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Generation and management of excess histones during the cell cycle

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1. ABSTRACT

Histones are essential proteins that package the DNA in all eukaryotes into chromosomes. However, histones can accumulate upon a decrease in DNA synthesis that occurs at the end of S-phase or following replication arrest. These positively charged histones can associate non-specifically with the negatively charged DNA and other cellular biomolecules, impairing their normal function. Hence, cells have evolved numerous strategies to limit the generation of excess histones and prevent deleterious effects due to their accumulation. Such strategies for histone regulation are discussed here, with particular emphasis on recent studies that implicate the DNA damage checkpoint kinases in the regulation of histone levels, especially in response to replication inhibition. We have also focused upon the recently discovered regulatory mechanism involving histone proteolysis in the budding yeast. Additionally, we speculate that cells may possess a surveillance mechanism for sensing histone levels, particularly in the G1 and S-phases of the cell cycle. Proper regulation of histone levels has major implications for the maintenance of epigenetic marks on chromatin, genomic stability and the packaging of sperm DNA.

2. INTRODUCTION: THE NEED FOR REGULATING HISTONE LEVELS IN CELLS

The DNA in all eukaryotic cells is wrapped around basic histone proteins to form nucleoprotein filaments called chromatin (1). This packaging of DNA into chromatin not only enables the cell to fit its lengthy genome inside the nucleus, but also provides it with the means to regulate access to the information contained within the DNA (2). Two molecules each of the core histones H4, H3, H2A and H2B form an octameric protein core around which 147bp of DNA is wrapped to give rise to the nucleosome core particle that forms the basic repeating unit of chromatin (3). In higher eukaryotes, one molecule of the linker histone H1 associates with each nucleosome core particle to seal two turns of the DNA around the nucleosome (4). Cells exhibit a tight coordination between histone and DNA synthesis. Histones are synthesized mainly during the S-phase when they are rapidly deposited onto the newly replicated DNA with the help of histone chaperones (5, 6). The uncoupling of DNA synthesis and histone deposition during S-phase leads to a loss of viability in the budding yeast *Saccharomyces cerevisiae* (7). Inhibition of histone synthesis triggers

spontaneous DNA damage and S-phase arrest in human cells (8, 9). Further, yeast cells lacking optimal amounts of histone H4 are prone to genomic instability (10). Genomic instability is characterized by the increased rate of acquisition of alterations in the genome and is associated with most if not all human cancers (11, 12). Hence, S-phase cells need to maintain an optimal supply of newly synthesized histones to ensure rapid assembly of nucleosomes behind the replication fork. However, when present in excess, the positively charged histones can potentially interact non-specifically with the negatively charged DNA as well as other negatively charged cellular components and interfere with many cellular processes, particularly those that require access to the genetic information contained within the DNA. Even a slight stoichiometric excess of histones over DNA is sufficient to trigger chromatin aggregation and block transcription as demonstrated by *in vitro* experiments (13). Histone overexpression greatly increases the incidence of mitotic chromosome loss in yeast (14). Further, mutations in *Drosophila* that cause inappropriate accumulation of histone mRNAs are lethal (15, 16). Excess histone accumulation can occur due to the slowing down of DNA synthesis at the end of every S-phase or when DNA synthesis has been inhibited due to DNA damage or replication arrest during S-phase (17, 18). However, despite the huge demand for histones for chromatin assembly, significant pools of "free" histones are not found in cells under normal growth conditions. This is because free histone pools can be extremely harmful to cells. During each S-phase, large quantities of replication-coupled histones are synthesized for bulk chromatin assembly. At the same time, smaller quantities of specialized histone variants are also synthesized that need to be incorporated into chromatin either outside of S-phase (such as the replacement variant H3.3 or Htz1), or into specialized chromatin as in the assembly of centromere specific H3 variant Centromeric Protein-A (CENP-A) in centromeres. An excess of replication-coupled histone variants could swamp out the minor variants and displace them from chromatin with a disastrous outcome for the cells. Another reason to avoid having large pools of histones that are not incorporated into nucleosomes is that these histones could potentially sequester crucial chromatin modifying enzymes and prevent them from acting on their normal physiologically relevant substrates. Not surprisingly, yeast cells lacking subunits of certain chromatin modifying complexes are sensitive to histone overexpression ((19, 20) and Singh and Gunjan, unpublished results). Further, eviction of histones from inducible promoters has become established as a general feature of transcriptional initiation (21, 22). If large pools of free histones existed, it is possible that the machinery responsible for the transcriptional eviction of histones could get clogged up with free histones resulting in disastrous consequences. Consistent with this hypothesis, yeast cells were found to accumulate excess histones when the normal metabolism of transcriptionally evicted histones was abolished (23). Hence, cells need to achieve a very delicate balance between histone synthesis and the demand for chromatin assembly and they have evolved a variety of transcriptional, posttranscriptional and posttranslational strategies to achieve this balance throughout the cell cycle. This review will focus on these strategies, with particular emphasis on the contribution of DNA damage checkpoint kinases in the regulation of histone levels in the budding yeast.

3. SOURCES OF EXCESS FREE HISTONES

3.1. Multiple histone genes

All eukaryotes have multiple genes encoding each histone protein, some of which are non-allelic variants (24). For example, the unicellular eukaryote budding yeast has two gene copies encoding each core histone protein, while the diploid human genome contains 28 copies encoding histone H4 alone (25). Fruit flies, *Xenopus* and sea urchins carry hundreds of genes encoding each core histone (24). Why do eukaryotes carry multiple histone genes? Since histones are essential for viability, one possibility is that the multiple histone genes simply serve as a backup in case of inactivating mutations in one or more genes (7). However, this is unlikely as the majority of genes essential for viability in eukaryotes have no additional gene copies to serve as backup. A second possibility is that the multiple histone genes may have some unique functions. This appears to be the case for non-allelic core histone variants, which are usually present as single gene copies that often serve as replacement histones and are expressed throughout the cell cycle. Histones are primarily synthesized in S-phase and deposited by chromatin assembly factors or histone chaperones on to the replicating DNA to form chromatin in a process known as chromatin assembly (6). A third possibility could be that the high demand for histones for chromatin assembly on newly replicated DNA during S-phase can only be met by the simultaneous expression of multiple histone genes. Cells appear to have very limited quantities of histone chaperones compared to the number of nucleosomes that must be assembled. Nucleosome assembly on newly replicated DNA occurs about 200bp behind the replication fork, which is almost as soon as enough DNA has been synthesized to be incorporated into a single nucleosome (26). Hence, to allow the histone chaperones to be recycled efficiently after each round of histone deposition on the DNA, a high concentration of histone proteins is required, which could be readily achieved by high levels of expression from multiple histone genes. This might indeed be the case for eukaryotes with extensive genomes and those with very short embryonic cell cycles as in *Xenopus*. However, it has been demonstrated that both budding yeast and chickens only require half their complement of histone genes for viability (27, 28). In fact, our previous studies have revealed that the full complement of histone genes in the budding yeast synthesize an excess of histones which are deleterious to cells and render them more sensitive to a variety of DNA damaging agents (17). Further, a reduction in histone gene dosage appears to be beneficial for yeast cells by making them resistant to DNA damaging agents. Whatever the reasons behind the existence of multiple histone genes in eukaryotes, it is quite likely that they synthesize histones in excess of what is required for chromatin assembly during S-phase (Figure 1). This line of thinking is amply supported by experimental data regarding the number of histone molecules per cell in the budding

