Insulators: A “Safety Guard” for Genome Stability in *Drosophila melanogaster*

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ABSTRACT

Chromatin insulators are DNA-protein complexes that assist in the formation of chromatin loop structures by mediating long-range contacts between distant sites, which regulate proper organization of the chromatin fiber within the tridimensional space of the nucleus. It is considered that this function of insulators is required for the regulation of gene expression during development and in differentiated cells. This thesis focuses specifically in the Suppressor of Hairy wing [Su(Hw)] insulator of Drosophila and its associated proteins, and explores the possibility that chromatin insulators are not only significant for regulation of gene expression, but are also essential for DNA replication and for the maintenance of genome stability.

Su(Hw) is one of the best characterized insulators in Drosophila and requires the insulator proteins Su(Hw), Modifier of mdg4 [Mod(mdg4)] and Centrosomal protein 190 (CP190) to accomplish its function.

Traditionally, there are two well-known properties that are shared by chromatin insulators: they have the ability to block the communication between enhancers and promoters when located between them and they can function as heterochromatin barriers. However, previous studies have revealed that not all insulator sites in the genome share the enhancer-promoter blocking property, leaving open the possibility that, in addition to the establishment of chromatin

loops and gene transcription regulation, insulators might play yet unexplored roles in the genome.

Chapter one introduces a new role for the phosphorylation of H2Av (γH2Av), traditionally a marker for DNA damage, in insulator function. This work shows that γH2Av is required for the Su(Hw) enhancer-blocking function, and suggests that the ATM and ATR kinases modulate insulator function through phosphorylation of H2Av at insulator sites. Chapter two explores the possibility that Su(Hw) and HIPP1 (HP1-Insulator Protein Partner 1) may play a role in the regulation of DNA replication in the genome. Findings in this work suggest that insulators regulate activation of origins of replication by cooperation with other proteins such as HP1 and H4K20me1. Altogether these findings provide new insights into insulator function and suggest that Su(Hw) and likely other insulators are critical for DNA replication and for the maintenance of genome stability.

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INTRODUCTION

Chromatin organization and gene transcription regulation in eukaryotes

Genome complexity and genome size significantly increase as organisms evolve from prokaryotes to eukaryotes and from single celled eukaryotes to metazoans. This evolutionary trend is driven by the synergy between two innovative aspects of the eukaryotic genome: chromatin on the one hand and the seemingly unlimited ability of the genome to grow in size on the other.

Since increase in genome size is not paralleled by an expansion in the number of genes, higher eukaryotes have produced genomes in which the DNA coding regions are reduced to a proportionally small fraction embedded within the remainder sequences, which correspond to a mix of repetitive, intron and intergenic DNA. As a result, we find that even relatively small genomes such as the Drosophila genome can be as long as 10 cm, and large genomes such as the human genome can measure 2 meters or longer, when aligned in a single thread of DNA. In contrast, the size of the cell nucleus -where the DNA has to be packed- has remained relatively small and is only 5 um and 10 um, in flies and humans, respectively.

Organizing eukaryotic chromosomes into chromatin is the solution to packing these extremely large genomes into very small nuclear volumes. Similarly, the

development of sophisticated regulatory gene networks in eukaryotes, capable of sustaining complex developmental processes, is only possible because of the extra non-coding DNA available, which can be used to produce additional regulatory sequences that can multiply the possibilities for spatio-temporal regulation of gene expression during development and in differentiated tissues. Since having regulatory sequences in close proximity to the promoter only allows for a limited number of regulatory sites, the novel regulatory sequences are often found distantly from the promoter, sometimes thousands of bp away. Because distant regulatory sequences require direct contact with promoters in order to activate transcription, higher eukaryote genomes are forced to adopt architectures and a three-dimensional organization that promotes the formation of DNA loops and facilitate contacts between distant sites in the genome. This nuclear organization is only possible in eukaryotes where chromosomes form a supramolecular structure known as chromatin, which results from interactions of DNA with proteins and is build around the nucleosome as a basic repeating structural and functional unit.

Nucleosomes strongly contribute to the ability of eukaryote genomes to be packaged into small nuclear spaces. Nucleosomes consists of 145-147 bp of DNA wrapped around a protein complex known as the histone octamer, which includes 2 copies of the core histones H2A, H2B, H3 and H4 (Kornberg and Lorch, 1999). The highly basic amino (N)-terminal tails of histones are rich with lysine residues and protrude from their own nucleosome core, facilitating

interactions with adjacent nucleosomes. Arrangement of DNA into nucleosomes plays a dynamic role in altering DNA accessibility by experiencing various forms of post-transcriptional modifications (PTMs), such as phosphorylation, acetylation and methylation. These PTMs function as epigenetic “histone codes” and govern a number of important biological processes either by changing the interactions between histone core and DNA within nucleosomes, or by affecting the combination of nucleosomes and its binding proteins to control different cellular events, including heterochromatin formation, transcription silencing or activation, DNA repair, replication and recombination (Bannister and Kouzarides, 2011; Yao et al., 2012). In a chromatin fiber, short linker DNA connect adjacent nucleosomes, which form nucleosomal arrays that in turn form the 10nm chromatin fiber, organizing the primary structure of the chromatin. Specific interactions between individual nucleosomes drive the folding of nucleosomal arrays into the 30nm fiber, and the subsequent fiber-fiber interactions further contribute to the large-scale configurations that build an entire chromosome, including non-compacted euchromatin, and condensed heterochromatin (Luger et al., 2012; Tremethick, 2007). However, since so far the 30nm “fiber-like” structure can be visualized (or can form) only after the chromatin fragments are isolated or released from the nuclei in vitro, the link between how histone modifications result in the assembly of nucleosomes that produce particular chromatin structures or organizations is not clear (Tremethick, 2007).

Based on the composition of the chromosome, one way to regulate gene expression is via histone modifications on the nucleosome. For example, CpG islands are regions of high CpG density found at promoters of most human genes, and cytosine methylation at CpG islands ensures the long term silence of gene expression, which is commonly found in the imprinted genes on the X chromosome in eukaryotes (Deaton and Bird, 2011). DNA methylation recruits nucleosome-remodeling complexes that modify chromatin organization in these regions such that DNA promoters are no longer accessible to transcription factors.

Position Effect Variegation (PEV) is a phenomenon that reflects the role of chromatin in gene transcription regulation in eukaryotes. The most classical example was firstly described by Muller in 1930 and was found after a translocation of the *Drosophila white* gene into a heterochromatin region. Generally, the *white* gene of *Drosophila melanogaster* is located at the euchromatin on the X chromosome and controls the red pigment expression in the fly's eye. A chromosomal inversion induced by X-rays juxtaposed the *white* gene close to the boundary between heterochromatin and euchromatin. Although the DNA sequence of the *white* gene remained unchanged, the juxtaposition of the gene close to heterochromatin resulted in a variegated pattern in the color of the eye, indicating that some cells where able to express the White protein whereas in other cells the *white* gene was silenced. This finding suggested the neighboring heterochromatin could partially silence the expression of the *white*

gene. This phenomenon describes the ability of certain type of histone PTMs normally found in heterochromatin to spread through the chromatin fiber. Though many conserved epigenetic factors, including the histone H3 lysine 9 methyltransferase Suppressor of Variegation 3-9 (SuVar 3-9), Heterochromatin protein 1 (HP1) and histone H3 methylated at lysine 9 (H3K9me2/3) have been identified as factors that help in the creation of a core memory system, specific details of the mechanism of heterochromatin formation, remodeling and maintenance are still not clear (Elgin and Reuter, 2013).

Another category of factors important for gene expression regulation is that of regulatory DNA elements. Enhancers, on the one hand, are regulatory DNA elements that bind to transcription factors to activate the promoter of target genes. Enhancers can activate gene transcription at promoters with independence of orientation and over great distance separations, both in *cis* and in *trans*. On the other hand, silencers are regulatory elements that have the opposite effect and can suppress gene expression by a variety of mechanisms that generally include recruiting of nucleosome remodelers to chromatin and the formation of chromatin that is refractory to transcription activation. Finally, there is a third type of regulatory elements that help confining the ability of chromatin to activate or repress transcription specific chromatin boundaries, which are traditionally defined as chromatin insulators (Kolovos et al., 2012).

Chromatin insulators and their associated proteins

Chromatin insulators are traditionally defined as a group of functional genomic elements bound by specific proteins, which form and delimit functional chromatin domains participating in transcription regulation or gene expression (Kyrchanova and Georgiev, 2014; Labrador and Corces, 2002a; Schoborg and Labrador, 2014). Generally, insulators display two properties when bound by insulator proteins: they can either interfere with enhancer-promoter interactions when present between these elements, or they serve as boundaries to prevent heterochromatin spreading, which buffer transgenes from chromosomal position effects (Gerasimova and Corces, 2001). The former property contributes to the primary role that insulators play in repression and activation of transcription, and the latter property suggests insulators regulate the global nuclear organization to minimize the effects of PEV. Both properties are essential for insulator function in the control of proper gene expression, temporally and spatially, during development (Gaszner and Felsenfeld, 2006). Although great details of the mechanism of insulator function remain obscure, genetic and molecular evidence support a model in which insulator function by stabilizing interactions between long-range insulator sites in the genome. These interactions promote the establishment of chromatin loops, which further forms higher order organizations known as Topologically Adjacent Domains (TADs) via interactions between insulator proteins and perhaps other proteins (Van Bortle et al., 2014).

Though insulators are diverse in different organisms, they have been found in almost all eukaryotes, including yeast, plants, sea urchins and vertebrates, with a conserved function that requires the recruitment of their associated insulator binding proteins. So far, six Drosophila insulators have been identified and defined by their binding proteins, including Suppressor of Hairy-wing [Su(Hw)], dCCCTC-binding factor (dCTCF), GAGA factor, Zeste-white 5 protein (Zw5), Boundary Element Associated Factor of 32kDa (BEAF-32) and transcription factor IIIC (TFIIIC) (Gerasimova and Corces, 2001; Gurudatta and Corces, 2009; Heger and Wiehe, 2014).

Reviewing the timeline of insulator history, the first described chromatin insulator was the scs and scs' (specialized chromatin structure) in *Drosophila* in 1985. Both sites flank a 14kb region containing five genes including two genes encoding the heat shock protein 70 (Hsp70). Two proteins are required for scs and scs' insulator function: the Zeste-white 5 (Zw5) protein binds scs, while BEAF-32 proteins bind scs' (Kuhn et al., 2004; Udvardy et al., 1985). The interaction between Zw5 and BEAF proteins provide the evidence for their involvement in the formation of a chromatin loop between the scs and scs' insulators (Kyrchanova et al., 2013). For Zw5, an insulator protein preferring binding at the promoter region, previous limited study found that strong loss of function of mutations in *zw5* arrest development at the first instar larval stage, though Zw5 is dispensable during embryogenesis. Weak *zw5* alleles mutant males are sterile and display a variety of eye, bristle and wing phenotypes,

indicating Zw5 might be involved in cell proliferation and differentiation (Gaszner et al., 1999).

BEAF-32 is an interesting protein, since BEAF-32 binds hundreds of sites independent of scs' binding sites, several of which have been shown to have insulator activity, suggesting that BEAF-32-dependent insulators are common in *Drosophila* rather than being a unique property of scs' (Jiang et al., 2009). The BEAF-32 gene encodes two different isoforms, BEAF-32A and BEAF-32B, which differ in 80 amino acids at the N-termini. Both isoforms contain an atypical C2H2 zinc finger DNA binding domain, termed BED finger domain at the N-termini, and a C-terminal domain that is required for trimerization. The trimer structure of BEAF-32 contributes to its high binding affinity to clusters of CGATA motifs. Genome-wide CHIP analysis shows that BEAF-32 prefers to bind to such motifs in the promoter regions of active genes and is required for stimulating their transcription, suggesting that BEAF-32 plays a role in maintaining most associated promoter regions in an environment that facilitates high transcription (Jiang et al., 2009; Kyrchanova and Georgiev, 2014).

Though insulator elements have also been identified in vertebrates, the proteins responsible for their activity has been elusive. In 1999, the insulator protein CTCF was identified to bind at the insulator previously described in the β-globin locus in humans. CTCF is the first and only characterized vertebrate insulator binding protein, and is highly conserved in eukaryotes including Drosophila

(dCTCF)(Bell et al., 1999; Schoborg and Labrador, 2010). Though conserved in most bilaterian phyla, CTCF is absent in yeast, *Caenorhabditis elegans* and plants (Heger et al., 2012). It contains a highly conserved DNA-binding domain with 11 zinc fingers and coordinates chromatin organization via 66800 binding sites at a genome-wide scale. CTCF was originally identified as a transcription factor involved in both transcriptional activation and repression, and also implicated in X chromosome in activation. The insulator function of CTCF has also been implicated in the imprinting at the Igf2/H19 locus (Cuddapah et al., 2009). In Drosophila, the dCTCF orthologue binds insulator sites in the highly conserved homeotic gene cluster (Hox cluster), which code for transcription factors that determine body patterning along the anteroposterior axis of the organism. Bithorax complex (BX-C) is one of the Hox cluster, which consists of three homeotic genes Ultrabithorax (Ubx), abdominal A (abd-A) and Abdominal B (Abd-B), and specifies the third thoracic segment and all eight abdominal segments of the fly. Fab-7 and Fab-8 are insulators identified within the Hox cluster and regulate Hox gene expression in a dCTCF dependent manner (Herold et al., 2012). Though the mechanism of dCTCF-dependent insulator function remains enigmatic, recent studies indicate that dCTCF also has a broad function of in cell cycle regulation and cell proliferation. A recently published work analyzed *Drosophila* ChIP data to examine the changes in dCTCF-binding sites during the cell cycle, and they found the conservation and intensity of dCTCF binding are cell cycle regulated, further suggesting dCTCF might contribute to the

establishment of the three-dimensional architecture of the genome by maintaining local chromatin compartments (Shen et al., 2015).

GAGA factor is another important insulator in eukaryotes, which was originally found as an transcriptional activator to regulate of Ubx, one of the Hox cluster genes in Drosophila (Biggin and Tjian, 1988). It is encoded by the gene *Trithorax-like* (*Trl*), and prefers to associate with the promoters of many genes. Generally, the protein is composed of an N-terminal BTB/POZ domain that mediates the interaction with other proteins, allowing GAGA factor to homodimerize or heterodimerize with other factors; a C2H2 DNA binding zinc finger domain, and a polyQ domain (Melnikova et al., 2004). In addition to enhancer blocking function at the gene promoter region, GAGA factor is also suggested to play a role in establishing paused polymerase, regulate the level of promoter paused polII, and maintaining nucleosome free regions to regulate gene transcription (Fuda et al., 2015).

Gypsy **insulators and alternative endogenous insulators in** ***Drosophila***

In *Drosophila*, the *gypsy* insulator is one of the best characterized chromatin insulators. The gypsy insulator consists on a 340bp DNA fragment located at the 5’ untranslated region of the *Gypsy* retrotransposon. Retrotransposons are eukaryotic genome repetitive elements that can replicate through cycles of transcription, reverse transcription and integration into the genome. Generally, the mechanism of retrotranspositon is similar to the infections cycle of

retroviruses, with the difference that the retrotransposons do not form infectious particles, and in general lack the ability to infect new cells (Havecker et al., 2004). Gypsy is a member of the family of retrotransposons with two long terminal repeats (LTRs) which are the most abundant constituents of eukaryotic genomes. The LTRs are the sequence repeats that flank the internal coding region of the retrotransposon's genome. This region consists of three retrotransposon genes: *gag*, which codes for the GAG proteins, responsible for the structural organization of the viral capside that contains the retrotransposon RNA; Pol, is a polyprotein encoding gene containing the Reverse transcriptase, Integrase, RNase-H and Protease enzymatic functions; Finally, the *envelope* (*env*) gene encodes for the proteins that form the envelope of retroviral particles, which allow retroviruses an extracellular phase in the cycle and infect other cells. Most retrotransposons lack an *env* gene (Marlor et al., 1986). In *Drosophila*, *Gypsy* is stable and does not transpose with detectable frequencies in most strains. Gypsy transposition is regulated by the *flamenco (flam)* gene, which has a strict maternal effect on gypsy mobilization, since transposition occurs at high frequency only in the germ line of the progeny of females homozygous for *flam* mutations (Prud'homme et al., 1995; Touret et al., 2014). *flam* is transcribed into a long non-coding RNA containing copies of clusters of multiple retrotransposons including *Gypsy*, and functions in the piwiRNA pathway, which activates a dedicated RNA-interference mechanism that silences retrotransposons (Mevel-Ninio et al., 2007; Van Bortle et al., 2014).

A variety of mutations have been identified that are caused by insertion of *gypsy* into *Drosophila* genes, such as *yellow* (y^2) and *cut* (ct^6) (Harrison et al., 1989; Mongelard et al., 2002). Genetics and molecular studies identified Su(Hw) binding sites in *Gypsy*, and found that phenotypes resulting from *Gypsy* insertions can be completely rescued by mutations in the *su(Hw)* gene, indicating that binding of Su(Hw) protein is required for the *gypsy* mutagenic effect in flies (Geyer and Corces, 1992; Holdridge and Dorsett, 1991; Modolell et al., 1983; Smith and Corces, 1992). Su(Hw) is a DNA binding protein containing twelve zinc finger domains that can recognize the twelve repeated copies of a 12bp motif in the 5' untranslated region of the *gypsy* retrotransposon, and are required for the protein's function; a leucine zipper motif at the C terminal region that is also involved in the enhancer blocking effect; and a dispensable acidic domain located at the carboxy-terminal end, since it is absent from other species except for *Drosophila melanogaster* and could be compensated by a second acidic domain at the amino-terminal region. Null mutations of su(Hw) cause loss of female fertility and suppress the phenotype of *gypsy*-induced mutations (Gdula and Corces, 1997; Harrison et al., 1993).

In addition to Su(Hw), Mod(mdg4), which interacts with Su(Hw), is also required for the gypsy insulator function in *Drosophila.* This protein has numerous isoforms that are produced by alternative splicing, including a 2.2kb transcript [Mod(mdg4)67.2] which is suggested to be the only isoform mediating the enhancer blocking function of the gypsy insulator activity. Sequence analysis

reveals that all Mod(mdg4) isoforms contain a Bric-a-brac, Tramtrack and Broad-complex and a zinc finger (BTB/POZ) domain at the N-terminal end that is evolutionarily conserved protein-protein interaction motif. This domain of is required for the interaction of Mod(Mdg4)67.2 with Su(Hw). The C-terminus are diverse when comparing different Mod(mdg4) isoforms and possesses an acidic domain that contains 50% Asp and Glu residues that in Mod(Mdg4)67.2 mediates the interaction with the C-terminus of Su(Hw) (Ghosh et al., 2001). Loss of all mod(mdg4) locus is recessive lethal, while mutations specific for Mod(mdg4)67.2 isoform have no known phenotype, except for the partial loss of the gypsy insulator function (Gerasimova et al., 1995; Golovnin et al., 2007; Mongelard et al., 2002). Since Mod(mdg4)67.2 does not contain a DNA binding domain, binding of Mod(mdg4)67.2 to insulator sites depend on other DNA binding proteins. Though *Drosophila* polytene chromosomes show an almost perfect colocalization between Mod(mdg4)67.2 and Su(Hw), the genome Chip-chip shows other insulator binding sites for Mod(mdg4)67.2, such as dCTCF and BEAF-32 in embryos, suggesting the common proteins are involved in different insulator binding sites.

An additional component of the *gypsy* insulator is CP190, which was identified in a genetic screen for dominant enhancers of *mod(mdg4)67.2* mutations. CP190 was originally identified and characterized as a result of its association with centrosomes and microtubules during mitosis (Whitfield et al., 1995). The protein contains an N-termial BTB/POZ domain and an aspartic acid D-domain, both of

which are required for association with insulator binding proteins. In addition, there is a CENT domain necessary for the function associated with centrosomes, three C2H2 zinc finger motifs and a C terminal E-rich domain that is required for disassociation from the target sites during heat-shock (Ahanger et al., 2013). Loss of CP190 rescues the phenotype of *gypsy* induced mutations, suggesting CP190 is required for the insulator enhancer blocking activity (Pai et al., 2004). Moreover, CP190 is also found to bind to other insulators as cofactors, such as dCTCF and BEAF-32, and recruit other insulator proteins for the completed insulator function (Maksimenko et al., 2015). However, the function of CP190 seems more complicated than other insulator proteins. Early studies suggest that CP190 is involved in the construction of the nuclear skeleton during cell cycle, and complete loss of CP190 causes pharate lethality, suggesting CP190 is required for fly development. However, depleting CP190 in culture cells does not significantly interfere with cell division or even centrosomes and microtubule organization (Butcher et al., 2004; Oegema et al., 1997). Furthermore, the formation of insulator bodies during osmotic stress is also dependent of CP190 is (Schoborg et al., 2013b). Finally, these and other data suggest that in addition to function as part of various chromatin insulators CP190 might function as a transcription regulator (Ahanger et al., 2013).

Insulator body formation during osmostress and apoptosis

The pattern distribution of insulator proteins in diploid cells has been studied for many years. In some cases, insulator proteins show a uniform and diffused

distribution in the nuclei. However, is also common to see insulator proteins aggregated into a small number of large discrete foci located at the nuclear periphery forming nuclear speckles that were named “insulator bodies” (Gerasimova and Corces, 1998; West et al., 2002). Insulator bodies were considered to contain multiple individual insulator sequences from distant sites in the chromosome, which were brought together by interactions between insulator proteins. These evidence has been considered to support the rosette-like model in which chromatin loops mediated by insulator proteins converge together in the center of a flower-like structure (Gurudatta and Corces, 2009; Labrador and Corces, 2002a). However, recent work has shown that insulator bodies form in response to osmotic stress and during cell death, rather than during normal conditions. Insulator bodies induced during osmotic stress contain a defined structural arrangement of insulator proteins in which the DNA component of the genomic insulators is likely missing. The question of whether such insulator bodies are relevant for genuine genome insulators or their function remains uncertain (Golovnin et al., 2008; Schoborg and Labrador, 2014; Schoborg et al., 2013b). So far all known insulator proteins are components of the insulator bodies, and only Mod(mdg4)67.2 appears to be directly required for the association of Su(Hw) to insulator bodies *in vivo*, though CP190 seems to be necessary for the formation of insulator bodies in S2 cells (Schoborg et al., 2013b).

Insulators are involved in cell proliferation and DNA replication

As mentioned before, the primary role of chromatin insulators is to contribute to efficient transcription regulation (Van Bortle and Corces, 2013). However, recent findings revealed evidence supporting additional roles for chromatin insulators in DNA replication and cell proliferation. During mitosis, most transcription factors, RNA polymerases and other regulatory elements are absent from the highly condensed mitotic chromosomes. However CTCF, has been described as an insulator protein that remains bound to mitotic chromosomes at the Igf2/H19 locus, though the enhancer-promoter loop that is normally found at the same locus is lost (Burke et al., 2005). Moreover, cell type specific CTCF binding sites are enriched within the early -and middle- DNA replication zones and at the corresponding boundaries in human genome, further suggesting insulators are involved in DNA replication (Chen et al., 2012; Van Bortle and Corces, 2013). In addition to CTCF, Additional evidence shows that replication of the chicken FR/β-globin region initiates early in S phase at three G+C-rich sites localized close to the 5' HS4 β-globin insulator, indicating that origin of replication activity might be required for insulator function or vice versa (Prioleau et al., 2003). However, only recently new evidence in Drosophila has emerged showing a similar effect of insulators in *Drosophila*. CHIP data analysis of a 100 kb region of the genome suggests that the Drosophila DNA Replication-related Element binding Factor (DREF), a homodimeric transcription factor that binds to DNA replication related elements that regulate many genes involved in DNA replication and cell proliferation, has binding sites similar to insulator protein binding sites, including

Su(Hw), BEAF-32, and dCTCF. In addition, the CHIP-seq data using DREF as an anchor shows the binding density of different insulators are directly corrected to DREF, though DREF and BEAF-32 shows antagonism interaction (Gurudatta et al., 2013). Additional publications further show insulator protein Su(Hw) recruits proteins involved in replication, such as GCN5, ORC3 and Brahma, and constitutes part of origin recognition complex-binding sites in the Drosophila genome, although no direct evidence or mechanism linking Su(Hw) with the Cell cycle has been documented so far(Vorobyeva et al., 2013).

CHAPTER I

ATR and ATM Modulate Chromatin Insulator Activity Through Phosphorylation of H2Av at Insulator Sites

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Author contributions:

(1). Ran An: Conceiving and designing experiments; performing experiments; collecting and analyzing data; making figures and writing the manuscript.

(2). Todd Schoborg: Designing and making the HIPP1::mcherry and H2Av::mcherry transgenic flies.

(3)Mariano Labrador: Conceiving and designing experiments; performing experiments; analyzing data and revising the manuscript.

Abstract

Chromatin insulators mediate the formation of higher order chromatin structures by stabilizing long-range interactions between distant genomic sites along the chromatin fiber. The properties that traditionally define chromatin insulators is to function as chromatin boundaries by preventing the spreading of heterochromatin and their ability to block enhancer-promoter interactions when located between enhancers and promoters. Insulators contribute to gene transcription regulation by promoting contacts between gene regulatory regions and promoters, and help maintaining the stability of the tridimensional organization of the genome. However, the mechanism of insulator function, or the specific role that insulators play in genome architecture and how this architecture is maintained during cell division and differentiation, remains unclear. Here, we find that the Suppressor of

Hairy wing (Su[Hw]) insulator protein in *Drosophila* co-localizes with the phosphorylated form of the histone variant H2Av (γH2Av) in chromosomes. γH2Av is a marker for double strand breaks (DSB), and functions in the DNA damage repair pathways regulated by ATR and ATM, two kinases that phosphorylate H2Av. We also found that loss of the enhancer-blocking function of the *Gypsy* insulator correlates with loss of γH2Av, which partially restores the phenotype of *Gypsy* induced mutations to wild type. These results suggest that phosphorylation of H2Av at insulator sites is required for insulator function. Supporting this notion, we found that mutations in ATM and ATR affect the expression of insulator proteins and also produce different degrees of phenotypic suppression on the y^2 and ct^6 allele. Our findings suggest that ATR and ATM are required for the insulator enhancer blocking function by mediating phosphorylation of γH2Av, which is significant for maintenance of the genome stability.

Introduction

Chromatin insulators are involved in the formation of higher order chromatin structures by mediating long-range interactions between distant genomic sites along chromatin fibers (Gerasimova and Corces, 2001; Labrador and Corces, 2002a; Yang and Corces, 2012). These interactions lead to the formation of chromatin loops along chromatin fibers, which are critical for the maintenance of

genome architecture within the nucleus of the cell. Generally, insulators play two distinct roles in the genome: they can function either as enhancer-promoter blocking elements when located between enhancers and promoters or as barriers against the spreading of proteins that promote the formation of condensed chromatin onto active chromatin (Valenzuela and Kamakaka, 2006). Both properties contribute to the regulation of gene transcription, as well as to the stability of the architectural organization of the genome within the nucleus(Yang and Corces, 2011). Chromatin insulators are defined as sequence of genomic DNA bound by DNA binding proteins, plus additional proteins that are specifically associated to the insulator through protein-protein interactions. Chromatin insulators have been found in various organisms from yeast to human (Schoborg and Labrador, 2014). In *Drosophila melanogaster*, the *gypsy* insulator is one of the best-characterized insulators. The *gypsy* insulator is found in a 340-430bp fragment located in the 5' untranslated region of the *Gypsy* retrotransposon (Cai and Levine, 1995; Geyer and Corces, 1992). The Su(Hw) protein specifically binds the Gypsy insulator binding sites, and in combination with Modifier of Mdg4[Mod(mdg4)67.2] and Centrosomal protein 190 (CP190), forms an insulator protein complex that is essential for insulator activity. In addition to Su(Hw), a number of insulator DNA binding proteins have been identified, which have insulator function properties. These proteins are the Drosophila CCCTC- binding factor (dCTCF), GAGA factor (GAF), the boundary element associated factor (BEAF-32) and Zeste white 5 (ZW5), all of which have shown to be involved in the organization of intervening chromatin loop structures possibly via both

homotypic and heterotypic interactions (Bushey et al., 2008; Schoborg and Labrador, 2010; Spana and Corces, 1990).

