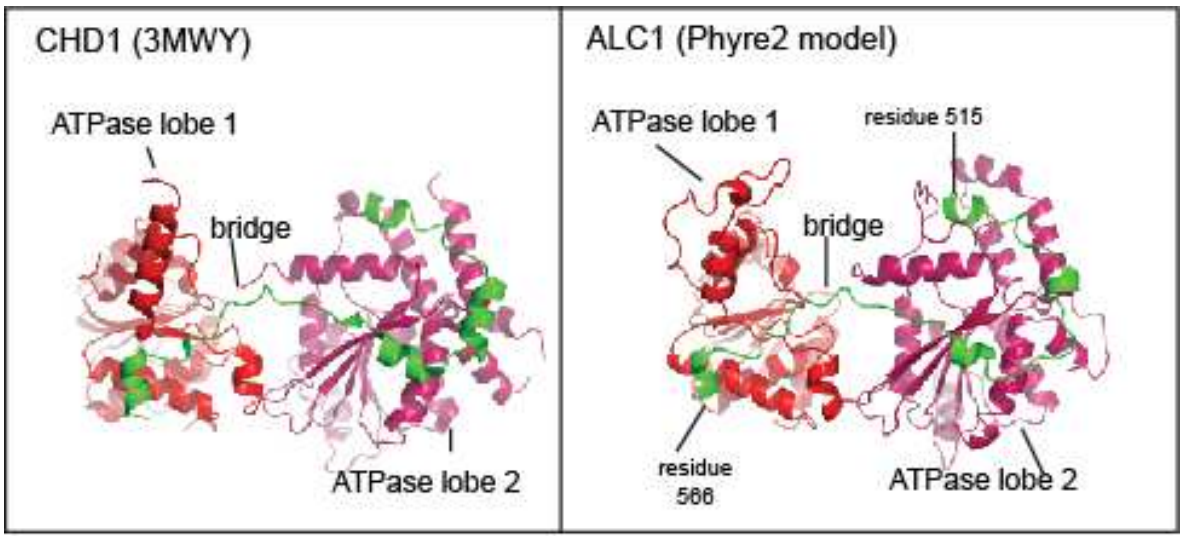


Figure 34 High degree of structural similarity between Chd1, Iswi and Alc1. Comparison of structure of CHD1 SNF2 ATPase and bridge regions (residues 871-922, PDB accession number 3MWY) [138] with a model of the corresponding region of ALC1 (residues 515-566). The ALC1 model was generated using Phyre2 (www.sbg.bio.ic.ac.uk/phyre2/) and depicted with PyMol. These proteins bear remarkable homology in their SNF2 ATPase lobes and the subsequent section of amino acids called the C-terminal Bridge in Chd1. Alc1 also has a predicted C-terminal bridge like fold at the C-terminus of its ATPase.

In light of the similarity between ALC1, CHD1, and ISWI, we considered the possibility that the ATPase domain of ALC1 might also be intrinsically active. If that were the case, we predict that removing potential inhibitory region(s), including the macrodomain and the portion

of the protein between the SNF2 ATPase and the macrodomain, might generate a constitutively active enzyme. To test this hypothesis, we generated a series of ALC1 C-terminal deletion mutants. These mutants all contained the ALC1's annotated SNF2 ATPase (residue 1-515) and varying amounts of ALC1 extending through the bridge and continuing until the beginning of the macrodomain (residue 666). As diagrammed in Fig. 35A, these ALC1 mutants included ALC1 [1-515], ALC1 1-527], ALC1[1-551], ALC1[1-584], ALC1[1-606], ALC1[1-617], ALC1[1-635], ALC1[1-666] which extend the annotated Alc1 SNF2 [1-515] construct by ~18 amino acid increments. Wild type ALC1 and ALC1 mutants were expressed in baculovirus-infected insect cells with an N-terminal FLAG epitope tag, purified by sequential anti-FLAG agarose immunoaffinity and DEAE-NPR ion exchange chromatography (Fig 35). Each was then assayed for ATP-dependent nucleosome remodeling and DNA-dependent ATPase activities, using concentrations of DNA or nucleosomes 20-fold greater than needed for maximal activation of wild type ALC1.

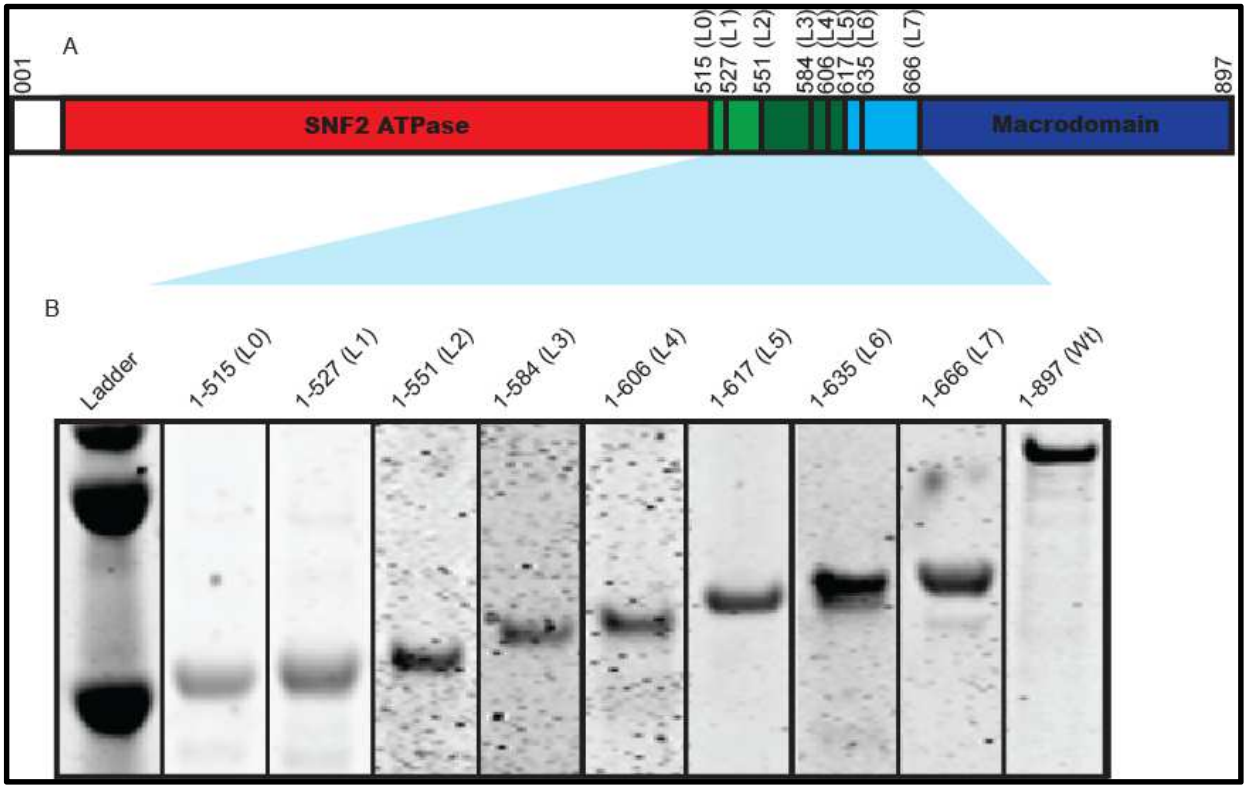


Figure 35 Purification of ALC1 C-terminal deletion mutants. All proteins were purified by FLAG immune-purification and DEAE-NPR HPLC. A, Diagrammatic representation of Alc1 truncation constructs indicated by residue number. B, Colum purified protein was quantified using Imperial™ Protein Stain.

Like the full-length ALC1 protein, all of these ALC1 mutants lacked detectable ATPase activities in the absence of PARP1 and NAD^+. In parallel experiments, we assayed each ALC1 mutant for DNA and nucleosome-dependent ATPase in the presence of PARP1 and NAD^+. Unlike the wild type ALC1, none of the ALC1 mutants that lacked the macrodomain exhibited any detectable activity in either the presence or absence of PARP1 and NAD^+ ((Fig. 36 and data not shown), arguing that the macrodomain is strictly required for ALC1 activity. In addition, we found that the macrodomain did not activate ALC1's ATPase in *trans*, since addition of free

purified macrodomain to ALC1 [1-666] did not restore PARP1/NAD^+-dependent ATPase activity (data not shown). Thus, activation of ALC1's ATPase by the macrodomain requires a covalent linkage between the SNF2 ATPase domain and the macrodomain.

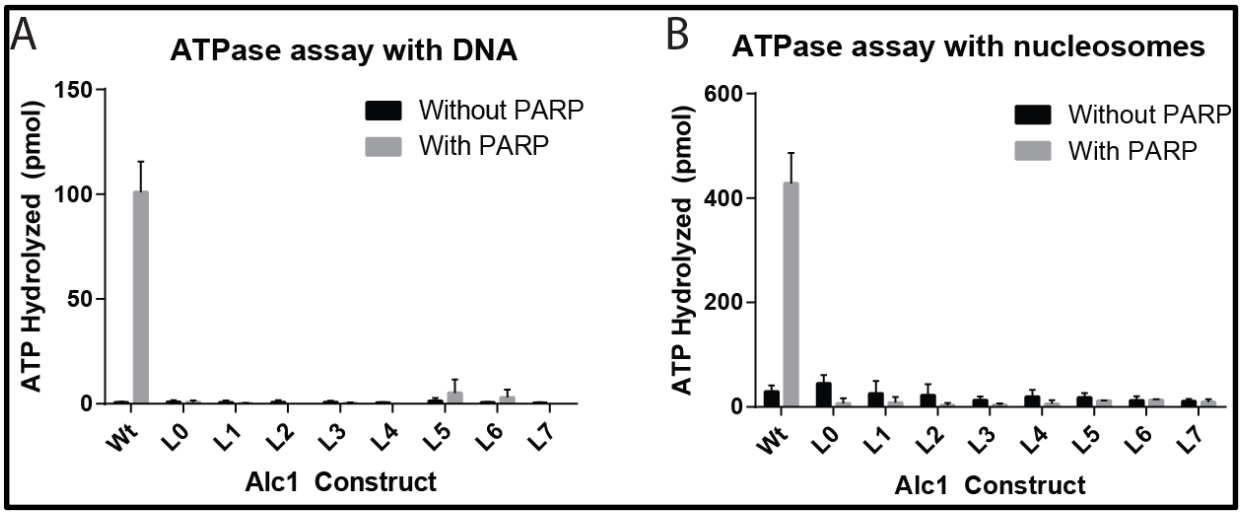


Figure 36 ATPase activity of ALC1 C-terminal deletion mutants. ATPase activity of ALC1 and ALC1 C-terminal deletion mutants containing the residues shown on the X axis. ATPase activity is represented as pmols hydrolyzed during 20 min reactions. Assays contained 1 pmol ALC1 and 2.5 µg of a 215 bp DNA fragment containing the core 601 nucleosome positioning sequence (A) or 1 µg of Hela cell mononucleosomes (B). WT, wild type ALC1. All experiments were performed three or more times. Error bars are ± standard deviation.

The observation that none of the panel of ALC1 mutants that lacked the macrodomain exhibited detectable activity in either the absence or presence of PARP1 and NAD^+ is consistent with the possibility that, unlike CHD1's and ISWI's SNF2 ATPase, the ALC1 SNF2 ATPase is not intrinsically active and is unlikely to be regulated by a simple "de-inhibition" mechanism, in which the ALC1 macrodomain or other region of the protein serves an inhibitory function similar to the chromo-wedge of CHD1. We also considered the possibility that in the absence of the

macrodomain, the ALC1 SNF2 ATPase may be inactive because it has a very low affinity for DNA or nucleosomes and that a major function of the macrodomain is to aid in recruiting the enzyme to its substrate. Arguing against this possibility, even though assays were performed at a very high DNA concentrations, we were still not able to measure any detectable ATPase, in either the presence or absence of PARP1 and NAD.

NMAC functions as an ATPase Coupling domain

Although the data presented thus far argues that neither the macrodomain nor the linker, region between the SNF2 and the macrodomain, functions alone as a negative regulator, it did not rule out the possibility that all or part of the region linking ALC1's annotated SNF2 domain and the macrodomain might cooperate with the macrodomain to negatively regulate the enzyme. If this were the case, one might expect to be able to generate a constitutively active enzyme by deleting some or all of the linker region. As shown in the multiple sequence alignment in Fig. 37b, the linker region includes several blocks of sequence highly conserved throughout evolution, including a block of sequence that immediately follows the SNF2 ATPase and falls within the predicted bridge region, a second block of sequence that extends beyond the predicted bridge, and a third, highly charged region, which we refer to as NMAC (N-terminal to the macrodomain ATPase Coupling) and which is conserved in most species but is missing from green pufferfish.

