Abstract

Accurate DNA replication during cell proliferation is necessary for proper human development as well as the avoidance of many diseases. Replication is an asynchronous event, as many sites across the genome carry out initiation at various times throughout S-phase of the cell cycle, a process called the replication-timing (RT) program. Aberrations in RT have been associated with cancer, yet the exact mechanisms underlying the control of RT remain largely unknown. We hypothesize that the modulation of chromatin structure by histone post-translational modifications (PTMs) plays an important role in the regulation of RT. Heterochromatic PTMs (i.e. H3K9me) are associated with late S-phase replication while euchromatic PTMs (i.e. H4K16ac) are associated with early replication. A previously-developed histone gene replacement platform in Drosophila was employed to mutate specific histone residues (e.g. H3K9R and H4K16R) and to prevent certain histone PTMs. First, the effects of histone mutation were assessed in diploid cells, using flow cytometry to observe cell cycle progression from G1 to S phase. H3K9R mutants had an abnormal proportion of cells in G and S-phase whereas H4K16R mutants did not, demonstrating that the H3K9 residue is necessary for proper cell cycle progression in Drosophila. Second, to more closely examine S-phase progression itself, the effects of histone mutation were assessed in polyploid cells, using immunofluorescence to detect when various regions of the genome replicated. H4K16R mutants performed abnormally synchronous replication of both euchromatic and heterochromatic regions, suggesting that the H4K16 residue plays a role in regulating the RT program. Additional findings show that H4K16R may disrupt RT by hindering DNA repair mechanisms. Since abnormal RT is error-prone, RT dysregulation may contribute to the emergence of cancer. Therefore, exploring
the interrelationships among histone PTMs, chromatin structure, and regulation of RT may provide a greater understanding of the underlying causes of cancer.

**Introduction**

Precise coordination of DNA replication is essential for transmitting identical genetic content from generation to generation. The replication, or duplication, of the entire genome is carried out during S-phase of each cell cycle and begins at specific initiation sites called “origins of replication” along every chromosome [1]. Replication origins become competent for DNA synthesis, or “licensed,” in G1-phase by recruiting many trans-acting licensing factors, beginning with origin recognition complexes (ORCs). As an ORC is bound to a DNA site, it establishes a pre-replication complex (pre-RC) by recruiting licensing factors Cdc6, Cdt1, and MCM2-7 helicases [2,3]. All pre-RCs have the potential to become active origins; however, only a fraction of them actually proceed to the next stage of licensing – formation of a pre-initiation complex (pre-IC) via kinase-mediated phosphorylation [1]. The pre-ICs then recruit DNA polymerase and primase, both of which are important proteins within the DNA replisome that facilitate bidirectional DNA synthesis away from each origin [3].

Given the massive size of the human genome, the process of replication requires hundreds of these active origins per chromosome [1]. These numerous initiation events do not, however, take place simultaneously. Rather, the firing of origins is asynchronous, occurring at various time points throughout S-phase that, altogether, feed into a well-regulated spatial and temporal scheme – the replication-timing program [3]. This program has been conserved throughout eukaryotic evolution, and aberrations in normal replication timing have been
associated with many cancers, including leukemia [1]. Although conserved, the exact mechanism underlying this temporal regulation remains poorly understood.

Recent work has begun to emphasize the significance of chromatin structure as a possible means of regulating replication timing [1,3,4]. To form a single unit of chromatin, approximately 146 bp of DNA are spooled around an octamer of histone proteins. These units, or nucleosomes, are coiled together across the genome into higher-order chromatin structures. Differential chromatin structures result in a distinction between more-condensed chromatin regions, termed heterochromatin, and less-condensed regions, called euchromatin [5]. With histone proteins at the core of every chromatin unit, differential chromatin organization is under the control of histone post-translational modifications (PTMs), including histone methylation (me) and acetylation (ac) [6]. Specifically, histone PTMs can recruit chromatin remodelers that modulate the strength of electrostatic interactions holding chromatin DNA and histones together [5]. Evidence has shown that several histone PTMs are enriched in euchromatin (H3K4me, H3K36me, H4K16ac) while others are enriched in heterochromatin (H3K9me, H3K27me, H4K20me) [3,8].

