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New functions for an old variant: no substitute for histone H3.3

Simon J Elsaesser, **Aaron D Goldberg**, and **C David Allis**
Laboratory of Chromatin Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA

Abstract

Histone proteins often come in different variants serving specialized functions in addition to their fundamental role in packaging DNA. The metazoan histone H3.3 has been most closely associated with active transcription. Its role in histone replacement at active genes and promoters is conserved to the single histone H3 in yeast. However, recent genetic studies in flies have challenged its importance as a mark of active chromatin, and revealed unexpected insights into essential functions of H3.3 in the germline. With strikingly little amino acid sequence difference to the canonical H3, H3.3 therefore accomplishes a surprising variety of cellular and developmental processes.

Introduction

Histone proteins form the core of the nucleosome – the fundamental repeating unit of chromatin. In a single nucleosome, approximately two superhelical turns of DNA wrap around an octamer of the core histone proteins H2A, H2B, H3, and H4. A wealth of discoveries in recent years has transformed our view of histones from static scaffolding proteins to modulators of virtually all processes that act on or depend on DNA, including replication and repair, regulation of gene expression, and maintenance of centromeres and telomeres. Apart from the four core histones, metazoans have a number of histone variants such as H3.3, H2A.Z, and H2A.X that contain a distinct amino acid sequence and are expressed in different patterns throughout the cell cycle. Like histone posttranslational modifications (PTMs) and nucleosome remodeling, the use of histone variants contributes to the regulatory repertoire of chromatin.

Histone H3 variants have distinct sequences and expression patterns

In metazoans, three main classes of histone H3 genes encode distinct H3 proteins: the ‘canonical’, replication-dependent histone H3, the replication-independent histone variant H3.3, and the centromeric H3 variant CENP-A [1,2]. Apart from the replication-dependent histone H3 shared by all metazoans (called by its systematic name H3.2 hereafter), mammals possess another replication-dependent variant H3.1 with a single amino acid substitution (Figure 1a). Two major exceptionally conserved differences account for unique functions of H3.1/2 and H3.3: differential expression during the cell cycle and amino acid variation in residues 87-90 of the histone core region (Figure 1a). As discussed below, the rather subtle difference in primary sequence between H3.3 and H3.1/2 in this region

Corresponding author: Simon J Elsaesser, selsaesser@rockefeller.edu.

('AAIG' vs. 'SAVM') is necessary and sufficient to account for selective deposition and enrichment at specific loci in the genome [3]. In the yeast species *S. cerevisiae* and *S. pombe*, all non-centromeric H3 genes encode for an identical H3.3-like protein sequence. Phylogenetic relationships (Figure 1b) suggest that the metazoan H3.3 and yeast H3 share a common ancestor with conserved functions.

Most higher eukaryotes organize their genes for all four canonical histones H2A, H2B, H3.1/2 and H4 in repeats with a total of 10-50 intronless copies of each histone gene [4,5]. Organisms at the base of the metazoan tree, such as *Trichoplax adhearens*, have only one or few copies of H3.1/2, arguing for a later expansion of the canonical histone genes (Figure 1c). H3.1/2 transcripts from these clusters lack a poly(A) tail but share a conserved 3′ stem loop [6]. These unique features are thought to be responsible for the tight restriction of replication-dependent histone gene expression to S phase [7]. In contrast, H3.3 genes are present in single copies, often contain introns, and give rise to classical polyadenylated mRNAs. Unlike H3.1/2, the expression of H3.3 genes is replication-independent, and H3.3 has long been established as the predominant H3 variant in quiescent, G1, and G2 cells [8] Consequently, its cell cycle-independent expression enables H3.3 to serve as a substrate for both replication-dependent deposition and histone replacement processes that occur outside of S phase.

H3.3 is enriched at active genes, promoters, and regulatory elements

The bulk of newly synthesized histones are incorporated during DNA replication. Once assembled into nucleosomes, the H3/H4 tetramer has been observed to be much more stable in chromatin than the H2A/H2B dimers, measured by global levels of displacement during replication and transcription [9]. However, pioneering cytological studies of H3 variant deposition in *Drosophila* provided evidence for rapid H3/H4 exchange at specific loci in euchromatin [3]. While low levels of H3.3 are deposited together with H3.2 during replication, H3.3 was specifically enriched within actively transcribed genes by a replication-independent replacement process dependent on active transcription [3,10]. Conversion of the H3.3 variant region 87-90 ('AAIG') to the H3.1/2 sequence 'SAVM' abolished replication-independent incorporation [3]. These findings underscore the importance of the variant H3.3 sequence in addition to its cell-cycle independent expression. Interestingly, the single replacement of a 'S' with 'A" at position 31 of the histone H3 tail did not have any influence on the deposition pathway, suggesting that H3.3 S31 and its phosphorylation do not play a role in H3.3 deposition [11].

