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HAL Authorization

1 **Title**

2 **Transcription-coupled H3.3 recycling: a link with chromatin states.**

3
4 **Affiliations**

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9
10
11 **Keywords**

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13 histone post-translational modifications

14
15
16
17 **Abstract**

18 Histone variant H3.3 is incorporated into chromatin throughout the cell cycle and even in non-
19 cycling cells. This histone variant marks actively transcribed chromatin regions with high
20 nucleosome turnover, as well as silent pericentric and telomeric repetitive regions. In the past
21 few years, significant progress has been made in our understanding of mechanisms involved in
22 the transcription-coupled deposition of H3.3. Here we review how, during transcription, new
23 H3.3 deposition intermingles with the fate of the old H3.3 variant and its recycling. First, we
24 describe pathways enabling the incorporation of newly synthesized vs old H3.3 histones in the
25 context of transcription. We then review the current knowledge concerning differences
26 between these two H3.3 populations, focusing on their PTMs composition. Finally, we discuss
27 the implications of H3.3 recycling for the maintenance of the transcriptional state and underline

28 the emerging importance of H3.3 as a potent epigenetic regulator for both maintaining and
29 switching a transcriptional state.

30

31 **Main text**

32 *1. Introduction*

33 An adult human body is composed of around 37.2 trillion cells, ensuring the provision of the
34 variety of cells that make up all the tissues, yet sharing a common genome [1]. The variety of
35 cell types are characterized by different transcriptional programs, thus understanding the
36 mechanisms governing the establishment, maintenance and dynamic changes in these programs
37 is of great scientific interest and therapeutic potential [2]. Histone proteins, initially viewed as
38 a monotonous scaffolding system providing compaction of the genetic information in
39 chromatin within the nucleus of the cell, also contribute to a functional versatility of chromatin
40 organization. Indeed, while each nucleosome is composed of two copies of four types of
41 histones, H2A, H2B, H3 and H4 [3], most of these histones are represented by several variants
42 which, when incorporated into the nucleosome, add variability within the basic module
43 (reviewed in [4]). Focusing on the H3 family, the main variants include two replicative histones
44 H3.1 and H3.2, and two non-replicative ones - H3.3 and centromeric CENP-A (Figure 1a).

45 While CENP-A diverges significantly from other H3 variants (Figure 1a), H3.3 differs from its
46 replicative counterparts only by 5 (for H3.1) and 4 (for H3.2) amino acids (Figure 1a). Three
47 of these residues located in the histone fold domain confer a specificity of interaction with the
48 DNA in the nucleosome particle and with unique sets of histone chaperones (Figure 1ab, [9–
49 11]). Moreover, a single amino acid Serine31 present uniquely in the N-terminal tail of H3.3
50 (Figure 1a) plays an important regulatory role [5–8] (discussed further in section 7). The
51 emerging view now defines a chromatin landscape that includes a choice of histone variants
52 along with the post translational modifications (PTMs) and defines distinct chromatin states
53 that correspond to specific cellular types and functions (reviewed in [6]). Recent data
54 uncovered particular histone dynamics during transcription challenging the maintenance of a
55 given state in a cellular lineage. Histones bound to chromatin, when displaced, can get
56 reincorporated. However, this reincorporation relies on a distinct deposition pathway as
57 compared to the one involved in the deposition of newly synthesized H3.3 [7]. This finding
58 underlines a capacity to differentiate between the two H3.3 subpopulations (which we refer to
59 as new and old H3.3). In this review, we summarize our current knowledge concerning new
60 and old H3.3 incorporation during transcription. We highlight the key differences between
61 pathways enabling newly synthesized H3.3 deposition and re-incorporation of old H3.3

62 histones, respectively. We review how PTMs can provide a means to distinguish new and old
63 H3.3. This leads us to discuss how regulating the deposition of old and new histones with
64 distinct PTM signatures could impact chromatin states, either in maintenance or switch of
65 transcriptional programs.

66

67 2. Definition and detection of new and old H3.3 histones

68 Various approaches have been used to study the fate of histone proteins (summarized in [8]).
69 Among them, the epitope-tagging of histones with SNAP proved especially insightful in the
70 context of histone deposition because it enables distinguishing newly synthesized and
71 previously existing populations of histones. The SNAP-tagging takes advantage of a mutated
72 DNA repair protein, an O⁶-alkylguanine-DNA alkyltransferase, fused with the protein of
73 interest (Figure 2a). In the cell culture context, the simple addition of a cell-permeable reagent
74 to the media, namely a benzylguanine (BG) derivative, allows for the rapid and irreversible
75 labeling of the SNAP-tagged protein [9,10] (a method recently developed also *in vivo* in mice
76 [11]). This provides a convenient means to carry out pulse-chase experiments to label distinct
77 populations and access new and old histones (Figure 2bc). The method has been used initially
78 to follow the centromeric H3 variant CENP-A [12]. Stable cell lines expressing tagged versions

79 of other H3 variants provided mechanistic insights into H3 deposition pathways [13,14] by
80 tracking H3 histones and their dynamics via single cell imaging and super resolution
81 microscopy [7,15] (Figure 2d, method detailed in [16]), as well chromatin
82 immunoprecipitations [17] (method detailed in [18]). The two distinct old and new H3.3
83 subpopulations in the context of transcription could be traced recently (Figure 2bc, [7]). Old
84 H3.3 represents histones present in the nucleus before the chase, and new H3.3 the histones
85 synthesized during and after the chase (Figure 2bc). A major fraction of old H3.3 gets evicted
86 from the chromatin co-transcriptionally [7]. However, they are either recycled locally or
87 replaced by new H3.3. The recycling and new H3.3 deposition involve distinct pathways /
88 distinct protein partners [7]. This opens up questions of how and why this differential
89 recognition occurs and what consequences ensue for the chromatin states.

90

91 3. *New H3.3: Mechanisms for newly synthesized H3.3 deposition*

92 Since in this review we wish to focus more specifically on the recycling of H3.3, we will
93 provide only a brief background. For details about the deposition of new H3.3 and the histone
94 chaperones see [19].

