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Direct assessment of histone function using histone replacement

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Abstract

Histones serve many purposes in eukaryotic cells in the regulation of diverse genomic processes, including transcription, replication, DNA repair, and chromatin organization. As such, experimental systems to assess histone function are fundamental resources towards elucidating the regulation of activities occurring on chromatin. One set of important tools for investigating histone function is histone replacement systems, in which endogenous histone expression can be partially or completely replaced with a mutant histone. Histone replacement systems allow systematic screens of histone regulatory functions and the direct assessment of functions for histone residues. In this review, we will describe currently existing histone replacement systems in model organisms, benefits and limitations of these systems, and opportunities remaining for future research with histone replacement strategies.

Keywords

epigenetics; chromatin; unicellular eukaryotes; Drosophila; Arabidopsis

Histone replacement as a strategy to search for novel histone functions

In eukaryotic cells, genomic DNA is wrapped around histone octamers (see Glossary) to form nucleosomes. Histones can regulate cellular processes through post-translational modifications (PTMs), or conversely, the absence of PTMs, as well as through the incorporation of histone variants. Thus, histones play critical roles in regulating various genomic processes with widespread effects on organismal development and fitness [1]. A major strategy for determining the function of histone PTMs has historically been to analyze phenotypes incurred by the mutation of histone-modifying or histone-reading proteins (for example, see [2, 3]). While this approach has been used to identify functional

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consequences for a multitude of histone modifications, limitations remain with this strategy. For example, analyzing the contributions of histone-modifying enzymes to the regulation of different phenotypes can be complicated by the fact that these enzymes often target non-histone substrates as well. Moreover, histone-modifying enzymes can mediate essential roles through their actions on histones and/or on non-histone substrates, making it difficult to study loss-of-function mutants in these situations (for example, see [4, 5]). Another issue is that there can be multiple histone-modifying enzymes with redundant functions, in which case phenotypes may not be observed if all of the relevant histone-modifying enzymes are not mutated (for example, see [2]). The presence of redundant histone-modifying enzymes is especially confounding when all of the enzymes targeting a specific histone residue are not known. Finally, only relying on discovering new histone-modifying enzymes to uncover PTMs is a strategy that can preclude (or at the very least slow down) the discovery of novel functions for specific histone residues.

As a solution to many of these challenges, researchers have developed histone replacement systems, in which expression of endogenous wild type histones is compromised and replaced with histones mutated at a specific residue (or multiple residues) to allow the direct functional assessment of histones (Figure 1). Histone replacement systems can be partial, if only some copies of the endogenous histone genes have been mutated or silenced, or complete, if all endogenous expression of the relevant histone is eliminated (Figure 2). Moreover, while not strictly histone replacement systems, histone mutants have also been expressed in wild-type backgrounds of many organisms. Expressing histone mutants in backgrounds retaining various amounts of endogenous wild-type histone expression can provide information about the dominance of histone mutations.

Clearly, the expression of histone mutants *in vivo* provides a complementary and sometimes essential strategy to the investigation of the many cellular roles of histones. In addition to circumventing many of the aforementioned obstacles with the mutation of histone-modifying or histone-reading proteins, histone replacement systems also provide a method for researchers to study the role of non-modifiable histone residues, thus expanding the scope of this strategy to assess histone function. In this review, we compare and highlight established histone replacement systems where endogenous histone expression has either been partially or completely replaced in model systems used for chromatin research.

Establishment of the first histone replacement systems in unicellular eukaryotes

Histone replacement systems were first generated in unicellular eukaryotes: the budding yeast *Saccharomyces cerevisiae* [6–9], the fission yeast *Schizosaccharomyces pombe* [10], and the ciliated protozoan *Tetrahymena thermophila* [11, 12] (Table 1). The earliest histone replacement experiments probed histone function in *S. cerevisiae* by segmental deletion, mainly focusing on the N-terminal tail region [9, 13, 14]. These experiments demonstrated that much of the H2B N-terminal tail is dispensable for viability [9], that at least one of the H2A or H2B N-terminal tails is required for viability [14], and that the H4 N-terminal tail plays a role in transcriptional regulation [13]. Further experiments assessed the effects of H3

and H4 N-terminal tail small deletions on viability, H3K56 acetylation, H3K4 methylation, H3K79 methylation [15], and chromosome loss [16]. Additionally, deletion of pairwise combinations of histone N-terminal tails and the combination of histone point mutations with N-terminal tail deletions were used to determine redundant functions between histone elements in the regulation of viability, the cell cycle, chromatin structure, DNA damage [17] and DNA repair [18]. These early experiments were groundbreaking in that they allowed researchers to assess the functions of different histone tails *in vivo*, and as genetic tools improved, more sophisticated methods developed to facilitate both the functional assessment of many histone residues simultaneously and the analysis of individual histone residues.

Many initial histone replacement experiments assessed specific histone residues that were hypothesized to be involved in regulating a biological process of interest due to PTMs on those residues. Three fundamental studies assessed *S. cerevisiae* strains with point mutations in H4 N-terminal tail residues to reveal that single point mutations in K16 to R19 cause a loss of transcriptional silencing at specific loci and defects in mating efficiency and growth [6–8]. Additionally, multiple PTMs were studied when individual point mutants targeting modifiable residues on H3 (i.e. R8, K9, S10, K14, K16) were expressed in *S. pombe* to study the effect of these mutations, or pairwise combinations of these mutations, in the regulation of chromosome segregation and centromeric silencing [10]. Finally, *T. thermophila* strains with a complete replacement of canonical H3 with H3 serine 10 mutated to alanine (H3S10A) were generated to show that a lack of H3S10 phosphorylation causes defects in chromosome segregation during mitosis and meiosis [11] and *T. thermophila* mutations targeting phosphorylation sites on histone H1 were utilized to assess the impact of H1 phosphorylation on transcriptional regulation [12, 19]. These experiments allowed researchers to investigate individual roles for specific histone residues in a way that, in some cases, would not be possible through the mutation of histone-modifying proteins. For example, as lysine acetyltransferases target multiple histone residues for acetylation [20, 21], separating functions for individual lysine residues is difficult through this traditional method of analysis. However, as demonstrated above, histone replacement systems provide a solution to this problem. In this way, these early replacement systems facilitated the assessment of various functions for a pre-defined set of histone residues.

After these initial experiments assessing specific histone residues, a number of systematic screens examining large sets of histone mutants were conducted in *S. cerevisiae*. For example, a screen was performed targeting histones H3 and H4 to identify mutants displaying aberrant transcriptional silencing in *S. cerevisiae* [22]. Replacement mutants targeting every residue on H3 and H4 that were confirmed targets for PTMs [23] mimicking either constitutively unmodified or modified states were also generated [24] in order to screen for roles in regulating gene silencing and DNA damage sensitivity. Additionally, alanine-scanning mutagenesis experiments were performed where all residues on the canonical nucleosome [25], all residues on the surface of the canonical nucleosome [26], and all residues buried within the canonical nucleosome [27] were individually targeted to conduct an unbiased screen for roles of these residues in various processes such as cell viability, DNA damage, replication, transcriptional elongation, and chromosome bi-orientation [28]. A different approach, in which every residue on histones H3 and H4 that could in theory be a target for PTMs (i.e. serine, threonine, lysine, and arginine)

was individually substituted with alanine, was utilized to systematically assess the role of chromatin changes during gametogenesis [29]. These extensive experiments assessing histone function with an unbiased approach demonstrate the utility of histone replacement towards the comprehensive analysis of histone function. Rather than having to identify and mutate every protein that modifies a certain set of histone residues, researchers are instead able to develop a pipeline towards assessing the role of different histone residues through mutational analysis. As such, these experiments revealed many novel roles for modifiable and unmodifiable histone residues, as this approach importantly allows the assessment of histone residues that are not targets for PTMs.

Recently, synthetic biology approaches have been applied to study fundamental and translational questions surrounding histone biology. For example, “humanized” budding yeast strains, which express either human H3 and H4, human H2A and H2B, or human H2A, H2B, H3, and H4, were generated to assess whether yeast can utilize human core histones [30]. These experiments demonstrated that human histones poorly complement yeast histones, leading to interesting implications about the function of the relatively few divergent histone residues between species. In response to recent studies demonstrating the link between histone mutations and human cancers [31, 32], oncohistone mutations were also recently studied with a humanized budding yeast library to evaluate the functional significance of these mutations *in vivo* [33]. Histone replacement in unicellular eukaryotes provides a method to rapidly generate and assess new oncohistone mutations, although more translational work in a metazoan system would allow further assessment into the disease mechanism of these mutations. Synthetic biology approaches have also been used to assess the functions of histone variants. For example, H2A-H2A.Z hybrids were expressed in yeast depleted of H2A.Z in order to identify three histone regions that confer H2A.Z identity [34]. Additionally, homologous recombination (HR) was used to replace H2A.X in fission yeast with chimeric histones combining H2A with the C-terminal tails of the plant-specific H2A.W and H2A.M variants [35]. This replacement system allowed researchers to study a fundamental evolutionary question about histone variant diversification, providing evidence that variant-specific motifs can alter specific chromatin states (in this case, heterochromatin) prior to the segregation of those variants within chromatin. These approaches demonstrate the diverse implications of histone replacement systems from the elucidation of disease mechanisms for histone mutations to the assessment of foundational questions about histone evolution in eukaryotes.

