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Chromatin Challenges during DNA Replication and Repair

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Inheritance and maintenance of the DNA sequence and its organization into chromatin are central for eukaryotic life. To orchestrate DNA-replication and -repair processes in the context of chromatin is a challenge, both in terms of accessibility and maintenance of chromatin organization. To meet the challenge of maintenance, cells have evolved efficient nucleosome-assembly pathways and chromatin-maturation mechanisms that reproduce chromatin organization in the wake of DNA replication and repair. The aim of this Review is to describe how these pathways operate and to highlight how the epigenetic landscape may be stably maintained even in the face of dramatic changes in chromatin structure.

Introduction

A number of epigenetic phenomena, such as X-chromosome inactivation, genomic imprinting, centromere function, and gene silencing, rely on the establishment and faithful maintenance of specific chromatin structures. These structures are defined by DNA methylation, histone posttranslational modifications (PTMs), histone variants, and chromatin-binding proteins (i.e., HP1 and Polycomb; see also Reviews by B.E. Bernstein et al., page 669 of this issue, T. Kouzarides, page 693 of this issue, and B. Schuettenguber et al., page 735 of this issue). Epigenetic states defined by chromatin structure can be propagated with high fidelity through DNA replication, mitosis, and, at least in some cases, even meiosis. In addition to being relatively stable through cell division, epigenetically defined chromatin structures also need to be sufficiently plastic to allow programmed changes in transcription patterns during development and differentiation of multicellular organisms (see also Review by M.A. Surani, page 747 of this issue). Chromatin status that determines specific patterns of gene expression has a reversible nature that provides a basis for epigenetic reprogramming as a means to generate biomedically useful pluripotent cells. Because of the genome-wide alterations in chromatin structure that occur during replication, S phase may provide a unique window of opportunity for cells to modify chromatin structures that influence gene expression patterns and, thus, cell fate (Figure 1). Consistent with this, transcriptional activation of certain developmentally regulated genes such as the *HoxB* cluster depends upon DNA replication (Fisher and Mechali, 2003). In addition, chromatin reconfiguration during the first round of DNA replication is necessary to activate enhancer-driven gene expression in the early mouse embryo (Forlani et al., 1998). Eukaryotic cells also need to remodel chromatin structure to access and repair potentially lethal DNA lesions that continuously challenge the genome. Many of the same players are involved in repair- and replication-coupled chromatin modulation. However, chromatin dynamics during DNA repair are distinct in that faithful restoration of the original organization is essential to avoid unscheduled epigenetic changes (Figure 1). In this Review, we focus on chromatin dynamics involved in propagation of chromatin organization during replication and restoration following repair. We emphasize how these mechanisms may have evolved to meet the dual challenge of orchestrating transient changes in chromatin structure while preserving the epigenetic fabric of the genome.

Duplicating Nucleosomal Organization

The basic building block of chromatin is the nucleosome core particle, which contains 147 base pairs of double-stranded DNA wrapped in 1.65 left-handed superhelical turns around the surface of an octamer of histone proteins (Davey et al., 2002; Polo and Almouzni, 2006). The histone octamer consists of a central $(H3\text{-}H4)_2$ tetramer that is flanked on either side by two H2A-H2B dimers. Two fundamentally distinct processes affect chromatin structure during DNA replication (Figure 2). The first is the transient disruption of pre-existing nucleosomes that are located ahead of replication forks and their transfer onto nascent DNA, which is a reaction known as parental histone segregation. The second is the deposition of newly synthesized histones through a pathway known as replication-dependent de novo nucleosome assembly (Figure 2). Parental histone segregation and de novo assembly affect the whole genome during each passage through S phase. Therefore, these two processes potentially have a

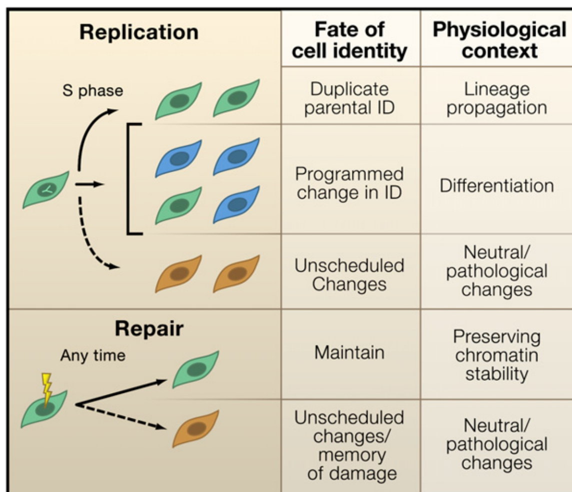

Figure 1. Replication- and Repair-Coupled Chromatin Dynamics and Cell Identity

The process of DNA replication is accompanied by genome-wide disruption and reassembly of chromatin. Depending on whether the parental chromatin organization is reproduced or reconfigured, the daughter cells either will be identical to their mother (lineage propagation) or will have changed their gene-expression profile (differentiation). Differentiation (change in "ID") can occur through either symmetrical or asymmetrical division, with the latter giving rise to two distinct cells (one potentially being epigenetically identical to the mother). Unscheduled chromatin changes can arise if cells fail to duplicate the parental epigenetic ID or to follow a differentiation program. This may have pathological consequences in terms of genetic stability and gene expression. The principal theme of chromatin disruption/restoration during DNA repair is similar. However, a major difference is that DNA damage may occur throughout the genome at any time during the life of the cell. Failure to restore chromatin organization following DNA repair may thus lead to unscheduled changes that potentially threaten epigenetic stability.

widespread and profound impact on the ability of proliferating cells to propagate or modify epigenetic states that depend upon specific chromatin structures.

Disruption of Parental Nucleosomes

Progression of the replication fork leads to disruption of parental nucleosomes ahead of the fork. Current evidence suggests that the nucleosome is disrupted into two parental H2A-H2B dimers and a $(H3\text{-}H4)_2$ tetramer (Figure 2). It remains an unsolved issue whether the force of the moving fork alone suffices to evict the parental histones or whether other factors are involved. However, by analogy to other chromatin-based processes (i.e., repair and transcription; see Review by B.E. Bernstein et al., page 669 of this issue), it can be hypothesized that nucleosome disruption is facilitated by ATP-dependent chromatin-remodeling enzymes and chaperones acting as histone acceptors. RNAi experiments in cultured mammalian cells indicate that chromatin remodelers are required for replication through chromatin. Depletion of WSTF, which forms a complex with the mammalian ISWI homolog SNF2h, reduces the rate of DNA replication throughout S phase (Poot et al., 2004). In contrast, another SNF2h subcomplex, ACF-SNF2h, appears to be particularly important for efficient replication through heterochromatin domains (Collins et al., 2002). However, the slow replication speed seen in these experiments could also reflect a function of the enzymes in chromatin formation behind the fork (Fyodorov et al., 2004). Thus while these complexes seem important during replication, an unresolved issue is how they operate at the fork; do they work on parental nucleosomes ahead or on nascent chromatin behind?

