Histone Acetyltransferase 1 is Required for DNA Replication Fork Function and Stability

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ABSTRACT

The replisome functions in a dynamic environment that is at the intersection of parental and nascent chromatin. Parental nucleosomes are disrupted in front of the replication fork. The daughter duplexes are packaged with an equal amount of parental and newly synthesized histones in the wake of the replication fork through the action of the replication-coupled chromatin assembly pathway. Histone acetyltransferase 1 (Hat1) is responsible for the cytosolic diacetylation of newly synthesized histone H4 on lysines 5 and 12 that accompanies replication-coupled chromatin assembly. Analysis of the role of Hat1 in replication-coupled chromatin assembly demonstrates that Hat1 also physically associates with chromatin near sites of DNA replication. The association of Hat1 with newly replicated DNA is transient but can be stabilized by replication fork stalling. The association of Hat1 with nascent chromatin may be functionally relevant as loss of Hat1 results in a decrease in replication fork progression and an increase in replication fork stalling. In addition, in the absence of Hat1, stalled replication forks are unstable and newly synthesized DNA becomes susceptible to Mre11-dependent degradation. These results suggest that Hat1 links replication fork function to the proper processing and assembly of newly synthesized histones.

INTRODUCTION

The central event in the division of a cell is the duplication of its chromosomes. Chromosome duplication requires the proper functioning of two interconnected processes. The first is the replication of the genomic DNA. The second is the duplication of the chromatin structure that governs the correct packaging and architecture of the chromosomes in the nucleus. The successful coordination and completion of these processes is essential to ensure genome stability, maintain correct patterns of gene expression and properly regulate cell proliferation.

DNA replication occurs in a unique and highly dynamic chromatin environment. The replication fork must navigate through the chromatin structure in front of the fork by disrupting nucleosomes in its path. In the wake of the replication fork, the nascent daughter duplexes must be rapidly assembled into nucleosomes. Nascent chromatin on the daughter duplexes is assembled from two distinct pools of histones; parental and newly synthesized.

The parental histones are derived from the nucleosomes disrupted during the passage of the replication fork. These nucleosomes dissociate into stable H3/H4 tetramers and H2A/H2B dimers. Regulation of parental histone recycling, mediated by the histone chaperone Asf1, is critical for proper replication fork function and stability (1). Asf1 functions in conjunction with the MCM2-7 replicative helicase and RPA to remove parental histones H3 and H4 from in front of the replication fork and transfer them to the newly replicated DNA near their original genomic location(2-6). Loss of Asf1 or disruption of Asf1 activity through histone over-expression impedes DNA unwinding and replication fork progression (7-9). Other factors, such as FACT and the POLE3-POLE4

complex are also involved in processing parental histones at the replication fork and may be involved in the association of H2A/H2B dimers with the H3/H4 tetramers(10-12).

To maintain nucleosome density, an equal quantity of newly synthesized histones must be delivered to sites of DNA replication. This is accomplished through the replication-coupled chromatin assembly pathway. The replication-coupled chromatin assembly pathway begins with the processing of new newly synthesized H3 and H4 in the cytoplasm, where there is a large burst of histone synthesis to meet the needs of chromosome duplication(13). The histone methyltransferase SetDB1 is associated with the ribosome and mono-methylates histone H3 lysine 9 co-translationally(14). H3 and H4 then form stable heterodimers in a chaperone-mediated process(15). The H3/H4 dimers are then bound by the Hat1 complex, which consists of the histone acetyltransferase Hat1 and the histone chaperone Rbap46, resulting in the acetylation of H4 on lysines 5 and 12(16-20). The modified H3/H4 dimers are then transferred to the histone chaperone Asf1 through the direct association of Asf1 with the Hat1/Rbap46/H3/H4 complex(15,21). The Asf1/H3/H4 complex can associate with specific importins/karyopherins to facilitate the nuclear import of the H3/H4 dimers(21-26). Once in the nucleus, additional processing occurs on some H3/H4 complexes with the acetylation of H3 lysine 56 by CBP/Rtt109 and additional lysine residues in the H3 NH_2-terminal tail by Gcn5(21,27-38). The modified H3/H4 complexes are then transferred to the CAF-1 complex (Chromatin Assembly Factor 1), which facilitates the deposition of H3/H4 tetramers near replication forks through a physical interaction with PCNA(39-47).

Several recent studies have shown that replication-coupled chromatin assembly is required for replication fork function. In these studies, the supply of histones to the replication fork was blocked, either by preventing new histone protein synthesis or disrupting histone deposition by depleting CAF-1 or Asf1(39,48-51). There is also evidence that the post-translational modifications on newly synthesized histones can influence replication fork function. Histone H3 lysine 56 acetylation has been shown to positively regulate binding of histones to CAF-1(38). Consistent with this, loss of H3 lysine 56 acetylation and CAF-1 have similar effects on DNA replication in *S. cerevisiae*(49). Histone deacetylases, HDAC1 and HDAC2, which have been proposed to deacetylate newly synthesized histones following their assembly into chromatin, have been shown to be important for replication fork function and for the stabilization of stalled replication forks in conjunction with the WRN helicase(52-56).

