Post-translational Modifications of Newly Synthesized Histones H3 and the Role of H3 K56 Acetylation on Chromatin Assembly in Mammalian Cells

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POST-TRANSLATIONAL MODIFICATIONS OF NEWLY SYNTHESIZED HISTONES H3 AND THE ROLE OF H3 K56 ACETYLATION ON CHROMATIN ASSEMBLY IN MAMMALIAN CELLS

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Abstract

The project I am presenting aimed to: 1. Elucidate the pattern of post-translational modification on the different variants of newly synthesized histones H3 in mammalian cells; 2. Reveal whether the acetylation of residue K56 on newly synthesized H3 histones plays a role in the incorporation of the histone into chromatin in mammalian cells; and 3. Determine whether the acetylation of residue K56 on newly synthesized H3 histones plays a role in the incorporation of the histone specifically in replicating chromatin in mammalian cells. The experiments to answer these questions were performed using HEK293 cells with inducible expression of FLAG-histones, enabling us to control the synthesis of new histones of interest and to detect and analyze their presence and relative levels in the cells. The results suggest that the acetylation of lysine 56 on histone H3 may play a positive role in the incorporation of the histone into new chromatin, and lack of acetylation may be reducing the efficiency of incorporation compared to acetylated histones.

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1. Chromatin

In eukaryotes, DNA is packaged around proteins to form a compact, organized, yet dynamic structure called chromatin. The basic unit of chromatin is the nucleosome, consisting of an octamer of four types of histones (two of each): H2A, H2B, H3, and H4 (1). Each of these histones contains a globular histone fold domain, and an N-terminal tail extending out away from the fold domain. The globular region is made up of three alpha-helices, separated by two loops, which are involved in intranucleosomal interactions between the histones. Within the nucleosome, histones are arranged in a tetramer of two H3/H4 heterodimers, held together by α-helix interactions between the two H3 molecules, and two H2A/H2B heterodimers interacting between the H2B and H4 molecules. Approximately 147 bp of DNA are wrapped around the nucleosome core 1.8 times (1-3). The globular domains of the histone proteins forms electrostatic interactions with the surrounding DNA, and the N-terminal tails of histones protrude out of the nucleosome core and interact with nearby nucleosomes to form higher-order chromatin folding (1, 4). This structure functions predominantly to condense the DNA. By interactions among the histones within different nucleosomes, the structure of chromatin can become more condensed, preventing DNA binding proteins from acting on the given region of DNA. Alternatively, fewer interactions between nucleosomes can result in more relaxed chromatin, allowing regulatory factors to come in contact with the DNA. The

nucleosome density is one important factor in such internucleosomal interactions, and it varies in different regions of chromatin, depending on the need for local activity at a given time of the cell cycle or during given conditions, such as DNA replication in S-phase, gene transcription, and double stranded break repair (5-10). During replication, nucleosomes are disrupted by helicases in order to allow the replication machinery full access to the DNA (11, 12). Behind the replication fork, nucleosomes are reassembled on both sides of the fork, and both pre-existing and newly synthesized histones are incorporated into chromatin (13-15).

During replication, newly synthesized histones are directly deposited onto DNA (16, 17). This deposition is mediated by histone chaperones and controlled by nucleosome assembly factors (18, 19). The mechanism of de novo nucleosome assembly involves the deposition of two H3/H4 dimers onto DNA to form a tetramer, followed by the addition of two H2A/H2B dimers to complete the histone octamer (1, 16, 18-24).

2. Histone H3 Variants

Higher eukaryotes have four evolutionarily conserved histone H3 variants: the replication-coupled H3.1 and H3.2, the replication-independent H3.3, and the centromere-specific CENP-A (25, 26). Histones H3.1 and H3.3 differ in sequence at only four amino acid residues, three of which have been found responsible for

the difference in deposition. Changing these sequences on H3.1 to match those of H3.3 results in the replication-independent deposition of H3.1 (26-29). The replication-coupled H3.1 is expressed only during S-phase, while the replication-independent H3.3 is expressed at basal levels throughout the entire cell cycle (30-34). It is incorporated into chromatin during transcription and following DNA repair. Enrichment of H3.3 is particularly observed in transcriptionally active genes and in the gene promoters of both active and repressed genes, as well as in telomeric repeats (29, 34-38). However, it is not found in satellite DNA and Y-chromosomal repeat DNA. Changing the amino acid sequence of histone H3.3 alters its genome enrichment to a pattern more similar to that of H3.1. Incorporation of H3.3 inside the body of the gene correlates with transcriptional activity; in transcriptionally repressed genes, H3.3 is only found in the promoter region, and not in the body of the gene (29). In embryonic stem cells, pluripotency genes incorporate histone H3.3, and it is lost upon differentiation, when these genes are no longer expressed, but housekeeping genes continue to incorporate H3.3 even after differentiation, correlating with their continuous activity.

3. CAF-1 and HIRA Chromatin Assembly Factors

Histone chaperones and chromatin assembly factors are important in regulating the nucleosome assembly and eviction (18, 39-41). The main protein that

mediates the incorporation of H3/H4 during replication-coupled assembly is CAF-1 (22, 42-46). CAF-1 consists of three subunits, called Cac1, Cac2, and Cac3 in yeast, or p150, p60, and p48 in *Drosophila* and human cells (46). During DNA synthesis, CAF-1 interacts directly with PCNA, a DNA polymerase processivity factor, targeting histones to be deposited at the site of replication (41, 47, 48). CAF-1 also mediates nucleotide excision repair-coupled and ssDNA break repair-coupled nucleosome assembly outside of S-phase (49-53).

CAF-1 is important for heterochromatin maintenance, and mutating it impairs gene silencing of telomeres; however telomeres still incorporate nucleosomes even in the absence of CAF-1 (45, 54-56). In yeast, CAF-1 is not essential for viability, though *cac1* deletion mutants are slightly sensitive to genotoxic stress, such as UV radiation (45, 54, 57). Loss of CAF-1 also increases sensitivity to DNases, indicating a decrease in the efficiency of nucleosome assembly in the absence of the protein (58).

Like other chromatin assembly factors, the p60 and p48 subunits of CAF-1 contain seven WD repeats (45, 59-63). These are 4-10 amino acid repeats ending in pairs of Trp-Asp (WD) residues, and are highly conserved in eukaryotes, but not found in prokaryotes. They are involved in protein-protein interactions, mediating signal transduction, RNA processing, gene expression, vesicular trafficking, and cell division (63-65). The presence of WD repeats

results in a propeller structure forming 4-10 internal beta-strands each ending in a WD pair, and these structures are specialized loops where protein interactions take place (63, 67-69).

The nucleosome assembly factor involved with replication-independent nucleosome assembly is called Hir in yeast, and HIRA in humans (24, 29, 41, 70). HIRA specifically binds and mediates H3.3 incorporation into chromatin, in association with the chromatin remodeling factor CHD1 (70-74). HIRA is required for transcriptional regulation in *Drosophila*, and is responsible for H3.3 deposition at both active and repressed genes, but not on transcription factor binding sites and telomeres, where the H3.3 incorporation is not dependent on HIRA but on other factors (29, 75-78). The normal H3.3 enrichment pattern, including transcription start sites and house keeping genes, is not observed in cells lacking HIRA. Depletion of HIRA prevents replication-independent histone deposition, but not nucleotide excision repair-coupled nucleosome assembly (70).

HIRA contains seven WD-repeat sequences, which share 27% identity with the CAF-1 WD region (59, 61, 62, 70). The protein is phosphorylated in a temporal manner throughout the cell cycle (79, 80). Yeast Hir1 and Hir2 repress transcription of H2A and H2B genes during most of the cell cycle, and recruit SWI/SNF nucleosome remodeling complex at the G1/S boundary, allowing H2A

and H2B transcription (81-84). HIRA has overlapping function with CAF-1 in telomeric and mating type silencing (59).

Both CAF-1 and HIRA bind H3/H4 directly (85, 86) and require histone chaperone Asf1 (Anti-silencing factor 1) for most efficient nucleosome assembly, in yeast, *Drosophila*, and human cells (57, 87). In yeast, Hir proteins associate with Asf1 to promote silencing (88).

4. Asf1 Histone Chaperone

Asf1 is a histone chaperone involved in both replication-coupled and replication-independent nucleosome assembly (49, 57, 58, 88-92). In mammals, there are two proteins, Asf1a and Asf1b, that share 71% homology and whose function is highly redundant (92-94). The N-terminal end of Asf1 consists of 155 extremely highly conserved residues, arranged in defined secondary structures. This is the functional part of the protein, involved in protein-protein interactions with H3/H4 dimers as well as other chromatin assembly factors (91, 95). The C-terminal end is highly acidic, but not as well conserved and has an undefined, random coiled structure. It is not functionally essential, but is thought to stabilize interactions with proteins bound to Asf1 (95). *Drosophila* and human Asf1 lack the acidic C-terminal tail, but instead the histone chaperone is phosphorylated in a replication-dependent manner (94). The protein has a conserved concave hydrophobic

groove, flanked by electronegative acidic surface (95). This site is thought to be the histone binding site.

Asf1 is highly important in histone deposition, as histones that are not able to bind to Asf1 are not deposited onto chromatin (96). In yeast, *Drosophila* and humans, when bound to H3/H4, Asf1 directly binds the Cac2/p60 subunit of CAF-1 and mediates histone deposition (49, 57, 97, 98). Similarly, Asf1 binds to HIRA directly, mediating replication-independent/transcription-coupled nucleosome assembly (59, 70, 79, 88, 95, 99, 100). Asf1 does not tightly associate with chromatin, indicating that it has a transient role in escorting the histones to the correct location (49).

Aside from replication- and transcription-coupled assembly, Asf1 has been implicated in escorting histones to sites of DNA damage repair for incorporation post-repair (49). Asf1 interacts directly with cell cycle checkpoint kinase Rad53, mediating nucleosome assembly following DNA damage (101-103). CAF-1 is essential for Asf1-mediated nucleosome assembly following nucleotide excision repair, but independent of repair, Asf1 alone in high concentrations can also deposit histones onto chromatin (49, 92).

Yeast Asf1 has also been shown to mediate chromatin disassembly, and there has been evidence supporting a role of Asf1 in recycling histones as H3/H4

dimers (11, 93, 104). Binding of Asf1 to H3/H4 dimers prevents $[H3/H4]_2$ tetramer formation (105). Asf1 recruited to chromatin binds H3/H4 dimers, disrupting the tetramer and removing the nucleosomes from the DNA (106). Asf1 interaction with helicases is essential for replication fork progression (13, 93, 106). This indicates another role for Asf1 in DNA replication by removing nucleosomes before the replication fork.

Overexpression of Asf1 interferes with telomeric silencing and mating type silencing in yeast (57, 107-109). Loss of Asf1 in yeast decreases sensitivity to DNases due to increase in nucleosome density and chromatin compaction in the absence of the protein. This further supports Asf1's activity in disassembly of nucleosomes. In addition, Asf1 interacts with transcription factor TFIID and chromatin remodeling enzyme Brahma, possibly mediating the removal of nucleosomes from transcriptionally active sites (110-112). Deletion of Asf1 results in high sensitivity to genotoxic stress, increases doubling time and cell cycle progression, and also alters histone gene expression (57, 99, 107).

Asf1 binding to newly synthesized H3/H4 is important in mediating some post-translational modifications specific to new histones. Asf1 itself has no acetyltransferase activity, but it transports histones in close proximity and favorable orientation to HATs (95). It has been shown to be important for the acetylation and methylation of H3 K9, as deletion of Asf1 reduces the abundance

of this modification (58, 93). Asf1 is also absolutely essential for the acetylation of H3 K56 by Rtt109 (to be discussed in detail below) (9, 113-116).