Finally, the most novel histone replacement systems developed in unicellular eukaryotes allow complete histone replacement at all endogenous histone gene loci, compared to previous systems in which the replacement histone is either expressed from an episomal plasmid or integrated at a single histone gene locus by HR, with the other histone gene deleted. A sophisticated system in *S. cerevisiae* utilized a CRISPR/Cas9-based histone shuffle strategy that allowed for complete replacement of histones H3 and H4 at all endogenous loci [36] (Figure 3). This system was used to generate individual H3 and H4 point mutants as well as combinatorial mutants of H3 and H4 with high efficiency, focusing on major methylation sites (H3K4, H3K36, and H3K79) and residues that have previously been shown to compromise growth when mutated (H3K56 and H4K59). Similarly, a CRISPR/Cas9-based histone replacement system has been developed in *Trypanosoma brucei*

allowing complete *in situ* histone replacement; as proof-of-concept validation, the H4K4R mutant was generated [37]. Importantly, a recent study in *S. cerevisiae* found that two copies of integrated mutant histone genes can rescue growth deficiencies or lethality associated with a single copy of the mutant histone gene [38]. Thus, replicating endogenous conditions as closely as possible is important for the development of histone replacement systems. Complete histone replacement and histone gene replacement at all endogenous loci via gene targeting are ideal parameters for a histone replacement system, but as demonstrated by the research process in *S. cerevisiae*, establishing a system with these parameters may take many iterations upon previous work to develop.

Using histone replacement in metazoans

While histone replacement systems in unicellular eukaryotes provide valuable information about histone function, there are likely additional roles for histone residues in multicellular eukaryotes due to their greater complexity and need for regulation across different cell types and developmental stages. For example, methylation at H3K27 is critical for development in all multicellular eukaryotes, but this PTM is absent in *S. cerevisiae* [39]. *Drosophila melanogaster* was the first multicellular eukaryote in which histone replacement systems were generated [40] and resulting systems have been applied to study histone function in the context of a metazoan organism.

Utilizing the fact that all replication-dependent histone genes are clustered at a single genomic locus in *D. melanogaster* [41], an elegant histone replacement system was established in which the entire canonical histone complement could be replaced with modified histone transgenes [40]. Flies were generated that were homozygous for a chromosomal deletion, which removes the entire replication-dependent histone gene cluster (Δ*HisC*), and a transgene cassette was introduced into Δ*HisC* mutants providing 12 copies of the replication-dependent histone gene cluster encoding either wild-type histones or particular histone mutants (unless otherwise specified, H3 in this section refers to replication-dependent H3.2) [40]. H3K27R replacement mutants depleted of endogenous wild-type H3 exhibited a derepression of genes that are normally repressed by Polycomb Repressive Complex 2 (PRC2) and homeotic transformations similar to PRC2 mutants, while the H3K27R mutation expressed in a dominant context (i.e. with endogenous wild-type H3 still expressed) failed to cause these phenotypes [40, 42]. Thus, assessing complete histone replacement mutants has the potential to reveal functions for histone residues that are concealed in a dominant context. A variation of this genetic system was also utilized to study the effect of individual mutations that prevent the addition of PTMs on three histone residues with proposed roles in well-characterized epigenetic pathways: H4K20, H3K27, and H3K36 [43]. These experiments further supported that H3K27 is required for the repression of Polycomb target genes, consistent with prevailing hypotheses about the function of H3K27me3 [44–46]. In contrast, H2A replacement mutants were used to demonstrate that the loss of H2A monoubiquitylation by PRC1 is not responsible for phenotypes associated with null mutations in PRC1, providing critical evidence against a prevailing hypothesis [47]. Finally, H3K56, H3K115, H3T118, and H3K122 replacement mutants allowed the assessment of several PTMs within the H3 globular domain, which were previously rather poorly characterized due to the fact that

the enzymes generating these PTMs had numerous histone and/or non-histone targets [48]. These results exemplify the utility of histone replacement mutants as a complementary strategy to the mutation of histone-modifying proteins that can either further support conclusions from these experiments, shed light on important new information that conflicts with previous understandings, or even allow the study of PTMs when the relevant enzymes present complications for analysis.

Histone replacement studies assessing replication-independent histone variants have also been conducted in *D. melanogaster*. For example, studies have been conducted replacing the coding sequences of all gene copies of H3.2 with H3.3 or vice versa [49]. These experiments provided evidence that H3.2 and H3.3 can replace each other for at least some functions, as clones exclusively expressing either H3.2 or H3.3 differentiated into normally patterned adult tissues and displayed repression of typically silenced gene states. In addition, one study found that the complete absence of H3.3, or replacement of H3.3 with the mutant H3.3K4A, caused sterility but did not affect viability [50]. The complete replacement of H3.3 with H3.3K4R in contrast was found to rescue the fertility defects of H3.3-deficient flies, suggesting that the non-modified state of H3.3K4, rather than a specific PTM at H3.3K4, is critical for fertility [51]. Studies have also been conducted replacing H3.2, H3.3, or both H3.2 and H3.3 with mutants that cannot be methylated at either K4 or K9 [49, 52, 53]. These mutants were used to demonstrate redundancy between K9ac at H3 and H3.3 for gene activation during development, while they were also utilized to show specific roles for H3.3K9 methylation in repressing the expression of heterochromatic elements. H3.3K27R mutants have been assessed to counteract a prevailing model that H3K27ac is required for transcriptional activation, as these mutants fail to display global transcriptional defects [54]. However, a very small number of genes, notably containing several Polycomb targets, exhibit transcriptional derepression. As demonstrated above, histone replacement systems are ideal tools to study the role of minor sequence differences between histone variants on the regulation of chromatin-regulated processes *in vivo* in multicellular eukaryotes.

Recently, a novel histone mutagenesis platform in *D. melanogaster* was engineered using a CRISPR/Cas9-mediated HR pathway to knock in the replacement histone genes [55] (Figure 4). This platform provided an improvement over previous systems in that it was less labor intensive to utilize, as previous strategies required four plasmids for transgenic complementation and complex crossing protocols, compared to a single plasmid for transgenic complementation with this strategy. Additionally, the CRISPR/Cas9-based strategy allows the replacement gene to be targeted *in situ* to the endogenous histone locus, eliminating positional effects associated with randomly integrated plasmids. As such, this strategy provides a much higher-throughput strategy for generating histone replacement mutants and offers greater control over mutant lines. This system was used to generate 40 alanine substitution mutations, covering all known modified residues on histones H3 and H4 in *D. melanogaster* [55]. The mutants were screened for various phenotypes including viability, fertility, DNA damage sensitivity, and gene silencing defects to demonstrate roles for H4K16 in the regulation of male viability and female oogenesis, H3K27 in late embryogenesis, and H3R26 in PRC2-mediated gene silencing. Similar to recent histone replacement systems developed in unicellular eukaryotes, this histone mutagenesis platform

allows histone replacement at the endogenous locus through HR and provides a method for the systematic assessment of histone function.

In sum, in their initial usages, histone replacement systems in *D. melanogaster* were predominantly used to study specific residues on histone H3 that had previously been demonstrated to play important roles in the regulation of development and disease [40, 42, 43, 56–58]. However, optimizations implemented in more recent systems have allowed for more systematic screens of histone residues in this organism due to their higher efficiencies [55]. While the different histone replacement strategies have varied slightly in the number of histone replacement gene units required for complete rescue (e.g. 12 histone gene units required in the first system [40] and 20 histone gene units required in the most recent system [55]), these results indicate dosage compensation occurring in the histone replacement backgrounds, as the genome of *D. melanogaster* contains approximately 100 endogenous copies of the histone gene unit [59].

Expanding the use of histone replacement to study chromatin in plants

In addition to evaluating histone replacement systems in animals, assessing histone function in plant model organisms provides important information about the conservation of epigenetic regulatory mechanisms across eukaryotes and the roles of histones in plant-specific pathways. Additionally, plant model systems such as *Arabidopsis thaliana* can deliver a higher-throughput strategy towards the assessment of histone mutants compared to animal organisms such as *D. melanogaster* due to the relative ease of maintaining stable transgenic lines.

