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Fly Fishing for Histones: Catch and Release by Histone Chaperone Intrinsically Disordered Regions and Acidic Stretches

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Abstract

Chromatin is the complex of eukaryotic DNA and proteins required for the efficient compaction of the nearly two-meter long human genome into a roughly ten-micron diameter cell nucleus. The fundamental repeating unit of chromatin is the nucleosome: 147bp of DNA wrapped about an octamer of histone proteins. Nucleosomes are stable enough to organize the genome yet must be dynamically displaced and reassembled to allow access to the underlying DNA for transcription, replication, and DNA damage repair. Histone chaperones are a non-catalytic group of proteins that are central to the processes of nucleosome assembly and disassembly, and thus the fluidity of the ever-changing chromatin landscape. Histone chaperones are responsible for binding the highly basic histone proteins, shielding them from non-specific interactions, facilitating their deposition onto DNA, and aiding in their eviction from DNA. Though most histone chaperones perform these common functions, recent structural studies of many different histone chaperones reveal that there are few commonalities in their folds. Importantly, sequence-based predictions show that histone chaperones are highly enriched in intrinsically disordered regions (IDRs) and acidic stretches. In this review, we focus on the molecular mechanisms underpinning histone binding, selectivity, and regulation of these highly dynamic protein regions. We highlight new evidence suggesting that IDRs are often critical for histone chaperone function and play key roles in chromatin assembly and disassembly pathways.

Graphical abstract

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INTRODUCTION

The nucleosome is the fundamental repeating unit of chromatin: the physiological form of the eukaryotic genome. Early studies of chromatin revealed this repeating unit of histones and DNA [1,2], and higher resolution structural studies that followed yielded exquisite insights into the arrangement and interactions of nucleosomal components [3,4]. Nucleosomes do not form spontaneously, therefore the construction and destruction of a single nucleosome requires the coordinated actions of many proteins within the cell [5–11]. Central to this process are both ATP-dependent chromatin remodeling complexes and ATP-independent histone chaperones (reviewed in [12,13]). ATP-dependent chromatin remodelers can be divided into four families: the SWI/SNF, the NuRD/Mi-2/CHD, the ISWI, and the INO80/SWR1 family (reviewed in [14]). Despite differences in their mechanisms and subunit compositions, all chromatin remodeling complexes contain a structurally similar catalytic ATPase core. This catalytic core is necessary to convert the chemical energy of ATP into rotational movement leading to nucleosome sliding, eviction or exchange.

The large family of histone chaperones are key players in chromatin assembly and remodeling processes, despite them not consuming ATP [9,15–21]. Histone chaperones are defined as a group of proteins that bind to histones and stimulate their transfer onto DNA or to other proteins [5,22,23]. Histone chaperones perform many functions in the cell, including: binding to histones [5], shielding charge and preventing aggregation [24], using their nuclear localization signals (NLSs) along with histone NLSs to aid in cytoplasmic-nuclear shuttling [25,26], maintaining a soluble pool of histones [27], regulating their deposition onto DNA [28], and aiding in histone sliding, nucleosome destabilization and histone eviction [29,30] (Figure 1). As histone chaperones are non-catalytic, they rely solely on multiple energetic minima to guide histone through the deposition pathways and onto DNA [24]. Surprisingly, histones chaperones have evolved a multitude of highly divergent molecular structures to perform relatively similar tasks [31–34].

Histones are extremely basic proteins, with predicted isoelectric points (pI) of human histones ranging from 10.3–11.4. Histones are also very small, contain a high degree of

hydrophobic amino acids, and are prone to interact non-specifically and aggregate with many other macromolecules within the cell. One of the most important responsibilities of a histone chaperone is to partially neutralize the charge of histone proteins to prevent non-functional and detrimental interactions. To do this, histone chaperones are thought to rely on their acidic nature to bind histones and neutralize charge [5]. Interestingly, acidic amino acids are not evenly distributed throughout most histone chaperones, but instead tend to cluster into “acidic stretches” in their primary sequences. The importance of acidic stretches appears to vary between different histone chaperones, with some chaperones requiring acidic stretches to function (such as Npm) [35,36], and other chaperones in which the deletion of acidic stretches does not have an effect on *in vitro* histone deposition activity (such as yeast Nap1) [37], although the *in vivo* necessity is still unclear. It has long been assumed these regions in histone chaperones only serve to increase affinity via non-specific electrostatic interactions with histones. In this “electrostatic steering” model, disordered acidic stretches of a chaperone would interact with the basic histone proteins non-specifically to bring them into proximity of the folded core of the chaperone, which would then make more specific contacts such as hydrogen bonds and van der Waals interactions (concept reviewed in [38]). Consistent with this hypothesis, deletion of acidic stretches in many chaperones lead to an overall decrease in affinity for histone proteins [36,39–41]. However, more recently many acidic stretches have also been shown to make very specific contacts with histones and, in some cases, may actually act as drivers of chaperone specificity [42,43].

Intrinsically disordered regions (IDRs) are also highly prevalent on histone chaperones. Intrinsically disordered proteins (IDPs) and IDRs are defined as extremely flexible and dynamic proteins, or regions of proteins, resulting from a lack of stable secondary structure (reviewed in [44,45]). IDRs do not fold spontaneously into a single stable structure, but rather sample an ensemble of different conformations. Many disordered proteins and proteins with disordered regions fall into the “dark proteome”: the regions of proteins whose conformation remains unknown [46]. IDRs are generally very highly charged and contain few hydrophobic amino acids [47,48], therefore these regions can be accurately predicted directly from the primary sequence of a protein [49]. Proteome wide surveys show that predicted IDRs are highly enriched on many nuclear and chromatin binding proteins [50,51], suggesting that these highly dynamic regions may play key roles in histone binding and chromatin organization. Furthermore, the enrichment of IDRs in nuclear and chromatin proteins, and their lack of enrichment in mitochondrial and more ancient classes of proteins, suggests that functional disorder is largely a eukaryotic innovation.

IDRs in proteins confer many advantages compared to well-ordered proteins, particularly in protein:protein interactions. Many IDRs are able to extend far from the protein core and have large capture radii to interact with potential ligands [52]. These regions also have intrinsic kinetic advantages compared to ordered proteins whose association rates are often diffusion limited. For example, highly charged IDRs can make many transient contacts with potential ligands to form an encounter complex with fast on and off rates, and can often fold only upon binding to their specific ligand. This “fly casting” mechanism facilitates fast association rates and highly specific binding between IDRs and their protein targets, such as the interaction between the intrinsically disordered p53 transactivation domain (TAD) and CREB [53,54]. Additionally, by adopting different secondary structures upon binding

different ligands, one IDR can facilitate interactions with multiple structurally different ligands (reviewed in [55]). This unique property of IDRs allows for a greater number of potential interactions compared to what a well-ordered protein can achieve and is essential for highly complex protein interaction networks. IDRs are also enriched in known protein hubs, consistent with their importance in multivalent protein interactions [56]. Finally, many IDRs can remain disordered even when bound to a ligand. These “fuzzy complexes” are often formed using multivalent, transient interactions between protein and ligand and are of great importance in many processes such as FGNUP-mediated nuclear import [57,58] and Npm1-mediated local phase separation during nucleolus formation [59,60].

Despite the high prevalence of acidic stretches and IDRs in histone chaperones, their roles in histone binding, deposition, and regulation of chaperone function have remained elusive. Due to their dynamic nature, IDRs are notoriously difficult to study at the molecular level. Despite these difficulties, in this review we highlight recently published structural and functional analyses of acidic stretches and IDRs in histone chaperones showing that these regions are not only important for increasing affinity, but play a multitude of specific and diverse regulatory roles. This review is not an exhaustive list detailing all known chaperone:histone interactions and complete deposition pathways, which have been recently surveyed elsewhere (reviewed in [61–63]). Rather, the studies highlighted in this review are prime examples from the recent literature that shed light on the functions and regulation of highly dynamic regions in human histone chaperones and chromatin-binding proteins, including how these regions may influence chromatin structure, gene expression and epigenetic inheritance.

Conservation of IDRs and Acidic Stretches in Histone Chaperones

Due to their structural plasticity and ability to bind specifically to multiple ligands, IDRs are thought to be critical for establishing and fine-tuning highly complex, multivalent protein interaction and complex signaling networks [56,64]. Consistent with this hypothesis, previous comparative proteome-wide disorder predictions have shown that IDRs are more prevalent among higher eukaryotes compared to both bacteria and archaea [65]. In addition, IDRs are predicted to be unequally distributed throughout the human proteome [50]. Classification of predicted disordered regions by gene ontology and subcellular localization show that IDRs are, not surprisingly, enriched in processes specific to eukaryotic cellular function including nuclear and chromatin binding proteins, as well as those involved in nucleosome assembly [50,51]. On the other hand, proteins involved in processes that function in all three kingdoms of life, such as metabolic enzymes, are predicted to be highly ordered. These predictions have led to the hypothesis of an active molecular evolution of protein disorder (reviewed in [66]).

Using the Regression-based Accurate Prediction of Protein Intrinsic Disorder content (RAPID) [51], we compared the disorder content of 28 human histone chaperones against annotated human chromosomal, nuclear, cytoplasmic, mitochondrial, and transmembrane proteins by one-way ANOVA followed by multiple comparisons. We find that, as a group, histone chaperones have a high content of intrinsic disorder (mean of 38%) and are significantly more disordered than nuclear (27%), cytoplasmic (23%), mitochondrial (16%),

and transmembrane (11%) proteins, but are not significantly different compared to chromosomal phosphoproteins (35%), chromosomal proteins (33%), and nuclear phosphoproteins (29%) (Figure 2). We next used DISOPRED3 software to predict the by-residue disorder content of these 28 human histone chaperones [49,67]. We find that IDRs greater than 25 amino acids are present in 26 out of 28 human histone chaperones, with the WD-repeat proteins RbAp48 and RbAp46 being the two exceptions (Figure 3). However, both of these proteins are known to be constituents of chaperone complexes and have not been shown to transfer histones when isolated. Together, these results indicate that histone chaperones as a group are highly enriched in predicted disordered regions, and that these regions likely play important roles in their function.