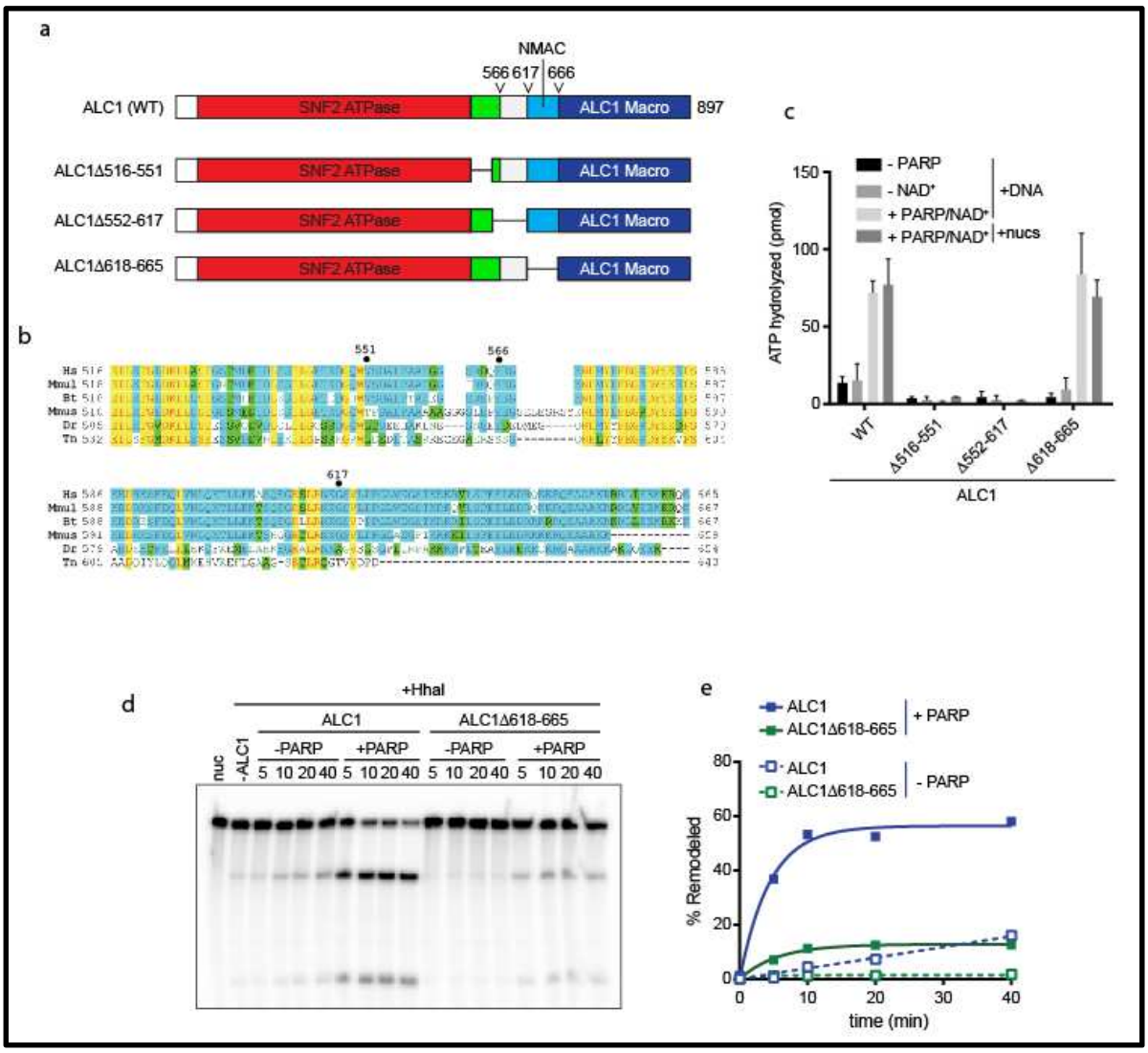


Figure 37. NMAC is an ATPase Coupling domain. A region of ALC1 immediately N-terminal to its macrodomain couples ATPase to nucleosome remodeling activity. (b) Diagram showing ALC1 internal deletion mutants. SNF2 ATPase, bridge-like region, and macrodomain are colored as in Fig. 1a. A region N-terminal to the macrodomain (NMAC) is highlighted in cyan. Deleted regions are indicated by thin lines. (c) DNA- and nucleosome-stimulated ATPase activities of ALC1 internal deletion mutants. All experiments were performed three or more times. Error bars, ± standard deviation. (d) Mono-nucleosomes containing HeLa cell histones were assembled on a 215 bp DNA fragment containing the core 601 nucleosome positioning sequence (diagrammed at bottom) and monitored for restriction enzyme accessibility after incubation with ATP, NAD^+ and either no ALC1 (-ALC1), ALC1 or ALC1Δ618-665, with or without PARP1. nuc, undigested nucleosome. (e) Quantitation of remodeling assays shown in panel (d).

Based on this observation we designed three deletion mutants, ALC1Δ515-551, ALC1 551-617, ALC1ΔNMAC (lacking residues 618-665), each missing a different conserved part of the linker (Fig. 37a). As shown in Fig. 37c, like the full-length ALC1 protein, none of the ALC1 deletion mutants had any ATPase activity in the absence of PARP1 and NAD^+. Hence, deletion of these regions failed to generate a constitutively active enzyme. In the presence of PARP1 and NAD^+, ALC1Δ515-551 and ALC1Δ552-617 had no detectable ATPase activity (Fig. 37c) or nucleosome remodeling activity (data not shown), indicating that sequences within the predicted bridge region and perhaps extending beyond are required for ALC1 activity. In contrast ALC1Δ NMAC had DNA and nucleosome stimulated activity comparable to that of wild-type ALC1, arguing that there is not a strict requirement for a specific length of sequence between the SNF2 ATPase and macrodomain of ALC1.

To determine if deletion of NMAC had any impact on remodeling activity, we performed a time-course comparing the nucleosome remodeling activities of ALC1 and ACL1ΔNMAC. As shown in Figs 37d and 37e, nucleosome remodeling by both wild type ALC1 and ALC1ΔNMAC was strongly dependent on PARP1 and NAD+. Notably, however, while wild type and ALC1ΔNMAC had indistinguishable ATPase activities, the nucleosome remodeling activity of ALC1ΔNMAC was much lower than that of its wild type counterpart. All the three constructs generated above were able to bind PAR through their macrodomain. An additional construct with the double deletion of residues 552-617 and NMAC showed no ATPase activity or PAR binding (data not shown). This could be due to poor folding of the truncated construct of steric clashes that do not allow the ATPase lobes and the macrodomain to be in a functional conformation. We suspected that although all the three constructs deletion constructs had a functional PAR binding

macrodomain, their association with the nucleosome may be affected, thereby generating their respective ATPase outcomes.

We used the immobilized nucleosome template binding assay to see if the ALC1Δ515-551 and ALC1Δ551-617 constructs were able to interact with nucleosomes (Fig. 38). As shown in Fig. 38, the ALC1Δ515-551 construct was recruited to the nucleosome independently in absence of PARP1. The ALC1Δ552-617 construct also behaved similarly albeit with lower recruitment. In presence of PARP1 and NAD^+, ALC1Δ515-551 and ALC1Δ552-617 recruited to the nucleosome at levels similar to that of wild-type ALC1. In case of ALC1ΔNMAC, recruitment to the nucleosome was less than wild-type ALC1's but still showed dependence on PARP1 and NAD^+ for recruitment to the nucleosome.

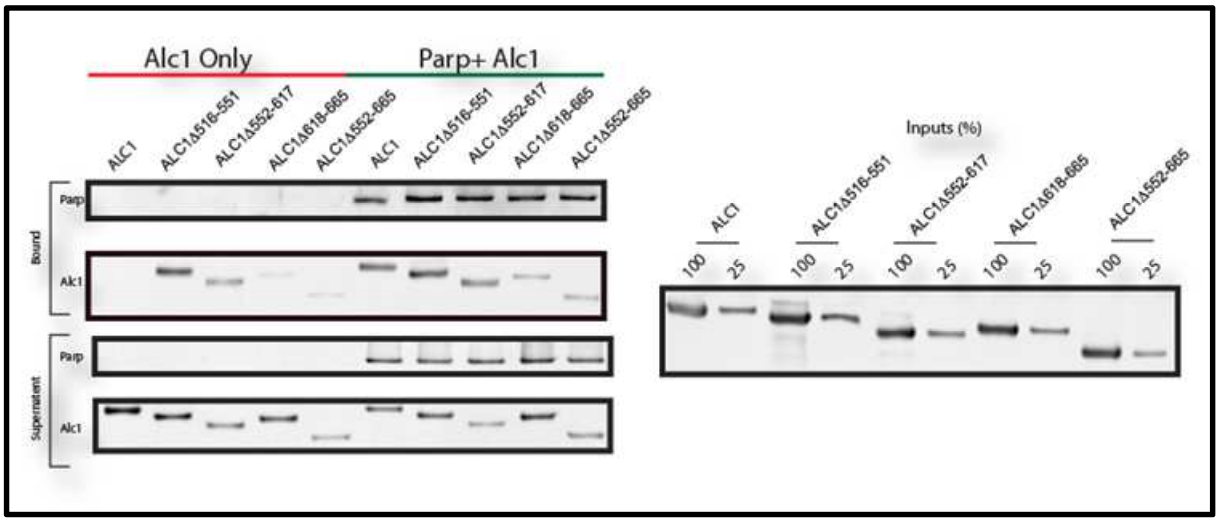


Figure 38 Immobilized nucleosome template binding assay of ALC1 and Linker deletion constructs. Mononucleosomes reconstituted with HeLa cell histones on biotinylated DNA and immobilized on streptavidin beads were incubated with wild-type or mutant Alc1, NAD^+, ATP, with or without PARP1. Bound fractions were analyzed by anti-Flag western blotting. All experiments were performed three or more times.

Although we did not succeed in identifying a constitutively active internal deletion mutant of ALC1, we did gain a number of important insights from analysis of these mutants. In particular, our results argue that activation of the SNF2 ATPase of ALC1 depends on (i) sequences that fall within and extend beyond the predicted bridge region and (ii) a functional macrodomain. In addition, they identify a region immediately N-terminal to the macrodomain, NMAC, that is required to couple the energy of ATP hydryolysis to nucleosome remodeling. Taken together, our results argue that the ALC1 macrodomain does not function simply as a tether that brings ALC1 to PARP1-bound nucleosomes. In addition, they argue that the macrodomain does not act as a negative regulatory domain whose effect is relieved upon binding to poly-ADP-ribosylated PARP1. Instead, they suggest that the macrodomain and the region between the SNF2 ATPase and macro-domains might participate more directly as an "allosteric" activator of the ALC1 ATPase.

Macrodomain of macroH2A1.1 is an activator of ALC1's SNF2 ATPase

Although the data presented thus far is most consistent with the idea that the macrodomain is not needed simply for recruitment but instead may have a positive regulatory role in ALC1's activation, we wished to test this model using an independent approach. If the major function of the ALC1 macrodomain is to enhance recruitment of ALC1 to its substrate, one would predict that an ALC1 chimeric construct in which the normal macrodomain is replaced with another PAR binding macrodomain, but not with a PAR non-binding macrodomain, would exhibit PARP1- and NAD^+-dependent activity (Fig 39a). We chose to replace ALC1's macrodomain with the PAR binding macrodomain from the histone variant

macroH2A1.1, and the macrodomain from its non-PAR binding splice variant macroH2A1.2. As controls, we generated two more ALC1-macrodomain chimeras, one containing a point mutant of the macroH2A1.1 macrodomain (G224E) that renders it PAR non-binding, and the other containing the non-PAR binding macrodomain from another histone variant, macroH2A2 (Fig 39a). As expected, ALC1 containing the PAR binding macrodomain of Macro1.1 was able to bind PAR in a similar fashion to wild type ALC1 (Fig 39b), while the remaining ALC1-macrodomain chimeras showed no detectable PAR binding under the reaction conditions. In addition, in the presence of PARP1 and NAD^+, the PAR binding ALC1-Mac1.1 bound immobilized nucleosomes similarly to ALC1 while its PAR non-binding version ALC1-Mac1.1G224E did not.