Histone chaperones can potentially facilitate disruption of parental nucleosomes by acting as histone acceptors and hence aid the transfer of the histones onto the daughter strands. Such a function may be critical to ensure recycling of parental histones, as opposed to their loss. FACT is an evolutionarily conserved H2A-H2B chaperone complex that facilitates progression of the RNA polymerase during transcription by mediating H2A-H2B transfer (Belotserkovskaya and Reinberg, 2004). Evidence from both in vitro studies in *Xenopus* and genetic studies in *S. cerevisiae* suggests a similar role for FACT in replication (Formosa, 2003). Importantly, FACT was recently identified in complex with MCM proteins in yeast and human cells (Gambus et al., 2006; Tan et al., 2006). The MCM complex is widely regarded as the helicase that unwinds DNA ahead of the DNA polymerase (Takahashi et al., 2005). This interaction with the replicative helicase places FACT in a key position to facilitate nucleosome disruption and potentially aid redeposition of H2A-H2B. An important unresolved question is how the parental tetramer $(H3\text{-}H4)_2$ is released and transferred. Is it a passive process or is it supervised by a chaperone?

Transfer of Parental Histones

Parental histones are transferred behind the replication fork onto either the leading or the lagging strand (Jackson, 1988; Sogo et al., 1986). This transfer occurs almost as soon as enough DNA has emerged from the replisome to allow the formation of nucleosomes (Sogo et al., 1986). The parental histones carry PTMs that in theory could serve as a blueprint for copying epigenetic information onto newly synthesized histones. If this is the case, the exact mechanism by which parental histones are reassembled onto nascent DNA likely has a major impact on the ability of cells to stably propagate PTM-based epigenetic information through DNA replication. Histone segregation during chromatin replication has been studied using cell-free DNA-replication systems and in vivo density-labeling techniques (see below). The consensus from these studies is that parental nucleosomes are disrupted into two H2A-H2B dimers and one $(H3\text{-}H4)_2$ tetramer. The latter is then transferred onto one of the nascent DNA strands to form a subnucleosome structure onto which either old or newly synthesized H2A-H2B dimers are added to complete the nucleosome. This transfer appears rapid and very efficient, as only extreme excess of naked DNA can compete with the daughter strands for parental histone binding during chromatin replication in

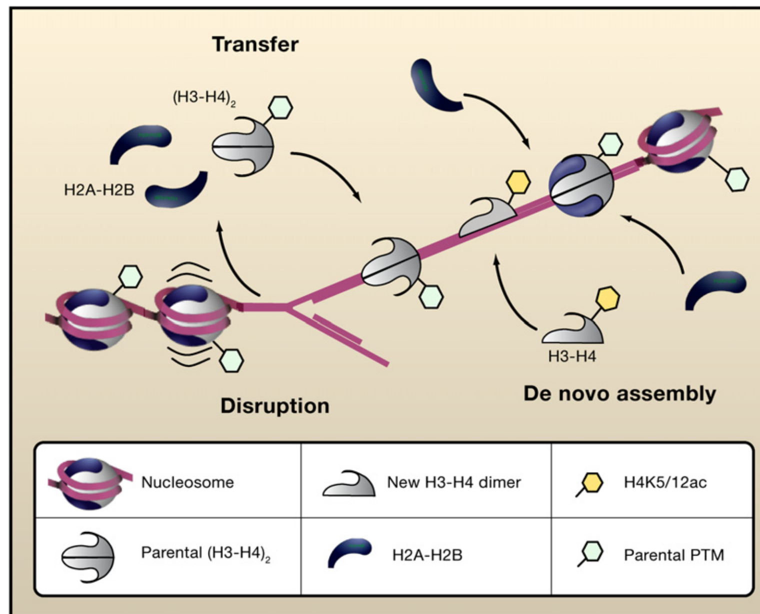

Figure 2. Chromatin Challenges at the Replication Fork
Packaging of DNA into chromatin presents a dual challenge at the replication fork. First, the DNA template has to be accessed, and, second, nucleosomal organization has to be reproduced on daughter strands. Ahead of the moving fork, parental nucleosomes are disrupted into H2A-H2B dimers and $(H3\text{-}H4)_2$ tetramers. The latter are transferred onto the daughter strands in a random fashion that may allow PTMs and histone variants to be maintained. This segregation mechanism operates together with de novo nucleosome assembly to fully reproduce nucleosomal density on daughter strands. Basic substrates for de novo deposition are H3-H4 dimers, which are diacetylated at H4K5/K12. A major question is how these three events—disruption, transfer, and de novo assembly—are coordinated to preserve genetic stability and to reproduce the epigenetic landscape, possibly by using the PTMs on parental histones as a blueprint for de novo-assembled nucleosomes.

cell-free systems (Gruss et al., 1993; Randall and Kelly, 1992). Throughout much of the genome, the $(H3\text{-}H4)_2$ tetramer is segregated as a stable entity, such that newly synthesized H3-H4 generally is not found within the same nucleosome as parental H3-H4 (Jackson, 1988; Prior et al., 1980; Figure 2). However, the available data do not exclude the possibility that the deliberate use of a different segregation mechanism during replication of specific regions of the genome might lead to dissociation of parental $(H3\text{-}H4)_2$ tetramers into two H3-H4 dimers (Tagami et al., 2004). The parental H3-H4 dimers would then associate with newly synthesized H3-H4 dimers brought by histone chaperones. In principle, the presence of both parental and newly synthesized H3-H4 molecules in the same nucleosome could facilitate the duplication of pre-existing PTMs onto the new histones (Tagami et al., 2004). Alternatively, segregation of parental histones may be coupled to de novo deposition in a manner that could ensure the duplication of PTMs onto newly synthesized histones. It is conceivable that cells may have evolved enzymes that discriminate parental and new H3-H4 molecules and simply copy the PTMs of parental $(H3\text{-}H4)_2$ tetramers onto the new histones. This could occur during transfer or after deposition. The discrimination may be possible either on the basis of the multiple lysine acetylations carried by newly synthesized histones (see below) or because new H3-H4 molecules are dimeric, whereas the parental histones $(H3\text{-}H4)_2$ may be tetrameric (see below). However, much work lies ahead before we can fully comprehend the histone segregation process and how it contributes to the propagation of histone variants and their PTMs.

