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Propagating Epigenetic States During DNA Replication

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1. Introduction

The propagation of epigenetic states during DNA replication is critical for maintaining gene expression patterns across cell generations. Phenotypically diverse, but genetically identical, cells within a multicellular organism originate from a single cell, the zygote. During development, the cells derived from this zygote will divide and differentiate along multiple developmental pathways until reaching their final cell fates, with each cell-type expressing a different subset of their common genetic information. Differential gene expression in alternate cell-types in both unicellular and multi-cellular organisms is dependent, in part, upon post-translational modifications to DNA and histones. When the DNA itself is replicated, these post-translational modification patterns must also be replicated in order for epigenetic states to be inherited. The replication of epigenetic states involves many proteins acting in a concerted manner. Failure of one protein to act may have devastating effects on the cell and organism including loss of cell identity, inviability or disease. In this review, we will explore how multiple chromatin and gene expression states are transmitted epigenetically from mother cell to daughter cell during DNA replication and the contribution of replication factors to this process, taking examples from *S. cerevisiae*, *Drosophila* and mammals.

2. Nucleosomes as a minimal unit for carrying epigenetic information

The minimal repeating unit of chromatin in eukaryotes is the nucleosome, which is composed of approximately 147 bp of DNA wrapped 1.7 times around an octamer of histones containing two each of H2A, H2B, H3, and H4 (Luger et al., 1997). In chromatin, nucleosomes are separated from each other by differing lengths of linker DNA that can be bound by linker histones, such as H1 and H5 in mammals (Kornberg, 1977). This minimal, or "beads on a string", order of chromatin conformation can then be folded into several higher order structures containing numerous other chromatin-associated proteins and RNAs. Some of the most dramatic of these structures include centromeres, highly condensed metaphase chromosomes and Barr bodies in mammals. Information integral to epigenetic processes and written onto these nucleosomes is found in the form of post-translational modifications to histones and chemical modifications to DNA. These modification patterns are critical for regulating diverse cellular processes ranging from gene expression and DNA repair to chromatin compaction. When DNA is replicated to pass on

genetic information to daughter cells, these nucleosomal modifications must also be duplicated to ensure regulatory and structural information related to the accessibility of that genetic information is also inherited.

Distinct histone modification patterns are found on newly synthesized histones relative to parental histones, which display altered modifications reflecting their locus-specific functions. H4 found within newly synthesized histone H3.1-H4 dimers are diacetylated at K5 and K12 (Sobel et al., 1995, Loyola et al., 2006). This H4 modification pattern is initiated in the cytoplasm, is highly conserved across evolution and is mediated by the acetyltransferase HAT1 (Sobel et al., 1995, Parthun, 2007). However, once H4 is incorporated into chromatin, these marks are removed within ~20 min and other patterns are then created (Taddei et al., 1999). In budding yeast, acetylation of H4 K91 also occurs prior to incorporation into chromatin (Ye et al., 2005). H4 K91 lies at the site of interaction between H3/H4 dimers and H2A/H2B dimers (English et al., 2006). The role of this modification in regulating nucleosome formation is not yet understood. However, nucleosomes containing H4 K91A mutants are more easily digested by micrococcal nuclease (Ye et al., 2005), raising the possibility that this modification regulates nucleosome stability.

In contrast to H4, the modifications present on newly synthesized H3 vary somewhat across organisms. In *Drosophila*, H3 is primarily acetylated on H3 K9 and/or K23 (Sobel et al., 1995). In contrast, in mammals, newly synthesized H3 is monomethylated on K9 (Loyola et al., 2006) and may also be acetylated at K56 prior to assembly (Das et al., 2009, Xie et al., 2009). In *S. cerevisiae*, newly synthesized H3 is acetylated at numerous sites including K9, K27 and K56 (Masumoto et al., 2005, Kuo et al., 1996, Adkins et al., 2007, Burgess et al., 2010). These modifications may aid in distinguishing parental from newly synthesized histones and facilitate nucleosome assembly by the chromatin assembly machinery (See Sec. 3). Revision of the modification patterns on newly synthesized histones to match pre-existing locus-specific patterns must occur during or shortly after DNA replication. Such changes mediated by enzymes targeted to the replication fork or those loci ensure successful propagation of epigenetic states from mother to daughter cell.

3. Chromatin disassembly and assembly during DNA replication

3.1 Nucleosome organization and dynamics at the replication fork

During DNA replication, 4 ± 1 nucleosomes are transiently destabilized at the replication fork by the DNA replication machinery. These nucleosomes must be disassembled in front of the fork for DNA polymerase to gain access to its template and then, after replication, be reassembled behind the fork to repackage the newly synthesized DNA into chromatin. During SV40 replication *in vitro*, this process destabilizes a region of ~650-1100 bp of DNA involving approximately two nucleosomes in front of the replication fork and a short region behind the fork on the daughter strands (Gasser et al., 1996). In SV40 minichromosomes, 0 - ~380 bp of unpackaged DNA lies ahead of the branchpoint signifying the replication fork and ~260 - ~440 bp of unpackaged DNA is present in the daughter strands behind the fork. The region comprising the nucleosomes adjacent to either side of the fork likely lacks linker histones. The nucleosome immediately upstream of the fork is partially disassembled, and may exist as a H3/H4 tetramer. After replication, nucleosome assembly occurs on the daughter strands once the length of DNA needed to wrap around the histone octamer has passed through the replication machinery (Sogo et al., 1986, Gasser et al., 1996).

This chromatin disassembly and reassembly process during DNA replication requires the removal of nucleosomes containing parental histones and the assembly of both parental and newly synthesized histones into nucleosomes. Parental (pre-replicative) H3/H4 have been shown to be removed from in front of the replication fork as H3/H4 dimers and then reassembled together with other parental H3/H4 dimers to reform parental $(H3/H4)_2$ tetramers on both daughter strands through the use of density labeling of parental H3/H4 and sedimentation analysis (Jackson, 1990, Yamasu & Senshu, 1990, Gruss et al., 1993) or stable isotope labeling of "old" H3.1 variants and mass spectrometry (Xu et al., 2010). In contrast, H2A/H2B dimers appear to be removed from in front of the fork prior to H3/H4, and then, along with newly synthesized H2A/H2B dimers, to be randomly incorporated onto nucleosomes containing either parental or newly synthesized $(H3/H4)_2$ tetramers behind the fork. The assembly of the basic structure of chromatin is then completed by the loading of a linker histone, e.g. H1 (Jackson, 1990). This pattern of nucleosome disassembly and assembly during DNA replication implicates parental H3/H4, and the modifications on these histones, as being the direct and critical instructions for the reformation of preexisting epigenetic states after passage of a replication fork through a chromosomal locus. These patterns on the parental nucleosomes may act as a guide for generating similar patterns on adjacent nucleosomes that contain newly synthesized histones, but direct demonstration of how this occurs has yet to be accomplished. Other key instructions will be found on the parental DNA strands themselves in the form of methylated and hydroxymethylated cytosines (See Sec. 4.2).