95 Histones are small proteins carrying strong positive charges enabling their close association
96 with negatively charged DNA molecules in chromatin. However, these charges may also lead
97 to promiscuous interactions with other proteins or nucleic acids present in the cytoplasm (where
98 they are synthesized), as well as during their trafficking in and out of the nucleus. Thus, when
99 not incorporated into chromatin, histones are constantly escorted by accompanying proteins,
100 histone chaperones ([20], reviewed in [21,22]). Newly synthesized H3 histones are transferred
101 to the nucleus either as monomers or H3-H4 dimers ([23], reviewed in [24]). They first
102 associate with general chaperones, such as heat shock protein 90 (HSP90) and heat shock
103 cognate protein 70 (HSC70) [23]. In the nucleus, newly synthesized H3.3 associates with
104 distinct, major protein complexes, ensuring deposition at specific chromatin regions [25]. The
105 interaction with chaperones recognizing a distinct motif in the histone fold domain of the H3.3
106 variant enables the specific H3.3 deposition as opposed to the replication coupled deposition
107 of H3.1 [19]. In the first case, an AAIG domain at residues 87-90 is critical for H3.3
108 recognition, in the second case, it is an SVM motif for H3.1/ H3.2 (Figure 1ab) [26–28].
109 Concerning the key proteins involved in H3.3 deposition, two major complexes have been
110 identified enabling distinct enrichment at various genomic loci. The death domain associated
111 (DAXX) and alpha-thalassemia/mental retardation, X-linked protein (ATRX) complex [5,29]

112 favors H3.3 enrichment at satellite repeats of pericentric and telomeric regions [26,29], as well
113 as endogenous retroviral elements (ERVs) [30,31] and imprinted loci [32] (Figure 3 left side).
114 Interestingly, the interaction of DAXX with a GHKL type ATPase MORC3 can regulate
115 accumulation of H3.3 at ERVs [33]. Moreover, the histone regulator (HIRA) complex proved
116 critical for new H3.3 incorporation at high nucleosome turnover loci, especially at
117 transcriptional start sites, active gene bodies and promoters [5,17,34,35], as well as
118 transcription factor binding sites [5,36,37], and DNA damage sites [38] (Figure 3 right side).
119 Given its capacity to bind naked DNA, the HIRA complex could additionally enable random
120 incorporation onto any region lacking nucleosomes via a gap filling mechanism, thereby
121 maintaining chromatin integrity (Figure 3 right side) [36,39]. Mechanistically, the HIRA-
122 mediated new H3.3 deposition requires the trimerization of the HIRA subunit [13]. This trimer
123 associates with two other subunits - calcineurin binding protein 1 (CABIN1) and ubinuclein 1
124 (UBN1) [13,40,41]. In addition to these core components, the association with the Anti-
125 Silencing Factor 1 (ASF1) [29,42] facilitates the transfer of the H3-H4 dimers to UBN1 for
126 deposition onto DNA (Figure 3 right side) [32,43,44]. ASF1 is present in mammalian cells in
127 the form of two paralogous proteins - ASF1a and ASF1b [45], which interact with any of the
128 H3 variants. However, they do show chaperone partner preferences with ASF1a preferentially

129 associated with HIRA, and ASF1b with chromatin assembly factor 1 (CAF1) - a chaperone of
130 replication-dependent H3 variants [46]. Recent data suggest that ASF1 can transfer new H3.3
131 directly to UBN-1, without requiring the interaction with HIRA [7], although the details of this
132 interaction require further analysis.

133

134 4. Old H3.3: Mechanisms for transcription-coupled recycling of old H3.3

135 To date, the re-incorporation of previously chromatin-bound histones has been considered
136 mostly in the context of DNA synthesis. It is clear that during the obviously disruptive process
137 of DNA replication, repair and recombination, recycling of parental histones along with new
138 histone deposition can challenge the maintenance of nucleosome density and the chromatin
139 states (reviewed in [47]). While we have learnt a lot about the replicative H3 variants in the
140 DNA synthesis-coupled deposition, we only recently gained insight into the H3.3 variant
141 deposition during S phase [15,48]. Furthermore, H3.3, given its expression throughout the cell
142 cycle, as opposed to replication-coupled expression of H3.1 and H3.2, also offers the possibility
143 to explore the balance between new incorporation and recycling outside S-phase. This aspect
144 is particularly interesting considering histone eviction associated with transcription. In fact, we
145 know relatively little about the histones evicted from chromatin in a transcriptional context;

146 how much is evicted? how? Are they discarded for degradation? Are they recycled back onto
147 chromatin at their original genomic site or elsewhere? Or even simply stored outside of
148 chromatin? Transcription-coupled eviction of the H3 family has been linked to p300-mediated
149 acetylation operating on the H3 N-terminal tail, more specifically H3K14 [49]. The histone
150 chaperone ASF1 itself has also been implicated in the H3 eviction during the transcript
151 elongation by RNA Polymerase II (RNA PolII) [50], possibly as a hand over for histones, in a
152 manner that compares to models proposed in the context of DNA replication [15]. Concerning
153 histone degradation, the recruitment of the Ring Finger Protein 8 (RNF8) ubiquitin ligase,
154 which in turn marks histones for proteasome-mediated degradation, exploits the specific
155 phosphorylation of H3T11 in the N-terminal tail [51]. Finally, to prevent accumulation of
156 excess levels of soluble histones, the histone chaperone NASP directs their degradation via the
157 chaperone-mediated degradation pathway [52]. However, how and what proportion of histones
158 gets degraded upon eviction and, more importantly, what happens to the ones that remain in
159 the nucleus, still needs to be defined.

160 What determines the level of recycling may also depend on the context. A recent report
161 indicates that recycling can potentially increase when the concentration of newly synthesized
162 histones is low [53], suggesting that the level of recycling is not necessarily steady. This may

163 be particularly important to consider in post-mitotic cells that will rely mainly on non-
164 replicative histones. Importantly, as stated before, the process of transcription results in a
165 massive eviction of H3.3 and a large fraction of this evicted H3.3 is recycled back into
166 chromatin [7]. Remarkably, the process requires the involvement of some, but not all, of the
167 components of the typical HIRA chaperone complex [7]. More specifically: (i) it does not
168 require the trimerization of the HIRA subunit itself or UBN1 interaction, yet (ii) it does strictly
169 require association with the ASF1 histone chaperone (Figure 4) [7,13]. Whether this pathway
170 uniquely involves the engagement of HIRA and ASF1 alone, or as part of the typical complex
171 associated with new H3.3 deposition, remains an open question. One could even consider that
172 recycling of histones could be differentially regulated at distinct genomic regions and/or be
173 influenced by the PTM status of the histones. Recent results identifying UBR7 as a novel
174 chaperone interacting specifically with the evicted H3.1-H4 dimers [54] can substantiate the
175 latter possibility. Indeed, recognition of evicted H3.1 by UBR7 depends on the presence of
176 H3K4me3 or H3K9me3 mark on these histones [54].

177 Schematically, the distinct outcomes of transcriptional-coupled old H3.3 recycling are: (i)
178 precise maintenance of states when recycled back to the same location (Figure 5a), (ii) loose
179 maintenance by staying in the chromatin vicinity as part of the local H3.3-enriched subnuclear

180 region (“conglomerate”) (Figure 5b) [22], or (iii) switch of states when moved ectopically to a
181 different genomic region (Figure 5c). In terms of preserving local chromatin states, the first
182 two scenarios would be more conservative. The latter, however, has the potential to promote a
183 change in the PTMs at the new locus, given the qualitative differences in the PTM landscape
184 between new and old H3.3 (discussed in the following section). Given that HIRA proves crucial
185 for the chromatin states’ dynamics, especially in systems relying on robust and coordinated
186 transcriptional regulation (i.e. embryonic development and embryonic stem cells (ESCs)
187 differentiation) [55], it will be important to disentangle how new and old H3.3 histones are
188 channeled through distinct deposition pathways in the nucleus.