These regions of histone chaperones, like most IDRs, are enriched in polar and charged amino acids with very few hydrophobic amino acids. We note that regions of different charge tend to cluster into acidic and basic stretches. We define an acidic stretch as a region in the primary sequence of a protein at least 8 amino acids in length and composed of at least 75% glutamate or aspartate residues, though stretches of more than 15 tandem acidic residues exist in many histone chaperones. Based on this definition, acidic stretches, predicted to be important mediators of histone interactions, are found in 23 out of the 28 human histone chaperones listed (Table 1) and are consistently found in regions that are predicted to be disordered (Figure 3). Despite little conservation in the primary sequences, there has likely been selective pressure to maintain these highly charged IDRs in histone chaperones. Additionally, many of the five chaperones that don't have acidic stretches in their primary sequence do have acidic regions on their folded structures, further indicating acidic regions as key mediators in histone interactions [34,68–70]. Below, we present recent evidence for roles of IDRs and acidic stretches, in relationship to previously characterized ordered domains, within individual chaperone families.

Examples of IDRs and Acidic Stretch Function in H2A/H2B Chaperones

FACT—Facilitates Chromatin Transcription (FACT) is a heterodimeric H2A/H2B specific chaperone composed of Spt16 and SSRP1 (known as Pob3 in yeast) subunits [71,72]. FACT was originally identified as a factor that enhances RNA Pol II transcription through chromatin templates [73]. Subsequent analyses demonstrated that FACT is a histone chaperone responsible for disassembly and reassembly of nucleosomes during transcription and DNA replication [74,75]. More recent single molecule FRET studies show that FACT binds to mono-nucleosomes and can induce partial uncoiling of nucleosomal DNA from histones, consistent with a role for FACT in activating transcription elongation through chromatinized DNA templates [76]. Structural studies on the FACT complex show that it is composed of four distinct domains: Spt16 N-terminal domain (Figure 4A), the dimerization domains of Spt16 and SSRP1, the middle domains of Spt16 and SSRP1 (Figures 4B and 4C, respectively), and the long C-terminal domains (CTD) of Spt16 and SSRP1, which are predicted to be disordered and have acidic stretches (Figure 3) [77–79]. Structural work has shown that the middle domain of Spt16 is a major site of H2A/H2B interaction [80], whereas SSRP1 is thought to interact mainly with H3/H4 and DNA to aid in H2A/H2B displacement from intact nucleosomes [74,79]. Comprehensive quantitative histone binding studies using Spt16, SSRP1, and the FACT heterodimer show that FACT binds specifically

to histones H2A/H2B mainly through its Spt16 subunit. This interaction is promoted by the presence of histone tails, and deletion of the acidic CTD of the Spt16 subunit decreases histone affinity by ~6 fold though loss of the CTD did not completely abolish binding [81]. Recently, it was also shown that FACT can bind to, and is critical for the deposition of, the H2A/H2B-like CENP-T/-W complex at centromeres [82]. The disordered CTD of the Spt16 subunit is also necessary for this interaction, possibly indicating that there may be alternative ligands that can be recognized by acidic regions of many histone chaperones. This “ligand switching” mechanism may provide additional levels of cellular regulation by acidic stretches of histone chaperones.

A recent study used scanning alanine mutations in yeast Spt16 and Pob3 (homologue of vertebrate SSRP1) to identify regions of the disordered, acidic CTDs that are important for cell proliferation and histone binding [83]. Interestingly, the authors identify not only acidic residues, but also conserved aromatic residues adjacent to acidic stretches as key mediators of histone interactions. A crystal structure determination of the major Spt16 peptide (^{966}EEEVSEY^{972}) bound to H2A/H2B shows a strikingly conserved mode of interaction; with Tyr^{972} anchored into a hydrophobic groove of the H2A/H2B dimer created by H2B residues Tyr^{45} and Met^{62}. In addition, Glu^{968} of Spt16 makes electrostatic and hydrogen bond contacts capping the basic dipole created by the N-terminus of α2 of H2B (Figure 5A). This “anchoring and capping” mechanism appears to be extremely well conserved among at least H2A/H2B interacting proteins, as the Spt16 peptide overlays nearly perfectly with previously solved crystal structures of peptides from the H2A.Z/H2B chaperone ANP32E (Figure 5B) and the chromatin remodeler SWR1 (Figure 5C). Importantly, all of these peptides bind sites that are at the histone:DNA interface in the nucleosome structure, suggesting that these acidic peptides directly compete with DNA for binding histones. Furthermore, the authors identify a region in the CTD of Pob3 critical for binding H2A/H2B that has an extremely similar aromatic-adjacent-to-acidic composition (^{507}SEVEDF^{512}), and that mutation of many of these residues in Spt16 and Pob3 showed moderate Spt null phenotypes *in vivo*. Taken together with structural studies of other chaperone:histone complexes, these results strongly suggest a conserved mode of interaction. Furthermore, the anchoring of single aromatic residues within the chaperone may provide an efficient means of achieving histone specificity, otherwise not achievable by low-complexity acidic stretches. The structures presented in Figure 5 represent small regions of histone chaperone IDRs that become ordered upon binding histones. There may also exist histone chaperone IDRs that remain disordered in the functional complex, but this has yet to be described in detail.

Nap1 and Nap1L1—Nucleosome Assembly Protein 1 (Nap1) is one of the major H2A/H2B and linker histone chaperones first identified in yeast [29,84]. The crystal structure of the central ordered region of yeast Nap1 (residues 70–370) was solved over a decade ago [85] (Figure 4D). This structure shows that Nap1 exists as a stable dimer held together by an unusual non-coiled coil motif, similar to the later solved structures of the histone chaperones SET and yeast Vps75 [86,87]. Absent from the structure of Nap1 are the N-terminal 70 and C-terminal 47 amino acids, both of which are predicted to be disordered. Deletions of both the N and C-terminal disordered regions independently and in

combination suggest that both regions contribute to binding H2A/H2B and likely act synergistically [24]. The C-terminal region, in particular, is highly acidic and has thus been named the C-terminal acidic domain (CTAD). Though truncations of the CTAD of yeast Nap1 did not completely abolish its histone binding or deposition activity *in vitro* [37], the CTAD is necessary for chaperone dependent dissociation of H2A/H2B from nucleosomes and nucleosome sliding [29], indicating that this region may be important in competing for histone:DNA interactions. Consistent with this hypothesis, mechanistic studies of yeast Nap1 indicate that it thermodynamically favors proper nucleosome formation by disfavoring non-nucleosomal histone:DNA interactions and that *in vivo* deletion of Nap1 caused increased H2A/H2B occupancy on DNA and altered gene expression [24,88]. This mechanism of disfavoring non-nucleosomal histone:DNA interactions may be a general feature of non-catalytic chaperones. While an atomic-resolution structure of the Nap1:H2A/H2B complex has not yet been solved, hydrogen-deuterium exchange mass spectrometry (HDX-MS) and low resolution crystallography studies indicate direct interactions between H2A/H2B and acidic residues on both the central region of Nap1 and CTAD, though these studies conflict on the oligomerization state of the complex [89,90].

Compared to yeast Nap1, the human homologue Nap1-like 1 (Nap1L1) contains a very similar domain layout, with a highly conserved and ordered central region between two disordered regions (Figure 3). Nap1L1 has been demonstrated to be a histone chaperone for both H2A/H2B and linker histone H1 [91,92]. Recent binding studies comparing human Nap1L1 with yeast Nap1 indicate that deletion of the CTAD has a greater negative effect on the human protein than on the yeast homologue [93]. The same study showed that two distinct regions in the Nap1L1 CTAD can each independently bind to an H2A/H2B dimer, and that these regions are both composed of acidic residues adjacent to a single tyrosine (^{349}DDDDDYDE^{356} and ^{372}ENDPDYDP^{379}), similar to anchoring and capping motifs in peptides from Spt16, ANP32E and SWR1 [30,83,94], again suggesting a conserved mechanism of histone recognition.

Npm Family—Nucleoplasmin (Npm2) is the archetypal molecular chaperone and the first protein to be called a chaperone [5,95]. Npm2 is the major H2A/H2B storage chaperone present in vertebrate oocytes and early embryos, but is not found in any other somatic cells [96]. *Xenopus laevis* Npm2 contains a highly stable core domain (residues 15–118), whose structure has been solved and assembles into a homopentamer composed of antiparallel beta-sheets (Figure 4E) [31], extremely similar to the structure of the human protein [97]. This homopentameric core domain fold is also found in a variety of other chromatin-related proteins in *Drosophila*, such as FKBP39 and many others with unknown function [98]. In Npm2, the core domain is flanked by the short N-terminal tail (residues 1–14) and longer C-terminal tail domain (residues 119–195), both of which are predicted to be disordered (Figure 3). The C-terminal tail contains multiple acidic and basic stretches, including the longest acidic stretch in *X. laevis* Npm2 (called “A2” and comprised of 18 glutamates and aspartate residues in a 25 amino acid stretch). Negative stain electron microscopy studies have suggested the importance of the Npm2 C-terminal tail domain in histone interactions [42,99]. Quantitative binding studies using C-terminal truncations of Npm2 have shown that though it is not required for binding, the C-terminal tail domain dramatically enhances

affinity of Npm2 for H2A/H2B and H3/H4 [36,100]. Interestingly, truncation mutants that retain A2, but lose the remainder of the tail domain seem to have increased affinity toward histones and more robust deposition in *in vitro* chromatin assembly assays, suggesting that the extreme C-terminal region negatively regulates the function of Npm2 [35,101]. The molecular mechanisms underlying this regulatory function of the disordered tail domain of Npm2 remain unknown.

Npm1 (nucleophosmin/numatrin/B23/NO38) is an Npm-family member found in somatic cells and is the most commonly mutated gene in normal karyotype acute myeloid leukemia (AML) [102]. Npm1 has a similar domain layout as Npm2, including an N-terminal core pentamerization domain and a disordered C-terminal tail domain with acidic stretches (Figure 3) [103]. Npm1 also contains an extreme C-terminal 3-helix bundle with a nucleolar localization signal (NoLS), which is not found in other Npm family members (reviewed in [104]). AML-associated mutations in this 3-helix bundle lead to unfolding and loss of nucleolar localization of Npm1 [105,106]. The disordered C-terminus of Npm1 has also been implicated in organizing subcompartments of the nucleolus through phase-separation [59,60]. Npm1 was originally described as a histone chaperone, but has a multitude of other functions in the cell including nucleic acid binding, ribosome biogenesis, transcriptional regulation, and regulation of the ARF/p53 pathway [104,107–109]. Pull down assays show that Npm1 interacts with both H3/H4 and H2A/H2B, and C-terminal truncations show that the tail is necessary for binding H2A/H2B, linker histone H1, and histone deposition, whereas the core domain is sufficient for H3/H4 binding [40,110]. A separate study showed that C-terminal truncation mutants can act in a dominant negative fashion to inhibit rRNA transcription and cell proliferation [111]. Npm1 was also shown to regulate its RNA binding activity through intra and intermolecular interactions in the tail domain, implicating this disordered region as a site of regulation potentially similar to Npm2 [112].