To test the effect of the new macrodomains on the activities of the ALC1-macrodomain chimeras, we first performed ATPase assays. As expected, the PAR non-binding ALC1Mac1.2 and ALC1Mac2A2 had no ATPase activity in absence or presence of PARP1 and NAD^+ (Fig. 40a and data not shown). To our surprise, however, both PAR-binding ALC1-Mac1.1 and PAR non-binding ALC1-Mac1.1G224E were constitutively active, showing strong ATPase activity in the presence of DNA, with or without PARP1 and NAD^+ (Fig. 4a and data not shown). We performed two kinds of assays to rule out the possibility that the PARP- and NAD+ independent activity was due to some contaminating ATPase. First, we generated ATPase dead versions of the ALC1-Mac1.1 and ALC1-Mac1.1G224E enzymes by introducing a Walker B mutation, which interferes with ATP binding. ATPase dead versions of the enzyme had no activity in presence or absence of PARP1 and NAD^+ (Fig. 40a). Second, we showed that NAD^+-dependent ATPase activity co-purified with ALC1-Mac1.1G224E protein during high resolution HPLC on a DEAE-NPR column (Fig. 40b).

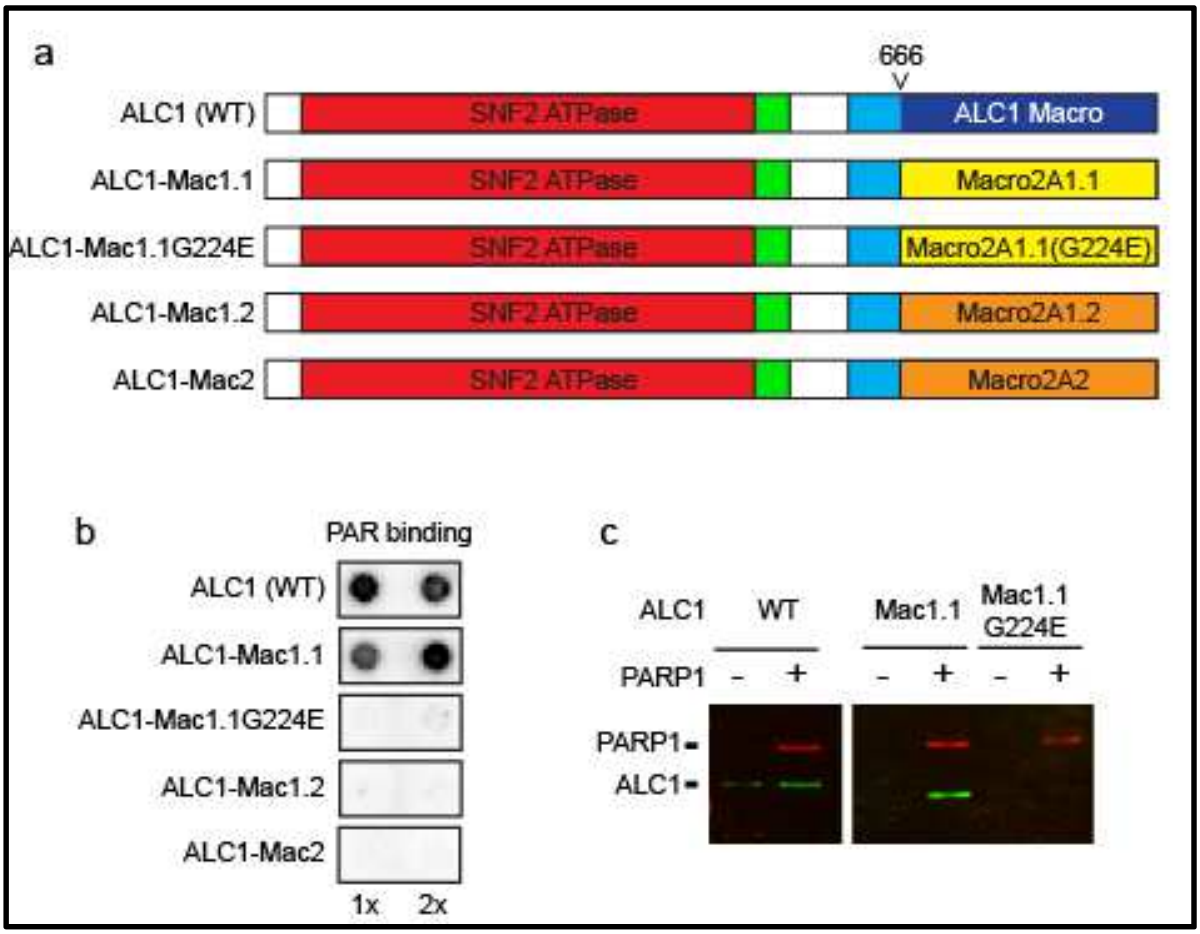


Figure 39. **PAR binding activities of ALC1-macrodomain chimeras.** (a) ALC1-macrodomain chimeras include ALC1 residues 1-666 fused to the macrodomains of histone H2A variants macroH2A1.1 (ALC1-Mac1.1), macroH2A1.2 (ALC1-Mac1.2), or macroH2A2 (ALC1-Mac2). An additional chimera, ALC1-Mac1.1G224E, contains the macroH2A1.1 macrodomain with an point mutation that prevents binding to ADP-ribose and ADP-ribosylated PARP1 (b) PAR binding was assayed using nitrocellulose filter binding assays. Binding reactions contained ~100ng (1x) or 200ng (2x) of wild-type ALC1 or ALC1-macrodomain chimeras and ^{32}P-labeled PAR. (c) Nucleosome binding activities of ALC1-macrodomain chimeras. Mononucleosomes reconsituted with Hela cell nucleosomes on biotinylated DNA and immobilized on streptavidin beads were incutated with NAD^+ and wild type ALC1 or ALC1-macrodomain chimeras, with or without PARP1.

We also tested ALC1Mac1.1 and ALC1Mac1.1G224E for their abilities to support nucleosome remodeling. Both of these ALC1-macrodomain chimeras were able to robustly remodel nucleosomes in the presence or absence of PARP1 and NAD^+ (Fig. 40 d and e and data

not shown). This suggested that unlike ALC1ΔNMAC, the two constitutively active mutants were able to engage the nucleosome productively and remodel them.

Taken together, these observations provide strong support for the idea that the macrodomain does not serve simply as a tether that increases the affinity of the ALC1 SNF2 ATPase domain for its substrate by interacting with DNA- or nucleosome-bound, PARylated PARP1, since replacing ALC1's macrodomain with PAR or non-PAR binding versions of the macrodomain of macroH2A1.1 renders the enzyme constitutively active. Instead, they suggest that the macrodomain plays some more active role as a positive activator, most likely via intramolecular interactions with the SNF2 ATPase or other conserved domain of the enzyme. This highlighted the fact that the Macro1.1 had a specific interaction with the SNF2 ATPase and the other conserved domains that was able to render it constitutively active. More importantly, this interaction that rendered it constitutively active was independent of a PAR-binding event.

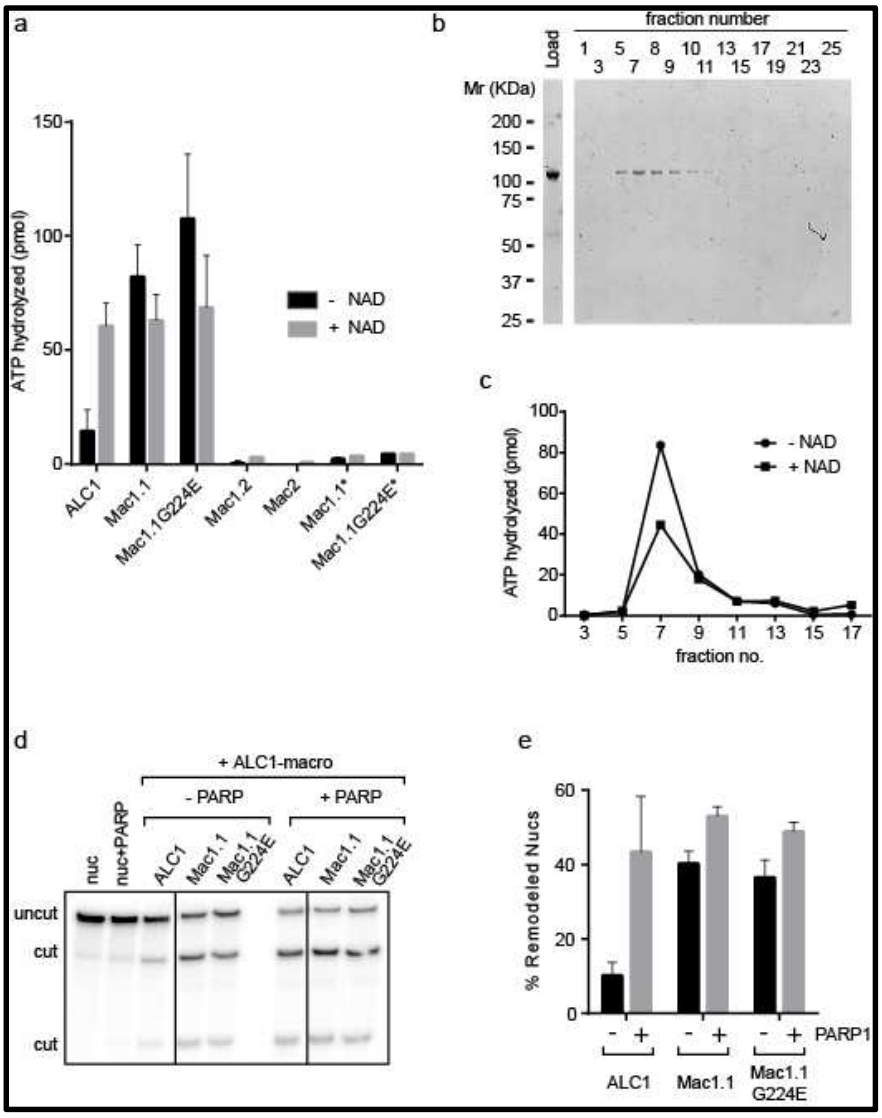


Figure 40: **Macrodomain of macroH2A1.1 is positive activator of ALC1's SNF2 ATPase** (a) ATPase activity assay of Alc1 chimeric constructs represented as pmols of ATP hydrolyzed. Construct names are indicated on the x-axis. Assays are performed with a 2.5 µg of a 215 bp DNA fragment containing the core 601 nucleosome positioning sequence at one end. (b) Alc1-Mac1.1 column fractions from a DEAE-NPR HPLC on a coomassie stained protein gel. (c) ATPase activity assay of Alc1-Mac1.1 column fractions represented as pmols of ATP hydrolyzed. Fraction numbers listed on the x-axis show activity coincides with the fractions containing the purified protein. (d) Nucleosome sliding activities of Alc1 fusion/chimeric proteins. Nucleosomes reconstituted with purified HeLa cell histones were monitored for restriction enzyme accessibility after incubation with ATP and wild-type or mutant Alc1, Parp1 and NAD^+. (e) Quantitation of nucleosome sliding shown in fig 4d. The percent shifted was calculated as the ratio of the intensity of the larger cleaved band relative to the sum of intensities of the intact and cleaved fragments. All experiments were performed three or more times. Error bars, ± standard deviation.

ALC1 ATPase activation with different nucleic acid substrates

In addition to the above mentioned points of intradomain control in the protein, we also wished to investigate in more detail the kinds of DNA effectors that can affect ALC1 activity. We tested ATPase activity with DNA that had different cytosine modifications. As shown in Fig. 41 we observed that there was no significant difference between the ATPase activity of Alc1 using different types of DNA under the conditions of our assay. Methylated cytosines and hydroxymethylated cytosines had no effect on enhancing or diminishing the ATPase activity of Alc1.

