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Histone variants in the specialization of plant chromatin

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Abstract

The basic unit of chromatin, the nucleosome, is an octamer of four core histone proteins (H2A, H2B, H3, and H4) and serves as a fundamental regulatory unit in all DNA-templated processes. The majority of nucleosome assembly occurs during DNA replication when these core histones are produced *en masse* to accommodate the nascent genome. In addition, there are a number of non-allelic sequence variants of H2A and H3 in particular, known as histone variants, that can be incorporated into nucleosomes in a targeted and replication-independent manner. By virtue of their sequence divergence from the replication-coupled histones, these histone variants can impart unique properties onto the nucleosomes they occupy and thereby influence transcription and epigenetic states, DNA repair, chromosome segregation, and other nuclear processes in ways that profoundly affect plant biology. In this review we discuss the evolutionary origins of these variants in plants, their known roles in chromatin, and their impacts on plant development and stress responses. We focus on the individual and combined roles of histone variants in transcriptional regulation within euchromatic and heterochromatic genome regions. Finally, we highlight gaps in our understanding of plant variants at the molecular, cellular, and organismal level, and we propose new directions for study in the field of plant histone variants.

Introduction

Throughout eons of evolution, the nucleosome has remained a defining characteristic of eukaryotes. As the fundamental unit of chromatin, the nucleosome acts as a barrier between DNA and interacting proteins, making it an integral regulatory component in virtually every DNA-templated process. The nucleosome consists of ~147 bp of DNA wrapped around a histone octamer containing a core $(H3\text{-}H4)_2$ tetramer flanked by two H2A-H2B dimers. Histone-histone and histone-DNA interactions contribute to nucleosome stability, while the tails of each histone provide a substrate for posttranslational modifications and protein binding. During replication, DNA content doubles and so does the demand for nucleosomes. To accommodate this demand, canonical histone genes evolved into often intronless multigene families whose expression is tightly linked to the cell cycle with highest expression during S phase. In contrast, histone variants have introns and often show replication-independent expression and deposition. The naming conventions for histone

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variants generally consist of a prefix indicating the histone protein family (e.g. H2A) to which they belong, followed by a period and a number or letter indicating a specific variant type (e.g. H2A.Z) (130).

Some histone variants such as H2A.Z are conserved throughout eukaryotes while others are lineage-specific, such as the flowering plant-specific H2A.W variant. By changing nucleosome composition, histone variants can change the internal stability of a nucleosome, DNA-histone interactions, internucleosomal interactions, accessibility to chromatin binding proteins, as well as potential posttranslational modifications. All of these changes alter the chromatin landscape and influence key nuclear processes. Therefore, histone variants represent a wealth of currently untapped information that will contribute to answering several of the outstanding questions of eukaryotic epigenetics.

In this review, we assess the current understanding of plant variant histones with a focus on the roles they play in transcriptional control. The eukaryotic genome can be partitioned into transcriptionally permissive euchromatic regions and transcriptionally repressive heterochromatic regions of both facultative and constitutive types. Histone variants H3.3, H2A.Z and H2A.X are found in euchromatic regions. Recent reviews of H3.3, H2A.Z, and H2A.X can be found in Borg et al. and Lei and Berger (9, 69). Here, we discuss how H3.3 is implicated in promoting chromatin accessibility in ways potentially unique to plants. H2A.Z has a more complex relationship with gene expression and we discuss evidence that implicates the variant as both a transcriptional activator and repressor. While H2A.X is known primarily for its role in DNA damage response, we focus on recent evidence pointing toward a role for H2A.X in transcriptional control. Other histone variants contribute to heterochromatin function and we highlight H2A.W and H1, which have also been recently reviewed in Lei and Berger, Kotlinski et al., and Probst et al. (63, 69, 107). H2A.W serves as the heterochromatic counterpart to H2A.X with respect to the DNA damage response, and its structure is thought to promote chromatin condensation. Finally, we call attention to the oft overlooked linker histone H1 and analyze how chromatin structure is dependent on H1 in both heterochromatin and euchromatin (See Figure 1).

Euchromatin-Associated Histone Variants

H3.3

In plants, histone H3 proteins are categorized into 4 groups: canonical histone H3.1, H3.3 variants, centromeric H3 variants, and H3-like histones. Centromeric H3 defines the centromere and is essential for kinetochore assembly and proper cell division, while the function of H3-like variants is largely unknown (152). Plant H3.3 contains many features typical of histone variants including introns, replication-independent deposition into chromatin, and expression in terminally differentiated tissue (52, 99, 127). Despite these differences, H3.3 differs from H3.1 at only 4 amino acids (written H3.1→H3.3): A31T, F41Y, S87H and A90L (Figure 1B). The *Arabidopsis* genome possesses three H3.3 genes encoding identical proteins (Table 1) (130). Evolutionary analysis of H3 proteins shows that H3.3 evolved independently in plants and animals (139, 140). Despite their independent origins, H3.3 differs from H3.1 at three of the same amino acids in both plants and animals, with H3.1 in flowering plants having an additional amino acid substitution at residue 41

(139). This evidence of convergent evolution strongly points toward the importance of H3.3 to the function of the eukaryotic genome.

Few studies have investigated exactly how H3.3 is deposited into plant chromatin. In mammals, H3.3 variants are incorporated into genic regions by the HIRA (Histone Transcriptional Regulator A) and in nongenic regions such as pericentromeric repeats and telomeres by ATRX/DAXX (Alpha Thalassemia-mental Retardation X-linked syndrome/ Death-domain Associated protein) (Table 1) (70, 111). *Arabidopsis* ATRX mutants do indeed have altered global H3.3 levels (33, 95). While *atrx* mutants are viable, *hira atrx* double mutants result in partial lethality and show strong developmental defects in the surviving plants, indicating a potential cooperation between ATRX and HIRA. (33). Interestingly, *atrx* mutants display loss of H3.3 in genic regions while H3.3 enrichment at transposable elements (TEs) and pericentromeric regions is unchanged (33). This is counter to observations in mammals where ATRX deposits H3.3 at non-genic regions, suggesting a functional divergence of ATRX-dependent H3.3 localization between plants and mammals (33).

H3.3 localization and relationship with gene expression—While the H3.3 genomic distribution pattern is different from H3.1 in both plants and animals, their respective distribution patterns are highly similar across species (127). In *Arabidopsis*, immunofluorescence and ChIP-seq experiments show that H3.1 is generally enriched at transposable elements, pericentromeric heterochromatin, and heterochromatin domains in the arms, while H3.3 is associated with euchromatic and nucleosome-depleted regions (NDR) (122, 123, 127, 144). Recent evidence indicates that this distinction in H3.1 and H3.3 distribution is caused in part by sequence variation at amino acid 41: Phe in H3.1 and Tyr in H3.3. Alignment analysis of monocot, dicot and ancient plant histone H3 reveals that the Phe41 residue first appeared in fern H3.1 and established in land plants (76). Lu et al. showed that while Tyr41 is not important for the genomic distribution of H3.3, a Phe41Tyr point mutation in H3.1 causes the protein to lose its heterochromatin-specific localization and spread into active regions (76). This is especially surprising considering that animal H3.1 and H3.3 both have Tyrosine at position 41, and are still able to maintain distinct localization patterns. Tyrosine differs from Phenylalanine in its ability to be phosphorylated. In human cells, H3 is known to be phosphorylated at Tyr41 and this is thought to help prevent heterochromatic proteins from binding active regions (27). Therefore, one hypothesis drawn from these results is that Phe41 evolved to differentiate H3.1 from H3.3 in plants where phosphorylation at Tyr41 has not yet been reported. Alternatively, these results could indicate that H3.1 Phe41 evolved to achieve an additional degree of chromatin targeting unique to vascular plants.