The specific role that long-range interactions, and formation of chromatin loops play in nuclear processes, other than regulation of gene expression, remains relatively unexplored. Recent findings suggest that insulator function may be required also for DNA replication, and regulation of cell proliferation In *Drosophila* (Gurudatta et al., 2013; Vorobyeva et al., 2013). In addition, indirect evidence suggests that the Su(Hw) insulator may also be involved in the DNA damage repair. For example, previous work shows that Double Strand Breaks (DSBs) induced by P-element excisions on the X chromosome are more efficiently repaired in the germ line of *su(Hw)* mutants, suggesting that Su(Hw) has an inhibitory effect in the repair of DSBs (Lankenau et al., 2000). Furthermore, recent work in our laboratory has shown that null mutations in su(Hw) result in an increase in DNA damage during oogenesis in *Drosophila*. These findings show that the levels of the γH2Av significantly increase in ovaries from *su(Hw)* mutant females. H2Av is the Drosophila equivalent to H2Ax, which is universal marker for DNA damage in the genome, from yeasts to Humans. In addition, these results how that DNA damage is not meiotic in origin and is not induced by transposable element activation, which suggests that likely originates from malfunction of the DNA replication process and that Su(Hw) is involved in regulation of DNA replication to maintain the genome stability in the *Drosophila* ovary (data not published) (Hsu et al., 2015). Albeit these advances in our

understanding of chromatin insulators, differences in function and/or organization between somatic or germline cells, the role of insulators in DNA repair and DNA replication, or the mechanism of insulator function in regulation of gene transcription remains unclear.

In this work, we explored the link between insulator proteins and γH2Av in somatic cells and we find a high level of co-localization in the distribution of insulator sites and γH2Av throughout the genome and in insulator bodies after osmotic stress. This co-localization depends on the protein components of each *Drosophila* insulator. Furthermore, we used the insertion of the *Gypsy* retrotransposon into *Drosophila* genes, such as *yellow (y)* and *cut (ct),* which result in a clear phenotype that can be used to study the insulator function. We find that the presence of γH2Av depends on the insulator proteins, since mutation in any of the genes that encode insulator proteins found at the *Gypsy* insulator results in the loss of γH2Av at the *Gypsy* insulator sites. Also, removing γH2Av from insulator sites by either overexpression of HP1 and insulator partner protein-1 (HIPP1), or treatment with ATR and ATM inhibitors such as caffeine, leads to a failure of the enhancer blocking property of the *Gypsy* insulator, suggesting that phosphorylation of H2Av is required for the insulator function. These results provide a novel framework for elucidating the mechanism of chromatin insulator in enhancer-promoter blocking property and to investigate the

possibility that insulators might be involved in the coordination of transcription and replication to maintain the genome-wide stability.

Materials and Methods

Drosophila Stocks

All fly stocks and crosses were maintained using standard cornmeal-agar media and yeast in a 25°C incubator. The fly stocks used in this paper included: microinjection to generate transgenic lines yw; P{HIPP1::mcherry, w+} and yw; P{H2Av::mcherry, w+} were performed by GenetiVision; the lines obtained from the Drosophila Bloomington Stock Center at Indiana University: w*; P{GAL4-vg.M}2; TM2/TM6B, Tb^1(Stock #6819), *w1118; PBac(RB)su(Hw)^{e04061}/TM6B, Tb^1*(Stock #18224), *y1 sc* v1; P{TRiP. HMS00845}attP2* (Stock #33903 CP190 RNAi) and *mei41^{D5} f^1; sv$^{spa\text{-}pol}$* (Stock #4236); the lines from V. Corces (Emory Univerisity): *y^{2}wct^6; cp190$^{H31\text{-}2}$/TM6B, Tb^1*, *cp190^{p11}/TM6B, Tb^1*, *su(Hw)^v/TM6B, Tb^1*, *mod(mdg4)^{u1}*; the lines from K. McKim (Rutgers University): M29: *w*; P{neoFRT}82B tefu$^{atm\text{-}3}$ e^1/TM6B, Tb^1*(Stock #8625); the lines from B. McKee (University of Tennessee): *HisAv$^{J3(810)}$/TM3;* the lines generated by our lab: *mei41^{D5}, f^1/FM7a; su(Hw)^{e041061}/TM6B, Tb^1* (Hsu et al., 2015), *y^{2}wct^6; PBac(RB)su(Hw)^{e04061}/TM6B, Tb^1*, *y^{2}wct^6; P{ry[+t7.2]=neoFRT}82B tefu$^{atm\text{-}3}$/TM6B, Tb^1, su(Hw)^{e04061}, su(Hw)^{e04061}HisAv$^{J3(810)}$/TM6B, Tb^1, mei41^{D5}; P{neoFRT}82B tefu$^{atm\text{-}3}$ e^1//TM6B, Tb^1*, *y^{2}wct^6; cp190^{p11}/TM6B, Tb^1, y^{2}wct^6; mod(mdg4)^{u1}/TM6B, Tb^1*, , *y^{2}wct^6;* P{GAL4-vg.M}2/*Cyo*;

PBac(RB)su(Hw)^{e04061}/TM6B, Tb^{1}; y^{2}wct^{6}; P{TRiP. HMS00845}attP2/TM6B, Tb^{1} (CP190 RNAi).

Polytene Chromosome 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 cover slide. Salivary glands were squashed on a microscope slide until the polytene chromosomes are spread out. Slides were dipped in liquid nitrogen to remove cover slides. 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 at 1:200 dilution, and incubated overnight at 4°C in a humidified chamber. Primary antibody were removed by incubating in washing buffer (PBS+0.1%NP40) for 10 minutes at RT. Secondary antibodies were then diluted in blocking solution (1:200) and incubated for 1 hour at RT, and washed as described before. DAPI (4', 6-diamidino-2-phenylindole 0.5μg/ml) was used to counter stain the DNA for 30 seconds and was rinsed with PBS. Slides were mounted with Vectashield mounting medium (Vector Laboratories) and sealed with nail polish.

Slides were analyzed using a wide-field epifluorescence microscope (DM6000 B; Leica) equipped with a charge-coupled device camera (ORCA-ER; Hamamatsu Photonic) and a HCX Plan Apochromat (Leica) 100×/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.

Immunostaining Intensity Quantification with Fiji

Open the image with Fiji, and select the region of interest (polytene chromosome) using Image-Adjust-Color Threshold; then separate each channel by using Image-Type-RGB Stack; finally use Analyze-Measure to quantify the immunostaining intensity in each channel. The channel of DAPI is used as the internal control.

S2 Cells Immunostaining

For normal conditions, S2 cells were incubated in insect medium (HyClone SFX; Thermo Fisher Scientific) supplemented with penicillin and streptomycin at 25°C. In order to induce osmotic stress, NaCl was added to the cell culture to a final concentration of 250mM for 30 minutes. Controls were treated with medium only. Cover slides were pretreated with ethanol and coated with concanavalin A, which allowed S2 cells to adhere to the glass surface. Cells were dropped on treated coverslips and were allowed to spread for 30 minutes. Cells were fixed with 4% PFA for 10 minutes at RT, followed by 3 washes with PBS. Fixed Cells were permeabilized with 0.2% Triton X-100 for 5 minutes, and washed twice with PBS.

Permeable cells were then incubated in the blocking solution (3% milk in PBS) for 10 minutes at RT. Primary antibodies diluted in blocking solution overnight at 4°C in a humidified chamber. Washing buffer (PBS+0.1% Triton X-100) was used to wash off unbound antibodies. Secondary antibodies incubation, DAPI staining, and mounting were performed as described above.

Antibodies

Rat and rabbit anti-Su(Hw), anti-Mod(mdg4)67.2 and anti-CP190 polyclonal IgG antibodies were generated in our laboratory (Schoborg et al., 2013a; Wallace et al., 2010). Mouse anti-γH2Av IgG (Developmental Studies Hybridoma Bank) and rabbit anti-RFP IgG (A00682, GenScript) are commercially available. All the primary and secondary antibodies were diluted as a ratio of 1:200 for immunostaining. The following secondary antibodies were used: Donkey FITC-conjugated anti-mouse IgG and Texas red-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.); Donkey Alexa Fluor 488-conjugated anti-rabbit IgG (A-21206, Life Technologies), and Donkey Alexa Fluor 555-conjugated anti-rabbit IgG (A31572, Life Technologies). Peroxidase-conjugated affinity purified goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) and Peroxidase-conjugated affinity purified goat anti-Rb IgG were used as a ratio of 1:5000 for western blot.

Western Blot

Early third instar stage larvae were collected and homogenized in RIPA lysis buffer with protease inhibitor (Roche) and phosphatase inhibitors (Sigma) on ice. Lysates were resolved in a 8%-15% acrylamide gel, wet transferred at 4°C overnight (10-15V) and probed with primary antibodies and secondary antibodies as described above.

Caffeine and Okadaic Acid (OA) Treatment

For salivary glands treatment, salivary glands were incubated in either 20mM caffeine or 50nM OA with insect medium for 3 hours at 25°C. This treatment was followed by the immunostaining procedure as described above. Flies were also fed with 2.5mM caffeine mixed into the standard cornmeal-agar media. Adult flies were allowed to feed and breed in food containing caffeine, and flies feeding with normal food were used as control. The phenotypes of the offspring were analyzed.

Results

γH2Av co-localizes with insulator proteins in chromosomes

Analysis of the distribution of insulator proteins using novel new generation sequencing approaches has shown that Su(Hw) binding sites frequently correspond to origins of replication, and that Su(Hw) protein may interact with

ORC proteins and components of the replication machinery (Vorobyeva et al., 2013). Earlier reports also suggested that Su(Hw) might have a role in DNA repair by homologous recombination in the germline of Drosophila females (Lankenau et al., 2000). Moreover, previous work in our laboratory showed that null mutations in Su(Hw) result in increasing levels of γH2Av in the ovary, and in the activation of an ATR DNA damage response and spindle checkpoints which recruits other proteins for repair and/or replication pathways (submitted). Collectively, these results indicate that chromatin insulators may play an still uncharacterized role in the maintenance of genome stability.

In order to further investigate this possibility, we asked first whether there is a correlation between insulator proteins and the distribution of γH2Av in the genome of untreated flies. To detect γH2Av in chromosomes we used a newly generated monoclonal anti-γH2Av that has shown to be highly specific both, in immunostaining experiments using ovaries and S2 cells, and in western blots (Lake et al., 2013). We used this anti-γH2Av antibody to immunostain polytene chromosomes, and asked whether there is a correlation between γH2Av and the distribution of all three insulator proteins found in the Gypsy insulator (Su(Hw), Cp190 and Mod[mdg4]). Antibodies anti-Su(Hw), anti-Cp190 and anti-Mod(mdg4)67.2 were used to co-immunostain chromosomes. Remarkably, our results showed that γH2Av co-localizes with every single binding site of Su(Hw) in polytene chromosomes (Fig.1.1A). Since Mod(mdg4)67.2 always binds to

Su(Hw) in wild type flies, we found identical co-localization pattern between γH2Av and Mod(mdg4)67.2 (Fig.1.1C). In addition, a number of bands appear not to co-localize with Su(Hw) binding sites. We found that these γH2Av sites are mostly located at interbands and co-localize with binding sites of CP190 (Fig.1.1B). Our results also show a high level of γH2Av in the chromocenter that does not co-localize with insulator sites, indicating that his pool of γH2Av sites are likely unrelated with insulator function (Fig.1.1).

To further confirm the specificity of the γH2Av antibody in polytene chromosomes and to assess whether γH2Av signals associated to insulator sites are not technical artifacts, we performed immunostaining using salivary glands from *HisAv*^{l3810} mutants. *HisAv*^{l3810} is a mutant allele of the H2Av gene that determines a 311bp deletion in this gene which removes the second exon and is homozygous lethal at the late third instar (Clarkson et al., 1999; Lake et al., 2013; van Daal and Elgin, 1992). We also used larvae overexpressing a transgenic form of H2Av fused to mcherry-H2Av::mcherry (Schoborg et al., 2013b). As expected, polytene chromosomes from mutant *HisAv*^{l3810} showed no signal for γH2Av, and γH2Av and H2Av::mcherry completely co-localize in larvae overexpressing of the H2Av::mcherry transgene (Fig.A1.1A). These data confirms the specificity of the monoclonal γH2Av antibody in polytene chromosomes, and strongly supports the notion that γH2Av extensively co-

localize with chromatin insulator protein binding sties in polytene chromosomes and may play a role in insulator function.

γH2Av forms insulator bodies in S2 cells during osmotic stress

In addition to function in nuclear architecture and gene transcription regulation, chromatin insulator also play a role in the cell response to heat shock and osmotic stress (Schoborg et al., 2013b; Wood et al., 2011). Particularly, the nuclear distribution of all known insulator proteins in *Drosophila* is dramatically reorganized in response to osmotic stress. We have previously shown that after cells are exposed to 250mM NaCl, insulator proteins rapidly dissociate from chromatin and form large protein aggregates, known as insulator bodies, which appear as multiple foci located in inter-chromatin spaces during live imaging experiments, or in immunostaining experiments using antibodies against insulator proteins (Schoborg et al., 2013b). Since we have shown that γH2Av co-localizes with insulator proteins on polytene chromosomes, and since γH2Av plays multiple roles in maintaining genome stability via DNA repair after DNA damage and replication stress, we next asked whether γH2Av also participates in the cell response to osmotic stress by forming insulator bodies (Madigan et al., 2002). Surprisingly, under normal conditions, γH2Av shows a diffused pattern similar to that of other insulator proteins in S2 cells (Fig. 1.2B). However, under osmotic stress conditions, γH2Av forms protein foci that perfectly co-localize with insulator bodies by Su(Hw) proteins (Fig. 1.2A). These data shows that γH2Av

also forms insulator bodies and, together with all known insulator proteins in *Drosophila*, participates in the cell response to osmotic stress.

Phosphorylation of H2Av depends on insulator proteins genome-wide

The overlapping distribution of insulator proteins and γH2Av in chromosomes and in insulator bodies suggests that γH2Av may have a role in insulator function. To investigate this possibility, we first asked whether phosphorylation of H2Av at insulator sites depends on insulator proteins. To test this possibility, we performed immunostaining experiments on polytene chromosomes from homozygous null mutant larvae for genes encoding insulator proteins. Interestingly, results show that in homozygous *su(Hw)*^{e04061}, a su(Hw) null mutation induced by a piggyback transposon insertion, the overall γH2Av distribution changes such that most γH2Av bands observed in wildtype disappear in the mutant (Hsu et al., 2015; Schoborg et al., 2013a; Schoborg et al., 2013b). It forms instead a diffused distribution pattern along all chromosomes with only a few clear signals mainly located at polytene bands, and showing no co-localization with Mod(mdg4)67.2 (Fig. 1.3A). We quantified the change in signal levels by measuring the fluorescence intensity associated to chromosomes and comparing between wildtype and mutants (see materials and methods). This quantitative analysis confirmed our initial observation at the microscope, and shows that the amount of γH2Av is significantly reduced in *su(Hw)*^{e04061} when compared with wildtype (Fig. A1.2A).

Interestingly, we found that in *su(Hw)*^{e04061} mutant larvae, Mod(mdg4)67.2 is still bound to chromosomes, but it associates now with CP190 sites. The association of Mod(mdg4)67.2 with CP190 in Su(Hw) null mutants is likely mediated by interactions between the BTB domains of both proteins and, to our knowledge, it has not been previously reported. The reduced amount of γH2Av and the lack of γH2Av co-localization with Mod(mdg4)67.2 suggests a decreasing amount of the γH2Av associated with CP190 sites (Fig.A1.2C). Indeed, a remarkably reduced level of co-localization between γH2Av and CP190 was detected in *su(Hw)*^{e04061} mutant (Fig.1.3B). Similarly, in *mod(mdg4)*^{u1} mutant, γH2Av appears diffused and associates mainly to bands, rarely showing co-localization with Su(Hw) or CP190 (Fig. 1.3C and 1.3D). On the other hand, Su(Hw) shows a dramatically reduced level of co-localization with γH2Av in *cp190*$^{P11/H31-2}$ mutant, indicating CP190 probably plays a role in H2Av phosphorylation at Su(Hw) insulator sites as well (Fig. 1.3E). In addition, mutations in insulator proteins correlated with an accumulation of γH2Av signal in telomeres of polytene chromosomes, indicating that loss of insulator protein activity may also trigger DNA damage and activation of DNA repair mechanisms at telomeres (Fig. A1.2B). Taken collectively, these data suggest that phosphorylation of H2Av at insulator sites depends on specific protein components of the insulator proteins complexes, and that γH2Av may play a role in insulator function. On the other hand, loss of insulator function leads to new chromosomal sites of γH2Av, such as telomeres, where is likely

targeted for activation of DNA repair pathways, which in turn suggests chromatin insulators play a role in maintenance of genome stability.

Non-functional gypsy insulators lack γH2Av

Since γH2Av co-localizes with every single binding site of Su(Hw) in polytene chromosomes, we next asked whether phosphorylation of H2Av is necessary for the *gypsy* insulators enhancer blocking function. To address this question, we firstly analyzed y^2 and ct^6 mutants, which are induced by integration of the *Gypsy* retrotransposon in the regulatory region of the *yellow(y)* and *cut(ct)* genes (Georgiev and Kozycina, 1996; Geyer and Corces, 1992; Pai et al., 2004). The gypsy retrotransposon contains a 400bp sequence in the 5' untranslated region of the genome that carries 12 Su(Hw) binding sites, which provide gypsy with its insulator properties (Modolell et al., 1983). Using antibodies against the insulator proteins Su(Hw), Mod(mdg4)67.2 and CP190, a strong immunofluorescence signal can be detected at both y^2 and ct^6 sites in polytene chromosomes, which reflects the presence of the gypsy insulator and binding of these proteins at these sites. Likewise, we have shown that γH2Av co-localizes with the insulator proteins at the same y^2 and ct^6 sites, which confirms that the gypsy insulator follows the same rule as all other Su(Hw) binding sites, and is also associated with γH2Av as described before genome-wide (Fig. 1.4A).

The y^2 and ct^6 phenotypes are rescued, or partially rescued, in homozygous mutant flies for any known gene encoding a gypsy insulator protein, given that the enhancer-blocking function of gypsy is impaired by the absence of any of its insulator protein components (Mongelard et al., 2002; Pai et al., 2004). To test whether phosphorylation of H2Av at *Gypsy* insulator sites depends on insulator proteins, we performed immunostaining experiments to detect γH2Av at y^2 and ct^6 sites in polytene chromosomes from insulator mutant third instar larvae. Results show that the intense γH2Av signal observed at y^2 and ct^6 sites in wildtype is totally absent in either $su(Hw)^{e04061}$ or $mod(mdg4)^{u1}$ mutants (Fig 1.4B and 1.4C). Results showing no γH2Av in su(Hw) null mutant suggest that γH2Av is not just associated with the DNA at or near insulator DNA sequences (for example, as an independent histone core component of nucleosomes), and that Su(Hw) is required for recruitment or phosphorylation of H2Av at Su(Hw) binding sites and at the gypsy insulator. The absence of Mod(mdg4)67.2 in $mod(mdg4)^{u1}$ mutants, however, still allows Su(Hw) binding to the gypsy insulator, whereas its enhancer-blocking activity is largely but not completely impaired(Mongelard et al., 2002). Results showing the absence of γH2Av at y^2 and ct^6 in $mod(mdg4)^{u1}$ null mutants indicate that binding of Su(Hw) to gypsy insulators alone is not sufficient to allow phosphorylation of H2Av, and suggest that non-functional insulators lack γH2Av.

To further explore whether γH2Av is required for enhancer blocking function, we tested the effect of CP190 on γH2Av at y^2 and ct^6 sites. With this purpose, we used trans-heterozygous mutants $cp190^{H31\text{-}2/P11}$, which are normally viable until pupal stage, and allow microscopic analysis of polytene chromosomes (Pai et al., 2004). Previous reports have shown that enhancer blocking does not function in these mutants, corroborating that cp190 is required for gypsy insulator fuction(Pai et al., 2004). We used $y^2/+ct^6/+;cp190^{P11/H31\text{-}2}$ mutant female larvae to test whether γH2Av was present at y^2 and ct^6 sites. Results show that γH2Av does not bind gypsy insulators at y^2 and ct^6 sites in the absence of CP190 (Fig. 1.4D, A1.3B and A1.3C), reinforcing the notion that gypsy insulators require γH2Av for proper enhancer-blocking function. Taken together, these data shows that phosphorylation of H2Av at the *Gypsy* insulator depends on the proteins that bind the insulator DNA, and that γH2Av is absent in non-functional insulators.

HIPP1 overexpression induces loss of γH2Av while maintaining other insulator proteins bound to chromatin and rescuing y^2 and ct^6 phenotypes

In addition to Su(Hw), Mod(mdg4)67.2 and CP190, a novel protein-Heterochromatin protein 1 Insulator Partner Protein 1 (HIPP1)- has recently been identified as a potential component of the Su(Hw) insulator (Alekseyenko et al., 2014). HIPP1 co-immunoprecipitates with Su(Hw) and with Heterochromatin protein 1 (HP1), and localizes to Su(Hw) binding sites genome-wide, suggesting that it might be a partner of Su(Hw) and may have a role in insulator function

(Alekseyenko et al., 2014). We have generated a protein fusion between HIPP1 and mcherry (HIPP1::mcherry), which we have used to transform S2 cells and to generate UAS>HIPP1::mcherry transgenic flies that can express this transgene under the control of a GAL4 driver. First, we asked whether HIPP1::mcherry behaves like all other known insulator proteins in their response to osmotic stress, by forming insulator bodies. We expressed HIPP1::mcherry in S2 cells under the control of the copper-responsive metallothionein promoter, and induced osmotic stress by increasing media salt concentration to 250mM NaCl. Results show that HIPP1::mcherry remains diffused in the nucleoplasm before stress, with a distribution that perfectly overlaps that of a Su(Hw)::GFP transgene that is co-expressed with HIPP1::mcherry (Fig. 1.5A). After stress, HIPP1::mcherry forms insulator bodies that perfectly overlap with those formed by Su(Hw), confirming that HIPP1 indeed shares this property with all other chromatin insulator proteins in *Drosophila* (Fig. 1.5B). We expressed the HIPP1::mcherry in flies, but all the general expression drivers used, including *tubulin*, ubiquitin and *hsp70*, generated larvae that could not reach third instar stage and could not develop into adult flies (data not shown).

Next, we developed a genetic assay that would allow us testing gypsy insulator activity in *Drosophila* adult tissues in the background of lethal mutations, such as overexpression of HIPP1::mcherry. Our assay consist on the overexpression of a protein, or a specific RNAi of interest, using the vestigial Boundary Enahncer-gal4 driver (vgBE-Gal4), which drives GAL4 expression in a stripe of cells at the

dorsal/ ventral boundary in the developing wing and haltere imaginal discs (Schoborg et al., 2013a; Schoborg et al., 2013b; Williams et al., 1994). In our assay, flies expression transgenes in the wing margin are also mutant for y^2 and ct^6. If the expressed transgene has an effect on the gypsy insulator function, we expect to see an enhancement or a supreesion of the y^2 and ct^6 phenotypes in the wing blade color and margin, respectively. Additionally, vgBE-Gal4 also drives expression of UAS transgenes in salivary glands, which allows for a simultaneous analysis of the distribution of the protein of interest in polytene chromosomes.

We first used CP190-RNAi to assess the validity of the assay, since the effect of null CP190 mutations is known and the CP190-RNAi line was successfully used previously in our laboratory (Schoborg et al., 2013b). Results show that y^2ct^6; UAS>CP190-RNAi vgBE-Gal4 flies exhibit black wings and an almost perfectly round wing margin, indicating that the y^2 and ct^6 mutant phenotypes induced by gypsy are rescued because of lack of CP190 function in CP190-RNAi expressing cells (Fig. A1.3D and A1.3E). Indeed, we also confirmed our previous results by showing that γH2Av at y^2 and ct^6 sites in polytene chromosomes is not detectable in most nuclei, though some chromosomes show weak γH2Av signals, likely occurring because of unequal efficiency of the CP190 RNAi in different cells (Fig. A1.3A).

We performed this assay using y^2ct^6; UAS>HIPP1::mcherry vgBE-Gal4 flies to test the effect of HIPP1::mcherry in *gypsy* insulator function, and to determine the distribution of HIPP1::mcherry in polytene chromosomes, compared with that of γH2Av. Results show that both y^2 and ct^6 are partially rescued by overexpression of HIPP1::mcherry (Fig. 1.6E). Moreover, immunostaining on polytene chromosomes also shows that γH2Av is missing at y^2 and ct^6 sites, while the insulator proteins Mod(mdg4)67.2, and CP190 remain associated to the gypsy insulator. These results suggest that HIPP1::mcherry impairs the insulator enhancer-blocking function at y^2 and ct^6 sites independently of Mod(mdg4)67.2 and CP190 which results from the missing of γH2Av (Fig. 1.6A-1.6C and Fig. A1.4). In addition, HIPP1::mcherry is found at y^2 and ct^6 *gypsy* insulator binding sites on some of the polytene chromosomes, indicating HIPP1 might cause removal or de-phosphorylation of γH2Av to inhibit insulator function (Fig. 1.6D). In summary, these data supports the hypothesis that phosphorylation of H2av is required for insulator enhancer blocking function, and that HIPP1 functions by inhibiting insulator function. We speculate that HIPP1 may inhibit insulator function by antagonizaing the γH2Av role in the insulator.

ATM and ATR phosphorylate H2Av at Su(Hw) insulator sites

Since we showed that under normal conditions the phosphorylated form of H2Av colocalizes with insulator sites and insulator bodies, and that loss of insulator activity correlates with lack of γH2Av, we explored the possibility that

phosphorylation of H2Av could be part of a mechanism that regulates the activity of chromatin insulators. Interestingly, H2Av in Drosophila and H2Ax in mammals, are well-known phosphorylation targets for ATR (Ataxia Telangiectasia Related and ATM (Ataxia Telangiectasia mutated), which are two protein kinases belonging to the phosphatidylinositol 3' kinase-like kinase (PIKK) enzyme family, and are conserved throughout eukaryotes (Cimprich and Cortez, 2008; Joyce et al., 2011; Song et al., 2004).

The most common role of ATM and ATR are the regulators of the DNA damage response (DDR) pathway, which is a signal transduction pathway that coordinates cell cycle transitions, DNA replication, DNA repair and apoptosis (Cimprich and Cortez, 2008). Activation of ATM or ATR by DNA damage has dual effects that are essential for repair. On one hand, checkpoint is activated by ATM or ATR until the damage is repaired (Chowdhury et al., 2005; Freeman and Monteiro, 2010). On the other hand, the DNA damage is recognized by the sensor proteins, including MRN (Mre11-Rad50-Nbs1) and 9-1-1 (Rad9-Rad1-Hus1), that initiate the activation of DDR on chromatin. One of the earliest events of DDR is phosphorylation of histone H2AX at Ser139 by ATM, which is specific for DSB repair and telomere maintenance, or ATR, that is activated by single-stranded DNA ends generated during processing of DSBs or collapsed replication forks (Bensimon et al., 2011; Freeman and Monteiro, 2010). Though ATM and ATR are responsible for different damage response, ATR is also found involved in the DSB repair pathway overlapping with ATM (LaRocque et al.,

2007; Xue et al., 2015). γH2Av plays an important role in recruiting the repair proteins to the damage focus. Once DNA repair accomplishes, the phosphorylation marker would be removed by protein phosphatase 2A (PP2A) (Chowdhury et al., 2005).

So far, in addition to the DNA damage marker to recruit other DNA repair machinery, ATM and ATR are also found to have novel functions. For example, ATM is involved in DNA condensation in human cells (Burgess et al., 2014). In addition, recent study also reports that ATR mediates a checkpoint at the nuclear envelope in response to the mechanical stress in both human and mouse cells (Kumar et al., 2014).

Therefore, we asked whether mutations in ATR and ATM have an effect on the distribution of γH2Av in polytene chromosomes. First, we used the *tefu*$^{atm-3}$ allele, a mutation at the kinase domain of the *Drosophila* ATM homolog that results in a premature stop codon (Pedersen et al., 2010). Results show that in *y*^{2}*ct*^{6}*;tefu*$^{atm-3}$ individuals the overall γH2Av signal in chromosomes is only slightly reduced (Fig. 1.7A). The lack of a significant reduction in γH2Av throughout the genome in this mutant was also confirmed at the y^{2} and ct^{6} sites, where γH2Av is not significantly different from wildtype (Fig. 1.8A). One possibility to explain these results is that phosphorylation of H2Av, particularly at y^{2} and ct^{6} sites, may be independent of ATM and possible may require ATR kinase activity. Alternately,

ATM and ATR activities may be redundant, and both kinases may be able to phosphorylated H2Av at insulator sites. To distinguish between these two possibilities, we first tested the presence of γH2Av at insulato.r sites in the background of the ATR homozygous mutant *mei41*^{D5}. *mei41* is the *Drosophila* homolog of ATR, and *mei41*^{D5} is a mild mutant allele, which consist on a point mutation that changes proline^{2159} in the kinase domain to leucine, but that is sensitive to DNA damage. Homozygous *mei41*^{D5} adult flies are viable and fertile under normal conditions (Laurencon et al., 2003). Results show that the distribution and intensity of γH2Av of γH2Av in *mei41*^{D5} polytene chromosomes are not significantly different from wildtype (Fig. 1.7B). However, altogether these results suggest that ATR is not sole contributor to H2Av phosphorylation at the insulator sites.

