ABSTRACT

Accurate duplication and regulation of the eukaryotic genome requires precise coordination among multiple replication factors. *RecQL4*, the least characterized RecQ protein, is a 1208 amino acid protein containing a centrally located and highly conserved helicase domain. Mutations in *RecQL4* may lead to distinct clinical diseases with premature aging symptoms and increased cancer rates. Recently, efforts have focused on elucidating specific protein-protein interactions to shed light into the cellular processes in which RecQL4 may be involved. Previously, others have shown a direct interaction between RecQL4 and Mini-chromosome-maintenance protein 10 (Mcm10), a highly conserved protein with essential roles in DNA replication and heterochromatin formation. Work in 293T cells suggests that Mcm10 mediates the association of RecQL4 with the Mcm2-7 helicase; however, the biological ramifications of this interaction in a multicellular context remain unclear. Using both yeast two-hybrid and genetic approaches, we have shown that the RecQL4-Mcm10 interaction is conserved in *Drosophila melanogaster* and is important for alternate modes of replication. This study highlights the key roles of RecQL4 and Mcm10 in cell viability and further demonstrates their cooperative function in DNA replication.

Investigating the Interaction of RecQL4 and Mcm10 in *Drosophila melanogaster*

by

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Department of Biology
East Carolina University

Thesis submitted in partial fulfillment of the requirements for the degree of Master of Molecular Biology and Biotechnology in the Department of Biology in the Graduate School of East Carolina University

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CHAPTER 1: DETERMINING IF THE MCM10-RECQL4 INTERACTION IS CONSERVED IN *DROSOPHILA MELANOGASTER*

INTRODUCTION

The faithful completion of the highly regulated events of the cell cycle is essential in maintaining cell viability, the integrity of the genome, and in preventing many known clinical disease states. For example, cancer, the second leading cause of death in the United States, is the result of both intrinsic factors (inherited mutations, hormones, and immune conditions) and environmental/acquired factors (tobacco, diet, radiation, and infectious organisms) (Anand *et al* 2008). Additionally, mutations leading to failures in the DNA replication machinery have been seen in many different types of cancers; these failures include, but are not limited to, unregulated cell proliferation, failure to differentiate, and defects in chromosome biology (Jones *et al* 2007). Investigations regarding the mechanisms controlling DNA replication will improve our understanding of cancer biology, and may aid in the development of new treatment therapies.

DNA replication is one part of the highly orchestrated events of the cell cycle. The eukaryotic cell cycle consists of two consecutive processes, interphase and mitosis, primarily characterized by DNA replication and segregation of replicated chromosomes into two separate daughter cells, respectively (Vermeulen *et al* 2003). DNA replication occurs during the synthesis (S) stage of interphase. The S-phase is preceded by Gap 1 (G_1) in which the cell grows and prepares for DNA synthesis, and is followed by Gap 2 (G_2), during which the cell undergoes another growth period and prepares itself for the onset of mitosis. Dormant cells in G_1, before proceeding to DNA replication, may enter into a resting state called Gap 0 (G_0) (Vermeulen *et al* 2003). Mitosis, the process of nuclear division, includes the stages of prophase, metaphase, anaphase and telophase. It is the progression of these coordinated events of the cell-cycle that

must occur in a timely and efficient manner in order for cell proliferation to be properly sustained.

The accurate replication of genomic DNA requires an intricate coordination among multiple replicative factors prior to the onset of S-phase (Thu and Bielinsky 2013). Eukaryotic DNA replication relies upon the combined actions of the origin recognition complex (ORC), the cell division cycle 6 (Cdc6) protein and DNA replication factor Cdt1 to load head-to-head, double hexameric complexes of a helicase, Mcm2-7, onto replication origins. Helicases hydrolyze ATP to translocate along DNA, separating the two DNA stands and dislodging proteins in their path (Croteau *et al* 2012). The successful loading of these replication components, occurring during the G_1-phase of the cell-cycle, is often referred to as 'origin licensing,' and together, these factors constitute the pre-replication complex (pre-RC). At the G_1-to-S-phase transition, the protein kinases Cdc7/Dbf4 and Cdk2/Cyclin E are required to transform the Mcm2-7 double hexameric complex into an active replicative helicase, leading to the melting of origin DNA and the onset of DNA replication initiation (Zhu *et al* 2007). Cdc7/Dbf4 phosphorylates the Mcm2-7 complex, thereby allowing the recruitment of Sld3 and, the helicase coactivator, Cdc45. Cdk2/Cyclin E promotes formation of the pre-loading complex (pre-LC) composed of Sld2, Dpb11, DNA polymerase (Pol)-ε, and a second helicase coactivator complex, GINs (go-ichi-ni-san) (Thu and Bielinsky 2013). It is important to note that Pol-ε is not equipped to initiate DNA synthesis but, another enzyme, Pol-α/primase, exists for this purpose; however, the association of Pol-α/primase requires unwound DNA. The complicated cascade of events leading to origin unwinding is under precise coordination and Mcm10 appears to be a crucial component in the execution of these steps. When exactly Mcm10 associates with replication origins is still an open question for investigation.

Mcm10 is a highly abundant nuclear protein conserved in eukaryotic organisms ranging from yeast to humans (Figure 1-1). It is closely associated with the Mcm2-7 complex, but it is not part of the same protein family (Liachko and Tye 2008). Mcm10 has been shown to interact with members of the pre-RC and elongation complex (Christensen and Tye 2003). Mcm10 is important for mediating interactions between other replication proteins (Liachko and Tye 2008); indeed, yeast two-hybrid studies have demonstrated binding of Mcm10 to itself, Mcm2, Orc2, and Hp1 (Apger *et al* 2010). Recent findings also indicate that Mcm10 is required for a novel step during activation of the Cdc45-MCM-GINS (CMG) helicase at DNA replication origins (Deursen *et al* 2012). Taken together, these observations suggest a role for Mcm10 in DNA replication as a helicase activator and/or a component of the elongation machinery.

RecQ like protein 4 (RecQL4/ RecQL4) is a member of the RecQ family of helicases first identified by Kitao and colleagues (Kitao *et al* 1998). All prokaryotes and eukaryotes possess at least one member of the RecQ family. Bacteria and yeast possess a single RecQ protein, while five orthologs exist in humans and as many as seven have been reported in certain plant species (Macris *et al* 2005). The RecQ protein family contains a highly conserved SuperFamily II (SFII) helicase domain that is essential for viability but outside of this homology the proteins vary significantly (Figure 1-2; Capp *et al* 2009). Loss of function of human family members, WRN and BLM, lead to autosomal recessive diseases Werner syndrome and Bloom syndrome, respectively (Croteau *et al* 2012). Mutations in *RecQL4* result in three autosomal recessive diseases: Rothmund-Thomson syndrome (RTS), RAPADILINO, and Baller-Gerold syndrome. These disease states present with a wide variety of symptoms including growth retardation, cataracts, a characteristic skin rash (poikiloderma), skeletal abnormalities, and a predisposition to cancer, specifically osteogenic sarcomas.

Of the three RecQ proteins implicated in human disease, RecQL4 is the least well characterized. Early reports, by two independent groups, showed sequence homology between the *Saccharomyces cerevisiae* DNA replication initiation factor, Sld2, and the N-terminus of the Xenopus RecQL4 homolog, xRTS (Matsuno et al 2006; Sangrithi et al 2005). These observations demonstrate RecQL4 to be the only known metazoan homolog of Sld2. The same groups went on to demonstrate that the N-terminus of xRTS is necessary for replication initiation in Xenopus oocyte extract and that ~20% of wild-type replication levels are restored when xRTS-depleted Xenopus oocyte extracts are supplemented with the N-terminus of human RecQL4. Further, Xu and colleagues (Xu *et al* 2009) used ChIP to report that human RecQL4 is a primary interacting partner to replisome factors Mcm10, the Mcm2-7 complex, Cdc45 and GINS. Similar studies demonstrated that the CMG complex, in human cells, requires RecQL4 and Mcm10 (Im *et al* 2009). Additionally, multiple reports show RecQL4 dynamically associating with foci produced by DNA damaging agents including hydrogen peroxide (H_2O_2), ionizing radiation, topoisomerase inhibitors, and UV (Croteau *et al* 2012). Taken together, these studies suggest that RecQL4 is a key player in replication and damage repair.

For nearly 100 years, the fruit fly has been used as a model to answer questions relating to mechanisms of inheritance, the construction of the animal body plan, the formation of complex biological patterns, the function of the nervous system and the forces acting on genetic variation in natural populations (Matthews *et al* 2005). The fruit fly has also been widely used as a model to investigate gene function, as the entire *Drosophila* genome has been fully sequenced and annotated; a resource that is publicly available. Additionally, it is currently known that 75% of all human disease genes have related sequences in *Drosophila melanogaster* (Bier 2005). With this in mind, I intend to use *Drosophila melanogaster* as a model to analyze the interaction

of Mcm10 and RecQL4 to better characterize their function physically and genetically. The findings from this study could fundamentally improve our understanding of these clinical diseases and the processes of DNA replication as a whole.

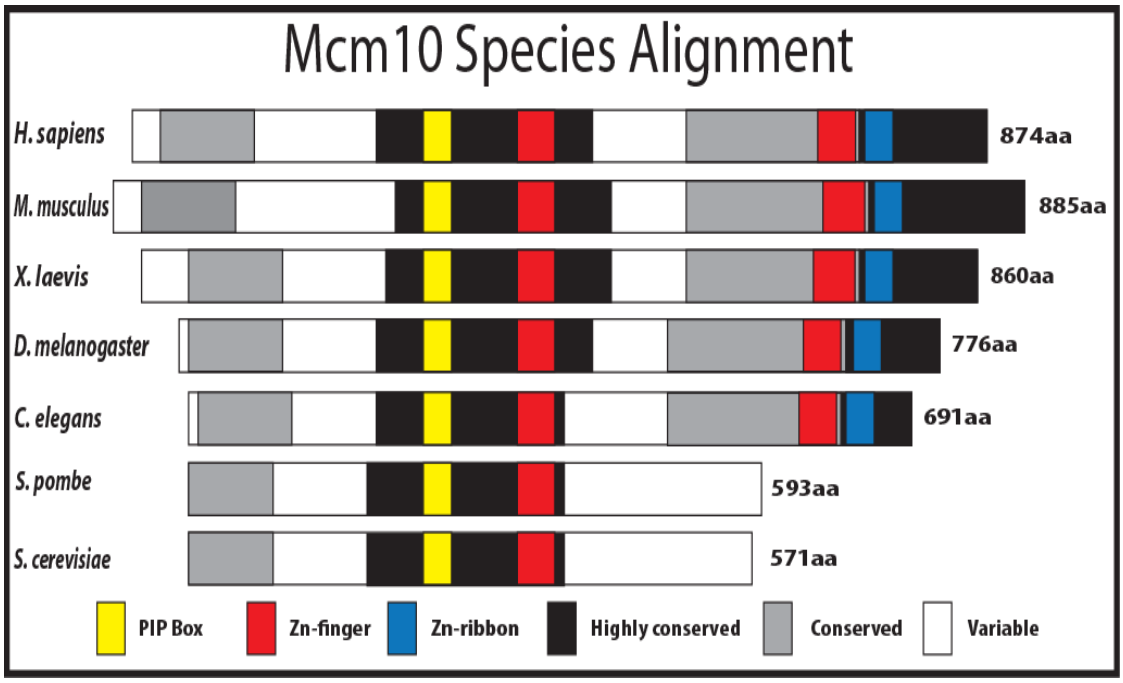


Figure 1-1: Schematic representation of the conserved Mcm10 protein across multiple species depicting conserved zing finger motifs (red and blue), highly conserved regions (black), moderately conserved regions (grey), and the PCNA-interacting protein (PIP) box (yellow). In addition, metazoan species harbor an extended C-terminus that has been demonstrated to facilitate key protein-protein interactions.

