BIOCHEMICAL AND DEVELOPMENTAL CHARACTERIZATION OF A SNF2-LIKE ATPASE AMPLIFIED IN LIVER CANCER 1 (*ALC1*)

BY

Aaron J. Gottschalk

Submitted to the graduate degree program in Biochemistry and Molecular Biology
and the Graduate faculty of the University of Kansas Medical Center
in partial fulfillment for the degree of
Doctor of Philosophy.

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Chairperson

Committee members* _____*

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Date defended: _____

The Dissertation Committee for Aaron J. Gottschalk certifies
that this is the approved version of the following dissertation:

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Chairperson*

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest thanks and gratitude to my mentors Joan and Ron Conaway. During my tenure in their lab as a graduate student I have been afforded an amazing opportunity to learn and grow as a scientist while independently developing this project. It is their extreme kindness, steadfast encouragement, guidance, and humor that have made even the toughest of problems both scientific and in life seem very surmountable, and for that I thank them profoundly. Next, I would like to thank my committee members Drs. Glen Andrews, Jennifer Gerton, Leslie Heckert, and Ken Peterson for all their useful advice and suggestions throughout the course of my graduate career. Not only did my committee members provide great scientific direction but they also made bi-annual meetings fun and engaging. I would like to thank all of the members of the Conaway Lab including: Charles Banks, Margaret Banks, Yong Cai, Jingji Jin, Stephanie Kong, Nawel Mahrour, Ana Pedraza, Chieri Sato, Shigeo Sato, Henrietta Szutorisz, Hide Takahashi, Tingting Yao, Dotan Sela, Lu Chen, and Merry McClaird. The members of the lab provided me with an infinite amount of 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 labmates. I would like to particularly thank the core faclities here at Stowers Institute including Mike Washburn, Laurence Florens, and Selene Swanson in Proteomics; Tari Parmely, Maria Katt, and Valerie Neubauer in Tissue Culture; and countless members of the molecular biology and aquatics core facilities who have through their collaborative services allowed my research to flourish. I would like to thank Andreas Ladurner and

Gyula Timinzsky (EMBL), and Lee Kraus and Raga Krishnakumar (Cornell University) for collaborative efforts on this project. I would particularly like to thank Jim and Virginia Stowers for providing such an amazing institute. Lastly, and perhaps most importantly, I would like to thank my parents and family members for their unconditional love and support.

ABSTRACT

Post-translational modifications play a key role in recruiting chromatin remodeling and modifying enzymes to specific regions of chromosomes to modulate chromatin structure. Alc1 (Amplified in Liver Cancer 1), a member of the SNF2 ATPase superfamily with a carboxy-terminal macrodomain, is encoded by an oncogene implicated in the pathogenesis of hepatocellular carcinoma. Using a variety of biochemical techniques we show that Alc1 interacts transiently with chromatin-associated proteins, including histones and the poly-(ADP-ribose) polymerase Parp1. Alc1 ATPase and chromatin remodeling activities are strongly activated by Parp1 and its substrate NAD and require an intact macrodomain capable of binding poly-(ADP-ribose). Alc1 is rapidly recruited to nucleosomes *in vitro* and to chromatin in cells when Parp1 catalyzes PAR synthesis. We propose that poly-(ADP-ribosyl)ation of chromatin-associated Parp1 serves as a novel mechanism for targeting a SNF2 family remodeler to chromatin. Using zebrafish as a model organism, we aimed to study possible roles of *ALC1* in early organismal development. We found through qPCR and whole mount *in situ* analysis, that *ALC1 is* expressed ubiquitously within the blastomere prior to gastrulation, with peak expression observed during the Dome stage, and later at 24hpf that *ALC1* is expressed within the anterior central nervous system. The injection of embryos with morpholinos targeting *ALC1* resulted in pleiotropic phenotypes that were partially rescued by co-injection of either human or zebrafish mRNA. These findings suggest that Alc1 and its associated enzymatic activities are most likely required for proper organismal development.

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Chapter I. Introduction

Chromatin Structure and Nuclear Architecture

Initial studies on chromatin structure began in the early 1960's when Joseph Gall used electron microscopy to describe the contents of nucleated newt erythrocytes as highly flexible fibers of uniform diameter and was the first to suggest they were interphase chromosomes [1]. Throughout the 1960's it was largely accepted that eukaryotic chromatin was composed of a linear strand of DNA coated with an evenly repeated arrangement of 5 histone proteins forming a 100Å fibers. Nearly a decade later, biochemical studies performed by Hewish and Burgoyne using DNAse to digest chromatin suggested that chromatin consisted of a more periodic particulate structure [2]. Moreover, electron microscopy analysis on interphase nuclei performed by Olins and Olins in 1974, and Woodcock and Stanchfield in 1976 suggested the existence of multiple forms of chromatin, including both 70Å fibers and 15Å strands resembling "beads on a string" [3-5]. Together, these pivotal studies suggested that chromatin was not a monolithic entity as once thought but may be highly dynamic, taking on various forms and structures depending on biological need.

In 1974, a landmark paper concerning chromatin structure and the oligomerization of histones was published by Roger Kornberg and Jean Thomas[6]. With the use of biochemical techniques such as chemical cross-linking of proteins followed by sedimentation analysis, they deduced that the fundamental unit of chromatin was composed of an octamer of histones wrapped in DNA. Furthermore, the octamer itself consisted of a tetramer of histone proteins H3 and H4, and two copies of a dimer

formed between histone H2A and H2B. They noted approximately 1 copy of each subunit per 100 base pairs of DNA, and ,through the observed ratios of histones, deduced that the repeating unit of chromatin includes approximately 200bp of DNA. Thus the nucleosome hypothesis was born and consequently a great paradigm shift occurred in the understanding of how nuclear processes are governed.

More than two decades later the structure of the nucleosome was finally solved after many years of tireless work by Karolyn Luger and Tim Richmond[7]. Using x-ray crystallography to map the peptides within the nucleosome to a resolution of 2.8 Å, they discovered that 146 base pair of DNA are wrapped around an octamer of histones, making 14 histone-DNA contacts through the phosphodiester backbone. The structure was in accord with Kornberg's and Thomas's hypothesis, 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. Structural studies by Luger and colleagues 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. While normally an unstructured region of a protein may be of least interest to a structural biologist, many studies throughout recent decades have suggested these N-terminal tails also play important regulatory roles in a variety of nuclear processes, many of which we will cover later.

The current view concerning the hierarchical structure of chromatin starts with the most simple chromatin structure consisting of nucleosomes evenly spaced across DNA with roughly 50 base pair of linker DNA separating them forming a "beads on a string" structure (Fig. 1). Nucleosomes, with the help of linker histones, such as histone H1 [8] and other non histone proteins, are in turn folded into higher order arrays to form chromatin fibers of roughly 30nm in diameter[9]. These chromatin fibers are further woven together to form chromosomes. This elaborate and highly efficient packaging allows the ~2 meters of DNA that make up the human genome to fit into nuclei with diameters on the order of 2-6 microns. While the organization of chromatin into a structure of repeating nucleosomes brings many benefits in terms of compaction and topology, it also comes at a cost because this wrapping of DNA around the nucleosomes makes the sequences within the octamer relatively inaccessible to certain DNA-binding regulatory proteins and accordingly is refractory to necessary nuclear processes such as DNA replication, DNA repair, and transcription. Not surprisingly, eukaryotes have evolved an elaborate system of enzymes including histone modifiers, histone chaperones, and chromatin remodelers that act in concert to change the landscape of chromatin and allow regulatory proteins access to DNA and necessary nuclear processes to ensue.

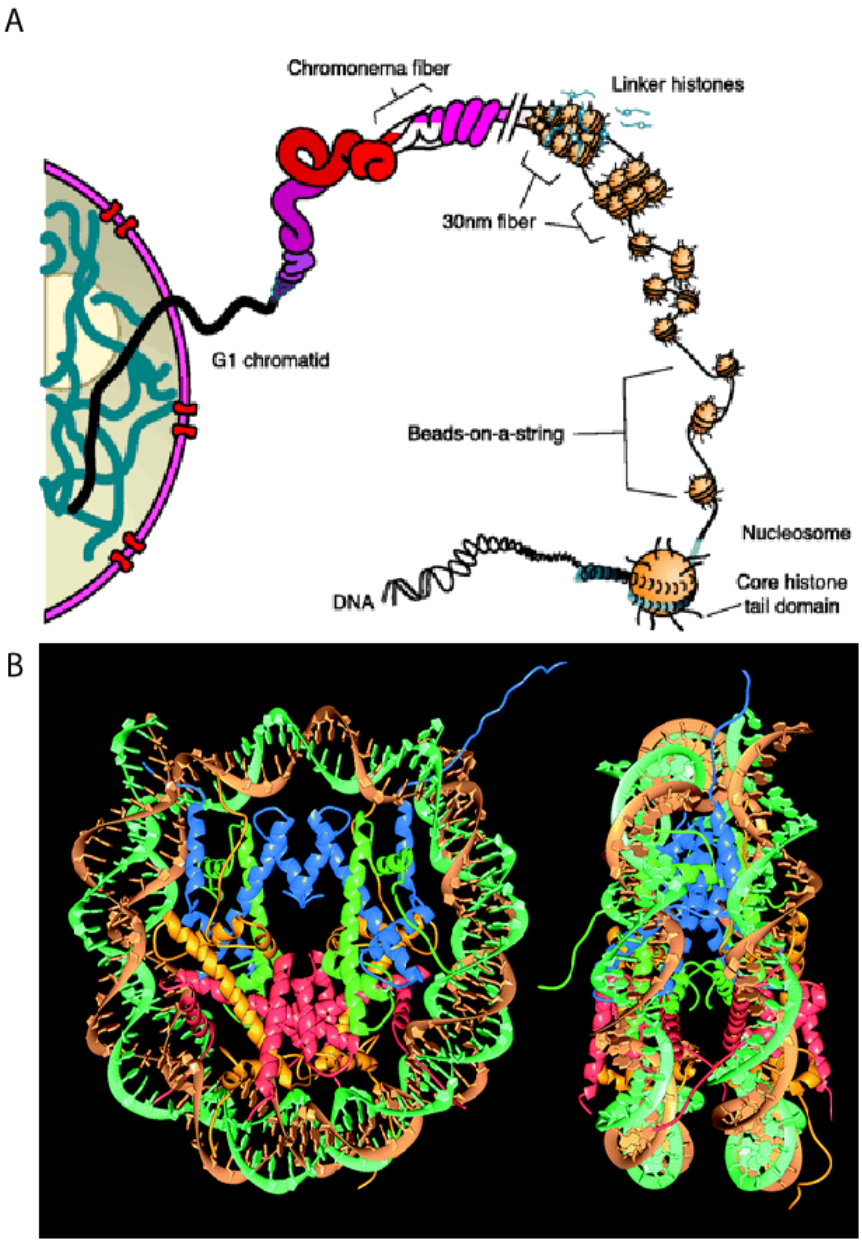


Figure 1. Multiple levels of chromatin folding. A. Compaction of DNA within the interphase nucleus occurs in a hierarchical fashion beginning with the most basic structure of chromatin, the nucleosome. Strings of nucleosomes can be further compacted into 30nm fibers through histone tail-mediated nucleosome-nucleosome interactions. Finally, tertiary structures such as chromanema fibers are formed through tail-mediated interaction of individual fibers. B. 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. Figure adapted from (Horn, PJ & Peterson, CL. *Science* 297(5588):1824 (2002)) and (Luger, K et al. *Nature* 389(6648):231 (1997)).

Histone Modifying enzymes and their Modifications

One of the first studies suggesting nucleosomes had a function outside of packaging DNA came from the Kornberg lab in 1987. It was found that promoters assembled with nucleosomes were refractory to transcriptional initiation by both SP6 polymerase and the mammalian RNA polymerase II complex *in vitro*. Interestingly, while nucleosomes inhibited transcriptional initiation *in vitro,* both polymerases, once engaged, were capable of reading through a nucleosome [10]. Moreover, as both polymerases transcribed through nucleosomes, there seemed to be a concomitant loss of nucleosomes, suggesting displacement by an unknown mechanism. These findings suggested that nucleosomes may distinctly regulate the earliest steps in the context of RNA transcription. Despite this interesting finding, chromatin was still largely ignored by scientists until a year later when Han and Grunstein showed nucleosome loss at select promoters, through histone H4 depletion, resulted in transcriptional activation of multiple genes [11]. After these two complimentary reports were published it was apparent that 1) nucleosomes likely to regulated many biological processes; and 2) the chromatin field was about to gain much more attention.

Within the chromatin field intense focus was now building around nucleosomal regulation of biological processes. How do nucleosomes regulate these processes? Is regulation imparted by simply the presence or absence of a nucleosome on a given sequence of DNA? Or could there be more intricate mechanisms of nucleosomal regulation that serve to "fine tune" nuclear processes? A major leap forward in understanding of how nucleosomes regulate nuclear processes came from the Grunstein

lab, who probed nucleosomal function by using *Saccharomyces cerevisiae* strains expressing histones with deleted segments *in vivo*. While they found that deletions within the hydrophobic core of histone H4 were lethal and blocked chromosomal segregation, they found that N-terminus is dispensable for growth but essential for repressing the silent mating loci in *S. cerevesiae*. Moreover, the derepression of the mating loci was specific, as other regulated genes were repressed and induced normally. Using this same strategy they found no effects of N-terminal deletions of histones H2A and H2B on mating. Interestingly, a second study published by the Grunstein lab two years later suggested that loss of a region in the H4 N-terminus encompassing a number of extremely conserved lysines resulted in drastic reduction of *GAL1* and *PHO5* activation in *S.cerevisiae* [12]. These findings now seem quite trivial, but at the time were the first to suggest that: 1) histones in and of themselves contribute to unique biological functions; 2) highly conserved histone tails had specific biological roles; and 3) conserved residues on histone tails may play specific roles in regulation of chromatin function.

In recent years it has been shown by many groups that residues in the N-terminal tail of histones along with defined positions within the globular domain can carry post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP ribosylation [13-20] (Fig. 2). The first evidence that these modifications could be reversible came from the Allis and Schreiber labs in 1996 [21-22]. The Allis lab first reported the purification of a *Tetrahymena* protein strikingly homologous to yeast *GCN5*, which had been shown genetically to behave as a

transcriptional activator. The group found that Gcn5 could act as a histone acetyltransferase *in vitro* and that its enzymatic activity was required for transcriptional activation *in vivo*. Schreiber and colleagues purified mammalian histone deacetylases using matrices formed with known HDAC inhibitors, and, using microsequencing, found that one of the proteins was highly homologous to yeast *RPD3,*which had been shown genetically to behave as a transcriptional repressor [22]. To date, every one of the 4 canonical histones has been reported to be reversibly acetylated with different functional outcomes dependent on the residue acetylated. Generally it is believed that acetylation of histones results in a much more "decondensed" chromatin that is permissible for ensuing nuclear processes, and hypoacetylated regions of chromatin tend to be "condensed" and are transcriptionally inactive.

Jenuwein and colleagues were the first to report a functional link between histone methylation and chromatin structure [23]. Using genetic screens in fruitflies and fission yeast they identified a protein Su(Var)3-9 that was capable of propagating heterochromatin, a higher-order structure of chromatin that is generally not permissible for transcription. Moreover, this protein proved to be a histone methyltransferase with activities specific for histone H3K9. Finding an enzyme capable of removing methylation marks, and H3K9 in particular, proved difficult until Zhang and colleagues discovered that a large group of proteins containing a jumonjiC-domain acted as histone demethylases and, in particular, that the protein JHDM2A could specifically remove H3K9 methylation [24]. Methylation events unlike acetylation events which generally result in open chromatin, are proving to be more of a specialized modification tailored to

regulate specific steps in nuclear processes and often result in different biological outcomes depending on the residue modified and extent of modification.

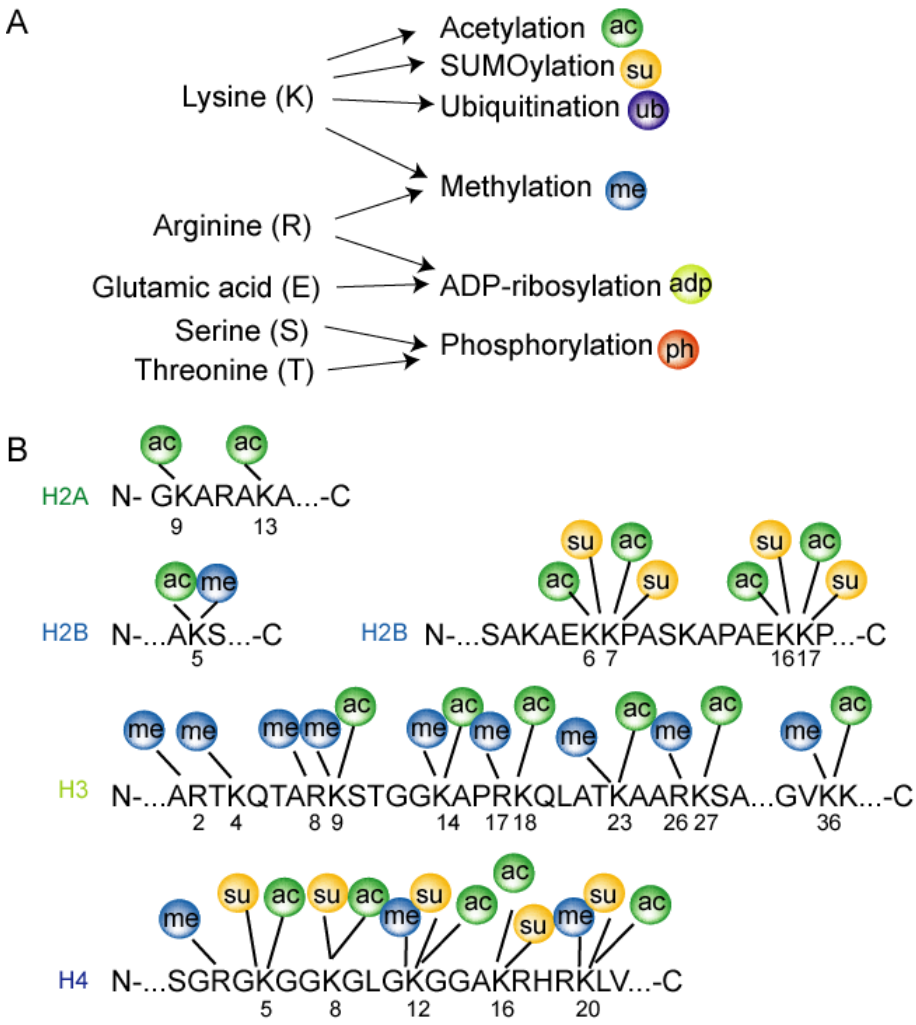
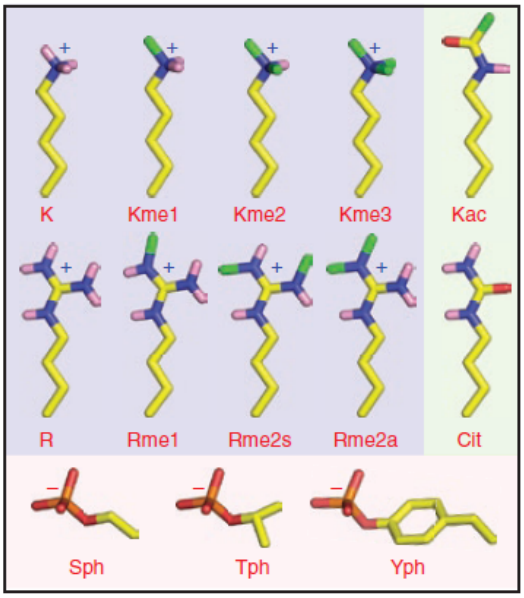


Figure 2. Flexible and highly charged N-terminal histone tails are post-translationally modified. A. residues and their corresponding modifications B. Primary structure of histone N-terminal tails and the modifications mapped to date.

During the mid 1990's it was becoming readily clear that histone modifications including acetylation and methylation were important regulators of chromatin structure and nuclear processes, but it was still unclear how these effects were imparted. Many

speculated that modification of the highly basic histone N-terminal tails merely acted to change local chromatin structure through charge neutralization, ultimately leading to disruption of histone-DNA contacts. While this is certainly true in the case of acetylation of lysine residues, methylation of lysine groups does not affect the overall charge of the histone molecule, indicating these modifications may exert their effects in other manners[25]. One of the first clues to the how histone modifications impart effects on chromatin structure and nuclear processes came from the Grunstein and Gasser labs in 1995 [26]. They found that silent information regulator 3 (Sir3) and Sir4, which were known to be required for repression of yeast silent mating-type loci, could bind to methylated histone tails through a chromodomain. This binding event was required for the targeted recruitment of SIRs within the genome. Importantly, they also found that when histone tails were acetylated, Sir3 and Sir4 failed to bind, suggesting the possibility of cross-talk between histone modifications. In 1999, the Zhou group solved the crystal structure of the bromodomain, a domain commonly found in transcriptional activators and at the time known to bind acetylated lysine residues [27]. With the identification of the chromo- and bromo- domains, the race to find additional histone reader modules began. Today researchers have identified a growing number of domains (Fig. 3) recognizing a variety of modifications that serve as binding platforms for regulatory factors such as chromatin modifiers and chromatin remodelers that contribute to maintenance of local chromatin structure and regulation of cellular processes including transcription, replication, DNA repair and cell cycle progression.



	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)

Figure3. Histone Tail binding regulates chromatin structure and nuclear processes. A molecular depiction of modified histone residues and list of known reader modules and the corresponding post-translational modifications they recognize. Figure adapted from Taverna et al. *Nat Struct Mol Biol* 14, 1025-1040 (2007).

Conservation and diversification of SNF2 ATPase superfamily

Nearly twenty years ago Gorbalenya and Koonin [28-29] discovered a large family of proteins sharing a series of short ordered motifs. At the time, the majority of family members that had been studied had been shown to act enzymatically as nucleic acid strand separating helicases. Consequently, these short ordered motifs became known as helicase motifs and were labeled sequentially I, Ia, II, III, IV, V, and VI (Fig. 4). Recent bioinformatics approaches have used primary sequence similarity to further subdivide proteins containing helicase motifs into several superfamilies [30], and structural characterizations of two helicase-like superfamilies (SF1 and SF2) have revealed that a common core of two recA-like domains is highly conserved [31]. More recently it was found that these helicase-like enzymes hydrolyze ATP in an active site cleft located between the two recA-like domains, consequently leading to a change in the relative orientation of these domains [32]. It is this change in orientation of the two domains, shown by mutagenesis and structural studies, that leads to mechanical motion required to fulfill the general function of helicases.

Within the helicase-like 2 superfamily (SF2), there are a group of proteins with highly similar primary sequence to *S. cerevisiae* Snf2p. These comprise the Snf2-like family (Fig. 5). Much like Snf2p, many of the enzymes in the Snf2-like family were first identified as ATPases residing within chromatin remodeling complexes, and it is now widely recognized that these proteins serve as a core subunit of these multi-subunit complexes and are required for ATP dependent chromatin remodeling processes [33]. The Snf2-like family is now known to include a large group of proteins ubiquitously

found in eukaryotes and to a lesser extent present in both eu- and archaeabacteria. Comprehensive bioinformatic analysis of completed genomes has recently been performed in attempts to catalogue Snf2-like members [30]. By scanning for proteins containing spans of similarity in sequence over helicase-like regions it was found that 24 distinct Snf2-like subfamilies exist. Interestingly, many of the subfamilies correlated with known biological function. This amazing diversity within the Snf2-like family and currently known functional linkages strongly suggest that the Snf2 family helicase–like region has specifically evolved to execute distinct functions unique to each subfamily.

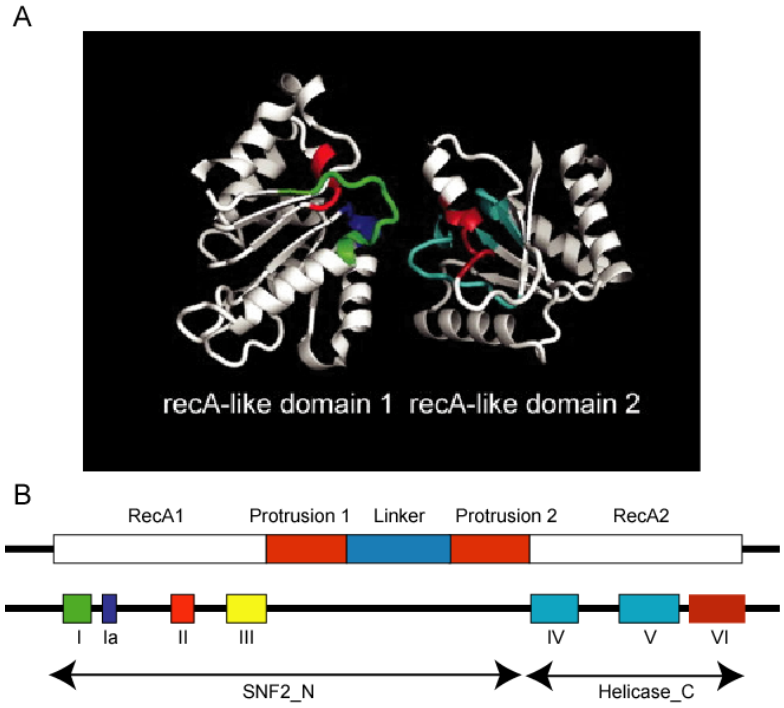


Figure 4. Conservation of structural features within Snf2 family proteins. A. Crystal structure of Rad54 and structural features found within. B Schematic depicting structural components of Snf2 family of helicases. Figure adapted from Flaus, A.et al. *Nucl. Acids Res*., 2006. 34(10):2887-2905 [30].

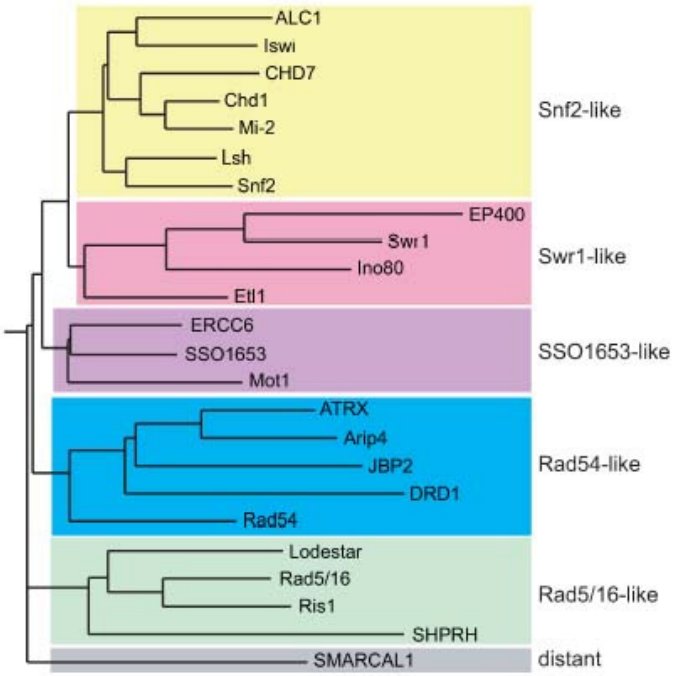


Figure 5. SNF2 superfamily is highly conserved. Schematic Diagram depicting relationships between Snf2-like subfamilies according to sequence similarity across helicase-like regions. There are 24 distinct subfamilies that are named for their member, such as *S.cerevesiae* Snf2p (Snf2 subfamily), *D.melanogaster* Iswi (Iswi subfamily), *M. musculus* Chd1(Chd subfamily). Figure adapted from Flaus, A.et al. *Nucl. Acids Res*., 2006. 34(10):2887-2905 [34].

SNF2 ATPases and their influence on nuclear processes

By the late 1980's and early 1990's it had already been reported in numerous studies that there was a strong correlation between changes in transcription and alteration of chromatin structure [35]. For example, it was reported that altering histone stoichiometry in yeast restored transcription to promoters disrupted by transposon insertion, and it was known that loss of histone H4 resulted in increased levels of transcription. In addition, there were suggestions that transcription might be directly affected by nucleosome positions; however, the cause-and-effect relationship between altered chromatin structure and transcriptional outcome was not clearly understood [11, 36]. The first direct evidence suggesting a functional interaction between transcriptional activators and chromatin came from the Winston lab in 1992. It had been previously reported that mutations in Snf5 affected transcription of a large set of genes including *SUC2*, a glucose repressible gene that encodes the enzyme invertase [37]. Winston's lab reported; 1) that mutation of the Snf2 and Snf5 genes resulted in transcriptional repression of *SUC2*; and 2) that these transcriptional effects could be reversed by introducing mutations into the genes encoding histones H2A and H2B [38]. In addition, biochemical analysis of local chromatin structure surrounding the *SUC2* gene suggested that localized structural changes in chromatin observed in Snf2 and Snf5 mutant yeast could be rescued by mutations in *H2A/H2B* genes. These findings provided the first clue that the transcriptional activators Snf2 and Snf5 were involved in changes in local chromatin structures and that these changes in structure had direct bearing on transcriptional outcome. Exactly how these regulated changes in chromatin structure

were occurring on a biochemical level was not known until a few years later, when multiple groups including the Peterson, Kingston, and Green laboratories, independently purified the 10 subunit Swi/Snf complex from both yeast and mammalian cells [39-40]. These groups showed that the multisubunit complex Swi/Snf was able to remodel nucleosomes in an ATP dependent manner *in vitro,* as evidenced by the altered nuclease cleavage pattern and DNA topology. Furthermore, this remodeling of local chromatin by Swi/Snf was shown to directly affect transcriptional outcome by allowing the transcriptional activator Gal4 access to its binding site within a promoter (Fig. 6).

A year after the first characterization of SWI/SNF the Wu laboratory used an *in vitro* system to characterize an ATP-dependent chromatin remodeling factor that exhibited greatly enhanced activity in the presence of GAGA factor. A complex of 4 proteins, which they named named NURF (Nucleosome Remodeling Factor) turned out to be responsible for the remodeling activity in the fraction used in their assay [41], and a report later that year suggested the complex's catalytic subunit, a 140kDa protein named Iswi, contained an ATPase domain with a strikingly high degree of homology to Snf2, the catalytic subunit of the previously described Swi/Snf complex. The high degree of homology between the Snf2 and Iswi ATPase domains not only provided a link between these two complexes but inspired the search for additional chromatin remodeling enzymes.

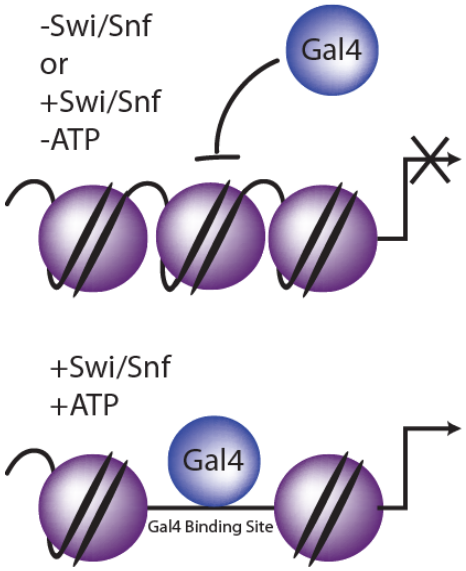


Figure 6. Control of transcriptional activator binding by chromatin remodeling. SWI/SNF chromatin remodeling complex in the presence of ATP is capable of remodeling promoter chromatin (by various mechanisms see Fig 8) and thus allowing the binding of the Gal4 activator to its consensus sequence. Once Gal4 is bound, coregulatory proteins and RNA pol II can then be recruited, leading to transcriptional activation.

Today, members of the SNF2 superfamily, bound by a common ATPase domain, have been reported to play roles in nearly every nuclear process imaginable. Most Snf2 family remodelers, including those of the Snf2, Iswi, Chd, and Mi-2 subfamilies, have roles in transcriptional regulation; where they can function as activators, repressors, or both [30]. Chromatin remodelers play roles in earlier steps of transcriptional activation, including promoter remodeling, to allow activators access to DNA [39, 41-42] and facilitate of access of TBP and general transcriptional machinery to promoters [43-44]. In addition, remodelers such as Iswi have established roles in chromatin assembly and formation of nucleosome arrays with well ordered spacing, which could promote repression through occlusion of transcription factors and the general transcriptional machinery from promoters [45-46]. Subsequent to transcriptional initiation, Swi/Snf

complex and the orthologous Rsc complex have been shown to facilitate elongation of RNA polymerase II through nucleosomes *in vivo* and *in vitro* in a way that depends on their remodeling capabilities [47-49]. Furthermore, it has been suggested that the Chd-Mi2 type remodeler Hrp1 remodels chromatin structure near the 3' end of genes to ensure proper transcriptional termination [50]. Thus it appears that, during each step of transcription, chromatin remodelers contribute to proper regulation.

In addition to reported transcriptional roles [51-59], it is well documented that the Snf2-related chromatin remodeling complexes Ino80 and Swr1 have essential roles in DNA damage and recombination [60]. An interesting characteristic of Ino80 and Swr1 type remodeling complexes is that each includes Ruv-B like helicases as subunits [61-62]. These Ruv-B like helicases are highly conserved from bacteria to humans and are known to play a DNA repair role in bacteria, consistent with the possibility that the Ino80 and Swr1 type remodelers have evolved functions that help maintain genomic integrity. In yeast, plants, flies, and mammals, recent research has shown that DNA damage responses are impaired when components of the Ino80 subfamily are mutated [51, 63-68]. In *S. cerevesiae,* Ino80 and Swr1 complexes bind through association with phosphorylated histone variant H2AX directly to sites of double strand break (DSB) [64-66], where they are required for proper processing of DNA ends (Fig. 7). Numerous reports suggest that Ino80 plays a role in the eviction of nucleosomes proximal to the DSB [69-71] and in chromatin of the homologous donor locus [71]. Impaired nucleosome eviction by loss of Ino80 has been shown to lead to reduced association of repair and checkpoint factors, such as Mre11 with the site of DSB [64, 66, 72], leading to

a decrease in single-stranded DNA production through processing events and subsequent loss of invasion of the single-stranded DNA into the homologous donor locus [71]. While the Swr1 complex does not affect nucleosome eviction at DSBs [68], it is required for transient deposition of histone variant H2AZ at sites of DNA damage. The presence of the variant histone H2AZ at DSBs is required for the sustained recruitment of DNA repair factors such as Rad51 [73], Mec1, and Ku80, to DSBs which are required for non-homologous end joining (NHEJ) [70]. Additionally, loss of H2AZ leads to inability of DSBs to localize to the nuclear periphery [73]. Thus, it seems these chromatin remodeling complexes play a pivotal role in maintenance of genomic integrity by both "lighting the landing strip" and "clearing the way" so that the required DNA damage machinery can "land and deploy safely".