3.2 Chromatin disassembly at the replication fork

Several proteins have been implicated in nucleosome disassembly in front of the replication fork, including FACT and Asf1p (Fig. 1). FACT, or Facilitator of Chromatin Transcription, consists of Spt16p and Pob3p in yeast and SPT6 and SSRP1 in mammals. FACT is best understood for its role in transcription elongation where FACT replaces H2A/H2B dimers upon passage of the transcription machinery. FACT can also bind H3/H4 through Spt16p to promote nucleosome disassembly and reassembly during both transcription elongation and DNA replication (Stuwe et al., 2008, Belotserkovskaya et al., 2003). FACT localizes to replication foci in mammals (Hertel et al., 1999) and is required for DNA replication in *Xenopus* egg extracts (Okuhara et al., 1999). FACT is thought to remove H2A/H2B from in front of the replication fork, thereby facilitating DNA replication. Consistent with this function, Spt16p of FACT interacts with the Mcm4p subunit of the replicative MCM helicase (Tan et al., 2006). FACT also co-purifies with DNA polα (Wittmeyer & Formosa, 1997) and promotes replication fork progression (Gambus et al., 2006). Also, Pob3p interacts with Rfa1p, a subunit of RPA that binds ssDNA during DNA replication (VanDemark et al., 2006). These interactions implicate FACT in promoting nucleosome disassembly and deposition, respectively, on either side of the replication fork.

Anti-Silencing Factor 1, Asf1p (mammalian Asf1a & Asf1b), is an evolutionarily conserved chromatin assembly factor that was discovered in a screen for genes, which when overexpressed, led to silencing defects in *S. cerevisiae* (Le et al., 1997). Asf1 binds H3/H4 dimers through a surface on H3/H4 that associates with a second H3/H4 dimer in the context of a nucleosome. This Asf1-H3/H4 interaction may prevent premature $(H3/H4)_2$ tetramer formation prior to nucleosome assembly (English et al., 2006, Tagami et al., 2004). Asf1p participates in chromatin assembly during both transcription and replication (Green

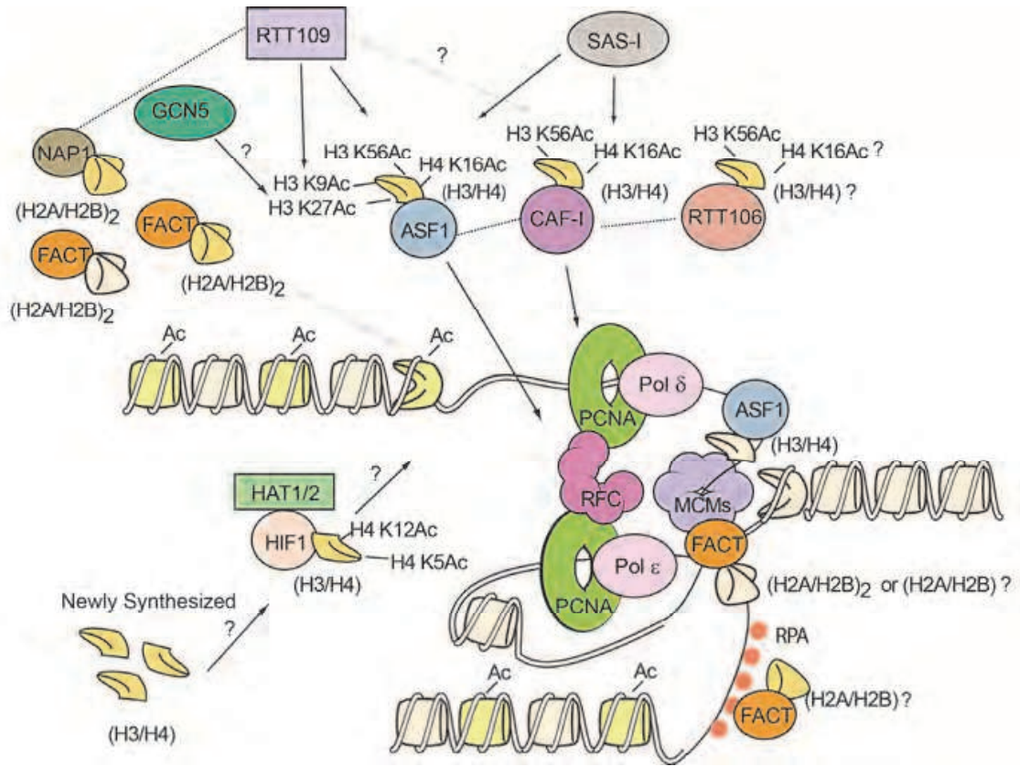

Fig. 1. Replication-coupled chromatin assembly in *S. cerevisiae*. Parental histones (beige) are removed from in front of the fork by assembly factors FACT (orange) and Asf1p (blue), which both interact directly or indirectly with the MCM helicase (blue-grey). FACT also binds to single stranded DNA binding protein Rpa1p of the RPA complex (coral). Whether FACT binds H2A/H2B dimers or tetramers is unclear. Newly synthesized H3/H4 dimers (yellow) are acetylated at H4 K5 and 12 by Hat1p in complex with Hat2p (dull green) and assembly factor Hif1p (peach). Rtt109p (light purple) binds to Asf1p bound to newly synthesized H3/H4 dimers (yellow) and acetylates H3 K56. If, where, and how newly synthesized H3/H4 are transferred from Hat1p/Hat2p/Hif1p to Asf1p or other assembly factors is unknown. CAF-1 (magenta) binds H3/H4 containing H3 K56ac, and possibly H3 K9ac and H4 K16ac, and interacts with Asf1p through the Cac2p subunit (dotted line). Assembly factor, Rtt106p (light red), binds newly synthesized H3/H4 containing H3 K56ac, and possibly H3 K9ac and H4 K16ac, and also interacts with CAF-1 through the Cac1p subunit. SAS-I (grey) associates with CAF-1 or Asf1p bound to H3/H4. Sas2p of SAS-I acetylates H4 K16. PCNA (bright green) tethers Polδ (light pink) and Polε (light pink) to the replication fork. PCNA is loaded onto DNA by RFC (dark pink). CAF-1 and Asf1p associate with the replication fork through interactions between Cac1p and PCNA and Asf1p and the Rfc2-4p subunits of RFC. Gcn5p (turquoise) and Rtt109p acetylate residues including H3 K9 and K27 and influence chromatin assembly during replication. Assembly of new and parental histones behind the fork is facilitated by FACT, and likely Nap1p (brown), which bind H2A/H2B, as well as CAF-1, Rtt106p, Asf1p, and possibly Hif1p, which bind H3/H4. Current models predict that Asf1p transfers H3/H4 dimers to CAF-1 for assembly. However, Asf1p may also directly assemble H3/H4 dimers. Question marks indicate where mechanisms are unclear.

