Analysis of Drosophila Insulator Protein Function in Replication Timing and the Osmotic Stress Response

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I am submitting herewith a dissertation written by Emily Stow entitled "Analysis of Drosophila Insulator Protein Function in Replication Timing and the Osmotic Stress Response." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry and Cellular and Molecular Biology.

Mariano Labrador, Major Professor

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(Original signatures are on file with official student records.)
Analysis of Drosophila Insulator Protein Function in Replication Timing and the Osmotic Stress Response

A Dissertation Presented for the Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Emily Christine Stow
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ABSTRACT

Chromatin insulators contribute to the three-dimensional organization of the eukaryotic genome. Insulators and their associated proteins form boundaries between differing chromatin environments, regulate enhancer-promoter interactions, and contribute to the formation of distal genomic contacts. Growing evidence suggests chromatin insulators play roles in cellular processes that extend beyond genome organization alone. The collection of work presented here investigates the involvement of insulators in the timing of DNA replication, the nuclear response to osmotic stress, and the maintenance of genome stability. Chapter 1 characterizes a novel component of the Su(Hw) insulator protein complex in Drosophila melanogaster and its contributions to insulator function, genome stability, and the replication timing program. Chapter 2 analyzes conserved properties of the osmotic stress response pathway in Drosophila and human genomes. Chapter 3 identifies a role for Su(Hw) in maintenance of genome stability and demonstrates that Su(Hw) mutants undergo elevated DNA damage possibly due to replication stress. These findings provide evidence that chromatin insulators play a multifaceted role in cellular function that intersects with their role in genome organization.
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INTRODUCTION

Interactions between proteins and DNA organize eukaryotic chromosomes in three-dimensional space within the nucleus. The chromatin fiber is the primary form of genome organization and consists of a linear string of nucleosomes, which are DNA-protein complexes containing 146 base pairs of DNA wrapped around eight histone proteins, connected by linker DNA of variable length between 20 and 60 base pairs (Luger et al., 1997). Chromatin can exist in two structurally distinct environments designated as euchromatin and heterochromatin. Euchromatin compartments cluster near the center of the nucleus, are sites of active transcription, and undergo DNA replication during early synthesis phase (S-phase) of the cell cycle. Heterochromatin compartments are preferentially associated with the nuclear periphery, contain few transcriptionally active genes, and replicate during late S-phase (Gonzalez-Sandoval and Gasser, 2016; Lubelsky et al., 2014). Heterochromatin and euchromatin domains alternate along the linear chromosome and domains with similar transcriptional properties cluster together by forming interactions across the three-dimensional space of the nucleus (Dixon et al., 2012; Nora et al., 2012; Smith et al., 2016). Chromatin insulators are a diverse class of genetic elements that direct the binding of specialized insulator protein complexes and contribute to transcription regulation, chromatin organization, and higher order genome structure. Recent studies add additional levels of complexity to the role of insulators in nuclear function. Here, we analyze the mechanism of insulator function in nuclear processes such as the DNA replication timing program, genome stability during oogenesis, and the nuclear response to osmotic stress.
I. Drosophila Chromatin Insulators

Insulators have been identified in most eukaryotes but exhibit the greatest diversity in

*Drosophila melanogaster* (Chung et al., 1997; Farrell et al., 2002; Guo et al., 2008; Heger et al., 2013; Heger et al., 2012; Heger et al., 2009; Hily et al., 2009; Ishii et al., 2002; Ishii and Laemmli, 2003; Palla et al., 1997; Schoborg and Labrador, 2010). Early experimental evidence revealed the ability of insulators to regulate enhancer-promoter interactions and serve as boundaries between heterochromatin and euchromatin (Chung et al., 1993; Geyer and Corces, 1992; Kellum and Schedl, 1991; Kellum and Schedl, 1992). More recent advances expand the list of insulator functions to include forming contacts between distal genomic sites and spatially organizing chromatin in the three-dimensional space of the nucleus (Byrd and Corces, 2003; Hou et al., 2012; Labrador and Corces, 2002). Insulators are defined by their DNA-specific sequence and associated proteins. The *Drosophila melanogaster* genome contains five known insulator proteins including Zeste White 5 (Zw5), Boundary Element Associated Factor (BEAF 32A and 32B), CCCTC Binding Factor (CTCF), GAGA Factor (GAF), and Suppressor of Hairy Wing [Su(Hw)], whereas only a CTCF insulator protein has been identified in mammals (Burcin et al., 1997; Gomez-Diaz and Corces, 2014; Moon et al., 2005). Each insulator protein is able to perform multiple functions and the designated function depends on cell type and insulator location. Components of insulator protein complexes are also cell type and location dependent, suggesting that insulator complex components specify the function of the insulator at a given site (Gurudatta and Corces, 2009). The following three examples illustrate the various functions of insulator proteins discovered during early studies of *Drosophila* insulators.

The *Drosophila* bithorax complex controls the development of thoracic and abdominal body segments. *Abdominal-B (Abd-B)* gene expression patterns control development of the
posterior parasegments. The expression pattern of *Abd-B* in posterior parasegments is regulated by initiators located in cis regulatory regions identified as *infra-abdominal 5-9 (iab-5-iab-9)*. Analysis of the bithorax complex revealed the ability of the *Fab-7* and *Fab-8* genetic elements to determine parasegment identity by forming boundaries between the *iab* initiators and their action on *Abd-B* expression patterns in each parasegment. Deletion of *Fab-7* or *Fab-8* leads to the misregulation of Abd-B expression and fusion of parasegments (Barges et al., 2000; Zhou et al., 1996). Later, the GAF insulator protein was found to bind *Fab-7* and CTCF was found to bind *Fab-8*, leading to the identification of *Fab-7* and *Fab-8* as insulator elements (Holohan et al., 2007; Wolle et al., 2015). The binding of insulator proteins to insulator sites allows the insulator to carry out position-specific regulation of enhancer-promoter interactions.

The *Drosophila* 87A7 heat shock locus provides an example of another canonical role of insulators, their ability to form transcriptionally distinct domains. Upon exposure to high temperatures, two heat shock genes in a heterochromatin region near the 87A7 locus become transcriptionally active with no changes to the surrounding chromatin. An early study identified two unique chromatin structures that flanked the heat shock gene region and named them *specialized chromosome structures (scs and scs’)* (Udvardy et al., 1985). The Zw5 and BEAF-32 insulator proteins were later identified to bind *scs* and *scs’*, respectively (Gaszner et al., 1999; Zhao et al., 1995). These structures were amongst the earliest characterized insulators and highlight the ability of insulators to mark the boundaries of transcriptionally distinct compartments.

*Gypsy* is a well-characterized retrotransposon in *Drosophila* and contains twelve binding sites for the Su(Hw) insulator protein. All insulator complexes rely on partner proteins to carry out basic insulator functions. Common insulator partner proteins are Centrosomal Protein 190
(CP190) and Modifier of mdg4 [Mod(mdg4)], which exists in multiple splice variants that each interact with different insulator proteins (Dorn et al., 1993). Insulator complexes may contain multiple combinations of partner proteins. CP190 and Mod(mdg4), are Broad-complex, Tramtrack and Bric-a-brac/Poxvirus, and Zinc Finger (BTB/POZ) domain-containing proteins that often form a complex with Su(Hw) (Georgiev and Gerasimova, 1989; Pai et al., 2004). Both CP190 and Mod(mdg4) are required for the enhancer blocking function of the Su(Hw) insulator (Kurshakova et al., 2007; Pai et al., 2004). CP190 was originally discovered due to its association with centrosomes and microtubules during mitosis (Oegema et al., 1997). It was later found that mutations in CP190 cause suppression of gypsy-mediated mutant phenotypes, suggesting it is required for the enhancer blocking ability of Su(Hw). CP190 is also able to interact with insulator proteins BEAF-32 and CTCF (Liang et al., 2014). The ability of CP190 to interact with multiple insulator proteins suggests CP190 plays a global role in insulator function. Modifier of mdg4 is another essential component of the gypsy insulator. Mod(mdg4) 67.2 was identified as the Su(Hw)-specific isoform because of its frequent association with Su(Hw) in microscopy analysis of Drosophila polytene chromosomes (Gerasimova and Corces, 1996; Ghosh et al., 2001). Loss of the Mod(mdg4) 67.2 isoform alone only causes defects in gypsy-related phenotypes, whereas loss of Mod(mdg4) altogether is recessive lethal (Gause et al., 2001; Gerasimova et al., 1995b). Mutant alleles that arise from ectopic insulator insertion and display obvious phenotypes provide a useful way to assay for insulator function and the contributions of insulator binding proteins to insulator function.

The yellow² and cut⁶ mutant alleles contain an insertion of the gypsy insulator sequence between tissue specific enhancers and the promoters, allowing the Su(Hw) insulator protein to bind and block transcription activation in a tissue specific manner (Gerasimova et al., 1995b;
Jack et al., 1991; Parkhurst and Corces, 1986a). In \( y^2 \), \textit{gypsy} is positioned between the \textit{yellow} body and wing enhancers and the promoter. The \( y^2 \) flies appear yellow in body color and wing color. In \( ct^6 \), \textit{gypsy} is positioned between the wing margin enhancer of \textit{cut} and the promoter, blocking transcription activation. The \( ct^6 \) flies have wings with cut-like aberrations in the wing margin. Mutations in \( su(Hw) \) reverse these phenotypes. This model of insulator function provides another illustration of the canonical enhancer blocking role of insulator proteins and provides an elegant system to study insulator function.

A recent study identified yet another member of the \textit{gypsy} insulator complex called Heterochromatin and Insulator Partner Protein, 1 (HIPP1) (Alekseyenko et al., 2014). This study revealed the presence of HIPP1 in Su(Hw) and CTCF insulator complexes as well as the presence of HIPP1 at Su(Hw) and CTCF binding sites. HIPP1 also displayed a strong association with CP190, a component of both Su(Hw) and CTCF insulator complexes. HIPP1 interacts with Heterochromatin Protein 1a (HP1a) as well. A phenotypic analysis of \textit{hipp1} mutants revealed no obvious phenotypes, suggesting a more thorough investigation of the involvement of HIPP1 in insulator function is required (Glenn and Geyer, 2018). However, since the only obvious phenotype of \( su(Hw) \) mutants is female sterility resulting from defects in germline development, it is not surprising that mutations in \textit{hipp1}, a Su(Hw)-interacting protein, would not result in readily noticeable phenotypes (Klug et al., 1968; Klug et al., 1970). HIPP1 has also been identified as a homolog of the human protein Chromodomain Y Like (CDYL). HIPP1 and CDYL contain a similar crotonase like domain however HIPP1 lacks the chromodomain that is present in CDYL (Glenn and Geyer, 2018). Crotonase domains contain an active site called an oxyanion hole that stabilizes the enolate anion intermediate from an acyl-CoA substrate (Wu et al., 2009). CDYL has been linked to a variety of cellular processes including DNA damage.
repair, epigenetic inheritance, and recruitment of histone remodelers (Abu-Zhayia et al., 2018; Escamilla-Del-Arenal et al., 2013; Franz et al., 2009; Liu et al., 2017b; Mulligan et al., 2008; Wu et al., 2013; Zhang et al., 2011). Homology between HIPP1 and CDYL suggests HIPP1 may participate in functions similar to CDYL. Since Su(Hw) has been proposed to play a role in recruiting histone remodelers, investigating a role for HIPP1 in that function of the insulator is a promising avenue of research (Vorobyeva et al., 2013b).

Su(Hw) was recently found to colocalize with replication origins in regions of heterochromatin. Co-immunoprecipitation studies reveal the ability of Su(Hw) to recruit the histone acetyltransferase complex, SAGA, and the nucleosome remodeling complex, Brahma to future replication origins. These complexes modify existing chromatin modifications to create an open environment that can be accessed by replication machinery. Thus, the origin recognition complex (ORC) was found to bind in close proximity with a subset of Su(Hw) binding sites. Additionally, Su(Hw) was also shown to co-immunoprecipitate with the ORC3 subunit of the ORC, suggesting a close relationship between the Su(Hw) insulator protein and replication machinery (Vorobyeva et al., 2013b). Due to the similarities between CDYL’s recruitment of histone remodelers and Su(Hw)’s ability to recruit remodelers to replication origin sites, we have chosen to investigate a role for the Su(Hw)-HIPP1 complex in DNA replication.

II. Eukaryotic Replication Program
Eukaryotic chromosomes require the establishment and activation of many replication origins to replicate the genome within the timing constraints of the cell cycle (Mechali, 2010). During early development, activation of replication origins is widespread in order to accommodate rapid growth of the organism. As cells divide and differentiate to accept various cell fates, the genome
adopts regulatory mechanisms to ensure appropriate gene expression and the number of active origins consequently decreases (Fragkos et al., 2015). Transcriptionally active regions contain many origins of replication and replicate earlier during S-phase compared to transcriptionally repressed regions (Goldman et al., 1984). How the cell responds to cues from the chromatin environment to choose origins of replication and the appropriate time to activate them during replication is an active area of research that is essential to understanding the replication timing program.

Origins of replication require licensing and activation to initiate DNA synthesis. Although many possible replication origins are licensed during replication, only a subset are activated (Fragkos et al., 2015). Origin licensing occurs during the G1 to S phase transition and begins with nucleosome remodeling to prepare origins for the binding of the six-subunit origin recognition complex (ORC 1-6). Licensing factors, cell division cycle 6 (CDC6) and cdc10-dependent transcript 1 (CDT1), subsequently bind the origin and allow the double hexamer mini-chromosome maintenance (MCM2-7) complex to be loaded onto the DNA. After the origin is licensed, activation of the origin relies on the formation of the pre-initiation complex (pre-IC) during the G1-S phase transition and activation of the two MCM hexamers during S-phase. The phosphorylation of several MCM residues leads to the release of MCM hexamers and the release of DNA polymerase bidirectionally from the replication origin, initiating DNA synthesis (Yeeles et al., 2015). Release of MCM from the origin leads to the recruitment of other components of the DNA replication machinery, such as the proliferating cell nuclear antigen (PCNA), which assist in the processivity of the replication forks (Tardat et al., 2010).

Each replication origin should be activated only once per cell cycle. Re-replication at an origin results in improper DNA synthesis, causing DNA damage that must be resolved to ensure
integrity of the genome. The eukaryotic cell has multiple ways to prevent re-replication by ensuring an activated origin is appropriately delicensed. The prevention of re-replication relies on the degradation of the origin licensing proteins. Interactions between PCNA and the licensing proteins target them for proteasomal degradation (Jorgensen et al., 2011).

A topologically associating domain (TAD) is a region of chromatin that frequently interacts and possesses similar transcriptional properties. TADs are consistent within a given cell type and correlate with replication domains (Pope et al., 2014; Smith et al., 2016). During DNA replication, the cell replicates transcriptionally active domains first, followed by the transcriptionally repressed domains. One possible explanation for this timing program is the difference in nucleosome density between active and repressed domains. Low nucleosome density is a requirement for ORC binding. Because repressed domains of the genome are characteristically more nucleosome dense than active domains, repressed domains require a greater extent of nucleosome remodeling to prepare for replication origins (Gomez-Diaz and Corces, 2014).

In addition to low nucleosome density, specific chromatin marks and protein determinants can target the establishment of replication origins. Histone 4 Lysine 20 (H4K20) methylation is associated with many cellular processes including DNA transcription and replication. PR-Set7 is a SET domain containing methyltransferase that mediates the monomethylation of H4K20, a chromatin mark commonly found at replication origins (Fang et al., 2002; Nishioka et al., 2002; Tardat et al., 2010). Artificial PRSet7 monomethylation of H4K20 is capable of recruiting components of the pre-replication complex to random genomic loci that would not otherwise function as origins (Beck et al., 2012). H4K20 methylation is also a component of origin delicensing. Once an origin has been activated, PCNA recruits the E3
ubiquitin ligase, cullin ring finger ligase-4 complex containing Cdt2 (CRL4\textsuperscript{Cdt2}), to tag PR-Set7 for ubiquitination. CRL4\textsuperscript{Cdt2} mediated PR-Set7 ubiquitination leaves H4K20me1 to be dimethylated by the H4K20 specific methyltransferase, Suv4-20 (Abbas et al., 2010). The conversion of H4K20me1 to H4K20me2 signifies delicensing of the replication origin (Tardat et al., 2010). The failure to degrade PR-Set7 leads to an increase in H4K20me1 and re-replication, leading to DNA damage and stalling of DNA replication (Li et al., 2016). The importance of H4K20 regulation during DNA replication provides an example of how replication machinery relies on cues from the chromatin environment to successfully replicate the genome.

Replication origins are carefully regulated to protect against re-replication, a common source of replication-dependent DNA damage. If re-replication at an origin occurs, the DNA damage accumulated activates a replication-specific repair pathway. Ataxia Telangiectasia-mutated (ATM) and Ataxia Telangiectasia-Related (ATR) are serine/threonine protein kinases that respond to various types of DNA damage (Sancar et al., 2004). These kinases phosphorylate histone variant H2Av in Drosophila and H2AX in humans (γH2Av and γH2AX, respectively), to signal sites of DNA damage. ATM and ATR also phosphorylate checkpoint proteins to specify the appropriate repair pathway. Although ATM and ATR have overlapping roles in DNA damage response, ATM is primarily associated with response to double-strand breaks while ATR is associated with response to single-strand breaks. ATM activation leads to phosphorylation of the Check 2 (Chk2) check point protein (Drosophila \textit{mnk}), while ATR phosphorylates Check 1 (Chk1) (Drosophila \textit{grapes}). Stalling of replication forks during S-phase is one source of single-strand DNA breaks, identifying ATR as the primary responder to replication-associated DNA damage (Centore et al., 2010; Zeman and Cimprich, 2014).
Although the mechanisms of origin licensing and activation are well understood, it remains unclear how metazoan nuclei determine origin placement and differentiate between active and inactive origins. DNA replication must be carefully timed to coordinate replication of the entire genome without causing DNA damage (Zannini et al., 2014). Thousands of bidirectional replication origins must be licensed and released in an organized manner (Lubelsky et al., 2014). The eukaryotic genome utilizes differences in chromatin environments to sort the genome into early and late replicating domains. The correlation between replication domains and chromatin-dependent TADs suggests genome organization is critical to the replication timing program (Dixon et al., 2012). The importance of insulators in genome organization makes them an interesting target of research to better understand the relationship between nuclear structure and the replication timing program.

Recent studies in mammalian systems establish a role for DNA replication in the establishment and maintenance of TADs. Surprisingly, deletion of insulators in mouse embryonic stem cells does not affect compartmentalization of active and inactive regions of the genome or replication timing. Instead, the deletion of insulator independent, intra-TAD elements resulted in differences in replication timing that coincide with changes in compartmentalization of active and inactive domains (Sima et al., 2019). Additionally, inhibition of DNA replication, rather than inhibition of transcription, prevents TAD formation during early mouse embryogenesis, further suggesting an important link between replication programs and genome structure that should be further explored (Ke et al., 2017).
III. Mammalian Chromatin Insulators

Since only the CTCF insulator has been identified in mammals, the study of mammalian insulator is more straightforward, compared to Drosophila. Mammalian insulators are positioned between topologically associating domains (TADs), where they segregate regions undergoing frequent interactions and prevent interactions between adjacent TADs (Smith et al., 2016; Symmons et al., 2014). The removal of insulator elements results in loss of boundary strength and promotes intermingling of TADs (Nora et al., 2012). Mammalian insulator elements are also found within TADs to mediate gene regulation. Investigating the role of Drosophila and mammalian insulators in transcriptional regulation, DNA replication, and genome stability will lead to a better understanding of genome organization and function.

Recent advances in the analysis of mammalian genome folding has led to the development of a model to explain how the mammalian insulator protein CTCF both regulates gene transcription and shapes the genome into series of adjacent TADs (Moon et al., 2005; Rao et al., 2014). Instrumental to these studies was the development of genome-wide chromosome conformation capture (Hi-C), a procedure that uses next-generation sequencing to generate a map of DNA-DNA interaction frequencies across the genome (Belton et al., 2012). Analysis of this data can infer the three-dimensional structure of the genome. Using Hi-C data and polymer simulations, the model of loop extrusion has been developed as a model of mammalian genome folding. In this model, G1 cohesin complexes extrude loops of DNA until they encounter two CTCF molecules bound in opposing orientation (Fudenberg et al., 2016; Nora et al., 2017). Once an interaction between cohesin and two CTCF proteins is formed, a stable loop domain is formed until proteins are turned over (Hansen et al., 2017). Through this process, interactions within the loop domain are possible, bringing together distal enhancers and their target promoters.
Additionally, an extruded domain is prevented from interacting with an adjacent domain due to the boundaries provided by CTCF sites located at the base of the loop. A/B compartmentalization partitions the human genome at the megabase scale into regions of transcriptionally active (A) compartments and transcriptionally inactive (B) compartments. Compartments can be subdivided into topologically associating domains (TADs) which range in size from <250kb-1Mb and are formed by loop extrusion.

Hi-C analysis of the Drosophila genome reveals the presence of TAD structures similar to those found in mammalian systems. However, a model of loop extrusion similar to the one described in mammals has yet to be unveiled in Drosophila and the existence of insulator-mediated loop domains in Drosophila remains unclear (Hou et al., 2012; Rowley et al., 2017b; Tanay and Cavalli, 2013). An important difference between mammalian and Drosophila loop domains is that CTCF and cohesin alone form loop domains in mammals whereas a variety of factors mediate loop formation in Drosophila. In addition to insulator proteins, loop domains are formed by the Zelda transcription factors during early development and polycomb group proteins in regions of heterochromatin (Eagen et al., 2017; Entrevan et al., 2016; Hou et al., 2012; Hug et al., 2017; Ogiyama et al., 2018). The existence of Drosophila loop domains in transcriptionally active regions appear to rely on transcription activity alone, further suggesting loop domains in Drosophila differ significantly from mammals (Rowley et al., 2017b; Ulianov et al., 2016). The ability of multiple factors to form loop domains makes it more difficult to develop a comprehensive model of genome folding. Therefore, it is important to investigate the different mechanisms of loop formation in Drosophila to develop a full understanding of genome organization.
IV. Nuclear Response to Osmotic Stress

Comparing nuclear processes between *Drosophila* and mammalian genomes provides a way to understand differences in genome structure and function. The osmotic stress response has proven to be a simple and useful tool for understanding genome function in both *Drosophila* and mammalian systems and has unveiled important similarities and differences in the way insulator proteins respond to stress in the different organisms (Schoborg et al., 2013b). In both organisms, insulator proteins exit chromatin and accumulate in nuclear space upon treatment with media that contains a high salt osmolality. Additionally, the nuclear volume decreases and chromatin condenses (Berga-Bolanos et al., 2010; Schoborg et al., 2013b). In Drosophila, the insulator proteins form aggregates in the nuclear periphery, called insulator bodies, while in mammals, CTCF becomes homogenously distributed in the nuclear space but excludes chromatin-occupied territory (Amat et al., 2019). It is not yet known why insulator proteins behave differently during osmotic stress in Drosophila and mammals. One possibility is that the variety of insulator proteins in Drosophila are better suited to form aggregates whereas CTCF alone is not able to form aggregates in mammals. Here, we analyze the osmotic stress response in human cells and find that additional components of chromatin architecture exit chromatin during osmotic stress treatment including members of the cohesin complex. We also find that cohesin participates in the osmotic stress response in Drosophila, suggesting cohesin may contribute to nuclear structure in Drosophila as well as mammalian genomes.