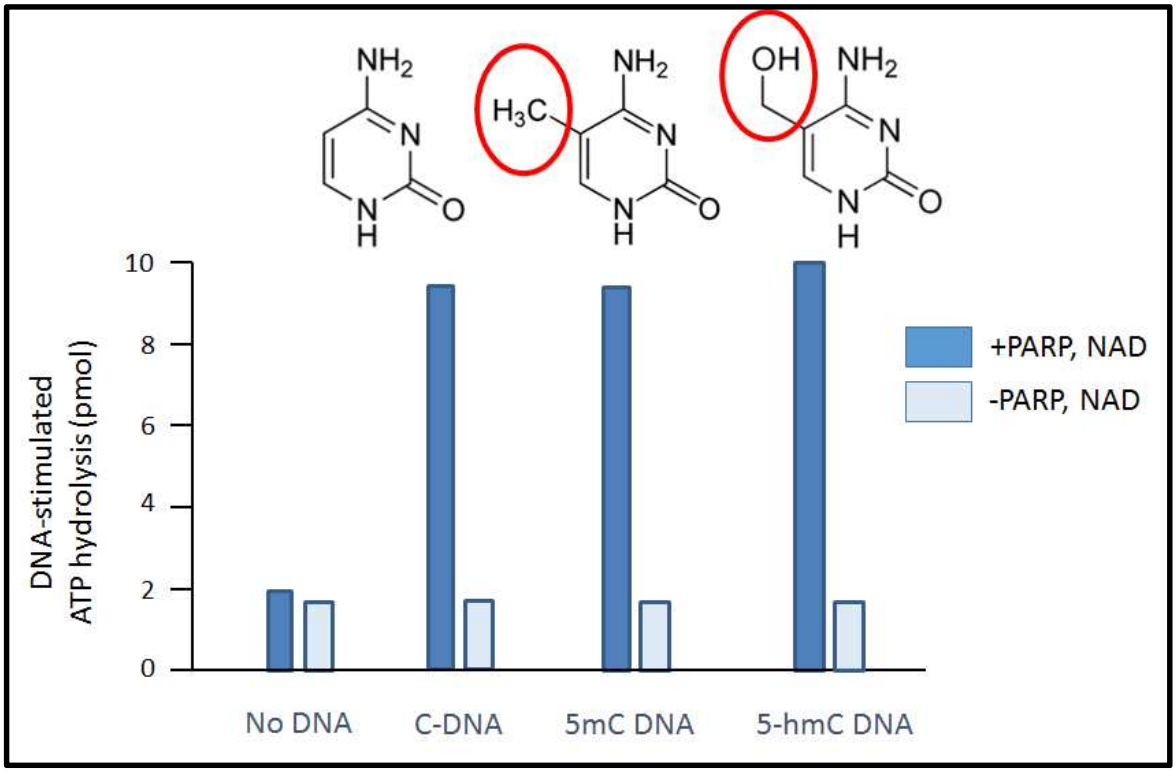


Figure 41 Alc1 ATPase activity with different Cytosine modified DNA. ATPase activity is represented as pmols hydrolyzed during 20 min reactions. Assays contained 1 pmol ALC1 and 100 ng of a 215 bp DNA fragment containing the core 601 nucleosome positioning sequence that has modified cytosines incorporated wherever indicated. C-DNA, unmodified DNA; 5mC DNA, 5-methylated cytosine labelled DNA; 5-hmC DNA, 5-hydroxymethylated cytosine labelled DNA.

In particular, we asked whether ALC1's ATPase could only be activated by dsDNA, or whether ssDNA or RNA would also activate the ATPase. Double-stranded DNA and ssDNA of varying lengths were able to stimulate the Alc1's ATPase, but ssRNA was unable to accomplish the same result. (Fig. 42).

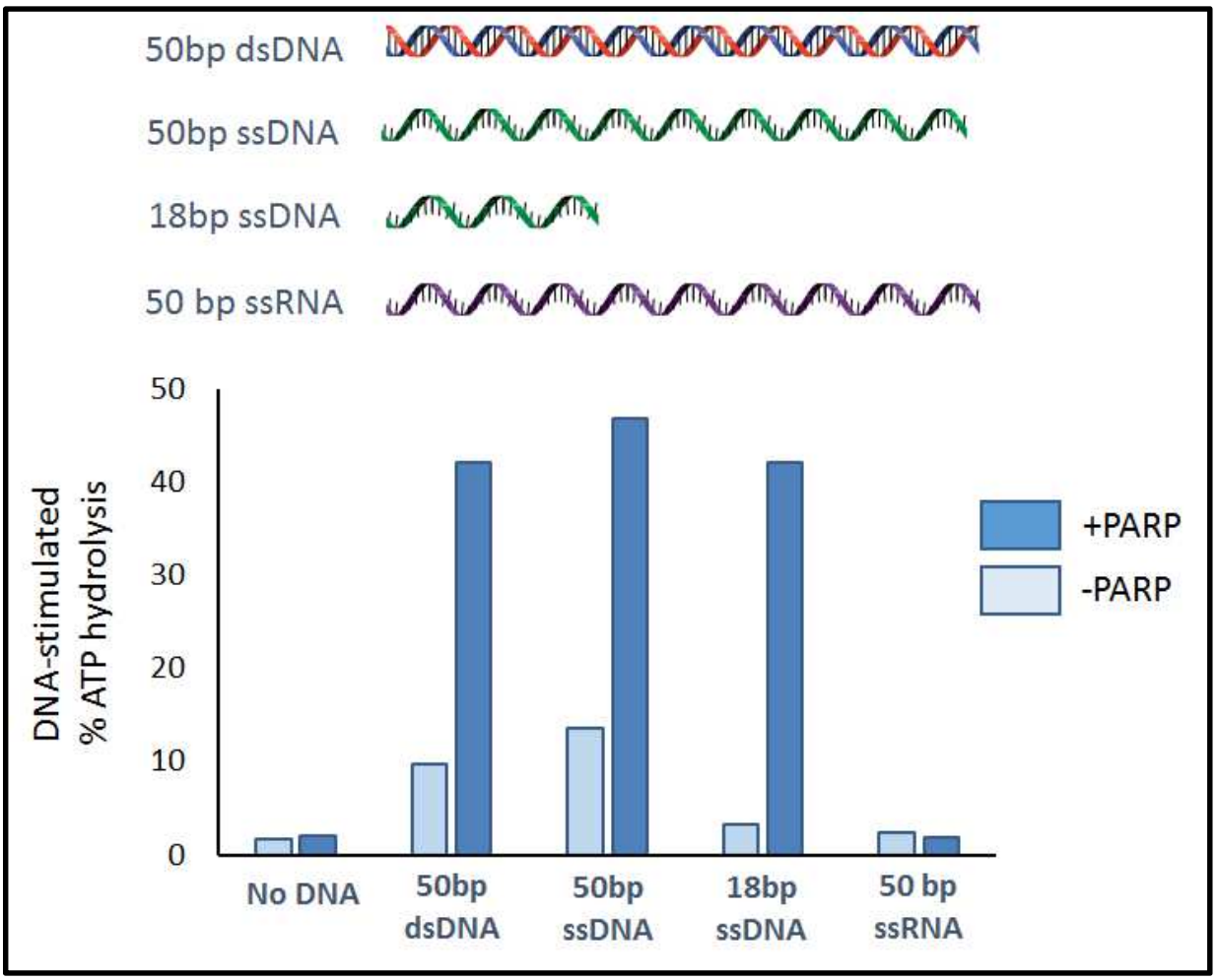


Figure 42 Stimulating Alc1's ATPase with different lengths and types of nucleic acids. Graph shows the activity of Alc1 ATPase in presence and absence of PARP1 with ds and ssDNA and ssRNA. ATPase assays were performed with 100ng of DNA.

Having observed that single stranded DNA that contained all four bases stimulated ATPase, we wished to determine whether a homopolymer ssDNA which cannot form transient secondary structures, could stimulate the ATPase. In these experiments we used 50 bp PolydA and 50bp PolydT to activate ALC1. As shown in Fig 43, PolydA and PolydT were not sufficient to stimulate ALC1, suggesting that truly single stranded DNA is unable to stimulate ALC1's ATPase in presence or absence of PARP1 and NAD^+.

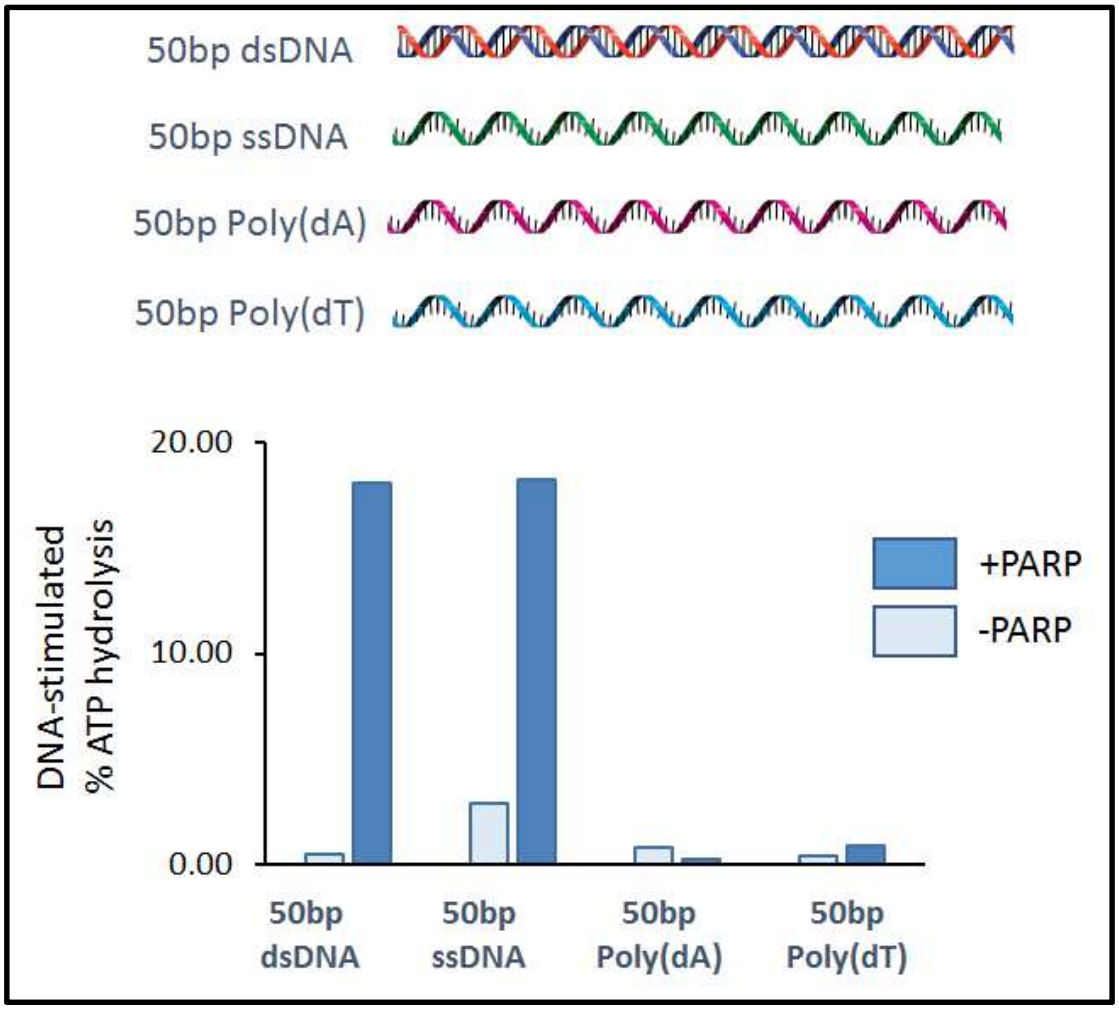


Figure 43 Stimulating Alc1's ATPase with PolydA and PolydT. Graph shows the activity of Alc1 ATPase in presence and absence of PARP1. ATPase assays were performed with equimolar amounts of nucleic acid substrate.

To determine whether single stranded DNA failed to activate ALC1 because the SNF2 ATPase cannot use it or whether it does not activate PARP1, We used the constitutively active chimera of ALC1 that we had generated (ALC1-Mac1.1) to test the above. As shown in Fig. 44, the constitutively active ALC1-Mac1.1 chimera was not able to show any appreciable ATPase activity in presence or absence of PARP1 providing further support for the idea that ALC1's SNF2 ATPase functions only with dsDNA.