Recent advances in chromatin immunoprecipitation (ChIP) technologies have allowed a more detailed map of H3.3 deposition, revealing specific H3.3 incorporation throughout the gene body of transcribed genes as well as highly enriched foci at the promoter region in *Drosophila* and mammalian cells (Figure 2a) [12-20]. H3.3 enrichment at promoter regions has been observed not only at active genes but also at inactive genes, possibly accounting for a 'poised' state of these genes [13,18]. Furthermore, H3 replacement also occurs at genic and intergenic regulatory regions in various metazoans (Figure 2a) [13,14,20].

Mechanism of H3.3-specific deposition in metazoans

HIRA, the homolog of yeast Hir1 in higher eukaryotes, has been shown to assemble chromatin independent of replication and to interact with ASF1a/b in a multisubunit complex specific for H3.3 [21,22]. HIRA and the SWI/SNF family chromatin remodeler CHD1 have also been implicated in H3.3 deposition *in vivo* [23]. Globally, H3.3 continues to be incorporated into chromatin even in the absence of HIRA or CHD1 [23,24]. To this end, we have recently found that, while HIRA is required for H3.3 deposition at genic regions in mouse embryonic stem (ES) cells, H3.3-enrichment at telomeres and most

regulatory elements is HIRA-independent [25]. Instead, the SWI/SNF-type chromatin remodeler ATRX mediates localization of H3.3 to telomeres [25].

Thus, a number of factors required for proper H3.3 localization at specific genomic regions have now been identified, while the precise mechanisms of H3.3 replacement remain obscure. How are the subtle differences between H3.3 and H3.1/2 interpreted by and translated into to a site-specific deposition? To date, there are no structural details known on the recognition of H3.3. While several H3.3-associated factors have been identified [22], none of them have been shown to directly and specifically bind H3.3.

Mechanism of H3 replacement in yeast

Based on the homology in sequence, we expect a common structural theme in chaperones that recognize yeast H3 and metazoan H3.3 in a way that would allow specific discrimination of H3.3-specific sequence features. Despite relying on a single H3.3-like species, both replication-dependent and replication-independent H3 deposition pathways are found in yeast: in *S. pombe*, H3 expressed outside of S phase is preferentially incorporated in euchromatin [26,27]. A number of studies in *S. cerevisiae* detected H3 replacement at active [28] and also inactive [29,30] promoters, but only to a small extent throughout transcribed gene bodies. Genetic studies in yeast delineated a pathway comprising the SWI/SNF family chromatin remodeler Snf2 and the histone chaperone Asf1, as well as Hir1 or Spt6 for H3 exchange at the promoter region [30-34]. H3 deposition at the gene body required active transcription, Hir1, and Asf1 [29,30,35]. Hir1, Hir2, Hir3 and Hpc2 constitute the HIR repressor complex that has been shown to catalyze replication-independent histone deposition together with the H3/H4 chaperone Asf1 *in vitro* [36,37]. Spt6 has also been shown to facilitate nucleosome assembly *in vitro* [38]. Therefore, chromatin remodelers, histone chaperones and deposition factors cooperate in the eviction of old and deposition of new histones in yeast (Figure 2b). Interestingly, the elongation complex FACT (Spt16/Pob3) redeposit H3/H4 units in the wake of RNAPII, favoring recycling of ‘old’ histone over exchanging them with ‘new’ H3/H4 [39]. When Spt16 is deleted, a Hir1-dependent pathway takes over to deposits more ‘new’ H3 [39,40]. In conclusion, yeast genetics of replication-independent histone exchange processes might yield clues to yet undiscovered components of metazoan H3.3-deposition pathways. Moreover, novel pathways for histone exchange might exist uniquely in higher eukaryotes (Figure 2b).