The related Npm3 protein has a conserved core domain, but a shorter C-terminal disordered tail domain than either Npm1 or Npm2, and contains a single acidic stretch (Figure 3). Few studies have probed the function of the somatic Npm3 protein, but it binds to both H2A/H2B and H3/H4 with a modest preference for H3/H4 *in vitro*, and its domain arrangement suggests a similar model of histone binding to the disordered acidic stretch [113]. Interestingly, Npm3 was also shown to form hetero-oligomers with Npm1, which can function to suppress the RNA binding activity of Npm1 [114]. Further studies into the roles of Npm3 are necessary to delineate the function of this protein and its hetero-oligomers with family members Npm1 and Npm2.

Examples of IDRs and Acidic stretch Function in H2A Variant Chaperones

Histone chaperones are generally specific for either H2A/H2B dimers, H3/H4 dimers or H3/H4 tetramers, however histone variants pose a unique challenge. Histone variants serve highly specific functions on chromatin, but often only differ from their canonical counterparts by a few amino acids. Variant-specific chaperones recognize, ensure accurate deposition and aid in the removal of these non-canonical histone variants. Below are examples of how disordered regions and acidic stretches of histone chaperones can aid in specifically recognizing, depositing, or displacing these variants.

H2A.Z Chaperones—H2A.Z is a variant of canonical H2A that is incorporated into nucleosomes at promoters of actively transcribing genes. H2A.Z has been shown to alter nucleosome stability and is thought to facilitate more open chromatin forms [115,116]. H2A.Z incorporation and DNA methylation are found in mutually exclusive areas of the genome of *Arabidopsis* and are antagonistic to one another, indicating a complex mechanism for maintenance of gene expression by histone variant incorporation [117]. Though similar to canonical H2A (64% identity), the cell specifically recognizes and incorporates H2A.Z in the proper genomic location, ensuring accurate and stable gene expression.

The chaperone Chz1 was identified in yeast as the major H2A.Z/H2B specific chaperone [118]. The core domain of Chz1 has a bipolar charge distribution, with acidic residues near the N-terminus and basic residues near the C-terminus, and is predicted to be almost completely disordered. The NMR structure of the Chz1:H2A.Z/H2B complex shows a largely extended conformation for Chz1 draped over the H2A.Z/H2B dimer and capped by 2 short helices (Figure 5E), similar to the structure of the MCM2:$(H3/H4)_2$ complex (Figure 5G) [119]. This structural study and follow up biophysical studies indicate that Chz1 relies predominantly on its bimodal charge distribution to make electrostatic contacts with both basic and acidic surfaces of the H2A.Z/H2B dimer for its high affinity [43,120]. Some of these surface residues are only present in the H2A.Z/H2B dimer, but not in canonical H2A, and are likely important for the specificity of the chaperone.

A metazoan homologue for Chz1 has not been identified, however it was recently demonstrated that the protein ANP32E is a histone chaperone that can specifically remove H2A.Z/H2B dimers from chromatin [30,121]. In these studies, the authors independently solve the crystal structure of a short acidic peptide derived from ANP32E bound to H2A.Z/H2B (Figure 5B). This complex is strikingly similar to the Spt16 peptide bound to H2A/H2B dimers [83], with a single tyrosine anchor and multiple acidic residues capping the H2A.Z/H2B dimer, along with additional non-polar contributions from Leu^{218} and Met^{222} of ANP32E. Compared to the Chz1:H2A.Z/H2B complex (Figure 5E), the ANP32E peptide binds to a different site on the H2A.Z/H2B dimer, specifically recognizing H2A.Z/H2B through a single glycine residue (Gly^{92}) in the C-terminal helix of H2A.Z. Binding of ANP32E also leads to a conformational extension of the C-terminal helix of H2A.Z, promoting further contacts between the histone variant and chaperone. Comparison of this structure with the structure of a nucleosome containing H2A.Z/H2B indicates that this chaperone-induced helix extension likely hinders nucleosomal interactions between the C-terminus of H2A.Z and the C-terminus of H4, consistent with ANP32E assisting in the removal of H2A.Z/H2B dimers from nucleosomes [115]. Interestingly, a peptide derived from the catalytic subunit of the chromatin remodeler SWR1 was also crystallized in complex with a H2A.Z/H2B dimer, where it adopts an almost identical conformation and binding site as ANP32E (Figure 5C) [94]. This study suggests that certain ATP-dependent chromatin remodelers interact with histones in a similar fashion to chaperones.

Another H2A.Z/H2B chaperone, YL1, was recently identified in complex with H2A.Z/H2B dimers in the soluble portion of cell extracts [122]. While ANP32E and SWR1 appear specifically aid in the removal of H2A.Z from chromatin, YL1 acts as a depositor of this variant histone. Two independently solved crystal structures of YL1 (human and *Drosophila*

proteins) bound to H2A.Z/H2B (Figure 5D) share many similarities to the ANP32E:H2A.Z/H2B complex structure (Figure 5B), including the same anchoring and capping mechanism, as well as a similar extension of the C-terminal helix of H2A.Z [123,124]. YL1 also has some unique features not seen in the ANP32E:H2A.Z/H2B structure, such as more extensive hydrophobic interaction with the C-terminal helix, particularly Tyr^{30} of YL1 that is positioned at the interface of the extended C-terminal helix and interacts directly with the H2A.Z specific residue Asp^{97}. YL1 continues to extend around the H2A.Z/H2B dimer, following the path of nucleosomal DNA and ending with its C-terminal region making contacts with the α1 helix of H2A.Z. The positioning of this C-terminal region is similar to that of the N-terminal region of Chz1 (Figure 5E), suggesting that YL1 may use a combination of the binding mechanisms observed for both ANP32E and Chz1. Together, these four structures of H2A.Z/H2B specific chaperones (Chz1, ANP32E, SWR1, and YL1) give key insights into commonalities and differences in molecular mechanisms used by chaperones to specifically recognize, remove and deposit this variant histone.

APLF—MacroH2A is a histone variant of H2A that has a C-terminal macro domain linked to a H2A homology domain and is generally found in transcriptionally repressed regions of the genome, including throughout the inactive X chromosome [125,126]. Aprataxin-PNK-like Factor (APLF) is an acidic nuclear protein that is recruited to sites of DNA damage via interactions with PARP-1 catalyzed poly-ADP ribose chains [127–129]. APLF participates in chaperoning histones at DNA damage sites [130]. In the same study the authors use a variety of truncations to identify the C-terminal acidic domain, which shares homology to the Nap1L1 CTAD, as a major interaction site for both H2A/H2B and H3/H4. Interestingly, the C-terminal acidic domain is predicted to be disordered and has acidic and aromatic residues (Figure 3). In particular, its ^{480}DEDSDWE^{486} region is reminiscent of anchoring and capping motifs present in Spt16, ANP32E and SWR1 peptide structures [30,83,94]. The authors of this study further show that APLF deficient cells are unable to recruit macroH2A to sites of DNA damage, suggesting APLF is the first identified chaperone specific to macroH2A. Pull-down experiments using truncations of the C-terminal acidic domain lose the ability to bind all histones, including macroH2A. The molecular mechanisms underlying the specificity of APLF toward macroH2A remain largely unknown.

Examples of IDRs and Acidic Stretch Function in H3/H4 Chaperones

Asf1 and MCM2—Antisilencing Factor 1 (Asf1) is a somatic H3/H4 chaperone that was originally identified in yeast as a protein which, upon overexpression, could de-repress silent loci [131]. Asf1 is responsible for binding the dimeric form of H3/H4 [132], aiding in histone acetylation prior to nuclear import [133], and coordinating with either CAF-1 for replication-dependent deposition of H3.1/H4, or HIRA, DAXX or DEK for replication-independent deposition of H3.3/H4 [134–137]. CAF-1 likely tetramerizes H3.1/H4 prior to deposition, whereas it is less clear if H3.3/H4 are deposited as dimers or tetramers [23,41,135,138]. In mammals, Asf1 is present in cells in two distinct isoforms, Asf1a (also known as CIA) and Asf1b. Structurally, human Asf1a and Asf1b are composed of an N-terminal core of antiparallel beta-sheets (residues 1–156) (Figure 4F) and a C-terminal disordered domain (residues 157–204). Multiple NMR and x-ray crystallography studies of

Asf-1 N-terminal core bound to H3/H4 or the H3 C-terminal helix yield insights into how Asf-1 blocks the tetramerization of two H3/H4 dimers by using both hydrophobic and acidic residues to compete for the dimer:dimer interaction surface [32,132,139–141]. Though human Asf1a and Asf1b lack distinct acidic stretches, the C-terminal disordered domains are slightly acidic (Figure 3), whereas the yeast Asf1 C-terminus is both longer and contains multiple acidic stretches (Figure 3 insert). Genetic disruption of the acidic C-terminus in yeast shows phenotypes consistent with loss of H3/H4 binding [142]. Binding studies using serial truncations of the yeast Asf1 C- terminal domain showed a ~200-fold reduction in affinity upon loss of the entire C-terminal domain, but no effect upon truncation of the C-terminal 69 amino acids [39], indicating a major site of H3/H4 interaction within residues 155–210. Further cross-linking experiments in the same study implicate a direct interaction between Glu^{210} and H3/H4. In contrast, introduction of a truncated Asf1 lacking the C-terminal acidic stretch into a *S. pombe* Asf1 null strain was sufficient to rescue the lethality phenotype, suggesting that this region may be dispensable *in vivo* for *S. pombe* yeast [143].