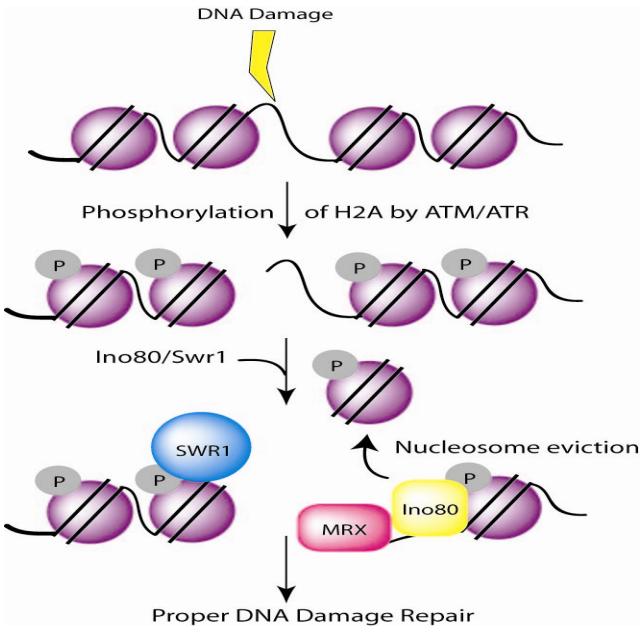


Figure 7. Ino80 and Swr1 complexes regulate double-strand break repair. Upon DNA damage, kinases ATM/ATR (Ataxia telangiectasia (A-T) mutated (ATM) and A-T and RAD3-related (ATR) phosphorylate H2AX. Ino80 and Swr1 are recruited to DSB site via phosphorylation of H2AX. Upon recruitment Ino80 evicts nucleosomes proximal to break and allows repair machinery Mre11-Rad50-Xrs2 (MRX) complex access so that DNA damage can be properly repaired.

Not surprisingly the SNF2 superfamily of helicases has its hands on the process of DNA replication as well. Modulation of chromatin is crucial during DNA replication, particularly when the replication fork is impeded upon replicative stress [74]. Stalled replication forks can result from encounters with damaged DNA or depleted levels of nucleotides. If these situations are not resolved in a timely manner, the replication machinery may disassemble, leading to replication fork collapse, which in turn can result in deleterious DNA damage [75]. Ino80 is known to be enriched at replication forks, particularly at replication forks that are stalled or under replicative stress, and loss of Ino80 results in replication fork collapse and replisome integrity defects [57, 76-79]. Research on Ino80's role during DNA replication is in its infancy, and not nearly enough is understood; it is thought, that Ino80 aids replication by stabilizing the replisome and relaying damage signals upon encountering stress to activate the inter-S-phase checkpoint, thus promoting the correct repair of encountered DNA lesions.

Along with contributing to such nuclear processes as RNA transcription, DNA damage, and DNA replication, Snf2 family members play specialized roles in telomere regulation [80-82], centromere stability and segregation [83-85], cell cycle regulation [82] and regulation of early developmental processes [86]. While it is nearly impossible to cover all reported cellular roles of Snf2 remodelers, it's safe to say that any nuclear process in which chromatin is an impediment will require the aid of a Snf2 remodeler.

SNF2 ATPases: Fine-tuned Motors for specific functions

Comprehensive biochemical characterization of the entire Snf2-like ATPase family has not been completed to date, but it is known that members of the Snf2, Iswi, Mi2, Chd, Ino80, and Swr1 subfamilies exhibit different modes of chromatin remodeling [87]. These chromatin remodelers can affect nucleosome structure in a variety of ways, including (a) sliding, or moving the histone octamer to a new position leading to exposure of DNA [88-90]; (b) octamer ejection, leading to a complete displacement and exposure of DNA [91-94]; (c) removal of H2A-H2B dimers, resulting in destabilization of the nucleosome and exposure of DNA [95-96]; and (d) dimer swapping (i.e. exchanging resident canonical H2A-H2B dimers for dimers containing H2B and the histone H2A variant H2A.Z) (Fig 8) [52].

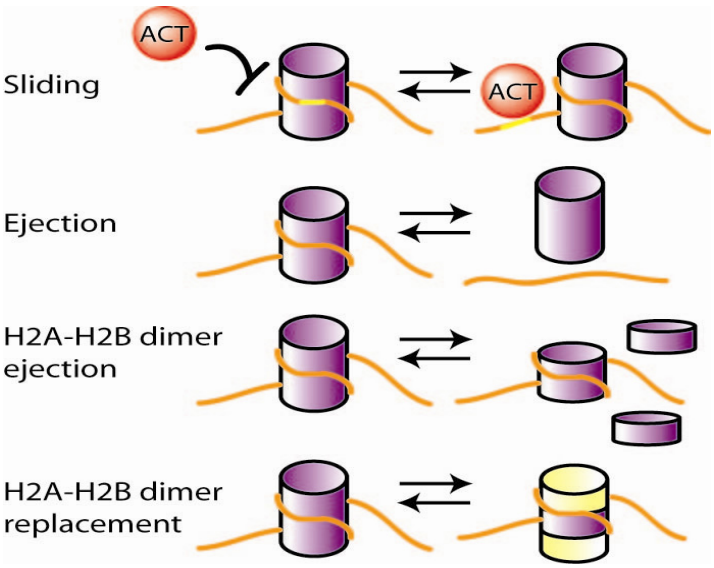


Figure 8. SNF2 ATPases have specialized modes of chromatin remodeling. Nucleosome remodeling can occur in a variety of ways including nucleosomal sliding, ejection, dimer removal and replacement. Remodeling results in increased accessibility of underlying nucleosomal DNA to DNA-binding regulatory factors.

Most Snf2-like ATPases are capable of sliding nucleosomes, but they have very different efficiencies of remodeling and often display different outcomes. For example, SWI/SNF complexes randomize the positions of nucleosomes on templates that were evenly spaced initially [97], while ISWI complexes promote the equal spacing of DNA between nucleosomes on a template [98-99]. SWI/SNF complexes also have the ability to eject nucleosomes [93, 100]; while ISWI complexes lack this activity. Furthermore, all SWI/SNF remodeling complexes tested to date are capable of ejecting histone H2A-H2B dimers, but only a distinct subset of ISWI type remodelers have this activity [95-96]. Interestingly, to date only SWR1 and INO80 type remodelers exhibit histone-variant exchange. SWR1 is capable of swapping out canonical H2A-H2B dimers with H2AZ-H2B dimers *in vitro* [52, 61], and there is evidence that Ino80 complex may to able to reverse the reaction *in vivo* [101].

Mechanisms of nucleosome remodeling reactions such as sliding and octamer ejection by Snf2-like ATPases have been studied recently by many groups and are currently a subject of much debate. The active remodeling of nucleosomes, whether by sliding or octamer ejection, requires the breakage (and reformation in the case of sliding) of 14 histone-DNA contacts. Histone-DNA contacts are known to be extremely stable, and nucleosomes have been shown *in vitro* to exhibit very slow rates of spontaneous translational movement on DNA, disassembly (loss of histone H2A-H2B dimers), or octamer ejection [102]. It is has been estimated that the energy required for breaking all 14 histone-DNA contacts is approximately 12-14 kcal mol^{-1} (1 kcal mol^{-1}/contact) [103]. To overcome such an energetic barrier, nucleosome remodelers couple the hydrolysis of

ATP to the breakage of histone-DNA contacts. Initially, it was thought that remodeling was due to ATP dependent movement of remodelers around the nucleosome, or due to an imposed conformational change in the octamer itself [104]. Second generation models suggested that the ATPase domain of nucleosome remodelers could generate torsional stress and/or themselves undergo a conformational change that would expose nucleosomal DNA [105-109]. More recently, a significant advance was made towards a mechanistic understanding when it was demonstrated that SF2 family remodelers are actually DNA translocases rather than strand separating helicases [110-112] and can track along the phosphate backbone of one of the two DNA strands [32, 113]. Furthermore, remodelers such as SWI/SNF and ISWI have been shown in triple-helix displacement assays to track in a 3'-5' direction along one strand of DNA [111-112, 114].

The notion that remodelers can translocate on DNA raised the possibility that translocation could act as a motor force that pumps DNA around the octamer surface. Several elegant biochemical studies used chemical crosslinking and exonuclease protection assays to attain evidence that ISWI ATPase binds the nucleosome both on the linker DNA near the entry/exit site, and within the histone octamer at a defined internal location about two turns from the nucleosomal dyad. Interestingly, both ISWI and SWI/SNF remodeling activity is inhibited when nicks or DNA gaps are introduced within this same internal location [112, 114-116], suggesting that; 1) the remodeler may engage the nucleosome near these gaps, and 2) must be able to track continuously along DNA for efficient remodeling to occur. Furthermore, remodeling of mononucleosomes by SWI/SNF or RSC results in movement of the free DNA end to a position roughly two

turns from the nucleosomal dyad [112, 114-116]. These findings suggest that remodelers engage the octamer in a fixed position and forcibly pump DNA around the octamer.

Currently models postulate that both ISWI and SWI/SNF type remodelers use a "DNA inchworm" translocation mechanism that involves the coordinated movement between two domains: the DNA-binding domain (DBD) and a DNA tracking domain [32, 113]. The two recA-like motifs, which include a platform for DNA interaction and the ATP hydrolysis cleft, are thought to make up the tracking domain, while the DBD binds within the linker DNA region for ISWI and internal to the nucleosome for SWI/SNF. When the remodeler engages the nucleosome, the tracking domain binds tightly to a fixed position within the nucleosome two turns of DNA from the nucleosome dyad. The DBD of Snf2 subfamily remodelers bind loosely about 10bp (or 1 turn of DNA) ahead, whereas the DBD of Iswi subfamily remodelers binds to the linker region. Translocation ensues as the tracking domain pulls 1bp of DNA toward (SWI/SNF) or away (ISWI) from the nucleosomal dyad. Next, the DBD steps forward 10bp along the DNA, and binds tightly to a new position. Lastly, the tracking domain releases its grip on the DNA, the DBD undergoes a conformational change, pulling in 10bp of DNA as it returns to its original position on the histone octamer (Fig. 9). The net result of the cycle is propagation of an 11bp wave of DNA around the nucleosome by one-dimensional diffusion [87] leading to translational movement of the nucleosome along linear DNA.

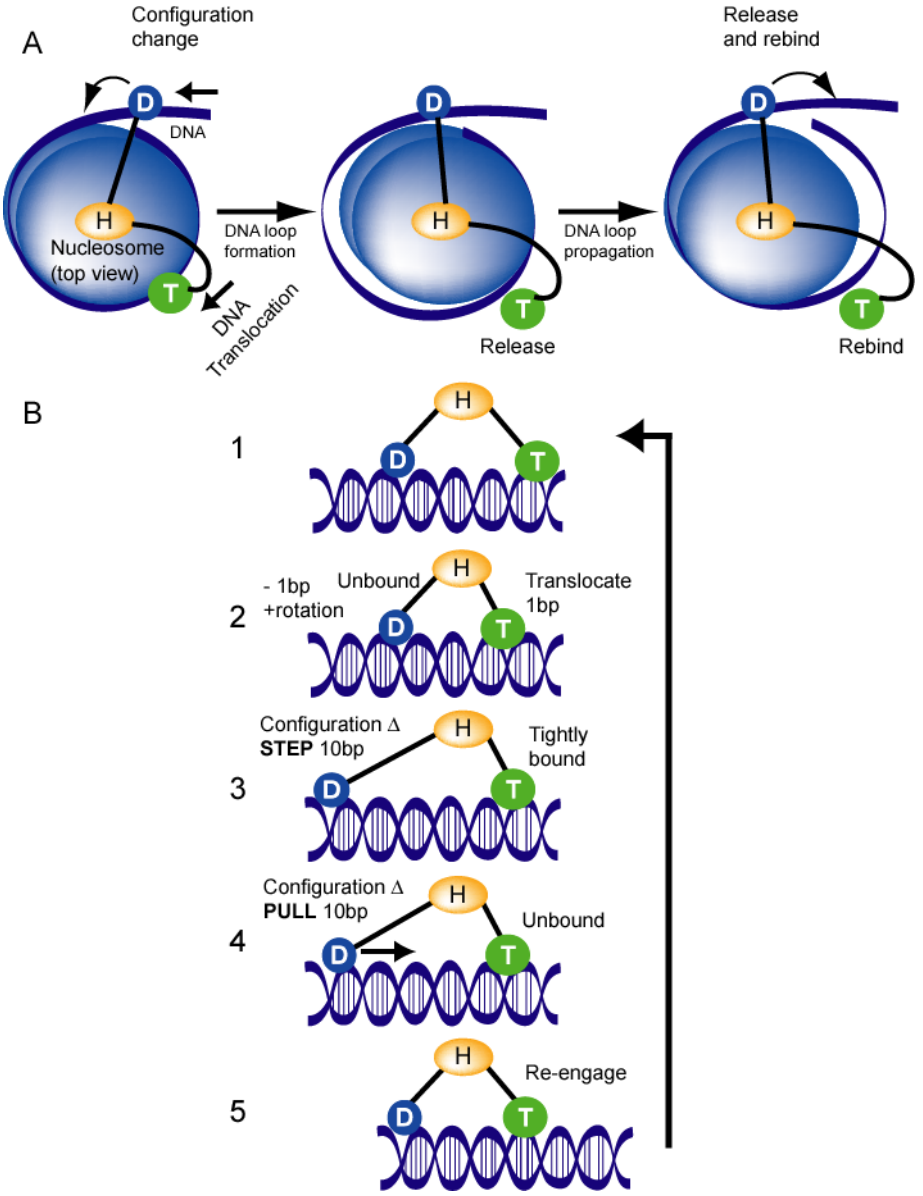


Figure 9. Model for translocation of DNA around nucleosomes. a) Iswi remodels nucleosomes by the formation of a DNA loop on the nucleosomal surface. Remodeling is achieved through the cooperative action of a DNA translocase (T) domain near located near the nucleosomal dyad and a DNA binding domain (D) within the linker region, linked through a hinge domain (H). Swi/Snf remodels nucleosomes in a similar fashion but has a DNA binding domain (DBD) that helps to facilitate translocation within the nucleosome. b)Translocation of DNA around the nucleosomal surface is a multistep process that begins with the tracking domain tranlocating 1 base pair. Subsequent to translocation the DBD undergoes a conformational change and binds DNA tightly 10bp away from the translocase domain. Lastly, the DBD undergoes a final conformational change and pulls the DNA towards the translocase domain resulting in a net translocation of 11 base pairs.

Poly(ADP-ribosyl)ation in the Nucleus

The existence of poly-ADP-ribose (PAR) was first reported in 1963 by P. Chambon and coworkers, who found that addition of NAD^+ to hen liver extracts stimulated the incorporation of labeled ATP into an acid-insoluble fraction of poly(A)-containing products [117]. The enzyme responsible for the synthesis of poly-ADP-ribose was in turn named PARP (Poly-ADP-ribose Polymerase). The structure of poly-ADP-ribose was subsequently solved by three independent laboratories [118-121], and it is now known that PAR is a homopolymer of ADP-ribose units linked by glycosidic ribose-ribose 1'-2' bonds. Polymers synthesized both *in vitro* and *in vivo* can be extremely heterogeneous, reaching lengths of 200-400 units. These long polymers are also irregularly branched approximately once per linear section of 20 to 50 units of ADP-ribose [122-126], and branch sites are chemically linked in the same manner as linear links [126] (Fig 10). Interestingly, it has been postulated that certain types of long poly-ADP-ribose chains may form a helicoidal secondary structure similar to the structures of RNA and DNA [127-128]. Currently, it is not known whether the structural heterogeneity of PAR carries any functional significance, but one could imagine that this heterogeneity could play a role in determining functional outcomes *in vivo.*

PARylation itself can take place in two manners, resulting in either; 1) the generation of free poly-ADP-ribose or 2) the covalent attachment of poly-ADP-ribose to an acceptor protein. Production of free poly-ADP-ribose has been shown to occur both *in vitro* and *in vivo* and has been suggested to play a role in stress dependent signaling process [129-132], however, the overwhelming percentage of poly-ADP-ribose

synthesized is covalently attached to acceptor proteins. Furthermore, it has been shown that the vast majority of poly-ADP-ribose (estimated at >90%) in cells is covalently linked to Parp1 [133]. Residues reported to be targeted for covalent trans- and auto-modification most frequently include lysine, glutamic acid, and aspartic acid. Covalent linkage to PAR occurs through schiff base formation with the ε-amine of lysines, and ester formation with carboxyl groups of aspartic and glutamic acids. Unlike other modifications such as acetylation, phosphorylation and ubiquitination, there seems to be a lack of consensus sequence surrounding acceptor residues, thus it has not been possible to predict proteins that may be poly-ADP-ribosylated. Indeed, despite nearly a half-century of research, no specific glutamic or aspartic acid residues on acceptor proteins have been confirmed *in vitro* or *in vivo*. This inability to confirm such sites may be attributed to one of three factors including; 1) the instability of the ester bond between aspartic and glutamic acid residues and poly-ADP-ribose under alkaline conditions [134], 2) the inability to use mass spectrometry to perform analysis because of PAR's heterogeneity, and 3) the possibility that poly-ADP-ribosylation may in fact just be a promiscuous modification. To date more than 200 nuclear proteins, most of which are chromatin associated, have been proposed to be covalently modified by poly-ADP-ribose *in vitro* [135]. Substrates reported to be poly-ADP-ribosylated include PARP family members, histones, HMG proteins, topoisomerases 1 and 2, nuclear scaffold proteins, and transcription factors including p53 and CTCF (Table 1).

Nuclear Substrates	Functional Relevance
Histones (H1, H2A, H2B, H3, H4)	Modulation of chromatin structure
High Mobility Group Proteins	Unknown
Low Mobility Group Proteins	Unknown
Poly(ADP-ribose) polymerases	scaffolding for recruitment of factors, inhibition of DNA binding?
Topoisomerases I and II	Unknown
A24 Protein	Unknown
hnRNPs (A1, A2/B1, C1/C2, G, H, K)	Unknown
Lamin A/C	Unknown
Numatrin/B23	Unknown
Nucleolin/C23	Unknown
Centromere-binding proteins CENPA, CENPB, Bub3, MARCKS F52/GAP43	Unknown
p53	Inhibition of DNA binding
DNA polymerase alpha	Inhibition of catalytic activity
PCNA	Inhibition
Telomeric repeat binding factor-1	Inhibition of DNA binding
CTCF	Enhancement of DNA binding
NuMA	Unknown

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Table 1. Nuclear proteins reported to be poly-ADP-ribosylated in vivo. Many nuclear proteins have been proposed to be poly(ADP-ribosylated), but the functional relevance of most modifications is unknown.

The synthesis of free and/or covalently bound poly-ADP-ribose is a result of multiple enzymatic steps including: 1) initiation or covalent modification of acceptor protein by Parp; 2) subsequent elongation of the polymer using the previously attached mono-ADP-ribose as a starting unit; 3) branching of the polymer; and, if needed, 4) release of the covalently bound , branched poly-ADP-ribose from the acceptor protein by the enzymatic activity of PARG (Poly-ADP-Ribose Glycohydrolase), the only human

enzyme biochemical experiments indicate that catabolyzes PAR. Results of *in vitro* biochemical experimental indicate that the archetypal PARP enzyme, Parp1, can facilitate autonomously the initiation, and subsequent elongation and branching of poly-ADP-ribose. The activation of Parp1 has been shown to be largely dependent on Parp1 binding to DNA. Upon binding to DNA ends, hairpins, and cruciforms, along with other DNA structures the enzyme is allosterically activated and begins PAR synthesis. Alternatively, Parp1 has also been shown to be activated by binding to proteins and through a phosphorylation event catalyzed by Erk2 [136].

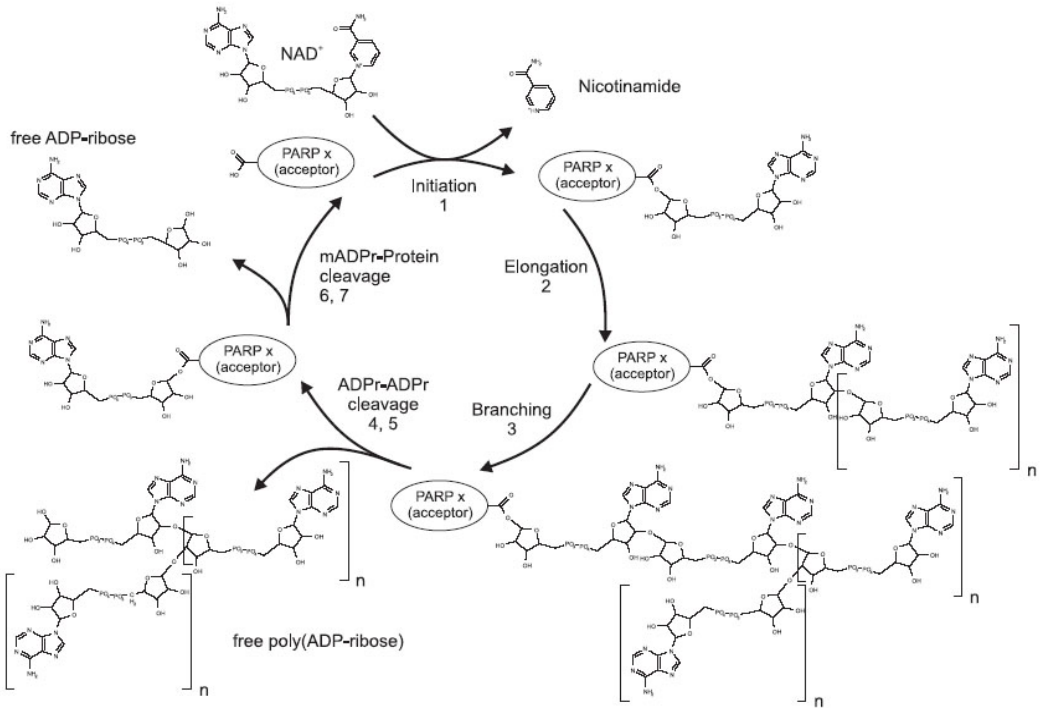

Figure 10. Poly-ADP-ribosylation reaction occurs through multiple steps. Reaction begins with an (1) initiation in which a single mono-ADP-ribose unit is covalently attached to acceptor protein; (2) elongation proceeds as successive ADP ribose units are attached to the initial unit; (3) branching occurs randomly every 20-50 ADP-ribose units; (4,5) poly(ADP-ribose) is cleaved by Parg, an endo- and exonuclease that acts specifically on PAR; (6,7) mADPr-Protein bond is cleaved via protein lyase. Figure adapted from Hassa et al. *Microbiol Mol Biol Rev*, 2006. 70(3):789 [135]

Normally, the constitutive levels of poly-ADP-ribose are low in unstimulated cells [137-140]. However, it is reported that PAR levels may increase up to 500 fold upon activation by mitogenic stimuli or DNA damage. This increase in poly-ADP-ribose corresponds to a drastic decrease in the cellular levels of NAD^+, as NAD^+ is the immediate precursor utilized for PAR formation. NAD^+ concentration within the cell directly controls the constitutive and activated levels of PAR formation [139, 141-143]. Poly-ADP-ribose exists fairly transiently *in vivo,* as the half life of polymers synthesized upon genotoxic stress is between >30 s to 6 min [141, 144-146]. Interestingly, activated levels of PAR decay in a biphasic manner with approximately 85% of polymers turned over in less than 30 s, while the remaining polymers are catabolyzed within 6 min [141, 146]. Unlike the fast turnover of polymers in response to external stimuli, the constitutive fraction of poly-ADP-ribose has a half life of nearly eight hours [132]. Differences in the amount of polymer synthesized and in turnover rate between the active and constitutive fractions likely are a result of the varying biological roles played by the two fractions of poly-ADP-ribose.

The PARP Family of Proteins

For many years it was thought that Parp1, the founding member of the PARP family, was the only enzyme with poly-ADP-ribosylation activity in cells. Following the development of knockout mice lacking the PARP1 gene [147-148], this view has changed, since primary cells from these mice can still synthesize poly-ADP-ribose upon induction of genotoxic stress [149]. Moreover, five new genes encoding poly-ADP-ribosylating enzymes were identified [150-154], further indicating that Parp1 belongs to a family of poly-ADP-ribosylating polymerases.

PARP family members can be divided into three subgroups according to their domain structures, sequences of their catalytic domains, and their enzymatic activities [135] (Fig. 11). Subgroup 1 includes Parp1, Parp1b (an isoform of Parp1 resulting from initiation of transcription from an alternative site in the Parp1 gene) [155], Parp2, and Parp3. Both Parp1 and Parp2 play a significant biological role in response to distinct stress response pathways [156-157]. The second subgroup contains a single member, Parp4, or vault-Parp. Parp4 is the largest member of the PARP family and is a component of a cytoplasmic ribonucleoprotein complex (vault complex) that includes an untranslated RNA (vault RNA) and two additional proteins, major vault protein and telomerase associated protein-1 [151]. The biological function of the vault complex is unknown to date. Parp5 and Parp6, alternatively known as Tankyrase 1 and 2 belong to subgroup 3, and are both reported to be components of the telomeric complex [150, 152, 157].

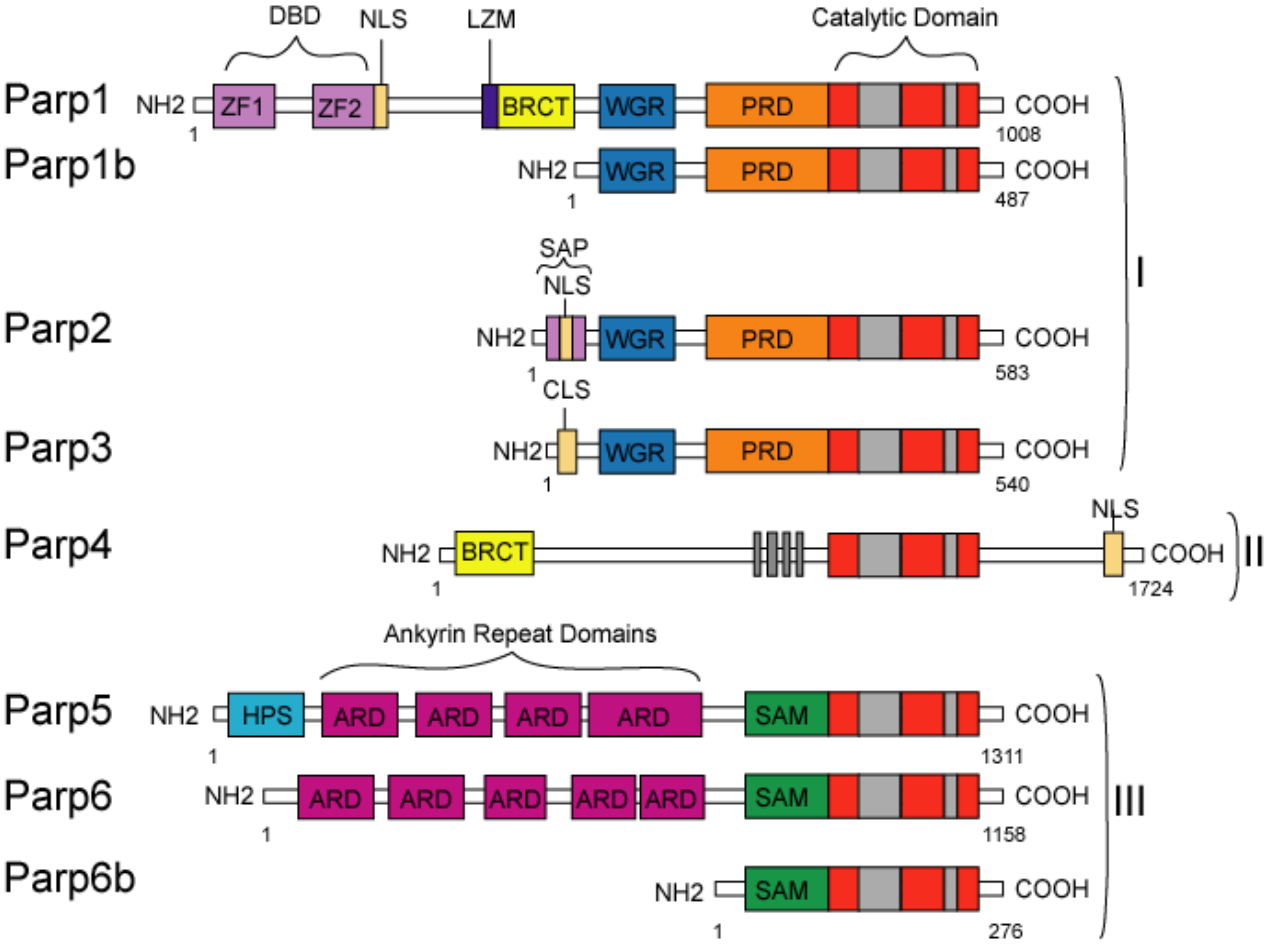

Figure 11. Parp family members and their structural domains. The most significant domains are highlighted and include: the WGR domain which is found in a variety of poly(A) polymerases and is named after a conserved central motif (W/G/R); the PRD domain, or PARP regulatory domain, which may play a role in regulation of PARP branching activities; the BRCT domain named after the breast cancer suppressor protein-1 (BRCA1) carboxy-terminal domain found in many proteins involved in DNA repair processes and cell cycle checkpoint processes [158]. The ankyrin repeat domain (ARD) mediates protein-protein interactions in diverse sets of proteins [159]; the sterile alpha motif (SAM) is a domain involved in signaling and has been reported to mediate homo- and hetero-dimerization. Other domains include the Parp1 ZF-I and ZF-II domains, which act as DNA nick sensors and general DNA-binding domain [156]; SAP, SAF/Acinus/PIAS-DNA binding domain; LZM, a putative leucine zipper-like motif; NLS, nuclear localization domain; CLS, centriole localization signal; and HPS, His-Pro-Ser region.

While all members of the PARP family are reported to have automodification activity [150-152, 154-155, 160], Parp1 has the strongest such activity *in vitro*. 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 so called "automodification domain" (AD) [161-162]. Recently, there has been some disagreement within the field regarding the identity of Parp1 residues targeted for automodification. Earliest reports suggested that 25 to 30 glutamic acid residues within the automodification domain were targeted for poly-ADP-ribosylation [161, 163], but, more recently, it was shown that loss of these glutamic acid residues had no effect on automodification of Parp1 *in vitro* [164]. It was further reported that 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 [164]. Interestingly, these residues are in close proximity to the catalytically active site, and 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[135]. 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[135].

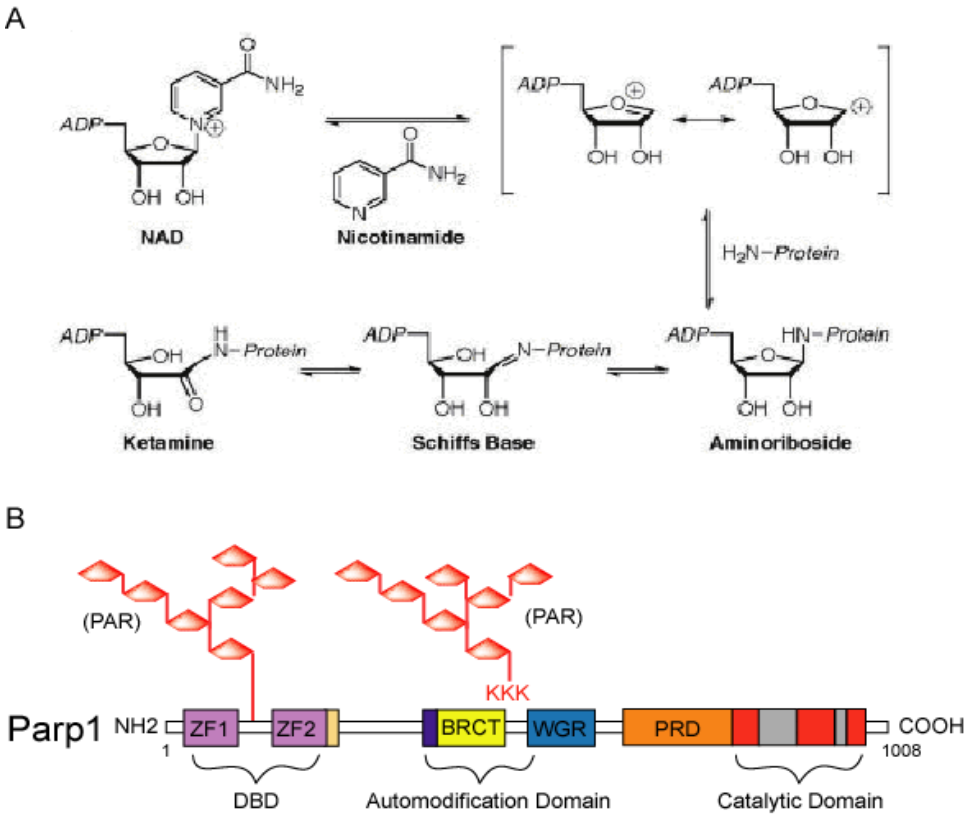

Figure 12. Parp1 automodification occurs on lysine residues (a) Mechanism proposed for the NADase-dependent automodification of lysine residues by Parp1 through Schiff base formation (b) Schematic of Parp1 including sites of auto-ADP-ribosylation. Figure adapted from Hassa et al. *Microbiol Mol Biol Rev*, 2006. 70(3):789 [135]

Parp1 and its role in DNA Damage Repair

The earliest evidence that Parp plays a supportive role in DNA damage repair came in 1956, when it was observed that cellular levels of NAD^+ decreased dramatically in cells treated with DNA-damaging agents [165]. Yet it was not until the late 1970's that the poly(ADP-ribosylation) reaction was causally linked to depletion of NAD^+ in cells suffering from DNA damage [166-167], and the specificity of the relationship of Parp activation and resulting decrease of NAD^+ were established through use of specific Parp inhibitors. A role for Parp in DNA damage repair was established by Shall and collaborators [168-170], who showed that specific inhibition of Parp1 resulted in an increase in cytotoxicity through an increase in the half-life of DNA double-strand breaks (DSBs). Inhibition of Parp results in accumulation of single-strand breaks that are normally repaired through homologous recombination (266-269), or sister chromatid exchange [171-173]. Furthermore the inhibition of Parp1 has been shown to promote carcinogenic induced gene amplifications [174-176]. Interestingly, for reasons we will discuss later, Parp inhibition also led to an increased rate of apoptosis [177] and resulting decrease of cellular necrosis [178-179],

Multiple lines of evidence suggest Parp is involved in repair of DNA damage by the base excision repair (BER) pathway. To begin with, base damage and DNA single-strand breaks (SSBs) are the major types of DNA damage inducing poly(ADP-ribosyl)ation *in vivo* [166-167, 180-190]. Furthermore, Parp has been shown to interact with multiple components of the BER pathway, including XRCC1, DNA ligase, and DNA polymerase β [191–192]. Moreover, it has been demonstrated that repair of SSBs

requires the presence of NAD^+ both *in vivo* and within cell-free DNA BER assays [193]. $Parp1^{-/-}$ mice are known to be extremely sensitive to γ-irradiation and exposure to *N*-methylnitrosurea [194], and after exposure exhibit increased genomic instability marked by increases in the levels of sister chromatid exchanged and chromatid breaks. Cell lines derived from these $Parp1^{-/-}$ mice have difficulty proceeding through mitosis after being treated with DNA-damaging agents and rapidly undergo apoptosis [195].