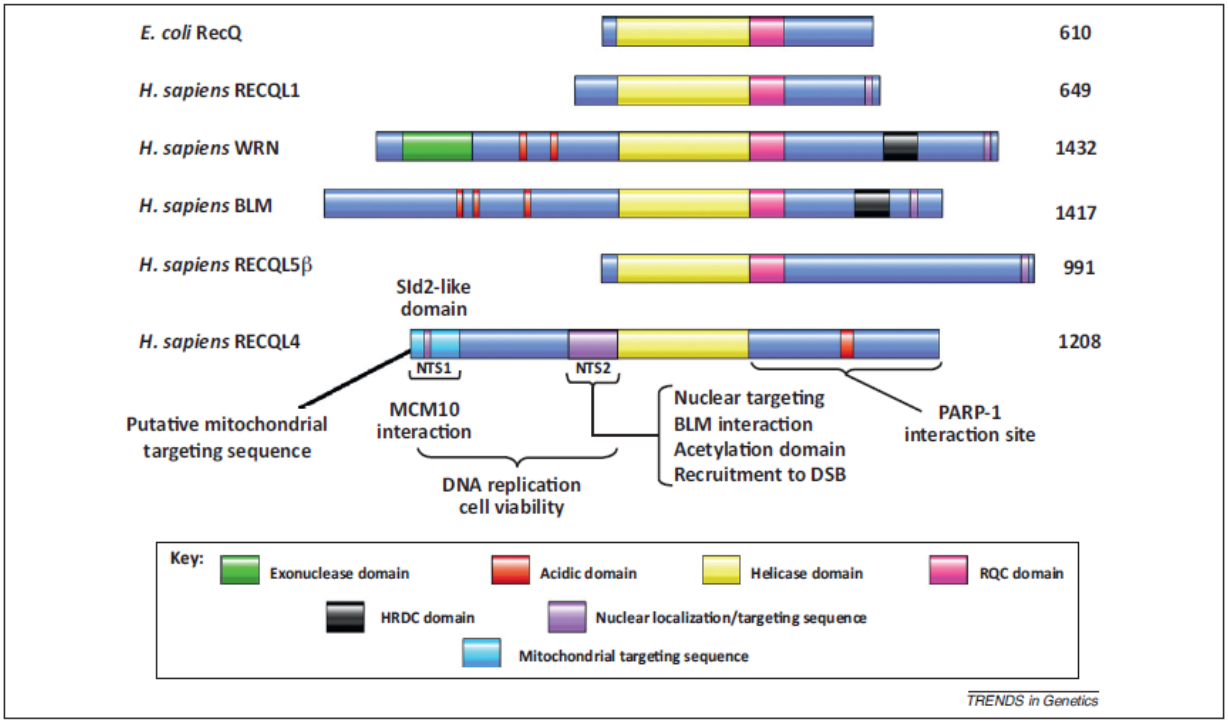


Figure 1-2: Schematic representation of human RecQ helicase family members. A single RecQ protein is found in bacteria, three in *Drosophila*, five in humans, and seven in certain plant species. RecQ proteins share a highly conserved internal domain (yellow) possessing ATP-dependent 3'-5' helicase activity. RecQL4 contains a unique N-terminal domain which shares sequence homology with the yeast replication initiation factor, Sld2. (Croteau *et al* 2013)

OBJECTIVE

The specific purpose of this research was to determine whether the Mcm10-RecQL4 protein interaction is conserved in *Drosophila melanogaster*. A study in human kidney embryonic cells (293T) has suggested a direct interaction between RecQL4 and Mcm10 using FLAG tagged co-immunoprecipitation and western blotting (Xu *et al* 2009). Xu and colleagues further demonstrated that the Sld2-like domain of RecQL4 is responsible for this direct interaction and that Mcm10 acts to mediate the association of RecQL4 with the Mcm2-7 replicative DNA helicase and GINS complex in a cell-cycle dependent manner. Through the manipulation of the yeast GAL4 transcription factor, in the context of the yeast two-hybrid system, we wish to determine if the interaction between RecQL4 and Mcm10 is conserved in *Drosophila melanogaster.*

In order to use the yeast two-hybrid system to test for protein-protein interactions, the genes encoding our proteins of interest must first be cloned into vectors for entry into the Gateway® System and subsequently transformed into our yeast strain, AH109. The Gateway® cloning system exploits the site-specific recombination system utilized by bacteriophage lambda to shuttle sequences between plasmids bearing flanking compatible recombination attachment (*att*) sites. Once captured by our entry clone, pENTR, the genes encoding our proteins of interest can be recombined into our destination vectors, pGBKT7 and pGADT7, resulting in expression clones geared for protein-protein interactions analysis (Liang *et al* 2013). Once these protein constructs are successfully transformed into yeast, cells containing the fusion proteins are plated on permissive media and restrictive media lacking histidine. Growth on the permissive media is expected, while growth on the restrictive media indicates the presence of a physical protein-protein interaction. For our analysis, we will utilize the *HIS3* reporter gene, encoding the

essential amino acid histidine which is necessary for yeast survival, to act as the selectable marker for a positive protein interaction. A positive interaction of our two proteins of interest will promote the recruitment of RNA Polymerase II, thereby allowing the onset of transcription of our reporter gene (Figure 1-3).

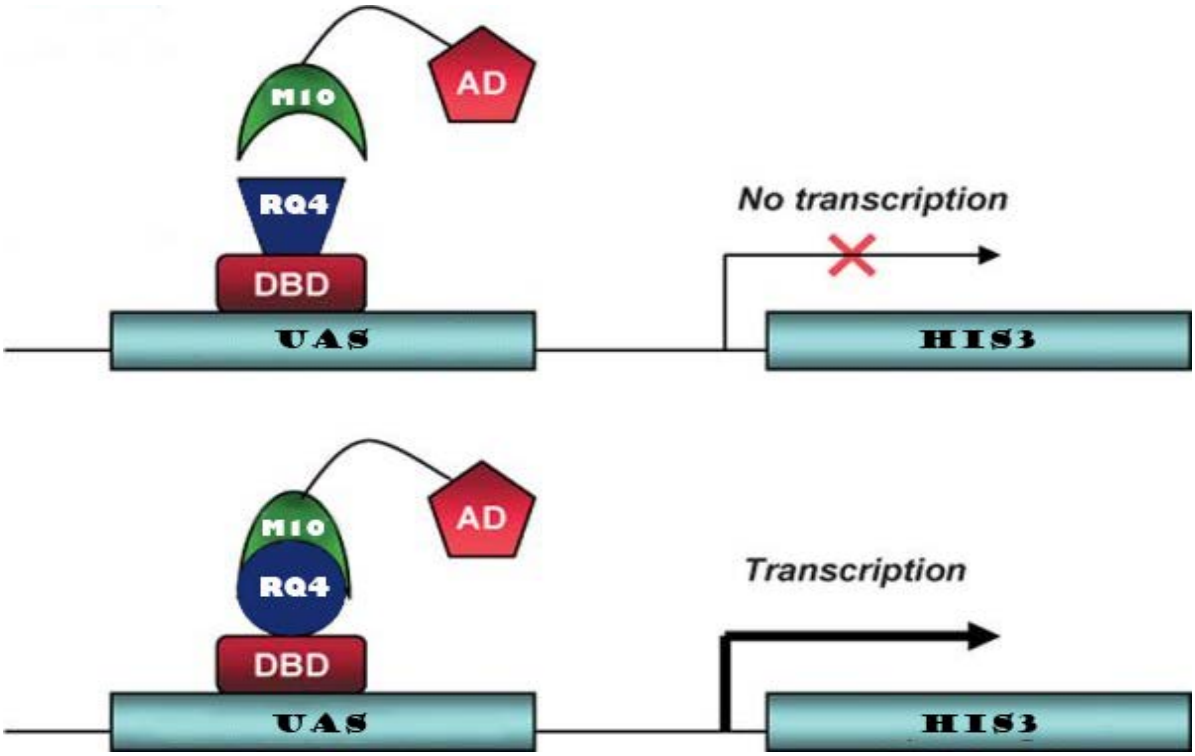


Figure 1-3: Yeast two-hybrid system. Genes encoding Mcm10 (M10) and RecQL4 (RQ4) are fused to the GAL4 activating domain (AD) and binding domain (BD), respectively.

MATERIALS AND METHODS

Yeast Strains, Media, and Transformations: *Saccharomyces cerevisiae* strain AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 :: GAL1*$_{UAS}$ *GAL1*$_{TATA}$*-HIS3, GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2, URA3 :: MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ)* was grown in YPD complete medium containing 1% yeast extract, 2% peptone, and 2% D-glucose. Plasmid DNA bacterial transformations were conducted as described in the α-select chemically competent cells general use protocol (Bioline); cells were grown in medium lacking leucine, tryptophan, or histidine as necessary to select for the presence of various plasmids.

Reverse Transcription-PCR Cloning of *recql4* cDNA: To obtain cDNA for *Drosophila recql4*, total RNA from *Drosophila* early embryos (0-8hrs) was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit. The first-strand cDNA was amplified by PCR with the Elongase® Enzyme mixture (Life Technologies) using an in-frame forward primer (5'-CACCGCCGACATGGACG-3') and reverse primer (5'-GAGTAAAATGTTATTGAATGG-CATCAC-3'). The *recql4* cDNA was confirmed by DNA sequencing to be free of errors that would alter the RecQL4 protein. The 4.8-kb PCR product was subsequently cloned into the entry vector pENTR/D (Clontech).