Recent evidence suggests that Hat1 also has the potential to influence replication fork function. Studies in a wide range of eukaryotes show that loss of Hat1 sensitizes cells to DNA double strand breaks and causes HU sensitivity and genome instability in mammalian cells (19,57,58). In addition, it was recently reported that Hat1 is transiently recruited to chromatin during replication-coupled chromatin assembly and affects the protein composition of nascent chromatin(59). Therefore, we investigated whether Hat1 provides a link between the processing and assembly of newly synthesized histones and replication fork function. We confirm that Hat1 transiently associates with newly replicated DNA. We show that loss of Hat1 induces a dramatic reduction in replication fork progression and increases replication fork stalling. We also demonstrate that stalling of replication forks stabilizes the association of Hat1 with newly replicated DNA

and that loss of Hat1 leads to destabilization of stalled forks and MRE11-dependent degradation of newly synthesized DNA.

MATERIALS AND METHODS

Cell culture conditions. Mouse embryonic fibroblasts were prepared as previously described (19). Cells were grown in DMEM (Sigma) supplemented with 10%FBS (Sigma) and Penicillin/Streptomycin (Gibco).

Chromatin assembly assay. Three Hat1$^{+/+}$ or Hat1$^{-/-}$ MEF cell lines were seeded in equal quantities on coverslips and allowed to attach for 24 hours. For N-acetyl cysteine experiments (NAC), cells were seeded and allowed to grow for 48 hours in 5 mM NAC (Sigma; cat# A9165). Cells were then incubated with 10 uM IdU (Sigma; cat# I7125) for 30 minutes. For thymidine chases, IdU containing media was replaced with fresh media for indicated times. For cells treated with hydroxyurea (HU), IdU containing media was replaced with fresh media containing 4 mM HU (Sigma; cat# H8627) for designated times. 100 uM MIRIN (Sigma; cat# M9948) was added simultaneously where specified. Cells were then permeabilized with 0.5% TritonX-100 and fixed with 4% PFA simultaneously for 15 min, rinsed with PBS, and fixed again with 4% PFA for 10 minutes at room temperature. After several PBS washes, cells were incubated with 1N HCl for 10 minutes, washed with PBS until pH neutralizes, and blocked with 5% BSA for 1 hour at room temperature. BSA was removed with PBS washes and primary antibodies detecting IdU and a protein of interest were diluted in 1% BSA/0.3% TritonX-100 and added to cells overnight at 4^{o}C (IdU: mouse anti-BrdU, 1:20, Becton Dickinson; Total Histone H4: rabbit anti-Total Histone H4, 1:500, Upstate Cell Signaling Solutions 05-

858; Hat1: rabbit anti-Hat1, 1:1000, Abcam ab12163; H4K5Ac: rabbit anti-Histone H4 Lysine 5 Ac, 1:250, Abcam ab51997; H4K12Ac: rabbit anti-Histone H4 Lysine 12 Ac, 1:250, Abcam ab46983; PCNA: rabbit anti-PCNA, 1:50, Santa Cruz Biotechnology sc-7907; RAD51: rabbit anti-RAD51, 1:200, Abcam ab63801). The following day, primary antibodies were removed with PBS and cells were subjected to the Duolink™ Proximity Ligation Assay protocol according to manufacturer instructions (Sigma: DUO92008, DUO92004, DUO92002, DUO82049). After amplification, cells were incubated with AlexaFluor 488–conjugated anti-mouse secondary antibody (1:250, Molecular Probes) for 1 hour at room temperature, antibody was removed with PBS, nuclei were stained with 20 mM Hoechst 33342 Fluorescent Stain and mounted on slides using Vectashield. Slides were analyzed under an Andor Spinning Disk Confocal fluorescence microscope. Images were acquired using MetaMorph version 7.8.10 and quantification was completed using ImageJ version 1.52t according to a previously described protocol (63).

DNA fiber assay. DNA was labeled with 50μM and 250μM for 20 minutes each. HU (Sigma) was used at 4mM for 5 hours; Mirin (Sigma) was used at 100μM for 5 hours. After labeling and treatment, cells were collected by trypsinization and resuspended in PBS. 2μL of the cells suspension were spotted on a glass slide and lysed with lysis buffer (0.5% SDS, 200 mM Tris-HCl, pH 7.4, 50 mM EDTA) for 10min, slides were then tilted to 15° to stretch the DNA fibers and fixed with Methanol/Acetic Acid (3:1) overnight at 4 degrees. Next day DNA was denatured with 2.5N HCl for 30min and wash several times with PBS before blocking with 1%BSA/PBS for 30min. Rat anti-BrdU (1:50, AbD Serotec) was used to detect CldU, and mouse anti-BrdU (1:20, Becton Dickinson) to detect IdU. Antibodies were diluted in blocking buffer and incubated for 1 hour at room

temperature. AlexaFluor 594–conjugated anti-rat (1:250, Molecular Probes) and AlexaFluor 488–conjugated anti-mouse (1:250, Molecular Probes) were used as secondary antibodies and incubated for 1 hour at room temperature. Slides were mounted with Vectashield with DAPI.