Along with BER, there are multiple other pathways of DNA damage repair, and it is likely that Parp plays a significant role in all. It is known that Parp associates with both SSB and DSB lesions [196-198] and binds electrostatically to DNA ends. Upon end binding, Parp covers a region of seven nucleotides on each side [199-200] suggesting that Parp binds to DNA strand breaks as a dimer. While Parp activity is induced by DNA DSBs, the amount synthesized is much less than the activation due the DNA SSBs [196, 201]. These differences could highlight both qualitative and quantitative differences in the requirement for PARylation in different DNA repair pathways. Although the precise function of Parp in these pathways is not known, reports suggest that PARylation activity is not required for excision of damaged bases [202-204], or for the resynthesis of DNA after the excision [203, 205]. Multiple models have been proposed to explain the role of PARylation in maintenance of genomic integrity. These include: 1) the recruitment model, 2) the chromatin-dependent repair mode, and 3) the signaling model [137]. Importantly, these models are not mutually exclusive, and it is highly likely aspects of each mechanism may contribute *in vivo.*

The recruitment model is based on the observation that Parp is one of the first nuclear factors to recognize lesions in DNA, and is therefore capable of contributing directly to recruitment of DNA repair machinery to sites of DNA damage *in vivo* [206] (Fig. 13). The association of Parp1 with the BER complex (XRCC1, DNA ligase III, and DNA polymerase β) further supports this model [191-192, 207] and suggests that Parp1 can facilitate DNA repair through recruitment of these important factors. Moreover, 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 [208]. Indeed, it is very likely that production of long chains of PAR, through either extensive automodification of Parp1 or modification of local chromatin structures could indeed target enzymes to sites of DNA strand breaks much faster than individual factors searching for damage themselves. This model is compatible with the known kinetics of Parp1 action, both before and after excision of damaged DNA.

The chromatin-dependent repair model is based on the fact that DNA repair (and all nuclear processes for that matter) is greatly influenced by packaging of DNA into chromatin. There are many reports that histones are PARylated in response to DNA damage [140, 209-210], suggesting Parp may play a role in DNA repair through its activity on chromatin. It is possible that by modifying itself and histones in the vicinity, Parp1 alters the local higher-order structure of chromatin surrounding the DNA damage in a manner conducive to DNA repair. A report suggesting that in the absence of Parp activity, DNA repair is less efficient in non-transcribed heterochromatic regions of the genome, suggests that condensed local chromatin structure can be quite an impediment to

DNA damage repair [211]. Furthermore it has been shown that extensive PARylation of chromatin leads to a greater accessibility of DNA to nucleases [212]. It could easily be envisioned that Parp1 could act enzymatically to relieve the steric restraints of condensed chromatin and grant access to the required repair enzymes, many of which are components of large multi-subunit complexes. Future *in vitro* analysis using chromatin templates in DNA damage assays will no doubt shed light on this hypothesis.

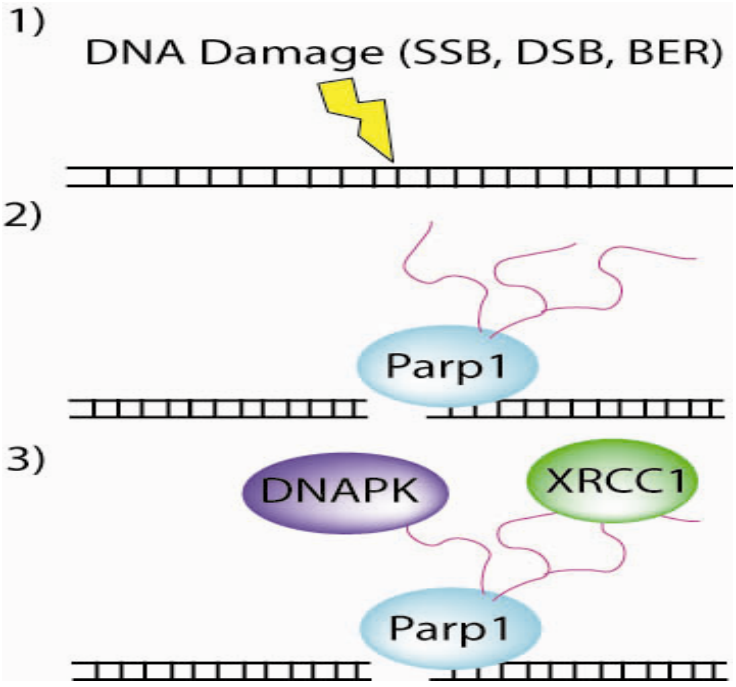

Figure 13. Parp1 contributes to DNA damage repair through recruitment of repair factors. 1) Parp acts as a DNA damage sentinel and is one of the first responders to DNA damage. 2) Upon binding to DNA damage through its N-terminal zinc fingers, Parp1 is enzymatically activated and automodifies itself. 3) Through recognition of PAR, DNA damage repair factors such as DNAPK and XRCC1 are recruited to sites of damage.

Lastly, the signaling model proposes that as Parp detects damaged DNA, extensive modification of Parp 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 [137]. Although conceptually similar to the recruitment model, this model is sustained by the observation that p53 function is regulated through cellular PARylation. Parp inhibition with chemicals significantly suppresses the accumulation of p53 in response to ionizing radiation [213-215]. Similarly, knockdown of Parp expression results in a significant delay in p53 induction in response to γ–irradiation [216]. Furthermore, *PARP*$^{-/-}$ cells have considerably lower levels of p53 in the absence of genotoxic stress [217-218]. How Parp regulates levels or activity of p53 is not known. It is possible that Parp1 activity contributes to either the level of p53 mRNA transcribed or the stability of p53 protein levels. Furthermore, it is possible that Parp1 facilitates p53 activity, as p53 could bind PAR, or Parp itself [214, 219] in a non-covalent manner directing it towards sites of DNA damage [220]. Future studies looking into the interaction of Parp and p53 could provide nice insight into cellular signaling responses during DNA damage.

Parp1, NAD^+ metabolism and Cell Death

For two reasons, NAD^+ is an essential cofactor within the world of energy metabolism. First, it is required for the synthesis of ATP and acts to balance the overall cellular redox potential [221]. Second, NAD^+ is also an immediate substrate for the synthesis of poly(ADP-ribose) polymers, and the level of PARylation within the cell inversely determines the cellular level of free NAD^+ as it has been shown that catabolism of NAD+ in mammalian cells occurs mainly through PARylation reactions [222-225] (Fig. 14). To illustrate this point, it is estimated that the concentration of free NAD+ within cells, under normal physiological conditions, is around 400μM-500μM with a half life of approximately 1hour [137, 226], but that upon exposure to genotoxic agents the cellular levels of NAD+ can drastically decrease to less than 10% of normal levels within 5 minutes [227-228]. The physiological consequences of both NAD+ and ATP depletion during DNA damage have been studied and have been found to directly affect cell death decisions. The process of apoptosis requires sufficient ATP for proper execution and if the cellular ATP concentration is lowered drastically through over-stimulation of Parp enzymatic activity, the cell will enter energy-failure-induced necrosis [229-230]. Upon entering energy-induced cellular necrosis, the cell lyses and generates further damage to the surrounding tissue. This process is thought to contribute to pathogenesis of both ischemia and diabetes [179, 231]. Interestingly, eukaryotic cells appear to have devised a mechanism to promote apoptosis and prevent the Parp dependent switch to necrosis. It is known that upon modest DNA damage, death proteases known as caspases cleave Parp in its bipartite nuclear localization signal and effectively produce two fragments, a 24-KDa DNA binding fragment and an 84kDa catalytic fragment rendered inactive through lack

of DNA binding capability [232-234]. By inactivating Parp1, the cell can spare the levels of NAD+ and ATP and allow for proper execution of apoptosis to ensue. These findings suggest that Parp plays a pivotal role in the delicate balance of cell death decisions.

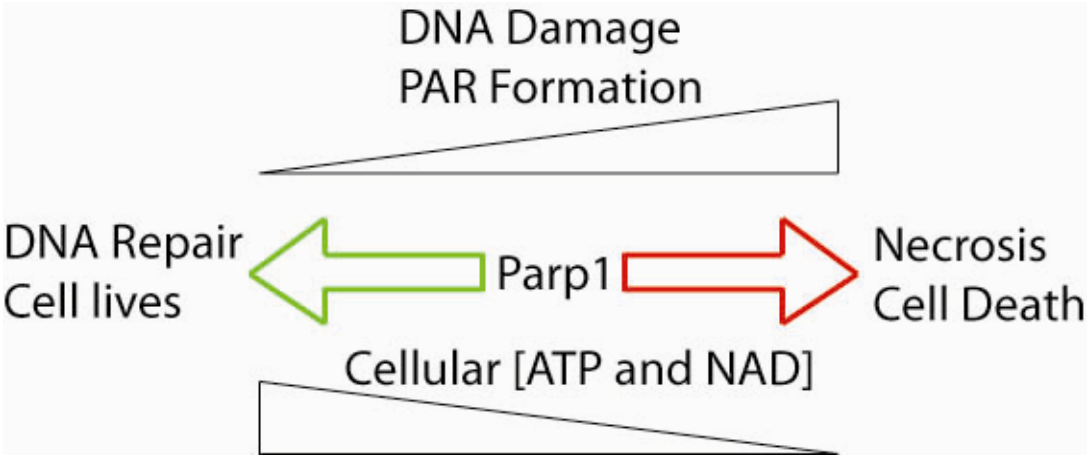

Figure 14. Parp1 helps to mediate matters of cell death. Too much DNA damage results in a rapid increase in cellular levels of PAR and concomitant decrease in the cellular levels of ATP and NAD+, which can result in cell death by energy-failure-induced necrosis

Modulation of Chromatin Structure by Parp

Parp and its associated enzymatic activities are thought to contribute to regulation of chromatin structure in both the presence and absence of DNA damage. It has been shown through biochemical studies from multiple groups that Parp can catalyze the covalent modification of histones *in vivo*, histone PARylation is thought to contribute to destabilization of nucleosomes and, in turn, to affect higher-order chromatin structure by decondensation [235-237]. It has been reported that all histones can be modified [137], but *in vivo* evidence suggests that histones H1 and H2B act as the major acceptors for modification by Parp1 and Parp2 [235, 237]. While the modification of histones as a means to modulate chromatin is an attractive model, it should be kept in mind that over 90% of the nuclear PAR is attached to Parp1 [133]. This finding suggests modification of histones *in vivo* may contribute to modulation of chromatin structure in some instances, but most likely does not represent the major mode of chromatin modulation by Parp1. A second model regarding contribution of Parp1 and PARs to chromatin structure was suggested by the observation that PAR, either free or attached to Parp1, could act as an attractive matrix for histones released from destabilized nucleosomes [212, 236]. This histone-shuttling model also contends that the highly charged PAR moieties could strip basic proteins, such as histones, from DNA [236, 238-239].

Interestingly, studies in *D. melanogaster* suggest that Parp may have differing roles in chromatin modulation, depending on whether it is associated with euchromatic or heterochomatic regions of the genome. If Parp expression is abolished or enzymatic activity is chemically inhibited using 3-aminobenzamide (3-AB), PAR accumulation is

blocked, and both localized chromatin decondensation or "puffing" and transcription at genes induced by heat shock and ecdysone is disrupted [240]. While the targets of PARylation under these circumstances were never determined, it appears that upon activation by external stimuli, Parp1 can promote decondensation of euchromatin and allow transcription to ensue. Interestingly, the genetic disruption of Parp1 in *Drosophila* results in a decondensation of heterochromatin marked by increased accessibility to micrococcal nuclease [241]. This increase in nuclease sensitivity was not noted within euchromatic regions, suggesting that Parp1 has opposing roles in regulation of chromatin structure that are highly dependent on genomic localization. Kraus and colleagues recently have used a variety of techniques including biochemical, cell-based, and cytological approaches to characterize Parp1 effects on chromatin structure. They report 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 [242-243]. Interestingly, Kraus and colleagues found that in the presence of NAD^+, Parp1 automodifies itself and promotes the decondensation of chromatin structures in vitro, allowing transcription to occur (Fig. 15). Importantly, Kraus and colleagues showed that Parp1 could be activated in the absence of DNA damage and that these chromatin modulations proceed without the direct modification of chromatin. These findings further emphasize the dual nature of chromatin modulation by Parp1 and suggest that local NAD+ levels along with other nuclear signaling may regulate both Parp1 activity and local chromatin structure. Further

studies on regulation of Parp1 activity in the absence of DNA damage will provide insight into Parp1-dependent regulation of transcription and chromatin structure.

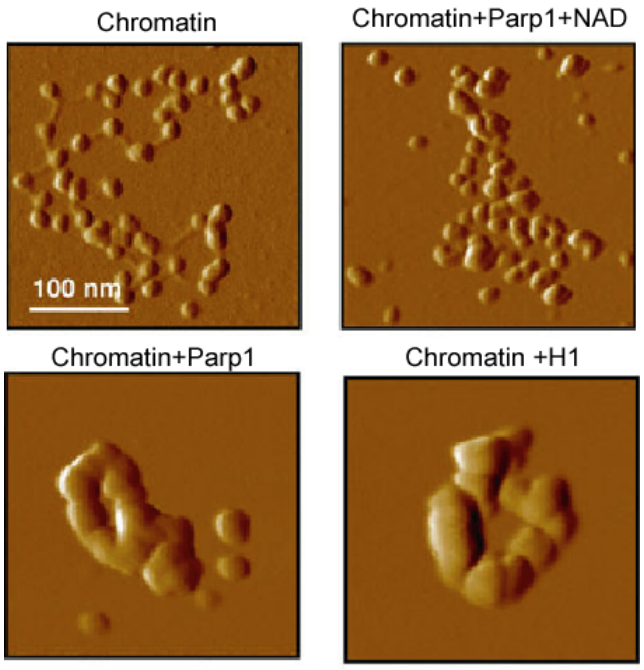

Figure 15. Parp1 and its dual nature of chromatin modulation. In the absence of NAD+ Parp1 assembles into chromatin and promotes a condensed transcriptionally repressive structure similar to chromatin in the presence of histone H1. With the addition of NAD+, enzymatic activity of Parp1 facilitates decondensation of chromatin and promotes transcription. Figure borrowed and adapted from Kim, MY et al.Cell, 2004. 119(6):803) [242].

Parp and its roles in transcriptional regulation

In recent years, many studies using a variety of experimental approaches have suggested Parp1 and its associated enzymatic activity can contribute directly to transcriptional regulation [137, 244-245]. Parp1 has been reported to contribute to transcriptional regulation by acting as a promoter-specific coregulatory factor for a number of different transcriptional regulators, including NF-κB, nuclear receptors, Hes1, B-Myb, HTLV Tax-1, Sp1, NFAT, Elk1, and others [136, 156, 243, 245-249]. In the majority of these cases Parp1 is thought to be recruited to target promoters via these DNA-binding transcription factors. At least two different mechanisms have been proposed for its role in coregulation of transcription. The first mechanism involves modulation of chromatin as discussed in the previous section. In the second mechanism, Parp1 acts as a component of enhancer/promoter binding complexes, in some examples stimulating transcription with the aid of its PARylation activities, and in others inhibiting transcription [245, 250-251]. Interestingly, in a few reports Parp1 activity is not required for its role as a coregulator for NF-κB, B-Myb, and HTLV Tax, suggesting that different and undefined mechanisms of regulation by Parp1 have yet to be discovered.

A few reports suggest that Parp1 may act as a coregulatory factor by functioning as a regulated "promoter-specific exchange factor". In 2005, Pavri and colleagues reported that Parp1 acts as a regulated exchange factor for retinoic acid receptor α (RAR/RXR) regulated genes [247]. Using a purified *in vitro* transcription system with chromatin templates, they were able to demonstrate that Parp1 is required for retinoic acid induced transcription dependent on RARα/RXR. Interestingly, Parp1 activity in this

case was not required, as catalytically inactive Parp1 still stimulated activation of transcription. The mechanism of RARα/RXR coregulation by Parp1 was revealed when it was demonstrated *in vivo* through ChIP analysis using *PARP1*$^{+/+}$ and *PARP1*$^{-/-}$ MEFs that Parp1 promotes the conversion of Mediator from an inactive form (+Cdk8) to an active from (-cdk8), however, the exact mechanism of this conversion remains unclear and needs to be further investigated. Another report, by Ju and colleagues in 2004, further suggested that Parp1 may act as a "promoter-specific exchange factor". These authors observed that Parp1 is required for the dissociation of the TLE (Transducin-like enhancer of Split) corepressor complex at the *MASH1* promoter in neuronal stem cells [246]. They found that upon treatment of the cells with PDGF, which initiates differentiation of the cells, the TLE complex is released from the promoter but the transcription factor Hes1 and Parp1 remain. Furthermore, they observed that calcium/calmodulin-dependent protein kinase (CaMKIIδ) is recruited to the promoter upon PDGF treatment and is also required for the release of the TLE corepressor complex. These findings lead the authors to propose that the CaMKIIδ facilitates the activation of *MASH1* by stimulating Parp1 activity, leading to PARylation of the TLE corepressor complex, the subsequent dissociation of TLE then allows transcription activation to ensue. Recently, another intriguing report about Parp1 regulation of hormone regulated genes further illustrate this promoter-specific exchange model [252]. The Rosenfeld group reported that Parp1 can recruit topoisomerase IIB (TopoIIB) to hormone-regulated promoters. Upon recruitment of TopoIIB, promoter DNA is cleaved, and an NCoR corepressor complex is exchanged for a Parp1 coactivator complex that includes TopoIIB, Asc2, Ku70/80, and DNA-PK, leading to the repair of DNA and

transcriptional activation. The authors suggest that DNA cleavage is needed to resolve topological constraints, which lead to favorable structural changes at the promoter. Examination of a large set of genes by the Rosenfeld group concluded that this seemed to be a common mechanism of transcriptional activation shared amongst ER, AR, RAR, T_3R, and AP-1 regulated genes. While this mode of coregulation of transcription through DNA cleavage within a promoter region is intriguing, the proposed mechanism remains controversial; and needs to be investigated further. How Parp promotes the exchange of coregulators is not understood. The regulated exchange of transcription factors by PARylation is likely to be mediated through a localized change in net charge, which could conceivably contribute to dissociation of factors through disruption of DNA binding capabilities or by conformational changes of target proteins accrued upon modification. Further studies on Parp1 regulation of transcription through its catalytic activity will provide more mechanistic insight.

Ultimately, gaining more insight into the regulatory roles Parp1 plays in transcription will require understanding the location of Parp1 within the genome under various conditions. Recently, Kraus and colleagues performed ChIP-chip analysis and found Parp1 binding is enriched at the promoters of as many as 90% of expressed RNA polymerase II transcribed genes in MCF-7 breast cancer cells [253]. Furthermore, the enrichment of Parp1 at these promoters correlated with depletion of histone H1, and those promoters with high Parp1/histone H1 ratio were found to be mostly actively transcribed genes. Interestingly, upon knock-down of *PARP1* in these cells the authors noted a 3-5 fold increase in histone H1 binding at promoters, and a resulting decrease in expression

of genes that had previously been highly occupied by Parp1. This suggests Parp1 can regulate transcription of a large set of genes (>1000), by virtue of excluding histone H1 from promoter regions. In a more recent publication, the Kraus Lab reported the effects of both Parp1 and Parg enzymes on transcriptional regulation. While there have been many reports that Parp1 has a role in transcriptional regulation, only a few studies had investigated the role of Parg in transcriptional regulation [242, 254-255]. Using the same shRNA targeted knockdown system in MCF-7 cells as used in their previous report [253], the authors found that ~1200 genes were regulated by Parp1 and ~1100 genes were regulated by Parg [256]. The majority of robustly regulated genes were enriched for roles in either stress response or metabolic functions. Interestingly, correlation analysis revealed that the majority of genes affected by knockdown by one factor were similarly affected by knockdown of the other factor [256]. These results were counterintuitive as one might have expected that two enzymes with opposing actions might also have opposing functions in transcriptional regulation. Nevertheless, chromatin immunoprecipitation analysis of both factors suggested that the promoter occupancy by Parp1 and Parg was highly correlated, further suggesting the two enzymes may work cooperatively to regulate transcription. Consistent with previous reports, the authors also found that Parp1 and Parg enzymatic activities are required for some but not all target genes through reintroduction of shRNA resistant catalytic mutants. While there have been many case-by-case examples reported by various labs regarding Parp1 and transcriptional regulation, these studies performed in recent years by the Kraus lab have been the first to utilize a genomics approach to portray perhaps a more general than expected role for Parp1, Parg and NAD^+ metabolism in transcriptional regulation.

Aside from acting as a promoter-specific coregulatory protein, Parp1 also has been shown to regulate transcription and genome organization through a role in insulator formation. Insulators are DNA elements that aid in the organization of the genome by forming discrete regulatory units that act to limit the effects of enhancers and promoters or by the preventing the spread of heterochromatin [257]. Recent studies by the Ohlsson lab suggest that CTCF, a DNA binding protein required for the function of insulators, is PARylated [258-260]. This PARylation of CTCF is required for CTCF DNA binding capability and thus is required for insulator function. Interestingly, if Parp1 is specifically inhibited by 3-AB, insulator function is lost *in vivo* [258, 260]. While it is not completely clear at the moment, it has been proposed that Parp1 may mediate insulator association with the nuclear matrix, as Parp1 associated proteins (including (DNAPK, nucleophosmin, topoisomerase II and Ku70/80) are known to associate with components of the nuclear matrix [259, 261-263]. The role Parp plays in insulator function is not completely understood, but Parp1's function in insulator formation is consistent with its roles in other processes that are mediated by the establishment of local chromatin structures. By providing dynamic flexibility within local chromatin and larger-scale nuclear architecture Parp1 aids in the facilitation of necessary nuclear processes.

Alc1 and its role in pathogenesis of Hepatocellular Carcinoma

Hepatocellular Carcinoma (HCC) is one of the most frequently diagnosed human cancers affecting more than 1 million individuals annually [264]. Along with a high frequency of occurrence, the diagnosis of HCC perhaps has one of the poorest prognoses, as it is estimated that the overall 5-year survival rate is less than 5% [265]. While there are a few etiological factors known to contribute to the pathogenesis of HCC including, hepatitis B and C virus infection, and alcohol-induced liver cirrhosis, the genetic events required for HCC pathogenesis are just being discovered. Much like other cancers, it is thought HCC develops only after the accumulation of mutations within multiple cancer-related genes that are normally responsible for regulation of biological processes such as cellular proliferation and apoptosis. One of the most frequently detected genetic alterations in HCC is the amplification of the long arm of chromosome 1 [266-269]. Interestingly, amplification of 1q has also frequently been reported within other solid tumors in bladder, breast, nasopharyngeal, and esophageal tissues, suggesting this genetic alteration may play a role in the pathogenesis of multiple forms of cancer [270-272]. More recently, it was discovered that the minimal amplified region could be narrowed down to 1q21 [269, 273], and this amplification is found in 58%-78% of HCC patients by comparative genomics hybridization. The high rate of 1q21 amplification in HCC patients suggested that a candidate oncogene might be found within this region of the genome. This candidate oncogene was identified when Guan and colleagues in 2008 isolated *ALC1* (Amplified in Liver Cancer 1; Gene Bank accession number AF537213) from 1q21 using microdissected DNA [274]. Using fluorescent *in situ* hybridization, amplification of *ALC1* was detected in ~50% of HCC cases (Fig. 16). Furthermore,

ALC1 mRNA was overexpressed in greater than half of tumor samples taken from HCC patients when compared with their respective non-tumor liver samples.

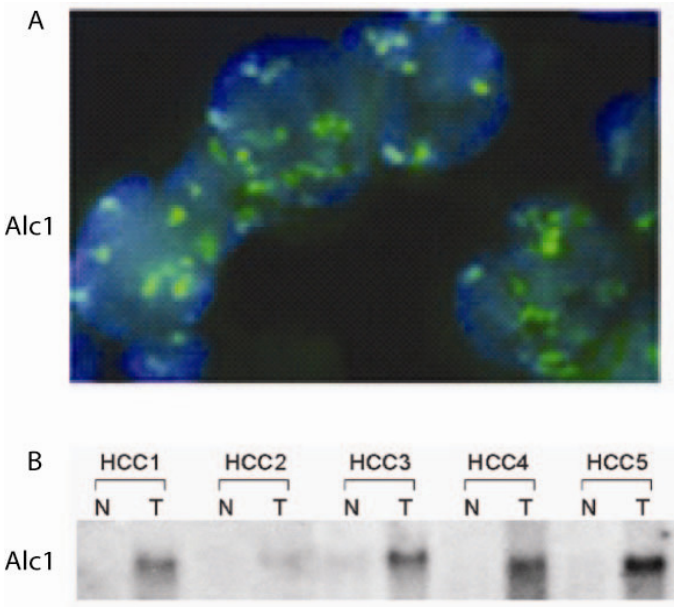

Figure 16. Chromosomal region 1q21 amplification contributes to onset of Hepatocellular Carcinoma. a) Fluorescent *In Situ* hybridization analysis of *ALC1* in HCC cell line (Blue = DAPI staining of DNA, Green = probes hybridized to *ALC1* genomic sequence). b) Northern Analysis of ALC1 expression in HCC patients (N = non tumor tissue, T = tumor tissue). Figure borrowed and adapted from Ma, NF et al. *Hepatology*, 2008. 47(2):503 [274].

Guan and colleagues found that introduction and overexpression of full length ALC1 cDNA in LO2 human liver cell line promoted an increase in colony formation in soft agar assays [274], demonstrating its tumorigenic abilities. Meanwhile, tumor xenograft experiments in nude mice suggested that ALC1 could dramatically increase tumorigenicity of immortalized liver cell lines. Further suggesting that ALC1 has tumorigenic capabilities, Guan and colleagues recently reported that transgenic ALC1 expression in mouse induces spontaneous tumors [275]. Nearly 25% of transgenic mice

had cancerous lesions while no lesions were found in control mice. Spontaneous tumor formation was observed only in older transgenic mice (over 20 months old), implying that HCC carcinogenesis caused by abnormal CHD1L expression is a long process. Interestingly, these tumors were not limited but also appeared within the abdomen wall, neck, uterus, gall bladder, and colon. This may be due to the fact that the expression of the transgene was under the control of a CMV driven enhancer leading to the ubiquitous overexpression of *ALC1*. Nonetheless these findings strongly suggest that *ALC1* acts as an oncogene and could be instrumental in the pathogenesis of HCC and other cancers.

Alc1: A SNF2 ATPase with an interesting accessory domain

While the current evidence strongly suggests that *ALC1* may act as an oncogene in the pathogenesis of HCC, the molecular mechanisms by which Alc1 protein contributes to oncogenesis remain to be determined. Some insight was gained when Guan and colleagues through used flow cytometry experiments to investigate the effect of Alc1 overexpression in cells synchronized through serum starvation. They observed that Alc1 promotes G1/S phase transition after release, as nearly 12% more cells were in S-phase after transfection with *ALC1* than vector only controls. Interestingly, through the use of further overexpression and knockdown experiments, the obtained evidence suggests that Alc1 promotes the G1/S phase transition, and ultimately cellular proliferation, by down regulating the expression of members of the p53 pathway (p53, $p21^{Waf1/Cip1}$), while upregulating Cdk2, and cyclin E [274]. While more detailed experiments certainly need to be completed, it is therefore possible that Alc1 could contribute to the pathogenesis of HCC at least in part by regulation of the p53 pathway.

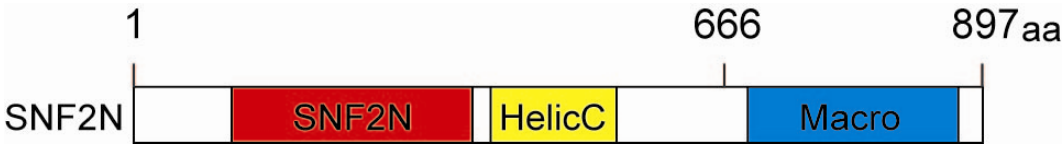

Figure 16. Schematic diagram depicting the structural domains of Alc1. SNF2N, SNF2 family N-terminal domain; HelicC, Helicase superfamily C-terminal domain; Macro, macrodomain

While the *in vivo* characterization of *ALC1* and its potential role as an oncogene may prove to be a cornerstone in the understanding of HCC pathogenesis, at the time we began our studies Alc1 protein had yet to be biochemically characterized. What was known was that Alc1 is a member of the SNF2 ATPase superfamily as it contains a Snf2-like ATPase domain highly similar in primary sequence to Snf2, Iswi, and Chd1 (Fig. 5). In fact, it was so similar to these members across the helicase motifs that it was given the alternative name *CHD1L* (Chromodomain helicase DNA binding 1-like, which is currently still the official name according to HUGO). This alternative name *CHD1L*, however, is a bit of a misnomer as Alc1, unlike Chd1 and all other Chd-type subfamily remodelers, is lacking an identifiable chromodomain.

In lieu of an accessory domain such as the chromodomain found in Chd-type remodelers, Alc1 has a C-terminal macrodomain (Fig. 16). Macrodomains were first discovered over twenty years ago upon the cloning of macroH2A [276], a histone variant highly similar to canonical histone H2A except for the presence of a C-terminal macrodomain. It is now known that macrodomains are ancient highly conserved domains that are found in prokaryotes, eukaryotes, and single-stranded RNA viruses that replicate within animal cells. [276-278]. Structurally, macrodomains are composed of a 'macro fold' that is approximately 190 amino acids in size and is formed from a mixed $\alpha-\beta$ fold that is similar to the P-loop of nucleotide triphosphate hydrolases [279-280] (Fig. 17). Macrodomain function had not previously been tied to specific cellular processes, but it was suggested by Ladurner and colleagues [279, 281], to function as a binding module for metabolites of NAD^+, including free mono-ADP-ribose, poly(ADP-ribose), and *O*-

acetyl-ADP-ribose (OAADPR), a product of protein deacetylation by SirT proteins. Ladurner and colleagues observed that the Af1521 macrodomain containing protein, found in the thermophile *Archaeoglobus fulgidus*, exhibits a high affinity for ADP-ribose, as isothermal titration calorimetry suggests and equilibrium dissociation constant (K_D) of 126 nM. The macrodomain affinity for ADP-ribose was nearly 45-fold greater than observed for ADP [282]. 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 [281]. Interestingly, some macrodomains are capable of hydrolyzing phosphate groups from nucleotides of ADP-ribose derivatives, suggesting that the domain may be evolving to provide new functions [280-281, 283].

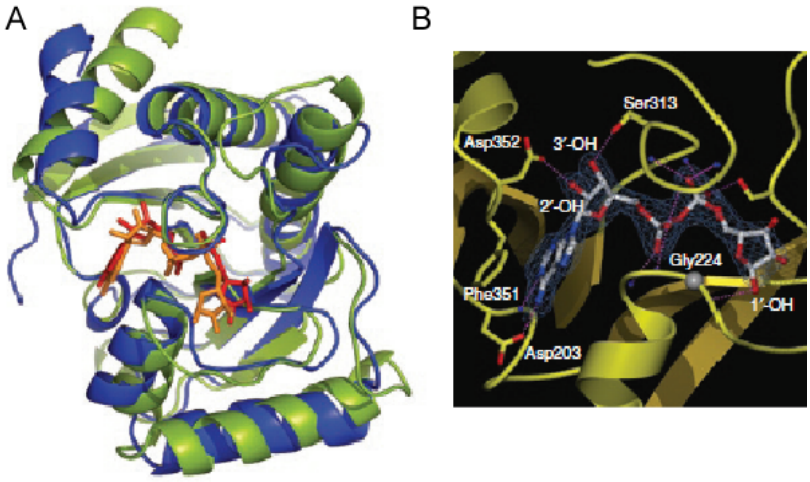

Figure 17. Macrodomains are highly conserved structural domains that bind NAD+ metabolites. X-ray crystallographic structure of macrodomains from A. *A. fulgidus* (Archaea) (PDB 2BFQ)[280] and B. human MacroH2A1.1 (PDB311B)[284] binding ADP ribose. Figure adapted from Kraus, WL et al. *Nat Struct Mol Biol*, 2009. 16(9):904 [277] and Timinszky, G. et al. *Nat. Struct. Mol. Biol*, 2009. 16 [284].

While *ALC1* is the only gene that encodes both a SNF2 ATPase domain and a C-terminal macrodomain, at least ten other genes in the human genome encode a macrodomain. In addition to Alc1 and the three isoforms of MacroH2A (MacroH2A1.1, MacroH2A1.2, and MacroH2A2), there are three other proteins C6orf130, MacroD1, and MacroD2, that carry macrodomains and that do not currently have described molecular functions. These proteins lack additional functional domains that might otherwise provide insight into cellular function. The remaining macrodomains encoded by the human genome are found in proteins with PARP catalytic domains. These include the recently discovered Parp9, Parp14, and Parp15 [285], which are unusual in that they include multiple macrodomains in tandem which is not the case with other proteins. That these newly discovered proteins contain both PARP catalytic domains and tandem macrodomains suggests these two interesting domains may be evolutionarily tied to each other and may contribute together to a variety of cellular processes.

As described in chapter II, the efficient packing of chromatin within the nucleus presents a formidable barrier to the enzymes required for proper initiation and completion of nearly all nuclear processes. To bypass this impediment and allow processes to ensue, enzymes such as Snf2-like ATPases and Parp1 efficiently modulate chromatin structure. The focus of the remaining chapters revolves around Alc1, a Snf2-like ATPase and potential oncogene, with unknown function. In beginning our studies on Alc1, our aim was to gain some insight into the molecular mechanisms by which Alc1 works and how it contributes to normal cellular processes. By gaining insight into these mechanisms through biochemical characterization of Alc1, we hoped also to gain insight into how

aberrant expression of *ALC1* could lead to the pathogenesis of cancer. In chapter III, I will describe a series of experiments that characterize Alc1 function through a series of biochemical experiments. Through this rigorous biochemical characterization we find that Alc1, and its associated enzymatic activities, potentially serve to functionally link the fields of chromatin remodeling and NAD^+ metabolism.

Chapter II. Biochemical studies of Alc1

Investigation of Alc1 Protein-Protein interactions

In order to gain insight into the biological roles and pathways in which Alc1 may contribute, we began by investigating possible Alc1 protein-protein interactions. To do this, we used a very straightforward approach that began with the generation of an HEK293/FRT cell line stably expressing *ALC1* with an N-terminal FLAG tag (F-Alc1). After establishment of the cell line and clonal selection, nuclear extracts were made according to Dignam's method [286], and immunoprecipitations using M2 (anti-FLAG) agarose were performed. The initial results suggested, that unlike many SNF2 superfamily members, Alc1 does not reside in a stable multi-subunit complex (Fig. 18); however, MudPIT mass spectrometry indicated that preparations of F-Alc1 contained substoichiometric amounts of histones, Parp1, and several Parp1-interacting proteins (Table 2, see F-Alc1 (wt)).

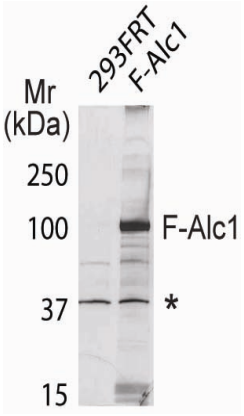

Figure 18. SDS-PAGE analysis of F-Alc1 from HEK293/FRT cells. Aliquots of Flag-immunoprecipitates from equivalent amounts of nuclear extract from HEK293/FRT cells or HEK293/FRT cells stably expressing F-Alc1 were analyzed by SDS-PAGE and silver staining. The asterisk in denotes a protein that non-specifically binds and is eluted from Flag agarose beads.

