

Chromatin dynamics and DNA replication roadblocks

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Running title: New histone modifications and the DNA damage response

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Abstract

A broad spectrum of spontaneous and genotoxin-induced DNA lesions impede replication fork progression. The DNA damage response that acts to promote completion of DNA replication is associated with dynamic changes in chromatin structure that include two distinct processes which operate genome-wide during S-phase. The first, often referred to as histone recycling or parental histone segregation, is characterized by the transfer of parental histones located ahead of replication forks onto nascent DNA. The second, known as *de novo* chromatin assembly, consists of the deposition of new histone molecules onto nascent DNA. Because these two processes occur at all replication forks, their potential to influence a multitude of DNA repair and DNA damage tolerance mechanisms is considerable. The purpose of this review is to provide a description of parental histone segregation and *de novo* chromatin assembly, and to illustrate how these processes influence cellular responses to DNA replication roadblocks.

Keywords: histone modifications, *de novo* chromatin assembly, DNA replication, DNA repair, histone chaperones

1. Chromatin replication

Chromatin structure and histone post-translational modifications exert a major influence on DNA-dependent processes in eukaryotic cells. During DNA replication, pre-existing histones present in chromatin during the preceding G1 (hereafter referred to as parental histones) are transferred to nascent sister chromatids behind replication forks, resulting in a two-fold dilution of nucleosomes along replicated DNA[1]. This must be compensated via *de novo* assembly of nucleosomes which, in turn, creates a demand for synthesis of new histones. While the generation of new chromatin is mainly coupled to DNA replication in proliferating cells, some nucleosome assembly/re-assembly also occurs during the DNA synthesis step of DNA repair processes (*e.g.* nucleotide excision repair and DNA mismatch repair), as well as in the context of gene transcription. *De novo* DNA replication-coupled nucleosome assembly is essential for cell viability[2], and non-lethal mutations that cripple this process engender persistent DNA lesions accompanied by perturbation of DNA replication[3–5].

Consumption of new histones via nucleosome assembly behind replication forks must be coupled with the synthesis of histones and their deposition onto DNA by histone-binding proteins known as histone chaperones[6]. Because of their basic nature, excess free histones (*i.e.* histones that are not associated with chaperones or assembled into nucleosomes) can interact non-specifically with nucleic acids thereby potentially leading to insoluble aggregates[7,8]. To avoid this, histone gene expression and protein stability are diminished when DNA replication is blocked upon *e.g.*, treatment with genotoxic drugs[6]. In addition, free histones are tightly bound by histone chaperones (Kds in the low to sub-nanomolar range) after their import into the nucleus by karyopherins, which prevents aggregate formation[9]. Chaperones and other chromatin assembly factors then act in concert to promptly assemble nucleosomes behind DNA replication forks using new histones.

Newly synthesized histones harbor specific patterns of post-translational modifications acquired prior to their deposition onto nascent DNA that differentiate them from parental histones[10–16]. After assembly into nucleosomes, new histones are

progressively modified such that they become indistinguishable from parental histones. A large body of literature has emerged linking modifications of new histones with nucleosome assembly, gene expression, DNA repair, and DNA replication. Here, we will review the impact of such modifications and of *de novo* chromatin assembly on the cellular response to DNA lesions that interfere with replication, hereafter referred to as replicative stress. This has been extensively studied using the budding yeast *Saccharomyces cerevisiae* as model organism. Most of the results discussed in this review originate, unless otherwise specified, from experiments performed in yeast and relate to yeast proteins. Comparison with other species/systems will be discussed when appropriate (see Table 1): to avoid confusion, factors/genes from organisms other than yeast will be labeled in some instances in the text, e.g., hsYFG1 refers to human YFG1.

Yeast	Human	Function
FACT (Spt16, Pob3, Nhp6)	FACT (SPT16 and SSRP1)	Chaperone involved in nucleosome dis/re-assembly at replication forks, interacts with DNA polymerase α , CMG complex and RPA[17–19]
Dpb3	POLE4	Subunit of DNA polymerase ϵ , leading strand nucleosome reassembly, binds H3-H4[20,21]
Dpb4	POLE3	Subunit of DNA polymerase ϵ , leading strand nucleosome reassembly, binds H3-H4[20,21]
Mcm2	MCM2	DNA helicase subunit involved with DNA polymerase α and Ctf4 in lagging strand parental nucleosome reassembly[22–24]
DNA polymerase α	DNA polymerase α	Lagging strand nucleosome reassembly with MCM2 and Ctf4[24]
Ctf4	WDHD1/AND-1	Replisome component required for Mms22 recruitment to replication forks, involved with DNA polymerase α in lagging strand parental nucleosome reassembly[24,25]
Asf1	Asf1 A/B	Histone chaperone for new H3-H4 dimers, required for Rtt109-dependent H3K56 acetylation [26,27]
Vps75	N/A	Histone chaperone for H3-H4, forms a stoichiometric complex with Rtt109[28,29]
CAF-1 (Cac1, 2 and 3)	CAF1 (p150, p60 and p48)	Chromatin assembly complex[30]
Rtt109	N/A	Histone acetyltransferase for H3K56 acetylation of new histones[31]
Rtt106	N/A	Histone chaperone, interacts with CAF-1[32–34]

PCNA	PCNA	DNA polymerase processivity clamp involved in CAF-1-dependent histone deposition[35]
HAT1 (RbAp46)	HAT1	Histone acetyltransferase of new histone H4 on K5 and K12[36]
Gcn5	KAT2A/B	Catalytic subunit of ADA and SAGA histone acetyltransferase, acetylates new histone H3[37,38]
Kap123	IPO4	Karyopherin involved in nuclear import of newly synthesized histone H3[39–41]
Kap121	IPO5	Karyopherin involved in nuclear import of newly synthesized histone H4 and H2A-H2B[39–41]
Kap95, 104, 114	KPNB1, TNPO1/2, IPO9	Karyopherins involved in nuclear import of newly synthesized histone H2A-H2B[39–41]
Rtt101	N/A	Cullin subunit of E3 ubiquitin ligase complex, ubiquitylates previously K56 acetylated H3 on K121-122-125[42,43]
Mms1	N/A	Adaptor subunit of E3 ubiquitin ligase complex, binds to acetylated H3[42,43]
Mms22	Distant orthologue of MMS22L	Subunit of the rtt101 ubiquitin ligase complex[42,43]
Hst3	N/A	Sirtuin, deacetylase for H3K56 in late S/G2[44]
Hst4	N/A	Sirtuin, deacetylase for H3K56 in late G2/M and G1[44]
N/A	SET8/Pr-SET7/KMT5A	Histone methyltransferase for H4 lysine 20, mono-methylates histones in G2[45,46]
N/A	SUV4-20H1/H2	Histone methyltransferase for H4 lysine 20, di- and tri-methylates histones [47]
N/A	TonsL	Forms homologous recombination repair complex with Mms22L, contains ARD domain binding to unmodified H4K20[48–53]
Distant ortholog of Mms22	Mms22L	Homologous recombination repair complex with TonsL, interacts with both Rad51 and the ssDNA-binding complex RPA[48–53]

Table 1: Comparison between chromatin assembly regulators in yeast and human cells.

2. Recycling of pre-existing histones from parental nucleosomes upon passage of DNA replication forks

DNA replication is initiated at genomic sequences called “origins”, which serve as platforms for stepwise assembly of the replication machinery[54–56]. The first step, known as origin licensing, consists of loading the MCM replicative helicase onto origin DNA. This is mediated by Cdc6, Cdt1, and the *origin recognition complex* (ORC), a multi-subunit protein complex that binds to origin DNA[55,57,58]. Together with the MCM helicase, these proteins constitute the pre-replicative complexes (pre-RCs). Because cyclin-dependent kinases (CDKs) interfere with pre-RC formation via multiple mechanisms[55,58,59], pre-RCs can only assemble in late M-phase and G1 when CDK activity is low. The fact that replication origin firing during S-phase disrupts pre-RCs, and that CDKs prohibit pre-RC formation, ensures that no origin can be fired twice within the same cell cycle.