In addition to learning more about genome structure, studying the osmotic stress response contributes to our knowledge about health conditions involving high osmotic pressure or mechanical stress. Components of the signaling cascades initiated by osmotic stress are essential for viability (Berga-Bolanos et al., 2010). Persistent osmotic stress may lead to a host of
diseases and disorders such as eye disease, diabetes, irritable bowel syndrome, cardiovascular disease, and liver disease (Brocker et al., 2012). Studying the role of insulator proteins in the response to nuclear osmotic stress may be critical to understanding how the nucleus adapts during osmotic stress to increase chances of cell survival.

Chromatin insulator proteins contribute to genome organization in both *Drosophila* and mammals. The studies presented here provide an in-depth analysis of insulator function in non-canonical roles such as timing of DNA replication, response to osmotic stress, and maintenance of genome stability during oogenesis. These studies complement a growing body of evidence that chromatin insulator proteins are equally critical for both genome organization and genome stability in Drosophila and mammals alike.
CHAPTER 1: A *Drosophila* Insulator Interacting Protein Suppresses Enhancer-Blocking Function and Modulates Replication Timing
This chapter has been submitted for publication:


My contribution included: (1) performing experiments, (2) data collection and analysis, (3) writing manuscript and making figures, and (4) devising experiments. Ran An’s contribution included (1), (2), and (4). Todd Schoborg’s contribution included (1) and (4). Nastasya Davenport’s contribution included (1) and (2). James Simmon’s contribution included (2) and (3). Mariano Labrador’s contribution included (3) and (4).
Abstract

Insulators play important roles in genome structure and function in *Drosophila* and mammals. More than six different insulator proteins are required in *Drosophila* for normal genome function, whereas CTCF is the only identified protein contributing to insulator function in mammals. Interactions between a DNA binding insulator protein and its interacting partner proteins define the properties of each insulator site. The different roles of insulator protein partners in the *Drosophila* genome and how they confer functional specificity remain poorly understood. Functional analysis of insulator partner proteins in *Drosophila* is necessary to understand how genomes are compartmentalized and the roles that different insulators play in genome function. In *Drosophila*, the Suppressor of Hairy wing [Su(Hw)] insulator is targeted to the nuclear lamina, preferentially localizes at euchromatin/heterochromatin boundaries, and is associated with the *Gypsy* retrotransposon. The properties that the insulator confers to these sites rely on the ability of the Su(Hw) protein to bind the DNA at specific sites and interact with Mod(mdg4)-67.2 and CP190 partner proteins. HP1 and insulator partner protein 1 (HIPP1) is a recently identified partner of Su(Hw), but how HIPP1 contributes to the function of Su(Hw) insulator complexes has not yet been elucidated. Here, we find that mutations in the HIPP1 crotonase-like domain have no impact on the function of Su(Hw) enhancer-blocking activity but do exhibit an impaired ability to repair double-strand breaks. Additionally, we find that the overexpression of either HIPP1 or Su(Hw) causes defects in cell proliferation by limiting the progression of DNA replication. We also find that HIPP1 overexpression suppresses the Su(Hw) insulator enhancer-blocking function.
Introduction

Chromatin insulator proteins function by coordinating the regulation of gene expression with chromosome structure. Canonical roles of insulator proteins include their ability to prevent communication between enhancers and target promoters and their role in forming boundaries between regions of heterochromatin and euchromatin. (Wallace and Felsenfeld, 2007; West et al., 2002; Yang and Corces, 2012). The *Drosophila melanogaster* genome encodes a diverse array of insulator proteins, each with unique roles and binding sites contributing to both genome structure and transcriptional regulation. Accessory proteins that interact with DNA binding insulator proteins are essential for insulator function, and the discovery of novel insulator partner proteins may contribute to our understanding of insulator function and mechanisms.

The 5’ untranslated region of the *Gypsy* retrotransposon contains 12 binding sites for the Suppressor of Hairy wing [Su(Hw)] protein, allowing *Gypsy* to function as an insulator in *Gypsy* insertion sites. Flies with mutations in *su(Hw)* have no discernable phenotype other than female sterility due to oogenesis-specific phenotypes (Hsu et al., 2019; Hsu et al., 2015; Klug et al., 1968; Klug et al., 1970; Soshnev et al., 2013b). All insulator binding proteins rely on partner proteins to carry out basic insulator functions. Thus, Centrosomal Protein 190 (CP190) and Modifier of mdg4 67.2 [Mod(mdg4)] are Broad-complex, Tramtrack and Bric-a-brac/Poxvirus, and Zinc Finger (BTB/POZ) domain-containing proteins that interact with Su(Hw) to form the Su(Hw) insulator complex (Georgiev and Gerasimova, 1989; Gerasimova et al., 1995b; Pai et al., 2004). Both Mod(mdg4) and CP190 are required for the enhancer blocking function of the Su(Hw) insulator (Gerasimova et al., 1995b; Kurshakova et al., 2007; Pai et al., 2004).

A recent study identified yet another member of the *Gypsy* insulator complex called Heterochromatin protein 1 and Insulator Partner Protein 1 (HIPP1) (Alekseyenko et al., 2014).
HIPP1 is found to interact with multiple DNA-binding protein complexes, including a high-confidence association with Heterochromatin Protein 1a (HP1) and insulator proteins CTCF and Su(Hw). Another recent study investigated the localization and developmental patterns of HIPP1 (Glenn and Geyer, 2018). This study found that HIPP1 is primarily recruited to euchromatin regions in a Su(Hw)-dependent manner and that hipp1 null mutants have no discernable phenotype and that mutation of hipp1 does not affect the function of the Su(Hw) insulator. This study also identified HIPP1 as the fly homolog of the human Chromodomain Y-like protein (CDYL). Both HIPP1 and CDYL contain a crotonase-like domain (CLD), which is able to mediate interactions with histone modifiers to prevent the addition of transcriptionally activating histone modifications such as acetylation and crotonylation, while promoting the addition of repressive histone modifications (Wu et al., 2009). CDYL exists in multiple isoforms. The CDYLb isoform contains an N-terminal chromodomain in addition to its C-terminal CLD. HIPP1 does not contain a chromodomain and therefore shares more similarities with the CDYLa and CDYLc isoforms, which also lack a functional N-terminal chromodomain (Glenn and Geyer, 2018; Wu et al., 2013).

The human CDYL protein has been found to associate with histone remodeling complexes to promote heterochromatin formation and maintenance, including interactions with the Polycomb Repressive Complex 2 (PRC2) and chromatin assembly factor-1 (CAF-1) (Liu et al., 2017b; Zhang et al., 2011). CDYL specifically associates with H3K9me3 as well as di- and tri-methylated H3K27 and negatively regulates lysine crotonylation, a modification associated with active promoters (Franz et al., 2009; Liu et al., 2017a; Zhang et al., 2011). CDYL is also a component of the Repressor element-1 silencing transcription factor (REST) complex. CDYL contributes to REST-mediated transcriptional silencing of target genes by mediating the interaction between REST and the H3K9-specific G9a methyltransferase (Zhang et al., 2011).
In addition to its insulator activity, Su(Hw) appears to function as a transcriptional regulator of neural genes and has previously been suggested as a functional homolog of mammalian REST (Lakowski et al., 2006; Soshnev et al., 2012). Additionally, the sterility phenotype of \textit{su(Hw)} mutant females has been linked to the de-repression of neural genes in the female germline (Soshnev et al., 2013b). The association of HIPP1, a CLD-containing protein, with Su(Hw) adds to the similarities between Su(Hw) and the mammalian REST complex, however another study (Glenn and Geyer, 2013) did not find a significant effect on the expression of Su(Hw)-regulated genes in HIPP1 mutants. This suggests that the CLD function of HIPP1 may contribute to alternative roles of the Su(Hw) complex.

One recent study demonstrates that CDYL localizes to sites of double strand breaks (DSBs) to promote recruitment of the Polycomb Repressive Complex 2 subunit Enhancer of Zeste 2 (EZH2), leading to transcriptional repression through trimethylation of H3K27 (Abu-Zhayia et al., 2018). This study further reveals that CDYL recruitment to DSBs occurs normally in mutants for the chromodomain and concludes that this role for the CDYL protein is dependent on the CLD. Additionally, CDYL-depleted cells exhibit a reduced accumulation of H3K27me3 at DSB sites as well as a heightened sensitivity to DNA damage-inducing agents such as ionizing radiation and the chemotherapy drug cisplatin. CDYL-depleted cells also exhibit a significant reduction in homology directed repair (HDR) frequency, with no significant change in the frequency of break sites repaired by non-homologous end joining (NHEJ). Since the \textit{Drosophila} HIPPI protein contains a CLD homologous to the CLD of human CDYL and lacks a functional chromodomain, it is possible that the HIPPI protein plays a role similar to the CDYL protein in transcriptional silencing at DSBs and in promotion of the HDR pathway (Abu-Zhayia et al., 2018).
Chromatin insulators are important components of genome architecture across Eukaryotes (Chung et al., 1997; Farrell et al., 2002; Guo et al., 2008; Heger et al., 2013; Heger et al., 2012; Heger et al., 2009; Hily et al., 2009; Ishii et al., 2002; Ishii and Laemmli, 2003; Palla et al., 1997; Schoborg and Labrador, 2010). Recent advances in mammalian systems have pinpointed roles for the insulator protein CTCF in shaping genome architecture in a flexible manner to allow for changes in gene transcription and dynamics of the DNA fiber as the cell cycle progresses (Belton et al., 2012; Naumova et al., 2013; Pekowska et al., 2018). The loop extrusion model involves the extrusion of DNA loops by the cohesin complex until the complex encounters and forms a stable interaction with two CTCF molecules bound to DNA in opposing orientation (Fudenberg et al., 2016). The formation of stable loops creates topologically associating domains (TADs) with CTCF sites located at the border between consecutive TADs, in which the CTCF-cohesin loop anchor colocalizes with break point cluster regions (BCRs) mediated by Topoisomerase 2B (TOP2B) (Canela et al., 2017a). BCRs at loop anchors are thought to occur due to the torsional strain accumulated during transcription, replication, and folding of the genome. The colocalization between loop anchors and BCRs suggests loop extrusion mediated by cohesin and CTCF places a conformational strain on the nucleus that must be alleviated by TOP2B forming nicks in the DNA, allowing the chromatin fiber to relax, and rejoining the ends at the break site.

Although the loop extrusion process has not yet been described in Drosophila, similarities between mammalian CTCF and Drosophila insulator proteins encourage investigation into conserved mechanisms. It has been shown that function and stabilization of the Su(Hw) insulator complex relies on Topoisomerase 2 (TOP2) activity, specifically through an interaction between TOP2 and Mod(mdg4)2.2 that stabilizes the association of Mod(mdg4)2.2 with the Su(Hw) complex (Ramos et al., 2011). Additionally, Su(Hw) interacts with Drosophila Topoisomerase I-
Interacting Protein (dTopors), a protein that binds the nuclear lamina and directs Su(Hw) binding sites to positions along the nuclear lamina in order to define lamina associated domains (LADs) (Capelson and Corces, 2005). The association of genomic sites with the nuclear lamina confines movement of the DNA fiber during processes such as transcription and replication (Gonzalez-Sandoval and Gasser, 2016). It has not yet been shown how Su(Hw) binding is regulated to allow transcription or replication of Su(Hw)-bound sequences located in LADs.

In agreement with the well-documented association of Su(Hw) binding sites at band and interband transitions, it has been shown that Su(Hw) binding sites are enriched in malachite chromosome fragments, which are regions that make up 11% of late replicating domains and are positioned between two contrasting chromatin environments (Khoroshko et al., 2016). Malachite chromatin can be found in regions flanking intercalary heterochromatin domains (IH). IH domains resemble pericentric heterochromatin but are found interspersed in the euchromatic regions of the genome. These domains were originally identified as the bands along arms of polytene chromosomes from Drosophila salivary glands (Belyaeva et al., 2008; Kaufmann, 1939). Replication of IH domains occurs late during the replication timing program and is initiated by origins in the surrounding euchromatin (Lubelsky et al., 2014; Pope et al., 2014). The flanking malachite regions containing Su(Hw) replicate first, followed by the internal IH content. The positioning of Su(Hw) binding sites in these transition regions between euchromatin and heterochromatin suggests that the Su(Hw) protein complex may be regulated in a cell cycle-specific manner to allow entry of replication machinery into intercalary heterochromatin. In agreement with this model, we recently reported that mutations in su(Hw) contribute to replication stress in developing Drosophila egg chambers and dividing neuroblasts (Hsu et al., 2019). The
mechanism by which Su(Hw) is required to maintain genome stability during DNA replication has not yet been elucidated.

The identification of a novel Su(Hw)-interacting protein such as HIPPI provides an opportunity to further investigate Drosophila insulator mechanisms and functions. Here, we analyze the relationship between HIPPI and Su(Hw) and provide evidence of novel roles for the Su(Hw) insulator complex in cell proliferation and genome stability. We have developed fly lines overexpressing Su(Hw), lines overexpressing HIPPI, and a CRISPR-generated mutant of endogenous hipp1 with a deletion of the CLD domain (hipp1\textsuperscript{ACLID}). We show that Su(Hw) and HIPPI overexpression result in severe cell proliferation defects. Overexpressing HIPPI also results in the excess accumulation of larval brain cells in the early phase of DNA replication, suggesting HIPPI expression levels may regulate phases of the replication timing program in Drosophila. We additionally provide evidence that larval brain cells from hipp1\textsuperscript{ACLID} mutants are deficient in DNA damage repair following X-ray irradiation. We also show that overexpression of HIPPI results in suppression of Su(Hw)-mediated enhancer blocking with no disruption of Su(Hw) binding to DNA. This disruption of insulator function correlates with the displacement of cohesin from the Su(Hw) sites. These results provide additional evidence that Su(Hw) plays a role in cell proliferation that may be dependent on a role in DNA replication. This study also provides compelling evidence that HIPPI functions in the DNA damage repair pathway in a similar manner as the human CLD-containing protein, CDYL. Taken together, these findings further suggest insulator proteins contribute important functions to the processes of genome replication and genome stability, raising new and intriguing questions about the mechanisms mediating such functions.
Results

*hipp1* mutants lacking the crotonase-like domain are deficient in DNA repair

HIPP1 has been identified as the homolog of the human CDYL protein (Glenn and Geyer, 2018). The HIPP1 and CDYL proteins both contain a C-terminal crotonase-like domain (CLD) while CDYL also contains an N-terminal chromodomain. The presence of conserved features between the CDYL and HIPP1 crotonase-like domains suggests that the function of this domain is consistent between the two proteins. Both CDYL and HIPP1 contain critical residues to form an oxyanion hole which is required for stabilizing an anion intermediate produced during reactions with an acyl-CoA substrate (Glenn and Geyer, 2018; Wu et al., 2009). Figure 1.1A shows the alignment of these critical residues, indicated by red boxes, between HIPP1 and CDYLb, the most abundant isoform of CDYL (Abu-Zhayia et al., 2018). The function of human CDYL in promoting the HDR pathway occurs normally in chromodomain mutants, suggesting this role relies on the crotonase-like domain. Therefore, it is possible that HIPP1 shares this role with CDYL.

To investigate whether the CLD of CDYL and HIPP1 play a similar role in the HDR pathway, we generated mutants by specifically removing the CLD domain of HIPP1 using CRISPR-Cas9. We targeted guide RNAs to sequences flanking the region encoding the CLD and generated a deletion and stop codon early in the sequence. The gRNAs used are reported in Table A1. We generated two different CLD deletion fly lines (HIPP1 31.2 and HIPP1 14.3, Figure 1.1A). Both fly lines contain a frameshift followed by a stop codon early in the sequence and lack the residues critical for the formation of an oxyanion hole (Glenn and Geyer, 2018; Wu et al., 2009). The generation of early stop codons and deletion of the CLD in both alleles was confirmed by DNA and cDNA sequencing (data not shown). Figure 1.1A shows the alignment of our mutant alleles with the CLD from Oregon-R, *hipp1*, and human CDYLb. We performed experiments using
flies trans-heterozygous for the HIPP1 31.2 and 14.3 to limit any effect from possible off-target mutations induced by the CRISPR Cas-9 method.

Next, to determine whether HIPP1 participates in the HDR pathway, we evaluated the ability of dividing cells from hipp1\textsuperscript{A\textsubscript{CLD}} larval brains to recover from DNA damage by quantifying the occurrence of chromosomal aberrations (CABs) following X-ray treatment and recovery (Gatti and Goldberg, 1991; Merigliano et al., 2017). Larval brains contain many dividing cells, making them useful for cell cycle-related studies. CABs were quantified by counting the number of metaphasic nuclei containing one or more aberrations and comparing this number to the total number of metaphases, including those with no CABs (Figure 1.1B). We found that hipp1\textsuperscript{A\textsubscript{CLD}} samples contained a significantly higher number of metaphases with one or more CABs following X-ray treatment and recovery compared to the Oregon-R control (Figure 1.1D, p=0.0017). This result suggests that a role for the CLD in DNA damage repair is conserved between the human CDYL and Drosophila HIPP1 proteins. How this role relates to HIPP1-containing complexes, such as CTCF and Su(Hw) insulator complexes and HP1 complexes, remains unknown. It will require additional studies to determine a link between this role of HIPP1 and the Su(Hw) insulator complex, however this conserved function further supports the idea that HIPP1 and CDYL are homologous proteins.

**Su(Hw) and HIPP1 colocalize and dynamically bind to polytene chromosomes**

Functional analysis of different su(Hw) mutations and the genomic distribution of Su(Hw) binding sites suggests that different binding sites may have different functions, depending on the genomic location and the partner proteins associated with Su(Hw) at the given site (Soshnev et al., 2012). To further characterize the interaction between Su(Hw) and HIPP1, we developed Gal4-inducible
transgenic constructs fused to fluorescent proteins to observe localization patterns of HIPPI
(P{HIPPI::mC, w+}) relative to Su(Hw) (P{Su(Hw)::GFP, w+}) binding sites (Figure 1.2A). We
drove the expression of the transgenic constructs with a vestigial Gal-4 promoter (vg-Gal4) that
specifically drives transgenic expression in larval wing discs but also induces significant
expression in salivary glands (Barwell et al., 2017; Schoborg et al., 2013b). We immunostained
with antibodies specific for GFP and RFP (mCherry) to observe the localization patterns of
Su(Hw)::GFP and HIPPI::mC on polytene chromosomes from larval salivary glands. Under these
conditions, we observed a significant overlap between Su(Hw) and HIPPI signal (Figure 1.2B).
Next, we analyzed localization patterns of HIPPI::mC and Su(Hw)::GFP in S2 cells in both
normal media and osmotic stress media (growth media supplemented with 250mM NaCl). Osmotic
pressure drives the formation of insulator bodies in Drosophila cells, and all known Drosophila
insulator proteins associate with these bodies (Schoborg et al., 2013b). Therefore, we can indirectly
ask whether HIPPI is associated with insulator function by determining whether HIPPI also
localizes to insulator bodies. We found that Su(Hw)::GFP and HIPPI::mC staining patterns
overlap with insulator bodies, following the addition of osmotic stress media, supporting the notion
that HIPPI is closely associated with insulator function (Figure 1.2C).

We also observed that the distribution of Su(Hw) and HIPPI binding sites, relative to the
band/interband structure of polytene chromosomes, was different among nuclei. In some nuclei
Su(Hw) and HIPPI localize exclusively to bands while in other nuclei they localize exclusively to
interbands (Figure 1.3A and B). These observed changes in binding patterns suggest that Su(Hw)
and HIPPI binding is dynamic and is possibly regulated in a cell cycle-specific manner. We also
observed that some nuclei lack HIPPI::mC altogether (Figure 1.3C). This suggests that HIPPI
association with Su(Hw) is dynamically regulated and may contribute to a function of Su(Hw)
only during specific stages of the cell cycle. We hypothesize that the interaction between HIPPI and Su(Hw) occurs transiently during the cell cycle, and that HIPPI contributes to cell cycle-specific aspects of insulator activity such as regulating Su(Hw) function during DNA replication.

**Overexpression of Su(Hw) and HIPPI disrupts cell proliferation**

To explore the hypothesis that Su(Hw) may play a role in replication, we next determined whether driving overexpression of the same Su(Hw) and HIPPI transgenic constructs impacts cell cycle progression. Driving the expression of Su(Hw)::GFP with a vg-Gal4 driver revealed significant cell proliferation defects in the adult wing margin, while driving the expression of HIPPI::mC with the same driver revealed no wing margin defects (Figure 1.4 A and B, p=0.0003). Driving the expression of both Su(Hw)::GFP and HIPPI::mC in the same individuals resulted in wing margin defects that do not significantly differ from Su(Hw)::GFP expression alone (Figure 1.4 A and B).

To confirm that these defects in wing morphology were not due to apoptosis, we overexpressed p35 along with Su(Hw)::GFP (Figure 1.4A). p35 is a potent caspase inhibitor in *Drosophila* (Miller, 1997). Defects in the wing margin persisted, suggesting that the lack of cells in the wing margin are due to a lack of cell proliferation rather than apoptosis induced by elevated levels of Su(Hw) protein. Similar phenotypes are produced by mutations in the Notch pathway which also result in inhibition of cell proliferation in the wing margin (Baonza and Garcia-Bellido, 2000).