The two human isoforms of Asf1 share 71% identity. Interestingly, the core of Asf1a and Asf1b share a high degree of homology (84% identity), whereas their C-terminal tails are far more divergent (28% identity). Despite not having an acidic stretch, human Asf1a has histone chaperone activity *in vitro* [144]. It was also recently shown that Asf1b, but not Asf1a, is necessary for cellular proliferation, and that Asf1b levels correlate with breast cancer disease outcomes [145]. Further evolutionary analyses showed that Asf1a and Asf1b arose from a duplication in the ancestor of jawed vertebrates, and that the C-terminus of both Asf1a and Asf1b have undergone positive selection consistent with distinct functional roles for the C-termini in the cell [146]. More complete experiments are necessary to understand the differences in the histone chaperone functions of these two isoforms at the molecular level.

Another question that remains is why the human Asf1 C-terminal domains have lost so much of their acidic character compared to yeast Asf1 (Figure 3 insert). One hypothesis is that another disordered acidic protein in human cells can compensate for this lack of acidic stretches to aid in Asf1-mediated chaperoning of H3/H4. In support of this hypothesis, previous functional analyses show that Asf-1 cooperates with MCM2-7 subunits of the human DNA helicase to bind histones at the replication fork, and this mechanism can regulate replication fork progression by histone supply and demand in human cells [137]. In addition, the direct interaction between human MCM2 and histone H3 has been known for 20 years [147]. More recent NMR and crystallography studies have yielded molecular insights into the mechanism of cooperation between the MCM2 subunit of human DNA helicase and Asf1 in chaperoning H3/H4 at the replication fork [148,149]. The heterotetrameric (Asf1 Core, MCM2, and H3/H4 dimer) crystal structures reveal cooperativity between the ordered region of Asf1 and the highly extended and acidic MCM2 in binding opposite faces of the H3/H4 dimer (Figure 5F). MCM2 makes extensive contacts with H3/H4 using mainly electrostatic interactions and with a single tyrosine (Tyr^{90}) anchored into a hydrophobic pocket created by H3 helices α1 and α2, reminiscent of anchoring and capping motifs in other H2A/H2B chaperones. These studies also support a model in which parental H3/H4 tetramers are dissociated into dimers and chaperoned separately onto the DNA of daughter strands, suggesting that histone chaperones may play

important roles in the processes of epigenetic inheritance. However, MCM2 can also bind the tetrameric form of H3/H4 (Figure 5G) [148], and other studies suggest that H3.1/H4 tetramers are largely not split into dimers during replication-dependent deposition whereas H3.3/H4 tetramers are split during both replication-independent and replication-dependent deposition [150,151]. Together these studies suggest that H3/H4 splitting decisions during replication may be dependent on functionally distinct chromatin regions. A better understanding of the network of chaperone:histone interactions during DNA replication is necessary to give mechanistic insight into these conflicting observations.

CAF-1—Chromatin Assembly Factor 1 (CAF-1) is the major H3.1/H4 chaperone that acts synergistically with Asf1 to deposit H3.1/H4 during DNA replication [135,152,153]. CAF-1 is a heterotrimeric chaperone composed of p150, p60 and RbAp48 (also known as p48) subunits, also known as Cac1, Cac2, and Cac3 in yeast, respectively. Early studies suggested that human CAF-1 binds to and deposits H3.1/H4 dimers *in vivo* [23], however more recent evidence suggests that yeast CAF-1 forms tetramers of two H3/H4 dimers and deposits the tetrameric form onto DNA [154,155], though the mechanisms of tetramerization in the human protein remains an active area of research. The Cac1/p150 subunit is largely disordered and contains a large acidic stretch (the glutamate/aspartate or ED domain) in the middle of the protein sequence (Figure 3). Pull-down studies using GST-tagged individual subunits show that Cac1 is the only subunit able to pull-down endogenous H3/H4 from HeLa cells [156]. Recent quantitative binding studies using individual subunits show that Cac1 has the highest affinity for H3/H4, though this affinity was an order of magnitude weaker than the trimeric CAF-1 complex indicating that other subunits likely aid in coordinating H3/H4 [138]. In the same study the authors show that Cac1 alone is sufficient to promote H3/H4 tetramerization and assemble tetrasomes on DNA, and by using truncation mutants they go on to show that the C-terminus, including the structured domain and disordered acidic stretch, is sufficient to induce H3/H4 tetramerization. Though a complete structure of the CAF-1:H3/H4 complex remains elusive, this study utilizes a hybrid of x-ray crystallography, hydrogen-deuterium exchange mass spectrometry (H/DX-MS) and chemical cross linking to provide novel information about the architecture of the complex.

Though Cac1/p150 is the largest subunit and independently binds histones and forms tetrasomes, the two smaller subunits (p60 and RbAp48) also aid in histone chaperone function of CAF-1. RbAp48 is the smallest subunit of the CAF-1 heterotrimer and is a highly ordered WD-repeat protein that forms a seven bladed β-propeller structure. RbAp48 is not an obligate subunit of CAF-1, as it can be found in complex with a multitude of other proteins [157,158]. The structure of RbAp46, a homologue of RbAp48 with 90% sequence identity, was solved along with a peptide derived from residues 25–41 of H4 (Figure 4G) [34]. This very ordered complex structure shows α1 of H4 inserted into a pocket on the side of the donut-shaped β-propeller forming extensive contacts with mainly hydrophobic and acidic residues from helices at the N and C-terminal regions and an acidic loop in RbAp46. Importantly, these key interacting residues in RbAp46 are 100% conserved in the CAF-1 subunit RbAp48, and it was previously shown that both RbAp46 and RbAp48 bind to the first helix of H4, making it very likely that the interaction observed in the RbAp46:H4 crystal structure is conserved in RbAp48 [159]. Additionally, the *Drosophila* protein

Nurf55/p55 is nearly identical to mammalian RbAp46/48 and functions as a non-catalytic member of multiple chromatin remodeling complexes, including Polycomb Repressive Complex 2 (PRC2), to increase affinity toward histones [160]. Nurf55 was also crystallized with the first helix of H4 where it forms very similar contacts compared to RbAp46 [70,161]. In the same studies, the authors demonstrate that an acidic surface of Nurf55 can also interact specifically with the N-terminal tail of H3, and that the strength of this interaction is modulated by lysine 4 trimethylation (H3K4me3), demonstrating that these WD repeat proteins can bind multiple targets to increase affinity and specificity of chaperone and chromatin modifying enzyme complexes. These studies and others show that RbAp48 mainly binds the dimeric form of H3/H4 and that there is a conformational change in the H3/H4 dimer upon binding RbAp48 which inhibits tetramerization, suggesting a mechanism for exchange between Asf-1 and CAF-1 during H3/H4 deposition [162]. Taken together with studies of subunit p150, these studies indicate that there is likely cooperativity between ordered and disordered regions of the CAF-1 heterotrimer to bind to, tetramerize, and deposit H3/H4.

Spt Family of Transcriptional Regulators—Much of the research that identified new histone chaperones was originally done in yeast. Classic examples are experiments using Ty transposons to disrupt gene expression followed by forward genetic screens for secondary mutations that rescue gene expression. These suppressor of Ty (Spt) mutants are often involved in eukaryotic transcription processes, such as Spt15 (now known as TATA-binding protein), Spt3, 7, and 8 (members of the SAGA chromatin modifying complex), and Spt11 and 12 (H2A and H2B, respectively). Other Spt genes, such as Spt16 of FACT, Spt2, and Spt6 have been identified as histone chaperones in yeast [71,163–165].

More recent pull-down studies using H3/H4 conjugated beads and HeLa cell extracts identified a novel protein as a possible histone chaperone in metazoan cells [166]. Though this protein as a whole is basic (pI=9.79), pull-down studies using truncation mutants implicate the acidic C-terminus in interactions with histones H3/H4. This C-terminal region also shares homology to known yeast histone chaperone Spt2, and since no other known motifs were identified it was named human Spt2 (hSpt2). This study showed that hSpt2 can also assembly nucleosomes *in vitro* and is critical for RNA PolI-mediated transcription of rRNA, suggesting it functions as a histone chaperone in the nucleolus. The C-terminal Spt2 homology domain of hSpt2 was later crystallized bound to a H3/H4 tetramer (Figure 5H) [167]. The structure shows hSpt2 C-terminal domain bound around the periphery of a H3/H4 tetramer, mimicking the trajectory of nucleosomal DNA, and making contacts using both acidic and hydrophobic amino acids. The structure of Spt2 is mainly composed of short helices and loops, similar to structures of MCM2 and Chz1 bound to histones. In addition, hSpt2 also has a long C-terminal helix that makes key contacts with the four-helix bundle formed by the H3-H3 interface in the tetramer, consistent with this chaperone specifically recognizing H3/H4 tetramers.

Examples of IDRs and Acidic Stretch Function in H3 Variant Chaperones

H3.3 Chaperones—H3.3 is a H3 histone variant that is 96% identical to canonical H3.1. H3.3 incorporation into nucleosomes occurs largely in a replication-independent fashion and

at gene promoters is generally associated with transcriptional activation, however it is also specifically deposited in heterochromatic regions. It is therefore the responsibility of a network of histone chaperones specific to this histone variant to incorporate it into these distinct regions of chromatin to ensure proper and stable gene expression.

HIRA is a highly conserved histone chaperone that was originally identified in yeast and was the first chaperone demonstrated to be specific to H3.3/H4 [23]. In humans, the HIRA protein was shown to be a member of a functional complex, along with proteins Ubinuclein-1 (UBN1) and Calcineurin Binding Protein 1 (CABIN1) [168,169]. The human HIRA complex (HIRA/UBN1/CABIN1) is known to cooperate with both Asf1a and RNA PolII, as well as a variety of transcription factors and chromatin remodelers to mediate replication-independent deposition of H3.3/H4 mainly into gene bodies, intergenic and regulatory regions of actively transcribing genes [69,170,171]. Though the HIRA protein is necessary for the function of the HIRA complex, UBN1 and CABIN both play critical roles in histone recognition and deposition by the HIRA complex. UBN1, in particular, was demonstrated to be necessary for the stability and function of the HIRA complex [172]. More recent x-ray crystallography of a small peptide of UBN1 (residues 122–142) bound to the Asf1:H3.3/H4 complex showed that UBN1 makes contacts with a number H3.3 specific residues, especially Gly^{90} (Figure 5I), and shares some similarities with the previously solved DAXX:H3.3/H4 structure (Figure 4H) [173,174]. Mutation and quantitative binding analyses in the same study showed that UBN1 is the major determinant of H3.3 specificity of the HIRA complex, and that mutation of Gly^{90} to the H3.1 residue Met^{90} caused loss of specificity. Though this region of UBN1 is not predicted to be fully disordered (Figure 3), it contains aromatic residues Tyr^{132} and Phe^{138} which are inserted into a hydrophobic groove in the H3.3/H4 dimer and polar residues Asp^{133}, Asp^{136}, Asp^{140}, and Asn^{141} that make key contacts to the basic histone surface, similar to anchoring and capping motifs observed in many H2A/H2B chaperones [30,83,94,123].