Locus	Description	NSAF x 10^4 F-Alc1 (wt)	F-Alc1 (E175Q)	F-Alc1 (D723A)
gi|148612870|ref|NP_004275.3|	Alc1	2574	1537	1382
gi|4503841|ref|NP_001460.1|	Ku70	137	21	ND
gi|156523968|ref|NP_001609.2|	Parp1	111	12	ND
gi|106775678|ref|NP_001035807.1|	Histone H2A	105	33	ND
gi|15617199|ref|NP_254280.1|	Histone H2A	105	33	ND
gi|10863945|ref|NP_066964.1|	Ku80	102	14	ND
gi|4504253|ref|NP_002096.1|	Histone H2AX	83	38	ND
gi|4506587|ref|NP_002938.1|	RPA3	57	21	ND
gi|6005757|ref|NP_009123.1|	Spt16	51	ND	ND
gi|169167131|ref|XP_001720197.1|	Histone H3	46	15	ND
gi|4504255|ref|NP_002097.1|	Histone H2AZ	44	20	ND
gi|4506585|ref|NP_002937.1|	RPA2	39	ND	ND
gi|13654237|ref|NP_008835.5|	DNAPK	38	14	1
gi|110825961|ref|NP_005475.2|	Parp2	29	19	ND
gi|18105048|ref|NP_542160.1|	Histone H2B	20	12	ND
gi|10800140|ref|NP_066406.1|	Histone H2B	20	12	ND

Table 2. Identification of possible protein-protein interactions through MudPIT analysis. Whole cell lysates from HEK 293/FRT cells expressing wild type or mutant FAlc1 were immunoprecipitated with anti-FLAG (M2) agarose. Immunopurified proteins were identified using a modification of the multidimensional protein identification (MudPIT) procedure (1, 2). Shown are the most abundant proteins that were detected by MudPIT masspectrometry in Flag immunopurified material from cells expressing wild type F-Alc1 but not the macrodomain mutant F-Alc1 (D723A). The normalized spectral abundance factor (NSAF) is proportional to the amount of protein present in the sample (3, 4) and is calculated using the formula:

$$(\mathrm{NSAF})_k = \frac{(SpC/L)_k}{\sum_{i=1}^{N} (SpC/L)_i},$$

where *SpC* = spectal count, *L* = protein length in amino acids, and *i* = all proteins detected in the MudPIT runs. ND, not detected.

Expression and Purification of wild-type and mutant Alc1

The presence of Snf2-like ATPase and macrodomains in Alc1 raised the possibility that both domains might contribute to its function and, perhaps, that one might regulate the activity of the other. To address these possibilities, we prepared wild-type and mutant versions of Alc1 that could be used as components of defined biochemical assays. In particular, we expressed and purified recombinant wild-type F-Alc1; a DEAH box mutant F-Alc1(E175Q), which is mutated at a position expected to prevent ATP binding and hydrolysis; a macrodomain mutant F-Alc1(D723A), which is mutated at a position shown previously to decrease substantially the affinity of ADP-ribose binding by AF1521, a macrodomain-containing protein from *Archaeoglobus fulgidus* [281]; and the Alc1 macrodomain (amino acids 666-897) (Fig. 19).

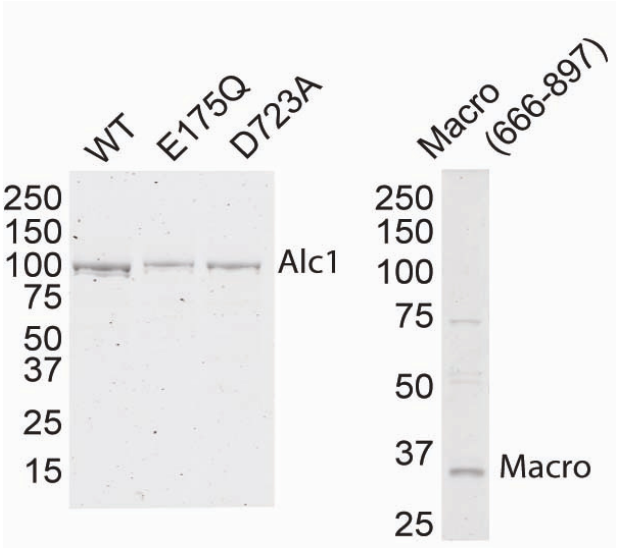

Figure 19. SDS-PAGE analysis of recombinant wild type and mutant versions of Alc1. Proteins were expressed in insect cells using baculovirus expression system and purified by Flag-immunopurification via M2 agarose. Subsequent to separation by SDS-PAGE, proteins were coomassie stained and scanned with the Typhoon imaging system.

Alc1 Macrodomain is a PAR binding Motif

Because of the presence of a macrodomain in Alc1, and because our MudPIT analysis performed on F-Alc1 immunopurifications suggested an interaction with Parps 1, we set up experiments to determine if Alc1 can possibly bind poly(ADP-ribose) (PAR). To test for this possibility, we used nitrocellulose filter binding assays, which rely on the fact that PAR binds nitrocellulose only when bound by proteins. To begin, ^{32}P-labeled PAR was produced by incubation of recombinant Parp1 with ^{32}P-labeled NAD^+ and sonicated DNA for 30 minutes. After incubation, ^{32}P-labeled PAR was purified from Parp1 through incubation with proteinase K, followed by phenol-chloroform extraction. To remove any residual free ^{32}P-labeled NAD^+ remaining, the sample was passed over multiple G-25 sephadex columns. PAR binding assays were performed by incubating radiolabeled PAR with 1 pmol of purified recombinant proteins, including wild-type, E175Q, and D723A F-Alc1, and Alc1 macrodomain (residues 666-897), for 30 minutes and subsequently dot-blotting the reactions on nitrocellulose (Fig. 20). The membranes were then washed extensively with Tris-Buffered Saline with Tween-20 (TBST) and analyzed with a phosphoimager. Our initial results indicated that F-Alc1 (wt) can bind PAR in a salt sensitive manner, with optimal binding occurring below 300 mM NaCl (Fig. 20B). This binding could be abolished by heat treatment suggesting that a proper protein structure is required for the binding (Fig. 20C). The DEAH box mutant F-Alc1(E175Q) was found to bind PAR similarly as F-Alc1 (wt), suggesting that ATPase activity is not required for PAR binding. Importantly, however, the introduction into the macrodomain of a single point mutation, D723A, was enough to abolish binding of PAR by Alc1. Further indicating that the Alc1

macrodomain is necessary and sufficient for PAR binding, the isolated Alc1 macrodomain bound PAR (Fig. 20C).

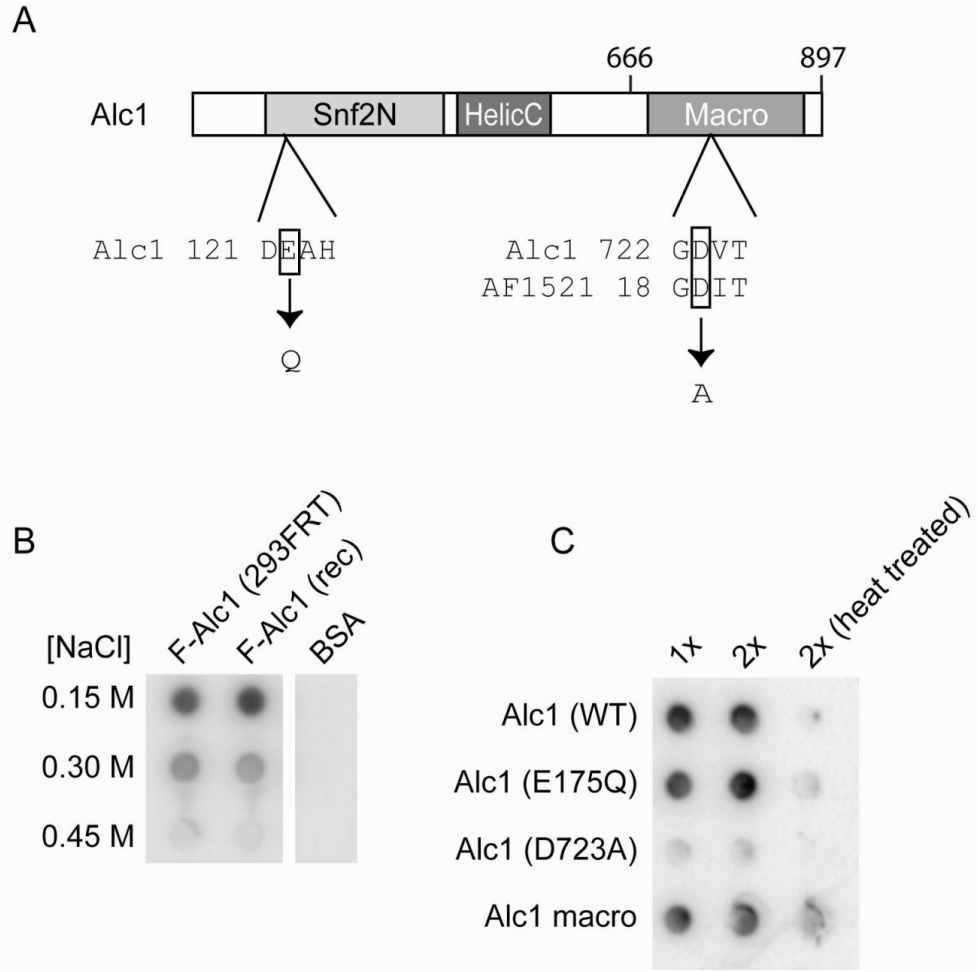

Figure 20. Alc1 binds poly-(ADP-ribose). (A) Alc1 domain structure. Sequences below diagram show amino acid changes in catalytically inactive Alc1 mutant E175Q and macrodomain mutant D723A. The mutated macrodomain region is compared to the homologous sequence from the AF1521 macrodomain. Snf2N, SNF2 family N-terminal
domain; HelicC, Helicase superfamily c-terminal domain; associated with DEXDc-,DEAD-, and DEAH-box proteins; macro, macrodomain. (B) ~100 ng of each protein was incubated with ^{32}P-labeled PAR in buffer containing the indicated NaCl concentrations. PAR binding was detected with a nitrocellulose filter binding assay. (C) ~100 ng (1X) or 200 ng (2X) wild type or mutant Alc1 was incubated with PAR in buffer containing 0.15M NaCl, and PAR binding was measured as in panel (B). F-Alc1, Flag epitope-tagged Alc1; BSA, bovine serum albumin; rec, recombinant.

Studies on Alc1 ATPase activity

Many SNF2 superfamily members have both DNA- and nucleosome-activated ATPase activities that enable their enzymes to contribute to the variety of biological processes detailed earlier. To determine whether Alc1 has similar activities, we assayed anti-Flag agarose eluates from F-Alc1 expressing HEK293/FRT cells and wild type and mutant versions of recombinant F-Alc1, expressed in and purified from Sf21 cells, for ATPase activity. A fraction containing F-Alc1 from HEK293/FRT cells exhibited robust nucleosome-dependent ATPase; however, recombinant F-Alc1 lacked activity (Fig. 21, compare lanes 2 and 3), and the ATPase activity associated with F-Alc1 immunopurified from HEK293/FRT cells was lost after size exclusion chromatography (see Fig.28B, C), suggesting a requirement for an activating factor or cofactor.

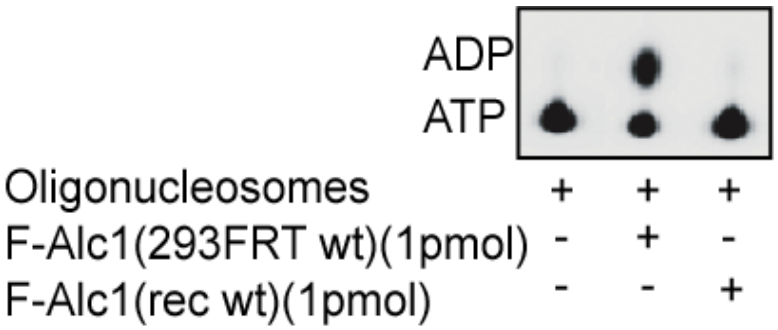

Figure 21. Purified recombinant Alc1 lacks detectable ATPase activity. ATPase activity comparison of F-Alc1 immunopurified from HEK293/FRT and recombinant F-Alc1 expressed and purified from SF21 insect cells.

Our observation that the Alc1 macrodomain binds PAR, together with evidence from MudPIT mass spectrometry that anti-FLAG agarose eluates from F-Alc1 expressing

HEK293/FRT cells contained substoichiometric amounts of Parp1 (and proteins known to associate with Parp1), raised the possibility that addition of NAD^+ and Parp1 to reactions might stimulate ATPase. Indeed, we observed that the ATPase activity of recombinant F-Alc1 was strongly stimulated by addition of Parp1 and NAD in the presence of either DNA or nucleosomes (Fig. 22A). Furthermore, ATPase was not activated in the absence of DNA or nucleosomes or when either NAD or Parp1 were omitted from reactions, suggesting Parp1-dependent PAR synthesis is required for the reaction (Figure 22B).

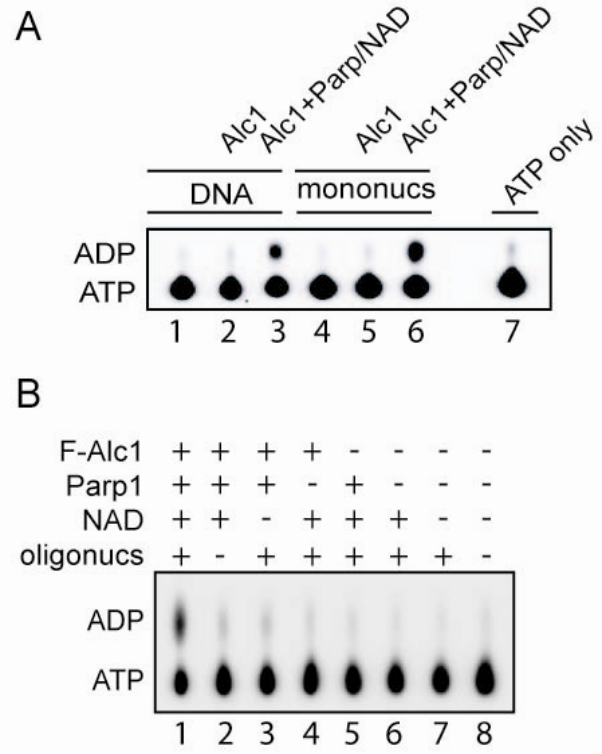

Figure 22. Alc1 has Parp1- and NAD-dependent ATPase activities A) ATPase assays performed with recombinant F-Alc1, with or without Parp1 and NAD, in the presence of DNA or an equimolar amount of mononucleosomes assembled on the same DNA with HeLa histones. B) ATPase assays performed as in panel A with the indicated combinations of recombinant F-Alc1, Parp1, NAD, and oligonucleosomes.

Suggesting a coupling of ATPase and PAR binding activities, we found that ATPase activity depends on an intact macrodomain. F-Alc1 (D723A), which does not bind PAR, lacks ATPase activity in either the presence or absence of Parp1 and NAD (Fig. 23, compare lanes 6 and 10). PAR binding is not, however, sufficient to activate ATPase, since the addition of free PAR does not activate Alc1 ATPase (Table 3). In addition, our data is consistent with the idea that ATPase activity associated with Alc1 immunopurified from mammalian cells might be due to the presence of residual Parp and NAD in the fraction, and indeed, observations we will discuss later further support this idea (see Fig. 28)

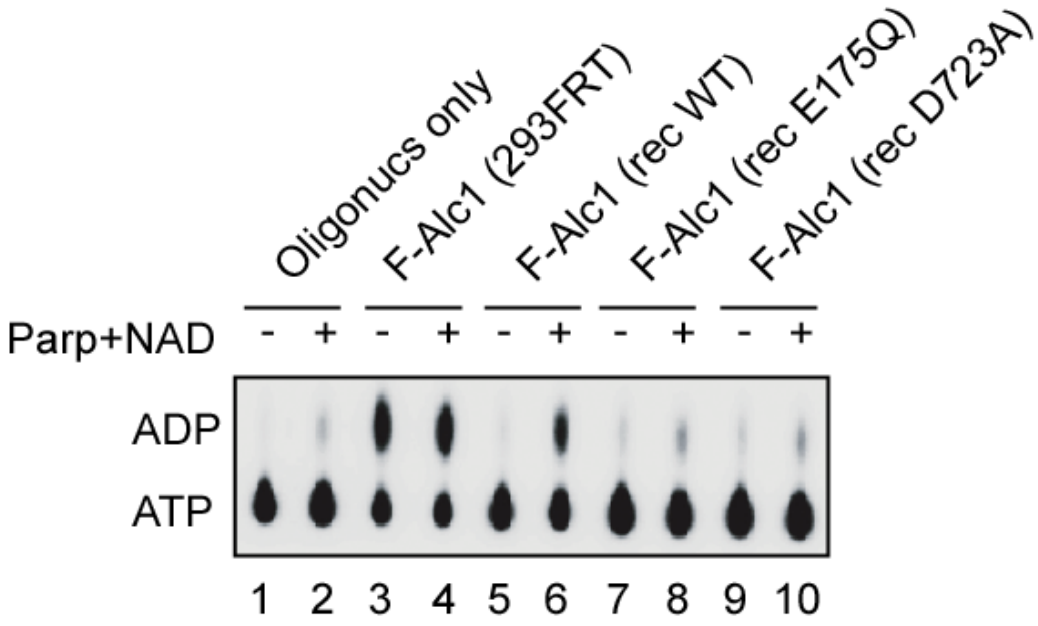

Figure 23. Stimulation of Alc1 ATPase by Parp1 and NAD$^+$ requires an intact macrodomain. ATPase assays performed with ~100ng of wild-type or mutant recombinant (rec) F-Alc1, or F-Alc1 from HEK 293/FRT (293FRT) cells and 150ng of HeLa cell oligonucleosomes, with or without the presence of Parp1 and NAD.

Our observations that ATPase was not activated in the absence of DNA or nucleosomes or when either NAD or Parp1 were omitted from reactions, suggested Parp1-dependent PAR synthesis is required for the reaction (Fig 22B). Consistent with this possibility, addition of poly-(ADP-ribose) glycohydrolase (Parg), an enzyme known to catalyze the hydrolysis and breakdown of PAR, blocks activation of Alc1 ATPase by Parp1 and NAD (Figure 24A).

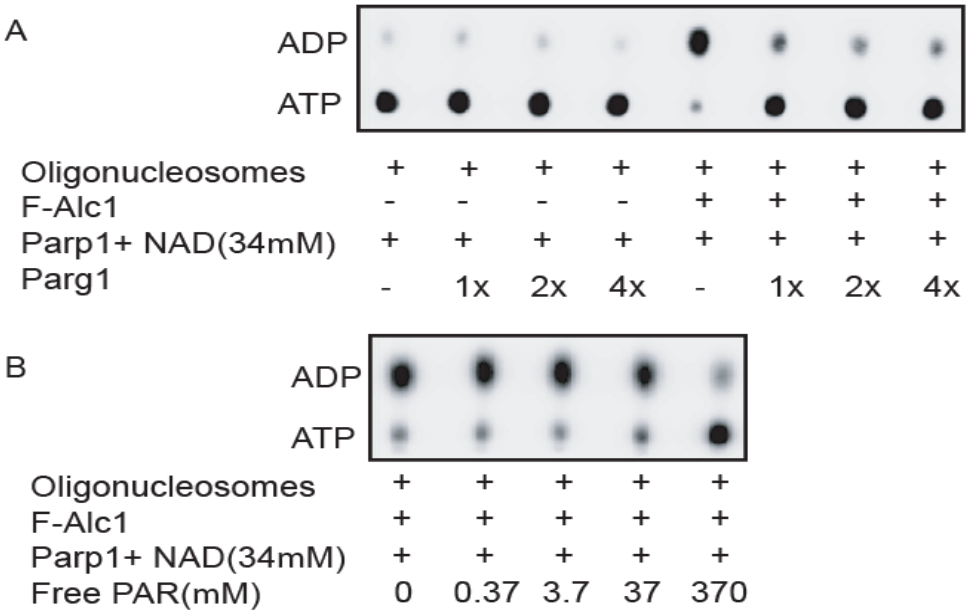

Figure 24. Alc1 ATPase activity is inhibited by enzymatic activites of Parg or by addition of free PAR. A. Parp1- and NAD-dependent Alc1 ATPase is inhibited by Parg1. Recations were performed as described in Materials and Methods with or without the addition of 1ng (1x), 2ng (2x), or 4ng (4x) purified recombinant Parg1 enzyme. B**.** Free PAR can competitively compete away Alc1 Parp- and NAD-dependent ATPase activity.

Interestingly, we found that titration of free PAR into the ATPase reaction at the beginning can inhibit Parp1- and NAD^+- dependent Alc1 ATPase activity (Fig. 24B). Furthermore, neither free PAR nor ADP-ribose activate Alc1 ATPase, even when present

at concentrations (expressed in mole equivalents of adenosine) nearly five times higher than the maximal amount of poly-(ADP-ribosyl)ated species that could be synthesized in reactions containing Parp1 and NAD (Table 3). These findings suggest that simple binding of Alc1to PAR or ADP-ribose is not sufficient for ATPase stimulation. Taken together, our data suggests that Alc1 ATPase activity depends on automodification of Parp1 and/or on PARylation of Alc1 itself. As discussed later, our data is most consistent with the former possibility.

Reaction components	**ATP hydrolysis (pmol/min)**
Alc1, Parp-1, NAD (34 μM), nucleosomes	4.6
Parp-1, NAD (34 μM), nucleosomes	0.9
Alc1, PAR (1.5 μM), nucleosomes	0.8
Alc1, PAR (15 μM), nucleosomes	1.0
Alc1, PAR (150 μM), nucleosomes	0.9
PAR (150 μM), nucleosomes	0.8
Alc1, ADPr (1.5 μM), nucleosomes	1.2
Alc1, ADPr (15 μM), nucleosomes	1.0
Alc1, ADPr (150 μM), nucleosomes	1.0
ADPr (150 μM), nucleosomes	1.1
Nucleosomes	0.9

Table 3. Neither poly(ADP-ribose) nor ADP-ribose activate Alc1 ATPase activity. 30 minute ATPase reactions were performed as described in Methods with or without 1 pmol Alc1, 1 pmol Parp-1, and the indicated concentrations of NAD, free ADP(ribose) (ADPr), or free PAR. All reactions contained 1 pmol Hela cell long oligonucleosomes, 40 μM ATP. Concentrations of PAR and ADPr are expressed as mole equivalents of adenosine determined using the extinction coefficient of adenosine (A_{260nm} = 15 O.D. (cm^2/μmol) at pH 7). PAR was prepared as described [281].

Studies on Alc1 chromatin remodeling activities

Many Snf2 superfamily members, including Chd1, Iswi, and Ino80, can catalyze the ATP-dependent remodeling of nucleosomes *in vitro* [30, 51, 287-288]. To determine if Alc1 also has chromatin remodeling activity we employed a previously described assay [114, 289-290] that takes advantage of the fact that DNA on the octamer surface is largely protected from cleavage by restriction enzymes, while DNA outside the nucleosome boundary is accessible.

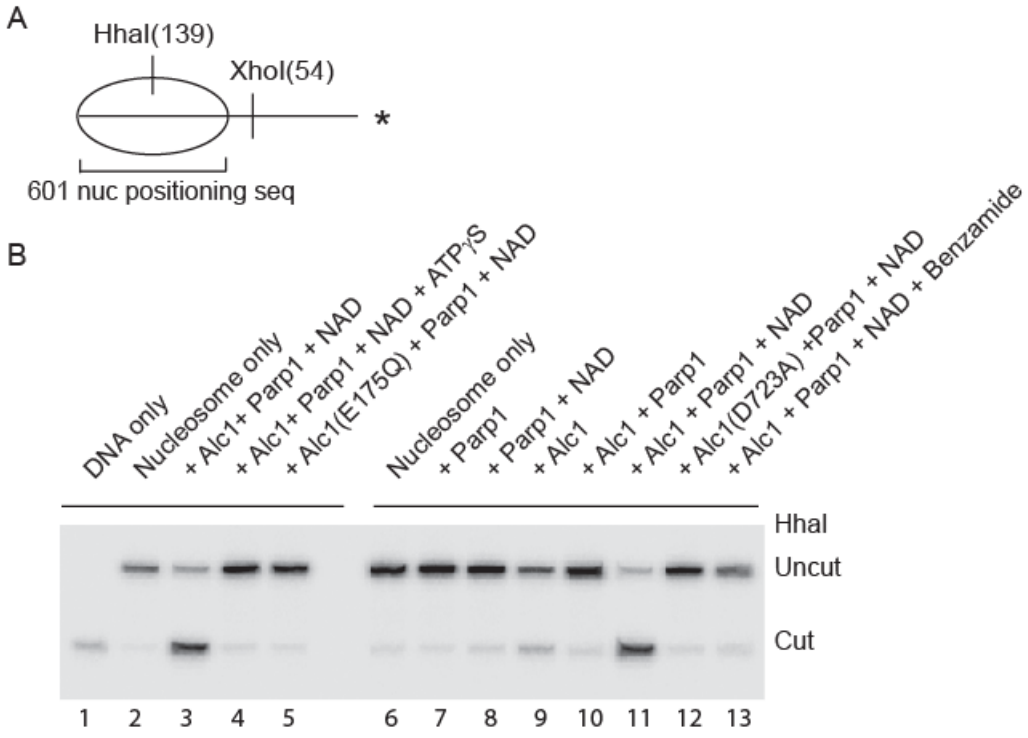

Figure 25. Alc1 has Parp1- and NAD- dependent nucleosome remodeling activities. A) Schematic showing location of positioned nucleosome (nuc) and length of *Hha*I and *Xho*I cleavage products. Asterisk, ^{32}P-labeled DNA end. B) DNA or nucleosomes reconstituted with recombinant histones were monitored for restriction enzyme accessibility after incubation with ATP (lanes 3, 5, 7–13) or ATPγS (lane 4) and wild-type or mutant Alc1, Parp1, NAD, or 2 mM benzamide.

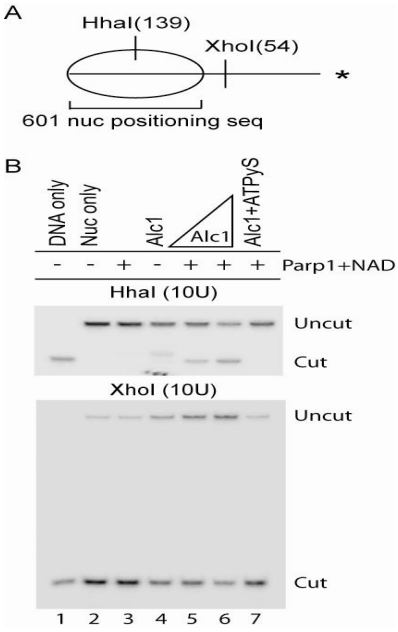

Figure 26. Alc1 slides nucleosomes directionally in an ATP- Parp1- and NAD- dependent manner. (*A*) Schematic showing location of positioned nucleosome (nuc) and length of *Hha*I and *Xho*I cleavage products. Asterisk, ^{32}P-labeled DNA end. (*B*) DNA or nucleosomes reconstituted with HeLa cell histones were monitored for restriction enzyme accessibility after incubation with ATP (lanes 3–6) or ATPγS (lane 7) and Alc1, Parp1, and NAD as indicated.

We assayed for nucleosome remodeling using mononucleosomes assembled from purified recombinant histones or HeLa oligonucleosomes on a ^{32}P end-labeled DNA probe containing a nucleosome positioning sequence (Fig 25A) [291] We found that nucleosome remodeling activity, much like ATPase activity, depends strongly on Parp1 and NAD (Fig. 25B, compare lanes 11 and 9) and is inhibited by benzamide, a potent inhibitor of Parp1 (Fig. 25B, lane 13). Furthermore the remodeling is absolutely ATP dependent, as the DEAH box mutant F-Alc1 (E175Q) fails to remodel mononucleosomes (Fig. 25B, compare lanes 3 and 5), nucleosome remodeling by F-Alc1 is inhibited by ATPγS (Fig. 25B, compare lanes 3 and 4). In addition, the macrodomain mutant F-

Alc1(D723A), which exhibits reduced PAR binding, is inactive in our nucleosome remodeling assays (Fig. 25B, lane 12).

To determine how chromatin remodeling is achieved by Alc1 we utilized the same restriction enzyme accessibility assay, but monitored the assay simultaneously with two restriction enzymes, HhaI and XhoI. If remodeling events orchestrated by Alc1 resulted in partial disassembly of the octamer, or eviction of the entire octamer, one would predict that the HhaI site would be more accessible while XhoI accessibility would remain unchanged (Fig. 26A). If, however, Alc1 remodels via a nucleosome sliding mechanism, one would expect to see an increase in HhaI accessibility and a corresponding decrease in XhoI accessibility. Our results demonstrate that the accessibility of a Hha I site, initially protected by the positioned nucleosome, is increased after incubation with recombinant F-Alc1, Parp1, and NAD. Arguing that Alc1 moves the nucleosome from its initial lateral position toward a more central position on the DNA, we observe a concomitant decrease in accessibility of an XhoI site outside the initial nucleosomal boundary (Fig. 26B). These observations argue that the mode by which Alc1 remodels chromatin involves the sliding of nucleosomes.

Co-fractionation of Enzymatic Activities with Alc1

Our observation that ATPase and remodeling activities depend on intact Alc1 catalytic and macrodomains argues that the observed enzymatic activities could be attributed to Alc1. We used further chromatographic techniques to further confirm the association of these activities with Alc1, and to examine the possibility that other cofactors and/or contaminants could be contributing. Accordingly, we subjected anti-FLAG agarose eluates from F-Alc1 expressing HEK293/FRT cells to size exclusion chromatography, and followed with SDS-PAGE and western analysis of the fractions with antibodies directed against Flag-Alc1(Fig. 27).

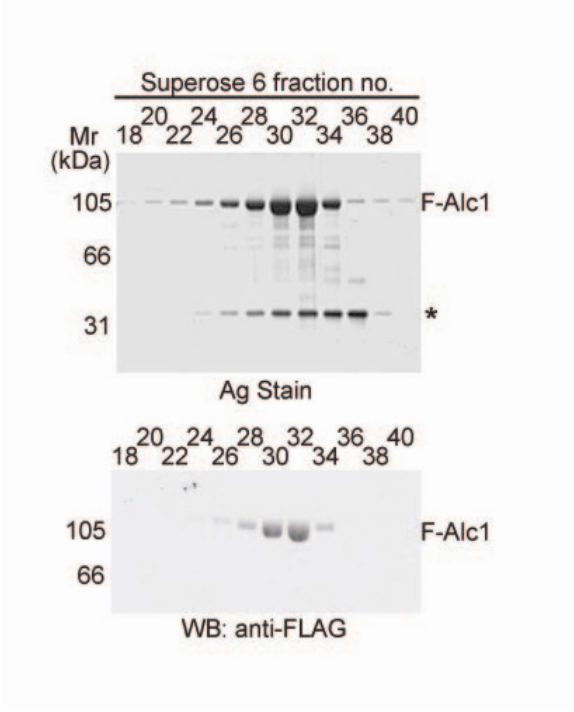

Figure 27. Fractionation of Alc1 purified from HEK293/FRT cells by size-exclusion chromatography. The indicated fractions were characterized by SDS-PAGE and silver staining (upper panel) or anti-FLAG western blotting (lower panel), which suggested F-Alc1 ran nicely as a monodisperse peak. The asterisk denotes a protein that non-specifically binds and is eluted from Flag agarose beads.

Subsequent to SDS-PAGE and western analysis we performed both ATPase and nucleosome remodeling assays with the collected fractions. These assays demonstrated that Parp1- and NAD-dependent ATPase and nucleosome remodeling activities co-eluted with F-Alc1 from the column as a mono-disperse peak (Fig. 28A, B, and C). Taken together, our findings argue that Alc1 possesses ATP-dependent nucleosome remodeling activity and that nucleosome remodeling, like ATPase, is closely coupled to PAR binding.

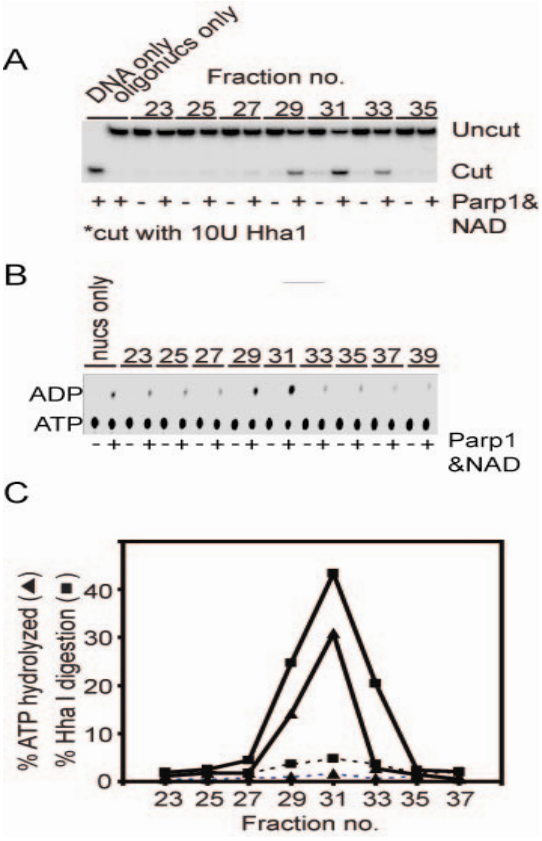

Figure 28. ATPase and nucleosome remodeling activities co-purify with Alc1. A) Equal volumes of F-Alc1 eluates chromatographically fractionated via superose 6 were used to evaluate nucleosome remodeling capability in the presence or absence of Parp1 and NAD. B) Indicated fractions were used to evaluate ATPase activity in the presence or absence of Parp1 and NAD. C) Graph depicting the cofractionation of ATPase (triangles) and nucleosome remodeling (squares) activities with Alc1 in the presence (solid lines) and absence (dashed lines) of Parp1 and NAD.

Parp automodification is the critical event required for Alc1 enzymatic activation

At this point in our studies we had determined that Alc1 is in fact an ATPase that is activated in a Parp1- and NAD- dependent manner to remodel nucleosomes, but how this enzymatic activation unfolded was not completely clear. There were three formal possibilities for Alc1 activation. First, Parp1 could modify Alc1, leading to activation of Alc1 enzymatic activities; second, Parp1 might automodify itself, after which Alc1 binds this modification and is enzymatically activated or; third, Parp1 could modify histone proteins, after which Alc1 could bind the modification and be enzymatically activated. While it has been documented that histones are modified *in vivo,* we have failed to detect histone modification *in vitro* under our assay conditions that make use of purified oligonucleosomes. PARylation of histones may very well contribute to Alc1 function *in vivo*, but, we can definitively say that histone modification by Parp1 is not required for Alc1 activation, as Alc1 ATPase is activated in the presence of Parp1, NAD, and DNA (Fig. 22A).

To determine how production of ADP-ribose is contributing to Alc1 activation we first scaled up an assay, with conditions highly similar to ATPase and remodeling assays, and examined the outcomes with SDS-PAGE and western analysis. As expected, we found that PAR production by Parp1 is highly dependent on the presence of NAD and DNA (Fig. 29). Upon addition of DNA to the reaction, Parp1 is enzymatically activated. Indicating that Parp1 becomes extensively modified, most of the Parp1 runs much more slowly appearing as a high molecular mass smear on SDS-PAGE. Importantly, we did not detect a similar change in migration of Alc1, suggesting that Alc1 is either not

modified or that it is subject to only limited PARylation under our assay conditions (Fig. 29).