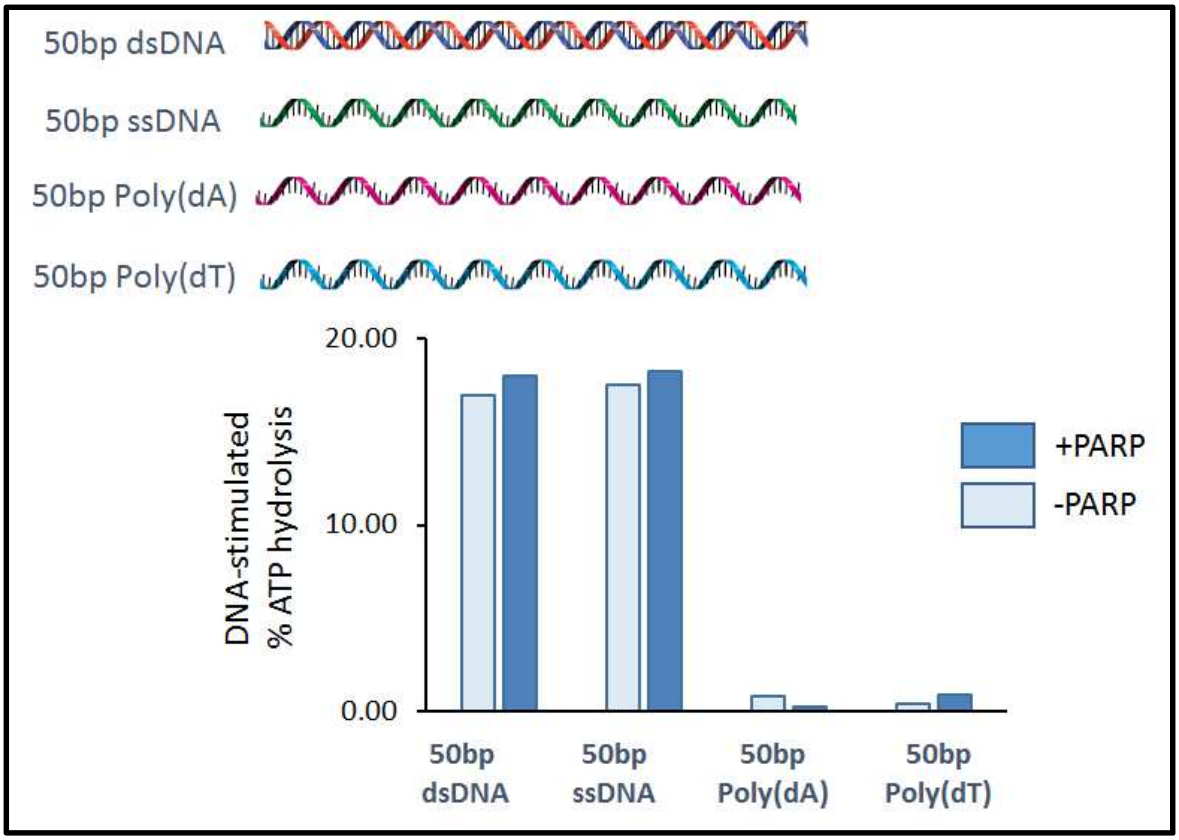


Figure 44 Alc1Mac1.1 ATPase stimulation with PolydA and PolydT. Graph shows the activity of Alc1 ATPase in presence and absence of PARP1. ATPase assays were performed with equimolar amounts of nucleic acid substrate.

To further reassert out observation that Alc1's SNF2 ATPase can be stimulated only by dsDNA, we performed an experiment where we mixed the PolydA and PolydT in the same tube to generate dsDNA structures *in vitro*. We discovered that the mixture of PolydA and PolydT was able to stimulate the ATPase of ALC1 suggesting that homopolymeric DNA, if double stranded, was sufficient to stimulate ALC1's ATPase (Fig. 45).

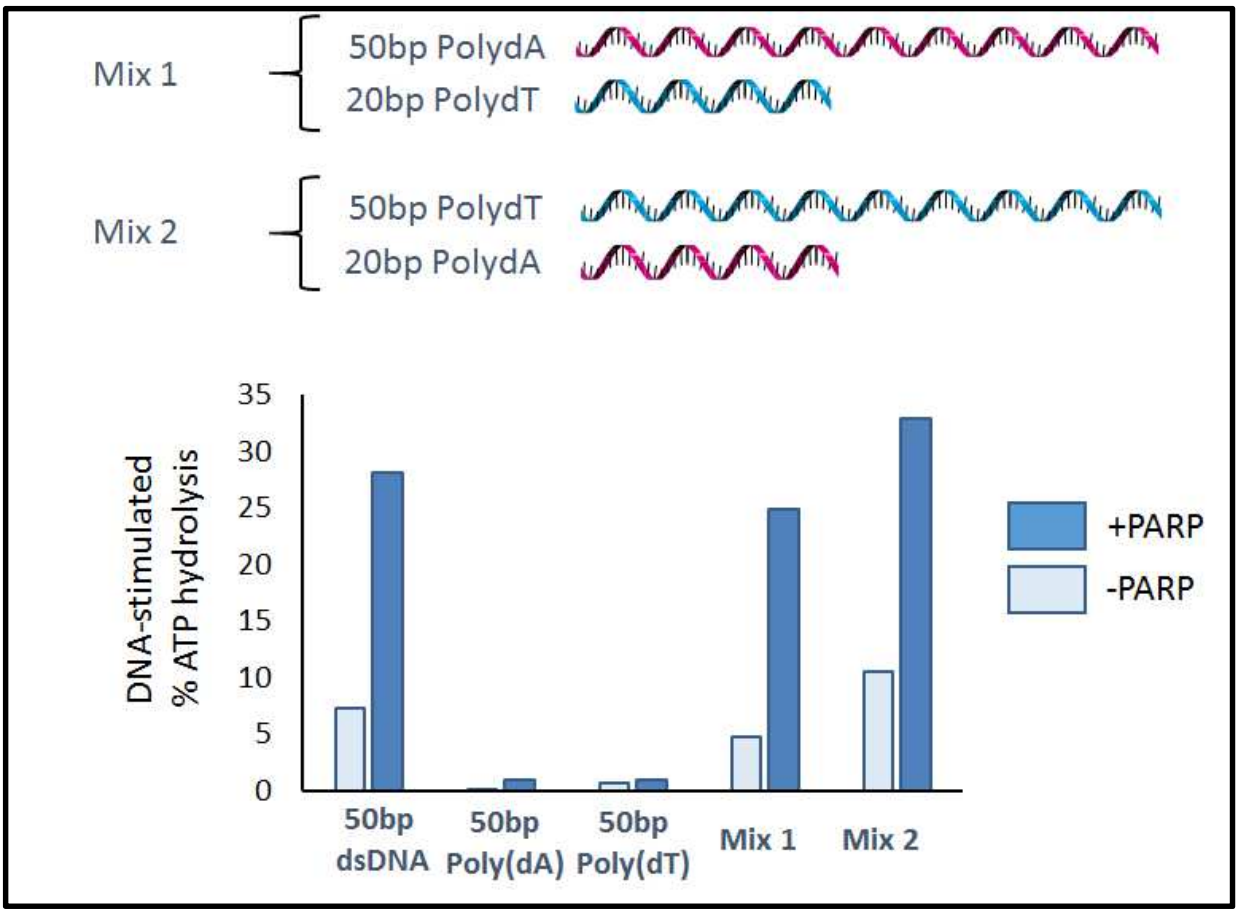


Figure 45 Alc1 ATPase stimulation with mixture of PolydA and PolydT. Graph shows the activity of Alc1 ATPase in presence and absence of PARP1. ATPase assays were performed with equimolar amounts of nucleic acid substrate.

We also tested the constitutively active Alc1Mac1.1 chimera to make sure that this activity can be attributed solely to the SNF2 ATPase of ALC1 and is not a result of any modification in the activation of PARP1 or subsequent PAR formation using ss or ds DNA. As shown in Fig. 46, we discovered that the chimera was stimulated by the mixture of PolydA and PolydT.

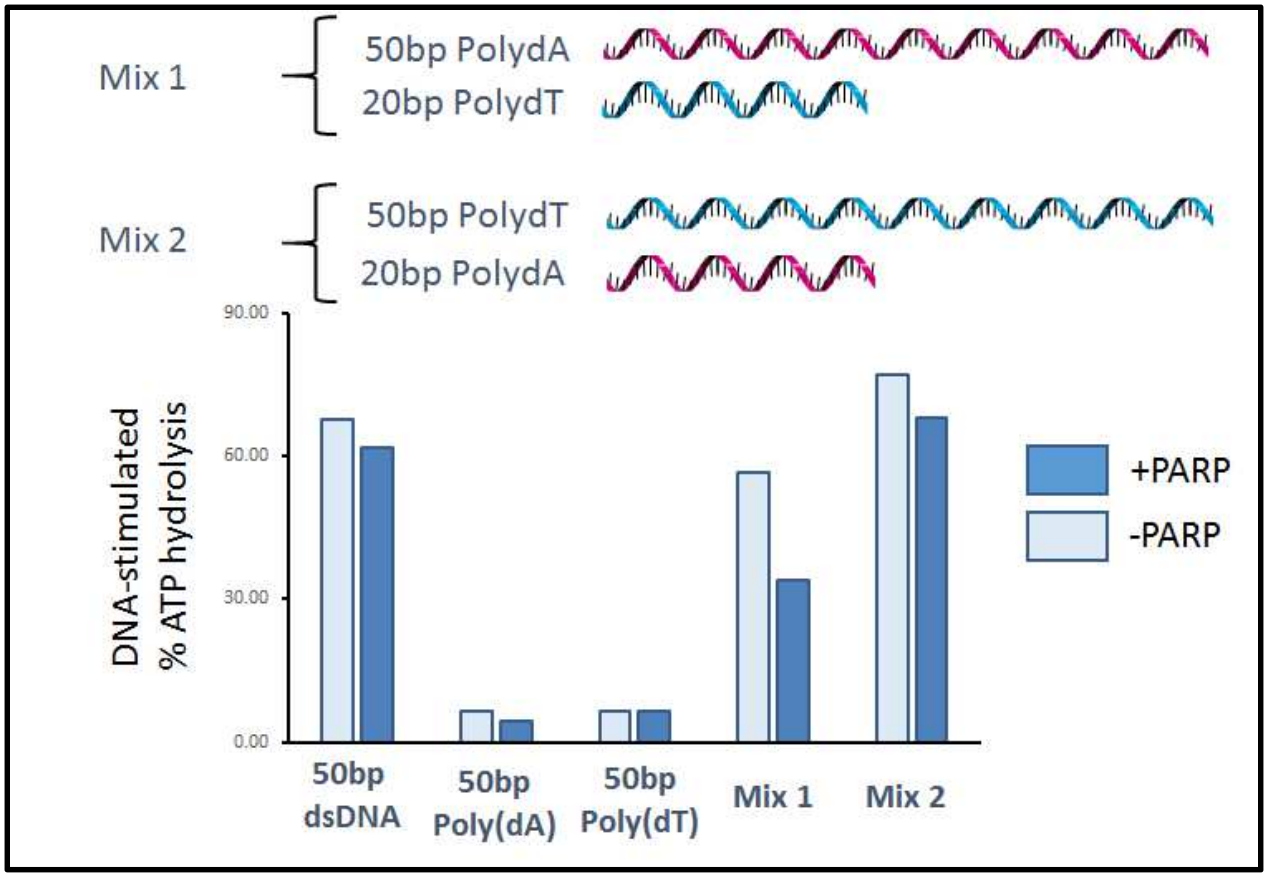


Figure 46 Alc1Mac1.1 stimulation with mixture of PolydA and PolydT. Graph shows the activity of Alc1 ATPase in presence and absence of PARP1. ATPase assays were performed with equimolar amounts of nucleic acid substrate.

Discussion

In the previous chapter we show that our findings are most consistent with the model that activation of ALC1 occurs through direct and stable binding of ALC1 to autoPARylated PARP1. Our new findings suggest that there are multiple modes of control for ALC1's SNF2 ATPase. Comparing CHD1 regulation to ALC1, we see some striking similarities and also some salient and unique features of ALC1. CHD1 has a Snf2 ATPase that has been shown to be the minimal active unit of CHD1. CHD1 possesses a pair of chromodomains that bind methylated histones and regulate its activity in an allosteric fashion. The regulation is not directly mediated by the chromodomains, but by a linker domain present between the tandem chromodomains known as the chromo-wedge (Fig. 47) [138].

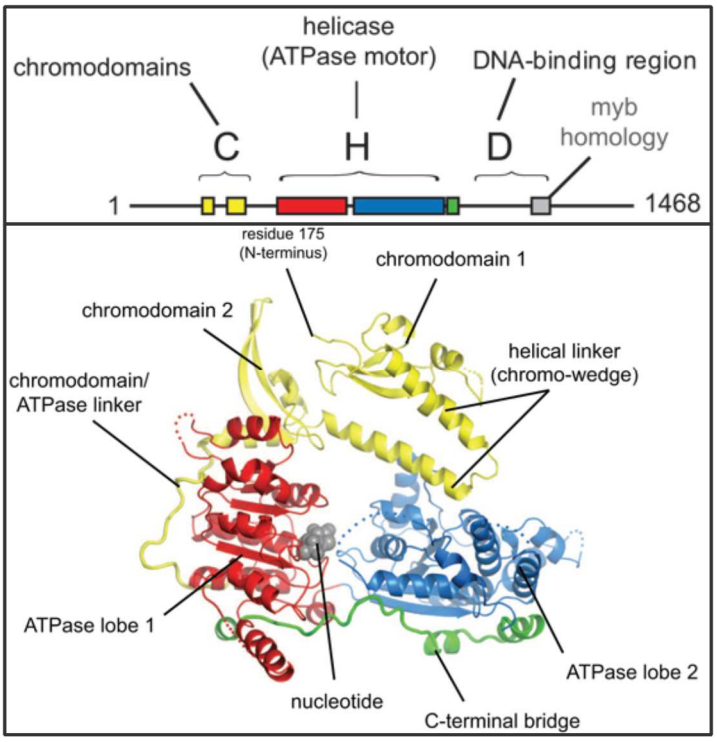


Figure 47. Domain Organization of Chd1. Top: Schematic representations of CHD1. Bottom: The two ATPase lobes of CHD1 are flanked by the double chromodomains (yellow) and the extended C-terminal bridge (green). Figure adapted from [138].