H3.3 function: a balancing act between facilitating and repressing transcription?

As a highly conserved replacement variant, does H3.3 have a conserved function at promoters, coding regions, and regulatory elements? Two recent studies assessing inducible gene expression suggest that incorporation of H3.3 promotes initial gene activation [18,41]. One possibility is that nucleosome eviction and H3.3 deposition may serve as a mechanism for the rapid removal of inhibitory histone posttranslational modifications and/or replacement with activating marks as suggested by others [3]. However, even though nucleosomal H3.3 is enriched in activating modifications such as H3K4me3, these modifications in particular seem to be established only after nucleosomal deposition [42,43]. Rather than introducing a particular set of PTMs, ongoing histone exchange could therefore contribute to a highly dynamic steady state of establishment and removal of histone PTMs at specific genomic locations. Continuous histone exchange and H3.3-incorporation at boundaries of chromatin domains has therefore been proposed to limiting the spreading of certain histone modifications [13,29].

Based on the apparent lability of H3.3 nucleosomes in chromatin extracts, it has been proposed that nucleosome-destabilizing properties could help promote and propagate an active chromatin state [20,44]. As *in vitro* studies found little stability difference in recombinant H3.1/2 and H3.3 nucleosome [45,46], this effect might be potentiated by histone PTMs or inherent to CG-rich promoter DNA sequences that often coincide with H3.3-enrichment [47]. Furthermore, cooperative effects with H2A.Z and exclusion of the linker histone H1 could account for some of the properties of H3.3-containing nucleosomes [44,48].

Is H3.3 a general marker of active chromatin? Notably, the HIR complex has been shown to have a repressive role on transcription in yeast [44,49-53]. Hir1 was first identified as a potent repressor of the canonical histone genes in *S. cerevisiae* [49], and recently its repressor function in *S. pombe* has been mapped to a large number of promoters and also to suppression of cryptic transcripts from within coding regions [52], likely by repopulating nucleosome-free regions [31]. It is tempting to speculate that replication-independent H3.3 deposition in metazoans is similarly used to replenish nucleosome-free regions. Indeed, H3.3 knockdown leads to a slight decrease in nucleosome density [48]. Despite its predominant enrichment in euchromatin, H3.3 might also play significant roles in heterochromatic regions. HIRA, ASF1a and the mammalian Hir2 homolog Ubinuclein-1 have been implicated in the formation of facultative heterochromatin [54,55], and H3.3 has been observed in pericentric heterochromatin and at telomeres [11,56].

Biological significance of replication-independent H3.3 deposition

Clues for the functional significance of H3.3 come from genetic studies in flies and mice. Loss of both genes of H3.3 in flies leads to complete sterility, mild transcriptional defects, particularly at highly expressed genes, and partial but incomplete lethality (~42% viability) [57]. Intriguingly, the grossly normal development to adulthood of the surviving H3.3-deficient flies indicates that H3.3 is not absolutely required for transcription and development [57,58]. Indeed, while expression of a subset of genes in adults was perturbed, the precisely timed and localized expression of developmental key factors was not affected in H3.3-deficient flies. Similarly, although HIRA is required for fertility, adult HIRA null flies have no phenotypic abnormalities [24]. In mice, targeted mutagenesis of HIRA resulted in gastrulation defects and patterning abnormalities of mesendodermal derivatives prior to early embryonic lethality [59], suggesting a more prominent role for replication-independent chromatin assembly during mammalian development. Although HIRA may have various H3.3-independent functions, H3.3 itself is also important for mammalian development: a retroviral gene trap insertion into the murine H3.3A gene generated an H3.3 hypomorph that caused developmental defects and neonatal lethality [60].

How do flies compensate so well for the loss of H3.3 or HIRA? Intriguingly, an unknown mechanism seems to allow the cells to sense overall histone levels, as replication-dependent histone H3.2 genes are upregulated in H3.3-deficient flies. Furthermore, upregulated endogenous replication-dependent histone gene transcripts were found to be polyadenylated to some extent, likely achieved by a known alternative histone mRNA processing mechanism [57,61]. Importantly, viability and wildtype expression of most genes are fully restored when an additional H3.2 transgene is introduced [57]. Thus, elevated levels of H3.2 can largely rescue the transcriptional phenotype in adult H3.3 null flies. In rapidly dividing cells, replication-dependent deposition of H3.1/2 could compensate for loss of nucleosomes during transcription (Figure 3a). Alternatively, replication-independent pathways could tolerate H3.1/2 as substrates in the absence of H3.3 (Figure 3b).