Highly expressed genes have enrichment of H3.3 over the transcribed region, or gene body, with a bias toward the 3’ end (123, 127, 144). However there is no correlation between H3.3 occupancy and transcriptional changes in *h3.3* knockdown (*h3.3kd*) plants, and H3.3 appears to be dispensable for general transcription. This is particularly surprising considering that complete loss of H3.3 is lethal (145). However, a reduction in H3.3 in some stress-responsive genes was associated with reduced transcript levels in *h3.3kd* mutants. Thus, H3.3 likely plays a specific role in the activation of groups of genes that are involved

in environmental responses, while not impacting transcription globally (145). Also, a recent study demonstrated that H3.3 inhibits flowering by increasing H3K4me3 and H3K36me3 levels at the *FLOWERING LOCUS C* (FLC) gene (156). The authors found that an interaction between FRIGIDA (FRI) and the HIRA chaperones results in the deposition of H3.3 at the 3' end of *FLC*. Consequently, increased H3.3 at the 3' of *FLC* aids in formation of a gene loop, increasing the interaction between the 5' and 3' end, thereby promoting transcriptional activation (156).

While H3.1 overlaps with several repressive chromatin modifications including DNA methylation, H3K9me2, and H3K27me1/3, H3.3 overlaps with several active chromatin marks like H3K4me3, H3K36me3, H3K9me3, H2B ubiquitylation, and RNA Pol II occupancy (127, 144). Despite these correlations, genome-wide patterns of H3K4me3 and H3K36me3 are relatively unchanged between *h3.3kd* mutants and wild type *Arabidopsis* (145). However, H3.3 was shown to promote H3K4me3 at a subset of genes with shorter length (<1 kb) (156). Interestingly, loss of H3.3, specifically over gene bodies, is associated with a decrease in DNA methylation and an increase in H1 occupancy (Figure 2A) (145). Additionally, chromatin accessibility assays showed that H3.3-containing nucleosomes are more sensitive to DNase I activity (123). Since H1 has been shown to prevent binding of DNA methyltransferases in pericentromeric heterochromatin, H3.3 may serve as a foil to H1 in euchromatic regions, with gene body H3.3 increasing chromatin accessibility to DNA methyltransferases by preventing H1 deposition (145, 153). Crystal structures of H3 methyltransferases ATXR5/6 reveal their ability to methylate lysine 27 of H3.1 but not H3.3. Therefore, H3.3 could also attenuate the Polycomb pathway of gene repression, of which H3K27 methylation is a key element (56). This difference also suggests that H3.3 can not only stimulate relaxed chromatin but can also perpetuate this chromatin state across cell divisions by preventing the establishment of heterochromatic marks.

H3.1 replacement by H3.3 is also a marker for cell fate transitions. Cells undergoing their last cell cycle before differentiation have a lower H3.1/H3.3 ratio and a higher rate of H3.1 eviction compared to dividing cells. This ratio is thought to change in the cells exiting the root meristem because H3.1 replacement with H3.3 occurs during the G2 phase, a phase that is longer in this last cell cycle than in earlier cycles, allowing more time for H3.1 eviction (101). This phenomenon is found in several plant developmental processes, including the stomatal and hypocotyl cell lineages, suggesting H3.1 eviction is a general feature in cell proliferation and organogenesis (49, 101).

H2A.Z

H2A.Z can be traced to a single evolutionary origin and its maintenance through nearly all branches of Eukarya underscore its vital role in multicellular development (80). Since the discovery of H2A.Z, it has been linked with numerous biological processes like plant immunity, germline development, and stress response as well as cellular processes including genome stability, DNA repair, as well as both transcriptional activation and repression (Table 1) (57, 82, 84, 85, 108, 113, 149). H2A.Z composes on average 15% of total H2A cellular content and loss of H2A.Z is lethal in most multicellular and some unicellular eukaryotes including *Tetrahymena*, *Drosophila*, mice and humans (17, 37, 57, 74, 136). This

surprisingly is not the case for plants, where loss of H2A.Z in *Arabidopsis* is not lethal but does lead to a severe and pleiotropic phenotype including stunted growth, early flowering, and reduced fertility (16, 20, 82, 94). This tolerance of H2A.Z loss makes plants an exciting model to probe the mechanisms of this histone variant in transcriptional regulation.

H2A.Z is deposited into the post-replicated nucleosome as an H2A.Z/H2B dimer by the SWI2/SNF2-related 1 complex (SWR1), a member of the INO80 subfamily of chromatin remodelers (Table 1) (62, 87, 137). At the level of primary structure, H2A.Z varies from H2A in three prominent ways; the docking domain, the L1 loop and the acidic patch (Figure 1B). The implications of these differences with respect to chromatin binding and gene regulation have been reviewed by Bönisch and Hake (8). Briefly, the extended acidic patch of H2A.Z is speculated to increase the opportunity for interactions between adjacent nucleosomes as well as secondary protein binding (31, 38, 104). Taken individually, the changes to the docking domain and the L1 loop appear to have opposing effects on nucleosome stability. The docking domain of H2A.Z exhibits less hydrogen bonding with H3, suggesting nucleosome destabilization, while the 4 amino acid substitutions found in the L1 loop have been shown to increase histone octamer stability (1, 8, 129). Additionally, amino acid substitutions in the H2A.Z C-terminus reduce binding of linker histone H1 to the core nucleosome particle (158).

While all plants appear to have H2A.Z, they do differ in the number of H2A.Z paralogs, and in some cases distinct splice variants exist within organisms (32, 61). The *Arabidopsis* genome encodes three expressed H2A.Z proteins; HTA8, HTA9, and HTA11 (Table 1). Although mutant analysis in plants indicates substantial redundancy between isoforms, they do exhibit distinct expression levels and patterns (124). As sub functionalization has been shown in some animals, future investigations will reveal any unique roles between paralogs in plants (34, 97). Interestingly, we found that the expression pattern across tissue types of the various *Arabidopsis* H2A.Z paralogs is synchronized with corresponding somatic H2B isoforms, with HTA11 and HTB2 having matched expression profiles, as do HTA9 and HTB4. This suggests that H2A.Z isoforms have preferred dimerization partners when deposited in the nucleosome (124).

H2A.Z localization and relationship with gene expression—There is a wealth of evidence that implicates H2A.Z as an essential player in transcriptional responses. However, understanding the exact mechanisms dictating H2A.Z-dependent transcriptional regulation are complicated by the fact that H2A.Z has reported roles as both a transcriptional activator and a repressor. In this section, we discuss how analyses of H2A.Z mutants, structural features, and localization patterns support a role for H2A.Z as a transcriptional activator. H2A.Z's role as a repressor is discussed in a later section.