The MCM helicase separates DNA strands at the time of origin firing but its ability to trigger initiation depends on the contribution of other proteins that are not part of the pre-RCs[60]. These additional proteins form a complex with MCM known as CMG, which is composed of Cdc45, MCM, and the 4 polypeptides of the GINS sub-complex[61]. Unlike pre-RCs, the formation of CMG depends on the activity of S-phase CDKs. These kinases therefore exert two opposing functions in the control of DNA replication: i) they promote initiation of DNA replication at the onset of S-phase, and then ii) prevent re-replication of DNA at later stages of the cell cycle[58]. The helicase activity of CMG is activated by phosphorylation of MCM subunits by Cdc7-Dbf4 (known as the Dbf4-dependent kinase or DDK), leading to melting of origin DNA and recruitment of DNA polymerases and other replisome components[58].

Parental nucleosomes are transiently disassembled into (H3-H4)₂ tetramers and (H2A-H2B) dimers upon passage of DNA replication forks and are reassembled rapidly onto replicated daughter chromatids[62–64]. For example, during replication of SV40 minichromosomes in mammalian cells, nucleosomes are formed within approximately 225 ± 145 bp on the leading strand and 285 ± 120 bp on the lagging strand, which is sufficient to accommodate histone octamers[63]. Two distinct processes contribute to restore nucleosome density behind replication forks: i) parental histone recycling, and ii) *de novo* chromatin assembly i.e. the incorporation of newly synthesized histones onto

nascent DNA. The bulk of the evidence obtained in several model systems indicates that “intact” parental (H3-H4)₂ tetramers are generally transferred to daughter chromatids, whereas parental histone H2A-H2B dimers can reassociate with either parental or new (H3-H4)₂ tetramers[65–69]. Nevertheless, it cannot be excluded that a subset of parental nucleosomes might be split into H3-H4 and H2A-H2B dimers upon passage of replication forks[70]. The above considerations imply that histone chaperones involved in the recycling of parental histones likely accommodate (H3-H4)₂ tetramers, rather than H3-H4 dimers.

The histone-binding complex FACT (Facilitates chromatin transcription) plays important roles in the disruption of pre-existing nucleosomes and/or the recycling of parental histones behind replication forks. FACT comprises three polypeptides in yeast, Spt16, Pob3 and Nhp6 (the latter two are replaced by a single polypeptide, hsSSRP1, in humans)[71]. The Spt16 subunit, also known as Cdc68, was identified through genetic screens designed to uncover transcriptional regulators in *S. cerevisiae*[72–74]. The human FACT complex, consisting of hsSPT16 and hsSSRP1, was later purified by virtue of its ability to disassemble and reassemble nucleosomes during transcriptional elongation *in vitro*[17]. Three lines of evidence indicate that, independently of its role in transcription, FACT also plays a role in DNA replication. First, the Spt16 and Pob3 subunits of FACT interact with the catalytic subunit of DNA polymerase α [75]. Second, mutations in genes encoding FACT subunits genetically interact with hypomorphic mutations affecting DNA replication enzymes[76]. Third, mutant alleles of *SPT16* and *POB3* confer sensitivity to conditions that impede DNA replication, such as the depletion of dNTPs caused by hydroxyurea[76].

In addition to DNA polymerase α [75], FACT interacts with the CMG complex[77] and Replication protein A (RPA)[78]. FACT is therefore present at the heart of the replisome and current evidence suggests that Spt16 binding to H2A-H2B and H3-H4 promotes nucleosome disassembly and reassembly at replication forks[17–19]. FACT engages in extensive contacts with DNA while maintaining multiple components of a partially disassembled nucleosome in close proximity[79–81]. This transient disruption of histone-DNA interactions facilitates MCM activity *in vitro* as well as replication fork

progression *in vivo*[82,83]. Since evidence from both yeast and mammalian cells indicate that FACT and Pol α interact with H2A-H2B[17–19,84], it is also plausible that the former factors might contribute to recycling H2A-H2B during replication. Intriguingly, FACT activity is dispensable for viability in fission yeast[85] and in certain differentiated vertebrate cells[86], implying that this complex is not essential for either replication or transcription. As other proteins have been shown to promote replication through nucleosomal substrates, e.g., the simian virus 40 helicase[87], it seems plausible that other replisome components allow nucleosome disruption and replication fork progression in the absence of FACT.

Although parental histones can be segregated onto both sister chromatids, there exists a modest strand bias for deposition of parental histones behind replication forks, with yeast[20] and human[22] showing opposite bias toward the lagging and leading strand, respectively. Importantly, specific histone-binding replisome components have been shown to direct the transfer of parental histone toward the leading or lagging strand. The Dpb3 (hsPOLE3) and Dpb4 (hsPOLE4) subunits of the leading strand DNA polymerase ϵ bind histones H3 and H4[20,21], and have been shown in yeast to promote transfer of parental histones to the leading strand. The N-terminal region of both the yeast and human MCM2 subunit of CMG possesses a histone-binding domain that plays roles in transferring (H3-H4)₂ tetramers behind replication forks [23,88]. In both yeast and human, histone binding by MCM2 promotes parental histone segregation toward the lagging strand[22,24]. Interestingly, mutation that cripple the interaction between CMG and the lagging strand DNA polymerase α promotes accumulation of parental histones on the leading strand[24], suggesting that these factors are functioning together in restricting strand asymmetry during parental histone segregation behind replication forks.

Consistent with the fact that parental H2A-H2B can reassociate with either pre-existing or new (H3-H4)₂ tetramers[65,69], hsMCM2 binding blocks the interaction surface between H3-H4 and H2A-H2B, implying that at least one H2A-H2B dimer must dissociate from parental octamers for hsMCM2 to gain access to H3-H4. Crystallographic data also indicate that hsMCM2 can bind H3-H4 dimers in conjunction with the hsAsf1 histone chaperone *in vitro*[23,89,90]. Yeast and human Asf1 binds H3-H4 dimers via a

region of H3 that serves as an interface for formation of (H3-H4)₂ tetramers, which is *a priori* incompatible with the fact that parental (H3-H4)₂ tetramers are transferred behind replication forks as intact units. We note that Asf1/MCM complexes purified from human cells lack components of the active CMG complex, specifically Cdc45[90], raising the possibility that Asf1/MCM complexes are not formed at active replication forks. Nevertheless, it cannot be excluded that transient disruption of parental (H3-H4)₂ tetramers occurs before their reformation on daughter chromatids. Another possibility is that MCM2 might interact with dimers made of new histones H3-H4 bound by Asf1[23].

3. Assembly of nucleosomes from newly synthesized histones

The partition of parental histones among the two daughter chromatids implies that, in order to restore a normal nucleosome density, nucleosomes containing newly synthesized histones must be assembled behind DNA replication forks by histone chaperones (**Figure 1**).