These observations led us to conclude that Su(Hw) overexpression limits cell proliferation in the wing margin and that the lack of cell proliferation persists with the combined overexpression of Su(Hw)::GFP and HIPPI::mC. These data also show that overexpression of HIPPI does not rescue cell proliferation defects arising from Su(Hw) overexpression.
Based on these observations, we tested how Su(Hw) and HIPPI overexpression may affect *Drosophila* growth. Driving the expression of HIPPI::mC and Su(Hw)::GFP with a ubiquitous actin-Gal4 driver revealed a significant decrease in larval body size. We compared the sizes of HIPPI::mC and Su(Hw)::GFP overexpressing larvae to larva sizes from a line expressing H2Av::mC with the same actin-Gal4 driver. Both HIPPI::mC and Su(Hw)::GFP expression resulted in a decrease in larval body size, with Su(Hw)::GFP expression exhibiting a greater reduction in size (Figure 1.4 C and D), suggesting that Su(Hw) overexpression serves as a more potent inhibitor of cell proliferation compared to HIPPI. Both HIPPI::mC/actin-Gal4 and Su(Hw)::GFP/actin-Gal4 larvae die without reaching sizes larger than shown in Figure 1.4, and never enter pupation stage.

We additionally measured growth in larvae expressing HIPPI::mC with an actin-Gal4 driver in a *su(Hw)v/e041061* mutant background. These larvae exhibit a reduced size, significantly smaller than HIPPI overexpression alone. The inability of mutations in *su(Hw)* to rescue the effects of HIPPI overexpression on larval growth suggests roles for HIPPI in cell proliferation that extend beyond interactions with the Su(Hw) insulator complex alone. HIPPI interacts with other protein complexes including CTCF and HP1 (Alekseyenko et al., 2014). Therefore, HIPPI overexpression may contribute to cell proliferation defects through interactions with CTCF and HP1, as well as through interactions with Su(Hw). Interestingly, mutations in *su(Hw)* have been linked to developmental defects and replication stress (Hsu et al., 2019; Klug et al., 1968; Klug et al., 1970). These results suggest that HIPPI overexpression combined with mutations in *su(Hw)* promote more severe cell proliferation defects compared to HIPPI overexpression alone.

Next, we measured the mitotic index of dividing neuroblasts in larval brains expressing HIPPI::mC to further assess whether defects observed in wing development and larval growth are
the result of cell cycle disruption. We measured the mitotic index in HIPPI1::mC/actin-Gal4 larval brains and found they have a significantly lower mitotic index compared to the Oregon-R control (Figure 1.4E, p=0.0410). This result suggests HIPPI1-overexpressing cells complete the cell cycle less frequently than Oregon-R cells, causing insufficient cell proliferation and providing an explanation for the reduced size previously noted in HIPPI1 overexpressing larvae.

**HIPPI1 overexpression delays the transition of DNA replication between early and late replicating regions of the genome**

Our observations of delays in the cell cycle when Su(Hw) and HIPPI1 proteins are overexpressed in the nucleus suggests that these proteins serve as barriers to normal cell cycle progression. The association of Su(Hw) with the nuclear lamina and regions flanking intercalary heterochromatin domains suggests Su(Hw) plays a role in maintaining functional boundaries between LADs and actively transcribed TADs in the nuclear interior (Khoroshko et al., 2016). One possibility is that in addition to functioning as a boundary, the Su(Hw) insulator complex also mediates the detachment of chromatin from LADs and from euchromatin/heterochromatin transition sites, thereby facilitating access into these domains by cellular machinery during genome replication. Since heterochromatin domains flanked by Su(Hw) binding sites should be late replicating domains, we asked whether changes in Su(Hw) or HIPPI1 expression levels alter the rate of DNA replication within each domain.

Due to the cell proliferation defects observed in HIPPI1-overexpressing conditions, we hypothesized that HIPPI1 may affect the progression of replication forks when present in its overexpressed form. To test this hypothesis, we quantified the amount of single strand DNA (ssDNA) in Oregon-R, *hipp*1*ACLD*, and HIPPI1::mC/actin-Gal4 larval brains using ssDNA as a
marker for active replication forks (Zellweger et al., 2015) (Figure 1.5A). BrdU is a nucleotide analog used to monitor nucleotide incorporation during DNA replication. The detection of BrdU incorporation into DNA relies on the binding of a BrdU-specific antibody. The anti-BrdU antibody, however, can only detect incorporated BrdU within the DNA if the DNA is single stranded, thus DNA must be denatured prior to antibody incubation. Detecting BrdU incorporation in non-denaturing conditions provides a way to measure the amount of ssDNA present at stalled or active replication forks (Despras et al., 2010). We incubated Oregon-R, hipp1^{ACLD}, and HIPPI::mC/actin-Gal4 larval brains with BrdU for one hour, followed by fixation and immunostaining. We found a significant increase in BrdU accumulation in HIPPI overexpression larval brains compared to the Oregon-R control (Figure 1.5B). This suggests that overexpression of HIPPI leads to an accumulation of active and stalled replication forks. Taken together with our observation that HIPPI overexpression causes a decrease in the mitotic index, the observed accumulation of ssDNA suggests that HIPPI overexpression disrupts DNA replication, possibly leading to the activation of checkpoints and stalling of the cell cycle. We hypothesize that the interaction between HIPPI and Su(Hw) alters insulator activity in a cell cycle-specific manner and that HIPPI overexpression prolongs this change in insulator function, thereby misregulating insulator properties and possibly aspects of replication timing.

Next, we quantified the level of DAPI intensity in the larval brain samples from our BrdU experiments to observe differences in cell cycle stages. DAPI intensity is a common method to determine phases of the cell cycle (Stohr et al., 1977). Cells in G1 contain only one copy of each chromosome and cells in S and G2 contain greater than one copy of each chromosome. By quantifying the frequency of cells that contain different ranges of DAPI intensity, we observed that HIPPI::mC/actin-Gal4 cells were biased towards a greater DAPI content, compared to
Oregon-R or hipp1^{ACLD} cells (Figure 1.5C). This data suggests that a large fraction of HIPP1::mC/actin-Gal4 cells are arrested during S or G2 phases, possibly a consequence of replication fork stalling. We also observe a shift towards less DNA content in hipp1^{ACLD} cells, suggesting replication is suppressed in hipp1^{ACLD} mutants, potentially through a Su(Hw)-dependent mechanism (Figure 1.5C).

To further investigate the influence of Su(Hw) and HIPP1 on DNA replication, we used 5-ethynyl-2-deoxyuridine (EdU) as a marker for DNA replication to monitor the progression of DNA replication in HIPP1-overexpressing organisms. EdU is a thymidine analog that incorporates during DNA replication and can be detected by activation of an EdU-specific label. Incubation of tissues for a fixed amount of time allows us to observe the number of cells undergoing S-phase as well as the genomic location of active DNA replication. We incubated larval brains in EdU for ten minutes including brains dissected from Oregon-R, su(Hw)^{v/e04061}, hipp1^{ACLD}, and HIPP1::mC/actin-Gal4 larvae (brains from Su(Hw)::GFP/actin-Gal4 larvae do not develop to a large enough size to allow dissection). We then detected EdU incorporation using an EdU-specific label and fluorescence microscopy (Figure 1.5D). DNA replication occurs in distinct phases dependent upon chromatin state (Armstrong et al., 2018; Lubelsky et al., 2014). Euchromatin replicates during early S-phase, a combination of euchromatin and heterochromatin replicate during middle phase, and constitutive heterochromatin replicates during late S-phase. Based on EdU labeling of larval brains, we quantified the number of cells in each phase for each genotype. EdU labeling that overlaps with light DAPI stain indicates early S-phase, EdU labeling that partially overlaps with light DAPI stain and partially with dark DAPI stain indicates middle S-phase, and EdU labeling that overlaps exclusively with dark DAPI stain indicates late S-phase (Figure 1.5D). We found that there are significantly more HIPP1::mC/actin-Gal4 cells in the early S-phase category, compared to Oregon.
R (Figure 1.5E, Early S-phase, p=0.0073). We also found that significantly fewer HIPPI1::mC/actin-Gal4 cells were in the late replication compared to Oregon-R (Figure 1.5E, Late S-phase, p=0.0095). This result suggests that HIPPI1 overexpression stalls progression of DNA replication in the early replication phase and delays the entry of replication machinery into middle and late replicating regions. Additionally, we noticed no significant change in su(Hw)\textsuperscript{w04061} or hipp1\textsuperscript{ΔCLD} mutants. This suggests that the delay in the replication timing program is dependent upon HIPPI1 overexpression.

We next aimed to observe nucleotide incorporation at a higher resolution to better understand the slow progression of S-phase in HIPPI1::mC/actin-Gal4 cells. To do so, we visualized the incorporation of 5-Bromo-2'-deoxyuridine (BrdU) relative to Su(Hw) and HIPPI1 in the salivary glands of HIPPI1::mC/vg-Gal4 and Su(Hw)::GFP/vg-Gal4 organisms. We found that in some genomes both Su(Hw)::GFP and HIPPI1::mC signals occurred opposed to BrdU signal. This pattern of BrdU incorporation suggests that HIPPI1 and Su(Hw) may be barriers to DNA replication at certain stages during genome replication, possibly slowing down the S-phase and cell proliferation when overexpressed (Figure 1.5F).

To better understand the effect of Su(Hw) and HIPPI1 on the progression of S-phase, we next observed the time that it takes for Oregon-R, hipp1\textsuperscript{ΔCLD}, HIPPI1::mC/vg-Gal4, and Su(Hw)::GFP/vg-Gal4 to complete each stage of the S-phase using polytenes from third instar larvae salivary glands. Polytenes undergo endocycling in which they only participate in G and S-phases (Smith and Orr-Weaver, 1991). Chromatin is distributed into condensed heterochromatin-like bands and euchromatin interbands along the arms of polytene chromosomes, with pericentric heterochromatin located at the chromocenter. We timed stages of S-phase in polytenes by incubating salivary glands in cell culture media for periods of time, followed by fixation and
antibody staining. Specifically, we incubated salivary glands with EdU for 10 minutes at the beginning of each experiment. We then washed the EdU from salivary glands and allowed them to incubate in media for 2, 4, 6 or 8 hours. Following incubation, tissues were immediately fixed and labeled using a PCNA antibody. By comparing areas of EdU incorporation with areas of PCNA immunostaining, we were able to estimate a progression time between different S-phase stages in each of the lines. For instance, if a polytene displayed EdU in early/late phase pattern and PCNA in an end S-phase pattern after 6 hours of incubation, we concluded that it took the chromosome 6 hours to progress from the early/late phase of replication to the end S-phase replication stage (Figure 1.6A). We assigned EdU and PCNA staining patterns to phases of DNA replication according to a previous study (Kolesnikova et al., 2013). This study labeled polytene chromosomes with PCNA and characterized early S-phase (I) as continuous PCNA signal, early to late phase (II) as PCNA signal in polytene arm bands, late S-phase (III) as PCNA in the chromocenter and intercalary heterochromatin, and end S-phase (IV) as very weak PCNA signal in the chromocenter and intercalary heterochromatin. Stage V is assigned to chromosomes in G phase and show no PCNA signal. Under these conditions, we found that HIPP1::mC/vg-Gal4 chromosomes take significantly more time to progress between replication phases when compared with Oregon-R cells. This result further suggests HIPP1 plays a role in DNA replication and that misregulation of HIPP1 by overexpression causes replication to progress more slowly (Figure 1.6 A and B).

**HIPP1 overexpression disrupts Su(Hw) insulator function**

Together, our results suggest that the effects of HIPP1 on cell cycle progression could be both dependent and independent from Su(Hw) insulators. This conclusion is not unexpected since, in
addition to Su(Hw), HIPP1 interacts with other insulator proteins such as dCTCF, as well as with HP1. In each one of these interactions HIPP1 may have a putative role on the progression of the cell cycle. To uncover the role of HIPP1 in insulator function, we analyzed Su(Hw)-dependent phenotypes in flies overexpressing HIPP1. The yellow\textsuperscript{2} and cut\textsuperscript{6} mutations (\(y^2\) and \(ct^6\)) are caused by an insertion of the Gypsy retrotransposon between tissue specific enhancers and the promoter of these genes, allowing the Su(Hw) insulator protein to bind and disrupt normal enhancer promoter interactions and gene transcription activation (Jack et al., 1991; Parkhurst and Corces, 1986a). These mutations result in flies with yellow body and yellow wings (\(y^2\)), and cuts in the wing margin (\(ct^6\)). Interestingly, we observed that driving HIPP1 overexpression with a vestigial-Gal4 promoter in flies with \(y^2\)ct\textsuperscript{6} background results in the restoration of wild-type black wing blades and rounded wing margins (Figure 1.7A). Immunostaining of polytene chromosomes from larvae with the same genotype reveal that Su(Hw) remains bound to the yellow locus in HIPP1-overexpressing polytene chromosomes, despite the suppression of insulator function (Figure 1.7B). This result suggests overexpression of HIPP1 suppresses the enhancer blocking activity of the Su(Hw) insulator without disrupting the binding of Su(Hw) to DNA. We also observe that \(y^2\)ct\textsuperscript{6}; hipp1\textsuperscript{4CLD} mutants display no rescue of the \(y^2\) and \(ct^6\) phenotypes, suggesting that the activity of the CLD is not necessary for insulator function (Figure 1.7A).

*Drosophila* insulators lack a comprehensive model that combines the canonical roles of insulator proteins such as enhancer blocking and boundary formation with genome-wide organizational properties such as loop formation. Recent computer modeling and work in mammalian systems have provided a model of loop extrusion by cohesins to explain the multifaceted role of mammalian CTCF in shaping the genome while contributing to gene transcription (Fudenberg et al., 2016). The presence of loop extrusion-like domains in *Drosophila*
has been disputed (Rowley et al., 2017b), but recent work analyzing Hi-C maps of the *Drosophila* genome at a resolution of ~200 bp reveals the presence of TADs defined by insulator binding sites, suggesting the same organizational principles are conserved between insects and mammals (Wang et al., 2018). These advances in elucidating the involvement of the cohesin complex in driving loop formation and the confirmation of TAD organization in *Drosophila* motivated us to further explore whether the cohesin complex plays a role in the insulator function of the gypsy insulator.

Using fluorescence microscopy in polytene chromosomes, we observed that WAPL, a component of the cohesin complex, colocalizes with Su(Hw) and other insulators at a number of sites including γ² sites (Cunningham et al., 2012) (Figure 1.7B). However, in HIPPI::mC/vg-Gal4 overexpression conditions, we observe that WAPL no longer colocalizes with Su(Hw). Although the formation of loops mediated by cohesin and insulators have not directly been observed in *Drosophila*, similarities between organizational properties in human and *Drosophila* genomes point towards a conserved mechanism. We hypothesize that cohesin contributes to the insulator function of Su(Hw). Under this assumption, when HIPPI is overexpressed and WAPL leaves the insulator site, Su(Hw) is no longer able to act as an insulator. Altogether these data suggest HIPPI modulates replication timing by regulating insulator activity in a cell cycle- or genome replication-dependent manner.

**Discussion**

Insulator binding sites are abundant in the genome and play critical roles in genome structure and function. Null mutations in mammalian CTCF and in most insulator proteins in *Drosophila*, including dCTCF, often result in lethality. Therefore, it is surprising that mutations in *su(Hw)* have no discernible effect during development or in the adult organism other than female sterility (Hsu
et al., 2015; Klug et al., 1968; Klug et al., 1970; Soshnev et al., 2013b). Interestingly, a null mutation of HIPP1 is also viable with no discernible phenotype in adults (Glenn and Geyer, 2018). This raises the question of whether these proteins truly play a role in important 3D genome organization and function, in addition to contributing to tissue specific gene transcription regulation. Results presented here and elsewhere show that mutant and overexpressing genotypes of Su(Hw) and HIPP1 generate phenotypes related with defects in DNA repair and genome replication (Hsu et al., 2019).

Coinciding with the findings of Glenn and Geyer (2018), we found that the CLD specific mutant produced in this report (hipp1^{ACLID}) also has no obvious effect on development or insulator function. However, we do find that hipp1^{ACLID} has an impaired ability to repair double-strand breaks produced following X-ray treatment. Glenn and Geyer (2018), previously identified HIPP1 as the Drosophila homolog of the human CDYL protein. However they found that hipp1 mutants did not have the same consequences on viability and male fertility as mutants for human cdyl (Glenn and Geyer, 2018). Here, we investigated whether the CLD of HIPP1 and CDYL share a role in DNA repair. The CLD of human CDYL is an important component of the homology directed repair pathway (Abu-Zhayia et al., 2018). We find that hipp1^{ACLID} mutants display an impaired response to DNA damage. Additional work will be required to establish to what extent the role of HIPP1 in DNA repair in Drosophila is conserved with that of CDYL in humans. Although a connection between the Su(Hw) insulator complex and DNA damage repair has not yet been established, it has been reported that the CTCF boundary function in humans is linked to double-strand break formation by topoisomerase activity (Canela et al., 2017a). Additionally, it has been shown that topoisomerase II modulates Su(Hw) insulator function in Drosophila (Ramos et al., 2011). This evidence suggests the possibility that insulator binding sites in Drosophila may also be sites that
accumulate torsional stress, which may lead to replication forks stalling during DNA replication and rely on HIPP1 for efficient torsion relief and/or repair.

Supporting these observations, we also find that WAPL, a component of the cohesin complex, colocalizes with the Su(Hw) insulator complex at the y^2 locus. The cohesin complex has been identified as a critical component of CTCF-mediated loops in mammalian systems and through the loop extrusion mechanism is thought to be responsible for accumulation of torsional stress at CTCF sites in mammals (Canela et al., 2017a). Our finding that WAPL colocalizes with the gypsy insulator suggests insects have a conserved mechanism of insulator mediated DNA looping (Fudenberg et al., 2016). Our results show that upon HIPP1 overexpression, y^2 and ct^6 phenotypes are reversed to wild-type while simultaneously WAPL no longer colocalizes with the corresponding Su(Hw) insulator sites. The correlation between WAPL presence and the enhancer-blocking activity of the Su(Hw) insulator suggests cohesin plays a role in stabilizing the interactions required for enhancer-blocking in the Su(Hw) insulator complex. These observations indicate that the role of HIPP1 binding to Su(Hw) may be transient, i.e. its role may be to temporarily destabilize cohesin’s association with the insulator complex and transiently suppress the compartment boundary activity of the insulator.

Here we show that an increase in HIPP1 expression both inhibits phenotypes mediated by the Su(Hw) insulator and delays cell cycle progression. These findings, taken together with our observations that overexpression of Su(Hw) and HIPP1 delays cell cycle progression suggest that HIPP1 antagonizes insulator function as a part of a mechanism that regulates progression of replication forks through different genome compartments. Earlier studies establishing the association of Su(Hw) with sites flanking intercalary heterochromatin and the initial observation that Su(Hw) colocalizes with multiple replication origins directed our attention to the question of
whether the Su(Hw)-HIPPI interaction may play a role in DNA replication (Khoroshko et al., 2016; Vorobyeva et al., 2013b). We observe that Su(Hw) overexpression serves as a potent inhibitor of cell proliferation, causing a greater decrease in cell proliferation than HIPPI overexpression. Additionally, we find that HIPPI overexpression has a negative effect on DNA replication, resulting in an accumulation of early replicating cells and fewer late replicating cells. Our analysis of DNA replication in polytene chromosomes reveals that HIPPI overexpression specifically delays the transition between stages of replication. These findings suggest that the transition between replicating domains is sensitive to the levels of HIPPI expression. Since Su(Hw) binding sites are enriched in regions flanking intercalary heterochromatin, or late replicating domains, we hypothesize that HIPPI modulates Su(Hw) insulator activity during S-phase perhaps by timing the replication entry into chromatin domains guarded by Su(Hw) binding sites. Higher amounts of HIPPI may cause an imbalance in this process and result in misregulation of the transition between euchromatin and heterochromatin by replication machinery. The replication defects caused by HIPPI overexpression, however, are quite dramatic and most likely cannot be accounted for by HIPPI-Su(Hw) interactions alone. HIPPI has a variety of binding partners other than Su(Hw), including other insulator proteins such as dCTCF and HP1 (Alekseyenko et al., 2014). Future experiments are needed to address the mechanistic details of the relationship between HIPPI and HP1 and other insulators.

The evidence presented here suggests HIPPI interacts with Su(Hw) to regulate yet unknown aspects of replication timing in the *Drosophila* genome, but would appear to contradict recent findings demonstrating that deletion of insulator sites in mouse embryonic stem cells does not significantly affect compartmentalization of active and inactive regions of the genome or replication timing (Sima et al., 2019). Moreover, this finding would appear to be in line with the
observations that Su(Hw) and HIPPI null mutations are viable, or that dCTCF mutants, although ultimately lethal, allow for full embryo development in Drosophila (Gambetta and Furlong, 2018). On the other hand, detailed studies analyzing genome structure during development have identified a role for DNA replication in the establishment and maintenance of TADs by demonstrating that inhibition of DNA replication, rather than inhibition of transcription, prevents TAD formation during early mouse embryogenesis, further suggesting a link between replication programs and genome structure (Ke et al., 2017). In this context, a role of genome structure in normal genome replication is also reinforced by our previous finding that mutations in su(Hw) lead to replication stress in nurse cells and dividing neuroblasts (Hsu et al., 2019). The observation that Su(Hw)-deficient cells present replication defects challenges the accepted notion that su(Hw) null mutations allow for complete normal development. It is possible that the replication defects originating from the lack of su(Hw) alone are insufficient to prevent development. Perhaps it is the combination of multiple architectural proteins in Drosophila, or a combination of functionally different CTCF sites in mammals, which collectively shape the structure of the genome, that is needed for normal replication progression through genome compartments and normal replication timing.

In summary, we have presented evidence that a Su(Hw)-interacting protein has the ability to regulate insulator activity and alters the rate of cell proliferation and the replication timing program when ectopically expressed. We propose that HIPPI is a regulator of insulator activity. When the Su(Hw) insulator sites are knocked out genome-wide, flies are viable but actively replicating cells undergo replication stress, which suggest insulator function is required for normal replication timing (Hsu et al., 2019). When HIPPI is overexpressed, we observe misregulation of
genome replication and replication timing, possibly resulting from the ectopic inactivation of insulator function. These findings strengthen notion that genome replication is supported by a mechanism in which insulator function must be regulated to allow normal replication timing and cell-cycle progression.