Yeast two-hybrid system: Yeast manipulation and growth were conducted using standard protocols as in Christensen and Tye (2003) and as found in manufacturer's protocols (Clontech; Matchmaker Yeast Two-Hybrid System). Yeast strain AH109 (Clontech) was used as the reporter strain. Plasmids used were pGBKT7 and pGADT7 (Clontech) except that both wre converted to the Gateway cloning system (Invitrogen) by insertion of the Gateway cassette into the multiple cloning site. In addition, the Kan^R gene in pGBKT7 was disrupted by insertion of the Amp^R gene to facilitate use in the Gateway system. Entry clones were all sequence verified

prior to LR reactions with two-hybrid plasmids. Resulting two-hybrid clones were then sequence verified to ensure the proper reading frame was maintained and no ectopic mutations were introduced. Dilution series (1:5) were plated to quantify interaction strength.

RESULTS

The main objective of this investigation was to determine if the Mcm10-RecQL4 interaction is conserved in *Drosophila melanogaster*. Emerging evidence suggests that interaction between Mcm10 and RecQL4 is important for maintaining the integrity of the genome, with cooperative roles in DNA replication (Xu *et al* 2009; Im *et al* 2009). To conduct this analysis, we utilized the yeast two-hybrid system which takes advantage of the separable domains found in the yeast GAL4 transcription factor. Full length Mcm10 was fused to the GAL4 activating domain (pGADT7) while full length RecQL4 was fused to the GAL4 binding domain (pGBKT7) (Figure 1-3). These fusion constructs were subsequently transformed into our yeast strain AH109 and plated on permissive and restrictive complete media lacking histidine. Interactions were indicated by growth on the restrictive media as a result of the transcription of the HIS3 reporter construct. As expected, we observed growth on our restrictive media containing both Mcm10 and RecQL4; although, growth was seen on restrictive media with pGBKT7 RecQL4 lacking pGADT7 Mcm10 (data not shown). We believed this to be the result of one-hybrid activity and decided to switch vector orientations so that full length Mcm10 was fused to the GAL4 binding domain (pGBKT7) and full length RecQL4 was fused to the GAL4 activating domain (pGADT7). By switching vector orientation, we were able to show an interaction that was specific to Mcm10 and RecQL4 (Figure 1-4).

In order to determine the domains of Mcm10 responsible for the observed protein interaction, a yeast two-hybrid analysis was performed using 200 amino acid fragments of Mcm10, overlapping by 100 amino acids, against full length RecQL4 (Figure 1-6). This allows for a 100 amino acid resolution with respect to interaction domains. Fragments of Mcm10 were fused to the GAL4 binding domain (pGBKT7) and full length RecQL4 was fused to the GAL4

activating domain (pGADT7). Our results reveal that the last 176 amino acids of Mcm10 contribute to the interaction with *Drosophila* RecQL4. It should be noted the first 100 amino acids of Mcm10 has been reported to have one-hybrid activity when fused to the GAL4 binding domain (pGBKT7) (Figure 1-6; Apger *et al* 2010). Therefore, in order to eliminate the one-hybrid activity in these interaction studies, clones were constructed without the first 100 amino acids when Mcm10 was fused to the GAL4 binding domain.

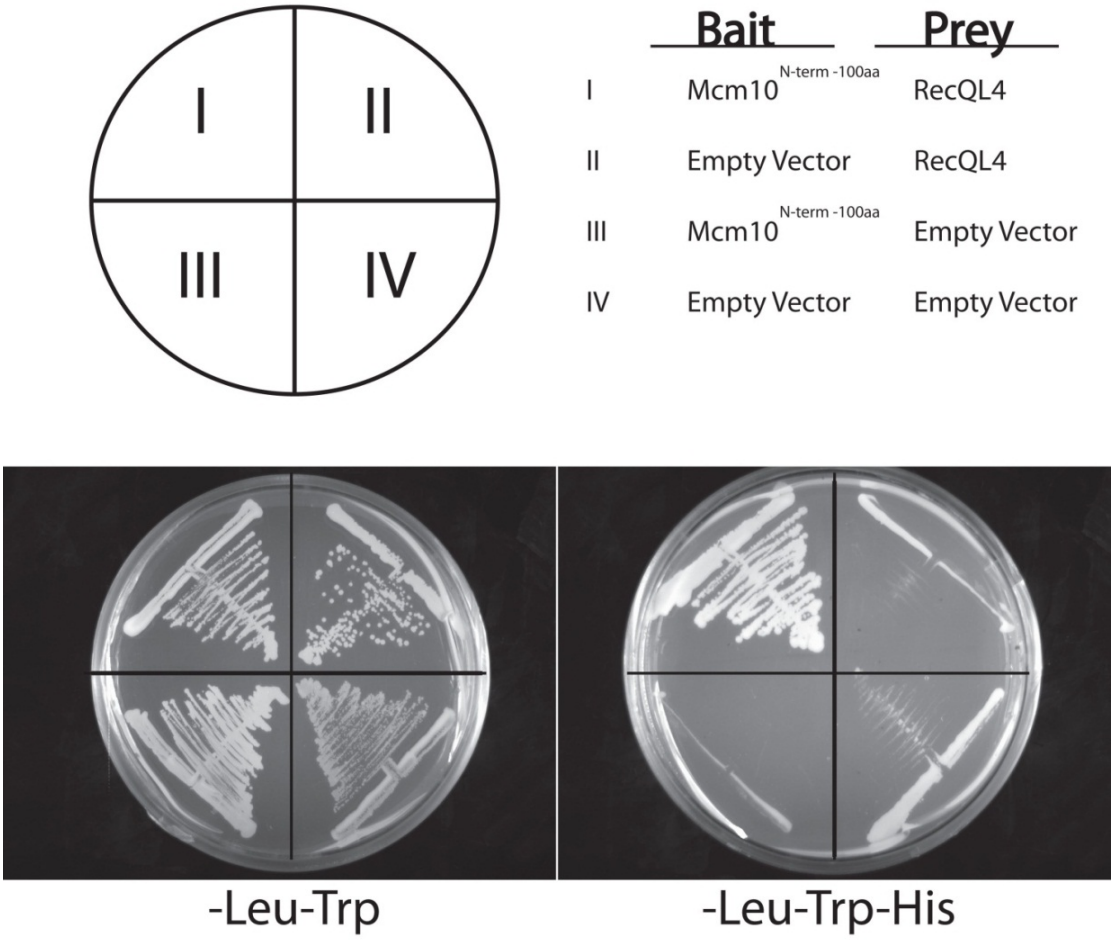


Figure 1-4: *Drosophila* Mcm10 directly interacts with RecQL4. Top panel: Schematic diagram of the proteins fused to the GAL4 activation domain (GAL4AD) in pGADT7 vector and GAL4 binding domain (GAL4BD) in pGBKT7 vector for prey and bait constructs, respectively, indicated in the top panel. Bottom panel: The growth of yeast co-transformants containing both GAL4BD and GAL4AD fusion proteins on the SD permissive medium (lacking Leu and Trp) and restrictive medium (lacking Leu, Trp, and His).

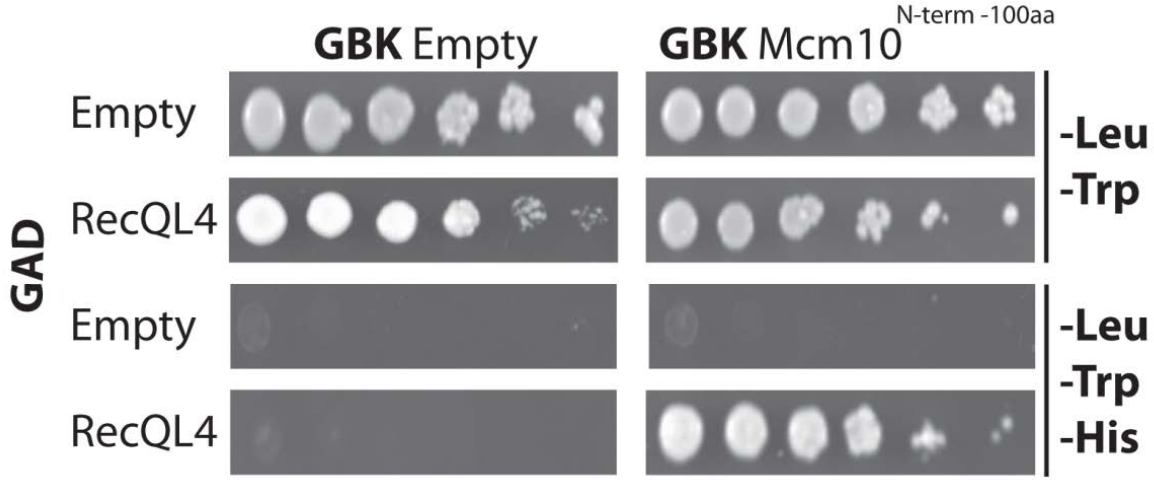


Figure 1-5: Yeast two-hybrid 5-fold dilution series showing the interaction of full length Mcm10 with full length RecQL4. Two-hybrid interactions indicated by growth on media lacking histidine between Mcm10 and RecQL4. Growth was not observed on empty controls. Mcm10 constructs were made to lack the first 100 aa due to its previously reported one-hybrid activity.