To ask whether both kinases, ATM and ATR, contribute to H2Av phosphorylation, we generated a double mutant *mei41*^{D5}*; tefu*$^{atm-3}$ third instar larvae and determined the distribution of γH2Av in polytene chromosomes. Interestingly, the double mutant *mei41*^{D5}*; tefu*$^{atm-3}$ shows a drastic reduction in the levels of γH2Av throughout all polytene chromosomes (Fig. 1.7C), which suggests that phosphorylation of H2Av in insulator sites results from the kinase activity of both ATM and ATR. This result suggest that both kinases can phosphorylate H2Av at insulator sites, and that one kinase can compensate for absence of the second, explaining our previous results using single mutants. To

further verify these findings, we used caffeine to further confirm whether phosphorylation of H2Av is required for the insulator enhancer-promoter blocking function. Caffeine is universally recognized as a strong inhibitor of kinase activities including *Drosophila* ATM and ATR (Blasina et al., 1999; Hall-Jackson et al., 1999; Sarkaria et al., 1999). As expected, immunostaining experiments showed that the levels of γH2Av are dramatically reduced and barely detectable in polytene chromosomes of the salivary glands after incubation with 20mM caffeine medium (Fig 1.8B-1.8D). Collectively, these results support a model in which the kinase activities of ATR and ATM control the phosphorylation of H2Av at insulator sites.

ATM and ATR modulate levels of insulator proteins

Interestingly, during the analysis of the distribution of γH2Av in single mutants *tefu*$^{atm-3}$ and *mei41*^{D5}, we found that insulator proteins were often missing from insulator sites when compared with wildtype (Fig. 1.7A and 1.7B). This observation suggested that ATR and ATM may have a role in the stability of insulator proteins at insulator sites. To further explore this possibility we asked whether mutations in ATR and ATM might influence the expression or the distribution of insulator proteins in chromosomes. We performed immunostaining experiments using antibodies against insulator proteins on salivary glands from *tefu*$^{atm-3}$ and *mei41*^{D5} mutants. Results show a dramatic reduction in the level of Su(Hw), Mod(mdg4)67.2 and CP190 insulator proteins in *tefu*$^{atm-3}$ chromosomes (Fig. 1.9A-1.9D). Western blot analysis of third instar larva confirms a similar

reduction in the total amount of insulator proteins in *tefu*$^{atm-3}$ (Fig. 1.9G) (Pedersen et al., 2010). On the other hand, *mei41*^{D5} mutants do not show a significant reduction in insulator proteins bound to chromosomes, and western blots only show a decrease in the amount of CP190 (Fig. 1.9E-1.9F, and 1.9H) (Laurencon et al., 2003). This may indicate that either ATR is only a mild mutation of ATR or that ATM is able to compensate for the malfunction of ATR. Overall, these data suggest that the level of insulator proteins is sensitive to the activity ATM and ATR.

ATM and ATR control gypsy insulator activity through phosphorylation of H2Av

Our findings so far revealed that γH2Av is present at insulator sites across the genome, particularly at Su(Hw) and gypsy insulators. Importantly, we have shown that ATM and ATR phosphorylate H2Av, which opens the prospect that H2Av phosphorylation may be part of a mechanism that regulates the activity of chromatin insulators. Our hypothesis is that insulator function might be activated by H2Av phosphorylation and inhibited by H2Av dephosphorylation. To test this hypothesis we used caffeine to confirm whether phosphorylation of H2Av is required for the insulator enhancer-promoter blocking function. As mentioned before, caffeine is universally recognized as a strong inhibitor of ATM and ATR kinase activities, including in *Drosophila* (Katzenberger et al., 2006; Sarkaria et al., 1999). Our immunostaining experiments showed that the levels of γH2Av are dramatically reduced and barely detectable in polytene chromosomes after

incubation of salivary glands with 20mM caffeine in culture media (Fig. 1.8B-1.8D). This result further supports a model in which the kinase activities of ATR and ATM control the phosphorylation of H2Av at insulator sites.

Second, we fed y^2 ct^6 homozygous males and females with fly food containing 2.5mM caffeine, and allowed their offspring to develop to adults in the same caffeine containing media. Offspring generated in this manner takes a much longer time to complete their development, and there is a high rate of lethality at the pupal stage. However, escapers revealed a partially rescued phenotype on both y^2 and ct^6, which appeared as spotted dark-pigmented abdomen cuticle in males and wing margins with less pronounced cuts and with bristles more evenly distributed (Fig. 1.10A). These results suggest that the combined inhibition of the kinase activity of ATR and ATM can inactivate the enhancer blocking activity of the gypsy insulator. In addition, immunostaining of salivary glands from third instar larvae grown in 2.5 mM caffeine also showed a dramatically reduced level of γH2Av on polytene chromosomes (Fig. 1.10B). However, the immunostaining did not show a complete elimination of γH2Av, suggesting that phosphorylation of H2Av was only partially reduced, unlike in salivary glands directly incubated in caffeine, explaining the partial nature of the rescued y2 and ct6 phenotypes (Fig. 1.10B and 1.8B-1.8D).

In our model for a regulatory role of γH2Av in the regulation of enhancer blocking activity of insulators, inactivation is mediated by the loss of γH2Av at insulator sites, and we predict that this loss is produced by dephosphorylation of γH2Av. It has been reported that during DNA repair of DSB PP2A dephosphorylates γH2Ax (Chowdhury et al., 2005). To explore the possibility that γH2Av at Drosophila insulator sites can also be dephosphorylated by PP2A we used Okadaic Acid (OA), a potent inhibitor of the phosphatase activity of PP2A (Freeman and Monteiro, 2010; Hamilton et al., 2009; Nowak et al., 2003). In previous study, OA is used as the inhibitor of PP2A, which dephosphorylates ATM to inactivate the kinase activity (Cho et al., 2014; Lee et al., 2015). Thus, treatment with OA would decrease dephosphorylation of ATM, which results in more γH2Av (Chowdhury et al., 2005). Interestingly, in wild type, immunostaining signals for γH2Av appear at insulator binding sites as it does in untreated chromosomes, although they are much stronger in intensity (Fig. 1.11). This result suggests that OA inhibits PP2A, and prevents dephosphorylation of γH2Av.

Next we asked whether the lack of γH2Av at insulator sites in the background of insulator protein mutants could result from an active process of dephosphorylation by PP2A. We performed immunostaining of salivary glands from third instar mutant larvae from *su(Hw)*^{e04061}, *mod(mdg4)*^{u1} and *cp190*$^{p11/H31-2}$ and treated with OA. Surprisingly, in *su(Hw)*^{e04061} mutant, γH2Av is now detectable at the Mod(mdg4)67.2 binding sites, which correspond to binding sites

of CP190, since Mod(mdg4)67.2 moves to the CP190 binding sites in *su(Hw)*^{e04061} mutant (Fig. 1.12A-B and S1.2C). γH2Av is not detectable in at CP190 sites in untreated *su(Hw)*^{e04061} mutants (Fig. 1.3B). Similarly, in *mod(mdg4)*^{u1} mutants, γH2Av locates now at Su(Hw) and CP190 binding sites, whereas in untreated salivary glands, γH2Av is absent from all insulator sites (Fig. 1.12C-D and 1.3C-D). Finally, in *cp190*$^{p11/H31-2}$ mutant salivary glands treated with OA, γH2Av locates at Su(Hw) binding sites, whereas the untreated *cp190*$^{p11/H31-2}$ mutant show less γH2Av at Su(Hw) insulator sites (Fig. 1.12E and 1.3E). Collectively, these data supports a mechanism of insulator function, in enhancer blocking is facilitated by the presence of γH2Av. Insulators are activated through phosphorylation of H2Av by ATM and ATR and are inactivated by dephosphorylation of γH2Av by PP2A.

Discussion

Our results provide evidence supporting that the activity of the Su(Hw) chromatin insulator can be regulated by ATM and ATR through phosphorylation of the histone variant H2Av. Importantly, these results suggest that phosphorylation of H2Av by ATM and ATR may function to regulate chromatin insulators activity either globally, at insulators genome-wide for example during cell cycle, or at particular sites in the genome, in response to specific regulatory demands. Evidence comes primarily from experiments showing that γH2Av is associated to

Su(Hw) insulator sites throughout the genome, as measured by colocalization of insulator proteins with γH2Av, using fluorescence microscopy and immunostaining in polytene chromosomes. The significance of these results is reinforced by findings that mutations in genes encoding insulator proteins result in lack of γH2Av at insulator sites, or in a redistribution of γH2Av to sites that do not appear to be related with insulator sites. More importantly, we provide evidence that the same association occurs at gypsy insulator sites found at the y^2 and ct^6 loci and that lack of insulator function at y^2 and ct^6, correlates with lack of γH2Av. Finally, our results support the notion that phosphorylation of H2Av depends on ATR and ATM kinase activity, whereas dephosphorylation depends on phosphatase activity of PP2A, suggesting that the activity of insulators is modulated by the phosphorylation status of H2Av at insulator sites.

ATM and ATR roles in the stability of genome architecture.

ATM and ATR play a major role in the control of the stability of the genome and in the signal pathways required for DNA damage repair, cell cycle checkpoint activation and apoptosis. In addition to these important roles in the maintenance of the homeostasis of the genome, ATR and ATM function in response to mechanical stress, and can sense changes in the condensation of the genome as well. Is not clear how ATR and ATM and the role of H2Av phosphorylation in DNA damage response relates to our findings that γH2Av is found at insulator sites. The normal sources of inherent and non-induced DNA damage are well

characterized and consist on replication stress, oxidative damage and DNA damage resulting from transcriptional activity. In addition DNA damage and repair are necessary for the normal process of DNA recombination and proper chromosome segregation during meiosis in the germline (Jones and Petermann, 2012; San Filippo et al., 2008). However, it cannot be ruled out that γH2Av in insulator sites could correspond to the signaling of DNA damage repair caused by an unidentified type of DNA damage that is connected with insulator function. In this respect, our laboratory has reported that the levels of phosphorylated H2Av increased significantly during oogenesis in *su(Hw)* mutant females. This increase in γH2Av, is concomitant with malformation of the MTOC (Micro Tubule Organization Complex) and leads to dorsal-ventral malformations on embryos. All these phenotypes are the signature of an excess of unrepaired DNA in the germline of Drosophila, suggesting the possibility that Su(Hw) could may have a role in DNA repair in the germ line (Klattenhoff et al., 2007; Lankenau et al., 2000) (Hsu, et al, submitted). Interestingly, we also found that these phenotypes, and the classical sterility phenotype of Su(Hw) mutants in the female germline were partially rescued by mutations in the $mei41^{D5}$, the Drosophila ATR homolog. New findings in this work now support the existence of interactions between insulators and γH2Av, and that ATR, as well as ATM, are involved in the regulation of the insulators activity, also in somatic cells.

However there is no evidence supporting that γH2Av at insulator sites is nucleosomal in nature, a requirement in order to argue that phosphorylation of H2Av at insulator sites is involved in a DNA repair pathway. Interestingly, Su(Hw) insulators are unlike all other insulator counterparts in Drosophila or in mammals, in the sense that Su(Hw) insulators are found in nucleosomal-rich DNA sequences, whereas dCTCF, Cp190, GAGA, Mod of Mdg4 and BEAF are always associated with DNA poor in nucleosomal content (Negre et al., 2010). The significance of the association of insulators with nucleosomes is not clear, but it does not appear to be a factor that correlates with the presence of γH2Av in insulators, since a large fraction of Cp190 insulators also co-localize with γH2Av. On the other hand, there are no reports, to our knowledge, of H2Av or other histones that perform a function in the cell as part of a non-nucleosomal protein complex. Therefore, determining whether γH2Av in insulator sites is a component of the insulator protein complex or in fact a component of nucleosomes associated to insulator sites, is necessary to further understand the role of γH2Av, ATM and ATR in insulator function. Next experiments in our laboratory will address these questions, in part by analyzing the distribution of γH2Av in chromosomes at a genome sequence resolution using Chromatin IP and new generation sequencing technologies.

An intriguing possibility is that ATM and ATR might be part of a global surveillance mechanism, in which in addition to ensuring the integrity of the

genome through signaling repair pathways after DNA damage and replication stress, ATM and ATR also participate in the maintenance of the three-dimensional organization of the genome. There is strong evidence supporting a role for insulator proteins in the response to stress (Gerasimova and Corces, 1998; Gerasimova et al., 2007; Lei and Corces, 2006). Particularly, our laboratory recently reported data showing that insulator proteins dissociate from chromatin during osmotic stress, forming large nuclear foci known as insulator bodies, that localize in inter-chromosomal spaces and are formed by the coalescence of all known insulator proteins (Schoborg et al., 2013b). Experimental evidence suggests that chromatin loops are lost after the rearrangement of insulator proteins during osmotic stress. These loops most likely reorganize after stress recovery, which would suggest there are mechanisms in place to preserve the three-dimensional organization of the genome during certain stress conditions that may compromise the integrity of this organization. Interestingly, both osmotic stress and mechanical stress exerted in mammalian cells activate a signaling response mediated by ATR, although the significance of this response remains unknown (Kumar et al., 2014). Here we have shown that γH2Av is also found at insulator bodies formed after osmotic stress, suggesting the intriguing possibility that phosphorylation of H2Av by ATR and ATM at insulator sites may be a direct response to stress that leads to the inactivation of the insulators and to the formation of insulator bodies.

Collectively our results have revealed the existence of a new mechanism, by which the kinase activity of ATR and ATM regulate insulator activity through phosphorylation of the histone variant H2Av at insulator sites. We speculate that this mechanism may lead to changes in the global organization of the genome, for example during episodes of stress or during preparation for DNA replication and mitosis, allowing the reset of the long-range interactions that are mediated by insulator proteins. In addition, by targeting the activity of ATR, ATM or phosphatase PP2A at individual insulator sites, this mechanism might potentially regulate the activity of a specific insulator during development or in response to signal transduction pathways. The involvement of ATM and ATR in this mechanism is particularly intriguing, since the major role of these proteins is maintenance of the integrity of the genome, and it brings the question of whether insulators play an unanticipated role in genome instability. In addition to increase our further understanding of this mechanism, future studies should elucidate the functional connections between this role, cell cycle, DNA replication and repair and the better characterized functions of insulators in genome organization and gene transcription regulation.

CHAPTER I APPENDIX

Figure 1.1. γH2Av co-localizes with insulator proteins in polytene chromosomes. A. Immunostaining performed on polytene chromosomes from wildtype early third instar larvae shows co-localization between γH2Av and Su(Hw). The bottom figures below each main figure amplify specific regions of the chromosomes to magnify the image showing co-localizations. γH2Av is shown in green, insulator proteins in red, and DAPI in blue. B. Co-localization between γH2Av and Mod(mdg4)67.2 in polytene chromosomes. C. Co-localization between γH2Av and CP190 in polytene chromosomes.

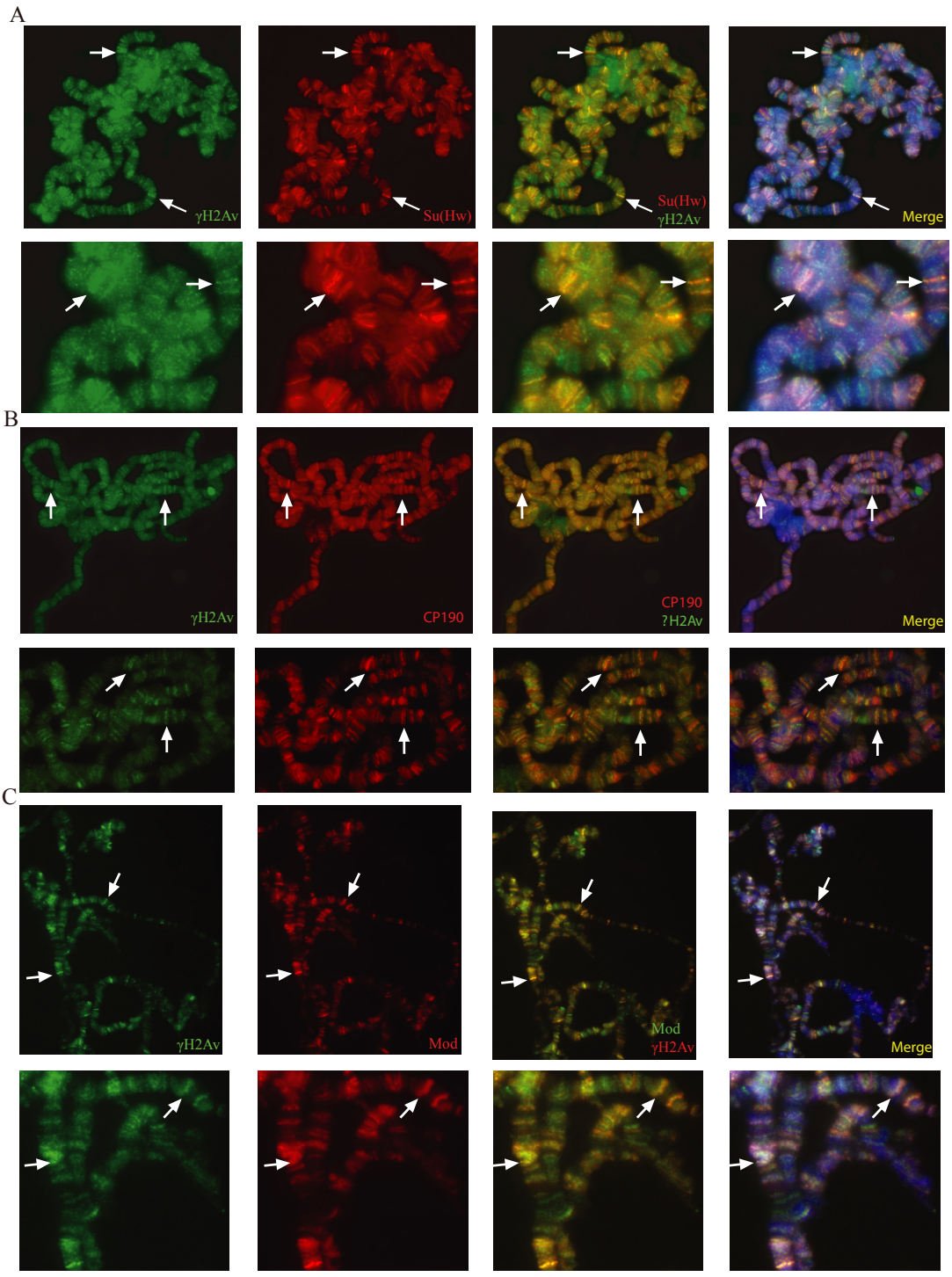

A
γH2Av
Su(Hw)
Su(Hw)
γH2Av
Merge
B
γH2Av
CP190
CP190
?H2Av
Merge
C
γH2Av
Mod
Mod
γH2Av
Merge

Figure 1.2. Phosphorylated H2Av forms insulator bodies in S2 cells during osmotic stress. A. γH2Av co-localizes with Su(Hw) insulator bodies in S2 cells under osmotic stress (treated with 250mM NaCl). B. In control S2 cells, without osmotic stress, both Su(Hw) and γH2Av show diffused immunostaining pattern. Bottom figures below show specific cells to magnify the distribution of the proteins.

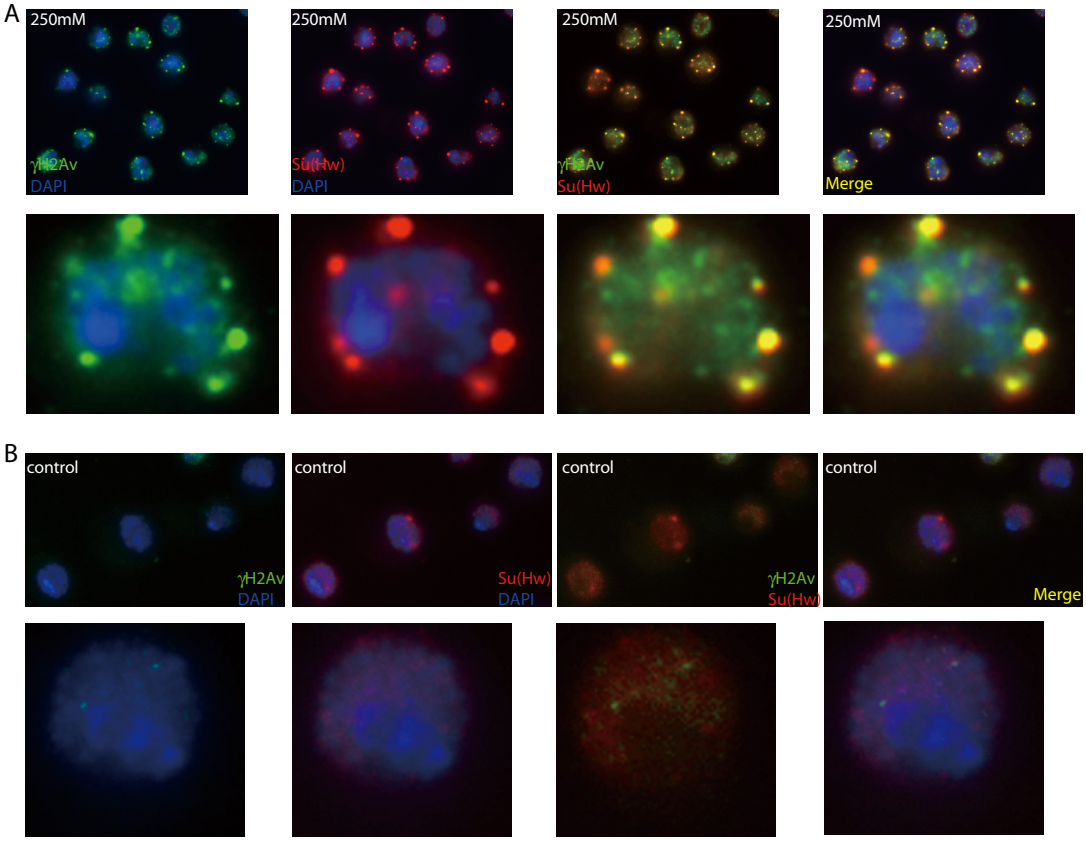

A
250mM
γH2Av
DAPI
250mM
Su(Hw)
DAPI
250mM
γH2Av
Su(Hw)
250mM
Merge
B
control
γH2Av
DAPI
control
Su(Hw)
DAPI
control
γH2Av
Su(Hw)
control
Merge

Figure 1.3. Phosphorylation of H2Av depends on insulator proteins genome-wide. A-B. γH2Av shows no co-localizations with Mod(mdg4)67.2 (A) and CP190 (B) in polytene chromosomes in *su(Hw)*^{e04061} mutant. C-D. γH2Av shows almost no co-localizations with Su(Hw) (C) and CP190 (D) in polytene chromosomes in *mod(mdg4)*^{u1} mutant. E. γH2Av shows a dramatically reduced level of co-localizations with Su(Hw) in polytene chromosomes in *cp190*$^{p11/H31-2}$ mutant.

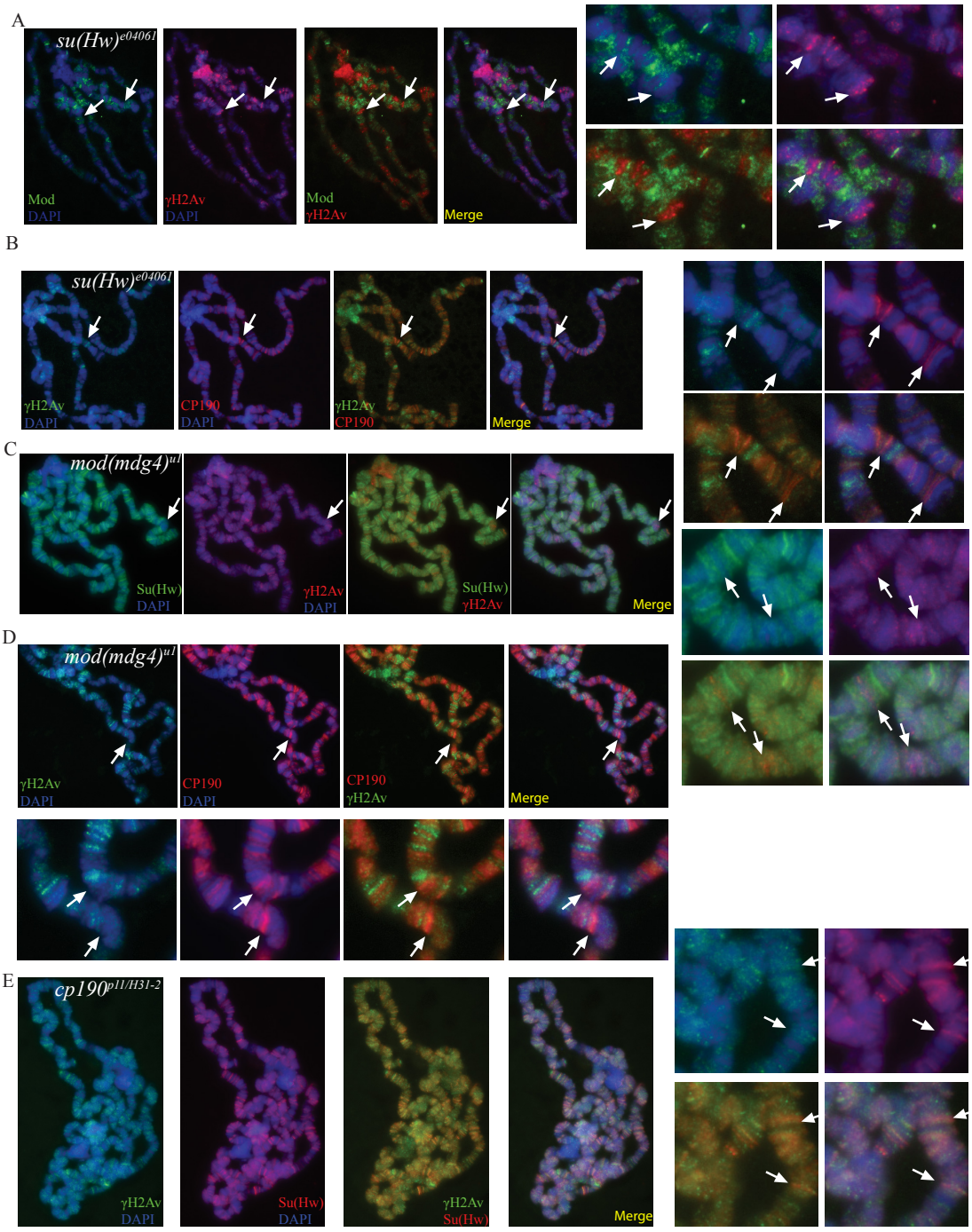

A
su(Hw)e04061
Mod
DAPI
γH2Av
DAPI
Mod
γH2Av
Merge
B
su(Hw)e04061
γH2Av
DAPI
CP190
DAPI
γH2Av
CP190
Merge
C
mod(mdg4)u1
Su(Hw)
DAPI
γH2Av
DAPI
Su(Hw)
γH2Av
Merge
D
mod(mdg4)u1
γH2Av
DAPI
CP190
DAPI
CP190
γH2Av
Merge
E
cp190p11/H31-2
γH2Av
DAPI
Su(Hw)
DAPI
γH2Av
Su(Hw)
Merge

Figure 1.4. Phosphorylation of H2Av correlates with gypsy insulator function. A. γH2Av co-localizes with all insulator components at y^2 and ct^6 binding sites in polytene chromosomes. B-D. γH2Av is absent from y^2 and ct^6 sites in $su(Hw)^{e04061}$, $mod(mdg4)^{u1}$ and $cp190^{P11/H31-2}$ mutants in polytene chromosomes.