**Materials and Methods**

**Drosophila stocks**: All fly stocks and crosses were maintained on standard cornmeal-agar media and yeast in a 25°C incubator. The fly stocks used in this work included: microinjection to generate transgenic lines yw; P{HIPP1::mC, w\(^+\)}, yw; P{SuHw::EGFP, w\(^+\)} (Schoborg et al., 2013b), and yw; P{H2Av::mC, w\(^+\)} were performed by GenetiVision; the lines obtained from the *Drosophila* Bloomington Stock Center at Indiana University: *su(Hw)*\(^{d041061}/TM6B\); w\(^+\); P{GAL4- vg.M}\(^2\); TM2/TM6B; the lines from V. Corces (Emory University): *su(Hw)*\(^+/TM6B\), Tb1, *mod(mdg4)*\(^{u1}\). The mutant lines *hipp1*\(^{\Delta CLD31.2}\) and *hipp1*\(^{\Delta CLD14.3}\) were generated by our lab using CRISPR Cas-9; microinjection of guide RNAs was performed by Gentivision.

**Antibodies**: Rabbit anti-Su(Hw) polyclonal IgG antibody was generated in our laboratory (Wallace et al., 2010). Rabbit anti-WAPL polyclonal IgG was generated in the laboratory of Dr. Judith Kassis and used according to prior reports (Cunningham et al., 2012). The following commercially available primary antibodies were used: Mouse anti-GFP IgG (Developmental Studies Hybridoma Bank #12A6), rabbit anti-RFP IgG (A00682, GenScript), mouse anti-PCNA IgG (Abcam ab29), and mouse anti-BrdU IgG (Developmental Studies Hybridoma Bank G3G4). Antibodies were used at a concentration of 5 \(\mu g/ml\). The following secondary antibodies were used: Donkey FITC- conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.),
Donkey Alexa Fluor 488-conjugated anti-rabbit IgG (A-21206, Life Technologies), and Donkey Alexa Fluor 488-conjugated anti-mouse IgG (A-21202, Thermo Fisher).

**Expression vector construction:** Expression vectors for S2 cells and P-elements were created as previously described (Schoborg et al., 2013b). The S2 cell dual-expression constructs contain Su(Hw)-EGFP and HIPP1-mCherry sequences, including introns, under the control of the copper-responsive metallothionein promoter in the pMK33-CTAP tag vector backbone. Fly expression constructs were created as previously described (Schoborg et al., 2013b). Su(Hw)-EGFP and HIPP1-mCherry were amplified from pMK33 and inserted into the pUAST-Y vector backbone.

**Polytene chromosomes immunostaining and quantification:** Salivary glands from early third instar larvae were dissected in insect media (HyClone SFX; Thermo Fisher Scientific), and fixed immediately with 4% PFA; 50% acetic acid on a coverslip. Salivary glands were squashed on a microscope slide until the polytene chromosomes were well spread. Slides were dipped in liquid nitrogen to facilitate coverslip removal. Polytene chromosomes were blocked for 10 minutes at room temperature (RT) in blocking solution (PBS, 0.1% NP40, 3% nonfat milk). Primary antibodies were diluted in blocking solution to a concentration of 5 μg/ml and incubated overnight at 4 °C in a humidified chamber. Primary antibodies were removed by incubating in washing buffer (PBS, 0.1% NP40) for 10 minutes at room temperature. Secondary antibodies were then diluted in blocking solution (1:200) and incubated for 1 hour at room temperature and washed as previously described. DAPI (4’, 6-diamidino-2-phenylindole 0.5 μg/ml) was used to counter stain the DNA for 30 seconds before rinsing with PBS. Slides were mounted with Vectashield mounting medium (Vector Laboratories) and sealed with nail polish.
**Stress treatment and immunostaining:** S2 cells 3–5 days after subculture were allowed to adhere to a poly-l-lysine coverslip for 30 min in a covered 35 mm cell culture dish. To induce osmostress, media was removed and quickly replaced with fresh SFX media supplemented with the indicated concentration of NaCl (from a 5M stock). Controls were kept in conditioned media. Cells were stressed for 20 min and then immunostained as previously described (Rogers and Rogers, 2008; Schoborg et al., 2013b). In brief, cells were fixed with 4% PFA for 10 min at RT, rinsed 3x with PBS, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with 3% nonfat milk for 10 min at room temperature. Primary antibodies were diluted in 3% nonfat milk, and coverslips were incubated for 1 hour at room temperature in a humidified chamber followed by a 3x wash with PBS/0.1% Triton X-100 for 10 min each. Secondary antibodies were then diluted in 3% nonfat milk and incubated for 1 hour at room temperature, and coverslips were washed as described. 0.5 μg/ml DAPI was added to counterstain DNA; slides were then rinsed twice with PBS, and mounted in Vectashield.

**Cytology for mitotic indexing:** Mitotic spreads from larval brains were scored for mitotic indices as described in (Gatti and Goldberg, 1991). In brief, larval brains were dissected in insect media (HyClone SFX; Thermo Fisher Scientific), incubated in 0.5% sodium citrate, pH 6.0, for 10 minutes followed by fixation with 4% PFA; 50% acetic acid, and softly squashed between a coverslip and slide. Slides were stained with 4′, 6-diamidino-2-phenylindole (DAPI, 0.5 μg/ml) and were mounted in Vectashield mounting medium (Vector Laboratories).
X-ray sensitivity assessment: Mitotic chromosomes from larval brains were observed for the presence of aberrations following X-ray treatment and recovery in a similar manner as (Gatti and Goldberg, 1991; Merigliano et al., 2017). In short, third instar larvae, Oregon-R and hipp1ACLD, were irradiated with 7.5 Gy. Irradiation was performed in a Rad-Source RS-2000 Biological Irradiator. 2 hours after X-ray exposure, larval brains were dissected and placed in insect media (HyClone SFX; Thermo Fisher Scientific) supplemented with 0.1 mM colchicine for 1 hour, followed by a 10-minute incubation in 0.5% sodium citrate, pH 6.0. Brains were fixed with 4% PFA; 50% acetic acid. After the incubations, brains were then softly squashed and stained with 4′, 6-diamidino-2-phenylindole (DAPI, 0.5 μg/ml), rinsed twice with PBS and mounted in Vectashield mounting medium (Vector Laboratories). Brains were mounted on individual slides and observed. Slides were scanned for metaphasic nuclei and approximately 50 metaphases were collected for each slide. Metaphases were then scored for the presence of CABs.

EdU incorporation and detection: Brains or salivary glands dissected from larvae were labeled with EdU according to the manufacturer’s protocol (Click-iT EdU Alexa Fluor 488 Imaging Kit; C10337, Invitrogen). In brief, tissues were incubated in 10 μM EdU diluted in insect media (HyClone SFX; Thermo Fisher Scientific) for the indicated amount of time. The tissues were fixed in 4% PFA; 50% acetic acid and adhered to a microscope slide. Slides were treated with the Click-iT reaction cocktail containing the Alexa Fluor azide for 30 minutes. Slides were washed in a blocking solution (PBS, 0.1% NP40, 3% nonfat milk) and labeled with the indicated primary and secondary antibodies and DAPI (0.5 μg/ml) before observation.
**Microscopy:** Slides were analyzed using a wide-field epifluorescence microscope (DM6000 B; Leica) equipped with a charge-coupled device camera (ORCA-ER; Hamamatsu Photonics) and an HCX Plan Apochromat (Leica) 40X or 100X/1.35 NA oil immersion objective. Image acquisition was performed using SimplePCI (v6.6; Hamamatsu Photonics). Image brightness and contrast adjustments were performed using Fiji (Schindelin et al., 2012). Samples were processed and imaged under identical conditions of immunostaining, microscope, camera, and software settings.

**BrdU labeling and analysis:** For the BrdU incorporation assay, brains dissected from third instar larvae were incubated for 1 hour in 0.1 mg/ml BrdU at room temperature. Tissues were then fixed with 4% PFA; 50% acetic acid, washed in PBS, and incubated in blocking solution. Brains were then labeled with 2 μg/ml BrdU primary antibody (Developmental Studies Hybridoma Bank, G3G4) and DAPI (0.5 μg/ml). BrdU signal intensity was quantified using Image J analysis software. Regions of interest (ROIs) were determined for each nucleus using signal from the DAPI channel. Background subtraction using a rolling ball algorithm was performed prior to taking measurements. To sort cells based on DNA content, DAPI intensity was measured from ROIs determined by DAPI staining. Line plots were generated by quantifying the frequency of nuclei that fell within ranges of fluorescent intensity.

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Chapter 1 Appendix

Figure 1.1. Mutations in HIPPI disrupt DNA damage repair pathways

(A) A wild-type and two hippI mutant alleles are shown. The crotonase-like domain is shown in blue. Mutant alleles for hippI, 31.2 and 14.3, contain a frameshift (followed by an early stop codon) preceding the crotonase-like domain. (B) A sequence alignment of human CDYLb, wild-type HIPPI, and our hippI mutant alleles are shown. The critical residues for forming the oxyanion hole are outlined in red (L403, L452, D483). (C) Representative images of an Oregon-R chromosome spread (top) and hippI^{ACLD} chromosome spread (bottom) from Drosophila brains are shown. The Oregon-R spread contains no chromosome aberrations (CABs) while the hippI^{ACLD} chromosomes show one CAB indicated by the white arrow. (D) The percent of metaphases containing one or more CABs for each condition is shown. Both Oregon-R and hippI^{ACLD} show an increase in CABs following irradiation (IR) and recovery. hippI^{ACLD} displays a significant increase in the number of CABs, compared to Oregon-R following irradiation and recovery (p=0.0017). Statistical Significance was determined using an unpaired t-test.
Figure 1.2. Su(Hw) and HIPPI colocalize in polytene chromosomes and in insulator bodies during osmotic stress

(A) Diagrams of wild-type and transgenic HIPPI are shown. The HIPPI used for transgenic constructs in this work contains a C-terminal mCherry (mC) domain. (B) A polytene chromosome from larvae P{Su(Hw)::GFP, w/+}, P{HIPPI::mC, w/+}, vg-Gal4, labeled with anti-GFP and -RFP antibodies. Su(Hw)::GFP is shown in green, HIPPI::mC is shown in red, and DAPI is shown in blue. (C) S2 cells expressing transgenic Su(Hw)::GFP and HIPPI::mC transgenic constructs. Su(Hw)::GFP is shown in green and HIPPI::mC is shown in red. The top panel contains cells grown in normal media. The bottom panel shows cells treated for 20 minutes with media containing 250 mM added NaCl.
Figure 1.3. Binding patterns of Su(Hw) and HIPPI to polytene chromosomes is variable in different nuclei suggesting their binding may be dynamic and cell cycle-dependent

(A) Polytene chromosomes from P{Su(Hw)::GFP, w+}/vg-Gal4 larvae expressing Su(Hw)::GFP. Su(Hw), labeled with an anti-GFP antibody, localizes to either all interbands (left) or all bands (right) of the polytene chromosome. In zoom in images arrows point to interbands and arrowheads point to bands. (B) Polytene chromosomes from P{HIPPI::mC, w+}/vg-Gal4. HIPPI larvae expressing HIPPI::mC. HIPPI labeled with an anti-RFP antibody localizes to either all interbands (left) or all bands (right) of the polytene chromosome. Arrows and arrowheads as in A. (C) In addition to the exclusive band or interband binding pattern, many nuclei show no chromosome binding of HIPPI::mC. Polytene chromosomes from P{HIPPI::mC, w+}/vg-Gal4 immunostained with anti-RFP antibody at 10x (top) and 100x (bottom).
A) Su(Hw)::GFP DAPI Interbands Bands

B) HIPPI::mC DAPI Interbands Bands

C) HIPPI::mC DAPI 10X DAPI HIPPI::mC

100X HIPPI::mC DAPI DAPI HIPPI::mC
Figure 1.4. Su(Hw) and HIPP1 overexpression disrupts cell proliferation

(A) Images of wings from Oregon-R, P{Su(Hw)::GFP, w+}/vg-Gal4, P{HIPP1::mC, w+}/ vg-Gal4, or P{Su(Hw)::GFP, w+}; P{HIPP1::mC, w+}/vg-Gal4 flies are shown. Cuts in the wing margin suggesting cell proliferation defects can be seen in P{Su(Hw)::GFP, w+}/vg-Gal4 and P{Su(Hw)::GFP, w+}; P{HIPP1::mC, w+}/vg-Gal4 wings. (B) A bar graph quantifying the wing blade area from Oregon-R, P{Su(Hw)::GFP, w+}/vg-Gal4, P{HIPP1::mC, w+}/vg-Gal4, and P{Su(Hw)::GFP, w+}; P{HIPP1::mC, w+}/vg-Gal4 (***, p=0.0003). (C) Images showing the relative size of larvae, including control P{H2Av::mC, w+}/actin-gal4 over expressing H2Av::mC, P{Su(Hw)::GFP, w+}/actin-Gal4, overexpressing Su(Hw)::GFP or P{HIPP1::mC, w+}/actin-Gal4 overexpressing HIPP1::mC. Scale bar: 2 mm (D) A bar graph of the measured lengths of Oregon-R, P{HIPP1::mC, w+}/actin-Gal4, and P{Su(Hw)::GFP, w+}/actin-Gal4 larvae (****, P<0.0001; ***, p=0.0006). (E) A graph showing the mitotic index, ratio of mitotic nuclei to total nuclei, for Oregon-R, P{HIPP1::mC, w+}/actin-Gal4, and hipp1ACLD larval brains. P{HIPP1::mC, w+}/actin-Gal4 brains show a significant reduction in the mitotic index, compared with Oregon-R (p=0.0410). P values were determined using an unpaired t-test.
Figure 1.5. HIPP1 overexpression alters replication timing in larval brain cells

(A) Representative images of brain cells from Oregon-R and P{HIPP1::mC, w+}/actin-Gal4 larvae labeled with DAPI (blue) and incorporated BrdU (green). (B) Quantification of BrdU fluorescent intensity per nuclei in Oregon-R, hoppel^{AclD}, and P{HIPP1::mC, w+}/actin-Gal4 larval brain cells. (P<0.0001). (C) Frequency of nuclei with different levels of DAPI intensity from Oregon-R, hoppel^{AclD}, and P{HIPP1::mC, w+}/actin-Gal4 larval brain cells are charted. P{HIPP1::mC, w+}/actin-Gal4 larval brain cells exhibit a shift towards a higher level of DAPI intensity. (D) Representative images of larval brain cells labeled with DAPI (blue) and EdU (green) in Early, Middle, and Late S-phase. (E) P{HIPP1::mC, w+}/actin-Gal4 larval brains have a significantly larger ratio of cells in the early replication phase, compared to Oregon-R (p=0.0073). su(Hw)^{e04061/v} mutants, and hoppel^{AclD} mutants were also considered. P{HIPP1::mC, w+}/actin-Gal4 larval brains have a significantly lower ratio of cells in the late replication phase, compared to Oregon-R (p=0.0095). P values were determined using an unpaired t-test. (F) Polytenic chromosomes from P{Su(Hw)::GFP, w+}/vg-Gal4 larvae showing Su(Hw)::GFP in green, labeled using anti-GFP antibody, and BrdU in red, labeled with anti-BrdU antibody. BrdU incorporation is enriched at sites of low Su(Hw)::GFP staining. (G) Polytenic chromosomes from P{HIPP1::mC, w+}/vg-Gal4 larvae showing HIPP1::mC in green, labeled with anti-RFP antibody, and BrdU in red. BrdU incorporation is enriched at sites of low HIPP1::mC staining.
A OR P(HIPP1::mc, w+)/actin-Gal4

B

** ****

C

D Early S-phase Middle S-phase Late S-phase

E

Ratio of stage specific cells to EdU positive cells

F Su(Hw)::GFP / vg-Gal4

G HIPP1::mc / vg-Gal4
**Figure 1.6. HIPPI overexpression delays the progress of replication in polytene chromosomes**

(A) Images of polytene chromosomes from Oregon-R (top) and P{HIPPI::mC, w⁺}/vg-Gal4 (bottom) salivary glands. Chromosomes were initially labeled with EdU, followed by fixation and staining with an anti-PCNA antibody after certain time points, specifically 2, 4, 6, and 8 hours after the EdU incubation. In the example, Oregon-R chromosomes progress from early S-phase (I) to the end of S-phase (IV) in 4 hours. P{HIPPI::mC, w⁺}/vg-Gal4 chromosomes remain in the early S-phase (I) after the 4-hour incubation. (B) Quantification of how many chromosomes from each time point were able to complete each phase transition (I-II, II-III, III-IV, IV-V). P values using an unpaired t-test are as follows: I-II, ***, p=0.0007, **, p=0.0011; II-III, **, p=0.003, *, p=0.0230, **, p=0.0014; III-IV, *, p=0.0199, **, p=0.006, ***, p=0.0001.
A

OR, 4hr incubation

P{HIPP1::mC, w+}/vg-Gal4, 4hr incubation

B

I-II

Length of replication phase transition (hours)

**  

II-III

***  ***  **

III-IV

**  *  *  ***

IV-V

n.s.

OR, 4hr incubation

P{HIPP1::mC, w+}/vg-Gal4, 4hr incubation
Figure 1.7. HIPPI overexpression rescues $y^2$ and $ct^6$ phenotypes

(A) $y^2ct^6$, $y^2ct^6$; P{HIPPI::mC, w$^+$}/vg-Gal4, and $y^2ct^6$; hipp$^{1}_{ALD}$ wings are shown. Only wings from $y^2ct^6$; P{HIPPI::mC, w$^+$}/ vg-Gal4 flies look phenotypically normal, showing almost perfectly round margins and black blades, indicating that overexpression of HIPPI::mC suppresses gypsy enhancer-blocking activity. (B) Polytene chromosomes from $y^2ct^6$ and $y^2ct^6$; P{HIPPI::mC, w$^+$}/ vg-Gal4 larvae, showing the tip of the X chromosome and the Su(Hw) band on the $y^2$ gypsy insertion. Su(Hw) is shown in green, WAPL is shown in red, and DAPI is shown in blue. Su(Hw) and WAPL colocalize at the $y^2$ site (arrow). Su(Hw) and WAPL do not colocalize at the $y^2$ site in the $y^2ct^6$; P{HIPPI::mC, w$^+$}/ vg-Gal4 chromosome (arrow).
CHAPTER 2: Architectural Proteins Modulate the Nuclear Response to Osmotic Stress in Drosophila and Mammalian Genomes
Abstract

Chromatin insulator proteins regulate enhancer-promoter interactions as well as mediate three-dimensional genome organization. Drosophila insulator proteins form boundaries between euchromatin and heterochromatin and function as insulators to both block and facilitate enhancer-promoter interactions. The mammalian insulator protein CTCF partitions the nucleus into topologically associating domains correlating with regions of similar transcription properties. Recent evidence reveals that chromatin insulator proteins in Drosophila and mammals contribute to nuclear stability through functions that extend beyond genome structure and regulation of transcription. Here, we find that insulator proteins play a key role in modulating the nuclear response to osmotic stress. We observe that architecture proteins, including insulator proteins and cohesin, exit chromatin during osmotic stress. The disassembly of architecture results in significant three-dimensional conformation changes observed by Hi-C. We conclude that insulator proteins are critical components of the osmotic stress response and their removal during osmotic stress possibly promotes cell survival.
**Introduction**

Three-dimensional genome architecture is formed by chromatin insulator proteins that facilitate cis and trans chromosome interactions (Fujioka et al., 2016; Maass et al., 2018; Monahan et al., 2019; Schoborg et al., 2013a). Insulator proteins bind specific DNA sites called insulator elements to regulate transcription and form interactions between distal sites (Gerasimova and Corces, 1996; Spana et al., 1988a). Insulators were first discovered in *Drosophila melanogaster* and were defined by their ability to regulate gene expression by insulating transcriptionally active gene regions from surrounding regions of heterochromatin (Kellum and Schedl, 1991; Udvardy et al., 1985). Further analysis identified five different Drosophila insulators plus their associated proteins, revealing the ability of insulators to regulate enhancer-promoter interactions at specific gene loci (Gomez-Diaz and Corces, 2014; Schwartz and Cavalli, 2017). Drosophila insulator proteins mediate enhancer-promoter interactions by both preventing enhancer-promoter interactions when located between the enhancer and target promoter and facilitating enhancer-promoter interactions by forming a complex with another insulator site, thereby bringing together distal genomic loci (Cai and Shen, 2001; Muravyova et al., 2001; Parkhurst and Corces, 1986a).

Genome-wide chromosome conformation capture analysis (Hi-C) has revealed that CTCF, the only known mammalian insulator protein, works with cohesin complexes to partition mammalian genomes into topologically associating domains (TADs) (Hadjur et al., 2009; Kagey et al., 2010; Kurukuti et al., 2006; Moon et al., 2005; Parelho et al., 2008; Wendt et al., 2008). TADs contain frequently interacting regions of chromatin that also share transcriptional properties (Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012). The process of loop extrusion leads to the formation of TADs. Interphase cohesin complexes extrude chromatin loops
until encountering two CTCF insulator proteins bound in opposing orientation (Fudenberg et al., 2016). Once an interaction between the cohesin complex and two CTCF sites in opposite orientation and bound by CTCF proteins occurs, a stable complex is formed until the residency time of the proteins on chromatin expires (Hansen et al., 2017). This dynamic process provides an opportunity for many enhancer-promoter interactions to occur as the loop is extruded, as well as explains how differential gene expression occurs throughout the cell cycle. It is not yet clear whether the model of loop extrusion applies to any aspect of insulator protein function in the Drosophila genome.

Hi-C analysis of Drosophila nuclei established the presence of chromatin loop domains and TADs similar to those found in mammals (Hou et al., 2012; Rowley et al., 2017b; Tanay and Cavalli, 2013). A number of different factors have been found to contribute to Drosophila loop domains including the transcription factor Zelda during zygotic genome activation, polycomb group proteins in transcriptionally silent domains, and insulator proteins to regulate local gene expression (Eagen et al., 2017; Entrevan et al., 2016; Hou et al., 2012; Hug et al., 2017; Ogiyama et al., 2018). Since classes of proteins other than insulators can form loop domains in Drosophila, it is unclear whether insulator proteins are essential to higher order genome structure. Strikingly, transcription activity alone appears to play a fundamental role in maintaining genome organization. The inhibition of RNA Pol II causes TADs and loop domains to fade and upon removal of the inhibitor, structure returns (Rowley et al., 2017b; Ulianov et al., 2016).