In *Arabidopsis*, most genes contain a prominent H2A.Z peak at the +1 nucleosome beyond the TSS. With respect to this enrichment pattern in plants, no gene has been studied more than *FLOWERING LOCUS C* (*FLC*). The most prominent and unifying phenotype of all H2A.Z and SWR1 mutants is the accelerated transition from vegatative to reproductive growth. Expression analysis reveals that these mutants display decreased transcript levels of the floral repressor *FLC* which leads to early flowering (15, 16, 28, 29, 68, 82, 86, 98,

124). H2A.Z-containing nucleosomes at this locus follow the pattern expected for expressed genes with a characteristic peak of enrichment directly downstream of the TSS (Figure 1D). ChIP analysis revealed that SWR1 subunits PIE1 and ARP6 are required for deposition of H2A.Z at *FLC* as well as *FLC* paralogs *MAF4* and *MAF5* (29). This finding, coupled with the observation that loss of HTA9 and HTA11 also result in decreased expression of these genes indicates that H2A.Z itself is required for their proper activation (82).

Recently, several protein interaction assays from independent groups have provided new insight into the composition of the plant SWR1 complex and H2A.Z interactors (77, 94, 106, 124). Understanding how these new subunits influence SWR1 activity will help in deconvoluting the various and sometimes contradictory functions of H2A.Z in plant transcription. Of several newfound subunits, the most studied in relation to H2A.Z deposition has been Methyl-CpG Binding Domain 9 (MBD9). ChIP-seq analysis of H2A.Z enrichment in *Arabidopsis* seedlings shows that about 20% of H2A.Z enriched sites become depleted in *mbd9* mutants (124). Comparison of FLAG-tagged MBD9 enrichment with corresponding ATAC-seq data indicate that MBD9 localizes primarily to areas of open chromatin and suggests that MBD9 promotes H2A.Z deposition at the 5' end of highly active genes (106). Future experiments will be needed to determine exactly where and when during plant development this MBD9-containing SWR1 complex is performing its function, and whether specific SWR1 subtypes may be related to the activating and repressive roles of H2A.Z.

H2A.Z enrichment at the +1 nucleosome of genes in most organisms leads many to speculate that H2A.Z may help in the targeted recruitment of transcription initiation machinery. However, the presence of a second H2A.Z peak at the −1 nucleosome in some organisms suggests that H2A.Z may serve as a mere mark of transcription as opposed to a targeting factor. Bagchi et al found that while the level of H2A.Z at the +1 nucleosome did not correlate with gene activity in yeast, it did correlate with upstream antisense transcript levels, indicating that the bimodal profile of H2A.Z at the +1 and −1 nucleosomes found in yeast is the reflection of a transcription event rather than an initiator of that event (4). This observation is corroborated by H2A.Z enrichment patterns in other organisms. For instance in humans, where bidirectional transcription is common, H2A.Z peaks are found on both sides of the NDR (134). Whereas in *Drosophila*, where bidirectional transcription is not frequently observed, there is a pronounced lack of H2A.Z enrichment at the −1 nucleosome (23). Colino-Sanguino et. al reviewed recent work in mammals highlighting the contradictory results found from experiments aimed at uncovering the relationship between RNA polymerase pausing at the TSS and H2A.Z (21). In *Arabidopsis*, GRO-seq data indicate a lack of bidirectional transcription and we again observe a lack of H2A.Z enrichment at the −1 nucleosome (48). While H2A.Z has been implicated in gene activation across organisms, the idea that promoter H2A.Z enrichment reflects the direction of transcription implies that H2A.Z incorporation perhaps does not facilitate targeted initiation but may help reinforce existing transcription patterns.

While H2A.Z is clearly required for high level transcription of many genes, the presence of an H2A.Z-containing nucleosome alone is likely not sufficient for transcriptional activation. For instance, recent evidence points toward acetylation of H2A.Z as a modulator of

flowering time. Crevillen et al revealed for the first time in plants the occurrence of H2A.Z acetylation and showed that this acetylation is required for proper *FLC* expression (25). While this study marks an exciting insight into H2A.Z mediated activation, future studies are needed to determine how universal this mechanism of activation is across the plant genome. NAP1-RELATED PROTEIN 1 and 2 were recently identified as inhibitors of H2A.Z deposition (138). Unexpectedly, *nap1;nap2* double mutants displayed an increase in H2A.Z enrichment at the TSS of *FLC* but a decrease in *FLC* expression. Wang et al. use an observed increase in nucleosome density around the TSS to explain this reduced expression and thus provide further evidence that TSS H2A.Z enrichment is likely not sufficient for activation. Additionally, *arp6* mutants also display alterations in H3K4me3 enrichment at *FLC*, but it is unclear whether this observation is a direct consequence of H2A.Z loss in the *arp6* mutant (Figure 2C) (86). Based on current evidence it is still unclear if H2A.Z's role in active transcription is one of initiation, maintenance, or both. Indeed, various seemingly contradictory results have been reported regarding the role of H2A.Z nucleosomes as a barrier to Pol II elongation and in modulating chromatin accessibility (19, 90, 92, 142).

H2A.X

H2A.X, the most similar histone variant to H2A, differs from canonical H2A only via a C-terminal SQL(E/D) motif in animals and a SQEF motif in plants (Figure 1B). H2A.X is present in most eukaryotes; however, unlike H2A.Z, it has evolved multiple times (61, 131). *Arabidopsis* and rice each encode two constitutively expressed and functionally redundant H2A.X genes (Table 1) (67). The H2A.X variant is best known for its role in coordinating DNA damage responses in both animals and plants. The presence of phosphorylated H2A.X is considered a hallmark of DNA damage repair (110). However, a number of studies suggest that phosphorylated H2A.X is also required for gene activation (30, 125, 146).

The mechanisms underlying the genome-wide distribution of H2A.X remain largely unknown in both plants and animals (105). In humans, FACT (FAcilitates Chromatin Transcription) plays an important role in both removal and incorporation of H2A.X (Table 1) (35, 46). Studies in the mammalian system showed that H2A.X is incorporated *de novo* into damaged chromatin by FACT (46, 105). However the involvement of FACT chaperone in plant H2A.X deposition has not been investigated and we do not know if *de novo* deposition of H2A.X in response to DNA damage occurs in plants. Since the FACT chaperone is conserved among eukaryotes and domain organization of both FACT proteins, SSRP1 and SPT16, is similar between plant and mammalian, FACT may also play a role in plant H2A.X deposition (45).

H2A.X localization and relationship with gene expression—Cytologically, *Arabidopsis* H2A.X is excluded from chromocenters and primarily enriched over euchromatin (75). Chromatin immunoprecipitation experiments reinforce this observation showing an enrichment of H2A.X over the bodies of expressed genes (150). The relatively ubiquitous distribution of H2A.X in euchromatin is consistent with its role as a platform for DNA damage repair (DDR), as sites of DNA damage preloaded with H2A.X allow for a rapid response.