De Novo Histone Deposition

The deposition of newly synthesized histones is critical to fully restore nucleosome density onto the two daughter strands. In *S. cerevisiae*, passage through S phase in the absence of core histone synthesis results in a loss of viability that cannot be rescued by re-expression of histones in G2 (Kim et al., 1988). Chromatin-Assembly Factor 1 (CAF-1) is an evolutionarily conserved three-subunit protein with the unique ability to preferentially deposit newly synthesized H3-H4 onto replicating DNA (Shibahara and Stillman, 1999). Consistent with this, human CAF-1 is found in a specific predeposition complex containing the major S phase histones H3.1 and H4 (Tagami et al., 2004). Notably, CAF-1 is not associated with the replacement histone variant H3.3, which is incorporated into chromatin independently of DNA replication. CAF-1 is targeted to sites of DNA synthesis that are associated with either replication or repair via a direct interaction with PCNA (Moggs et al., 2000; Shibahara and Stillman, 1999). PCNA is a ring-shaped homotrimeric protein that encircles DNA and serves as processivity factor for DNA polymerases. Interestingly, the interaction of CAF-1 with PCNA depends upon phosphorylation of the large subunit of CAF-1 (known as p150 in human cells) by Cdc7-Dbf4 (Gerard et al., 2006), a protein kinase that is essential for DNA replication. The p150 subunit of CAF-1 contains both the PCNA-binding motif and a dimerization domain (Gerard et al., 2006; Moggs et al., 2000). The phosphorylation of p150 by Cdc7-Dbf4 enhances CAF-1 binding to PCNA by disrupting the p150 dimer interface (Gerard et al., 2006). These findings raise the exciting possibility that the ability of CAF-1 to bind PCNA may be tightly regulated to ensure that H3-H4 deposition does not interfere with other important PCNA-dependent processes that constitutively occur behind replication forks. Alternatively, Cdc7-Dbf4 may coordinate CAF-1-mediated histone deposition with ongoing DNA replication. This would be consistent with the finding that histone deposition and uptake

by CAF-1 are immediately blocked upon treatment of human cells with replication inhibitors (Groth et al., 2005).

A conceptually important point is that newly synthesized H3-H4 dimers do not associate as tetramers prior to their deposition onto DNA (Polo and Almouzni, 2006). This conclusion was initially drawn from the fact that epitope-tagged H3.1 (replication-dependent variant) or H3.3 (replacement variant) did not copurify with non-tagged H3 when affinity-purified from the pool of soluble histones that are not incorporated into chromatin (Tagami et al., 2004). A similar finding was recently reported for soluble epitope-tagged histone H4 (Benson et al., 2006). An important concept that emerges from these studies is that the basic building blocks for de novo nucleosome assembly (during replication and repair) are H3-H4 dimers, rather than tetramers (Figure 2). Whether both dimers are delivered by CAF-1 is currently unclear. CAF-1-mediated histone deposition is aided by anti-silencing function 1 (Asf1), a highly conserved histone H3-H4 chaperone that has emerged in the limelight due to its multiple roles in histone dynamics. Asf1 can synergize with CAF-1 in repair- and replication-coupled nucleosome assembly in vitro (Mello et al., 2002; Tyler et al., 1999), although it alone cannot promote DNA-synthesis-dependent histone deposition. In proliferating human cells, the two human Asf1 isoforms (Asf1a and Asf1b) are the major chaperones controlling the flow of newly synthesized S phase histones (Groth et al., 2005). As such, CAF-1-mediated histone deposition may be fine-tuned both by the regulated binding to PCNA and by histone availability through Asf1. In yeast *S. cerevisiae* replication still occurs in the absence of both CAF-1 and Asf1 (Tyler et al., 1999), but the double mutants show severe defects in genome stability (Myung et al., 2003). An important point concerning Asf1 is that this chaperone binds H3-H4 in a manner that physically blocks tetramer formation (English et al., 2005, 2006; Mousson et al., 2005). NMR studies initially showed that Asf1 binds to the C-terminal helix of histone H3 (Mousson et al., 2005), which is a region of H3 that plays an important role in tetramer formation (Davey et al., 2002). Consistently, Asf1 was found to form a stable complex with dimeric H3-H4 when the three proteins were coexpressed in bacteria (English et al., 2005), and the crystal structure of this trimeric Asf1-H3-H4 complex has been reported recently (English et al., 2006). This structure reveals details of the Asf1-histone interaction that provide important insight into nucleosome assembly and disassembly processes. Asf1 envelops the C-terminal domain of histone H3 to occlude interaction with another H3-H4 dimer. Furthermore, Asf1 also contacts and causes a conformational change of the C terminus of histone H4, which otherwise interacts with H2A within the nucleosome. On this basis the authors suggest a "strand-capture" mechanism whereby Asf1 uses the H4 C-terminal tail to break-up $(H3\text{-}H4)_2$ tetramers during nucleosome disassembly. In yeast Asf1 has been suggested to facilitate histone eviction during transcription (Mousson et al., 2006), and it is thus crucial to determine whether this involves splitting of the parental tetramer into dimers by Asf1. If so, it is tempting to speculate that Asf1 could also facilitate occasional splitting of parental $(H3\text{-}H4)_2$ tetramers into dimers during replication to allow pairing with newly synthesized dimers.

Here we have discussed the three critical steps (disruption, transfer, and de novo deposition) necessary for duplication of nucleosome organization during replication. These steps should not be considered as independent events because disruption and transfer must be coordinated with de novo deposition as well as replication-fork progression in order to preserve both genetic and epigenetic stability. Elucidating how these different processes are orchestrated represents a major challenge for future research.

Duplicating PTMs and Epigenetic Domains

DNA methylation and histone PTMs are thought to provide a chromatin-based memory system, given that these marks can orchestrate formation and maintenance of epigenetic domains through recruitment of modifying enzymes and structural proteins. The major question is how and to what extent PTMs are maintained on parental nucleosomes during segregation and subsequently duplicated onto de novo-assembled nucleosomes. At least certain PTMs are maintained during segregation (Benson et al., 2006), which is consistent with the idea that PTMs on parental nucleosomes participate to reproduce the epigenetic state. Here we discuss how transfer of parental histones and chromatin factors, DNA methylation, and de novo nucleosome assembly may be coordinated to maintain epigenetic memory.