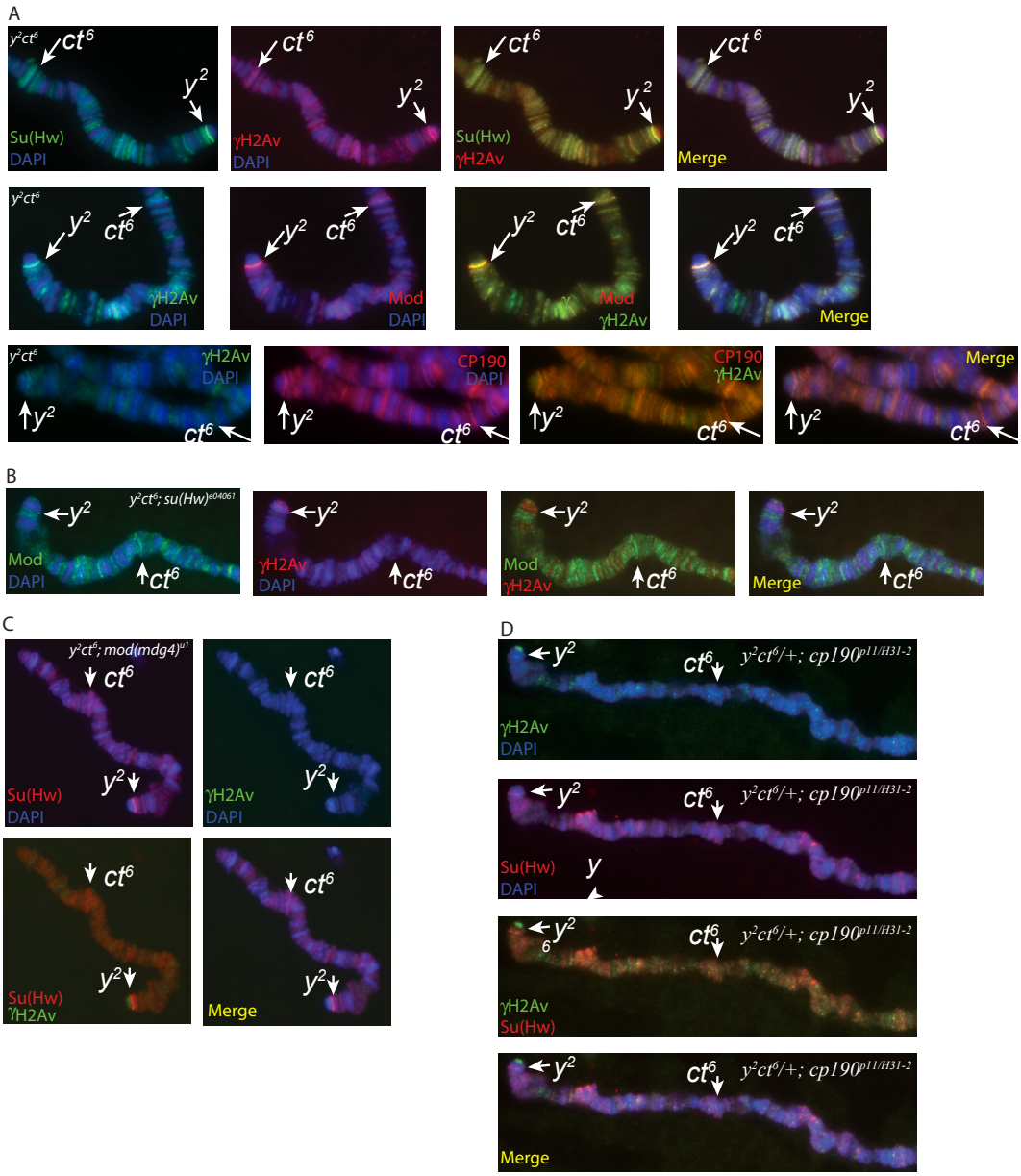

A
y²ct⁶
ct⁶
y²
Su(Hw)
DAPI
γH2Av
Mod
Merge
CP190
B
y²ct⁶; su(Hw)e04061
C
y²ct⁶; mod(mdg4)u1
D
y²ct⁶/+; cp190p11/H31-2

Figure 1.5. HIPP1 is a component of the insulator bodies. A. HIPP1::RFP and Su(Hw)::GFP show diffused pattern in S2 cells under normal conditions. B. HIPP1::RFP co-localizes with insulator bodies in S2 cells after osmotic stress.

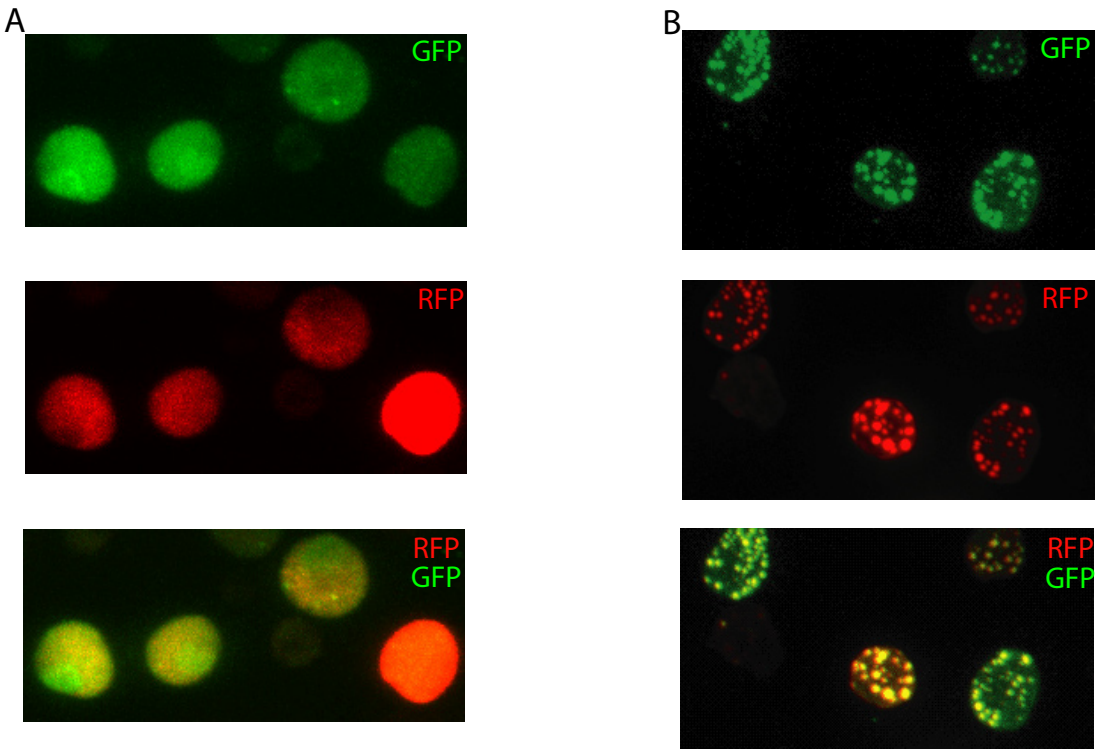

A
GFP
RFP
RFP
GFP
B
GFP
RFP
RFP
GFP

Figure 1.6. Overexpression of HIPP1 induces loss of γH2Av while maintaining other insulator proteins bound to chromatin and rescuing *y*^{2} and *ct*^{6} phenotypes. A-C. γH2Av disappears at *y*^{2} and *ct*^{6} sites with overexpression of HIPP1, while Su(Hw), Mod(mdg4)67.2 and CP190 still bind to the polytene chromosomes. D. HIPP1 is found to co-localize with Su(Hw) at *y*^{2} and *ct*^{6} binding sites in the polytene chromosomes. E. Overexpression of HIPP1 in the wing using *vg-gal4* driver partially rescues both *y*^{2} and *ct*^{6} phenotype on the wing. Arrows indicate *y*^{2} and *ct*^{6} sites.

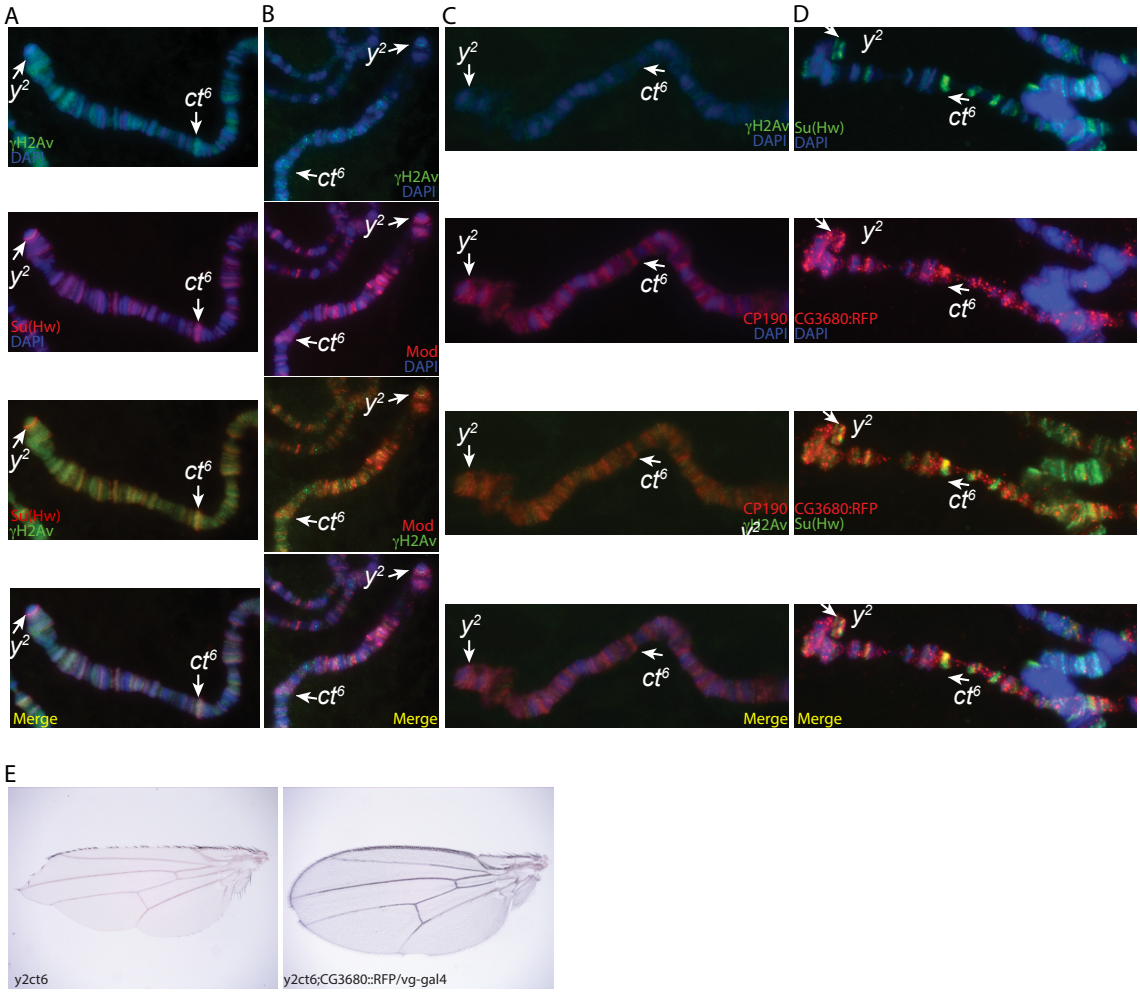

A
B
C
D
y^2
ct^6
γH2Av
DAPI
Su(Hw)
Mod
CP190
CG3680:RFP
Merge
E
y2ct6
y2ct6;CG3680::RFP/vg-gal4

Figure 1.7. Phosphorylation of H2Av results from the kinase activities of ATM and ATR . A-B. γH2Av is only slightly changed on polytene chromosomes from *tefu*$^{atm-3}$ and *mei41*^{D5} single mutants. Arrows indicate the insulator sites without γH2Av in single mutants. C. γH2Av almost totally disappears on polytene chromosomes in *tefu*$^{atm-3}$ and *mei41*^{D5} double mutant.

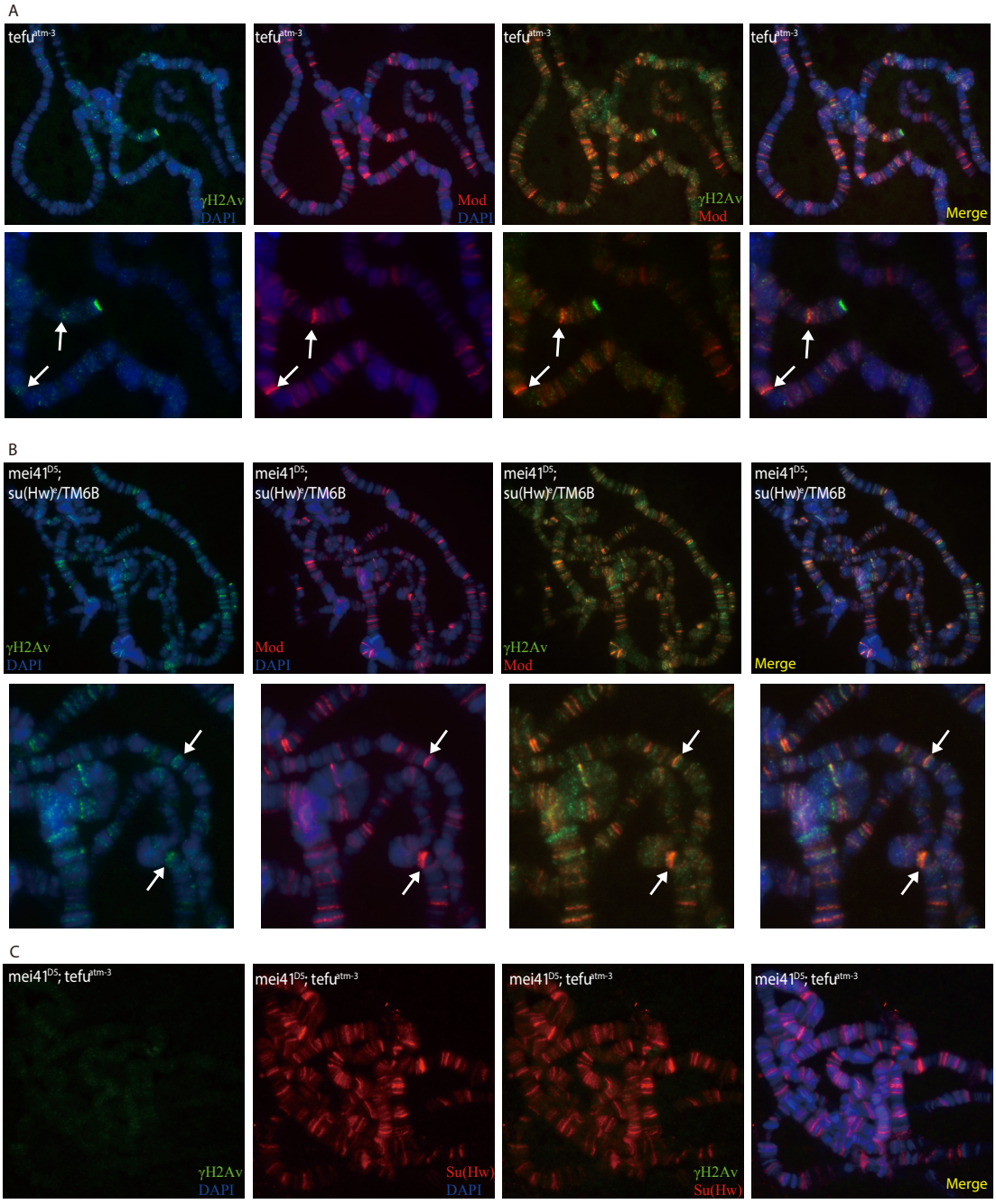
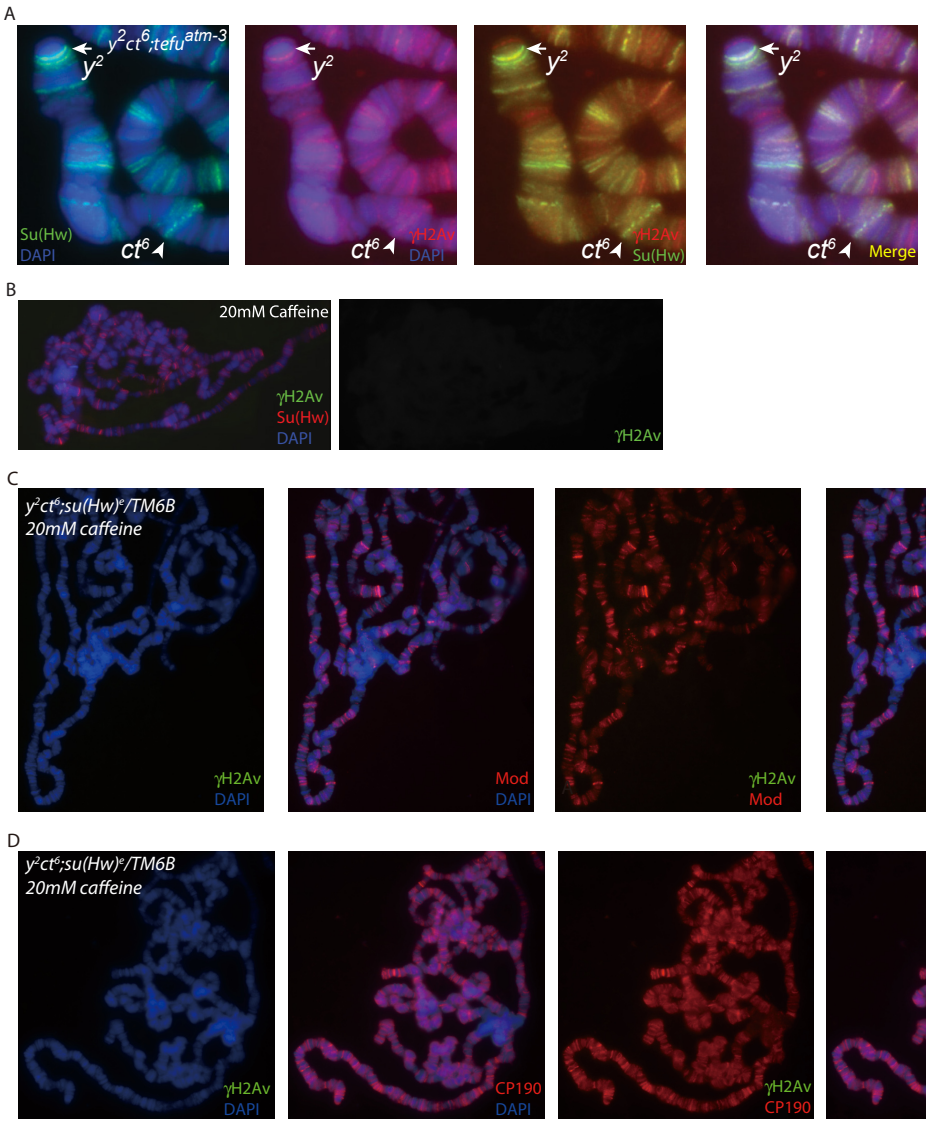

A
tefu atm-3
γH2Av
DAPI
tefu atm-3
Mod
DAPI
tefu atm-3
γH2Av
Mod
tefu atm-3
Merge
B
mei41 D5;
su(Hw) e/TM6B
γH2Av
DAPI
mei41 D5;
su(Hw) e/TM6B
Mod
DAPI
mei41 D5;
su(Hw) e/TM6B
γH2Av
Mod
mei41 D5;
su(Hw) e/TM6B
Merge
C
mei41 D5; tefu atm-3
γH2Av
DAPI
mei41 D5; tefu atm-3
Su(Hw)
DAPI
mei41 D5; tefu atm-3
γH2Av
Su(Hw)
mei41 D5; tefu atm-3
Merge

Figure 1.8. ATM mutant alone does not inhibit phosphorylation of H2Av at *y*^{2} and *ct*^{6} binding sites, while caffeine inhibits the overall phosphorylation of H2Av. A. γH2Av is still at *y*^{2} and *ct*^{6} sites, and co-localizes with Su(Hw) in *tefu*$^{atm-3}$ mutant. B-D. γH2Av totally disappears when the salivary gland is treated with 20mM caffeine for 3 hours, while insulator proteins Su(Hw) (B), Mod(mdg4)67.2 (C) and CP190 (D) still bind to chromosomes. Arrows indicate *y*^{2} and *ct*^{6} sites.


A
y2 y2ct6;tefuatm-3
Su(Hw)
DAPI
ct6
y2
γH2Av
DAPI
ct6
y2
γH2Av
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ct6
y2
ct6
Merge
B
20mM Caffeine
γH2Av
Su(Hw)
DAPI
γH2Av
C
y2ct6;su(Hw)e/TM6B
20mM caffeine
γH2Av
DAPI
Mod
DAPI
γH2Av
Mod
Merge
D
y2ct6;su(Hw)e/TM6B
20mM caffeine
γH2Av
DAPI
CP190
DAPI
γH2Av
CP190
Merge

Figure 1.9. ATM and ATR modulate levels of insulator proteins. A-B. Immunostaining on polytene chromosome with Su(Hw) and CP190 antibodies in *tefu*$^{atm-3}$*/TM6B* and *tefu*$^{atm-3}$ homozygous mutant. C-D. Immunostaining on polytene chromosome with Mod and Su(Hw) antibodies in *tefu*$^{atm-3}$*/TM6B* and *tefu*$^{atm-3}$ homozygous mutant. E-F. Immunostaining on polytene chromosome with Mod, Su(Hw) and CP190 antibodies in *mei41*^{D5}*;su(Hw)/TM6B* mutant. G-H. Western blot with 3rd instar larvae shows insulator protein levels change in *tefu*$^{atm-3}$ mutant (G) and *mei41*^{D5}*;su(Hw)/TM6B* single mutant (H).

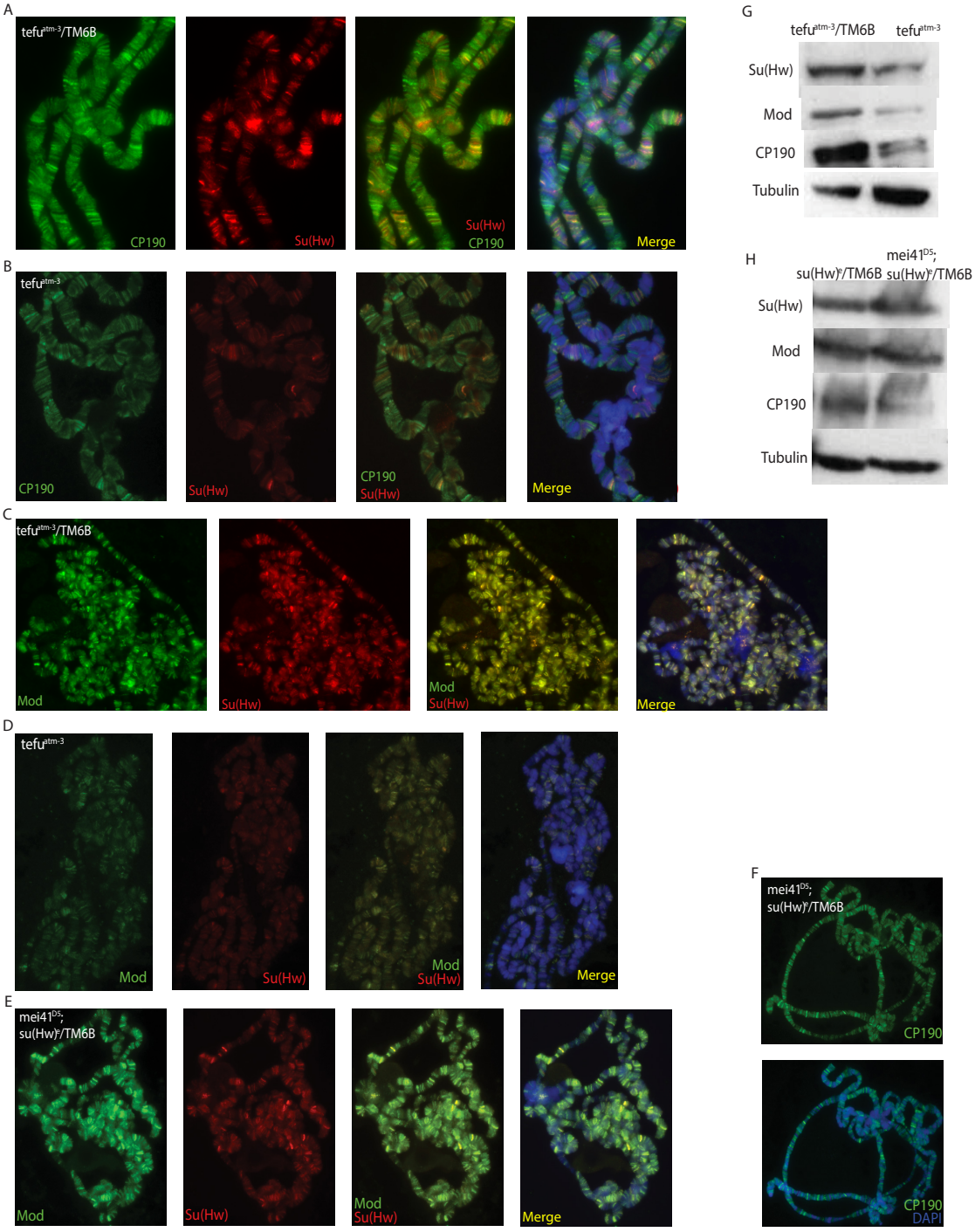
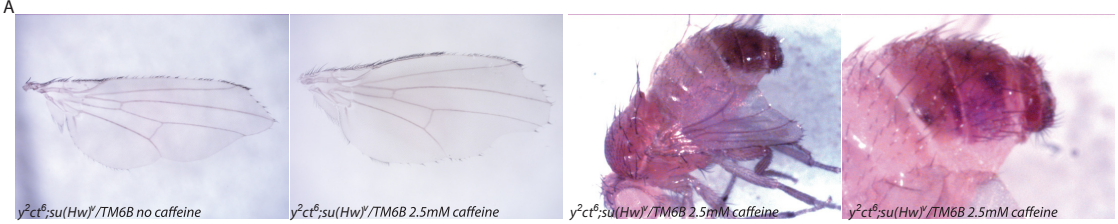

A
tefu^atm-3/TM6B
CP190
Su(Hw)
Su(Hw)
CP190
Merge
B
tefu^atm-3
CP190
Su(Hw)
CP190
Su(Hw)
Merge
C
tefu^atm-3/TM6B
Mod
Su(Hw)
Mod
Su(Hw)
Merge
D
tefu^atm-3
Mod
Su(Hw)
Mod
Su(Hw)
Merge
E
mei41^D5;
su(Hw)^v/TM6B
Mod
Su(Hw)
Mod
Su(Hw)
Merge
G
tefu^atm-3/TM6B
tefu^atm-3
Su(Hw)
Mod
CP190
Tubulin
H
mei41^D5;
su(Hw)^v/TM6B
su(Hw)^v/TM6B
Su(Hw)
Mod
CP190
Tubulin
F
mei41^D5;
su(Hw)^v/TM6B
CP190
CP190
DAPI

Figure. 1.10 Caffeine partially rescues y^2 and ct^6 mutant phenotypes. **A.** y^2 and ct^6 phenotypes are partially rescued by feeding with 2.5mM caffeine. B. γH2Av shows dramatically reduced levels in the polytene chromosomes from larvae generated by treatment with 2.5mM caffeine.

A


y2ct6;su(Hw)v/TM6B no caffeine
y2ct6;su(Hw)v/TM6B 2.5mM caffeine
y2ct6;su(Hw)v/TM6B 2.5mM caffeine
y2ct6;su(Hw)v/TM6B 2.5mM caffeine

B

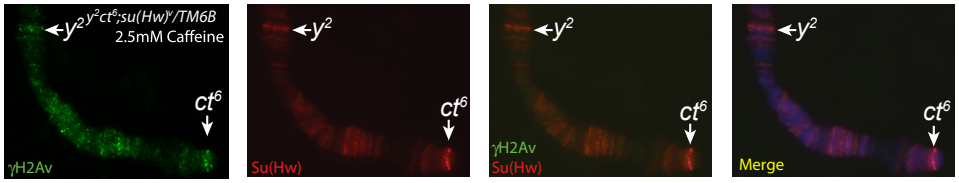

y2 y2ct6;su(Hw)v/TM6B
2.5mM Caffeine
ct6
γH2Av
y2
ct6
Su(Hw)
y2
ct6
γH2Av
Su(Hw)
y2
ct6
Merge

Figure 1.11. Okadaic Acid induces high levels of γH2Av at insulator sites. A-D. An increased level of γH2Av is shown in samples treated with OA (B) compared to the control (A). B. OA induced γH2Av co-localizes with Su(Hw). C. OA induced γH2Av co-localizes with Mod(mdg4). D. OA induced γH2Av co-localizes with and CP190. E. Immunostaining quantification confirms a significant increase in γH2Av after OA treatment.