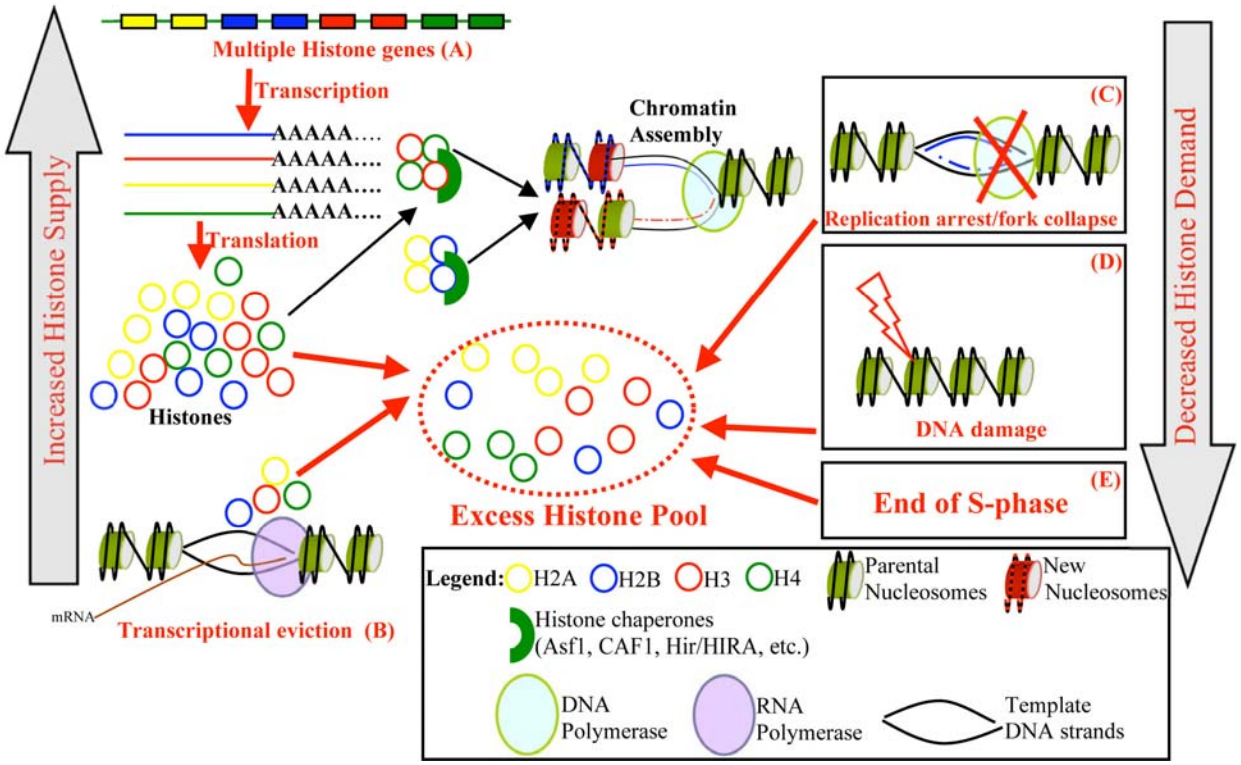

Figure 1. Potential sources of excess histones. Red lettering and red arrows denote processes that can potentially contribute to the generation of excess histones in the cells. Excess histones result from either an increase in histone supply or a decrease in the demand for histones. (A) Excess histones could result from the simultaneous expression of several copies of histone genes that synthesize more histones than what is required for chromatin assembly. (B) They could also arise as a result of histones evicted due to the passage of RNA polymerase through a chromatin template during transcription. (C) Replication arrest results in a drop in the demand for histones which would then accumulate. (D) DNA damage results in the localized remodeling of chromatin resulting in the loss of histones from the damaged site, presumably to allow access to repair factors. (E) Due to a drop in the rate of DNA synthesis and the concomitant low demand for chromatin assembly at the end of S-phase, excess histones may accumulate. See text for details.

yeast. Theoretically, with a genome size of 12,495,682bp and a nucleosome every 160bp, each budding yeast cell would have about 78,100 nucleosomes and require only 156,200 molecules of each core histone to package its entire genome into chromatin. However, estimates for the number of molecules of each core histone per cell based on experimental evidence are considerably higher and range from a high of 1,205,000 molecules of histone H4 (an 8-fold excess over the required amount) to a low of 461,000 molecules of histone H3 (still a 3-fold excess) per cell (29). Even if these estimates for the number of histone molecules per yeast cell are only roughly correct, they further highlight the crucial role played by posttranslational mechanisms in regulating histone levels, since *in vitro* data suggest that even a slight stoichiometric excess of histones over DNA is sufficient to trigger chromatin aggregation and block transcription (13). In fact, to ensure that all the genome is fully packaged into chromatin following DNA replication, the default state in eukaryotes may indeed be to synthesize excess histones during S-phase and get rid of unincorporated histones at the end of S-phase.

3.2. Transcriptionally evicted histones

The presence of histone proteins on the DNA is likely to impose a significant hindrance to the passage of the transcriptional machinery through chromatin. It is now widely accepted that this problem is resolved *in vivo* by the eviction of histones from the gene by the transcribing RNA polymerase (21, 30). In fact, the most highly transcribed genes, such as the rDNA genes, appear to be devoid of nucleosomes (31). However, the fate of these evicted histones is far from being clear. One possibility is that these histones are recycled to re-establish a proper chromatin structure on the template DNA following the passage of the RNA polymerase. Alternatively, these evicted histones may be targeted for degradation and replaced with histone variants that specifically mark transcriptionally active chromatin. Factors that specifically facilitate transcription from chromatin, such as the FACT (Facilitates Chromatin Transcription) complex may do so by either helping with the disassembly of histones, keeping the histones sequestered during that round of transcription, reassembling the chromatin following completion of transcription, or any combination of the above (32). The histone chaperone Asf1 (Anti Silencing Function 1) may

also participate in the disassembly and reassembly of a proper chromatin structure in the context of transcription (33). Additional factors contributing to this process may include the budding yeast histone chaperone HIR (Histone Regulation) complex and its human counterpart HIRA that have been shown to be involved in replication independent chromatin assembly (34). Recent evidence from a collaborative study between our laboratory and those of Drs. Sebastián Chávez and Vincent Géli has indeed uncovered a role for the budding yeast FACT complex subunit Spt16 in preventing the accumulation of transcriptionally evicted histones (23). Further, overexpression of Hir2, a component of the yeast HIR complex appeared to partially suppress the defects arising due to the loss of spt16 function. This study underscores the importance of mechanisms that ensure that transcriptionally evicted histones are dealt with promptly to avoid the deleterious effects of excess histone accumulation.

3.3. Histone accumulation upon replication arrest

The vast majority of histone synthesis is strictly coupled to DNA synthesis and occurs during S-phase (24). This ensures the rapid incorporation of the newly synthesized DNA into chromatin. However, replication inhibitors lead to a drastic drop in DNA synthesis and chromatin assembly, resulting in an accumulation of unincorporated newly synthesized histones (18). DNA damage during S-phase also results in DNA replication slowing down or coming to a standstill, either due to the physical impediment posed by the DNA lesions to the passage of the DNA polymerase, or due to the activation of the intra-S-phase DNA damage checkpoint that prevents the firing of new origins (35, 36). The accumulation of histones on histone chaperones in response to DNA damage and replication arrest has been demonstrated in the budding yeast, as well as in cultured mammalian cells in response to replication inhibition with hydroxyurea (17, 37). Although cells are unlikely to suffer frequent replication arrest due to such replication inhibitors under normal conditions, replication inhibitors are an important class of anti-cancer drugs and their effects on histone accumulation may impact their therapeutic effects.

3.4. Histone removal during DNA damage, repair and recombination

The packaging of DNA with the help of histones to form chromatin reduces/regulates its accessibility to processes and factors that require access to the DNA or to the information contained within. The accessibility problem is perhaps most acute when DNA damage occurs in the context of chromatin and repair factors have to gain access to the damaged site to carry out necessary repairs. DNA damage may result in the marking of the chromatin adjacent to the damaged site with specific posttranslational modifications on the histones, such as the phosphorylation of gamma H2A, extending over several megabases (38). Recent evidence suggests that histones may be evicted locally from the site of a DNA double strand break (DSB) to allow access to the repair machinery (39). It is unclear at present if other forms of DNA damage also involve removal of histones from the site of the break, but preliminary evidence suggest that this may indeed be the case since overexpression of histones in the budding yeast results in increased sensitivity to a variety of DNA damaging agents ((17) and Liang and Gunjan, in preparation). The extent of histone removal is likely to vary depending on the type of DNA damage and the repair pathway used. For example, a DSB can be repaired via the Non-Homologous End Joining (NHEJ) pathway wherein the broken ends are simply ligated together, thus requiring minimal, if any, removal of histones. Alternatively, the DSB can be repaired by the Homologous Recombination (HR) pathway involving extensive strand exchange and thus require extensive removal/remodeling of nucleosomes. The fate of histones removed from the site of DNA damage is unclear at present and these histones may either be degraded or sequestered by histone chaperones and subsequently reassembled after DNA repair is completed. Since a typical mammalian cell experiences several DSBs per cell per day, it is likely that DNA repair and recombination processes contribute small to moderate amounts of free histones that need to be tackled by the appropriate cellular machinery promptly.