Immunofluorescence. Cells were seeded on coverslips and allowed to attach for 24 hours. Next day the cells were fixed with 4% PFA at room temperature for 10 minutes, washed several times with PBS and permeabilized with 0.5% Triton X-100/PBS for 15 minutes at room temperature, after several PBS washes cells were blocked with 5% BSA in PBS for 30 minutes at room temperature. Anti-phosphorylated ATR (Ser 428) (Cell Signaling #2853 1/100) was incubated overnight at 4 degrees. Next day after several washes, secondary AlexaFluor 594–conjugated anti-rabbit was diluted 1/250 and incubated 1 hour at room temperature. Antibody excess was extensively washed and slides were mounted with Vectashield with DAPI.

Comet Assay. The Comet Assay kit (Trevigen, Gaitherburg,MD) was used according to the manufacture instructions. Briefly, MEFs were resuspended in ice cold PBS (Ca2+ and Mg2+ free) to a concentration of 1×10^5 cells/ml. 5 µl cells were mixed with 50 µl of warm low melting Agarose and 50 µl were evenly spread onto the special comet slides. Slides were stored at 4 °C in the dark and transferred to pre-chilled lysis solution for 60 minutes at 4 °C. Next, slides were transferred to alkali unwinding solution at room temperature for 60 minutes. Slides were transferred to electrophoresis tank which contained pre-chilled Alkaline electrophoresis solution and run at 1 Volt/cm, 300 mA for 45 minutes at 4 degrees. The slides were immersed twice in deionized water for 5 minutes intervals and washed in 70% ethanol for 5 minutes. Then cells were stained

with 100 µl of SYBR Green I for 5 minutes in the dark and slides were analyzed under Zeiss Axiophot fluorescence microscope. Images were taken using Metavue software version 6.3r2 software and comet tails were analyzed using opencomet by Imagej.

RESULTS

Hat1 transiently localizes to newly replicated DNA. Current models of replication-coupled chromatin assembly predict that Hat1 associates with, and modifies, newly synthesized histone H4 in the cytoplasm before transferring the modified histones to Asf1 for subsequent nuclear import and deposition. However, recent results using iPOND (isolation of proteins on nascent DNA) suggested that Hat1 becomes transiently associated with newly replicated DNA(59). As this has the potential to significantly expand the role of Hat1 in genome duplication, we sought to confirm this observation.

Proximity ligation assay-based chromatin assembly assays (CAAs) have recently been developed and serve as a powerful method for analyzing protein dynamics on newly replicated DNA(60-63). The proximity ligation technique determines whether two molecules reside close to each other in the cell by employing two species-specific secondary antibodies that are fused to oligonucleotides. If the secondary antibodies recognize primary antibodies that are in close proximity, the oligonucleotides can both bind to a nicked circular DNA, creating a template for rolling circle replication. This amplifies sequences that can be bound by a fluorescent probe and visualized. To adapt this for use as a chromatin assembly assay, newly replicated DNA is labeled by the incorporation of the thymidine analog IdU. The proximity of proteins to newly replicated DNA is detected using antibodies against the protein of interest and antibodies

recognizing IdU. To validate the CAA, we monitored the localization of PCNA, H4 lysine 5 acetylation and H4 lysine 12 acetylation to newly replicated DNA in Hat1$^{+/+}$ and Hat1$^{-/-}$ MEFs (mouse embryonic fibroblasts). As seen in Figure 1A, quantitation of the CAA precisely mirrored the results previously obtained with iPOND. The localization of PCNA to newly replicated DNA was Hat1-independent and the acetylation of H4 lysines 5 and 12 required Hat1(19,59).

Using α-Hat1 antibodies, we tested whether Hat1 is in proximity to newly replicated DNA. There is abundant CAA signal in Hat1$^{+/+}$ cells and only background in the Hat1$^{-/-}$ cells (Figure 1B). We next asked whether Hat1 is transiently associated with newly synthesized DNA or whether it is stably bound to chromatin. We performed CAA assays immediately following a pulse of IdU and after 15, 30 and 60 minutes of a thymidine chase. As seen in Figure 1C, the level of Hat1 on newly replicated DNA is significantly reduced after a 15 minute chase and is completely lost after 30 minutes. Intriguingly, if replication forks are stalled by the addition of HU, Hat1 association with newly replicated DNA is stabilized for extended periods of time (at least 5 hours). These data verify that Hat1 is transiently associated with nascent chromatin near sites of DNA replication and becomes stably associated when replication forks stall.