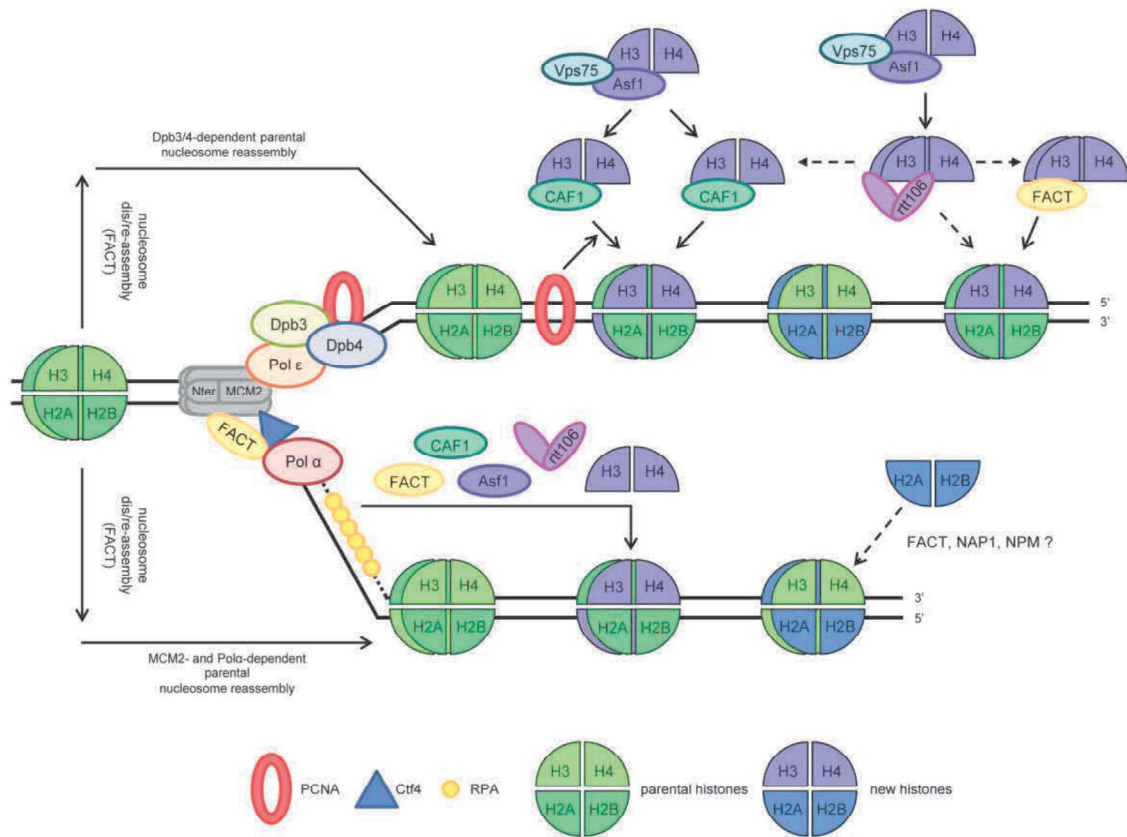


Figure 1: Recycling of histones from pre-existing nucleosomes and assembly of nucleosomes from newly synthesized histones upon passage of DNA replication forks (see text for details).

In cell-free systems, new nucleosomes can be assembled via deposition of H3-H4 followed by eventual addition of H2A-H2B[91,92]. While several H2A-H2B chaperones have been described, such as FACT, polymerase α [84], NAP1[93,94] and nucleoplasmin (NPM)[95] family members, replication-coupled assembly of new H2A-H2B into nucleosomes is less well characterized than that of H3-H4. Asf1 is an “upstream” chaperone that interacts with H3-H4 dimers. This occurs via binding of Asf1 to the C-terminus of H3, thereby blocking the ability of H3-H4 to form tetramers[27]. The fact that parental H3-H4 tetramers largely remain intact during parental histone segregation

implies that Asf1 predominantly interacts with new histones *in vivo*. We note that *in vitro*, Asf1 can bind histones in conjunction with other H3-H4-binding proteins including hsMCM2, yeast Vps75, hsTONSL, as well as subunits of the CAF1 complex[96]. The interaction between Asf1, H3-H4 dimers, and Vps75 is of interest here: it allows acetylation of new histones in yeast[29] since Vps75 forms a 2:1 stoichiometric complex with the acetyltransferase Rtt109 [44,97,98] (described in detail in section 4). The Vps75-histone complex adopts several conformations *in vitro*[29,99] with Vps75 forming dimers or tetramers that bind H3-H4 dimers or (H3-H4)₂ tetramers, although it is unclear which form of the Vps75-histone complex predominates *in vivo*.

Asf1/Vps75 are thought to transfer new histone H3-H4 dimers to downstream chaperones[43], including the CAF1 complex[30] and Rtt106[32], for assembly into nucleosomes. CAF1 comprises three subunits: p150 (CHAF1A), p60 (CHAF1B), and p48 (RpAb48 or RBBP4) in humans[100], and Cac1 (Rlf2), Cac2 and Cac3 (Msi1) in *S. cerevisiae*[101]. CAF1 is the only protein complex that has been formally demonstrated to promote DNA replication-coupled deposition of H3-H4[91]. CAF1 interacts with the DNA polymerase processivity clamp PCNA[35,102–104], which is essential for the ability of this chaperone to deposit histones at replication forks. CAF1 also interacts with Asf1 *in vivo*[70], which may facilitate the deposition of new H3-H4 onto nascent DNA. Recent structural analyses indicate that CAF1 associates with DNA and one H3-H4 dimer, and that two such complexes can act together to form (H3-H4)₂ tetramers on newly replicated DNA[105,106]. Currently available data are consistent with the notion that once new H3-H4 reach CAF1, no further intermediates are required for the assembly of new nucleosomes.

While Rtt106 interacts directly with CAF1[32], deletion of genes encoding these two factors causes a synergistic disruption of gene silencing in yeast[32]. Nevertheless, it is unclear whether these factors act in a same or distinct chromatin assembly pathways since detailed mechanistic data linking these two chaperones are lacking. Dimers of Rtt106 bind (H3-H4)₂ tetramers both *in vivo*[33] and *in vitro*[107], although in contrast to CAF1[105,106] the precise intermediates formed between Rtt106/H3-H4 and nascent DNA have not yet been characterized. Interestingly, Rtt106 can transfer new histones to

FACT *in vivo*, and consequently deletion of *RTT106* diminishes the amount of histones bound to FACT[108]. The fraction of histones associated with Rtt106 that are transferred to FACT versus CAF1, and whether or not Rtt106 assembles nucleosomes on its own *in vivo*, i.e., in a FACT- and CAF1-independent manner is unclear and therefore requires further study. We note that CAF1- or Rtt106-independent pathways for replication-coupled deposition of new H3-H4 molecules must exist in yeast since *cac1Δ rtt106Δ* cells are viable, albeit slow growing and sensitive to drugs that interfere with replication[5,32]. This is in contrast to the situation in human cells where depletion of CAF1 causes DNA damage, accumulation of cells in S-phase, and cell death[109–111]. This suggests either that human cells are more sensitive than yeast to chromatin assembly defects, or that there exists a lesser degree of redundancy at this level of the chromatin assembly cascade in human versus yeast.

In yeast, the single-stranded DNA binding complex RPA can interact with histone H3-H4 and deposit them on adjacent double-stranded DNA, as well as interact with the histones chaperones FACT, CAF1, Asf1 and Rtt106[112]. RPA is therefore proposed to act as a bridge between H3-H4/chaperones and DNA. While a fraction of RPA-bound histones was determined to be new H3-H4, on the basis that they contained H3K56ac, H4K5ac and K12ac, it is unclear whether RPA also binds parental histones and contributes to their recycling behind replication forks. The contribution of other histone-binding proteins present at replication forks, including DNA polymerase α and MCM2, to the assembly of nucleosomes comprised of newly synthesized histones is also incompletely characterized.

4. Modifications of newly synthesized histones

Early experiments performed in several eukaryotic systems documented that new histones H3 and H4 are acetylated prior to their deposition onto DNA[11,16], and that new nucleosomes assembled behind DNA replication forks harbor acetylated lysines[113]. Subsequent data revealed that lysines located in both the N-terminal tails and the globular domains of H3 and H4 were acetylated in new histones[114–118]. Treatment with histone deacetylase inhibitors was also shown to increase

deoxyribonuclease I sensitivity in newly replicated DNA[113] and to cause elevated chromatin acetylation after S phase (see for example[44]). These results were interpreted to mean that new histones are eventually modified after deposition, in this case deacetylated, such that they progressively become indistinguishable from parental histones.

Previous studies suggest that H3 and H4, which are rapidly acetylated after their synthesis[11,16], might be modified in the cytoplasm prior to nuclear import[36]. While cytosolic extracts from duck erythroblasts and other cell types allow acetylation of H4[11], the HAT1 enzymatic complex that acetylates new H4 molecules is predominantly nuclear[117,119,120]. Although this nuclear localization does not preclude that the functionally active form of the enzyme may also be present in the cytoplasm in lesser amounts, cytoplasmic acetylation of new histones has yet to be formally shown *in vivo* rather than in extracts. Of the four lysine residues that are acetylated in the N-terminal tail of H4 (positions 5, 8, 12, 16), K5 and K12 are acetylated at high levels in new histones among the eukaryotic species examined to date[10,121]. Acetylation of lysines in the N-terminal tail of new H3 molecules is more variable across species, with K9, K14 and K23 being major targets[10,14,28,122–124]. In yeast, new H3 are acetylated at every lysine in their N-terminal tail, with a preference for H3K9[124], while certain lysines located in the globular domains of new histones are also acetylated, e.g., K56 of H3[114,125,126] and K91 of H4[116]. In comparison with new H3-H4 molecules, modifications of newly synthesized H2A-H2B have been far less studied.