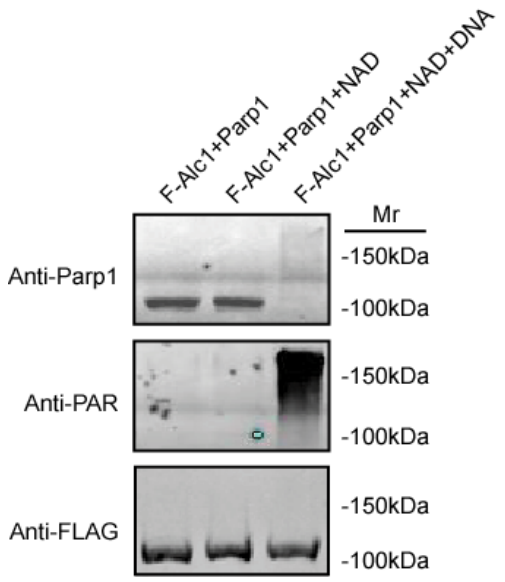

Figure 29. Alc1, unlike Parp1, is not detectably PARylated in vitro. *In vitro* PARylation reactions were set up with conditions similar to remodeling and ATPase assays. After 30min incubation reactions were subjected to SDS-PAGE and western analysis. Western blots were scanned and analyzed with Li-COR imaging system.

To address further the alternative possibility that modification of Alc1 leads to its activation, we performed order of addition experiments using the Parp1 inhibitor benzamide (30). When added at the beginning of the reaction, benzamide blocked nucleosome remodeling (Fig. 30); however, when Parp1 was preincubated with nucleosomes and NAD before addition of benzamide and Alc1, robust chromatin remodeling activity was detected (Fig. 30), suggesting that the essential PARylation events occur before Alc1 addition. These results demonstrate that Alc1 enzymatic

activity is most likely activated by the binding of Alc1 to PARylated Parp1 in the presence of DNA and/or chromatin.

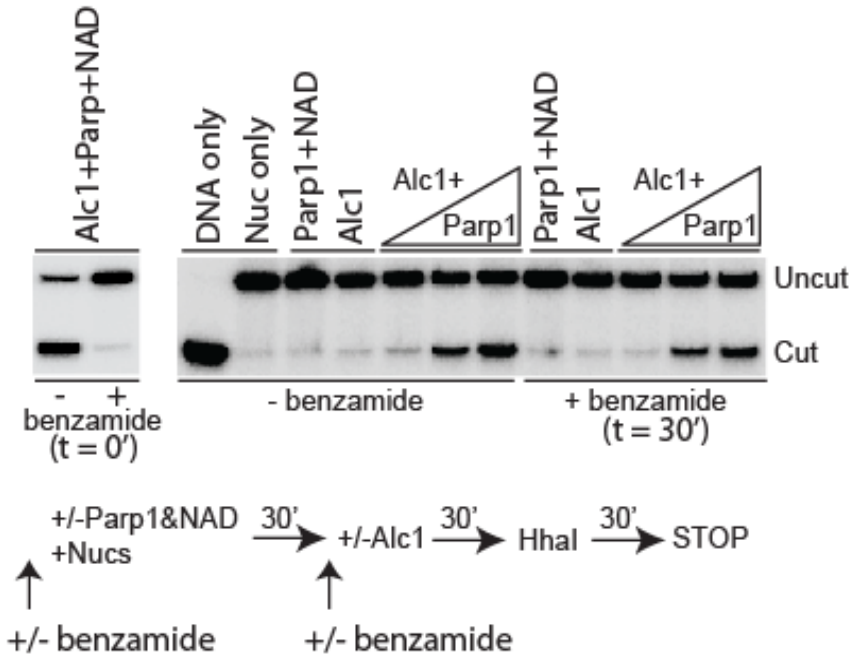

Figure 30. Alc1 PARylation is not required for nucleosome remodeling. Nucleosome remodeling assays were performed as described in the depicted experimental diagram with nucleosomes reconstituted with HeLa cell histones. Nucleosomes were preincubated for 30 min with Parp1 and NAD before the addition of Alc1, with or without 2mM benzamide.

Alc1 recruitment to chromatin is mediated by PARylated Parp1

Alc1, unlike other chromatin remodeling and modifying enzymes or complexes, lacks targeting domains, such as bromo- or chromo-domains, that contribute to targeted recruitment to regions of specifically marked chromatin. However, our observation that Alc1 ATPase and chromatin remodeling activities require Parp1 and NAD raises the possibility that Alc1 could be targeted to chromatin by PARylation *via* its macrodomain. We tested this hypothesis using both biochemical and *in vivo* assays.

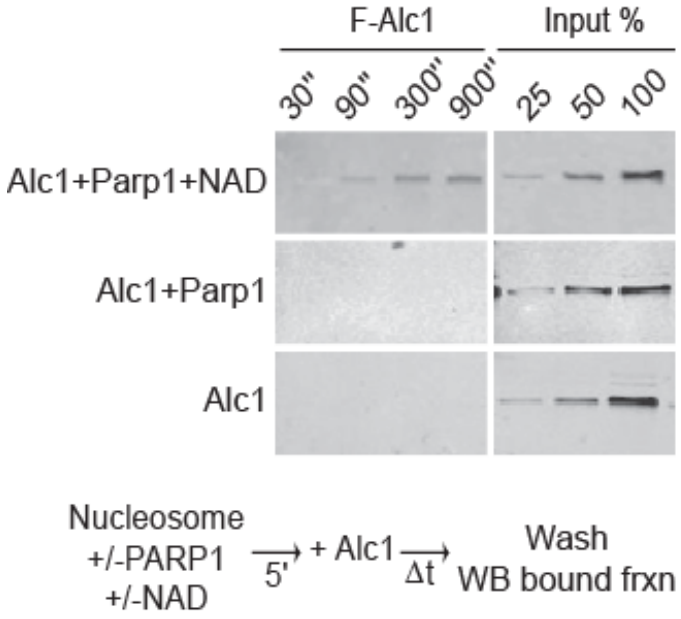

Figure 31. Alc1 binds nucleosomes in a Parp- and NAD- dependent manner. Mononucleosomes reconstituted with HeLa cell histones on biotinylated DNA and immobilized on streptavidin beads were incubated for the indicated times with recombinant F-Alc1, with or without Parp1 and NAD. Bound fractions were analyzed by anti-Flag western blotting.

First, we set up *in vitro* assays to test Alc1's ability to bind mononucleosomes formed on biotinylated DNA and immobilized on streptavidin beads (Fig. 31). In these assays we first incubated Parp1, in the presence or absence of NAD, with immobilized

nucleosomes for five minutes. Subsequently, we added F-Alc1 to the reaction mixtures, and continued the incubation for varying periods of time. The immobilized templates were then washed multiple times, and the remaining bound fractions were examined through western analysis. Interestingly, we found that in the presence, but not in the absence, of Parp1 and NAD, Alc1 was rapidly recruited to nucleosomes and remained bound after extensive washing (Fig. 31). In addition, we found that F-Alc1 (D723A) fails to be recruited to nucleosomes in the presence of Parp1 and NAD, suggesting that proper recruitment of Alc1 to chromatin requires both PARylation and an intact macrodomain capable of binding PAR (Fig. 32).

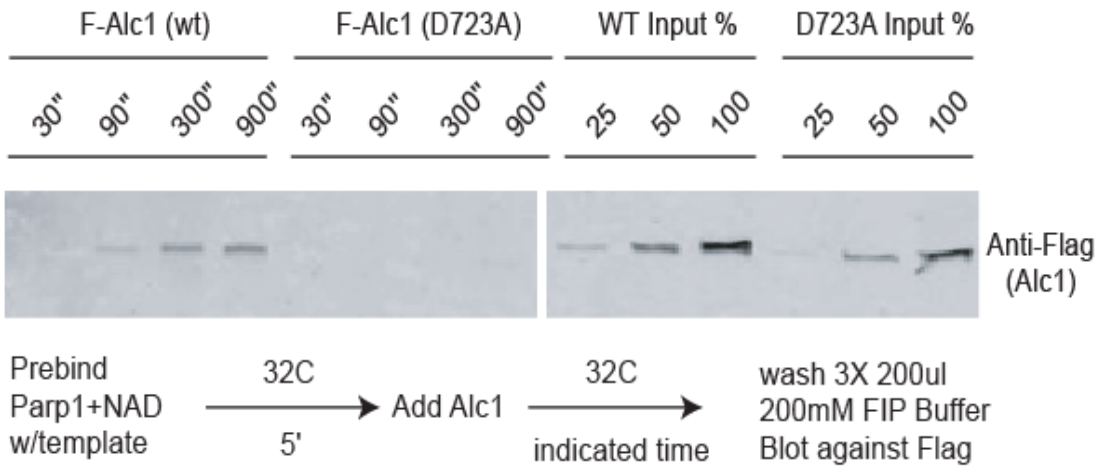

Figure 32. An intact macrodomain is required for recruitment of Alc1 to nucleosomes *in vitro* via Parp1. Using the biotinylated nucleosomal template as described before we compared F-Alc1 (wt) and F-Alc1 (D723A) ability to bind chromatin in the presence of Parp1 and NAD. Antibodies recognizing FLAG were used to monitor presence or absence of F-Alc1.

To determine whether Alc1's association with chromatin *in vivo* might be mediated through binding to chromatin associated, PARylated Parp1, we developed Hek293/FRT cell lines expressing F-Alc1 (wt), F-Alc1 (E175Q), and F-Alc1 (D723A). F-Alc1 was then purified from nuclear extracts prepared from each cell line and eluates were examined by western analysis and MudPIT mass spectrometry.

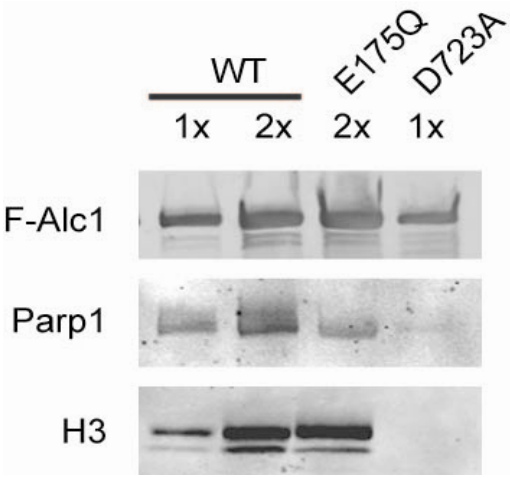

Figure 33. Alc1 protein-protein interactions are mediated through PAR binding via the macrodomain. Whole cell lysates from HEK 293/FRT cells expressing wild-type or mutant F-Alc1 were immunoprecipitated with anti-FLAG (M2) agarose. Precipitated proteins were analyzed by western blotting.

As expected we found that F-Alc1 (wt) and F-Alc1 (E175Q) associated with both Parp1 and histones, however, F-Alc1 (D723A) exhibited greatly reduced association with Parp1 or histone proteins (Fig.33). Furthermore, many of the proteins known to interact with Parp1 (DNA-PK, Ku70, Ku80 etc.) that were detected by MudPIT in the F-Alc1 (wt) eluates were not detected in F-Alc1 (D723A) eluates (Table 2). Thus, an intact Alc1 macrodomain mediates interaction of Alc1 with Parp1 *in vivo*, and this observation is

consistent with the model that PAR binding is required for recruitment of the Alc1 ATPase to chromatin.

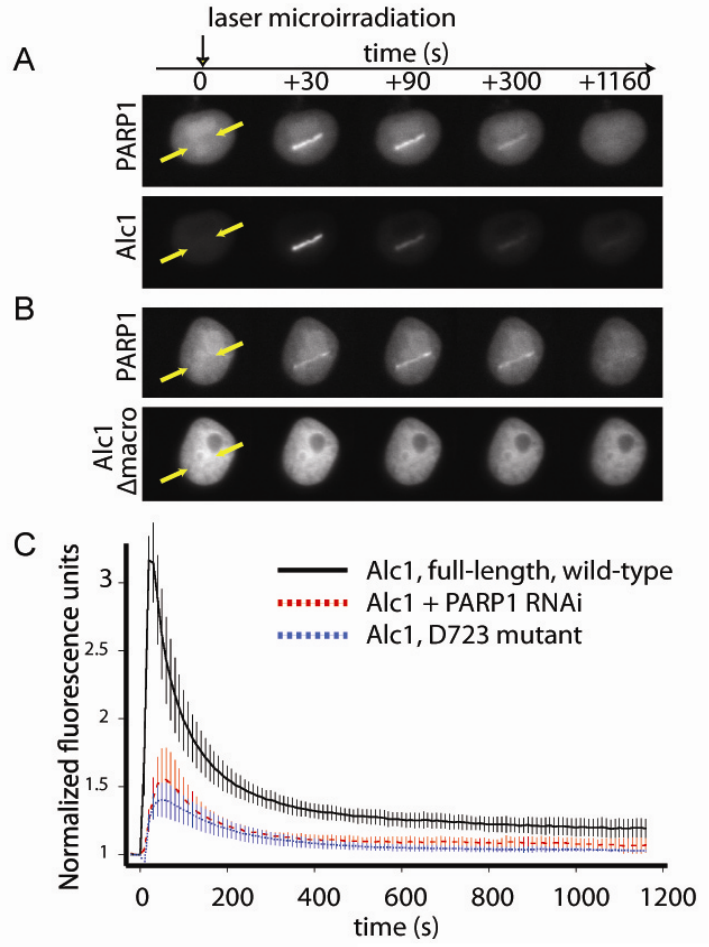

Figure 34. Targeted recruitment of Alc1 *in vivo* depends on its macrodomain and on PARP1 activity. Microirradiated HeLa cells were imaged for recruitment of EYFP-Alc1 wild type or EYFP-Alc1Δmacrodomain (Δmacro) and PARP1-mCherry. (A) Recruitment of EYFP-Alc1 and PARP1-mCherry to site of micro-irradiation (between arrows). (B) Loss of Alc1's macrodomain abrogates PARylation-induced recruitment of Alc1 to chromatin. The background in Alc1 images is lower because the integration time of the CCD camera was lowered to allow accurate quantitation of the recruitment kinetics. (C) Kinetics of recruitment ($n \geq 6$) to micro-irradiated sites of wild-type (black) and D723A macrodomain mutant (blue) Alc1, or recruitment of wild-type Alc1 after Parp1 knockdown (red).

Next, we established a collaboration with Gyula Timinzsky and Andreas Ladurner to determine whether Alc1 is recruited to locally-induced PARylation sites in living cells. To test this model, full-length Alc1 cDNA was fused to EYFP, and a pulsed-laser was used to micro-irradiate a small section of DNA in a human cell nucleus. The laser rapidly induces a highly localized region of DNA damage that recruits and enzymatically activates cellular PARP1 [292-293]. Parp1 and Alc1 are recruited rapidly to the micro-irradiated region. Alc1 and Parp1 fluorescence appears within seconds, and most is lost from the irradiated site within 10 minutes (Fig. 34 A and C). In further experiments, our collaborators observed that deletion of the macrodomain results in a complete loss of recruitment to the micro-irradiated region (Fig. 34B), while the macrodomain point mutant Alc1(D723A), which exhibits greatly reduced PAR binding *in vitro*, also exhibits reduced recruitment to the micro-irradiated region (Fig. 34C). Consistent with data indicating that the Alc1 macrodomain alone binds PAR *in vitro*, they observe that the Alc1 macrodomain is recruited to sites of microirradiation in cells (*data not shown*).

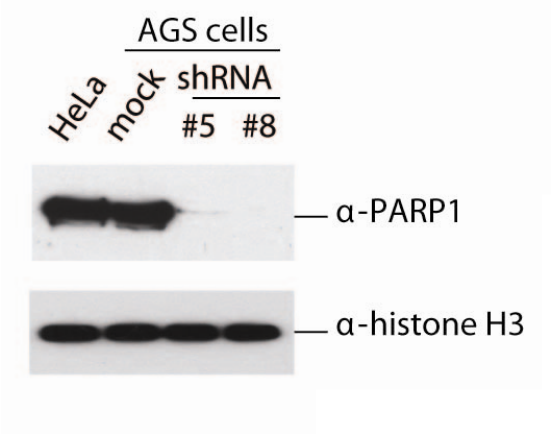

Figure 35. Parp1 is efficiently knocked down. Anti-Parp1 and histone H3 western blots of lysates from Hela cells or from AGS cells expressing two different shRNAs targeting *PARP1* or a nontargeting shRNA (mock).

Arguing that Alc1 recruitment requires the presence of Parp1 protein and PAR synthesis, they also observed a substantial reduction in Alc1 recruitment when endogenous Parp1 was knocked down using short-hairpin-mediated RNAi (Fig. 34C, Fig. 35) or in the presence of the Parp inhibitor PJ34 (Fig. 36). Thus, Parp1 and Alc1 are co-recruited to irradiation-induced sites of localized PAR synthesis in living cells, and Alc1 association with chromatin *in vivo* depends on an intact macrodomain.

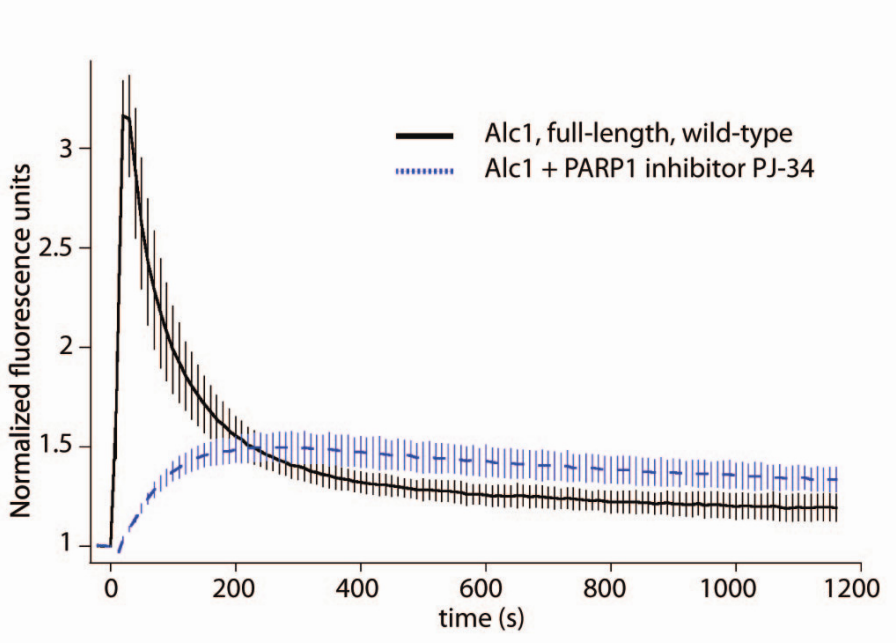

Figure 36. The effect of PARP inhibitor PJ-34 on Alc1 recruitment kinetics. (A) Real-time recruitment kinetics ($n \geq 6$) of wild-type Alc1 at the site of laser micro-irradiation in HeLa cells in the presence (blue dashed line) and absence (black line) of the PARP inhibitor PJ-34.

Chapter III. Biological studies of Alc1

Alc1 conservation amongst species

When we began our studies on Alc1, bioinformatic analysis and homology searches across the currently sequenced genomes suggested Alc1 was highly conserved within higher eukaryotes as it could be found in mammals such as chimpanzees (*P. troglodytes*), mice (*M.musculus*), and rats (*R. norvegicus*), and other vertebrates with sequenced genomes such as chicken (*G.gallus*) and zebrafish (*D. rerio*), Among vertebrate species, Alc1 was found to be conserved with 39% identity and 83% consensus with respect to amino acid sequence (see Fig.37 and appendix 1). With Alc1's strong conservation in higher eukaryotes, and in particular vertebrates, we thought perhaps Alc1 plays a fundamental role required for vertebrate development and therefore elected to pursue studies into possible Alc1 functions.

Figure 37. Conservation of Alc1 ATPase domain across species. Alc1 is highly conserved amongst higher plants and animal species. Weblogo analysis depicting Alc1 conservation across vertebrate species.

With advances in technologies the complete sequencing of an organism's genome has become much simpler, resulting in an exponential growth of annotated genomes in recent years. With the increase of genomes annotated it is now known that Alc1 is evolutionarily conserved beyond vertebrate species, as homologues of Alc1 can be found in non-vertebrate metazoans such as sea anemone, and sea urchin. Furthermore, Alc1 homologues can be found within higher plants such as Arabidopsis and rice. Yet, unlike other similar ATPases within the Snf2, Iswi, and Chd subfamilies, the Alc1 ATPase is not found within lower eukaryotes such as fruit fly (*D.melanogaster*), worm (*C.elegans*), or yeast (*S. cerevesiae* or *S. pombe*). The apparent lack of a clear Alc1 orthologue in lower eukaryotes is particularly interesting, and highly puzzling, given that both higher animals and higher plants encode for *ALC1*. Possible explanations for the perplexing evolution of Alc1 includes 1) evolutionary convergent events that combined two formerly separate gene products together in higher eukaryotes and higher plants, or 2) evolutionary divergent events that lead to the split of a single functional gene product into two functional gene products in lower eukaryotes. When looking at the evolution of Alc1 from this perspective it is easiest to focus on the pairing of functional domains. Alc1 contains both a functional SNF2 ATPase and a macrodomain that have been genetically and functionally linked, as Alc1 is the only protein known to contain both domains. The case for divergent evolution leading to the production of two gene products acting in concert as a functional orthologue is entirely possible as lower eukaryotes such as fly, worm, and yeast do have proteins that are primarily composed of only a macrodomain. Until now there has not been a functional link between proteins encoding for macrodomains and proteins encoding for ATPases. It will be interesting if future studies

show proteins carrying these two domains can functionally interact within lower eukaryotes, and perhaps contribute to the same biological processes as Alc1.

Studies on Alc1 expression during zebrafish development

Because *ALC1* is specifically present in vertebrates, we speculated it might play a role in vertebrate development. Zebrafish (*Danio Rerio*) provides a great model for studying the potential function of Alc1 in vertebrate development for several reasons. Most early developmental processes can be studied within the first 24 hrs post fertilization in zebrafish. The zebrafish developing embryo is transparent. In addition, the zebrafish embryo develops external to the mother, making observation extremely easy, and a single mating of zebrafish produces a large number of embryo per clutch (>200), allowing for easier statistical analysis and greater confidence in observations. Last, and most importantly, the expression of zebrafish genes can be readily manipulated through microinjection of embryo with morpholinos, which can block gene expression by interfering with either RNA splicing or protein translation.

Zebrafish early development can be broken up chronologically into different periods including: Cleavage period (0.7-2.2 hpf), Blastula Period (2.2 -5.25 hpf), Gastrula Period (5.25-10 hpf), Segmentation period (10-24 hpf), Pharyngula Period (24-48 hpf), Hatching period (48-72 hpf). After fertilization the cleavage period begins and cells in the animal pole or blastomere divide rapidly and synchronously and symmetrically every 15 minutes. After 7 successive divisions there are 128 cells and the embryo moves into the blastula period (Fig. 38). During this period the blastodisc begins to look "ball-like" and is perched on top of the yolk cell. Early in the blastula period cell divisions are still synchronized and occur every 15 minutes but become less synchronized around the time the midblastula transition begins (~512 cells). During the midblastula transition (MBT)

cell cycles lengthen gradually, cells become motile, and a global wave of transcription ensues. After the onset of the MBT, the marginal tier of blastomeres (blastocyst cells closest to the yolk cell), undergo a collapse, releasing their cytoplasm and nuclei into the adjoining yolk cell. This process leads to the formation of the yolk syncytial layer (YSL). The YSL is an extraembryonic tissue and makes no contribution to the embryo, it does, however, play a nutritive role and is now thought to be a major motor for the process of epiboly. Epiboly begins at the end of the Blastula Period, during Dome stage (Fig. 38C), as both the YSL deep within the blastodisc begins to dome towards the animal pole.

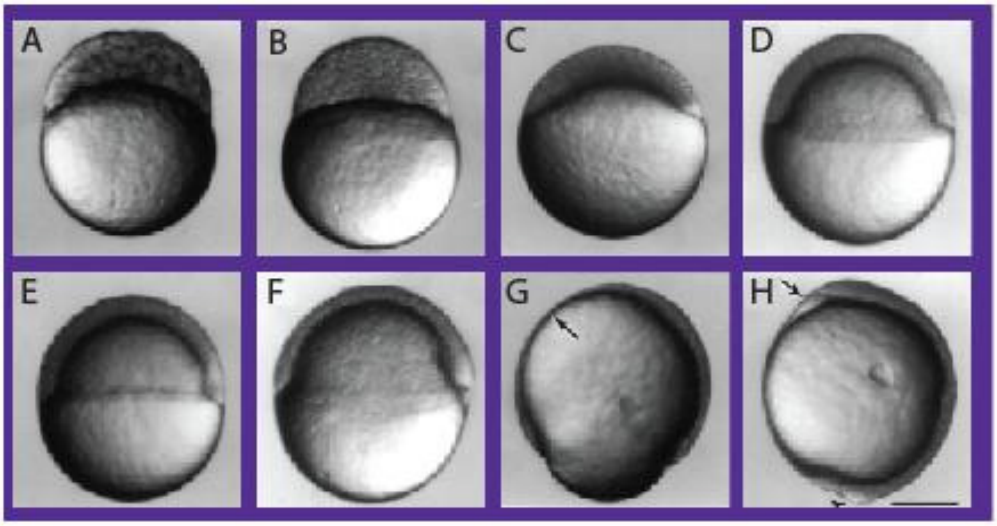

Figure 38. Highlighted developmental stages during the Blastula and Gastrula periods. Left side views with anterior up and dorsal to the left. A) 256-cell stage (2.5 h). B) High stage (3.3 h). C) Dome stage (4.3 h). D) 50%-epiboly stage (5.25 h). E) Germ ring stage (5.7 h). F) Shield stage (6 h). The embryonic shield, marking the dorsal side is visible as a thickening of the germ ring to the left. G) 75%-epiboly stage (8 h). H) Tail Bud stage (10 h). The arrow shows the polster (anterior end of embryo), and the arrowhead shows the tail bud. A distinctive region just ventral to the tail bud (i.e. just to the left in this view) shows where the yolk disappears as epiboly ends. Scale bar: 250 µm. Figure adapted from Kimmel et al. (1955) [294]

During epiboly the blastodisc and YSL are thinned and spread uniformly over the yolk cell to produce the blastoderm, in a process akin to pulling a knitted ski cap over your head. When the embryo reaches 50% epiboly (that is, the blastoderm covers 50% of the yolk cell) (Fig. 38D), the Blastula Period ends, and the process of gastrulation begins. The Gastrula Period in zebrafish occurs during a time period spanning approximately from 5.25 hpf to 10 hpf, during which the process of epiboly continues, and the complex morphogenetic cell movements of involution, convergence, and extension occur, producing the primary germ layers and the embryonic axis. Five and a half hours post fertilization, cells near the blastoderm margin move under each other, or involute, forming a germ ring consisting of two cell layers, the epiblast and hypoblast, that encompass the entire blastoderm (Fig. 38E). Within the next half hour, coordinated movements of cells produce a local thickening on one side of the animal pole, near the germ ring, forming a structure called the shield (Fig. 38F). At this time it is finally possible to distinguish both the dorsal-ventral axis as well as the anterior-posterior axis. During the next four hours, continued convergent and extension of cells within the animal pole produce the neural plate, brain, and notochord rudiments along with a prominent tail bud (Fig. 38H). After the Gastrulation Period is complete, the Segmentation Period (10 1/3-24h), commences and development of somites occur leading to the rudiments of many primary organs including the optic vesicle, brain neuromeres, and pronepheros. Subsequent to completion of segmentation, the Pharyngula Period (24-48h) begins, and the heart begins beating, fins develop, and embryos become motile within the confines of the chorion (or transparent egg like shell).

In our initial experiments with zebrafish, we studied the temporal patterns of *ALC1* expression during early development. By setting up timed matings between wild-type (AB strain) zebrafish we were able to collect embryos at various developmental stages (Fig. 39A&B). These included the 8 to 16 cell stage, 128 cell stage, high stage, dome stage, 50% epiboly, 24 hours post fertilization, and 2.5 days post fertilization. RNA was trizol extracted twice from the corresponding staged embryos and further purified with an RNeasy column. After reverse transcription reaction reactions to generate cDNA libraries, real-time qPCR analysis was performed to detect overall levels of *ALC1* expressed. To control for sample to sample variation, *ALC1* levels were normalized to levels of *ODC* (Ornithine Deoxycarboxylase), a housekeeping gene that is expressed at fairly constant levels throughout zebrafish development. We observed that *ALC1* expression levels were highest within the first 4 hours post fertilization and dramatically tailed off as the embryo proceeded through gastrulation and later stages of development (50% epiboly-2.5dpf) (Fig. 39A).

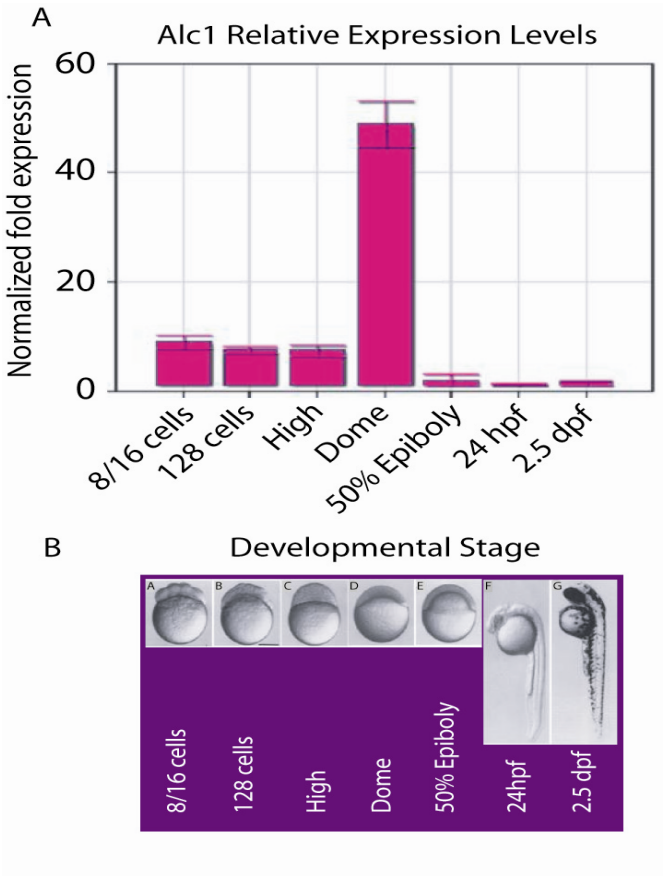

Figure 39. ALC1 expression in zebrafish during early embryogenesis. Timed matings were set up and wild-type (AB) embryos were collected at various early developmental stages. Levels of *ALC1* mRNA were detected with quantitative real-time PCR and normalized to expression of *ODC* (Ornithine Decarboxylase), a housekeeping gene commonly used within the field.

Perhaps the most striking observation made was the vast increase in *ALC1* expression seen during Dome stage (approximately 4.25 hpf) (Fig. 39A). While many genes are upregulated during early embryogenesis during the mid-blastula transition, the increase in expression of *ALC1* is not due to the rapid burst of transcription that takes place during MBT, as levels of *ALC1* mRNA at high stage would certainly be more pronounced as well. While we cannot rule out the role of maternally inherited *ALC1*, this observation suggests that Alc1 protein derived from zygotic expression is not required for

the first wave of transcription associated with the mid-blastula transition. The peak expression of *ALC1* during the Dome stage coincides with the beginning of epiboly and precedes the onset of gastrulation, and therefore suggests that Alc1 could be involved in the processes of gastrulation. During gastrulation the coordinated morphogenetic cell movements of involution, convergence and extension to produce the primary germ layers and to orient the embryonic axis. These primary germ layers ultimately lead to the formation of specific tissues and organs. To determine if *ALC1* exhibited a germ layer or tissue specific expression pattern we performed whole mount *in situ* hybridization on staged wild-type (AB strain) zebrafish embryo (Fig. 40).

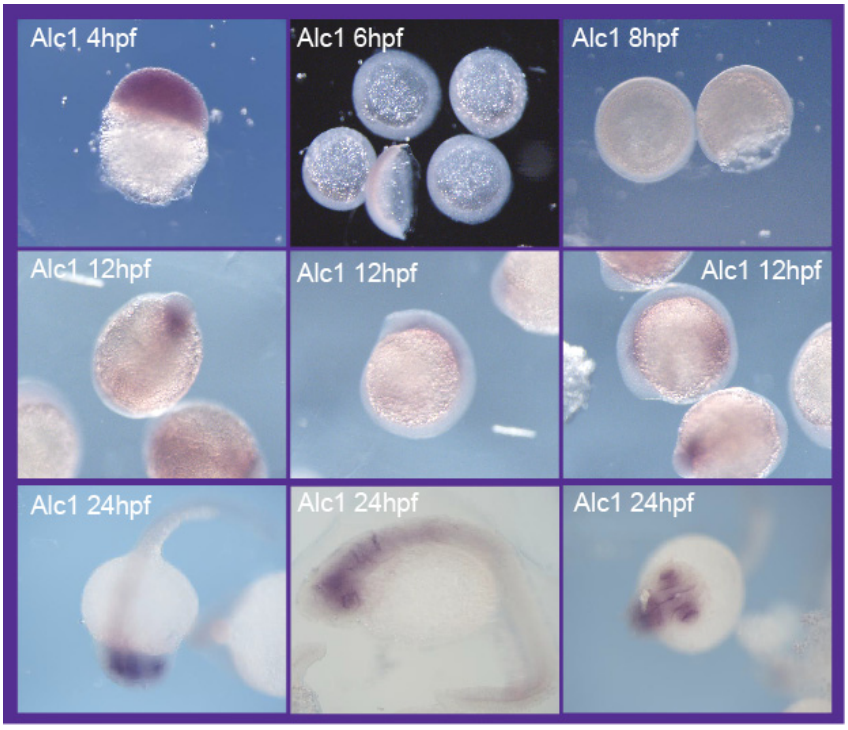

Figure 40. ALC1 mRNA expression pattern during early zebrafish development. Whole mount *In situ* hybridization was performed with probes designed against Alc1 (Chd1L) on zebrafish of different developmental stages. *ALC1* expression pattern can be visualized by purple staining. During earlier stages (4 and 6hpf) we found it particularly difficult to maintain the integrity of the yolk, through the stringent high salt/higher temperature hybridizations, therefore only animal poles are depicted in some cases.

The results of our *in situ* hybridization studies reinforce our real-time qPCR findings, as we found that Alc1 (Chd1L) was highly expressed during Dome stage (~4hpf) (Fig. 40). Interestingly, this expression is not specific to the YSL during Dome stage as *in situ* analysis of embryo suggests a more ubiquitous expression within the blastodisc. Much like the qPCR analysis suggested *ALC1* expression is absent at 6 hours post fertilization, and is not detected by *in situ* analysis until later during segmentation and pharyngula periods (12 and 24hpf). Fascinatingly, *in situ* analysis of embryo 24hpf suggests *ALC1* expression within the anterior central nervous system opening the possibility that Alc1 may have some role in proper nervous system development.

ALC1 and its potential role in early development

Due to the intriguing expression pattern of *ALC1* during early development of the zebrafish, we asked whether Alc1 plays a role in proper organismal development. To determine if loss of Alc1 protein affects proper development of zebrafish we designed anti-sense morpholinos oligonucleotides against *ALC1* mRNA. Morpholinos are highly-stable synthetic oligonucleotides around 25 bp in length that bind complementary sequences of RNA through standard nucleic acid base pairing. Unlike other antisense genetic manipulation technologies (e.g. siRNA and shRNA), morpholinos do not act by inducing the degradation of the target mRNA. Instead, they act sterically to hinder protein access to the targeted pre mRNA sequence. We designed morpholinos to affect Alc1 protein expression in two ways: 1) by targeting the 5' untranslated region near the start codon to block translation or 2) by targeting exon-intron junctions to interfere with pre-mRNA processing events through prevention of splice directing small nuclear ribonucleoprotein (snRNP) complex formation (Fig. 41). The use of morpholinos as such not only would allow us to decrease the overall level of Alc1 protein, but also could in principle give us the opportunity to alter which exons are included in the mature mRNA.