et al., 2005, Sanematsu et al., 2006). Asf1p also functions in chromatin disassembly; Asf1p increases the rate of histone eviction at the yeast *PHO5* and *PHO8* promoters during transcription (Korber et al., 2006) and globally removes H3/H4 from chromatin (Adkins & Tyler, 2004). In *Drosophila*, Asf1 localizes to replication foci during S phase and depletion of Asf1 from *Drosophila* or mammalian cells results in delayed progression through S phase and inefficient DNA replication (Tyler et al., 1999, Groth et al., 2005, Sanematsu et al., 2006, Schulz & Tyler, 2006). These defects are related, in part, to a proposed role of Asf1p in facilitating nucleosome disassembly in front of the replication fork in conjunction with the FACT complex. Consistent with this model, Asf1 associates with the MCM helicase via H3/H4, and depletion of Asf1p slows DNA duplex unwinding by MCMs (Groth et al., 2007a, Groth et al., 2007b). Moreover, when fork progression is inhibited by treating cells with hydroxyurea, MCMs continue to unwind DNA and complexes of Asf1-H3/H4-MCMs accumulate. Consistent with disassembly, H3/H4 in these complexes carry marks associated with parental histones, H4 K16ac and H3 K9me3, rather than newly synthesized H3/H4 (Groth et al., 2007a). Other factors must also participate in disassembly of H3/H4 during replication as *ASF1* is not essential in *S. cerevisiae*.

3.3 Chromatin assembly at the replication fork

Several chromatin assembly factors have been linked to replication-coupled assembly behind the fork including Asf1p, CAF-1, Rtt106p and FACT (Fig. 1). In *Drosophila* and other organisms, the histone variant H3.1 is assembled into nucleosomes during replication–coupled chromatin assembly in S phase whereas the variant H3.3 is incorporated into chromatin throughout the cell cycle (Ahmad & Henikoff, 2002). The human Asf1 homologs, Asf1a and Asf1b, associate with both H3.1 and H3.3 (Tagami et al., 2004), consistent with their dual roles in transcription and replication-coupled chromatin assembly. In contrast, Chromatin Assembly Factor 1, CAF-1, comprised of p150, p60, and p48 in mammals and Cac1p, Cac2p, and Cac3p in *S. cerevisiae*, associates with newly synthesized H3/H4 (Kaufman et al., 1995) and mediates their incorporation into chromatin during DNA replication (Smith & Stillman, 1989, Verreault et al., 1996). In mammals, CAF-1 associates with H3.1, but not H3.3 (Tagami et al., 2004), and is required for progression through S phase (Hoek & Stillman, 2003, Ye et al., 2003), consistent with CAF-1 playing a critical role in assembly during DNA replication and repair (Smith & Stillman, 1989, Moggs et al., 2000, Kamakaka et al., 1996, Gaillard et al., 1996). CAF-1 is recruited to replication forks through binding of the Cac1p subunit of CAF-1 to PCNA (Zhang et al., 2000, Shibahara & Stillman, 1999). Cac1p contains a PCNA-binding motif and mutations in this region disrupt CAF-1-PCNA interactions in pull down experiments as well as result in silencing defects in budding yeast (Krawitz et al., 2002).

In *S. cerevisiae*, *Drosphila*, and human cells, Asf1p functions with CAF-1 to promote rapid nucleosome assembly during DNA replication and repair (Sharp et al., 2001, Tyler et al., 1999, Mello et al., 2002). However, CAF-1 does not enhance replication-independent histone deposition, implying CAF-1 may be exclusively involved in replication-coupled chromatin assembly (Sharp et al., 2001). Asf1p is proposed to transport H3/H4 dimers to CAF-1 for deposition onto DNA. Consistent with this model, CAF-1-dependent nucleosome assembly is stimulated by Asf1p. In the absence of Asf1p, H3/H4 are not readily transferred to CAF-1 (Tyler et al., 1999, Sharp et al., 2001, Mello et al., 2002, Groth et al., 2005). Asf1 interacts with CAF-1 via the Cac2p subunit (Krawitz et al., 2002, Mello et al., 2002, Tyler et al., 2001), and H3 mutants that do not bind to human Asf1 can associate with CAF-1 (Galvani et al., 2008). In

addition, Asf1p binds to Replication Factor-C, RFC, which loads PCNA onto DNA and this interaction may localize Asf1p behind the fork. RFC loaded onto nicked templates is sufficient to target Asf1p to DNA, and the Rfc2-5p subunits of RFC co-precipitate with Asf1p (Franco et al., 2005). *In vivo*, *asf1Δ rfc1-1* mutants exhibit synthetic growth defects and accumulate between S phase and the metaphase-to-anaphase transition. Similar slow growth phenotypes are not observed in *cac1Δ rfc1-1* mutants (Kaufman et al., 1998, Kaufman et al., 1997, Franco et al., 2005).

In *S. cerevisiae,* CAF-1 also interacts with the assembly factor Rtt106p through Cac1p (Huang et al., 2005), and Rtt106p and Asf1p co-purify from *in vivo* extracts (Lambert et al., 2010). Rtt106p binds H3/H4 dimers through PH domains, similar to Pob3p, and exhibits chromatin assembly activity *in vitro* (Huang et al., 2005, Li et al., 2008). Chromatin assembly by CAF-1 and Rtt106p is stimulated by H3 K56ac (Li et al., 2008), a modification catalyzed by the acetyltransferase Rtt109p while H3/H4 dimers are bound by Asf1p (Tsubota et al., 2007, Driscoll et al., 2007). Cells lacking CAF-1, Asf1p or Rtt106p exhibit defects in Sir-mediated silencing in *S. cerevisiae* (Huang et al., 2005, Huang et al., 2007, Kaufman et al., 1997, Tyler et al., 1999). Silencing defects are more severe in *cac1 rtt106*, and *asf1 cac1* mutants relative to single mutants, indicating that multiple assembly pathways impact epigenetically silenced chromatin (Huang et al., 2005, Tyler et al., 1999).