5. Post-translational Modifications

Histones are most often post-translationally modified on their N-terminal tails, which protrude out of the nucleosome core and are accessible for interactions with histone modifying proteins (1, 4, 66). Such modifications include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ribosylation and occur at lysine, arginine, serine, and threonine residues (66, 73). The pattern of modifications is responsible for regulating chromatin structure and activity, not directly, but by recruiting and interacting with regulatory proteins that recognize the specific modifications (117-119). Such proteins contain domains that read the pattern and bind to the histone carrying it: chromodomains bind methylated lysine residues, bromodomains bind acetylated lysine residues. Proteins containing bromodomains generally recognize a range of acetylated lysines, while chromodomains show greater specificity in recognizing a given methylation pattern. The HP1 chromodomain only binds dimethylated H3 K9, while the Polycomb chromodomain binds dimethylated H3 K27 (66). The chromatin remodeling complex SWI/SNF has a bromodomain, and binds acetylated histone N-tails to destabilize the nucleosome (7).

Specific post-translational modification patterns are correlated with particular chromatin activities. It is important to note that these patterns of modification can have differential effects based on the cellular context (66). For example, the combination of H4 K8 acetylation + H3 K14 acetylation + H3 S10 phosphorylation has been linked to transcription, while H3 K9 trimethylation + H3 and H4 un-acetylation represses transcription. In addition, H4 K5,8,12 acetylation is involved in histone deposition of newly synthesized histones, a modification that is evolutionarily conserved (66, 120). Centromeric histone H3 variant CENP-A differs from canonical H3 in residues that would normally be phosphorylated and acetylated in other H3 variants (41, 66). The lack of these residues is associated with the maintenance of chromatin condensation in regions incorporating CENP-A. Another mark of chromatin condensation, particularly during mitosis, is H2A S1, T119 phosphorylation + H3 T3, S10,28 phosphorylation.

A number of histone modifying enzymes are responsible for the post-translational modifications on histones throughout the cell cycle. These include histone acetyltransferases (HATs), which acetylate lysine and arginine residues, histone methyltransferases (HMTs), which methylate lysine and arginine residues, and histone kinases, which phosphorylate serine and threonine residues (66). Some histone modification enzymes interact with DNA regulatory proteins, targeting their activity to specific locations on chromatin. For example, ATM is a DNA damage checkpoint kinase, which is recruited to and activated at sites of dsDNA

breaks, phosphorylates histone H2A at these sites (66, 121).

While some modifications are involved in recruitment of regulatory proteins, others are important for regulating the modifications of other residues. Methylation of H4 R3 promotes H4 K5,8,12 acetylation (66). Subsequently, acetylation of H4,5,18 promotes H3 R2,17,26 methylation. Phosphorylation of H3 S10 inhibits methylation of H3 K9.

Histone acetylation is particularly interesting, as it has been shown to regulate a wide range of processes, including DNA replication, chromatin assembly, chromosome condensation, transcription, DNA repair (66, 117, 118, 122-125). New histones H3 are acetylated on various residues, promoting their interaction with chromatin assembly factors for incorporation into chromatin (94, 126-129). Acetylation of new histones H3 and H4 is important for nucleosome assembly, and preventing acetylation, such as by deletion of the H3 and H4 N-tails, results in decreased nucleosome density and loss of viability (41, 130, 131). This importance is further supported by the redundancy in function of acetylating the H3 and H4 tails: in yeast, acetylation of H4 K8 is sufficient to make up for the lack of acetylation on H3 due to N-tail deletion (41, 129, 131). Di-acetylation of H4 K5,12 in newly synthesized histones is highly evolutionarily conserved, and has been implicated in nucleosome assembly (66, 93, 120, 132).

Another modification, acetylation at K56 of histone H3, is also found on newly synthesized histones H3, and will be discussed in greater detail later (10, 133). Many post-translational modifications found on new histones are removed following their incorporation into chromatin (21, 134). Fewer PTMs are observed in histones outside of S-phase (135). In addition, pericentric heterochromatin contains no acetylation at H4 histones, supporting the fact that acetylation is involved in active chromatin (86, 136). Lack of deacetylation of pericentric H4 interferes with kinetochore function and chromosome segregation (134). Ultimately, the balance of HAT and HDAC activity is critical for the proper regulation of chromatin activity.

6. H3 K56

The lysine 56 residue of histone H3 is located within the globular domain of the histone, at the point where DNA enters and exits the nucleosome. (10, 137,138). This residue makes direct contact with the phosphate backbone of the surrounding DNA (116, 139). Because of its prominent location within the nucleosome, extensive studies have been done to determine if this lysine and its acetylation are structurally significant for the nucleosome itself as well as for chromatin in general (140-142). It has been hypothesized that this modification destabilizes the nucleosome, resulting in a number of downstream events, further discussed shortly. Studies have determined that acetylation of H3 K56 has no

effect on the formation or stability of the nucleosome (140). Chromatin fiber compaction is also not affected significantly by the modification of this residue (140, 141). However, acetylation of this lysine does weaken interaction with DNA at that point, resulting in localized “DNA breathing” (10, 116, 140). Thus, other, more complex mechanisms must be involved in the downstream events involving this modification.

In yeast cells, H3 K56 is abundantly acetylated in a cell-cycle dependent manner (133, 143-147). The modification occurs only on newly synthesized histones during S-phase, and is removed shortly after incorporation of the histones into chromatin (10, 147-149). Removal of the modification is important for genome stability (148, 149). However, complete lack of the modification also results in sensitivity to genotoxic agents (10). The same modification occurs in *Drosophila*, *Tetrahymena* and low, but significant levels are also observed in mammalian cells (5, 9, 125, 144, 145, 150). However, unlike in yeast, H3 K56ac is detected throughout the entire cell cycle in both *Drosophila* and mammals. In yeast and human cells, the modification has been found to be gene-specific, and is particularly prevalent at histone gene promoters (125). The modification occurs on all histone H3 variants (6, 125, 151).

Acetylation of H3 K56 has implicated roles in replication, DNA repair, transcriptional regulation, homologous recombination, as well as chromatin

assembly (5, 6, 7, 9, 10, 90, 113, 115, 116, 125, 137, 138, 146-148, 152-156). Histone chaperone Asf1 is required for the acetylation of H3 K56, and the modification is not detected in *asf1* deletion mutants (114, 116, 146, 149, 157, 158). Asf1 binds the H3/H4 heterodimer at the C-terminus of H3, a site away from the K56 residue, allowing it to be in solution and accessible to HATs and other proteins (150, 159). Histones deposited by CAF-1 are acetylated at K56, and CAF-1 binding to chromatin is enhanced by the presence of the modification on CAF-1-bound H3 (10, 115). CAF-1 binds H3 K56 directly via its Cac1 subunit. Acetylation of H3 K56 does not affect binding of H3/H4 to Asf1, but it directly affects the binding affinity with CAF-1 (115). These facts support the role of H3 K56 acetylation in replication-coupled nucleosome assembly in yeast.

In yeast, H3 K56 acetylation is shown to have a critical role in packaging DNA into chromatin following DNA replication and repair (5, 10, 113, 115, 116, 146, 147, 155, 160). In *Drosophila* and HeLa cells, acetylation of H3 K56 increases with an increase in DNA damage, and it has been shown in human cells to co-localize with the DNA-damage specific histone variant H2AXp (9). In yeast, K56 acetylation is not removed post-assembly at sites of DNA damage, and this delay of deacetylation promotes DNA repair (10). The modification regulates reassembly of nucleosomes after DSB repair and is necessary for the completion of repair (5). The fact that *asf1* deletion mutants and *asf1 cac1* double deletion mutants are highly sensitive to genotoxic stress supports the involvement of H3

K56 in DNA repair (57).

Increased levels of H3 K56 acetylation have also been correlated with tumorigenicity, as well as pluripotency! (9, 125, 160). H3 K56 has been defined as marker for the epigenetic difference between pluripotent and differentiated cells (125). In *Drosophila*, the chaperone responsible for mediating the acetylation of H3 K56, Asf1, is highly abundant in embryos and less so in adults, suggesting that the modification is also found at higher levels in undifferentiated cells and indicating evolutionary conservation of H3 K56 as a pluripotency marker (57). In human embryonic stem cells, canonical histone gene promoters contain H3 K56ac, while variant histone genes do not. In differentiated cells, variant histone gene promoters also contain the modification, while the levels detected in the canonical histone genes are reduced.

H3 K56 acetylation is observed in the promoter regions of histone genes and genes involved in core transcriptional network (90, 125, 154). In yeast, H3 K56 acetylation occurs prior to the expression of histone genes, and the presence of the modification in histone gene promoters is absolutely necessary for the transcription of the histone genes (7). This correlates with the observed effect of Asf1 on expression of histone genes (93). Consistent with the implicated role in transcriptional regulation, the modification has been shown to be essential to the recruitment of chromatin remodeling factor SWI/SNF (7). A hypothesized model

suggests that in yeast, acetylation of H3 K56 in histone gene promoters recruits SWI/SNF to the promoter. SWI/SNF destabilizes the nucleosome and activates gene transcription (7).

7. Rtt109 HAT

An important histone acetyltransferase that has been implicated in acetylating H3 K56 on newly synthesized histones is the yeast Rtt109 (116). It shares structural homology to the mammalian HAT p300 (146, 147, 161-168). Binding to histone chaperones (Asf1 or Vps75) is essential for the HAT function of Rtt109 (116, 146, 147, 169). Histone chaperone Asf1 is required for H3 K56 acetylation of nascent H3 by Rtt109 (113-116, 146). Asf1 escorts the histone in close proximity to the HAT and presents it in the correct orientation for acetylating the residue. Rtt109 acetylates H3 K56 only when associated with both Asf1 and H4 in a transient Rtt109-H3/H4-Asf1 complex (116). The HAT itself interacts with, but does not stably bind histones (116, 169).

Rtt109 requires AcCoA to acetylate H3 K56, and both AcCoA and H3 must enter the active site of Rtt109 in order for acetylation to occur (116, 146, 147, 161, 170). The order of binding is not important and likely random. Rtt109 lacks AcCoA binding motif, but has been shown that functionally it binds the molecule similarly to other HATs (116). The reaction occurs between the ε-amino group of the lysine and the acetyl group of AcCoA (170). In mammalian p300, the binding

of AcCoA to the HAT is required prior to H3 binding, and the involvement of a histone chaperone is not necessary (9, 165, 171).

Rtt109/Asf1 is important for genome stability (116). Deletion of Rtt109 results in chromosomal rearrangement and genomic instability, hypersensitivity to DNA damage and activation of DNA damage checkpoints (146). *rtt109* deletion mutants lack H3 K56 acetylation (146, 172). The similarity in the profile of *rtt109* deletion mutants and *asf1* deletion mutants confirm that Rtt109 acts in the same pathway as Asf1 (146, 173, 174). The current model suggests that Asf1 binds new H3/H4 dimers, binding the C-terminus of H3, and presents the H3 K56 residue to Rtt109 for acetylation. Following acetylation, the heterodimer is transferred to CAF-1 for deposition onto chromatin (113, 115, 147, 150).

8. H3 K56 HDACs

The balance between acetylation and deacetylation of the H3 K56 residue is essential to the genomic stability of cells. Yeast cells that cannot undergo K56 acetylation are sensitive to genotoxic stress, but this sensitivity is even higher in cells that cannot deacetylate the residue (10, 57, 99, 146, 148, 151, 180). This puts special importance to histone deacetylases involved in removing the modification at the appropriate time in the cell cycle. The HDACs responsible for this are the Hst3 and Hst4 proteins (Sir2 in *Drosophila*; SIRT1 and SIRT2 in

humans), members of the Sirtuin family (9, 148, 149, 152, 175-180).