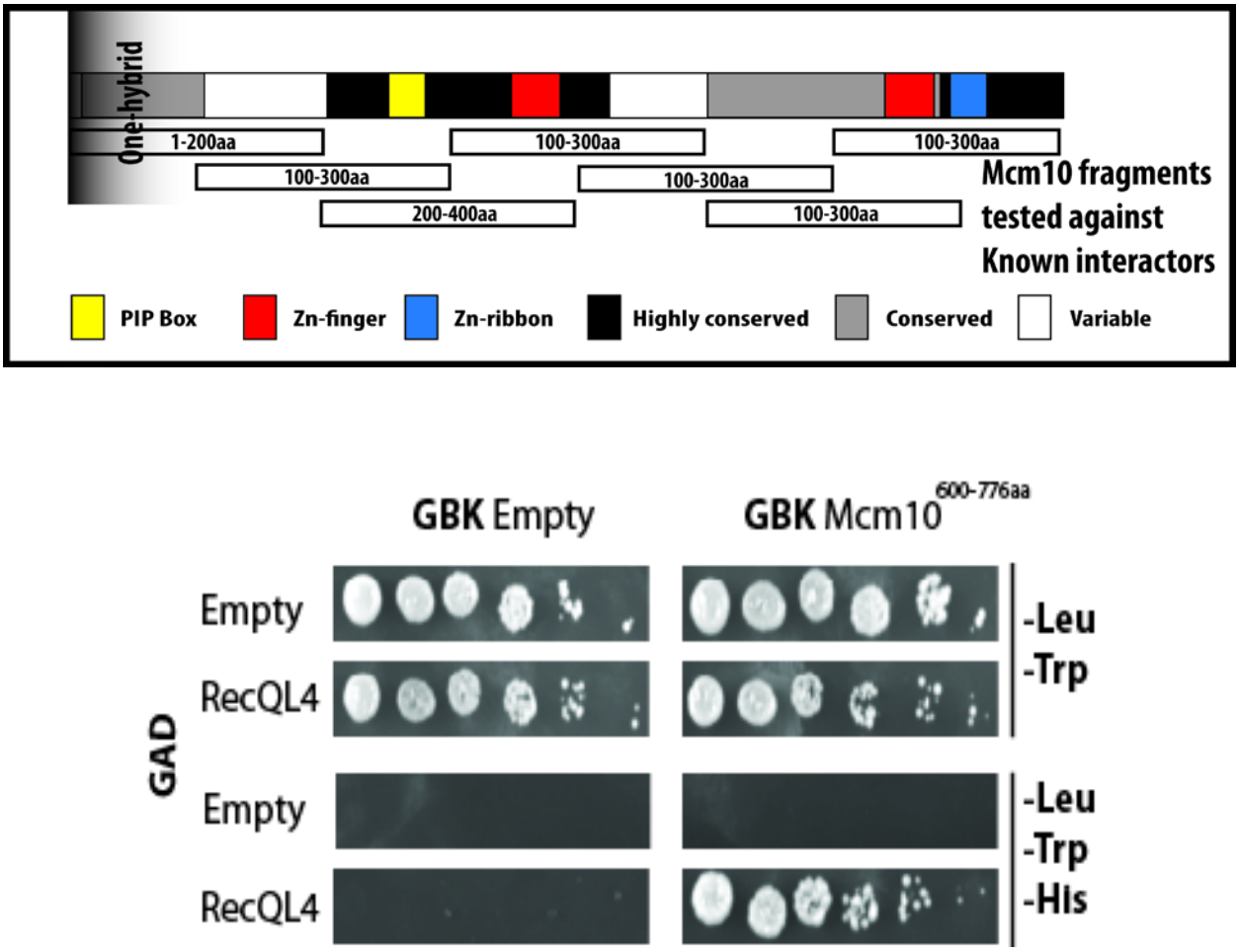


Figure 1-6: Yeast two-hybrid dissection of Mcm10 interaction domains responsible for the Mcm10-RecQL4 interaction in *Drosophila melanogaster*. Two-hybrid interactions indicated by growth on media lacking histidine. Growth was not observed on empty controls.

DISCUSSION

The yeast two-hybrid system, first described by Fields and colleagues, was devised to identify genes encoding proteins that physically associate with a given protein *in vivo* (Fields and Song 1989). Although numerous modifications, improvements, and multitudinous applications of the technique have been brought forth, undoubtedly the most valuable use of the two-hybrid system has been to identify protein partners for a protein of interest (Liu 1998). In this report, we have utilized the two-hybrid technology to show that the Mcm10-RecQL4 interaction is conserved in *Drosophila melanogaster* (Figure 1-4). This novel finding is consistent with results of other laboratories that have shown the Mcm10-RecQL4 interaction to exist in human cell culture using coimmunopreciptation and mass spectrometry (Xu *et al* 2009; Im *et al* 2009). It appears that the Mcm10-RecQL4 interaction is present in metazoan species which may be the result of the unique Mcm10 C-terminus found in higher eukaryotes.

The versatile and powerful yeast two-hybrid system not only allows identification of proteins that interact, but also can be used to define and test the domains and/or residues necessary for the interaction of two proteins (Li and Fields 1993). To determine if the unique C-terminal region of Mcm10 is responsible for the interaction with *Drosophila* RecQL4 we created a protein construct containing the last 176 amino acids of Mcm10 to test against full length RecQL4. Our analysis demonstrates that the Mcm10-RecQL4 interaction is at least partly due to the last 176 amino acids of Mcm10 (Figure 1-6) which is consistent with the findings of previous reports detailing the importance of the Mcm10 C-terminal domain in facilitating protein interactions with essential replication factors (Apger *et al* 2010). The C-terminal extension of Mcm10 manifests not only in *Drosophila* but also in other metazoan species (Figure 1-1; Thu and Bielinsky 2014). In contrast, the C-terminal domain varies highly from uni-to multicellular

organisms with *Saccharomyces cerevisiae* and *Saccharaomyces pombe* lacking the C-terminal interface (Figure 1-1; Thu and Bielinsky 2014). The expansion and conservation of Mcm10's C-terminal domain may be a consequence of evolutionary pressures associated with DNA replication through the more complex and varied genomic contexts that are present in the larger genomes and differentiated tissues of higher eukaryotes (Apger *et al* 2010). Nevertheless, the biological implication of an additional DNA-binding domain in higher eukaryotes is not yet clear and remains a topic of interest to dissect differences in function across species. Our findings support the notion that the two Zn-coordinating structures residing within the C-terminal domain of higher eukaryotes are essential and provide the main interaction surfaces for DNA and protein binding (Thu and Bielinsky 2014).

CHAPTER 2: PHENOTYPIC CHARACTERIZATION OF THE *DROSOPHILA MELANOGASTER* MCM10-RECQL4 INTERACTION THROUGH AN EXTENSIVE DOUBLE MUTANT ANALYSIS

INTRODUCTION

Understanding how the interaction between RecQL4 and Mcm10 contributes to DNA replication will require thorough examination. Wu and colleagues (2008) first established *Drosophila* as a model system for probing RecQL4's functions in DNA replication through the analysis of two *P*-element transposon-induced mutant alleles, $recq4^{19}$ and $recq4^{23}$. Additionally, our group (Apger *et al* 2010) has previously analyzed two *Drosophila* Mcm10 mutants, $Mcm10^{Scim19}$ and $Mcm10^{d08029}$, which has suggested a role for Mcm10 in DNA replication, chromosome condensation, and heterochromatin formation. To gain insight into the fairly uncharacterized interaction, we will generate flies harboring both the described before RecQL4 and Mcm10 mutant alleles. Single and double mutant flies will subsequently be examined for abnormalities in cell cycle progression and chromosome morphology within the 3rd instar larval brain (Reubens *et al* 2014); chromosome compaction and DNA content in the endoreplicating salivary glands (Chmielewski *et al* 2011); and synchrony of the mitotically dividing cells undergoing early embryogenesis (Apger *et al* 2010).

OBJECTIVES

Considering the lethality and female sterility phenotypes seen in our homozygous *RecQL4* mutants (Wu *et al* 2008), we intend on using balanced heterozygotes to generate double mutants flies. Balancer chromosomes suppress recombination with their homologues allowing for the maintenance of lethal and sterile mutants as balanced heterozygotes while carrying dominant markers that are visible in adult flies (Lattao *et al* 2011). Both previously described RecQL4 mutants were received balanced over *TM3* (Third Multiple 3; FBba0000047) carrying the *Sb* marker (*Stubble*; FBgn0003319). This dominant marker, located on the right arm of the third chromosome, presents with stout and short bristles found on the superior adult thorax. As reported in Apger *et al* 2010, our Mcm10 mutants are homozygous viable; albeit showing low viability, as homozygotes are underrepresented when compared to their heterozygous siblings (coined as a semilethal phenotype). These mutant alleles will be balanced over *CyO* (*Curly of Oster*; FBba0000025) carrying the *Cy* marker (*Curly*; FBgn000403). Adult flies harboring the *CyO* present with wings curled upward and outward with expressivity of this phenotype shown to increase with temperature. The generation of these double mutant flies will aid in our understanding of the relationship between Mcm10 and RecQL4 through an extensive phenotypic analysis using various *Drosophila* tissues.

The brain of third-instar, wandering *Drosophila melanogaster* larva is a highly useful tissue for cytological studies investigating the mitotic phenotypes of mutations thought to impact cell-cycle progression and chromosome morphology. This tissue is unique in that, unlike most other larval organs, it persists into the adult stage (Truman *et al* 1990). To add to the growing body of evidence supporting a role for Mcm10 and RecQL4 in DNA replication (Wu *et al* 2008; Apger *et al* 2010; Croteau *et al* 2012; Thu and Bielinsky 2010), we will take advantage of the

cells in the third instar larval brain containing proliferative centers that produce characteristic patterns of cell cycle progression. Specifically, a phospho-histone-3 antibody will be incorporated in to our previously established protocol (Apger *et al* 2010) to obtain mitotic indices (Reubens *et al* 2014). Knowing that the initiation of histone-3 (H3) phosphorylation occurs at different phases of cell division with a global H3 dephosphorylation observable upon exit of mitosis (Hans and Dimitrov 2001), we can use this approach to generate data sets amendable to parametric testing. Samples will be prepared for quantitative analysis as described in Reubens *et al* 2014.

With the advent of molecular genetics, it is now understood that eukaryotes possess multiple different cell cycle variations. In particular, endoreplication is a variant form of replication in which cells increase their genomic DNA content without dividing (Edgar and Orr-Weaver 2001). The endoreplicating nuclei found in *Drosophila melanogaster* salivary glands provide an avenue for the study of mechanisms controlling chromosome condensation and DNA replication. By the time larvae reach third instar, their nuclei complete ten successive rounds of replication; reaching an average ploidy of 1024n (Edgar and Orr-Weaver 2001). Using the well-studied *Drosophila* salivary glands, we wish to quantitate the chromosome compaction and DNA content of our RecQL4 and Mcm10 mutants. As described in Chmielewski and Christensen 2011, using the Qubit® dsDNA assay kit in combination with our novel method for determining the nuclear volume of DNA, we can assess the quantity of DNA per unit volume ($pg/\mu m^3$).

The *Drosophila melanogaster* embryo provides a unique model to study developmental patterns of mitotically dividing cells (Foe and Alberts 1983). Cell-cycles in the *Drosophila* early embryo occur in a syncytium (i.e., cells share the same cytoplasm and are not divided into separate cells by plasma membranes), lack gap phases, proceed in synchrony, and occur rapidly.

Previous work using a RecQ4-GFP transgene located on the second chromosome has shown that RecQL4 is nuclearly localized during interphase (coincident with DNA staining) and subsequently dispersed into the cytoplasm during prophase, metaphase, and anaphase (Wu *et al* 2008). RecQ4-GFP nuclear localization is then seen again upon the formation of new nuclear envelopes during telophase. These observations suggest a role for *Drosophila* RecQL4 in DNA replication. Additionally, it has been reported that Mcm10 is maternally loaded and present at highest levels in the early embryo (Arbeitman *et al* 2002). Taken together, we wish to determine if these two proteins are important for the coordinated cellular development of the *Drosophila* early embryo. Early embryos will be collected after 8 hours and DAPI stained to visualize the behavior of nuclear DNA.

