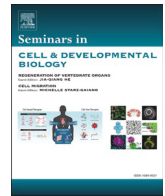




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Review

Seminars in cell and development biology on histone variants remodelers of H2A variants associated with heterochromatin

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ABSTRACT

Variants of the histone H2A occupy distinct locations in the genome. There is relatively little known about the mechanisms responsible for deposition of specific H2A variants. Notable exceptions are chromatin remodelers that control the dynamics of H2A.Z at promoters. Here we review the steps that identified the role of a specific class of chromatin remodelers, including LSH and DDM1 that deposit the variants macroH2A in mammals and H2A.W in plants, respectively. The function of these remodelers in heterochromatin is discussed together with their multiple roles in genome stability.

1. Introduction

The distribution of core histone protein variants constitutes an important layer of epigenetic information that differentiates regions of the eukaryotic genome. These variants are deposited and organized in a dynamic manner that is integrated into essential nuclear processes, such as DNA replication and transcription. This volume includes a number of reviews and perspectives highlighting the functional and structural diversity of histone variants. Here, we focus on H2A variants with an emphasis on those involved in formation of the specialized chromatin regions grouped under the umbrella term, heterochromatin. In this discussion, we will consider recent evidence demonstrating that one class of chromatin-remodeling ATPase proteins deposits specific H2A variants to chromatin, and then we will tie these findings to the experimental history of these chromatin remodelers.

2. Types of histone H2A variants

Most multicellular eukaryotes share at least three types of histone H2A variants: H2A.Z, H2A.X and replicative H2A while often only two types are present in unicellular eukaryotes (e.g. budding and fission yeast) [1–5]. The term “replicative” alludes to the fact that many, although not all variants in this group, are expressed during S phase in the cell cycle and are incorporated immediately after DNA replication. At the protein sequence level, H2A variants are primarily distinguished

by a few conserved residues in the core domain and sequence variations in their C-terminal tail. Generally, the C-tail is the shortest in H2A.Z, while replicative H2A and H2A.X are distinguished by the motif SQ[E/D]Φ in H2A.X (where Φ stands for a hydrophobic amino acid). In plants and yeast, serine phosphorylation of SQ[E/D]Φ is also essential for DNA damage response [3,6]. In this volume, other chapters are dedicated to H2A.X and H2A.Z, and these variants will not be discussed here further.

Unique types of H2A variants that are associated with transcriptional repression have also evolved in animals and plants [5,7]. Metazoans encode macroH2A, which contains a ~25 kDa ‘macro-domain’ at its C-terminal and is associated with domains of chromatin that are transcriptionally repressed [8,9]. The linker between the core histone and the macro domain corresponds to the C-terminal tail of other H2A variants, is enriched in K residues, and promotes compaction of nucleosome arrays *in vitro* [10]. The positive impact of macroH2A on chromatin condensation and its association with heterochromatin suggest further a role for macroH2A in transcriptional repression (see other chapters in this volume). Land plants also evolved two classes of H2A variants with long C-terminal tails enriched in K residues, H2A.M in bryophytes and H2A.W in vascular plants, that are further distinguished by the C-terminal KSPK motif [11,12]. Similar motifs are also found in macroH2A variants [5], and in sperm-specific H2B variants in sea urchins [13], as well as in linker histone H1 [14]. H2A.W confers distinct properties to the nucleosome through differences in its primary amino acid sequence

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in the L1 loop, the docking domain, and the KSPK motif in the extended C-terminal tail [12,15]. H2A.W exclusively occupies constitutive heterochromatin [11,16] in plants, while the bodies of expressed genes are covered by H2A and H2A.X, with the first nucleosomes occupied by H2A.Z [11,17]. In mammals, macroH2A can be found at constitutive or facultative heterochromatin, and it is frequently associated with transcriptional silencing, but a positive effect on transcription for a subset of genes has been reported as well [9,18,19]. The specific genomic location of each type of variant suggests dedicated mechanisms of deposition for each variant.

3. Chromatin remodelers and chaperones

At the DNA replication fork, nucleosomes are deposited by dedicated chaperones that assemble a tetramer (2H3-2H4) and two heterodimers H2A-H2B. The general chaperone proteins, such as Nucleosome Assembly Protein 1 (Nap1) and FAcilitates Chromatin Transcription (FACT), mediate deposition of the H2A-H2B heterodimer irrespective of the H2A variant [20–22]. To date, no chaperone has been identified that deposits a specific type of H2A variant at the DNA replication fork. Thus, additional undiscovered mechanisms must exist that explain why histone variants such as H2A.Z, macroH2A and H2A.W show enrichment at specific genomic locations. The incorporation of H2A variants into chromatin at specific genomic regions is generally not coupled to replication [3,9,23,24], so localized deposition *via* differential replication timing is not possible.