4. STRATEGIES EVOLVED BY CELLS TO DEAL WITH EXCESS FREE HISTONES

Cells have evolved a number of surveillance mechanisms to preserve genomic integrity. Defects in these mechanisms increase the incidence of mutations and genome instability, which are implicated in oncogenesis (40). Upon DNA damage or replication stress, checkpoint responses arrest the cell cycle to provide additional time for efficient repair (41). Cell cycle progression resumes after the damage has been repaired. In the budding yeast, two essential protein kinases, Mec1 and Rad53, play multiple roles in the DNA damage and replication arrest response (42, 43). Mec1, the central DNA damage response kinase in the budding yeast belongs to the conserved family of phosphatidylinositol-3 kinases (PI-3 kinases) and is related to fission yeast Rad3, and human ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia-Rad3-related). Rad53, the main effector kinase in the DNA damage response pathway, has homologs in fission yeast (Cds1) and humans (CHK2). All DNA damage responses of Rad53 are strictly dependent upon the upstream kinase Mec1 or in some instances upon another related PI-3 kinase Tel1 (44). DNA damage leads to the Mec1/Tel1 dependent hyperphosphorylation of Rad53 and a dramatic increase in its kinase activity. Upon activation in response to DNA damage, Rad53 triggers a phosphorylation mediated cascade of events that lead to cell cycle arrest, activation of downstream effectors such as the Dun1 kinase, expression of DNA repair genes and stimulation of DNA repair. Mec1 and Rad53 protect cells against DNA damage via a number of other functions. DNA damage during S-phase slows down the rates of replication fork elongation independently of checkpoint kinases and triggers a Rad53/Mec1-dependent block in the firing of late origins (45). As a result, DNA damage leads to an abrupt decrease in DNA synthesis (35). In addition, Mec1 and Rad53 are required to prevent both spontaneous and DNA damage-induced collapse of replication forks, presumably through their

ability to phosphorylate replication and repair proteins at stalled replication forks (36, 46). The essential function of Mec1 and Rad53 in the budding yeast is to promote deoxyribonucleotide triphosphate (dNTP) production during S-phase to coincide with DNA replication. This is achieved via phosphorylation and degradation of Sml1, a stoichiometric inhibitor of ribonucleotide reductase (Rnr) (47). The lethality, but not the DNA damage sensitivity of *mec1* and *rad53* mutants is rescued by elevating dNTP levels through disruption of the *sml1* gene or overexpression of Rnr large subunits (46, 47). Most of the known functions of Rad53 in the DNA damage response are dependent upon its activation by the upstream kinases Mec1 and Tel1. However, the fact that *rad53 sml1*double deletion mutants have a slow growth phenotype, which is absent in *mec1 sml1* double deletion cells, argues that Rad53 has a Mec1-independent role during normal cell cycle progression (47).

4.1. The role of checkpoint kinases in the DNA damage and replication arrest response pathway of budding yeast

The coupling of histone synthesis to DNA synthesis has been known for half-a-century now (48, 49). Upon inhibition of replication, histone transcripts were shown to be downregulated due to a combination of transcriptional inhibition of histone genes and enhanced histone mRNA degradation in mammalian cells and largely due to transcriptional inhibition in the budding yeast, though there may be some contribution from the posttranscriptional regulation of histone mRNAs (19, 50-53). However, the molecular details of the pathways that lead to the downregulation of histone mRNAs and the factors involved therein are only now being elucidated in mammalian cells and in the budding yeast (19, 54-57). Differences in the details of histone transcript regulation between the budding yeast and metazoans are to be expected as budding yeast histone transcripts are polyadenylated, while most metazoan histone transcripts are not polyadenylated, but end in a 3' stem loop structure. This stem loop is recognized by the Stem Loop Binding Protein (SLBP) which facilitates histone pre-mRNA processing as well as its subsequent translation (58, 59). In recent years, the roles of checkpoint kinases in the replication arrest or DNA damage dependent transcriptional repression of histone genes has received a significant amount of attention. Following ionizing radiation treatment of human cells which mainly results in DNA strand breaks, an ATM, p53 and p21 dependent G1 checkpoint is activated, which results in repression of histone gene transcription due to the loss of phosphorylation on NPAT, a known activator of histone genes and a cyclin E/Cdk2 substrate. On the other hand, ATR along with Upf1 ((Up frameshift 1), a regulator of the nonsense-mediated decay pathway that degrades mRNAs with premature stop codons arising from genes with introns), was shown to be required for the degradation of histone transcripts following replicational stress (56, 60). A 3' exonuclease designated as 3'hExo has also been identified in human cells that interacts specifically with 3' ends of histone mRNAs and may be capable of initiating their degradation (61). More recently, a role for another PI-3 kinase DNA-PK (DNA activated Protein Kinase) has been suggested in the downregulation of histone transcripts in response to replication inhibition with aphidicolin in cultured human cells (57). With the exception of p53, NPAT and Upf1, the downstream effectors of histone transcript regulation by these checkpoint kinase signaling pathways have not yet been identified in metazoans and would benefit greatly from further studies.

In budding yeast, inhibition of DNA replication triggers concerted transcriptional repression of the four gene pairs encoding core histones (27). Three of the four divergent histone gene promoters contain a *neg*ative cis-acting element (*NEG*) that is required for histone gene repression both outside of S-phase and in response to drugs that interfere with replication. A group of four functionally related Histone Regulation proteins (Hir1, Hir2, Hir3 and Hpc2) act together with the histone H3/H4 chaperone Asf1 to promote histone gene repression through the *NEG* element outside of S-phase and in response to replication arrest (27, 62). The ATP-dependent multi-subunit chromatin remodeling factor RSC (Remodel the Structure of Chromatin) has been found to be associated with the histone gene promoters when they are repressed, however a cause and effect relationship has not yet been established (63). Although earlier studies had suggested that histone transcript downregulation during the cell cycle and upon replication arrest in the budding yeast is mainly due to transcriptional repression of the histone genes, a recent study indicates that the regulation of histone mRNA stability via polyadenylation by the poly (A) polymerases Trf4/Trf5 and the exosome (a complex of 3'-5' exoribonucleases) may also play a role (19, 53, 64). A role for the budding yeast checkpoint kinase Rad53 in the repression of cyclin genes has been reported in response to DNA damage in the G1 phase of the cell cycle (65, 66). In our laboratory, we have explored the potential roles of yeast DNA damage checkpoint kinases in the repression of histone mRNAs caused by genotoxic agents that interfere with replication. We have uncovered that kinase activity of Rad53 and its upstream kinases Mec1/Tel1 are necessary for histone gene repression in response to genotoxic agents that interfere with DNA replication (54). We are currently dissecting the molecular details of how checkpoint kinases instigate the downregulation of histone transcripts. It will be interesting to see how the interplay between the checkpoint kinases, the Hir proteins, the RSC chromatin remodeler and the Asf1 histone chaperone leads to repression of the histone gene promoters.

4.2. Posttranslational regulation of histone protein levels through regulated proteolysis

In stark contrast to the numerous studies over the past half-a-century on the transcriptional and posttranscriptional regulation of histone mRNAs, until recently very little was known about the posttranslational regulation of histones. This was largely due to early studies that suggested histones to be extremely stable proteins with half-lives up to 4-5 months depending on the histone (67, 68). However, these half-lives probably reflect the stability of chromatin bound histones, as it has been recently shown that excess non-chromatin associated histones are rapidly