In yeast, Hst3 is present in low levels in S phase and increases in G2/M, opposite to the levels of H3 K56 acetylation (109, 148). It has been shown to deacetylate H3 K56 in mature chromatin. It is thought that K56 deacetylation in G2 might signal Hst3 degradation in G2/M, in order for K56 acetylation to accumulate again in G1 (148). The importance of sirtuins in deacetylating H3 K56 was shown by blocking sirtuin function, and a subsequent increase of acetylated K56 levels were observed; double deletion of Hst3 and Hst4 showed HDAC specificity to this residue, since acetylation at other residues was not affected (148).

Yeast Hst3 and Hst4 act redundantly and double deletion has severe effects (180). Double deletion increases the levels of acetylated H3 K56 even after S-phase (148). However, replication-induced DNA damage was observed by the presence of the DNA damage marker, phosphorylated H2A, long after DNA replication, indicating that deacetylation of H3 K56 is important for completion of double-stranded break repair (148). Failure to deacetylate K56 also leads to defective silencing in telomeres (151).

9. Specific Aims

It is apparent that H3 K56 is an important residue, though very little is known about its function in mammalian cells. The primary part of my project aimed to reveal more information about the role of this residue in human cells, mainly in nucleosome assembly during DNA replication. In addition, I attempted to construct an informational map of the post-translational modifications found on newly synthesized H3 histone variants.

Materials and Methods

Reagents

Buffer A
10 mM Tris, 1.8 mM BME, 3 mM $MgCl_2$,10 mM Sodium Butyrate, pH 7.6

HB Buffer
20 mM HEPES free acid, 5 mM KCl, 2 mM $MgCl_2$, 10 mM NaButyrate, pH 7.5

SDS Sample Buffer
0.1 M Tris, 2% SDS, 2 M urea, 23% glycerol, 5 M EDTA, 0.3 M BME, 0.002% BoBlue

SDS-PAGE Running gel
18% Acrylamide / 0.09% Bis-acrylamide, 0.75 M Tris pH 8.8, 0.1%SDS, 0.05% APS, 0.05% TEMED.

SDS-PAGE Stacking gel
6% Acrylamide / 0.16% Bis-acrylamide, 0.125 M Tris pH 6.8, 0.1% SDS, 0.05% APS, 0.1% TEMED

SDS-PAGE Running Buffer
50 mM Tris, 380 mM glycine, 0.1% SDS

SDS-PAGE Transfer Buffer
25 mM Tris, 192 mM Glycine, 20% Methanol

Blocking Buffer
50 mM Tris, 150 mM NaCl, 0.1% Tween-20, 0.2% w/v I-Block (Applied Biosystems, Cat# T2015).

1X PBS (Phosphate Buffered Saline)
0.008 M sodium phosphate, 0.002 M potassium phosphate, 0.14 M sodium chloride, 0.01 M potassium chloride, pH 7.4

1X TBS (Tris Buffered Saline)
50 mM Tris, 150 mM NaCl, pH 7.5

3E Buffer
0.12 M Tris, 6 mM NaOAc-3H2O, 3 mM EDTA, pH 7.6

4% SDS – DNA Sample Buffer
33% 3E Buffer, 5% glycerol, 1% SDS, 0.01% BoBlue

4% SDS - DNA Gel
4% Acrylamide / 0.2% Bis-acrylamide, 33% 3E Buffer, 0.1% SDS, 0.08% TEMED, 0.05% APS.

4% SDS – DNA Gel Running Buffer
26% 3E Buffer, 0.1% SDS

IP Wash Buffer
20 mM Tris pH 8.5, 0.5 M NaCl, 10 mM NaButyrate, 1 mM EDTA, 1% Triton X-100

High Salt TSE
1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris, pH 8.1

Medium Salt TSE
1% Triton X-100, 2 mM EDTA, 400 mM NaCl, 20 mM Tris, pH 8.1

Low Salt TSE
1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris, pH 8.1

2.5% SDS extraction buffer
1 mM EDTA pH 7.2, 10 mM Tris pH 7.4, 10 mM NaButyrate, 50 mM NaCl, 0.3 M BME, 2.5% SDS

0.5% SDS extraction buffer
1 mM EDTA pH 7.2, 10 mM Tris pH 7.4, 10 mM NaButyrate, 50 mM NaCl, 0.5% SDS

Methods

Cell culture

HEK 293 cells were grown at 37°C, 5% CO_2 in HyClone High Glucose DMEM supplemented with 9% Fetal Bovine Serum, Pen/Strep (100 U/mL penicillin, 0.1 mg/mL streptomycin), 2 mM L-Glutamine, 0.11 mg/mL G418, (0.055 mg/mL hygromycin, for transfected cells).

Creating wild type and mutant clones

Cells expressing FLAG-tagged histones were created using a HEK293 Tet-ON Advanced system, in which the addition of the tetracycline-derivative, doxycycline, induces the expression of FLAG-tagged proteins. FLAG-H3.1 clones were created by transfecting HEK293 Tet-ON Advanced cells with a DNA plasmid encoding the specific FLAG-histone. Hygromicin resistance marker was used to select for successfully transfected clones. Transfection medium was prepared by combining 500 µL Opti-1 DMEM, 10 µL of Lipofectamine 2000, 0.25 ug linear hygromycin marker, 3.75 ug plasmid DNA, incubated at room temperature for 20 min. Cells were grown to 90% confluency in Complete non-hygromycin DMEM in 6-well plates. Growth medium was removed and replaced by transfection medium to incubate for two days at 37°C. The medium was replaced with Complete hygromycin DMEM to select for transfected cells. Hygromicin-resistant clones were tested for induction.

Mutants

Cell lines containing mutant FLAG-histones were created by Cheryl Doughty by point-directed mutagenesis using the Stratagene QuikChange kit.

Test induction

Cells were grown in Complete hygromycin DMEM at 37°C in 6-well plates to 90% confluency. Cell were induced with 2 ug/mL doxycycline and incubated at 37°C for 4 hours. Cells were harvested and centrifuged at 2000 RPM for 2 min to remove the growth medium. Total cell extract was prepared as described below and samples were analyzed by Western Blot. Induction was confirmed by probing the blot with anti-FLAG antibody (Sigma Cat # F7425, 1:1000 dilution).

Total cell extract

Cell pellets were washed in 1 mL Buffer A and resuspended in 500 µL 2 mM EDTA with 0.2 M H_2SO_4. The samples were sonicated for 5 seconds to break up the plasma and nuclear membranes and incubated overnight at 4°C with constant rotation to allow acid-extraction of the proteins. Samples were centrifuged in a microcentrifuge at max speed for 10 min at 4°C, and the proteins in the supernatant were TCA-precipitated with 25% Trichloroacetic acid. Samples were washed with acetone to remove residual TCA, dried, and resuspended in SDS sample buffer to run on SDS-PAGE and transfer for Western blot.

Synchrony

Cells were treated with 5 mM Thymidine in Complete DMEM for 24 hours at 37°C, 5%CO_2 to block the cell cycle at the G1/S-phase boundary. Cells were then washed with unsupplemented DMEM, and released with DMEM containing 30 μM deoxycytidine. At the time of the release, cells were also induced by supplementing the release medium with 2 ug/mL doxycycline. Cells were kept at 37°C, 5% CO_2 and harvested in mid- S phase, 4 hours after release. Samples were taken prior to thymidine treatment, prior to release, and at the time of harvest, in order to confirm the effectiveness of the synchrony by flow cytometry.

Flow Cytometry

Cells were obtained from culture and centrifuged at 1000 RPM for 2 min to remove the growth medium. Cells were washed in 1X phosphate buffered saline (PBS) supplemented with 20 mM EDTA, and resuspended in 70% ethanol overnight at 4°C to fix them for flow cytometry.

At the time of analysis, fixed cells were centrifuged at 2000 RPM for 2 min to remove the ethanol. Cells were washed in 1X phosphate buffered saline supplemented with 20 mM EDTA, and resuspended in the same buffer. Cells were prepared for staining and flow cytometry by adding 0.1% Triton X-100, 5% RNAse II (10 μg/mL), and 5% propidium iodide. Cells were incubated at 37°C for 30 min. Cells were analyzed by flow cytometry using FACSCanto cyometer (BD

Bioscience) and BD FACSDiva Software.

Cytoplasmic S100 Isolation

Cells were harvested and centrifuged at 1500 RPM for 2 min in order to remove the growth medium. Cells were washed once with 30 mL ice cold Buffer A, followed by two washes in 30 mL ice cold HB Buffer. The cell pellet was centrifuged at 4000 RPM for 2 min at 4°C and resuspended in 500 µL HB Buffer. The cells were homogenized and incubated on ice for 30 min. The sample was centrifuged at 12000 RPM for 10 min at 4°C. The spin was repeated with the supernatant to ensure complete sedimentation of the pellet. The supernatant was transferred to an ultracentrifuge tube and centrifuged at 43000 RPM for 1.5 hours at 4°C in a Beckman TL-100 Ultracentrifuge, using a TLA-45 Rotor.

Immunorecipitation of FLAG-histones

Preparing beads

1x 150 µL of mouse-M2 (anti-FLAG) beads (Sigma, Cat# F2426) per IP (for every 70 A_{260} of nuclei)

2x 150 µL of mouse-IgG beads (Sigma, Cat # A0919) per IP (for every 70 A_{260} of nuclei)

Beads were washed 6x in 1 mL of 1X TBS. When immunoprecipitating chromatin, an additional wash with 1mL of 2 mM EDTA was performed to remove any salt.

Samples were prepared for IP by adding 0.25% Triton X-100, 1 mM PMSF, 1 mM EGTA. Samples were pre-cleared by incubating in non-immune IgG beads for 1 hour at room temperature, with constant rotation. Samples were centrifuged at 2000 RPM for 2 min and allowed to sit on ice for 5 min to settle.

Sequential IP was performed by incubating the pre-cleared supernatant with the non-immune IgG beads for 1 hour at room temperature, with constant rotation, to serve as a negative control. The samples were centrifuged at 2000 RPM for 2 min and allowed to sit on ice for 5 min to settle. The supernatant was then incubated with the M2 anti-FLAG beads overnight at 4°C with constant rotation. The IgG beads were stored in 2mM EDTA at 4°C overnight.

Treatment of S100 samples following FLAG-IP

The IP sample was centrifuged at 2000 RPM for 2 min and allowed to sit on ice for 5 min to settle. The supernatant, containing the unbound fraction, was saved. The M2 and IgG beads were washed 5x for 10 min in 1 mL of IP wash buffer supplemented with 1 mM PMSF, and 1x for 10 min in 10 mM Tris, pH 8.1.

Post IP-extraction from beads

After washing the beads post IP, they were extracted in an equal volume of 2.5% SDS extraction buffer at 70°C for 1 hour. Samples were centrifuged at 2000 RPM for 2 min and allowed to sit for 5 min at room temperature to settle. The

supernatant was transferred into a new tube, and the beads were washed with 150 µL of 0.5% SDS extraction buffer. The beads were centrifuged at max speed for 2 min and allowed to sit for 5 min at room temperature to settle. The supernatants were combined. Phenol/chloroform/alcohol (25:24:1) was added to the sample at a volume 58.6% of the sample volume, and the samples were shaken vigorously for 15 min. Samples were centrifuged at max speed for 10 min at 4°C. The aqueous layer was removed, and the organic layer was adjusted to 0.1 M HCl. 12 volumes of acetone were added, and the sample was incubated overnight at -20°C to precipitate the proteins. Samples were washed with acetone to remove residual phenol/chloroform, dried, and resuspended in SDS sample buffer to run on SDS-PAGE and transfer for Western blot.