Hat1 is required for normal replication fork progression. The physical association of Hat1 with the highly dynamic chromatin at sites of DNA replication greatly expands the spectrum of potential functions for this enzyme in genome duplication. In particular, this raises the possibility that Hat1 plays a direct role in replication fork function or stability. To test this, we used DNA fiber analysis in Hat1$^{+/+}$ and Hat1$^{-/-}$ MEFs (Fig. 2*A*). Hat1$^{+/+}$ and Hat1$^{-/-}$ cells were incubated with CldU, followed by IdU incubation for equal

times and replication fork progression was measured by DNA fiber analysis in which antibodies targeting the CldU (red) and IdU (green) are used to label the newly replicated DNA with different colors. The relative rates of replication fork progression were determined by measuring the lengths of the IdU tracts that are located at junctions with CldU labeled DNA, as this ensures that the replication fork was functional at the beginning of the IdU incubation. We observed a significant decrease in the length of labeled DNA fibers in the Hat1$^{-/-}$ cells, indicating that DNA replication progressed more slowly in the absence of Hat1. Consistent with an effect of Hat1 loss on replication fork function, analysis of PCNA dynamics at the replication fork by CAA showed that PCNA dissociation is significantly delayed in the absence of Hat1 (Figure 2B).

Loss of Hat1 increases replication fork stalling. A decreased rate of DNA replication can be due to decreases in the velocity of the replication fork or increases in the frequency of replication fork stalling. To test the latter possibility, we stained Hat1$^{+/+}$ and Hat1$^{-/-}$ cells with antibodies against phosphorylated ATR (Ser428). ATR is recruited to single stranded DNA at sites of replication fork stalling where it is activated by phosphorylation. Loss of Hat1 resulted in an increased number of cells positive for phospho-ATR foci (Fig. 3A).

To confirm the increase in replication fork stalling, we used the CAA to measure the association of Rad51 with the single strand DNA that is created at stalled replication forks(64). As seen in Figure 2B, there was a significant increase in the association of Rad51 with newly synthesized DNA in the absence of Hat1. As a positive control, treatment with HU results in a large increase in Rad51 CAA signal in the in both Hat1$^{+/+}$ and Hat1$^{-/-}$ cells.

The effect of Hat1 loss on replication fork stalling could be the result of increased levels of DNA damage inhibiting replication fork progression. Hat1$^{-/-}$ MEFs contain increased levels of γ-H2AX, a marker of DNA damage(19). It was recently shown that Hat1$^{-/-}$ MEFs display defects in mitochondrial function that result in elevated levels of ROS (reactive oxygen species). The mitochondrial defects are linked to the elevated levels of DNA damage as growth of Hat1$^{-/-}$ MEFs in the presence of N-acetylcysteine (NAC) eliminated the elevated levels γ-H2AX staining(65). To determine whether Hat1 loss indirectly affects replication fork stalling through increased ROS and DNA damage, we performed Rad51 CAAs in Hat1$^{+/+}$ and Hat1$^{-/-}$ cells grown in the presence and absence of NAC. As seen in Figure 3C, growth in NAC led to a partial decrease in the Rad51 signal in Hat1$^{-/-}$ cells. This suggests that loss of Hat1 effects replication fork stalling through both DNA damage-dependent and DNA damage-independent mechanisms. Together, these data indicate that Hat1 is necessary for proper replication fork function and the prevention of replication stress.

Hat1 is critical for the stability of stalled replication forks. As seen in Figure 1C, Hat1 is stably associated with stalled replication forks. To determine whether Hat1 is involved in maintaining the stability of stalled replication forks, we analyzed the stability of newly replicated DNA at stalled replication forks using the DNA fiber assay. Hat1$^{+/+}$ and Hat1$^{-/-}$ cells were treated with CldU and IdU sequentially for equal lengths of time. HU was then added to induce replication fork stalling. After 5 hours, the lengths of the IdU and CldU tracts were measured. If the stalled replication forks remain stable, the ratio of IdU tract length to CldU tract length will be 1. If the newly replicated DNA (represented by the IdU labeled DNA) at the stalled forks is unstable, the ratio of IdU

tract length to CldU tract length will be less than 1. As seen in Figure 4A, there was a significant decrease in the IdU tract length in the absence of Hat1. We conclude that Hat1 is required for the protection of newly replicated DNA at stalled replication forks.

The degradation of newly replicated DNA at stalled replication forks is the result of Mre11 nuclease activity (66)((67). To determine whether the instability of nascent DNA in the absence of Hat1 is also Mre11-dependent, $Hat1^{+/+}$ and $Hat1^{-/-}$ cells were sequentially treated with CldU and IdU for equal lengths of time. The cells were then treated with HU in the presence of Mirin, a specific inhibitor of Mre11 activity. As seen in Figure 4B, newly replicated DNA is equally stable in $Hat1^{+/+}$ and $Hat1^{-/-}$ cells when Mre11 activity is inhibited. These data indicate that Hat1 functions to protect newly replicated DNA from Mre11-mediated degradation.

We used a comet assay to determine whether Hat1-dependent replication fork instability led to a decrease in the ability of cells to recover from replication stress. $Hat1^{+/+}$ and $Hat1^{-/-}$ cells were treated with HU for 3 hours and then allowed to recover for 12 hours in the absence of HU. As seen in Figure 4C, $Hat1^{+/+}$ cells were better able to recover from prolonged replication stress than the knock out cells, consistent with a loss of replication fork integrity in the absence of Hat1.