MATERIALS AND METHODS

Fly husbandry/stocks: Fly stocks (w^{118} FlyBase ID, FBst0003605; $Mcm10^{Scim19}$ FlyBase ID, FBst0013070, y[1] w[67c23]; $Mcm10^{d08029}$ FlyBase ID, FBst1011557, P{XP}Mcm10[d08029]; $RecQ4^{23}$ FlyBase ID, FBst0041120, w[*], RecQ4[23]/TM3, Sb[1]; $RecQ4^{19}$ FlyBase ID, FBst0041119, w[*], RecQ4[19]/TM3, Sb[1]) were obtained from the Bloomington Fly Stock Center and the Exelixis *Drosophila* Stock Collection at Harvard Medical School. Production of experimental strains harboring mutations in both *Mcm10* and *RecQ4* were generated using the chromosomal translocation strain ap^{Xa} (FlyBase ID, FBab0007867, $T(2;3)ap^{Xa}$). *Mcm10* and *RecQ4 P*-element insertions were confirmed by PCR (data not shown). All fly stocks were maintained at 25°C on *Drosophila* Diet Medium K12 (U.S. Biological no. D9600-07B).

Larval brain squash/mitotic index: Third instar wandering larvae were harvested from age- and density-matched bottles and dissected in a 16-well dissecting dish containing 100 µl 1X PBS, pH 7.2, with 1% PEG 8000 using No.5 tweezers (Electron Microscopy Sciences, Hatfield, PA). After removing attached imaginal discs, third instar wandering larval brains were transferred to a hypotonic solution (0.5% sodium citrate) and incubated for 10 minutes. Brains were then fixed with 4% formaldehyde in dH_2O for 30 minutes. Fixed samples were transferred to a cleaned microscope slide, overlaid with a siliconized coverslip, and subsequently squashed using a 4" vise. Following the squash, the sample slide was lowered into liquid nitrogen for 1 minute allowing the removal of the siliconized coverslip and then incubated in 75 µl 1X PBS for 6 minutes. The sample was further incubated in 75µl 0.5% Triton-X in 1X PBS for 15 minutes and then 75 µl BSA in membrane wash buffer (10X PBS and 2% Tween-20) for 30 minutes at room temperature. Using a 20cc syringe equipped with a 22 gauge blunt fill needle filled with

Vaseline, a single line of petroleum jelly was dispensed encircling the squashed sample. The following primary antibody was incubated in 3% BSA overnight at 4°C: rabbit anti-phosphohistone H3 (Upstate Biotechnology/Millipore, 1:750). Following overnight incubation, squashed samples were washed three times with 1X PBS and subsequently incubated in 100 µl secondary antibody (anti-rabbit 468; 1:500 in 3% BSA) in the dark for 1 hour at room temperature. The sample was again washed three times with 1X PBS, counterstained with 3 µg/mL DAPI (Sigma), rinsed an additional three times with 1X PBS, and mounted in Vectashield (Vector Labs). Mitotic index determinations were performed on these squash preparations by selecting 10 random, well-populated fields of view for each brain squash, using a 63X objective on the Zeiss LSM700 confocal microscope equipped with Zeiss's ZEN Black software package. Total nuclei were counted for each field and the total was divided by the total number of phospohistone H3 positive cells observed in each field to generate the fraction of cells in mitosis. Mitotic indices were calculated and square root transformations were conducted in Excel® 2010. Mitotic indices were organized by slide ID so that comparisons could be made on a slide by slide basis. Datasets were imported into the JMP®10 software package to visualize the distributions, conduct Levene's test of variance, and carry out the paired t-test. Data was imported into R statistical software to conduct Kolmogrov-Smirnov Goodness of Fit analyses.

Chromosome compaction and DNA content analysis: DNA compaction and DNA content analysis were performed as described previously (Chmielewski and Christensen 2011). Briefly, for salivary gland whole mounts to acquire DNA volume, salivary glands form third instar wandering larvae were dissected in 100 µl 1X PS, fixed in 4% formaldehyde in 1X PBX (1X PBS with 1% Triton X-100), and stained with DAPI. After DAPI staining, removed salivary glands were transferred to a slide prepared with Vaseline to serve as a support for the coverslip

and mounted in Vectashield Mount Medium (Cat. No. H-1000, Vector Labs). Montage images were acquired using the 20X objective on the Zeiss LSM700 confocal microscope equipped with the ZEN Black software package. The areas of polytene nuclei were established using Adobe® Photoshop® element CS5 software. To determine DNA content per salivary gland pair, salivary glands were dissected out of wandering third instar larvae in 150 µl of HyQ Graces's Unsupplemented Insect Cell Culture Medium (cat. No. 30610.01, HyClone, Logan, UT, USA). Once dissected, glands pairs were transferred to a PCR tube (Fisher, cat. No. 14230225) prefilled with 3-5X 1 mm glass beads (Biospec Products, Inc., cat. No. 11079110) along with 300 µm glass beads (Sigma, 212-300 µm) and 25 µl of squishing buffer (20 µg/ml proteinase K, 100mM Tris-Base, 25 mM NaCl, and 1mM EDTA). Glands were vortexed to facilitate the breakup of the salivary gland then incubated at 37ºC for 30 minutes and 85ºC for 10 minutes, vortexed again for 15 seconds, and spun at 12,000 rpm for 2 minutes. DNA content was determined using the Qubit® dsDNA HS Assay Kit (Invitrogen, cat. No. Q32854) along with the Qubit® 2.0 Florometer (Invitrogen, cat. No. Q32866) following manufacturer's instructions.

Early embryo collection and developmental staging: Embryos were collected, fixed with methanol/EGTA, prepared, and stained as in Kellum and Alberts (1995). Establishment of developmental stages was based on the number of DAPI stained nuclei in each individual syncytial embryo. Microscopy was performed using the Ziess LSM700 laser confocal controlled by Zeiss ZEN Black software.

Egg count and hatch rates: Egg production by wild-type and mutant females after a single mating was scored by counting the number of eggs laid in successive 24-hour periods every other day for a total of 10 days. Five pairs of adult flies of the same genotype were placed on grape agar media containing yeast paste for the duration of the experiment. To calculate the

rate of egg hatching, fifty freshly laid eggs were lined on grape ager and incubated at 25ºC for 48 hours. Four trials were conducted for each genotype, resulting in a total of 200 eggs examined. After the incubation period, the number of unhatched and hatched eggs was determined.

RESULTS

Genomic organization of *recq4* and *mcm10* mutant alleles: RecQL4 mutant alleles, $recq4^{19}$ and $recq4^{23}$, were generated through *P*-element imprecise excision. The $recq4^{19}$ mutant allele contains a remnant insertion of 3.5 kb mapped to 43 nucleotides upstream of the translation initiation codon of *RecQL4* (Wu *et al* 2008). In addition, $recq4^{19}$ has a deletion of 3220 bp from the nucleotide 43 bp upstream to nucleotide 3174 downstream of the translation initiation codon of *RecQL4*. Due to the nature of this deletion, removing most of the coding region of *RecQL4,* this allele is expected to act as a null mutant (Figure 2-1). The null mutants present with homozygous lethality within the first instar larval stages. Further, $recq4^{23}$ has an insertion of 3.8 kb mapped to 43 nucleotides upstream of the translation initiation codon and expected to act as a hypomorphic mutant (Figure 2-1). Homozygous $recq4^{23}$ flies present with low viability and female sterility (Wu *et al* 2008).

Mcm10 mutant alleles, $Mcm10^{Scim19}$ and $Mcm10^{d08029}$, have previously been studied in our lab and our results support a role for Mcm10 in DNA replication (Apger *et al* 2010). $Mcm10^{Scim19}$ was generated in a screen designed to identify genes responsible for the transmission of a centromere-attenuated minichromosomes (Dobie *et al* 2001). $Mcm10^{Scim19}$ was found to be a *P*-element insertion 76 bp upstream of the translation start codon and are homozygous viable (Apger *et al* 2010; Figure 4). $Mcm10^{d08029}$, first identified in the Exelixes *P*-element insertion collection, contains a *P*-element insertion in the second exon of *Mcm10* which is expected to truncate the C-terminus by 71 a.a. (Apger *et al* 2010; Figure 4). Before double mutant flies can be generated, we must first verify the presence of our *P*-element transposon-induced mutant alleles in our recently obtained fly strains. Standard genomic prep procedures was conducted to isolate genomic DNA which was subsequently amplified through PCR using

P-element and gene-specific primers. DNA sequencing of appropriate sized DNA products visualized after gel electrophoresis demonstrated that our mutant strains still contained the described *P*-element insertion at the appropriate location (data not shown).

Generation of double mutants: Double mutant flies were generated using balancers in combination with the second and third chromosome fusion strain, ap^{Xa}, as described by the pre-made cross scheme. To our fascination, we observed a genetic interaction between Mcm10 and RecQL4 through the rescue of the aberrant phenotypes associated with homozygous *recql4* mutant alleles when placed in either a hetero- or homozygous *Mcm10* mutant background (Figure 2-3B). This observation, taken with our observed physical interaction through two-hybrid analysis, further demonstrates the conserved interaction of Mcm10 and RecQL4 in *Drosophila melanogaster*.

Cytological phenotypes of *recql4* and *mcm10* mutant alleles: Different modes of programmed DNA replication can occur in an organism in varying tissues at specific developmental stages. In *D. melanogaster*, a variant cell cycle is well pronounced during early embryogenesis, where the first 13 cycles occur rapidly in a shared cytoplasm consisting of only S-phase and mitosis without intervening gap phases (Swanhart *et al* 2005). Additionally, the nuclei found in the *Drosophila* larval salivary glands undergo endocycles in which cells successively replicate their genomes without segregating chromosomes during mitosis and thereby become polyploidy (Edgar *et al* 2014). Given the previously reported roles for Mcm10 and RecQL4 in DNA replication (Apger *et al* 2010; Im *et al* 2009; Xu *et al* 2009; Christensen and Tye 2003), analysis of our mutant alleles will provide an opportunity to understand the relationship between Mcm10 and RecQL4 in the context of the cell cycle.

Canonical cell-cycle progression and chromosome morphology: To begin understanding the impact of our *recql4* and *mcm10* mutant alleles on DNA metabolism, we first sought to test the effect of cell-cycle progression with our single and double mutants during the more canonical cell cycle in the brain tissues of the wandering third instar larvae. To assess cell-cycle delay, mitotic indexes were determined for the genotypes indicated in Figure 2-4 by using the specificity of a phospho-histone H3 antibody. Squash preparations were visualized on the Zeiss LSM700 confocal microscope allowing the calculation of mitotic indexes by dividing the number of mitotically dividing cells (positive phospho-histone H3 labeling) by the total number of observable cells. Prior to statistical comparison, derived datasets were subsequently square root transformed to meet the assumptions required for parametric testing (Reubens *et al* 2013). Surprisingly, even with an observable variance between genotypes, our results indicate that neither our *mcm10* or *recql4* mutant alleles impact the normal mechanisms controlling cell-cycle progression ($p>0.05$) (Figure 2-4). Additionally, for each prepared squashed sample, visual inspection of metaphase chromosomes were conducted and no apparent chromosomal abnormalities were observed. Our results indicate that Mcm10 and RecQL4 are not essential for the progression of the canonical cell cycle in the cell types found in the *Drosophila* larval brain.

