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The Histone Domain of macroH2A1 Contains Several Dispersed Elements that Are Each Sufficient to Direct Enrichment on the Inactive X Chromosome

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Histone variants replace the core histones in a substantial fraction of nucleosomes, affecting chromatin structure and impacting chromatin-templated processes. In many instances incorporation of histone variants results in formation of specialized regions of chromatin. Proper localization of histone variants to distinct regions of the genome is critical for their function, yet how this specific localization is achieved remains unclear. macroH2A1 is enriched on the inactive X chromosome in female mammalian cells, where it functions to maintain gene silencing. macroH2A1 consists of a histone H2A-like histone domain and a large, globular C-terminal macro domain that is not present in other histone proteins. The histone domain of macroH2A1 is alone sufficient to direct enrichment on the inactive X chromosome when expressed in female cells, indicating that sequences important for correct localization lie in this domain. Here we investigate whether divergent sequences of the H2A variant macroH2A1 contribute to its correct localization. We mapped the regions of the macroH2A1 histone domain that are sufficient for localization to the inactive X chromosome using chimeras between H2A and the histone domain of macroH2A1. Multiple short sequences dispersed along the macroH2A1 histone domain individually supported enrichment on the inactive X chromosome when introduced into H2A. These sequences map to the surface of the macroH2A1/H2B dimer, but are buried in the crystal structure of the macroH2A1 containing nucleosome, suggesting that they may contribute to recognition by macroH2A1/H2B deposition factors.

Keywords: macroH2A; H2A; chromatin assembly; histone variants; X inactivation

In eukaryotes, chromatin is the basic platform for all DNA associated processes, such as replication, transcription, and genome segregation. The nucleosome is the fundamental particle of chromatin, consisting of DNA wrapped around two copies each of four core histones, H2A, H2B, H3 and H4. One mechanism by which chromatin structure is regulated is by changing nucleosome composition, through the incorporation of histone variants.[1] Assembly of nucleosomes containing histone variants into distinct regions of the genome is important for the formation of functional chromosomal domains, such as centromeres, as well as to delineate expressed or silent chromatin. How histone variants are correctly localized to their regions of action is poorly understood.

Nearly all of the core histones have variants, with histone H2A showing the greatest diversity in metazoans.[2] H2A exhibits uniform nuclear distribution.[3] The variant H2A.X is also present throughout the genome and its phosphorylation in response to

Abbreviations used: GFP, green fluorescent protein; FISH, fluorescence *in situ* hybridization; Xi, inactive X chromosome.

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DNA damage promotes the recruitment of DNA repair machinery.[4] H2A.Z is enriched on heterochromatic foci and is necessary for faithful chromosome segregation.[5,6] H2A.Bbd is depleted on the inactive X chromosome (Xi) in mammalian female somatic cells.[7] The macroH2A variants are enriched on the Xi in mammalian female cells, where they contribute to stable gene silencing.[8–12] Enrichment of macroH2A1 on the Xi requires *XIST* RNA, a non-coding RNA that coats the Xi and recruits a number of chromatin modifying activities to mediate stable transcriptional silencing of the Xi.[13,14] The molecular mechanisms underlying the *XIST* RNA-dependent enrichment of macroH2A1 on the Xi are unknown.