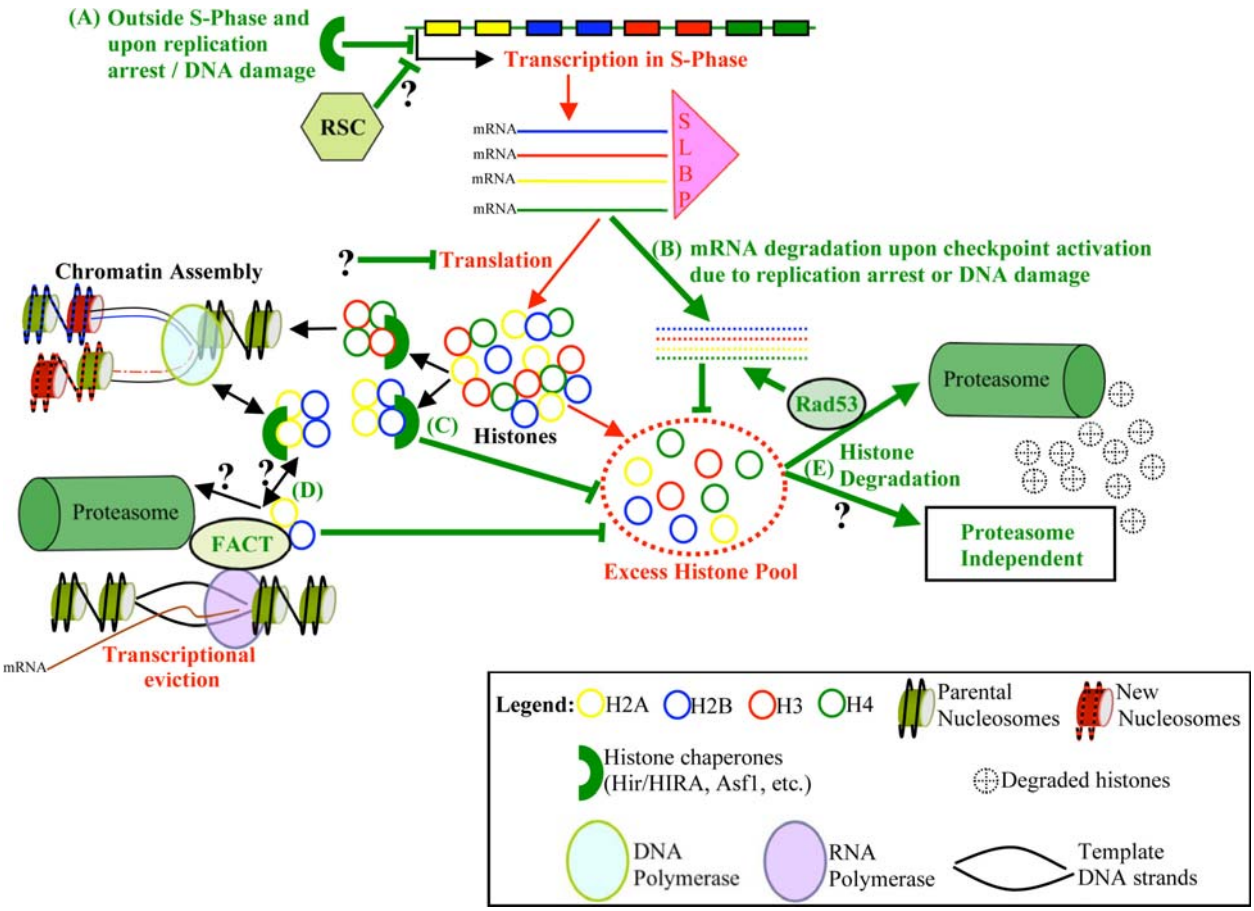

Figure 2. Management of excess histones. Green lettering and green arrows denote processes that contribute to the management of excess histones in the cells. Question marks denote hypothetical or suspected pathways whose significance has not yet been demonstrated. (A) The bulk of histone synthesis coincides with DNA replication and is limited to the S-phase of the cell cycle. HIR proteins in yeast and their homolog HIRA in human cells repress the majority of histone genes outside of S-phase and upon replication inhibition or DNA damage. Other factors such as Asf1 in yeast may help in the repression of histone genes. (B) Histone mRNAs are downregulated upon the activation of the replication arrest or DNA damage checkpoint. It is not clear to what extent the regulation of translation of histone mRNAs contributes to preventing histone excess, although the metazoan SLBP is required for efficient translation and is upregulated in late G1 through S-phase and degraded as cells enter G2. (C) Histone chaperones buffer excess histones, which may be the first line of defense against transient histone excess. (D) The FACT complex either by itself or in cooperation with histone chaperones may deal with transcriptionally evicted histones by either sequestering them for subsequent reassembly or potentially targeting them for degradation via its interactions with the proteasome sub-particles. (E) Excess histones are targeted for degradation by the Rad53 kinase in budding yeast, presumably via an ubiquitylation and proteasome dependent pathway. However, degradation via ubiquitin and proteasome independent pathways is also possible. The existence of a posttranslational mechanism for the regulation of histone protein levels has not yet been demonstrated in metazoan cells. See text for details.

degraded with a half-life of around 30-40 minutes in the budding yeast (17). This degradation of histones was dependent upon the DNA damage checkpoint kinase Rad53 and this process was shown to be important not only for the removal of histones left-over after completion of DNA replication at the end of every S-phase, but also to prevent excess histone accumulation upon DNA damage or replication arrest (Figure 2). The regulated destruction of many cellular proteins takes place via a cascade of reactions initiated by the phosphorylation of the proteins, followed by their polyubiquitylation and subsequent degradation by the multi-subunit, multi-functional protease known as the proteasome (69, 70). Since Rad53 is a kinase and can efficiently phosphorylate histones *in vitro*, the degradation of excess histones may also occur via a phosphorylation / ubiquitylation / proteasome pathway (71). *In vitro* experiments have indeed revealed that all four core histones can be ubiquitylated efficiently by purified E2 enzymes in an E3-ligase independent manner (72, 73). Further, Rad6, an E2 enzyme that normally monoubiquitylates histone H2B *in vivo*, was shown to be capable of efficiently ubiquitylating histone H3 in the absence of E3 *in vitro*, and this ubiquitylated H3 was degraded by purified 26S proteasome complexes (74).

However, the relevance of such studies to the *in vivo* regulation of histone levels has remained unclear, especially since polyubiquitylation, which is required for efficient recognition and degradation by the proteasome *in vivo*, has never been reported to occur *in vivo* on histones (75, 76). Preliminary data from our laboratory strongly suggests that the Rad53-mediated degradation of histones does indeed occur via a phosphorylation, ubiquitylation and proteasome dependent pathway, although the molecular details of this pathway are still awaited (77).

Further evidence for the involvement of the ubiquitin / proteasome pathway in the regulation of histone proteins comes from the degradation of the centromere specific histone H3 variant Cse4 (homolog of mammalian CENP-A) in the budding yeast. Cse4 was recently shown to be degraded in a Rad53-independent manner via the ubiquitin / proteasome pathway (78). The ubiquitylation mediated degradation of excess Cse4 is likely to contribute to its specific localization on the yeast centromeres by preventing association with non-centromeric regions. Surprisingly, when all the lysines present in Cse4 were mutated to arginines to remove potential ubiquitylation sites, the mutant protein was still degraded, albeit slowly. Addition of an epitope tag on the N-terminus of Cse4 in an effort to block alpha amino group ubiquitylation did result in the lack of detectable Cse4-ubiquitin conjugates but not its degradation. Core histones occur in pairs *in vivo* with H3 paired with H4 and H2A with H2B (1). It is possible that ubiquitylation of the H4 bound to Cse4 can function "in trans" to promote degradation of Cse4. This certainly does not occur in the N-end rule pathway whereby proteins with certain amino acids in their N-terminus are selectively targeted for destruction via the ubiquitin / proteasome pathway (79). This allows the selective degradation of individual subunits of a multi-subunit protein complex by N-terminus “degron” tagging, while sparing the rest of the members of complex, as has been shown for subunits of the MCM (Mini Chromosome Maintenance) complex involved in DNA replication (80). However, it is possible that under certain circumstances it is beneficial for the cell to degrade all the subunits of a complex simultaneously. A degradation pathway may exist *in vivo* to deal with such substrates and would be capable of targeting all the subunits of a large protein complex simultaneously for degradation when only one or few subunits are sufficiently polyubiquitylated. Although core histones occur as [H3/H4] or [H2A/H2B] dimers, when present in excess, the positively charged histones are all individually capable of associating inappropriately with the negatively charged DNA (81, 82). Hence, in order to efficiently deal with histone excess, cells may have evolved pathways to degrade all non-chromatin bound histone molecules present in multimeric complexes, even if only one of them carries the degradation signal.

The results obtained for the degradation of Cse4 are highly reminiscent of the enigmatic degradation of p21, a potent inhibitor of cyclin dependent kinases (83, 84). The degradation of p21 in an ubiquitylation-independent, but proteasome dependent manner has been suggested and is supported by the observation that certain proteasome subunits can directly associate with the C-terminal region of p21 (85, 86). In a similar manner, it is possible that the degradation of Cse4 and other histones can occur via multiple redundant mechanisms. However, the redundant degradation mechanisms may not be easy to uncover as one degradation pathway may be preferred over the rest and so a disruption of the preferred pathway would be required to detect any redundant mechanisms. The degradation of histones by the proteasome in the absence of ubiquitylation has been suggested following oxidative damage to these proteins (87). The 20S catalytic proteasome core was shown to be capable of specifically degrading both free and DNA associated (though not necessarily nucleosomal) oxidatively damaged histones, but not undamaged histones (88). It is entirely possible that cells have evolved multiple redundant pathways for the proteolysis of non-chromatin bound histones, which would further highlight the potential importance of such mechanisms in maintaining cell viability and genomic stability.

Degradation of histones may be of special significance for the repair of DNA damage. DNA replication comes to a standstill upon DNA damage in S-phase and leads to the accumulation of excess histones (17, 35). These histones can interfere with DNA repair processes by competing directly with the DNA repair factors for binding to DNA. Consistent with this hypothesis, histone levels strongly influence the DNA damage sensitivity of budding yeast cells (17). Overexpression of histones in the budding yeast results in increased DNA damage sensitivity, whereas a reduction in histone gene dosage results in resistance to DNA damaging agents. Nucleosomes can also be an impediment for the DNA repair factors to gain access to the DNA lesion. Not surprisingly, several chromatin remodeling factors have been shown to be recruited to the sites of double strand breaks (DSBs) via a potential interaction with phosphorylated H2A which marks extensive stretches of chromatin on both sides of a DSB (89). Remarkably, phosphorylated H2A has never been found to localize very close to the actual DSB where DNA repair factors such as Mre11 and Rad51 are localized (89, 90). A simple explanation for these observations could be that histones are removed from the vicinity of a DSB to allow access to the repair factors, as has been demonstrated in the budding yeast (39, 91). Histones disassembled from the site of a DSB need to be either sequestered by histone chaperones or degraded, perhaps in a Rad53 dependent manner, to prevent them from associating non-specifically with the DNA. Interestingly, it has been reported that the proteasome is required for efficient DSB repair and is recruited to DSB sites in the budding yeast, though its substrates are not known (92). One class of proteasomal substrates could potentially be the histones removed from the sites of DNA damage.