Several additional factors participate in nucleosome assembly during DNA replication, including FACT (and potentially Nap1p), the INO80 complex, and ACF1-SNF2H. As outlined above, FACT-RPA interactions via binding of Pob3p to Rfa1p may promote H2A/H2B deposition behind the replication fork (VanDemark et al., 2006), but the understanding of H2A/H2B disassembly and assembly during DNA replication lags behind that of H3/H4. Also, the ATP-dependent chromatin remodeler Ino80p localizes to origins of replication and replication forks during entry into S phase, is required continuously for fork progression under replication stress (Papamichos-Chronakis & Peterson, 2008) and functions in DNA repair (Morrison et al., 2004, van Attikum et al., 2004). How these proteins interact with other chromatin assembly factors during DNA replication remains to be explored. Currently, it is unclear whether certain factors are targeted to replication forks as they pass through some regions of the genome but not others, or the extent to which specialized factors to promote certain epigenetic processes. In support of some factors being critical for replication through epigenetically silenced loci, ACF1-SNF2H facilitates replication through heterochromatin in mammals (Collins et al., 2002). And, CAF-1 and Rtt106p contribute to the recruitment and spreading of Sirs in silent chromatin in budding yeast (Huang et al., 2007).

4. Propagation of chromatin modifications and epigenetic states

4.1 Histone modifications and replication factors in heterochromatin formation

The integrity of silent chromatin is influenced by the composition of nucleosomes at silenced loci as well as elsewhere throughout the genome. In budding yeast, transcriptionally active loci are enriched in acetylated histones and certain methylated forms of histones (e.g. methylated H3 K4 and H3 K36), whereas histones in silenced loci (rDNA locus, telomeres and silent mating-type loci *HML* and *HMR*) are hypoacetylated and hypomethylated (Bernstein et al., 2002, Braunstein et al., 1996, Katan-Khaykovich & Struhl, 2005, Rusche et al., 2002, Suka et al., 2001). Overexpression or loss of histone modifying enzymes often results in silencing defects. During silent chromatin formation, the Sirs, Sir1-4p, are

recruited to silencers flanking the *HM* loci. Sir2-4p then spread across *HMR* as the deacetylase Sir2p removes acetyl groups from H3 and H4 to facilitate nucleosomal binding by the Sirs. Once formed, silent chromatin is inherited efficiently as the genome is duplicated each S phase (Rusche et al., 2002, Hoppe et al., 2002) (see also Luo et al., 2002). Inappropriately modified histones can prevent Sirs from interacting stably with silent loci. This can occur either through disrupting Sir binding to nucleosomes or, upon global loss of histone modifications, through redistribution of Sirs to other genomic regions containing hypoacetylated and/or hypomethylated nucleosomes. Re-localization can deplete the pool of Sirs available for forming silent chromatin at appropriate loci as well as result in silent chromatin formation at inappropriate sites in the genome (Singer et al., 1998, van Leeuwen et al., 2002). Once formed, this "off target" silent chromatin can be propagated epigenetically during DNA replication.

Defects several factors involved in DNA replication and replication-coupled nucleosome assembly affect silencing, including Pol30p (PCNA), Rfc1p, Dna2p, Orc1p, Orc2p, Orc5p, Cdc7p, Cdc45p, Polε, Hif1p, CAF-1, Asf1p and Rtt106p (Axelrod & Rine, 1991, Foss et al., 1993, Kaufman et al., 1997, Loo et al., 1995, Smith et al., 1999, Zhang et al., 2000, Singer et al., 1998, Ehrenhofer-Murray et al., 1999, Huang et al., 2005, Poveda et al., 2004, Triolo & Sternglanz, 1996). These factors impact silent chromatin in multiple ways. For example, Orc1p binds Sir1p and facilitates Sir recruitment to silencers containing ARS elements adjacent to the *HM* loci (Gardner et al., 1999, Zhang et al., 2002, Rusche et al., 2002). Other ORC subunits also affect silencer function, but the role of ORC in silencing and replication initiation can be genetically separated (Dillin & Rine, 1997). The mechanisms by which some factors, including Cdc7p, Cdc45p and Polε, contribute to silencing have yet to be elucidated.

Silencing defects associated with CAF-1, Asf1p, PCNA, Rfc1p, Hif1p and Rtt106p, are linked to replication-coupled chromatin assembly and the misregulation of replication-coupled histone modifications (Fig. 1). Yeast *pol30* mutants with silencing defects have defects in CAF-1 and *ASF1*-dependent pathways (Zhang et al., 2000, Sharp et al., 2001). The silencing defects in *pol30, cac1*, *asf1* and *rfc1-1* mutants reflect, in part, misregulation of histone acetylation by SAS-I, Rtt109p and/or Gcn5p leading to hypoacetylation of at least H3 K9, H3 K56 and H4 K16 throughout the genome (Miller et al., 2010, Miller et al., 2008).

CAF-1 and Asf1p both bind to the H4 K16 acetyltransferase complex SAS-I (Meijsing & Ehrenhofer-Murray, 2001, Osada et al., 2001) and loss of the catalytic subunit of SAS-I, Sas2p, alters the chromosomal distribution of Sirs and results in silencing defects (Kimura et al., 2002, Meijsing and Ehrenhofer-Murray, 2001, Osada et al., 2001, Reifsnyder et al., 1996, Suka et al., 2002). Asf1p also binds the H3 K56 acetyltransferase Rtt109p, stimulates H3 K56ac *in vitro* and is required, along with *RTT109,* for H3 K56ac in S phase *in vivo* (Driscoll et al., 2007, Recht et al., 2006, Schneider et al., 2006, Tsubota et al., 2007, Han et al., 2007). Misregulation of H3 K56ac leads to silencing defects (Hyland et al., 2005, Miller et al., 2008, Xu et al., 2007, Sharp et al., 2001) and SAS-I and *rtt109* mutants have silencing phenotypes similar to those of *cac1*, *asf1* and *pol30* mutants (Ehrenhofer-Murray et al., 1999, Meijsing & Ehrenhofer-Murray, 2001, Miller et al., 2010, Miller et al., 2008, Osada et al., 2001). PCNA interacts with Rtt109p and SAS-I *in vivo*, but this interaction is lost in *pol30* mutants with defects in CAF-1- and Asf1p-dependent pathways (Miller et al., 2010), implying that acetylation of H3 K56 and H4 K16 are coupled to DNA replication. In addition, Asf1p binds the histone chaperone complex Hif1p/Hat1p/Hat2p in a Hat2p-dependent manner

(Fillingham et al., 2008). Cells lacking the chromatin assembly factor Hif1p or the acetyltransferase Hat1p have telomeric silencing defects, implicating this complex and Hat1p-dependent modifications in regulating silencing (Kelly et al., 2000, Poveda et al., 2004). Whether SAS-I, Rtt109p and/or Hat1p associate with Asf1p independently, simultaneously or sequentially, and how these complexes interact with Rtt106p and CAF-1 remains to be clarified.