Replication-Coupled Memory

How and to what extent are epigenetic domains reproduced in a replication-coupled manner? Certain domains such as pericentric heterochromatin must be accurately duplicated to ensure proper chromosome segregation (Ekwall et al., 1997; Peters et al., 2001; Taddei et al., 2001). Hallmarks of these domains in mammalian cells are the presence of DNA methylation, hypoacetylated histones, histone H3 trimethylated at lysine 9 (H3K9me3), histone H4 trimethylated at lysine 20 (H4K20me3), and HP1 as well as the prevalence of the H3.1 histone variant (Loyola et al., 2006; Wallace and Orr-Weaver, 2005). The maintenance of these marks through faithful reproduction on daughter strands can represent a form of memory. Several "replication-coupled" mechanisms that can participate in the propagation of this silent state have now been discovered (see below). It is more questionable whether the transcriptionally active state is similarly duplicated. Replication of transcriptionally active loci may not necessarily entail duplication of active PTMs onto de novo-assembled nucleosomes. Rather, active marks on parental nucleosomes could suffice to maintain a permissive state such that new nucleosomes will acquire active marks when transcription resumes (Kouskouti and Talianidis, 2005). Indeed, enrichment of the replacement histone variant H3.3 in these regions is likely to rely on transcription-coupled

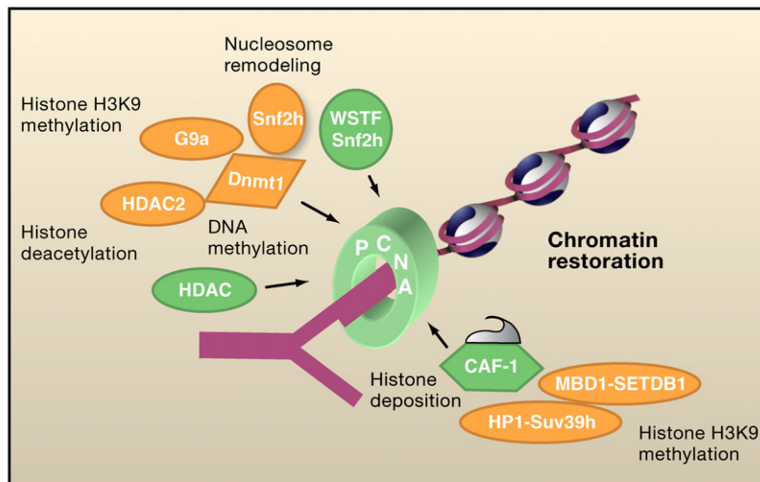

Figure 3. PCNA at the Fork: A Hub for Chromatin Restoration

PCNA forms a bridge between genetic and epigenetic inheritance through its dual role as a DNA polymerase processivity factor and as a platform for chromatin restoration. Here we illustrate how PCNA at the replication fork directs DNA methylation, de novo histone deposition, histone deacetylation, and nucleosome remodeling at the daughter strands by recruitment of multiple chromatin-modulating factors. Several of these factors can themselves bring in additional activities (i.e., CAF-1 and DNMT1). This may ensure coordinated propagation of epigenetic marks such DNA methylation and histone H3K9 methylation (DNMT1/G9a; Esteve et al., 2006) and a coupling between histone deposition and imposition of PTMs (CAF-1/SETDB1; Sarraf and Stancheva, 2004). The scheme represents factors recruited to the replication fork through PCNA (directly or indirectly). General chromatin maturation factors are shown in green, and factors operating in a locus-specific fashion are shown in orange.

propagation (Ahmad and Henikoff, 2002). A central issue in the maintenance of active loci during replication may be to prevent creation of repressive marks.

An emerging theme for replication-coupled memory is the use of PCNA as a hub that couples chromatin restoration to replication (Figure 3). PCNA can thus be viewed as a bridge between genetic and epigenetic inheritance (Zhang et al., 2000). High-resolution microscopy studies indicate that PCNA (Sporbert et al., 2002) and CAF-1 (Taddei et al., 1999), which itself uses PCNA as a landing pad, are retained on newly synthesized DNA for a period of 20 min (about 40 kb). This may well represent a window of opportunity for chromatin maturation. PCNA recruits a large number of chromatin-modulating enzymes to sites of DNA replication; these enzymes include DNMT1 (a maintenance DNA methyltransferase; Chuang et al., 1997; Leonhardt et al., 1992), CAF-1 (Krude, 1995; Shibahara and Stillman, 1999), HDACs (Milutinovic et al., 2002), and WSTF-SNF2h (Poot et al., 2004; Figure 3). Whereas some of these enzymes can be considered as general chromatin-maturation factors (i.e., CAF-1 and HDACs), others must operate in a domain-specific manner (i.e., DNMT1). This could be achieved through regulated recruitment or by modulation of enzymatic activity at the site. A major question concerning replication-coupled memory is how recruitment of these enzymes is orchestrated to allow formation of distinct chromatin domains. Given that nucleosomes present a barrier to enzymes operating on DNA, it seems likely that the actions of DNMT1, SNF2h-WSTF, and CAF-1 are coordinated to ensure efficient DNA methylation prior to chromatin maturation. DNMT1 re-establishes symmetrical CpG methylation on newly synthesized hemimethylated DNA (Chuang et al., 1997; Leonhardt et al., 1992). Incorporation of at least some imprinted DNA sequences into nucleosomes inhibits the ability of DNMT1 to methylate target CpGs (Okuwaki and Verreault, 2004). Thus, the physiological substrate of DNMT1 is unlikely to be DNA wrapped around fully mature nucleosomes.

An additional layer of complexity appears when considering that DNMT1 and CAF-1 themselves have the ability to recruit a number of enzymes implicated in chromatin maturation (Figure 3). DNMT1 interacts with HDACs (Fuks et al., 2000; Rountree et al., 2000), the ATP-dependent chromatin-remodeling enzyme SNF2h (Robertson et al., 2004), the polycomb protein EZH2 that directs H3K27me (Vire et al., 2006), and the H3K9 methyltransferase G9a (Esteve et al., 2006). This interplay likely ensures that replication-coupled propagation of DNA methylation coincides with formation of a repressive chromatin state. Together with G9a, DNMT1 forms a binary complex, which is required for recruitment of G9a to replication sites and for the maintenance of H3K9 methylation at epigenetically silenced rDNA repeats (Esteve et al., 2006). This binary memory module may thus be important for coordinating DNA methylation with H3K9 methylation at silenced euchromatic loci. In contrast to DNMT1, CAF-1 acts on replicated DNA throughout the genome to reproduce nucleosomal density. Nevertheless, several lines of evidence indicate that CAF-1 plays a role in setting up the repressed state (see below), illustrating that propagation of silenced chromatin is intimately linked to the histone deposition process. Consistently, loss of CAF-1 function causes heterochromatin abnormalities and loss of viability during development in mouse (Houlard et al., 2006), *Xenopus* (Quivy et al., 2001), and *Drosophila* (B. Klapholz and N. Dostatni, personal communication).