189

190 5. *PTMs composition as a proxy for distinguishing new and old H3.3 histones*

191 When considering potential functional differences between new and old H3.3, the nature of the
192 PTMs present on their amino acids is revealing. Global analysis of chromatin showed that the
193 bulk H3.3 is enriched in active chromatin marks, including K4 and K79 methylation, as well
194 as K9, K14 and K18 acetylation [57]. However, to identify newly synthesized histones, which
195 represent only a small fraction of the total histone pool which is usually ignored, it is important
196 to use biochemical fractionation that can separate soluble from chromatin bound pools [58,59].

197 These fractionation experiments provided a map of the PTMs from histone synthesis to
198 deposition, distinct for each individual histone variant [59].

199 Newly synthesized histone H4 shows a conserved dual K5, K12 acetylation pattern, unique
200 characteristic of the new H4 fraction [60]. In the case of the H3 family, analysis carried out in
201 HeLa cells showed that both H3.1 and H3.3 in the soluble fraction are largely unmodified -
202 among examined residues on average about 80-100% of histones carry no detectable
203 modifications [59]. Among the rare detected modifications, the only methylation observed
204 corresponds to lysine 9, more specifically H3K9me1 (present on both H3.1 and H3.3) and
205 H3K9me2, present specifically on the H3.3 variant (Figure 6 left side) [59]. Since H3K9
206 trimethylation is important for heterochromatin, this observation is particularly interesting in
207 the context of DAXX/ATRX-mediated heterochromatic deposition of H3.3. It is possible that
208 the small fraction of H3.3 methylated on lysine 9 is being targeted to pericentric and telomeric
209 regions, especially given that H3K9me1 is a preferential substrate of Suv39 to reach
210 trimethylation [59]. Interestingly, newly synthesized H3.3-H4 dimers can be sequestered for a
211 later deposition into PML bodies in a DAXX-dependent manner [61], and it is tempting to
212 envisage that H3K9 methylated H3.3 could be the one preferentially recognized in this process.

213 Other than marks on K9, lysine 14, 18 and 23 show low levels of acetylation on newly

214 synthesized H3.3 (with 20-30% of the residues carrying the modification) (Figure 6 left side)
215 [59]. These modifications, associated with differentiation and tissue-specific gene activation
216 [62–64], reflect the euchromatic profile of bulk H3.3 [57,65]. Notably, some of the marks on
217 newly synthesized H3.3 are imposed sequentially and can be mutually exclusive - such as
218 heterochromatic H3K9me1 and euchromatic H3K14K18ac [23]. Thus, several subpopulations
219 exist that could possibly preferentially engage with distinct chaperones. In summary, the newly
220 synthesized H3.3 population is largely unmodified and acquires most of the PTMs after the
221 chromatin incorporation, at least in the systems studied to date.

222 In contrast to newly synthesized H3.3, old, chromatin-bound H3.3 is massively enriched in
223 methylation-based active marks, mostly H3K4me3 (present on over 70% of the old histones),
224 as well as methylation of lysine 36 and 79 (Figure 6 right side) [59]. It also shows some
225 acetylation on residues K9, K14 and K23 - all common markers of the active transcriptional
226 state (Figure 6 right side) [59].

227 Therefore, while new histones are mostly unmodified, the old, chromatin bound H3.3 is
228 extensively methylated, especially at lysine 4. In light of the recognition of post-nucleosomal
229 H3.1 via direct binding to H3K4me3 by UBR7 [54], it is possible that a similar mechanism
230 exists to specifically recognize and deposit the old H3.3. Thus, the striking differences in

231 methylation levels between new and old H3.3 provide not only a reliable means to distinguish
232 them experimentally but also a potential mechanism to sort out which histones to incorporate,
233 sequester or degrade (Figure 5).

234

235 6. *Histone H3.3 distribution and local modification landscapes*

236 Within chromatin, the density of H3.3 shows a pattern averaging one H3.3 nucleosome for
237 every three H3.1 nucleosomes. This dispersed nature is not mirrored by the PTMs present on
238 the histones in shared genomic locus - biochemical analysis of short oligonucleosome arrays
239 showed that if an array contains H3.3 nucleosomes, the active PTMs (e.g. H3K4me3 and
240 H3K9ac) are similarly enriched on all H3 histones within that array, and not only the H3.3 [59].
241 The shared local PTM status of both H3.3 and H3.1 indicates it is the modification landscape,
242 rather than the histone variant composition, that has a dominant role in controlling the
243 chromatin state. In support of the central role of the PTMs, direct targeting of an H3K4me3
244 writer, PRDM9 methyltransferase, to the promoter region can effectively re-initiate silenced
245 gene expression [66]. This suggests that the introduction of specific PTMs, either by recruiting
246 appropriate chromatin modifiers or perhaps by incorporating already modified histones, can
247 indeed be sufficient for a transcriptional switch. Given that local inheritance and spreading of

248 repressive marks, specifically H3K27me3, from parental histones was previously put forward
249 in the context of replication (reviewed in [67]), it is tempting to speculate that the observed
250 dispersed nature of H3.3 may reflect its function in promoting/seeding and propagating of open
251 chromatin-related PTMs outside of S-phase. If transcription-coupled recycling of methylated
252 H3.3 indeed serves the purpose of introducing and/or reinforcing the active state of the locus,
253 even a small number of such histones would suffice, thanks to the read-write mechanism-based
254 spreading of marks such as H3K4me3 (reviewed in [68]). This would explain (i) why the cell
255 retains a fraction of evicted H3.3 and (ii) evolved pathways allowing for specific recognition
256 and deposition of these histones back onto the chromatin. Interestingly, ESCs carrying an
257 alanine substitution of lysine 4 in H3.3 lose the capacity to differentiate, while they can still
258 self renew [69]. These cells lose H3.3 occupancy at ESCs-specific enhancers, thereby
259 disrupting their transcriptional state [69]. Therefore, in addition to the classical AIG
260 recognition motif for H3.3 chaperones, some regulatory elements in the N-terminal tail may
261 require a proper K4 site for accurate deposition and/or maintenance of H3.3 in specific
262 chromatin regions. It will be exciting to uncover and understand the mechanisms of modified
263 H3.3 recognition and their role in chromatin states maintenance.