DISCUSSION

Contrary to the predictions of current models of replication-coupled chromatin assembly, our results demonstrate that Hat1 localizes to chromatin at sites of DNA replication. There are several models to explain the localization of Hat1 to nascent chromatin. First,

Hat1 may not transfer H3/H4 dimers to Asf1. Rather, Hat1 may remain associated with the H3/H4 dimers throughout the entire replication-coupled chromatin assembly process and load onto newly replicated DNA through CAF-1-mediated deposition of H3/H4/Hat1 complexes. This model is consistent with numerous proteomic studies that have identified the Hat1 complex as major components of soluble H3 and H4 complexes(22,26,68-72). Alternatively, following transfer of H3/H4 dimers to Asf1, Hat1 may enter the nucleus independently and bind to nascent chromatin after histone deposition. Finally, Hat1 may participate in an additional chromatin assembly pathway distinct from the Asf1/CAF-1 pathway. One potential pathway may utilize the histone chaperone NASP. A distinct nuclear yeast Hat1 complex contains histones H3 and H4 and a histone chaperone, Hif1, which is the yeast homolog of NASP(15,21,68). Subsequent experiments have shown that NASP also interacts with the Hat1 complex in mammalian cells(22). NASP is important for buffering the pools of soluble H3/H4, particularly under conditions of replication stress, and can form a multi-chaperone complex with Asf1. Several studies have shown that NASP can function as a nucleosome assembly factor *in vitro*(73-75). Hence, this model predicts that Hat1 localizes to newly replicated DNA in conjunction with NASP-mediated deposition of H3/H4.

It is clear that DNA replication is coupled to newly synthesized histone deposition. This link was originally suggested by studies demonstrating that DNA replication required active protein synthesis(76-78). More recently, histone supply was more directly linked to replication fork function by experiments that specifically limited histone production(48,50). The assembly of newly synthesized histones into chromatin was

directly implicated in replication fork function through the identification of DNA replication defects in cells lacking components of the replication-coupled chromatin assembly pathway, such as Asf1 and CAF-1(39,49,51).

While histone deposition in the wake of the replication fork is clearly important for fork progression, histone deposition occurs with normal kinetics in Hat1$^{-/-}$ cells, leaving open the question of how Hat1 influences replication fork progression(59). There are a number of possibilities. While histones are still deposited onto newly replicated DNA in the absence of Hat1, nucleosomes may not be assembled properly. Proteomic analysis showed that nascent chromatin assembled in the absence of Hat1 was associated with increased levels of topoisomerase 2 and Hat1$^{-/-}$ cells are hypersensitive to topoisomerase 2 inhibition(59). Therefore, topological defects in nascent chromatin assembled in the absence of Hat1 may impede replication fork progression. The nascent chromatin proteomics also identified a number of proteins that are depleted from nascent chromatin assembled in Hat1$^{-/-}$ cells. These include the bromodomain proteins, Baz1a, Brg1 and Brd3(59). Decreased levels of these proteins in the proximity of replication forks may create an altered chromatin structure that negatively affects replication fork function or they may be directly involved in replisome function. Indeed, it was recently shown that Brd2, Brd3 and Brd4 function at the replication fork to antagonize the ATAD5-mediated unloading of PCNA, which is consistent with our observation that PCNA unloading is slowed in Hat1$^{-/-}$ cells(79). Finally, the presence of Hat1 on nascent chromatin near replication forks suggests that Hat1 may directly modify and regulate components of the replisome.

The association of Hat1 with nascent chromatin is transient but becomes stable if replication forks stall. The physical association of Hat1 with stalled forks is likely to be functionally relevant as Hat1 is required for the stability of stalled replication forks. An attractive mechanism for the role of Hat1 in replication fork stabilization involves the recruitment of Rad51 to stalled forks. Rad51 binds to single strand DNA at stalled replication forks and plays a central role in maintaining replication fork stability. Hat1 forms an S-phase-specific complex with Rad51 and is involved in the recruitment of Rad51 to DNA double strand breaks (80). However, we do not detect any decrease in Rad51 localization to stalled replication forks in $Hat1^{-/-}$ cells, suggesting that the mechanisms for Rad51 recruitment to DNA double strand breaks and stalled replication forks are distinct.

It has also been suggested that Hat1 is involved in the initiation of DNA replication. Studies in yeast showed that Hat1 physically interacts with the origin recognition complex (ORC). In addition, combining mutations in Hat1 with temperature sensitive alleles of ORC components or CDC45 resulted in synthetic growth defects. Hat1 was also recruited to origins of replication at the time of origin activation. Despite these connections, there were no defects in replication origin firing in Hat1 mutants in yeast(81).

Our results suggest an update to current models of replication-coupled chromatin assembly to incorporate the localization of Hat1 to nascent chromatin at sites of DNA replication. In addition, our results indicate that Hat1 lays a direct and integral role in both genome and epigenome duplication.

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FIGURE LEGENDS

Figure 1. **Hat1 localizes to newly replicated DNA**. A) Quantitation of CAAs for PCNA, H4 lysine 5 acetylation and H4 lysine 12 acetylation performed in $Hat1^{+/+}$ and $Hat1^{-/-}$ cells (*** indicates p value < 0.01). B) Images of CAAs for Hat1 in $Hat1^{+/+}$ and $Hat1^{-/-}$ cells. Cells were visualized for IdU, Hat1 CAA and DAPI as indicated. C) Quantitation of Hat1 CAAs following a thymidine chase of the indicated length.