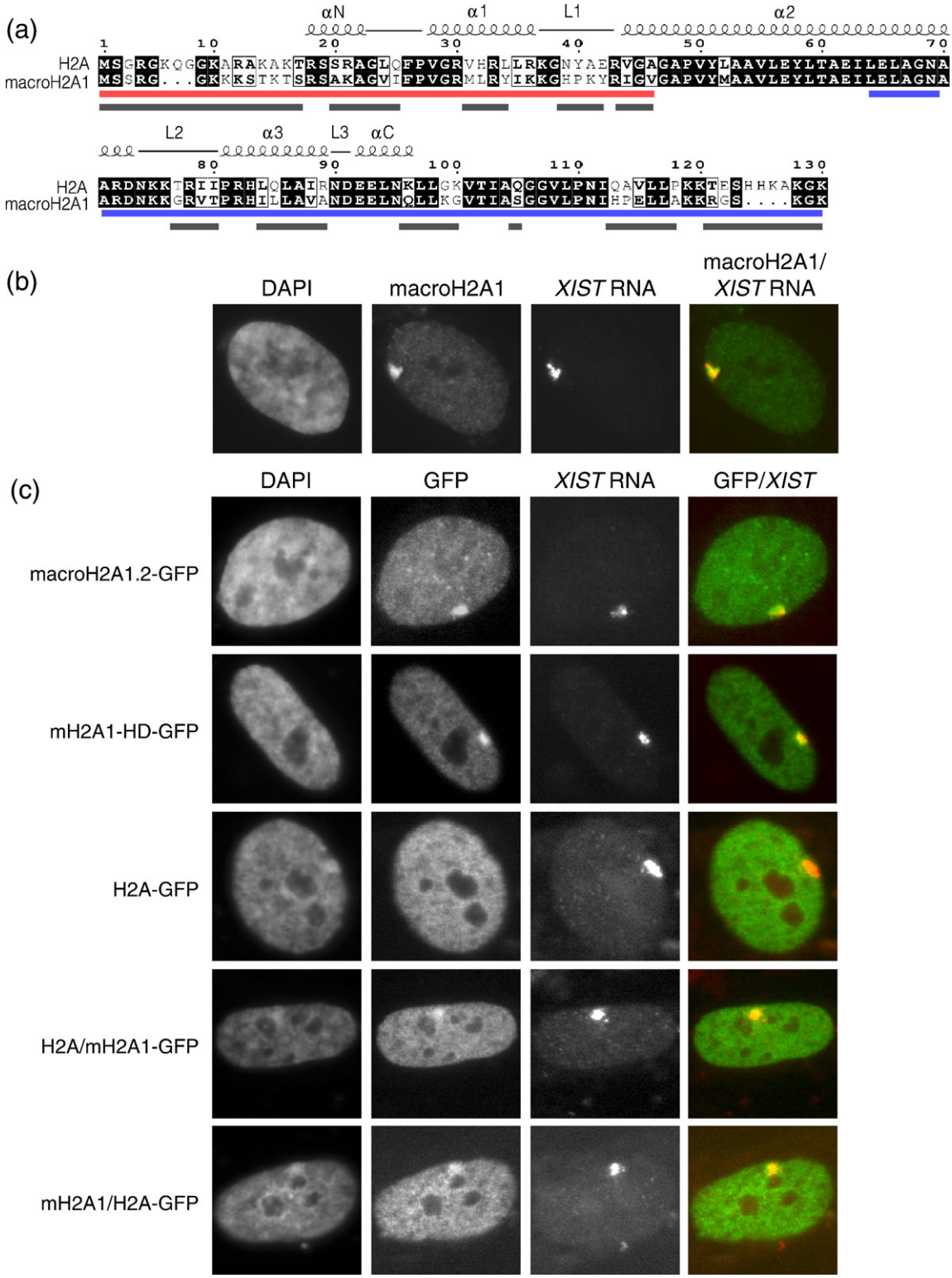

Figure 1 (*legend on next page*)

macroH2A variants consist of an N-terminal histone domain and a large C-terminal macro domain.[15] MacroH2A1.1 and macroH2A1.2 are produced by alternative splicing and differ only in a small region of the macro domain.[16] Histone H2A and the histone domain of macroH2A1 (mH2A1-HD) are highly conserved in primary sequence (Figure 1(a)) and nearly identical in structure.[15,17] Despite this conservation, H2A and the mH2A1-HD show dramatically different localization patterns when expressed as green fluorescent protein (GFP) fusions in female cells. While H2A exhibits uniform nuclear distribution, the mH2A1-HD is enriched on the Xi.[18] Alanine scanning mutagenesis revealed that no single mutation in the mH2A1-HD was sufficient to disrupt its localization,[18] suggesting multiple redundant localization elements. Here, we identify individual amino acids or small clusters of amino acids in the mH2A1-HD that are sufficient to direct the chimeric proteins to the Xi. The regions that are individually sufficient to support enrichment on the Xi included the N and C-terminal tails, a region within the α1 helix, the L1 loop, the docking domain, and a short stretch of residues that lie adjacent to the C-terminal tail. These residues are predicted to be surface exposed in the macroH2A1/H2B dimer and align together on a single side of the dimer. These regions may provide H2A variants with individual signatures that direct specific interactions with different deposition factors or histone exchange machinery, thus ensuring that each variant is uniquely localized to the regions of the genome at which it functions.