HIRA is largely responsible for depositing H3.3/H4 into actively transcribing regions of the genome, however another chaperone pathway must exist to mediate deposition into telomeres and pericentric heterochromatin. To fulfill this role, the death-associated protein 6 (DAXX) cooperates with the alpha-thalassemia X-linked mental retardation (ATRX) chromatin remodeler in chaperoning H3.3/H4 independent of HIRA at telomeres and pericentric DNA repeats [175,176]. DAXX is composed of a core domain (residues 183–417) that is predicted to be ordered and sufficient to interact with H3.3/H4 [176], a partially ordered N-terminal domain (residues 1–182), and a long disordered C-terminal domain (residues 418–740) that contains a large acidic stretch (residues 433–488) (Figure 3). Pull-down experiments using DAXX truncations show that the central domain of DAXX that includes the acidic stretch (residues 302–495) interacts with H3.3/H4 more strongly than the N-terminal region (residues 1–302) [41], suggesting that the acidic stretch is an important mediator of H3.3/H4 interactions. The structure of the DAXX core domain in complex with a H3.3/H4 dimer shows that the core of the chaperone is highly helical and envelopes the histone dimer covering ~40% of the histone surface area (Figure 4H) [174]. This structure and mutational analysis show that DAXX also specifically recognizes Gly^{90} of H3.3, similar to the later solved Asf1:UBN1:H3.3/H4 structure. Though this structure is highly ordered, more recent HDX-MS analysis of DAXX alone and in complex with H3.3/H4 indicate that

both DAXX and H3.3/H4 likely co-fold upon binding, and that though the core domain of DAXX is predicted to be ordered, it is likely highly dynamic in solution [177]. The exact contribution from the disordered, acidic C-terminal domain in binding to and specifically recognizing H3.3/H4 remains unclear.

Genetic screens in *Drosophila* identified the transcriptional coactivator DEK as a histone chaperone specific for H3.3/H4 upon phosphorylation by CK2 [178]. Staining of polytene chromosomes showed that DEK is associated with more open chromatin, suggesting that DEK deposits histones in transcriptionally active regions of the genome, in contrast to DAXX. Further studies show that H3.3/H4 dimers are normally recruited to PML nuclear bodies by Asf1 and DAXX [179], where they are stored by PML and DEK proteins prior to deposition, and that loss of DEK causes a global redistribution of H3.3 from telomeres to chromosome arms [180]. These studies indicate that there are multiple independent pathways to mediate H3.3/H4 deposition in functionally distinct chromatin regions, H3.3/H4 can be stored in PML body “triage” centers, and supply of this histone variant can be mediated by “gatekeeper” chaperones such as DEK. This may be a general phenomenon of histone chaperones, as many other histone variants, such as macroH2A, are found in functionally distinct domains of chromatin [181].

CENP-A Chaperones—CENP-A is a more divergent H3 variant histone (roughly 50% identity with canonical H3 in humans) that is incorporated specifically at the centromere [182]. Studies in *S. cerevisiae* have shown that the centromere is dictated by a ~125bp sequence of DNA that is necessary for the incorporation of Cse4 (the yeast homologue of CENP-A) into nucleosomes [183,184]. Cse4, in turn, is necessary for the recruitment of key complexes that make up the kinetochore to bridge mitotic spindles to centromeric chromatin during mitosis [185]. In metazoans, this process is much more complex, with much larger centromeric regions (0.3–5Mbp DNA) composed of highly variable repeated arrays of a 171bp sequence known as α satellite DNA, which is interspersed with both H3 and CENP-A containing nucleosomes [186]. Further biophysical analyses showed that CENP-A/H4 tetramers are more compact than canonical H3/H4 tetramers, and impart significant rigidity when incorporated into nucleosomes at the centromere, possibly important for maintaining centromeric identity in metazoans [187].

How CENP-A is specifically targeted to and deposited in these highly variable regions remains an area of active investigation. Analyses in yeast showed that the acidic domain of protein Scm3 is necessary to recruit Cse4 to centromeres and assemble centromere-specific nucleosomes [188,189]. NMR structural analysis of the histone-binding domain of Scm3 shows that this region is intrinsically disordered in solution and, by making a single chain Cse4-Scm3-H4 fusion protein, the structure was solved (Figure 5J) [190]. The structure shows that Scm3 wraps the Cse4/H4 dimer via a long irregular structure capped by a long N-terminal and shorter C-terminal helices, similar to the structure of Chz1 (Figure 5E). The chaperone packs it’s longer N-terminal helix against α2 of Cse4 and makes largely hydrophobic contacts to multiple Cse4-specific residues, though there are many acidic residues present in the loop and C-terminus of the Scm3. The structure also shows that Scm3 binding induces a major conformational change in the Cse4/H4 dimer that blocks a DNA binding site of Cse4/H4.

Bioinformatic and functional analyses showed that the protein Holliday Junction Recognition Protein (HJURP) is the human homologue of yeast Scm3 and is also necessary for assembly of CENP-A containing nucleosomes at the centromere [191–193]. Previous structural and mutational analyses showed that loop 1 and α2 of the histone fold region form a CENP-A targeting domain (CATD) and are necessary to direct CENP-A to centromeres, suggesting that this region is specifically recognized by the chaperone HJURP [194,195]. Truncation analysis showed that the N-terminal 80 amino acids of HJURP are necessary and sufficient for preferential recognition of CENP-A/H4 [196]. The crystal structure of the N-terminal region of HJURP bound to a CENP-A/H4 dimer was solved and shows that HJURP forms a long N-terminal helix, followed by a 15 amino acid loop and a three-stranded antiparallel β-sheet (Figure 4I) [33]. The amphipathic N-terminal helix packs against α2 of CENP-A in an antiparallel arrangement, in a similar position to the N-terminal helix of yeast Scm3 (Figure 5J), making contacts with many acidic residues on the CENP-A surface. The loop and 3-stranded β-sheet of HJURP make largely hydrophobic and van der Waals contacts with both CENP-A and H4, which help to prevent tetramerization of two CENP-A/H4 dimers. In addition, the authors find a number of contacts outside of the previously described CATD of CENP-A, particularly Ser^{68}, that are important for the specificity of HJURP. It's also worth noting that, in contrast to many other chaperone structures, this region of HJURP is very basic (predicted pI=10.5) and makes contacts with many acidic residues of the histone variant. This may represent a unique mechanism of this variant chaperones function, though many of these acidic residues are found in both CENP-A and canonical H3.1.

Finally, more recent functional analyses showed another protein, acidic nucleoplasmic DNA-binding protein 1 (And-1), interacts with both CENP-A and HJURP and is necessary for assembly of CENP-A containing nucleosomes at the centromere in mammalian cells [197]. This protein is highly acidic and contains WD40 and HMG domains similar to those found in other histone chaperones RbAp46/48 and the SSRP1 subunit of FACT, suggesting that it may function as a novel CENP-A variant chaperone. Further analysis of these CENP-A chaperone pathways may help delineate new mechanisms of centromere establishment and maintenance.

Post-Translational Modifications and the Functional Regulation of Histone Chaperones

Structural states of IDRs/IDPs are easily perturbed due to their extreme structural plasticity and shallow energy barriers between multiple different states [198,199]. These unique features allow IDRs/IDPs to act as responsive sensors to even subtle changes in environment or to post-translational modifications (PTMs). One of the most common, and the most thoroughly studied, protein PTMs is phosphorylation. Charge-shifting addition of a phosphate group is an exquisite mechanism for controlling the function of IDRs on many proteins. Many histone chaperones are also regulated by cellular kinases by acting as substrates for phosphorylation on their IDRs. We and others previously showed that *Xenopus* Npm2 is heavily phosphorylated on its disordered N and C-terminal tails during development, and that this modification increases acidic stretch solvent accessibility leading to increased histone affinity and sequestration [35,200–202]. The family member Npm1 is also heavily modified by phosphorylation and acetylation, which alter its histone chaperone

activity, RNA binding activity, oligomerization state, and subcellular localization [40,203–206]. Additionally, the H3.3/H4 chaperone HIRA was shown to be dynamically phosphorylated by Akt1 on its disordered region during muscle cell development [207]. This phosphorylation leads to a suppression of H3.3 incorporation into chromatin, and disappears in response to differentiation cues, leading to increased H3.3 deposition and activation of muscle genes. Phosphorylation may also act to up regulate the activity of a histone chaperone. This is the case for DEK, another H3.3/H4 chaperone, whose chaperone activity is dependent on CK2 phosphorylation on multiple sites along its predicted disordered regions [178,208]. Altering the stability of the protein is another mechanism by which phosphorylation can control histone chaperone function. This was demonstrated for the H3/H4 chaperone Asf1 in a study that showed that Tousled-like kinases (TLKs) phosphorylate its disordered C-terminus leading to increased stability of the protein and decreased proteosomal degradation [209]. Together, these studies demonstrate mechanistically distinct ways by which histone chaperones can be regulated via phosphorylation.

Another lesser-known PTM commonly found on histone chaperones is glutamylation. Glutamylation is the isopeptide addition of one or more glutamates onto a backbone glutamate of a protein. This unique PTM was originally discovered on the disordered acidic tails of α and β tubulin, and is catalyzed by the tubulin tyrosine ligase-like (TTLL) family of enzymes [210,211]. Glutamylation on tubulin tails is known to regulate the binding of microtubule associating proteins (MAPs), including Tau [212]. More recently, it was shown that this modification is not unique to tubulin, but that many acidic nuclear proteins are subject to post-translational glutamylation [213]. In this study, the authors identify many known histone chaperones as substrates for TTLL4 and TTLL5-mediated glutamylation, including Nap1&2, Npm1, and ANP32E. Our mass spectrometry of *Xenopus* oocyte and egg Npm2 also identified multiple glutamylation sites on its largest acidic stretch in the intrinsically disordered C-terminal tail domain [35].