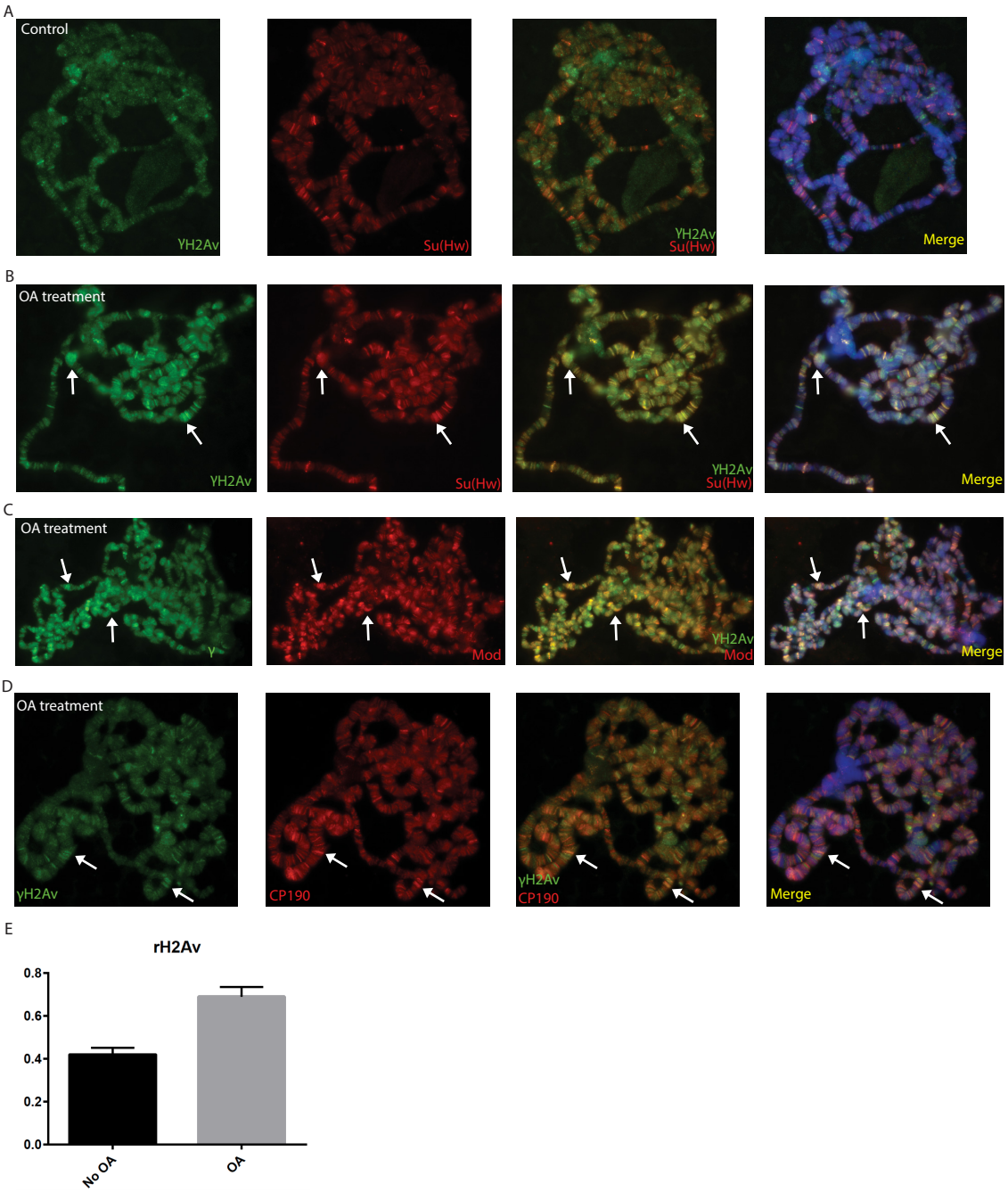
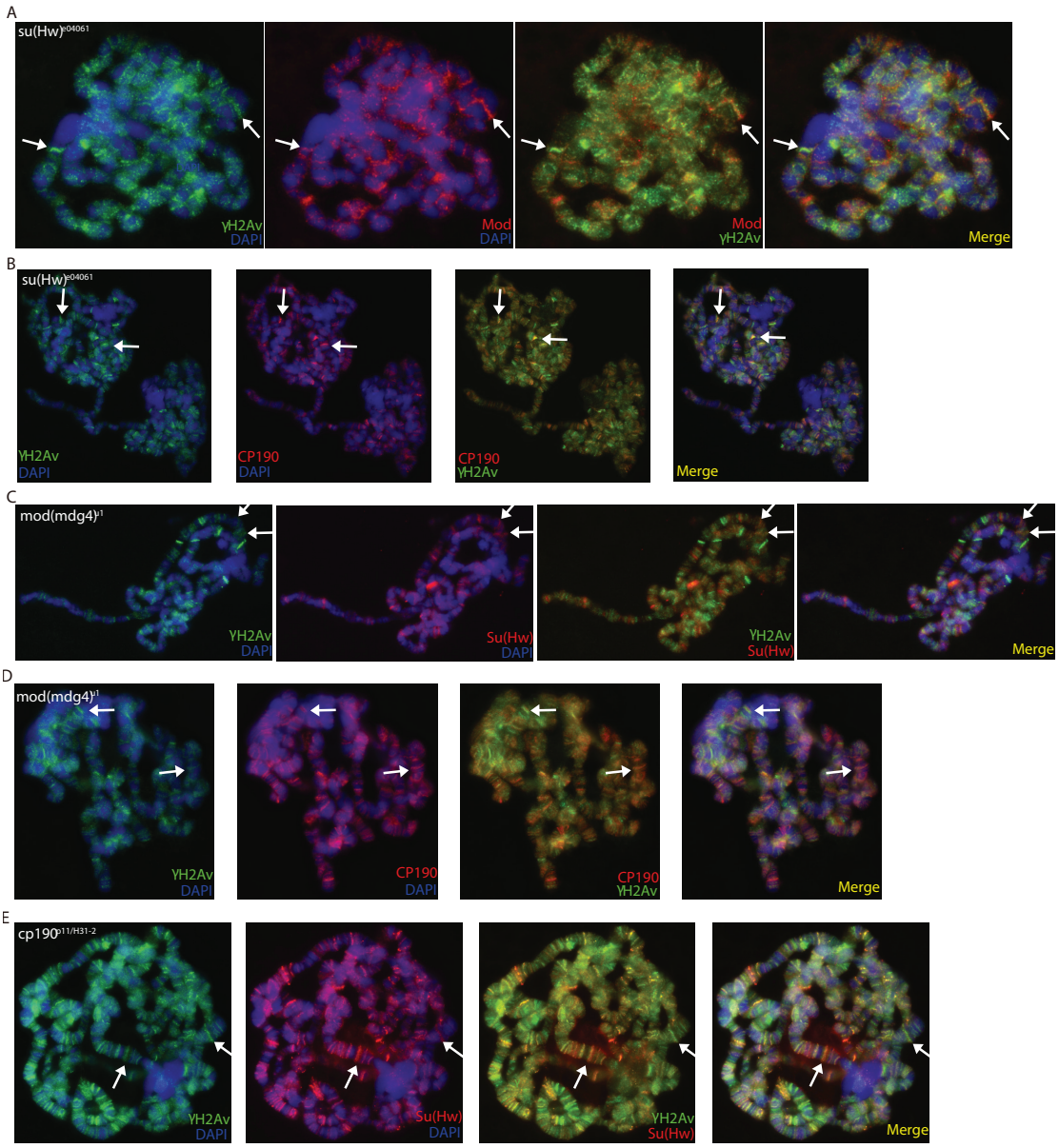

A
Control
γH2Av
Su(Hw)
γH2Av
Su(Hw)
Merge
B
OA treatment
γH2Av
Su(Hw)
γH2Av
Su(Hw)
Merge
C
OA treatment
γ
Mod
γH2Av
Mod
Merge
D
OA treatment
γH2Av
CP190
γH2Av
CP190
Merge
E
rH2Av
0.8
0.6
0.4
0.2
0.0
No OA
OA

Figure 1.12. γH2Av induced by OA treatment co-localizes with insulator proteins in *su(Hw)^{e04061}*, *mod(mdg4)^{u1}* and *cp190$^{p11/H31-2}$* mutants. A-B. Salivary glands dissected from larvae treated with OA for 3 hours in *su(Hw)^{e04061}* mutant. C-D. Salivary glands dissected from larvae treated with OA for 3 hours in *mod(mdg4)^{u1}* mutant. E. Salivary glands dissected from larvae treated with OA for 3 hours in and *cp190$^{p11/H31-2}$* mutant. Polytene chromosomes were co-immunostained with different insulator protein antibodies as indicated in the figure. Arrows indicate co-localizations between insulators and induced γH2Av.


A
su(Hw)e04061
γH2Av
DAPI
Mod
DAPI
Mod
γH2Av
Merge
B
su(Hw)e04061
γH2Av
DAPI
CP190
DAPI
CP190
γH2Av
Merge
C
mod(mdg4)u1
γH2Av
DAPI
Su(Hw)
DAPI
γH2Av
Su(Hw)
Merge
D
mod(mdg4)u1
γH2Av
DAPI
CP190
DAPI
CP190
γH2Av
Merge
E
cp190c11/H31-2
γH2Av
DAPI
Su(Hw)
DAPI
γH2Av
Su(Hw)
Merge

CHAPTER II

Chromatin Insulators Regulate DNA Replication and Cell Cycle Progression in Drosophila

Authors: Ran An, Todd Schoborg and Mariano Labrador

Author contributions:

(1). Ran An: Conceiving and designing experiments; performing experiments; collecting and analyzing data; making figures and writing the manuscript.

(2). Todd Schoborg: designing and making the HIPP1::mcherry, Su(Hw)::eGFP and H2Av::mcherry transgenic flies.

(3)Mariano Labrador: Conceiving and designing experiments; performing experiments; analyzing data and revising the manuscript.

Abstract

Chromatin insulators assist the proper organization of chromatin loops and genome architecture by mediating long-range interactions between distant genomic sites. Because the enhancer-promoter blocking and the barrier against heterochromatin spreading properties of insulators, they are considered genomic elements involved in the regulation of gene expression, and very little is known about their possible role in other genome functions. However, recent studies have shown a genome-wide overlap between insulators sites and DNA replication-related factors, as well as the co-immunoprecipitation of Suppressor of Hairy Wing (Su[Hw]) with proteins involved in the formation of origins of replication in *Drosophila*. These data link insulators with DNA replication, but no direct evidence is available involving insulators with DNA replication. In this work,

we provide additional evidence suggesting that Su(Hw) has a role in cell proliferation. We utilize polytene chromosomes as a tool to analyze the new HIPP1 insulator protein (HP1 and insulator partner protein-1) together with other insulator proteins and chromatin markers that are involved in DNA replication. We suggest that overexpression of Su(Hw) or HIPP1 in polytene chromosomes is an efficient system to study the role of different chromatin proteins during cell cycle. Our results provide strong evidence suggesting that HIPP1 and Su(Hw) may play a role in the activation of origins of replication, and provide new insights of how chromatin insulators may coordinate the process of DNA replication through the different stages of S phase and between euchromatin and heterochromatin in endoreplication.

Introduction

Chromatin insulators, or boundary elements, are specific DNA sequences bound by proteins that function by establishing functional chromatin domains in the genome. Insulators partition the genome in independent compartments arranging the tree-dimensional organization of the genome within the nucleus, which is required for proper gene expression regulation (Ghosh et al., 2001; Labrador and Corces, 2002b). Chromatin insulators were initially characterized by two common properties: the ability to block enhancer-promoter communication when located between enhancers and promoters, and the ability to prevent the spreading of

heterochromatin (Gerasimova and Corces, 2001; Gurudatta and Corces, 2009). These two properties function in the genome to counteract the spreading of silenced DNA into active DNA and to facilitate the proper interactions between distant regulatory sequences with their cognate promoters (Brasset and Vaury, 2005; Gaszner and Felsenfeld, 2006; Yang and Corces, 2012). Although the precise mechanism of insulator function remains unclear, all known insulator proteins function as structural scaffolds that facilitate DNA-protein and protein-protein interactions, such that they can stabilize contacts between distant insulator sequences in the genome, therefore creating chromatin loops (Labrador and Corces, 2002a). It is thought that these loops facilitate and prevent selective contacts between distant sites in the genome, therefore orchestrating interactions between DNA promoters and transcription factors that enable specificity in the transcription program during development and in differentiated tissues. However, little is known whether this chromatin organization facilitates or inhibits the process of DNA replication through the doubling of the genome during the cell cycle.

Insulator function is conserved in eukaryotes, from yeasts to humans (Schoborg and Labrador, 2010). In *Drosophila*, one of the best characterized insulators is the gypsy insulator, which was initially described as a structural component found at the 5' untranslated sequence of the *Gypsy* retrotransposon genome (Spana et al., 1988). The gypsy insulator contains 12 binding sites specific for the suppressor of Hairy wing [Su(Hw)] protein, which in addition to *Gypsy*

retrotransposons, binds at thousands of endogenous Su(Hw) insulator sites through the genome (Parkhurst and Corces, 1986; Spana et al., 1988). In addition to Su(Hw), the gypsy insulator as well as multiple Su(Hw) insulators through the genome, require the function of other insulator protein components, such as Modifier of mdg4-67.2 [Mod(mdg4)67.2] and Centrosomal Protein 190 (CP190) (Gerasimova et al., 2000; Gerasimova and Corces, 1998; Pai et al., 2004). Furthermore, the HIPP1 protein (HP1 and Insulator Partner Protein 1) has been recently shown to co-immunoprecipitate with Su(Hw) and co-localize with Su(Hw) insulators sites through the genome, suggesting HIPP1 is a new component of the Su(Hw) insulator (Alekseyenko et al., 2014). The HIPP1 sequence predicts the presence of a crotonase domain closely related to the crotonase domain found in the human CDY protein. Interestingly, human CDY has been shown to be able to acetylate histones H4 and H2A in vitro (Holden et al., 2001; Lahn et al., 2002; Wu et al., 2009). Although the specific function of this domain in chromatin in vivo is still unclear, the structure of the crotonase and its in vitro activity suggests that it may be involved in histone acetyltransferase activity, which, if confirmed, will make HIPP1 the first example of a protein with an enzymatic activity present in a chromatin insulator complex.

Besides their well established role in transcription regulation, recent published findings suggest that insulators may also be involved in DNA replication and cell proliferation, given the significant overlap in the binding site distribution of several insulator proteins with binding sites of replication factors such as DREF and

GCN5 in the genome (Gurudatta et al., 2013; Vorobyeva et al., 2013). In support of this idea it has also been shown that Su(Hw) co-immunoprecipitates with origin replication complex proteins (ORC) and that Su(Hw) binding sites overlap with origins of replication through the genome (Vorobyeva et al., 2013). More interestingly, recent data from our laboratory has revealed that ovaries from *su(Hw)* mutant females show a significant increase in the levels of histone H4K20 monomethylated (H4K20me1), simultaneously with a significant increase in phosphorylated histone H2Av. Both observations, in combination with other findings supporting that these mutants are undergoing DNA damage and repair, suggest that lack of Su(Hw) may lead to replication stress during oogenesis in *Drosophila* (submitted for publication) (Tardat et al., 2010).

The finding that H4K20me1 is elevated in these mutants is particularly interesting, because H4K20me1 has been shown to have an important role in maintaining genome stability (Beck et al., 2012b; Jorgensen et al., 2013; Wu and Rice, 2011). Monomethylation of H4K20 is mediated by the PR-Set7/ SET8, a phylogenetivally conserved methyltransferase that is required for normal cell cycle progression, and mutations of *pr-set7* result in DNA damage and S phase arrest in *Drosophila* (Jorgensen et al., 2007b). On the other hand, overexpression of PR-Set7 causes accumulation of H4K20me1 at origins of replication and leads to replication stress (Jorgensen et al., 2007b; Tardat et al., 2010a). Depletion of PR-Set7 in Drosophila S2 cells also causes defects in chromosome compaction and triggers DNA damage response (Sakaguchi et al.,

2012; Sakaguchi and Steward, 2007). Additionally, mutations in *pr-set7* lead to small size tissues such as wing discs and salivary glands in Drosophila, and contain fewer cells due to improper cell division during development (Karachentsev et al., 2007; Karachentsev et al., 2005). These evidence suggest that misregulation of H4K20me1 can lead to genome instability in a manner dependent on cell cycle and DNA replication.

The finding that loss of Su(Hw) causes an increase in H4K20me1 supports the idea that Su(Hw) may play a role in DNA replication and cell cycle progression. Here we show that HIPP1 protein co-localizes with Su(Hw) in polytene chromosomes and is also found in insulator bodies after osmotic stress, and that both Su(Hw) and HIPP1 play a role in DNA replication and cell proliferation in *Drosophila*. Overexpression of both Su(Hw) and HIPP1 result in arrest of the cell cycle, which is helpful for the detection of cell cycle regulated proteins. Our findings reveal novel insights into the possible function of Su(Hw) and HIPP1 regulating the activity of origins rereplication in Drosophila.

Materials and Methods

Fly Stocks & Husbandry

All stocks and crosses were cultured using standard cornmeal-agar media at 25°C. Transgenic lines generated by Genetivision (Houston TX): yw; P{Su(Hw)::eGFP,w^+}, yw; P{HIPP1::mcherry, w^+} and yw; P{H2Av::mcherry, w^+}.

The lines obtained from the Drosophila Bloomington Stock Center at Indiana University: w*; P{GAL4-vg.M}2; TM2/TM6B, Tb^1(Stock #6819). *w^{1118}; PBac(RB)su(Hw)^{e04061}/TM6B, Tb^1*(Stock #18224), w^{1118}; P{UAS-GFP.E2f1.1-230}32 P{UAS-mRFP1.CycB.1-266}13/CyO, P{en1}wg^{en11}; MKRS/TM6B, Tb^1(Stock #55117). Other lines given as gifts: *y^2wct^6; cp190^{H31-2}/TM6B, Tb^1*. *cp190^{p11}/TM6B, Tb^1*. *su(Hw)^v/TM6B, Tb^1*. *mod(mdg4)^{u1}*(Victor Corces, Emory University); *yw; Hsp70-Gal4/Cyo. yw; Actin-Gal4/TM6C, Sb^1, Tb^1* (Bruce McKee, University of Tennessee). GMR-Gal4 (Tom Dockendorff, University of Tennessee). UAS-p35; Dr^{Mio}/TM6B, Tb^1 (Jae Park, University of Tennessee, Knoxville).

Polytene Chromosome Immunostaining

Salivary glands from early third instar were dissected in insect media (HyClone SFX; Thermo Fisher Scientific), and fixed immediately with 4% PFA/50% Acetic acid on a cover slide. Cover a microscope slide on the fixed salivary gland and squash it until the polytene chromosomes are well spread out under the microscope. Dip the microscope slide in the liquid nitrogen to remove the cover slide. Block the polytene chromosomes for 10 minutes at room temperature (RT) in the blocking solution (PBS+0.1%NP40+ 3%nonfat milk). Primary antibodies were diluted in the blocking solution with ratio of 1:200, and incubated with the slide overnight at 4°C in a humidified chamber. Once finished, wash out the primary antibody with washing buffer (PBS+0.1%NP40) for 10 minutes at RT. Secondary antibodies were then diluted in the blocking solution with ratio of

1:200, incubated with the slide for 1 hour at RT, and washed as described before. 4', 6-diamidino-2-phenylindole (0.5μg/ml DAPI) was used to stain the DNA for 30 seconds and resin with PBS. Finally mount the slide with Vectashield mounting medium (Vector Laboratories) and seal the slides with nail polish.

Slides were analyzed using a wide-field epifluorescence microscope (DM6000 B; Leica) equipped with a charge-coupled device camera (ORCA-ER; Hamamatsu Photonic) and a HCX Plan Apochromat (Leica) 100×/1.35 NA oil immersion objective. Image acquisition was performed using SimplePCI (v6.6; Hamamatsu Photonics). Samples were processed and imaged under identical conditions of immunostaining, microscope, camera and software settings.

S2 Cells Staining with Osmotic Stress

S2 cells were incubated in insect medium (HyClone SFX; Thermo Fisher Scientific) supplemented with penicillin and streptomycin in a 25°C incubator. Sodium Chloride (NaCl) was added to the cell culture to make the final concentration to 250mM for 30 minutes. Cover slides were pretreated with ethanol and coated with concanavalin A, which allowed S2 cells to adhere. Drop cells on treated coverslips and let cells spread. Cells were fixed with 4% PFA for 10 minutes at RT, followed by 3 washes with PBS. Fixed Cells were permeabilized with 0.2% Triton X-100, and washed twice with PBS. Permeable cells were then incubated in the blocking solution (3% milk in PBS) at RT, and added primary antibodies diluted in the blocking solution overnight at 4°C in a

humidified chamber. Washing buffer (PBS+0.1% Triton X-100) were used to wash off unbound antibodies. Secondary antibodies incubation, DAPI staining, and slides mounting are the same as described before.

Antibodies

The antibodies generated in our laboratory include rat and rabbit anti-Su(Hw), anti-Mod(mdg4)67.2 and anti-CP190 polyclonal IgG antibodies(Schoborg et al., 2013a; Wallace et al., 2010). The antibodies are commercial available including rabbit anti-RFP IgG (A00682, Genscript), monoclonal anti-α-tubulin IgG, rabbit anti-Histone H4 (mono methyl K20) IgG (ab9051, Abcam), monoclonal anti-PCNA IgG (PC10, Abcam), monoclonal C1A9 (Heterochromatin Protein 1) (DSHB) and rabbit anti-GFP (A11122, Invitrogen) antibodies. All the primary antibodies were diluted as a ratio of 1:200 for immunostaining, and 1:5000 for western blot. Secondary antibodies Donkey FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.), Texas red-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.); Donkey Alexa Fluor 488-conjugated anti-rabbit IgG (A-21206, Life Technologies), and Donkey Alexa Fluor 555-conjugated anti-rabbit IgG (A31572, Life Technologies) were used as a ratio of 1:200 for immunostaining. Peroxidase-conjugated affinipure goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) and Peroxidase-conjugated affinipure goat anti-Rb IgG (Jackson ImmunoResearch Laboratories) were used as a ratio of 1:5000 for western blot.

Western Blot

Early third stage instars were collected and homogenized in RIPA lysis buffer with protease inhibitor (Roche) and phosphatase inhibitors (Sigma) on ice. Lysates were resolved in a 8%-15% acrylamide gel, wet transferred at 4°C overnight (10-15V) and probed with primary antibodies and secondary antibodies as described above.

Results

HIPP1 co-localizes with Su(Hw) in polytene chromosomes and forms insulator bodies

HIPP1 is a newly identified protein that binds pericentric heterochromatin and Su(Hw) insulator sites in S2 cells in *Drosophila* (Alekseyenko et al., 2014). HIPP1 contains a crotonase-fold domain, which is conserved in mammals and catalyzes a wide range of metabolic reactions employing different mechanisms (Alekseyenko et al., 2014; Holden et al., 2001). A structural study of the human CDY protein, the closest relative to HIPP1, shows the presence of an “oxyanion hole”, which is the feature that defines the crotonase superfamily. The oxyanion hole is essential to stabilize an enolate anion intermediate derived from an acyl-CoA substrate, which mediates acetylation processes but little is known about its function(Holden et al., 2001; Wu et al., 2009).

In *Drosophila*, pull-down experiments have shown that HIPP1 co-immunoprecipitates with Su(Hw), Mod(mdg4), Cp190 and HP1 among other proteins(Alekseyenko et al., 2014). These interactions suggest that HIPP1 may have a role in insulator function. To address the question of whether HIPP1 has insulator function, we started by generating transgenic flies expressing HIPP1 fused to mCherry, under the control of an UAS promoter (UAS-HIPP1::mc). Experiments presented elsewhere (manuscript in preparation) revealed that expression of HIPP1::mc in a wildtype background can disrupt the insulator enhancer-blocking function of the gypsy insulator at the $yellow^2$ and cut^6 loci (y^2 and ct^6) respectively. We used this construct to co-express HIPP1::mc with Su(Hw)::eGFP, which we previously developed in the laboratory (Hsu et al., 2015; Schoborg et al., 2013a). We co-expressed HIPP1::mc and Su(Hw)::eGFP using *vestigial-Gal4 (vgBE-Gal4)* driver expressed in the salivary glands and a metallothionein promoter induced with 500 μM $CuSO_4$, in transgenic flies and S2 cells, respectively. We used antibodies directed against mcherry and eGFP, to detect HIPP1::mc and Su(Hw)::GFP, respectively. As expected, HIPP1::mc and Su(Hw)::GFP perfectly co-localize on polytene chromosomes, suggesting that polytene chromosomes could be used to study the distribution and function of Su(Hw) and HIPP1 *in vivo* (Fig. 2.1A). Expression of HIPP1::mc and Su(Hw):GFP in S2 cells, show a uniform nuclear distribution (Fig. 2.1C), identical to the distribution of insulator proteins normally observed in unstressed cells that are in interface (Schoborg et al., 2013b).

We have previously shown that all Drosophila insulator proteins, including Su(Hw), dissociate from chromatin to form large protein foci in the interchromosomal spaces of the nucleus in response to osmotic stress (Schoborg et al., 2013b).Then we asked whether HIPP1 also forms insulator bodies in response to osmotic stress. We treated S2 cells co-expressing HIPP1:mc and Su(Hw)::GFP with 250mM NaCl for 30 min, and used antibodies anti-mCherry and anti-GFP to determine the location of both proteins during osmotic stress. Results show that HIPP1:mc co-localizes Su(Hw)::GFP forming bodies identical to the insulator bodies previously identified for all known insulator proteins (Capelson and Corces, 2005; Schoborg et al., 2013b). This result further supports that HIPP1 has insulator protein properties and responds to osmotic stress forming insulator bodies (Fig. 2.1B).

HIPP1 and Su(Hw) expression and localization in chromosomes might be regulated by cell cycle

Next, we analyzed the localization of HIPP1::mc in polytene chromosomes. Surprisingly, HIPP1 shows a variable distribution on chromosomes when different nuclei are compared. There are 4 distinct major distribution patterns of HIPP1 in polytenes: A) It binds consistently in the interband region of the chromosomes; B) it binds only to band DNA; C) appears as a diffused pattern that spans bands and interbands equally; and D) some nuclei show a complete absence of the protein (Fig. 2.2A-C). More surprisingly, in salivary glands overexpressing of HIPP1::mc that are wildtype for Su(Hw), we found the

endogenous Su(Hw) protein in some nuclei localized exclusively in interbands, a pattern that is never found in wildtype nuclei (Fig. 2.2D). These data suggests that HIPP1 is post-translationally regulated and that its genome-wide binding sites may be variable in a manner that depends on the specific stages of the cell cycle, and can affect the binding sites of Su(Hw).

Subsequently, we analyzed the distribution of overexpressed Su(Hw)::GFP fusion protein using anti-GFP antibodies. Interestingly, we find that Su(Hw)::GFP shows a localization pattern that is different from that of the endogenous Su(Hw). In most cells Su(Hw) appears to be associated exclusively with DNA in interbands (Fig. 2.2E), a localization that is different from that found in wildtype. In wildtype polytene cells, Su(Hw) localizes mostly at the boundaries between bands and interbands, but it frequently appears directly associated with bands, and only in some sites it associates with interbands (Ghosh et al., 2001; Wallace et al., 2010). In a manner similar to HIPP1::mc, the distribution pattern observed in cells overexpressing Su(Hw)::GFP is not common to all nuclei, and some nuclei show a localization of Su(Hw) that is identical to that of wildtype, losing the predominant association with interbands. Remarkably, colocalization of HIPP1::mc with Su(Hw) only occurs in certain nuclei, suggesting that interactions are also regulated by the cell cycle. These results suggest that the levels of Su(Hw), as well as its location in chromosomes, not only depends on the transcriptional output, but the protein is also regulated post-transcriptionally. Collectively these data suggests that both Su(Hw) and HIPP1 are post-

translationally regulated and that their binding sites are variable, seemingly depending on the genetic background or on the specific stages of the cell cycle.

Overexpression of Su(Hw) and HIPP1 results in cell proliferation defects

Experiments described above suggested that changes in the amount and distribution of insulator proteins could respond to changes in the cell cycle stages, and were performed in salivary glands from third instar larvae and S2 cells, expressing Su(Hw):GFP and HIPP1::mc under the control of mild Gal4 drivers or inducible promoters, respectively. To further study the role of HIPP1 in insulator function we utilized Gal4 drivers that specifically direct transgene expression in different tissues and during development. First, we used an actin-Gal4 driver that express Gal4 in developing embryos as well as in larvae and adult tissues. We collected first stage larvae expressing either actin-Gal4>HIPP1::mc or actin-Gal4> Su(Hw):GFP, at 24 hours after ovoposition, and let larvae grow during 4 days in cornmeal-agar before assessing their relative growth, and used actin-Gal4>H2Av::mc larvae as a control. Surprisingly, whereas the 4 days control actin-Gal4>H2Av::mc larvae grew to a normal size of approximately 5-6 mm, 4 days larvae expressing either actin-Gal4>Su(Hw):GFP or actin-Gal4>HIPP1::mc transgenes, produced larvae significantly smaller in size. Specifically, larvae expressing actin-Gal4>HIPP1::mc grew to a size of approximately 3-4 mm or 50% of the normal size, whereas actin Gal4>Su(Hw)::GFP produced larvae of approximately 1 mm, barely larger than

1st instar larvae. In both instances larvae appear healthy and with normal movements but they were unable to produce viable pupae (Fig. 2.3A).