Evidence has mounted in recent years for the degradation dependent and independent roles of the 19S regulatory and the 20S proteolytic core sub-particles of the 26S proteasome in transcription (93, 94). The 19S regulatory particle of the proteasome has been shown to facilitate transcriptional elongation as well as interact with

the FACT complex involved in chromatin assembly and disassembly during transcriptional elongation, though the significance of this interaction is unknown (95). The recruitment of the 19S particle to chromatin depends on H2B monoubiquitylation that is also known to stimulate FACT activity, which in turn promotes H2B monoubiquitylation (96). Our recent results suggest that FACT may be involved in regulating the flow or availability of histones during the chromatin assembly / disassembly process involved in transcriptional elongation (23). This raises an intriguing possibility that the interaction between the 19S particle and FACT may serve to potentially degrade unwanted histones, in a Rad53 dependent or independent manner, by recruiting the 20S proteolytic core of the proteasome. Degradation of such transcriptionally evicted histones could potentially aid in keeping the highly transcribed genes largely nucleosome free by preventing their reassembly (21). It would also serve to erase the existing epigenetic marks carried on the degraded histones which would force chromatin reassembly using newly synthesized histones, possibly including replacement histone variants, thus resetting or fine tuning the potential for subsequent transcriptional activation (30, 97).

Regulated histone proteolysis probably plays a major role in the packaging of sperm DNA. During the differentiation of vertebrate spermatids, the majority of histones are replaced first by transition proteins and, subsequently, by protamines. This massive structural reorganization is necessary to package the haploid genome within the narrow confines of the sperm head. The histones displaced from DNA are extensively ubiquitylated and, ultimately, proteolysed (98, 99). The ubiquitin conjugating enzyme UBC4 and a testis-specific isoform of UBC4 are induced during the late stages of spermiogenesis (100, 101). Further, a ubiquitin ligase known as either LASU1 or $E3^{Histone}$, was recently purified from bovine testis as an E3 enzyme that functions in concert with UBC4 to mediate extensive ubiquitylation of the four core histones *in vitro* (102). Further studies are necessary to test whether UBC4 and $E3^{Histone}$ are needed for histone degradation during spermiogenesis *in vivo*, as well as to investigate if related enzymes in other eukaryotes are involved in the degradation of excess histones in general.

Any histone degradation pathway needs to be tightly regulated to avoid degradation of chromosomal histones, failing which the gross chromatin structure could be seriously disrupted and precious epigenetic marks in the form of histone modifications may be lost. As such, cells are likely to have evolved mechanisms to ensure that its histone degradation pathways do not target chromosomal histones. One simple way of achieving this would be to carry out histone degradation in the cytoplasm, away from the chromatin. This would be a good way to dispose of excess newly synthesized histones or histones that may accumulate in the cytoplasm upon DNA damage, when nuclear histone chaperones get saturated with histones bound to them. However, histones displaced from the chromatin during transcription or DNA repair would have to be exported out of the nucleus to be degraded in the cytoplasm. Since proteasomes are present both in the nucleus and the cytoplasm of yeast cells, it is possible that histone degradation occurs in both these cellular compartments (103, 104). Nuclear degradation of histones would need to be very tightly controlled to protect the chromosomal histones. Rad53 is a nuclear protein and it appears to selectively target excess non-chromatin associated histones for degradation by associating with them (17). Rad53 is not known to associate with chromatin and this presumably allows the chromosomal histones to escape degradation by the Rad53-mediated histone degradation pathway. However, both free and DNA-associated oxidatively damaged histones (but not undamaged histones) have been reported to be degraded by the 20S catalytic core of the nuclear proteasome in an ubiquitylation-independent manner (88). Interestingly, Poly-ADP ribose polymerase (PARP) which ADP-ribosylates proteins, is activated by DNA damage and has been reported to ADP-ribosylate histones as well as enhance the activity of the 20S proteasome core (105, 106). Hence, certain histone modifications, such as phosphorylation by Rad53, oxidation or ADP-ribosylation may be a prerequisite for histones to be targeted for destruction by the proteasome in an ubiquitin dependent or independent manner. Newly synthesized histones are known to carry certain acetylation marks that are promptly removed upon their incorporation into chromatin (107). However, the function of these acetylation marks on newly synthesized histones is unclear and one possibility is that they serve to distinguish unincorporated histones from chromatin associated histones (108). Such posttranslational modifications would allow unincorporated newly synthesized histones and a limited amount of chromatin-associated histones at specific sites (for example, damaged histones or histones at a DSB) to be selectively targeted for destruction. The Rad53-independent degradation of Cse4 may also involve some kind of modification by another factor prior to it being targeted for degradation. Future studies should uncover the exact nature, sites and enzymology of these modifications on histones that may target them for degradation.

4.3. Cell cycle responses to the presence of excess histones

Due to potential cytotoxity upon the non-specific binding of excess histones to negatively charged cellular components, it is possible that the presence of excess histones may perturb cell cycle progression. In order to avoid such a scenario, cells may have evolved specific surveillance mechanisms to detect the presence of excess histones and make appropriate adjustments to cell cycle progression while the excess histone are tackled by the degradation machinery. Evidence for the existence of such mechanisms comes from studies in mammalian cells and the budding yeast. In mammalian cells, inducible overexpression of histones led to the accumulation of cells in S-phase and at later time points a late-S/G2 arrest (109). This was similar to the effects observed upon the knockdown of the histone chaperone Asf1, which presumably helps buffer excess histones (37, 109). Overexpression of histones or depletion of Asf1 led to the delocalization of the single stranded DNA binding

Replication Protein A (RPA) from the replication foci during S-phase (109). It is possible that the presence of excess histones upon Asf1 depletion or histone overexpression results in the binding of these histones to the single stranded DNA present at the replicating fork and thus prevents the binding of RPA. RPA is known to play a major role in the sensing and signaling of DNA damage by virtue of its interaction with single stranded DNA leading to the activation of the DNA damage checkpoint (110). Excess histones do not appear to cause DNA damage directly or activate the DNA damage checkpoint in yeast or mammalian cells (17, 109). Hence, how S-phase progression is slowed down or arrested in the presence of excess histones is not clear at present. It is possible that a signaling pathway exists to sense the presence of excess histones and triggers a slowing down or arrest of cell cycle progression in a manner similar to a DNA damage checkpoint. It is likely that some known members of the cellular replication, DNA damage response, histone regulatory or even the dNTP pool regulatory machineries will be involved in a signaling pathway for sensing and responding to excess histone levels in mammalian cells.

Preliminary work from our collaborative study in the budding yeast hints at the existence of a signaling pathway which appears to sense the presence of excess histones in the G1 phase of the cell cycle and may delay G1 progression or entry into S-phase (23). Conditional mutants in the essential Spt16 subunit of the FACT complex which facilitates transcriptional elongation appear to accumulate high levels of endogenous histones (23, 32). These histones most probably arise from the transcription dependent eviction of histones since expression of histone genes in yeast is undetectable outside of S-phase ((21, 27, 30) and our unpublished data). Spt16 function is presumably required for the reassembly of these histones onto the DNA following the passage of the RNA polymerase (32). In temperature sensitive (*ts*) *spt16* mutants, the transcriptionally evicted histones accumulate at the restrictive temperature and appear to require Rad53 activity for their elimination (23). Conditional *spt16 rad53* double mutants accumulate rapidly in G1 at the restrictive temperature concomitant with the accumulation of excess endogenous histones. Upon release from alpha factor mediated G1 synchronization, the double mutants also exhibited a delay in entry into S-phase. These effects were recapitulated in wild type cells upon the induction of histone overexpression, suggesting a direct link between the presence of excess histones and the delays observed in G1 progression and S-phase entry. These delays were linked to the dramatic downregulation of the G1 cyclin *CLN3*, as well as *CLN1* and *CLN2* to a lesser extent (23). Taken together, these data suggest that the presence of excess histones in G1 can result in the targeting of the critical machinery required for cell cycle transitions to bring about cell cycle delays. This is reminiscent of the manner in which yeast cells delay G1 progression by downregulating GI cyclins *CLN1* and *CLN2* transcripts in response to DNA damage by Methyl Methane Sulfonate (MMS) (65). In this case Rad53 is not required for the initial downregulation of the G1 cyclin transcripts, but is required to prevent the reaccumulation of G1 cyclins. Although it is not yet known how excess histones are sensed and how this signals the downregulation of G1 cyclins, it is possible that Rad53 and/or histone chaperones are involved in some aspect of this regulation. Future studies are likely to uncover the molecular framework for the sensing and signaling of excess histones, as well as the cell cycle response to histone excess.