Interestingly, global H3K4me3 levels in flies lacking H3.3 were comparable to wild-type but drastically reduced in flies with a H3.3K4A transgene [57], indicating that only in the absence of H3.3, H3.2 becomes the major carrier for this mark. We and others speculate that in the absence of a replacement variant, re-deposition of histones in *cis* partially substitute for replication-independent incorporation of new histones [62], which is analogous to the competing pathways for 'new' and 'old' histone observed in yeast (Figure 3c)[39]. Thus, if no 'new' histones are available, more 'old' histones with 'old' marks might be retained.

Histone replacement by H3.3 is essential for reproduction in metazoans

Despite potential compensatory mechanisms, HIRA and H3.3 play critical roles in sexual reproduction in all studied metazoans [24,57,58]. Both male and female H3.3 null flies are sterile. In mammals, the hypomorphic gene trap of H3.3A described above also led to male sub-fertility [60]. Strikingly, H3.3 is substrate for several large-scale chromatin remodeling events during metazoan reproduction, in particular gametogenesis and fertilization [63]. Meiotic sex-chromosome inactivation in mammalian male germ cells also involves massive incorporation of H3.3 into the X and Y chromosomes and subsequent silencing [64], a process that could be mechanistically related to the formation of facultative heterochromatin [54,55].

Meiosis is partly impared in H3.3 null flies due to a defect in chromosome segregation [57]. After meiosis, the condensation of sperm chromatin requires removal of most histones and replacement with protamines, although some pool of H3.3 is retained in mammalian and *C. elegans* sperm chromatin [64,65]. After fertilization, a maternal pool of H3.3 is used to rechromatinize the paternal genome in the male pronucleus [23,24,65,66]. This asymmetrical distribution of H3 variants could be important in epigenetic distinction of maternal and paternal information.

Critically, all remodeling events in the germline seem to be exquisitely specific to H3.3, as a H3.2 transgene under the H3.3 promoter cannot rescue the fly's sterility [58]. It is therefore likely that the phenotype is a direct consequence of impaired large-scale chromatin remodeling rather than a secondary effect due to gene expression changes related to transcriptional defects in the absence of H3.3. Consistent with this notion, H3.3 incorporation in the male pronucleus precedes onset of transcription and relies on HIRA and CHD1 activity [23,24]. The essential germline functions of H3.3 therefore likely created the strong evolutionary pressure that drove the exceptional conservation of the H3.3 protein in higher eukaryotes.

Conclusions

Why is the use of H3.3 so diverse and widespread while not all of its functions are essential in metazoans? We speculate that both germline and somatic functions of H3.3 have evolved from the single H3.3-like ancestor present in unicellular organisms. Differential timing of H3 gene expression might have allowed some tailoring of H3 variants for replication-dependent and independent functions, but ultimately the diversification of H3.1/2 amino acid sequence efficiently excluded these replication-dependent histones from H3.3-specific pathways. The separation of replication-dependent and -independent pools of H3/H4 might have allowed subsequent multiplication of the replication-dependent histone genes to fuel the growing need for bulk histones during replication of larger genomes without affecting fine-tuned histone replacement processes. We will need more detailed studies on how these H3.3-specific pathways affect chromatin structure and function to ultimately understand why metazoans evolved this exquisite specificity. Interestingly, H3 variants similar to H3.1/2 and H3.3 have emerged by convergent evolution in plants, multicellular fungi and

even the protozoans *Tetrahymena* and *Trypanosoma brucei* [67,68], suggesting a universal theme in chromatin regulation by histone variants.