Zebrafish ALC1 is annotated by Ensembl (ENSDART00000020505) to be 3977 bp long and encode for a protein that includes 1026 residues. This annotation suggests that zebrafish Alc1 is slightly longer than the rest of the vertebrate Alc1 (most being around 900 residues) as it includes an extended c-terminal region. Zebrafish *ALC1* is predicted to include 26 exons and have the same two functional domains, the ATPase domain (SMART: DEAD-like_N and DNA/RNA_helicase C) and the macrodomain

(SMART: A1pp). The ATPase domain is spread over a large portion of the protein and spans exons 2-13, while the macrodomain is found within the c-terminal portion and spans exons 18-22.

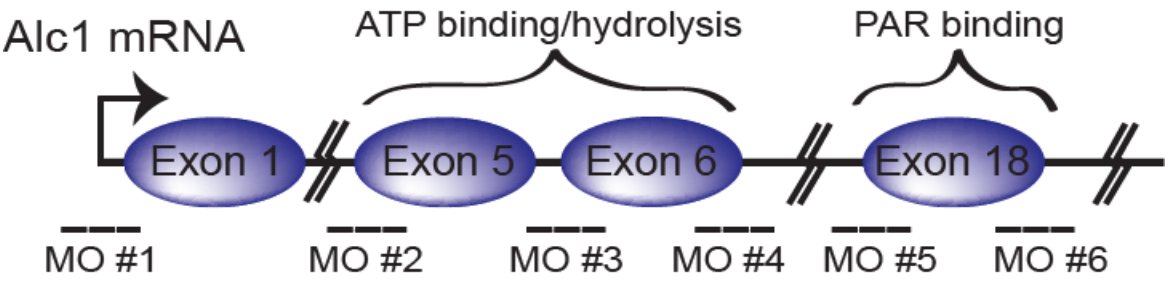

Figure 41. Morpholino design strategy to investigate role of Alc1 in early zebrafish development. Anti-sense morpholinos were developed to target zebrafish *ALC1* mRNA. A translation blocking morpholino (MO#1), along with morpholinos targeted to interfere with correct splicing of the ATPase region (MO#2-4). and macrodomain (MO#5&6) were used to investigate Alc1 function during early development.

Along with designing a translational blocking morpholino (MO#1), we decided to selectively target the exons that encode ATPase and PAR binding motifs. Normally, by targeting the junction at the 5' end of an exon with morpholinos one can cause this exon to be spliced out, while targeting the 3' end of an exon can cause inclusion of downstream intron sequence within the mature mRNA. While splice-blocking morpholinos can at times be very effective, many times splicing machinery can minimalize the splicing defects by using downstream cryptic splice-sites, therefore it is optimal to try several morpholinos directed at an exon of interest to increase chances of success. To potentially disrupt the ATPase domain we designed morpholinos that are

anti-sense to the intron 4:exon 5 junction (MO#2), the intron 5:exon6 junction(MO#3), and the exon 6:intron 6 junction (MO#4). To potentially disrupt the PAR binding motif we designed morpholinos against both exon-intron junctions for exon 18 (MO#5 and MO#6).

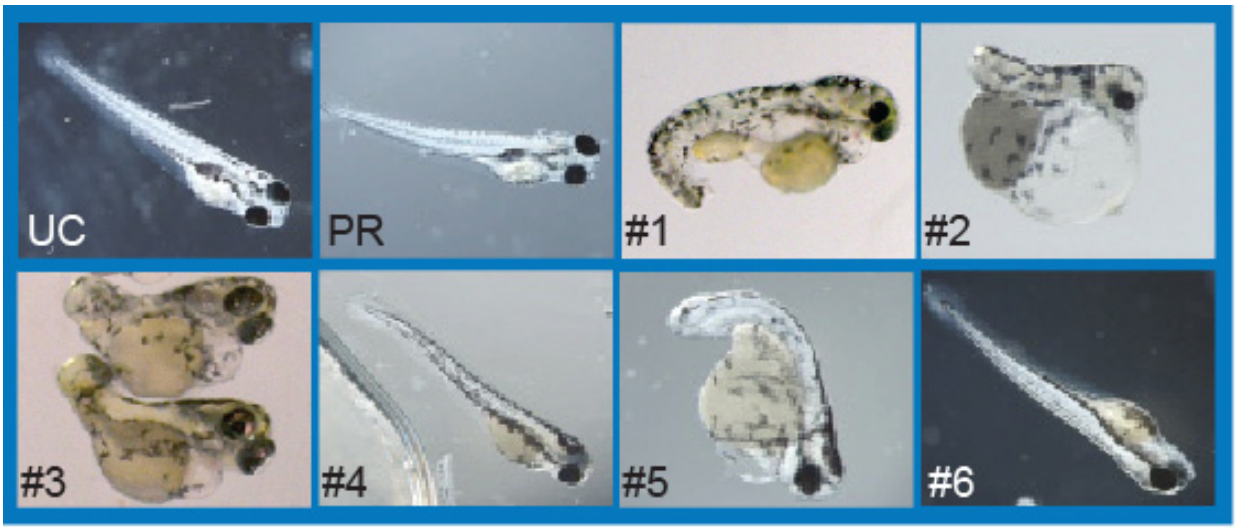

Figure 42. Phenotypes associated with disruption of Alc1 gene expression by morpholinos. Representative photos of embryos 3.5 days post fertilization: UC= uninjected control, PR= phenol red only, 1= MO#1(translation blocker), MO#2-4(splice blocker against DEAD box), MO#5-6 (Splice blocker against PAR binding motif). Where indicated ~7.5 ng of morpholinos were injected with phenol red as a visual aid.

Using a microinjection apparatus we injected embryos with morpholinos into the yolk cell near the animal-vegetal margin prior to 16-cell stage and preferably within the first two divisions. Injections were performed with haste as it has been shown that after the 16-cell stage morpholinos are no longer able to be efficiently transported from the yolk cell into the developing blastomere. Initial results with injection of a moderate amount of morpholinos (7.5 ng) suggested that the translation blocking morpholino

MO#1 could contribute to a phenotypic effect not seen with either uninjected control fish from the same clutch (batch of embryo from a single mating), fish injected with phenol red only (a non-toxic dye used for visual aid during injection) (Fig. 42), or non-targeting control morpholino (data not shown). Interestingly, while phenotypically distinct from uninjected control, we found that most of the embryos injected with translation blocker were able to survive to at least 5 days post fertilization regardless of the amount of morpholino injected (Fig. 43), at which time animal use regulations required us to euthanize the animals. Gross phenotypic analysis of embryos injected with the translational blocking morpholino suggested that decreased levels of Alc1 contribute to defects in the overall organization of the organism. The most common phenotypes noted are shortened anterior-posterior axis and curled tail appearance, both of which could be a result of decreased dorsalization or increased ventralization. Morevover, further observations suggest there may be a cranio-facial defect as well as many of the morphants have eyes that are shifted anterior and ventral, and some appear also to lack a functional jaw.

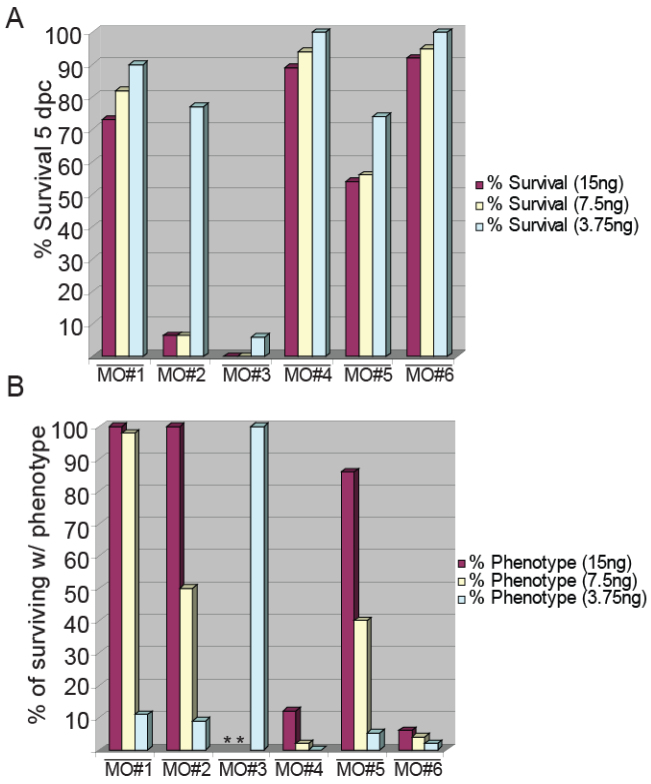

Figure 43. Statististics of morpholino effect on zebrafish early development. Titrated amounts of each morpholino (15ng, 7.5ng, or 3.75ng) were injected into recently fertilized eggs ($n \geq 100$, for each morpholino titration) and embryo were allowed to develop. At 5 dpf embryo survival rates were determined (those with a beating heart were considered alive) and surviving fish were phenotypically scored. For each titration uninjected controls were performed and survival scores were normally greater than 98% with phenotypes rarely noted.

Our observations suggest that embryos injected with splice-blocking morpholinos directed at exons required for ATPase activity (MO#2 and MO#3), exhibited more robust developmental problems than embryos injected with MO#1 (Fig. 42 and 43). When using larger amounts of morpholinos (15ng and 7.5ng) we noticed that most embryo did not survive past 24 hrs much less 5 days. Interestingly, embryos injected with MO#2 and MO#3 exhibited severe delays in development, particularly during the onset of epiboly and gastrulation, and most failed to successfully progress through epiboly and remained

stalled around Dome stage. Using smaller amounts of these morpholinos (typically ~3.5ng, and >1ng in case of MO#2, data not shown) increased the survival of animals through 5 days and allowed for the progression of embryo through epiboly and gastrulation. Interestingly, gross phenotypic analysis of the surviving embryos suggests that most exhibited phenotypes much like those of embryos injected with the translation blocking morphants (MO#1), including the shortened anterior-posterior axis and craniofacial abnormalities (Fig. 42). In addition to these phenotypes, embryos injected with splice-blocking morpholinos also exhibited large amounts of edema, a symptom that is commonly noted within morphants but that can be due specifically to defects within the circulatory system.

Analysis of embryos injected with a morpholino (MO#5) directed against the exon contributing to the PAR binding macrodomain had effects strikingly similar to the translation blocker phenotype (Fig. 42). The majority of these morphants, regardless of the amount of morpholino amount used, were able to progress through early development and survive to 5 days post fertilization (Fig. 43). While the percentage of surviving morphants (MO#5) exhibiting phenotypes was slightly less than the translation blocker (Fig. 43), the common phenotypes noted with the translation blocker such as curling tail, shortened A-P axis, and edema we observed as well. Interestingly, we found that morpholinos directed against the 3' end of exons (MO#4 and MO#6) were largely ineffective at producing defects that were noticeable with gross phenotypical analysis. While these morpholinos did not produce a noticeable developmental defect, the absence of phenotype helped to reassure that phenotypes noted with the other morpholinos were

most likely not due to non-antisense "off target" effects, due simply to injection of morpholino.

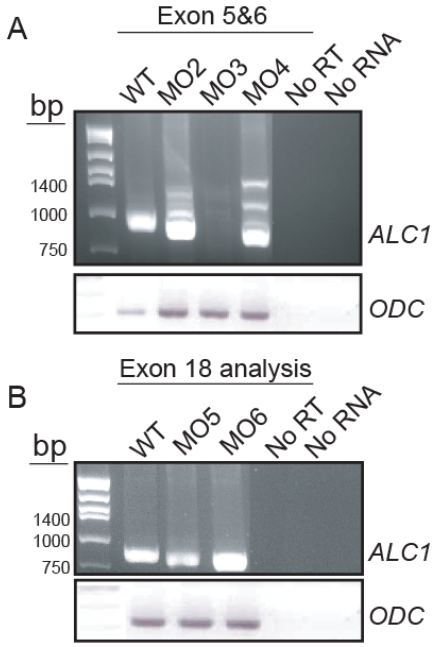

Figure 44. Verification of morpholino effects on correct splicing of ALC1 mRNA. Using primers flanking exons targeted by splice-blocking morpholinos we can qualitatively assess the effects of morpholinos on proper maturation of mRNA.

To confirm that the splice-blocking morpholinos we designed were targeting *ALC1* mRNA we injected 20 embryos with each morpholino as noted earlier. We collected embryos at 24 hours, homogenized them, and double Trizol extracted the RNA. Subsequent to purification, the RNA was then reverse transcribed using oligo-dT primers to amplify poly-adenylated transcripts. Primers were designed to amplify the region surrounding the targeted exons. By amplification of a region encompassing exon 1 through exon 8 we were able to analyze effects on mature mRNA transcripts by morpholinos 2, 3, and 4 (Fig. 44A). Using this method we observed that injection of

morpholinos 2 and 4 resulted in shortened transcripts when compared to uninjected control (Fig. 44A). This suggested that morpholinos had effectively altered the transcript by complete of partial exclusion of exons, normally included within the mature transcript. Interestingly, injection of embryos with MO#3 resulted in complete loss of transcript, as signal was non-detectable after amplification with flanking primers (and primers specific to other regions within transcript, data not shown). It is formally possible that morpholino #3 destabilizes the transcript to such an extent that it is lost through RNA quality control mechanism(s) such as nonsense mediated decay. Primers flanking exon 18 were also used to analyze the effects of MO#5 and MO#6 on transcript maturation. Our observations suggest that morpholinos 5 and 6 also alter the transcript length in comparison to wild-type, suggesting that proper maturation of the mRNA has been disrupted. Taken together these results suggest that our splice-blocking morpholinos are correctly targeting the *ALC1* transcript.

To determine if the gross phenotypic effects observed in developing embryos upon morpholino injection is due to the loss or alteration of Alc1, and not simply due to off-target effects, we set up rescue experiments. As the observed phenotypic effects of morpholino injections are pleiotropic and can vary from slight to extreme, we set up a phenotypic scoring method based on several factors before attempting the rescue experiments (Fig. 45). Class 1 fish, when compared to their uninjected controls, may exhibit mild cardiac edema, have a slightly shortened anterior-posterior axis, may have smaller eyes, but are otherwise largely normal. Class 2 fish have the same shortened A-P axis as class 1 fish but also exhibit tail curling. Class 3 fish may have an extremely

shortened A-P axis, more robust tail curling, and craniofacial defects. Class 4 fish exhibit vast tissue disorganization (Fig. 45).

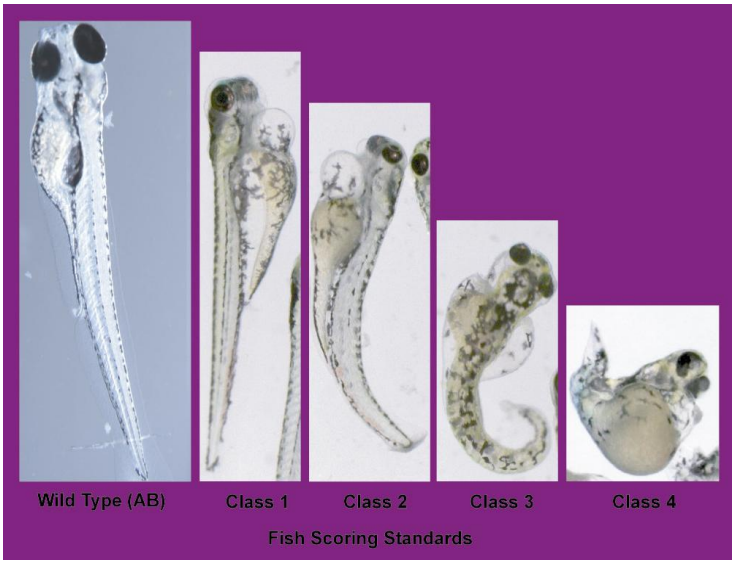

Figure 45. Standards set for scoring of zebrafish phenotypes. Injection of morpholinos targeting Alc1 produce pleiotropic phenotypes. In order to assay for partial rescue, morphants were phenotypically scored based on a variety of attributes including anterior-posterior axis length, cardiac edema, tail curling, and craniofacial defects.

From earlier experiments we knew that injection of lower amounts of the translation blocking morpholino (MO#1) consistently elicited "milder" phenotypes at lower amounts injected, which were very similar to the phenotypes associated with exon 18 splice-blocking morphants (Fig. 43&44). We also knew that embryos were particularly sensitive to MO#3, the splice-blocking morpholino directed against Alc1 ATPase domain, as most fish did not survive, and those that did had more extreme phenotypes (Fig. 43&44). Because of the range in phenotypes produced by injection of these two morpholinos and the differences in their mechanisms of action (MO#1 being a translation blocker and MO#3 being a splice blocker) we decided to focus our rescue

experiments on these two morphants. In these rescue experiments we co-injected small amounts (50pg) of either human or zebrafish *ALC1* full length transcript with 7.5 ng of MO#1 or 3.0ng of MO#3 into greater than 100 embryo each for analysis of rescue capability. We had to use small amounts of mRNA because we found through titration of mRNAs that larger amounts themselves can contribute to gross phenotypic effects as well (data not shown). When injected with morpholino #3, less than 10% of embryos survived to 5 days post fertilization, and, as observed in earlier experiments, most the embryos did not proceed through epiboly (Fig. 43&46). The co-injection of either human or zebrafish full length transcripts with MO#3 did not noticeably alter the total number of embryos that survived to 5 dpf or the number of embryos that properly proceeded through epiboly and gastrulation (data not shown); however, it did noticeably alter the range of phenotypes found in embryo that were able to survive. The vast majority of fish that survived the injection of only MO#3 (>80%) had severe phenotypes and were scored as class 3 or 4 (Fig. 45&46). Meanwhile, with the co-injection of human or zebrafish *ALC1* mRNA, we observed reduced numbers of fish scored as class 3 or 4 (~50% with human mRNA co-injection, and 20% with zebrafish co-injection) (Fig. 45&46).

Co-injection of *ALC1* mRNA with the translation blocking morpholino did not drastically affect the survival rates of animals. This is perhaps not surprising as fish were much less sensitive to the effects of the translation blocking morpholino and greater than 85% survived upon injection of MO#1 only (Fig. 46). Upon coinjection of human *ALC1* mRNA and MO#1, we did observe an increase in the number of fish scored as class 1 and a decrease in fish scored as class 3 or 4. We observed that zebrafish *ALC1* full length

transcript was unable to rescue phenotypes produced from injection of translation blocking morpholino #1. This was to be expected though as the morpholino should still be able to hybridize and target any zebrafish full-length transcript introduced, rendering it incapable of being translated. Taken together these findings suggested to us: 1) that human Alc1 can functionally compensate for a loss of zebrafish Alc1; 2) extreme differences noted among the phenotypic effects observed with MO#1 and MO#3 may reflect loss of protein versus possible dominant negative effects (will discuss later); and 3) phenotypic consequences of morpholino injections are likely the result of direct effects on Alc1 protein rather than off-target effects.

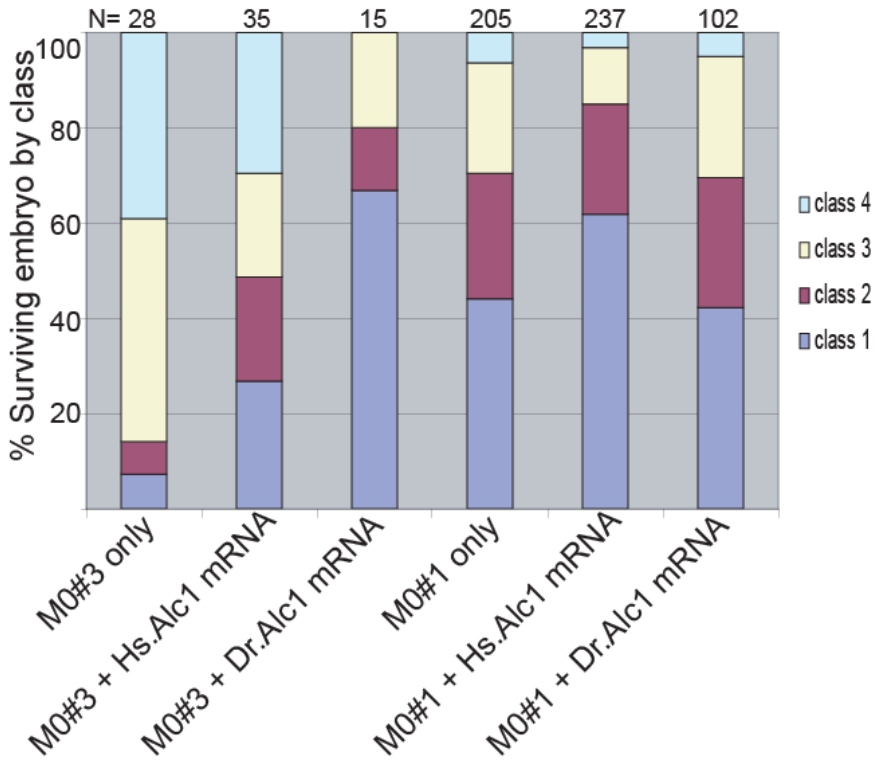

Figure 46. Partial rescue of morpholino induced phenotypes with co-injection of *ALC1* mRNA. Embryo were injected with translation-blocking (MO#1, 7.5 ng) or splice-blocking morpholino (MO#3, 3 ng) in the presence or absence of Dr.*ALC1* mRNA (50pg) or Hs.*ALC1* mRNA (50pg). N= number of fish phenotypically scored at 5 days

Chapter IV. Discussion

Alc1 Enzymology: Mechanism and Implications

Our biochemical analysis of Alc1 has provided many advances in the understanding of how Alc1 might contribute to the regulation of nuclear processes. By using a straightforward approach to characterize Alc1 through its functional domains, we have made key advances that not only serve to provide insight regarding Alc1 mechanism and function, but also serve to functionally link the fields of chromatin remodeling and the NAD^+ metabolism. Our studies were initiated through the MudPIT analysis of Alc1 immunoprecipitated from HEK293/FRT nuclear extracts. Eluates were found to contain a sizeable amount of Parp1, proteins previously reported to interact with Parp1 (e.g. Parp2, Ku70, Ku80, DNA-PK) (Table 2), and an abundance of histone proteins (visible by silver-stain, see Fig. 18), suggesting that Alc1 worked in the context of chromatin. These associated proteins, along with the fact Alc1 contained a macrodomain, prompted us to investigate the nature of this Alc1-Parp1 connection. Prior to our studies on Alc1, little was known about macrodomains or how they contributed to functions of proteins containing them. There were isolated reports suggesting that macrodomains could serve as binding modules for NAD^+ metabolites (including ADPR, PAR, and *O*-acetyl-ADP-ribose), but exactly how these binding events might contribute to the control of nuclear functions remained to be determined [279, 281, 295]. Using *in vitro* PAR binding experiments we found that Alc1, much like the histone variant macroH2A1.1[284] , binds poly(ADP-ribose), and this binding is mediated by a functional macrodomain (Fig. 20). While this finding in itself was exciting, the question still remained…how does PAR

binding mediated by its c-terminal macrodomain of Alc1 contribute to possible activities of Alc1?

The first clue that Parp and NAD^+ metabolites could directly affect the enzymatic activity of Alc1 came from ATPase assays. Prior to finding that Alc1 was capable of binding PAR, we performed numerous ATPase assays under a myriad of titrated conditions with purified recombinant F-Alc1 (wt). These assays, all done in the presence of both nucleosome or DNA substrates, failed miserably to detect activity (Fig. 21). It was further perplexing that F-Alc1 preparations purified from 293FRT cells did exhibit DNA and nucleosome dependent ATPase activity(Fig. 21), but that we could not recapitulate this activity with the purified recombinant protein. Initially we simply attributed this ATPase to contaminating activities. We were convinced that: 1) we were using the wrong substrate for stimulation of ATPase activity; 2) we were somehow missing an activating cofactor; or 3) despite the presence of a Snf2-like domain, this protein had somehow lost its ATPase activity over millions of years of evolution.

After finding that Alc1 bound PAR (Fig. 20), our research endeavors were invigorated with fresh ideas and new hypotheses. With new found enthusiasm we set up the same tired ATPase assay we had done previously so many times with purified recombinant F-Alc1 (wt) and oligonucleosomes, but this time we fortified the assay with purified recombinant Parp1 and NAD^+ as well. To our amazement, we found a robust stimulation of F-Alc1 (wt) ATPase activity (Fig. 22A and B) upon addition of both Parp1 and NAD^+ suggesting that a PARylation reaction catalyzed by Parp1 was stimulatory. Furthermore, this ATPase activity was dependent on PAR binding as F-Alc1(D723A)

lacked detectable ATPase activity (Fig. 23). Subsequent to finding that Alc1 exhibited ATPase activity in the presence of Parp, and NAD^+, and a suitable substrate (ie. oligonucleosomes or DNA), we were interested in exploring the nature of this ATPase activity. How might Alc1 ATPase be contributing to nuclear processes, and through what mechanisms might these contributions be mediated? In light of the facts that 1) many of the SNF2 family ATPases we had previously studied including Ino80 and SRCAP [61-62], had chromatin remodeling activities and 2) that Alc1 interacted with chromatin proteins (Table 2), we decided to investigate if Alc1 ATPase activity contributed to chromatin remodeling. Initially, we set up assays with classic gel shift-based nucleosome sliding assays with ^{32}P labeled-mononucleosomes that had been assembled by the salt dilution method with octamers donated from purified HeLa oligonucleosomes. To our dismay, we quickly found out that this method of assay was unsuitable to characterize chromatin remodeling via Alc1. After running the assay we found that in the presence of Parp1 and NAD, Alc1 would supershift the labeled nucleosomes despite the presence of 500-fold competitor oligonucleosomes and DNA. While this finding was disheartening, as we now needed to find a different assay to address the question whether Alc1 drives/catalyzes chromatin remodeling, useful information regarding Alc1 recruitment to chromatin was extracted from this finding, much of which we will discuss later.

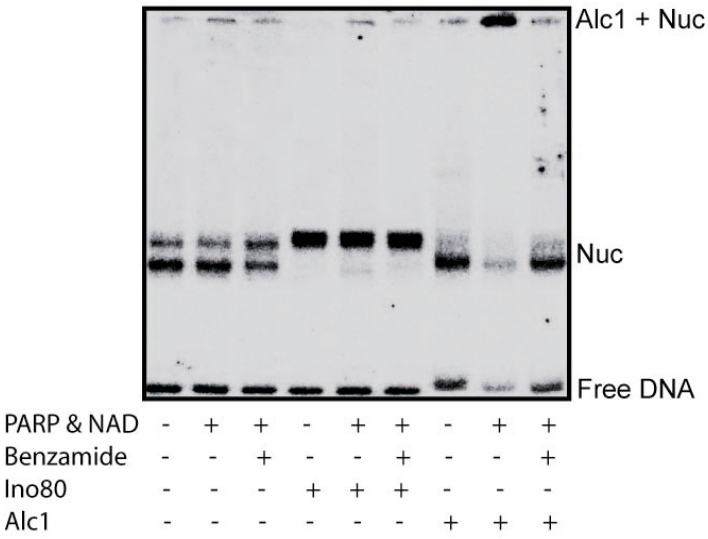

Figure 47. Alc1 supershifts labeled nucleosomes and DNA in the presence of Parp1 and NAD. ATPases (Alc1 and Ino80) were incubated with radiolabeled nucleosomes in the presence or absence of Parp and NAD. After incubation 500x competitor, both CT-DNA and oligonucleosomes, were added and reaction was allowed to incubate for 30 minutes. After final incubation the reactions were seperated on native gels and exposed to phosphorimager.

After shifting to restriction enzyme accessibility based assays for detection of nucleosome remodeling we quickly found that, Alc1 exhibited not only ATPase but also chromatin remodeling activity in the presence of Parp1 and NAD (Fig. 25). Moreover, these remodeling events were also dependent on an intact macrodomain capable of binding PAR (Fig.25). Providing more mechanistic insight, we found that Alc1 uses the force generated by its ATPase to catalyze the directional movement, or sliding, of nucleosomes on linear DNA (Fig. 26). While we cannot completely rule out the possibility that Alc1 may use other mechanisms of chromatin remodeling *in vivo*, such as octamer ejection or histone dimer ejection, our *in vitro* observations suggest that Alc1 may remodel chromatin through a simple nucleosome sliding mechanism.

It was evident Alc1 was a chromatin remodeler but the mechanism by which Parp1 and its cofactor NAD^+ stimulated Alc1 activity remained outstanding. While it was conceivable that modification of Alc1 by Parp1 could contribute to Alc1 enzymatic activation, we were unable to detect PARylation of Alc1 in our reactions (Fig. 29). Furthermore, western analysis with antibodies directed against Alc1 revealed a homogenous population of protein migrating as a single band at the predicted mass of 105 kDa (Fig. 29). Given that PARylation is extremely bulky and heterogenous, the PARylation of Alc1 would likely produce a slower migrating, heterogenous "smear" of a signal, much like what is observed with antibodies directed against Parp1. Nonetheless, western analysis of our *in vitro* reactions could not rule out the possibility that Parp1 may be mono(ADP-ribosyl)ating Alc1, as the antibodies we used specifically recognized PAR ($ADPR_n$, $n \geq 2$), and mono(ADP-ribosyl)ation of Alc1 by Parp1 would be unlikely to produce a noticeable change in Alc1 migration with SDS-PAGE analysis. To rule out the possibility that modification of Alc1 was required for stimulation of chromatin remodeling, we set up order of addition experiments using the potent Parp1 inhibitor benzamide. Our observation that Alc1 can still remodel nucleosomes, when added subsequent to benzamide, suggested that the critical PARylation event required for stimulation of chromatin remodeling was occurring prior to Alc1 addition, and further suggested that modification of Alc1 by Parp1 is not required for enzymatic activation (Fig.30)

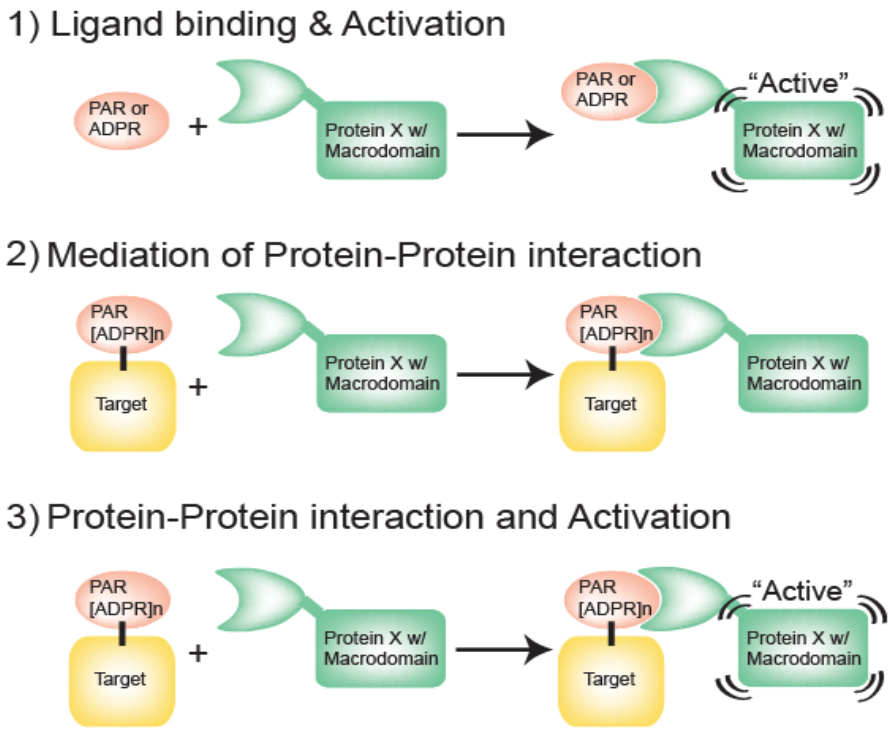

Figure 48. Binding of NAD+ metabolites and possible contributions to protein function. The binding of NAD metabolites by macrodomain containing proteins could contribute to protein function in a variety of ways including; 1) allosteric activation upon ligand (free ADPR or PAR) binding; 2) mediation of protein-protein interactions through post-translational modification of target protein; and 3) mediation of protein-protein interaction and simultaneous allosteric activation of macrodomain containing protein.

The observation that F-Alc1 (D723A) lacked detectable remodeling activity suggested that a PAR binding event, facilitated by the c-terminal macrodomain, was somehow mediating Parp1 and NAD stimulation of Alc1 enzymatic activities. Macrodomain binding of NAD^+ metabolites could conceivably contribute to protein function in a variety of ways including; 1) by regulating protein function through allosteric mechanisms upon ligand binding; 2) by mediating protein-protein interactions through post-translational modification of the target protein; or 3) by mediating protein-protein interactions and simultaneously affecting the macrodomain containing protein function through allosteric mechanisms (Fig. 48). By setting up ATPase assays that

include various NAD^+ metabolites, including monomeric ADP-ribose (mADPR) and free PAR in the presence of oligonucleosome substrate, we were able to test if the stimulation of Alc1 could be explained by an allosteric mechanism brought about by a simple ligand-binding event (Fig. 48). We found that addition of mADPR and free PAR failed to stimulate Alc1 ATPase activity in the presence of a nucleosomal substrate. These observations suggested that the mechanism of Alc1 activation could not be explained by activation though a simple ligand-binding event (Table 3).

Prior to our studies, macrodomains had been shown to interact with NAD^+ metabolites, but there was no evidence suggesting that macrodomains could mediate protein-protein interactions. As Parp1 is the most extensively PARylated protein within the cell, we thought it might be likely interaction of Alc1 with Parp1 could be mediated through PAR binding. Supporting the idea that Alc1-Parp1 interaction is mediated through PAR binding, we found that immunopurification of F-Alc1(D723A) from HEK293/FRT cells, unlike immunopurifications of F-Alc1 (wt) and F-Alc1 (E175Q), resulted in eluates lacking Parp1 via MudPIT and western analysis (Table 2, Fig. 33). Furthermore, interactions with many proteins previously reported to interact with Parp1 were abrogated upon mutation of the macrodomain. These findings argued that macrodomain mediated PAR binding might facilitate the bulk of Alc1 interactions within the nucleus.