Though the precise function of histone chaperone glutamylation remains unknown, a recent study shows that glutamylation of the CTAD of *Xenopus* Nap1 increases maternal linker histone deposition and alters H1 dynamics on chromatin [214]. It is likely that histone chaperone glutamylation alters histone affinity, specificity and/or deposition rates, as this unique PTM adds both additional negative charge and bulk onto acidic stretches of multiple histone chaperones. It is also possible that glutamylation of non-histone chaperones could provide additional negative charge needed to activate their histone chaperone function. This would be similar to the chromatin modifying protein PARP-1, which activates its own histone chaperone function via auto poly-ADP ribosylation (PARylation) [215]. Given the abundance and importance of acidic stretches in histone chaperones, further studies of this unique acidic modification are needed to delineate its function. Other modifications such as lysine methylation, lysine acetylation, arginine methylation, and ubiquitination also likely regulate histone chaperone function.

Common Themes, Open Questions and Perspectives

The recent studies highlighted in this review have begun to uncover diverse roles for IDRs and acidic stretches in histone chaperones and have illuminated some common themes. These include:

1. The cooperation between ordered and disordered regions of histone chaperones in binding to and chaperoning histones.
2. Acidic stretches in histone chaperones shielding histone charge and often hijacking nucleosomal histone:DNA interaction sites.
3. The use of aromatic residues adjacent to acidic stretches in a conserved “anchoring and capping” histone binding mechanism.
4. The use of IDRs as optimal sites for post-translational modification and chaperone regulation.

Despite IDRs and acidic stretches being common to most histone chaperones, many questions remain unanswered. The exact molecular mechanisms underlying histone transfer from chaperone to DNA *in vivo* remain largely unexplored. Additionally, histone handoff pathways between many chaperones remain unclear or completely undefined, particularly in the case of H2A/H2B chaperones. What roles do chaperones play in the process of epigenetic inheritance of histone PTMs and variants? How do modifications to IDRs and acidic stretches of chaperones affect their activity and global chromatin organization?

Though high-resolution studies of IDRs are inherently difficult to due to their dynamic nature, x-ray crystallography of small peptides derived from these regions bound to histones have provided exquisite insights into mechanisms of histone recognition. In addition, NMR is poised to provide information on even transient structural states of histone chaperone IDRs at the atomic level in solution, as well as probe the potential for these regions to adopt different structures upon binding different histone ligands, though this mode of regulation has currently not been shown for a histone chaperone. Hydrogen-deuterium exchange mass spectrometry (H/DX-MS) and chemical cross-linking have also emerged as powerful solution techniques to gain valuable insights into the architecture and dynamics of chaperone:histone complexes. Cryo-EM is another emergent technology poised to yield high-resolution insights into how chaperones cooperate with larger complexes, such as chromatin remodeling complexes or the DNA replication machinery. Quantitative histone binding studies using truncation mutants have, and will continue to, provide new insights into the functional roles of these regions. The emergent CRISPR/Cas9 genetic engineering technology will provide an efficient means to create targeted knockouts, truncations, and even point mutations in living cells, which will deepen our understanding of the roles of histone chaperones, their IDRs and acidic stretches *in vivo*. In-depth genetic analyses of these highly divergent regions of histone chaperones may also help to provide further insights into their roles and illuminate their evolutionary history. Finally, further investigations into the functions of PTMs that occur on histone chaperones will undoubtedly deepen our understanding of the many diverse mechanisms of regulation. By applying combinations of these biochemical, biophysical, structural, and genetic techniques, the field is more prepared than ever to delineate the functional roles of these dynamic regions in

histone chaperones, and to gain a more complete understanding of the pathways governing histone deposition and chromatin maintenance.

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HIGHLIGHTS

- Intrinsically disordered regions (IDRs) and acidic stretches are common features among the structurally diverse histone chaperone family.
- These regions are often directly involved in histone binding and regulation of chaperone function.
- Recent studies have begun to shed light on both common and unique mechanisms of IDRs and acidic stretches in mediating chaperone:histone interactions.

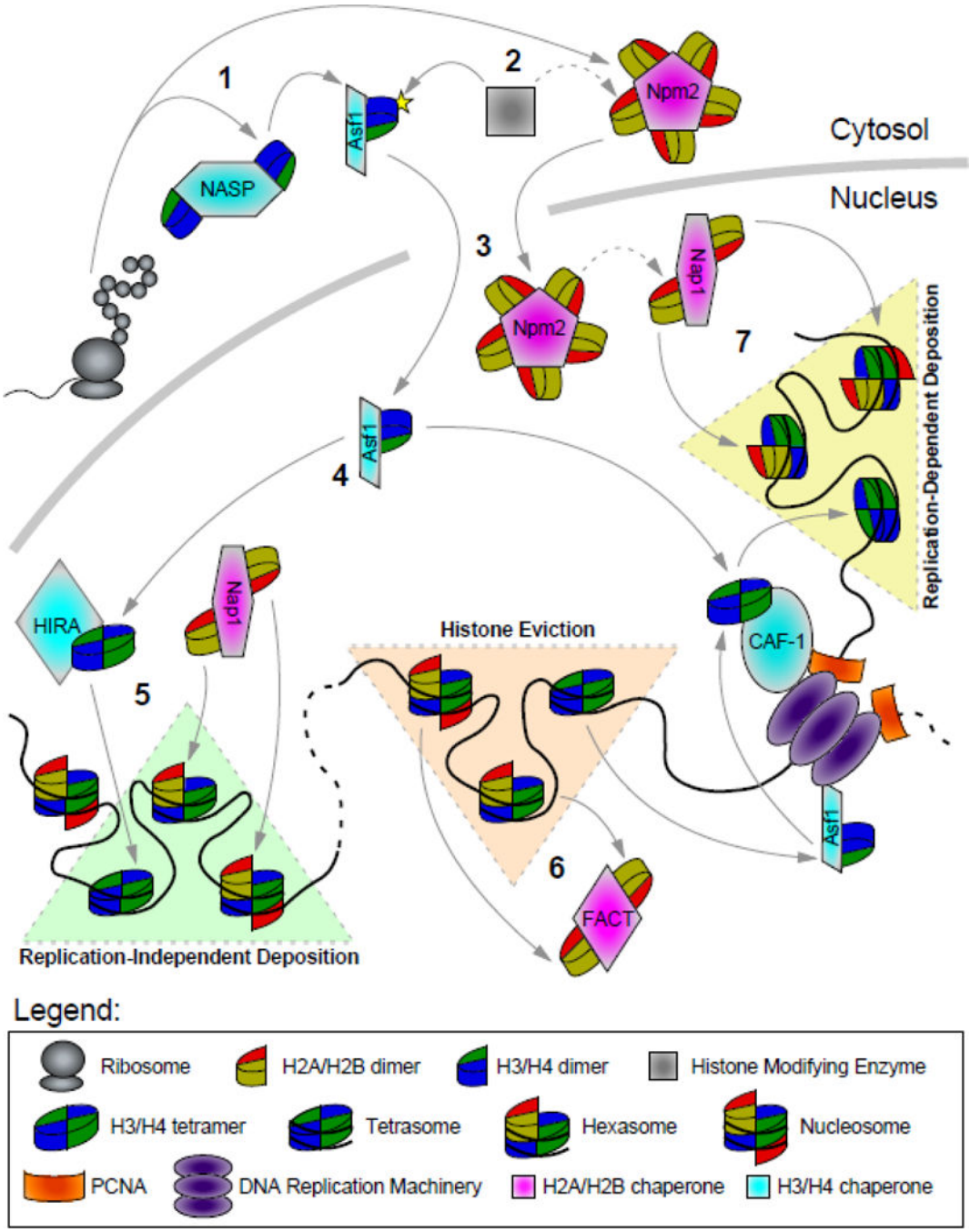

Figure 1. Roles of Histone Chaperones in the Cell

1. Heat-shock proteins (HSPs) along with histone chaperones, such as NASP, Npm2, and Asf1 bind to the newly translated histones and shield them from non-specific interactions in the cytosol.

2. Histone chaperones, such as Asf1 and RbAp46, provide a scaffold for pre-deposition histone modifications, such as HAT1-mediated acetylation of H4 tails. These modifications are crucial for nuclear import and transfer of H3/H4 to other chaperones. Less is known about the function of pre-deposition H2A/H2B modifications (dashed arrow).

3. Histone chaperones, such as Npm2, NASP, Nap1, and Asf1 use their nuclear localization signals (NLS) to interact with importins to aid in shuttling histones between the cytosol and nucleus. Histones also interact with import factors via NLS's on their disordered tails. Chaperones also store and provide a soluble pool of histones.

4. Histone chaperones transfer histones to other chaperones. Asf1 transfers H3.1/H4 dimers to CAF-1 and H3.3/H4 dimers to HIRA, DAXX, or DEK for deposition. CAF-1 tetramerizes H3.1/H4 prior to deposition, whereas HIRA, DAXX and DEK may bind and deposit both the dimeric and tetrameric forms of H3.3/H4 depending on the chaperone bound and chromatin context (only the tetramer deposition model is shown for simplicity). Less is known about transfer among H2A/H2B chaperones (dashed arrow).

5. Replication-independent histone deposition of H3.3/H4 by HIRA, DAXX and DEK, and H2A/H2B by Nap1 and FACT. Histone variants can be incorporated by other variant-specific histone chaperones, such as YL1, HJURP and APLF.

6. Chaperones, along with ATP-dependent chromatin remodelers, mediate histone eviction prior to DNA replication. H2A/H2B is displaced by FACT and Nap1. H3/H4 is displaced by Asf1 and recycled by CAF-1. Large-scale histone eviction occurs during DNA replication (shown), transcription, and DNA repair. The MCM2 subunit of the replisome aids in Asf1-mediated chaperoning at the replication fork, and CAF1 also interacts with the sliding clamp PCNA. Histone variants can be displaced by variant-specific chaperones, such as ANP32E and ATP-dependent chromatin remodelers such as SWR1.