The actions of these histone PTMs and their effects on chromatin architecture may be important for the regulation of replication timing [6,8]. For this project, we chose to investigate one PTM for heterochromatin (H3K9me) and one for euchromatin (H4K16ac). These histone PTMs are able to regulate replication timing in two ways. First, PTMs may serve as recruiting sites for certain factors that can modulate replication timing. For example, H3K9 methylation serves as an “anchor” for the binding of Heterochromatin Protein 1 (HP1), which in turn recruits additional effectors (ORC2 and ORC3) that are components of the ORC licensing complex [1,3]. Second, differences in chromatin condensation may affect the amount of trans-acting licensing
factors that can be recruited to those regions [4]. Unlike condensed heterochromatic regions of the genome, euchromatic regions are more "open" and have been shown to exhibit greater accessibility to licensing factors. Euchromatic regions therefore have a higher frequency of origin firing than heterochromatin, ultimately leading to variability in replication timing [4,8]. Hence, regions associated with euchromatic H4K16ac accompany early-replicating sequences, while those of heterochromatic H3K9me accompany late-replicating sequences [3,4,8]. Yet, the role of histone PTMs in the regulation of replication timing has never been directly tested. In this project, we will explore the possible effects that histone PTMs have on the replication-timing program.

The function of histone PTMs in regulating replication can be analyzed by preventing PTMs and examining subsequent changes in phenotype. Previous work has focused on mutating histone-modifying enzymes, which are responsible for adding or removing PTMs. However, these enzymes often have non-histone targets that can complicate analysis of mutant phenotypes [6,9]. A more direct approach involves the mutation of the histone residues themselves. This approach is challenging to perform in animals, whose histone genes are spread out among various loci in the genome. *Drosophila* are an exception, with all of their histone genes clustered at a single location [6]. A histone replacement platform was established with *Drosophila*, in which existing endogenous histones were removed and replaced with a tandem array of either wild-type (HWT) or mutated transgenic histones [6]. Our laboratory previously engineered a lysine-to-arginine (K → R) mutation at the ninth residue of histone H3 (H3K9R) and a separate mutation at the sixteenth residue of histone H4 (H4K16R) [10]. Mutations at H3K9 perturb proper heterochromatin formation, likely by disrupting PTM methylation [5,6,10]. Mutations at H4K16 are predicted to perturb euchromatin.
To explore the relationship between histone PTMs and replication timing, we examined both diploid and polyploid replication in H3K9R and H4K16R mutants. First, in diploid cells, we analyzed the overall cell cycle via flow cytometry. Fewer diploid H3K9R cells were observed in G1 and S-phase, and more were observed in G2/M-phase. In contrast, diploid H4K16R cells did not exhibit abnormal cell cycle phasing. Therefore, H3K9 is necessary for proper cell cycle progression while H4K16 may not be. Second, in polyploid cells, we focused specifically on S-phase replication itself in H4K16R mutants. These mutants carried out simultaneous replication of euchromatic and heterochromatic regions, an abnormal phenotype which suggests that H4K16 regulates the replication-timing program. Aberrations in H4K16R replication may have been a result of DNA damage, specifically double-strand breaks, during S-phase.

**Materials and methods**

*Flow cytometry preparation*

All fly stocks were initially provided by our laboratory, which we raised on standard cornmeal media for all experiments. For flow cytometry experiments, females with the genotype $\frac{His^\Delta}{Cyo}$, $\frac{twi^GALA}{Tm6B}$ were previously crossed with male $\frac{His^\Delta}{Cyo}$, $\frac{PCNA^{-2xYFP}}{12xK9R}$ or $\frac{12xK16R}{12xK16R}$ to produce H3K9R and H4K16R offspring (see Penke et al. 2016 for crossing scheme and culture conditions) [10]. We selected third instar larvae for imaginal wing disc dissection, followed by a nuclei isolation method adopted from Ma and Weake, 2014 [13]. All flow cytometry experiments were performed using an LSR II Flow Cytometer (Becton, Dickinson and Company), through which the amount of DNA at a certain stage (from DAPI) was assessed. Phasing of the cell cycle in Fig. 1A-C was accomplished using FlowJo software (Dean-Jett-Fox model).
**Immunofluorescence preparation**