The acetyltransferase Gcn5p has been proposed to facilitate replication-coupled chromatin assembly through modifying the N-terminal tail of H3. Consistent with this model, co-precipitation of H3 with the Cac2p subunit of CAF-1 is dramatically reduced in *gcn5* or H3 K5R mutants (Burgess et al., 2010). And, in *gcn5* or H3 K5R mutants, reduced levels of H3 containing modifications of newly synthesized histones, K9ac, K27ac and K56ac, are incorporated into chromatin adjacent to an early firing replication origin in cells arrested in early S phase (Burgess et al., 2010). *RTT109* and *GCN5*-dependent H3 K9ac (Fillingham et al., 2008, Adkins et al., 2007, Berndsen et al., 2008, Kuo et al., 1996) is also defective in *po30* mutants (Miller et al., 2010). This loss of H3 K9ac is consistent with loss of interactions between pol30p mutants and Rtt109p (Miller et al., 2010), but could also indicate the activity of Gcn5p during chromatin assembly was compromised in *pol30* mutants.

PCNA localizes numerous factors to the replication fork to propagate epigenetic states in mammals as well (Fig. 2). PCNA binds the maintenance DNA methyltransferase Dnmt1 (Chuang et al., 1997, Iida et al., 2002) (See Sec. 4.2). PCNA also recruits CAF-1 to DNA and promotes CAF-dependent chromatin assembly *in vitro* (Moggs et al., 2000, Shibahara & Stillman, 1999). MBD1, a methyl CpG binding protein and SETDB1, a H3 K9 methyltransferase, are, in turn, targeted to replication foci by CAF-1 and together with 5-mC DNA, MBD1 and SETDB1 promote stable heterochromatin formation (Sarraf & Stancheva, 2004, Moldovan et al., 2007). In addition, a mammalian H4 K20 methyltransferase, SET8, binds PCNA and co-localizes with PCNA at replication foci *in vivo* (Huen et al., 2008). Monomethylation of H4 K20 by SET8 is important for progression through S phase (Huen et al., 2008) and methylated H4 K20 is enriched in heterochromatic regions in multiple species (e.g. Schotta et al., 2004). In *Drosophila*, mutants of *mus209,* a PCNA ortholog, also suppress position-effect variegation (Henderson et al., 1994), but why this occurs is unclear.

Another illustrative example of silent chromatin formation can be found during development in *Drosophila*. Polycomb group (PcG) proteins maintain transcriptional repression patterns of the homeotic (*Hox*) genes, which control segmental identities and body patterning. PcG proteins are recruited to *cis*-acting PcG response elements, PRE, at target loci. There, Polycomb Repressive Complexes 1 and 2, PRC1 and PRC2, establish silent chromatin, which is then propagated over multiple cell generations to maintain *Hox* genes in a silenced state during development. Variants of PRC2 complexes contain a H3 K27 methyltransferase Ezh2, ESC, Suz12 and the histone binding protein Nurf55 (p55 of dCAF-1). The Polycomb, Pc, subunit of PRC1 can bind H3 K27me3, implying the catalytic function of PRC2 reinforces the association of PRC1 with chromatin (Min et al., 2003, Fischle et al., 2003b, Francis, 2009). Consistent with chromatin structure containing PcG proteins being inherited during DNA replication as opposed to re-established *de novo* after replication, recent studies using an *in vitro* SV40 replication system have demonstrated PRC1 remains associated with both naked DNA and chromatin templates upon DNA replication (Francis et al., 2009), implying PRC1 was passed from in front of to behind the fork along with parental H3/H4.

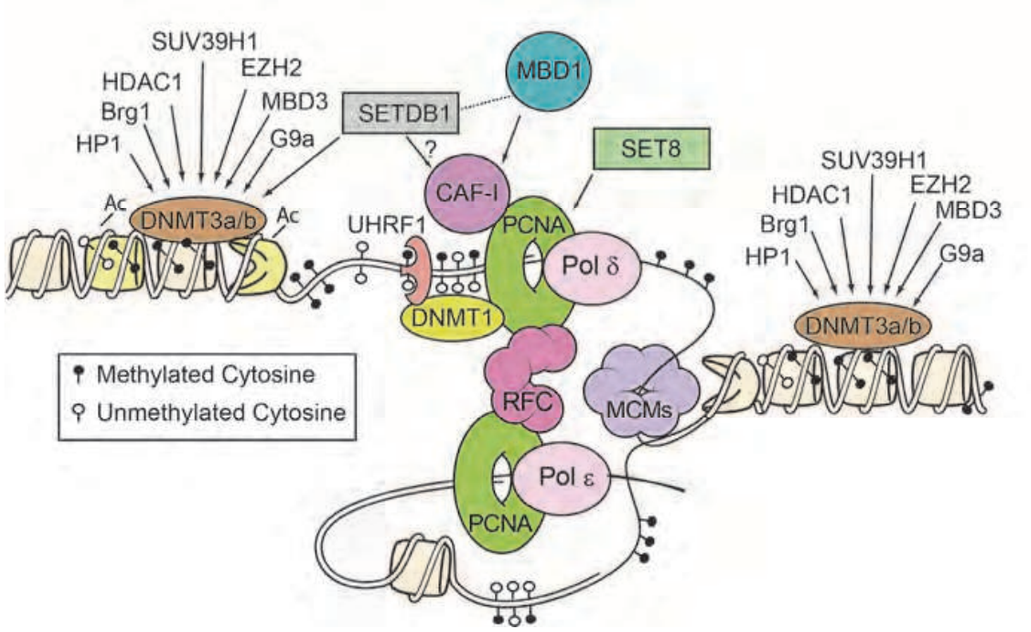

Fig. 2. Propagation of DNA methylation. Hemimethylated cytosines on newly synthesized DNA are recognized by UHRF1 (pink). The unmethylated daughter strand is methylated by the maintenance methyltransferase, Dnmt1, (yellow) which is localized to the replication fork through interactions with PCNA (bright green) and UHRF1. Dnmt3a/3b (brown) are are *de novo* methyltransferases that localize to nucleosome-bound DNA and also interact with UHRF1 (not shown). Factors interacting with Dnmt3a/3b, include heterochromatin protein HP1, chromatin remodeler Brg1, histone deacetylase HDAC1, H3 K9 methyltransferases SUV39H1 and G9a, H3 K27 methyltransferase EZH2 and methyl DNA binding protein MBD3. The H3 K9 methyltransferase SETDB1 (grey) also interacts with Dnmt3a/3b and is localized to the replication fork through the chromatin assembly factor CAF-1 (magenta). CAF-1, which binds PCNA, also recruits the methyl DNA binding protein MBD1 (teal) that directly interacts with SETDB1. The mechanism for interaction between SETDB1 and CAF-1 is unclear and denoted by the question mark. The H4 K20 methyltransferase SET8 (dark green) is also targeted to the replication fork through interactions with PCNA.