CAF-1 can integrate a series of activities necessary for propagation of H3K9 methylation. A replication-coupled memory mechanism that links DNA methylation and histone H3K9 methylation was recently uncovered (Reese

et al., 2003; Sarraf and Stancheva, 2004). A key finding was that MBD1, a protein that binds to methylated CpG dinucleotides, forms a replication-dependent complex with the H3K9 methyltransferase SETDB1 and CAF-1 (Sarraf and Stancheva, 2004). This complex is needed for heritable maintenance of H3K9 methylation and stable silencing at certain genes in proliferating cells. The authors proposed that the transient displacement of MBD1-SETDB1 from methylated DNA in front of the fork facilitates SETDB1-mediated K9 methylation of newly synthesized H3 deposited behind the fork by CAF-1. H3K9me3 is a bona fide repressive mark present in pericentric heterochromatin and stably silenced genes, where it forms a platform for HP1 (Feldman et al., 2006; Maison and Almouzni, 2004). In mouse cells, a significant fraction of MBD1 is concentrated in pericentric heterochromatin, where it binds to Suv39h1 and HP1 (Fujita et al., 2003). Thus, a similar mechanism may also be required for H3K9 methylation of new histones deposited by CAF-1 during heterochromatin replication. CAF-1 is also implicated in the delivery of HP1 to foci of heterochromatin replication. The largest subunit of CAF-1 directly binds to HP1 proteins (Murzina et al., 1999) and is needed to establish a replication-specific pool of HP1 molecules that can be incorporated during heterochromatin maturation (Quivy et al., 2004). HP1 molecules in these replication-specific structures could represent either pre-existing and/or newly synthesized HP1. As HP1 itself binds to the H3K9 methyltransferase Suv39h (Aagaard et al., 1999), this may be a complementary mechanism for propagation of the H3K9me3 mark (Figure 3). An attractive theory is that CAF-1, through interactions with chromatin-bound enzymes (i.e., Suv39h) released ahead of the replication fork, can facilitate modification of newly synthesized and deposited histones on daughter strands. Such a mechanism could ensure memory of the repressed state through CAF-1 without jeopardizing active domains that also require CAF-1-mediated de novo assembly. It will be important to understand whether the dual roles of CAF-1 in histone deposition and delivery of chromatin factors (i.e., HP1-Suv39h) can be separated. If so, what is then the balance of their contribution to the essential function of CAF-1 in higher eukaryotes?

PTMs on Newly Synthesized Histones

An important point concerning the mechanism of nucleosome assembly and epigenetic inheritance is when and how PTMs are imposed onto newly synthesized histones. The PTMs present on histones prior to their incorporation into chromatin may influence the final epigenetic state or be transient and removed during chromatin maturation. Here we discuss recent insight into the role of acetylation and methylation marks present on newly synthesized histones.

In a wide range of eukaryotic organisms, newly synthesized H3 and H4 are transiently acetylated at multiple lysine residues within their amino-terminal tails (Benson et al., 2006; Sobel et al., 1995). Mutations of multiple lysine residues that compromise the acetylation of both H3 and H4 lead to a loss of cell viability that is associated with severe defects in chromatin structure during passage through S phase (Ma et al., 1998). This underscores the importance of these acetylations in replication-coupled histone dynamics. The acetylation pattern on H3 varies between different species and even between the human histone H3 variants (Benson et al., 2006; Loyola et al., 2006; Sobel et al., 1995). This flexibility suggests that the charge of the H3 tail may be important for proper handling of H3-H4 dimers prior to assembly. In contrast acetylation of newly synthesized histone H4 at lysines 5 and 12 is highly conserved among species (Sobel et al., 1995). Consistently, mass-spectrometry analysis of soluble HeLa histone H3.1-H4 and H3.3-H4 dimers showed an almost exclusive enrichment of H4K5/K12 diacetylation (Loyola et al., 2006; Table 1). It has been speculated that the transient acetylation of histone tails may participate in nuclear import (Mosammaparast et al., 2002). However, at least for the H4 tail, replacement of the four acetylatable lysines (K5, K8, K12, and K16) by arginine residues does not impair nuclear import, which supports the argument that nuclear import does not require lysine acetylation (Glowczewski et al., 2004). In vertebrates, the acetylation of newly synthesized H4 at K5/K12 is catalyzed by a two-subunit enzyme known as HAT1-RbAp46 (Barman et al., 2006). This enzyme is dispensable for replication-coupled nucleosome assembly, but its absence confers sensitivity to drugs that damage DNA during replication (Barman et al., 2006). Thus, it is possible that the presence of acetylated histones on newly replicated DNA may create a favorable environment for repair and reactivation of stalled replication forks. However, the functional significance of the H4K5/K12 diacetyl mark still remains largely an enigma.

The H4K5/K12 diacetyl mark is transient since it is removed 20 to 60 min after replication (Taddei et al., 1999). It is likely that the transient presence of acetylated histones on newly synthesized DNA regulates the kinetics of nascent chromatin maturation (Annunziato and Seale, 1983) by providing a window of opportunity for histone-modifying enzymes to maintain or alter specific chromatin structures. The importance of this deacetylation event for maturation of pericentromeric heterochromatin is illustrated by experiments in fission yeast and mouse cells where treatment with HDAC inhibitors interferes with HP1 binding and proper chromosome segregation (Ekwall et al., 1997; Taddei et al., 2001).