Figure 2. Hat1 is required for replication fork progression. A) Left top, schematic diagram of CldU/IdU labeling of DNA fibers. Left bottom, representative images of replication forks from $Hat1^{+/+}$ and $Hat1^{-/-}$ MEFs. Right, violin plot of the quantification of the fork distances traveled during CldU pulse (green fiber). At least 250 fibers were scored per cell line, P-value was calculated using the Wilcoxon test. B) Quantitation of PCNA CAAs following a thymidine chase for the indicated lengths of time.

Figure 3. Hat1 loss causes replication fork stalling. A) phospho-ATR immunofluorescence (red) in $Hat1^{+/+}$ and $Hat1^{-/-}$ MEFs. Cells were also stained with DAPI. B) Quantitation of CAAs measuring localization of Rad51 to newly replicated DNA at stalled replication forks in $Hat1^{+/+}$ and $Hat1^{-/-}$ MEFs. C) Quantitation of Rad51 CAAs in Hat1+/+ and Hat1-/- cells grown in the absence and presence of 5 mM N-acetylcysteine (NAC).

Figure 4. Hat1 protects newly replicated DNA from Mre11 digestion at stalled forks A) Left top, schematic of CldU/IdU pulse-labeling followed by HU treatment. Left bottom, representative images of CldU and Idu replication forks in HU treated cells. Right, violin plot of IdU to CldU fiber length ratios for individual replication forks in HU treated Hat1$^{+/+}$ and Hat1$^{-/-}$ MEFs. At least 250 fibers were scored per cell line, P-value was calculated using the Wilcoxon test. B) Cells were treated as in (A) in the presence of 100μM Mirin. Left, representative pictures of both Hat1$^{+/+}$ and Hat1$^{-/-}$ DNA fibers. Right, violin plot of IdU to CldU fiber length ratios for individual replication forks. Statistical analysis was performed as in (A). C) Comet assay in Hat1$^{+/+}$ and Hat1$^{-/-}$ MEFs untreated or treated with HU and then released into fresh media for recovery.

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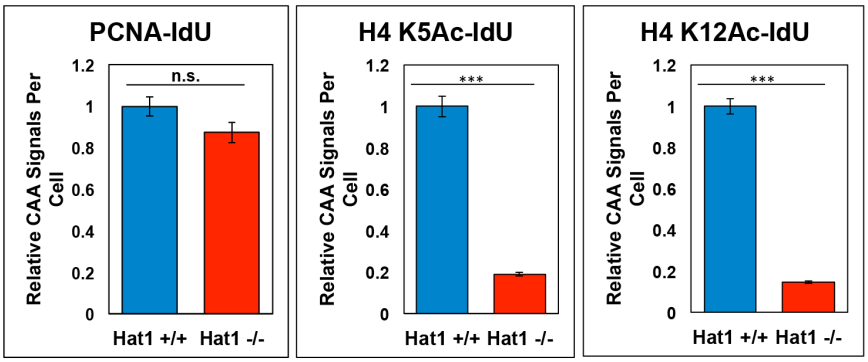
A



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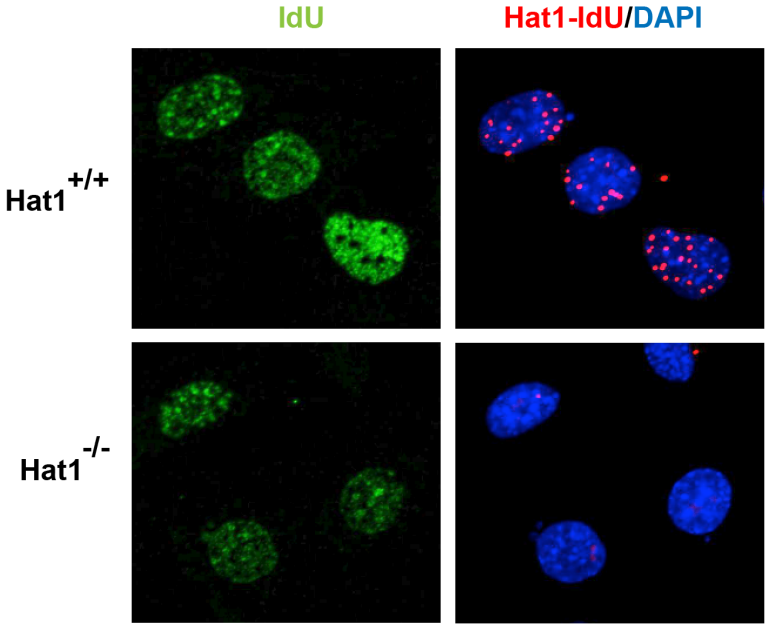