Although H2A.X is best known as a platform for DDR in plants and other eukaryotes, for the first time in plants, phosphorylated H2A.X (γH2A.X) was recently found to be required for transcriptional activation. Xiao et. al found that expression of the *ABA Insensitive 4* (*ABI4*) gene is repressed by Oxidative Stress 3 (OXS3) family proteins during seed germination. They went on to show that this repression is in fact due to an interaction between OXS3s and H2A.X that prevents H2A.X phosphorylation and subsequent ABI4 activation. Given γH2A.X's well characterized role in DNA damage response, it will be interesting to investigate whether this γH2A.X-dependent activation involves other elements of DNA damage repair. Future experiments measuring the occurrence of double-stranded breaks (DSBs) and the localization of DDR machinery around the ABI4 promoter during activation will help determine how γH2A.X-dependent activation relates to our previous understanding of γH2A.X function (146).

Although rare, there is some evidence of a non-canonical role for H2A.X in gene regulation from other eukaryotes as well. In the mammalian fibroblast cell, the *High Mobility Group AT-Hook 2* (*HMGA2*) gene also depends on γH2A.X for activation (30). Dobersch et al found that γH2A.X precedes DNA demethylation and transcription initiation. These results would indicate that chromatin conformation changes during activation involve DNA breakage. However, not all studies support a role for H2A.X in gene activation. Recently, Eleuteri et al found that H2A.X curbs embryonic stem cell proliferation by repressing rRNA transcripts. They found that H2A.X, independent of H2A.X phosphorylation, at rDNA promoters is responsible for the targeted recruitment of the nucleolar remodeling complex, a complex known to establish heterochromatic features at rDNA (36).

While there is still no unifying mechanism for H2A.X/γH2A.X in transcription, evidence does suggest that H2A.X involvement in transcription is highly dependent upon cell type. Interestingly, H2A.X is maximally enriched in highly proliferative cell types compared to differentiated cell types and the enrichment patterns tend to favor transcribed genes (117, 118). Seo et al have shown that endogenous H2A.X occupancy is positively correlated with Pol II density at a given TSS in the proliferative Jurkat cancer cell line, while they are inversely correlated in differentiated CD4 cells. Thus, non-canonical functions of H2A.X may arise from unique enrichment patterns present in unique cell types.

Given the apparent connection between DDR and H2A.X phosphorylation, there are surprisingly few studies profiling the mark in plants. However, profiling in mammalian cells shows γH2A.X spreads in *cis* over large domains surrounding a DSB (53). Interestingly, the boundaries of γH2A.X domains often correspond to the native topological associated domains (TADs), suggesting γH2A.X propagation is compartmentalized by the 3D conformation of chromatin in the nucleus (22). This will be a particularly interesting avenue to explore in plants considering the fact that *Arabidopsis* has significantly fewer TAD boundaries than animal models, or even other plant species like rice (72).

Heterochromatin-associated histone variants

H2A.Z in facultative heterochromatin

Since H2A.Z incorporation at the TSS has been shown to be important for proper transcription in many organisms, it is puzzling at first to realize that the mere level of H2A.Z at this site does not reliably reflect expression level. Coupling H2A.Z ChIP-seq data with RNA-seq in *Arabidopsis* seedlings reveals that this TSS enrichment has a parabolic correlation with expression. That is, the highest and lowest expressed genes have lower levels of TSS H2A.Z enrichment than those that are moderately expressed (20, 151, 159). This correlation is also found to a lesser extent in rice, where total genic H2A.Z is parabolically correlated with expression similar to promoter H2A.Z in *Arabidopsis* (151). Looking at H2A.Z in the promoter region of genes offers a limited perspective. Recent works discussed in the following section analyzing genic H2A.Z beyond promoter enrichment help to paint a more complete picture of H2A.Z as a transcriptional regulator.

Several recent studies in plants have revealed a role for H2A.Z in gene repression. While initial experiments revealed some H2A.Z-dependent repression within specific genes or gene families, no genome-wide relationship between H2A.Z and repression had been established (66, 126). However, in 2012 Coleman-Derr and Zilberman found that H2A.Z enrichment beyond the TSS and into the gene body is anticorrelated with transcriptional output and that these lowly transcribed genes are enriched in pathways involving environmental or developmental responses (20). Since then, several papers have been published validating a repressive role of H2A.Z in gene transcription. Particularly, gene body H2A.Z was shown to play a repressive role in response to light, drought stress, salt stress, and heat stress response in *Arabidopsis*, as well as phosphate deficiency in rice and heat stress in Brachypodium distachyon (7, 24, 81, 93, 128, 151). A recent study in rice showed that reductions in both H2A.Z and H3K4me3 correlated with increased expression under phosphate starvation while decreases in H3K4me3 alone did not (40). Additionally, loss of the INO80 chromatin remodeling complex (responsible for H2A.Z eviction from chromatin) leads to decreases in both the deposition of H3K4me3 and transcription elongation typically observed at thermomorphogenesis genes during a high temperature induction (147). These results suggest that a coordination between H3K4me3 and H2A.Z may be required for proper activation of certain responsive genes.

Despite this recent focus on H2A.Z-mediated repression, no model has been proposed to sufficiently account for the genome-wide association between H2A.Z and repression. One idea is that gene body H2A.Z facilitates repression in a reversible manner serving as a more dynamic alternative to DNA methylation (20). This notion is supported by findings that the SWR1 complex is required for trimethylation of H3K27 at most H2A.Z enriched sites, a key step in the Polycomb pathway of gene silencing (Figures 1D and 2B) (12). However, several recent reports indicate that H2A.Z may use Polycomb proteins to achieve silencing outside of the accepted Polycomb pathway, raising several questions about the canonical pathway of Polycomb silencing. While SWR1 is required for H3K27me3, the small number of overlapping upregulated genes between *hta9 hta11* and PRC2 catalytic subunit CURLY LEAF (CLF) mutants suggest that H2A.Z-mediated repression is independent of PRC2

activity (43, 65). Even more evidence that H2A.Z achieves repression via an unexplored Polycomb pathway comes from Cai et al, who found that H2A.Z enrichment is required for repression of several anthocyanin biosynthesis genes. Interestingly, while H2A.Z is required for the deposition of H3K27me3 at these genes, H3K27me3 is not necessary for their repression (11). Recently, our group, as well as others, identified an interaction between the SWR1 complex and several ALFIN1-LIKE family proteins (AL5, AL6, and AL7) (77, 106, 124). Little is known about this plant-specific family of proteins but the few studies of ALFIN1-LIKE proteins in *Arabidopsis* implicate them in Polycomb-mediated silencing. Molitor et. al. identified the same AL proteins found in SWR1 pulldowns as interactors with POLYCOMB REPRESSIVE COMPLEX 1 (PRC1) (88). They went on to show that *al6/al7* double mutants cause a delay in the chromatin state switch from active H3K4me3 to repressive H3K27me3 in key seed developmental genes (88). This led authors to propose that ALs bind H3K4me3 via a plant-homeodomain (PHD) and recruit PRC1 to initiate Polycomb mediated silencing. How exactly the ALs are targeted to these genes destined for repression is still unclear, and given H2A.Z's relatively newfound role in repression of responsive genes, it will be interesting to see how the interaction between SWR1 and ALs influences where silencing occurs.