In addition to N-terminal tail acetylation, newly synthesized histones are acetylated at lysine 56 of H3 and lysine 91 within the globular domain of H4 prior to their incorporation into chromatin (Masumoto et al., 2005; Ye et al., 2005; Zhou et al., 2006). These two residues are found in strategic locations within the nucleosome core particle, suggesting that their acetylation could regulate maturation of nascent chromatin and nucleosome stability. H4K91 lies within surfaces of interaction with H2A-H2B dimers and conspicuously close to the H4 interaction surface with Asf1 (English et al., 2006), whereas the side chains of H3K56 contribute to weak contacts with DNA at the

Table 1. PTMs on Soluble Non-Nucleosomal H3.1 and H3.3 in Proliferating Cells

Histone	Modification	Residue	Modification State	Non-Nucleosomal	
				H3.1	H3.3
H3	Methylation	K9	unmod	56%	52%
			me1	35.5%	17%
			me2	0.5%	4%
			me3	—	—
	Acetylation	K14	Ac	7%	20.5%
		K9/K14	diAc	0%	5%
H4	Acetylation	—	monoAc	18%	28%
		K5/K12	diAc	72%	50.5%

(—) Not detectable. Modified from Loyola et al. (2006).

entry and exit points of the nucleosome core (Davey et al., 2002). Mutations that abolish H3K56 or H4K91 acetylation cause hypersensitivity to genotoxic agents that interfere with DNA replication in a manner that is epistatic with mutation of the Asf1 histone chaperone (Celic et al., 2006; Recht et al., 2006; Ye et al., 2005). The fraction of new H4 molecules that are K91 acetylated in nascent chromatin has not been determined, but virtually all the new H3 molecules that are deposited throughout the genome in *S. cerevisiae* are K56 acetylated (Celic et al., 2006). Replication in the absence of this PTM on de novo-deposited histones leads to spontaneous DNA damage and chromosome rearrangements. Conceivably, the ubiquitous presence of acetylated histones behind replication forks enables cells to repair DNA lesions independently of the chromatin environment in which replication-blocking lesions occur.

The H3K56ac mark is transient similar to the tail acetylations, yet the deacetylation occurs with strikingly different kinetics. During a normal cell cycle, H3K56 is deacetylated in G2/M phase (Maas et al., 2006; Masumoto et al., 2005). The deacetylation requires Hst3 and Hst4, two proteins that are related to the NAD^+-dependent histone deacetylase Sir2 (Celic et al., 2006; Maas et al., 2006). Failure to deacetylate histone H3K56 severely compromises chromosome physiology. Cells lacking Hst3 and Hst4 are sensitive to genotoxic agents that impede replication-fork progression and exhibit a high incidence of mitotic chromosome loss and replication-linked spontaneous DNA damage. Thus, the acetylation of new histones is a double-edged sword. Its presence is needed for efficient repair of DNA lesions that block replication forks, but its continuous presence throughout the genome has even more disastrous consequences for the maintenance of genomic stability. It is thus not simply the presence or absence of these modifications that is important but rather the proper coordination of acetyl addition and removal as part of a regulated cycle that is essential. This has important implications for the use of histone deacetylase inhibitors in cancer chemotherapy (Dokmanovic and Marks, 2005). Chemicals that nonspecifically inhibit the enzymes that deacetylate new histones likely have undesirable effects, such as spontaneous DNA damage and chromosome rearrangements, in normal cells.

Compared to acetylation, histone methylation is generally a stable mark. Even though several histone demethylases have now been identified, histone lysine methylation has a low turnover rate and is most likely important for memory (Volkel and Angrand, 2007). A significant fraction of soluble dimeric histone H3.1 and H3.3 was recently found to carry specific methylation marks (Loyola et al., 2006; Table 1). This raises an important point concerning the mechanism of de novo nucleosome assembly and epigenetic inheritance; are histone PTMs imposed prior to (on soluble histone dimers) or after their incorporation into chromatin (on nucleosomal histones)? Analysis of PTMs on non-nucleosomal (predeposition) H3-H4 dimers compared with those found on $(H3\text{-}H4)_2$ in mononucleosomes supports the general view that most histone methylations are imposed after deposition (i.e., H4K4me, H3K27me, and H3K36me; Loyola et al., 2006). Yet, intriguingly, a significant fraction (36%) of soluble H3.1 was monomethylated at lysine 9, whereas soluble H3.3 in addition to K9me also presented significant amounts of K9me2 and K9Ac. Thus, some methylation marks can be established at a step prior to nucleosome assembly. Furthermore, since H3.1 and H3.3 are incorporated into chromatin via different pathways (Ahmad and Henikoff, 2002; Tagami et al., 2004), it can be suggested that the assembly line may determine the predeposition PTM signature. This initial PTM pattern may direct the activity of modifying enzymes acting at the nucleosomal level (i.e., Suv39h) and thereby influence the final PTM pattern. Curiously the Suv39h histone methyltransferase preferentially operates on H3 tails monomethylated at lysine 9 (Loyola et al., 2006). An important issue will be to resolve at what stage in the methylation process each of the enzymes are working, as mono-, di-, and trimethylation occur in separate steps during nucleosome assembly and chromatin maturation (Loyola et al., 2006). Given that several of these enzymes are present at the site of de novo histone deposition behind the replication fork (Figure 3), they could