How could these macrodomain mediated protein-protein interactions contribute to the nuclear function of Alc1? A couple lines of evidence had suggested that PAR binding could mediate Alc1 interaction with chromatin. First, we had observed that F-

Alc1 (D723A), unlike F-Alc1 (wt), did not co-immunopurify with histone proteins (Fig.33), and, our initial attempts at a classic nucleosome sliding assays suggested the enzymatic activity of Parp1, as evidenced by the observed supershift, may have enhanced the affinity of Alc1 for a mononucleosome (Fig. 47). The idea that PARylation could be contributing to the targeted recruitment of Alc1 was particularly interesting. Many chromatin remodeling enzymes that carry Snf2-like ATPases similar to Alc1 also contain themselves, or physically interact with other proteins, that have functional post-translational binding modules such as bromo-, chromo-, and tudor domains (Fig. 3). If the PAR binding macrodomain was contributing to the targeted recruitment of Alc1, it would suggest a novel mechanism of targeted chromatin remodeling. To test this hypothesis *in vitro* we used an immobilized nucleosome binding assay. Our suspicions were quickly confirmed; Alc1 exhibited Parp1- and NAD^+-dependent binding of chromatin (Fig. 31). Furthermore, this enhanced nucleosome binding observed in the presence of Parp1 and NAD^+ also required an intact macrodomain as F-Alc1 (D723A), confirming our hypothesis, at least *in vitro* that the PAR binding macrodomain was required for proper recruitment of Alc1 to chromatin (Fig. 32). *In vivo* studies performed by our collaborators in the Ladurner lab further confirmed our recruitment hypothesis. By using microirradiation of nuclei to produce damaged DNA, leading to accumulation of concentrated amounts of PAR at damage sites, our collaborators found that Alc1 was rapidly recruited to sites of induced DNA damage in a Parp1 dependent manner (Fig. 34). Moreover, this recruitment was observed to be dependent on an intact macrodomain (Fig 34), and, on PAR synthesis, as treatment of cells with PJ-34, a potent enzymatic inhibitor of Parp1, abrogates Alc1 recruitment (Fig. 36).

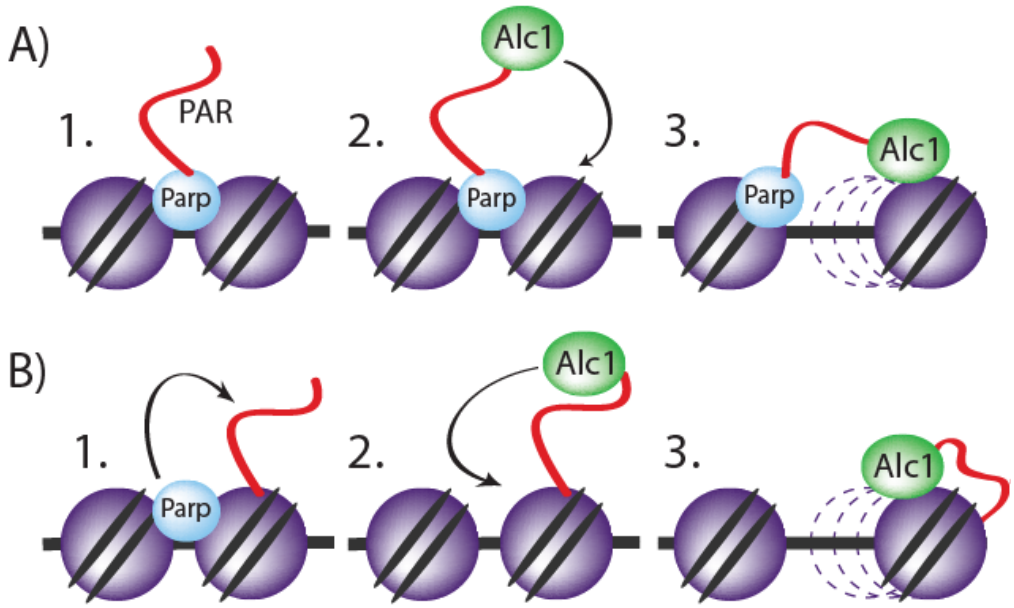

Figure 49. Model depicting Alc1 targeted recruitment and chromatin remodeling a) Automodifciation of Parp1 leads to recruitment and activation of Alc1. b) trans-poly(ADP-ribosyl)ation of histone proteins (and other chromatin proteins such as transcription factors, not depicted) leads to recruitment and activation of Alc1

Targeted recruitment of Alc1 to chromatin through PARylation events could concievably occur in a couple of ways including: A) Allosteric activation of Parp1 leading to automodification and recruitment of Alc1; and/or B) trans-poly(ADP-ribosyl)ation of local chromatin proteins by Parp1 after allosteric activation resulting in recruitment and activation of Alc1. While our data does not rule out that transmodification of local chromatin proteins (such as histones, see Fig. 49b) could contribute to recruitment and activation of Alc1 *in vivo*; the observation that Alc1

ATPase is activated *in vitro* in the presence of DNA, suggests that modification of histones is not required.

Whether the apparent PARylation-dependent increase in the affinity of Alc1 for nucleosomes is sufficient to explain the activation of its ATPase and nucleosome remodeling activities in the presence of Parp1 and NAD remains to be determined. It is tempting to speculate that upon the macrodomain binding of PAR, an allosteric activation of the enzyme could occur through induction of conformational changes. The region of Alc1 that connects the ATPase domain to macrodomain is predicted to be composed of coiled-coil domains and highly unstructured loop-like regions. Perhaps this unstructured region could facilitate communication between the macrodomain and ATPase domain upon PAR binding events. Interestingly, our collaborators in the Ladurner lab, have found that when Alc1 (Δmacro, 1-666aa), is coexpressed with the isolated Alc1 macrodomain (macro only, 666-897aa), they can be copurified together (*personal communication from Andreas Ladurner*). In light of this finding, it would be interesting to determine if the copurified products would exhibit PARylation dependent recruitment kinetics similar to Alc1 (wt), and, if so, exhibit enzymatic activities similar to Alc1 (wt). Such future experiments could help to determine whether binding of a PARylated species, most likely Parp1 itself, to the Alc1 macrodomain results in allosteric activation of the enzyme.

Alc1: biological roles under normal physiological circumstances

Currently there is little known about the function of Alc1 under normal physiological circumstances. Most of the work done by other labs has focused on detailing the role of Alc1 in the context of hepatocellular carcinoma [269], and the vast majority of our work has focused on detailing the enzymatic mechanisms of Alc1[296]. Our observation that Alc1 is a Parp1- and NAD-dependent chromatin remodeler opens the possibility that Alc1 could be involved in many of the nuclear processes regulated by Parp1, including DNA damage repair and transcriptional regulation.

There are several lines of evidence that indirectly suggest a role in DNA damage repair for Alc1. First, analysis of multiple eluates from immunopurifications of F-Alc1 from HEK293/FRT cells by MudPIT mass spectrometry suggest that Alc1 associates with many proteins known to be involved in DNA damage repair pathways (e.g. DNA-PK, Ku70, Ku80, RPA2 and 3, Parp2, and XRCC1) (Table 2). Moreover, these interactions are dependent on a functional macrodomain. While it is tempting to suggest that these interactions provide a clear link to DNA damage repair roles for Alc1, it should be noted that many of these proteins (e.g. DNA-PK, Ku70, and Ku80) have reported roles in other nuclear processes such as transcriptional regulation. Therefore we cannot infer of a role for Alc1 in DNA damage solely from these results. The second line of evidence we have that indirectly suggests a role in DNA damage repair for Alc1 (although some would argue directly suggests, depending on what field their research focus resides), is colocalization of Alc1 with Parp1, and associated machinery [297] at sites of DNA damage produced by microirradiation of nuclei (Fig. 34). Interestingly, the Alc1

macrodomain by itself shows prolonged occupancy at sites of DNA damage *(personal communication, Andreas Ladurner)* and our collaborators in the Ladurner lab published work suggesting the macrodomain containing variant histone mH2A1.1 is also recruited to DNA damage with similar kinetics as full length Alc1 [284]. Furthermore, they report histone mH2A1.1 has a role in compaction of chromatin surrounding DNA damage breaks. Boulton and colleagues recently reported results attained from comet assays that suggest that increase or decrease of cellular levels of Alc1 can enhance the sensitivity of U2OS cells to various DNA damaging agents [297]. While these types of experiments are a step in the right direction, they use surrogate indicators of DNA repair and do not directly assess the role of Alc1 and other macrodomain containing proteins in DNA damage repair processes. As many chromatin remodelers have roles in both transcription and DNA damage repair, careful controls are required to rule out the possibility that secondary effects may be contributing to observed phenomena.

Nonetheless, it is tempting to speculate at the moment how Alc1 may contribute to DNA damage repair. Although the aforementioned findings do not rule out the possibility of roles in transcription contributing to an observed DNA damage repair phenotype, future studies are warranted focusing on how Alc1 modulation of chromatin contributes to DNA damage repair. Interesting phenomenological points worth addressing include; 1) kinetics of Alc1 recruitment to sites of DNA damage and; 2) proximity of Alc1 to lesions. While other SNF2-like ATPases such as Ino80 and Swr1 are reported to contribute to DNA damage repair, these chromatin remodeling complexes seem to exhibit much slower kinetics of recruitment than Alc1, reaching maximal

enrichment at DNA damage, or sites of HO exonuclease cleavage, around 4 hours post damage [65]. Through our microirradiation studies we have found Alc1 is recruited to DNA damage sites with kinetics similar to Parp1, and maximal enrichment is observed around 30 seconds with signal detectable to around 15 minutes post induction of damage. While this perceived difference in kinetics among chromatin remodelers such as Alc1 and Ino80 could be due to differences of DNA damage induction and detection, it is nonetheless striking, and yet, not completely surprising. Alc1 enjoys the benefit of being recruited to sites of DNA damage by Parp1, an enzyme known to be an early sensor of DNA damage. Ino80 recruitment to DNA damage sites is in large part dependent on a cascade of events that lead to the phosphorylation of histone H2A, a histone modification that Ino80 has been shown to specifically recognize [64]. This cascade of events, while incredibly efficient, takes nearly 30 minutes for maximal enrichment of phosphorylated H2A surrounding the site of DNA damage. The disparity in observed kinetics of recruitment suggests that if Alc1 is directly involved in DNA damage repair it is likely to play an "immediate responder" role that is much more upstream of the role Ino80 plays in repair of lesions. Differences in targeted recruitment among these remodelers may also lead to distinct populations of remodeled chromatin based on proximity to lesions. Ino80 specifically recognizes the phosphorylation of H2A, and it is recruited to a large region estimated by some to cover more than 10 kilobases of DNA flanking the lesion to be repaired. Conversely, with the findings that Parp1 binds directly to DNA ends, and upon binding automodifies itself with PAR chains reaching 400 mono-ADPR units, one might expect chromatin modulation by Alc1 to be confined to a small region (>1000 bp) from the lesion to be repaired (Fig. 50). It will be of interest to determine in the future if

chromatin remodeling enzymes such as Alc1 and Ino80 contribute to DNA repair processes through different means or perhaps through partially redundant and cooperative means. Much could be learned by looking into the kinetics of recruitment of known repair factors as well as the overall kinetics of damage repair with loss of Alc1. By characterizing possible effects on repair kinetics, it should be possible to begin determining if, when, and where, Alc1 enzymatic activity may be required.

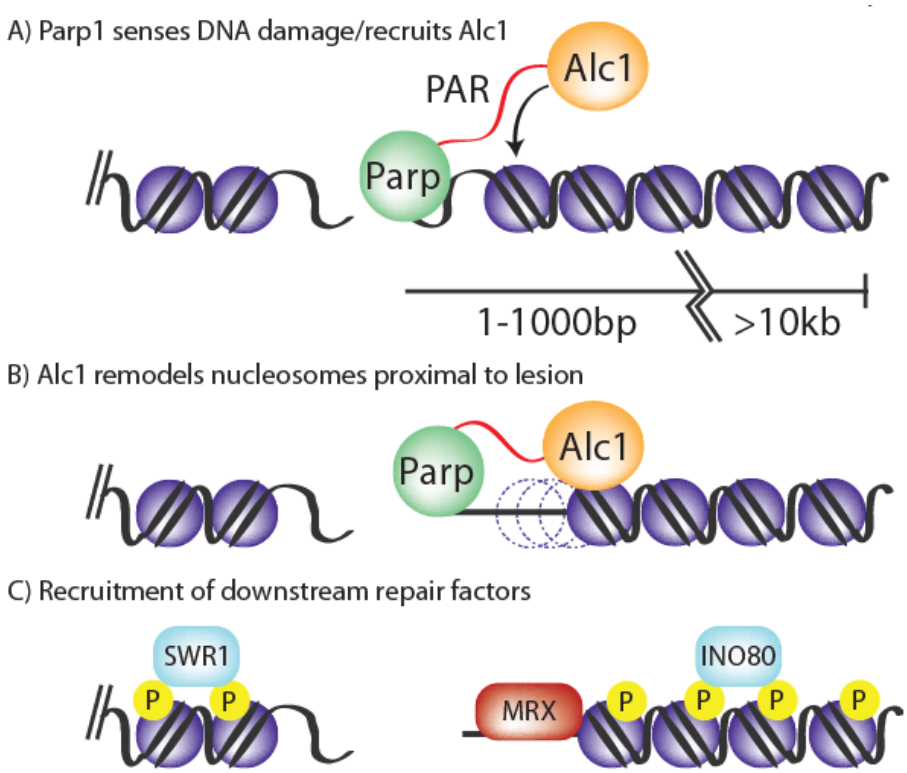

Figure 50. Speculative model depicting Alc1 role in DNA damage repair. A) Upon sensing DNA damage (both SSB and DSB), Parp1 binds to DNA ends and allosterically activates itself, leading to its automodification. Alc1 binds PAR and is recruited to site of DNA damage. B) Within 15 minutes Alc1 works to remodel local chromatin structure allowing repair machinery access to regions flanking the DNA damage lesion. C) Downstream repair factors are recruited through signalling cascade resulting in phosphorylation of histone H2A; efficient and timely recruitment results in proper repair.

In addition to possible roles in DNA damage it is also likely that Alc1 may contribute to transcriptional regulation. There are several lines of evidence suggesting that Alc1 can contribute to transcriptional regulation. Guan and colleagues, while studying the tumorogenic properties of Alc1, found that overexpression of Alc1 resulted in decreased levels of p53 and p21 transcripts and increased levels of cyclin E and cdk2 transcripts in hepatoma cells [274]. While these results could be due to indirect effects, a more recent publication by Guan and colleagues suggests that overexpressed Alc1 binds to the *ARHGEF9* promoter in hepatoma cells and stimulates increase in *ARHGEF9* transcript levels. They further suggest that overexpression of *ARHGEF9*, a gene that encodes for the Rho small GTPase Cdc42, can contribute to filapodia formation and promote epithelial-mesenchymal-transition leading to metastasis of hepatoma cells [298]. In addition to these findings, our lab has preliminary results from microarray analysis of HepG2 cell lines expressing doxycyclin-inducible shRNAs targeting *ALC1*. Our data suggests that, upon induction of *ALC1* shRNA, a small set of transcripts (<200 total) are reproducibly affected. Taken together, these findings suggest that it is likely that Alc1 may contribute to transcriptional regulation of at least some genes.

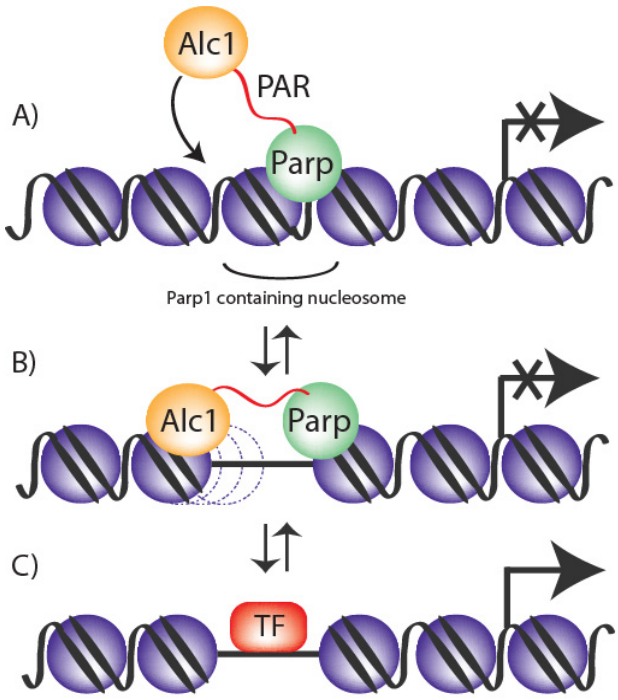

Figure 51. Speculative model depicting Alc1 role in transcriptional regulation. a) Parp1, integrated within a nucleosome, PARylates itself and effectively recruits Alc1. b) Upon recruitment Alc1 disrupts local chromatin structure through utilization of its ATPase. c) with local chromatin structure disrupted DNA binding transcription factors can now localize to promoter and initiation of transcription can ensue.

Unlike Alc1, the role of Parp1 in transcription is fairly well characterized, as Parp1 has been shown by many groups to act as a transcriptional coregulator for a large set of genes. Interestingly, Kraus and colleagues have recently found that Parp1 binds to the promoters of many actively transcribed genes in MCF7 cells [253, 256]. At these promoters, Parp1 is thought to bind in the linker DNA between nucleosomes, much like histone H1, forming a Parp1-containing nucleosome. Interestingly, this localization of Parp1 at actively trancribed genes anti-correlates with the presence of histone H1, suggesting that these proteins may play opposing roles. Kraus and colleagues have found the majority of genes regulated robustly by Parp1 were enriched for roles in either stress

response or metabolic functions. A cooperative role for Alc1 and Parp1 in transcriptional regulation, to date, has not been established within the literature; however, we are currently investigating the possibility. In chromatin immunoprecipitation experiments using anti-Alc1 polyclonal antibodies that we have generated, we have obtained preliminary evidence that in both MCF7 and HepG2 cells, Alc1 bound to several promoters shown by the Kraus lab to be bound and regulated by Parp1. While these results are preliminary, and further investigation is certainly required, it is tempting to speculate that Parp1 could integrate at promoters, and possibly contribute to trancriptional regulation by recruiting Alc1. Upon recruitment, Alc1 could utilize its nucleosome remodeling capabilities to change the local chromatin structure to promote either activation or repression of the target gene (Fig. 51).

Many transcription factors have been reported to be targeted for poly(ADP-ribosyl)ation by Parp1. Therefore it is formally possible that Alc1 could be recruited to modulate chromatin through interaction with modified transcription factors. One such interesting possibility includes targeted recruitment of Alc1 to chromatin through PARylated CTCF. CTCF, a transcription factor required for the formation of insulator boundary elements, is reportedly required to be PARylated for efficient DNA binding and establishment of boundary elements [258]. A recent collaborative study from the Weng and Peterson lab, using deep-sequencing and high-density tiling arrays, suggests that the insulator binding protein CTCF positions up to 20 nucleosomes around its binding sites across the human genome[299]. They further suggest that CTCF acts as an anchoring point for positioning of the nucleosomes and that chromatin remodeling is an important

component of CTCF function. While they did not specify a chromatin remodeling enzyme responsible for the regular spacing of nucleosomes that flank CTCF, it is tempting to speculate that Alc1 may be a suitable candidate. We have not yet investigated Alc1 effects on nucleosome arrays *in vitro,* but our simple mononucleosome remodeling assays suggest that Alc1 nucleosome remodeling is more similar to Iswi than Swi/Snf, in that it moves nucleosomes from a lateral to medial position. Iswi and other enzymes such as Chd1, both known to move nucleosomes in a lateral to medial fashion, also have been shown to potentiate the regular spacing of nucleosomes on an array. Given these findings, it is easy to speculate Alc1 may also potentiate the regular spacing of nucleosomes *in vitro*, and thus could perform such a role *in vivo*, directed by an anchoring, PARylated, insulator protein such as CTCF (Fig. 52).

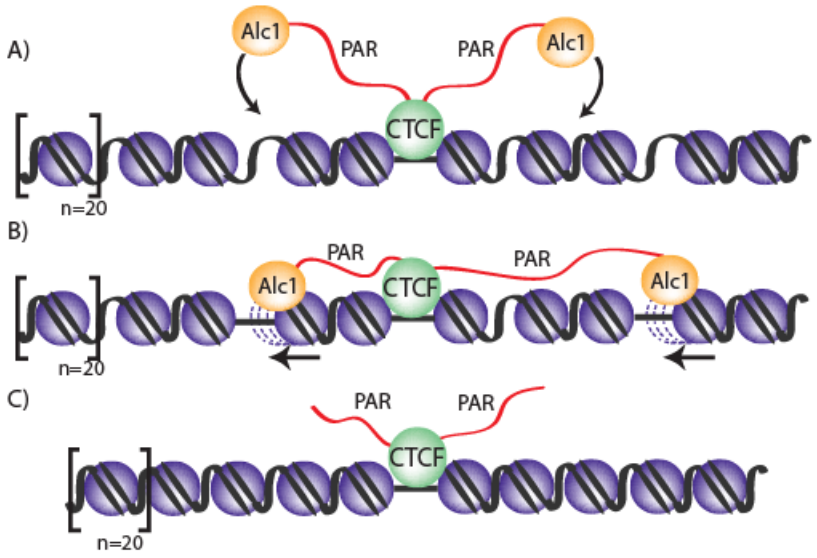

Figure 52. Model depicting possible role for Alc1 in insulator formation. a) PARylation of CTCF by Parp1 leads to recruitment of Alc1 to insulator elements b) Alc1 remodels nucleosomes creating an evenly spaced array c) CTCF maintains functional insulator

Role of Alc1 in proper organismal development

Our studies into Alc1 function in early zebrafish development suggest that Alc1 may play an important role in organismal development. Both qPCR analysis of *ALC1* transcript levels, and whole mount *in situ* analysis across early zebrafish development, suggest *ALC1* is expressed early during zebrafish embryogenesis, primarily during the blastula period prior to the onset of gastrulation (Fig. 39 and 40). Peak expression is observed upon commencement of epiboly during Dome stage. Our observations that perturbation of Alc1 protein levels and transcript splicing suggest that Alc1 is required for proper development as well (Fig. 42). While there are a few caveats with our studies using morpholinos, including the chance of off-target effects and possible induction of p53 pathway (a fairly high percentage of morpholinos induce p53 pathway non-specifically), it is important to note that we have used multiple morpholinos targeting *ALC1* in attempts to gain confidence in our observations (Fig. 41). Gross phenotypic analysis of morphants suggests that perturbations of Alc1 lead to a pleiotropic phenotype that often includes shortened anterior-posterior axis, craniofacial developmental defects, edema, and curling of the tail (Fig. 42). Our observation of partial rescue, upon co-injection of full-length human and zebrafish cDNA with morpholinos suggests that the effects noted are in fact specific to the perturbation of Alc1 protein levels (Fig. 46)

Our strategy to target different portions of the *ALC1* transcript including the ATPase domain and macrodomain yielded interesting results that may provide some clues to Alc1 biological function. Interestingly, we have found that the translation blocking morpholino (MO#1) exhibited phenotypes and survival rates most similar to the

morpholino designed to disrupt proper splicing of the macrodomain (MO#5). These findings suggest that disruption of PAR binding of Alc1 creates a similar phenotypic effect as loss of the protein. When comparing these findings with our biochemical data, these biological findings make perfect sense, as we would expect loss of PAR binding to produce an Alc1 enzyme unable to be properly targeted to chromatin, thus mimicking a null phenotype. Interestingly, we find that embryo have greatest sensitivity to morpholinos (MO#2 and MO#3) directed against exons contributing to the ATPase domain (Fig. 43). In light of these findings, it is tempting to speculate that disruption of the ATPase domain may be leading to formation of a fragment of the Alc1 protein that acts as a dominant negative. One could imagine that recruitment of Alc1, lacking a functional ATPase, to a particular target could be detrimental to cellular processes such as transcriptional regulation and/or DNA damage repair. Taken together, these observations suggest that Alc1, and its associated ATPase and PAR binding domains, are required for proper organismal development.

With such a range in phenotypes observed it is difficult to suggest that loss of Alc1 contributes to defects in a particular developmental process or pathway (Fig. 45). It is worth noting that many of these phenotypes we observe within *ALC1* morphants are also typical of embryo exhibiting problems proceeding through gastrulation. Currently, however, our lack of understanding of the basic biology of Alc1 presents problems when trying to speculate about the function of Alc1 during early zebrafish development. Could Alc1 be contributing to proper development by regulating expression of key transcription factors for particular developmental pathways? Possibly. Are the effects we observe

upon perturbation of Alc1 cell autonomous effects? We don't currently know. Could loss of Alc1 be effecting proper development through mechanisms not including transcriptional regulation? Absolutely. Although there are probably too many outstanding questions to suggest a particular role for Alc1 in development, careful observation of *ALC1* expression pattern may provide insight into biological function.

The expression patterns of *ALC1* noted within early zebrafish development is particularly interesting as it has been recently suggested, in a study published by Efroni and colleagues looking at global transcription of pluripotent embryonic stem (ES) cells , that *ALC1* exhibits markedly increased expression (~8.9 fold greater) in undifferentiated mouse ES cells compared to differentiated cells [300]. Furthermore Efroni and colleagues observe decreased proliferation of ES cells upon knockdown of *ALC1,* suggesting *ALC1* may promote proliferation of embryonic stem cells. Prior to the onset of gastrulation, the cells within the blastomere of the developing zebrafish are largely undifferentiated, much like mouse ES cells, as the formation of germ layers has not taken place. During the Blastula period the cells within the zebrafish embryo are proliferating rapidly and divide on average once every 15 minutes only slowing slightly after onset of the mid-blastula transition (MBT). MBT is accompanied by a global wave of transcription that continues throughout development. Much like cells in the developing zebrafish blastula, mouse embryonic stem cells are also proliferating at an extremely fast rate, and according to experiments using whole genome tiling arrays exhibit a hyperactive transcriptional landscape, compared to cells that have been differentiated [300]. Moreover, transcriptional hyperactivity in ES cells is apparently accompanied by

a disproportionate expression of chromatin remodeling genes such as *ALC1* [300]. *ALC1* expression is associated with high levels of proliferation, whether it be during the normal development of animals, or the onset of hepatocellular carcinoma. How Alc1 may contribute to this increase in proliferation is poorly understood at the moment, but given the observed association of Alc1 and Parp1, it is likely that these two proteins will cooperatively contribute to the high level of proliferation that occurs during early development. As Parp1 has a well established role in DNA damage repair, one mechanism by which Alc1 may contribute to increased proliferation is through the promotion of genomic stability by contributing to DNA damage repair. When cells divide at such a rapid rate, as observed during early development, it is likely that there is going to be an excessive amount of replication induced DNA damage that a chromatin remodeling enzyme such as Alc1 could help mitigate. Alternatively, as Parp1 also has well documented transcriptional roles, Alc1 could be contributing to proper organismal development through transcriptional control of genes that regulate cell cycle progression or cellular proliferation. Many of these questions regarding the basic biology of Alc1 need to be addressed prior to speculating on Alc1 function in early development. Future cell-based assays looking into Alc1 biological function will provide the groundwork, and serve as a cornerstone, on which to build a working hypothesis pertaining to the role Alc1 plays in development.

Role of Alc1 in Carcinoma Pathogenesis

Overexpression of *ALC1* is likely to contribute to pathogenesis of cancer through a cellular pathway or process, that under normal circumstances, is governed by a PARylation event. This event is also likely to include, but is not limited to, an Alc1 interaction with PARylated Parp1. Parp1 is an extremely abundant protein that is involved in many cellular processes; its interaction with Alc1 is probably not required for all processes. One could imagine that an excessive amount of Alc1 within the cell could be extremely detrimental to processes not normally involving Alc1. For instance, if Alc1 is not normally involved in DNA damage repair, the inappropriate recruitment of Alc1 to DNA damage could result in defects in repair, possibly through promoting a chromatin environment not suitable for proper repair to convene (Fig. 53). By promoting condensed chromatin flanking DNA damage, one could imagine repair factors required for the processing of DNA ends could be denied access, while alternatively promoting too open a structure flanking lesions might promote inappropriate access to other factors normally not involved in repair processes. Ultimately, by hindering DNA damage repair the rate of genetic mutations within the cell would likely increase dramatically thus resulting in further "hits" required for the transformation of the cell.

Alternatively, it is also possible that overexpression of Alc1 could contribute to pathogenesis of carcinoma through the unwarranted opening or closing of chromatin structure outside of the context of DNA damage. For instance, if Alc1 is recruited to promoters inappropriately by Parp1, it is conceivable that unwarranted changes in target gene expression could occur. This could be particularly detrimental, and likely to also

promote cellular transformation, if tumor suppressor genes, oncogenes, cell cycle regulated genes, or genes required for apoptosis are misregulated. As Guan and colleagues have already discovered a few genes within the p53 pathway that seem to be misregulated upon overexpression of *ALC1*[274], it will be interesting to investigate these genes as possible direct targets.

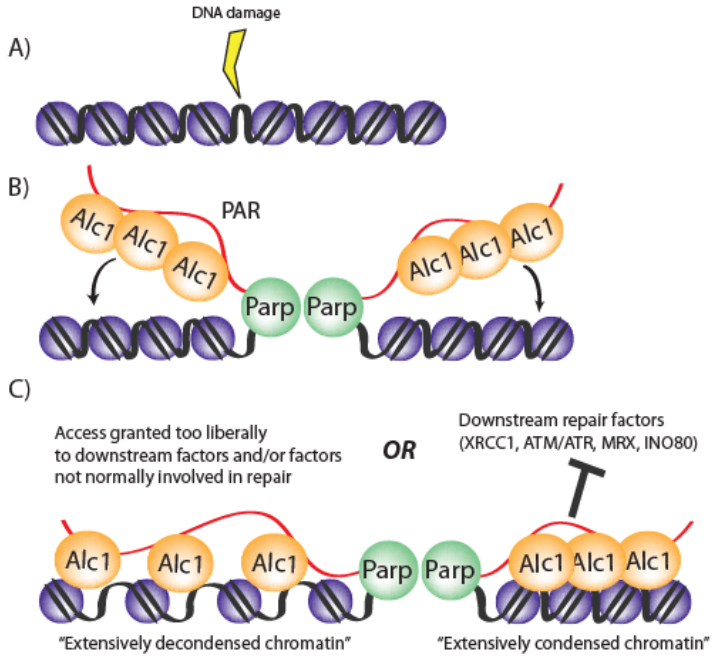

Figure 53. Effects of *ALC1* overexpression on DNA damage repair *a*) DNA damage occurs through a number of mechanisms b) Parp1 senses DNA damage, leading to the unwarranted recruitment of Alc1 to DNA damage c) Alc1 through its enzymatic activities establishes a local chromatin environment unsuitable for proper repair.

Our establishment of the functional interaction of Alc1 with Parp1 is particularly intriguing, as many small molecule inhibitors of Parp1 are now being found to be effective cancer treatment options. Parp1 is involved in single-strand break repair, and inhibiting Parp1 catalytic activity results in the accumulation of single-strand breaks.

When these breaks are encountered during DNA replication, replication forks stall and double-stranded DNA breaks occur. Under normal circumstances these double-stranded breaks are repaired through the error-free mechanism of homologous recombination. Patients without defects in homologous repair do not normally exhibit a phenotype upon treatment with Parp1 inhibitors, and interestingly, mice that are homozygous null for Parp1 show no increased rates in tumor formation. However, cells that have mutations within double-strand break repair, such as mutations in *BRCA1* or *BRCA2* genes, are particularly sensitive to inhibition of Parp1 and undergo apoptosis upon treatment. It is this selectiveness that currently makes Parp1 inhibitors such an attractive therapeutic option for patients with cancers resulting from mutations in the *BRCA* pathway. Likewise, we have shown that use of small molecule inhibitors of Parp1 can block Alc1 activity *in vitro* and *in vivo*. These findings suggest that small molecule inhibitors of Parp1 could in the immediate future, be a very attractive therapeutic option for patients suffering from hepatocellular carcinoma.

Future Directions

While our *in vitro* experiments detailing the Parp1- and NAD-dependent activation of Alc1 have provided much of the initial groundwork important for the understanding of Alc1 biology, many mechanistic questions remain to be answered regarding Parp1- and NAD- dependent activation of Alc1 ATPase. Evidence from the Kraus lab suggests that Parp1 can bind nucleosomes similarly to and in place of histone H1 [242, 253, 256]. Our studies have suggested that Alc1 binds tightly to nucleosomes in the presence of Parp1 and NAD. It is important to determine if Alc1 is a chromatin remodeler that is specific for Parp1-bound nucleosomes, and whether Parp1 must be bound to the nucleosome, in order for Alc1 to commence remodeling. To address this question *in vitro*, template binding assays similar to those described in chapter 2 can be performed and presence of Parp1 can be monitored. To further address this question *in vivo*, Alc1 could be also be immunopurified from chromatin pellet fractions subsequent to partial nuclease digestion. From this purification strategy it should be possible to not only determine if there is enrichment for Parp1-bound nucleosomes, but also determine if there is enrichment for particular chromatin marks, by following immunoprecipitations with SDS-PAGE and western analysis. In light of the findings that some SNF2 family remodelers such as SRCAP and SWR1 can catalyze the exchange of variant histones into nucleosomes, it will be interesting to test the possibility that Alc1 might facilitate the exchange of histone H1 for Parp1 or *visa versa*. To test for this enzymatic activity we could set up *in vitro* swapping assays our lab previously used to characterize the human SRCAP chromatin remodeling complexes.

There are many questions remaining that need to be addressed about Parp1 activation of Alc1 ATPase and chromatin remodeling activity. Can the enzymatic activation of Parp1, by means not including the presence of DNA or nucleosomes, also potentiate Alc1 enzymatic activation? Or put more simply, can PARylated Parp1, in the absence of DNA or nucleosomes activate Alc1. In our described *in vitro* assays we used DNA to stimulate Parp1 enzymatic activity to promote PARylation of Parp1 and ultimately potentiate Alc1 activation. This approach, while giving us much insight into cooperation of Parp1 and Alc1, does not allow for further dissection of the interaction. Parp1 can be activated in the absence of DNA, via activation through Erk2-dependent phophorylation [136]. Performing ATPase assays with pre-PARylated Parp1, acquired through Erk2-dependent activation, and Alc1, could provide much needed insight.

Further exploration of the basic properties of the nucleosome remodeling reaction catalyzed by Alc1 are also warranted. The *in vitro* chromatin remodeling assays we have used to explore Alc1 mechanism utilize mononucleosomes formed on short pieces of DNA, and therefore may resemble nucleosomes adjacent to double-strand breaks. If Alc1 contributes to Parp1-dependent transcriptional regulation, it would be predicted that Alc1 would be able to bind and remodel nucleosomes that are distant from DNA ends. Therefore, it is worth comparing the ability of Alc1 to remodel nucleosomes reconstituted, at defined places, on closed circular plasmids with various distances from DNA ends. Much like our earlier assays, we could use restriction site protection assays to monitor remodeling activity, or alternatively, utilize micrococcal nuclease digestion followed with indirect end-labeling assays.

In addition to further biochemical characterization of Alc1, we are interested in exploring potential roles of Alc1 in Parp1-dependent transcription. Kraus and colleagues have used genome-wide studies to demonstrate a genome-wide transcriptional role for Parp1 in MCF7 breast cancer cells. To study the role of Alc1 in regulating gene expression, we have generated both MCF7 and HepG2 cell lines expressing doxycyclin shRNAs targeting *ALC1*. We have found these lines, upon induction, to reduce protein levels of Alc1 by up to 80% compared to non-targeting shRNA control lines. Using these lines we are currently carrying out genome-wide expression analysis studies with Affymetrix arrays detailing the effects of Alc1 knockdown. The results of these analyses will be compared with previous studies on Parp1 by the Kraus lab, and candidate genes potentially regulated by both Parp1 and Alc1 will be further confirmed by rescue experiments using mouse *ALC1*. Further mechanistic studies into possible interdependent regulation by Alc1 and Parp1, including studies on colocalization using ChIP, can then be performed on identified target genes. Ultimately, these experiments can potentially identify genes whose transcription is regulated by Alc1, aid us in determining if Alc1 and Parp1 regulate an overlapping collection of genes, and, if so, can provide us with model genes we can use to explore whether and how Parp1 and Alc1 function together to control gene regulation in cells.