The first histone replacement systems generated in the model plant *A. thaliana* targeted histone H3 (either replication-dependent H3.1 or H3 variants). A centromeric H3 (CENH3) replacement system was developed where a *cenh3* null mutant was generated, followed by replacement with CENH3 expressed from a randomly integrated transgene [60]. GFP-tagged CENH3 replacement was demonstrated to rescue the *cenh3* embryonic-lethal phenotype, while *GFP-tailswap* plants (CENH3 histone fold domain fused to the H3.3 tail and an N-terminal GFP tag) showed greatly reduced fertility. A fraction of the progeny of *GFP-tailswap* plants pollinated by wild-type plants displayed a complete loss of chromosomes from the *GFP-tailswap* genome, resulting in an efficient strategy to generate haploid genomes used in crop breeding [60–63]. A similar system has also been used to study the diversification of CENH3 across plant species. For example, *A. thaliana* plants expressing the CENH3 histone fold domain merged to the *Zea mays* CENH3 tail (*GFP-maizetailswap*) were viable but showed a more severe sterility phenotype than *GFP-tailswap* plants, while plants expressing the full-length maize GFP-tagged CENH3 in place of endogenous CENH3 were embryonic lethal [64, 65]. However, full-length maize CENH3 lacking the GFP tag was able to functionally replace *Arabidopsis* CENH3, underscoring the importance of including untagged replacement histones in functional studies [66]. While the CENH3 replacement strategy remains to be utilized for functional studies of individual residues on this histone variant, this example demonstrates the diverse biotechnological applications that can be derived from histone replacement experiments.

Another early histone replacement system established in plants targeted canonical histone H3.1. To establish this partial replacement system, individual H3.1 substitution mutants were expressed from transgenes in a H3.1 quadruple mutant background, which has four out of the five endogenous histone H3.1 genes mutated (with two H3.1 genes knocked-out and two others being hypomorphic mutants) with T-DNA insertions [67]. This system was first used to study the role of alanine at position 31 in histone H3.1, as this residue demonstrates a conserved difference between H3.1 and H3.3 in plants and animals (with threonine being found at position 31 in the variant H3.3 in plants). H3.1A31T replacement mutants showed lower levels of H3.1K27 monomethylation compared to wild-type plants; moreover, biochemical experiments demonstrated that the H3.1A31T mutation inhibited the ability of H3K27 methyltransferases ATXR5 and ATXR6 to methylate H3.1K27. These results validated the hypothesis that the conserved difference at position 31 between H3.1 and H3.3 is responsible for the selective methylation of H3.1 by ATXR5 and ATXTR6 [67]. This partial H3.1 replacement system was also used to demonstrate that expression of the substitution mutant H3.1S28A, which cannot be methylated at H3.1K27 by ATXR5/6, exhibits similar genome instability phenotypes to *atxr5/6* mutants [68]. Similar experiments related to genome stability have also been performed with H3K27Q mutants in *T. thermophila* to assess the role of H3K27me1 in regulating replication stress [69]. These H3.1 studies in *A. thaliana* demonstrated again the utility of histone replacement systems for uncovering new roles for histone variants in multicellular organisms.

A more sophisticated H3.1 replacement system was recently developed that approximated complete histone replacement. A combination of traditional crossing and artificial microRNA was used in order to generate knockdown lines depleted of histone H3.1 [70]. H3.1 depletion mutants displayed developmental defects including enlarged inflorescence meristems, fasciated stems, ectopic leaflets, and reduced silique size. The developmental defects of these lines could be partially to completely rescued by the expression of microRNA-resistant H3.1. This system was used to study the effect of several H3.1 point mutants, including H3.1K4A, H3.1K9A, and H3.1K27A, on the propagation of H3K27me3 during DNA replication. Additionally, this system was utilized to assess whether the histone variant H3.3 could functionally replace canonical H3.1 and to precisely probe the impact of each of the four divergent residues between H3.1 and H3.3 on H3K27me3 propagation [70]. These experiments revealed that residue 31 is unique among the four divergent amino acids between H3.1 and H3.3 and that the H3.1K27A mutation is unique among the three lysine mutations assessed in their impact on regulating development and the maintenance of H3K27me3.

Two systems that allow the assessment of functions for histone H2A variants have also been described in *A. thaliana*. Partial H2A.Z replacement mutants were generated in order to assess the effect of H2A.Z mono-ubiquitination on transcriptional repression [71]. A double mutant containing mutations in two out of the three endogenous H2A.Z genes was generated by crossing and an N-terminal FLAG-tagged version of H2A.Z was then constitutively expressed under the control of the cauliflower mosaic virus 35S promoter. When wild-type H2A.Z was constitutively expressed in this system, it partially rescued the developmental phenotypes observed in the H2A.Z double mutant. This system was then used to assess the function of H2A.Z lacking mono-ubiquitination by expressing FLAG-

tagged H2A.Z with either K129 or both K129 and K132 mutated to arginine, and results showed that H2A.Z mono-ubiquitination plays an important role in mediating transcriptional repression. Additionally, a system was developed that expressed H2A.W from a transgene in a background completely deprived of endogenous H2A.W expression using a combination of T-DNA insertion mutants and CRISPR/Cas9-mediated mutagenesis [72]. This system was used to study the effect of differing regulatory and primary amino acid sequences of H2A.W isoforms, specifically by swapping the promoters, histone core domains, and C-terminal tails between the isoforms H2A.W.6 and H2A.W.7 and expressing these fusions in the *h2a.w* depletion background. Using this system, differences in primary amino acid sequences between the C-terminal tails of H2A.W.6 and H2A.W.7 were shown to regulate phosphorylation of the conserved KSPK motif within the C-terminal tail domain.

Finally, a complete histone H4 replacement system was recently described, allowing a systematic screen of histone H4 residues (Figure 5) [73]. The strategy used to create this system involved generating an H4 septuple mutant containing homozygous mutations in seven out of the eight endogenous H4 genes using CRISPR/Cas9. Next, every residue on H4 that could in theory be a target for PTMs (i.e. arginine, lysine, threonine, serine, and tyrosine) was individually mutated to alanine, valine, or phenylalanine. Additionally, each lysine residue was substituted for arginine and vice versa. These H4 point mutants were expressed in the H4 septuple mutant, while the remaining endogenous H4 gene was simultaneously targeted for mutagenesis with CRISPR/Cas9. This large library of H4 replacement mutants was screened to reveal roles for H4 in the regulation of flowering time. Using this system, a novel role for H4R17 in the modulation of flowering time was revealed, related to the function of this residue in regulating Imitation SWItch (ISWI) chromatin remodeling.

While many novel functions for H4 residues in the regulation of flowering time were revealed in the above screen, mutations in some H4 residues that have previously been speculated to play an important role in flowering time regulation were not observed to cause major flowering time phenotypes in these experiments [73]. For example, while mutations in Protein Arginine Methyltransferase 5 (PRMT5), the enzyme that catalyzes the symmetric dimethylation of H4R3, have been shown to cause late flowering [74], mutations of H4R3 were not observed to cause any significant flowering time differences in these screens. This result supports the hypothesis that the late flowering phenotypes in *prmt5* mutants are not caused by a loss of methylation on H4R3, but rather they are mainly caused by the action of PRMT5 on non-histone substrates such as RNA processing factors, leading to splicing defects in transcripts of flowering time regulators such as *FLK* [75]. This example demonstrates that histone replacement experiments can offer critical insights into mechanisms by which histone residues regulate diverse processes in eukaryotes.

Concluding remarks

Histone replacement has served over the last 30 years as a primary or complementary strategy for discovering new histone functions across diverse eukaryotic lineages. Compared to expressing mutant histones in a wild-type background, there are several advantages to completely replacing expression of endogenous histones with mutant histones. First,

complete histone replacement reveals all phenotypes caused by the histone mutation, instead of only dominant phenotypes. In certain cases, researchers may want to examine histone mutations in a dominant context, such as when studying some histone mutations that are known to cause diseases in humans but which comprise only a portion of chromatin. For example, H3K27M mutant histones were overexpressed in flies in a wild-type background as a model to study the role of this dominant gain-of-function mutation in pediatric brain cancers [76]. However, there are many cases where researchers may want to perform broader screens that can reveal roles for histone residues that are more subtle, in which case it would be beneficial to study genetic systems where the mutated histone is exclusively present in chromatin to eliminate extenuating factors in the experiment. In addition, it is possible that expressing mutant histones in wild-type backgrounds may lead to cytotoxicity due to the excess accumulation of histone supply [77]. However, various mechanisms exist to combat the accumulation of excess histones and can serve to regulate histone supply under conditions of histone overexpression [78–80].