SDS-PAGE

Gel and running buffer were prepared as previously described (181) and run at 37 mA constant current.

Western Blot

SDS-PAGE was transferred onto PVDF membrane overnight at 30 V constant voltage. Transfer buffer was prepared using standard recipe. Membrane was incubated at room temperature for 1 hour in blocking buffer, followed by 2 hours in primary antibody (rabbit anti-FLAG (Sigma, Cat# F7425, at 1:1000 dilution), rabbit anti-panH4 (Millipore, Cat# 07-108, at 1:1000 or 1:2000 dilution).

Membrane was washed twice in blocking buffer and incubated for 1 hour in secondary antibody (goat anti-rabbit-AP conjugated (Applied Biosystems, Cat# T2191, at 1:5000 dilution). Membrane was washed three times in blocking buffer, and incubated twice for 2 min each time in 1X Assay Buffer (Applied Biosystems, Cat# T2187), followed by a 5-minute incubation in CDP-star solution (Applied Biosystems, Cat# T2146) prior to exposure to film.

Radioactive Thymidine Labeling

Cells were harvested and centrifuged at 1500 RPM for 2 min in order to remove the growth medium. Cells were resuspended in 20 mL of Complete DMEM supplemented with 2 ug/mL doxycycline and 30 μM deoxycytidine, and labeled with 750 uCi ^{3}H-Thymidine (83.2 Ci/mmol; Perkin Elmer) for 1.5 hours at 37°C with frequent agitation.

NaButyrate Treatment

Cells were harvested and centrifuged at 1500 RPM for 2 min in order to remove the growth medium. Cells were resuspended in 20 mL of Complete DMEM supplemented with 2 ug/mL doxycycline and 30 μM deoxycytidine, and treated with 25 mM NaButyrate for 2 hours at 37°C with frequent agitation.

Nuclei Isolation

Cells were harvested and centrifuged at 1500 RPM for 2 min in order to remove

the growth medium. Cells were washed twice in 20 mL ice cold Buffer A, then resuspended in 10 mL Buffer A and incubated on ice for 15 min. Cells were homogenized and washed with 20 mL Buffer A. Nuclei concentration was determined by measuring the absorbance at 260 nm.

Preparing mononucleosomes

Isolated nuclei were resuspended in Buffer A at 40 A_{260}/mL. The sample was adjusted to 0.5 mM $CaCl_2$ and pre-incubated at 37°C for 5 min. Chromatin was digested with 2 U/mL Micrococcal Nuclease (Sigma, Cat# N3755) for 3.5 minutes at 37°C. The digestion reaction was stopped by adding 2 mM EGTA, pH 7.6, and incubating on ice for 20 min. The sample was centrifuged at max speed for 10 minutes at 4°C. The S1 fraction, containing the mononucleosomes, was transferred to a new tube and A_{260} was measured to calculate the mononucleosome concentration. The remaining pellet was resuspended in 1 mL of 2 mM EDTA and incubated on ice for 20 min. The sample was centrifuged at max speed for 10 min at 4°C. The S2 fraction, containing the oligonucleosomes, was transferred to a new tube and A_{260} was measured to calculate the oligonucleosome concentration. The remaining pellet, the P fraction, was resuspended in 1 mL of 2 mM EDTA.

Immunorecipitation of H4 acetylated at K5, 12

Preparing beads

For every sample to be immunoprecipitated, 3 x 150 µL of hydrated Protein A Sepharose beads (Amersham Scientific) per every 70 A_{260} of nuclei were prepared.

Protein A Sepharose beads were hydrated in 1 mL High Salt TSE for 30 min at room temperature with constant rotation. Following incubation, the beads were centrifuged at 2000 RPM for 30 sec to remove the supernatant, and washed 2x 5 min in 1 mL High Salt TSE, and 2x 5 min in 1:1 beads:Low Salt TSE. Per 300 µL of beads, 150 µL of antibody (the 7481 anti-H4 K5,12ac (Annunziato Lab) or RNIS) were added, and the beads were incubated at 37° for 1.5 hours with constant rotation. Following incubtation, the beads were washed 3x 5 min in 500 µL Low Salt TSE with 2 mM PMSF.

Samples were prepared for IP by adding 0.25% Triton X-100, 1 mM PMSF, 1 mM EGTA. Samples were pre-cleared by incubating in non-immune Rabbit Serum beads for 1 hour at room temperature, with constant rotation. Samples were centrifuged at 2000 RPM for 2 min and allowed to sit on ice for 5 min to settle.

Sequential IP was performed by incubating the pre-cleared supernatant with the non-immune beads for 1 hour at room temperature, with constant rotation, to

serve as a negative control. The samples were centrifuged at 2000 RPM for 2 min and allowed to sit on ice for 5 min to settle. The supernatant was then incubated overnight at 4°C with constant rotation with the 7481 beads, which contain the antibody that recognizes H4 acetylated at K5 and 12.

Treatment of ChIP samples following 7481-IP

The IP sample was centrifuged at 2000 RPM for 2 min and allowed to sit on ice for 5 min to settle. The supernatant, containing the unbound fraction, was saved. The 7481 and RNIS beads were washed 1x in 1 mL 2 mM EDTA to remove any salt, and 5x for 10 min in 1 mL of Medium Salt TSE supplemented with 1 mM PMSF, and 1x for 10 min in 10 mM Tris.

Post IP-extraction from beads

Post-IP extraction procedure for the ChIP samples was as described above.

Sample distribution

Part II Assembly into Chromatin Experiments, post-MNase digestion:

Of the S1 sample:

85% were prepared for IP

10% were prepared for Western Blot by $MgCl_2$/EtOH precipitation ("S1 input")

5 % were prepared for DNA gel by $MgCl_2$/EtOH precipitation

Of the S2 sample:

85% were prepared for IP

10% were prepared for Western Blot by $MgCl_2$/EtOH precipitation ("S2 input")

5 % were prepared for DNA gel by $MgCl_2$/EtOH precipitation

Of the P sample:

5 % were prepared for DNA gel by $MgCl_2$/EtOH precipitation

95% were frozen at -20°C

Part III Assembly onto new DNA Experiments, post-MNase digestion:

Of the S1 sample:

75% were prepared for IP

15% were prepared for Western Blot by $MgCl_2$/EtOH precipitation ("S1 input")

5% were TCA precipitated for scintillation counting

5 % were prepared for DNA gel by $MgCl_2$/EtOH precipitation

Of the S2 sample:

75% were prepared for IP

15% were prepared for Western Blot by $MgCl_2$/EtOH precipitation ("S2 input")

1% was TCA precipitated for scintillation counting

5 % were prepared for DNA gel by $MgCl_2$/EtOH precipitation

4% were frozen at -20°C

Of the P sample:

71% were prepared for Western Blot by $MgCl_2$/EtOH precipitation ("P")

6% were TCA precipitated for scintillation counting

5 % were prepared for DNA gel by $MgCl_2$/EtOH precipitation

18% were frozen at -20°C

Part III Assembly onto new DNA Experiments, post-ChIP:

Of the IgG and M2 beads:

75% were TCA precipitated for scintillation counting

25% were extracted for Western Blot

Of the unbound samples:

75% were prepared for Western Blot by $MgCl_2$/EtOH precipitation

25% were saved for TCA precipitated for scintillation counting (only a volume equivalent to that of the input sample was TCA precipitated. The rest was frozen at -20°C).

$MgCl_2$/EtOH precipitation

Samples were adjusted to 10 mM $MgCl_2$. Two volumes of 95% Ethanol were added, and the samples were incubated in a dry ice/Ethanol bath for 5 min. Samples were centrifuged at max speed for 10 min at 4°C. The supernatant was discarded and the sample was dried and resuspended either in SDS sample buffer to run on SDS-PAGE and transfer for Western Blot, or in DNA sample buffer to run on DNA gel and stain.

TCA precipitation for scintillation counting

200 µL of 10 mM EDTA, 50 ug BSA-DNA carrier, and the appropriate sample were incubated in 3 mL 10% TCA on ice for 30 min. The samples were filtered onto glass fiber filters using vacuum apparatus. Filters were washed 2x 3 mL cold 10% TCA, and 2x 3 mL cold 95% EtOH. Filters were dried in scintillation vials overnight.

DNA gel

Gel and running buffer were prepared as previously described (182) and run at 110 V constant voltage. Following electrophoresis, gel is washed for 20 min in dH2O, stained for 20 min with Ethidium Bromide, destained for 20 min in dH2O. Photograph of gel is taken in UV gel box

Results and Discussion

Part I: Post-translational Modifications of Newly Synthesized Histones H3.1 and H3.3

As described in the introduction, post-translational modifications of histones are of major regulatory importance in a number of cellular events, including chromatin assembly, transcription, silencing, and repair (117-119). The pattern of modifications may dictate the activity of the chromatin that incorporates the modified histones, and this pattern is dynamic and changes throughout the cell cycle in accordance to the cellular needs of chromatin activity. The acetylation of histone H4 at lysines 5 and 12, as well as the acetylation of H3 at lysine 56 in yeast (21, 111, 147-149), are such examples of transient post-translational modifications. Clearly, the acetylation and subsequent deacetylation of these residues is important in the genomic stability.

In order to study the effect of post-translational modifications on chromatin activity, it is important to have a map of the pattern that is found on a given histone at a given point in the cell cycle. The goal of this part of my project is to create such a map for newly synthesized replication-coupled (H3.1) and replication-independent (H3.3) variants of histone H3 in S-phase. HEK293 cells with inducible expression of wild-type FLAG-H3.1 or wild-type FLAG-H3.3 were used for this purpose. The FLAG-tag allows for easy detection by anti-FLAG

antibodies, and since its expression is only induced for a few hours prior to the experiment, its presence is indicative of newly synthesized histones. Thus, any FLAG-tagged histone present must be recently synthesized, rather than pre-existing.

Cells were synchronized in S phase in order to obtain the maximum number of histones containing modifications that are specific to newly synthesized histones in S phase. Synchrony was obtained by treating the cells with 5 mM Thymidine for 24 hours in order for them to accumulate at the G1/S boundary. Cells were then released by removing the thymidine and adding deoxycitidine at 30 μM for 4 hours, at which point the majority of cells were in mid-S phase. The synchrony was monitored by measuring the DNA content of cells through flow cytometry. Samples for flow analysis were taken at three time points throughout the experiment – prior to treatment, after 24-hour arrest, after 4-hour release. Cells were fixed for flow cytometry in 70% ethanol overnight at 4°C.

The clones expressing H3.1 synchronized to 68% in S phase, and the H3.3 clones synchronized to 84% in S (Fig. 1). It is not unusual for different clones to have different efficiency of synchrony, as there may be genetic variations that can account for that.

Expression of FLAG-histones was induced by treating with doxycycline at the time of release, for 4 hours to allow the synthesis of new histones FLAG-H3. Upon harvesting the cells, the cytoplasmic (S100) content, which includes the new histones, was extracted. Studying cytoplasmic histones ensures that the modifications being analyzed are specifically in the histones of the pre-deposition complex.

Following cytoplasmic extraction, FLAG-histones were isolated by immunoprecipitating with anti-FLAG M2 beads, as described in the Materials and Methods. Subsequent removal of the FLAG-histone from the IP beads was necessary in order to obtain a pure sample of newly synthesized FLAG-H3.1 or FLAG-H3.3. The efficiency of immunoprecipitation was monitored by analyzing 10% of the sample by Western blot probing with anti-FLAG/anti-pan-H4 antibody (Fig. 2). Comparing the intensities of the input and bound bands (lanes 2 and 5, each representing 10% of the sample), it can be said that the majority of the FLAG-H3.1 is pulled down. Lanes 4 and 6 are controls to show that following IP, the FLAG-H3.1 is bound by the anti-FLAG beads (lane 4), and after extraction, no more FLAG-H3.1 remains bound to the beads (lane 6).