Next, we used *GMR-gal4*, which drives transgene expression under the control of GMR (Glass Multiple Reporter) promoter elements specifically in the developing eye, and *vestigial-B-Enhancer-Gal4* (*vgBE-Gal4*), which drives expression of transgenes in a small stripe of cells at the dorsal/ventral boundary in developing wing and haltere imaginal discs. Both GMR-Gal4>Su(Hw)::GFP and GMR-Gal4>HIPP1:mc overexpressing flies show defects in the eye (Fig. 2.3B). Specifically, Su(Hw)::GFP expression causes the stronger effect, producing a rough eye, slightly smaller than wild type and with an irregular distribution of ommatidia. Interestingly, all interommatidial bristles are missing in GMR-Gal4>Su(Hw)::GFP eyes, altogether suggesting that overexpression of Su(Hw) causes a reduction in the number of cells in the eye that can have strong effect in specific lineages (Fig. 2.3B). GMR-Gal4>HIPP1:mc flies revealed a phenotype similar to that of GMR-Gal4>Su(Hw)::GFP flies, but less severe, showing slightly reduced eyes, with only patches of missing bristles (Fig.2.3B). Interestingly, flies overexpressing both GMR-Gal4>Su(Hw)::GFP and GMR-Gal4>HIPP1:mc, reveal a more dramatic phenotype that includes severely reduced size, irregular distribution of ommatidia producing a rough eye, and a complete absence of interommatidial bristles. In addition, eyes overexpressing both proteins also show formation of small patches of necrotic tissue that are missing when each protein is expressed individually (Fig. 2.3B).

Overexpression of Su(Hw)::eGFP driven by *vgBE-gal4* driver, revealed a "*cut*" like wing phenotype, showing missing bristles and scalloped wing margins, with most defects concentrated in the posterior part of the wing. This phenotype is very similar to phenotypes of *cut* mutants (Jack and DeLotto, 1992), which suggests a lack of cell proliferation similar to the one observed in the eye is occurring in the developing wing (Fig. 2.3C). To investigate the possibility that these defects may result from induction of apoptosis, we used co-expression of a UAS-p35 transgene. The baculovirus P35 protein expressed in *Drosophila* is able to completely inhibit apoptosis (Hay et al., 1994). Results show that the scalloped phenotype is only partially suppressed, suggesting that the developmental defects might include both, an increase in apoptosis and a defect in cell proliferation and that Su(Hw) may play a role in cell cycle regulation (Fig. 2.3C). On the other hand, overexpression of HIPP1::mc using vg-Gal4 driver did not show any visible phenotype in the margin of the wing, in consonance with our previous results showing that overexpression of HIPP1 produces milder phenotypes than overexpression of Su(Hw). In addition, we tested co-expression of both in vg-Gal4<Su(Hw)::eGFP; vg-Gal4<HIPP1::mc flies, and results support the notion that both insulator proteins may have additive effects, since the scalloped wing phenotype is more pronounced in wings with double expression. Collectively these results suggest that both Su(Hw) and HIPP1 likely function in parallel pathways involved in cell cycle, such that overexpression inhibits proliferation. However, it is difficult to discern the specific influence that apoptosis and cell proliferation have in these phenotypes.

To further dissect the contribution that defects in cell cycle, cell proliferation and apoptosis have in the production of these phenotypes, we decided to test overexpression of both Su(Hw) and HIPP1 in salivary glands. Salivary glands in *Drosophila* undergo apoptosis only during pupation and after metamorphosis. Before pupation, apoptosis is blocked in salivary glands by high levels of the *Drosophila* inhibitor of apoptosis 1 (DIAP1), such that ectopic expression of the inducer of apoptosis *reaper* (*rpr*) fails to produce cell death (reference: Viravuth P. Yin, Carl S. Thummel, and Arash Bashirullah JCB, 2007). Therefore, considering the results described above, we reasoned that overexpressing Su(Hw) or HIPP1 in salivary glands will have negligible activation of apoptosis effects, and the resulting phenotype might provide clues of the influence of these proteins in the cell cycle. Remarkably, overexpression of both HIPP1::mc and Su(Hw)::GFP using the actin-Gal4 driver produce very small salivary glands, which are difficult to dissect. For this reason, we used a *hsp70*-Gal4 driver that, in the absence of heat shock, is leaky enough only in salivary glands to produce healthy and normal size larvae with a significant expression of UAS transgenes in the salivary glands.

Interestingly, results show that both *hsp70*-Gal4<HIPP1::mc and *hsp70*-Gal4<Su(Hw)::GFP determine salivary glands of completely normal appearance, but significantly reduced in size (data not shown), where Su(Hw)::GFP glands are much smaller than HIPP1::mc overexpressing glands. In addition, we counted the number of nuclei in both samples and wildtype, and conclude that

the number of cells in HIPP1::mc and Su(Hw)::GFP overexpressing salivary glands is not significantly different from the number of cells in wildtype (data not shown). However, the size of each cell and each nuclei is significantly reduced in size, when compared with that of wildtype (data not shown). We concluded that apoptosis could not explain the difference in salivary gland size and that this difference is due to undereplication of cells overexpressing either HIPP1::mc or Su(Hw)::GFP. Since salivary glands appear to be healthy and we found no evidence of cell dead, and because the size of salivary gland cells correlates with the number of endoreduplications (Orr-Weaver, 2015), our results suggest that overexpression of Su(Hw) and HIPP1 extend the time required for genome duplication, completing a cell division cycle significantly less times than wildtype cells over the same time.

Overexpression of HIPP1 and Su(Hw) result in cell cycle progression defects

Results described above suggest HIPP1 and Su(Hw) overexpression result in a defect in cell proliferation in larval bodies, fly eyes, wings and salivary glands. To further understand the effect that overexpression of these insulator proteins have in cell cycle, we used a *Drosophila*-specific FUCCI expression system (Fly-FUCCI), which allows one to distinguish different phases in cell cycle (Zielke et al., 2014). The Fly-FUCCI relies on fluorochrome-tagged degrons from the Cyclin B and E2F proteins, which are degraded by the ubiquitin E3-ligases APC/C and $CRL4^{Cdt2}$, during anaphase and S phase respectively. The tagged degron

fragments of Cyclin B::mc and E2F::GFP are expressed under the control of a UAS promoter. We first used the *vg-gal4* driver to drive the expression of Fly-FUCCI Cyclin B::mc and E2F::GFP in salivary glands. Results show that very few nuclei show E2F::GFP signals, and CycB::mc could not be detected in the cytoplasm of any cell. We reasoned these results might in part be explained by the low ability of *vg-gal4* to drive gene expression on salivary glands (Fig. 2.4A).

However, in experiments co-expressing Su(Hw)::GFP simultaneously with Fly-FUCCI using the same *vg-gal4* driver, CycB::mc is clearly observed in the cytoplasm of numerous cells, indicating that these cells might now be arrested or temporarily arrested at the S phase(Fig. 2.4C). On the contrary, co-expression of HIPP1::mc with Fly-FUCCI results in E2F::GFP clearly observed in the nuclei of multiple salivary gland cells, indicating that these cells are arrested or temporarily arrested at the G1 phase or G1-S transition phase (Fig. 2.4B). As suspected, these results suggest that overexpression of HIPP1 and Su(Hw) can modify the normal progression of the cell cycle, for example by prolonging the duration of certain stages, such as G1 or S phase. To further understand the effect of insulator proteins in cell cycle progression, we used *hsp70-gal4* driver, which strongly drives gene expression on salivary glands. Results show that wild type salivary glands expressing Fly-FUCCI with hsp70-gal4 driver, E2F:GFP is detected in most nuclei, while very few cells show CycB::mc in the cytoplasm, confirming results obtained with vg-Gal4 and corroborating that in 3rd instar larva most nuclei are at G1 phase (Fig. 2.4D). However, overexpression of

Su(Hw)::GFP by *hsp70-gal4*, which produces small salivary glands, results in a large number of cells expressing CycB::mc in the cytoplasm, indicating that these cells are either in G1-S transition or in S phase (Fig. 2.4F). Interestingly, overexpression of HIPP1::mc using *hsp70-gal4*, which also causes cell proliferation defects and small salivary glands, results in salivary glands with most nuclei showing strong E2F::GFP signals, suggesting the nuclei are arrested at either G1 phase or at the G1-S transition phase. Collectively, these results suggest that overexpression of HIPP1 and Su(Hw) arrest progression of the cell at G1 or early S phase.

Though the Fly-FUCCI analysis results support that there are cell cycle progression defects associated with overexpression of Su(Hw) and HIPP1, it is still arguable whether the use of Fly-FUCCI is reliable in *Drosophila* endocyclic cells, since polytene chromosomes undergo endoreduplication without mitosis, and CycB is normally not expressed in cells undergoing endocycling (Fox and Duronio, 2013; Lilly and Duronio, 2005). Moreover, because HIPP1 and Su(Hw) are also tagged, only one marker can be used in the Fly-FUCCI system, making it impossible to determine for example if Su(Hw)::GFP overexpressing cells are also expressing E2F:GFP, making it difficult to determine whether the cell cycle is arrested at a certain phase, at a transition phase or whether E2F:GFP and CyB::mc are misregulated altogether and do not respond normally to the different phases of the cell cycle.

An alternative approach to test the effect of insulator proteins in the cell cycle and DNA replication is to use proliferating cell nuclear antigen (PCNA) as a marker. PCNA is a protein clamp that ensures high processivity in DNA synthesis during DNA replication and repair (Oda et al., 2010; Zielke et al., 2011). Using an antibody specific against PCNA, we first analyzed the distribution of PCNA in polytene chromosomes from wildtype third instar larvae. Results show that most nuclei lack significant signal, and in those nuclei with signal, PCNA locates in heterochromatic regions, mostly associated with condensed DNA at chromosome bands and at pericentric heterochromatin. After analyzing a large number of nuclei from different salivary glands, we could never detect PCNA in euchromatin, when we define euchromatin as chromatin associated with interband DNA (Fig. 2.5A). However, after overexpression of either Su(Hw)::GFP or HIPP1:mc with *vgBE-Gal4* driver, PCNA distribution is not found on heterochromatin or in bands. PCNA appears instead on the euchromatic region of chromosomes, suggesting the cell cycle is actually arrested at the S phase, once PCNA has initiated replication (Fig. 2.5B and 2.5C, Table. 1). Taken together, these data strongly suggests overexpression of Su(Hw) or HIPP1 result in cell cycle progression defects.

Overlapping distributions of monomethylated H4K20 and HP1 on polytene chromosomes depend on the cell cycle

To further analyze the role of Su(Hw) and HIPP1 in cell proliferation and cell cycle progression, we used proteins previously reported to be involved in DNA

replication as markers to help determine the effect of Su(Hw) and HIPP1 overexpression in the cell cycle. The histone H4 monomethylated at lysine 20 (H4K20me1), is essential for regulation of DNA replication (Karachentsev et al., 2005). Generally, the initiation of DNA replication is governed by the licensing of replication origins. The licensing process consists in the assembling of a pre-replication complex on replication of origins by ORC proteins and the MCM2-7 helicase complex. Initiation of DNA replication depends on a licensing mechanism that, among other events, requires the H4K20me1 by the histone monomethyltransferase Pr-Set7/Set8 during G1 phase. After firing, or activation of transcription, Pr-Set7 is degraded by a CRL4(Cdt2)-mediated PCNA-dependent process during S phase, which contributes to the removal of H4K20me1 at replication origins and the inhibition of further replication licensing until next cycle (Oda et al., 2010; Tardat et al., 2010). These processes were initially demonstrated in mammalian cells. However, a recent study suggests that dPr-Set7, the only H4K20 monomethyltransferase in *Drosophila*, interacts with PCNA, suggesting the existence of a similar mechanism in the role of H4K20me1 in *Drosophila* DNA replication (Sahashi et al., 2014). HP1, on the other hand, has been shown to be involved in firing of origins of replication in both in Drosophila and in fission yeasts (Fragkos et al., 2015; Hayashi et al., 2009; Schwaiger et al., 2010). In fission yeasts HP1 has been shown to recruit cdc7 to origins of replication, a step necessary for activation of pre-Replication complexes, and in Drosophila, HP1 seems to play a role in the activation of transcription in both euchromatin regions rich in DNA repeats and in pericentric heterochromatin

(Hayashi et al., 2009). Interestingly, it was early proposed that H4K20me2 mediated heterochromatin formation through interactions with (HP1) during replication of heterochromatin in *Drosophila* (Swaminathan et al., 2005), and H4K20me2 (H4K20 dimethylation) is only produced after methylation of H4K20me1 by Suv4-20 methytransferase.

Based on earlier studies showing that polytene chromosomes can be used to identify different stages of the cell cycle (Kolesnikova et al., 2013), we decided to characterize the distribution of H4K20me1 and HP1 during cell cycle using immunostaining in polytene chromosomes. Interestingly, we find that in wildtype, both H4K20me1 and HP1 show different distribution on chromosomes when different nuclei are compared: In some nuclei, both H4K20me1 and HP1 are found associated with bands on polytene chromosomes, whereas in other nuclei they associate with interbands. Finally, in certain nuclei we find the signal of both markers diffused and associated with bands as well as with interbands (Fig. 2.6). These results suggest that the distribution of H4K20me1 and HP1 could cell cycle regulated. More interestingly, except for the pericentromeric region, HP1 is always found in association with H4K20me1 in bands as well as in interbands. However this association does not reflect colocalization of both proteins but rather show a side-by-side juxtaposition of dots that align parallel to the bands in the chromosomes, suggesting that H4K20me1 and HP1 associate with the same DNA sequences, but not at the same time (Fig. 2.6).

Monomethylation of H4K20 and HIPP1 are regulated in a PCNA dependent manner

We have shown that the distribution of H4K20me1 in polytene chromosomes is variable in a manner that suggests it depends on the cell cycle. H4K20me1 has been shown to have a role in origins of replication (Tardat et al., 2010). Specifically, H4K20me1 has a role in the licensing of origins of replication, and subsequent H4K20me1, di- or tri- methylation is necessary for activation of replication (Beck et al., 2012). To prevent further activation of replication once an origin of replication has been activated in the S phase, Pr-Set7 (the methyltransferase that is responsible for H4K20me1) is degraded in a PCNA dependent pathway (Sahashi et al., 2014; Tardat et al., 2010). We performed immunostaining experiments to test whether H4K20me1 and PCNA show a dynamic association in their distributions. Interestingly, there is a clear co-localization between PCNA and H4K20me1. However, the strength of the signals that colocalize is opposite, such that a strong PCNA signal always colocalizes with a weak PCNA signal (Fig. 2.7A). This data suggests that the amount of H4K20me1 at specific sites in chromosomes depends on PCNA, such that binding of PCNA triggers lost of H4K20me1, and we speculate that the levels of H4K20m1 may decrease due to the degradation of PrSet-7 by a PCNA dependent mechanism.

We also performed immunostaining experiments on polytene chromosomes under overexpression of HIPP1 background to test whether HIPP1 distribution in

chromosomes is dependent on PCNA. Results show a co-localization between HIPP1 and PCNA similar to that observed for H4K20me1 and PCNA, such that when the signal of one protein is strong, the other is weak or disappear, which suggest that the stability of HIPP1 may also be regulated in PCNA dependent manner (Fig. 2.7B).

HP1 localization on polytene chromosomes changes in the background of overexpression of HIPP1 and *cp190* mutations

Given that HP1 and H4K20me1 localization in polytene chromosomes appears to change, likely obeying to the different phases of the cell cycle, we decided to use these two proteins as markers to test the effect of insulator mutations as well as overexpression of Su(Hw)::GFP and HIPP1::m in their distribution. Results show, that in *su(Hw)*^{e04061} or *mod(mdg4)*^{u1} mutants, which do not show a phenotype on larvae or adult flies, both HP1 and H4K20me1 appear to have a chromosomal distribution similar to that of wildtype (Fig. A2.1B-E and A2.2A-C). However, in Cp190 trans-heterozygote mutant (*cp190*$^{p11/H31-2}$), which results in larval lethality at the third instar stage, most H4K20me1 signals are found in interbands, unlike in wildtype where H4K20me1 is found in bands. In this mutant HP1 appears also in interbands and is much less concentrated on the chromocenter (Fig. A2.2D and Fig. 2.8A). These data suggests that null mutations of all insulator proteins do not equally affect the cell cycle, since only mutations in cp190, but not in *su(Hw)* or *mod(mdg4)*, appear to influence the distribution of HP1 and H4K20me1 in polytene chromosomes.

Similarly, we found that cells overexpressing Su(Hw)::mc do not show a significant difference in the distribution of H4K20me1 or HP1, which appears at the chromosome bands and with a high concentration of HP1 in the chromocenter (data not shown). However, in cells overexpressing of HIPP1::mc, the distribution of both, H4K20me1 and HP1, appears diffused and the concentration of HP1 at the chromocenter is remarkably reduced compared with wildtype (Fig. 2.8B). These data supports the idea that defects in insulator protein function, such as lack of Cp190 or overexpression of HIPP1 may result in changes in the normal progression of the cell cycle that translate in changes in the distribution of elements that participate in the DNA replication pathway, like H4K20me1 or HP1. As we have shown earlier, other insulator proteins, such as Su(Hw), can have similar effects in cell cycle progression, but without affecting the distribution of these markers.

Discussion

In this work, we provide evidence suggesting that the newly discovered insulator protein HIPP1, together with Su(Hw) and possibly other chromatin insulator partners, has a role in cell proliferation. This conclusion is supported by evidence from experiments showing that overexpression of both Su(Hw) and HIPP1 lead to defects in cell proliferation. These defects could be observed in experiments using global overexpression of both proteins, which determine viable but

significantly small size larvae. These larvae appear to be phenotypically normal with exception of their size and their inability to successfully pupae and give rise to adult flies. In addition, specific expression of Su(Hw) and HIPP1 in wing and eye imaginal discs produces adults with rough eyes, no interomatidial bristles and cut wings, suggesting that an excess of these insulator proteins may lead to a lack of cell proliferation, apoptosis, or both. We provide data supporting that these phenotypes result mainly from defects in proliferation, since inhibitors of apoptosis do not rescue the phenotype, and cell from salivary glands, where apoptosis is strongly inhibited, shows a reduced size with small nuclei, which indicates the number of rounds of replication is significantly smaller compared with wildtype cells.

HIPP1 functions as an insulator protein

Results presented here show that HIPP1 has a chromatin insulator related function. First, confirming previous results, we show here that HIPP1 colocalizes with insulator sites in polytene chromosomes (Alekseyenko et al., 2014). Second, we show that under conditions of osmotic stress, HIPP1 responds by forming insulator bodies, in the same manner as all other known insulator proteins (Schoborg et al., 2013b). Our laboratory has previously shown that under conditions of osmotic stress, but also during apoptosis, insulator proteins disassociate from chromatin to form large foci of proteins that localize to the interchromosomal spaces in the nucleus. These protein foci are known as insulator bodies (Schoborg et al., 2013b), and here we shown that HIPP1 also

associates with these insulator bodies during osmotic stress. The function of insulator proteins in the osmotic stress response remains unclear, but this finding further support HIPP1 shares this property with other insulator proteins.

Finally, we have shown in chapter I that overexpression of HIPP1 can inactivate the enhancer-blocking function of the gypsy insulator in the *yellow* and *cut* loci (y^2 and ct^6, respectively). This result indicates that a possible role of HIPP1 protein is to inactivate the function of insulators in the genome. Although the mechanism by which HIPP1 inactivates insulator function remains unknown, one possibility is that HIPP1 functions by breaking the long distant contacts between insulator sites, interfering with the formation of chromatin loops. Because HIPP1 has a crotonase domain, which is likely involved in histone acetylation(Alekseyenko et al., 2014), it could be speculated that acetylation of histones or other proteins related with insulator function, may promote disassociation of long-range contacts at insulator sites.

Insulator proteins may function by controlling the selection of origins of replication in the genome

Because HIPP1 shows both, an ability to inactivate insulator function and a cell proliferation phenotype, we analyzed the possible role that HIPP1, and insulators in general, might have in cell cycle and DNA replication. We have shown here that overexpression of both HIPP1 and Su(Hw) proteins have similar effects in the progression of the cell cycle, resulting in defects in cell proliferation.

Interestingly, Su(Hw) appears to have a stronger effect than HIPP1, producing smaller size larvae and more extreme phenotypes in the eye and in the wing. Coexpression of HIPP1, Su(Hw) and FUCCI markers revealed that that the cell cycle in salivary glands and wing imaginal discs is arrested at early S phase, by a mechanism that is still unclear. To further explore this phenotype we used markers such as HP1 and H4K20me1. Both proteins have been shown to have a role in origins of replication function.

Our results review that HIPP1 may localize at differ sites in different nuclei. For example in some nuclei it is found in bands, whereas in others it is found in interbands, and yet in others is completely missing; suggesting that these changes depend on specific stages of the cell cycle. In addition, overexpression of HIPP1 can in turn induce changes in the localization of other proteins such as Su(Hw), HP1 and H4K20me1. Thus, results analyzing the distribution of Su(Hw) when overexpressed suggest that Su(Hw) can be found in sites where is not normally found, such DNA in interbands. Interestingly, we observed the same distribution of wildtype Su(Hw) in cells overexpressing HIPP1 (Fig. 2.2D), which suggests this distribution may have physiological relevance. These global changes in the distribution of both proteins further support the notion that insulator proteins may have a role in the regulation of cell cycle progression.

More intriguing is the effect of overexpression of HIPP1 on the distribution of HP1 and H4K20me1 (See text box 1). Summarizing, in normal cells, HP1 and

Text box 1: Proposed model for HiPP1 and insulators in DNA replication

HIPP1 functions as a factor that inactivates insulators in a cell cycle dependent manner, to license and activate specific origins of replication and to allow replication of DNA at insulator sites. Overexpression of HIPP1 causes broad inactivation of chromatin insulators, leading to general activation of origins of replication, likely triggering replication stress and also causing cell proliferation defects. On the other hand, overexpression of Su(Hw) increases insulator activity at euchromatin (interbands), causing early stalling of replication forks and therefore also leading to replication stress and defects in cell proliferation.

Our data suggests that activation of origins of replication requires the presence of H4K20me1 at licensed origins of replication, followed by recruitment of HP1 and loading of PCNA.

We propose that this process is highly regulated by insulators, which would be involved in the mechanism that determines that only a small subset of origins of replication can be activated during the S phase of the cell cycle. Mutations in Su(Hw), or overexpression of Su(Hw) do not cause global activation of origins of replication. However overexpression of HIPP1 leads to a simultaneous activation of origins of replication with independence on whether origins of replication are in the euchromatin or in the heterochromatin.

H4K20me1 are found in small speckles frequently associated in most nuclei with condensed DNA in the bands of polytene chromosomes, and less frequently in the interbands. Careful analysis of this association shows that HP1 and

H4K20me1 do not precisely colocalize and instead associated with each other side by side, such that when HP1 shows a strong signal, H4K20me1 is missing or shows a weak signal. Similarly, HP1 and H4K20me1 associate with PCNA such that strong PCNA signals correlate with weak or no HP1 and H4K20me1 at the PCNA sites. Because H4K20me1 has been implicated with the licensing of origin of replication (Tardat et al., 2010), and HP1 has been implicated with the activation of replication in the origin of replication in yeast and in Drosophila (Hayashi et al., 2009; Schwaiger et al., 2010), we propose that in the sequence that leads to activation of DNA replication in Drosophila, H4K20me1 marks origins of replication that are licensed. These origins later recruit HP1, which activate replication by facilitating the loading of PCNA and other components of the replication machinery at the origin of replication. During this process, H4K20me1 becomes dimethylated or trymethylated (H4K20me2 or H4K20me3) and Pr-Set7 is degraded in a PCNA dependent manner. Removing Pr-Set7 from origins of replication during firing ensures that the same origin will not be licensed and activated twice during the same cell cycle (Beck et al., 2012).

To explain our results, we speculate that Insulator proteins have to be inactivated in order to allow progression of replication forks through genomic DNA. In addition, we suggest that chromatin insulators also regulate selection of the subset of origins of replication that are activated during each cell cycle, either because they function themselves as origin of replication (Vorobyeva et al., 2013), or because they introduce changes in chromatin organization that affect

origin of replication function. Specific mechanisms of how insulators may contribute to this mechanism are unknown, but HIPP1 is a candidate insulator protein that may convey such properties to chromatin insulators through its crotonase domain, which may be involved in histone acetylation (Wu et al., 2009). In metazoans, early stages of embryo development are mediated by maternal proteins and mRNAs, and initial cell divisions take place in absence of gene transcription, such that all origins of replication in the genome can be activated simultaneously. The use of all possible origins of replication, combined with the absence of transcription leads to replication cycles that are faster than cell cycles in cells fully differentiated or in the process of differentiation. In Drosophila, embryo transcription does not initiate until several consecutive replication cycles that occur significantly faster than once cells are differentiated. Once transcription is initiated in the embryo, and cell differentiation begins, every cell-type is redefined by a specific gene transcription program, which in turns responds to a reprograming of the epigenome after important changes in chromatin organization (Fragkos et al., 2015). In these differentiated cells replication cannot proceed by simultaneously activating all origins of replication, since licensing of origins of replication has to be coordinated with transcription. This coordination results in the sequential activation of origins of replication, starting with activation of early euchromatic origins and finalizing with activation of late origins at heterochromatin and condensed non-transcribed genes (Fragkos et al., 2015). In addition, only a fraction of the origins of replication activated during early embryo development are activated in differentiated cells.

The mechanism by which specific origins of replication are selected is unknown, and we propose that the role of HIPP1 is to inactivate insulator function in a stepwise manner to allow licensing and activation of specific origins of replication through the S phase of the cell cycle.

In our model, overexpression of HIPP1 induces inactivation of chromatin insulators likely triggering replication stress and cell proliferation defects, whereas overexpression of Su(Hw) increases insulator activity, causing stalling of replication forks and replication stress, and therefore also leading to defects in cell proliferation. We interpret these apparently contradicting effects by arguing that overexpression of HIPP1 leads to a global inactivation of insulators, which will produce a disordered licensing and activation of origins of replication, which would lead to replication stress. On the other hand, overexpression of Su(Hw) should have the opposite effect, since overabundance of this protein would make insulators stronger and more difficult to inactivate. Following our model, insulators that cannot be inactivated would lead to defects in licensing and activation of origins of replication and stalling of replication forks at active insulator sites, which will also contribute to replication stress.

One important observation of this work is that the localization of HIPP1 in the genome is variable, and most likely depends on particular stages of the cell cycle. Specifically, we hypothesize that HIPP1 binds different insulator sites at different stages of the S phase. For example, it binds CTCF and Cp190 insulator

sites during early replication of euchromatin in interbands, and Su(Hw) insulators associated with condensed DNA in bands (Wallace et al., 2010), at later stages of the S phase. This observation might be critical to further our understanding of insulator function, and can only be made by directly observing the HIPP1 distribution in the genomes of single cells. Analysis of single cell genome technology is not currently available and is only possible using immunostaining in polytene chromosomes. More powerful techniques, such us chromatin immunoprecipitation, require the use of a large number of cells and only provide a statistical distribution of binding sites, and cannot resolve whether a protein only binds a genome site temporarily or whether binding depends on cell cycle stages. Current data on the distribution of HIPP1 in the genome show it binds all insulator sites (Alekseyenko et al., 2014), and does not predict our findings showing a cell cycle dependent distribution.