5. EVOLUTIONARY CONSERVATION OF MECHANISMS INVOLVED IN HISTONE REGULATION

Given the importance of regulating histone levels, one would expect a strong conservation of histone regulatory mechanisms throughout eukaryotic evolution. This appears to be the case indeed as demonstrated by studies in the budding yeast and mammalian cells, especially in the regulation of histone mRNA levels in response to inhibition of replication or upon DNA damage (27, 54, 58, 59). A clear pattern has emerged from these studies which indicate a major role for the PI-3 kinases involved in the DNA damage response pathway in metazoan cells such as ATM (via its downstream effector, p53), ATR and DNA-PK, as well as their budding yeast homologs Mec1/Tel1 (via the downstream transducer kinase Rad53) in the downregulation of histone mRNAs upon inhibition of replication or DNA damage (54-57). The suspected involvement of ATM, ATR and DNA-PK in metazoan cells in the downregulation of histone mRNAs upon replication inhibition or DNA damage could be suggestive of in-built redundancy in histone mRNA regulatory mechanisms, or different kinds of DNA damage may activate specific PI-3 kinase dependent pathways, which ultimately converge on to a common set of effectors that bring about the downregulation of histone mRNAs (111). Interestingly, although budding yeast and metazoan histone mRNAs arise from genes that lack introns and differ in the presence or absence of the poly-A tail respectively, it is quite possible that their stability is at least partly regulated in common by the exosome (19, 56).

The budding yeast DNA damage transducer kinase Rad53 appears to be unique among the DNA damage checkpoint kinases in that it plays a key role in regulating histones by acting at the level of histone proteins to target any excess for degradation, as well as at the level of histone mRNAs to downregulate histone transcripts in response to replication arrest or DNA damage (17, 54). Interestingly, histone protein degradation mediated by Rad53 does not require its upstream activator kinases Mec1/Tel1, though the upstream kinases are required for the Rad53 mediated histone mRNA downregulation in response to replication inhibition or DNA damage (17, 54). The dual role of Rad53 in histone gene repression and degradation of excess histones raises an interesting issue. At least superficially, these two modes of regulation appear to be redundant with each other. However, early S-phase cells need to synthesize large amounts of histones at very high rates to meet the demand for rapid histone deposition behind the replication fork. The sudden decline in rates of DNA synthesis that follows DNA damage during S-phase therefore results in a substantial accumulation of excess

histones (17, 18, 37). Because they form a complex with Rad53, excess histones would eventually saturate the degradation machinery if they were allowed to be synthesized continuously in the absence of genotoxic agent-induced histone gene repression (17, 77). This may ultimately interfere with other important functions of Rad53, such as the phosphorylation of target proteins at sites of stalled replication forks. Hence, histone regulation at both the posttranscriptional as well as the posttranslational levels may actually complement each other and the presence of both regulatory mechanisms may be necessary for optimal control of histone levels.

Although Chk2, the mammalian homolog of yeast Rad53 may be playing a role in the downregulation of histone mRNAs in response to replication arrest or DNA damage via its downstream effector p53, there is no evidence yet for the existence of posttranslational regulation of histone protein levels in mammalian cells (55, 57). However, given the strong conservation of histone regulatory mechanisms throughout eukaryotic evolution and the universal need for regulating histone levels, there is a strong possibility that posttranslational regulation of histones does occur in mammalian cells. If the existence of such a pathway is demonstrated, it will be interesting to see if Chk2 or the highly related kinase Chk1, as well as upstream PI-3 kinases are involved in such a pathway. Although the different histone regulatory pathways in mammalian cells are expected to be more complex than their counterparts in the budding yeast, the basic principles as well as the major players are very likely to be conserved. Future studies will reveal if this is indeed the case.

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7. REFERENCES

1. van Holde, K. E.: Chromatin. Springer-Verlag, New York (1988)

2. Wolffe, A. P.: Chromatin: Structure and Function. Academic Press, San Diego (1995)

3. Richmond, T. J. and C. A. Davey: The structure of DNA in the nucleosome core. *Nature*, 423(6936), 145-50 (2003)

4. Brown, D. T.: Histone H1 and the dynamic regulation of chromatin function. *Biochem Cell Biol*, 81(3), 221-7 (2003)

5. Verreault, A.: De novo nucleosome assembly: new pieces in an old puzzle. *Genes Dev*, 14(12), 1430-8 (2000)

6. Gunjan, A., J. Paik and A. Verreault: Regulation of histone synthesis and nucleosome assembly. *Biochimie*, 87(7), 625-35 (2005)

7. Han, M., M. Chang, U. J. Kim and M. Grunstein: Histone H2B repression causes cell-cycle-specific arrest in yeast: effects on chromosomal segregation, replication, and transcription. *Cell*, 48(4), 589-97 (1987)

8. Ye, X., A. A. Franco, H. Santos, D. M. Nelson, P. D. Kaufman and P. D. Adams: Defective S phase chromatin assembly causes DNA damage, activation of the S phase checkpoint, and S phase arrest. *Mol Cell*, 11(2), 341-51 (2003)

9. Nelson, D. M., X. Ye, C. Hall, H. Santos, T. Ma, G. D. Kao, T. J. Yen, J. W. Harper and P. D. Adams: Coupling of DNA synthesis and histone synthesis in S phase independent of cyclin/cdk2 activity. *Mol Cell Biol*, 22(21), 7459-72 (2002)

10. Prado, F. and A. Aguilera: Partial depletion of histone H4 increases homologous recombination-mediated genetic instability. *Mol Cell Biol*, 25(4), 1526-36 (2005)

11. Lengauer, C., K. W. Kinzler and B. Vogelstein: Genetic instabilities in human cancers. *Nature*, 396(6712), 643-9 (1998)

12. Hoeijmakers, J. H.: Genome maintenance mechanisms for preventing cancer. *Nature*, 411(6835), 366-74 (2001)

13. Steger, D. J. and J. L. Workman: Transcriptional analysis of purified histone acetyltransferase complexes. *Methods*, 19(3), 410-6 (1999)

14. Meeks-Wagner, D. and L. H. Hartwell: Normal stoichiometry of histone dimer sets is necessary for high fidelity of mitotic chromosome transmission. *Cell*, 44(1), 43-52 (1986)

15. Sullivan, E., C. Santiago, E. D. Parker, Z. Dominski, X. Yang, D. J. Lanzotti, T. C. Ingledue, W. F. Marzluff and R. J. Duronio: Drosophila stem loop binding protein coordinates accumulation of mature histone mRNA with cell cycle progression. *Genes Dev*, 15(2), 173-87 (2001)

16. Berloco, M., L. Fanti, A. Breiling, V. Orlando and S. Pimpinelli: The maternal effect gene, abnormal oocyte (abo), of Drosophila melanogaster encodes a specific negative regulator of histones. *Proc Natl Acad Sci U S A*, 98(21), 12126-31 (2001)

17. Gunjan, A. and A. Verreault: A Rad53 kinase-dependent surveillance mechanism that regulates histone protein levels in S. cerevisiae. *Cell*, 115(5), 537-49 (2003)

18. Bonner, W. M., R. S. Wu, H. T. Panusz and C. Muneses: Kinetics of accumulation and depletion of soluble newly synthesized histone in the reciprocal regulation of histone and DNA synthesis. *Biochemistry*, 27(17), 6542-50 (1988)

19. Reis, C. C. and J. L. Campbell: Contribution of Trf4/5 and the nuclear exosome to genome stability through regulation of histone mRNA levels in Saccharomyces cerevisiae. *Genetics*, 175(3), 993-1010 (2007)

20. Pan, X., P. Ye, D. S. Yuan, X. Wang, J. S. Bader and J. D. Boeke: A DNA integrity network in the yeast Saccharomyces cerevisiae. *Cell*, 124(5), 1069-81 (2006)

21. Lee, C. K., Y. Shibata, B. Rao, B. D. Strahl and J. D. Lieb: Evidence for nucleosome depletion at active regulatory regions genome-wide. *Nat Genet*, 36(8), 900-5 (2004)

22. Schermer, U. J., P. Korber and W. Horz: Histones are incorporated in trans during reassembly of the yeast PHO5 promoter. *Mol Cell*, 19(2), 279-85 (2005)

23. Morillo-Huesca, M., M.-C. Muñoz-Centeno, R. K. Singh, G. U. Reddy, V. Oreal, D. Liang, D. Maya, A. Gunjan, V. Géli and S. Chávez: Accumulation of transcription-evicted histones induces a CLN3-dependent cell cycle delay in G1. *Submitted* (2008)

24. Stein G. S., J. L. Stein and W. F. Marzluff: Histone genes: Structure Organization and Regulation. John Wiley & Sons, New York. (1984)

25. Marzluff, W. F., P. Gongidi, K. R. Woods, J. Jin and L. J. Maltais: The human and mouse replication-dependent histone genes. *Genomics*, 80(5), 487-98 (2002)

26. Sogo, J. M., H. Stahl, T. Koller and R. Knippers: Structure of replicating simian virus 40 minichromosomes. The replication fork, core histone segregation and terminal structures. *J Mol Biol*, 189(1), 189-204 (1986)

27. Osley, M. A.: The regulation of histone synthesis in the cell cycle. *Annu Rev Biochem*, 60, 827-61 (1991)

28. Takami, Y., S. Takeda and T. Nakayama: An approximately half set of histone genes is enough for cell proliferation and a lack of several histone variants causes protein pattern changes in the DT40 chicken B cell line. *J Mol Biol*, 265(4), 394-408 (1997)