Multiple classes of proteins, as well as transcriptional activity, are able to mediate genome contacts in Drosophila whereas CTCF and the loop extrusion complex is responsible for forming a majority of contact domains in mammals (Wutz et al., 2017). The discrepancy in the
structural roles of insulator proteins between Drosophila and mammals raises the question of whether they display conservation in mediating other aspects of genome structure and function.

The osmotic stress response pathway provides a unique method for analyzing insulator-mediated genome architecture. Upon osmotic stress, nuclear space and cellular space shrink to accommodate changes in tonicity. Changes in nuclear morphology coincide with condensation of chromatin (Burg et al., 2007). Previous studies have made observations about the behavior of chromatin insulator proteins during osmotic stress. In Drosophila cells under osmotic stress, insulator proteins leave DNA and localize to the nuclear periphery where they form aggregates, known as insulator bodies (Schoborg et al., 2013b). This process is rapidly reversible upon return to isotonic media. A somewhat similar phenomenon has been observed in human breast cancer cells. Cells treated with hyperosmotic media causes DNA condensation and the departure of CTCF from binding sites. CTCF localizes to spaces void of chromatin but does not form insulator bodies. Hi-C analysis of human cells during osmotic stress reveals an increase in distal genome contacts and a decrease in short range interactions (Amat et al., 2019). The changes observed are rapidly reversed once the cells are returned to normal media. Further analysis of the similarities and differences in the osmotic stress response between Drosophila and mammals will lead to a better understanding of genome structure.

In addition to modeling properties of nuclear structure disassembly and reassembly, the osmotic stress response is significant due to the role of osmotic stress in the onset and development of common diseases such as kidney disease, eye disease, irritable bowel syndrome, cardiovascular disease, and liver disease (Brocker et al., 2012). Osmotic stress induces a conserved signaling cascade leading to the phosphorylation of transcription factors, such as nuclear factor of activated T cells-5 (NFAT5). Upon phosphorylation, NFAT5 translocates into
the nucleus and activates transcription of genes required for the cellular adaptation to a highly osmotic environment (Berga-Bolanos et al., 2010). nfat5 mutant mice are lethal, even without exposure to environmental stressors, highlighting the importance of the osmotic stress response pathway for preventing apoptosis and promoting organism survival. Investigating the role of insulator proteins in the osmotic stress pathway may contribute to the identification of novel therapeutic targets when treating osmotic stress-induced diseases.

Here, we compare the nuclear response to osmotic stress in Drosophila tissues and human cell culture. We find that Drosophila insulator proteins play a critical role in the nuclear response to osmotic stress and recovery from stress. We also identify that additional structural proteins, such as components of the cohesin complex, localize to insulator bodies during osmotic stress. In human cells, we observe an increase in γH2AX levels in cells after recovering from osmotic stress, indicating repair is inhibited during osmotic stress. Additionally, we observe changes in Hi-C contact maps and the removal of cohesin complex proteins during osmotic stress, suggesting that the removal of structural proteins allows DNA to adapt to environments of high osmolality.

**Results**

**WAPL, but not RAD21, colocalizes with gypsy insulator sites**

The mechanism of loop extrusion explains how a single insulator protein, CTCF, and a loop extruding factor, cohesin, can delimit TAD borders (Fudenberg et al., 2016). The presence of a similar loop domains or a loop extruding mechanism has not yet been discovered in Drosophila. Previously, we found that Wings Apart Like (WAPL) colocalizes with the Su(Hw) complex at the yellow² gypsy insulator site and that the presence of WAPL at the yellow² gypsy site correlates with
Su(Hw) enhancer-blocking ability (Chapter 1) (Stow et al., 2019). We next wanted to determine whether additional components of the cohesin complex colocalize with Su(Hw) sites, such as RAD21. We used a transgenic fly line with a tubulin-dependent expression driver for Rad21 fused to a myc tag (P\{tub-Rad21-myc\}). We labeled the chromosomes using Su(Hw) and myc antibodies. RAD21 was not present at gypsy sites, indicated by Su(Hw) staining at the yellow^2 locus (Figure 2.1A). However, RAD21 signal overlapped with some Su(Hw) sites, indicating that RAD21 may be present at a few Su(Hw) binding sites, but not all (Figure 2.1A). Su(Hw) complexes are known to vary in function depending on genomic location, so it is probable that RAD21 only localizes to Su(Hw) sites with specific functions (Soshnev et al., 2012). The presence of WAPL at gypsy sites and the absence of RAD21 suggests that WAPL alone or with other proteins may contribute to the enhancer-blocking function of the Su(Hw) complex in a cohesin complex-independent manner.

Cohesin responds to osmotic stress in Drosophila nuclei

Previous work analyzing the nuclear response to osmotic stress in Drosophila cells revealed that chromatin insulator proteins leave DNA binding sites and form insulator bodies in the nuclear periphery (Schoborg et al., 2013b). This response suggests that insulator proteins play an active role in adapting the nucleus to osmotic stress conditions by leaving native binding sites while chromatin undergoes condensation. Since G1 cohesin complexes play a critical role in shaping genome architecture in mammals, we asked whether cohesin responds to osmotic stress in a similar manner as insulator proteins in Drosophila.

To test whether cohesin responds to osmotic stress in Drosophila cells, we treated S2 cells with media containing 250mM NaCl for 20 minutes. After treatment, we fixed the cells and
immunostained for either WAPL or RAD21. We observe that before applying the osmotic stress media, WAPL is distributed throughout the nucleus in a somewhat uniform manner and forms distinct foci in the cytoplasm. Following treatment with osmotic stress media, WAPL forms foci in the nucleus that perfectly colocalize with Su(Hw)-labeled insulator bodies (Figure 2.1B). WAPL is a release factor for the cohesin complex and the removal of WAPL from human chromosomes using RNAi results in an increase in the residency time of cohesin on DNA and overall chromosome condensation (Wutz et al., 2017). To determine whether the DNA condensation observed during osmotic stress was due to the increased residency time of cohesin following the exit of WAPL, we next observed the localization of RAD21, a core subunit of the cohesin ring, following osmotic stress treatment. Before stress treatment, RAD21 signal is uniformly distributed throughout the nucleus, avoiding pericentric heterochromatin. Following osmotic stress treatment, RAD21 signal accumulates in the nuclear periphery and no longer colocalizes with DAPI. Interestingly, RAD21 does not form foci or colocalize with insulator bodies (Figure 2.1C). The departure of RAD21 and WAPL from chromatin during osmotic stress, along with insulator proteins, suggests that the cohesin complex is a part of Drosophila genome architecture and is at least partially disassembled to accommodate additional changes within the nucleus. This finding is in contradiction with the notion that cohesin drives chromosome condensation in the absence of WAPL (Wutz et al., 2017). Since RAD21 and WAPL leave DNA upon osmotic stress, additional factors in the osmotic stress response pathway must be responsible for driving chromosome condensation.

Polytene chromosomes provide a unique look at the osmotic stress response because changes in DNA structure can be readily observed (Schoborg et al., 2013b). We labeled polytene chromosomes under osmotic stress with antibodies for myc to detect the RAD21-myc fusion
protein as well as CP190, a component of the Su(Hw) insulator complex. We observe that RAD21 exits DNA during osmotic stress in polytene chromosomes, but still does not form bodies as seen with CP190 labeling, but RAD21 signal does partially overlap with CP190 foci. (Figure 2.1D). This provides additional evidence of the departure of a cohesin subunit during osmotic stress, but further suggests that RAD21 is not a component of insulator bodies. We conclude that RAD21 leaves DNA during osmotic stress, indicating that the cohesin complex is no longer associated with DNA. This further suggests that the chromosome condensation occurring in response to osmotic stress is due to a mechanism independent of the cohesin complex acting on DNA. However, it remains a possibility that departure of the cohesin complex and insulator proteins may spontaneously trigger chromatin condensation.

**Osmotic stress activates pathways similar to mechanical stress in human nuclei**

We next wanted to observe the osmotic stress response in human cells to determine whether the cohesin complex participates in the response as in *Drosophila* cells. Earlier observations of the osmotic stress response in human cells found that the nucleus undergoes severe mechanical stress due to cell and nuclear shrinkage (Burg et al., 2007). To confirm that our cell line, A375 (melanoma cells), undergoes similar shrinkage events, we began by observing to what extent the nuclear space shrinks upon osmotic stress treatment.

We analyzed the nuclear size of A375 cells in various salt concentrations ranging from 250mM to 600mM NaCl supplemented media and acquired DIC images of nuclei from data collected using an Amnis ImageStream Imaging Flow Cytometer. An example of images collected from this flow cytometer are presented in Figure A2.1. We found that the nuclear area significantly shrank in each treatment condition, compared to control (Figure 2.2A). The nuclear
area progressively shrank as the salt concentration increased, with the exception of the highest concentration, 600mM. This discrepancy may be due to an increase in apoptosis occurring in 600mM NaCl supplemented media. It has been previously noted that high NaCl concentrations lead to an increase in cell death (Dmitrieva et al., 2004). The overall shrinkage of A375 nuclei in response to osmotic stress confirms that A375 cells respond in a similar way to osmotic stress as cells used in prior studies. We also measured nuclear size in A375 cells that were allowed to recover in normal media following treatment with osmotic stress media. We found that the nuclear size of cells from each concentration tested did not vary significantly from the untreated sample after a one-hour recovery time (Figure 2.2A). This suggests that the osmotic stress response is a reversible process that is designed to help the cells survive hypertonic conditions rather than induce apoptosis.

Specific DNA sites associate with the nuclear lamina, thereby mediating interactions between the chromatin fiber and the nuclear envelope (Lanctot et al., 2007; van Steensel and Belmont, 2017). Regions of chromatin that have been found in close proximity to the nuclear lamina are referred to as Lamina Associated Domains (LADs). The association of LADs with the nuclear lamina is critical for spatially orienting the three-dimensional organization of the genome (Meuleman et al., 2013). Chromosome dynamics result in forces on the nuclear envelope through this relationship, and, in turn, external forces to the nuclear envelope are first sensed by lamina-associated domains (Kumar et al., 2014). Internal forces on the nuclear envelope arising from chromosome dynamics activate the DNA damage-sensing kinase, ataxia telangiectasia-related (ATR). ATR activation leads to the phosphorylation of H2AX (H2Av in Drosophila). Phosphorylation of H2AX by ATM or ATR is an early indicator of DNA damage and occurs when the nucleus experiences mechanical stress (Denais et al., 2016; Kumar et al., 2014).
Therefore, we asked whether the mechanical stress imposed on the nucleus during osmotic stress results in an increase of phosphorylated H2AX (γH2AX).

To observe levels of γH2AX before, during, and after osmotic stress treatment, we performed western blotting using A375 cell lysate. We prepared samples for cells incubated in normal media, cells treated for one hour in 250mM NaCl supplemented media, and cells that were allowed to recover from stress for one hour. The recovery cells produced the only visible band of γH2AX, suggesting that the DNA damage response is upregulated during recovery from osmotic stress, rather than during osmotic stress treatment (Figure 2.2B). This observation that γH2AX levels increase only during recovery from osmotic stress has previously been noted (Dmitrieva et al., 2011).

We next asked whether γH2AX levels change during or after treatment with different concentrations of NaCl in the osmotic stress media. We prepared samples of cells incubated in normal media, cells incubated in 250mM, 400mM, and 600mM NaCl supplemented media for one hour, and cells that underwent stress treatment at each concentration and recovered in normal media for one hour. We labeled the cells with a γH2AX antibody and analyzed them using the Amnis ImageStream Imaging Flow Cytometer. We then quantified the number of γH2AX foci present in the different conditions.

Cells treated with 400mM and 600mM NaCl supplemented media displayed a significant increase in γH2AX foci compared to wild type. Conversely, the 250mM NaCl treated sample had a slight reduction in γH2AX foci compared to wild-type, suggesting that the DNA damage response pathway is inhibited at low levels of salt stress or that the increase in γH2AX foci observed at higher concentrations is due to apoptosis. The 400mM and 600mM recovery samples contained a significantly higher number of γH2AX foci compared to control, but foci were
reduced compared to the osmotic stress samples (Figure 2.2C). Interestingly, the 250mM NaCl recovery sample contained an increased level of γH2AX foci compared to the control sample whereas the 250mM NaCl stress sample contained fewer γH2AX foci compared to control. This confirms the results of our western blot since cells treated with 250mM NaCl revealed no γH2AX band whereas cells that recovered for one hour from treatment with 250mM NaCl stress media showed a clear γH2AX band. These results support previous observations that elevated levels of γH2AX during osmotic stress are due to apoptosis whereas elevated γH2AX levels in recovery indicate repair of DNA damage accumulated during stress treatment (Dmitrieva and Burg, 2008; Dmitrieva et al., 2004; Sheen et al., 2006).

Previous work in our lab identified the presence of γH2Av, the Drosophila γH2AX homolog, in insulator bodies formed during osmotic stress as well as colocalization between insulators and γH2Av in normal conditions (An, 2016). This suggests that the DNA damage response pathway participates in adapting the nucleus to osmotic stress conditions. Although human nuclei do not form insulator bodies, it is possible that the recruitment of γH2Av to insulator bodies in Drosophila is a way to dampen activation of the DNA damage response pathways until return to normal media, just as human nuclei wait to significantly increase γH2AX levels until recovery from osmotic stress.

Cell migration studies have established a link between DNA damage and cell cycle suppression (Dasika et al., 1999; Pfeifer et al., 2018). An increase in DNA damage, especially during the G2/M phase transition, results in activation of checkpoints that stop the cell cycle and allow time for repair. In agreement with the observations from prior studies of the osmotic stress response, we observe cell cycle disruption during osmotic stress treatment with 250mM NaCl by analyzing cell sorting data collected from the Amnis ImageStream Imaging Flow Cytometer.
The cell cycle resumes normal distribution after recovery from osmotic stress treatment (Figure 2.2E). The observed increase in γH2AX foci during recovery from 250 mM NaCl treatment suggests the increase in DNA damage levels activate cell cycle checkpoints in a way that mimics cells that accumulate DNA damage while traveling through a small pore (Pfeifer et al., 2018). We hypothesize that the cell and nuclear shrinkage events that happen during osmotic stress activate similar pathways as those during cell migration through small spaces. This is supported by our evidence that γH2AX foci increase and the cell cycle is disrupted upon osmotic stress treatment.

Interestingly, the cell cycle appears to be less disturbed in cells treated with higher concentrations, 400 mM or 600 mM, of NaCl (Figure 2.2D). These cells also exhibit a high level of γH2AX foci during osmotic stress treatment, which differs from the 250 mM NaCl treatment condition (Figure 2.2C). It is not clear why higher levels of NaCl treatment would not result in complete disruption of the cell cycle. One study considering factors that prevent cell cycle disruption in cells experiencing mechanical stress by passing through small pores found that a combination of treatment with antioxidants, myosin II inhibitor, and overexpression of DNA repair factors leads to a mild rescue of cell cycle defects (Xia et al., 2019). In future studies, it will be interesting to consider whether factors contributing to myosin II inhibition or upregulation of DNA repair factors occur during hyperosmotic stress that may explain the apparent rescue of cell cycle defects at higher NaCl concentrations, compared to lower NaCl concentrations.

Nuclear architecture proteins exit chromatin during osmotic stress in human cells
It is well established that human cells have a programmed response to hyperosmotic environments (Brocker et al., 2012; Burg et al., 2007). Our observations that γH2AX levels rise in cells only after recovering from osmotic stress in normal media supports the idea that nuclei have a programmed response to repair the DNA damage accumulated during osmotic stress, however it remains unclear how nuclear structure shifts in response to this damage or to prevent an unnecessary accumulation of damage. We hypothesize that the nucleus releases higher order chromatin structure to accommodate the shrinking space and prevent DNA damage during osmotic stress.

Previously, we observed the release of insulator proteins and cohesin subunits from chromatin during osmotic stress in Drosophila cells (Chapter 1, Figure 1.1) (Schoborg et al., 2013b). Other work from our lab demonstrates that CTCF is released from chromatin during osmotic stress in human lung fibroblast cells (MRC5) (Garland, 2017). To test whether a similar event occurs in A375 cells, we analyzed immunostaining of nuclear architecture proteins before, during, and after osmotic stress. The CTCF insulator protein is uniformly distributed in the nucleus in control conditions. After incubating cells in osmotic stress media containing 250mM NaCl for 1 hr, CTCF is no longer bound to chromatin and localizes to the nuclear periphery as well as spaces in the nuclear interior devoid of DAPI stain (Figure 2.3A). Additional images of A375 cells during osmotic stress at different NaCl concentrations and after recovery from osmotic stress are presented in Figure A2.2. To confirm the separation between CTCF and DAPI signals, we analyzed cells labeled with CTCF and DAPI using the Amnis ImageStream Imaging Flow Cytometer. We then determined the Pearson’s correlation of CTCF to DAPI signal in the control, osmotic stress, and recovery samples. We found that the Pearson’s correlation between CTCF and DAPI is significantly reduced in osmotic stress samples compared to both control and
recovery samples (Figure 2.3B). During osmotic stress, DNA also appears more condensed and retreats away from the nuclear lamina (Figure 2.3C). The departure of CTCF from DNA is a similarity between Drosophila and humans, however it should be noted that CTCF in human cells does not form bodies as do insulator proteins in Drosophila. This may be due to the differing sizes of the genomes and the comparatively large amount of the CTCF insulator protein in humans.

We next tested whether components of the cohesin complex respond to osmotic stress in human cells. Since cohesin drives the formation of TADs through interactions with CTCF in the loop extrusion mechanism, we hypothesize that cohesin will exit DNA upon treatment with osmotic stress media in a similar manner as CTCF (Fudenberg et al., 2016). We observed control cells, cells treated with 250mM NaCl for one hour, and cells recovered from salt stress media for one hour. We labeled cells for the cohesin release factor WAPL, a cohesin regulatory subunit SA2, and the core cohesin SMC3. We found that all observed cohesin proteins are homogenously distributed in the nucleus prior to stress treatment. Remarkably, following stress treatment, WAPL, SA2, and SMC3 colocalize with CTCF, occupying spaces away from DAPI stain during osmotic stress (Figure 2.3C). WAPL, SA2, and SMC3 return to a normal distribution in the recovery samples. The departure of CTCF and cohesin from DNA suggests that genome structure is significantly altered during osmotic stress. Previous reports have shown that removal of WAPL from DNA locks cohesin onto DNA, driving mild DNA condensation (Wutz et al., 2017). Since we find that SA2 and SMC3, along with WAPL, exit DNA, we can conclude that the DNA condensation during osmotic stress is achieved by a mechanism other than continuous loop extrusion by cohesin. In combination with our observation that cohesin subunits WAPL and RAD21 exit chromatin in Drosophila cells, we conclude that the departure
of insulator proteins and cohesin may be necessary for DNA to condense during osmotic stress treatment. In the future, it would be interesting to test this hypothesis by observing the consequences of preventing the release of architecture proteins during osmotic stress.

**Osmotic stress alters three-dimensional chromosome structure**

The observed condensation of chromatin and departure of architecture proteins during osmotic stress indicates that the nucleus is significantly restructured during the osmotic stress response pathway. We next wanted to observe changes in the spatial organization of DNA in nuclei under osmotic stress. To do so, we performed genome-wide chromosome conformation capture (Hi-C) on A375 cells cultured in normal media, cells treated with 250mM NaCl supplemented media for one hour, and cells recovered from osmotic stress media for one hour. Hi-C data can be observed as a heat-map that represents the frequency of genomic contacts along the linear chromosome. Genomic features such as TADs can be clearly viewed on Hi-C heatmaps as discreet regions that undergo frequent interactions, indicated by a triangle-like pattern, and are isolated from neighboring regions, indicated by clear TAD borders. Multiple TADs cluster together across the three-dimensional space of the nucleus to form neighborhoods of gene regions that share similar transcription properties. These neighborhoods are called compartments and are visible as multi-Mb scale on Hi-C heatmaps. From heat map analysis, we observed that compartments become blurred and their borders become less defined during osmotic stress. This indicates a loss in domain organization and an increase in contact frequencies across domain boundaries. Compartment definition returns in the recovery sample (Figure 2.4A).

A canonical role of human CTCF is its ability to maintain boundaries between TADs and prevent interactions between sites in differing chromatin environments (Hadjur et al., 2009;
Kagey et al., 2010; Kurukuti et al., 2006; Moon et al., 2005; Parelho et al., 2008; Wendt et al., 2008). When a boundary is compromised, chromatin from one TAD may extend across TAD boundaries and form distal interactions with surrounding regions, which leads to ectopic transcription activation (Lupianez et al., 2016; Nora et al., 2017). Therefore, we hypothesize that, since boundaries are regulated by CTCF binding, the departure of CTCF and cohesin from DNA during osmotic stress will cause a weakening of boundary strength and an increase in interactions occurring over longer distances. We first considered the strength of all TAD boundaries in the control sample and compared changes in the strength of the same boundaries in the osmotic stress and recovery samples. Boundaries were determined by scanning the chromosome for regions that displayed a significant dip in interaction score. We found that in stress samples, there is a decrease in boundary strength compared to control, while the boundary strength returned to control levels in the recovery sample (Figure 2.4B). This result further suggests that during stress, CTCF leaves its binding sites, leading to the weakening of boundary insulation and the mixing of compartments. Additionally, our observation that cohesin departs chromatin during osmotic stress may contribute to the weakening of boundaries.

We next asked whether the weakening of boundaries altered the frequency of genomic contacts. By quantifying the number of interactions occurring over varying genomic distances, we found that the osmotic stress sample contained an enrichment of long-distance genomic interactions compared to the control and recovery samples (Figure 2.4C). We hypothesize that the loss of boundary strength, caused by the exit of CTCF and cohesin, leads to increased flexibility of the chromatin fiber and the ability of chromatin to interact over greater distances.