264

265 7. H3.3S31Phos - a specific H3.3 chromatin mark

266 Remarkably, it is possible to distinguish identical PTMs present in the context of distinct H3
267 variants in chromatin. This was first exemplified with the candidate tumor suppressor protein
268 ZMYND11 that recognizes trimethylation of lysine 36 (H3.3K36me3) specifically on H3.3 to
269 impact transcript elongation and mRNA processing [70,71]. This recognition can function
270 thanks to an inherent unique feature of H3.3 - the presence of a serine at the amino acid 31 that
271 can be modified by phosphorylation [72]. Interestingly, H3.3S31Phos, initially described as
272 metaphase-specific and important for proper chromosome condensation and protecting
273 centromeres from euchromatinisation [72], also links to transcriptional program control
274 [73,74]. In the context of murine macrophages, upon stimuli, the levels of H3.3S31Phos
275 increase and result in rapid transcriptional activation [73]. Mechanistically, in this case, the
276 transcriptional boost is mediated by ZMYND11 ejection initiated by H3.3S31 phosphorylation,
277 followed by binding and increased activity of SETD2 methyltransferase on the H3.3K36 locus
278 [73]. In mESCs, H3.3S31Phos acts as a nucleosomal cofactor of p300 acetyltransferase to help
279 regulate enhancer function and transcriptional reprogramming during differentiation [74].
280 Additionally, the mark proved essential for *early Xenopus* embryonic development during
281 gastrulation, further stressing its role in transcriptional reprogramming [75]. Most of these

282 regulatory functions of H3.3S31Phos link to the modification status of neighboring amino acids
283 (e.g. H3K36me3, or H3K27Ac/me), and future work should address the nature and extent of
284 all possible cross-talks. Importantly, whether the phosphorylation of H3.3S31 is retained upon
285 eviction from the chromatin and in turn could influence a target locus when recycled back to
286 the chromatin remains to be explored. In stimulated macrophages the H3.3S31Phos is enriched
287 strictly over gene bodies of genes responding to the bacterial lipopolysaccharide (LPS) [73].
288 Importantly, this enrichment is dependent on IkappaB kinase (IKK) association with elongating
289 RNA Pol II [73], demonstrating that the phosphorylation occurs co-transcriptionally, rather
290 than being brought by already modified histones. How is the kinase recruited to its target sites?
291 Is there a dedicated chaperone recognizing the phosphorylation specifically? Since similarly to
292 other histone phosphorylations [76], H3.3S31Phos has the potential to trigger rapid cellular
293 responses it will be important to further explore its function as a promising candidate for a
294 transcriptional programs' modulator.

295

296 8. Opened questions, outlook and future perspectives

297 We have come to appreciate the existence of co-transcriptional dynamics between deposition
298 of new and recycling of old H3.3. The question that ensues now is not only to understand the

299 mechanism but also functional consequences of this interplay for the physiology of a whole
300 organism. Lessons from H3.3-deficient organisms point to a high importance of this histone
301 variant across multicellular species (recently reviewed in [77]). In *C. elegans*, the H3.3
302 requirement only stands out upon heat shock to ensure an appropriate transcriptional activation
303 and the timely firing of replication origins [78,79]. In flies, H3.3 is mainly critical for male
304 germline development [80,81], while in *Xenopus* a specific requirement during gastrulation
305 could be revealed [56]. Finally, mice lacking H3.3 show phenotypes ranging from infertility to
306 lethality and embryonic development defects, depending on the number of H3.3 genes lost [82–
307 84]. While many of these defects can be linked with aberrant or failed transcriptional programs
308 at a specific time, the loss of H3.3 function experiments cannot address what would be the
309 outcome of failed deposition or recycling during transcription. To unravel this issue, the
310 question could thus be considered by exploring the cellular consequences of the disruption of
311 specific components of the HIRA complex with the capacity to disentangle H3.3 recycling
312 from new deposition.

313 The HIRA protein itself is clearly important for both new deposition and recycling of H3.3 [7].
314 In systems studied to date, HIRA activity was linked to the control of cell fate either to maintain
315 stemness or to enable differentiation, which may appear conflicting. Indeed, in C2C12

316 myoblasts, HIRA is required for both further differentiation into myotubes [85] and conversion
317 into osteoblasts [86]. Conversely, muscle stem cells in adult mice depend on HIRA to maintain
318 their self-renewal capacity rather than to enable their differentiation [87]. Conflicting
319 dependencies are also reported in the context of hematopoiesis - in some, but not all mouse
320 lineages, HIRA proved critical for transcriptional reprogramming [88,89]. Finally, in
321 hematopoietic transdifferentiation and neuronal differentiation of mESCs, HIRA also proved
322 critical - at early stages, to protect paternal lineage identity, and later on for the successful cell
323 fate change [55]. This seemingly dual and potentially opposing role of HIRA, whereby it can
324 contribute to both maintenance and switch in chromatin state, is a puzzle. A possible
325 explanation may relate to different chromatin outcomes when considering deposition of new
326 H3.3 vs recycling old H3.3, especially in light of the different marks that they may maintain or
327 lose. A way to test a key role for deposition could take advantage of the fact that HIRA
328 trimerization is strictly required for the new deposition but dispensable for the recycling.
329 Furthermore, another striking difference between the transcription-coupled mechanisms of new
330 and old H3.3 deposition directly relates to the distinct involvement of partners of HIRA, namely
331 ASF1 and UBN1 proteins. Indispensable for incorporation of old H3.3, ASF1 is not strictly
332 required for the new deposition [7]. Consistently, ASF1 is also important for histone recycling

333 during replication and associates with evicted histones during replication stress [90-92].
334 Moreover, ASF1 is necessary for the reprogramming of adult cells into iPCSs [93], as well as
335 mESCs differentiation, where it facilitates transcriptional activation of differentiation genes
336 [94]. In the case of UBN1, required only for the incorporation of new H3.3 [7], it is interesting
337 to note that its activity is important for H3.3 deposition at promoters of developmental genes
338 and successful neural differentiation of mESCs [95]. How the particular implications of ASF1
339 and UBN1 in new and old H3.3 deposition relate to these phenotypes will be an exciting area
340 to explore in order to uncover the details of these dependencies.

341 In this review, we discussed the ability of the cell to distinguish between newly synthesized
342 (new) and previously chromatin-bound (old) H3.3 in the context of transcription. We propose
343 that the functional significance of such distinction may lie with fundamental differences in the
344 PTMs landscapes detected at these histone subpopulations, in essence putting forward an
345 extension of the “H3 barcode hypothesis” [96]. Enrichment of H3K4me3 on old histones vs
346 largely unmodified status of new H3.3 may be leveraged by the cell to fine-tune chromatin
347 states. By recycling old H3.3 the cell has the potential to aid transcriptional regulation of key
348 genes - maintain the active transcription by being re-deposited back into the same locus, or
349 facilitate a switch in transcriptional state by incorporating it into regions previously silent.