Many short stretches of residues within the histone domain promote Xi- enrichment

To identify regions of mH2A1-HD that are sufficient for localization to the Xi, we generated GFP-tagged chimeras between H2A and the histone domain of macroH2A1. As both the primary sequence and three-dimensional structure of the mH2A1-HD and H2A are very similar (Figure 1(a)),[15,17] these chimeras are predicted to minimally perturb histone structure. We transfected chimeric constructs into IMR90 cells, a primary human female fibroblast line in which the macroH2A1 is enriched on the Xi, as demonstrated by immunostaining for macroH2A1 and fluorescence *in situ* hybridization (FISH) for *XIST* RNA (Figure 1(b)). The proportion of cells in which the GFP-tagged protein exhibited enrichment on the Xi (Figure 1(c)) was scored for each chimera (Figure 2). The mH2A1-HD-GFP was enriched on the Xi in 86% of IMR90 cells, comparable to the proportion observed for full-length macroH2A1.2-GFP (89%), and consistent with similar experiments in other human female cell lines.[18] In contrast, H2A-GFP exhibited uniform nuclear distribution in the majority of transfected cells. Two chimeras, one containing the first 43 amino acids of the mH2A1-HD fused to the last 83 of amino acids of H2A and the other containing the first 65 amino acids of H2A fused to the last 67 amino acids of the mH2A1-HD, also showed enrichment on the Xi in a proportion of cell comparable to full-length macroH2A1.2-GFP.

To more finely map the sequences of the mH2A1-HD that are sufficient for enrichment on the Xi, we constructed additional chimeras, each containing smaller portions of the mH2A1-HD. Short clusters of amino acids were individually exchanged from the mH2A1-HD into H2A-GFP. Swapped regions occurred in well-defined structural domains of H2A that are largely conserved in the crystal structures of H2A and mH2A1-HD, but are divergent in amino acid sequence.[17] The regions that were exchanged included the N and C-terminal tails, the helix and loop elements from the histone fold domain, and the docking domain, which defines an interface between macroH2A and the $(H3/H4)_2$ tetramer.[17] Each construct was transfected into IMR90 cells and Xi-enrichment was assessed by co-localization of the

Figure 1. Chimeras between the macroH2A1 histone domain and H2A are enriched on the Xi. (a) Sequence alignment of canonical H2A and the mH2A1-HD, generated using Clustal W software.[32] Identical residues are indicated by black boxes, similar residues by white boxes, while residues that are different are unboxed. Above the sequence is the core histone fold secondary structure, with the major loops (L) and helixes (α) indicated. The red line and blue line below the sequence indicates the region of the mH2A1-HD incorporated into the chimeras. The grey lines below the sequence indicate the regions from the mH2A1-HD exchanged into H2A in constructs analyzed in Figure 2. (b) Immunofluorescence for macroH2A1 and fluorescence *in situ* hybridization for *XIST* RNA demonstrates that macroH2A1 is enriched on the Xi in IMR90 primary human female fibroblasts. IMR90 cells were purchased from American Type Culture Collection, and cultured as instructed. The macroH2A1 antibody and protocol for co-immunofluorescence for macroH2A1 and FISH been published.[23,33] 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) stain delineates the location of the nucleus. (c) macroH2A1.2-GFP, mH2A1-HD-GFP, and the chimeras mH2A1-HD/H2A-GFP and H2A/mH2A1-HD-GFP are enriched on the Xi, while canonical H2A-GFP is not. Full-length macroH2A1.2, mH2A1-HD, and H2A were cloned into a backbone originating from pBOS-H2B-GFP (Clontech) using standard techniques. The resulting constructs express the histone residues tagged with GFP on the C terminus. The mH2A1-HD/H2A, H2A/mH2A1-HD chimeras, and all subsequent chimeras (Figure 2), were generated by replacing regions of H2A with the homologous regions of the mH2A1-HD. The integrity of each construct was confirmed by sequencing. PCR primers used to generate the chimeras in this study are listed in Supplementaary Data, Table 1. Eight hours after plating to glass coverslips, IMR90 cells were transfected using FuGENE 6 Transfection Reagent (Roche). Eighty-nine hours post-transfection, FISH for *XIST* RNA using directly labeled DNA probes was carried out as y described.[34] All microscopy was performed on an Olympus BX-60 Fluorescence microscope. Images were captured using a Hamamatsu ORCA-ER CCD camera and Openlab Digital Darkroom software (Improvision).