While histone replacement systems engender numerous benefits, confounding issues do still exist with these systems, so they are often supplemented with the traditional analysis of mutations in genes coding for histone-modifying or histone-binding proteins. For example, some histone residues can have multiple PTMs added to them, such as lysine mono-, di-, or trimethylation or lysine acetylation. Therefore, mutating said lysine residue could eliminate all possible PTMs from being added, and thus, determining what specific PTM is causing an observed phenotype in a histone replacement mutant would be difficult without more information. Additionally, mutating a particular histone residue may not only affect PTMs added to that specific residue, but also the ability of histone-modifying enzymes to add PTMs to other histone residues. This feature can be a benefit of these systems, but it also introduces confounding factors that often need to be addressed. In addition, histone mutations are more likely to cause lethality or sterility in a complete replacement background and consequently can be more difficult to study, although conditional knockout systems can allow the investigation of mutations with these effects [55, 59]. Finally, mutated histone residues can have dominant gain-of-function effects that can inhibit histone-modifying enzymes. For example, individual histone H3 lysine-to-methionine substitutions of H3K9, H3K27, and H3K36 have all been shown to inhibit the enzymatic activity of PRC2 complexes by capturing these histone-modifying enzymes [76, 81]. This final case represents a confounding factor, but it also presents a way to bypass some of the above obstacles presented for the traditional analysis of mutations in histone-modifying enzymes. Namely, histone gain-of-function mutations that inhibit modification pathways can be used to disrupt the addition of certain PTMs even if the relevant histone-modifying enzyme(s) have not been identified. However, if the sequestered histone-modifying enzymes have non-histone substrates and/or essential activities, these systems do not circumvent those complications. Nonetheless, the combination of evidence from a histone replacement system and evidence from mutating the relevant histone-modifying enzyme(s) often provides the clearest representation of the functional significance of a particular histone PTM given the current technological capabilities for *in vivo* work using model organisms.

In conclusion, histone replacement systems offer a valuable resource for epigenetics researchers to study histone function in an *in vivo* setting. Subsequent optimization of these

systems in unicellular eukaryotes, *D. melanogaster*, *A. thaliana*, and other biological models (for example, mammalian cell lines [82–85]) will further improve their utility for molecular biology research (see Outstanding Questions). In the future, these systems will continue to complement other strategies used for functional characterization of histones, such as classical genetic analyses of mutant alleles of histone readers/writers/erasers, or more recent high-throughput biochemical methods based on DNA-barcoded mononucleosome libraries [33, 86, 87]. Expected advancements in genetic engineering should accelerate the development of histone replacement systems that perfectly reflect *in vivo* expression of endogenous histones (for example, Cas9-mediated base editing of histone genes [88]), while also greatly expanding the number of biological systems in which this strategy can be deployed.

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Glossary

Alanine-scanning mutagenesis
site-directed mutagenesis technique where every amino acid in the protein is individually exchanged with alanine

Eraser proteins
proteins that remove chromatin marks

Histone
highly basic protein component of the nucleosome. Each histone contains a histone fold domain, which allows the histones to associate into the heterodimers H2A-H2B and H3-H4, and comprises approximately 75% of the histone protein mass. Each core histone also contains an unstructured N-terminal tail (and histones H2A and H2B also contain an unstructured C-terminal tail)—comprising approximately 25% of the histone protein mass—that protrudes from the nucleosome.

Histone octamer
protein complex composed of two copies of each of the positively charged core histones H2A, H2B, H3 and H4.

Histone post-translational modifications (PTMs)
covalent modifications of histone residues which can be recognized either individually or in a combinatorial manner to recruit reader, writer, and/or eraser proteins and indirectly achieve various outcomes within a cell. Histone PTMs are reversible and intrinsically dynamic, which allows them to change in response to external cues.

Histone replacement system

genetic system in which expression of endogenous wild type histones is compromised, and replaced with histones mutated at a specific residue (or multiple residues).

Histone variant
proteins that can replace the four core canonical histones (H3, H4, H2A, H2B) in nucleosomes in a replication-independent manner. Some histone variants can differ substantially in sequence from the canonical histones they replace, while others may vary by only a few amino acids, but in either case the incorporation of a histone variant to a region of chromatin can have significant consequences on chromatin states.

Oncohistone
Cancer-associated histone mutation

Nucleosome
the basic repeating unit of chromatin, composed of a histone octamer and approximately 150 base pairs of DNA

Reader proteins
proteins containing domains that interact with histone PTMs

Writer proteins
proteins that add chromatin marks

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Outstanding Questions

- Currently, systematic screens in multicellular eukaryotes have only targeted residues on the canonical histones H3 and H4 that can be targeted for post-translational modification. What additional functions will systematic screens assessing other histones and/or histone residues reveal in multicellular eukaryotes?
- How can we utilize currently existing libraries of histone replacement mutants to screen for additional functions for histone residues?
- Histone replacement systems are relatively labor-intensive to establish, but nonetheless, can serve as a valuable resource for answering questions in chromatin biology once the mutant lines are generated. What methods can we cultivate to facilitate the development of these systems?
- Due to the low efficiency of gene targeting technologies in plants, a strategy to introduce the histone mutations directly at the endogenous histone gene locus (as has been developed in yeast and flies) does not yet exist in these organisms. How far are we from developing *in situ* histone replacement systems in plants analogous to platforms already existing in other organisms?
- It is most reflective of endogenous cellular conditions to ensure that histone replacement mutants contain the same number of copies of histone genes as their wild-type counterparts. While such systems exist in yeast, they have not yet been developed in multicellular eukaryotes such as plants and flies. How can we develop more precise and efficient strategies to regulate the replacement histone dosage in these genetic systems?
- In systematic histone replacement screens, only a relatively small percentage of residues are found to be essential for viability. Why do we observe such a high level of evolutionary conservation of histones if the individual mutation of most histone residues results in a viable mutant that can be studied?

Highlights

- Histone post-translational modifications (PTMs) play critical roles in regulating various genomic processes with widespread effects on organismal development and fitness.
- A major strategy for determining the function of histone PTMs has historically been to analyze phenotypes incurred by the mutation of genes coding for histone-modifying or histone-reading proteins; however, complications such as redundant histone-modifying/reading proteins, non-histone substrates for these proteins, and essential roles for these proteins exist with this strategy.
- Histone replacement systems, in which some or all endogenous copies of a histone are replaced with a mutant histone, allow the direct functional assessment of histones and circumvent obstacles associated with previous strategies, and both modifiable and non-modifiable residues of histones can be studied with this strategy.

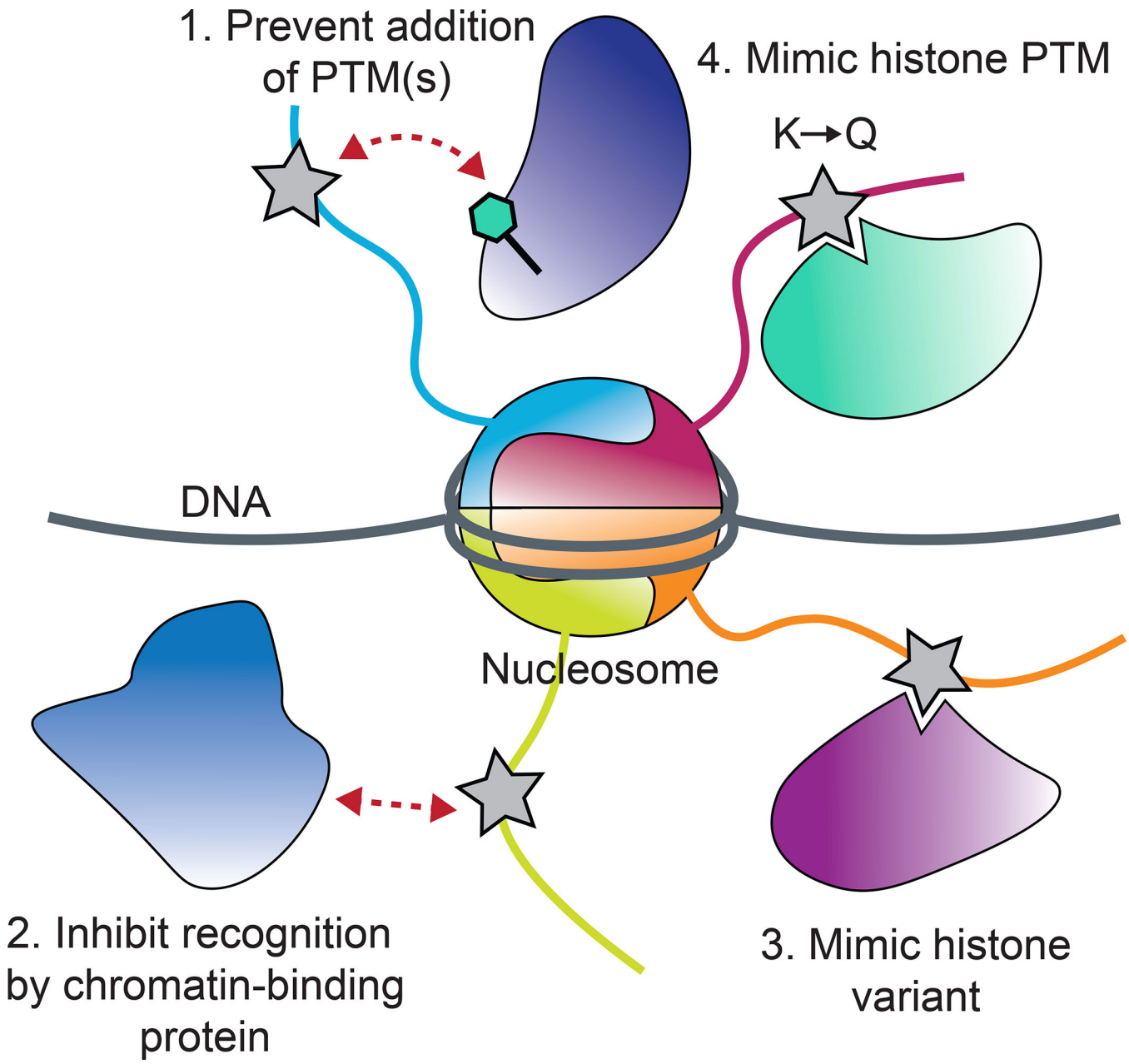

Figure 1: Functions for histone replacement mutants.