350 Refining and exploring this idea by identifying more interactors and revealing detailed
351 dynamics of old and new H3.3 on chromatin will allow for a deeper understanding of the
352 function of differential H3.3 deposition and its impact on chromatin plasticity in the context of
353 cell identity [97].

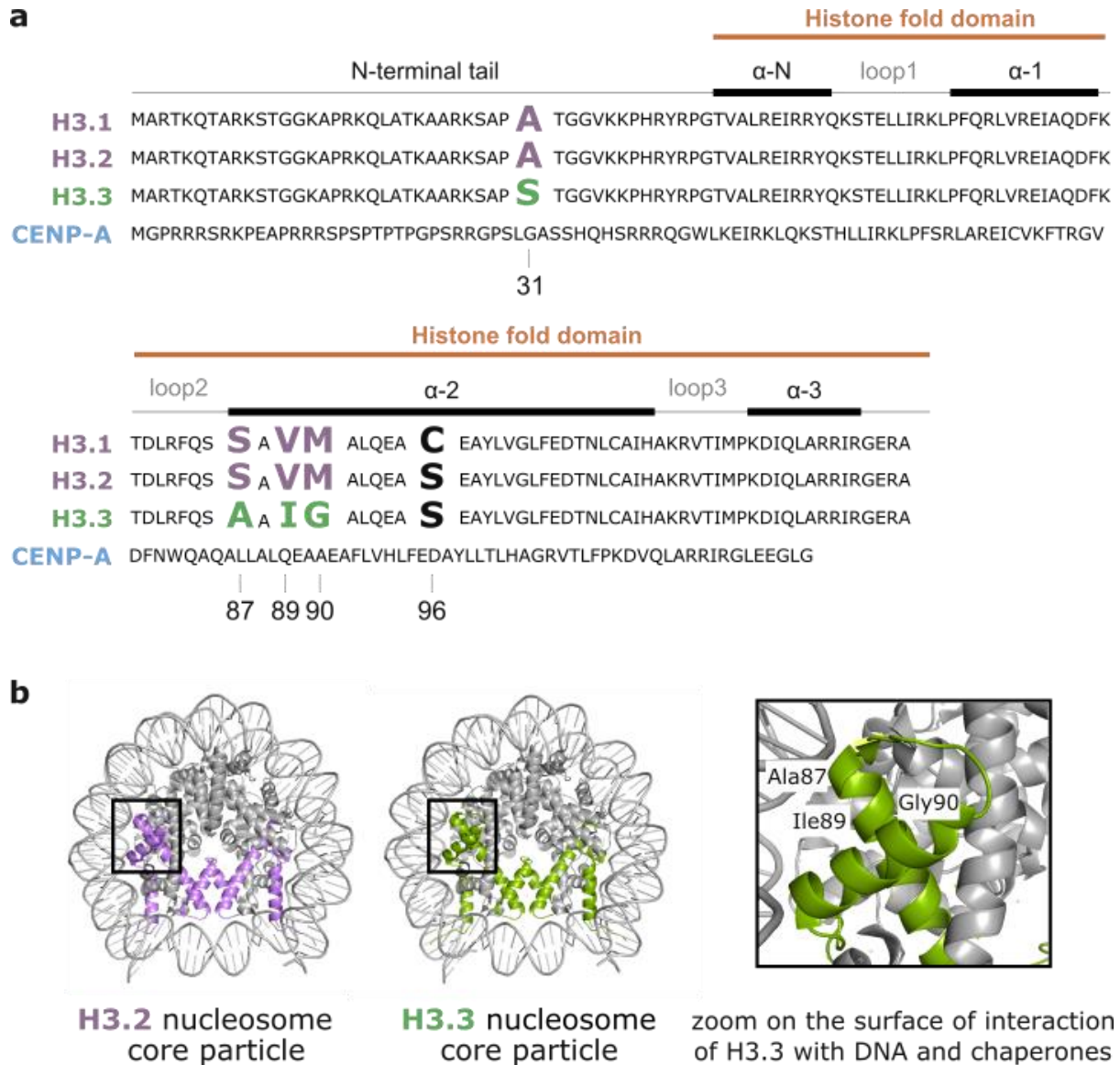
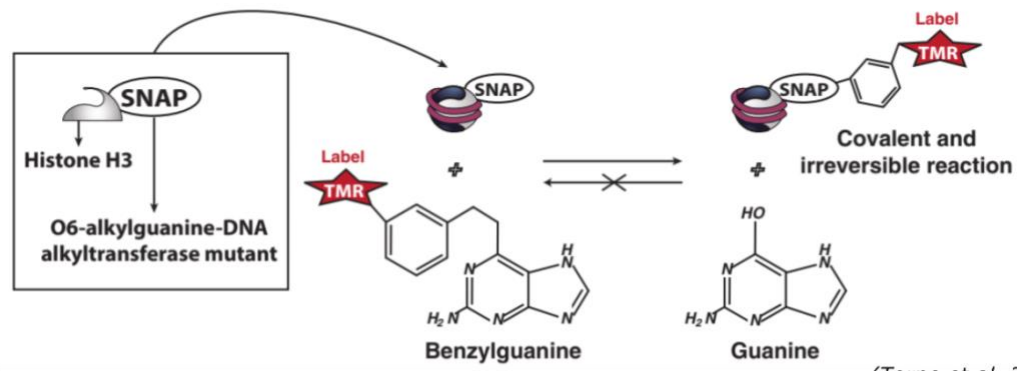


Figure 1. Sequences of major H3 variants and corresponding nucleosomal core particles

(a) Sequence alignment of major human H3 histone variants H3.1, H3.2 and H3.3. Highlighted are residues differentiating between H3.1, and H3.2 (purple residues) and H3.3 (green residues) variants - regulatory residue 31 on the N-terminal tail, as well as residues embedded in histone