The chromo wedge is a highly acidic patch of residues that directly interacts with a DNA-binding surface on the ATPase motor and interferes with DNA binding to the ATPase, thus negatively inhibiting the CHD1 ATPase. This inhibition by the chromodomains splays the two ATPase lobes wide open in a conformation that is not conducive to ATP hydrolysis. In the CHD1 crystal structure, the double chromodomains are clearly visible interacting with both ATPase. The interaction of the chromodomains with both ATPase lobes suggests that chromodomains would likely stabilize this open conformation, reducing the likelihood of ATPase closure and hydrolysis (Fig 48).

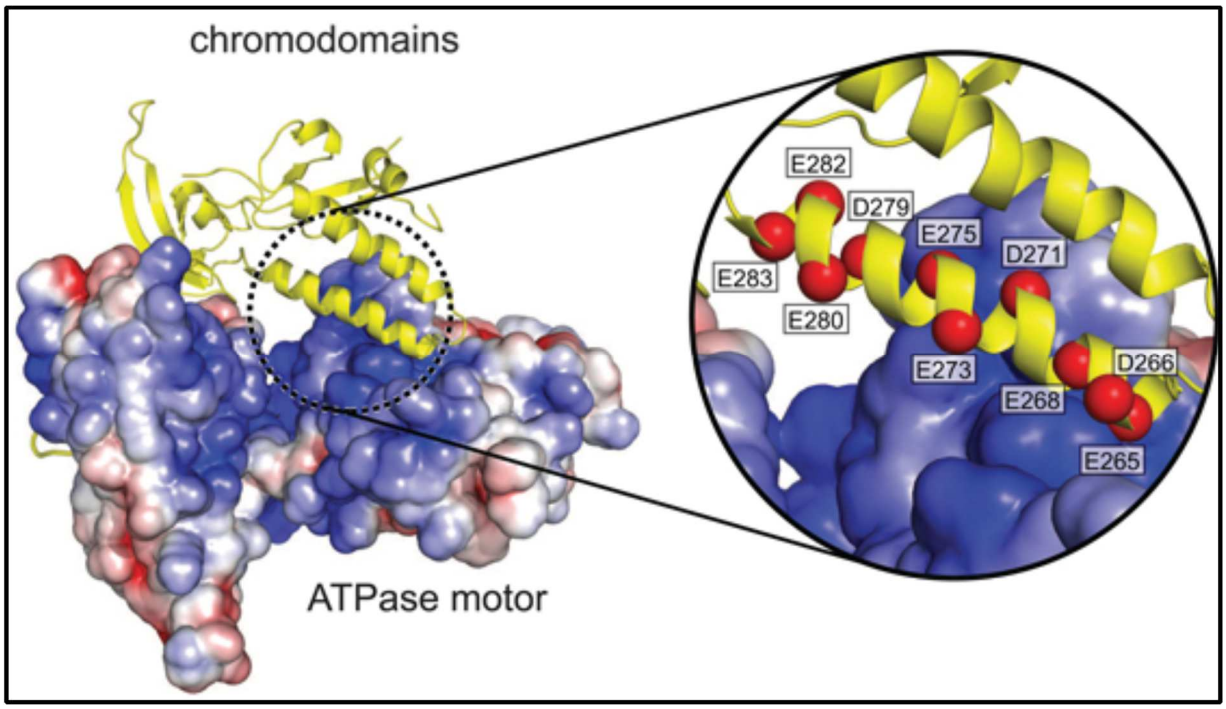


Figure 48 Electrostatic surface representation of CHD1 and a close-up of the chromo-wedge. Electrostatic surface representations of the ATPase motor, and a close-up of the contact between the helical linker of the chromodomains and the second ATPase lobe, with the Cα atoms of acidic residues shown as red spheres. Figure adapted from [138]

This mode of regulation allows CHD1 to control its ATPase activity in a manner such that its ATPase is only engaged once the chromodomains are out of the way and this solves the problem of abberant activation of this chromatin remodeler. A similar model is also seen in the case of ISWI where the ATPase is highly regulated by two accessory domains called the AutoN and NegC that negatively inhibit the SNF2 ATPase. Their inhibition is removed only in presence of the histone H4 tail, which counteracts the activities of both the AutoN and the NegC. Given that AutoN and NegC are also inhibitory elements that need to be inhibited to achieve an active ATPase, this model of governing the ATPase is called "inhibition of inhibition" (Fig. 49) [313].

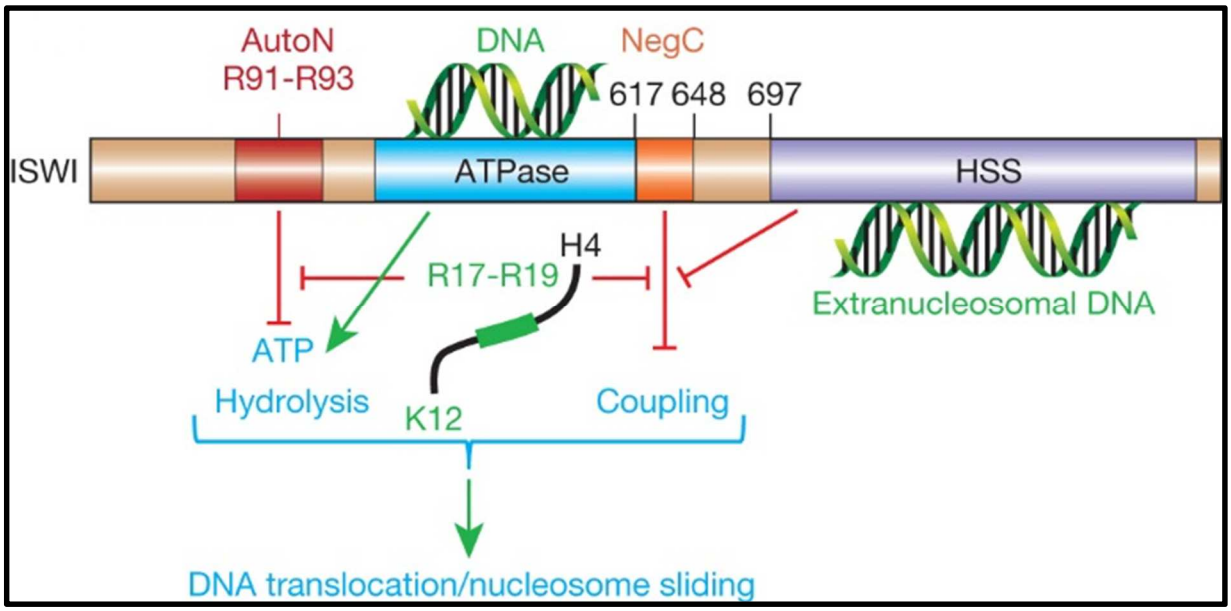
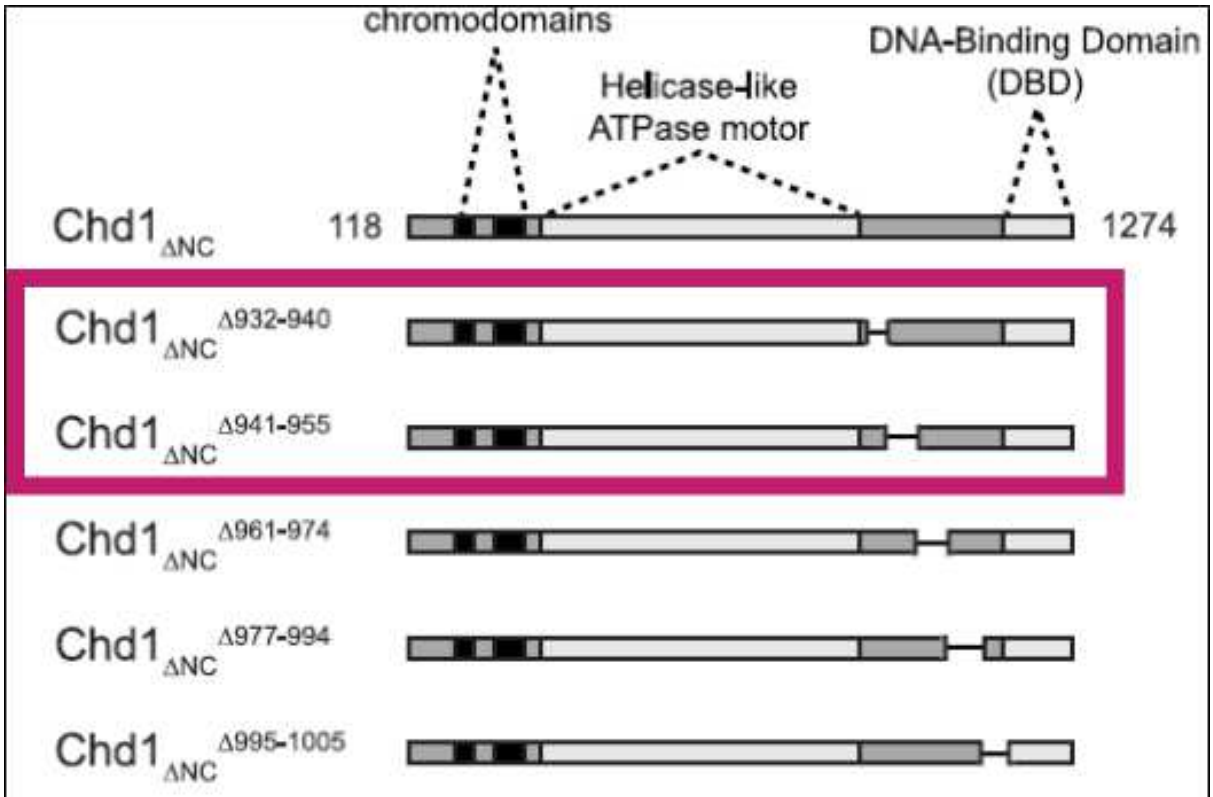


Figure 49 Inhibition of inhibition model of regulation in ISWI. Logic of ISWI ATPase regulation. The ISWI ATPase activity is positively regulated by DNA in the ATPase cleft. ISWI is also negatively regulated by two intrinsic domains: AutoN and NegC. AutoN inhibits the ATP hydrolysis rate, and is relieved by the basic patch (R17-R19) of the H4 tail, whereas NegC inhibits ATPase coupling to DNA translocation, and is relieved by the HSS domain binding to sufficient extranucleosomal DNA: defining the 'inhibition of inhibition' mode of ISWI regulation. Note: an additional lysine (H4K12) also promotes coupling. Figure adapted from [64].

Further, regions on CHD1 and the canonical SNF2 enzyme have been shown to play an active role in coupling the ATPase domain with their remodeling activities [138, 313-318]. The canonical SNF2 enzyme bears a region named SnAC (SNF2 ATPase Coupling) domain C-terminal to its Snf2 ATPase. This region has been shown to bind histone H3 and is highly conserved from yeast to humans. Deleting this region has no effect on the ATPase activity of the enzyme, but has a deleterious effect on the nucleosome remodeling capability [314]. A similar phenomenon occurs in CHD1, where deletion of the region C-terminus to the SNF2 ATPase renders the nucleosome remodeling activities to negligible and severely hampers the DNA stimulated ATPase activity as well (Fig. 50).

ALC1 has been shown to possess cryptic DNA-dependent ATPase and ATP-dependent nucleosome remodeling activities that are activated in the presence of PARP1 and NAD^+, its substrate for PAR synthesis. Second, an intermediate that activates ALC1 and that is resistant to the PARP inhibitor benzamide can be formed by pre-incubation of PARP1 and NAD^+ with nucleosomes or with naked DNA in the absence of ALC1 [305, 319]. Lastly, our studies have shown that activation of ALC1 occurs through direct and stable binding of ALC1 to autoPARylated PARP1. This activation was dependent on interactions between PAR and ALC1 that fall outside of the ADP-ribose binding pocket in its macrodomain and that contribute to ALC1 activation [319].