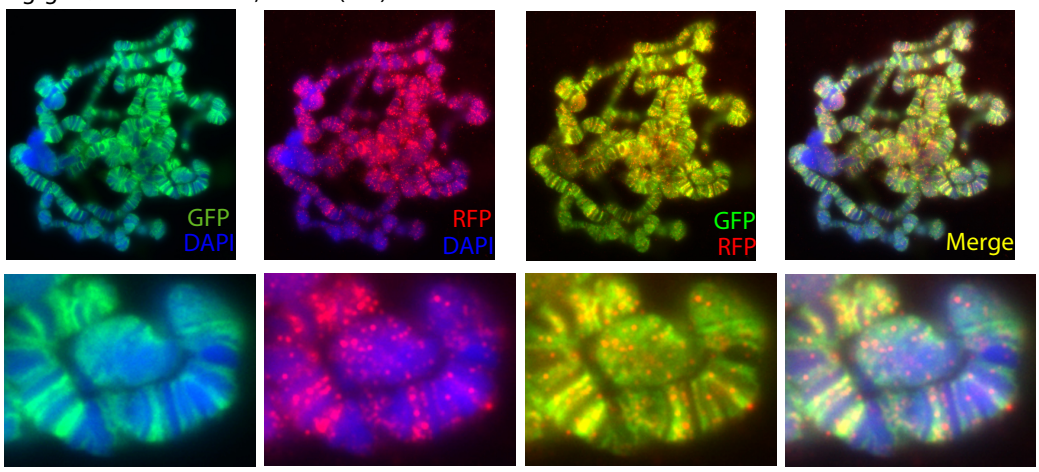
Although many questions remain about the role chromatin insulators in DNA replication and the control of the cell cycle, perhaps one of the most fundamental is the role that HIPP1 plays in insulator function and how it relates to DNA replication. Our proposal is that HIPP1, through its crotonase domain, is involved in histone acetylation, which may result in inactivation of insulator activity. It is essential to empirically determine whether the crotonase domain of HIPP1 actually has acetylation activity and to find out the specific histone or histones that HIPP1 modifies. Equally important is to determine the histone deacetylase that counteracts HIPP1 function, as well as the possible histone binding proteins

that bind HIPP1 acetylated histones. One candidate deacetylase is rpd3, which has already been described as involved in activation of origins of replication (Aggarwal and Calvi, 2004; De Rubertis et al., 1996). Future work should address all these questions and help further understand HIPP1 role in insulator function and the role of chromatin insulators in DNA replication and cell cycle.

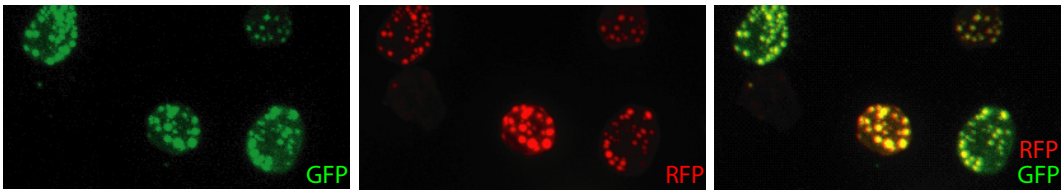
CHAPTER II APPENDIX

Figure 2.1. Su(Hw) and HIPP1 show colocalization on polytene chromosomes and S2 cells. Su(Hw)::GFP colocalizes with HIPP1::mcherry on polytene chromosomes (A). Su(Hw)::GFP and HIPP1::mcherry shows high co-localization the insulator bodies in S2 cells (B). Su(Hw)::GFP and HIPP1 shows diffused pattern in S2 cells under normal conditions (C).

A vg-gal4/UAS-HIPP1::mc;UAS-Su(Hw)::GFP



B



C

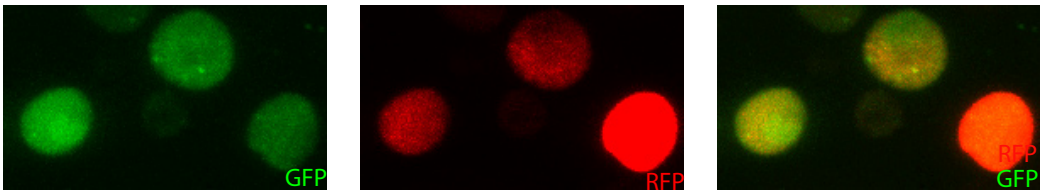


Figure 2.2. The distribution of HIPP1 and Su(Hw) in polytene chromosomes suggests that their expression and binding sites are cell cycle regulated. In different nuclei from a single salivary gland HIPP1 can localize to interbands (A), can appear with a diffused pattern that spans bands and interbands (B), or appear localizing exclusively to bands (C). Su(Hw) also shows different localization patterns under overexpression of HIPP1 background (D). Su(Hw) concentrates in interbands on polytene chromosomes from cells overexpressing Su(Hw) (E).

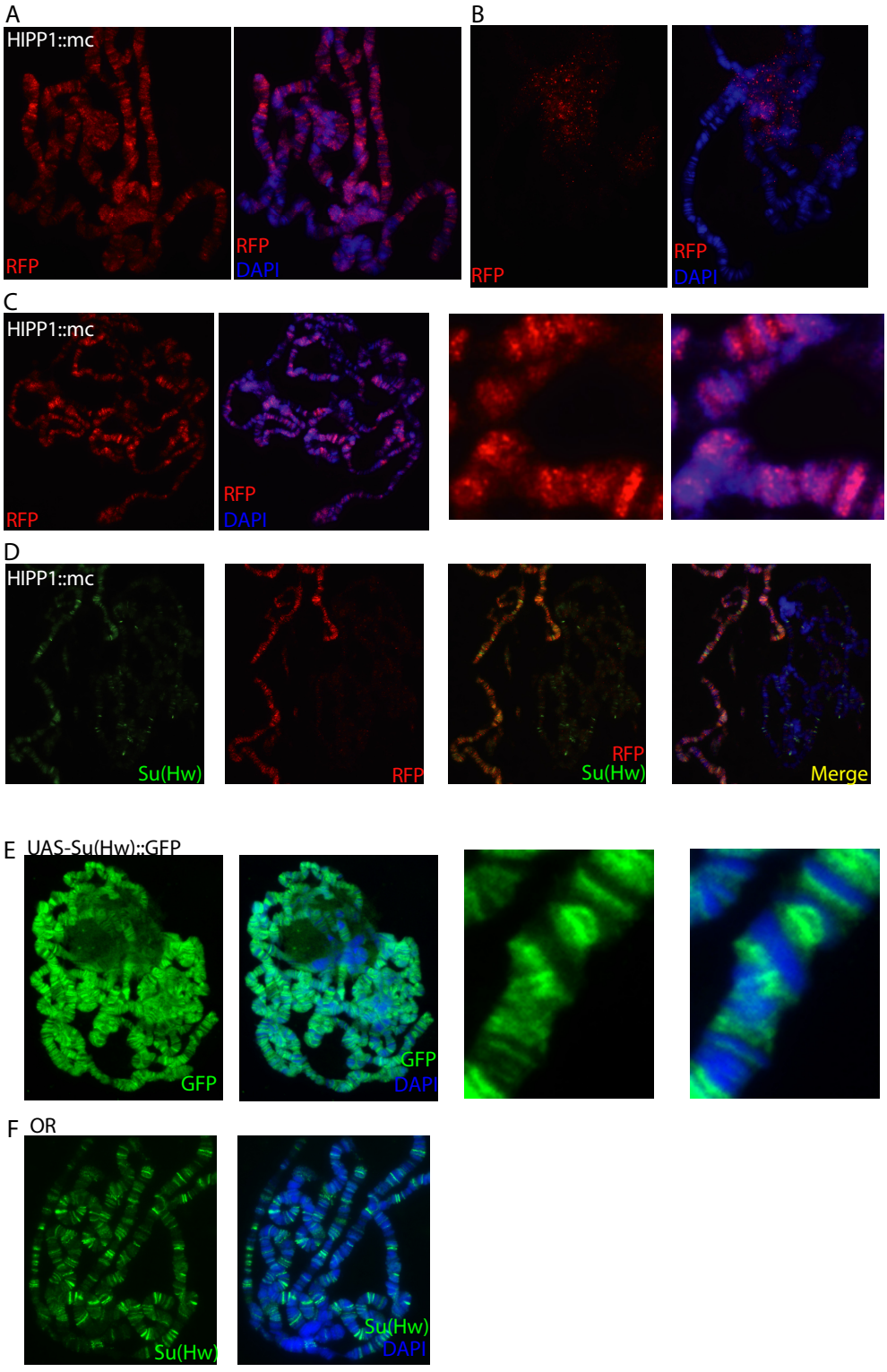

A
HIPP1::mc
RFP
RFP
DAPI
B
RFP
RFP
DAPI
C
HIPP1::mc
RFP
RFP
DAPI
D
HIPP1::mc
Su(Hw)
RFP
RFP
Su(Hw)
Merge
E UAS-Su(Hw)::GFP
GFP
GFP
DAPI
F OR
Su(Hw)
Su(Hw)
DAPI

Figure 2.3. Overexpression of HIPP1 and Su(Hw) results in defects on cell proliferation. Overexpression of either HIPP1 (mid) or Su(Hw) (right) with the actin-gal4 driver results in small third instar larvae compared with control wildtype larvae (left) (A). Overexpression of Su(Hw) (left: GMR-Gal4; UAS-Su(Hw)::GFP) leads to the formation of rough eyes; Overexpression of HIPP1 leads to a relatively mild rough eyes phenotype (mid: GMR-Gal4; UAS-HIPP1::mc); and overexpression of both HIPP1 and Su(Hw) (right: GMR-Gal4/UAS-HIPP1::mc; UAS-Su(Hw)::GFP) results in a more severe phenotype on the eyes (B). Overexpression of Su(Hw)::GFP with vg-Gal4 driver leads to defects on wing formation, while overexpression of HIPP1::mc have no effect on wing disc development; To exclude the possibility that these defects result from apoptosis, we coexpressed UAS-P35 with overexpression of Su(Hw). Results show similar defects on the wing margin. Overexpression of both HIPP1 and Su(Hw) results in a more severe phenotype on wing margins (C).

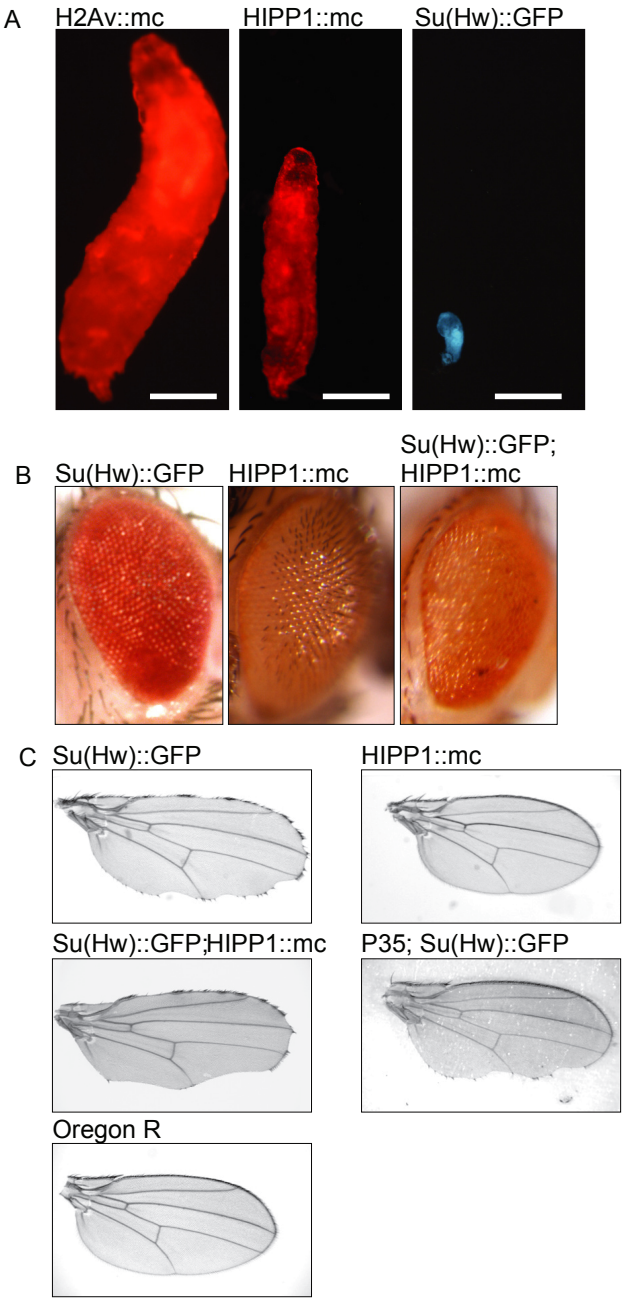
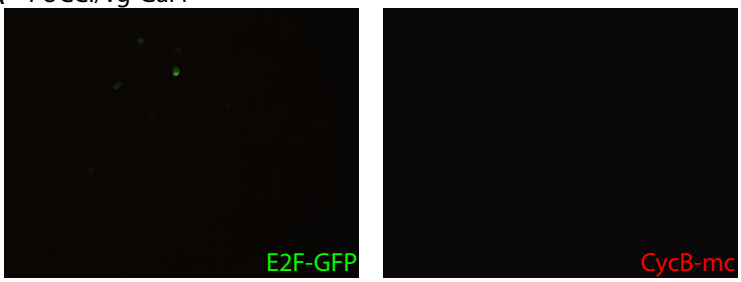
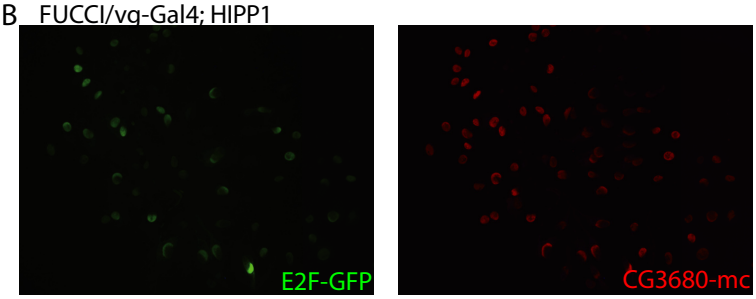

A
H2Av::mc
HIPP1::mc
Su(Hw)::GFP
B
Su(Hw)::GFP
HIPP1::mc
Su(Hw)::GFP;
HIPP1::mc
C
Su(Hw)::GFP
HIPP1::mc
Su(Hw)::GFP;HIPP1::mc
P35; Su(Hw)::GFP
Oregon R

Figure 2.4. FUCCI markers suggest cell cycle progression is altered under overexpression of Su(Hw) and HIPP1. A-C. FUCCI is expressed by the vg-gal4 driver in wildtype (A), overexpression of HIPP1 (B) and overexpression of Su(Hw) (C). D-E. FUCCI is expressed by the strong hsp70-gal4 driver in wild type (D), overexpression of HIPP1 (E) and overexpression of Su(Hw) (F).

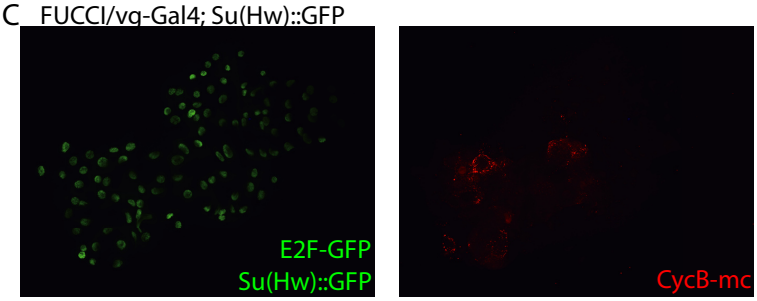
A FUCCI/vg-Gal4



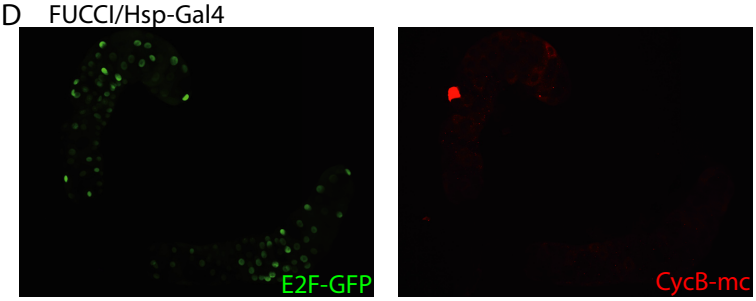
B FUCCI/vg-Gal4; HIPP1



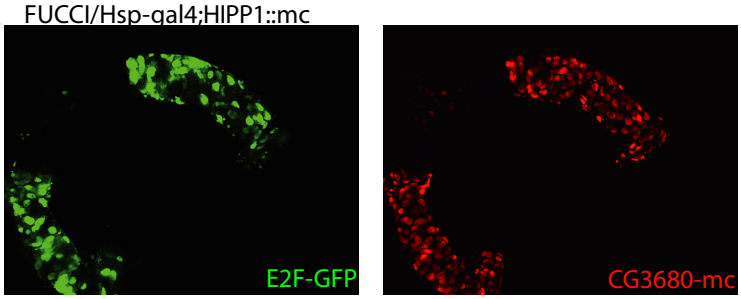
C FUCCI/vg-Gal4; Su(Hw)::GFP



D FUCCI/Hsp-Gal4



E FUCCI/Hsp-gal4;HIPP1::mc



F FUCCI/Hsp-Gal4;Su(Hw)::GFP

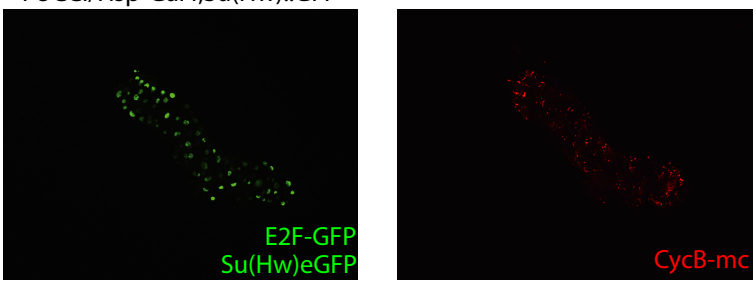
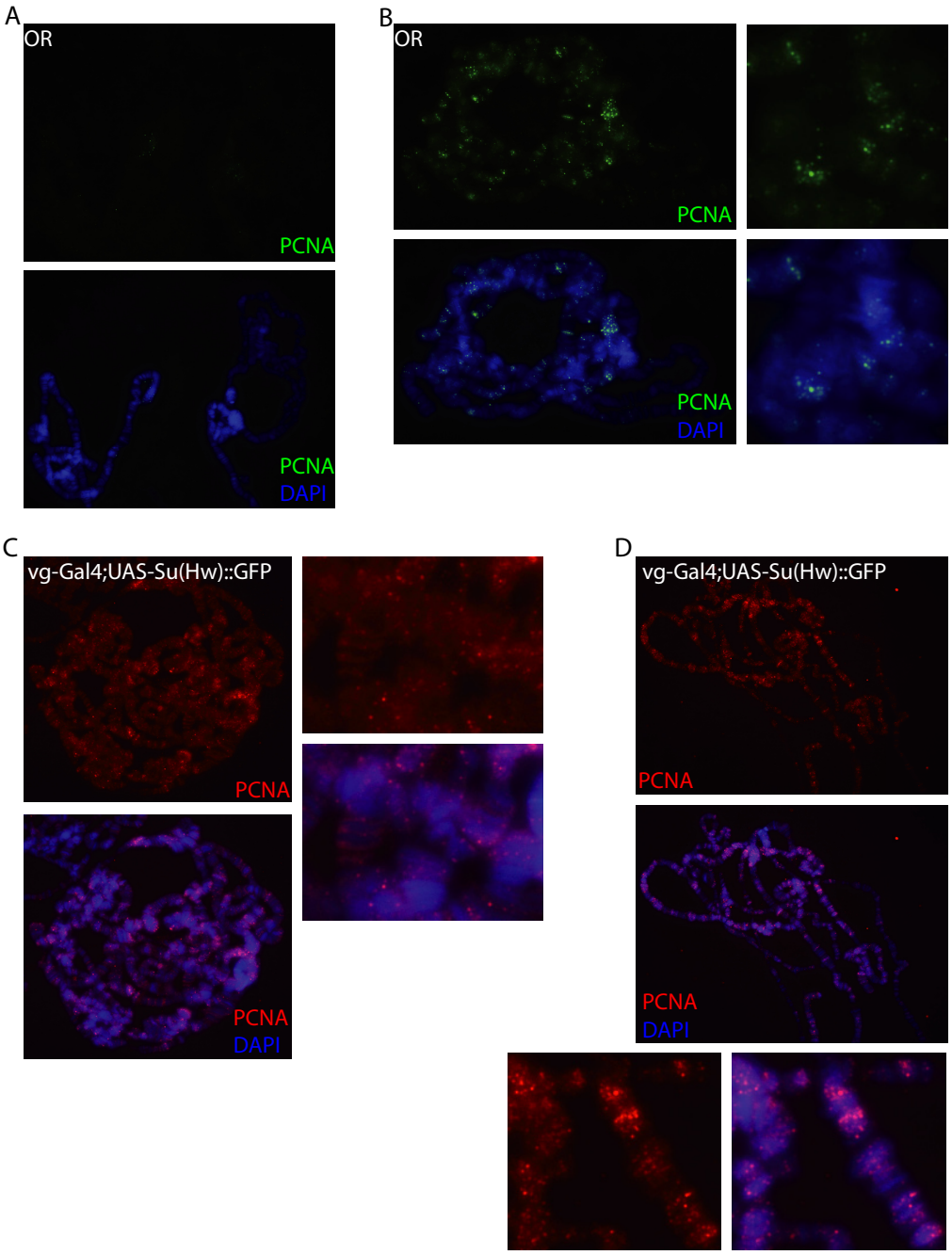


Figure 2.5. PCNA distribution in polytene chromosomes depends on Su(Hw) and HIPP1. The distribution pattern of PCNA in wildtype (A-B), after overexpression of Su(Hw) (C-D) and after overexpression of HIPP1 (E-F).


A
OR
PCNA
PCNA
DAPI
B
OR
PCNA
PCNA
DAPI
C
vg-Gal4;UAS-Su(Hw)::GFP
PCNA
PCNA
DAPI
D
vg-Gal4;UAS-Su(Hw)::GFP
PCNA
PCNA
DAPI

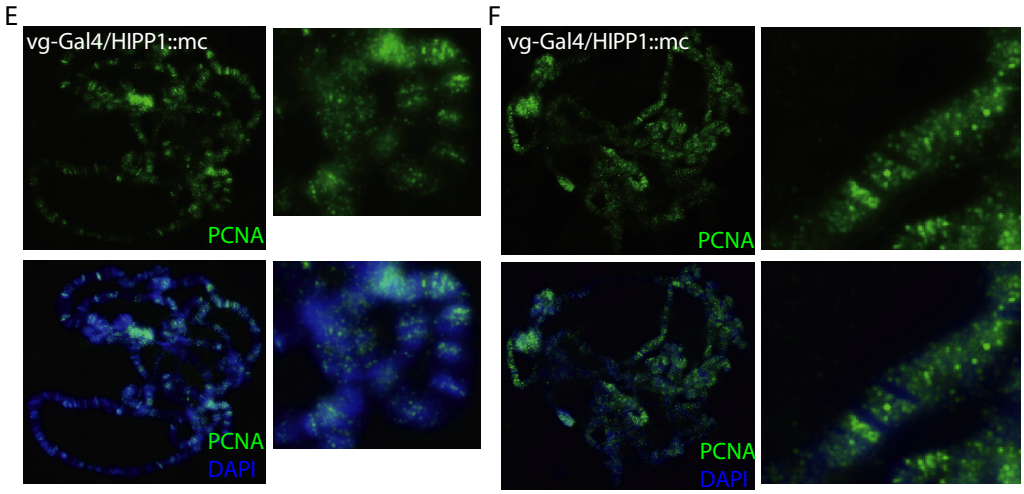

E
vg-Gal4/HIPP1::mc
PCNA
PCNA
DAPI
F
vg-Gal4/HIPP1::mc
PCNA
PCNA
DAPI

Figure 2.6. HP1 distribution in polytene chromosomes correlates with that of H4K20me1 and appear to be cell cycle regulated. HP1 and H4K20me1 associate with condensed DNA at bands in polytene chromosomes (A), appear as a diffused pattern associated with both bands and interbands (B), and specifically associate with interbands (C).

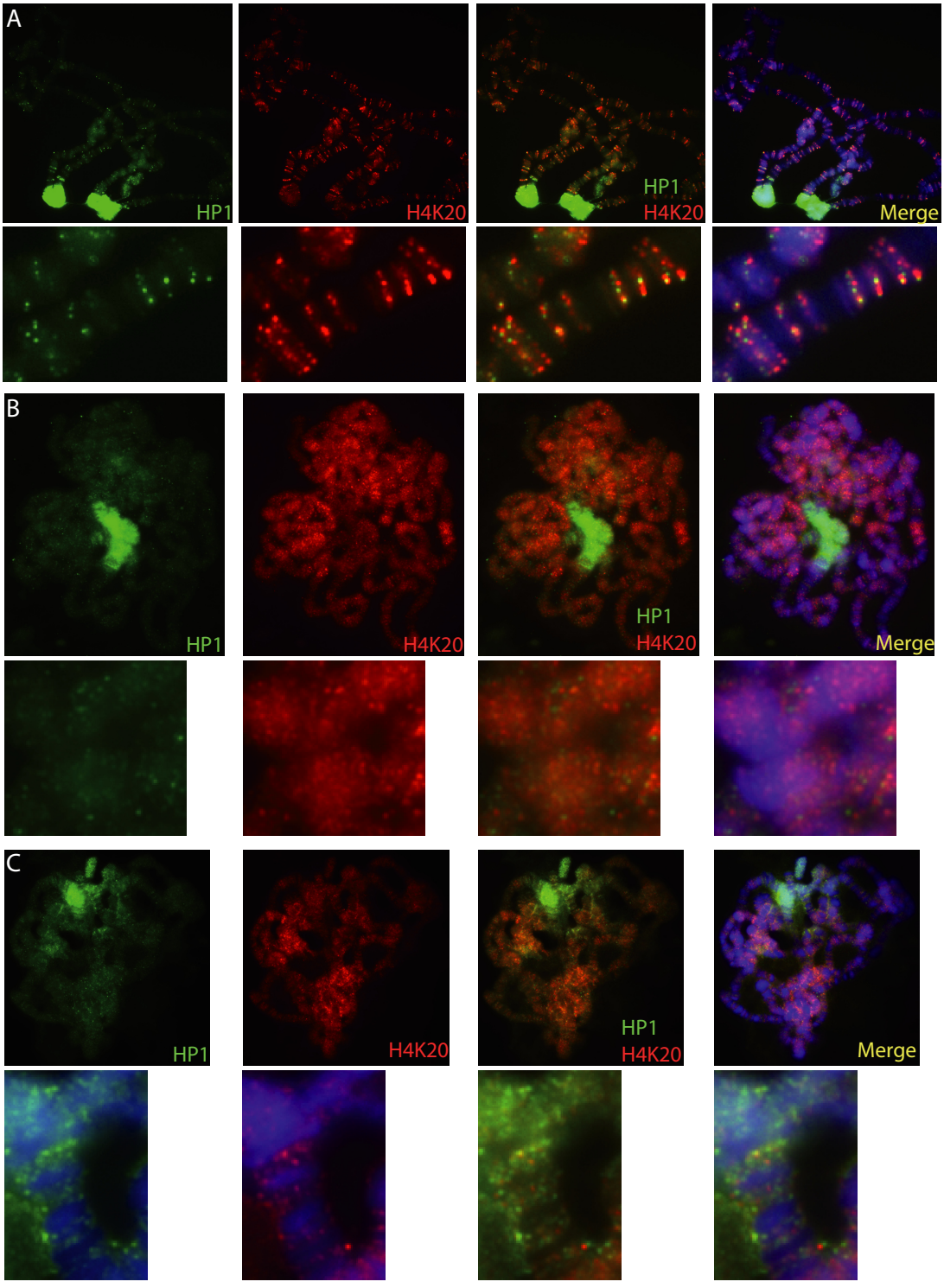

A
HP1
H4K20
HP1
H4K20
Merge
B
HP1
H4K20
HP1
H4K20
Merge
C
HP1
H4K20
HP1
H4K20
Merge

Figure 2.7. The distribution of H4K20me1 and HIPP1 suggest both are regulated in a PCNA dependent manner. PCNA shows binding sites on polytene chromosomes similar to H4K20me1, but the intensity of the signals are opposite (A). A similar correlation exists between PCNA and HIPP1 (B).