In addition to chaperones, chromatin remodeling complexes can regulate the deposition, as well as the removal, of H2A variants [3,25,26]. Chromatin remodeling complexes consist of multiple peptide subunits, and all contain at least one member of the SWI2/SNF2 subfamily of DNA-dependent ATPases, which belongs to the superfamily of SF2 helicases and translocases [27–30]. For example, the yeast SWR1 chromatin remodeling complex contains Swr1 as its DNA-dependent ATPase, and the yeast INO80 complex contains Ino80 as its SNF2-like protein [26]. Some chromatin remodelers have been shown to reposition nucleosomes on a small DNA fragment *in vitro*, while others promote disassembly of the histone octamer, alter nucleosome conformation, or perform exchange of histone variants. Some chromatin remodelers of the SWI2/SNF2 subfamily act only in a large multi-subunit complex, as has been reported for Swr1, whereas others can act *in vitro* on their own [31,32].

The highly conserved ATPase domain is found in all members of the SWI2/SNF2 family and is required for chromatin remodeling function [26,30,33]. SWI2/SNF2 members of the SF2 family share the ability of translocation along DNA with other helicases. However, SWI2/SNF2 members do not catalyze DNA strand separation as do *bona fide* helicases of the SF1 family because they lack the necessary wedge domain. One model suggests that DNA loops are formed during the process of directional translocation and that these loops are propagated on the nucleosome surface, resulting in repositioned nucleosomes.

SWI2/SNF2 family members are generally grouped into four or more subfamilies based on additional domains that confer specific functional properties [33,34]. For example, the INO80/SWR1 subfamily member Swr1 includes a helicase SANT-associated (HSA) domain that binds actin or actin-related proteins, while the ISWI subfamily contains a HAND-SANT-SLIDE (HSS) domain that recognizes unmodified histone H3 tails; other subfamilies are characterized by chromodomains or have additional bromodomains [30,34].

Several dedicated chromatin remodeling complexes have been identified that perform an exchange of the histone variant H2A.Z, including the yeast SWR1 complex [26,31,33]. The SWR1 complex shows a stepwise deposition of H2A.Z *in vitro*: first, one H2A-H2B dimer is exchanged with a H2A.Z-H2B dimer resulting in a heterotypic nucleosome, followed by another H2A.Z-H2B dimer deposition, resulting in a homotypic nucleosome [35]. The reaction of the SWR1 complex is unidirectional [31] and the reverse reaction, the removal of H2A.Z from

chromatin, can be performed by the INO80 complex [26].

A dedicated chromatin remodeler has not yet been identified for every histone variant. For example, no available evidence exists for chaperones or chromatin remodelers specific for deposition or eviction of H2A.B or H2A.X [30]. A few mechanisms have been reported that are responsible for extraction of macroH2A from chromatin. For example, ATRX, another member of the SWI/SNF family of DNA-dependent ATPases, is involved in the elimination of macroH2A1 at telomeres and the α -globin locus *in vivo* [36]. The chaperone FACT removes macroH2A2 at transcribed chromatin in a process termed "pruning" [9].

Until recently, no dedicated mechanism of macroH2A or H2A.W deposition had been identified [9,23]. Two recent reports demonstrate that DDM1 and LSH (see below) are the first chromatin remodelers identified that are involved in the deposition of H2A.W and macroH2A in the heterochromatin of plants and mammals, respectively. DDM1 and LSH are phylogenetically related and form, together with their human homolog HELICase Lymphoid Specific (HELLS), the DHL family of chromatin remodelers [37]. This review traces the discovery of these remodelers and reviews our current understanding of their mechanism and its functional implications.