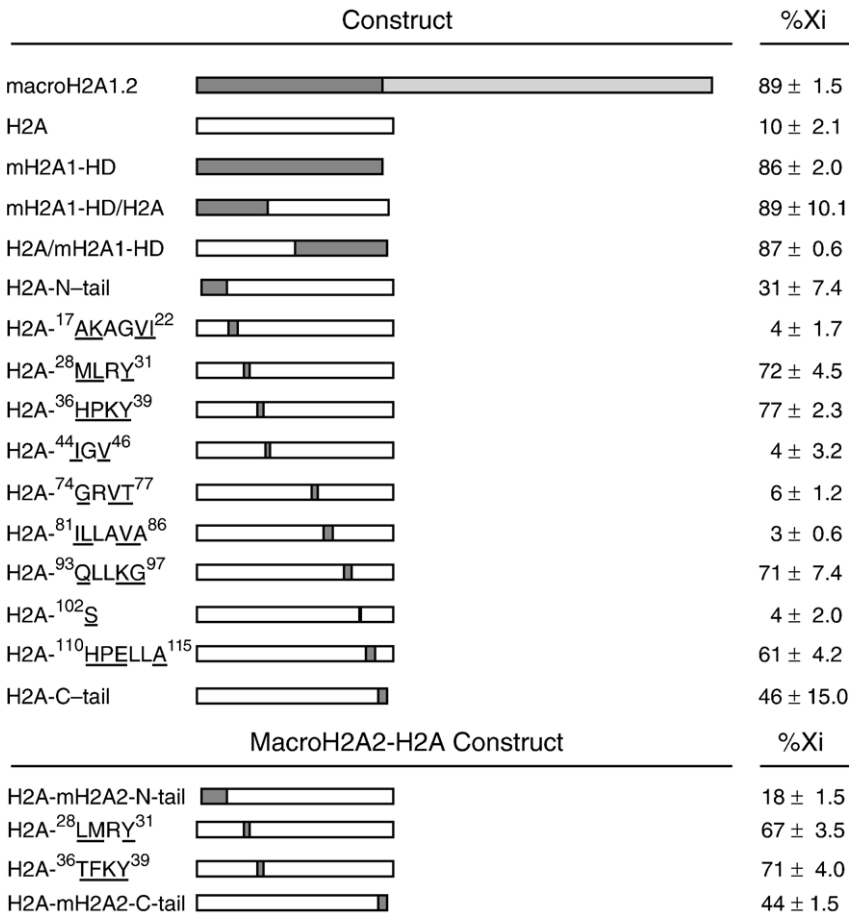

Figure 2. Multiple interspersed residues from the mH2A1-HD are sufficient to promote enrichment of H2A on the Xi. Left: Representations of the macroH2A1/H2A and macroH2A2/H2A constructs used in this study. H2A is represented in white and mH2A1-HD or mH2A2-HD are represented in grey. Right: The percentage of transfected cells showing Xi-enrichment. Numbers result from at least 100 transfected cells counted from each of three independent transfections. Xi-enrichment was assayed by co-localization with endogenous macroH2A1, using an affinity purified rabbit polyclonal serum generated against the macro domain of macroH2A1.[23]

histone-GFP signal with endogenous macroH2A1, detected by immunofluorescence using an antibody specific for the macro domain. The proportion of transfected cells with Xi-enrichment was determined for each construct (Figure 2).

Four residues in the α1 helix (^{28}MLRY^{31}, substituted residues are underlined, amino acid number from mH2A1-HD), four amino acid residues of the L1 loop (^{36}HPKY^{39}), and five amino acid residues within the docking domain (^{93}QLLKG^{97}) of macroH2A1 were each sufficient to cause H2A chimeras to become enriched on the Xi in a proportion of cells (≥71%) approaching that seen for the mH2A1-HD-GFP (89%; Figure 2). Mutation of residues in the same position of the docking domain of Htz1, the *Saccharomyces cerevisiae* H2A.Z homolog, prevents its efficient incorporation into chromatin.[19] These residues are critical for the interaction of Htz1 with the Swr1 complex, which is necessary for Htz1 deposition and exchange into chromatin, suggesting the possibility that the residues in the docking domain of macroH2A1 that direct Xi enrichment might also facilitate interaction with a deposition factor. Comparison of the crystal structure of nucleosomes containing H2A or the mH2A1-HD showed that the L1 loop is the only region of the mH2A1-HD that differs significantly from H2A in structure, the docking domain and α1 helix do not show major structural differences.[17,20,21] Therefore the primary sequence differences at the docking domain and α1 helix, as well as structural differences at the L1 loop, may play a role in mH2A1-HD localization.