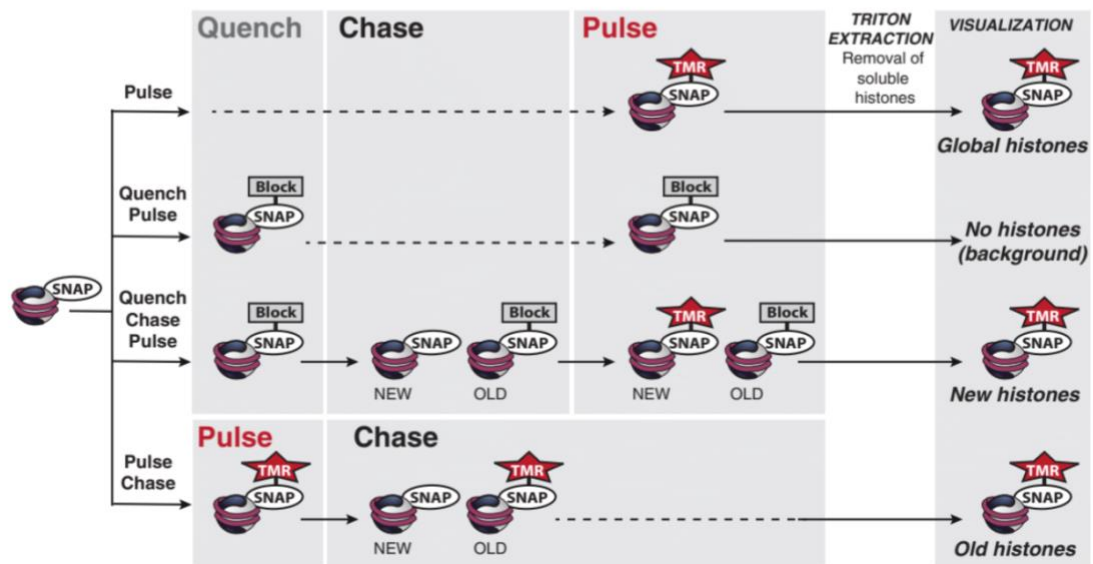
fold domain - residues 87, 89 and 90 conferring specificity of histone chaperones, and residue 96. Additionally, centromeric variant CENP-A, the most divergent from the rest family, is depicted at the bottom of the scheme to illustrate low sequence similarity with the remaining variants. (b) Crystal structures of nucleosome core particles containing H3.2 and H3.3 with highlighted residues essential for the chaperone recognition. With these residues being buried in the nucleosome core, the two structures are very similar. Crystal structure data sourced from [98].

a



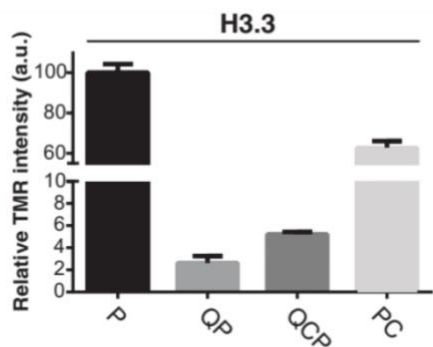
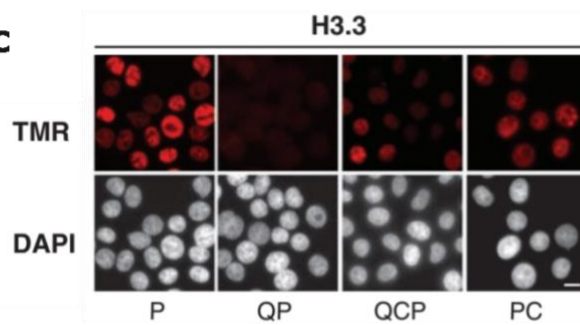
(Torre et al. 2018)

b



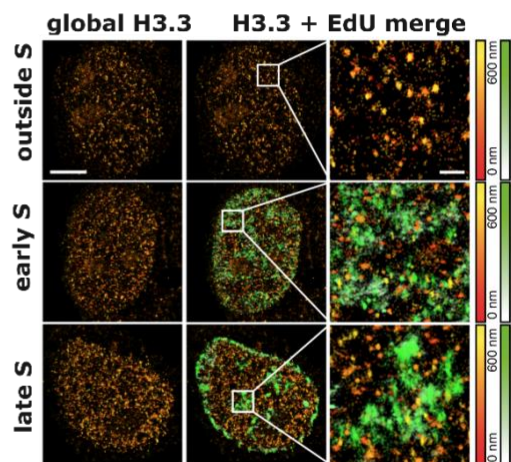
(Torre et al. 2018)

c



(Torre et al. 2018)

d



super-resolution imaging of H3.3 dynamics throughout S phase (modified from Clement et al. 2018)

Figure 2. SNAP-tagging technology to follow histone dynamics

Schemes and legends for panels (a), (b) and (c) are sourced and modified from [16], figure and legend for panel (d) is sourced and modified from [15]; (a) H3 histone variants are fused to the SNAP-tag, a 20-kDa mutant of the DNA repair protein O6-alkylguanine-DNA alkyltransferase that reacts specifically and covalently with benzylguanine derivatives *in vivo*. H3-SNAP histone variants incorporated into the nucleosome particle are irreversibly labeled with a synthetic probe such as the fluorescent TMR (exemplified in this figure) or the optically inert “Block”. (b) Protocol scheme for labeling of global histones (“Pulse”), no-labeling control (“Quench-Pulse”), new histones (“Quench/Chase/Pulse”) and old histones (“Pulse/Chase”). Triton extraction is performed to eliminate the soluble fraction and visualize chromatin associated/incorporated histones. c) Image visualization of H3.3 histones; (“P - Pulse”), no-labeling control (“QP - Quench-Pulse”), new histones (“QCP - Quench/Chase/Pulse”) and old histones (“PC - Pulse/Chase”). Scale bar=10µm. Bottom panel: Cell-by-cell signal intensity is quantified and average is normalized to “Pulse” levels. “Quench/Pulse” leads to dramatically reduced TMR intensity as no histones should be available for labeling after the “Quench” step (this serves as a control for quenching efficiency). The addition of a “Chase” period before the “Pulse” (“Quench-Chase-Pulse”) leads to increased TMR intensity, as newly synthesized

histones are available for labeling. The “Pulse-Chase” exhibits reduced signal intensity compared to the “Pulse” as only old retained histones remain labeled. (d) Example of data obtained using SNAP-tagging technology: Representative STORM images of global H3.3 (TMR, orange) and replicated DNA (EdU, green) in HeLa H3.3-SNAP. EdU labeling allows the selection of cells outside S phase, in early S phase and mid/late S phase. The color gradient corresponds to the z range. Scale bar=5 μ m. Insets represent enlarged images of selected area where scale bars=600 nm.