**Osmotic stress upregulates pro-inflammatory genes**
Specific genes are upregulated to help cells adapt to changes in the environment. To gain insight into the pathways upregulated in response to osmotic stress, we considered genes located in regions that were significantly remodeled during osmotic stress, compared to control. Restructuring of gene loci indicates an upregulation in gene transcription (Dixon et al., 2012). To identify changes in gene regions, we quantified the strength of TAD boundaries shared between the osmotic stress and control sample as well as the boundaries shared between the recovery and control sample. We then plotted the insulation score of the shared boundaries against one another and looked for boundaries that significantly differed (Figure 2.5A). Interestingly, pro-inflammatory genes Nuclear factor of the activated T cells-5 (NFAT5) and Nuclear factor kappa B1 (NFkB1) were located within TADs that were very strong in the control sample and weakened in the osmotic stress sample (Figure 2.5B). NFAT5 is a transcription factor specifically upregulated during osmotic stress (Brocker et al., 2012). NFAT5 is an essential gene and its expression, activation, and translocation into the nucleus leads to the activation of a number of genes involved in cell survival during osmotic stress (Berga-Bolanos et al., 2010). We considered frequencies of genomic contacts within and surrounding the nfat5 gene locus and found that this locus becomes less insulated upon osmotic stress, suggesting upregulation of nfat5 transcription (Figure 2.5B). This increase in transcriptional activity may contribute to the role NFAT5 plays in the osmotic stress response pathway.

Nuclear factor kappa B1 (NFkB1) is a transcription factor that mediates inflammatory responses. NFkB1 can be activated by NFAT5 and translocates from the cytoplasm to the nucleus upon activation, forming dimers in order to act as a transcription factor (Cohen and Hiscott, 1992; Shimizu et al., 1990). Heatmaps of interaction frequencies at the nfkb1 locus before, during, and after osmotic stress treatment revealed changes in TAD structure surrounding
the \textit{nfbkl} locus only in the osmotic stress sample (Figure 2.5B). The \textit{nfbkl} locus and the region immediately upstream show an insulated TAD during control and recovery conditions. During osmotic stress, the same region exhibits a loss in organization and an increase in surrounding interactions. This change suggests nearby genes may undergo changes in transcription rates.

Detecting changes in insulation surrounding genes known to participate in the osmotic stress response pathway suggests that the changes witnessed in Hi-C data following osmotic stress coincide with shifts in genomic structure that promote cell survival. Additional analysis of Hi-C data from osmotic stress samples will lead to the identification of more genomic features that promote cell survival during stress from the environment.

\textbf{Discussion}

Our results demonstrate that human and Drosophila genomes contain several conserved elements of the osmotic stress response pathway. In both organisms, core architectural proteins including insulator proteins and cohesin complex subunits exit chromatin. Although the process of loop extrusion has not yet been identified as a feature of Drosophila genome organization, we present evidence that cohesin subunits participate in the osmotic stress response by exiting chromatin and forming insulator bodies. Similarly, in humans, we observe the removal of insulator protein CTCF and the cohesin complex upon osmotic stress. Interestingly, in both organisms binding of these proteins to chromatin is achieved rapidly upon return to normal media, suggesting that the removal of proteins promotes cell survival.

Another similarity between the two pathways is the involvement of $\gamma$H2AX (humans), $\gamma$H2Av (Drosophila), in the response to osmotic stress. $\gamma$H2AX levels and foci are significantly upregulated in recovery samples, compared to control and stress samples. This aligns with
previous studies suggesting that DNA damage repair pathways are not activated to repair DNA damage accumulated during osmotic stress until cells are returned to normal media (Dmitrieva and Burg, 2008; Dmitrieva et al., 2004; Sheen et al., 2006). We hypothesize that a similar mechanism is present in Drosophila. Our previous observations that γH2Av colocalizes with osmotic stress bodies suggests that the DNA damage response and insulator proteins work together via an unknown mechanism to prevent premature DNA repair pathways and to maintain genome stability during osmotic stress (An, 2016).

It is not yet known why insulators leave DNA during osmotic stress. One possibility is that in response to the shrinkage of nuclear space, the nucleus triggers the release of higher order structure. Hypertonicity coincides with an accumulation of DNA damage and activation of G2 and G1 checkpoints (Mavrogonatou and Kletsas, 2009). Our data collected from flow cytometer experiments support the idea that cell cycle progression stalls upon osmotic stress (Figure 2.2D). Interestingly, previous studies establish connections between insulator proteins and the DNA damage response. Mutations in Drosophila su(Hw), a gene encoding the Su(Hw) insulator protein, cause replication stress in developing egg chambers, contributing to the sterility phenotype of su(Hw) mutants. Egg development is partially rescued in su(Hw) mutants by mutations of the checkpoint protein encoding gene, chk1, indicating that repression of the replication stress pathway in su(Hw) mutants leads to a partial rescue of su(Hw) mutant phenotypes (Hsu et al., 2019). Since osmotic stress also activates cell cycle checkpoints, the departure of insulator proteins from chromatin may contribute to checkpoint activation as a method of stalling the cell cycle to prevent errors during osmotic stress exposure. Conversely, our observations that the cell cycle does not stall and γH2AX levels increase in cells treated with 400mM or 600mM NaCl provides contradicting evidence to observations made with the 250mM
NaCl treated sample. Differences observed at higher concentrations of salt stress point toward a mechanism in which once a threshold of damage is accumulated during stress, the cell cycle resumes despite the accumulated stress. In the future it will be interesting to observe whether cells treated with higher concentrations of NaCl display increased rates of translocation due to rounds of DNA replication and cell division with unrepaired damage present (Bakhoum et al., 2018).

Finally, using Hi-C, we observe dramatic changes in nuclear architecture during osmotic stress. The departure of CTCF and cohesin correlates with weakening of TAD boundaries and an increase in distal interactions. Although these changes may rely solely on the removal of architecture proteins and the subsequent increase in chromatin fiber flexibility, we observe that these changes may lead to non-random changes in transcriptional programs indicated by changes in heatmap contacts surrounding pro-inflammatory genes. Genes encoding NFAT5 and NFkB1, participants in the osmotic stress response pathway, exhibit local restructuring, indicating changes in local organization, perhaps indicating changes in transcriptional activity. We propose that the chromatin dynamics during osmotic stress are non-random and promote cell survival by upregulating genes necessary for the osmotic stress response. Additional analysis of protein levels during osmotic stress are required to confirm this hypothesis.

These observations provide evidence of a conserved pathway for the tridimensional genome’s response to osmotic stress. Additionally, we provide evidence that cohesin participates in nuclear processes with insulator proteins in Drosophila. In the future, it will be interesting to further investigate the involvement of cohesin in Drosophila insulator function. Additionally, performing Hi-C on Drosophila cells under osmotic stress will allow us to draw more
conclusions about conserved elements of the osmotic stress response pathways in different organisms.

**Materials and Methods**

**Fly Stocks:** All fly stocks and crosses were maintained on standard cornmeal-agar media and yeast in a 25°C incubator. The fly stocks used in this work include: w*; y2ct6; vtdex8 P{tub-Rad21.271TEV-myc}, a gift from Bruce McKee (University of Tennessee).

**S2 Cell Culture:** Cells were maintained in HyClone SFX insect media (Thermo Fisher) supplemented with 1X Penicillin/Streptomycin at 25°C.

**Human Cell Culture:** A375 cells were maintained in Dulbecco’s Modified Eagle Medium (Thermo Fisher) supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin, 1% L-Glutamine at 37°C and 5% CO2.

**Stress Treatment and Immunostaining:** S2 cells 3–5 d after subculture were allowed to adhere to a poly-l-lysine coverslip for 30 min in a covered 35-mm cell culture dish. To induce osmotic stress, media were removed and quickly replaced with fresh SFX media supplemented with the indicated concentration of NaCl (from a 5M stock). Controls were kept in conditioned media. Cells were stressed for 20 min and then immunostained as previously described (Rogers and Rogers, 2008). In brief, cells were fixed with 4% PFA for 10 min at RT, rinsed 3×with PBS, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with 3% nonfat milk for 10 min at RT. Primary antibodies were diluted in 3% nonfat milk, and coverslips were incubated for 1 hr at
RT in a humidified chamber followed by a 3× wash with PBS/0.1% Triton X-100 for 10 min each. Secondary antibodies were then diluted in 3% nonfat milk and incubated for 1 hr at RT, and coverslips were washed as described. 0.5 μg/ml DAPI was added to counterstain DNA, rinsed 2× with H2O, and mounted in Vectashield.

A375 cells were grown on flame sterilized coverslips in a 6-well dish for 3 days prior to experiment. To induce osmotic stress, media were removed and replaced with fresh DMEM media containing the indicated concentrations of NaCl (from a 5M stock). Controls were placed in fresh DMEM media at the same time. Cells were stressed for 1 hour at 37°C and 5% CO2. Osmotic stress media was removed and replaced with fresh DMEM for 1 hour in recovery samples. Cells were then fixed in 2% PFA, prepared by diluting 16% PFA to 4% in DPBS, then adding 400uL 4%PFA to 400uL of the treatment media (Control or Osmotic Stress). Cells were fixed for 15 minutes at room temperature. Cells were then washed twice in cold DPBS and permeabilized with 0.25% Triton-X100 for 5 minutes. Next, cells were washed twice in cold DPBS and placed in 3mL of 80% ethanol and stored at -20°C for 1-7 days. Following ethanol permeabilization, cells were washed twice in cold DPBS and incubated in blocking buffer (10% Goat Serum, 0.25% Triton-X100) for 1hr at room temperature with rotation. Cells were labeled with primary antibodies diluted in blocking solution at 4°C overnight. Cells were then washed twice in cold DPBS and incubate with secondary antibodies diluted in the blocking buffer solution for 1 hour in the dark. Finally, cells were washed twice in cold DPBS, incubated in DAPI for 5 minutes, washed in ddH2O, and mounted on a slide using Vectashield mounting media.
**Antibodies:** Rabbit polyclonal IgG antibodies generated against full length Su(Hw) and CP190 lacking only the BTB domains were previously generated in our laboratory (Wallace et al., 2010) and used at a dilution of 1:200 for immunostaining. Rabbit anti-WAPL polyclonal IgG was generated in the lab of Dr. Judith Kassis and used according to prior studies (Cunningham et al., 2012). The following antibodies are commercially available: mouse α-myc (1:200; UPBio), mouse α-lamin (1:50, Hybridoma Bank), rabbit α-CTCF (1:250, abcam), mouse α-SMC3 (1:200, abcam), mouse α-SA2 (1:200, abcam), rabbit α-WAPL (1:100, abcam), mouse α-γH2AX (1:500, abcam) mouse α-CTCF (1:25, BD Biosciences). Secondary antibodies labeled with Alexa fluor 488 or Texas Red were obtained from Thermo-Fisher Scientific and used at 1:200.

**Microscopy:** Slides were analyzed using a wide-field epifluorescence microscope (DM6000 B; Leica) equipped with a charge-coupled device camera (ORCA-ER; Hamamatsu Photonic) and an HCX Plan Apochromat (Leica) 40X or 100X/1.35 NA oil immersion objective. Image acquisition was performed using SimplePCI (v6.6; Hamamatsu Photonics). Image brightness and contrast adjustments were performed by Fiji (National Institutes of Health). Samples were processed and imaged under identical conditions of immunostaining, microscope, camera and software settings.

**Chromosome Conformation Capture:** For each sample condition, 5 million cells were crosslinked using 1% formaldehyde and lysed. Chromatin was digested using high-fidelity HindIII restriction enzyme. Biotin was used to fill in digested ends, ends were then ligated together, followed by reversal of crosslinks by proteinase K treatment. DNA was purified using
phenol:chloroform isolation. After removing biotin from unligated ends, ligated ends with biotin were pulled-down using streptavidin coated beads. Illumina sequencing adapters from New England Biolabs were used to prepare samples for sequencing. (Belton et al., 2012; Golloshi et al., 2018).
Figure 2.1. Cohesin localizes to insulator binding sites and leaves chromatin during osmotic stress in Drosophila

(A) Images of polytene chromosomes from y^{2}ct^{6}; P{tub-Rad21-myc} flies and labeled with anti-Su(Hw) and anti-myc antibodies are shown. In the left panel, the white arrowhead indicates the yellow^{2} locus. In the right panel, the white arrowheads indicate sites of Su(Hw)-RAD21 signal overlap. (B) Images of S2 cells labeled with anti-Su(Hw) and anti-WAPL antibodies before osmotic stress (top panel) and after treatment with 250mM NaCl supplemented media (bottom panel). (C) Images of cells from third instar larvae CNS expressing P{tub-Rad21-myc} and labeled with anti-CP190 and anti-myc antibodies. Cells are shown before stress (top panel) and after osmotic stress treatment (bottom panel), and after recovery from osmotic stress. (D) Polytene chromosomes expressing P{tub-Rad21-myc} after 20 minutes of treatment with 250mM NaCl supplemented media are shown. Chromosomes are labeled with anti-CP190 and anti-myc antibodies.
Figure 2.2. Osmotic stress induces mechanical stress and DNA damage in human cells

(A) Nuclear size was determined for cells before stress, after treatment with NaCl supplemented media, and after recovery from osmotic stress media using images from the Amnis ImageStream Imaging Flow Cytometer. Significance was determined using ANOVA. Four asterisks indicate P<0.0001. (B) A Western Blot labeled with γH2AX for control (left), 250mM NaCl treated (middle), and recovery (right) cells. (C) Number of γH2AX foci were determined for cells before stress, after treatment with NaCl supplemented media, and after recovery from osmotic stress media using images from the Amnis ImageStream Imaging Flow Cytometer and a γH2AX spot count mask. Significance was determined using ANOVA. Four asterisks indicate P<0.0001. (D, E) Cell sorting graphs generated from the Amnis Image Stream Imaging Flow Cytometer. (D) For cells treated with different concentrations of NaCl, DAPI content shifts to the G/S-phase intermediate state in 250mM NaCl stress conditions. (E) In cells treated with osmotic stress media and allowed to recover for one hour, all cell populations shift back to a control-like distribution.
Figure 2.3. Osmotic stress triggers the release of nuclear architecture in human cells

(A) Images of A375 cells labeled with CTCF and DAPI before osmotic stress, during osmotic stress, and after recovery from osmotic stress. (B) Pearson’s correlation of CTCF and DAPI signal in cells before osmotic stress, during osmotic stress, and after recovery from osmotic stress. Pearson’s correlation determined using osmotic stress media using images from Amnis ImageStream Imaging Flow Cytometer software. Significance was determined using ANOVA. Four astersiks indicate P<0.0001. (C) Images of A375 cells labeled with Lamin and DAPI before osmotic stress, during osmotic stress, and after recovery from osmotic stress. (D) Images of A375 cells labeled with CTCF and WAPL antibodies (top panel), SA2 and CTCF (middle panel), and SMC3 and CTCF (bottom panel).
Figure 2.4. Boundary strength decreases, and distal interactions increase during osmotic stress

(A) Heatmaps of genomic contacts from Chromosome 2, 250kb bins are shown. The full chromosome is represented in the top panel and a magnified view of the the p arm of chromosome 2 is represented in the bottom panel. Maps shown include cells incubated in normal media (Control), cells incubated in media containing 250mM NaCl (250mM NaCl) for one hour, and cells incubated in 250mM NaCl for one hour, followed by normal media for one hour (Recovery). (B) A box plot of boundary strength differences is shown. Boundaries from stress sample were compared to control boundary strength (left). Boundaries from recovery sample were compared to control boundary strength (right). (C) A plot of interaction enrichment plotted against genomic distance for Control (black), 250mM NaCl (blue), and recovery (red) samples is shown.
Figure 2.5. Interaction frequencies at the *nfat5* and *nfκb1* gene loci change during osmotic stress

(A) Insulation scores for Control vs. Stress (left) and Control vs. Recovery are shown for gene regions. (B) Heat maps generated from control, stress (250mM NaCl), and Recovery samples at the *nfat5* locus and *nfκb1* gene loci with 2Mb flanking either side of the gene regions. 40kb resolution.
CHAPTER 3: Mutations in the Insulator Protein Suppressor of Hairy Wing Induce Genome Instability
Parts of this chapter have been submitted for publication:


My contribution included: (1) performing experiments, (2) data collection and analysis, (3) writing manuscript and making figures, and (4) devising experiments. Shih-Jui Hsu contribution included (1), (2), (3), and (4). James Simmon’s contribution included (1), (2), and (3). Heather Wallace’s contribution includes (1), (2), and (4). Andrea Lopez and Shannon Stroud’s contribution included (1) and (2). Mariano Labrador’s contribution included (3) and (4).
Abstract

Chromatin insulator proteins mediate the formation of contacts between distant insulator sites along chromatin fibers. Long-range contacts facilitate communication between regulatory sequences and gene promoters throughout the genome, allowing accurate gene transcription regulation during embryo development and cell differentiation. Lack of insulator function has detrimental effects often resulting in lethality. The Drosophila insulator protein Suppressor of Hairy wing [Su(Hw)] is not essential for viability but plays a crucial role in female oogenesis. The mechanism(s) by which Su(Hw) promotes proper oogenesis remains unclear. To gain insight into the functional properties of chromatin insulators, we further characterize the oogenesis phenotypes of su(Hw) mutant females. We find that mutant egg chambers have poorly formed microtubule organization centers (MTOCs) in the gerarium and show mislocalized Gurken (Grk) in later stages of oogenesis. Further analysis reveals an excess of DNA damage in egg chambers, which is independent of activation of transposable elements, and that Gurken localization defects and oogenesis progression are partially rescued by mutations in ATR (mei-41) and Chk1 (grapes) genes. In addition, we show that su(hw) mutant larval brains display an impaired ability to repair DNA damage following exposure to X-rays. Together, these findings suggest that Su(Hw) plays a critical role in maintaining genome integrity during germline development in Drosophila females as well as in dividing somatic cells.
Introduction

Chromatin insulators facilitate higher-order chromatin organization in the nucleus by stabilizing interactions between distant sites in the chromatin fiber. These long-range contacts help orchestrate interactions between regulatory sequences and gene promoters to accommodate the complex genomic networks of gene transcription required to promote cell and tissue differentiation during embryo development (Labrador and Corces, 2002; Lupianez et al., 2015; Yang and Corces, 2012). Insulator function is conserved throughout eukaryotes (Gurudatta and Corces, 2009; Heger and Wiehe, 2014; Schoborg and Labrador, 2014; Schoborg and Labrador, 2010; Van Bortle and Corces, 2012b).

Canonical insulator properties include the ability to prevent communication between distal enhancers and promoters when positioned in between them and function as boundaries to protect genes against heterochromatin-mediated silencing (Brasset and Vaury, 2005; Gaszner and Felsenfeld, 2006; Kellum and Schedl, 1991; Roseman et al., 1993; Udvardy et al., 1985; West et al., 2002; Zhao et al., 1995). These properties are mediated by insulator proteins, which bind the insulator DNA and may facilitate long-range DNA-DNA interactions (Phillips-Cremins and Corces, 2013; Van Bortle and Corces, 2012a). Recent progress in the study of insulator protein distribution and in the analysis of the three-dimensional organization of the genome within the nucleus has revealed a role of these proteins in the architectural organization of the genome (Lieberman-Aiden et al., 2009; Negre et al., 2010; Ong and Corces, 2014; Phillips-Cremins and Corces, 2013; Rao et al., 2014; Rowley et al., 2017a). Thus, a new paradigm has emerged where insulator proteins in combination with cohesin establish topologically associating domains of DNA, which correspond with topologically independent regions of gene transcription activation or repression throughout the genome (Fudenberg et al., 2016; Smith et al., 2016). However, although the functional principles behind this organization are strongly supported by experimental
ovservations in mammalian cells, the question of whether this model of genome organization is universal amongst eukaryotes, as well as the implications of the model in genome organization, genome function and genome integrity, remain an active focus of research (Canela et al., 2017b; Oomen et al., 2019; Rowley et al., 2017a).

The gypsy retrotransposon found in Drosophila contains one of the earliest characterized insulators. Gypsy can integrate at sites between enhancers and promoters, thereby disrupting enhancer-promoter communication and causing mutations that can be suppressed by mutations in the suppressor of Hairy wing gene [su(Hw)] (Modolell et al., 1983; Parkhurst and Corces, 1986b; Spana et al., 1988b). In addition to Su(Hw), which directly binds to the insulator DNA, two other proteins are required for gypsy insulator function: Modifier of mdg4 [Mod(mdg4)-67.2] and Centrosomal Protein 190 (CP190), which directly interact with Su(Hw) (Georgiev and Kozycina, 1996; Georgiev and Gerasimova, 1989; Ghosh et al., 2001; Pai et al., 2004). Unlike other insulator proteins in Drosophila such as dCTCF, CP190, BEAF, or GAGA factor, the function of both Su(Hw) and its binding partner Mod(mdg4)-67.2 are dispensable for viability (Butcher et al., 2004; Gerasimova et al., 1995a; Katokhin et al., 2001; Klug et al., 1968; Mohan et al., 2007; Roy et al., 2007). CP190 has insulator activity that is independent from Su(Hw) and forms insulators in the genome in association with other insulator proteins (Bushey et al., 2009; Mohan et al., 2007; Moshkovich et al., 2011). Homozygous su(Hw) loss-of-function mutations are viable with no evident phenotype other than female sterility (Klug et al., 1968; Klug et al., 1970). In ovaries, Su(Hw) is detected in the nucleus of both somatic follicle cells and germ cells (Baxley et al., 2011). Specifically, loss of Su(Hw) leads to suppression of yolk deposition in the oocyte and oocyte development is arrested at mid-oogenesis (Harrison et al., 1993; Klug et al., 1968; Klug et al., 1970). More recent findings have revealed that loss of Su(Hw) leads to an upregulation of
neuronal gene expression in germline tissue, suggesting that ectopic expression of these genes could be responsible for the sterility phenotype of su(Hw) mutations (Baxley et al., 2011; Harrison et al., 1993; Soshnev et al., 2013a; Soshnev et al., 2012). In fact, the oogenesis phenotype in su(Hw) mutant females can be partially suppressed by mutations that reduce the expression of the RNA-binding protein 9 (Rbp9), a protein expressed at higher levels in su(Hw) mutant ovaries that is involved in blood-brain barrier establishment (Kim et al., 2010; Soshnev et al., 2013a). Rescued females, however, do not produce viable offspring, and eggshells from laid embryos reveal strong dorsoventral transformations. Given the complexity of this phenotype, analysis of su(Hw) mutations in the female germ line could be instrumental for further understanding the role of Su(Hw) in oogenesis and the function of insulator proteins during development in general.