Mutated histones can 1) prevent the post-translational modification of histone residue(s), 2) inhibit recognition by a chromatin-binding protein, 3) partially or completely emulate a histone variant, and/or 4) mimic a constitutively modified state (e.g. lysine (K) to glutamine (Q) mutation to mimic the constitutively acetylated state of lysine residues). Importantly, as mutations in histones have been linked to human cancers ("oncohistones") and developmental disorders, histone replacement systems have broad applications for studying disease mechanisms *in vivo*. Additionally, due to the high conservation of histone proteins across eukaryotes, histone replacement systems established in model organisms remain relevant for translational applications towards human disease research. So far,

histone replacement systems have been implemented in several model organisms: the single-celled eukaryotes *S. cerevisiae, S. pombe, T. thermophila*, and *T. brucei* as well as the multicellular eukaryotes *D. melanogaster* and *A. thaliana.*

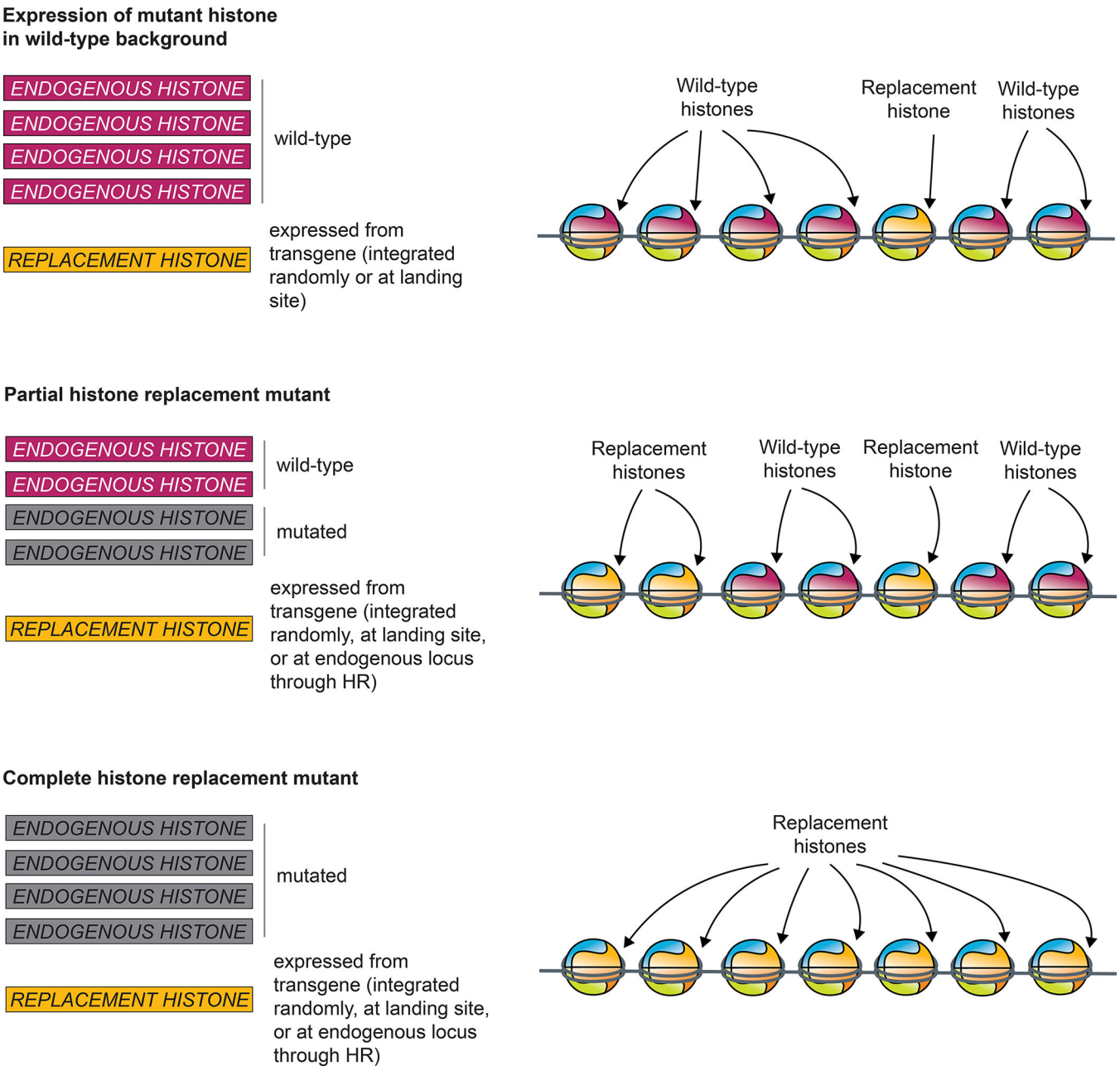

Figure 2:

Three types of histone replacement systems. (Top) Expression of a mutant histone in a wild-type background produces chromatin with minor insertion of mutant histones. (Middle) Partial histone replacement (some endogenous histone genes are mutated) produces chromatin with significant insertion of mutant histones. (Bottom) Complete histone replacement (all endogenous histone genes are mutated) produces chromatin that exclusively contains mutant histones. One major technical challenge with the generation of complete histone replacement systems is that the diploid genome of most organisms typically contains more than two copies encoding each histone.