Monoubiquitination of H2A.Z by the PRC1 catalytic subunit atBMI1 provides yet another connection between H2A.Z and Polycomb silencing with 68% of genes upregulated in *hta9/hta11* mutants being enriched for both H2A.Z and H2A121ub in WT (43). H2A.Z was also found as a mark of inactive enhancers in plants, with its presence being associated with lower expression of putative target genes and increased enrichment of H3K27me3, a finding that is in contrast to humans where enhancer H2A.Z instead colocalizes with activating marks H3K4me3 and H3K27ac (26, 51).

H2A.W in constitutive heterochromatin

H2A.W variants are exclusive to the plant lineage and are defined by an extended C-terminal tail containing an SPKK motif (61, 150)(Figure 1B). Since green algae and non-flowering land plants lack H2A.W variants, it is proposed that H2A.W evolved from early spermatophytes (61). Liverworts, mosses and lycophytes possess the novel H2A variant H2A.M as a potential alternative to H2A.W, with commonalities in the C-terminal tail and L1 loop (61). In contrast to other histone variants, H2A.W has S-phase expression in *Arabidopsis*. (152). Additionally, disruption of CAF-1, which regulates chromatin assembly after replication, results in reduced H2A.W levels, implying that its deposition is replication-dependent (Table 1) (5).

Phosphorylation dynamics of H2A.W variant HTA7 were found to play an essential role in the effective response to DNA damage in heterochromatic regions. Therefore, one proposed function of H2A.W is to serve as a functional complement to H2A.X in heterochromatin, providing a platform for phosphorylation in response to DNA damage (75). Monocot H2A.W contains multiple copies of the SPKK motif while eudicots have a single copy (61). This SPKK is known to promote chromatin condensation by binding to A/T rich sites on DNA generally found in the satellite repeats of constitutive heterochromatin. The presence of this motif as well as *in vitro* nucleosome assembly results indicate that H2A.W generally

promotes chromatin condensation (61, 150). However, recent studies highlighted below indicate that H2A.W's role in heterochromatin is perhaps more nuanced than previously expected.

H2A.W localization and relationship with heterochromatin accessibility— H2A.W is located primarily in constitutive heterochromatin, with correlation between the variant and H3K9me2, DNA methylation, and linker histone H1 (Figure 1C) (10, 75, 150). However, H2A.W deposition into heterochromatic regions does not depend on DNA methylation or H3K9me2 (150). New H2A.W triple mutants, *h2a.w-2*, created by crossing a CRISPR generated null *hta6* allele with *hta7* and *hta12* T-DNA lines reveal a potentially unique role for H2A.W in maintaining a level of accessibility in constitutive heterochromatin (10). Using ATAC and bisulfite sequencing analysis of *h1*, *h2a.w-2*, and *h1/h2a.w-2* double mutants, Bourguet et al concluded that H2A.W actually antagonizes the binding of H1 to linker DNA in constitutive heterochromatin (Figure 2D). They propose the SPKK motif of H2A.W competes with the two SPKK motifs found in H1 for binding on linker DNA. Therefore, the SPKK motif of H2A.W, which was thought to promote chromatin condensation when compared to other H2A variants, may actually function to prevent even further condensation by H1. This allows regions occupied by H2A.W to maintain a heterochromatic state while still being accessible to maintenance factors like DNA methyltransferases (10).

Of course, an analysis of H2A.W alone is incomplete without considering the chromatin remodelers that act on it. Recently, Osakabe et al identified DDM1 (DECREASE IN DNA METHYLATION 1) as a depositor of H2A.W in *Arabidopsis*. In stark contrast with *h2a.w-2*, *ddm1* mutants had significant derepression (40%) of pericentromeric TEs and no reported changes in H1 enrichment (Table 1) (100). While H3K9me2 and DNA methylation were reduced in *ddm1*, their effects on silencing TEs were found to be secondary to that of *ddm1*. The results of these two studies raise several exciting questions, namely; How does total H2A.W loss in *h2a.w-2* have a lesser effect on TE silencing than DDM1 loss? Is DDM1 acting independently of H2A.W to silence TEs in *h2a.w-2* mutants? Genomic profiling analysis of DDM1 enrichment in WT and *h2a.w-2* is one of many future experiments that will help answer these questions. Furthermore, deconvoluting the mechanisms behind DDM1 and H2A.W function may help to inform human disease, where Lymphocyte-Specific Helicase (LSH) and macroH2A appear to play a similar role in mammalian silencing (96).

H1

The linker histone H1 binds both the nucleosome core particle and the linker DNA to facilitate internucleosomal interactions and chromatin compaction. Interestingly, H1 and associated variants have a separate evolutionary origin from core histones, having evolved from bacterial proteins rather than archaeal ones (60). H1 histones are also more divergent across species compared to core histones (60). However, the general structure of a lysine rich C-terminal tail, a flexible and short N-terminus and a central globular domain are conserved across eukaryotes (158).

Due to the importance of H1 variants in chromatin dynamics, it is surprising to observe that H1 depletions in *Arabidopsis*, yeast, worms and fungi are viable while mutation of H1 variants in mouse and Drosophila are lethal (3, 39, 58, 78, 103, 119, 121, 135). Plant H1 variants are classified into two groups, main variants with ubiquitous and stable expression and minor variants, which accumulate in response to stress (59, 63, 130). In contrast to mammals with 11 H1 variants, only three nonallelic H1 variants are found in *Arabidopsis*: two highly similar major variants H1.1 and H1.2 and the shorter stress-induced minor variant H1.3 (Table 1) (2, 59). The key structural differences between H1.3 and H1.1/H1.2 are a decreased positive charge in H1.3, a shorter C-terminal domain, and a lack of (S/T)PXK DNA-binding motifs in both N- and C-terminal domains (Figure 1B) (115). While H1.1/2 variants are expressed in all cell types, H1.3 is expressed constitutively in guard cells with induced expression in other cell types during stress.

H1 localization—Genome-wide analysis of H1.1/2 in *Arabidopsis* show that linker histones are found in both heterochromatic and euchromatic regions and generally associate with methylated DNA sequences, with the strongest enrichment over hypermethylated TEs and lowly expressed genes. Genic H1 enrichment however is linked with methylation status rather than expression level, with similarly expressed genes only being enriched for H1 if methylated (14). At a closer look, gene body H1 enrichment is characterized by peaks at the 5' and 3' ends, just inside the nucleosome depleted regions. However, as genes increase in expression, total H1 occupancy falls as expected but enrichment takes on a new asymmetrical shape, with 5' ends having lower H1 levels with increasing enrichment towards the 3' ends. This asymmetry is not reported in Drosophila or mammals, and future investigations may uncover whether and how this pattern affects transcription in plants. The localization pattern of H1.3 is similar to H1.1/2 variants. However, compared to H1.1/2, H1.3 association with chromatin is far more dynamic and is more frequently associated with active chromatin marks such as H3K4me3 (116). Additionally, increased levels of DNA methylation, which are normally observed in response to stress, were significantly decreased in *h1.3* mutants under stress conditions. These distinctions suggest that H1.3 may outcompete H1.1/2 under stress allowing for increased accessibility to regulatory machinery like DNA methyltransferases (116).