4. Discovery of DDM1 and LSH

The discovery of DDM1 and LSH proceeded down two converging paths. DDM1 was first identified in a forward genetic screen conducted in 1990 using *Arabidopsis*. The rationale for the screen was a 1987 paper by Rattner and Lin [38] showing that chemically-induced DNA hypomethylation led to a dramatic decondensation of centromeric heterochromatin in mitotic chromosomes of the mouse. If centromere function was altered, which seemed likely given the treated chromosomes' grossly distorted structure, it was reasoned that mutations causing hypomethylation of centromeric DNA might prove useful to gain an understanding of this enigmatic region of the genome. At the time, it was known that *Arabidopsis* centromeres were composed of long tandem arrays of nucleosome-sized repeats, and that these repeats contained cytosine methylation as determined by differential digestion of restriction endonucleases whose cleavage was blocked by cytosine methylation [39]. The screen, which was based on DNA blots, netted three "decrease in DNA methylation" or *ddm* mutations, including two alleles of the *DDM1* gene [40]. Two surprising findings stood out from the outset: (i) DNA hypomethylation in *ddm1* mutants primarily affected repetitive DNA sequences, and (ii) the hypomethylation persisted after reintroduction of the sequences into a wild-type *DDM1* background by a genetic cross. These findings, published in 1993, suggested that *DDM1* did not encode a DNA methyltransferase, but the identity of DDM1 as a chromatin remodeler only became clear in 1999 after completion of a positional cloning effort [41]. In parallel to the screen just discussed, other *ddm1* alleles were recovered in a screen for loss of transcriptional gene silencing, which was accompanied by a loss of DNA methylation [42]. Despite the absence of a clear mechanism for DDM1 at the time of its discovery, the various *ddm1* alleles proved for the next two decades to be valuable tools to explore the maintenance of DNA methylation and its impact on transposons (see sections below).

In contrast to DDM1, LSH/HELLS was cloned based on its similarity to the superfamily of SF2 helicases [27,28,43]. Degenerate primers were designed comprising the highly conserved helicase domain I, which contains an ATP binding site, and Domain II, including a short 4 aa motif, DEXH. LSH/HELLS was cloned from T cell precursors and expression analysis by Northern blots suggested preferential expression in lymphocytes, hence the name "Lymphoid Specific Helicase" or "Helicase, Lymphoid Specific" [44,45]. Subsequently, a leukemia cell line served as an alternate source for the cloning of LSH/HELLS [46].

In primary cells, LSH is preferentially expressed in thymus, bone marrow, activated lymphocytes and testis, and it displays high expression in a number of cancer or leukemic cell lines [45–47]. Despite its preferential expression in lymphoid tissue, LSH also has non-immune

functions as newborns with a deletion of LSH die within hours after birth [47,48]. Since loss of LSH reduces DNA methylation levels (see below), the early death phenotype might be due to defects in the neuronal respiratory control, resembling the defects described for DNA methyltransferase DNMT1-mutant mice with DNA hypomethylation in neural tissue [49].

5. Investigation of how DDM1 and LSH affect the maintenance of DNA methylation

In flowering plants, cytosine methylation is found in three different nucleotide sequence contexts and there are specific DNA methyltransferases responsible for each [50]. For example, CpG methylation is maintained by METHYLTRANSFERASE1 (MET1), while CpHpG methylation (where H = A, C, or T) is deposited by the plant-specific CHROMOMETHYLASE 3 (CMT3). Plants also have cytosine methylation at asymmetric sites, CpHpH, which is modified by CHROMOMETHYLASE 2 (CMT2) and directed by an RNA-dependent DNA methylation (RdDM) pathway [51–53]. Loss of *DDM1* reduces both CpG and CpHpG methylation in Arabidopsis, rice and maize [52, 54–58] and primarily affects repetitive DNA sequences. This specificity was explained, in light of the subsequent discovery that DDM1 was a SF2 remodeler, by the hypothesis that the protein acted at the replication fork to mobilize nucleosomes and provide greater access of DNA methyltransferases to their substrate [41]. Evidence in support of this accessibility mechanism came from genetic studies showing that loss of H1 linker histones, and therefore reduced chromatin compaction, suppressed the hypomethylation phenotype of *ddm1* mutations [59]. This hypothesis is further strengthened by the retention of methylation on linker DNA, but not nucleosomal DNA, in Arabidopsis strains combining histone *h1* and *ddm1* mutations [60].

Because the loss of DDM1 is not lethal in Arabidopsis, the *ddm1* mutants were used to study the long-term consequences of depletion in DNA methylation. Inbred *ddm1* mutants show developmental abnormalities due to the accumulation of second-site mutations and epigenetic defects [54, 56, 61–63]. The latter are associated with either inappropriate loss, or ectopic gain, of cytosine methylation. Insight into the triggers for cytosine methylation came from study of successive generations of wild-type plants derived from backcrossed *ddm1* mutants, showing that *de novo* methylation in CpHpH contexts is directed by the RdDM pathway [64]. In this case, *de novo* methylation was deposited primarily on TE fragments often present in proximity of genes. Mutants deficient in DDM1 were also the foundation of epigenetic recombinant inbred lines, which have been used to study the potential impact of DNA methylation on TE and gene expression [65–67]. Hence, *ddm1* mutants have facilitated the exploration of the impact of DNA methylation on TE in adaptation [68].