In the case of new H4, the main acetyltransferase is a complex comprising the catalytic subunit HAT1 and the RbAp46 (RBBP7) histone-binding protein[120,127–129] which acetylates H4K5 and K12 *in vitro* and *in vivo*[127,130,131]. Histone H4 molecules that co-purify with HAT1 are also acetylated on K91[116], but the enzyme(s) that catalyzes this modification *in vivo* has not been formally identified. In yeast, new H3 molecules are modified prior to their deposition onto DNA by the acetyltransferase Gcn5[37,132]. The form of Gcn5 responsible for acetylation of new H3 molecules *in vivo* is not known: recombinant Gcn5 displays a preference for free versus nucleosomal histone H3 whereas the opposite is true for Gcn5-containing acetyltransferase complexes

including ADA and SAGA[38]. Moreover, deletion of genes encoding subunits of the ADA and SAGA complexes does not cripple the ability of Gcn5 to acetylate new histone H3 molecules *in vivo*. While the above seems to suggest that Gcn5 alone acetylates new histones, there is a discrepancy between the specificity of recombinant Gcn5, which almost exclusively acetylates H3K14 *in vitro*[38,124], and the patterns of N-terminal tail acetylation of new histones in yeast (which includes H3K9[123]). This implies that other enzymatic activities act to modify non-nucleosomal H3 *in vivo*, or that Gcn5 substrate specificity is different or modulated by other proteins *in vivo* versus *in vitro*.

The yeast HAT Rtt109 exclusively acetylates free histones[133] and its substrate specificity is modulated by the histone chaperones Vps75 and Asf1[26,28,97,122,133–135]. Asf1 is essential for acetylation of new H3 on K56 by Rtt109, whereas deletion of *VPS75* has no effect on this modification[28,44,98,135–138]. Remarkably, H3K56ac is present in virtually 100% of new H3 molecules in yeast[44,114]. This is in stark contrast to human cells where only small amounts of this mark can be detected by mass spectrometry[139–141], suggesting that its biological role has diverged significantly during evolution (more detail on this in section 8). Vps75 promotes Rtt09-mediated acetylation of K9 and other N-terminal lysines (*e.g.* K23 and K27) in new H3[26,28,122,134,142]. While Asf1 overexpression was also shown to increase Gcn5-mediated acetylation of new H3 on K9 *in vivo*, the precise mechanistic basis is unclear[123].

5. Modifications of new histones influence their binding to chaperones and karyopherins

Several modifications of new histones either directly or indirectly influence their interactions with chaperones and karyopherins, which in turn is expected to influence histone availability at replication forks and therefore the efficacy of *de novo* chromatin assembly. As will be discussed below, current evidence suggests that i) modifications are affixed to new histones in a stepwise manner[143], and ii) this ordered cascade promotes a “directional” flow of new histones toward deposition into nascent chromatin by influencing their binding to chaperones (**Figure 2**).

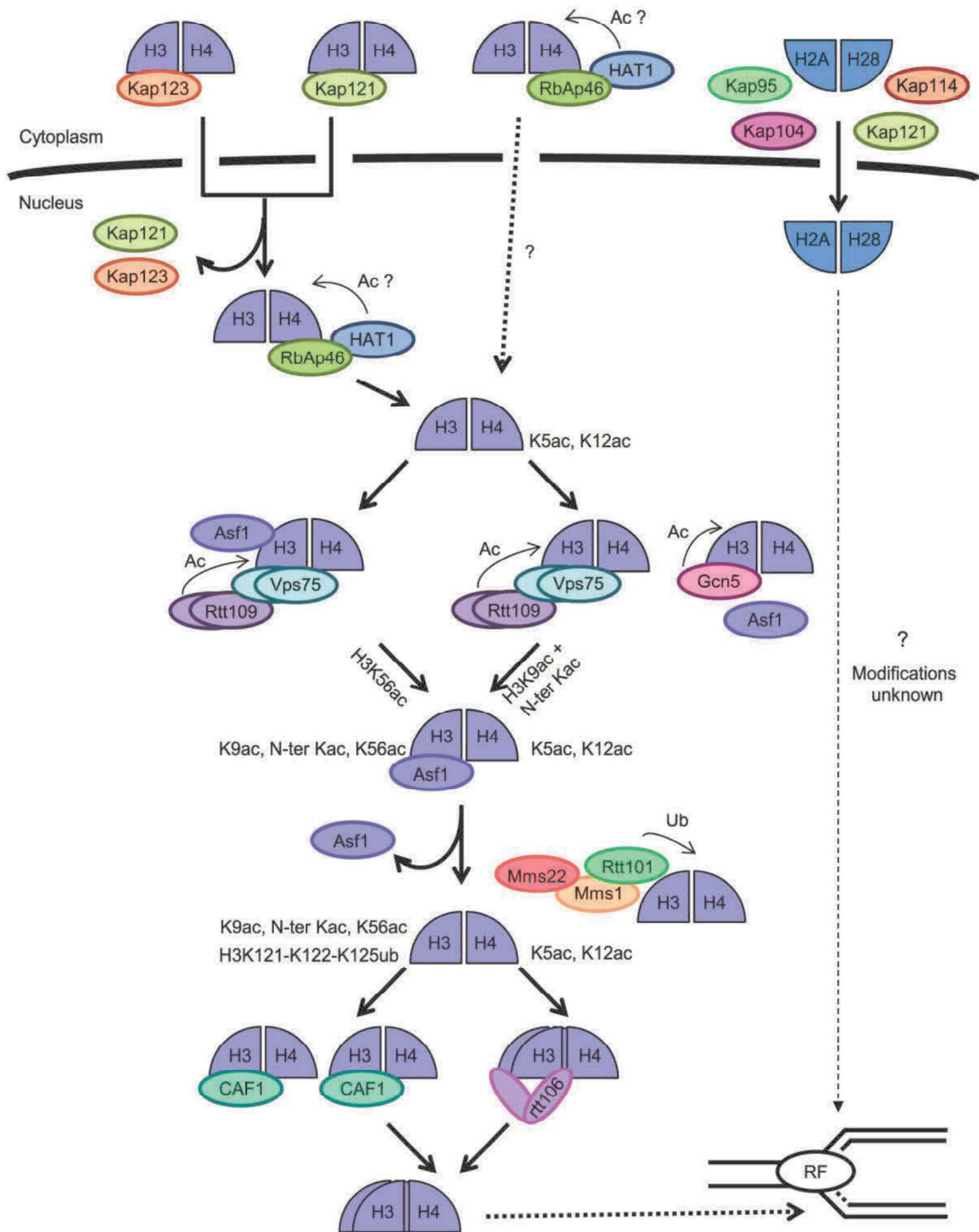


Figure 2: Modifications of new histones from synthesis to deposition into chromatin (see text for details). For clarity, Asf1 interactions with histones is only presented in the context of its role in promoting H3K56ac.

Histones are imported into the nucleus in association with karyopherins, which allow passage of cargo proteins through the nuclear pore complex[144,145]. In yeast cells newly synthesized H3 and H4 are imported in the nucleus by either of two abundant karyopherins, known as Kap123 and Kap121, whereas Kap95, 104, 114 and 121 import new H2A-H2B[39–41]. The nuclear localization signals of histones H3 and H4 are situated in their N-terminal tails[39,146,147]. The positive charge of N-terminal tail lysines in H3 and H4 promotes interaction with karyopherins and nuclear import, suggesting that charge neutralization via acetylation might influence this process[148]. Indeed, acetylation of N-terminal tail lysines reduces the affinity of histones for yeast and human karyopherins[148–150], raising the possibility that that these modifications may facilitate release of cargo histones from karyopherins upon entry in the nucleus.