H2A chimeras containing either mH2A1-HD N or C-terminal tail sequences or a six residue stretch immediately adjacent to the C-terminal tail (^{110}HPELLA^{115}) directed Xi enrichment at a frequency lower than that observed for wild-type macroH2A1 (31% to 61%). The histone tails protrude from the nucleosome and contain sites of post-translational modification,[22,23] suggesting that modification of macroH2A1 may also play a role in its correct localization. The decrease in percentage of cells with Xi-enrichment of chimeras containing mH2A1-HD tail sequences suggests that these chimeras may have altered affinities for factors that mediate macroH2A1 enrichment on the Xi.

Constructs that supported low levels of Xi-enrichment were not appreciably different from H2A-GFP, which showed enrichment on the Xi in only 10% of transfected cells. Enrichment of endogenous H2A or H2B on the Xi was not observed (Supplementary Data, Figure 1; and data not shown), consistent with a recent report.[3] H2A expression is normally restricted to S phase, and expression of H2A-GFP outside of S-phase may contribute to its ectopic enrichment on the Xi upon transient expression. The chimeras that were indistinguishable from H2A-GFP included residues in the N-terminal helix (^{17}AKAGVI^{22}), in the α2 helix (^{44}IGV^{46}) and (^{74}GRVT^{77}), and in the docking domain (^{81}ILLAVA^{86}

and ^{102}S). In contrast to the residues in the docking domain that promoted Xi-localization, the residues at similar positions to ^{81}ILLAVA^{86} in Htz1 were not critical for association with the Swr1 complex.[19] Because the regions of the mH2A1-HD that promoted enrichment on the Xi and the regions that did not promote enrichment on the Xi exhibited a comparable amount of sequence variation between mH2A1-HD and H2A (Figure 1(a)), primary sequence differences are not sufficient to predict which residues function in enrichment of macroH2A1 on the Xi.

A second member of the macroH2A family, macroH2A2, is also enriched on the Xi.[9,10] Of the mH2A1-HD residues that were sufficient to direct enrichment of H2A/mH2A1-HD chimeras to the Xi, the N and C-terminal tails, α1 helix, and L1 loop differ between mH2A1-HD and the macroH2A2 histone domain (mH2A2-HD). We generated chimeras containing the corresponding residues from mH2A2-HD and found that the mH2A2-HD N and C-terminal tails, α1 helix, and L1 loop were sufficient to direct enrichment of the H2A-mH2A2-HD-GFP chimeras to the Xi (Figure 2). These data indicate that localization function has been evolutionarily conserved in the regions that have diverged between these two macroH2A family members. This suggests the possibility that, although a subset of residues may be sufficient to direct enrichment to the Xi, the contributions of all residues may be necessary to ensure that these variants are correctly localized in a robust fashion.

Single amino acid substitutions further define Xi localization domains

Next, we asked whether individual amino acids of mH2A1-HD were sufficient to direct enrichment on the Xi. We generated chimeras in which single amino acids from the docking domain, L1 loop, α1 helix, or the domain adjacent to the C terminus were substituted into H2A-GFP and determined the proportion of transiently transfected cells showing Xi-enrichment (Table 1). In the docking domain cluster (^{93}QLLKG^{97}), the Xi-enrichment activity could be attributed primarily to K^{96}, as this was the only residue that resulted in Xi-enrichment when introduced into H2A-GFP. Replacement of Y^{31} from α1 helix (^{28}MLRY^{31}), directed enrichment of the chimeric histone to the Xi in a proportion of cells comparable to that seen with the entire α1 helix. M^{28} directed enrichment in a smaller fraction of cells, while L^{29} was not appreciably different from H2A-GFP. In the L1 loop (^{36}HPKY^{39}) or the domain adjacent to the C-terminal tail (^{110}HPELLA^{115}), multiple residues were involved in directing localization to Xi, as no single residue recapitulated the effect of the entire cluster. These data suggest that in some instances a single amino acid may be sufficient to determine which loader or exchange factor H2A or macroH2A1 interacts with, while in other instances the interaction face may be more extended.