In the work described in this chapter, we have applied a combination of approaches to determine in more detail the mechanism by which ALC1 nucleosome remodeling activity and ATPase activity is regulated by its domains. In particular we used assays that separate recruitment to the nucleosome, ATPase, and remodeling activity in an attempt to discover specific regions of the protein responsible for such regulatory effects In the course of our



Chd1 variant	normalized initial sliding rate (min^{-1})	nucleosome stimulated hydrolysis relative to ΔNC (%)	coupling efficiency (%)
ΔNC	100 ± 13	100 ± 10	100 ± 17
Δ932-940	0.029 ± 0.004	36 ± 3	0.081 ± 0.012
Δ941-955	1.2 ± 0.3	34 ± 3	3.5 ± 0.9
Δ961-974	50 ± 14	69 ± 10	72 ± 23
Δ977-994	81 ± 17	97 ± 9	84 ± 19
Δ995-1005	67 ± 10	63 ± 6	105 ± 18

Figure 50 Region of CHD1 responsible for coupling ATPase with remodeling. Deletion of residues 932-955 results in complete loss of nucleosome remodeling and significant reduction of ATPase activity Figure adapted from [317].

investigation, we considered three possible models for the regulation of ALC1's SNF2ATPase. First, ALC1's ATPase might be a constitutively active ATPase that is negatively regulated by

In the work described in this chapter, we have applied a combination of approaches to determine in more detail the mechanism by which ALC1 nucleosome remodeling activity and ATPase activity is regulated by its domains. In particular we used assays that separate recruitment to the nucleosome, ATPase, and remodeling activity in an attempt to discover specific regions of the protein responsible for such regulatory effects. In the course of our investigation, we considered three possible models for the regulation of ALC1's SNF2ATPase. First, ALC1's ATPase might be a constitutively active ATPase that is negatively regulated by internal domains including the macrodomain. This hypothesis could be consistent with the fact that a functional and covalently attached PAR-binding macrodomain is necessary for ALC1's activation. Alternatively, ALC1's ATPase could be a poor ATPase, unique amongst its family, and the conserved regions might play a role in its regulation while the macrodomain is merely a device to recruit the ATPase to its substrate. A third formal possibility is that ALC1's SNF2 ATPase is indeed an inactive ATPase that needs to be postively activated by its interaction with the macrodomain and other conserved regions.

Our findings are most consistent with the model that the SNF2 ATPase of ALC1 is positively regulated by the region between the SNF2 ATPase and the macrodomain and by the macrodomain itself. According to this model, these ALC1 regions work in a co-operative manner to yield a properly recruited, remodeling capable, ATPase active SNF2ATPase. Our data argues that in isolation, ALC1's SNF2 ATPase has no ATPase activity, at least under the reaction conditions we have used. Even with saturating concentrations of DNA or nucleosomes, we observe no activation of the SNF2ATPase in any mutant that lacks the macrodomain. These

observations are consistent with the idea that there are no negative regulatory elements in the conserved regions of the protein or in the macrodomain that inhibit an otherwise active SNF2 ATPase motor. This is in stark contrast to its close relatives CHD1 and ISWI, both of which follow a model where the SNF2 ATPase is inherently active and is inhibited by domains flanking the SNF2 ATPase [138, 313].

An important discovery of this study was the definition of NMAC (N-terminal to Macrodomain ATPase Coupling) region. Our study has shown that this region is actively involved in coupling the ATPase activity of ALC1 with its remodeling capabilities. In addition, we have evidence to suggest that it may functions as a putative DNA binding domain (data not shown). In that sense, NMAC is analogous to the SnAC domain of the canonical SNF2 enzyme. CHD1 also has a region analogous to the NMAC C-terminal to its SNF2 ATPase [314, 317]. This region plays a role in coupling the ATPase with its remodeling activity. However, unlike NMAC, deleting the coupling region of CHD1 reduces the ATPase activity of CHD1 drastically, whereas deleting NMAC has virtually no effect on the ATPase activity of ALC1. This robust and specific coupling role of NMAC could be due to a role of NMAC as a "hinge" that latches onto the nucleosome and gives the ATPase motor traction to successfully slide nucleosomes.

We believe regions predicted to be similar to the CHD1 C-terminal Bridge, are likely to be extensions of the SNF2 domain of ALC1. These could play a crucial role in guiding the conformation in which the SNF2 ATPase is held. Deletion of Bridge1 (ALC1 residues 515-551), which encompasses much of the bridge, causes association with the nucleosome independent of PARP1 and NAD^+. This domain appears to be analogous to the alpha-27 helix of Rad54 and an analogous region in INO80 that splay the two ATPase lobes in an open confirmation that does not allow it to engage its substrates [320]. The ATPase activity of INO80 increases dramatically

after the deletion of this region, but deleting Bridge1 renders the ATPase of ALC1 inactive [321]. However, we speculate the Bridge1 splays the ATPase lobes of ALC1 in a confirmation incapable of engaging the nucleosome. Upon interaction with autoPARylated PARP1, a conformational change allows it to interact with the nucleosome and engage the ATPase as well. We suspect that deletion of Bridge1 allows the ATPase lobes of ALC1 to engage the nucleosome, but disrupts the communication to the ATPase to process ATP. Bridge2 (ALC1 residues 552-617) has a similar effect on ALC1 but to a much lesser degree. Taken together, our observations suggest that Bridge1, Bridge2 and NMAC play an important role in inter-domain communication that positively regulates ALC1's SNF2 ATPase.

Lastly, replacing the ALC1 macrodomain with new macrodomains shed further light on the positive regulation of the ATPase by this accessory binding domain. The constitutively active nature of ALC1Mac1.1 and its PAR non-binding mutant version ALC1Mac1.1G224E is indicative of a greater role of the macrodomain in activating the ATPase of ALC1. Biologically, ALC1 plays an active role in DNA damage in conjunction with PARP1. It has also been shown that PARP1 recruits ALC1 and macrohistoneH2A1.1 via their PAR binding macrodomains to sites of DNA damage [290]. It is tempting to speculate that at sites of DNA damage, interaction of ALC1's SNF2 ATPase with the macrodomain of macroH2A1.1 may render it active independent of autoPARylated PAR. However, if this were the case, then any macrodomain with similar surface residues might be able to activate ALC1's ATPase. However, the macrodomain of macroH2A1.2 is almost identical to macroH2A1.1, differing only in a few residues (macroH2A1.2 has an E-I-S insertion at the mouth of the PAR binding pocket) that sterically block the interaction of PAR with the binding pocket [207]. If the surface interactions were the only crucial factor in this activation, then ALC1Mac1.2 would also be constitutively active. Our

observation that ALC1Mac1.2 is inactive suggested that there is a particular confirmation of Mac1.1 that allows it to activate the ATPase. Our experimental data further suggests that the conformation of the PAR binding pocket of Mac1.1 is able to constitutively activate the SNF2 ATPase. Not surprisingly, ALC1Mac2A2 did not have any detectable ATPase activity, as it shares neither the surface residues nor a PAR-binding pocket to activate the ATPase. The PAR binding, constitutively active mutant ALC1-Mac1.1 was recruited to nucleosomes in a PAR-dependent manner, while its PAR-non binding version ALC1-Mac1.1G224E, which is also constitutively active, is not recruited to the nucleosomes. This suggested that neither PAR binding or nucleosome recruitment is essential in the activation of ALC1, but that a conformational change induced in the ALC1 macrodomain after the PAR biding event might be crucial for ALC1's activation.

Taken together our observations allow us to conclude that ALC1 SNF2 ATPase may be inherently inactive and is positively regulated by conserved regions in the protein and the macrodomain conformation. In addition, we discovered a new region in the protein – NMAC that is able to couple ATPase and nucleosome remodeling activities. The bridge regions and sequences downstream also play an important role in relaying the communication to the SNF2 ATPase and are necessary for its activation. Their role in recruitment also highlights that the SNF2 ATPase may be in an inactive conformation in its resting state. This hypothesis is plausible as it has been observed that in the crystal structure of CHD1, the ATPase lobes are splayed open in an inactive conformation by a feature called the chromo-wedge [138]. More importantly the ATPase active nature of ALC1ΔNMAC, which is poorly recruited to the nucleosomes and PARP1 independent ATPase activation of the chimeric construct ALC1Mac1.1G224E, which is also poorly recruited to the nucleosome is indicative of the

independence of nucleosomal recruitment and SNF2ATPase activation, suggesting that ATPase activation does not require stable nucleosomal interaction. Transient interactions with the nucleosome and/or DNA are sufficient and necessary to activate the SNF2ATPase of ALC1.

The invention of a constitutively active Alc1 SNF2 enzyme by replacing its macrodomain with a macrodomain from macrohistoneH2A1.1 is also informative. Primarily, it tells us that the macrodomain is not just a tethering domain that increases the local concentration of the reactants. Instead, it plays an active role in activating the SNF2ATPase of Alc1. The constitutive active nature of ALC1Mac1.1 and ALC1Mac1.1G224E as opposed to the ATPase inactivity of ALC1Mac1.2, indicate that the PAR binding pocket of Mac1.1 has a specific confirmation that allows it to trigger the SNF2 ATPase activity. This interaction is independent of a PAR-binding event and is possibly relevant *in vivo* due to the inv lvement of macrohistoneH2A1.1 and Alc1 in PARP guided DNA damage repair. This also suggests that the Mac1.1 is able to activate the Alc1's SNF2ATPase by some very specific interactions. The Macro1.1 and Macro1.2 are splice variants and differ only by 30 amino acids. A prominent insertion of E-I-S residues in the PAR binding pocket of Macro1.2 renders it PAR non-binding. Further mutagenesis studies can shed light on the precise nature of the interaction between Alc1's SNF2 ATPase and the macrodomain of macrohistoneH2A1.1. With a thorough mutagenesis, it might be possible to mutate the macrodomain of ALC1 to mimic the macrodomain of macrohistone2A1.1 and produce another constitutively active version of the enzyme which may help understand the mechanism of its activation in further detail. Given the important biological roles of ALC1 and its involvement in several cancer pathogenesis, understanding its mechanistic control would be valuable information to design specific drug screens against ALC1.

I would like to close by presenting a composite view of all the information we have gained about the regulation of ALC1 (Fig. 51). The interplay of intermolecular and intramolecular interactions control the ATPase of ALC1 and may potentially be able to repurpose it in different cellular contexts. The fact that macroH2A1.1 can activate ALC1, suggests that there might a greater interplay between PARP, PAR, macrodomain containing histones and ALC1 in the context of DNA damage repair and transcription.

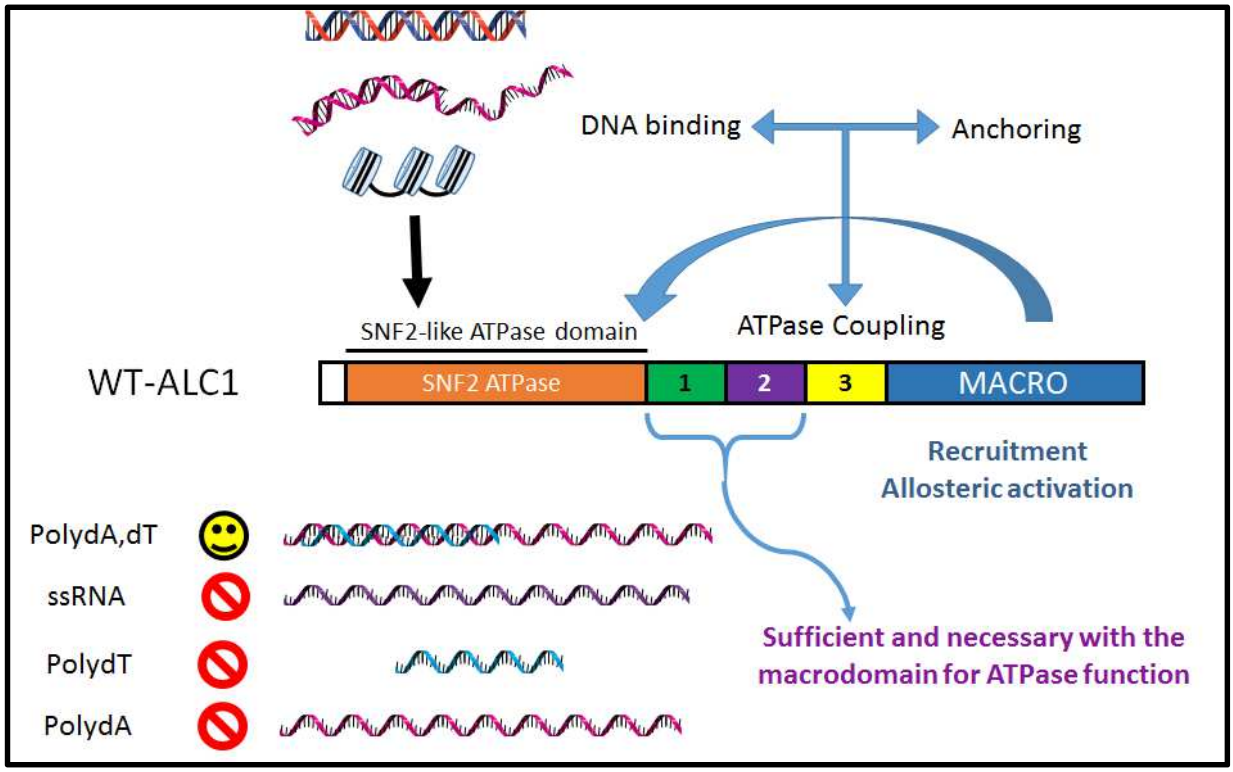


Figure 51 Working model for Alc1's ATPase activation. Logic of ALC1 ATPase regulation. The ALC1 ATPase activity is positively regulated by dsDNA or dsDNA with overhangs in the ATPase cleft. ALC1 is also positively regulated by two intrinsic domains: macrodomain and the linker between the ATPase and the macrodomain. NMAC couples ATPase activity with remodeling and also serves as an accessory DNA binding site.