29. Ghaemmaghami, S., W. K. Huh, K. Bower, R. W. Howson, A. Belle, N. Dephoure, E. K. O'Shea and J. S. Weissman: Global analysis of protein expression in yeast. *Nature*, 425(6959), 737-41 (2003)

30. Jamai, A., R. M. Imoberdorf and M. Strubin: Continuous histone H2B and transcription-dependent histone H3 exchange in yeast cells outside of replication. *Mol Cell*, 25(3), 345-55 (2007)

31. Dammann, R., R. Lucchini, T. Koller and J. M. Sogo: Chromatin structures and transcription of rDNA in yeast Saccharomyces cerevisiae. *Nucleic Acids Res*, 21(10), 2331-8 (1993)

32. Belotserkovskaya, R. and D. Reinberg: Facts about FACT and transcript elongation through chromatin. *Curr Opin Genet Dev*, 14(2), 139-46 (2004)

33. Williams, S. K. and J. K. Tyler: Transcriptional regulation by chromatin disassembly and reassembly. *Curr Opin Genet Dev*, 17(2), 88-93 (2007)

34. Loyola, A. and G. Almouzni: Histone chaperones, a supporting role in the limelight. *Biochim Biophys Acta*, 1677(1-3), 3-11 (2004)

35. Paulovich, A. G. and L. H. Hartwell: A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. *Cell*, 82(5), 841-7 (1995)

36. Tercero, J. A. and J. F. Diffley: Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. *Nature*, 412(6846), 553-7 (2001)

37. Groth, A., D. Ray-Gallet, J. P. Quivy, J. Lukas, J. Bartek and G. Almouzni: Human Asf1 regulates the flow of S phase histones during replicational stress. *Mol Cell*, 17(2), 301-11 (2005)

38. Downs, J. A., M. C. Nussenzweig and A. Nussenzweig: Chromatin dynamics and the preservation of genetic information. *Nature*, 447(7147), 951-8 (2007)

39. Tsukuda, T., A. B. Fleming, J. A. Nickoloff and M. A. Osley: Chromatin remodelling at a DNA double-strand break site in Saccharomyces cerevisiae. *Nature*, 438(7066), 379-83 (2005)

40. Kastan, M. B. and J. Bartek: Cell-cycle checkpoints and cancer. *Nature*, 432(7015), 316-23 (2004)

41. Weinert, T. A., G. L. Kiser and L. H. Hartwell: Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. *Genes Dev*, 8(6), 652-65 (1994)

42. Lowndes, N. F. and J. R. Murguia: Sensing and responding to DNA damage. *Curr Opin Genet Dev*, 10(1), 17-25 (2000)

43. Rouse, J. and S. P. Jackson: Interfaces between the detection, signaling, and repair of DNA damage. *Science*, 297(5581), 547-51 (2002)

44. Sanchez, Y., B. A. Desany, W. J. Jones, Q. Liu, B. Wang and S. J. Elledge: Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. *Science*, 271(5247), 357-60 (1996)

45. Santocanale, C. and J. F. Diffley: A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature*, 395(6702), 615-8 (1998)

46. Desany, B. A., A. A. Alcasabas, J. B. Bachant and S. J. Elledge: Recovery from DNA replicational stress is the

essential function of the S-phase checkpoint pathway. *Genes Dev*, 12(18), 2956-70 (1998)

47. Zhao, X., A. Chabes, V. Domkin, L. Thelander and R. Rothstein: The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage. *EMBO J*, 20(13), 3544-53 (2001)

48. Borun, T. W., M. D. Scharff and E. Robbins: Rapidly labeled, polyribosome-associated RNA having the properties of histone messenger. *Proc Natl Acad Sci U S A*, 58(5), 1977-83 (1967)

49. Gallwitz, D. and G. C. Mueller: Histone synthesis *in vitro* on HeLa cell microsomes. The nature of the coupling to deoxyribonucleic acid synthesis. *J Biol Chem*, 244(21), 5947-52 (1969)

50. Borun, T. W., F. Gabrielli, K. Ajiro, A. Zweidler and C. Baglioni: Further evidence of transcriptional and translational control of histone messenger RNA during the HeLa S3 cycle. *Cell*, 4(1), 59-67 (1975)

51. Gallwitz, D.: Kinetics of inactivation of histone mRNA in the cytoplasm after inhibition of DNA replication in synchronised HeLa cells. *Nature*, 257(5523), 247-8 (1975)

52. Sittman, D. B., R. A. Graves and W. F. Marzluff: Histone mRNA concentrations are regulated at the level of transcription and mRNA degradation. *Proc Natl Acad Sci U S A*, 80(7), 1849-53 (1983)

53. Lycan, D. E., M. A. Osley and L. M. Hereford: Role of transcriptional and posttranscriptional regulation in expression of histone genes in Saccharomyces cerevisiae. *Mol Cell Biol*, 7(2), 614-21 (1987)

54. Paik, J., G. U. Reddy, M. M. Kabbaj, A. Verreault and A. Gunjan: Checkpoint kinases repress histone gene transcription in response to genotoxic agents that impede replication. *Submitted* (2008)

55. Su, C., G. Gao, S. Schneider, C. Helt, C. Weiss, M. A. O'Reilly, D. Bohmann and J. Zhao: DNA damage induces downregulation of histone gene expression through the G1 checkpoint pathway. *EMBO J*, 23(5), 1133-43 (2004)

56. Kaygun, H. and W. F. Marzluff: Regulated degradation of replication-dependent histone mRNAs requires both ATR and Upf1. *Nat Struct Mol Biol*, 12(9), 794-800 (2005)

57. Muller, B., J. Blackburn, C. Feijoo, X. Zhao and C. Smythe: DNA-activated protein kinase functions in a newly observed S phase checkpoint that links histone mRNA abundance with DNA replication. *J Cell Biol*, 179(7), 1385-98 (2007)

58. Marzluff, W. F. and R. J. Duronio: Histone mRNA expression: multiple levels of cell cycle regulation and important developmental consequences. *Curr Opin Cell Biol*, 14(6), 692-9 (2002)

59. Marzluff, W. F.: Metazoan replication-dependent histone mRNAs: a distinct set of RNA polymerase II transcripts. *Curr Opin Cell Biol*, 17(3), 274-80 (2005)

60. Chang, Y. F., J. S. Imam and M. F. Wilkinson: The nonsense-mediated decay RNA surveillance pathway. *Annu Rev Biochem*, 76, 51-74 (2007)

61. Dominski, Z., X. C. Yang, H. Kaygun, M. Dadlez and W. F. Marzluff: A 3' exonuclease that specifically interacts with the 3' end of histone mRNA. *Mol Cell*, 12(2), 295-305 (2003)

62. Sutton, A., J. Bucaria, M. A. Osley and R. Sternglanz: Yeast ASF1 protein is required for cell cycle regulation of histone gene transcription. *Genetics*, 158(2), 587-96 (2001)

63. Ng, H. H., F. Robert, R. A. Young and K. Struhl: Genome-wide location and regulated recruitment of the RSC nucleosome-remodeling complex. *Genes Dev*, 16(7), 806-19 (2002)

64. Mitchell, P., E. Petfalski, A. Shevchenko, M. Mann and D. Tollervey: The exosome: a conserved eukaryotic RNA processing complex containing multiple 3'-->5' exoribonucleases. *Cell*, 91(4), 457-66 (1997)

65. Sidorova, J. M. and L. L. Breeden: Rad53-dependent phosphorylation of Swi6 and down-regulation of CLN1 and CLN2 transcription occur in response to DNA damage in Saccharomyces cerevisiae. *Genes Dev*, 11(22), 3032-45 (1997)

66. Sidorova, J. M. and L. L. Breeden: Rad53 checkpoint kinase phosphorylation site preference identified in the Swi6 protein of Saccharomyces cerevisiae. *Mol Cell Biol*, 23(10), 3405-16 (2003)

67. Commerford, S. L., A. L. Carsten and E. P. Cronkite: Histone turnover within nonproliferating cells. *Proc Natl Acad Sci U S A*, 79(4), 1163-5 (1982)

68. Tsvetkov, S., E. Ivanova and L. Djondjurov: Metabolic behaviors of the core histones in proliferating Friend cells. *Exp Cell Res*, 180(1), 94-105 (1989)

69. Henchoz, S., Y. Chi, B. Catarin, I. Herskowitz, R. J. Deshaies and M. Peter: Phosphorylation- and ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor Far1p in budding yeast. *Genes Dev*, 11(22), 3046-60 (1997)

70. Deshaies, R. J. and J. E. Ferrell, Jr.: Multisite phosphorylation and the countdown to S phase. *Cell*, 107(7), 819-22 (2001)

71. Hershko, A. and A. Ciechanover: The ubiquitin system. *Annu Rev Biochem*, 67, 425-79 (1998)

72. Haas, A. L., P. M. Bright and V. E. Jackson: Functional diversity among putative E2 isozymes in the mechanism of

ubiquitin-histone ligation. *J Biol Chem*, 263(26), 13268-75 (1988)