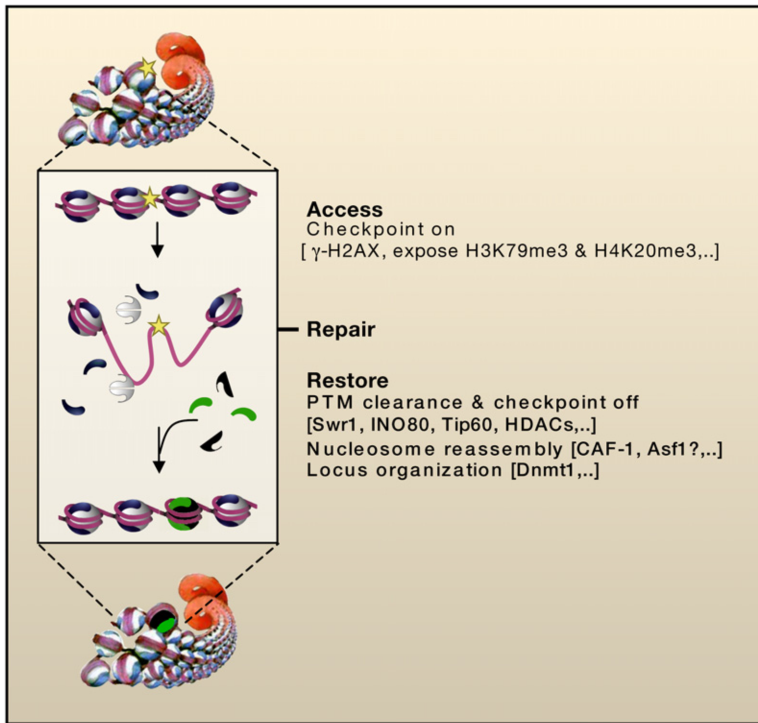

Figure 4. Resetting the Epigenetic Landscape after DNA Repair

Packaging of DNA into chromatin presents a dual challenge for DNA-repair processes. First, the lesion has to be made accessible for repair enzymes, and, second, chromatin organization has to be restored as described in the Access-Repair-Restore model (Green and Almouzni, 2002; Smerdon, 1991). During the repair process, chromatin surrounding the lesion is rearranged and modified to recruit checkpoint/repair factors. The restoration process can entail nucleosome reassembly through recycling or deposition of new histones, clearance of PTMs imposed during lesion detection and repair, and restoration of specific domains (i.e., through DNA methylation). Examples of factors involved in restoration are indicated (for details on the access process see van Attikum and Gasser, 2005; Wurtele and Verreault, 2006). The fidelity of the restoration process is not clear, and it is thus important to resolve whether genotoxic insults challenge epigenetic stability.

operate in a coordinated fashion to set up the final H3K9 methylation pattern.

Chromatin Restoration Coupled to DNA Repair

Coordination of DNA repair and chromatin dynamics is required to ensure maintenance of both genetic and epigenetic information in cells that experience DNA damage. To highlight that chromatin represents a challenge for DNA repair, Smerdon put forward the so-called "Access-Repair-Restore" model (Figure 4; Smerdon, 1991). During the access and repair processes nucleosomes are remodeled, extensively modified, and, in certain cases, even evicted (see below and Green and Almouzni, 2002; Peterson and Cote, 2004). In addition to these local changes, DNA damage can also lead to long-range effects on chromatin as illustrated in yeast, where relocalization of Ku and SIR proteins in response to double-strand breaks (DSBs) causes loss of telomeric silencing (Martin et al., 1999; McAinsh et al., 1999; Mills et al., 1999). PTMs imposed during lesion detection and repair may increase the plasticity of chromatin to facilitate repair and/or act as docking sites for repair and checkpoint proteins (van Attikum and Gasser, 2005; Wurtele and Verreault, 2006). Following successful completion of DNA repair the region must be cleared of these PTMs to restore pre-existing chromatin structure and turn off checkpoint signaling (Figure 4). Additionally, two other general restoration steps can be envisaged: (1) nucleosome reassembly involving either histone recycling or de novo deposition and (2) restoration of locus-specific organization. Here we discuss recent insights into mechanisms that operate to restore chromatin structure following DNA repair and highlight why epigenetic states may be challenged by genotoxic insults. If this is the case, epigenetic damage at postrepair sites may well contribute to chromosomal instability and aberrant gene expression in diseases, such as cancer, and aging.

As is the case for transcription, histone acetylation and ATP-dependent chromatin remodeling have been established, mainly through studies in yeast, as plausible mechanisms to open chromatin structure for the repair of both DSBs and UV-induced lesions (Peterson and Cote, 2004; van Attikum and Gasser, 2005; Wurtele and Verreault, 2006). For instance, histone-tail acetylation destabilizes the folding of chromatin into higher-order structures (Shogren-Knaak et al., 2006). Thus, at least in principle, chromatin opening to repair DNA has the potential to activate silenced genes (Yu et al., 2005). Interestingly, following the initial recruitment of histone acetylases (HATs), several histone deacetylases (HDACs) associate with chromatin near DSBs, and a clear decrease in histone acetylation takes place upon completion of repair (Tamburini and Tyler, 2005). HDACs could act to restore chromatin higher-order structure following DSB repair to prevent inappropriate gene activation. In addition, reduction of chromatin plasticity may be essential to inactivate the DNA-damage response by inhibiting further recruitment of checkpoint mediators that directly bind to constitutive histone marks, such as H3K79me3 and H4K20me3 (Huyen et al., 2004; Murr et al., 2006; Sanders et al., 2004).

One of the most intensively studied histone modifications associated with DSB repair is the phosphorylation of histone H2AX variants (H2AX in mammals, H2Av in *Drosophila*, and H2A in yeast). This phosphorylation, for

simplicity referred to as γ-H2AX, is induced very rapidly in large chromatin domains surrounding DSBs (van Attikum and Gasser, 2005). The significance of this PTM for repair and checkpoint signaling has been extensively reviewed elsewhere (van Attikum and Gasser, 2005; Wurtele and Verreault, 2006). Here we focus on recent studies highlighting the importance of histone dynamics in γ-H2AX maintenance and clearance. The emerging concept is that the timely removal of γ-H2AX from chromatin relies on a highly dynamic interplay between histone exchange and dephosphorylation of the evicted histone variant. In yeast the exchange is mediated by a complex interplay between the INO80 and SWR1 chromatin-remodeling complexes, likely in conjunction with the NuA4 HAT, all of which are recruited to the break partly through their binding to γ-H2AX itself (Bird et al., 2002; Downs et al., 2004; Morrison et al., 2004; Papamichos-Chronakis et al., 2006; van Attikum et al., 2004). The SWR1 complex replaces γ-H2AX with the H2AZ variant (Papamichos-Chronakis et al., 2006), which cannot be phosphorylated. This action is antagonized by the INO80 complex that maintains H2AX, such that it can continue to be phosphorylated at unrepaired DSBs (Papamichos-Chronakis et al., 2006). Although the exact purpose of this DSB-induced H2A-variant exchange is not known, it is likely important for DNA damage survival because loss of H2AZ and SWR1 function causes sensitivity to genotoxic agents (Downs et al., 2004). The human and *Drosophila* Tip60 complexes share many subunits with yeast INO80, SWR1, and NuA4 (van Attikum and Gasser, 2005), suggesting that the Tip60 complex is a hybrid of two or perhaps all three yeast complexes. The *Drosophila* Tip60 complex specifically acetylates nucleosomal phospho-H2Av and exchanges it with unmodified H2Av (Kusch et al., 2004). Given that H2Av shares similarity with both H2AZ and H2AX, the exchange resembles the situation in yeast, although it does not entail incorporation of a variant that cannot be phosphorylated. The mammalian Tip60 complex is likewise recruited to DSBs where it acetylates H4 and H2A (Murr et al., 2006), but it remains to be tested whether it also catalyzes γ-H2AX-H2B eviction. Following displacement from chromatin, soluble γ-H2AX-H2B is dephosphorylated by the HTP-C phosphatase complex in yeast (Keogh et al., 2006). Mutation of the phosphatase *Pph3* significantly delayed checkpoint recovery (Keogh et al., 2006), suggesting that the presence of soluble γ-H2AX influences checkpoint signaling, perhaps by being recycled. This highlights that persistence of certain PTMs on evicted histones may have hazardous consequences.