The best studied acetylation marks present on new histones in yeast are K9/56 for H3 and K5/12 for H4[26]. The situation is different in human cells, as H4 associated with Asf1 harbours K5/K12 acetylation, whereas H3.1 displays K14/K18ac and very low levels of K56ac[151]. While the role of these modifications is incompletely understood, one widely accepted model postulates that acetylation of new histones influences their interaction with chaperones, which in turns modulates histone availability for nucleosome assembly at replication forks. In a landmark paper, Li et al. showed that yeast H3K56ac increases both i) the abundance of histones bound to CAF1 and Rtt106 *in vivo*, and ii) the affinity of these chaperones for H3 *in vitro*[152]. Crystallographic evidence subsequently revealed that pleckstrin-homology (PH) domains in Rtt106 promote its binding to K56 acetylated H3[107,153]. In contrast, the structural determinants explaining the reported increased binding of K56 acetylated versus non-acetylated H3 to CAF1 *in vitro* are unknown. Gcn5- and Rtt109-dependent acetylation of N-terminal tail residues in H3 has also been found to promote the interaction of new H3 molecules with CAF1 *in vivo*[37], although the mechanistic basis is unclear.

In addition to promoting CAF1 and Rtt106 binding to new histones, K56ac has been reported to stimulate the ubiquitination of new histone H3 on several lysines[43]. This is explained at least in part by the increased affinity of the Rtt101-Mms1-Mms22 ubiquitin ligase complex for K56-acetylated versus non-modified H3. Rtt101-Mms1-Mms22 has previously been genetically linked to Rtt109, Asf1 and H3K56ac[42,136,154]. Rtt101-Mms1-dependent ubiquitination of new H3 molecules promotes their release from Asf1, thereby increasing the availability of new H3-H4 dimers for downstream chaperones[43]. Overall, the available data are consistent with a model in which new histone modifications promote their flow from entry into the nucleus to their deposition into nascent chromatin, by either increasing or decreasing histone binding to chaperones or karyopherins.

6. Modification of new histones, *de novo* chromatin assembly, and genomic stability

Several lines of evidence indicate that modifications of newly synthesized histones promote genomic stability. Mutations that abolish the acetylation of lysines in the N-terminal tails of either H3 or H4 sensitize yeast cells to genotoxins, and cause spontaneous activation of the DNA damage checkpoint with consequent accumulation of cells in G2/M[115,152,155,156]. Lysines located in the N-terminal tails of histone H3 and H4 appear to be functionally redundant in this regard, since mutating individual residues causes mild phenotypes in contrast to mutations affecting several lysines[157,158]. Yeast mutants lacking genes encoding acetyltransferases targeting the N-terminal tails of new histones, *i.e.* Gcn5 and Hat1, also cause synthetic sensitivity to genotoxins and abnormal cell cycle progression when combined with mutations of N-terminal tail lysines, further suggesting that acetylations of multiple residues act in a redundant manner to protect genomic integrity[156,159]. Mutations that prevent acetylation of globular domain lysines of histones, *e.g.* H3 K56 and H4 K91, cause strong sensitivity to genotoxic drugs[114,116] on their own, as well as synthetic sensitivity when combined with mutations of N-terminal tail lysines of H3 or H4[115,152]. Consistent with their role in *de novo* chromatin assembly during S phase, modifications of new histones, *e.g.*, H3K56ac, cause much stronger sensitivity to genotoxins that

interfere with DNA replication versus conditions such as ionizing radiation which damage cells irrespective of cell cycle phase[114].

It has been proposed that lack of modification of new histones causes sensitivity to genotoxins that interfere with replication as a result of defective or delayed histone deposition behind DNA replication forks. Consistent with this, inhibition of H4 or H2B expression during passage through S-phase causes yeast cells to accumulate in G2/M, consistent with replicative stress-induced activation of the DNA damage checkpoint[2,160]. Moreover deletion of the genes encoding yeast Rtt106 and subunits of the chromatin assembly factor CAF1 causes sensitivity to drugs that interfere with DNA replication[5], while lack of histone expression or conditional inactivation of CAF1 also cause DNA damage in human cells[3,111]. Yeast cells devoid of the histone chaperone Asf1 are also strongly sensitized to replicative stress[161], although genetic analyses indicate that this is due, for the most part, to the fact that Asf1 is essential for H3K56 acetylation. However, as mentioned previously, H3K56ac promotes chromatin assembly via its effect on i) CAF1 and Rtt106 binding to new histones[152], and ii) Rtt101-Mms1-Mms22-dependent ubiquitination of new histones leading to dissociation of H3-H4 dimers from Asf1 and consequent elevation of histone availability for downstream chaperones[43]. It is therefore likely that the sensitivity of cells lacking H3K56ac or other modifications of new histones to genotoxins arises at least in part via defective or delayed chromatin assembly.

The precise molecular mechanisms through which modifications of new histones and/or *de novo* chromatin assembly protect cells against drugs that interfere with DNA replication are poorly understood. In human cells, progression of DNA replication forks is coupled with ongoing histone synthesis and nucleosome assembly[3,90,111]. Moreover, recent reports indicate that inhibition of histone synthesis promotes the formation of replication-blocking R-loops in human cells, which might explain in part the impact of reduced nucleosome assembly on DNA replication[162]. Since stalled DNA replication forks can eventually “collapse” into DSBs[163], it seems plausible that the elevated DNA damage observed in cells with defective nucleosome assembly might result from abnormal DNA replication progression in human cells. In contrast to the

above, DNA replication fork progression can occur in the absence of *de novo* chromatin assembly in yeast, as lack of histone H2B or H4 expression does not prevent completion of the bulk of DNA synthesis during S phase[2,160]. Nevertheless, neutral 2D gel analyses indicate that DNA replication structures are unstable in *cac1Δ rtt106Δ* and *rtt109Δ* mutant cells upon exposure to genotoxins that interfere with DNA polymerases[5]. Moreover, cells lacking H3K56ac present i) DNA replication progression defects in response to the alkylating drug methyl methane sulfonate (MMS)[154] and, ii) destabilization of CAG/CTG repeats[164], which are known to form secondary structures that impede replication forks.

The fact that DNA replication fork progression is reduced upon genotoxic stress in cells lacking either acetylation of new histones or chromatin assembly factors could conceivably be due to either i) destabilization and eventual “collapse” of blocked DNA replication forks into DSB and/or ii) defective fork rescue/restart mechanisms. One important DNA repair mechanism that stabilizes and promotes restart of stalled DNA replication forks is homologous recombination (HR)[165]. Several yeast chromatin assembly mutants present persistent foci of the HR factor Rad52[5,154,166,167] which might reflect futile attempts at HR-dependent restart of stalled replication forks. Consistently, abnormal frequencies of HR-dependent genotoxin-induced sister chromatid exchange (SCE) were previously reported in cells lacking CAF1 subunits or H3K56 acetylation[168]. Cells devoid of H3K56ac also present elevated mutation rates[169] as well as a high frequency of aneuploidy and gross chromosomal rearrangements[170], which might reflect defective repair of replication-associated DNA damage. While the mechanisms are incompletely characterized, recent evidence indicates that histone deposition, via CAF1 and Asf1 (and therefore H3K56ac), promotes HR at least in part by stabilizing joint molecules formed at stalled replication forks[171]. On the other hand, replication-coupled chromatin assembly has also been shown to promote sister-chromatid cohesion[172], which is known to facilitate post-replicative HR[173]. The available data therefore supports the notion that HR repair of blocked replication forks is compromised in cells with defective chromatin assembly or modification of new histones.