Table 1. Percentage of cells exhibiting Xi-enrichment of chimeras containing single amino acid substitutions

Domain	Construct	Xi(%)[a]	SD[b]
α1 helix	^{28}MLRY^{31}	72	4.5
	^{28}MHRL^{31}	27	3.2
	^{28}VLRL^{31}	8	1.5
	^{28}VHRY^{31}	68	8.9
	^{28}VHRL^{31}	10	2.1
L1 loop	^{36}HPKY^{39}	77	2.3
	^{36}HYSE^{39}	39	13.1
	^{36}NPSE^{39}	3	2.1
	^{36}NYKE^{39}	7	1.0
	^{36}NYSY^{39}	36	11.1
	^{36}NYSE^{39}	10	2.1
Docking domain	^{93}QLLKG^{97}	71	7.4
	^{93}QLLGR^{97}	11	2.0
	^{93}KLLKR^{97}	64	2.5
	^{93}KLLGG^{97}	4	1.0
	^{93}KLLGR^{97}	10	2.1
C-terminal adjacent	^{110}HPELLA^{115}	61	4.2
	^{110}HAVLLP^{115}	37	2.3
	^{110}QPVLLP^{115}	26	2.1
	^{110}QAELLP^{115}	11	1.5
	^{110}QAVLLA^{115}	22	2.5
	^{110}QAVLLP^{115}	10	2.1

For comparison, the percentages for H2A-GFP (bottom of the list) or the chimera containing the cluster of mH2A-HD amino acids from which each single amino acid substitution is derived (top) are shown.

[a] Xi-enrichment was assayed by co-localization with endogenous macroH2A1. Numbers are averages from three independent transfections, in each of which at least 100 cells were counted.

[b] SD, standard deviation.

The elements that direct macroH2A1-like localization form an aligned surface-exposed domain

To gain insight into how dispersed sequences of the mH2A1-HD might promote localization to the Xi, we mapped the location of these residues onto the mH2A1-HD-containing nucleosome crystal structure.[17] During nucleosome assembly, H2A/H2B dimers are incorporated into nucleosomes rapidly after deposition of $(H3/H4)_2$ tetramers.[24] H2A and H2B form obligate dimers, as they are insoluble when expressed alone and are only folded and stable as dimers.[25–27] In addition, members of the Swr1 family of ATP-dependent chromatin remodeling factors exchange dimers containing the variant H2A.Z for H2A/H2B dimers in nucleosomes.[28–30] Thus, it is likely that mH2A1-HD/H2B dimers are the physiological substrate for the factors that incorporate macroH2A1 into nucleosomes. To examine the Xi-localization sequence elements in the context of the substrate likely to be relevant for chromatin assembly, we mapped the residues analyzed in chimeras on a single mH2A1-HD/H2B dimer portion of the macroH2A1 nucleosome structure, in the absence of $(H3/H4)_2$ tetramer or DNA (Figure 3(a)). The regions that did not promote localization were mostly buried or present on one face of the dimer (Figure 3(a), shown in yellow). All regions that promoted localization to the Xi aligned on the surface of the opposite face of the mH2A1-HD/H2B dimer (Figure 3(a), shown in