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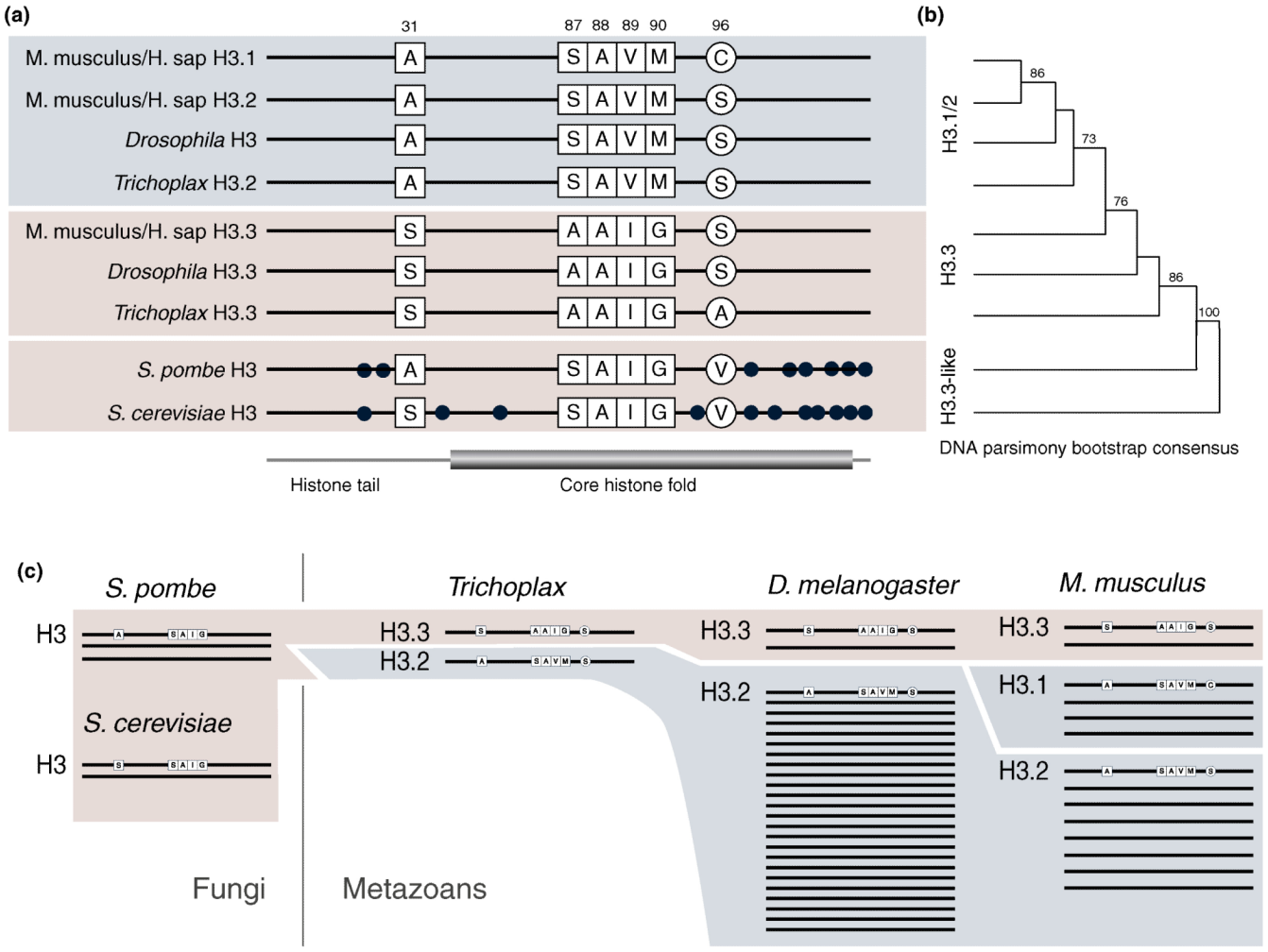

Figure 1. Protein sequences and gene complements of the non-centromeric histone H3 variants in fungi and metazoans

(**a**) Schematic representation of the major non-centromeric histone H3 protein sequences from human, mouse and *Trichoplax adhearens* (one of the most basal metazoan species), as well as budding and fission yeast. Amino acids that distinguish variants are highlighted with residue numbers, additional differences are indicated as dots. H3.1 only exists in mammals and only differs in position 96 from H3.2 present in all metazoans. H3.2 and H3.3 are distinguished by one amino acid difference at position 31 in the histone tail and three in amino acids 87-90 in the core histone fold. (**b**) Phylogenetic relationship of the respective histone H3 genes. An unrooted parsimony tree was constructed based on representative coding sequences (consensus tree of 100 bootstraps, excluding the wobble bases). The H3 genes of *S. pombe* and *S. cerevisiae* cluster with metazoan H3.3. (**c**) Schematic overview of the major non-centromeric gene complements of the indicated species. The placozoan *Trichoplax adhearens* has only one gene for H3.2 and H3.3 each, while higher metazoans have greatly expanded H3.1/2 gene complements.