In Drosophila, oogenesis begins with the asymmetric cell division of a germline stem cell located at the tip of the germarium, which gives rise to a daughter stem cell and a cystoblast. The cystoblast undergoes four incomplete mitotic divisions, forming an egg chamber containing sixteen germ cells that remain interconnected and are enclosed by an epithelium of follicle cells. Only one of the sixteen germ cells will adopt the oocyte cell fate while the remaining fifteen cells become nurse cells. Each egg chamber undergoes a developmental process that culminates with the formation of a mature oocyte at stage 14. As oogenesis progresses, at stage 6, the nucleus of nurse cells undergoes a dramatic change from a condensed five-blobs chromosome configuration to a decondensed chromosome morphology (Bate and Martinez Arias, 1993; Dej and Spradling, 1999). Before mid-oogenesis arrest, the only visible chromatin-configuration defect in su(Hw) mutant egg chambers is a delayed chromatin dispersal of nurse cell polytene chromosomes at stage 7 or 8. The prolonged development of defective egg chambers is
eliminated by mid-oogenesis arrest resulting in egg chamber degeneration around stages 9 to 10 (Baxley et al., 2011; Harrison et al., 1993; Klug et al., 1968). This defective chromatin dispersal is a common trait among a large number of unrelated mutants, such as genes encoding the spliceosome component prp22 (Klusza et al., 2013), the piwi-interacting RNAs (piRNAs) related protein, and rhino (Klattenhoff et al., 2009; Volpe et al., 2001), which complicates the identification of the mechanisms associated with Su(Hw) activity in the genome of egg chamber cells.

In this study, we conclude that the loss of Su(Hw) creates an accumulation of non-meiotic DNA damage in germline cells of ovaries, thereby activating DNA damage checkpoints. We show that mutations in meiotic checkpoint genes mei-41 (ATR) and grapes (chk1), but not mnk (chk2, loki), result in the rescue of the su(Hw) mutant spindle phenotype in ovaries. We conclude that the lack of su(Hw) expression in ovaries provokes an excess of unrepaired DNA breaks in germline cells and propose that this DNA damage is caused by replication stress. Our data supports that replication stress occurs in dividing somatic cells as well, in su(Hw) mutants.

Results

Microtubules are disorganized in su(Hw) mutant egg chambers

Previous analysis reveals a higher incidence of irregular nurse cell number in su(Hw) mutant egg chambers (Hsu, 2014). Abnormal nurse cell number in egg chambers also occurs in loss of function mutants of genes encoding proteins involved in piRNA-related pathways, including rihno and maelstrom (mael) (Sato et al., 2011; Volpe et al., 2001). mael encodes a γ-tubulin associated protein involved in the proper positioning of the MTOC, which is required to determine oocyte polarity and the precise localization of specific mRNAs within the Drosophila
Microtubule organization is critical at various stages of oogenesis. In stage one, formation of the MTOC, a structure with concentrated α-tubulin at the posterior of the oocytes, is required for oocyte differentiation. In stages 3 through 6, a microtubule array is extended from the MTOC through ring canals to the neighboring nurse cells. This polarized network of microtubules is required for intercellular transport from nurse cells to the oocyte. During stage 7 the microtubule network is reorganized, causing a shift in the polarity of the MTOC from posterior to anterior, and the growing microtubule network positions the oocyte nucleus to the anterior corner (Steinhauer and Kalderon, 2006; Theurkauf et al., 1992).

To assess whether su(Hw) mutants also show microtubule disorganization in addition to an irregular number of nurse cells, we previously used an anti α-tubulin antibody that allows detection of microtubule networks in egg chambers (Theurkauf et al., 1992). We found that, under our experimental conditions, MTOCs form properly in wildtype ovaries, exhibiting the typical concentration of α-tubulin at the posterior of the oocyte in the germarium (Figure 3.1A). However, in su(Hw) mutants, the α-tubulin signal is weaker, more diffuse, and less concentrated at the MTOC (Figure 3.1B and B1). Additionally, we were able to rescue the MTOC defect in the germarium of su(Hw) mutant ovarioles by ectopic expression of su(Hw)::eGFP driven by the nanos-GAL4 driver (Figure 3.1C and C1). In mod(mdg4) mutants, the α-tubulin signal appears to be as intense as wildtype (Figure 3.1D and D1). These images and observations were presented in a prior thesis but were never quantified (Hsu, 2014).

Here, to confirm our observations, we quantified the average integrated signal density of the α-tubulin signal in stage 1 egg chambers. The α-tubulin signal in su(Hw) mutants is significantly reduced compared to wildtype and the su(Hw)::eGFP driven by the nanos-GAL4
samples (Figure 3.1E). Interestingly, even though the average is higher in mod(mdg4)u1 mutants, the difference in α-tubulin signal between the su(Hw) and mod(mdg4) mutants is not significant, suggesting Mod(mdg4) may also partially contribute to this function of Su(Hw) in oogenesis (Figure 3.1E). These data suggest that proper formation of the MTOC is impaired upon loss of Su(Hw) and imply that the microtubule network is disorganized and may not function efficiently to facilitate egg chamber development. For example, it is well established that defects in MTOC regulation that affect establishment of polarity in early oogenesis result in the disruption of Grk signaling in later stages of oogenesis (Khurana and Theurkauf, 2010).

**Loss of Su(Hw) activates a DNA damage checkpoint during oogenesis**

Because of the similarity between spindle class and piRNA mutants with su(Hw) mutant phenotypes and the elevated levels of γH2Av seen in su(Hw) mutant egg chambers (Hsu, 2014), we hypothesize that su(Hw) mutations activate a DNA damage checkpoint during oogenesis. We first tested whether su(Hw) mutants activate a DNA damage signaling pathway by asking whether oocyte development is restored in females double mutant for su(Hw)v/e04061 and the *Drosophila* ATR allele, mei-41D5 (Brodsky et al., 2004; Laurencon et al., 2003). Results show that although double mutant females remained sterile, 54% of egg chambers recovered correct positioning of Grk around the oocyte nucleus at stage nine, (N=24, p<0.001, Fisher exact test), and had proper enlargement of the developing oocyte in stage nine and ten (Figure 3.2C, H, and I). These results show that loss of ATR function partially recovers oocyte development in su(Hw)v/e04061 mutants and suggests that loss of Su(Hw) function triggers a DNA-damage response through an ATR-dependent pathway.
Chk1 (Grp) and Chk2 (Mnk, Lok), two highly conserved downstream kinases in ATR/ATM DNA damage signaling, are both phosphorylated in response to DNA damage and participate in cell cycle checkpoint activation (Cimprich and Cortez, 2008). However, mutations of chk2, but not chk1, recover the dorsoventral patterning defects associated with mutants of spindle and piRNA pathway genes (Chen et al., 2007; Ghabrial et al., 1998; Klattenhoff et al., 2007). To determine the role of DNA repair pathways in su(Hw) mutant ovaries, we first tested the ability of chk1 (grp$^{z5170/209}$) chk2 (lok$^{30}$) double mutant flies to rescue the mutant phenotypes of su(Hw)$^{v/e04061}$ ovaries. We found that grp$^{z5170/209}$ lok$^{30}$ double mutants were able to rescue defective Grk localization but were unable to rescue the lack of fertility in su(Hw) mutants (Figure 3.2D). To test whether the loss of Su(Hw) activates a DNA damage response specifically mediated by ATR/Chk2, we generated double mutant flies using su(Hw)$^{v/e04061}$ and chk2 (lok$^{30}$, mnk$^{6006}$) mutations (Brodsy et al., 2004) and tested whether a mutation in chk2 is able to rescue Grk localization in su(Hw) mutants. The results showed that neither fertility nor Grk localization were rescued in these double mutants (Figure 3.2E and F). Finally, we generated double mutant flies using su(Hw)$^{v/e04061}$ and chk1 (grp$^{z5170/209}$) mutations. grp$^{z5170/209}$; su(Hw)$^{v/e04061}$ double mutants were able to rescue the Grk localization phenotype in 80% of egg chambers analyzed but were unable to rescue the sterility phenotype (Figure 3.2G and I). We measured egg chamber length for those used in this analysis to ensure consistency in staging (Figure A3.1). These findings show that the spindle-like phenotypes caused by loss of Su(Hw) are dependent on Chk1 (grapes) mediated checkpoint activity, downstream of the ATR mediated DNA-damage pathway, and are independent of Chk2 (mnk, lok). Since ATR/Chk1 activate a checkpoint in response to replication stress (Blythe and Wieschaus, 2015; Fogarty et al., 1997; Sibon et al.,...
1997), our results suggest that mutations of su(Hw) cause replication stress in developing egg chambers.

**Single-stranded DNA accumulate in su(Hw) mutant ovaries**

In previous reports, we observed an accumulation of H4K20me1 and γH2Av in su(Hw) egg chambers. Accumulation of γH2Av and H4K20me1 in su(Hw) mutant egg chambers suggests that the loss of su(Hw) results in replication stress (Hsu, 2014; Hsu et al., 2019). To directly observe whether su(Hw) mutant egg chambers display DNA replication defects, we quantified the amount of single-stranded DNA in su(Hw) \(^{/}TM6B\) and su(Hw)\(^{/}e04061\) nurse cells. An accumulation of single-stranded DNA indicates the stalling of replication forks (Zellweger et al., 2015). 5-bromo-2'‐deoxyuridine (BrdU) is a nucleotide analog that can be added to replicating DNA. Detection of incorporated BrdU with an antibody usually requires a denaturation step. Detection of BrdU in non-denaturing conditions allows for the observation of naturally occurring single-stranded DNA (Despras et al., 2010). We incubated su(Hw) \(^{/}TM6B\) and su(Hw)\(^{/}e04061\) egg chambers in BrdU for 30 minutes and labeled with BrdU antibody using non-denaturing conditions. We observe significantly higher levels of BrdU in stages 5-8 su(Hw)\(^{/}e04061\), compared to su(Hw)\(^{/}TM6B\) egg chambers (Figure 3.3). We also observe an increase in stage 2-4 su(Hw)\(^{/}e04061\) egg chambers, compared to su(Hw)\(^{/}TM6B\) egg chambers (Figure 3.3). Significant differences in BrdU incorporation between su(Hw) mutant and wildtype egg chambers are observed in both early and late stage egg chambers (Figure 3.3). We staged egg chambers based on egg chamber length (Figure A3.2). Altogether, our results suggest that DNA damage in su(Hw) mutants is caused by replication stress, which disrupts genome stability and eventually leads to checkpoint activation and contributes to the oogenesis phenotype of these mutants.
Su(Hw) contributes to ATR-mediated DNA damage repair in somatic cells

Partial rescue of su(Hw) mutant phenotypes by mei-41D5 mutants and the increase in γH2Av in su(Hw) mutant ovaries suggests Su(Hw) may also play a role in the DNA damage response pathway. To answer this question, we tested the ability of su(Hw) e04061 mutants and su(Hw) e04061; mei-41D5 double mutants to recover from DNA damage induced by X-ray irradiation. We first confirmed that larvae responded to our method of X-ray irradiation. Third instar larvae from mei-41D5 and su(Hw) e04061, mei-41D5 double mutants were exposed to 7.5Gy irradiation and allowed to recover for three hours. Levels of γH2Av were then measured in brains dissected from these larvae via western blot. We found that γH2Av levels were elevated in both single and double mutants following X-ray treatment (Figure 3.4A).

We next aimed to quantify the amount of damage accumulated in mei-41D5 mutants compared to su(Hw) e04061, mei-41D5 double mutants following X-ray treatment. To do so, third instar larvae were exposed to 7.5 Gray ionizing radiation. Larvae were allowed to recover from the exposure for two hours. After two hours, brains were dissected from larvae and incubated in a microtubule inhibitor for an additional hour, followed by a brief incubation in a hypotonic solution, making the total recovery time three hours. Chromosome aberrations (CABs) were quantified by counting the number of metaphase nuclei containing one or more aberration(s) and comparing this number to metaphases with no CABs (Figure 3.4B and C).

We find that su(Hw) e04061; mei-41D5 double mutants display a significant increase in the number of CABs following exposure to ionizing radiation compared to non-exposed samples and mei-41D5 single mutants. We observe that su(Hw) e04061; mei-41D5 double mutants have CABs in 49.6% of metaphasic nuclei at 3 hours post-irradiation exposure. mei-41D5 mutants alone have
CABs in 31.4% of metaphasic nuclei at 3 hours post-irradiation exposure. This result suggests that Su(Hw) is involved in the DNA damage repair pathway in somatic cells. Taken together, our results show that nurse cells from ovaries of su(Hw) mutant females undergo replication stress, which significantly increases the levels of γH2Av. Additionally, our results analyzing dividing somatic cells also suggest that the DNA damage response is diminished in su(Hw) mutants. These properties of Su(Hw) lead to the failure to repair DNA breaks and the activation of the ATR/Chk1 checkpoint as well as spindle-like phenotypes in the egg chambers of su(Hw) mutant developing oocytes.

**Discussion**

This study has uncovered a novel role for Su(Hw) in the maintenance of genome stability. This conclusion is supported by data showing that a DNA damage response is activated in su(Hw) mutant egg chambers as well as by an impaired ability to repair DNA damage following irradiation in dividing neuroblasts from su(Hw) mutant larval brains. Traditionally, gene mutations that lead to the activation of DNA damage response pathways in the germline of *Drosophila* females are recognized by the formation of dorsoventral patterning defects in eggshells (Abdu et al., 2002; Ghabrial et al., 1998). In su(Hw) mutant ovaries, however, oogenesis arrests and oocytes do not fully develop, preventing direct observation of whether dorsoventral patterning defects in eggshells are an element of the phenotype. This, in combination with the circumstance that all other phenotypes associated with the production of elevated intrinsic DNA damage cannot be directly observed without the appropriate experimental analysis, may explain why the phenotype of su(Hw) mutations has rarely been previously associated with DNA damage or the DNA damage response (Lankenau et al., 2000).
Previously, we have shown that an irregular number of nurse cells, MTOC disorganization, and Grk mislocalization are defects in su(Hw) mutant egg chambers. We have also shown previously that germline cells of su(Hw) mutants undergo an excessive accumulation of H2Av phosphorylation (Hsu, 2014; Hsu et al., 2019). Ataxia Telangiectasia-Related (ATR) and Ataxia Telangiectasia-mutated (ATM) are two highly conserved kinases with central roles in DNA repair, cell-cycle checkpoint progression, and cell fate determination (Sancar et al., 2004). In the DNA damage response, ATM primarily acts in repair of DSBs, whereas ATR is activated in response to various DNA lesions, particularly those generated by replication stress (Zeman and Cimprich, 2014). Both kinases are able to phosphorylate unique downstream effectors as well as a number of common targets, such as the human H2AX or the Drosophila equivalent H2Av. Phosphorylated H2AX (γH2AX) in humans, or phosphorylated H2Av (γH2Av) in Drosophila, is an indicator for ATR/ATM activity, and crosstalk between these two pathways occurs in response to DNA damage (Cimprich and Cortez, 2008; Sirbu and Cortez, 2013).

Although the detailed mechanism of how ATR and ATM coordinately function during oogenesis in Drosophila is still unclear, it seems that both proteins have distinct functions. Specifically, ATM is primarily involved in DNA damage repair, whereas ATR is also involved in cell-cycle checkpoint regulation (Joyce et al., 2011).

Due to the fundamental role that γH2AX (or γH2Av) has in all DNA damage repair pathways, the recognition of this modification has become a standard assay for DSB and DNA damage detection. In this study, we find a strong accumulation of γH2Av in mutant nurse cells, and reveal that mutation of the checkpoint gate keeper ATR/mei-41 rescues the spindle-like phenotype in su(Hw) mutants. Previous observations have shown that an elevated frequency of DSBs is generally produced by mutations in DNA repair genes or by retrotransposon activity.
induced by mutations in components of the pi-RNA pathway. Both phenomena can lead to DNA damage and the activation of checkpoint repair pathways mediated by ATR and Chk2, which are phenotypically characterized by microtubule disorganization and translational repression of Grk (Chen et al., 2007; Klattenhoff et al., 2007; Mohn et al., 2014; Sienski et al., 2012). Our data, however, shows that su(Hw) mutant egg chambers display microtubule disorganization and Grk mislocalization, and that mutants for ATR and Chk1 (grp), but not Chk2 (mnk, lok), partially rescue this phenotype. This important difference suggests that an alternative pathway, mediated by ATR and Chk1 is activated by a response to accumulation of DNA damage in su(Hw) mutant ovaries. This conclusion is further supported by our observation that DNA damage in su(Hw) mutant ovaries results from a pathway independent from transposable element activity.

A previous study reports that cutting the gene dosage of an RNA-binding protein, Rbp9, in half results in the rescue of female fertility in su(Hw)2/v mutants (Soshnev et al., 2013a). This study suggests that female sterility in su(Hw)2/v mutants is due to the derepression of neuronal genes such as rbp9 in the germline that are under the transcriptional control of Su(Hw) binding sites. Our results support the idea that Su(Hw) has roles during oogenesis that extend beyond transcriptional regulation. One possibility against this notion is that Su(Hw) may control the expression of genes involved in DNA damage signaling in the ovaries. However, published reports show no indication that Su(Hw) specifically controls the expression of genes involved in DNA repair or replication pathways (Baxley et al., 2011; Hsu et al., 2015). Instead, we suggest that null mutations of su(Hw) result in a complex multifactorial oogenesis phenotype. Sterility is caused mostly by the over-expression of neuronal genes such as rbp9, due to the lack of transcriptional repression activity of Su(Hw), whereas the spindle-like phenotype described here would be dependent on the structural activity of Su(Hw) in the genome through its insulator
function. Thus, a reduction of the levels of *rbp9* is enough to restore oogenesis development but not sufficient to eliminate the ventralization phenotype induced by replication stress and the activation of the *ATR/chk1* checkpoint. Likewise, expression of *su(Hw):eGFP* driven by *nanos-GAL4* can partially rescue fertility but does not completely eliminate the spindle-like phenotype, producing frequent mislocalization of Grk and ventralized eggshells.

DNA breaks in *su(Hw)* mutant females may be produced by two different mechanisms: an overall increase in the production of DNA breaks or a failure in the process of repairing DNA breaks. Intrinsic causes of DSBs in *Drosophila* female germline development are normally attributed to meiotic recombination, transposable element mobilization, or abnormal DNA replication. In ovaries, endoreplication is a specialized genome replication process that takes place within a limited time during oogenesis and produces polyploid nurse cells that supply nutrients for oocyte development. Cells undergoing endoreplication only pass through a G phase and a S phase, but do not undergo mitosis (M) (Lee et al., 2009). Euchromatin is duplicated early during endoreplication while heterochromatic sequences are duplicated at late S phase. Because S phase is shorter in each endoreplication cycle, some heterochromatic regions frequently lose the opportunity for replication. This loss of replication is called underreplication, and damaged DNA has been observed at the junction between replicated euchromatin and underreplicated heterochromatin regions (Hammond and Laird, 1985; Lilly and Spradling, 1996b; Yarosh and Spradling, 2014). In salivary gland polytene chromosomes, for example, the accumulated γH2Av signals are detected at local underreplication sites (Andreyeva et al., 2008).

Because Su(Hw) has been associated with heterochromatin-euchromatin boundaries (Khoroshko et al., 2016), one possibility is that the absence of Su(Hw) in mutants disrupts the endoreplication process and leads to an excess of DNA damage at these sites. DNA breaks may
result from stalled replication forks at the boundaries that fail to reinitiate replication (Lilly and Spradling, 1996a; Mirkin and Mirkin, 2007; Peng and Karpen, 2008). Interestingly, this mechanism has been suggested to explain the five lobes nuclear organization phenotype, which is typical in nurse cells during stages 1 to 5 of oogenesis, but that extends beyond stage 5 in su(Hw) and in other oogenesis mutants (Dej and Spradling, 1999). The 5 lobes structure is the result of homolog chromosome pairing during re-replication, and in normal nurse cells, these chromosomes separate and disperse in the nucleus after stage 5. The 5 lobes anomalous phenotype at later stages results from failure of chromosomes to disperse, which is likely due to unfinished replication forks that prevent sister chromatids from dispersing after stage 5 (Dej and Spradling, 1999).

Whereas research on insulator proteins has focused mostly on their roles in transcription, the possibility that chromatin insulators might also be involved in genome replication emerged recently after analysis of the genomic distributions of insulators and origin of replication proteins. This analysis revealed that a number of ORC and MCM2-7 helicase complex binding sites overlap with binding sites of Su(Hw). In addition, Su(Hw) is also capable of altering chromatin accessibility by recruiting the histone acetyltransferase SAGA and the BRAHMA chromatin remodeling complex, thereby creating a platform of low nucleosome density levels favorable for the recruitment of ORC and replication firing (Lu and Tower, 1997; Vorobyeva et al., 2013a). In line with these findings suggesting a role for Su(Hw) in the regulation of DNA replication, we have also found a significant accumulation of H4K20me1 in su(Hw) mutant ovaries. H4K20me1 is highly regulated during the cell cycle and plays an important role in the licensing of origins of replication. PR-Set7 is the histone methyltransferase responsible for the monomethylation of H4K20 (H4K20me1). Mutations in PR-Set7 in mice models suggest that
this protein has a role in DNA replication (Abbas et al., 2010; Tardat et al., 2010). Specifically, mutations in set7 that prevent PR-Set7 degradation during the cell cycle are lethal due to uncontrolled re-replication of the genome (Centore et al., 2010), and loss of PR-Set7 enzymatic activity also causes defects in origin of replication firing (Jorgensen et al., 2007; Tardat et al., 2007). Further experimental evidence has shown that the role of PR-Set7 in origins of replication depends on its specific mono-methyltransferase activity on H4K20. H4K20me1 functions as a substrate for subsequent methylation by Suv4-20h1 and Suv4-20h2 methyltransferases, which further dimethylate and trimethylate H4K20me1, respectively. H4K20me2 and H4K20me3 directly bind ORC and promote firing of replication forks at replication origins (Beck et al., 2012).

Our finding showing increased levels of H4K20me1 in ovaries is reminiscent of mutations that prevent PR-Set7 degradation, increasing the frequency of H4K20me1 and leading to re-replication. The observation that Chk1, and not Chk2, is the kinase activated by ATR in response to replication stress, suggests that at least two checkpoint pathways (ATR/Chk1 and ATR/Chk2) can independently trigger microtubule disorganization and dorsoventral transformations. Convergence of the two pathways is most likely explained because of the functional role that DNA repair proteins, including ATR, Chk1, and Chk2, have in the MTOC, coordinating the cell cycle with DNA repair functions (Golan et al., 2010; Katsura et al., 2009; Shimada and Komatsu, 2009).