H1-dependent silencing in euchromatin and heterochromatin—Recent evidence indicates that plant H1 contributes to the structural organization of both constitutive heterochromatin and euchromatin. Independent studies using H1 triple mutants (*3h1*) and double mutants both found chromocenter decondensation in *Arabidopsis* (14, 114). Despite this observation, H1 double and triple mutants had minimal TE derepression. This evidence is in conflict with the common view that chromatin compaction is required for efficient TE silencing and suggests that loss of H1 contributes to heterochromatin structure without any functional impact on silencing.

H1 variants impact the pattern of heterochromatic DNA methylation in CG, CHH and CHG contexts (109, 115, 154). *h1.1 and h1.2* mutants both show increased DNA methylation in heterochromatic TEs, suggesting that H1 variants inhibit heterochromatin accessibility to DNA methyltransferases. While further investigation is still needed, considering the

relationship between DNA methylation and H1 in plants as well as other eukaryotes may help to explain the surprisingly minimal impact H1 has on TE silencing. In mice, the situation is similar to plants, where *h1* mutants (mutation in both H1.1 and H1.2) show only partial TE upregulation (39). However in *Drosophila*, where cytosine methylation is absent, H1 loss does indeed induce general TE expression (55, 79). This suggests that while H1 contributes to TE silencing, organisms with DNA methylation are able to maintain this silencing despite H1-dependent changes in chromatin structure (14). This theory is supported by a small number of TEs in *Arabidopsis* that were found to be derepressed more in *met1;h1* double mutants than in either single mutant alone (14). Additionally, a recent study revealed that a family of TEs located in pericentromeric heterochromatin (where evidence suggests that silencing is achieved independently of DNA methylation) depend on H1 for their repression under heat stress. By contrast, a family of non-pericentromeric TEs affected by heat rely on DNA methylase CMT2 together with H1 for stable repression (73). H1 overexpression in vegetative *Arabidopsis* cells also predominantly leads to the repression of pericentromeric TEs (47). Similar to their effect on TE silencing, it was shown that loss of H1 intensifies the activation of antisense transcripts only at genes hypomethylated in *met1* (14).

As in heterochromatin, euchromatic H1 loss causes profound changes in chromatin structure with surprisingly little impact on gene expression. In WT plant cells there is a strong inverse correlation between nucleosome occupancy and transcription, with highly expressed genes having the lowest occupancy (114). Low nucleosome occupancy is often interpreted as a requirement for increasing accessibility of a transcribed gene to transcriptional machinery and lowering the energy barrier presented by nucleosomes to RNA polymerase procession. In H1-depleted cells this correlation is almost completely lost, with all genes having similar nucleosome occupancy regardless of expression level (114). Surprisingly, gene expression is relatively unchanged in these cells, with only about 3% of genes being misregulated. This result indicates that H1-mediated nucleosome occupancy is a consequence rather than a driver of steady state transcription. However, H1-depleted plants do have defects in several developmental and cellular transitions including seed dormancy control, flowering time control, and lateral root initiation (114). Collectively, these observations indicate that the massive structural alterations found in H1 mutants likely affect tight control of developmental and cellular transitions. Therefore, H1-dependent chromatin structures may have a more prominent role in transcriptional reprogramming rather than in fundamental expression. Supporting a role for H1 in transcriptional reprogramming is the observation that *3h1* mutant cells also have a dramatic reduction of nuclear H3K27me3, a hallmark of epigenetic silencing memory across plants and animals (114).

Conclusion and future directions

Most studies investigate histone variants by observing their genome-wide distributions before and after a disruption or exposure. However, it is clear that future studies will need to be performed at a higher temporal resolution to determine the exact order of events that take place during variant-mediated gene regulation. For instance, there is mounting evidence for a role of H2A.Z in regulating a majority of environmental responses but no data currently exists to explain how this repressive state comes about, or how it may

change during activation. Excitingly, Willige et al used temporally resolved H2A.Z profiling to find that gene body H2A.Z loss actually precedes activation of select red:far-red light sensitive genes, indicating that H2A.Z loss is not merely a consequence of their activation (143). Additionally, following enrichment of H3.3 and H1 through precise time points during cell fate determinations will help explain why these histones play fundamental roles in transcriptional reprogramming during development while being dispensable for general transcription. Similarly, conclusions in variant research have often been limited by assays profiling large cell populations. Emerging single cell data indicates that cells within these heterogeneous populations do not behave uniformly and meaningful changes in variant deposition may be masked by homogenized tissue samples. As chromatin profiling techniques advance and read depth requirements fall, single cell type profiling will uncover how these variants behave within uniform cell types and even single cells.

Modification of variants

It is reasonable to imagine that the apparent multifunctionality of H2A.Z is due in part to modifications to the histone itself. For instance, we know that H2A.Z acetylation is sufficient for gene activation at *FLC*. But what about acetylation at other genes with similar H2A.Z distribution profiles that appear inactive? Are those genes simply upstream of others in the process of activation or is there a compounding modification like methylation that is stifling activation? Future studies profiling these variant modifications genome-wide will be essential to closing the current knowledge gap between H2A.Z mediated activation and repression. Additionally, H3.3 K4 plays an essential role in mammalian embryonic stem cell differentiation, likely as a platform for methylation (41). This essential role for H3.3K4 in the stem cell could help explain the observation in plants that H3.3 is essential for viability while being dispensable for general transcription. This residue and others known to be modified in other species are conserved in plants meaning there is great potential for the future study of plant H3.3 modifications.

Role of chromatin remodelers in regulation

Histone variant chaperones are often used as proxies for the study of histone variants. However, these chaperones often have functions outside of just histone deposition. For instance, *swr1* mutants show a global depletion of H3K27me3 while this phenotype is much less severe in *hta9 hta11* double mutants (12, 43). Similarly, *ddm1* mutants have significant TE derepression while *h2aw-2* mutants do not. Future studies are needed to decouple the functions of these chromatin remodelers from the variants themselves. Additionally, while conservation and mutant analysis implicate other chromatin remodelers as variant chaperones in plants, H2A.X and H3.3 still do not have confirmed interactions with a chromatin remodeler or chaperone.

DNA methylation and histone variants

Each histone variant discussed in this review has some relationship with DNA methylation. H2A.Z and DNA methylation are mutually exclusive in the *Arabidopsis* genome, suggesting that gene body H2A.Z may serve to protect responsive genes from the more permanent effects of DNA methylation (20, 159). However, while loss of H2A.Z does cause hypermethylation over select regions, overall methylation patterns are unaffected (20, 94).

On the contrary, global reductions in DNA methylation in *met1* mutants result in an increase in H2A.Z enrichment at those sites, implying that it is DNA methylation which excludes H2A.Z rather than the inverse (159).