Mammalian cells, in contrast to plants, have cytosine methylation predominantly in the context of CpG sequences [69,70]. The DNA methyltransferases DNMT3A and DNMT3B are involved in the *de novo* establishment of DNA methylation patterns that are generated during embryogenesis, germ cell development and upon differentiation of embryonic stem (ES) cells [70,71]. Once established, methylation patterns are maintained at the replication fork via the ‘maintenance’ activity of DNA methyltransferase 1 (DNMT1), which prefers hemi-methylated DNA as a target [70].

Based on its similarity to DDM1 and the discovery that DDM1 is required for maintenance of cytosine DNA methylation patterns [40,41], LSH-deficient tissues and embryonic stem (ES) cells derived from knock-out mice were probed for DNA methylation. Genomic DNA derived from LSH-depleted embryos showed a 40–60% decrease of DNA methylation compared to wild-type embryos using diverse techniques, such as DNA blot analysis, thin-layer chromatography, and MeDIP (methylated DNA-immunoprecipitation) analysis [72–75]. Using bisulfite sequencing to determine methylation maps with single base resolution, it was found that methylation levels in the absence of LSH were

about 55% in murine embryonic fibroblasts (MEFs) compared to 85% in wild-type MEFs [76]. The level of methylation in LSH knockout MEFs resembles the degree of genomic methylation in undifferentiated murine ES cells [76].

LSH-depleted ES cells exhibit a greatly impaired capacity of *de novo* DNA methylation upon differentiation [77–79]. In contrast, the maintenance of methylation pattern of an episomal vector was largely unaffected. Further, the preservation of DNA methylation at most examined imprinted genes was unaltered in the absence of LSH [77,80]. These findings led to the hypothesis that LSH promotes *de novo* methylation rather than maintenance methylation, for example, by facilitating the association of DNMT3B with target chromatin [77–79, 81]. Alternatively, LSH could also facilitate DNMT1 recruitment through UHRF1, an accessory factor that targets DNMT1 to the replication fork [82,83]. Recent reports indicate that LSH depletion also reduces hydroxycytosine methylation (5hmC), an oxidative product of methylated cytosine generated by TET proteins [84–86]. This effect may be mediated by LSH interaction with TET2 or through regulation of TET2 mRNA [84,86] or possibly as a consequence of reduced cytosine methylation, which is a pre-requisite of 5hmC.

In ES cells, DNA methylation is thought to follow gene repression and to lock in gene silencing. LSH-depleted ES cells show impaired or delayed repression of stem cell genes – a phenomenon which is associated with reduced CpG methylation levels [77,78]. A reporter gene can be silenced upon recruitment of LSH [87,88] and some cancer cells show LSH-mediated silencing of selected genes associated with DNA methylation [89–92]. However, most protein-genes show normal expression in LSH-depleted MEFs, despite reduced CpG methylation, indicating possibly redundancy of silencing pathways in differentiated cells [74, 76]. An exception are subclasses of repetitive elements that are de-repressed in LSH-depleted cells [72,76,93,94]. These repetitive sequences, such as endogenous retroviral elements, typically undergo *de novo* DNA methylation during early mammalian embryogenesis [95]. However, in LSH-depleted ES cells these sequences show impaired *de novo* CpG methylation. This effect depends on the ATP binding site of LSH [79,96], indicating that the protein’s DNA translocase activity is essential to facilitate methylation of these repetitive sequences.

In conclusion, DHL remodelers impact DNA methylation through pathways that remain incompletely understood. Yet several findings suggest that the control of DNA methylation is not the ancestral or primary function of DHL remodelers. DDM1 and LSH orthologs exist in species that are devoid of DNA methylation, such as budding yeast [97]. In other species, these orthologs can be deleted without altering DNA methylation, as is the case in the fungus *Neurospora crassa* [98]. Moreover, recent evidence provides an explanation for this apparent disconnect between DHL remodelers and DNA methylation by demonstrating that DDM1 and LSH act directly on histone variant deposition and likely impact DNA methylation primarily through indirect mechanisms.

6. Deposition of histone variants by DDM1 and LSH into chromatin

While the ATP binding site is critical for DDM1 and LSH function *in vivo*, these remodelers do not contain any of the HSA, HSS, chromo- or bromo domains characteristic of other SWI/SNF2 subfamily members [79] and they belong to the same subfamily as ISWI [99]. Recombinant LSH and DDM1 support nucleosome sliding activity *in vitro* [100,101]. However, it remained unclear if this activity occurs *in vivo* and how DHL remodelers contribute to heterochromatin formation and repression of retroviral elements. Thus, the primary molecular function of DHL remodelers remained unknown until recently.