Persistent stalling of DNA replication forks can generate DSBs, which are repaired via non-homologous end joining (NHEJ) or homologous recombination (HR) (reviewed in [174]). There is considerable information about the roles of parental histone modifications in DSB break repair. For example, di-methylation of histone H4 lysine 20 (H4K20me₂) directly binds to the tandem tudor domains of hs53BP1 and its *S. pombe* homologue Crb2[175], which in turn promotes DSB repair by NHEJ. Ubiquitination of the N-terminal tail of H2A (H2AK13 and H2AK15) by RNF168 also binds directly to hs53BP1 and is inducible at DSB sites[176,177]. Lysine acetylation has also been reported to play roles in response to DSBs, *e.g.* modification of the N-terminal tail of H4 by the NuA4/Tip60 complex[178,179]. By comparison to modifications of pre-existing histones, much less is known about the role(s) of modifications of newly synthesized histones in response to DSBs. One exception is the enzyme that acetylates new H4 molecules at lysines 5 and 12 before their deposition onto nascent DNA[117]. Yeast and human cells lacking HAT1 are defective in some aspect of DSB repair by HR[156,180]. Moreover, in mammalian cells, HAT1-mediated histone acetylation may protect the DNA repair and replication intermediates against inappropriate degradation[181]. In any case, the fact that cells lacking some histone modifications that promote nucleosome assembly, *e.g.* H3K56ac, are far less sensitive to ionizing radiation-induced DSBs than to genotoxins that interfere with replication fork progression[114] suggests that these mutants are at least partly proficient in DSBs repair. Consistently, HR-dependent repair of HO endonuclease-induced DSB proceeds with normal kinetics in mutants lacking modifications on lysine residues in the N-terminal tails of new histones[115] or the globular domains of histones including H4K91ac[115] or H3K56ac[114]. Intriguingly, cells lacking H3K56ac, the histone chaperone Vps75, or the N-terminal tails of histone H3 or H4 present NHEJ defects [157,182], but the mechanistic basis is incompletely understood.

Upon DSB induction, resection of DNA ends was reported to be accompanied by loss of histones around the break[183]. CAF1 and H3K56ac were found to facilitate reassembly of histones around the DSB upon completion of HR-dependent repair which, via unknown mechanisms, appears to be coupled to deactivation of DSB-induced DNA damage checkpoint signalling and resumption of cell proliferation[183,184]. CAF1 is

also involved in chromatin reassembly after nucleotide excision repair of UV-induced DNA lesions[185], although it is unknown whether modification of new histones contribute to this. While the above data suggest that chromatin assembly and new histone modifications might promote cell cycle restart after DSB repair, the contribution of these effects to the sensitivity of H3K56ac mutants to replicative stress is unclear since pharmacological abrogation of the checkpoint kinase Rad53 does not rescue the sensitivity of these mutants to replicative stress[154]. We also note that MMS and HU exposure do not cause DSB at high frequency in yeast[186], raising doubts as to the functional relevance of DSB repair-coupled chromatin assembly to the sensitivity of cells exposed to these drugs.

Recent data indicate a role for H3K56ac-containing nascent chromatin in modulation of gene expression during DNA replication[187–189]. In wild-type yeast, the expression of genes replicated in early S is diminished after passage of replication forks such that their level of expression remains similar to those of late replicating genes. Proteins that promote H3K56ac, including Rtt109 and Asf1, as well as chromatin assembly factors such as CAF1 and Rtt106, contribute to this S phase-associated “gene expression buffering”[187–189], suggesting that *de novo* nucleosome assembly at gene promoters may play important roles in these phenomena[189]. However, the impact of such mechanisms on the DNA damage response is unclear.

7. Biological functions of nascent chromatin structure: H3K56ac as a model system

Several observations argue that modifications of new histones influence the DNA damage response via mechanisms not directly linked to their effects on chromatin assembly. Acetylation of new H3 on K56 has been used as a model to investigate these phenomena because of the abundance of this modification (virtually 100% of new histone H3 molecules are K56-acetylated in yeast[44]) and the fact that the enzymes that regulate H3K56 acetylation are well-characterized[31,44,136–138,190,191] (**Figure 2**). H3K56ac marks new histones deposited in nascent chromatin behind DNA replication forks[44,114] and in a replication-independent manner upon H3 exchange during transcription[192,193]. H3K56ac is deacetylated in chromatin by two partially redundant

sirtuin histone deacetylases, Hst3 and Hst4[44,191,194–197]. In *S. cerevisiae*, Hst3 expression is restricted to late S/G2 whereas Hst4 is expressed in late G2/M and G1[191]. Deletion of either of the genes encoding these deacetylases causes mild phenotypes, whereas double mutants present constitutive H3K56ac, spontaneous DNA damage, and HR foci, accompanied by extreme sensitivity to replicative stress[44,198–200]. We also note that Hst3 is degraded upon activation of the DNA damage checkpoint kinase Mec1, leading to persistence of H3K56ac in G2/M[114,201], although the impact of this phenomenon on the DNA damage response is unclear.

As alluded to above, constitutive H3K56ac (e.g. in *hst3Δ hst4Δ* cells) causes strong phenotypes, including sensitivity to temperature and replicative stress-inducing drugs, as well as spontaneous/persistent DNA damage markers including Rad53 checkpoint kinase activation, DNA repair foci (Rad52), and phosphorylated H2A (γ -H2A)[44,198–200]. The temperature and genotoxin sensitivity of *hst3Δ hst4Δ* cells is partially rescued upon introduction of mutations that prevent this modification (e.g. *rtt109Δ*, *asf1Δ* or *H3K56R*), indicating that the phenotypes of *hst3Δ hst4Δ* mutants depend on abnormal H3K56ac levels[44,136,198]. Upon pharmacological inhibition of Hst3/Hst4 with nicotinamide (NAM; a pan-sirtuin inhibitor), DNA damage markers appear only when cells enter a second S phase in the presence of the drug[44], suggesting that abnormal presence of H3K56ac in chromatin prior to the onset of S phase *i.e.*, in front of replication forks, causes the phenotypes of *hst3Δ hst4Δ* cells.

Toward understanding the biological functions of chromatin-bound H3K56ac, several mutations that suppress the phenotypes of *hst3Δ hst4Δ* cells without influencing H3K56ac levels have been identified. Preeminent amongst them are mutations in genes encoding the Rtt101-Mms1-Mms22 (RMM) ubiquitin ligase complex and its interacting proteins, e.g. the scaffolding proteins Ctf4 and Rtt107[42,136,154,200]. While mutations in this group of genes cause sensitivity to replicative stress-inducing drugs which is epistatic to mutations that abolish H3K56ac[154], the molecular mechanisms explaining these genetic links are not entirely clear. As mentioned previously, the RMM complex ubiquitinates new histones in a H3K56ac-dependent manner to promote nucleosome assembly[43]. However, Rtt107 and Ctf4 are not implicated in this process ([43] and our

unpublished observations) even though their deletion suppresses phenotypes caused by constitutive H3K56ac[200,202]. Moreover, it is conceptually difficult to imagine how crippling *de novo* nucleosome assembly might be beneficial to cells lacking Hst3 and Hst4. Interestingly, in addition to suppressing the phenotypes of *hst3Δ hst4Δ* cells, deletion of *CTF4* also partially suppresses the phenotypes of cells lacking H3K56ac or RMM-encoding genes[203], although the mechanisms are poorly understood.

One plausible model postulates that H3K56ac might influence the recruitment of RMM and perhaps other proteins to nascent chromatin behind replication forks. In such a scenario either lack of or constitutive H3K56ac would mislocalize and compromise the activity of RMM. Consistent with this, deletion of *RTT109*, which abolishes H3K56ac, also diminishes recruitment of Rtt107 and Rtt101 to chromatin upon treatment with replication-blocking genotoxins[204]. In addition, ChIP data indicate that Mms1 colocalizes with newly replicated DNA and replication proteins in an Mms22- and Rtt101-dependent manner[205]. Finally, the interaction between Mms22 and Ctf4, an integral component of replisomes[206], is important for the DNA damage response functions of H3K56ac[203]. While it is tempting, based on the above, to speculate that H3K56ac-dependent RMM recruitment to nascent chromatin behind stalled replication forks might promote DNA repair-related ubiquitination events, the identity of relevant RMM substrates is unknown. Apart from new histones[43], another known substrate of Rtt101 is the Spt16 subunit of the FACT complex[207]. However, Spt16 ubiquitination does not involve Mms1 or Mms22, and is therefore unlikely to be functionally related to H3K56ac. The activity of the Mus81-Mms4 nuclease involved in resolving HR intermediates has also been reported to be regulated by Rtt101/Mms1-dependent ubiquitination, but this depends on the interaction between Rtt101-Mms1 and Esc2, the latter of which does not have any known link with H3K56ac[208]. Interestingly, constitutive H3K56ac inhibits the elongation step of break-induced replication, a homology-dependent DSB repair mechanism that requires polymerisation of long stretches of DNA[209]. However, this phenomenon does not appear to be influenced by RMM and is therefore unlikely to fully explain the phenotypes of *hst3Δ hst4Δ* cells[209]. Finally, deletion of *MRC1*, a replisome component which promotes Mec1 activation in

yeast, partially suppresses the genotoxic drug sensitivity of cells lacking RMM or H3K56ac[202,210]. While the mechanisms remain to be fully elucidated, lack of Mrc1 appears to improve HR-dependent repair of stalled replication forks in H3K56ac pathway mutants[210].