For polytene chromosome experiments, female $\text{His}^\Delta \text{twi-} \text{GAL4} \text{CyO}$ flies were crossed with male $\text{His}^\Delta \text{UAS-}2\text{xFPF} \text{CyO}$; $12\text{xK9R}$ or $12\text{xK16R}$ flies. We selected third instar larvae of the H4K16R genotype for salivary gland dissection, and a subsequent polytene chromosome squash was performed according to a method described in Cai et al. 2010 [14]. Previous experiments had already conducted polytene squashes for the H3K9R genotype. In this experiment, for the H4K16R genotype, we used a 1:500 dilution of anti-PCNA (Abcam, 29) and a 1:1000 dilution of anti-HP1 (Developmental Studies Hybridoma Bank, C1A9) for antibody staining illustrated in Fig. 2A. In addition, we used a 1:5000 dilution of anti-$\gamma$H2Av for antibody staining illustrated in Fig. 3C. All images were collected from a Leica TCS SP5 AOBS spectral confocal scanner mounted on a DM IRE2 inverted fluorescent microscope.

**Results**

Histone PTMs may contribute to the regulation of genome duplication and the replication-timing program. To test this, we began by performing flow cytometry on third instar larval wing discs, which contain a relatively homogenous sheet of diploid cells. The purpose of flow cytometry was to determine the number of cells within a population in different phases of the cell cycle – G1, S, and G2/M-phase. This quantification method allowed us to analyze possible irregularities in cell cycle progression for PTM mutants (H3K9R and H4K16R), as aberrant progression patterns may be an indication that some aspect of replication in S-phase is malfunctioning.

To monitor progression patterns during flow cytometry, we looked at the strength of DAPI expression to categorize diploid cells into cell cycle phases based on DNA content (Fig.
1A). Noting the transition from 2c \( \rightarrow \) 4c DNA content, or G1 \( \rightarrow \) G2/M phase, we were able to estimate S-phase. For both HWT and H3K9R samples, the percentage of cells present in G1, S, and G2/M-phase were calculated. Results showed that H3K9R mutants had a significantly larger percentage of cells in G2/M-phase than HWT controls, accompanied by a significantly smaller percentage in S-phase (Fig. 1B). These defects signified that H3K9R had a notable influence on the progression of the cell cycle. For H4K16R mutants, there was no significant difference in cell cycle progression compared to HWT, as population percentages for G1, S, and G2/M-phase were relatively consistent (Fig. 1C).

Observed defects in cell cycle progression prompted further investigation of replication itself – exclusively S-phase. Replication-timing patterns can be analyzed in polyploid cells by utilizing polytene chromosomes. Obtained from third instar larval salivary glands, these chromosomes exhibit endoreduplication that causes them to continually re-enter S-phase and replicate without mitotic division, creating many copies of each chromosome per cell (polyploidy) [12]. Polytene chromosomes are useful because they can be easily observed using fluorescent microscopy and provide a visible distinction between euchromatic and
heterochromatic regions. Chromosome arms are primarily regions of less-condensed euchromatin and often denote sites of early S-phase replication. In contrast, compact chromatin complexes called chromocenters are mostly regions of heterochromatin and late replication.

During bidirectional DNA synthesis, PCNA serves as a molecular clamp that facilitates attachment of DNA polymerase to the replisome [11]. As a marker for replication, PCNA consequently provides a convenient way of determining the stage of S-phase occurring in a polytene chromosome at a specific time. We employed a three-stage quantification of PCNA polytene chromosome staining patterns to categorize chromosomes into early, mid, and late S-phase. In HWT control flies, early S-phase replication is represented by dense and wide PCNA-positive bands along chromosome arms; late S-phase is represented by sparse staining along arms but dense staining at the chromocenters. Mid S-phase replication falls between these two categories, often exhibiting partial staining in both regions (Supplemental Fig. 1A, see Appendix).