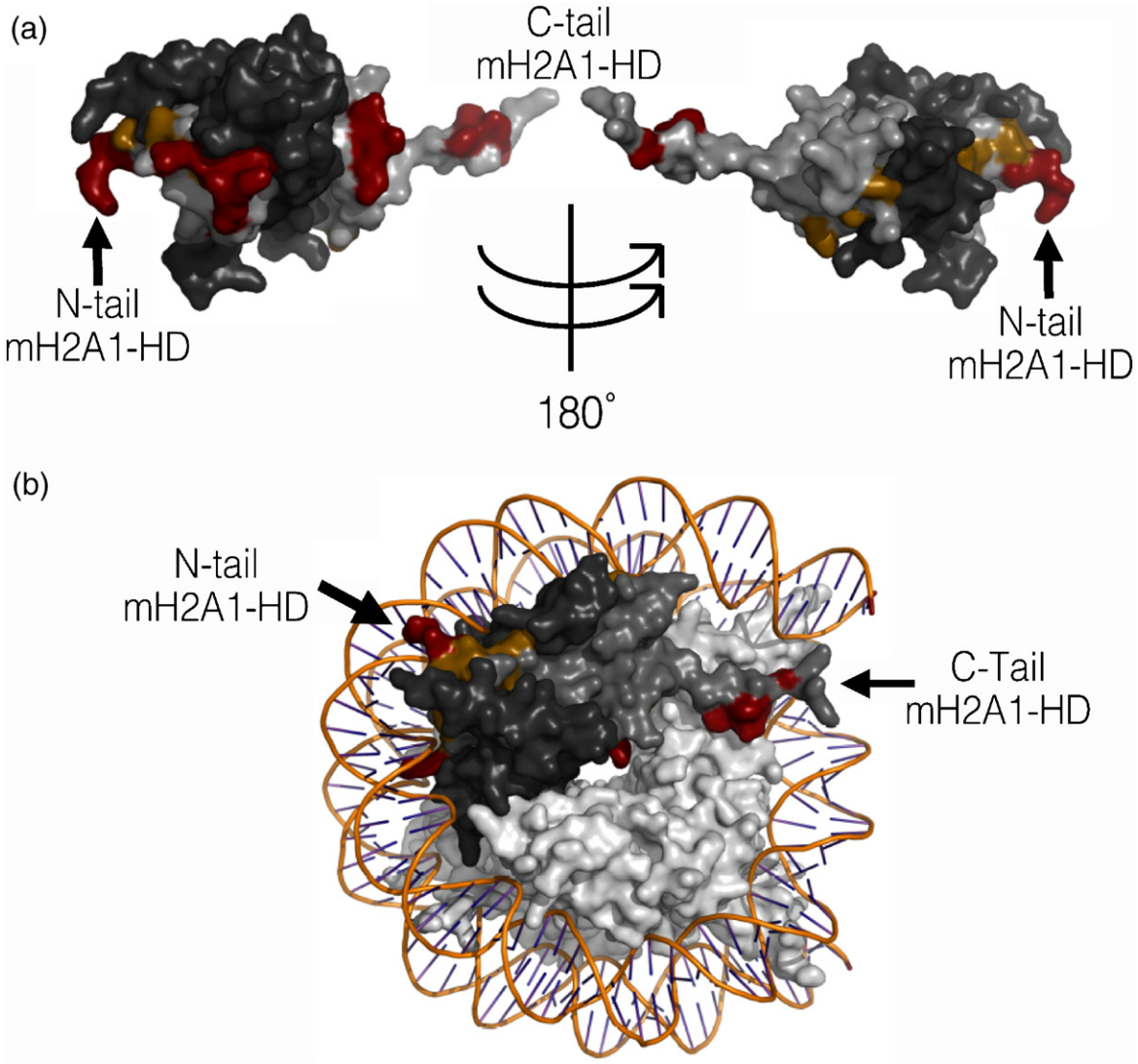

Figure 3. The residues that promote Xi-enrichment map on one face of the mH2A1-HD/H2B dimer. (a) The mH2A1-HD and H2B dimer from the mH2A1-HD containing crystal structure (PDB code 1A0I).[17] H2B is in dark grey and the mH2A1-HD is shown in medium grey. Residues that promoted Xi-enrichment are colored red and residues that do not are colored yellow. All crystal structure analysis was performed using Pymol v0.99 (DeLano Scientific: http://pymol.sourceforge.net/). (b) Top down surface view of the mH2A1-HD containing crystal structure (PDB code 1A0I).[17] H2B is dark grey, the mH2A1-HD is medium grey and all other histones are in light grey. Residues that induced Xi-enrichment are colored red and residues that did not are colored yellow. The DNA wrapped around the histone octamer is shown in ribbon format.

red). In contrast, many of these residues were buried within the nucleosome core when mapped onto the mH2A1-HD-containing nucleosome (Figure 3(b), shown in red). This clustering on the surface of the dimer suggests that these residues form an interface that may be recognized by factors responsible for incorporating macroH2A1/H2B dimers into nucleosomes.

H2A and H2A variants exhibit considerable sequence conservation; however, the regions in similar positions to those of mH2A1-HD that promoted Xi-enrichment (Supplementary Data, Figure 1, indicated by a red box) and those that did not (Supplementary Data, Figure 1, indicated by a yellow box) show considerable variability between variants. It seems likely that the non-conserved residues that promote localization could be used to discriminate between macroH2A and the other histone H2A variants during deposition/nucleosome assembly. Because the structures of the H2A variants determined to date are very similar to that of H2A,[17,20,31] the residues which align with those that promote Xi-enrichment would also cluster on a single face on other variants. It will be interesting to explore whether the homologous residues direct localization of other H2A variants, as they do for the macroH2A family. Such analysis may provide useful information toward the identification of the factors that mediate the distinct localization patterns of the H2A variants.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2007.05.063

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