Mutations abolishing H4K16 acetylation or H3K79 methylation (H3K79me), two abundant histone post-translational modifications of yeast euchromatin[211,212], suppress the phenotypes of *hst3Δ hst4Δ* cells without influencing H3K56ac levels[199]. The fact that lack of H4K16ac is associated with decreased global H3K79me3[199,213] suggests that the latter modification probably has a dominant influence on phenotypes caused by constitutive H3K56ac[199,200,214]. In addition to *hst3Δ hst4Δ*, the sensitivity of a multitude of mutants to drugs that compromise replication can be suppressed by deletion of *DOT1*, which encodes the sole H3K79 methyltransferase in yeast[199,215–217]. This effect was traced back to the Rad53-activating protein Rad9, which interacts with damaged chromatin via binding of its Tudor and BRCT domains to H3K79me3 and phosphorylated H2A, respectively[218,219]. Indeed, mutations crippling H3K79me3 diminish Rad53 activation which in turns alleviates sensitivity to replicative stress via mechanisms that remain unclear[200,216]. Consistent with this, inactivation of genes encoding proteins that promote Rad53 activation, (*e.g.* Rad9, the alternative PCNA ring 9-1-1 or the RFC-like clamp loading complexes), partially rescue the phenotypes of *hst3Δ hst4Δ* mutants[198–200]. We also note that a correlation between diminished Rad53 activation and elevated usage of error-prone translesion synthesis has been reported, which might promote survival of *hst3Δ hst4Δ* at the expense of increased mutagenesis[199,200,214,215,217].

Rad53 activation inhibits late origins of replication in response to DNA damage[220]. Constitutive Rad53 activity may therefore compromise completion of DNA replication and lead to long-lasting stretches of incompletely replicated DNA in *hst3Δ hst4Δ* cells[200,214,221]. Previous reports suggest that elevated H3K56ac might sensitize cells to such conditions, as lack of Hst3 destabilizes an artificial yeast chromosome engineered to have a greatly reduced number of efficient replication origins[222]. This is intriguing because *hst3Δ* cells are expected to present elevated

H3K56ac only in late S/G2, with H3K56ac being removed by Hst4 during the next G1. The above therefore indicate that abnormal persistence of H3K56ac during G2/M, which is also observed upon checkpoint activation leading to Hst3 degradation[201], might negatively influence the stability of chromosomes harbouring stretches of unreplicated DNA, although the mechanisms are unclear.

8. Non-methylated H4K20 presents functional similarities with yeast H3K56ac

The function of H3K56ac in metazoans is controversial. This modification has been ascribed roles in the regulation of gene expression, DNA damage response and chromatin assembly in non-fungal organisms, and a multitude of enzymes have been proposed to regulate its levels[139,223–231]. In contrast to the situation for asynchronously growing yeast which have a stoichiometry of $\approx 20\%$ H3K56ac[114], mass spectrometry analyses demonstrated that less than 0.08% of total H3 are H3K56ac in human cells[140]. This vast divergence indicates that the mechanisms through which metazoan cells regulate H3K56ac are likely to be very different than those of yeast. Variations in H3K56ac levels throughout the cell cycle were not consistently observed in metazoans[229,231–233]. Moreover, the stoichiometry of human H3K56ac is insensitive to pharmacological inhibition of histone deacetylases, casting doubts on the relevance of certain enzymes reported as H3K56ac modulators[140]. Finally, the deacetylases that mediate H3K56ac removal in yeast present fungal-specific sequence features that are not found in metazoan enzymes proposed to regulate this modification[194]. We note that a majority of metazoan/mammalian H3K56ac studies employed commercial antibodies for experimental methods such as immunoblotting, chromatin immunoprecipitation and immunofluorescence. Since publications from two different laboratories clearly demonstrated that these reagents present specificity issues[140,141], conclusions reached using these antibodies must be examined with caution.

One distinctive feature of yeast H3K56ac is its ubiquitous presence in newly synthesized histones leading to its accumulation in chromatin during S phase and removal in G2. In mammalian cells, methylation of histone H4 lysine 20 (H4K20me) behaves in a conceptually analogous albeit opposite manner (**Figure 3**): new histones deposited in chromatin during S-phase are non-methylated on H4K20 and progressively become

mono-, di- and tri-methylated after assembly into nucleosomes[48,234]. H4K20 is first modified by the mono-methyl transferase SET8/Pr-SET7/KMT5A[45,46], and is subsequently di- and tri-methylated by SUV4-20H1/H2[47,235]. In asynchronously growing cells, more than 80% of H4K20 is dimethylated, with lower abundance of other forms of this residue[234,236]. SET8 levels are tightly regulated by multiple E3 ubiquitin ligases leading to its degradation in G1 and S, which mostly restricts H4K20me0 presence in chromatin during S and early G2[237–239]. Set8 is ubiquitinated and degraded not only during S but also upon DNA damage in a manner that depends on the Crl4-Cdt2 ubiquitin ligase and on interaction of Set8 with PCNA via a specialized PCNA-interacting peptide (PIP)-degron sequence[240–243]. Cell cycle regulation of SET8 expression is important since removal of its PIP-degron leads to DNA damage checkpoint-dependent G2/M arrest[240–243]. Other pathways leading to SET8 degradation have also been reported: this enzyme can be ubiquitinated by SCF ^{β -TRCP} through CKI-dependent phosphorylation at Ser253 following DNA damage[244], and APC^{CDH1} was shown to promote SET8 proteolysis during mitosis[245]. Overall, the above highlights similarities between H4K20me0 and yeast H3K56ac including the presence in new nucleosomes assembled behind DNA replication forks, removal of the mark during G2, and persistence in chromatin upon DNA damage caused by degradation of enzymatic regulators.