DNA methylation and H3.3 are both enriched over the body of active *Arabidopsis* genes (18, 71, 155). Detailed characterization of *Arabidopsis h3.3kd* mutants revealed that the level of DNA methylation decreases exclusively at regions where H3.3 and DNA methylation overlap on active gene bodies (145). In the same *h3.3kd* mutants, these active gene bodies are also invaded by H1 and H2A.Z (Figure 2A). Therefore, reduced gene body methylation in *h3.3kd* might allow ectopic recruitment of H2A.Z-containing nucleosomes to gene bodies. Given H2A.Z's role in transcriptional repression, H3.3 enrichment over genes may be required to maintain suitable chromatin structure for transcription by antagonizing H1 invasion of active genes. Low H1 levels will therefore provide sufficient accessibility to DNA methyltransferases that methylate gene bodies and prevent invasion by H2A.Z. Similarly, *h2a.w-2* mutants also show an increase in H1 enrichment and a decrease in DNA methylation in constitutive heterochromatin. Therefore, H2A.W may serve as a functional complement to H3.3 with respect to maintaining the balance between H1 and DNA methylation specifically in constitutive heterochromatin.

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Summary Points

- H3.3 promotes DNA accessibility in part through an antagonistic relationship with H1.
- Phe41 is an amino acid substitution unique to plant H3.1 and may impart functions on H3.1 that are plant-specific.
- H3.3 cannot be methylated at K27, implying that H3.3 can interrupt the Polycomb pathway of gene repression and potentially perpetuate the euchromatic chromatin state across cell divisions.
- Eukaryotes without bidirectional transcription have peak H2A.Z enrichment downstream of the TSS while organisms with bidirectional transcription have bimodal H2A.Z enrichment. H2A.Z is therefore a marker for transcriptional direction.
- For the first time in plants, phosphorylation of H2A.X was found to be required for transcriptional activation of *ABI4*. It will be interesting to investigate whether this yH2A.X-dependent activation involves other elements of DNA damage response and repair.
- Recent evidence shows that H2A.Z enrichment within the gene body contributes to transcriptional repression likely through a non-canonical Polycomb pathway of gene silencing.
- H2A.W is a histone variant unique to plants which may promote accessibility of constitutive heterochromatin by competing with H1 for binding to linker DNA.
- Nucleosome occupancy depends on linker histone H1 and together with DNA methylation, promotes the silencing of TEs.
- H1-dependent chromatin structures may have a more prominent role in transcriptional reprogramming than in steady state expression.

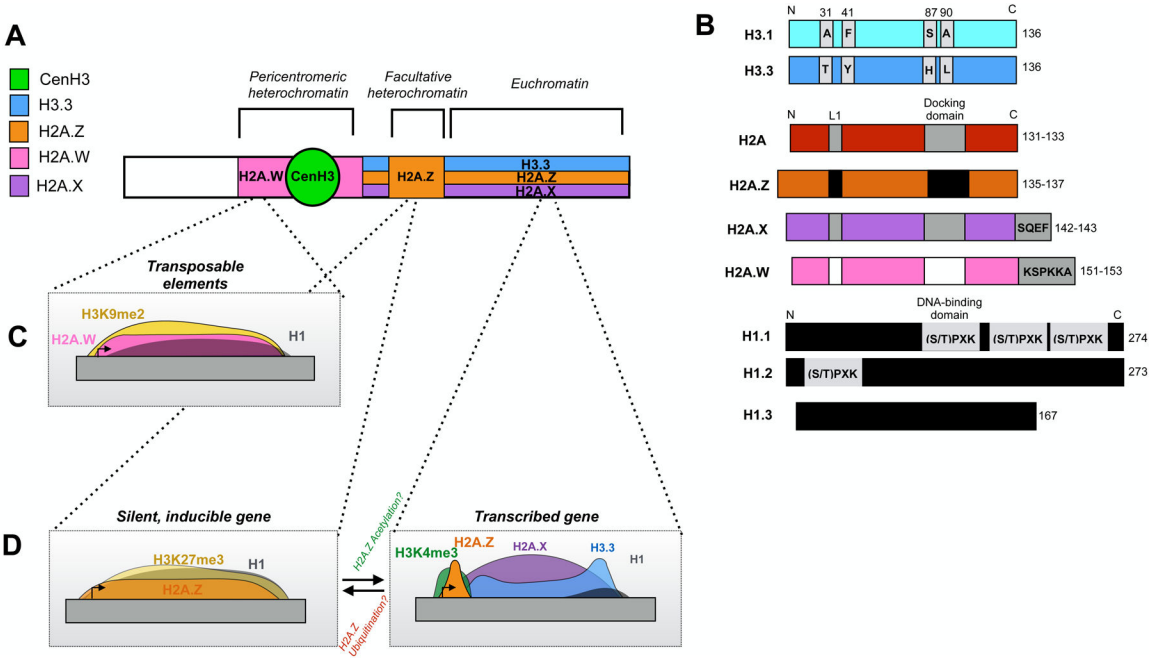

Figure 1. The nature of histone variants and their distribution in chromatin.

(**A**) Schematic diagram of a chromosome, showing the distribution of major chromatin types and the histone variants associated with each. (**B**) Diagrams comparing histone variants and their canonical counterparts. Regions of sequence differences or additions between variants and canonical types are shown as boxes. H3.1 and H3.3 differ by only four amino acid substitutions: two in the N-terminal tail and two in the histone-fold domain. H2A variants are distinguished mainly by sequence variation in the L1 loop and docking domain, while H2A.X and H2A.W have variant-specific C-terminal extensions. H1 subtypes vary in the occurrence of DNA-binding domains and their overall length. (**C**) Diagram showing the distribution of H2A.W on silent transposable elements and association with H1 and H3K9me2. (**D**) Two distinct, and perhaps interconvertible, distribution patterns of H2A.Z on silent genes in facultative heterochromatin (left) and active euchromatic genes (right). Silent genes show ubiquitinated H2A.Z nucleosomes across the gene body and are associated with H3K27me3 and H1, while active genes show acetylated H2A.Z in the +1 nucleosome and H3.3 in the gene body.