In prior experiments, this three-stage quantification method showed that H3K9R mutants had a larger percentage of chromosomes in early S-phase than HWT controls and corresponding smaller percentages in mid and late S-phase (Supplemental Fig. 1B). In this experiment, for H4K16R mutants, we applied a similar PCNA assay and three-stage quantification method at first (Fig. 2A). Data from multiple experiments were grouped into one set, and the percentage of cells out of the entire population was calculated for both mutant and control genotypes. H4K16R data showed changes in replication-timing patterns – an increased percentage of early and late S-phase cells and a decreased percentage of mid S-phase cells (Fig. 2B). However, to better monitor progression through S-phase with higher resolution, we opted for a different, more stringent quantification with five stages instead of three (see Supplemental Fig. 2 for
quantification criteria). This five-stage quantification method showed that, again, H4K16R mutants had an increased percentage of early (ER) and early-mid (E-MR) replicating cells and a decreased percentage of mid-late (M-LR) cells (Fig. 2C).

**Figure 2.** H4K16R mutants have altered RT patterns in certain chromosome regions. (A) Similar immunofluorescence and polytene chromosome preparation as HWT and H3K9R. Anti-HP1 staining to denote chromocenters. DAPI and PCNA were included in merge. (B) Quantification method representing the proportion of cells at three relative stages of replication. N represents sample size. (C) Alternative quantification method with five stages. (D) Alternative quantification method to consider partial/full staining of chromocenters in addition to stage of replication.
After reexamining the PCNA banding patterns of H4K16R polytene chromosomes, we found that many H4K16R chromosomes had a unique banding phenotype that differed from HWT and H3K9R chromosomes. In previous replication-timing assays, PCNA-stained regions were only dense in either the arms (early) or the chromocenter (late) at a specific time during replication (Supplemental Fig. 1A). However, for H4K16R mutants, many chromosomes originally considered “early” showed dense staining occurring in not only the chromosome arms but the chromocenter as well (Fig. 2A). This new phenotype did not fit into any of our categorization criteria, indicating that our initial quantification may not have been a sufficient representation of this mutation. As a result, we utilized an alternative quantification method that included a new categorization criteria – partial or full staining of the chromocenter (Fig. 2D).

Newly-quantified H4K16R data showed a larger percentage of the “Early, Full” phenotype, in which early S-phase chromosomes have simultaneously dense staining of both arms and chromocenter. Upon mutation in H4K16R chromosomes, early-replicating chromocenters may have begun to be replicate later, at the same time as heterochromatic arms. Therefore, although mutating the H3K9 and H4K16 residues originally appeared to yield similar replication-timing patterns, the unique H4K16R phenotype demonstrated that these residues in fact had different effects on S-phase progression.

We continued to investigate H4K16R polytene chromosomes in order to further explore why disruption of the H4K16 residue led to changes in S-phase progression. In polyploid cells, DNA repair is a crucial step as cells begin the transition from G to S-phase during endoreduplication; failure to repair damaged DNA at this step can lead to replication errors in S-phase [15]. When double-strand breaks (DSBs) occur in DNA, the histone variant H2Av becomes phosphorylated at its C-terminal tail, and this newly-phosphorylated form called γH2Av
becomes localized at this specific site of DNA damage [15]. Results showed that, during S-phase, H4K16R mutants had a significantly higher amount of γH2Av expression than HWT controls (Fig. 3A). Because of such high S-phase expression, H4K16R mutants did not have a significant difference in γH2Av expression from G to S-phase (Fig. 3A). In contrast, HWT controls had a significant decrease in γH2Av expression during this G to S-phase transition – a period of time when DNA repair mechanisms work to decrease DSBs. To expand upon these results, we then explored whether sites of DNA damage were occurring at the same locations as sites of replication. We co-stained polytene chromosomes with γH2Av as well as PCNA to assess co-localization of DSBs and replication. Our quantification comprised of three criteria: 1)
overlapping bands of γH2Av and PCNA, 2) non-overlapping bands of γH2Av, and 3) non-overlapping bands of PCNA (Fig. 3B-C). When comparing HWT and H4K16R cells, results showed approximately equal percentages of overlap and non-overlap – no significant difference in γH2Av and PCNA co-localization between genotypes (Fig. 3B). Therefore, the increased damage in S-phase may not have been strictly correlated with specific sites of replication. Our results were still able to show that disruption of the H4K16 residue had distinct effects on replication-timing patterns and significant increases in DSBs.