7. Replication-dependent histone deposition of H3.1/H4 by CAF1 and H2A/H2B by Nap1.

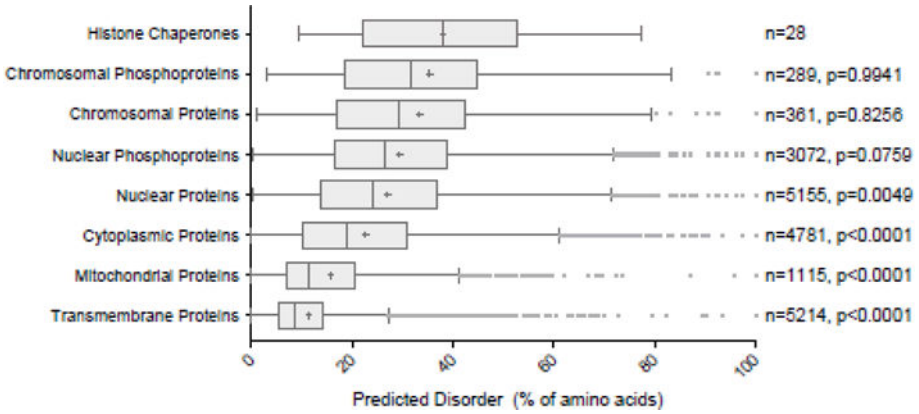

Figure 2. Histone Chaperones are Predicted to be Highly Disordered

A. Box-and-whiskers plot of RAPID disorder content predictions from 28 human histone chaperones (top) compared to annotated chromosomal phosphoproteins, chromosomal proteins, nuclear phosphoproteins, nuclear proteins, cytoplasmic proteins, mitochondrial proteins, and transmembrane proteins. Mean of each group is shown by +. Number of proteins in each group is shown by n value. p values shown from one-way ANOVA and multiple comparisons of each group compared to histone chaperones. Note: many histone chaperones are also included in the groups of nuclear proteins, nuclear phosphoproteins, chromosomal proteins, and chromosomal phosphoproteins.

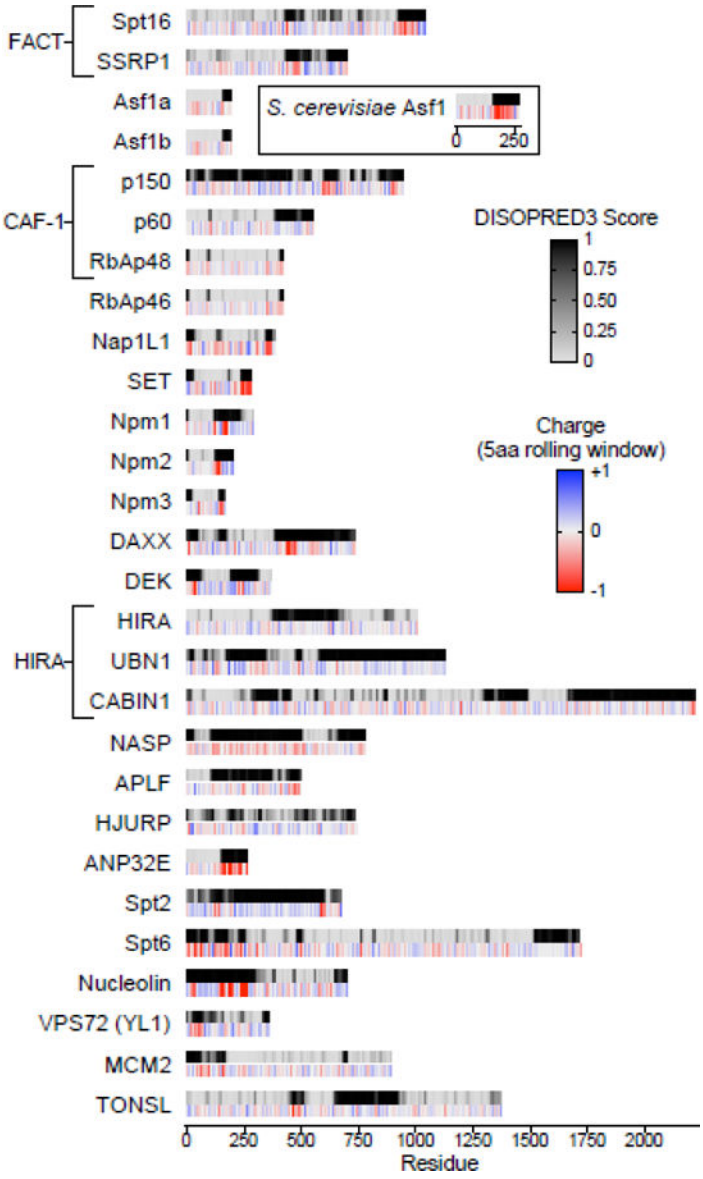

Figure 3. Many Histone Chaperones Contain Long Intrinsically Disordered Regions (IDRs) and Acidic Stretches

A. Top: By-residue DISOPRED3 disorder predictions for 28 human histone chaperones. Primary sequences colored light gray to black from 0 to 1 by DISOPRED3 score. 0=predicted to be highly ordered, 1=predicted to be highly disordered. Disordered regions >25aa are found in 26/28 human histone chaperones listed. Bottom: EMBOSS-calculated charge of the primary sequences of human histone chaperones, colored red to blue from −1 to +1. Charge calculated using a 5-amino acid sliding window. Acidic stretches are found in 23/28 human histone chaperones and cluster in predicted disordered regions. Insert: DISOPRED3 and EMBOSS charge analysis of yeast Asf1 showing that the yeast Asf1 has a longer and more acidic disordered C-terminus compared to human Asf1a and Asf1b.

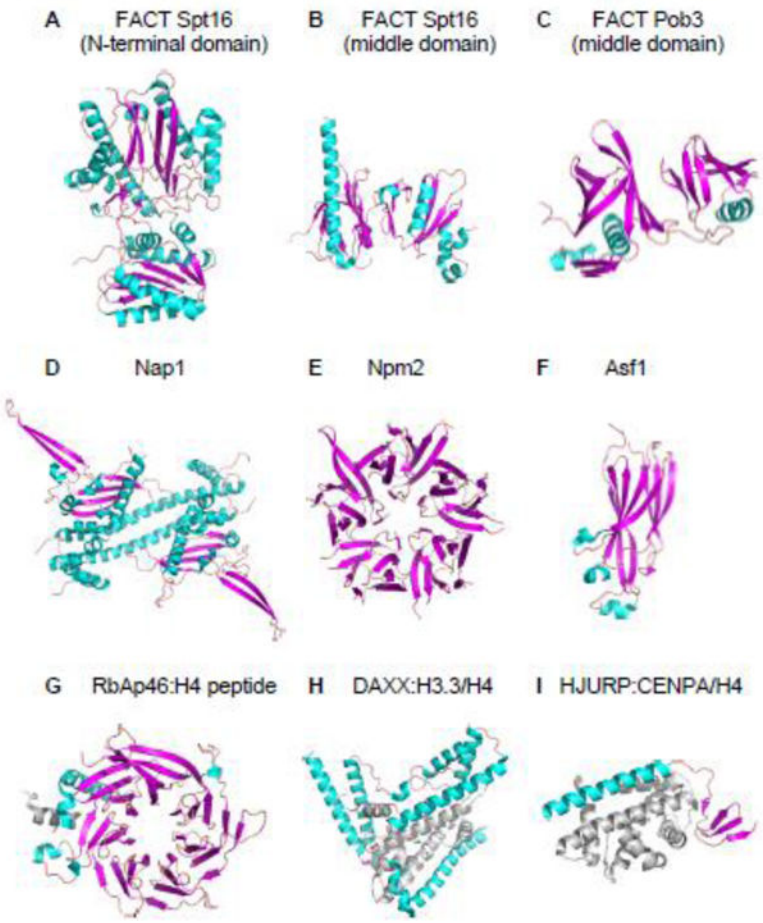

Figure 4. Diversity in the Structures of Various Histone Chaperones

A. Crystal structure of the N-terminal domain (residues 1–451) of yeast Spt16 subunit of FACT (PDB: 3BIT). Residues 452–1035 are not included. Helices, sheets and loops of histone chaperones are colored cyan, magenta, and light pink, respectively, throughout this figure.

B. Crystal structure of the middle domain (residues 653–944) of yeast Spt16 subunit of FACT (PDB: 4KHO). Residues 1–652 and 945–1035 are not included.

C. Crystal structure of the middle domain (residues 237–474) of yeast Pob3 (homologue of human SSRP1) subunit of FACT (PDB: 2GCL). Residues 1–236 and 474–552 are not included.

D. Crystal structure of the core domain (residues 74–365) of yeast Nap1 dimer (PDB: 2Z2R). Residues 1–73 and 366–417 are not included.

E. Crystal structure of the core domain (residues 16–118) of *Xenopus laevis* Nucleoplasmin pentamer (PDB: 1K5J). Residues 1–15 and 119–195 are not included.

F. NMR structure of the core domain (residues 1–156) of human Asf1a (PDB: 1TEY). Residues 157–204 are not included.

G. Crystal structure of human RbAp46 (residues 7–410) in complex with H4 peptide (residues 28–42) (PDB: 3CFV). Residues 1–6 and 411–425 of RbAp46 are not included. H4 peptide colored gray.

H. Crystal structure of the core domain (residues 181–387) of human DAXX in complex with a H3.3/H4 dimer (PDB: 4HGA). Residues 1–180 and 388–740 of DAXX are not included. H3.3/H4 dimer colored gray.

I. Crystal structure of human HJURP (residues 14–74) in complex with a CENP-A/H4 dimer (PDB: 3R45). Residues 1–13 and 75–748 of HJURP are not included. CENP-A/H4 dimer colored grey.