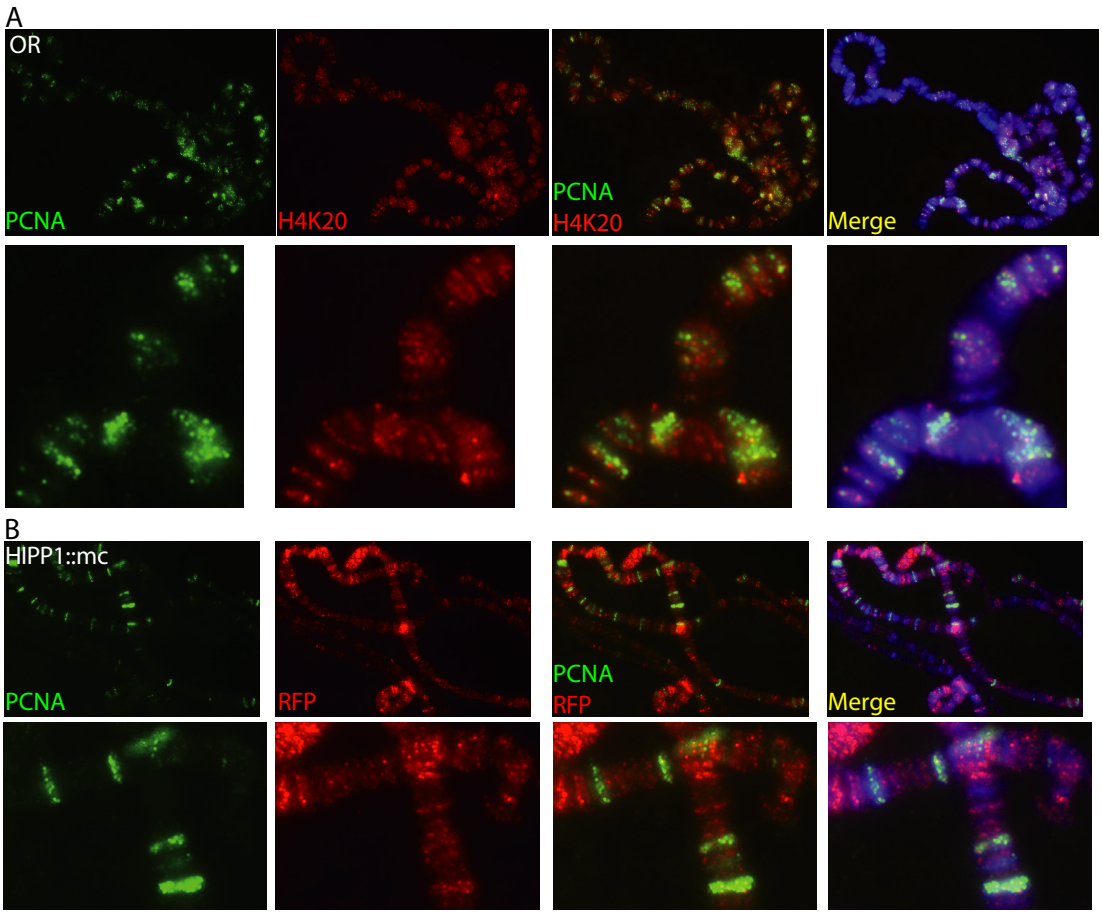

A
OR
PCNA
H4K20
PCNA
H4K20
Merge
B
HIPP1::mc
PCNA
RFP
PCNA
RFP
Merge

Figure 2.8. The distribution of HIPP1 and Su(Hw) in polytene chromosomes suggests that their expression and binding sites are cell cycle regulated. In different nuclei from a single salivary gland HIPP1 can localize to interbands (A), can appear with a diffused pattern that spans bands and interbands (B), or appear localizing exclusively to bands (C). Su(Hw) also shows different localization patterns under overexpression of HIPP1 background (D). Su(Hw) concentrates in interbands on polytene chromosomes from cells overexpressing Su(Hw) (E).

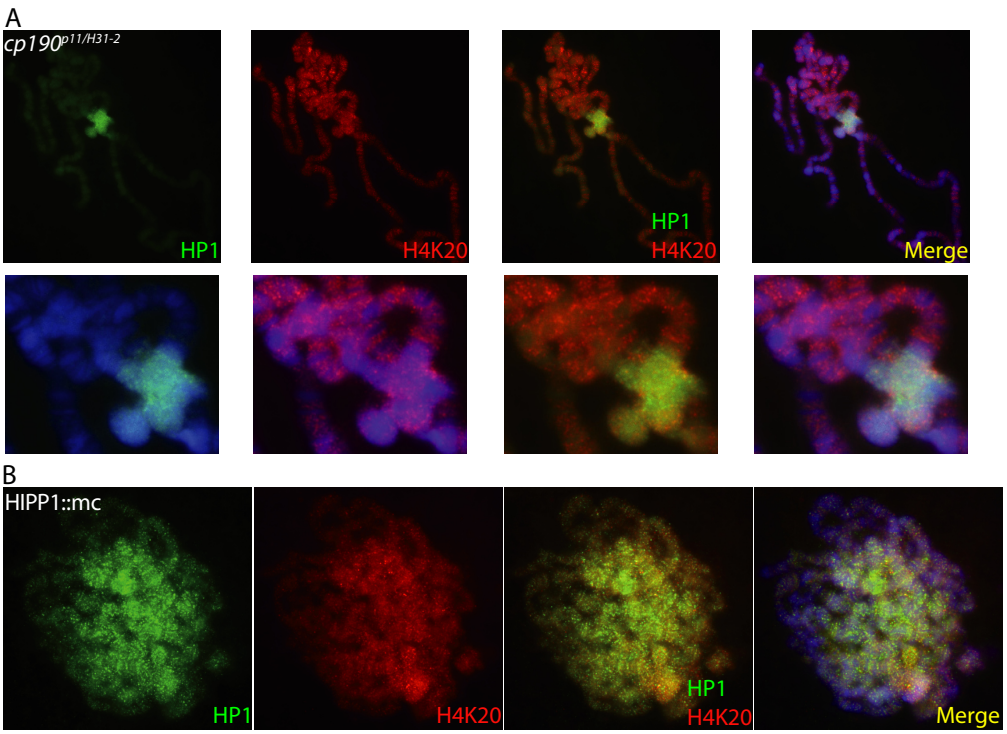

A
cp190p11/H31-2
HP1
H4K20
HP1
H4K20
Merge
B
HIPP1::mc
HP1
H4K20
HP1
H4K20
Merge

Table 1. Count of Nuclei with different genotype containing PCNA on polytene chromosomes from third instar larvae

Genotype	OR	HIPP1::mc	Su(Hw)::GFP	*su(Hw)*^{e}
PCNA/Total	9/57	35/48	33/42	6/26

CONCLUSIONS

The collection of work outlined here attempts to address two important questions regarding insulator biology from a hypothesis-driven perspective. Firstly, we addressed the question of what drives the enhancer-promoter blocking property of chromatin insulators and asked whether chromatin insulator function is regulated? Secondly, we asked whether chromatin insulators are involved in DNA replication and cell cycle regulation? The answers to these questions would not only reveal new roles for insulators in the genome, but also would bring novel insights into how these elements regulate genome function and genome stability.

In Chapter I, we found (i) γH2Av co-localizes with insulator proteins in polytene chromosomes. (ii) Co-localization between γH2Av and insulators depend on protein components of the insulator . (iii) γH2Av is found at insulator bodies after osmotic stress in S2 cells. (iv) Phosphorylation of H2Av is required for the enhancer-blocking activity of the gypsy insulator. (v) ATM and ATR phosphorylate H2Av at insulator sites and control gypsy insulator activity. (vi) H2Av can be dephosphorylated by PP2A at insulator sites. We concluded that ATM, ATR modulate insulator activity through phosphorylation of histone H2Av at insulator sites. In Chapter II, we found (i) Su(Hw) and HIPP1 overexpression causes defects in cell proliferation. (ii) Insulator protein expression is regulated during cell cycle. (iii) Chromatin insulators might modulate progression of the cell

cycle during S phase, and we concluded that chromatin insulator proteins regulate DNA replication and cell cycle progression in Drosophila.

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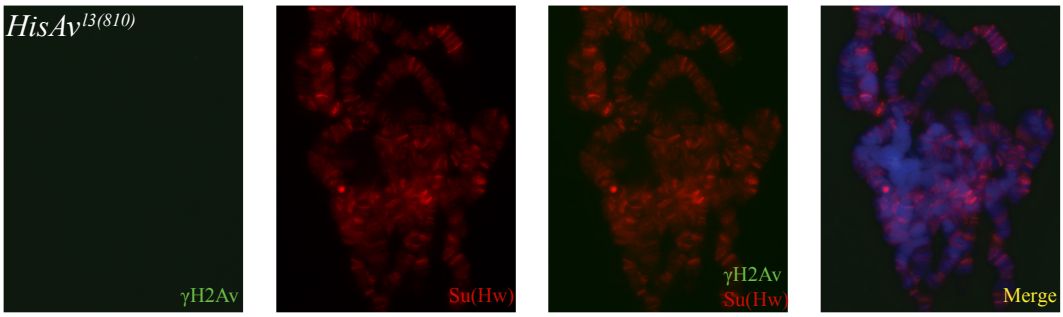
APPENDIX

Figure A1.1. The monoclonal antibody anti-γH2Av specifically detects phosphorylated H2Av on polytene chromosomes. A. Immunostaining on polytene chromosomes shows no detectable γH2Av signals in *HisAv*$^{J3(810)}$ mutant. B. γH2Av shows clearl co-localization with H2Av::mcherry with overexpression of H2Av::mcherry.

A


HisAv^l3(810)
γH2Av
Su(Hw)
γH2Av
Su(Hw)
Merge

B

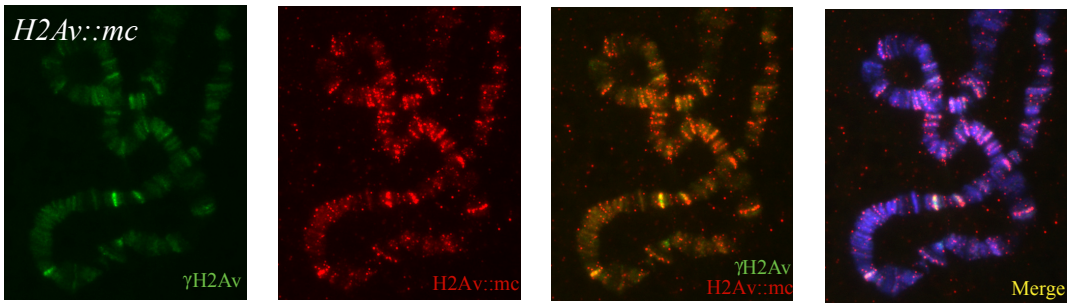
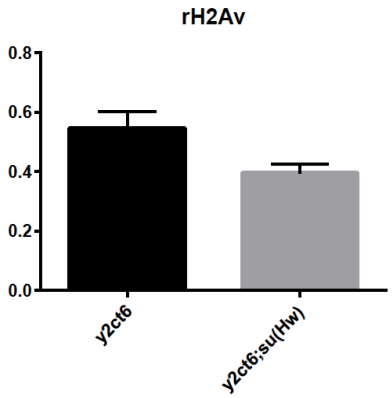
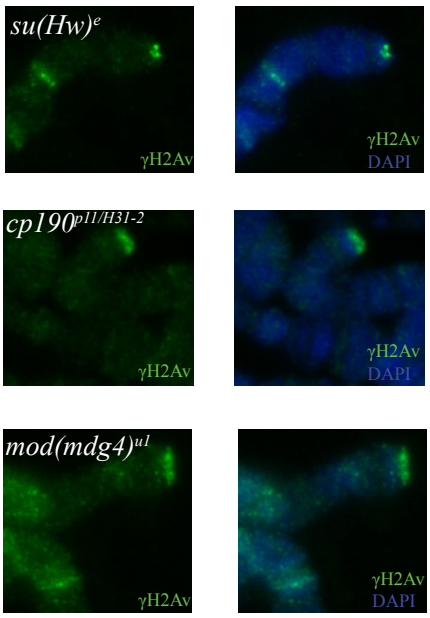

H2Av::mc
γH2Av
H2Av::mc
γH2Av
H2Av::mc
Merge

Figure A1.2. Binding sites of γH2Av in polytene chromosomes change in insulator mutants . A. Quantification of immunostaining images shows a significantly reduced level of γH2Av in *su(Hw)*^{e04061} mutant (p=0.0156). B. Telomere shows abundance of γH2Av in insulator mutants. C. Mod(mdg4)67.2 localizes to CP190 binding sites in *su(Hw)*^{e04061} mutant. Mod(mdg4)67.2 is shown in green, CP190 in red and DAPI in blue.

A


rH2Av
0.8
0.6
0.4
0.2
0.0
y2ct6
y2ct6;su(Hw)

B


su(Hw)e
γH2Av
γH2Av
DAPI
cp190p11/H31-2
γH2Av
γH2Av
DAPI
mod(mdg4)u1
γH2Av
γH2Av
DAPI

C

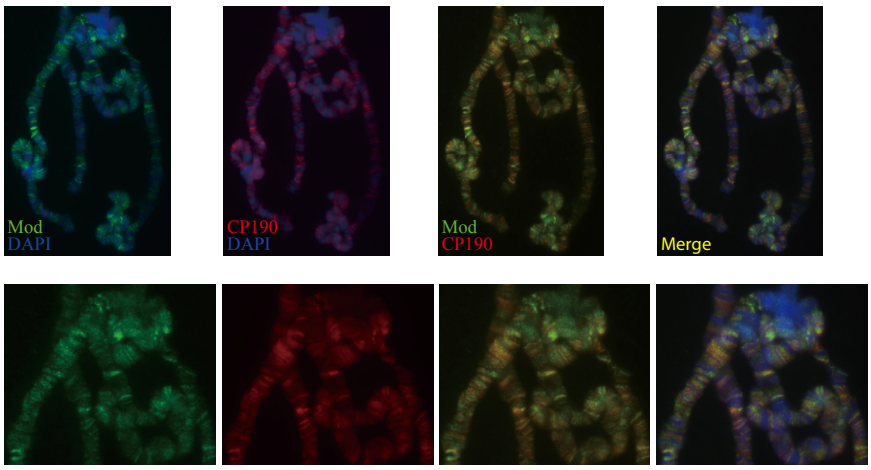

Mod
DAPI
CP190
DAPI
Mod
CP190
Merge

Figure A1.3. The presence of γH2Av at y^2 and ct^6 depends on the knock down efficiency of CP190 RNAi on polytene chromosomes. A. CP190 shows a dramatically reduced level after RNAi knock down, and γH2Av shows a diffused pattern similar to that in $cp190^{P11/H31-2}$ mutant. B. γH2Av totally disappears at y^2 and ct^6 sites, while Su(Hw) is still binding at the y^2 and ct^6 sites in $y^2ct^6/+;$ $cp190^{P11/H31-2}$ mutant. C. CP190 signals totally disappear in $y^2ct^6/+;$ $cp190^{P11/H31-2}$ mutant background. D. γH2Av shows a reduced level at y^2 and ct^6 sites after Cp190 knockdown by Cp190-RNAi driven by the vg-Gal4 driver. E. Both y^2 and ct^6 phenotypes are rescued by knocking down of CP190 by RNAi driven by the vg-Gal4 driver.

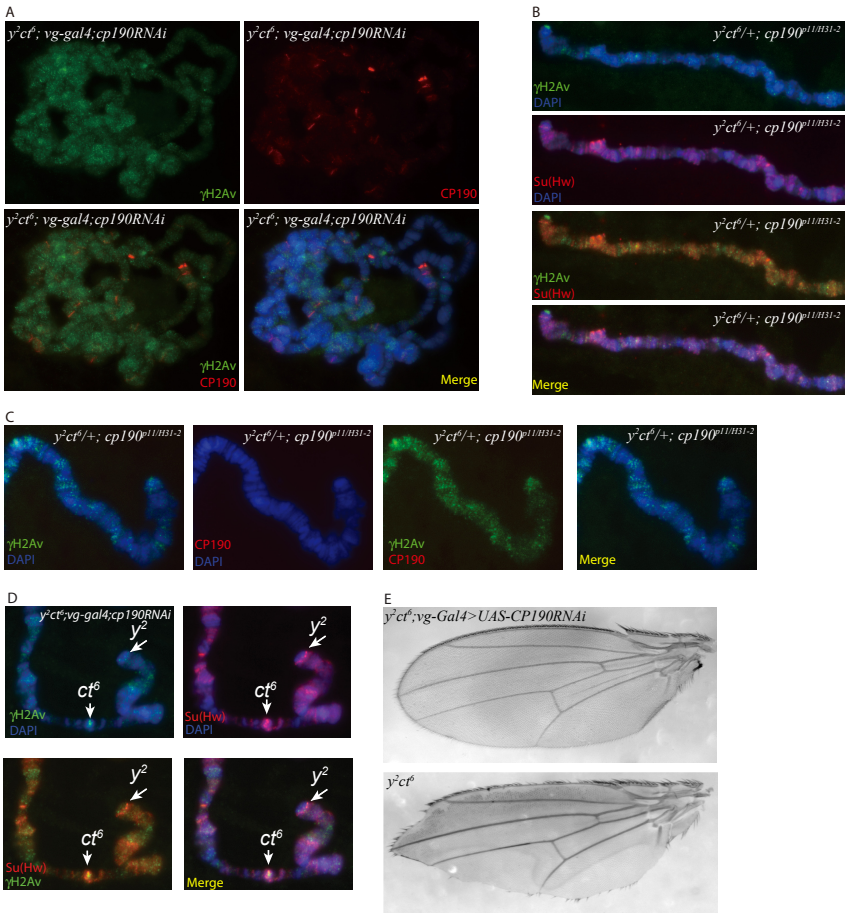

A
y2ct6; vg-gal4;cp190RNAi
γH2Av
CP190
Merge
B
y2ct6/+; cp190p11/H31-2
γH2Av
DAPI
Su(Hw)
Merge
C
CP190
D
y2ct6;vg-gal4;cp190RNAi
y2
ct6
E
y2ct6;vg-Gal4>UAS-CP190RNAi

Figure A1.4. Insulator proteins still bind to y^2 and ct^6 sites on polytene chromosomes after overexpression of HIPP1. Su(Hw) is shown in green, CP190 in red and DAPI in blue. Arrows indicate y^2 and ct^6 sites.

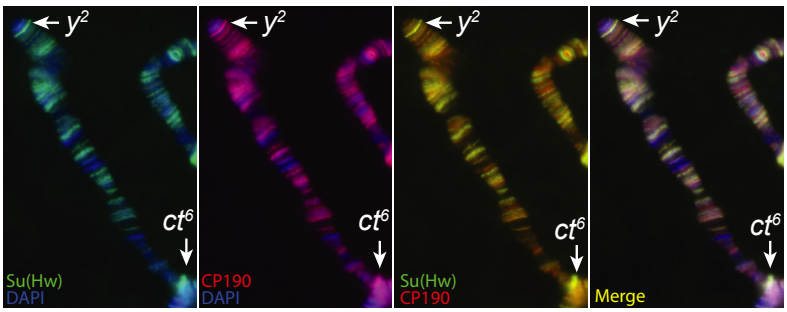

y2
ct6
Su(Hw)
DAPI
y2
ct6
CP190
DAPI
y2
ct6
Su(Hw)
CP190
y2
ct6
Merge

Figure A2.1. Localization of H4K20me1 on polytene chromosomes changes in *su(Hw)*^{e04061} mutants. A. Overview of H4K20me1 on wildtype polytene chromosomes, where is found in the condensed DNA in the bands. B-E. in *su(Hw)*^{e04061} mutants, H4K20me1 localizes to bands (B), interbands (D) or in a diffused pattern(E), and always associates with HP1 (C) .

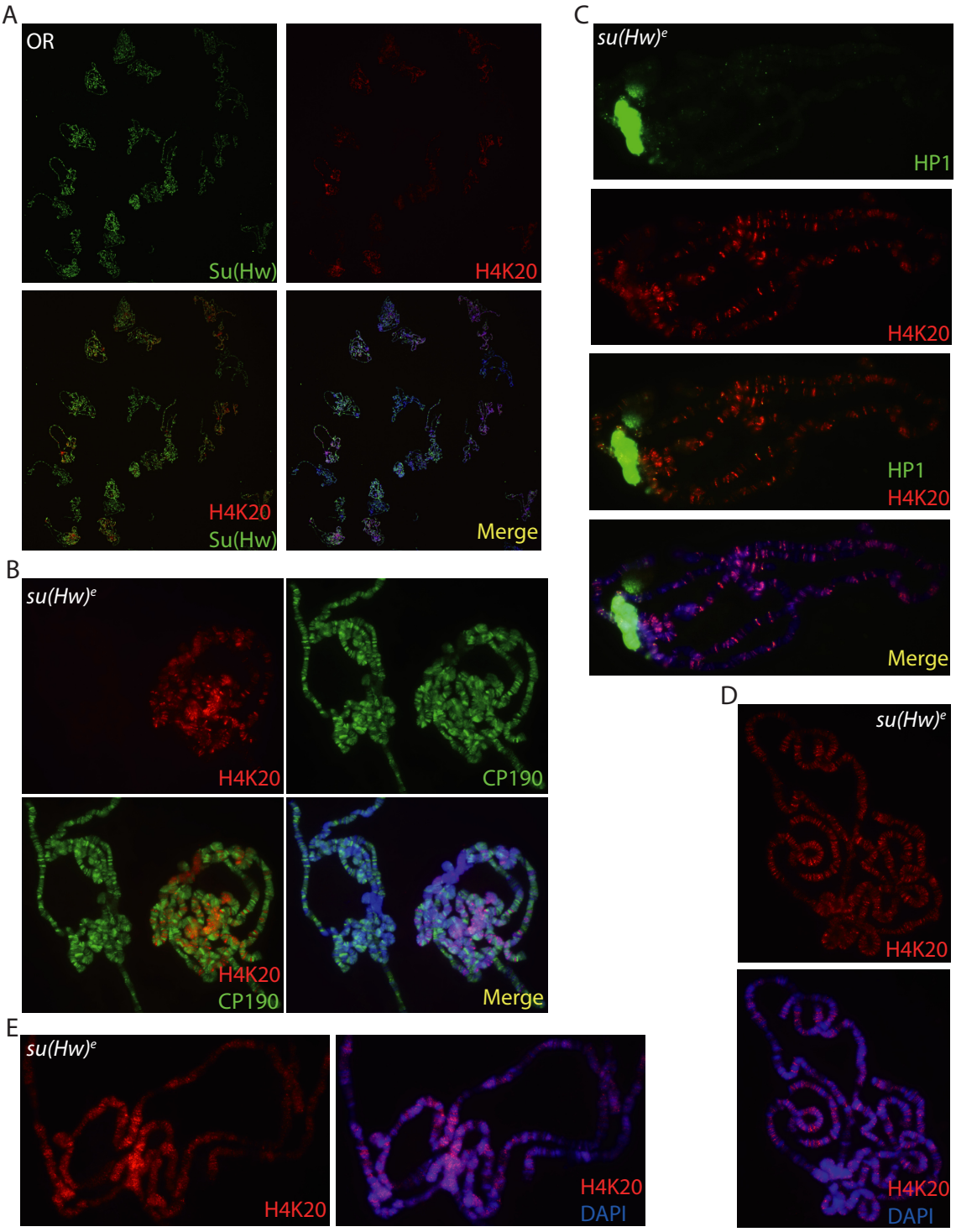
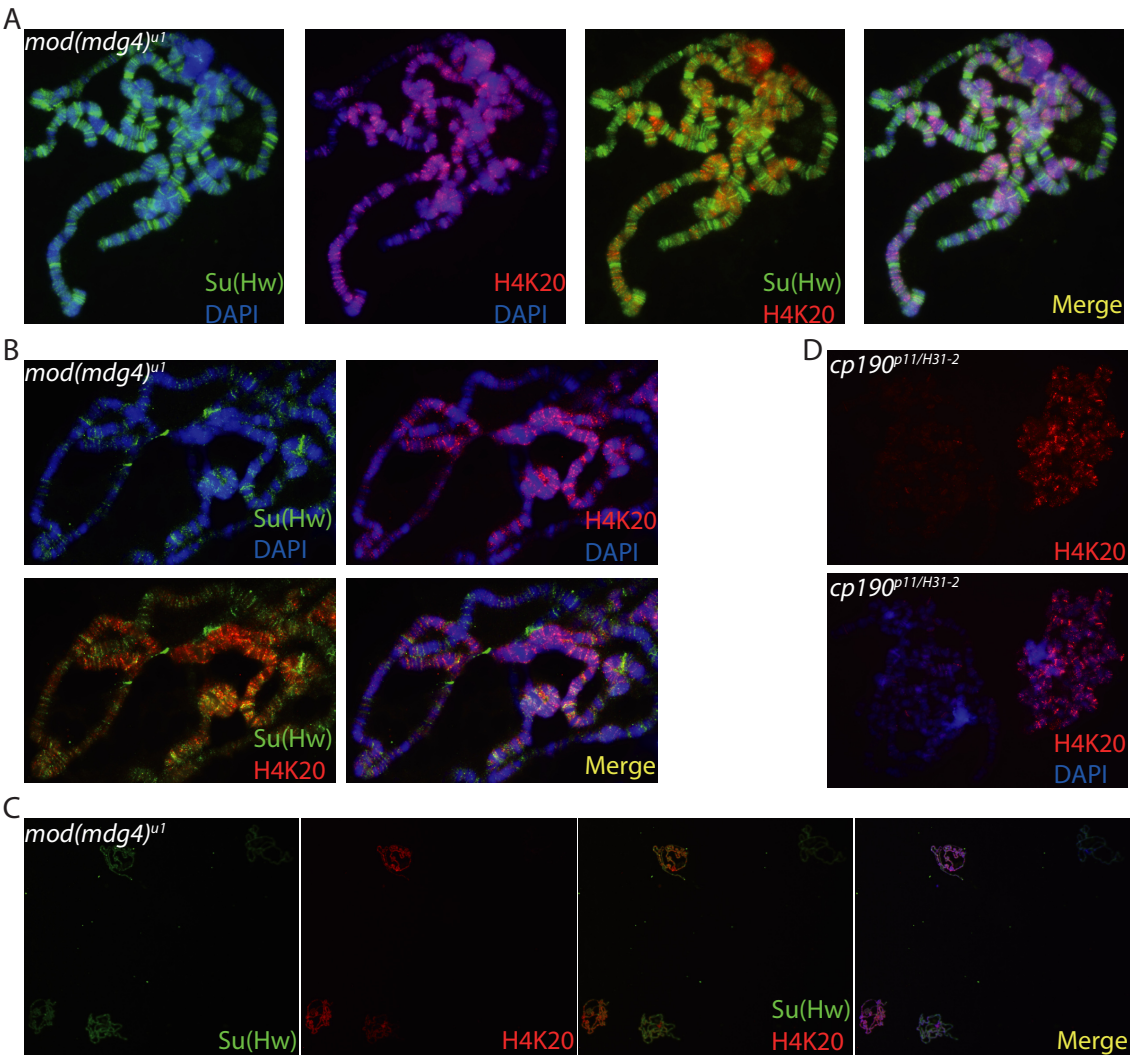

A
OR
Su(Hw)
H4K20
H4K20
Su(Hw)
Merge
B
su(Hw)e
H4K20
CP190
H4K20
CP190
Merge
C
su(Hw)e
HP1
H4K20
HP1
H4K20
Merge
D
su(Hw)e
H4K20
H4K20
DAPI
E
su(Hw)e
H4K20
H4K20
DAPI

Figure A2.2. Distribution of H4K20me1 on polytene chromosomes in *mod(mdg4)^{u1}* and *cp190$^{p11/H31-2}$* mutants. H4K20me1 is distributed with a different localization pattern on polytene chromosomes in *mod(mdg4)^{u1}* mutant (A-C), and *cp190$^{p11/H31-2}$* mutant (D).


A
mod(mdg4)u1
Su(Hw)
DAPI
H4K20
DAPI
Su(Hw)
H4K20
Merge
B
mod(mdg4)u1
Su(Hw)
DAPI
H4K20
DAPI
Su(Hw)
H4K20
Merge
D
cp190p11/H31-2
H4K20
cp190p11/H31-2
H4K20
DAPI
C
mod(mdg4)u1
Su(Hw)
H4K20
Su(Hw)
H4K20
Merge

VITA

Ran An was born in Qingdao, a seaboard city in China. She started to show her interest in biology when she played with the microscope to observe the amazing cell world in the hospital, where her mother worked as a doctor. This interest triggers her to choose Biological Sciences as her major in Shandong University without hesitation, and got her bachelor's in 2010. During her undergraduate study, she was selected to Yunnan University as an exchange student for one year, where she firstly learned that studying abroad could better realize her research dream and finally decided to apply for PhD study abroad after graduation. Fortunately, in 2010, she enrolled in the graduate program of Biochemistry, Cellular and Molecular Biology at the University of Tennessee, Knoxville, and joined in Dr. Mariano Labrador's lab as a PhD student. While doing research in the lab, she enjoyed the opportunities to teach senior undergraduate students as a teaching assistant, obtain a master degree in statistics to help her research and have internships in industries to broaden her views.