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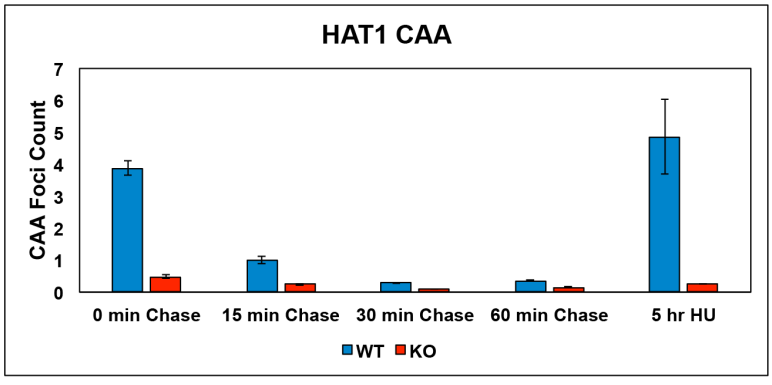


Figure 1

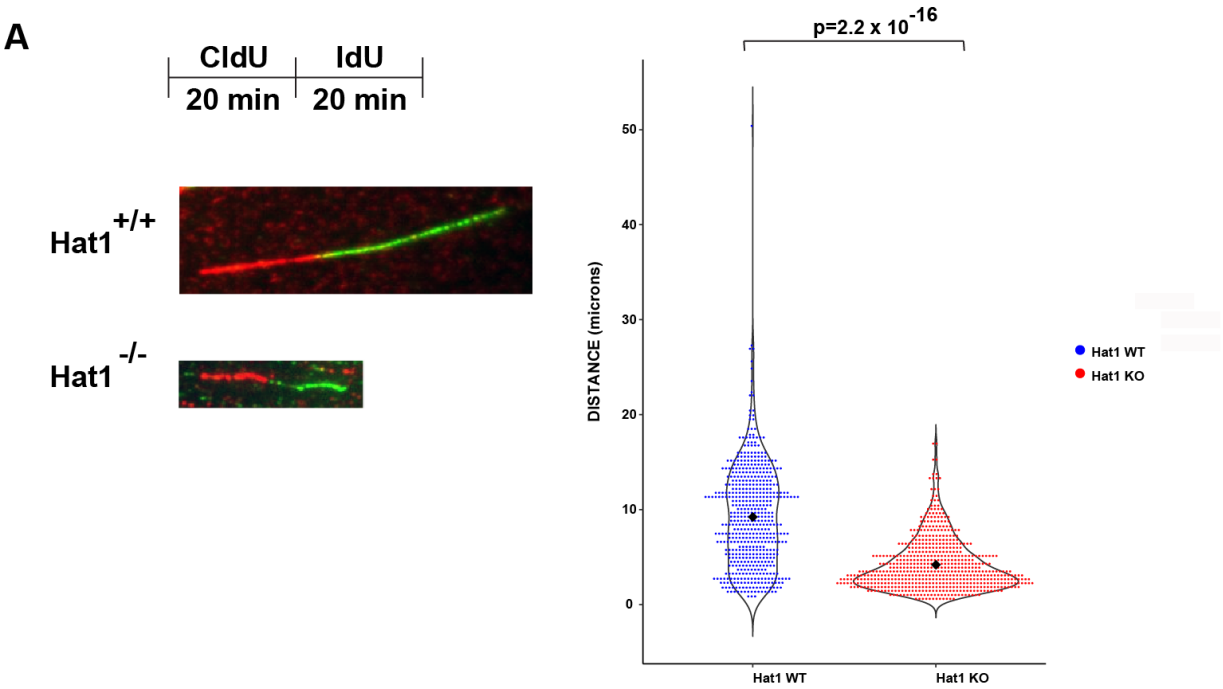
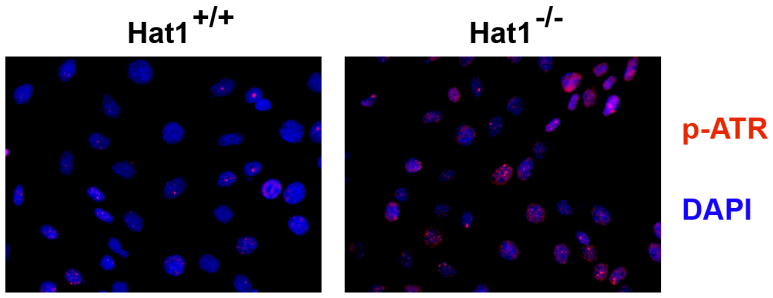


Figure 2

A



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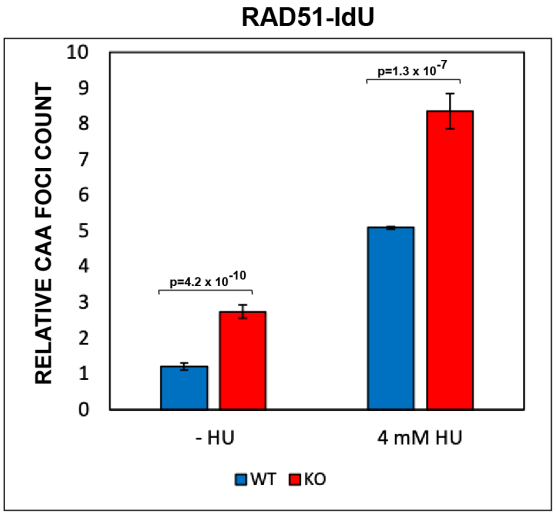