The induction of FLAG-histones in the HEK293 clones was routinely monitored in order to ensure that the cells do not constitutively express the protein in the absence of inducer, and that they still express when treated with doxycycline.

Figure 3 represents one such test, in which the sample in lane 1 is untreated, and the sample in lane 2 is treated with doxycycline for 4 hours. Total cell extracts were prepared by sonicating cells to break up the plasma and nuclear membranes and acid-extracting the proteins. Once in solution, proteins were TCA-precipitated and run on SDS-PAGE for Western blotting. Blots were probed with anti-FLAG/anti-pan-H4 antibodies. In a positive test, FLAG is not detected in the untreated sample, but is detected in the induced sample (Fig. 3).

The FLAG-H3.1 and FLAG-H3.3 samples obtained from this experiment are currently stored at -80°C and await their send-out to a Mass Spectrometry facility for analysis of the post-translational modifications found in each sample.

Beads Extraction

Anti-FLAG beads have very high affinity for the FLAG peptide, and extracting the FLAG-histones from the beads after immunoprecipitation required the development of a new extraction protocol (Fig. 4a). Following IP, the beads were washed extensively to remove any unbound or nonspecifically sticking material. Incubation at 70°C for 1 hour in a 2.5% SDS buffer containing 0.3 M 2-mercaptoethanol (ßME) was necessary to break the interactions between the anti-FLAG beads and the isolated proteins. The beads were subsequenty washed with 0.5% SDS buffer without ßME prior to the standard phenol-chloroform extraction described in the Materials and Methods (Fig. 4b).

Part II: FLAG-H3.1 S-phase assembly and the effect of K56 acetylation

In this part of the project, the questions of how efficiently the FLAG-H3.1 is assembled into chromatin and whether acetylation of K56 affects the efficiency were addressed directly. Two approaches were taken, the first of which aimed to detect whether FLAG-H3.1 associates with chromatin. This was done by comparing the ratios of FLAG-H3.1 to H4 in the total cell extract, the nuclei extract, and the chromatin of HEK293 cells. Differences in the ratios would indicate differences in the cellular distribution of FLAG-H3.1 compared to H4. Since H4 is essential for the assembly of all nucleosomes and chromatin formation, it is appropriate measurement to compare FLAG-H3.1 levels to.

Cells with inducible expression of wild type FLAG-H3.1 or a mutant version of FLAG-H3.1 were utilized. In the mutant histones, the K56 residue was changed to either Q or R. The K56Q mutant mimics constituitive acetylation of K56, while the K56R mutant mimics constituitively unacetylated K56. The cellular distribution of FLAG-H3.1 was compared among the wild type and two mutants, to determine the role of acetylation of K56 on deposition onto chromatin.

Cells were synchronized in S-phase to focus the study on the assembly in this point of the cell cycle. The synchrony was performed as described earlier, by blocking cells for 24 hours with 5 mM Thymidine and releasing for 4 hours with

30 uM deoxycitidine. Flow cytometric analysis confirmed that after the 4-hour release, 70% of the wild type, 68% of the K56Q, and 78% of the K56R cells were in S-phase (Fig. 5).

At the time of release, cells were treated with 2 ug/mL doxycycline in order to induce expression of FLAG-H3.1. Cells were harvested at 4 hours after release/induction, and an aliquot was saved as total cell sample. Proteins were extracted and tested for presence of FLAG-H3.1 following induction. Nuclei were isolated to analyze the chromatin content of the cells. An aliquot was saved to represent the nuclear content, and proteins were again extracted to test for FLAG-H3.1. Following nuclei isolation, chromatin was extracted by treating the sample with Micrococcal nuclease (MNase), which digests the linker DNA between the nucleosomes, leaving individual mononucleosomes intact. Because the amount of chromatin digested is directly proportional to the concentration of MNase and the incubation time, not everything is digested during the 3.5 minutes of incubation, resulting in two fractions of chromatin: S1 fraction, containing the mononucleosomes, and S2 fraction, containing oligonucleosomes. The degree of digestion is analyzed by running samples of each fraction on a 4% SDS-PAGE and staining with ethidium bromide (Fig. 6).

The ratios of FLAG-H3.1 to H4 found in each of the three fractions tested (total cell, nuclei, chromatin) were very similar to each other in cells expressing wild

type FLAG-H3.1, as well as cells expressing FLAG-H3.1 K56Q and FLAG-H3.1 K56R, suggesting that all of the FLAG-H3.1 detected in the cell is in the nucleus and is associated with chromatin (Fig. 7). No significant difference is observed in the levels of association of FLAG-H3.1 with chromatin between the unacetylatable K56R mutant and wild type FLAG-H3.1, suggesting that the lack of acetylation on the residue does not play a significant role in the efficiency of incorporation of the histone into chromatin.

The second approach to this part of the project directly tested whether FLAG-H3.1 is incorporated into nucleosomes. This involved the immunoprecipitation of H4 diacetylated at histones K5, 12. As mentioned previously, acetylation of these residues on H4 is a mark of newly synthesized histone H4 prior to incorporation into chromatin and shortly thereafter (42). By immunoprecipitating H4 K5,12ac$_2$ from chromatin and testing if FLAG-H3.1 is pulled down along with it, it can be determined with certainty whether or not (and to what degree) FLAG-H3.1 is incorporated into chromatin during S phase.

Cells were again synchronized in S phase and chromatin was isolated – the procedure used was as described above. However, following release of cells from thymidine block, the cells were treated for 2 hours with 25 mM sodium butyrate, an HDAC inhibitor. This treatment is done to ensure that the acetylation of newly synthesized H4 at lysines 5 and 12 will not be removed following

incorporation into chromatin and throughout the experimental procedure. The S1 and S2 fractions of chromatin were immunoprecipitated with anti- H4 K5,12ac$_2$ antibodies to immunoprecipitate all newly incorporated nucleosomes. Following IP, Western blot analysis probing with anti-FLAG demonstrated that FLAG-H3.1 was present in the immunoprecipitated nucleosomes (bound fractions) at H3/H4 ratios equivalent to those in the input and unbound fractions (Fig. 8). This was observed with the wild-type FLAG-H3.1 cells, as well as the cells expressing the K56Q and K56R FLAG-H3.1 mutants. The fact that the ratios of FLAG-H3.1/H4 K5,12ac$_2$ detected in the chromatin samples are equivalent is an indication that the FLAG-histones are incorporated into chromatin along with the new H4. Thus, consistent with the results shown in Figure 7, incorporation of the mutant histones into chromatin seems to occur similarly to wild-type FLAG-H3.1. Notably, the type of mutation, and consequently the presence or absence of acetylation on residue 56, does not seem to have a detectable effect on the degree of incorporation.

Part III: H3K56 assembly on newly synthesized DNA

The goal of this part of the project was to determine whether the acetylation of the H3 K56 residue is important for the deposition of histone H3 onto newly synthesized DNA. By comparing the efficiency of incorporation of each mutant histone into newly replicated DNA to that of wild type, it can be asked whether the presence of the modification on K56 affects nucleosome assembly specifically at the replication fork.

The experiment involved HEK293 cells with the capability to inducibly express either wild type or mutant FLAG-H3.1 in the presence of doxycycline. Cells were synchronized in S-phase, where DNA replication occurs and histone H3.1 is deposited on new DNA. The same synchrony procedure described earlier was used, and the synchrony was monitored by flow cytometry. Wild type cells synchronized on average to 70% in S phase at 3.5 hours after release (Fig. 9a), K56Q mutants synchronized up to 81% in S (Fig. 9b), and K56R mutants synchronized to 76% in S phase at the end of the release.

Cells were induced with 2 ug/mL doxycycline for a total of 5 hours from the time of release from the G1/S block, in order to express and incorporate FLAG-H3.1. To show that the induction was successful, a sample of cells was acquired just prior to the addition of doxycycline, and the total cell extract was tested by

Western blot to demonstrate that before the inducing agent, there is only trace FLAG-H3.1 present within the cells (Fig. 10, Lane 1). Another sample of cells, equivalent to the first, was acquired following the 5 hours of induction, prior to harvesting the cells, to show that after incubating with the inducing agent, FLAG-H3.1 is now present (Fig. 10, Lane 2).

At 3.5 hours into induction, the cells were treated for 1.5 hrs with 750 uCi of tritiated thymidine, which is incorporated into DNA as it replicates. Thus, any radiolabeled chromatin is known to be new. As DNA replicates, it needs to be wrapped around nucleosomes, and if the incorporated nucleosomes contain newly synthesized histones, FLAG-H3.1 will be detected in nucleosomes surrounded by radiolabeled DNA.

Following treatment, cells were harvested and nuclei were isolated, since the interest is in the nuclear histones. Chromatin was digested by incubating with micrococcal nuclease (MNase), which digests of the linker DNA between the nucleosomes resulting fractionation of the chromatin (Fig. 11). Chromatin was precipitated and tested for presence of FLAG-H3.1.

Both the S1 and S2 fractions were immunoprecipitated using anti-FLAG M2 beads in order to pull down any FLAG-H3.1 present in the samples. Non-immune IgG beads were used as a negative control. The bound and unbound fractions

from each IP were analyzed in as follows: 75% of the bound fractions and 25% of the unbound fractions were used for scintillation counting in order to determine the amount of radioactive DNA pulled down with the FLAG- histones; the remaining 25% of the bound and 75% of the unbound fractions were used for Western blotting in order to confirm efficient immunoprecipitation (see *Sample Distribution* in ***Materials in Methods***).

Consistently, all of the FLAG-H3.1 in the IP samples was pulled down, as FLAG was only detected in the bound fractions of S1 and S2 and not in the unbound (Fig. 12). No FLAG was detected in the non-immune IgG samples.

In quantifying the amount of radioactive DNA pulled down with the FLAG-histones, each sample was counted in duplicate and averaged, and the values were normalized by subtracting background counts. It is important to note that the counts obtained for the M2 bound fractions in these experiments were consistently about one order of magnitude lower than seen with FLAG-H4 (C. Doughty, personal communication).

Using the data for the amounts of radioactivity in the total samples, the percentage of radiolabeled DNA that was pulled down was calculated by dividing the counts of the bound M2 sample by the total (bound + unbound). The percentage of radiolabeled DNA pulled down by non-specific sticking in the non-

immune negative control sample (calculated the same way) was subtracted from the M2. This number represents the percentage of FLAG-H3.1 that was incorporated into radiolabeled (new) DNA.

The calculated percentages averaged between 0.05% and 0.25% (Fig. 13). It is not surprising to see such small percentages, as the amount of FLAG-H3.1 in the cell is minute compared to the amount of native H3.1, resulting in a higher probability of native histones being incorporated. However, for the purposes of this project, the absolute amount of FLAG-H3.1 incorporated is not important, as the comparison is among the incorporation of the different mutants, all of which are FLAG-tagged.

Based on the average percentages of incorporation of wild-type compared to mutant H3.1, the S1 fractions seem to show very slight preferential incorporation in the K56Q mutant (Fig. 13a). The opposite is seen with the K56R mutant, where there seems to be no incorporation on newly synthesized DNA (inconsistent with earlier western blot results!). The calculated percentages of incorporation for the wild type and different mutants are very close to each other, and due to the low scintillation counts, a definite conclusion cannot be made without additional experiments. If these data prove to be statistically significant, they may suggest that acetylation at that residue might positively influence the

deposition of H3.1 at the replication fork, however further analysis is necessary to determine the reason for inconsistency with the K56R results.