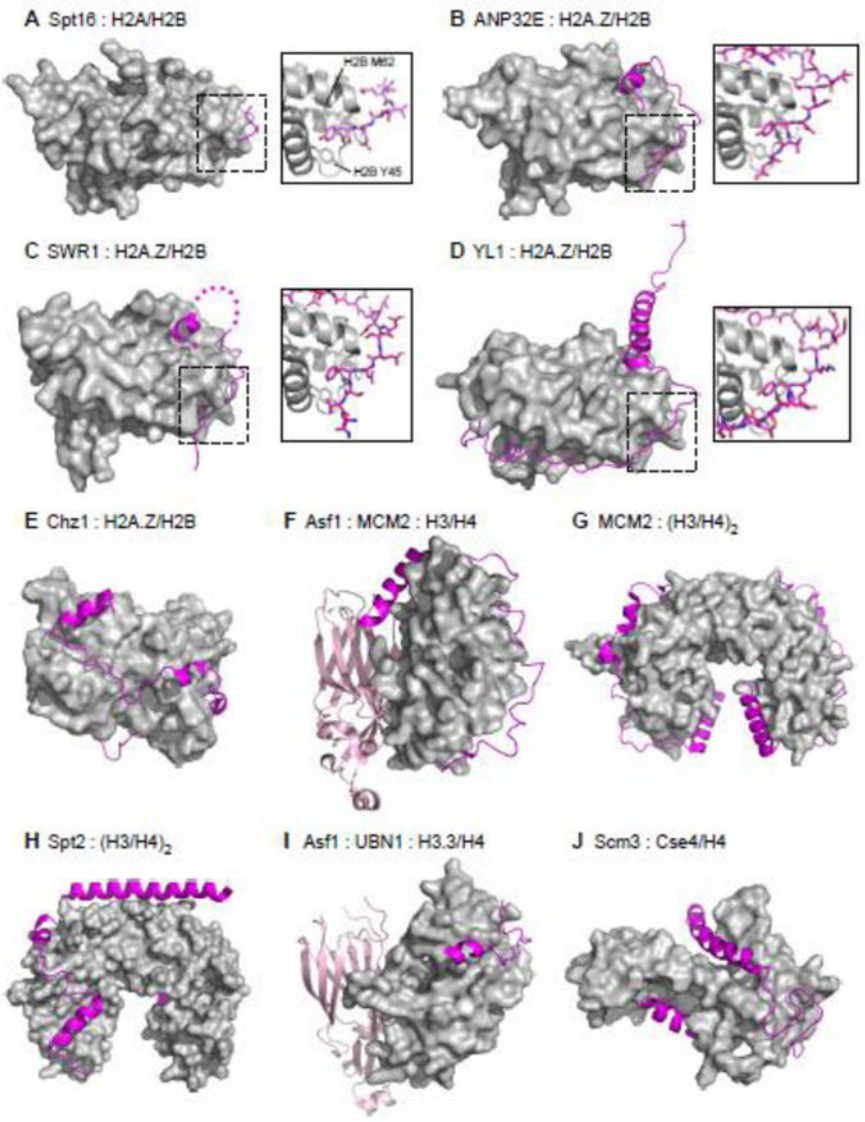

Figure 5. Recent Structural Insights into the Roles of Disordered Regions in Binding Histones

A. Crystal structure of a peptide derived from the acidic disordered C-terminus of yeast Spt16 (residues 967–972) bound to a H2A/H2B dimer (PDB: 4WNN). Chaperone peptide represented as a magenta ribbon, histones represented as grey surface. Boxed: Details of the interaction, with Spt16 peptide represented as magenta sticks and histones shown as gray ribbon with H2B residues Tyr^{45} and Met^{62} shown as sticks. Spt16 anchors a conserved tyrosine into the hydrophobic pocket created by H2B Tyr^{45} and Met^{62}, and uses neighboring acidic residues to cap helix 2 of H2B.

B. Crystal structure of a peptide derived from the acidic disordered C-terminus of human ANP32E (residues 215–237) bound to a H2A.Z/H2B dimer (PDB: 4CAY). Same coloring and representation used as in A. ANP32E also shows conserved “anchoring and capping” binding mechanism to the same site on the H2A.Z/H2B dimer. Additional contacts are made between H2A.Z specific residues and ANP32E, and are necessary for chaperone specificity.

C. Crystal structure of a peptide derived for the Z-domain of yeast SWR1 chromatin remodeler (residues 598–628) bound to a H2A.Z/H2B dimer (PDB: 4M6B). Same coloring and representation used as in A. SWR1 also shows conserved “anchoring and capping” binding mechanism to the same site on the H2A.Z/H2B dimer.

D. Crystal structure of a peptide derived from the acidic disordered N-terminus of Drosophila YL1 histone chaperone component of the SCRAP/SWR1 chromatin remodeling complex (residues 4–71) bound to a H2A.Z/H2B dimer (PDB: 5CHL). Same coloring and representation used as in A. YL1 also shows conserved “anchoring and capping” binding mechanism to the same site on the H2A.Z/H2B dimer.

E. NMR structure of yeast Chz1 histone chaperone core (residues 1–62) bound to a H2A.Z/H2B dimer (PDB: 2JSS). Same coloring and representation used as in A. Chz1 binds to H2A.Z/H2B via a long irregular extended conformation capped by short N and C-terminal helices. No clear anchoring a capping motif is apparent in this structure.

F. Crystal structure of the quaternary complex of human histone chaperone Asf1a core (residues 2–154) and replicative helicase MCM2 (residues 69–121) bound to a H3/H4 dimer (PDB: 5C3I). Same coloring and representation used as in A, but with Asf1 core represented as a light pink ribbon. MCM2 adopts an extended conformation capped by a C-terminal helix. Tyr^{90} of MCM2 is anchored into a hydrophobic pocket created by H3 helices α1 and α2, with adjacent acidic residues making electrostatic contacts with histone surface residues similar to conserved “anchoring and capping” binding motifs.

G. Crystal structure of human replicative helicase subunit MCM2 (residues 69–121) bound to a H3/H4 tetramer (PDB: 5BNV). Same coloring and representation used as in A. MCM2 adopts a very similar conformation as in F.

H. Crystal structure of the human histone chaperone Spt2 (residues 606–675) bound to a H3/H4 tetramer (PDB: 5BS7). Same coloring and representation used as in A. Spt2 binds to the H3/H4 tetramer in an extended conformation capped by helices, most notably a long acidic C-terminal helix which bridges the dimer-dimer interface in the H3/H4 tetramer. No clear anchoring a capping motif is apparent in this complex structure.

I. Crystal structure of the quaternary complex of yeast histone chaperone Asf1 core (residues 5–154) and a peptide from UBN1 (residues 122–142) bound to a H3.3/H4 dimer (PDB: 4ZBJ). Same coloring and representation used as in A, but with Asf1 core represented as a light pink ribbon. The UBN1 peptide adopts an extended conformation capped by a small N-terminal helix. Tyr^{132} and Phe^{138} of UBN1 are anchored into hydrophobic grooves of the H3.3/H4 dimer, and Asp^{133}, Asp^{136}, and Asp^{140} form electrostatic contacts similar to other anchoring and capping motifs.

J. NMR structure of the Cse4-binding domain of yeast Scm3 (residues 90–169) bound to a Cse4/H4 dimer. Same coloring and representation used as in A. Scm3 binds to Cse4/H4 in an extended conformation with N and C-terminal helices. The N-terminal helix of Scm3 contains residues Trp^{107}, Ile^{110}, Ile^{111}, Tyr^{114}, and I^{117} that make hydrophobic contacts with many Cse4-specific residues, and many acidic residues on the middle loop and C-terminus likely make electrostatic contacts.

Table 1

Intrinsic Disorder and Acidic Stretches in Human Histone Chaperones

Histone Chaperone	Uniprot Entry	Apparent Histone Specificity	% Disordered (RAPID prediction)	% Disordered (DISOPRED3 prediction)	Theoretical Isoelectric Point (pI)	Acidic Stretch?	Key References
FACT Spt16	Q9Y5B9	H2A/H2B	22	34	5.50	Yes	[71,73,83]
FACT SSRP1	Q08945	H3/H4	37	38	6.44	Yes	[71,73,79]
Asf1a	Q9Y294	H3/H4	14	22	4.29	No	[32,132,146]
Asf1b	Q9NVP2	H3/H4	9	21	4.46	No	[32,132,145,146]
CAF-1 p150	Q13111	H3.1/H4	45	72	5.69	Yes	[135,138,216]
CAF-1 p60	Q13112	H3.1/H4	24	34	7.18	No	[134,135]
CAF-1 RbAp48	Q09028	H3.1/H4	16	13	4.74	No	[34,157–159,162]
RbAp46	Q16576	H3.1/H4	12	13	4.89	No	[17,34,159]
Nap1L1	P55209	H2A/H2B	39	30	4.36	Yes	[85,88,89,93]
SET	Q01105	H3/H4 and H2A/H2B	60	35	4.22	Yes	[86,217,218]
Npm1	P06748	H3/H4 and H2A/H2B	53	48	4.64	Yes	[40,108,110,111]
Npm2	Q86SE8	H2A/H2B	68	49	4.97	Yes	[5,31,35,36,95]
Npm3	O75607	H3/H4 and H2A/H2B	30	36	4.55	Yes	[113,114]
DAXX	Q9UER7	H3.3/H4	41	63	4.79	Yes	[41,176,177]
DEK	P35659	H3.3/H4	77	55	8.69	Yes	[178,219]
HIRA	P54198	H3.3/H4	16	36	8.40	Yes	[23,69,207]
UBN1	Q9NPG3	H3.3/H4	50	76	9.37	Yes	[168,172,173]
CABIN1	Q9Y6J0	H3.3/H4	22	49	5.70	Yes	[169,220]
NASP	P49321	H3/H4	49	75	4.26	Yes	[27,221,222]
APLF	Q8IW19	Macro-H2A/H2B	49	69	4.98	Yes	[127–130]
HJURP	Q8NCD3	CENP-A/H4	29	47	9.40	Yes	[33,192,196]
ANP32E	Q9BTT0	H2A.Z/H2B	56	44	3.76	Yes	[30,121]
Spt2	Q68D10	H3/H4	67	83	9.79	Yes	[163,166,167]
Spt6	Q7KZ85	H3/H4	27	31	4.81	Yes	[165,223]
Nucleolin	P19338	H2A/H2B	62	58	4.60	Yes	[224,225]
VPS72 (YL1)	Q15906	H2A.Z/H2B	51	46	6.09	Yes	[122–124]

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Histone Chaperone	Uniprot Entry	Apparent Histone Specificity	% Disordered (RAPID prediction)	% Disordered (DISOPRED3 prediction)	Theoretical Isoelectric Point (pI)	Acidic Stretch?	Key References
MCM2	P49736	H3/H4	16	43	5.34	Yes	[137,148,149]
TONSL	Q96HA7	H3.1/H4	23	32	5.99	Yes	[226,227]