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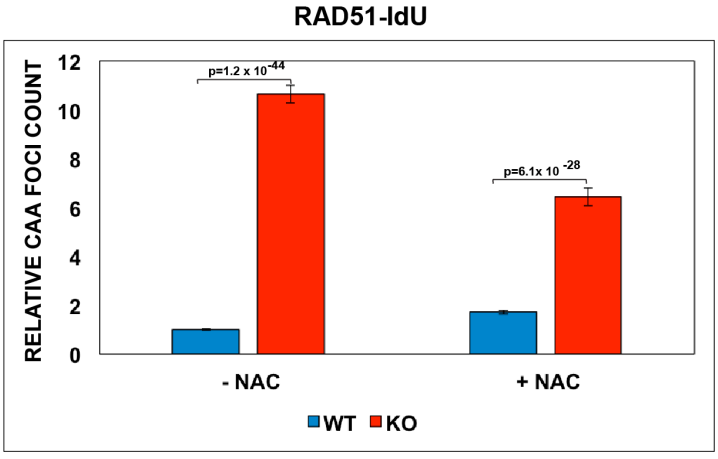

Figure 3

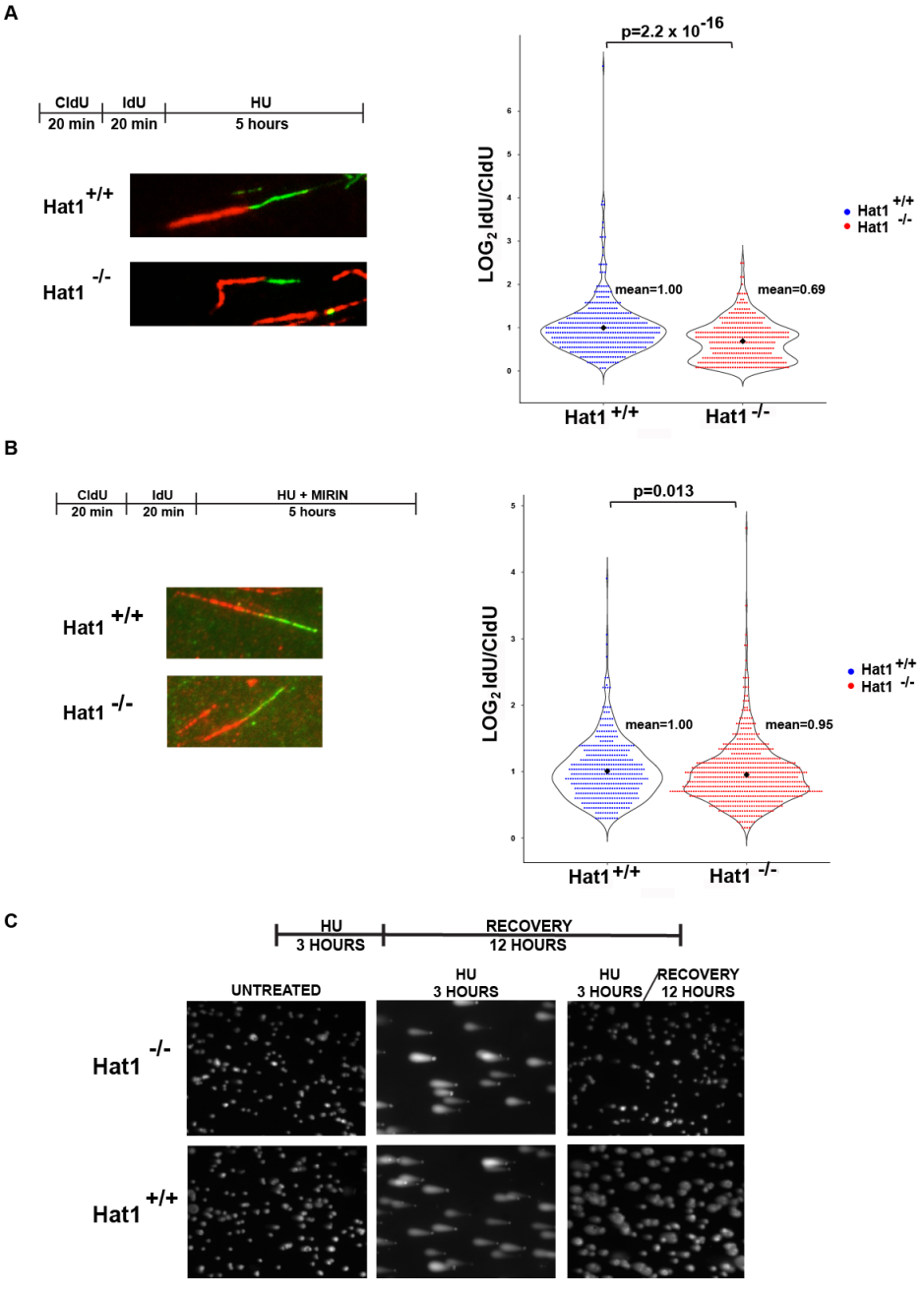

Figure 4