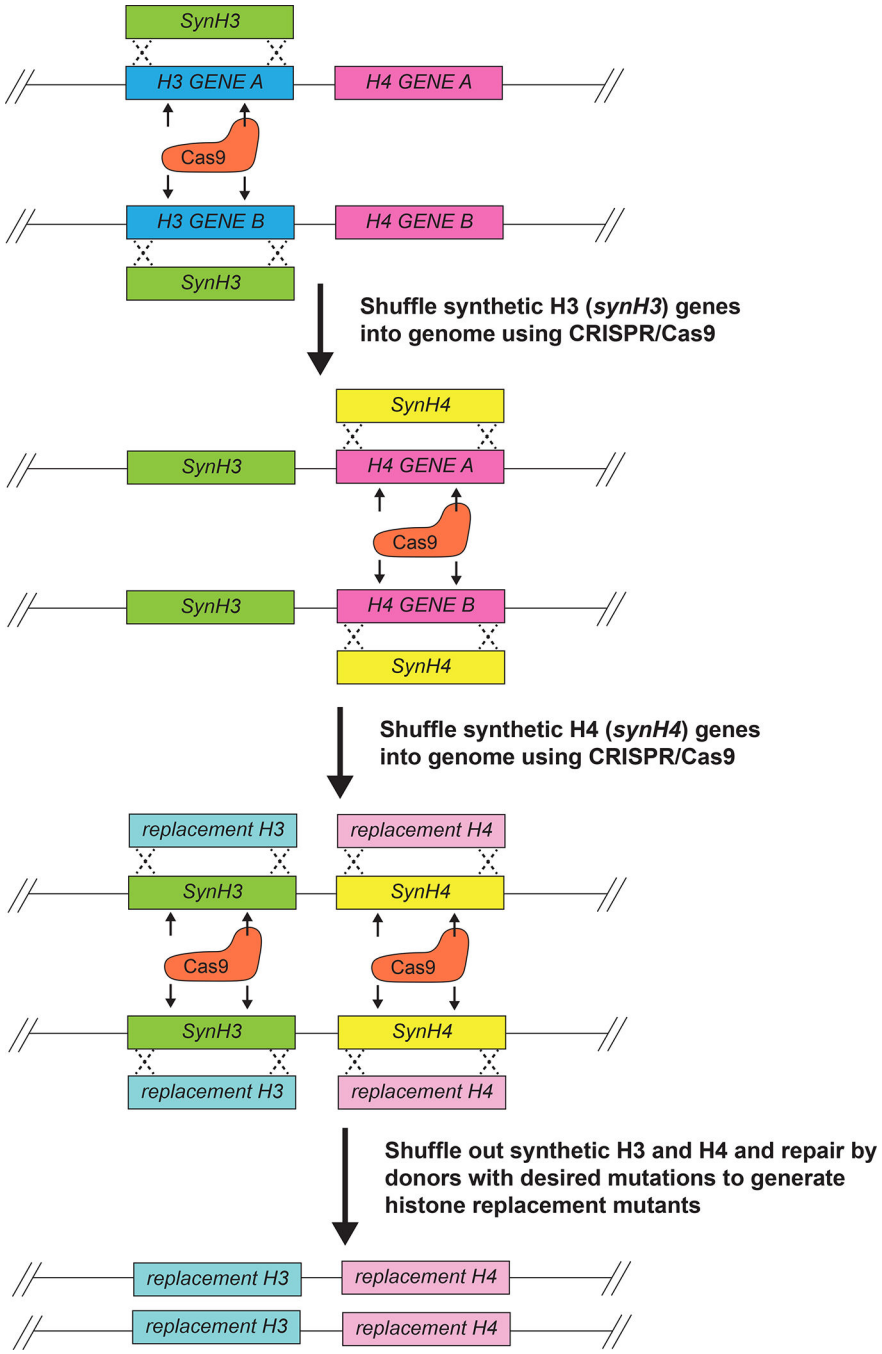

Figure 3: Strategy to generate histone H3 and H4 replacement mutants in *S. cerevisiae*.

First, synthetic H3 and H4 genes (containing the same protein sequences as endogenous H3 and H4 but different codons to minimize undesired homology-directed repair) are shuffled in to replace endogenous H3 and H4 *in situ* using CRISPR/Cas9. Next, synthetic H3 and H4 are targeted by CRISPR/Cas9 and donor templates containing endogenous H3 and H4 genes with the desired mutations are provided for repair, resulting in histone replacement mutants.

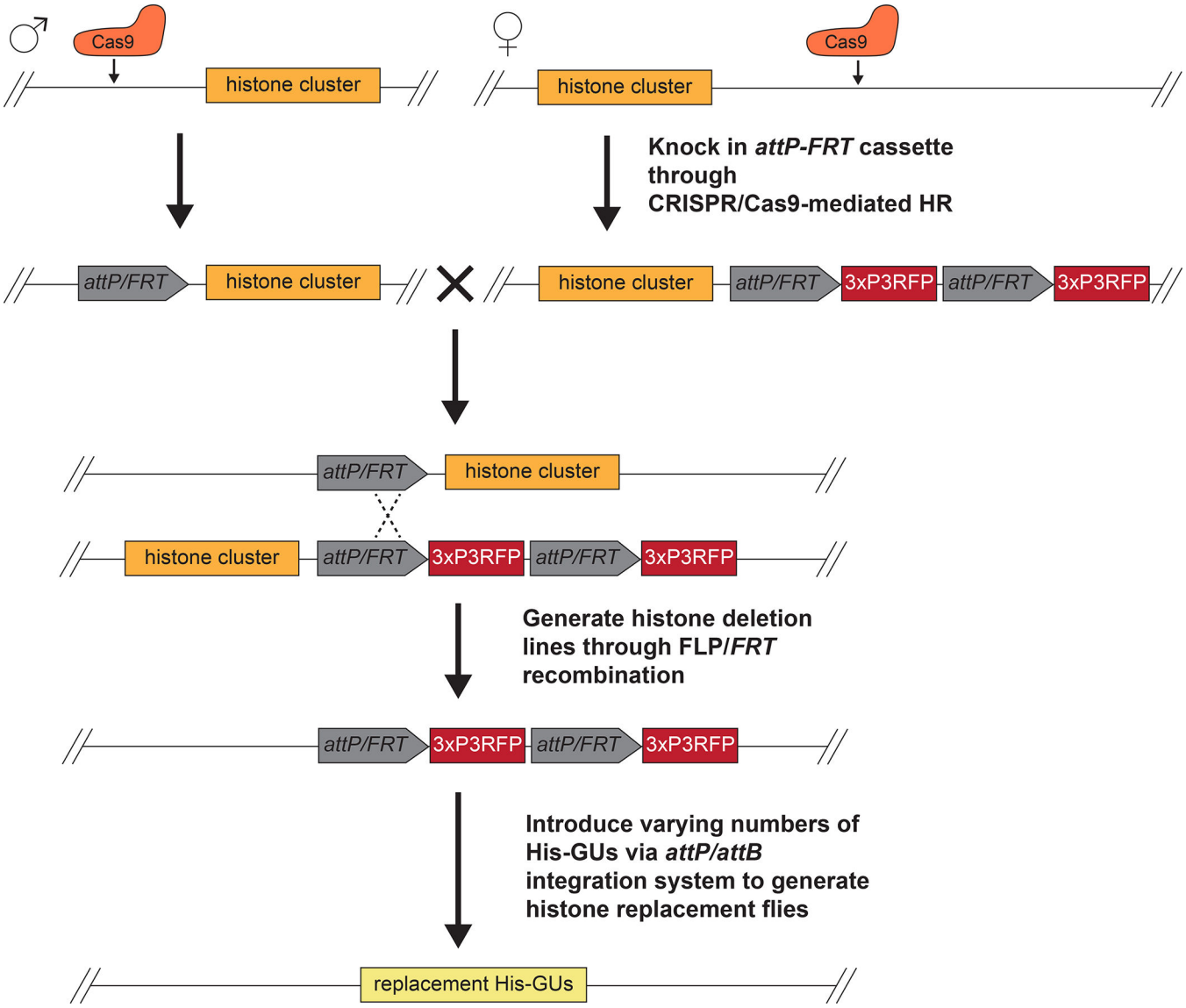

Figure 4: Strategy for *in situ* histone cluster replacement in *D. melanogaster*.

The histone cluster deletion (His^D) line is generated through a multistep process. First, the *attP-FRT* cassette is knocked in using CRISPR/Cas9-mediated HR on either side of the replication-dependent histone gene cluster (right side contains *attP-FRT* duplication). Next, these two fly lines are crossed and flippase activity is induced to generate the His^D line through HR. Varying numbers of histone gene units (His-GUs) are then introduced into the His^D background *in situ* through the *attP/attB* integration system to generate histone replacement lines. Viable flies are recovered with the introduction of 8 His-GUs and flies with 12 or 20 His-GUs show similar histone mRNA and protein levels to wild-type flies.