The impact of *ddm1* on chromatin accessibility at transposons suggested that this remodeler expels the linker histone H1 to maintain access to DNA methyltransferases [59,60,102]. However, it was later shown that DDM1 does not bind to H1 and does not impact its deposition

nor enrichment at transposons, leading to a search for a histone variant that could be deposited by DDM1 [37]. The catalytic site of DDM1 was identified and mutated to establish which activities of DDM1 are ATPase-dependent. *In vitro* assays showed that DDM1 binds the H2A.W-H2B dimer but not the most closely related variants H2A.X and replicative H2A [37]. A series of truncations on the N- and C-terminal regions identified two *circa* 30-aa long binding domains located on either sides of the ATPase central domain [37]. These domains are conserved amongst DDM1 homologs in plants, animals and fungi (Fig. 1). Western blots and genomic profiling of enrichment of H2A variants in *ddm1* showed that DDM1 is necessary for the deposition of H2A.W to pericentric heterochromatin. DDM1 deposition targets primarily families of transposons that are not methylated by the RNA-dependent DNA methylation pathway and comprise both DNA transposons and retrotransposons located in the pericentromeric regions. Gain-of-function experiments further showed that DDM1 is sufficient for the deposition of H2A.W even in absence of H3K9me1/2 and DNA methylation, epigenetic hallmarks of heterochromatin. Further genomic concluded that the primary function of DDM1 is the deposition of H2A.W to transposons that have the capacity to transpose rather than fragments of transposons [37]. Whether DDM1 catalyzes an exchange between H2A.W and another H2A variant remains unknown.

LSH is associated with heterochromatin, and LSH depletion induces DNA hypomethylation, alters MNase chromatin accessibility and changes histone modifications associated with repressed chromatin [76, 79, 88, 103–107]. To uncover LSH function in an unbiased way, a chemical-induced proximity assay was applied in which LSH was temporarily tethered to a genetically modified *OCT4* allele containing a GFP reporter [108]. An array of several DNA binding domains for the transcription factor ZFHD1 had been inserted upstream of the *OCT4* transcription start site using murine embryonic stem (ES) cells. The drug rapamycin was able to tether LSH to the locus via the DNA binding domain of ZFHD1. The system allowed a dynamic, reversible recruitment of LSH within minutes after drug treatment. Using this approach, it was found that wild-type LSH, but not the ATPase mutant, induced repression of the GFP reporter [108]. Common repressive marks, such as H3K27 methylation, H3K9 methylation or DNA methylation, were not apparent at the reporter locus. Instead, tethering of wild type LSH induced the accumulation of macroH2A1 and macroH2A2 and this deposition was dependent on the ATP binding site of LSH [108]. The reaction was histone variant specific, since H2AZ was not deposited, and other histones (H3 and H1) remained unchanged. Furthermore, suppression of the GFP reporter depended on macroH2A deposition, indicating that LSH mediated its transcriptional repression through macroH2A [108]. Thus, the *in vivo* system demonstrated that LSH was able to specifically deposit macroH2A upon recruitment in an ATP-dependent manner. In addition, LSH-depleted cells show genome-wide a 28% reduction of macroH2A domains and 53% reduction of peaks, indicating that LSH affects macroH2A deposition under physiologic conditions [108]. Yet, macroH2A was not completely absent in the genome, suggesting a redundancy in factors that promote macroH2A deposition [108].

To test whether LSH is directly or indirectly responsible for *in vivo*

macroH2A deposition, LSH interaction with macroH2A was examined [108]. LSH and macroH2A can be co-immunoprecipitated in cells, and furthermore LSH interacts with the H2A histone fold domain of macroH2A, as demonstrated using recombinant proteins *in vitro*. This interaction was specific, since LSH did not pull down H2A.Z [109]. *In vitro*, LSH directly induced the incorporation of macroH2A-H2B dimer in an ATP-dependent manner [109]. Furthermore, LSH mediated transfer activity is specific, since only macroH2A, but not H2A.Z was transferred. Both isoforms, macroH2A.1 and macroH2A.2, were equally utilized by LSH. The reaction was unidirectional, and LSH could not exchange macroH2A with replicative H2A or one macroH2A molecule with another macroH2A molecule [109]. A similar directionality had been reported for H2A.Z histone exchange mediated by SWR1 [31]. Kinetic analysis detected a two-step mechanism with generation of heterotypic nucleosomes followed by accumulation of homotypic macroH2A nucleosomes over time [109]. A stepwise dimer replacement had been also reported for SWR1 activity [35].