The S2 fractions show insignificant differences in the amount of incorporation of the wild type and mutants (Fig. 13b). This is not entirely surprising, as the amount of radiolabeled DNA in the S2 fraction represents the DNA pulled down in an array of oligonucleosomes. Thus, single FLAG-H3.1s can pull down arrays of various lengths, and the amount of labeled DNA is not directly proportional to the amount of FLAG, as it would be in a mononucleosome. Here, low levels of K56R incorporation are observed, although they remain lower than wild type. This may indicate that K56R mutants do incorporate into chromatin, but much less efficiently than wild type H3.1. If this is true, it can explain why previous results detected K56R mutants in chromatin.

The fact that mimicking constitutive acetylation at H3 K56 increases chromatin assembly at the replication fork is not surprising. Chromatin assembly factor CAF-1, known to be involved in the deposition of replication-coupled histone H3 variants, has been shown to preferentially bind H3/H4 heterodimers containing H56-acetylated H3.1, and that this modification directly affects the binding affinity of H3 to this chromatin assembly factor (10, 22, 42-46, 115). Since the levels of native H3.1 acetylated at K56 are low in mammalian cells (125), a constitutive modification of this residue will increase the amount of H3.1 that has higher

affinity for CAF-1, and thus higher deposition will be observed. CAF-1 associates with DNA polymerase processivity factor, PCNA, at the replication fork, further supporting the deposition of H3.1 at the replication fork (41, 47, 48).

FIGURES

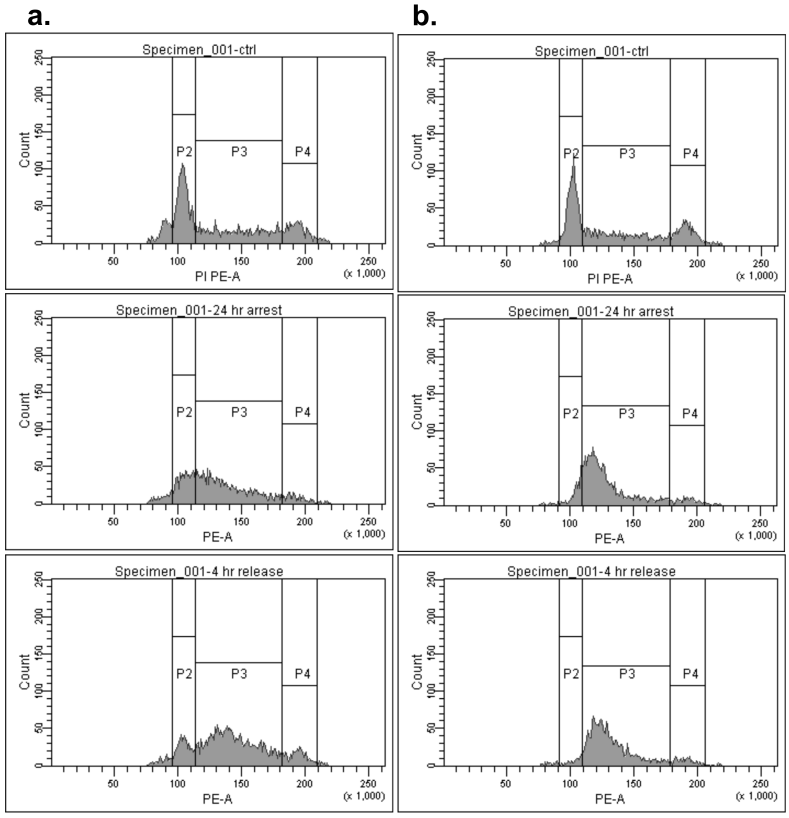

Figure 1: Flow cytometry analysis demonstrating progress of cell synchrony of HEK293 cells prior to cytoplasmic histone isolation for PTM analysis. Control samples consist of asynchronously cycling HEK293 cells encoding, but not expressing wild-type FLAG-H3.1 (a) and wild-type FLAG-H3.3 (b). The 24 hr arrest samples represent cells treated with 5 mM Thymidine for 24 hrs to block at the G1/S boundary. The 4 hr release samples represent cells treated with 30 uM deoxycitidine to release the block and 2 ug/mL doxycycline to induce FLAG-H3.1 expression.
(a). Of the control sample, 36% of cells are in G1 phase, 36% in S, and 17% in G2. After 24-hour arrest, 25% are in G1, 60% in S, 8 % in G2. At the end of the 4-hour release, 15% of cells are in G1, 68 are in S, 12% are in G2.
(b). Of the control sample, 38% of cells are in G1 phase, 38% in S, and 20% in G2. After 24-hour arrest, 10% are in G1, 80% in S, 8% in G2. At the end of the 4-hour release, 5% of cells are in G1, 84% in S, 8% in G2.

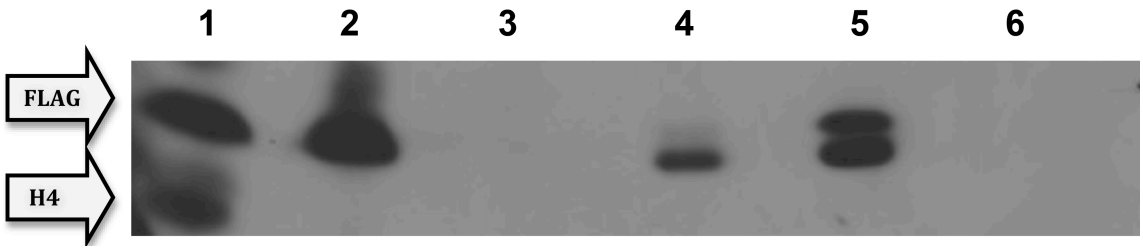

Figure 2: Western blot analysis of immunoprecipitation of S100 extract from S phase HEK293 cells expressing FLAG-H3.1. Lane content: 1. FLAG-H3.1 marker; 2. Input (10% of total S100 sample); 3. Unbound (100%); 4. Post-IP beads (10%) – beads immediately after IP; 5. Bound fraction after extraction from beads (10%); Upper band observed in Lane 5 (consistently observed in samples incubated with FLAG M2 beads), is thought to be resulting from the beads; 6. Post-extraction beads (90%) – beads following the extraction of FLAG-H3.1; Blot was probed with anti-FLAG (1:1000) / anti-pan-H4 (1:2000); 3.5-minute exposure.

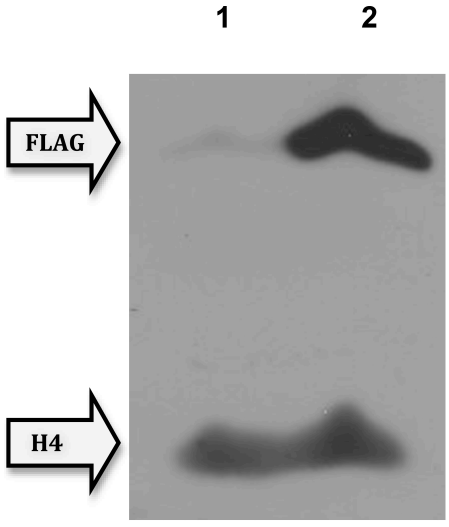

Figure 3: FLAG-H3.1 induction test by Western blot.
Total cell extract from (1) Uninduced and (2) Induced asynchronous HEK293 cells encoding wild-type FLAG-H3.1. Blot was probed with anti-FLAG (1:1000) / anti-pan-H4 (1:2000); 5-minute exposure.

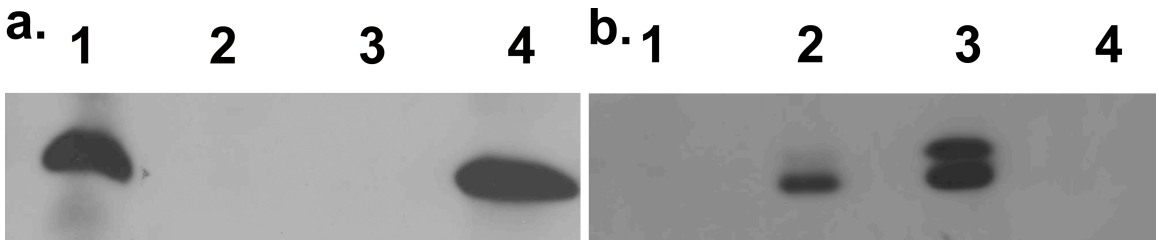

Figure 4: Western blot analysis of extraction efficiency of FLAG-histones from anti-FLAG M2 affinity beads.
(a). Unsuccessful extraction. Lane content: 1. FLAG-H3.1 marker; 2. Unbound (100%); 3. Bound fraction after extraction from beads (100%); 4. Post-extraction beads (100%) – beads following the extraction of FLAG-H3.1. Blot was probed with anti-FLAG (1:1000); 3-minute exposure.
(b). Successful extraction. Lane content: 1. Unbound (100%); 2. Post-IP beads (10%) – beads immediately after IP; 3. Bound fraction after extraction from beads (10%); 4. Post-extraction beads (90%) – beads following the extraction of FLAG-H3.1; Blot was probed with anti-FLAG (1:1000); 3.5-minute exposure.

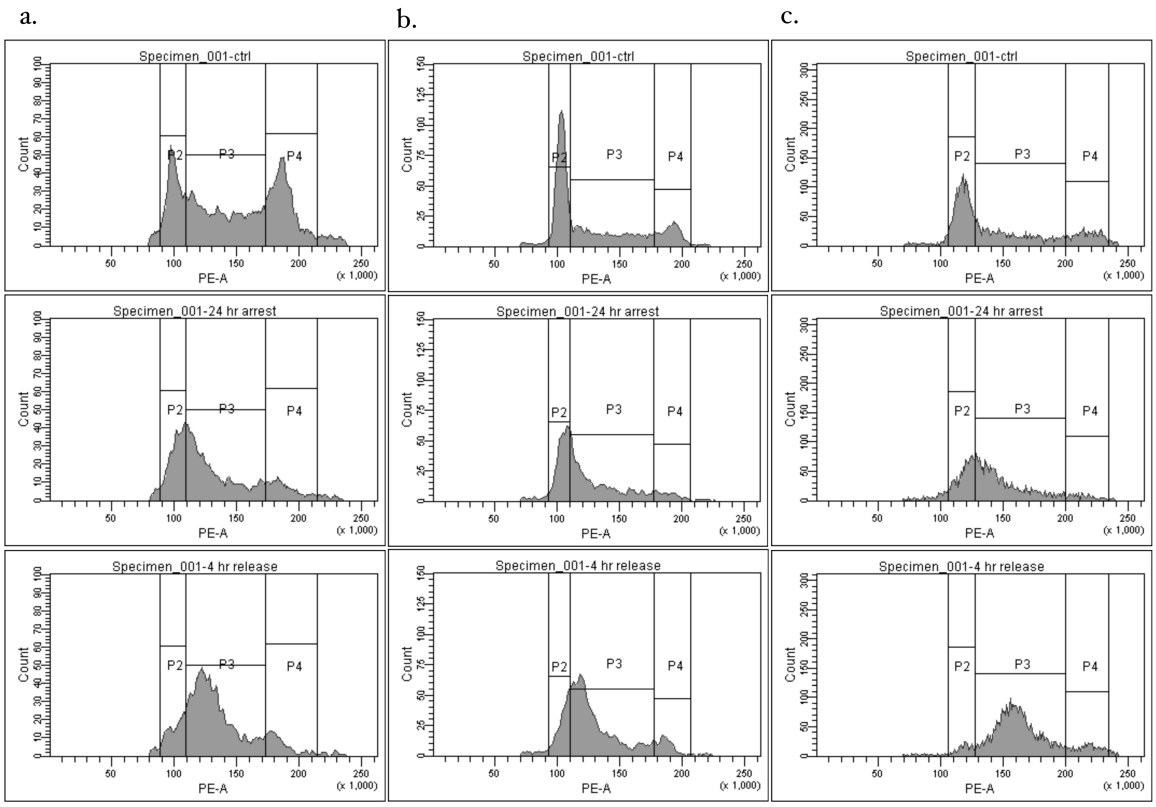

Figure 5: Flow cytometry analysis demonstrating progress of cell synchrony of HEK293 cells prior to ChIP for nucleosome assembly study. Control sample consists of asynchronously cycling HEK293 cells encoding, but not expressing, wild-type (a), K56Q (b), or K56R (c) FLAG-H3.1. The 24 hr arrest sample represents cells treated with 5 mM Thymidine for 24 hrs to block at the G1/S boundary. The 4 hr release sample represents cells treated with 30 uM deoxycitidine to release the block and 2 ug/mL doxycycline to induce FLAG-H3.1 expression.
(a) Control: 22% in G1, 40% in S, 32% in G2; 24-hr arrest: 28% in G1, 52% in S, 15% in G2; 4-hr release: 15% in G1, 70% in S, 12% in G2
(b) Control: 47% in G1, 33% in S, 16% in G2; 24-hr arrest: 34% in G1, 52% in S, 9% in G2; 4-hr release: 18% in G1, 68% in S, 10% in G2.
(c) Control: 43% in G1, 35% in S, 17% in G2; 24- hr arrest: 29% in G1, 58% in S, 6% in G2; 4-hr release: 7% in G1, 78% in S, 12%in G2.