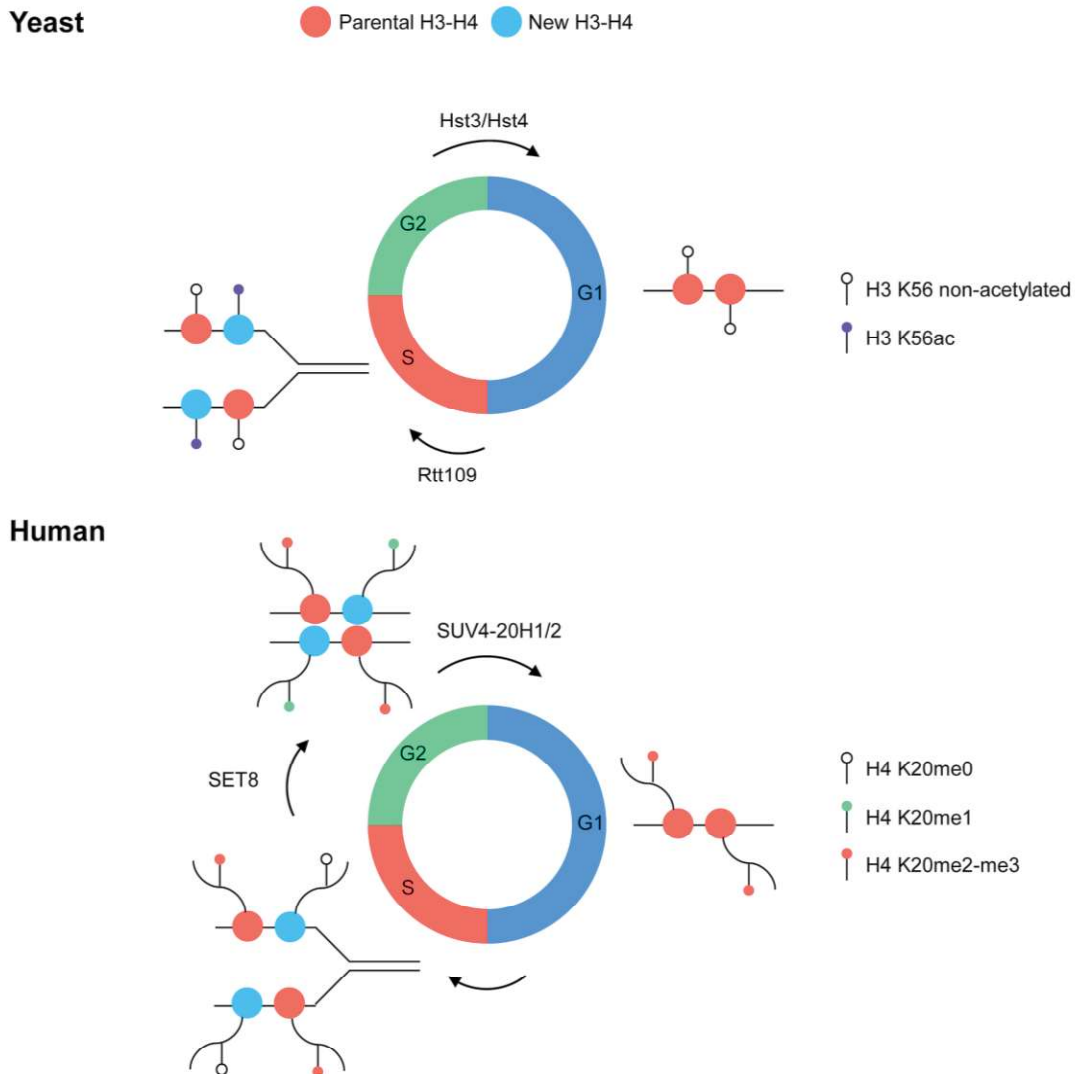


Figure 3: Comparison between the H3 K56ac and H4 K20me cycles in yeast and human, respectively.

Depletion of SET8 leading to elevated H4K20me0 and absence of H4K20me1 causes severe phenotypes including DNA damage and checkpoint-dependent cell cycle arrest, reduced S phase progression, and induction of senescence[237,239,246,247]. The phenotypic consequences of ablating the expression of SUV4-20H1/H2 that di- and trimethylate H4K20 are much less dramatic than those caused by lack of SET8[235,239]. This suggests that elevated H4K20me0 and/or absence of H4K20me1, rather than lack of H4K20me2-3 which is also a consequence of SET8 depletion, generate the severe phenotypes in cells lacking this enzyme. In mouse embryos, lack of SET8 during one

round of DNA synthesis/mitosis causes strong phenotypes when cells enter the next S phase[248], suggesting that events occur during G2/M or G1 in the presence of elevated H4K20me0 which compromise DNA replication in the subsequent S phase. Interestingly, the BAH domain of the ORC1 subunit of the ORC complex, which is not conserved in yeast, interacts directly with di-methylated H4K20 at origins of replication[249–251], a mark for which levels are expected to be diminished in cells lacking SET8. On the other hand, recent evidence indicates that SET8-mediated H4K20me1 during G2/M limits excessive ORC binding and origin licensing after mitotic exit by promoting chromatin compaction[252]. These seemingly contradictory results suggest that precise temporal regulation of H4K20 methylation during G2/M and G1 may be important to avoid replicative stress associated with abnormal DNA replication origin density in the subsequent S phase[253–255].

Initial observations revealed that DNA damage (*e.g.* accumulation of γ -H2AX) caused by SET8 depletion is partially rescued upon co-depletion of the HR factor Rad51[237]. TONSL and MMS22L form a complex that promotes the activity of this critical HR factor during repair of replication-associated DNA damage[49–53]. Interestingly, TONSL, through its ARD domain, preferentially binds histones and nucleosomes that are unmodified at H4K20[48], while MMS22L interacts with both Rad51 and the ssDNA-binding complex RPA[53]. Cells lacking Set8 also present increased binding of MMS22L/TONSL to chromatin *in vivo*[48,256], and lack of either MMS22L-TONSL or the latter's ARD domain sensitized cells to genotoxin-induced replicative stress[48–53]. Nevertheless, it is unknown whether MMS22L-TONSL activity directly influences the phenotypes of cells lacking SET8. We also note that hsMMS22L is a distant mammalian ortholog of the yeast H3K56ac pathway protein Mms22[49–52], reinforcing the notion that H4K20me0 might represent a functional metazoan ortholog of yeast H3K56ac.

The choice between DSB repair pathways is influenced by cell cycle phase, which in human results in part from the opposing activities of 53BP1 and BRCA1/2 that promote NHEJ and HR, respectively[257,258]. 53BP1 limits DNA end resection thereby tilting repair choice toward NHEJ, whereas increased resection upon BRCA1 binding

favors HR[259–262]. 53BP1 contains a tandem tudor domain which recognizes H4K20me2[176,263], and SET8 and other enzymes have been reported to methylate histones in the vicinity of DSBs to promote H4K20me2-dependent 53BP1 recruitment and NHEJ[264]. Moreover, the ability of 53BP1 to be recruited to DSBs was also recently shown to decline during S phase progression, presumably due to dilution of nucleosomes containing H4K20me2 dilution during chromatin replication[265,266]. On the other hand, BARD1, an obligate BRCA1 binding partner, contains an ARD domain similar to that of TONSL which specifically recognizes H4K20me0 on post-replicative chromatin during S/G2 to promote HR[267]. Finally, as mentioned previously, the MMS22L/TONSL complex also promotes HR and binds to H4K20me0-containing chromatin[48,53]. Taken together, the above indicates that the methylation status of H4K20 regulates DSB repair pathway choice via its opposite action on HR- and NHEJ-promoting activities.

9. Conclusions

The data discussed herein indicate that efficient *de novo* chromatin assembly and regulation of nascent chromatin structure are evolutionarily conserved determinants of genomic stability in eukaryotes. As noted throughout this text, several key questions remain unanswered. One pertains to the exact order of modifications acquired by new histones along the chromatin assembly pathway, and their biological importance. The precise nature of DNA lesions occurring in cells with defects in chromatin assembly or modification of new histones is also unclear. It is possible that reduced nucleosome formation behind replication forks might promote the aberrant formation of DNA replication/recombination intermediates, processing of which might lead to replicative stress and DNA damage. Alternatively, lack of histone modification in newly replicated chromatin might prevent or deregulate the recruitment or activity of DNA repair factors acting at stalled DNA replication forks. In the specific case of yeast H3K56ac, the molecular targets and mechanisms linking this modification to the Rtt101-Mms1-Mms22 ubiquitin ligase complex are incompletely characterized. Comprehensive identification of the targets of this ubiquitin ligase would provide valuable information on the spectrum of RMM-modulated molecular pathways. The relative contribution of gene expression regulation, *de novo* chromatin assembly, and recruitment of effector proteins to

chromatin on the phenotypes of mutants of the H3K56ac/RMM pathway is also currently unclear.

Given their biological importance, chromatin assembly pathways and nascent chromatin structure are likely to contribute to human health. In this regard, we note that lack of SET8-dependent methylation causes upregulation of p16Ink4 and p21 expression[246,247], thereby promoting cellular senescence, a mechanism known to limit the proliferative capacity of cancer cells[268]. Moreover, SET8 is downregulated upon oncogene-induced and replicative senescence, suggesting that reduced expression of this protein might be part of a feedback loop to strengthen the senescent phenotype[246,247]. The fact that SET8 is frequently overexpressed in several types of cancer[269] raises the possibility that selection for reduced senescence capacity via SET8 expression and H4K20 methylation-dependent inhibition of p16Ink4/p21 transcription might occur in tumors. We also note that SET8 methylates important cancer-relevant non-histone proteins including p53 and PCNA[269,270], and influences several critical biological processes including adipogenesis[271] and neurodevelopment[272]. Finally, modulation of *de novo* chromatin assembly, *e.g.* via Asf1 activity[273], has been reported to be associated with clinical outcome in certain cancers. It therefore seems likely that further investigation of modifications of new histones and *de novo* chromatin assembly will lead to identification of molecular targets for development of novel therapeutic strategies or of biomarkers to better categorize tumors and tailor anticancer treatments.

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11. Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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