DNA Content and Chromosome Packaging: DNA replication can occur without ensuing mitosis to generate polyploid tissues through a variant yet common mode of cell cycling known as endoreplication. In *D. melanogaster*, the endoreplicating nuclei found in wandering third instar larval salivary glands provides a well-suited system for dissecting defects in DNA replication and/or chromosome packaging. In our analysis, we utilized a novel method for determining the condensation of polyploid chromosomes and total DNA content of salivary gland nuclei (Chmielewski and Christensen 2011). Imaging of salivary glands from the

respective genotypes revealed that nuclei size from mutant larvae were approximately normal in comparison to heterozygous siblings and wild-type controls (data not shown). The average volume of homozygous *recq4*^{23} salivary gland nuclei was significantly reduced (Figure 2-5B), whereas a slight increase in nuclear volume was observed in homozygous *Mcm10*^{d08029} mutants (Figure 2-5A). Interestingly, the volume of nuclei in salivary glands of flies harboring both *recq4*^{23} and *Mcm10*^{d08029} mutant alleles were significantly increased and at comparable levels to that of the *Mcm10*^{d08029} single homozygous mutants (Figure 2-5C). Spectroscopic quantitation of salivary gland nuclear DNA revealed a 4.5-fold increase in total DNA per nuclei in *Mcm10*^{d08029} mutants (Figure 2-6A) and no change in *recq4*^{23} mutants (Figure 2-6B). However, in *Mcm10*^{d08209} and *recq4*^{23} double mutants, we observed a 3.5-fold increase in nuclear DNA (Figure 2-6C). Using volumes derived from the size analysis in conjunction with DNA content data, we were able to deduce the compaction ratio of the polytene chromosomes (Figure 2-7). Our results showed that the packing ratios of our single homozygous *Mcm10*^{d08029} mutants were 3.5-fold higher than our wild-type control while a 5-fold increase was seen in homozygous *recq4*^{23} mutants (Figure 2-7A&B). Similar to previous observations, the compaction ratio of our double mutants were at a comparable level to *Mcm10*^{d08029} single homozygous mutants with a 3-fold increase relative to wild-type (Figure 2-7C). Taken together, our findings suggest an epistatic relationship between our two proteins of interest with Mcm10 potentially functioning upstream of RecQL4.

Early embryonic developmental staging: The cell cycles of the *D. melanogaster* early embryo differ from those in the larval brain and salivary glands in that they occur very rapidly in a syncytium, proceed in synchrony, and consists of alternating S- and M-phases with no intervening gap phases. Examination of homozygous early embryos laid by homozygous females

0-8 hours after egg deposition (AED) in the respective *Mcm10* mutant alleles reveals that nuclear divisions occurred in normal synchrony (data not shown). In addition, this same observation was made in heterozygous *recql4* mutant embryos and double mutant embryos (data not shown). We next wished to quantitate the developmental progression of early embryonic cycles in our mutant embryos by counting the number of DAPI stained nuclei (Figure 2-8A). We found that after an 8 hour collection, 80% (n=119) of wild-type embryos had made it to gastrulation (Figure 2-8B). However, our homozygous $Mcm10^{d08029}$ and $Mcm10^{Scim19}$ mutants showed a significant reduction in gastrulating embryos (58% and 42%, respectively) with $Mcm10^{Scim19}$ homozygous mutant embryos showing an increased proportion of dead embryos (15%; Figure 2-8B). Unfortunately, we were unable to obtain homozygous *recql4* mutant embryos due to the nature of the mutations; thus, we analyzed heterozygous *recql4* mutant embryos which, as expected, did not deviate from wild-type in respect to embryos making it to gastrulation (Figure 2-8B). Analysis of double mutant embryos harboring the different combinations of our *Mcm10* and *recql4* mutant allele's revealed a notable reduction in gastrulating embryos and an increased amount of dead embryos, especially in flies possessing the homozygous $Mcm10^{Scim19}$ allele (Figure 2-8B). We were next prompted to determine if these embryonic phenotypes were the result of the relative dosage of our *Mcm10* alleles and found our heterozygous *Mcm10* and homozygous *recql4* double mutants to have less severe phenotypes (Figure 2-9). These findings highlight the importance of Mcm10 and RecQL4 during the rapid cell cycles of the *Drosophila* early embryo.

Egg production and hatch rates: To test the impact of Mcm10 and RecQL4 on egg laying, we compared the number of eggs laid every other day by young adult females of control and mutant genotypes. Over the course of 10 days in our initial experiment, we observed a

significant decrease in the amount of eggs laid by *Mcm10*^{d08029} homozygous females compared to wild-type controls. On average, wild-type females laid approximately 9 times more eggs than *Mcm10*^{d08029} females. Based on the results of our previous phenotypic characterization assays, we hypothesized that if the interaction of Mcm10 and RecQL4 was important for female egg laying we would see a rescue in egg production in our experimental flies harboring double mutations (Figure 2-10A). Interestingly, a small yet significant increase in total number of eggs laid was observed by *Mcm10*^{d08029}*;recql4*^{23} homozygous double mutant female flies with double mutants laying approximately 2 times more eggs on average than homozygous *Mcm10*^{d08029} single mutant females *(*Figure 2-10A). Similarly, this genetic rescue is consistent in respect to egg hatching (Figure 2-10B). After counting the number of freshly laid eggs, fifty eggs were subsequently transferred to grape agar plates and incubated at 25^{o}C for 48 hours prior to examination for hatching. After examining 200 eggs over the four trials for each genotype, we found an 80% hatch rate of eggs laid by *Mcm10*^{d08029}*;recql4*^{23} homozygous double mutant females while only ~10% of eggs laid by *Mcm10*^{d08029} homozygous single mutant females hatched (Figure 2-10B). Taken together, our results demonstrate that Mcm10 and RecQL4 function in the similar pathway regulating *Drosophila* female egg production and egg hatching.

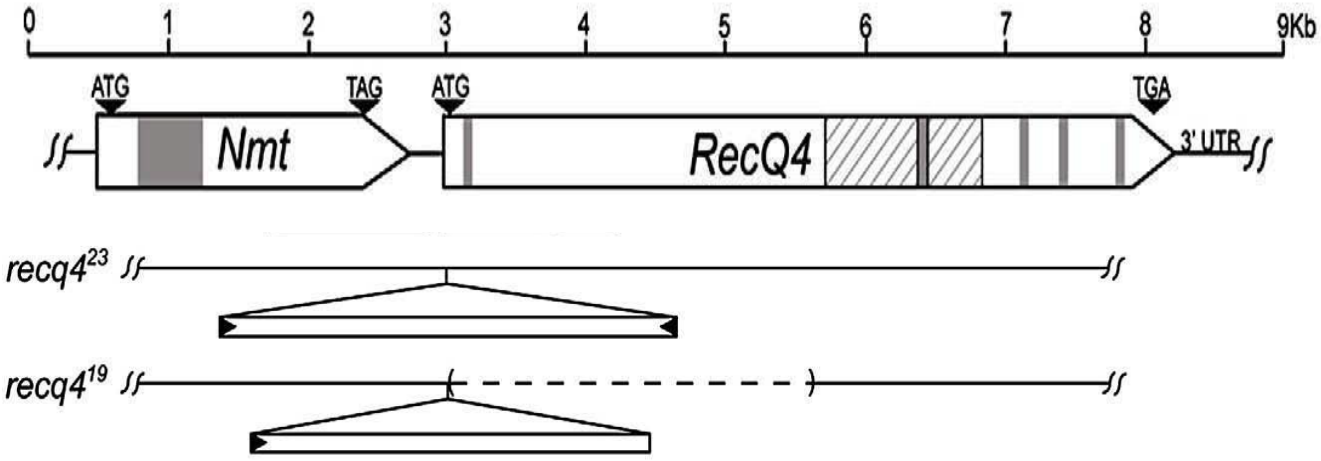


Figure 2-1: Schematic representation of the genomic structure of *Drosophila melanogaster* RecQL4 and the mutant alleles used in this report. Imprecise *P*-element excision gave rise to *recq4*^{23} and *recq4*^{19}. In *recq4*^{23}, a remnant insertion of ~3.8 kb insertion is found at the original P-element insertion site while recq4^{19} is left with a ~3.5 kb insertion at the same *P*-element insertion site (43 bp upstream of the translational start codon). In addition, *recq4*^{19} has a deletion of 3220 bp from the nucleotide 43 bp upstream of the translational initiation codon to nucleotide 3174 downstream of the translational initiation codon of *RecQL4*. Introns are indicated by gray. Dashed line shows the deletion. Hatched boxes represent the coding sequence of the putative helicase domain. (Wu *et al* 2008)

Figure 2-2: Schematic of the *Mcm10* gene region. *Mcm10* is located on the left arm of the second chromosome. *P*-element insertion sites are indicated for the two *Mcm10* alleles used in this analysis. $Mcm10^{Scim19}$ contains a *P*-element insertion 76 bp upstream of the translational initiation codon. $Mcm10^{d08029}$ contains a *P*-element insertion within the second exon resulting in a truncation of 71 amino acids.

A

Comparison of phenotypes observed in the two *Mcm10* and *RecQL4* alleles as described in the literature

	RecQL4 allele		*Mcm10* allele	
	RecQL4^{23}	*RecQL4*^{19}	*Mcm10* ^{d08029}	*Mcm10* ^{Scim19}
Nature of mutation	Hypomorphic, female sterile	Null, lethal	C-terminal truncation, semilethal	Hypomorphic, semilethal
Protein level	10% of wt	0% of wt	80% of wt	23% of wt

B

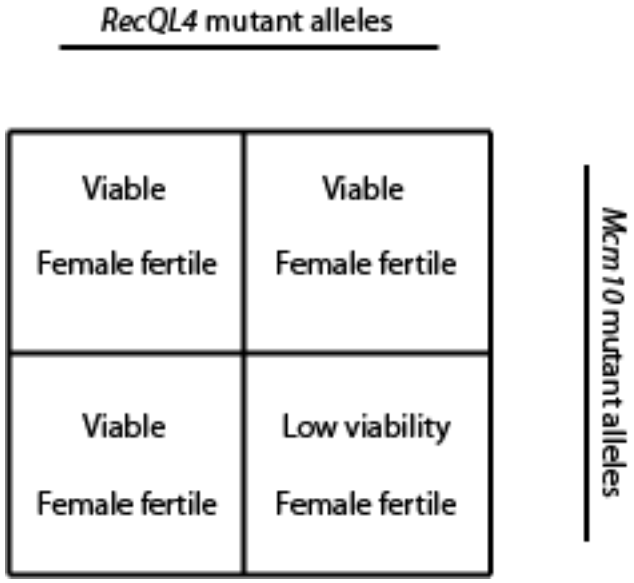


Figure 2-3: Genetic interaction indicated by the rescue of lethal and sterile phenotypes of *recql4* homozygous mutant alleles in a mutant *Mcm10* background. (A) Comparison of phenotypes observed in the two *Mcm10* and *recql4* alleles as described in the literature. (B) Schematic depiction of the observed rescue of lethal and sterile phenotypes associated with *recql4* mutant alleles in a *Mcm10* mutant background. All double mutant combinations result in viable and fertile adult flies.