Figure 6 (left): Efficiency of Mnase digestion.
Lane content: 1. S1 – mononucleosomes (0.4 OD); 2. S2 – oligonucleosomes (0.2 OD); 3. P – pellet (0.4 OD). Ethidium bromide staining.

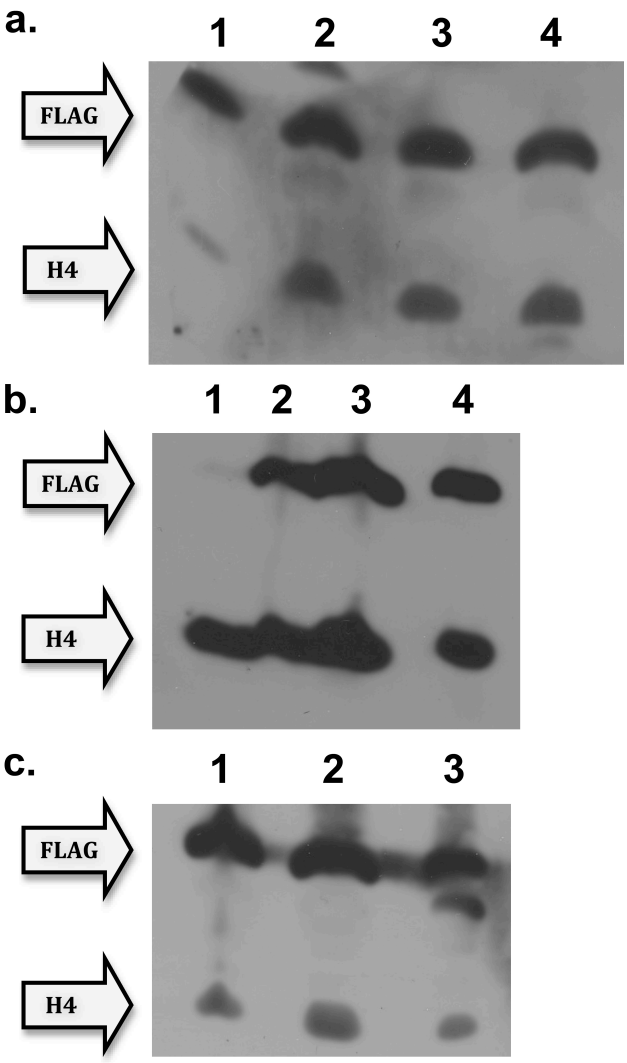

Figure 7 (right): Detection of wild-type FLAG-H3.1 (a), FLAG-H3.1 K56Q (b), and FLAG H3.1 K56R (c) in chromatin of HEK293.
(a) Lane content: 1. FLAG-H3.1 marker; 2. Total cell extract from HEK293 cells expressing wild-type FLAG-H3.1; 3. Nuclei; 4. chromatin fraction. Blot probed with anti-FLAG (1:1000) / anti-pan-H4 (1:2000); 5-minute exposure.
(b) Lane content: 1. Uninduced HEK293 cells encoding FLAG-H3 K56Q; 2. Total cell extract from cells expressing FLAG-H3.1 K56Q; 3. Nuclei; 4. Chromatin fraction. Blot probed with anti-FLAG (1:1000) / anti-pan-H4 (1:1000); 1-minute exposure.
(c) Lane content: 1. Total cell extract from cells expressing FLAG-H3.1 K56R; 2. Nuclei; 3. Chromatin fraction. Blot was probed with anti-FLAG (1:1000) / anti-pan-H4 (1:1000); 1.5-minute exposure.

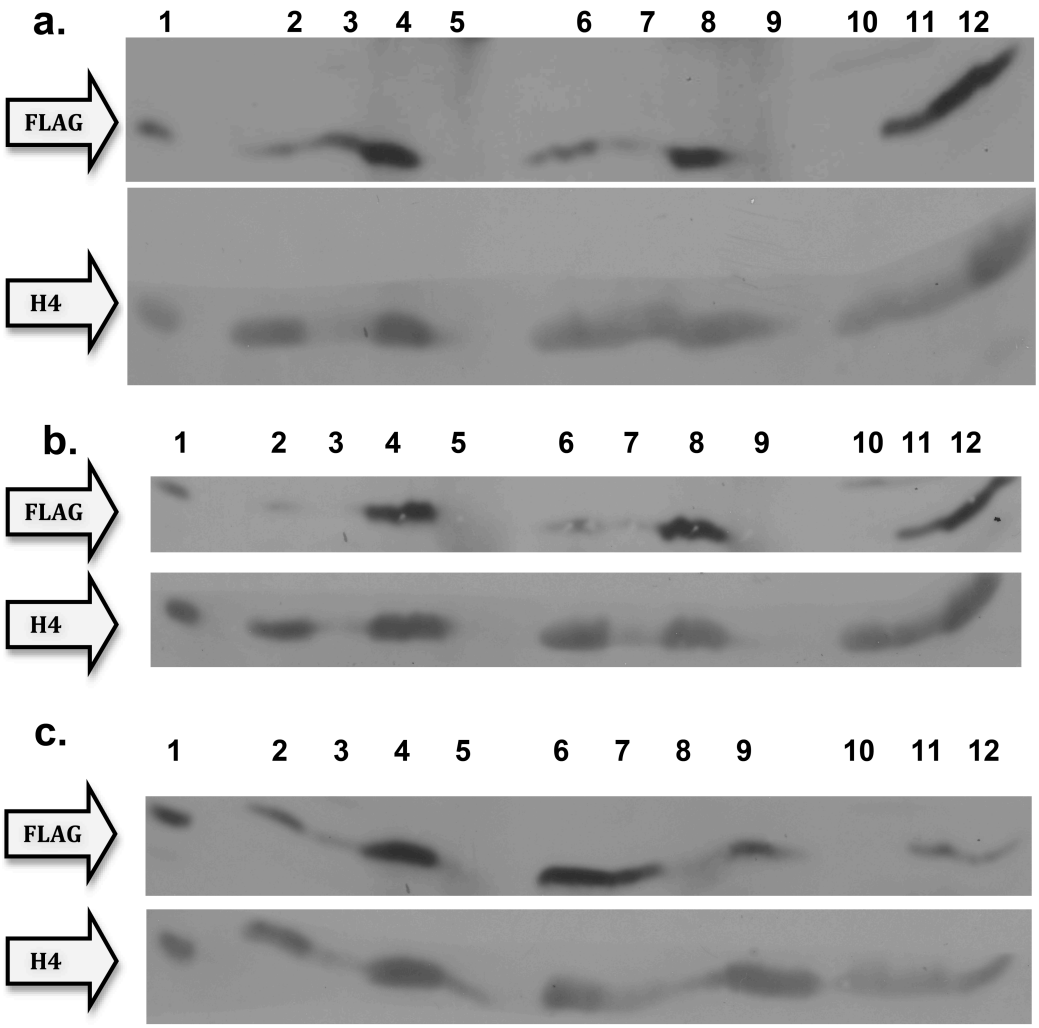

Figure 8: Detection of wild-type FLAG-H3.1 (a), FLAG-H3.1 K56Q (b), and FLAG H3.1 K56R (c) in chromatin of HEK293 cells following immunoprecipitation with anti-H4 K5,12 ac_2.
Lane content (a-b): 1. FLAG-H3.1 marker; 2. S1 input; 3. S1 unbound; 4. S1 Bound; 5. S1 non-immune; 6. S2 input; 7. S2 unbound; 8; S2 Bound; 9. S2 non-immune; 10. Uninduced; 11. Total cell extract; 12. Nuclei;
Lane content (c): 1. FLAG-H3.1 marker; 2. S1 input; 3. S1 unbound; 4. S1 Bound; 5. S1 non-immune; 6. S2 input; 7. S2 unbound; 8; S2 non-immune; 9. S2 Bound; 10. Uninduced; 11. Total cell extract; 12. Nuclei;
Top blots are probed with anti-FLAG (1:1000), bottom blots are probed with anti-H4 K5,12ac_2 (1:5000); 10-sec exposure.