4.2 Insights into mechanisms of epigenetic inheritance: DNA methylation

DNA methylation plays an important role in epigenetic processes during development by impacting a range of biological functions including gene expression, genome integrity, imprinting, and aging, as well as by contributing to diseases ranging from neuronal defects to cancer when misregulated. 5-methylcytosine, 5-mC, constitutes ~2-8% of the cytosines in human genomic DNA and occurs primarily within CpG dinucleotides, although non-CpG methylation can also occur (Gowher and Jeltsch, 2001, Ramsahoye et al., 2000). Maintaining the average methylation state of a locus during DNA replication, rather than the individual sites of DNA methylation is generally more important for maintaining the proper function, or expression state, of that locus. Consistent with this model, different regions of the genome tend to be either hypo- or hypermethylated. And, in methylated regions, slight variations in methylation patterns are commonly found at individual loci in both cell lines and tissues (Meissner et al., 2008, Zhang et al., 2009). Methylation events contributing to

these varied patterns have been quantified for the CpG island at the *FRM1* locus. At *FRM1*, the fidelity of maintenance methylation is ~0.96 and the probability of *de novo* methylation events having occurred per site per round of replication is ~0.17 (Laird et al., 2004). Thus, DNA methylation occurs stochastically (Riggs & Xiong, 2004) and can vary slightly from cell to cell without altering an epigenetic state.

DNA methylation is mediated by a family of DNA methyltransferases, Dnmts, which are classified as *de novo* (Dnmt3a/3b) or maintenance (Dnmt1) methyltransferases, according their primary role in establishing new methylation patterns or copying existing patterns onto newly synthesized DNA upon DNA replication. While methylating cytosines, Dnmts flip the target base out of the DNA helix and into a hydrophobic pocket to catalyze the transfer of the methyl group from S-adenosyl-L-methionine to the C5 position of cytosine to create 5-mC plus S-adenosyl-L-homocysteine, but their specificity for unmethylated versus hemimethylated DNA varies. The *de novo* methyltransferases Dnmt3a/3b readily methylate both unmethylated and hemimethylated DNA (Okano et al., 1998, Gowher & Jeltsch, 2001) and are critical for establishing proper DNA methylation patterns in early development in mammals (Okano et al., 1999). Dnmt3a/3b also help maintain DNA methylation within heterochromatin (Chen et al., 2003, Jeong et al., 2009, Liang et al., 2002). Dnmt3a/3b can interact with an additional Dnmt family member, Dnmt3L, which stimulates their catalytic activity *in vitro* and *in vivo,* despite Dnmt3L itself being catalytically inactive (Chedin et al., 2002, Chen et al., 2005, Gowher et al., 2005). In this role, Dnmt3L acts as a regulatory factor and is critical for proper methylation of imprinted genes and male germ cell development (Bourc'his et al., 2001, Hata et al., 2002, Webster et al., 2005).

Dnmt3a/3b also interact with several proteins to promote silenced epigenetic states (Fig. 2). Dnmt3a/3b binding partners include the histone deacetylase HDAC1 (Fuks et al., 2001), the histone methyltransferases SUV39H1 (Fuks et al., 2003), SETDB1 (Li et al., 2006), G9a (Epsztejn-Litman et al., 2008, Feldman et al., 2006) and EZH2 (Vire et al., 2006), the heterochromatin protein HP1 (Fuks et al., 2003, Smallwood et al., 2007), the 5-mC binding protein MBD3 and the chromatin remodeling factor Brg1 (Datta et al., 2005). These interactions all contribute to silent chromatin formation. For example, the H3 K9 methyltransferase G9a facilitates *de novo* DNA methylation and gene inactivation through recruiting Dnmt3a/3b and HP1 to multiple early embryonic genes to drive heterochromatin formation (Epsztejn-Litman et al., 2008, Feldman et al., 2006). Similarly, the H3 K27 methyltransferase Ezh2 recruits Dnmt3a/3b to chromosomal loci to promote DNA methylation and heterochromatin formation (Vire et al., 2006). Once formed, this heterochromatin and the associated modification patterns will be propagated epigenetically during DNA replication.

In addition to interacting with chromatin-modifying enzymes and structural components of heterochromatin, Dnmt3a/3b binds histones to promote DNA methylation. Dnmt3a/3b bind to the N-terminal tail of H3 lacking methylated K4 (Otani et al., 2009, Zhang et al., 2010, Ooi et al., 2007) and DNA methylation tends to be low at active promoters, which are enriched for H3 K4me3 (Hodges et al., 2009, Zhang et al., 2009). Dnmt3a/3b also preferentially bind H3 K36me3 and enhances DNA methylation of a nucleosomal substrate by Dnmt3a (Zhang et al., 2010). Like H3 K36me3, DNA methylation is enriched in bodies of active genes, especially in exons (Weber et al., 2007, Hodges et al., 2009, Kolasinska-Zwierz et al., 2009). Thus, Dnmt3a/3b-H3 interactions contribute to genome-wide chromatin modification patterns in transcriptionally active regions as well.

DNA methylation patterns established during development must be faithfully propagated throughout the lifespan of an organism via maintenance methylation during DNA

replication. The maintenance methyltransferase Dnmt1 localizes to replication foci in S phase (Leonhardt et al., 1992) and associates with the replication fork (Easwaran et al., 2004). Dnmt is loaded onto DNA through transient interactions with PCNA and binding to PCNA promotes the activity of Dnmt1 (Chuang et al., 1997, Iida et al., 2002, Schermelleh et al., 2007). By preferentially methylating hemimethylated DNA over unmethylated DNA (Fatemi et al., 2001, Goyal et al., 2006), Dnmt1 copies parental DNA methylation patterns onto newly synthesized daughter strands (Fig. 2). *In vitro,* Dnmt1 and PCNA form a complex with HDAC1, and Dnmt1/PCNA/HDAC1 co-localize *in vivo* (Chuang et al., 1997, Fuks et al., 2000, Milutinovic et al., 2002).