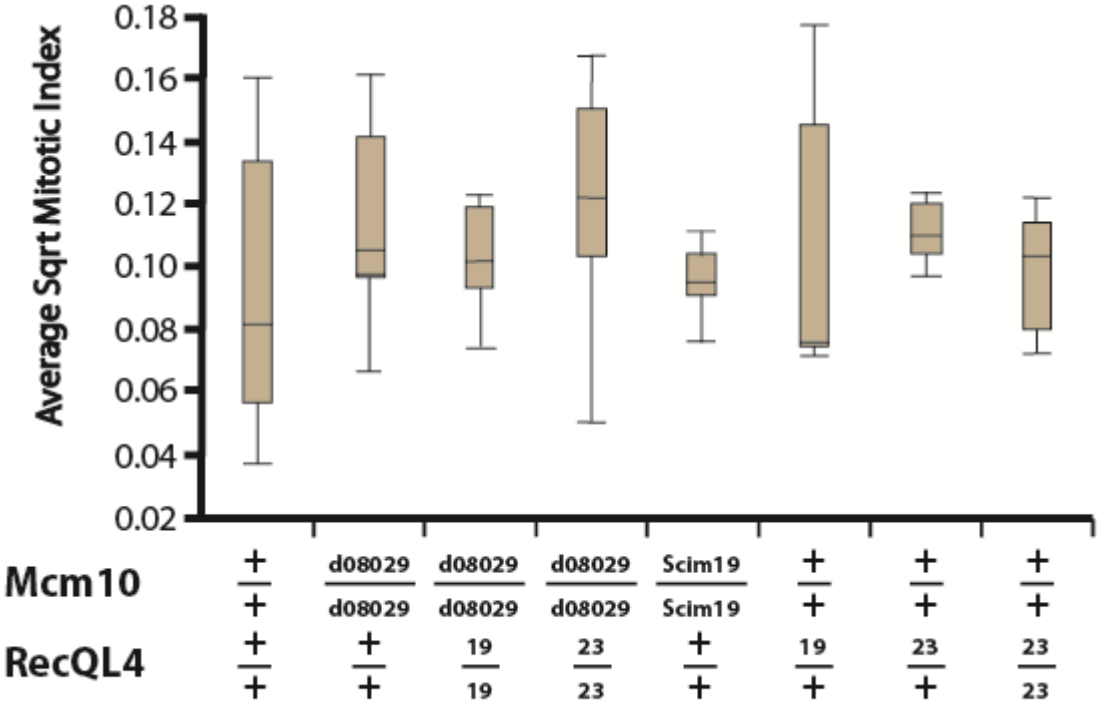


Figure 2-4: Mcm10 and RecQL4 are not essential for the progression of cell-cycles in the *D. melanogaster* larval brain. A phospho-histone H3 antibody was used to label mitotically dividing cells in the wandering third instar larval *Drosophila* brain to allow for the generation of a mitotic index for each genotype. After performing a square root transformation to allow for parametric testing we found that no genotype pair was significantly different from another indicating normal progression of the cell cycles found in the cells of *D. melanogaster* brains.

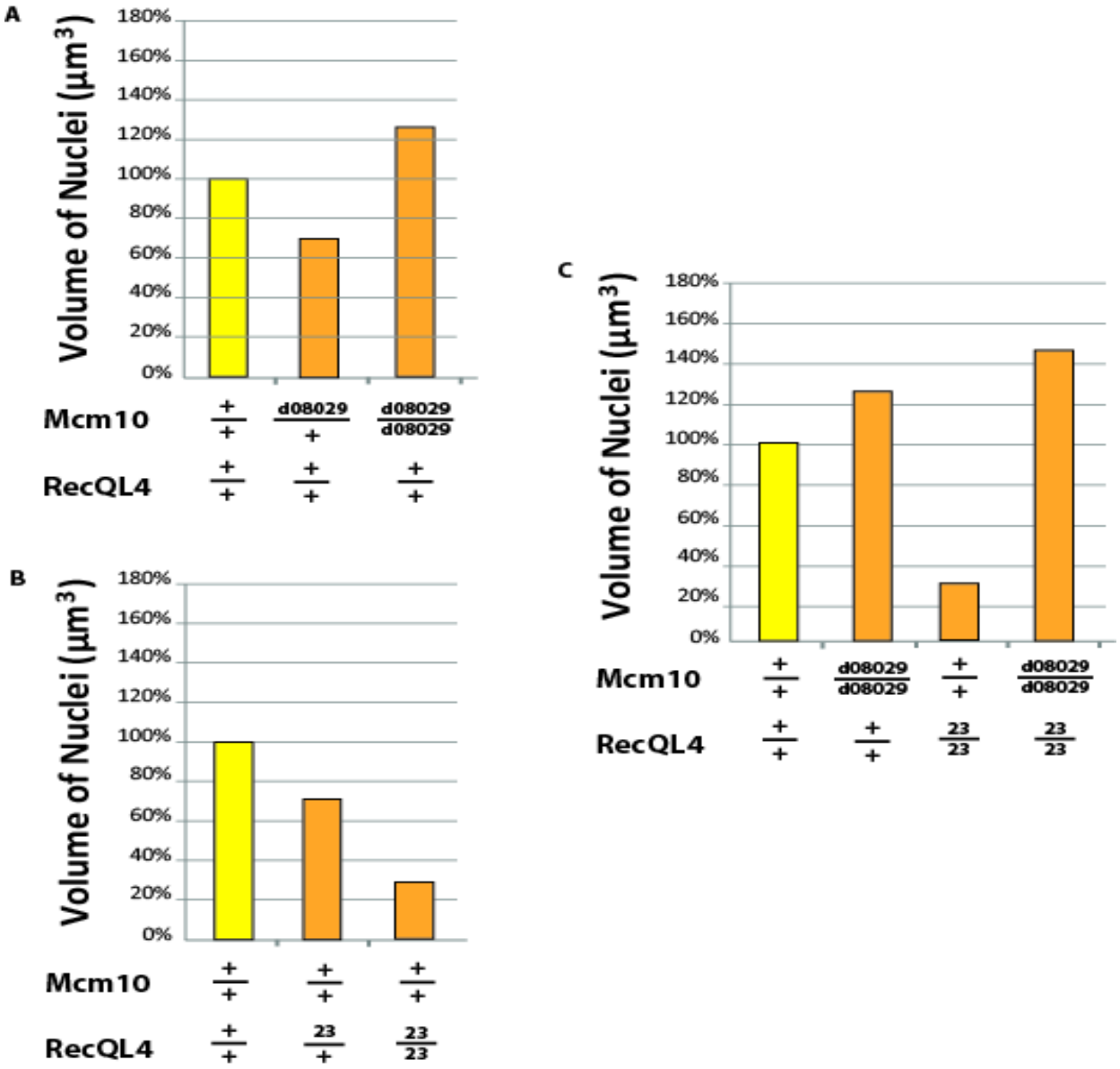


Figure 2-5: Analysis of *Mcm10*^{d08029} and *recql4*^{23} salivary gland nuclear volume suggests epistatic relationship. (A) Volume of salivary gland nuclei is determined in hetero- and homozygous *Mcm10*^{d08029} and compared to wild-type control. (B) Homozygous *recq4*^{23} salivary gland nuclear volume is significantly reduced compared to wild-type. (C) Salivary gland nuclear volume in *Mcm10*^{d08029}*;recql4*^{23} homozygous double mutants is brought to comparable levels of homozygous *Mcm10*^{d08029} single mutants.