**Discussion**

*Disruption of H3K9 Alters Normal Cell Cycle Progression*

In diploid H3K9R cells, flow cytometry data showed an increased percentage of cells in G2/M-phase and a decreased percentage in G1 and S-phase. These differences in cell cycle phasing may occur for a few reasons – a longer G2/M-phase, a shorter S-phase, or alternatively a longer S-phase – and could indicate problems with replication control mechanisms. To observe the efficiency of replication control in H3K9R mutants, we used flow cytometry to assess DNA re-replication, when cells spontaneously reactivate an origin and mistakenly fire a second round of replication [1]. However, we calculated the percentage of cells with greater than 4c DNA content (>4c) and found no significant re-replication population (Supplemental Fig. 3).

In addition to replication control, aberrations in cell cycle progression could also indicate problems with the mechanisms that regulate the transition of cells from phase to phase. Cellular conditions as well as the accuracy of replication must be assessed at several phase transitions in order to prevent amplification of damage. If signs of damage are detected, cells are kept in that phase until effectors can fix the problem [15]. Therefore, for H3K9R, the observed increase in
the proportion of cells in G2/M-phase may indicate that some type of defect had prompted cells to stay in G2/M-phase longer, corresponding with a shorter S-phase. These defects may have resulted from errors in replication, as diploid cells entering G2/M-phase come directly from the preceding S-phase. Alternatively, apparent elongation of G2/M-phase may have actually been the result of a longer, rather than shorter, S-phase; using our DAPI quantification, if replication errors occurred in late S-phase, these arrested cells could have mistakenly been categorized as early G2/M-phase cells. Live imaging of cells with a PCNA-RFP transgene would allow us to determine how long these cells are actually in late S-phase. Unlike the H3K9 residue, disruption of the H4K16 residue did not show notable changes in cell cycle progression. Thus, H3K9 is necessary for proper cell cycle progression in *Drosophila* while H4K16 may not be.

*Disruption of H4K16 Alters Normal Replication Timing*

H3K9R, a known heterochromatin disrupter, was predicted to change the structure of heterochromatin so that polytene chromosomes would no longer be late-replicating. Prior experiments in the Duronio laboratory with H3K9R supported these predictions, as results showed a decrease in the proportion of mid and late-replicating polytene chromosomes and an increase in the proportion of early-replicating regions of the genome. In this experiment, we focused on H4K16R, a potential euchromatic disrupter, and its effects on replication. We predicted that H4K16R would have somewhat opposite effects of heterochromatin-disrupting H3K9R – fewer early-replicating cells and more mid and late-replicating cells. However, data for H416R did not support these predictions; in both our three-stage and five-stage quantification, there was an increased proportion of cells in early replication and a decreased proportion in mid replication, similar to H3K9R.
Because of these unusual similarities in H3K9R and H4K16R replication patterns, we looked at the polytene chromosomes themselves to examine visual differences in phenotype. We noticed a unique phenotype in H416R chromosomes that showed simultaneously dense PCNA staining in both arms and chromocenters; therefore, we added a new quantification criteria – partial or full staining of the chromocenter. Compared to HWT controls, H4K16R mutants showed a greater proportion of “Early, Full” chromosomes that had both densely-stained arms and a full staining of the chromocenter. H4K16R also showed a greater proportion of the “Mid, Full” phenotype – intermediately-stained arms and a full chromocenter. Disruption of H4K16 therefore caused replication of euchromatic and heterochromatic regions at similar times. By mutating H4K16, we may have disrupted proper euchromatin formation, resulting in later replication as is typical of heterochromatin – less accessibility to licensing factors (ORCs) and a decrease in origin licensing. As a result, typical early-replicating, euchromatic regions (arms) were more inclined to replicate later in S-phase when chromocenters already replicate. Altered replication-timing patterns demonstrate that the H4K16 residue, as well as H3K9, are necessary for proper regulation of replicating timing.