Chapter V. Materials and Methods

Biochemical studies of Alc1

Purification of Flag- Alc1. For expression in human cells, Alc1 cDNA (accession number BC001171) was cloned into pcDNA5 with an N-terminal FLAG tag and introduced into HEK293/FRT cells as described [301]. Cells were grown to 70-80% confluence. Nuclear extracts were prepared according to the method of Dignam *et al.*[286], and FLAG-Alc1 and associated proteins were purified on anti-FLAG (M2) agarose beads (Sigma) as described . Alternatively, whole cell lysates were prepared as described in Supplemental Methods, and Flag-Alc1 and associated proteins were immunopurified as described [301] except beads were washed with 0.2 M KCl. For expression in Sf21 insect cells, Flag-Alc1 was cloned into a pBacPAK8 (Clontech) derivative, and purified from lysates of infected cells as described [302].

Purification of F-Alc1 from HEK293T cell whole cell extracts. To purify F-Alc1 and associated proteins from whole cell extracts, cells were washed with phosphate-buffered saline and then lysed by resuspension in 1 ml/dish of 40 mM Hepes-NaOH (pH 7.9), 0.2 M NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 0.2% Triton X-100. The resulting suspension was incubated with rotation at 4^{o}C for 30 min and spun at 40,000 rpm for 60 min at 4^{o}C in a 70.1 Ti rotor (Beckman-Coulter). Supernatants were subjected to anti-FLAG agarose chromatography as described in Materials and Methods except that beads were washed with buffer containing 0.2 M NaCl.

Poly-(ADP)ribose binding assays. Recombinant proteins (1 pmol) were incubated for 30 min at 32°C in 15 µl of 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 [281]. Reaction mixtures were applied to nitrocellulose and washed overnight with TBS-T containing 100 mM NaCl. Bound ^{32}P-labeled PAR was detected using a Typhoon phosphoimager.

ATPase Assays. ATPase assays were performed as described [301]. Where indicated, reaction mixtures contained ~100ng (1pmol) of Flag-Alc1 (wildtype, E175Q, or D723A) from HEK293/FRT cells or SF9 cells, ~115 ng (1 pmol) Parp1 (Trevigen, Inc., Gaithersberg, MD, USA), recombinant Parg (Trevigen, Inc., Gaithersberg, MD, USA), 34 μM nicotine adenine dinucleotide, and 150 ng of mono- or oligo-nucleosomes from HeLa cells [303].

Nucleosome Remodeling Assays. Mononucleosomes were reconstituted by dilution transfer from HeLa oligonucleosomes on a ^{32}P-end labeled 216bp DNA fragment (601-lat Gal4) generated by PCR from pGEM3Z-601-Gal4 [303-304]. 1 pmol F-Alc1 from HEK293/FRT or SF9 cells was incubated at 32^{o}C for 30 min with mononucleosomes (~0.01 pmol labeled mononucleosome, ~0.25 pmol unlabeled oligonucleosomes) in 20 mM Hepes-NaOH (pH 7.9), 50 mM NaCl, 4.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM PMSF, 45 μg/ml bovine serum albumin, 10% glycerol, 0.02% Triton X-100, 0.02% NP-40, and 2 mM ATP. Where indicated, reactions contained ATPγS (2 mM), Parp1 (1 pmol), NAD (34 μM), or benzamide (2 mM). Reaction products were incubated for a further 30 min with 10 U of either HhaI or XhoI and resolved on gels containing 7% polyacrylamide (19:1 acrylamide:bis), 7 M urea, and 45 mM Tris-borate/1 mM EDTA, pH 8.3 [305].

Size exclusion chromatography. Flag-immunopurified F-Alc1 (~35 μg in 100 μl) from HEK293/FRT cells was fractionated on a Superose6 sizing column using a SMART FPLC μSeparation system. The column was equilibrated in 40 mM Hepes-NaOH (pH 7.9), 0.1 M NaCl, 0.1 mM EDTA, 10% glycerol and was eluted using the same buffer at a flow rate of 50 μl/min. 50 μl fractions were collected and analyzed on silver-stained polyacrylamide gels and western blotting prior to use in assays.

Nucleosome Binding Assay. 40 pmol of mononucleosomes were assembled on a 5'-biotinylated 601-lat Gal4 fragment, bound to 400 μl streptavidin dynabeads, washed, and resuspended in a final volume of 400 μl (100 fmol mononucleosome/μl beads). 1 pmol recombinant F-Alc1 was incubated with 100 fmol immobilized nucleosomes in 45 μl of 20mM Hepes (pH 7.9), 50mM NaCl, 0.5% NP-40, 10% Glycerol, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 1 mM ATP and 100 μg/ml bovine serum albumin. Where indicated 1 pmol of Parp1 and NAD (34 uM) were included in reaction mixtures. Beads were washed 3 times with 200 μl of 40 mM Hepes-NaOH (pH 7.9), 0.2 M NaCl, 0.2% Triton X-100, and 10% glycerol, transferred to a fresh microcentrifuge tube, and bound proteins were eluted with 3x SDS sample buffer and analyzed by western blot.

Transient transfections. Hela-Kyoto and AGS cells were grown in Hepes-buffered DMEM-Glutamax-I (Invitrogen) containing 4.5 g/L glucose and 10% FCS US/certified (Invitrogen) and supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen) and MEM-non essential amino acids (MEM NEAA, Invitrogen). AGS cells stably expressing scrambled or two different short hairpin RNAs targeting PARP1 were generated at the IVBMB (Zurich, Switzerland) using a shRNA

SIN-lentivirus approach. Wild-type and mutant ALC1 cDNAs were amplified by PCR and cloned into the Bgl II and EcoRI sites of pEYFP-C1 (Clontech) for expression of EYFP-Alc1. PARP1 cDNA was amplified by PCR and introduced into the Nhe I and Sma I sites of pmCherry-N1 for expression of Parp1-mCherry. For pulsed-laser micro-irradiation experiments AGS cells were grown without puromycin. Where indicated, 1 μM PARP inhibitor PJ-34 (Alexis) was added 30 minutes prior to laser microirradiation.

Pulsed laser microirradiation, live imaging and image analysis. Pulsed laser microirradiaton was performed through a Zeiss C-Apo 63x/1.2 water immersion objective lens on a Zeiss Axiovert 200M epifluorescence microscope equipped with a frequency tripled 355 nm Nd:YAG pulsed laser (JDS Uniphase, Grenoble, France), scanned with galvo mirrors [306] and an ORCA Hamamatsu CCD camera (Hamamatsu Photonics KK, Hamamatsu, Japan). DNA damage was induced by focusing in the nucleus an ~ 6-8 μm line target including 40-42 points with a pulse energy of 200-300 nJ for three times. Cells were imaged every 10 seconds for 20 minutes. Cells were kept at 37 ºC in a CO_2 independent HEPES based imaging medium (Invitrogen) supplemented with 20% FBS (Invitrogen), 1 mM sodium pyruvate (Sigma), 2 mM L-glutamine (Sigma), 50 U/ml penicillin, 50 μg/ml streptomycin (Sigma) in MatTek (MatTek, Ashland, MA) glass bottom dishes. Live images were registered and analyzed using ImageJ. Igor Pro (WaveMetrics) was used for analyzing and plotting the data. Cell motions were corrected using ImageJ plug-in MultiStackReg [307]. To quantify protein recruitment following laser microirradiation, data were background subtracted, normalized to pre-microirradiation and corrected for fluorescence loss: $R_{(t)}=[(I_{(t)}-I_{back(t)})/(I_{(t0)}-$

$I_{back(t0)}$)]*[($T_{(to)}$-$I_{back(t0)}$)/($T_{(t)}$-$I_{back(t)}$)], where R: recruitment, I: Intensity acquired along the laser path region, I_{back}: Background region outside the cell of interest, T: Total fluorescence within the nucleus.

Biological studies of Alc1

Expression analysis of *ALC1* across developmental stages. Embryos (n=20/time point) were harvested at the designated developmental time points and total RNA was isolated, after homogenization, using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Complimentary DNA library was prepared from 2ug of total RNA using Superscript III reverse transcriptase enzyme (Invitrogen) and an oligo(dt) primer according to the manufacturers instructions. *ALC1* transcript levels were quantitated in triplicate using quantitative real time polymerase (qPCR) reactions containing the following: [1µl (20 pmol) of each primer listed below, 5 µl cDNA from previously mentioned reverse transcriptase reactions, 6.5 µl ddH20, and 12.5 µl of SyBR Green, 25 µl total reaction volume], and were normalized against *ODC* (Ornithine Decarboxylase) transcript levels. Reactions were cycled on MyiQ thermocyclers (BioRad) according to the manufacturer's instructions. Quantitation of gene expression was done through the ΔΔCt method with final analysis completed with the aid of iQ5 software (BioRad).

Primers used for Expression analysis of *ALC1* include:

Dr. Chd1L CDS Set 3 forward primer: 5'-CAACAAGTTGTCAGGCATTCGGCT-3'

Dr. Chd1L CDS Set 3 reverse primer: 5'-AATGACCAATCCGAGGCAGATGGA-3'

Dr. ODC forward: 5′ACACTATGACGGCTTGCACCG3′

Dr. ODC reverse: 5′CCCACTGACTGCACGATCTGG3′

Whole mount *in situ* hybridization. Staged embryos were fixed 24 hours in 4% paraformaldehyde 1′ PBS (phosphate-buffered saline), hand dechorionated and dehydrated overnight in methanol at -20°C. Then the embryos were then rehydrated stepwise in methanol/PBS and finally returned to 100% PBT (1′ PBS 0.1% Tween 20). Subsequent to rehydration, embryos older than the beginning of somitogenesis were treated 10 minutes with proteinase K (10 mg/ml in PBT). The reaction was stopped by rinsing in glycine (2 mg/ml in PBT). Embryos were postfixed in 4% paraformaldehyde 1′ PBS for 20 minutes and then rinsed in PBT 5 times for 5 minutes each. After postfixing the embryos were prehybridized for 1-3 hours at 70°C in hybridization buffer [50% formamide, 5′ SSC, 50 mg/ml heparin, 500 mg/ml tRNA, 0.1% Tween 20, 9 mM citric acid]. To make probe against Dr. Alc1, full length Dr. Alc1 (Ensembl ID: ENSDARP00000022305) was cloned into pCR2.1 TOPO vector and antisense mRNA was made through transcription reactions catalyzed by either T7 or Sp6 polymerase in the presence of digoxigenen labeled nucleotide. Hybridization took place using the same buffer as during the prehybridization, with 50 ng to 100 ng of probe added overnight at 70°C. Then the embryos were washed at 70°C for 10 minutes in [75% hybridization buffer, 25% 2′ SSC], 10 minutes in [50% hybridization buffer, 50% 2′ SSC], 10 minutes in [25% hybridization mix, 75% 2′ SSC], 10 minutes in 2′ SSC, 2 times 30 minutes in 0.2′ SSC. Further washes were performed at room temperature for 5 minutes in [75% 0.2′ SSC, 25% PBT], 5 minutes in [50% 0.2′ SSC, 50% PBT], 5 minutes in [25% 0.2′ SSC, 75% PBT], 5 minutes in PBT, and then 1 hour in [PBT with 2 mg/ml BSA (bovine serum albumin), 2% lamb serum]. After washes, the embryos were incubated overnight at 4°C with the preabsorbed alkalinephosphatase-coupled anti-digoxigenin antiserum

(described in Boehringer instruction manual) at a 1/5000 dilution in a PBT buffer containing 2 mg/ml BSA, 2% lamb serum. Finally the embryos were washed 6 times for 15 minutes each in PBT at room temperature. Detection was performed in alkaline phosphatase reaction buffer described in the Boehringer instruction manual. When the color was developed, the reaction was stopped in 1x PBS.

Microinjection of Morpholinos and mRNA. Morpholino oligos were purchased from Gene Tools, Inc. (Corvallis, OR) and diluted to 25 mg/ml in 1x Danieau's buffer (58mM NaCl, 0.7mM KCl, 0.4mM MgSO4, 0.6mM Ca(NO3)2, and 5mM Hepes (pH7.5)). Subsequent dilutions were made in 0.2MKCl and 0.2% Phenol Red and injected into the yolk of 1–4 cell zebrafish embryos of the AB line, approximately 5 nL per embryo (~3.75ng, 7.0ng, or 15ng total). Animals were raised to four days post fertilization and were then subjected to tricaine euthanasia. Morpholinos were designed to block translation of Dr. Alc1 (MO#1), and the correct splicing of exons 5 and 6 within Alc1 ATPase domain (MO#2, MO#3, MO#4), along with aims to disrupt the correct splicing of exon 18 within the Alc1 macrodomain (MO#5 and MO#6). Sequences for the morpholinos are as follows:

MO#1 (Translation blocker): 5'-GACTGCTCGAAGAAATGTGGAACATC-3'
MO#2 (5'exon 5 splice blocker): 5'-GGCACATCTATTAAATATGAGAAAG-3'
MO#3 (5'exon 6 splice blocker): 5'-CCAGCTGTGGGAAGAAAAAAGTCAC-3'
MO#4 (3'exon 6 splice blocker): 5'-AAAGTGGAATTGTGTAACCTCTTTG-3'
MO#5 (5'exon 18 splice blocker): 5'-CCATCCTGCCAATGAAAGACAAAGG-3'
MO#6 (3'exon 18 splice blocker): 5'-GTATTTAGTAGAGCCGTACCAACAC-3'

Rescue experiments required the coinjection of *ALC1* mRNA with morpholinos. To make mRNA, both human and zebrafish full length cDNA were cloned into pCR2.1 TOPO vector. *In vitro* transcription reactions were set up using the Ambion mMessage Machine Kit as follows (4ul DEPC treated dH2O, 2ul 10x T7 transcription buffer, 10ul 2x rNTP Mix (containing m7GpppG cap), 2ul linearized DNA template (0.5ug/ul), 2ul T7 polymerase, 20ul total volume). After 2 hours of incubation at 37C, reactions were treated with DNase for 10min to remove template. Next, mRNA was phenol:chloroform extracted and isopropanol precipitated. After resuspension in DEPC water, transcript was analyzed on 1X MOPS/Formaldehyde 1% agarose gel with ethidium bromide. Titrations of transcript were injected without the presence of morpholino to assess phenotype induction. It was found that 50 pg of transcript did not cause noticeable phenotype and therefore was a suitable amount for rescue trials. For rescue experiments, either human or zebrafish *ALC1* mRNA was coinjected with either MO#1 or MO#3, and phenotypes were assessed at 24 hours post fertilization and 5 days post fertilization.

Verification of Morpholino effect on *ALC1* transcript. Embryos were injected with 7.5ng of each morpholino as described above and allowed to develop for 24hrs. After 24hrs embryos (~20/morpholino) were harvested, homogenized, and RNA was extracted as described above. After RNA was purified, cDNA library was prepared from 2ug of total RNA using Superscript III reverse transcriptase enzyme (Invitrogen) and an oligo(dt) primer according to the manufacturers instructions. PCR reactions were set up

to qualitatively assess effects on mRNA by splice blockers directed against exon 5&6. Primers flanking exon 5&6 were designed and used to give an expected product of 999bp of length, amplifying a region from +16 of the transcript to +1015. Similarly, PCR reactions were set up to qualitatively assess effects on mRNA by splice blockers directed against exon 18. Primers flanking exon 18 were designed to give an expected product of nearly 1000 bp, amplifying a region from +1486-2515 within the *ALC1* trancript.

The sequences of the primers used were as follows:

Dr. Chd1L (+16) Forward Primer: 5'-CGAGCAGTCAGAGACAACATACCA-3'

Dr. Chd1L (+1015) Reverse Primer: 5'-AAGCTTTCCACTGGCCTCCACTAA-3'

Dr. Chd1L Set2 Exon 15 forward Primer : 5'-GGCCACCATGTTCTTCTGTTCTCT-3'

Dr. Chd1L Set3 Reverse Primer (exon 15): 5'-AATGACCAATCCGAGGCAGATGGA-3'

Morphant Scoring. In order to judge the phenotype of morphants with and without the coinjection of *ALC1* mRNA, embryos were tricaine euthanized 5 days post fertilization and injection, and sorted based on phenotypes. Gross phenotypic analysis was used for phenotypic scoring, therefore the most noticeable and common phenotypes were used as benchmarks. Greater than one hundred fish were injected with each morpholino, and fish were judged based on phenotypes such as: anterior-posterior axis length, craniofacial development (presence of jaw/normal eye development), tail curling, and cardiac edema. Fish with an observable heart beat prior to anesthesia treatment were considered alive.

Appendix

Appendix 1. Clustal alignment of Alc1 across vertebrate species.

Red = small hydrophobic, Blue = Acidic, Magenta = Basic, and Green = Hydroxyl + Amine + Basic – Q residues

```
H.sapiens Alc1                  MERAGATSRGGQAPGFLLRLHTEGRAEAAR--VQEQDLRQWGLTGIHLRS 48
P.troglodytes Alc1              MERAGAASRGGQAPGFLLRLHTEGRAEAAR--VQEQDLRQWGLTGIHLRS 48
M.musculus Alc1                 MASG--------LPRFLQALPAEHGPEPLRTRVQEPDLQQWGLTGIRLRS 42
G.gallus Alc1                   ------------MSRFYQALRRAGRARAGGLGVQEEDVSRWGLTGIKLRP 38
D.rerio Alc1                    ------------MSTFLRAVRDN-IPEKDKSELTENDLKKWGLGAIHLRP 37
                                             . *   :     ..     : * *: :*** .*:**.

H.sapiens Alc1                  YQLEGVNWLAQRFHCQNGCILGDEMGLGKTCQTIALFIYLAGRLNDEGPF 98
P.troglodytes Alc1              YQLEGVNWLAQRFHCQNGCILGDEMGLGKTCQTIALFIYLAGRLNDEGPF 98
M.musculus Alc1                 YQLEGVNWLVQCFHCQNGCILGDEMGLGKTCQTIALLIYLVGRLNDEGPF 92
G.gallus Alc1                   YQLDGVNWLVQCYQVQHGCILGDEMGLGKTCQTISLLLYLTKKLTNKERS 88
D.rerio Alc1                    YQLDGVKWLSLCMKNQQGCILGDEMGLGKTCQTISLLAYARGSLKMNGPF 87
                                ***:**:**    : *:******************:*: *    *. :

H.sapiens Alc1                  LILCPLSVLSNWKEEMQRFAPGLSCVTYAGDKEERACLQQDLKQESRFHV 148
P.troglodytes Alc1              LILCPLSVLSNWKEEMQRFAPGLSCVTYAGDKEERACLQQDLKQESRFHV 148
M.musculus Alc1                 LVLCPLSVLSNWKEEMERFAPGLSCVTYTGDKEERARLQQDLRQESGFHV 142
G.gallus Alc1                   LILCPLSVLSNWKEELERFAPGLSFVTYVGNKEERYKLQQNLKEQSHFRV 138
D.rerio Alc1                    LVLCPLAVLENWRQELERFCPSLSVICYTGDKEKRAELQQNLKSDPRFHV 137
                                *:****:**.**::*::**.*.** : *.*:**:*  ***:*:.:. *:*

H.sapiens Alc1                  LLTTYEICLKDASFLKSFPWSVLVVDEAHRLKNQSSLLHKTLSEFSVVFS 198
P.troglodytes Alc1              LLTTYEICLKDASFLKSFPWSVLVVDEAHRLKNQSSLLHKTLSEFSVVFS 198
M.musculus Alc1                 LLTTYEICLKDASFLKSFSWSVLAVDEAHRLKNQSSLLHRTLSEFSAVFR 192
G.gallus Alc1                   LLTTYEICLKDAAFLKFFDWAALVVDEAHRLKNQNSLLYETLTELPVGFS 188
D.rerio Alc1                    LLTTYEMCLKDARYLKSWKWKILVVDEAHRLKNQESLLHQTLKEFTVGFR 187
                                ******:***** :** : *  *.**********.***:.**.*:.. *


H.sapiens Alc1                  LLLTGTPIQNSLQELYSLLSFVEPDLFSKEEVGDFIQRYQDIEKESESAS 248
P.troglodytes Alc1              LLLTGTPIQNSLQELYSLLSFVEPDLFSKEEVGDFIQRYQDIEKEPESAS 248
M.musculus Alc1                 LLLTGTPIQNSLRELYSLLCVVEPDLFCREQVEDFVQRYQDIEKESKSAS 242
G.gallus Alc1                   LLLTGTPIQNSLQELYSLLSFIEPDIFPRKQVKEFVEYYQAVEKESEPAK 238
D.rerio Alc1                    VLLTGTPIQNNLQEVYSLLTFIQPSVFLPEAVEDFVNAYADIQTEPALVD 237
                                :*********.*:*:**** .::*.:*  : * :*:: *  ::.*.  ..

H.sapiens Alc1                  ELHKLLQPFLLRRVKAEVATELPKKTEVVIYHGMSALQKKYYKAILMKDL 298
P.troglodytes Alc1              ELHKLLQPFLLRRVKAEVATELPKKTEVVIYHGMSALQKRYYKAILMKDL 298
M.musculus Alc1                 ELHRLLQPFLLRRVKAQVATELPKKTEVVVYHGMSALQKKYYKAILMKDL 292
G.gallus Alc1                   ELHNLLQPFLLRRVKSEVTADLPKKVEVVLYHGMSALQRKYYKAILTKDL 288
D.rerio Alc1                    ELHQVLQPFLLRRVKAEVAAELPKKTELVVFHGLSALQKRYYKAILMRDL 287
                                ***.:**********::*:::****.*:*::**:****::****** :**

H.sapiens Alc1                  DAFENETAKKVKLQNILSQLRKCVDHPYLFDGVEPEPFEVGDHLTEASGK 348
P.troglodytes Alc1              DAFENETAKKVKLQNILSQLRKCVDHPYLFDGVEPEPFEVGDHLIEASGK 348
M.musculus Alc1                 DAFENETAKKVKLQNILTQLRKCVDHPYLFDGVEPEPFEVGEHLIEASGK 342
G.gallus Alc1                   DAFEGGTGRKVMLQNVLIQLRKCVAHPYLFNGVEPEPFEIGDHIVEASGK 338
D.rerio Alc1                    DAFRTDQSTKTRLLNVLMQLRKCVDHPYLFDGVEPEPFEMGEHLVEASGK 337
                                ***.   . *. * *:* ****** *****:********:*:*: *****
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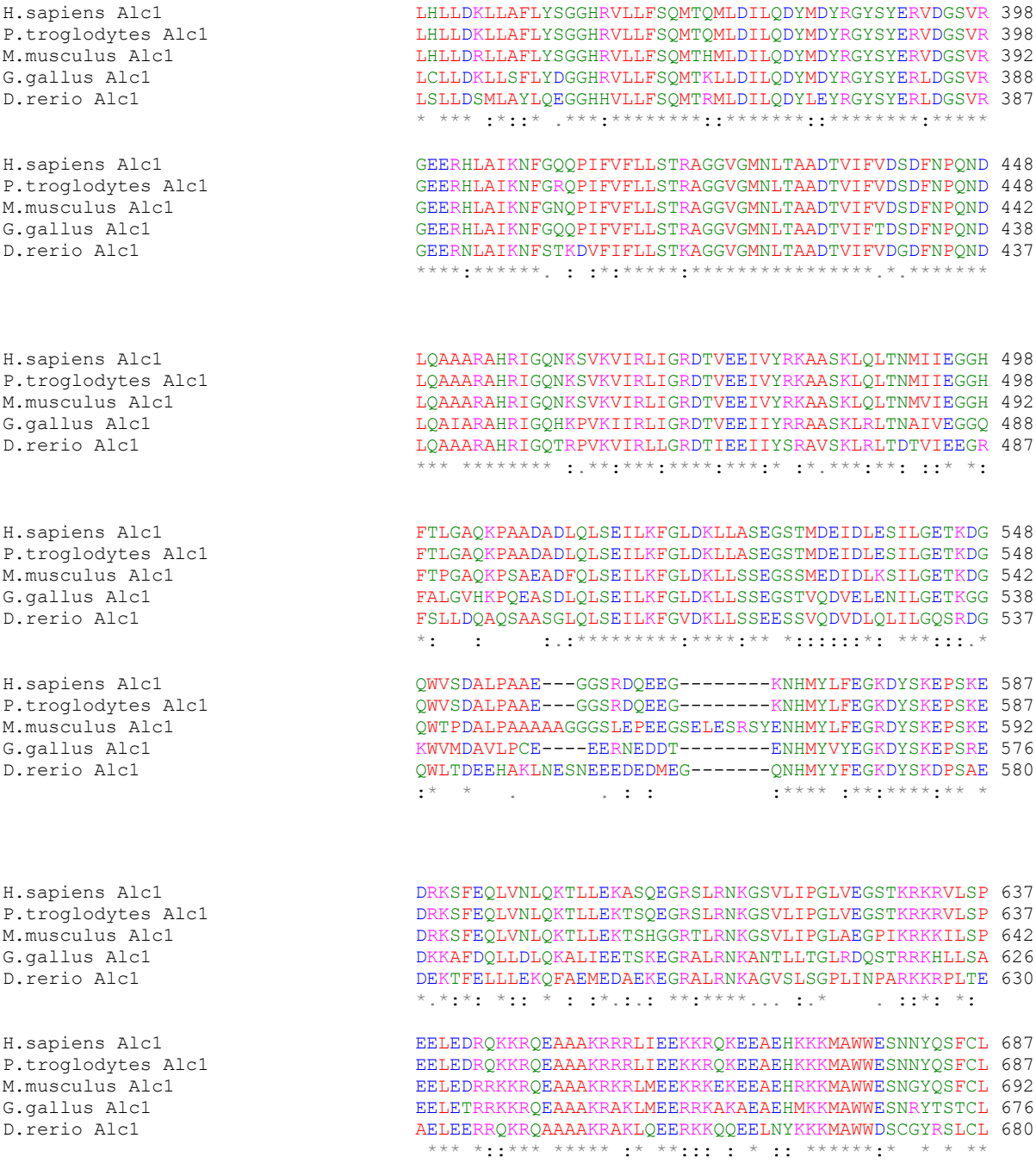
H.sapiens Alc1 LHLLDKLLAFLYSGGHRVLLFSQMTQMLDILQDYMDYRGYSYERVDGSVR 398
P.troglodytes Alc1 LHLLDKLLAFLYSGGHRVLLFSQMTQMLDILQDYMDYRGYSYERVDGSVR 398
M.musculus Alc1 LHLLDRLLAFLYSGGHRVLLFSQMTHMLDILQDYMDYRGYSYERVDGSVR 392
G.gallus Alc1 LCLLDKLLSFLYDGGHRVLLFSQMTKLLDILQDYMDYRGYSYERLDGSVR 388
D.rerio Alc1 LSLLDSMLAYLQEGGHHVLLFSQMTRMLDILQDYLEYRGYSYERLDGSVR 387
H.sapiens Alc1 GEERHLAIKNFGQQPIFVFLLSTRAGGVGMNLTAADTVIFVDSDFNPQND 448
P.troglodytes Alc1 GEERHLAIKNFGRQPIFVFLLSTRAGGVGMNLTAADTVIFVDSDFNPQND 448
M.musculus Alc1 GEERHLAIKNFGNQPIFVFLLSTRAGGVGMNLTAADTVIFVDSDFNPQND 442
G.gallus Alc1 GEERHLAIKNFGQQPIFVFLLSTRAGGVGMNLTAADTVIFTDSDFNPQND 438
D.rerio Alc1 GEERNLAIKNFSTKDVFIFLLSTKAGGVGMNLTAADTVIFVDGDFNPQND 437
H.sapiens Alc1 LQAAARAHRIGQNKSVKVIRLIGRDTVEEIVYRKAASKLQLTNMIIEGGH 498
P.troglodytes Alc1 LQAAARAHRIGQNKSVKVIRLIGRDTVEEIVYRKAASKLQLTNMIIEGGH 498
M.musculus Alc1 LQAAARAHRIGQNKSVKVIRLIGRDTVEEIVYRKAASKLQLTNMVIEGGH 492
G.gallus Alc1 LQAIARAHRIGQHKPVKIIRLIGRDTVEEIIYRRAASKLRLTNAIVEGGQ 488
D.rerio Alc1 LQAAARAHRIGQTRPVKVIRLLGRDTIEEIIYSRAVSKLRLTDTVIEEGR 487
H.sapiens Alc1 FTLGAQKPAADADLQLSEILKFGLDKLLASEGSTMDEIDLESILGETKDG 548
P.troglodytes Alc1 FTLGAQKPAADADLQLSEILKFGLDKLLASEGSTMDEIDLESILGETKDG 548
M.musculus Alc1 FTPGAQKPSAEADFQLSEILKFGLDKLLSSEGSSMEDIDLKSILGETKDG 542
G.gallus Alc1 FALGVHKPQEASDLQLSEILKFGLDKLLSSEGSTVQDVELENILGETKGG 538
D.rerio Alc1 FSLLDQAQSAASGLQLSEILKFGVDKLLSSEESSVQDVDLQLILGQSRDG 537
H.sapiens Alc1 QWVSDALPAAE---GGSRDQEEG--------KNHMYLFEGKDYSKEPSKE 587
P.troglodytes Alc1 QWVSDALPAAE---GGSRDQEEG--------KNHMYLFEGKDYSKEPSKE 587
M.musculus Alc1 QWTPDALPAAAAAGGGSLEPEEGSELESRSYENHMYLFEGRDYSKEPSKE 592
G.gallus Alc1 KWVMDAVLPCE----EERNEDDT--------ENHMYVYEGKDYSKEPSRE 576
D.rerio Alc1 QWLTDEEHAKLNESNEEEDEDMEG-------QNHMYYFEGKDYSKDPSAE 580
H.sapiens Alc1 DRKSFEQLVNLQKTLLEKASQEGRSLRNKGSVLIPGLVEGSTKRKRVLSP 637
P.troglodytes Alc1 DRKSFEQLVNLQKTLLEKTSQEGRSLRNKGSVLIPGLVEGSTKRKRVLSP 637
M.musculus Alc1 DRKSFEQLVNLQKTLLEKTSHGGRTLRNKGSVLIPGLAEGPIKRKKILSP 642
G.gallus Alc1 DKKAFDQLLDLQKALIEETSKEGRALRNKANTLLTGLRDQSTRRKHLLSA 626
D.rerio Alc1 DEKTFELLLEKQFAEMEDAEKEGRALRNKAGVSLSGPLINPARKKRPLTE 630
H.sapiens Alc1 EELEDRQKKRQEAAAKRRRLIEEKKRQKEEAEHKKKMAWWESNNYQSFCL 687
P.troglodytes Alc1 EELEDRQKKRQEAAAKRRRLIEEKKRQKEEAEHKKKMAWWESNNYQSFCL 687
M.musculus Alc1 EELEDRRKKRQEAAAKRKRLMEEKRKEKEEAEHRKKMAWWESNGYQSFCL 692
G.gallus Alc1 EELETRRKKRQEAAAKRAKLMEERRKAKAEAEHMKKMAWWESNRYTSTCL 676
D.rerio Alc1 AELEERRQKRQAAAAKRAKLQEERKKQQEELNYKKKMAWWDSCGYRSLCL 680

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H.sapiens Alc1                  PSEESEPED-LENGEE-SSAELDYQDPDATSLKYVSGDVTHPQAGAEDAL 735
P.troglodytes Alc1              PSEESEPED-LENGEE-SAAELDYQDPDATSLKYVSGDVTHPQAGAEDAL 735
M.musculus Alc1                 SSEDSELED-LEGGDE-SSAELAYEDLDSTSINYVSGDVTHPQAGEEDAV 740
G.gallus Alc1                   PSEESESEEEFEEGEAGLNVDLDYRDVDLNCIKYVMGDVTHPKAEEEDAI 726
D.rerio Alc1                    PRVDSEGED-MEPDED-DHVSFSSTDSDHTAIRYVLGDVTHPQADREDAI 728
                                .  :** *: :* .:    ..:   * * ..:.** ******:*  ***:

H.sapiens Alc1                  IVHCVDDSGHWGRGGLFTALEKRSAEPRKIYELAGKMKDLSLGGVLLFPV 785
P.troglodytes Alc1              IVHCVDDSGHWGRGGLFTALEKRSAEPRKIYELAGKMKDLSLGGVLLFPV 785
M.musculus Alc1                 IVHCVDDSGRWGRGGLFTALEVRSAEPRKIYELAGKMEDLSLGDVLLFPI 790
G.gallus Alc1                   IVHCLDDSGRWGRGGLFTALETRSDQPRKIYEMAGKMKDLQLGGTLLFPI 776
D.rerio Alc1                    IVHCVDDSGHWGRGGLFTALGLRSDEPRKQYELAGDMKDLELGNVLLFPV 778
                                ****:****:**********  ** :*** **:**.*:**.**..****:

H.sapiens Alc1                  DDKESRNKGQDLLALIVAQHRDRSNVLSGIKMAALEEGLKKIFLAAKKKK 835
P.troglodytes Alc1              DDKESRNKGQDLLALIVAQHRDRSNVLSGIKMAALEEGLKKIFLAAKKKK 835
M.musculus Alc1                 DDKESRDKGQDLLALVVAQHRDRTNVLSGIKMAALEEGLKKIFLAAKKKK 840
G.gallus Alc1                   DDKKSRRKGQDLLALIVAQHRDRSNNLSGIKLSALEKGLKKIYVAAKKRN 826
D.rerio Alc1                    DDKQSRLCGRDYLALIVAQQRDKANKLSGIRLTALDEGLKKIYKAAKQKK 828
                                ***:**  *:* ***:***:**::* ****:::**::*****: ***:::

H.sapiens Alc1                  ASVHLPRIGHATKGFNWYGTERLIRKHLAARGIPTYIYYFPRSKSAVLHA 885
P.troglodytes Alc1              ASVHLPRIGHATKGFNWYGTERLIRKHLAARGIPTYIYYFPRSKSAVLHS 885
M.musculus Alc1                 ASVHLPRIGHATKGFNWYGTERLIRKHLATRGIPTYIYYFPRSK--ARHS 888
G.gallus Alc1                   ATVHFPRIGYATKDFNWYGTERLIQKYLATRGIPTLIYYFPRNRGSASQP 876
D.rerio Alc1                    ASVHLPRIGHSTKGFNWYGTERLIRKHLATRGIFTSIYYYRRGSSHATVS 878
                                *:**:****::**.**********:*:**:*** * ***: *.   .  .

H.sapiens Alc1                  QSS----SSSSRQLVP----------------------------------- 897
P.troglodytes Alc1              QSS----SSS-RQLVP----------------------------------- 896
M.musculus Alc1                 QPA----SSSSAPLVP----------------------------------- 900
G.gallus Alc1                   SSS----VAASKP------------------------------------- 885
D.rerio Alc1                    STASTTTPSSSKPAASSPSESPHSSSPPANREGLTKSAELSTTSHEGPGA 928


H.sapiens Alc1                  -------------------------------------------------
P.troglodytes Alc1              -------------------------------------------------
M.musculus Alc1                 -------------------------------------------------
G.gallus Alc1                   -------------------------------------------------
D.rerio Alc1                    PGLADFMRGVHVYFYNMAATEKKKLTRYLITYDGDEEDLMSSHVTHIVGE 978

H.sapiens Alc1                  -------------------------------------------------
P.troglodytes Alc1              -------------------------------------------------
M.musculus Alc1                 -------------------------------------------------
G.gallus Alc1                   -------------------------------------------------
D.rerio Alc1                    VESPVHKQELQDLLHQYPQALLVKKNWLESCFASQRKVSVSKYVIRLT 1026
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Chapter VI. References

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