Dnmt1 also associates with newly synthesized DNA through binding UHRF1. UHRF1 is a multifunctional protein that reads hemimethylated DNA and histone modifications to facilitate inheritance of epigenetic states. UHRF1 targets Dnmt1 to newly synthesized DNA by specifically binding hemimethylated DNA (Arita et al., 2008, Avvakumov et al., 2008, Bostick et al., 2007, Hashimoto et al., 2008). UHRF1 co-localizes with PCNA and Dnmt1 at replicating heterochromatin and is required for maintaining DNA methylation in mammals (Bostick et al., 2007, Sharif et al., 2007). Disruption of Dnmt1/PCNA/UHRF1 interactions leads to global DNA hypomethylation and promotes tumor formation (Hervouet et al., 2010).

UHRF1 also binds H3 K9me2 and H3 K9me3, through a PHD domain. A second domain in UHRF1, SRA, also contributes to binding affinity (Karagianni et al., 2008). In addition, this SRA domain facilitates binding 5-mC DNA (Arita et al., 2008, Avvakumov et al., 2008, Hashimoto et al., 2008) and both domains are required to localize UHRF1 to pericentric heterochromatin. Consistent with H3 K9 methylation reinforcing DNA methylation during replication, localization of UHRF1 is reduced in cells overexpressing the H3 K9 demethylase JMJD2A (Karagianni et al., 2008). UHRF1 plays a second role in epigenetic processes by influencing histone modification states. UHRF1 contains a C3HC4 RING finger motif and acts as an E3 ubiquitin ligase targeting H3 *in vitro* and *in vivo.* This ubiquitination activity is important for maintaining higher order chromatin structure *in vivo* (Citterio et al., 2004, Karagianni et al., 2008). UHRF1/Dnmt1 also form a complex with the deacetylase HDAC1 and the H2A K5 acetyltransferase Tip60. Depletion of UHRF1 results in hypoacetylation of H2A K5 (Achour et al., 2009). Analyses how Dnmt1/PCNA/UHRF1 and UHRF1-hemimethylated DNA interactions regulate these histone modifications should clarify the extent to which deacetylation by HDAC1, ubiquitination by UHRF1 and acetylation of H2A K5 by TIP60 are coupled to DNA replication.

Although Dnmt1 and Dnmt3a/3b display preferences for different substrates, maintenance methyltransferases also participate in *de novo* methylation and *de novo* methyltransferases function in maintaining methylation patterns. Dnmt1 plays a secondary role in *de novo* DNA methylation at unmethylated loci through the conversion of hemimethylated DNA created by Dnmt3a/3b to fully methylated DNA (Fatemi et al., 2002, Feltus et al., 2003). Likewise, Dnmt3a/3b facilitate maintaining DNA methylation states upon DNA replication, particularly in chromosomal regions that are highly methylated or repetitive (Jones & Liang, 2009). Consistent with this, proliferating mouse embryonic stem cells lacking Dnmt3a/3b lose DNA methylation over time, despite the continued presence of Dnmt1 (Chen et al., 2003, Liang et al., 2002). Thus, although necessary, Dnmt1 alone is insufficient to maintain normal methylation levels on newly replicated DNA. Dnmt3a/3b likely aid in maintenance methylation through interacting directly with UHFR1 (Meilinger et al., 2009) as well as through catalyzing *de novo* methylation events within highly methylated regions, especially

in heterochromatic regions (Jeong et al., 2009). Thus, crosstalk between replication factors and chromatin modifying machinery reinforces propagation of DNA methylation, histone modifications and structural components of silent chromatin to maintain epigenetic states.

5. Switching of epigenetic states

5.1 Establishment, maintenance and inheritance of epigenetic states

Extensive reprogramming of epigenetic states occurs during primordial germ cell development, in mammalian early embryonic development and upon cell-type differentiation throughout an individual's lifespan (Sasaki & Matsui, 2008). Concepts for understanding the formation and stability of silent chromatin have been developed from the analysis of repression in the bacteriophage lambda (Ptashne, 1992). Studies in lambda showed a protein could facilitate the initial inactivation of a target gene, but once repressed, the maintenance of that the gene in its inactive state no longer required that protein. During silent chromatin formation, such proteins are said to be important for establishing, but not maintaining, the silenced state. In contrast, proteins that are required constantly to keep a repressed gene inactive are considered to be necessary for maintaining the silenced state. Proteins that are involved in the inheritance of silenced states facilitate the propagation of that state to subsequent cell generations.

An example of a protein important for establishing, but not maintaining, epigenetic states is Sir1p from budding yeast. Sir1p facilitates establishment by increasing the probability of Sir proteins being recruited to the *HM* loci (Rusche et al., 2002). Cells lacking *SIR1* can exist in two populations; transcriptionally active or silenced. Each population is stable over multiple generations, indicating that Sir1p is not required for maintaining or inheriting the different expression states (Pillus & Rine, 1989, Xu et al., 2006). These silenced and transcriptionally active cells will occasionally switch states, and often these switching events demonstrate a "grandmother effect". In these instances, all progeny ("granddaughters") derived from a single derepressed "grandmother" cell from two generations earlier will switch to a silenced state simultaneously, raising the possibility that an event linked to DNA replication in the grandmother was propagated to subsequent generations (Pillus & Rine, 1989). When mother and daughter cells switch epigenetic states simultaneously upon cell division, the daughter cell usually silences more rapidly than the mother. In instances where the switching event does not occur in both cells of a mother/daughter pair, the daughter cell is more likely to switch to a silenced state than the mother (Osborne et al., 2011). The mechanism behind this difference is unknown but could be linked to asymmetric inheritance of soluble proteins (e.g. Sirs) or the sister chromatids during cell division. Alternatively, asymmetric expression of proteins that inhibit (in the mother cell) or promote (in the daughter cell) silent chromatin formation could also contribute to this process (Osborne et al., 2011).

5.2 Switching histone modification patterns

Switching of histone modification patterns to promote different epigenetic states can occur several ways. Histone modifications can be actively removed by enzymes such as deacetylases or demethylases, exchange of histones via chromatin remodeling (Lu et al., 2009), or proteolytic cleavage of histone tails (Jenuwein & Allis, 2001, Bannister et al., 2002). Histone modifications at chromosomal loci can also be passively removed by dilution upon DNA replication. Alternatively, certain histone modifications can remain present at loci in cells with different epigenetic states, but the function of those modifications may be altered

through adding or removing modifications at neighboring residues (Bannister et al., 2002, Fischle et al., 2003a). Together, these changes can affect chromatin structure by altering interactions between histones and DNA or chromatin-associated proteins that bind nucleosomes, ultimately leading to switching of epigenetic states.