Effects of H4K16R May Arise From Errors In DNA Damage Repair

In H4K16R mutants, we proposed that alterations in the replication-timing program may be linked to disruption of DNA repair mechanisms. The expression of γH2Av was used to determine whether or not H4K16R mutants were repairing DSBs during a critical phase transition – from G to S-phase. We found that H4K16R mutants had a significantly higher amount of γH2Av expression in S-phase than HWT controls. These results suggest that high DNA damage in S-phase may explain why H4K16R mutants were unable to carry out normal
replication-timing patterns in polyploid cells – or that altered replication patterns, in turn, resulted in increased DNA damage. Furthermore, if DNA repair were carried out properly in polyploid cells, we expected to see a significant decrease in $\gamma$H2Av, or DSB, expression from G to S-phase. Results showed that H4K16R mutants did not have a significant decrease in $\gamma$H2Av expression from G to S-phase, which suggests potential errors in DNA repair in addition to abnormally high levels of S-phase damage. In experiments testing for co-localization of $\gamma$H2Av and PCNA in polytene chromosomes, we did not find significant differences between H4K16R mutants and controls. Therefore, damage in S-phase may not be strictly correlated with sites of replication. However, our results do not rule out the possibility that DNA damage was found at regions that were previously replicated and not yet repaired.

Our findings have shown that histone residues are essential for proper regulation of replication timing. Further investigation of the relationship between the replication-timing program and DNA repair is necessary, as high amounts of DNA damage may lead to dysregulation of replication. Aberrations in replication timing have been associated with the emergence of cancer in humans, yet control mechanisms of replication timing remain largely unknown. Therefore, we must continue to explore the interrelationships among histone PTMs, chromatin structure, and regulation of replication timing.

Acknowledgements

Generous funding by the National Institutes of Health. Special thanks to Dr. Taylor Penke, Robin Armstrong, and Dr. Bob Duronio for guidance during this project.
References

1. Donley, N., Thayer, M.J. DNA replication timing, genome stability and cancer: Late and/or delayed DNA replication timing is associated with increased genomic instability. Seminars in Cancer Biology, 2013. 23(2):80-89.


15. Lake, C.M., Holsclaw, J.K., Bellendir, S.P., Sekelsky, J., Hawley, R.S. *The development of a monoclonal antibody recognizing the Drosophila melanogaster phosphorylated histone H2A variant (γ-H2Av).* G3: Genes, Genomes, Genetics, 2013. **9**:1539-1543
**Supplemental Figure 1.** H3K9R mutants have a greater proportion of early-replicating cells than HWT non-mutants. (A) In previous experiments, immunofluorescence of polytene chromosomes was performed on larval salivary glands. DAPI was used for staining nuclei. Anti-PCNA staining denoted regions of replication. Three-stage quantification of chromosomes as early, mid, or late S-phase replication, based on fluorescent band density and width. (B) Quantification method representing the percentage of cells at three relative stages of replication.

**Supplemental Figure 2.** Immunofluorescence preparation same as Supplemental Fig. 1A. Alternative five-stage quantification of chromosomes as early (ER), early-mid (E-MR), mid-late (M-LR), late (LR), and very-late (VLR) S-phase replication. Quantification based on more stringent parameters of fluorescent band density and width.
Supplemental Figure 3. H3K9R mutants did not show a significant re-replication population. Quantification based on the “>4c” gate, which includes cells with greater than 4c DNA content.