H3-H4 Dynamics at Repair Sites

Within chromatin the overall exchange of H2A-H2B is rapid in comparison to that of H3-H4 (Kimura and Cook, 2001). Consistently, most known epigenetic marks are carried on the $(H3\text{-}H4)_2$ tetramer. Thus, exchange of H3-H4 is likely to have a more dramatic impact on the epigenetic status of a chromatin region as compared to H2A-H2B exchange. A recent study showed that DSB repair in yeast results in nucleosome depletion through a region spanning at least 2 kb on either side of the break (Tsukuda et al., 2005). This extent of chromatin disruption will necessitate a reassembly event. The mechanistic basis of this event is important, as de novo histone deposition will challenge the epigenetic state of the cell, whereas histone recycling may not. It is now clear that new histone H3-H4 is incorporated during repair of UV lesions (Polo et al., 2006). Stable de novo incorporation of histone H3.1 was visualized at sites of local UV damage, and it was dependent on proficient nucleotide excision repair (NER) and CAF-1 function (Polo et al., 2006). This is rather surprising given that NER only involves relatively short patches of DNA synthesis (30 nucleosides). Nonetheless, these data establish that major rearrangements of chromatin occur in vivo during NER and directly prove a role for CAF-1 in H3.1 deposition at repair sites. This is consistent with the fact that CAF-1 mediates nucleosome assembly onto UV-damaged DNA in vitro and is recruited to sites of UV repair in vivo (Gaillard et al., 1996; Green and Almouzni, 2003). Several studies in yeast point out Asf1 as a potential player in chromatin rearrangements at repair sites (Mousson et al., 2006). Moreover, there is evidence in mammalian cells that Asf1 can be subject to checkpoint control through the Tousled-like kinases (Groth et al., 2003; Krause et al., 2003). It will thus be important to examine in general how Asf1 can contribute to DNA-damage responses, whether it acts at repair sites, and, if so, whether it functions in access, restoration, or both. CAF-1-mediated de novo histone deposition likely contributes to chromatin restoration following a wide range of repair processes, as it is directly recruited to sites of single-strand breaks and DSBs that are marked by γ-H2AX (Lan et al., 2004; Nabatiyan et al., 2006; Polo et al., 2006). These findings highlight the fact that the loss of pre-existing histones during DNA repair potentially represents an important threat to the maintenance of epigenetic information. However, it is not yet established whether DNA repair is a major source of epigenetic instability. As is the case during replication, it is possible that cells possess mechanisms that allow repair to take place with minimal loss of epigenetic information. The existence of such mechanisms was recently illustrated by the finding that DNMT1 is recruited via PCNA to sites of DNA-repair synthesis, presumably to ensure that the newly synthesized DNA is appropriately methylated (Mortusewicz et al., 2005). Even when pre-existing histones are evicted to repair cytotoxic lesions, cells may have mechanisms to restore the histone modifications that are characteristic of a given locus onto the newly deposited histones. For instance, the restoration of PTMs onto histones deposited during DNA repair could be achieved through a self-sustaining mechanism dependent on a neighboring nucleosome or transcription. It is also formally possible that cells could use an intact sister chromatid or even a homologous chromosome as a source of epigenetic information to restore histone modifications following repair at the damaged site.

An important issue is the fate of parental histones that are displaced from chromatin as result of DNA repair or other processes. In *S. cerevisiae*, the checkpoint kinase Rad53 controls degradation of excess histones (Gunjan and Verreault, 2003). This is particularly important to limit accumulation of newly synthesized histones, but it may also provide a mechanism to get rid of evicted parental histones. No histone-degradation pathways have been described so far in mammalian cells; however, the CUL4-DDB-ROC1 ubiquitin ligase recently was found to mediate UV-induced H3 and H4 ubiquitylation and facilitate nucleosome eviction (Wang et al., 2006). It will be interesting to know whether any of these ubiquityl marks target evicted histones for degradation. In *S. cerevisiae*, some of the H3 molecules bound to CAF-1 are K79 methylated (Zhou et al., 2006). Because 90% of nucleosomal H3 is K79 methylated (van Leeuwen et al., 2002), there is a possibility that the K79-methylated molecules bound to CAF-1 originated from pre-existing histones that were evicted from chromatin by DNA metabolic events. Histone recycling presents a potential epigenetic hazard because the histones evicted from chromatin carry with them the PTMs typical of the locus from which they were displaced. Through its ability to bind PCNA, CAF-1 could inappropriately deposit methylated histones during replication or repair. To avoid this, it seems likely that the pool of soluble histones has to be sanitized, either through degradation of displaced histones and/or reversal of modifications that should be absent from newly synthesized histones. Conceivably, some of the histone lysine demethylases that were recently uncovered could protect the epigenetic landscape by removing methylation from the pool of histones available for de novo nucleosome assembly.

This section has focused on the mechanisms implicated in re-establishing chromatin organization following DNA repair; however, it is possible that complete resetting does not take place. Indeed, it may be important to keep memory of the repaired region by marking it with specific postrepair chromatin features, as discussed in Polo et al. (2006). The γ-H2AX-dependent loading of cohesin (that mediates sister-chromatid cohesion) provides one example of a postrepair feature of the damaged region (Strom et al., 2004; Unal et al., 2004). In relation to histone exchange, the potential persistence of acetyl marks on de novo-assembled histones may function as a recognition mark. Alternatively, incorporation of specific histone variants (i.e., H3.1) may change the local H3.1/H3.3 pattern and thereby provide a tracer in certain quiescent cell types where H3.3 dominates (i.e., neurons). In any case, such memory of damage may contribute to phenomena, such as radiation genome instability, that arise in progeny of damaged cells after several generations (Little, 2003).

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Note Added in Proof

H3K56 acetylation is detectable by mass spectrometry in human cells, albeit at lower stoichiometry than in yeast (Garcia, B.A., Hake, S.B., Diaz, R.L., Kauer, M., Morris, S.A., Recht, J., Shabanowitz, J., Mishra, N., Strahl, B.D., Allis, C.D., et al. (2006). Organismal differences in post-translational modifications in histones H3 and H4. J. Biol. Chem., in press. Published online December 28, 2006. 10.1074/jbc.M607900200.).