Several examples of histone modifications influencing the probability of establishing silencing have been observed in studies of yeast mating-type silencing. In the absence of the H4 K16 acetyltransferase Sas2p, the probability of establishing silencing in a given cell cycle decreases, whereas loss of the H3 K4 and H3 K79 methyltransferases encoded by *SET1* and *DOT1* increases the probability of establishment (Osborne et al., 2009). In the case of *DOT1*, H3 K79 methylation status has been demonstrated to influence this switching event (Osborne et al., 2011). The rate and mechanism of removal of different histone modifications can also vary during establishment. At *HMR* and telomeres, acetyl groups from lysine residues on the N terminal tail of H3 are rapidly removed by Sir2p, a structural component of silent chromatin. The rapid rate of loss of H3 K4me2 during silent chromatin formation also implicates an active process mediating removal of this mark. In contrast, H3 K79me2 appears to be removed passively via dilution during DNA replication over 3 to 5 cell generations (Katan-Khaykovich & Struhl, 2005).

5.3 Switching DNA methylation patterns

In mammals, high 5-mC levels are commonly found in transcriptionally silent loci and promote the formation and maintenance of heterochromatin. In contrast, loss of 5-mC can disrupt binding sites for methyl binding proteins, destabilize heterochromatin, and lead to gene reactivation. Like histone modifications, DNA methylation can be removed by both passive and active processes. Passive demethylation of the maternal genome during pre-implantation development is thought to reflect loss of 5-mC via dilution upon DNA replication (Howell et al., 2001, Rougier et al., 1998). 5-mC can also be actively removed by DNA glycosylases in plants and base excision repair and nucleotide excision repair have been implicated in DNA demethylation in multiple organisms (Chen & Riggs, 2011, Zhu, 2009).

Passive and active removal of DNA methylation and gene reactivation may also involve 5-hydroxymethylcytosine, 5-hmC, a modification recently identified in mammalian DNA (Tahiliani et al., 2009, Kriaucionis & Heintz, 2009, Munzel et al., 2010). 5-hmC is created through the conversion of 5-mC to 5-hmC by the oxygenase TET1 (Tahiliani et al., 2009). TET1 is a homolog of the trypanosome proteins JBP1 and JBP2 that oxidize 5-methyl thymine (Tahiliani et al., 2009). Two other mammalian TET family members, TET2 and TET3, are also predicted to convert 5-mC to 5-hmC. Passive demethylation via dilution during DNA replication may occur upon conversion of 5-mC to 5-hmC if Dnmt1 cannot use 5-hmC-containing DNA as a substrate, although evidence for this remains to be established. Alternatively, 5-hmC may function in replication-independent, or "active", demethylation through spontaneous conversion to cytosine or processing via base-excision repair, analogous to that catalyzed by DEMETER in plants during the removal of 5-mC (Gehring & Henikoff, 2007). Rapid demethylation of the paternal genome following fertilization involves conversion of 5-mC to 5-hmC, but how 5-hmC is later processed is unclear (Iqbal et al., 2011, Santos et al., 2002).

Currently, the genomic locations of 5-hmC are largely unknown, although 5-hmC is likely less prevalent than 5-mC in most cell types. 5-hmC is indistinguishable from 5-mC in bisulfite sequencing studies used to map genomic methylated CpGs sites as bisulfite

treatment converts 5-hmC into cytosine 5-methylenesulfonate, which is resistant to deamination and conversion to uracil (Huang et al., 2010). Thus, many chromosomal sites previously thought to contain 5-mC may actually contain 5-hmC. Identification of genomic regions enriched for 5-hmC will require alternative approaches such as chromatin immunoprecipitation coupled to next generation sequencing.

The impact of 5-hmC on gene expression and chromatin structure is also poorly understood. However, TET1 has been mapped to CpG rich sequences within hypomethylated promoters (Wu et al., 2011). TET1 association at these loci correlates with histone modification patterns signifying either repressed (e.g. H3 K27me3) or active (e.g. H3 K4me3 and H3 K36me3) chromatin, implying a role for TET1 in regulating multiple epigenetic states (Wu et al., 2011). Conversion of 5-mC to 5-hmC by TET oxygenases disrupts interactions between methyl binding domain (MBD) proteins and DNA, and thus, likely epigenetic states. MBD1, MBD2b, MBD2b/MBD3L and MBD4 bind to 5-mC DNA but not 5-hmC DNA *in vitro* (Jin et al., 2010). The Rett's syndrome protein MeCP2 is also predicted not to bind to 5-hmC DNA (Valinluck et al., 2004). Together, these observations imply TET oxygenases may function in maintaining hypomethylated chromosomal regions, but whether TET proteins can be directly coupled to DNA replication is not yet known.

6. Summary

Propagating epigenetic states of gene expression involves the concerted effort of a complex network of histone and DNA-modifying enzymes, histone chaperones and replication proteins to ensure efficient duplication and inheritance of chromatin modification patterns and chromatin associated proteins. In recent years, numerous chromatin modifications and corresponding enzymes have been identified and their links to epigenetic processes have been demonstrated. However, understanding their roles in regulating epigenetic processes upon DNA replication remains to be developed. Many questions still must be addressed to clarify how chromatin assembly is regulated by chromatin modifying enzymes, how histones and structural components of heterochromatin are removed from in front of the replication fork, and then rebuilt behind the fork during replication. Future progress will reveal how histone and DNA modification patterns contribute to locus-specific epigenetic states across the genome.

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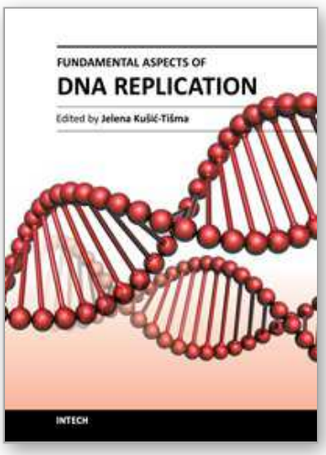

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DNA replication, the process of copying one double stranded DNA molecule to produce two identical copies, is at the heart of cell proliferation. This book highlights new insights into the replication process in eukaryotes, from the assembly of pre-replication complex and features of DNA replication origins, through polymerization mechanisms, to propagation of epigenetic states. It also covers cell cycle control of replication initiation and includes the latest on mechanisms of replication in prokaryotes. The association between genome replication and transcription is also addressed. We hope that readers will find this book interesting, helpful and inspiring.

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