Taken together, these *in vivo* and *in vitro* experiments demonstrate that DHL remodelers catalyze ATP-dependent deposition of heterodimers, including one H2A variant with H2B, into nucleosomes making LSH and DDM1 the first examples of a macroH2A/H2A.W deposition factor in the SWI/SNF2 family of chromatin remodeling enzymes.

7. Biological relevance

It has been suggested that DNA methylation is critical to suppress parasitic genes and their movement in plants and animals during development [68, 95, 96, 110–113]. Transposons and repetitive sequences derived from transposons represent up to two-thirds of mammalian genomes and up to 90% of the genomes of some plant species [68,114]. Mobility of transposable elements introduce mutations and *cis*-elements that participate in evolution and adaptation [115,116], but may be deleterious. Therefore, the transcriptional activity of transposons is tightly controlled and generally suppressed. LSH and DDM1 are critical to achieve silencing of endogenous retroviral elements in mammals [76,93,94] and the vast majority of transposon transcribed regions in plants [37,56,117,118], respectively. In plants, DDM1 also suppresses the movement of transposons [56,119,120]. Because depletion of DNA methylation activates subclasses of retroviral genes in mice [94] and several classes of transposons in plants [117,121], it was long assumed that DNA methylation is directly responsible for transcriptional repression because loss of the DNA methyltransferases MET1 and CMT3 also de-represses transposons [51,122,123]. Similarly, the loss of H3K9 methylation in *ddm1* mutants could explain the loss of transposon repression [51,124,125]. However, the variants deposited by LSH and DDM1, macroH2A and H2A.W have also been associated with repression of certain subclasses of transposons [126,127], suggesting that they might be directly responsible for the impact of these remodelers on transposon activity. This idea is supported by *de novo* repression of transposons, which were originally activated in a *ddm1* mutant, in F1 *ddm1/DDM1* hybrids without changes in levels of H3K9me2, DNA methylation and H1 [37]. It is also likely that macroH2A and H2A.W cooperate with other heterochromatic marks to silence transposons,

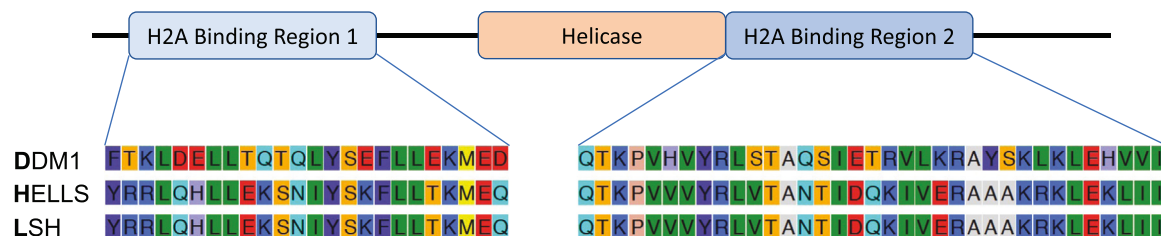


Fig. 1. Conservation of functional domains in DHL remodelers. We highlight the catalytic helicase domain flanked by two domains that have been shown to bind H2A.W-H2B heterodimers by DDM1, along with their alignments with the corresponding domains in HELLS and LSH. Note that the domains are not drawn to scale.

explaining the widespread impact of the loss of DHL remodelers on transposon silencing.

Depletion of DDM1 also has a strong impact on gene and transgene expression in plants [42, 128–130]. This effect might be explained by the presence of transposon-derived *cis*-acting elements that generate cryptic transcription start sites (TSS) that interfere with initiation of transcription at bona fide TSS [130]. The mechanism that prevents this spurious transcription has not yet been determined but the large impact on transcription likely explains the many facets of the phenotype of *ddm1* mutants. Similarly, mutation of LSH might affect globally transcription causing multiple defects including the ICF4 syndrome, a multi-organ disease with increased childhood lethality [131–133]. ICF is an acronym for immune deficiency, centromeric instability and facial anomalies. Mice with a deletion of LSH die perinatally and display multiple organ defects including stem cell defects and germ cell defects [47, 48, 73, 89, 134–136]. Some of the phenotypes in the LSH knockout mouse resemble the ICF disease; for example, reduced neural progenitor expansion in the absence of LSH may contribute to neural malfunction and mental retardation seen in ICF patients and abnormal Hox gene expression may be responsible for facial anomalies and congenital defects [137,138]. The immune deficiency defect in the absence of LSH is, at least in part, due to a defect in class switch recombination, a process that is required for the generation of immunoglobulin isotypes [139]. Upon LSH depletion B cell development and switch recombination efficiency are impaired resulting in a severe decrease of immunoglobulin production, one of the hallmark characteristics of ICF patients [139]. There is only a partial overlap in the phenotypes comparing macroH2A knockout mice [140] to LSH knockout mice, including reduced growth, reproductive problems in the C57BL/6 background and increased perinatal lethality (about 30% in macroH2A KO and 100% in LSH KO). This result may suggest that LSH has additional roles beyond macroH2A deposition. On the other hand, some phenotypes are not as overt and have been only recently described; for example, macroH2A depletion leads to a defect in B cell development and neurological defects [141–143].