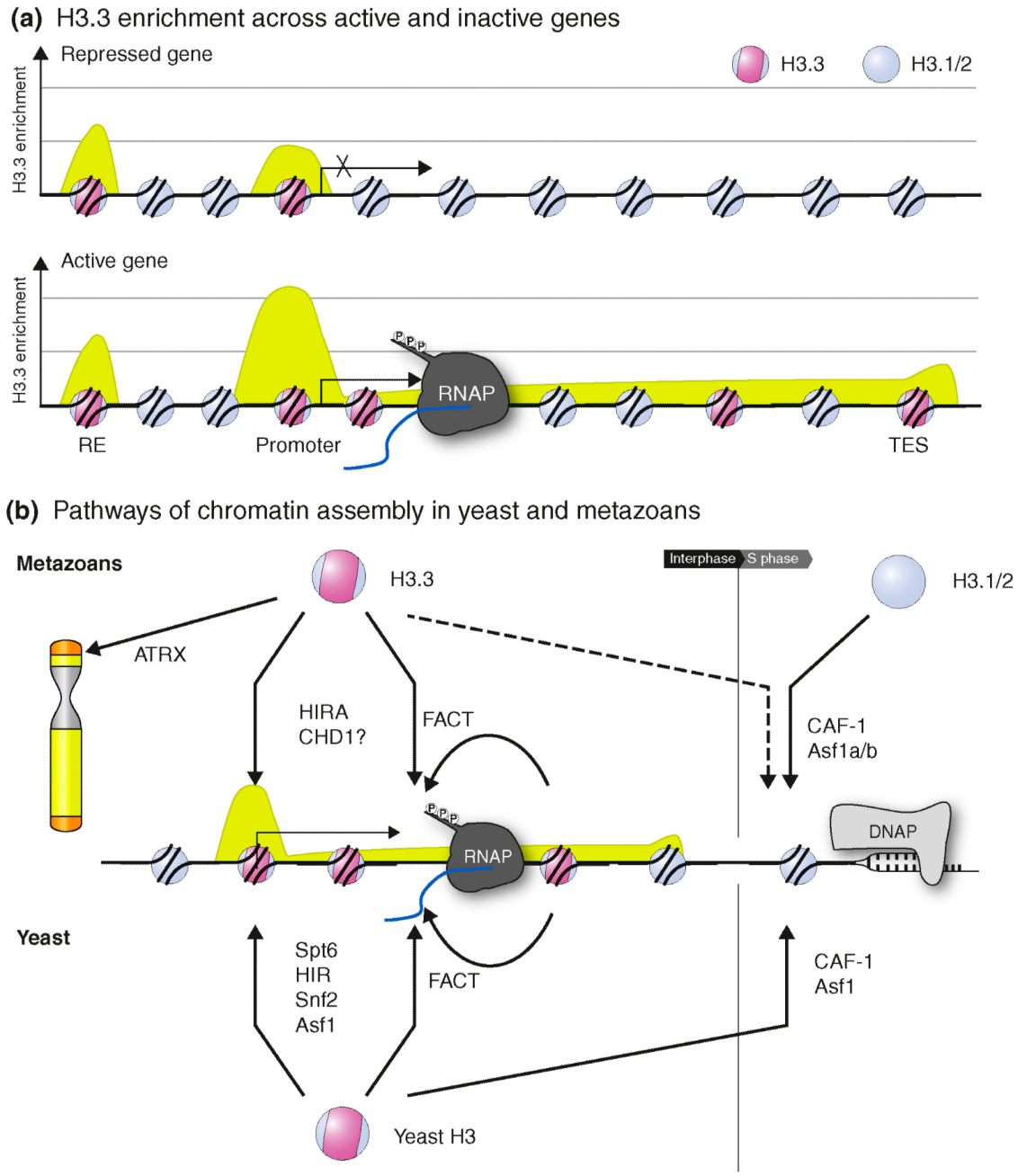

Figure 2. Genomic H3.3 localization and H3.3 deposition pathways

(**a**) Schematic map of an active and inactive gene locus comprising an upstream regulatory element (RE), transcription start site (Promoter) and transcription end site (TES). The distribution of histone H3.3 across the locus is shown in green, with representative H3.3 and H3.1/2 nucleosomes, as well as RNA polymerase II (RNAP). (**b**) Summary of the known factors involved in replication-dependent (right, S phase) and replication-independent (left, Interphase) chromatin assembly pathways, in metazoans and yeast. Replication-coupled assembly is thought to be mediated by the CAF-1 complex and Asf1 proteins in the wake of DNA polymerase (DNAP). H3.3-enrichment at telomeres is dependent on ATRX. Replication-independent deposition at promoters, regulatory elements and genic regions in metazoans requires HIRA, CHD1, and/or other factors, analogous to pathways in yeast