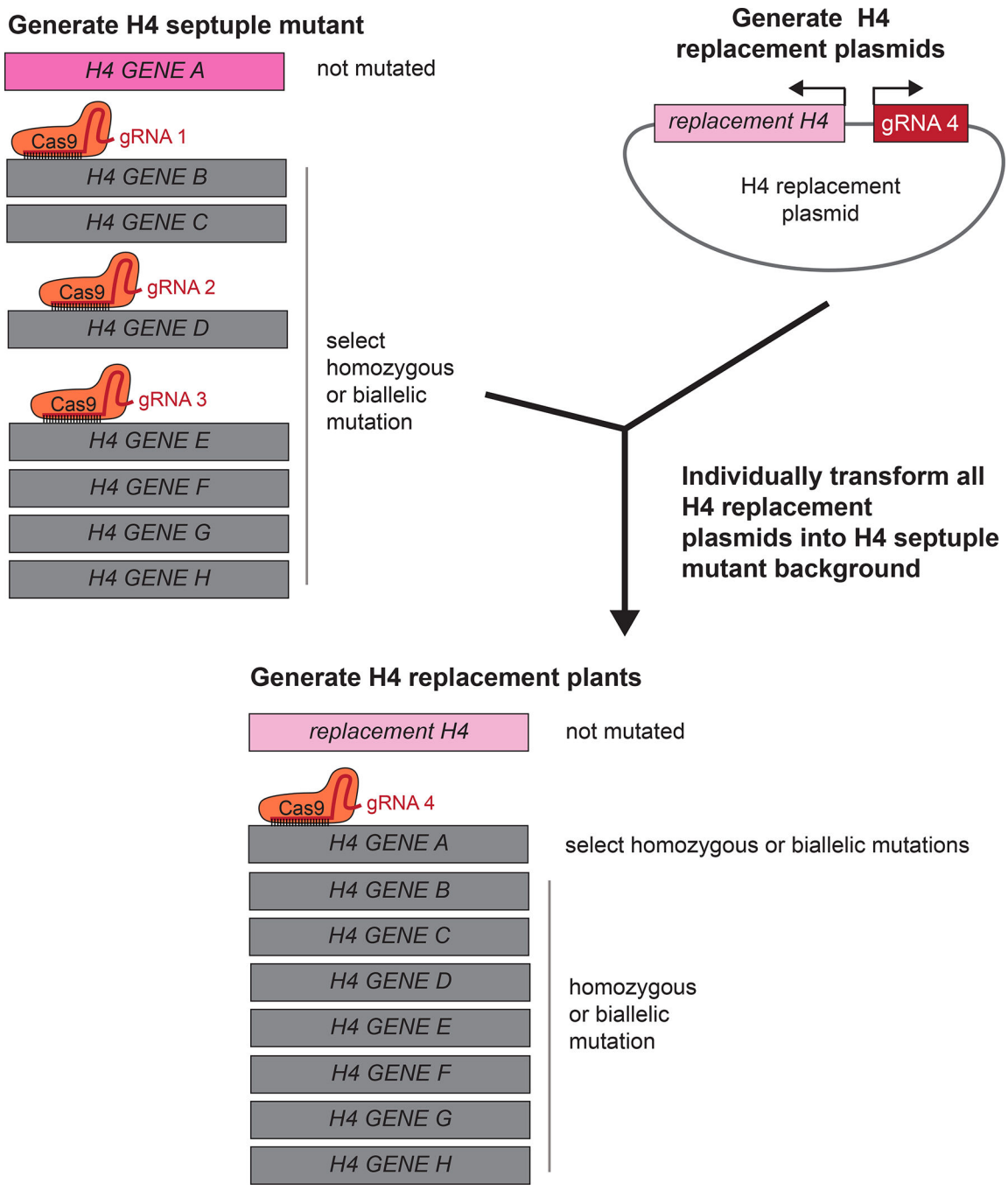

Figure 5: Strategy to generate H4 replacement mutants in *A. thaliana*.

Multiple guide RNAs (gRNAs) are used to simultaneously target all except one of the endogenous H4 genes for homozygous mutation and generate the H4 septuple mutant background. Sequence homology between H4 nucleotide sequences is taken advantage of to target multiple H4 genes with the same gRNA. H4 replacement plasmids, containing a replacement H4 gene and a gRNA targeting the remaining endogenous H4 gene are individually transformed into the H4 septuple mutant background. Resulting H4 replacement plants display a complete replacement of endogenous H4 with replacement H4 after

selecting plants with homozygous or biallelic mutations in the remaining endogenous H4 gene.

Table 1:

Histone replacement systems established in model organisms.

Organism	Targeted histone(s)	Mutants assessed	Type of replacement	Processes assessed	Reference
Saccharomyces cerevisiae	H2B	Small (5–58 amino acid) deletions distributed over protein, many in N-terminal tail; small insertions in the N-terminal tail	complete; provided on episomal plasmid	viability, mating efficiency	[9]
Saccharomyces cerevisiae	H2A, H2B	N- and C-terminal tail small (2–30 amino acid) deletions; H2A and H2B partial tailswap	complete; provided on episomal plasmid	viability	[14]
Saccharomyces cerevisiae	H4	Small (5–58 amino acid) deletions distributed over protein, many in N-terminal tail	complete; provided on episomal plasmid	viability, chromatin structure, cell cycle, mating efficiency, transcriptional silencing	[13]
Saccharomyces cerevisiae	H4	S1A, S1G, S1E, S1I, S1P, S1V, K5R/K8R/K12R/ K16R, K5R/K8R/K12R, K16R, K5A/K8A/K12A/ K16A, K5A/K8A/K12A, K16A	complete; provided on episomal plasmid	transcriptional silencing, mating efficiency, growth, sporulation	[6]
Saccharomyces cerevisiae	H4	K5G/K8G/K12G, K16G, K16Q, R17G, H18G, R19G, K20G K5R/K8R/K12R, K16R, R17K, H18R, R19K, K20R	complete; provided on episomal plasmid	transcriptional silencing, mating efficiency	[8]
Saccharomyces cerevisiae	H4	K5R, H18Y, K5R/K8R, K5R/K12R, K16R, K5Q/K8Q, K12Q/K16Q, K5R/K8R/K12R/K16R, K5Q/K8Q/K12Q/K16Q	complete; provided on episomal plasmid	gene expression, replication, nuclear division, growth, mating efficiency	[7]
Tetrahymena thermophila	H3	S10A	complete at endogenous locus	chromosome segregation, chromosome condensation, mitosis, meiosis, growth	[11]
Tetrahymena thermophila	H1	T35A/S43A/S45A/T47A/T54A; T35E/S43E/S45E/ T47E/T54E	complete at endogenous locus	gene expression	[12]
Tetrahymena thermophila	H1	K33, P34, T35, K38, K40, S43, A44, S45, T46, T47, V49, K50, K51, V53, T54 to Q, A, E, D, and/or K	complete at endogenous locus	gene expression	[19]
Saccharomyces cerevisiae	H3, H4	H3: R72G, A75V, F78L, K79R, K79E, R83A H4: R78G, K79M, T80A, V81A	complete; provided on episomal plasmid	transcriptional silencing	[22]
Schizosaccharomyces pombe	H3	R8A, K9A, K9R, S10A, K14R, K14A, K16G, K9R/ S10A, K9R/K14R, R8A/K16G	partial; provided on episomal plasmid	growth, centromeric silencing, sensitivity to thiabendazole, chromosome segregation	[10]
Tetrahymena thermophila	H3	K9Q	complete at endogenous locus	small RNA accumulation, DNA elimination	[89]
Saccharomyces cerevisiae	H3, H4	All modifiable residues identified in *Bos taurus* [23] to alanine, arginine, glutamine, and/or glutamate	complete; provided on episomal plasmid	transcriptional silencing, DNA damage	[24]
Saccharomyces cerevisiae	H2B	S10A, S10E, S33A, S33E	complete; provided on episomal plasmid	cell death, chromatin structure	[90]

Organism	Targeted histone(s)	Mutants assessed	Type of replacement	Processes assessed	Reference
Saccharomyces cerevisiae	H2A, H2B, H3, H4	All non-alanine surface residues to alanine	complete; provided on episomal plasmid	transcription, DNA damage	[26]
Schizosaccharomyces pombe	H3	K56R, K56Q	complete; provided on episomal plasmid	DNA damage, transcriptional silencing	[91]
Schizosaccharomyces pombe	H3	K56R, K56Q	complete; provided on episomal plasmid	growth, DNA damage	[92]
Saccharomyces cerevisiae	H3, H4	All non-alanine surface residues to alanine; alanine to serine; arginine to lysine and vice versa; lysine to glutamine; serine, threonine to aspartate; tyrosine to glutamate, phenylalanine; proline to valine; glutamate to glutamine and vice versa; aspartate to asparagine and vice versa; 4 to 36 residue N-terminal tail deletions	complete; provided on episomal plasmid or integrated at one endogenous locus	temperature sensitivity, DNA damage, transcription, transcriptional silencing, microtubule disruption, evolutionary fitness, NHEJ, H3K56 acetylation, H3K4 and H3K79 methylation	[15]
Saccharomyces cerevisiae	H2A, H2B, H3, H4	All non-alanine residues to alanine	partial; provided on episomal plasmid	H3K4 trimethylation	[25]
Saccharomyces cerevisiae	H2A, H2B, H3, H4	All non-alanine buried residues to alanine	complete; provided on episomal plasmid	transcription, DNA damage	[27]
Saccharomyces cerevisiae	Cse4 (Centromeric H3)	All non-alanine residues to alanine	partial; provided on episomal plasmid	Cse4 dimerization, viability	[93]
Saccharomyces cerevisiae	H2A, H2B, H3, H4	All non-alanine residues to alanine	complete; provided on episomal plasmid	histone deacetylation, DNA damage	[94]
Saccharomyces cerevisiae	H3, H4	every serine, threonine, lysine, and arginine residue to alanine; H4K5/8/12R	complete; provided on episomal plasmid	gametogenesis	[29]
Saccharomyces cerevisiae	H3	G44S	complete; provided on episomal plasmid	chromosome segregation, microtubule disruption, DNA damage, temperature-regulated growth, mitosis, transcription	[95]
Saccharomyces cerevisiae	H4	K16Q, K16R	complete; provided on episomal plasmid	fitness, chromosome segregation	[96]
Saccharomyces cerevisiae	H2A.Z, H2A	All non-alanine residues to alanine	complete; provided on episomal plasmid	DNA damage, mitosis, cell cycle	[97]
Saccharomyces cerevisiae	H2A, H2B, H3, H4	same as previous studies [26, 27, 94]	complete; provided on episomal plasmid	microtubule disruption, chromosome segregation, chromosome biorientation	[28]
Saccharomyces cerevisiae	H2A, H2B, H3, H4	pairwise combinations of tail deletions; pairwise combinations of H4K5/8/12R, H2AK4/7/13R, and H4 tail deletion; combination of H2A tail deletion with H3S1A, H3R2A, H3T3A, H3K4A, and H3Q5A	complete; provided on episomal plasmid	chromatin structure, cell cycle, cell growth, DNA damage	[17]
Saccharomyces cerevisiae	H2A, H2B, H3, H4	same as previous study [15]; individual N-terminal tail deletions	complete; integrated at one endogenous locus	chromosome loss, kinetochore biorientation, replication, chromosome segregation	[16]