CHAPTER IV

MATERIALS AND METHODS

Expression of Flag-ALC1 and ALC1 derived mutants used in the study.

Expression of Recombinant Proteins in Insect Cells. For expression of recombinant proteins in insect cells, wild-type human ALC1 (Accession NP_004275.4 | GI:373428660) and ALC1 mutants used in the study (ALC1 C-terminal and internal deletion mutants, ALC1 point mutants and ALC1-macrodomain chimeras) containing N-terminal FLAG epitope tag were subcloned into pBacPAK8 [322]. All ALC1-macrodomain chimeras included ALC1 residues 1-666. These were fused to residues 162-369 of histone macroH2A1.1 (Accession NM_138609.2 | GI:93141010) or a histone macroH2A1.1 with G224E mutation or residues 161-371 of histone macroH2A1.2 (Accession NM_004893.2 | GI:20336744) or residues 162-372 of histone macroH2A2 (Accession AF336304.1 | GI:14028706). Between the ALC1 residues and the macrodomains, are an additional two residues (Arg and Ile) encoded by an in frame EcoR1. Recombinant baculoviruses were generated with the BacPAK expression system (Clontech). Sf9 cells were cultured at 27°C in Sf-900 III SFM, supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. Flasks containing $1x10^8$ Sf9 cells were infected with the recombinant baculoviruses. Forty-eight hours after infection, cells were collected and lysed in 15 ml of ice-cold buffer containing 50 mM HEPES-NaOH (pH 7.9), 0.5 M NaCl, 5 mM $MgCl_2$, 0.2% Triton X-100, 20% (vol/vol) glycerol, 0.28 mg/ml leupeptin, 1.4 mg/ml pepstatin A, 0.17 mg/ml PMSF, and 0.33 mg/ml benzamidine. Lysates were centrifuged at 100,000 X g for 30 min at 4°C.

Purification of Recombinant Proteins.

FLAG-tagged proteins were purified from Sf9 cell lysates by anti-FLAG agarose immunoaffinity chromatography. Lysates from $1x10^8$ cells were incubated with 0.5 ml anti-

FLAG (M2) agarose beads for at least 8 h at 4°C. The beads were washed at least six times with Tris-buffered saline [50 mM Tris-Cl, pH 7.5] (TBS), which contained 0.8 M NaCl. Bound proteins were eluted from the beads with TBS containing 10% (vol/vol) glycerol and 0.3 mg/ml FLAG peptide. Where indicated, anti-FLAG agarose eluates prepared from Sf9 cells expressing recombinant FLAG-Alc1 proteins were further purified by ion exchange HPLC. Eluates were adjusted to a conductivity equivalent to that of 0.1 M NaCl and applied to a 0.6 ml TSK DEAE-NPR HPLC column (TosohBioSep) pre-equilibrated in buffer A [40 mM TrisHCl, pH 7.91 mM EDTA1 mM DTT 10% (vol/vol) glycerol] containing 0.1 M NaCl. The column was eluted with a 6-ml linear gradient from 0.1 to 1 M NaCl in buffer A, and 0.2-ml fractions were collected. Protein concentrations were estimated by comparing the intensities of Imperial™ Protein Stain (Thermo Scientific) bands with that of BSA in SDS-polyacrylamide gels scanned using an Image Studio® imaging system (LI-COR Biosciences).

PARP1 and PARG

PARP1 (high specific activity, >95% pure) and PARG (high specific activity, >95% pure) was obtained from Trevigen.

Generation of ^{32}P labelled 601-DNA Fragment

A 216 bp DNA fragment, "601" DNA^{32}P-labeled fragment, containing an end-positioned 601 nucleosome positioning sequence was amplified in a PCR reaction from pGEM-3Z-601 [323] in the presence of 6000Ci/mmol [α-^{32}P] dCTP. The forward and reverse primers used were a) 5′-ACAGGATGTATATATCTGACCGTGCCTGG and b) 5′-AATACTCAAG CTTGGATGCCTGCAG respectively. The 601 DNA fragment was amplified using 100 µl PCR

reaction that were set up by mixing, 67.5 µl deionized H_2O, 10 µl 10X PCR reaction buffer (Roche), 1µl pGEM-3Z-601 (10 ng/µl), 5 µl of 10 µm forward primer (a), 5 µl of 10 µm of reverse primer (b), 0.5 µl of dNTP stock solution containing 10 mM of each of the 4 dNTPs (dATP, dCTP, dGTP and dTTP), 1 µl Roche Taq DNA polymerase and 10 µl of [α-^{32}P] dCTP (6000 Ci/mmol, 3.3 µM)**.** The PCR reactions were performed in a thermal cycler (MJ Research, PTC 200) using the following program: Step 1: 1 min @ 96 ^{0}C; Step 2: 45 sec @ 94 ^{0}C; Step 3: 30 sec @ 57 ^{0}C; Step 4: 60 sec @ 72 ^{0}C; Step 5: Go to step 2 for 29 additional cycles Step 6: 7 min @ 72 ^{0}C; Step 7: forever @ 4 ^{0}C. Upon reaction completion, unincorporated nucleotides were removed by passing twice sequentially through NucAway™ spin columns (Ambion®, Cat. # AM10070) according to the manufacturer's instructions. To confirm the success of the PCR reaction, 5 µl of the reaction product was run on a 2% agarose gel. Detection of a single ~216 bp DNA band by ethidium staining confirmed a successful PCR reaction. DNA concentration was measured using a UV spectrophotometer.

Preparation of nucleosomes:

Nucleosomes were prepared from HeLa cells and transferred onto the ^{32}P-labeled or non-labelled 601 DNA by a serial dilution method essentially as described by Owen-Hughes *et al.* [324]. 50 pmol of ^{32}P-labeled 601 DNA fragment are mixed with 225 µg of HeLa nucleosomes in 1000 µl of a buffer containing 1.0 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 mM PMSF, and 1 mM DTT. After incubation at 30 °C for 20 minutes, the mixture was sequentially adjusted to 0.8, 0.6, and 0.4 M NaCl by dilution with 250 µl, 416 µl, and 832 µl, respectively, of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 mM PMSF, and 1 mM DTT, with a 30 min incubation at 30 °C after each dilution. Finally, the mixture was sequentially diluted to 0.2 M and

0.1 M NaCl by addition of 2.5 ml and then 5 ml of the same buffer containing 0.1% Nonidet P-40, 20% glycerol, and 200 μg/ml BSA. After reconstitution, the mononucleosome substrate can be stored at 4 °C for up to 3 months.

Poly ADP ribose Binding Assays.

Recombinant proteins (100 ng or 200 ng as indicated in the figure) were incubated for 30 min at 30 °C in 20 μL 40 mM HEPES-NaOH, pH 7.9, 0.1 M NaCl, 0.1 mM EDTA, 10% glycerol, and ^{32}P-labeled PAR, purified as described [145]. Reaction mixtures were applied to nitrocellulose and washed overnight with TBS-T (TBS and Tween 20), which contain 100 mM NaCl. Bound ^{32}P-labeled PAR was detected using a Typhoon phosphorimager.

DNA Binding Assays

Recombinant proteins (1 pmol) were incubated for 30 min at 30 °C in 15 μL 40 mM HEPES-NaOH, pH 7.9, 0.1 M NaCl, 0.1 mM EDTA, 10% glycerol, and ^{32}P-labeled 601 DNA fragment. Reaction mixtures were applied to nitrocellulose and washed overnight with TBS-T containing 100 mM NaCl. Bound ^{32}P-labeled 601 DNA fragment was detected using a Typhoon phosphorimager.

ATPase Assays.

ATPase assays were performed essentially as described [322]. Where indicated in figure legends, reaction mixtures contained ≈100 ng (1 pmol) DEAE-NPR fraction of Flag-Alc1 (wild-type or mutants) from Sf9 cells. Unless indicated otherwise, reactions contained 100ng of DNA,

~115 ng (1 pmol) Parp1 (Trevigen), 34 μM nicotine adenine dinucleotide, and 150 ng nucleosomes. Nucleosomes were prepared by salt dialysis as described above, except that 601 DNA was not radiolabelled [324]. Nucleic acid sequences used in the assays, wherever indicated, are as follows:

50 base pair dsDNA:

5'-CCGGCGCATAGAGCTCGTAGCCTCCTGTCAGGAGGCGCCTGGACG-3'

50 base pair ssRNA

5'-AUCCGCGUAUCUCGAGCAUCGGAGGACAGUCCUCCGCGG-3'

20 base pair dsDNA

5'-AGTGTCATATGCCTGAGATA-3'

20 base pair ssDNA

5'-TATCTCAGGCATATGACACT-3'

18 base pair ssDNA

5'-TATCTCAGGCATATGACA-3'

Nucleosome Remodeling Assays

Mononucleosomes were reconstituted by dilution transfer from HeLa cell oligonucleosomes on a ^{32}P-end-labeled 216-bp DNA fragment (601-lat Gal4) generated by PCR from pGEM3Z-601-Gal4 as described above [324, 325]. 1 pmol of ALC1 was incubated at 30 °C for 30 min with mononucleosomes (0.01 pmol of labeled mononucleosome and 0.25 pmol of unlabeled oligonucleosomes) in 20 mM HEPES-NaOH (pH 7.9), 50 mM NaCl, 4.5 mM $MgCl_2$, 2 mM DTT, 0.5 mM PMSF, 100 μg/ml BSA, 10% glycerol, 0.02% Triton X-100, and 0.02% Nonidet P-40. 2 μM ATP, 34 μM NAD^+, and benzamide were included in reactions as indicated.

Reaction products were incubated for a further 30 min with 10 units of HhaI and resolved on gels containing 7% polyacrylamide (19:1 acrylamide:bisacrylamide), 7 M urea, 45 mM Tris borate, and 1 mM EDTA (pH 8.3).

Immobilized Nucleosome and DNA Binding Assays

50 pmol of 5′-biotinylated 601 DNA fragment or mononucleosomes assembled on the same DNA fragment were bound to 500 µl of streptavidin-coupled Dynabeads® (Invitrogen), washed, and resuspended in a final volume of 500 µl (100 fmol of DNA or mononucleosome/µl of beads). 0.5 pmol of PARP1, with or without 1 pmol of ALC1, was incubated with 0.5 pmol of immobilized nucleosomes or DNA in 50 µl of buffer containing 20 mM HEPES (pH 7.9), 50 mMNaCl, 0.5% Nonidet P-40, 10% glycerol, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 1 mM ATP, 100 µg/ml BSA, and 34µM NAD^+ as indicated in the figures or figure legends. Nucleosome- or DNA-bound intermediates on Dynabeads® were collected using DynaMag^{TM} magnets (Invitrogen), washed with 200 µl of the same buffer, and transferred to a fresh microcentrifuge tube, and bound proteins were eluted with 1× SDS sample buffer. Template-bound intermediates were fractionated by SDS-PAGE gel and transferred to PVDF membranes, and visualized using a Typhoon PhosphorImager (Molecular Dynamics) to detect ^{32}P-PARylated proteins or by Western blotting using an Image Studio® imaging system.

Isolation and Analysis of PAR

ALC1·PARylated PARP1·nucleosome or PARylated PARP1·nucleosome intermediates were assembled in the presence of [^{32}P]NAD^+ (PerkinElmer Life Sciences) as described in the previous section. To isolate radiolabeled PAR, reaction products were digested in 60 µl of 20

mM EDTA, 0.1% SDS, 200 mM NaCl, 20 μg of GlycoBlue (Ambion), and 10 μg of proteinase K (Sigma) at 60 °C for 12 h. Following proteinase K digestion, reactions were brought to 0.08 M KOH and incubated for a further 3 h at 60 °C to remove any remaining peptides bound to the PAR species. PAR was precipitated with 70% ethanol; resuspended in loading buffer consisting of 6 M urea, 0.02% bromphenol blue, and 0.02% xylene cyanol; and analyzed on gels containing 20 or 25% polyacrylamide (19:1 acrylamide:bisacrylamide), 7 M urea, 45 mM Tris borate, and 1 mM EDTA (pH 8.3). Reaction products were visualized using a Typhoon PhosphorImager and analyzed using ImageQuant TL software (Molecular Dynamics).

CHAPTER V

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