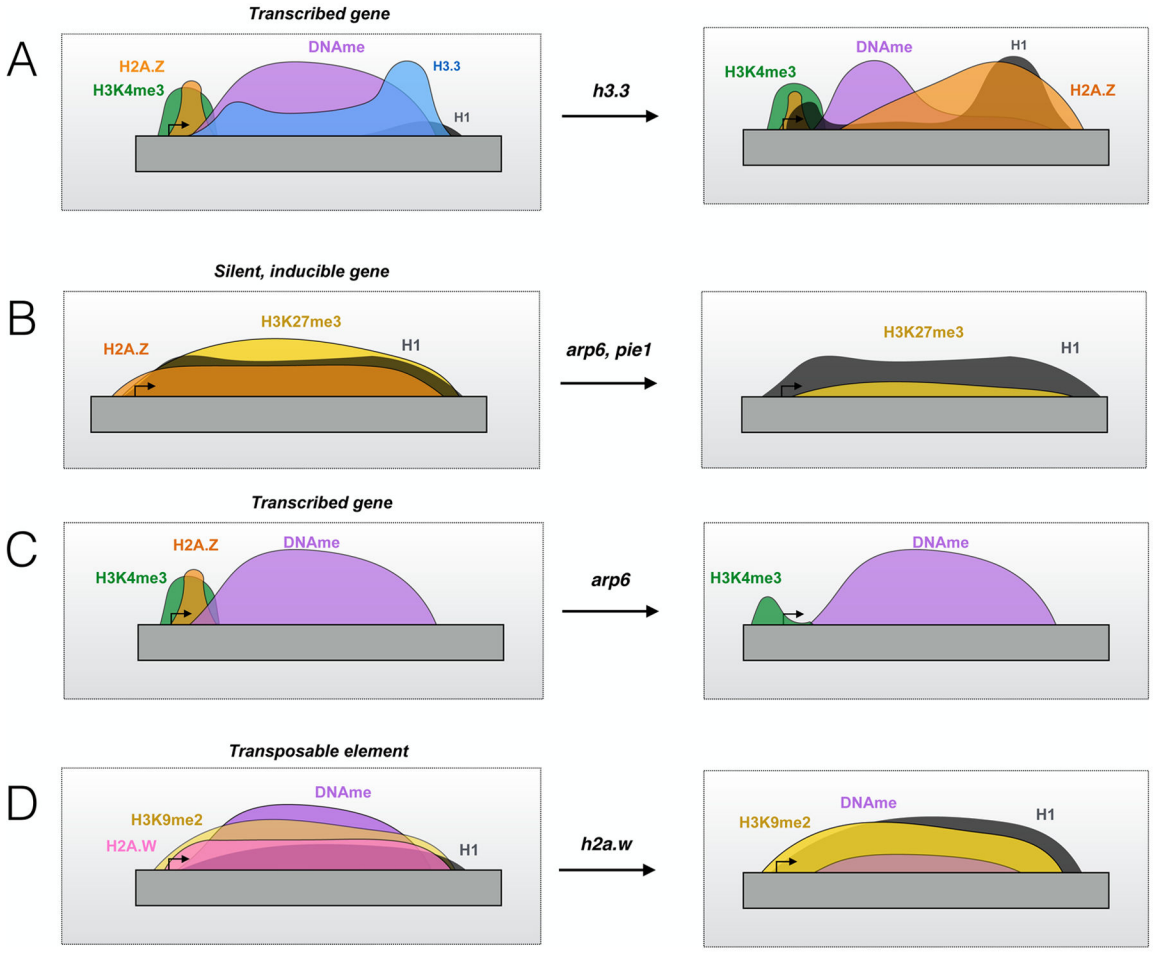

Figure 2. Chromatin landscape changes in response to histone variant depletion.

(**A**) H3.3 loss at transcribed genes results in reduced DNA methylation (DNAme) in the CG context, and increased H2A.Z and H1 in the downstream regions previously occupied by H3.3. Active histone marks such as H3K4me3 and H4K36me3 are generally unaffected. Given that H3.3 does not seem to be methylated at K27, genes targeted for polycomb repression may be subject to silencing when H3 nucleosomes become predominant in the absence of H3.3. (**B**) H2A.Z loss from silent, inducible genes in SWR1 mutants (*arp6* and *pie1*) results in reduced H3K27me3 without affecting H1 levels. These silent genes also lose H3K27me3 and generally become active upon H2A.Z loss. (**C**) In transcribed genes, H2A.Z loss in SWR1 mutants results in a reduction of H3K4me3, particularly around the +1 nucleosome, without changes in DNA methylation. H2A.Z loss from these genes generally corresponds to decreased Pol II occupancy and transcription as well. (**D**) Loss of H2A.W at transposable elements (TEs) results in reduced DNA methylation and increased H1 occupancy, while the repressive mark H3K9me2 is generally unaffected. Interestingly, one study showed that loss of H2A.W in *h2a.w* mutants did not result in widespread expression of silent TEs (10), while another study showed that H2A.W loss resulting from mutation of DDM1 did result in increased TE expression (98). In these *ddm1* mutants, there was also a reduction in H3K9me2 without changes in H1 enrichment. These contrasting results

suggest that H2A.W and the DDM1 remodeler have overlapping and distinct functions in heterochromatin.

Table 1.

Histone variant genes, proteins, and functions in *Arabidopsis thaliana*

Histone variants	Arabidopsis Genes	ArabidopsisLoci	Protein	Chaperones/ remodelers	Function			Knockout/ knockdown Arabidopsis phenotype	
					General	development	Stress responses		
H3.3	HTR4 HTR5 HTR8	At4g40030 At4g40040 At5g10980	H3.3 H3.3 H3.3	HIRA (95) ATRX(33)	Transcriptional activation (123, 127, 144, 156)	Flowering time(156); Male Gametogenesis (54)); Post-germination development (5); Cell proliferation and organogenesis (101)	Regulate the expression of responsive and hypervariable genes(145)	*htr5/htr8*, are phenotypically normal ; Triple mutants cause lethality; *htr4/ htr4;htr8/ htr8* plants that carried either *amiR-HTR5-I* or *amiR-HTR5-II* (*h3.3kd*) exhibit leaf serration, incomplete sterility, and growth defects (145)	H3.3
H2A.Z	HTA8 HTA9 HTA11	At2g38810 At1g52740 At3g54560	H2A.Z.8 H2A.Z.9 H2A.Z.11	SWR1 complex (42, 137)	transcriptional activation and repression	Flowering time (16, 29, 86, 98, 124); vegetative to reproductive phase transition (42, 148); Inflorescence architecture (11); Germline development (157); Circadian clock (133)	Temperature response (24, 66, 132); Phosphate deficiency (40, 126, 151) Drought stress (11, 128); Immunity (6); Salt stress (93)	Double/ triple mutants show developmental aberrations (83, 94); Triple mutants are viable but show reduced fertility (20); Knockdown of all three H2AZ genes cause early flowering (16)	H2A (25); mon (H2A SUM meth
H2A.X	HTA3 HTA5	At1g54690 At1g08880	H2A.X.3 H2A.X.5	FACT? (45)	DNA damage response (13, 67, 112); transcription activation (146)	H2A.X is unessential for *Arabidopsis* development (50)	genotoxic stress(141)	The single/ double mutants are viable, fertile and indistinguishable from wild type (50)	phos (67,
H2A.W	HTA6 HTA7 HTA12	At5g59870 At5g27670 At5g02560	H2A.W.6 H2A.W.7 H2A.W.12	DDM1 (100)	Chromatin condensation (150)	H2A.W is unessential for *Arabidopsis* development (10)	Not determined	H2A.W triple mutants are indistinguishable from the wild type (10)	phos (75,
H1	HON1 HON2 HON3	At1g06760 At2g30620 At2g18050	H1.1 H1.2 H1.3	NRP1 and NRP2?(91, 102)	Chromatin condensation (14, 114)	Flowering time, Seed dormancy, Lateral root, Stomata and callus development (114) male and female gametogenesis (120)	Drought stress (2) Combined light and water deficiency (116)	Triple mutants are viable but show extended dormancy, early flowering, increased root density and lateral root numbers, and altered stomata pattern (114)	phos acety and c form and p