LSH-depleted cells and *ddm1* mutants display signs of spontaneous DNA damage and genomic instability [136, 139, 144–147]. DDM1 also protects against telomere truncation [148]. It has been noted that LSH plays a role in DNA repair and that it promotes joining of broken DNA ends, but a precise mechanism remains unknown [83,139,147,149]. A recent study suggests that LSH or DDM1 depletion induces susceptibility to replication fork stalling and degradation of nascent DNA that leads to double-stranded DNA breaks [150,151]. The protection of stalled replication forks by LSH depends on its chromatin remodeling activity and is mediated by macroH2A deposition [151]. Deficiency of macroH2A has been previously found to confer susceptibility to replication stress [152]. The altered chromatin environment at the replication fork in LSH-depleted cells leads to an imbalance of histone modifications that are critical for recruitment of DNA repair factors [151]. Thus, LSH-depleted cells accumulate 53BP1 at the cost of BRCA1 resulting in impaired RAD51 loading and instability of stalled replication forks [151]. This pathway may contribute to the increased DNA damage and genomic instability that occurs in ICF4 cells. Controlling DNA repair may be an ancestral function of DHL remodelers because the fungus *Neurospora* DDM1/LSH ortholog MUS-30 is important for base excision repair pathway and interacts with RAD51-dependent homologous recombination [98]. Additional evidence in support of this hypothesis comes from budding yeast and the observation that mutation of its DDM1/LSH ortholog (IRC5/YFR038W) dramatically increases the numbers of spontaneous Rad52 DNA repair foci [153].

ICF syndromes can be caused by mutations in either Dnmt3b, ZBTB24, CDCA7 or LSH/HELLS [131–133]. The transcription factor ZBTB24 can regulate CDCA7 gene expression and may also participate in recruitment of DNMT3B to specific loci [154–156]. CDCA7 may promote association of LSH/HELLS with chromatin or act as co-factor in chromatin remodeling [101,157]. Thus ZBTB24, CDCA7 and

LSH/HELLS may share common pathways [147, 158–160]. LSH/HELLS has been shown to promote association of DNMT3B with chromatin targets and control DNA methylation [77–79, 81, 87]. All ICF patients share some DNA hypomethylation defects at pericentromeric repeats [158,159]. The relation between LSH/HELLS mediated macroH2A deposition and DNA methylation and the role of macroH2A in DNMT3B mutant cells is not yet understood. It has been suggested that DNA hypomethylation can induce reorganization of heterochromatic macroH2A [161]. A reduction of macroH2A deposition has been found in LSH/HELLS mutant cells but has not been examined as yet in other ICF models [108]. The precise molecular pathways underlying these complex diseases remain largely unknown.

8. Conclusion and outlook

Now that it has become clear that the effect of the mutants of DHL remodelers on DNA methylation was indirect, the discovery of the deposition of H2A variants to heterochromatin by DHL remodelers opens new and exciting avenues of research. One set of questions and experimental priorities will focus on understanding the mechanisms through which DHL remodelers act. For instance, what unique features distinguish DDM1 and LSH from other chromatin remodelers from the same clade, which also contains ISWI and less-studied remodelers? LSH exchanges replicative H2A to macroH2A, but whether DDM1 catalyzes such an exchange is unknown. Exchange of H2A to H2A.Z in yeast involves a protein complex with the catalytic subunit SWR1. Yet, no *bona fide* LSH complex or DDM1 complex has yet been identified, and it is unclear whether LSH and DDM1 co-operate with other factors *in vivo*. Such factors might help explain the targeting of DHL remodeler activities. LSH and DDM1 do not affect all regions of heterochromatin, and it will be important to elucidate how LSH or DDM1 are recruited to chromatin and why only certain genomic locations are affected. Since residual levels of macroH2A and H2A.W remain in the absence of LSH and DDM1, respectively, other as yet unidentified mechanisms must be able to deposit these variants in heterochromatin. We predict that different remodelers deposit macroH2A and H2A.W preferentially to distinct elements or locations within repressed domains of chromatin.