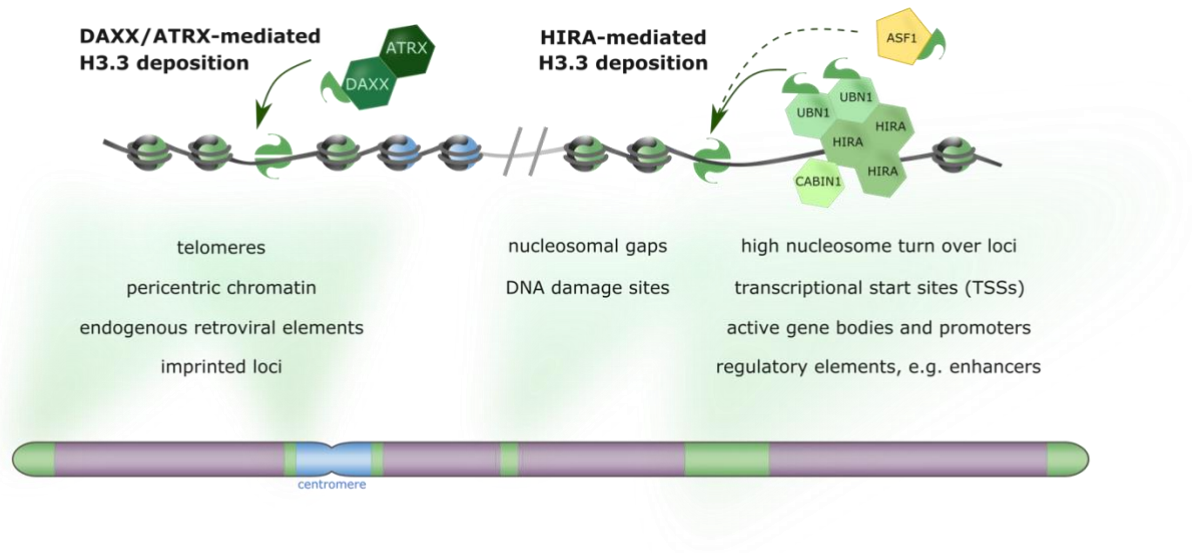


Figure 3. *De novo* deposition pathways of histone variant H3.3

De novo deposition of H3.3 (i.e. deposition of newly synthesized histones) occurs both in heterochromatic and euchromatic contexts. In heterochromatic regions, especially repeats present at telomeres, pericentric chromatin and endogenous retroviral sequences, as well as imprinted loci, H3.3 enrichment relies on the DAXX/ATRX complex and is linked to proper heterochromatin formation [5,27,31-33,99]. In actively transcribed regions and nucleosomal gaps, new H3.3 deposition relies on a replication-independent process linked to the HIRA chaperone complex [100,101]. The core HIRA complex consists of a trimer of HIRA associated with CABIN1 and UBN1 proteins [13,40,102]. While specific stoichiometry of these interactions remains to be determined, dimerization of UBN1 *in vitro* could indicate the joint deposition of two dimers of H3.3-H4 [103]. Besides these core members, the HIRA complex

also associates with the ASF1 protein, especially ASF1a [29,104]. Of note, the strict association with HIRA is dispensable since ASF1 can also deliver newly synthesized H3.3 directly to UBN1 for deposition without the need to associate with HIRA (dashed arrow) [7]. The schemes are based on [105] and [7].

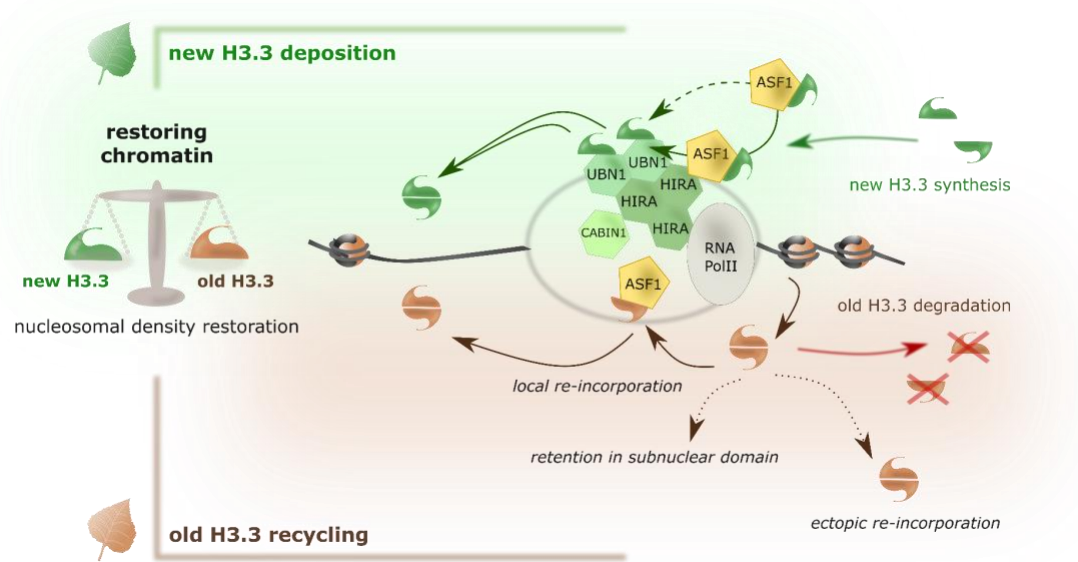


Figure 4. Old H3.3 recycling in the context of transcription

During transcription, significant nucleosome disruption occurs [7]. To restore nucleosome density after the passage of transcriptional machinery two options are envisaged - re-incorporation of previously evicted H3.3 or deposition of newly synthesized H3.3 (the latter described in Figure 3). Transcription-coupled chromatin re-incorporation of old H3.3 does not depend on the HIRA trimerization nor its interaction with UBN1. Instead, it critically depends on HIRA association with ASF1 [7]. Evicted histones can potentially be recycled and retained locally in H3.3-enriched subnuclear domains or re-incorporated within the same parental chromatin. Alternatively, they may get incorporated ectopically into a new location or

discarded for degradation. For transcribed loci, the balance between recycling old and bringing in new histones will ensure not only restoration of nucleosome density but, potentially, given different histone modification status of new and old H3.3, may also impact the chromatin state of the locus. Here we depicted both new and old H3.3 deposition pathways in the context of traditional HIRA complex, yet we can not exclude the possibility of the existence of separate HIRA complexes. Scheme based on [7] with a color code for old and new histones inspired by fresh and falling leaves.