mediated by Snf2, Asf1, HIR complex and/or Spt6. The FACT complex (Spt16 and Pob3/Ssrp1) might contribute to incorporation of new or recycling of old histones.

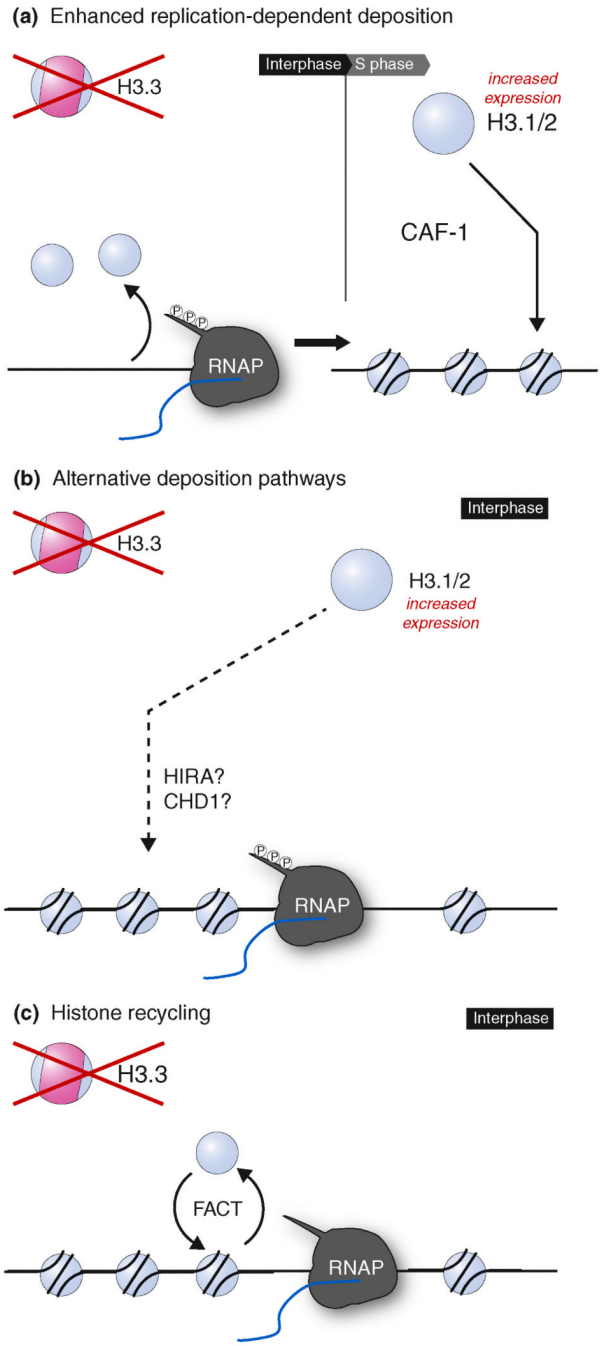

Figure 3. Putative compensatory mechanisms for the loss of H3.3

(**a**) Absence of H3.3 as substrate for replication-independent chromatin assembly could create nucleosome-free regions (see also ref. [62]). In rapidly dividing cells, these gaps could be filled during the next S phase via canonical replication-dependent chromatin assembly. (**b**) Elevated levels of H3.1/2 throughout the cell cycle could provide substrate for replication-independent chromatin assembly factors that are not restricted to H3.3. (**c**) In the absence of de-novo chromatin assembly, the FACT complex could favor transient eviction and redeposition of histone units in *cis*.