Answering the mechanistic questions just posed will also bring us closer to an understanding how DHL remodelers fulfill their larger roles in management of the genome. For example, how does DDM1 suppresses transposition? It is currently assumed that this suppression is the consequence of silencing transposase and other genes from transposons, but the impact of DDM1 on chromatin could directly prevent transposition. Recent work suggested that H2A.Z is a positive factor for transposon integration [162], so it is possible that H2A.W acts in an opposite manner. The complexity of the ICF4 syndrome in humans, even as manifested at the cellular level, and the diverse DNA repair and recombination defects exhibited by LSH-deficient mice and murine cell lines underscore how integral DHL remodelers are to the maintenance of genome stability.

Indeed, as discussed above, work in the fungi *Neurospora* and budding yeast suggest that the ancestral role of DHL remodelers may lie in DNA repair and replication pathways [98,153]. Our knowledge of these proteins, however, comes primarily from their participation in the establishment or maintenance of two very distinct chromatin features, namely histone variants and DNA methylation (see Fig. 2). It remains unclear if these functions represent evolution of distinct novel activities or an expansion of an ancestral function, and whether the process of macroH2A/H2AW deposition and DNA methylation are linked or represent independent events. At least it is clear that these convergent functions of DHL remodelers were acquired independently during evolution by an ancestral remodeler dedicated to the deposition of an H2A variant present in LECA because orthologs of DHL remodelers exist in organisms with no heterochromatin-specialized H2A variants. Functional studies of these orthologs are expected to reveal conserved elements of the mechanisms of action of DHL remodelers, which will be

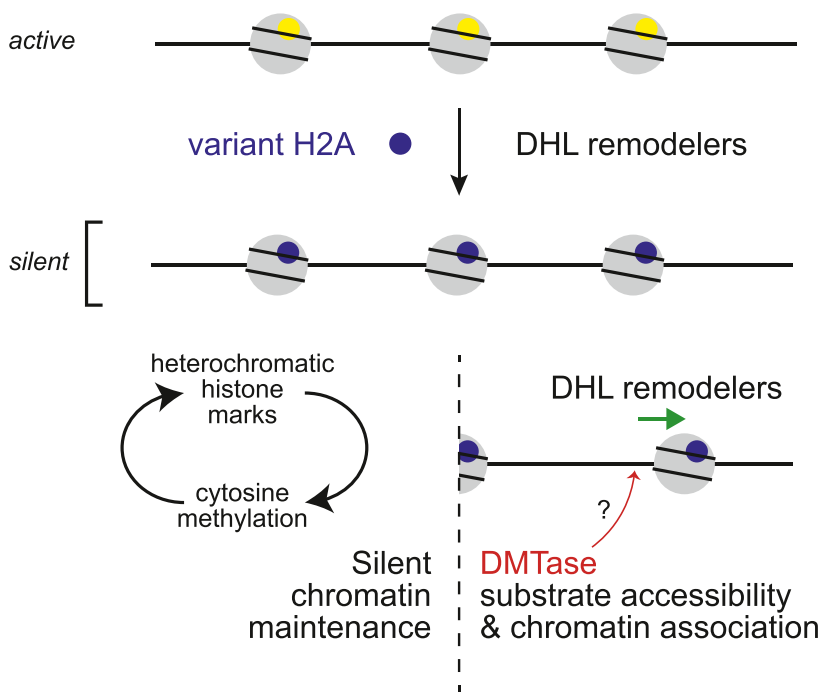


Fig. 2. DHL remodelers help initiate and maintain silent chromatin in plants and mammals. The DHL chromatin remodelers act to deposit histone H2A variants (blue circles) associated with silent chromatin, in exchange for replicative H2A proteins (yellow circles). Genomic regions with nucleosomes containing these variants are targeted for silent chromatin formation, reinforced by through a feed-forward cycle (lower left) involving the acquisition and maintenance of additional chromatin-level marks (e.g., histone H3K9 methylation). These marks may include cytosine methylation, and, consequently, the decrease in silent chromatin due to loss of DHL remodeler activity can lead indirectly to a reduction in DNA methylation. In addition, DHL remodelers appear to facilitate (lower right) the action of DNA methyltransferases, by increasing access to DNA substrates and/or by stabilizing interactions with chromatin.

essential to understand their functional diversification in flowering plants and mammals.

Declaration of interest

None.

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