73. Pickart, C. M. and A. T. Vella: Ubiquitin carrier protein-catalyzed ubiquitin transfer to histones. Mechanism and specificity. *J Biol Chem*, 263(29), 15076-82 (1988)

74. Haas, A., P. M. Reback, G. Pratt and M. Rechsteiner: Ubiquitin-mediated degradation of histone H3 does not require the substrate-binding ubiquitin protein ligase, E3, or attachment of polyubiquitin chains. *J Biol Chem*, 265(35), 21664-9 (1990)

75. Osley, M. A.: H2B ubiquitylation: the end is in sight. *Biochim Biophys Acta*, 1677(1-3), 74-8 (2004)

76. Jason, L. J., S. C. Moore, J. D. Lewis, G. Lindsey and J. Ausio: Histone ubiquitination: a tagging tail unfolds? *Bioessays*, 24(2), 166-74 (2002)

77. Singh, R. K., M. M. Kabbaj, J. Paik, and A. Gunjan: Histone levels are regulated by phosphorylation and ubiquitylation dependent proteolysis in *S. cerevisiae. Submitted* (2008)

78. Collins, K. A., S. Furuyama and S. Biggins: Proteolysis contributes to the exclusive centromere localization of the yeast Cse4/CENP-A histone H3 variant. *Curr Biol*, 14(21), 1968-72 (2004)

79. Varshavsky, A.: The N-end rule pathway of protein degradation. *Genes Cells*, 2(1), 13-28 (1997)

80. Labib, K., J. A. Tercero and J. F. Diffley: Uninterrupted MCM2-7 function required for DNA replication fork progression. *Science*, 288(5471), 1643-7 (2000)

81. English, C. M., M. W. Adkins, J. J. Carson, M. E. Churchill and J. K. Tyler: Structural basis for the histone chaperone activity of Asf1. *Cell*, 127(3), 495-508 (2006)

82. Tagami, H., D. Ray-Gallet, G. Almouzni and Y. Nakatani: Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell*, 116(1), 51-61 (2004)

83. Bloom, J., V. Amador, F. Bartolini, G. DeMartino and M. Pagano: Proteasome-mediated degradation of p21 via N-terminal ubiquitinylation. *Cell*, 115(1), 71-82 (2003)

84. Coulombe, P., G. Rodier, E. Bonneil, P. Thibault and S. Meloche: N-Terminal ubiquitination of extracellular signal-regulated kinase 3 and p21 directs their degradation by the proteasome. *Mol Cell Biol*, 24(14), 6140-50 (2004)

85. Chen, X., Y. Chi, A. Bloecher, R. Aebersold, B. E. Clurman and J. M. Roberts: N-acetylation and ubiquitin-independent proteasomal degradation of p21(Cip1). *Mol Cell*, 16(5), 839-47 (2004)

86. Touitou, R., J. Richardson, S. Bose, M. Nakanishi, J. Rivett and M. J. Allday: A degradation signal located in the C-terminus of p21WAF1/CIP1 is a binding site for the C8 alpha-subunit of the 20S proteasome. *EMBO J*, 20(10), 2367-75 (2001)

87. Shringarpure, R., T. Grune, J. Mehlhase and K. J. Davies: Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. *J Biol Chem*, 278(1), 311-8 (2003)

88. Ullrich, O., N. Sitte, O. Sommerburg, V. Sandig, K. J. Davies and T. Grune: Influence of DNA binding on the degradation of oxidized histones by the 20S proteasome. *Arch Biochem Biophys*, 362(2), 211-6 (1999)

89. Downs, J. A., S. Allard, O. Jobin-Robitaille, A. Javaheri, A. Auger, N. Bouchard, S. J. Kron, S. P. Jackson and J. Cote: Binding of chromatin-modifying activities to phosphorylated histone H2A at DNA damage sites. *Mol Cell*, 16(6), 979-90 (2004)

90. Unal, E., A. Arbel-Eden, U. Sattler, R. Shroff, M. Lichten, J. E. Haber and D. Koshland: DNA damage response pathway uses histone modification to assemble a double-strand break-specific cohesin domain. *Mol Cell*, 16(6), 991-1002 (2004)

91. Osley, M. A., T. Tsukuda and J. A. Nickoloff: ATP-dependent chromatin remodeling factors and DNA damage repair. *Mutat Res*, 618(1-2), 65-80 (2007)

92. Krogan, N. J., M. H. Lam, J. Fillingham, M. C. Keogh, M. Gebbia, J. Li, N. Datta, G. Cagney, S. Buratowski, A. Emili and J. F. Greenblatt: Proteasome involvement in the repair of DNA double-strand breaks. *Mol Cell*, 16(6), 1027-34 (2004)

93. Muratani, M. and W. P. Tansey: How the ubiquitin-proteasome system controls transcription. *Nat Rev Mol Cell Biol*, 4(3), 192-201 (2003)

94. Collins, G. A. and W. P. Tansey: The proteasome: a utility tool for transcription? *Curr Opin Genet Dev*, 16(2), 197-202 (2006)

95. Ferdous, A., F. Gonzalez, L. Sun, T. Kodadek and S. A. Johnston: The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II. *Mol Cell*, 7(5), 981-91 (2001)

96. Laribee, R. N., S. M. Fuchs and B. D. Strahl: H2B ubiquitylation in transcriptional control: a FACT-finding mission. *Genes Dev*, 21(7), 737-43 (2007)

97. Dion, M. F., T. Kaplan, M. Kim, S. Buratowski, N. Friedman and O. J. Rando: Dynamics of replication-independent histone turnover in budding yeast. *Science*, 315(5817), 1405-8 (2007)

98. M. L. Meistrich: Histones and Other Basic Nuclear Proteins. CRC, Orlando, FL (1989)

99. Chen, H. Y., J. M. Sun, Y. Zhang, J. R. Davie and M. L. Meistrich: Ubiquitination of histone H3 in elongating

spermatids of rat testes. *J Biol Chem*, 273(21), 13165-9 (1998)

100. Rajapurohitam, V., C. R. Morales, M. El-Alfy, S. Lefrancois, N. Bedard and S. S. Wing: Activation of a UBC4-dependent pathway of ubiquitin conjugation during postnatal development of the rat testis. *Dev Biol*, 212(1), 217-28 (1999)

101. Wing, S. S., N. Bedard, C. Morales, P. Hingamp and J. Trasler: A novel rat homolog of the Saccharomyces cerevisiae ubiquitin-conjugating enzymes UBC4 and UBC5 with distinct biochemical features is induced during spermatogenesis. *Mol Cell Biol*, 16(8), 4064-72 (1996)

102. Liu, Z., R. Oughtred and S. S. Wing: Characterization of E3Histone, a novel testis ubiquitin protein ligase which ubiquitinates histones. *Mol Cell Biol*, 25(7), 2819-31 (2005)

103. Enenkel, C., A. Lehmann and P. M. Kloetzel: Subcellular distribution of proteasomes implicates a major location of protein degradation in the nuclear envelope-ER network in yeast. *EMBO J*, 17(21), 6144-54 (1998)

104. Russell, S. J., K. A. Steger and S. A. Johnston: Subcellular localization, stoichiometry, and protein levels of 26 S proteasome subunits in yeast. *J Biol Chem*, 274(31), 21943-52 (1999)

105. Hassa, P. O., S. S. Haenni, M. Elser and M. O. Hottiger: Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? *Microbiol Mol Biol Rev*, 70(3), 789-829 (2006)

106. Ullrich, O., T. Reinheckel, N. Sitte, R. Hass, T. Grune and K. J. Davies: Poly-ADP ribose polymerase activates nuclear proteasome to degrade oxidatively damaged histones. *Proc Natl Acad Sci U S A*, 96(11), 6223-8 (1999)

107. Sobel, R. E., R. G. Cook, C. A. Perry, A. T. Annunziato and C. D. Allis: Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. *Proc Natl Acad Sci U S A*, 92(4), 1237-41 (1995)

108. Ma, X. J., J. Wu, B. A. Altheim, M. C. Schultz and M. Grunstein: Deposition-related sites K5/K12 in histone H4 are not required for nucleosome deposition in yeast. *Proc Natl Acad Sci U S A*, 95(12), 6693-8 (1998)

109. Groth, A., A. Corpet, A. J. Cook, D. Roche, J. Bartek, J. Lukas and G. Almouzni: Regulation of replication fork progression through histone supply and demand. *Science*, 318(5858), 1928-31 (2007)

110. Zou, L. and S. J. Elledge: Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science*, 300(5625), 1542-8 (2003)

111. Muller, B., J. Blackburn, C. Feijoo, X. Zhao and C. Smythe: Are multiple checkpoint mediators involved in a checkpoint linking histone gene expression with DNA replication? *Biochem Soc Trans*, 35(Pt 5), 1369-71 (2007)

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