Organism	Targeted histone(s)	Mutants assessed	Type of replacement	Processes assessed	Reference
Tetrahymena thermophila	H3	K27Q	complete at endogenous locus	replication stress, DNA damage	[69]
Saccharomyces cerevisiae	H2A, H2B, H3, H4	pairwise combinations of tail deletions	complete; provided on episomal plasmid	DNA damage, base excision repair, postreplication repair	[18]
Saccharomyces cerevisiae	H3, H4	same as previous study [15]	complete; integrated at one endogenous locus	aging	[98]
Saccharomyces cerevisiae	H3, H4	H3: K36 through K56 to alanine - generated in a previous study [15] H4: R35S	complete; provided on episomal plasmid	mitosis	[99]
Saccharomyces cerevisiae	H2A, H2B, H3, H4	All non-alanine surface residues to alanine; alanine to serine; arginine to lysine and vice versa; arginine to glutamate; lysine to glutamine; lysine to glutamate; serine, threonine to aspartate; tyrosine to glutamate, phenylalanine; proline to valine; glutamate to glutamine and vice versa; aspartate to asparagine and vice versa; aspartate to arginine; glutamate to arginine; individual N- or C-terminal tail deletion	complete; integrated at both endogenous loci	viability, fitness	[38]
Schizosaccharomyces pombe	H3	G34R	complete; provided on episomal plasmid	histone acetylation, H3K36 methylation, transcription, chromosome loss, chromosome segregation, DNA damage, DNA replication, homologous recombination	[100]
Saccharomyces cerevisiae	H2A, H2B, H3.1, H3.3, H4	divergent residues from human histones	complete; provided on episomal plasmid	viability, growth, nucleosome accessibility, transcription	[30]
Trypanosoma brucei	H4	K4R	complete at endogenous locus	NA	[37]
Saccharomyces cerevisiae	H2A, H2A.V, H2A.Z, H2B, H3.1, H3.3, H4	cancer-associated histone missense mutations, alanine substitutions at targeted residues	complete; provided on episomal plasmid	nucleosome stabilization, nucleosome sliding	[33]
Saccharomyces cerevisiae	H2A	H2A-H2A.Z and H2A.Z-H2A hybrids under native H2A.Z promoter (reporter tagged)	complete; provided on episomal plasmid	growth, fitness, genotoxic stress, interaction with histone chaperones and chromatin remodelers, transcription	[34]
Schizosaccharomyces pombe	H2A	H2A hybrids with (1) repetition of native C-terminal tail, (2) C-terminal tail of H2A.W with and without KPSK motif and (3) C-terminal tail of H2A.M	complete at endogenous locus	cell morphology, growth rate, stress responses, transcription, H3K9me2 accumulation	[35]
Saccharomyces cerevisiae	H3, H4	H3: K4R, K4R/K36R/K79R H4: K56Q, K59A, K56Q/K59A	complete at endogenous locus	DNA damage	[36]
Drosophila melanogaster	H3.2, H3.3	H3.3K4A, H3.3K9A (reporter tagged)	complete at transgene integration site	fertility, transcription	[50]
Drosophila melanogaster	H3.3	H3.3K4R, H3.3K9R, H3.3S31A	complete at transgene integration site	fertility, transcription, meiosis, chromatin structure	[51]

Organism	Targeted histone(s)	Mutants assessed	Type of replacement	Processes assessed	Reference
Drosophila melanogaster	H3.2	H3: K27R	partial at transgene integration site	development, fertility	[40]
Drosophila melanogaster	H3.2, H3.3	H3.3K4A and H3.2K4A; H3.3K4R and H3.2K4R	complete at transgene integration site	transcription, proliferation	[49]
Drosophila melanogaster	H3.2	H3: K27R	complete at transgene integration site	transcriptional silencing, development	[42]
Drosophila melanogaster	H3.2, H4	H3: K27R, K27A, K36R H4: K20A	complete at transgene integration site	development, DNA replication, fertility, transcriptional silencing	[43]
Drosophila melanogaster	H2A, H2Av	H2A: Kn7R/K118R/K121R/K122R H2Av: K120R/K121R	complete at transgene integration site	transcriptional silencing, development, viability, fertility	[47]
Drosophila melanogaster	H3.2	S28A	complete at transgene integration site	H3K27 methylation, development, mitosis, transcriptional silencing	[101]
Drosophila melanogaster	H3	K56R, K56Q, T80A, T80E, K115R, K115Q, T118A, T118E, T118I, K122R, K122Q, K115R/K122R, K115Q/K112Q	complete at transgene integration site	viability, development, cell cycle, apoptosis, transcription	[48]
Drosophila melanogaster	H3.2	H3.2K9R	complete at transgene integration site	development, transcription, nucleosome occupancy, HP1 binding, chromatin accessibility	[52]
Drosophila melanogaster	H3.2, H3.3	H3.3K9R, H3.3K9Q, H3.2K9R H3.3K9R	complete at endogenous locus (for H3.3)	development, transcription, fertility, chromatin structure	[53]
Drosophila melanogaster	H3.3	K27R	partial at endogenous locus	viability, H4K16ac, transcription, development	[54]
Drosophila melanogaster	H3.2, H4	All modifiable residues to alanine	complete at endogenous locus	fertility, DNA damage, transcriptional silencing	[55]
Arabidopsis thaliana	CENH3	CENH3 histone fold domain fused to H3.3 tail (reporter tagged)	complete at random transgene integration site	chromosome segregation, fertility	[60]
Arabidopsis thaliana	CENH3	*Arabidopsis arenosa* CENH3, *Brassica rapa* CENH3, *Zea mays* CENH3, *Homo sapiens* CENH3, *Caenorhabditis elegans* CENH3, *Saccharomyces cerevisiae* CENH3 (reporter tagged)	complete at random transgene integration site	kinetochore localization, embryonic lethality	[65]
Arabidopsis thaliana	CENH3	CENH3 histone fold domain fused to H3.3 tail, CENH3 histone fold domain fused to *Zea mays* CENH3 tail (reporter tagged)	complete at random transgene integration site	chromosome segregation, fertility	[64]
Arabidopsis thaliana	H3.1	A31T	partial at random transgene integration site	H3K27 monomethylation, transcriptional silencing	[67]
Arabidopsis thaliana	CENH3	*Lepidium oleraceum* CENH3, *Brassica rapa* CENH3, *Zea mays* CENH3, *Vitis vinifera* CENH3	complete at random transgene integration site	development, embryonic lethality, fertility, meiosis, chromosome segregation	[66]

Organism	Targeted histone(s)	Mutants assessed	Type of replacement	Processes assessed	Reference
Arabidopsis thaliana	H3.1	K4A, K9A, K27A	partial at random transgene integration site	development, transcription silencing, H3K27 trimethylation	[70]
Arabidopsis thaliana	H2A.Z	K129R, K129R/K132R (reporter tagged)	partial at random transgene integration site (expressed under constitutive promoter)	transcriptional silencing, flowering time, development	[71]
Arabidopsis thaliana	H2A.W	promoter, histone core domain and C-terminal tail swaps between H2A.W.6 and H2A.W.7	complete at random transgene integration site	H2A.W.6 phosphorylation	[72]
Arabidopsis thaliana	H3.1	K9R, K18R, K23R, K27Q, S28A, K36R, S28A/K9R, S28A/K18R, S28A/K23R, S28A/K36R	partial at random transgene integration site	DNA damage, transcriptional silencing, DNA content	[68]
Arabidopsis thaliana	H4	every serine, threonine, lysine, arginine, and tyrosine residue to alanine, valine, or phenylalanine; lysine to arginine and vice versa	complete at random transgene integration site	development, flowering time	[73]
mESCs	macroH2A	macroH2A1.1, macroH2A1.2 and macroH2A2 (reporter tagged)	complete by exogenous expression	reprogramming, H3K27 PTMs	[84]
mESCs	H3.3	K27A, S28A, S28E, S31A, S31E	complete by exogenous expression	H3K27ac	[82]
mESCs	H3.3	K27R	complete at endogenous locus	colony morphology, transcription, enhancer activity, chromatin accessibility, H3K4me1, histone acetylation	[85]
mESCs	H3.3	L126A/I130A	complete by exogenous expression	ATRX-Mcm6 association	[83]
mESCs	H3.1, H3.2, H3.3	K27R	complete at endogenous locus for H3.1/H3.2 and by exogenous expression for H3.3	transcription, differentiation, morphology	[88]