Altogether, our data suggest that DNA damage and genome instability may arise upon loss of Su(Hw) function in both germline and replicating somatic cells. Whether the DNA damage in su(Hw) mutants results from defects in genome organization that lead to replication stress, defects in the DNA repair pathway, or both is still unknown. However, our observation of
chromosomal aberrations in dividing neuroblasts in su(Hw) mutants suggest this phenomenon expands also to replicating somatic cells and is not limited to the germline or to polytene chromosomes. Our study opens a new avenue to further understand the role of architectural proteins in genome function and genome stability.

**Materials and methods**

**Fly genetics:** All fly stocks were cultured on cornmeal-agar food with yeast at 25°C. The fly stocks used in this study are: y^wct^6; su(Hw)^v/TM6B, a gift from Victor Corces (Emory University); mei-41^{D5}, and w^*; P{GAL4-nos.NGT40} (BDSC: 4442), which we refer to as nos-GAL4 through the text, were gifts from Laura Lee (Vanderbilt University). mei-w^68 and P{nanos-GAL4::VP16}, which we refer to as nanos-GAL4 throughout the text, were gifts from Bruce McKee (University of Tennessee). mnk^{6006}, was a gift from Bill Theurkauff (UMass Worcester). grp \textsuperscript{5170} lok^{30} and grp \textsuperscript{209} lok^{30} lines were a gift from Eric Weischaus (Princeton University). We also used Su(Hw)::eGFP[yw; P{su(Hw)::eGFP,w^*}] and w^{1118},PBac(RB)su(Hw)^{e04061}/TM6B (BDSC: 18224).

**Immunofluorescence staining of ovaries:** Three to five-day-old female ovaries were collected for ovary whole mount immunostaining as described previously (Page and Hawley, 2001). Briefly, tissues were fixed in 4% paraformaldehyde in 1:1 PBS and heptane (Sigma) and washed with PBST. Fixed tissues were incubated with blocking solution. Primary antibodies used for staining were as follows: FITC-conjugated mouse anti-tubulin (Sigma, 1:500), mouse anti-C(3)G (from Scott Hawley, Stowers Institute for medical research), rabbit anti-eGFP (Invitrogen, 1:100), rabbit anti-\(\gamma\)H2Av (Rockland, 1:5000), mouse anti-Orb, and anti-Grk (Developmental
Studies Hybridoma Bank, 1:200). The following secondary antibodies were used at 1:200 dilution: FITC-conjugated anti-rabbit IgG, TexasRed-conjugated anti-rabbit IgG, and FITC-conjugated anti-mouse IgG (The Jackson Laboratory). F-actin staining was performed using Texas Red-X phalloidin (Life Technologies).

For the BrdU incorporation assay, egg chambers were incubated for 30 minutes in 50µg/mL BrdU at RT. Tissues were then fixed with 4% PFA for 30 minutes, washed in PBST, and incubated in blocking solution. Egg chambers were then labeled with 2µg/mL BrdU primary antibody (Developmental Studies Hybridoma Bank, G3G4). Ovaries were stained with 4′, 6-diamidino-2-phenylindole (DAPI, 0.5 µ g/ml) and were mounted in Vectashield mounting medium (Vector Laboratories). Slides were analyzed under a Leica DM6000B wide-field fluorescence microscope equipped with a Hamamatsu ORCA-ER CCD camera and a HC PL FLUOTAR 20x/0.50NA objective. Image acquisition was performed using Simple PCI v6.6 (Hamamatsu Photonics). Image processing of raw Z-stacks for images in Figure 3.1 was performed using 3D Deconvolution Algorithm (AutoQuant) using an adaptive (blind) point spread function implemented into Deblur (v2.3.2) software (Leica). Final brightness/contrast adjustments after deconvolution were performed using ImageJ (v1.47b; National Institutes of Health) or Photoshop CS5 v 12.1 (Adobe). Wildtype and mutant samples were prepared and imaged under identical conditions of immunostaining, microscope, camera, and software settings. Egg chamber stage was determined based on size (Sullivan et al., 2000) measured in FIJI (Schindelin et al., 2012).

Integrated signal density was calculated by measuring the average fluorescence intensity and multiplying by the selected area. In the MTOC analysis, areas were selected as the insets in
Figure 3.1. In the BrdU incorporation analysis, areas were selected using DAPI staining of individual nurse cells from multiple egg chambers (Figure 3.2 C-D).

**X-ray sensitivity assessment:** Third instar larvae homozygous and heterozygous for *su(Hw)* *e04061* and *mei-41D5* mutations were irradiated with 7.5 Gy. Irradiation was performed in a Rad-Source RS-2000 Biological Irradiator. 3 hours after X ray exposure, larval neuroblasts were dissected and fixed. During the last hour of recovery, neuroblasts were dissected in SFX media (Thermo) and incubated in colchicine (10^{-5}M), for 1hr, followed by a 10min incubation in 0.5% sodium citrate, pH 6.0. Neuroblasts were then fixed for 1min in a 2:1:1 Acetic acid, 16% paraformaldehyde, H2O solution. After the incubations, neuroblasts were then softly squashed and stained with 4′, 6-diamidino-2-phenylindole (DAPI, 0.5 μg/ml) and were mounted in Vectashield mounting medium (Vector Laboratories). Slides were analyzed under a Leica DM6000B wide-field fluorescence microscope equipped with a Hamamatsu ORCA-ER CCD camera and a HC PL FLUOTAR 20x/0.50NA objective. Image acquisition was performed using Simple PCI v6.6 (Hamamatsu Photonics). Per each sample, 50 metaphases were collected.
Figure 3.1. The MTOC is impaired in su(Hw) mutants

Microtubules are labeled with alpha-tubulin antibody (green, aTub). (A-D) Images have been previously reported in an earlier thesis from our lab (Hsu, 2014). Germarium stage egg chambers with different genotypes. Arrowheads point to inset around stage 1 egg chambers. (A1-D1) Stage 1 egg chambers inset are magnified showing a dimmer and less bright MTOC (white arrowheads) in su(Hw)^vl e04061 mutant chambers. Scale bars are 10 μ m. (E) A bar graph quantifying the α -tubulin integrated signal density of stage 1 egg chambers from the indicated genotypes. P values were determined using a t-test. One asterisk indicates P<0.03.
Figure 2

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A

su(Hw)$^{Y/TM6B}$

aTub

DAPI

B

su(Hw)$^{Y/e04061}$

aTub

DAPI

C

nanosGal4>su(Hw)::eGFP; su(Hw)$^{Y/e04061}$

aTub

DAPI

D

mod(mdg4)$^{U1}$

aTub

DAPI

E

Average aTub Integrated Signal Density

* n.s.
Figure 3.2. Oogenesis failure in su(Hw)\textsuperscript{v/e04061} mutants is partially rescued by mei-41\textsuperscript{D5} (ATR) and grp\textsuperscript{z5170/209} (Chk1) mutations

Grk (green) was detected in egg chambers. (A-G) A su(Hw)\textsuperscript{v/TM6B} egg chamber with normal Grk signaling (A), su(Hw)\textsuperscript{v/e04061} egg chamber with Grk mislocalization (B), mei41\textsuperscript{D5}; su(Hw)\textsuperscript{v/e04061} egg chamber with rescued Grk localization (C), grp\textsuperscript{z5170/209}lok\textsuperscript{30};su(Hw)\textsuperscript{v/e04061} egg chamber with rescued Grk localization (D), lok\textsuperscript{30}mnk\textsuperscript{6006};su(Hw)\textsuperscript{v/e04061} egg chamber with Grk mislocalization (E), a mnk\textsuperscript{6006};su(Hw)\textsuperscript{v/e04061} egg chamber with Grk mislocalization (F), and a grp\textsuperscript{z5170/209};su(Hw)\textsuperscript{v/e04061} egg chamber with rescued Grk localization (G) are shown. (H) A mei-41\textsuperscript{D5}, su(Hw)\textsuperscript{v/e04061} double mutant egg chamber at stage 10. Scale bars are 20\textmu m. (I) Graph shows percentage of correct Grk localization in egg chambers from each genotype.
Figure 6

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 grp\^z5170/209; su(Hw)\^v/e04061

 lok\^30; su(Hw)\^v/e04061

 mei41\^D5; su(Hw)\^v/e04061

 A

 B

 C

 D

 E

 F

 G

 H

 I

 % correct Grk Position

 su(Hw) + - - - - - mei41 + + - + + grp + + + + - lok + + + - -
Figure 3.3. Levels of single stranded DNA are elevated in su(Hw) mutant ovaries

(A-B) Images of $su(Hw)^{y/TM6B}$ (A) and $su(Hw)^{w04061}$ (B) egg chambers labeled with anti-BrdU antibody (green) and DAPI (blue). Scale bars are 10 μm. (C) Graphs representing BrdU intensity per nurse cell. A graph representing BrdU intensity per nurse cell from stage 5-8 egg chambers (left). $p < 0.0001$. A graph representing BrdU intensity per nurse cell from stage 2-4 egg chambers (right). $p = 0.0009$. 
Figure 3.4. *su(Hw)* <sup>e04061</sup>, *mei-41<sup>D5</sup>* double mutants display an accumulation of DNA damage following irradiation

(A) Western blot analysis of *mei-41<sup>D5</sup>* single mutants and *mei-41<sup>D5</sup>; su(Hw)* <sup>e04061</sup> double mutants before and after 7.5Gy irradiation treatment. Irradiation treatment causes an accumulation of γH2Av in *mei-41<sup>D5</sup>* single mutants and *mei-41<sup>D5</sup>; su(Hw)* <sup>e04061</sup> double mutants. Non-phosphorylated H2Av was used as a loading control. (B) Examples of metaphasic chromosomes from *su(Hw)* <sup>e04061</sup> and *mei-41<sup>D5</sup>; su(Hw)* <sup>e04061</sup> neuroblasts (B1) example of an intact metaphase, (B2) example of an isochromatid deletion from *su(Hw)* <sup>e0406</sup>, *mei-41<sup>D5</sup>* double mutants, (B3) example of a chromatid deletion from *su(Hw)* <sup>e0406</sup>, *mei-41<sup>D5</sup>* double mutants. (C) Quantification if chromosome aberrations in *su(Hw)* <sup>e0406</sup> and *mei-41<sup>D5</sup>* mutants before irradiation and 3hrs post irradiation with 7.5Gy. Percent aberrations represents the percentage of metaphasic nuclei with one or more CABs out of the total population of metaphasic nuclei. 50 metaphases were considered per each sample. One asterisk indicates P=0.0445, two indicate P=0.0043, and four, P<0.0001, t test.
CONCLUSIONS

The collection of work presented here outlines roles for chromatin insulator proteins in cellular processes such as timing of DNA replication, response to osmotic stress, and maintenance of genome stability. A growing body of evidence suggests genome organization contributes to multiple aspects of cellular function and, similarly, processes such as transcription and replication have been found to play important roles in establishing and maintaining genome organization (Ke et al., 2017; Rowley et al., 2017b; Sima et al., 2019). Interestingly, features of genome organization are not consistent amongst eukaryotes, complicating the study of chromatin organization. Here, we have focused on specific insulator complexes and cellular processes to reach a better understanding of the relationship between nuclear organization and genome stability in both Drosophila melanogaster and humans.

Recent advances in next-generation sequencing techniques have provided a thorough understanding of how the human genome folds. CTCF, the only identified insulator protein in humans, interacts with cohesin to form chromatin loops that drive the formation of distal interactions and prevent mixing of differing chromatin environments (Fudenberg et al., 2016). Hi-C analysis of the Drosophila genome reveals the presence of TAD structures similar to those found in mammalian systems, however multiple mechanisms and protein complexes may lead to their formation (Hou et al., 2008; Rowley et al., 2017b; Tanay and Cavalli, 2013).

A number of different proteins and pathways have been shown to lead to TAD formation in Drosophila including the transcription factor Zelda during zygotic genome activation, polycomb group proteins in regions of heterochromatin, and active gene transcription (Eagen et al., 2017; Hug et al., 2017; Ogiyama et al., 2018; Rowley et al., 2017b). Drosophila insulator proteins are also thought to have a role in contact formation between distal enhancers and
promoters, forming loops over shorter distances compared to the capabilities of CTCF in the human genome (Fukaya et al., 2016; Lim et al., 2018). Given the variety of insulators in Drosophila, consideration of individual insulators in the context of specific roles will ultimately lead to a better understanding of their contributions to genome structure.

Chapters 1 and 3 have focused on Su(Hw), a well characterized Drosophila insulator protein, that regulates specific enhancer-promoter interactions and is required for proper oogenesis (Ghosh et al., 2001; Klug et al., 1968; Klug et al., 1970). Insulator proteins form complexes with partner proteins that contribute to insulator function. Su(Hw), CP190, and Mod(mdg4)-67.2 make up the Su(Hw) insulator complex, however additional proteins may associate with the complex to carry out specialized functions. Heterochromatin and Insulator Partner Protein 1 (HIPPI1) was recently identified as a component of the Su(Hw) and CTCF insulator complexes (Alekseyenko et al., 2014). Previous attempts to characterize the role of HIPPI1 in insulator function have been unsuccessful (Glenn and Geyer, 2018). Chapter 1 analyzes HIPPI1 function using a novel mutant construct and overexpression line.

Prior studies observe homology between HIPPI1 and the human protein CDYL due to the presence of a crotonase like domain (CLD) in both proteins (Figure 1.1A) (Glenn and Geyer, 2018; Liu et al., 2017a). Deletion of the CLD in HIPPI1 reveals similar deficiencies in DNA damage repair pathways, compared to the phenotypes of human CDYL mutants (Figure 1.1) (Abu-Zhayia et al., 2018). This suggests the two proteins function similarly to promote efficient DNA damage repair. Chapter 1 also investigates a role for HIPPI1 in DNA replication timing revealed by overexpressing HIPPI1. HIPPI1 overexpression leads to a delay in overall replication timing, specifically impeding the transition between early and late replicating domains (Figure 1.5-6). Additionally, HIPPI1 overexpression suppresses gypsy dependent phenotypes, suggesting
HIPPI serves as an antagonist to the role of Su(Hw) as an enhancer blocker (Figure 1.7). Taken together, these results suggest HIPPI regulates replication timing in a Su(Hw)-dependent manner and has a global effect on suppressing insulator activity.

Chapter 3 analyzes defects in egg chamber development in su(Hw) mutants. Through analysis of double mutants for su(Hw) and a number of DNA damage-related genes, we find that a mutation in checkpoint protein 1 (chk1) is able to partially rescue egg chamber development. Chk1 activates DNA damage checkpoints in response to DNA damage accumulated during replication (Figure 3.2) (Fogarty et al., 1997). The ability of mutations in chk1 to partially rescue su(Hw) mutant phenotypes suggests that DNA damage accumulated in su(Hw) mutants activates a DNA replication-specific checkpoint. Additionally, su(Hw) mutants display a heightened sensitivity to DNA damage induced by X-rays (Figure 3.4). The inability of su(Hw) mutants to repair DNA damage efficiently further suggests Su(Hw) plays a role in DNA damage pathways either by maintaining genome stability or by contributing to DNA repair processes.

Taken together, chapters 1 and 3 provide evidence that the Su(Hw) insulator complex is involved in aspects of DNA replication and genome stability. The relationship between Su(Hw) and HIPPI provides a possible role for insulator sites in the timing of DNA replication as the replication machinery transitions between early and late replicating domains. The ability of mutations in a replication-specific checkpoint protein to rescue su(Hw) mutant phenotypes further suggests genome organization created by insulator proteins is required for proper function of DNA replication programs. These newly identified roles for an insulator protein in DNA replication and repair highlight the importance of genome structure in mediating genome stability.
Chapter 2 analyzes the effects of osmotic stress on genome-wide organization. The osmotic stress response involves conformation changes in the cell and nucleus and a signaling cascade that promotes genome stability. This response is largely conserved and here we have described key similarities and differences in the ways Drosophila and mammalian insulators proteins handle genomic changes induced by osmotic stress. In agreement with previous studies, we observe the formation of insulator bodies in the nuclear periphery that appear as large aggregates of insulator proteins (Schoborg et al., 2013b). Interestingly, we discover that cohesin subunits also localize to insulator bodies during osmotic stress in Drosophila (Figure 2.1). This observation is particularly interesting in light of the loop extrusion model in mammalian systems. Although a similar model has not yet been shown in Drosophila, osmotic stress-induced changes in nuclear cohesin distribution suggest cohesin is a component of nuclear architecture in Drosophila that must be partially disassembled to adapt the cell to osmotic stress conditions. We also observe that cohesin subunits respond to osmotic stress in human cells. This further supports the model of loop extrusion and confirms prior observations that nuclear structure must be altered in response to mechanical forms of stress (Figure 2.3) (Amat et al., 2019; Schoborg et al., 2013a). In the future, it will be interesting to observe the consequences of insulator proteins remaining bound to chromatin during osmotic stress.

To gain insight into specific structural changes during osmotic stress, we employed Hi-C to generate a map of contacts across the human genome before, during, and after application of osmotic stress. This data reveals significant changes in genome structure during osmotic stress that are reversed upon return to control conditions (Figure 2.4). Boundaries mediated by CTCF are weakened and DNA contacts occur over a greater distance during osmotic stress treatment (Figure 2.4). We also observe heatmap signatures of transcription upregulation in loci
surrounding pro-inflammatory genes (Figure 2.5). These results demonstrate the extent to which genetic contacts are reshuffled to promote cell survival upon osmotic stress. The long-term consequences of osmotic stress on genome stability will be an important area of future investigation.

Taken together, the results presented here provide evidence that insulator proteins participate in cellular processes that extend beyond their canonical roles of transcription regulation, chromatin looping, and formation of boundaries. It is not surprising that genome structure is closely linked with processes such as DNA replication and the response to environmental stress. However, the complexity of factors contributing to genome structure presents a challenge to those investigating the contribution of specific components to nuclear function. Here, we analyzed the contributions of the Drosophila Su(Hw) insulator protein and find that mutations disrupting Su(Hw) function lead to genome instability and that a novel member of the Su(Hw) insulator complex functions in DNA replication timing. We also provide an analysis of the nuclear response to osmotic stress in Drosophila and humans and discover the involvement of chromatin architecture proteins in allowing the nucleus to adapt to osmotic stress conditions. These observations underline the importance of chromatin organization in genome stability and assign insulator proteins to previously unidentified roles in mechanisms essential for nuclear function and cell survival.


Lieberman-Aiden, E., N.L. van Berkum, L. Williams, M. Imakaev, T. Ragoczy, A. Telling, I. Amit, B.R. Lajoie, P.J. Sabo, M.O. Dorschner, R. Sandstrom, B. Bernstein, M.A. Bender,


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Table A1. Sequences of gRNAs used to generate HIPP1 mutants

The Crotonase 1 gRNA was successful in generating 17 and 2 base pair deletions upstream of the crotonase domain, resulting in a frame shift and generation of an early stop codon. The Null gRNAs and the Crotonase 2 gRNA were not successful in generating mutations.

<table>
<thead>
<tr>
<th>Name</th>
<th>gRNA Sequence (5’-3’)</th>
<th>PAM</th>
<th>Nucleotide position in HIPP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null 1</td>
<td>GGATGCGGCGTCCGAAGTTA</td>
<td>AGG</td>
<td>251-274</td>
</tr>
<tr>
<td>Null 2</td>
<td>CCCTGCTCAAGGGGACGGAT</td>
<td>ACC</td>
<td>433-456</td>
</tr>
<tr>
<td>Crotonase 1</td>
<td>GGCGAACAACGCTAGCGAAC</td>
<td>TGG</td>
<td>1,988-2,011</td>
</tr>
<tr>
<td>Crotonase 2</td>
<td>GGAACTGATACAGGGTTCCC</td>
<td>TGG</td>
<td>2,204-2,227</td>
</tr>
</tbody>
</table>
Figure A2.1. Example images collected from the Amnis Image Stream Flow Cytometer

(A) A375 cells incubated in normal media and labeled with DAPI dye and CTCF and γH2AX (gH2AX) antibodies. (B) A375 cells treated with 250mM NaCl media labeled with DAPI dye and CTCF and γH2AX (gH2AX) antibodies.
Figure A2.2. Osmotic stress is a reversible process that induces chromosome condensation, CTCF departure from DNA, and γH2AX foci accumulation

(A) Fluorescent images of CTCF (red) and γH2AX (green) in human A375 cells in media with increasing concentrations of added NaCl (250mM-600mM). DNA is stained with DAPI (blue, gray). (B) Fluorescent images of CTCF (red) and γH2AX (green) in human A375 cells placed in normal media for one hour following a one-hour incubation in the indicated concentration of NaCl. DNA is stained with DAPI (blue, gray).
Figure A3.1. Staging of egg chambers for Grk localization experiments

The length of stage 9 (S9) egg chambers was first measured in wildtype flies. Egg chambers used for localization of Grk in mutant females is not significantly different from S9 egg chambers in wildtype. Egg chamber length was measured in arbitrary units. Comparisons between genotypes were performed using two-tailed Mann-Whitney t-tests.
Figure A3.2. Staging of egg chambers for BrdU experiments

Graphs measuring the length of late stage (A) and early stage (B) egg chambers used for measuring BrdU intensity. The differences in length did not vary significantly in either category.
Emily Christine Stow grew up in Franklin, Tennessee. As a high school student at Brentwood Academy, she took a special interest in Biology classes and also excelled in courses on literature and history. In May 2014, she graduated from the University of Georgia with a Bachelor of Science in Cellular Biology. During her undergraduate studies, she investigated the role of membrane proteins in the propagation of viruses in the lab of Dr. Ralph A. Tripp in the Department of Infectious Diseases. Her training and experience in Dr. Tripp’s lab ignited her interest in research and encouraged her to pursue graduate studies. She was also inspired to begin a career in research by her aunt, Patricia Stow, who is a research scientist at St. Jude Children’s Research Hospital, and her sister, Dr. Sarah Stow, who is a research and development scientist at Agilent Technologies. She began graduate work at the University of Tennessee, Knoxville in August 2014 in the Department of Biochemistry and Cellular and Molecular Biology. Her research in Dr. Mariano Labrador’s laboratory focused on *Drosophila melanogaster* genetics and genome organization. During graduate school, she enjoyed exploring new research techniques, mentoring undergraduate students, and leading discussions for a molecular biology course. She was awarded the National Institutes of Health Program for Excellence and Equity in Research Fellowship for the first two years of graduate school.