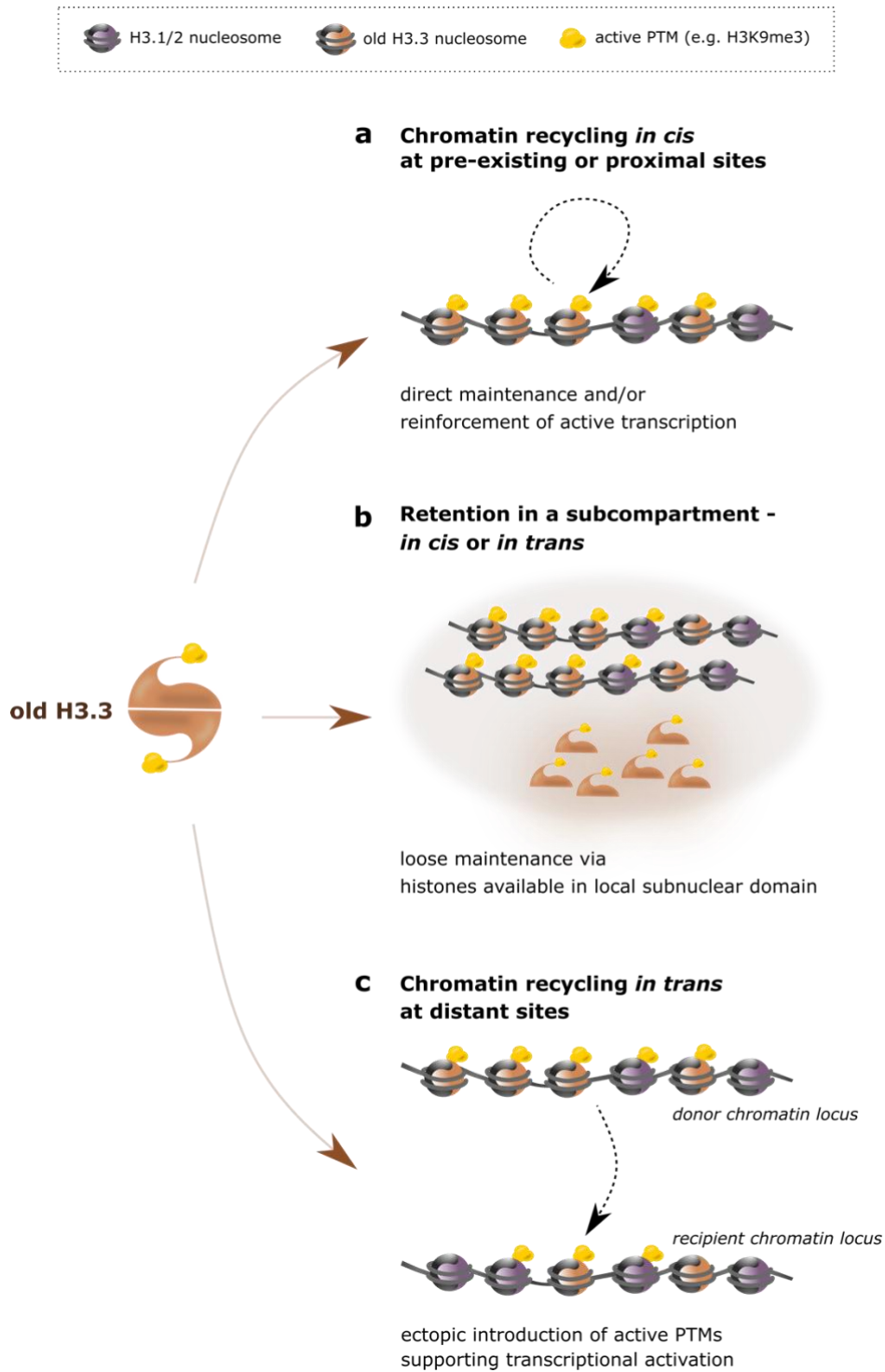


Figure 5. H3.3 recycling and its consequences - a model

Implications of H3.3 recycling for chromatin states: a model; (a) re-incorporation of evicted histones leading to reinforcement of the memory of the chromatin state, similarly like is the

case during replication; (b) retention of the old H3.3 in the local, H3.3-enriched domains, as observed with high resolution microscopy [15]; (c) ectopic re-incorporation of evicted histones into regions previously transcriptionally silent. It has a potential of introducing the active PTMs in the ectopic locus thereby seeding a new active state and facilitating its spreading into neighboring nucleosomes via interaction with chromatin remodelers responsible for recognition and introduction of the mark. For simplicity, the schemes refer only to the old H3.3 incorporation and H3K4me3 as a modification present on old H3.3 histones.

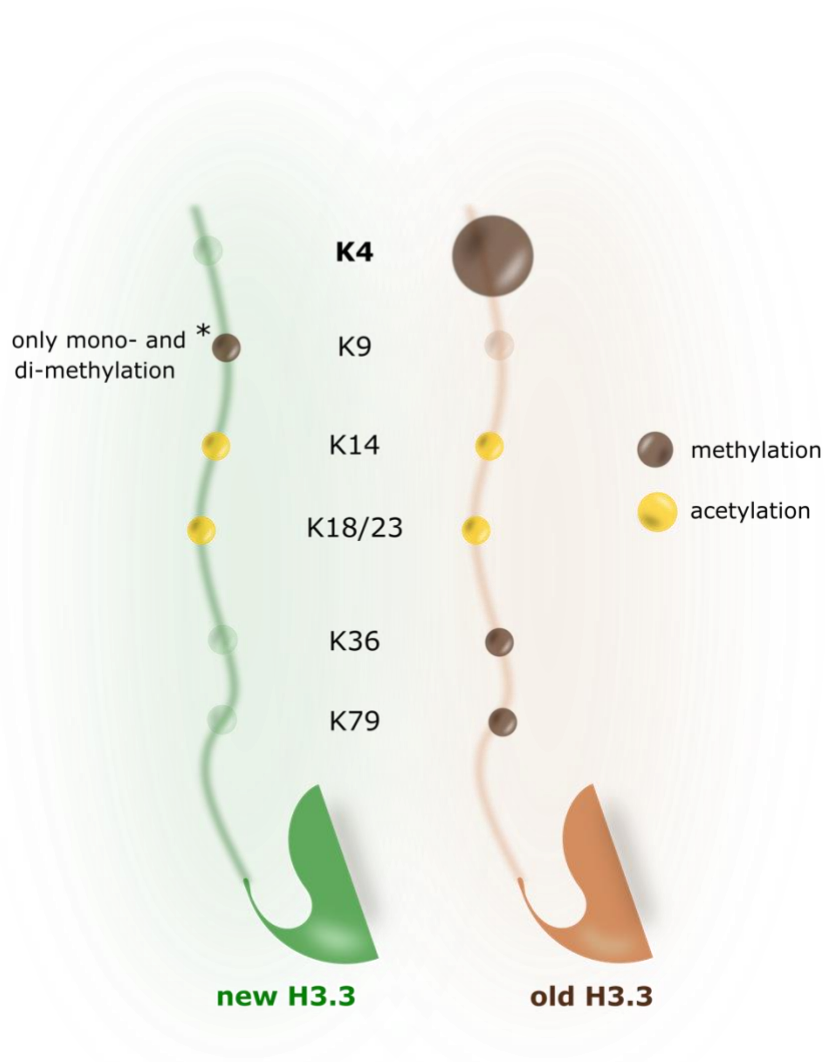


Figure 6. Major PTMs present on new and old H3.3 histones

The only methylation detected on new H3.3 are low levels of K9 methylation (only mono- and di-). In contrast, old H3.3 is heavily methylated on residues associated with active chromatin, especially K4me3. Both new and old histones also show some acetylation on residues generally associated with open chromatin. The size of each bead representing methylation or acetylation

at a specific locus is indicative of the extent of these modifications. Scheme based on data from

[59].

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