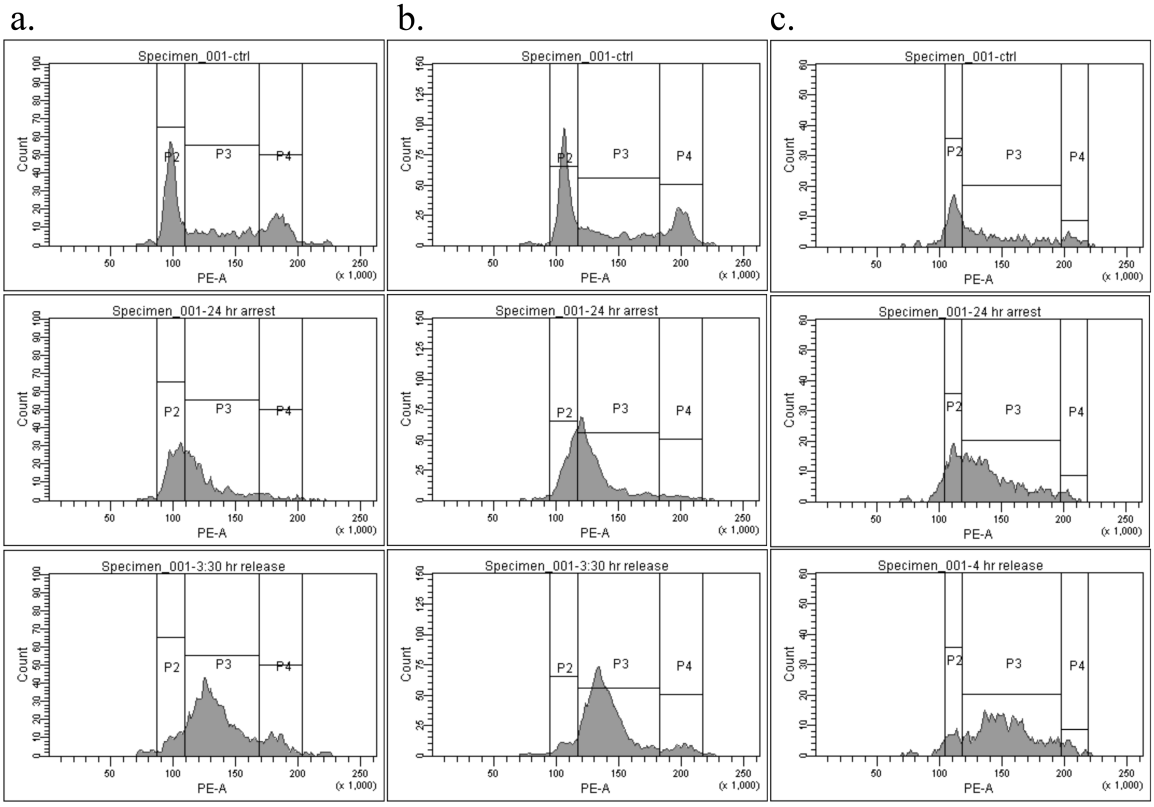

Figure 9: Flow cytometry analysis demonstrating progress of cell synchrony of HEK293 cells prior to radiolabeling experiment for replication fork analysis. Control samples consist of asynchronously cycling HEK293 cells encoding, but not expressing wild type (a), K56Q (b), or K56R (c) FLAG-H3.1. The 24 hr arrest samples represent cells treated with 5 mM Thymidine for 24 hrs to block at the G1/S boundary. The 3.5 hr release samples represent cells treated with 30 uM deoxycitidine to release the block and 2 ug/mL doxycycline to induce FLAG-H3.1 expression.
(a). Control: 41% in G1, 29% in S, 25% in G2; 24-hr arrest: 39% in G1, 50% in S, 7% in G2; 3.5-hr release: 11% in G1, 70% in S, 14% in G2.
(b). Control: 43% in G1, 29% in S, 26% in G2; 24-hr arrest: 30% in G1, 62% in S, 6% in G2; 3.5-hr release: 8% in G1, 81% in S, 9% in G2.
(c). Control: 30% in G1, 48% in S, 11% in G2; 24-hr arrest: 22% in G1, 64% in S, 5% in G2; 4-hr release: 11% in G1, 76% in S; 7% in G2.

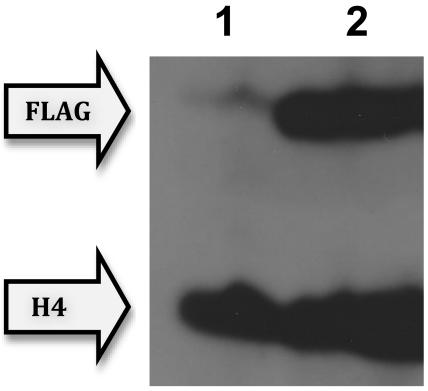

Figure 10: FLAG-H3.1 induction test by Western blot.

Total cell extract from 1. Uninduced and 2. Induced S-phase HEK293 cells encoding FLAG-H3.1 K56Q. Blot probed with anti-FLAG (1:1000) / anti-pan-H4 (1:1000); 1-minute exposure.

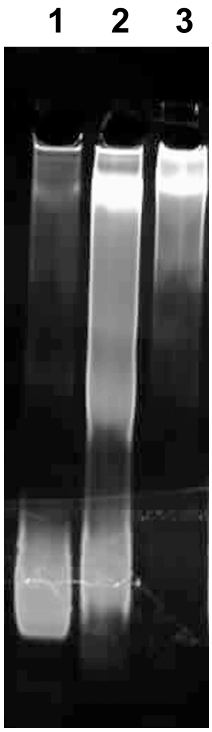

Figure 11: Efficiency of Mnase digestion.
Lane content: 1. S1 – mononucleosomes (0.4 OD); 2. S2 – oligonucleosomes (0.2 OD); 3. P – pellet (0.4 OD). Ethidium bromide staining.

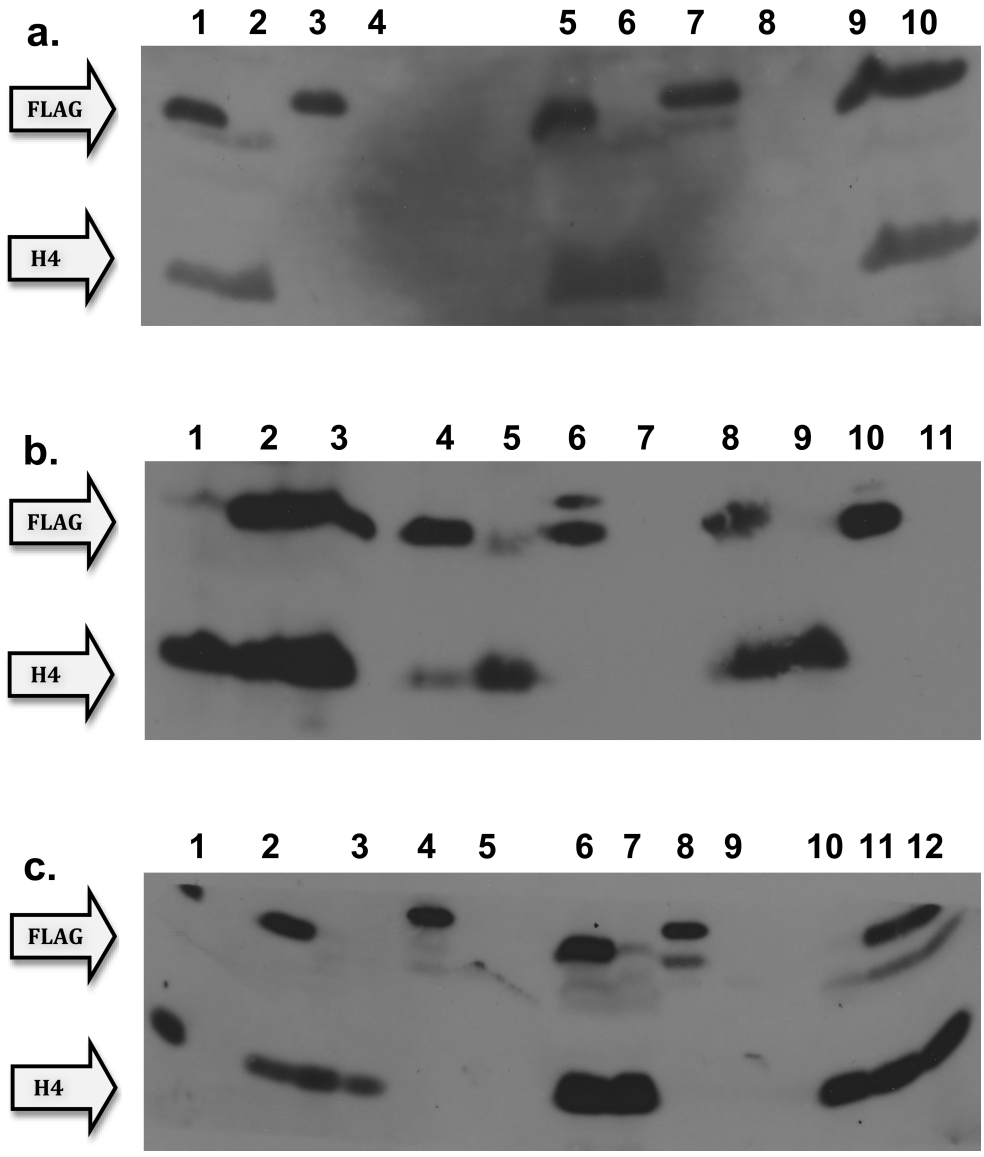

Figure 12: Western blot analysis of immunoprecipitation of thymidine radiolabeled mono- and oligonucleosomes from S-phase HEK293 cells expressing wild type FLAG-H3.1 (a), FLAG-H3.1 K56Q (b), and FLAG-H3.1 K56R (c).
(a). Lane content: 1. S1 input; 2. S1 unbound (75%); 3. S1 bound (25%); 4. S1 non-immune IgG (25%); 5. S2 input; 6. S2 unbound (75%); 7. S2 bound (25%); 8. S2 non-immune IgG (25)%; 9. Induced total cell extract; 10. Nuclei. Blot probed with anti-FLAG (1:1000) / anti-pan-H4 (1:2000); 5-minute exposure.

(b). Lane content: 1. Uninduced; 2. Induced; 3. Nuclei; 4. S1 input; 5. S1 unbound (75%); 6. S1 bound (25%); 7. S1 non-immune IgG (25%); 8. S2 input; 9. S2 unbound (75%); 10. S2 bound (25%); 11. S2 non-immune IgG (25%). Blot probed with anti-FLAG (1:1000) / anti-pan-H4 (1:1000); 1-minute exposure.
(c). Lane content: 1. FLAG-H3.1 marker; 2. S1 input; 3. S1 unbound (75%); 4. S1 bound (25%); 5. S1 non-immune IgG (25%); 6. S2 input; 7. S2 unbound (75%); 8. S2 bound (25%); 9. S2 non-immune IgG (25%); 10 uninduced; 11. Induced; 12. Nuclei. Blot probed with anti-FLAG (1:1000)/ anti-pan-H4 (1:1000); 1-minute exposure.

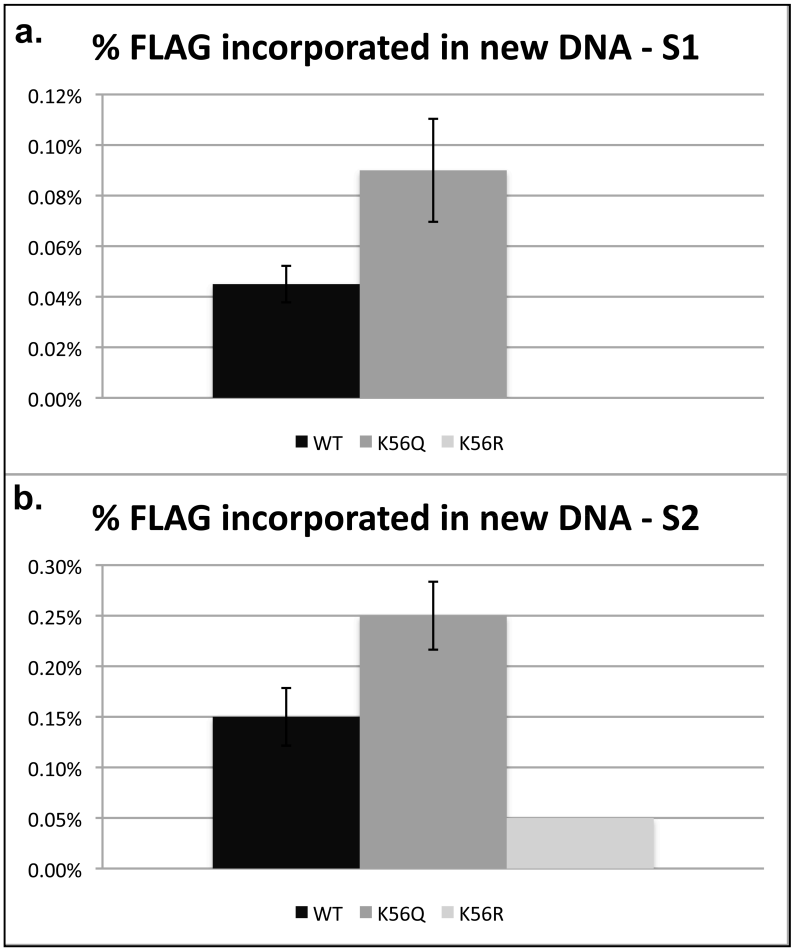

Figure 13: Average percentage of FLAG-H3.1 histones incorporated into newly synthesized DNA. Average of the percentages calculated from each experiment, based on the amount of radiolabeled DNA in the FLAG-bound fraction compared to the total IP amount. Equivalent to the percentage of FLAG-H3.1 incorporated into newly synthesized DNA. Error bars represent standard error. (a) based on data from S1 mononucleosomes sample; (b) based on data from S2 oligonucleosome sample.

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