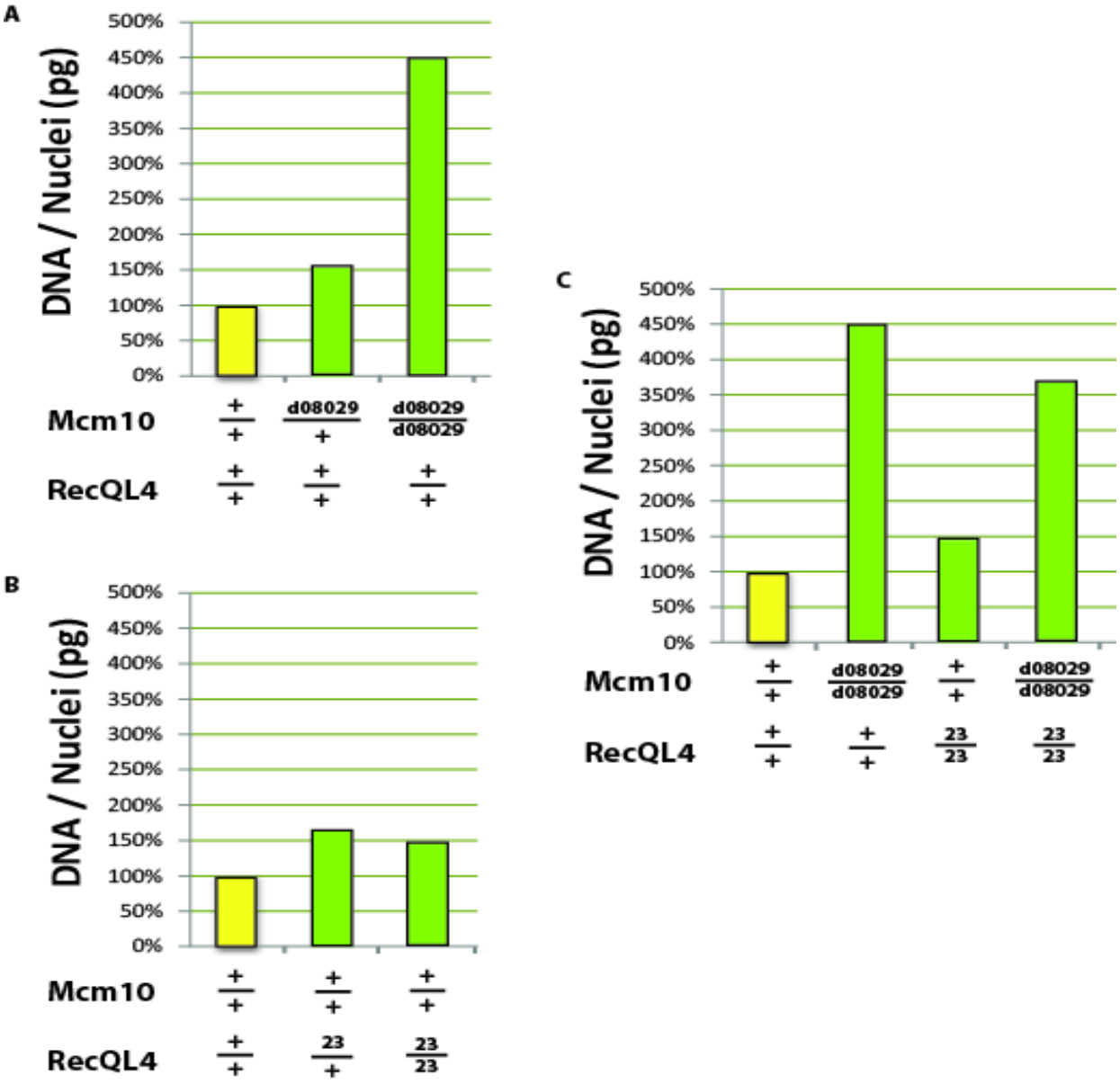


Figure 2-6: Analysis of *Mcm10*^{d08029} and *recql4*^{23} salivary gland DNA content per nuclei suggests epistatic relationship. (A) Quantitation of DNA content per nuclei of *Mcm10*^{d08029} homozygous mutants reveal a 4.5-fold increase in comparison to wild-type nuclei. (B) No significant difference in DNA per nuclei is seen between homozygous *recql4*^{23} mutants and wild-type. (C) DNA per nuclei in salivary glands of *Mcm10*^{d08029}*;recql4*^{23} homozygous double mutants is brought to comparable levels of homozygous *Mcm10*^{d08029} single mutants.

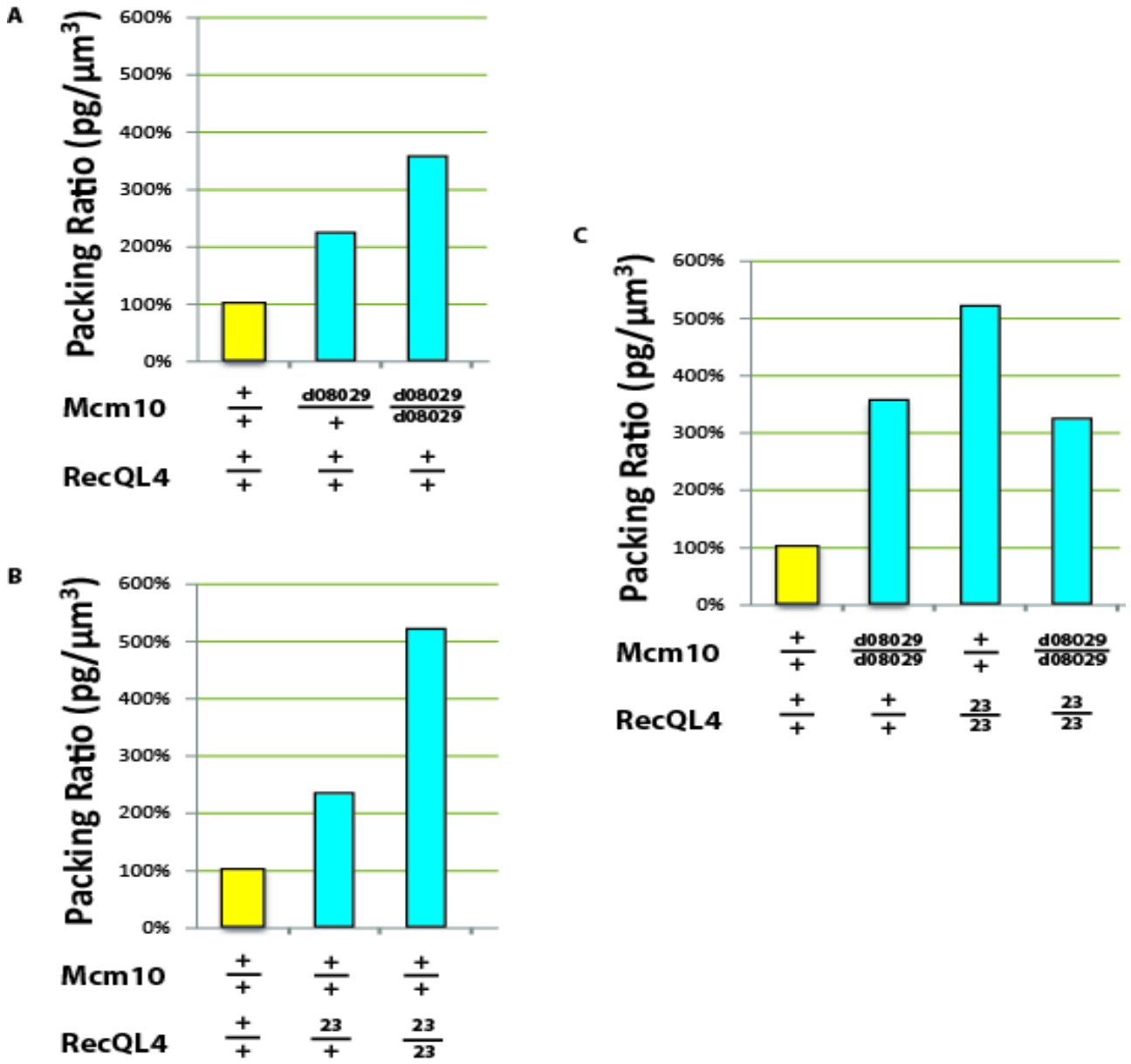
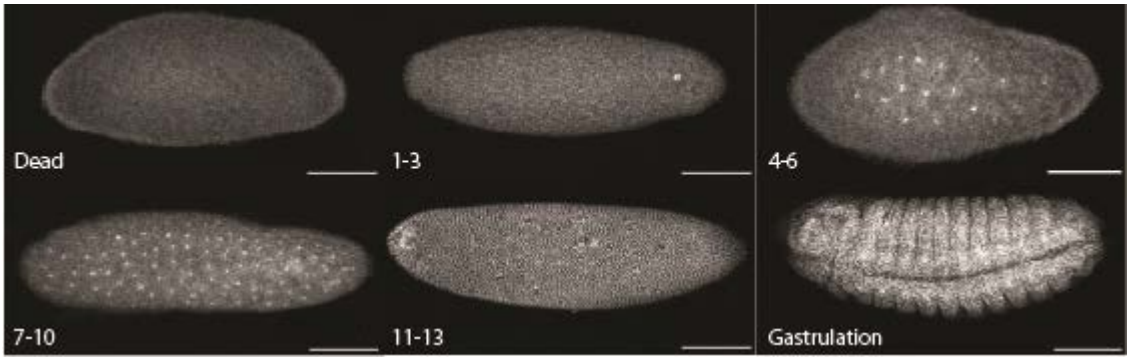


Figure 2-7: Polytene compaction ratios of *Mcm10*^{d08029} and *recql4*^{23} salivary gland nuclei suggest epistatic relationship. (A) Examination of *Mcm10*^{d08029} salivary gland polytene chromosome packing ratios show a 3.5-fold increase in comparision to wild-type. (B) A significantly pronounced 5-fold increase in chromosome packing is observed in homozygous *recql4*^{23} salivary gland nuclei. (C) Chromosome compaction is brought to similar levels of *Mcm10*^{d08029} in *Mcm10*^{d08029}*;recql4*^{23} double mutants.

A



B

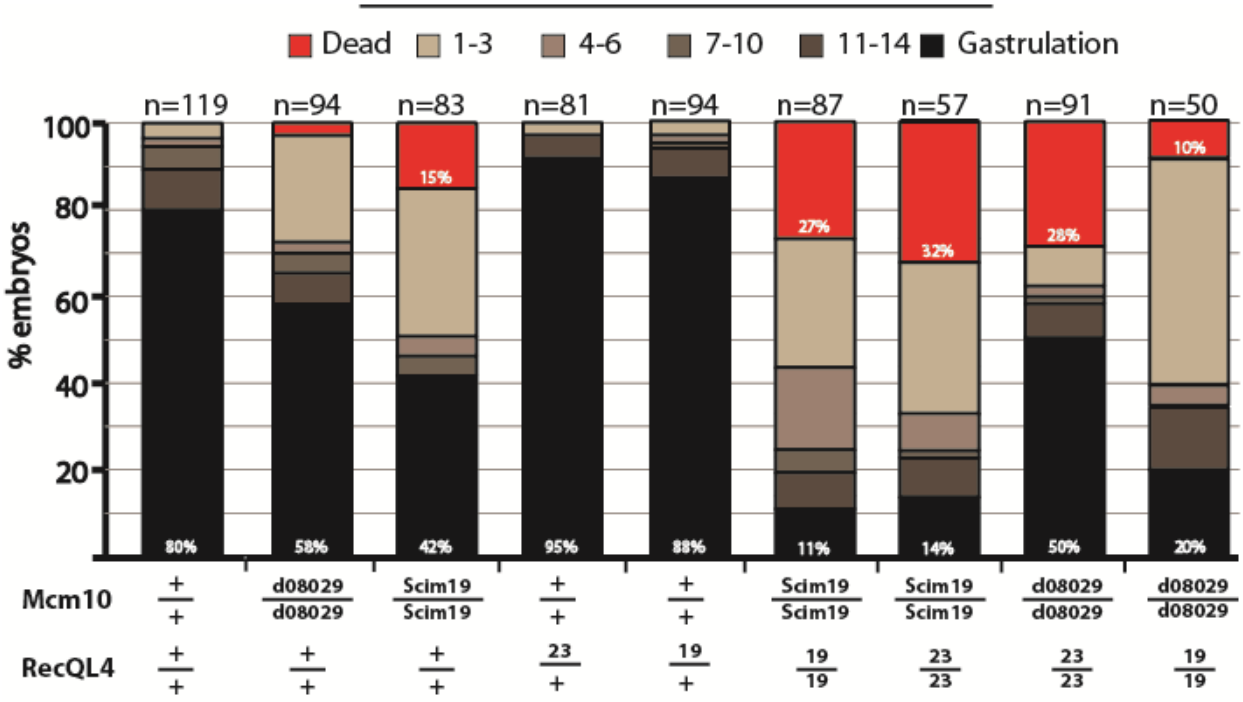


Figure 2-8: Delayed development in *D. melanogaster* early embryos of *Mcm10*;*recql4* double mutants. (A) Confocal images depicting embryos of a certain developmental periods based on the number of DAPI stained nuclei. (B) *D. melanogaster* early embryos of respective genotypes were scored on their developmental stage based the number of DAPI stained nuclei. Embryos harboring double *Mcm10*;*recql4* homozygous mutant alleles show delayed developmental progression with an increase incidence of embryonic death.

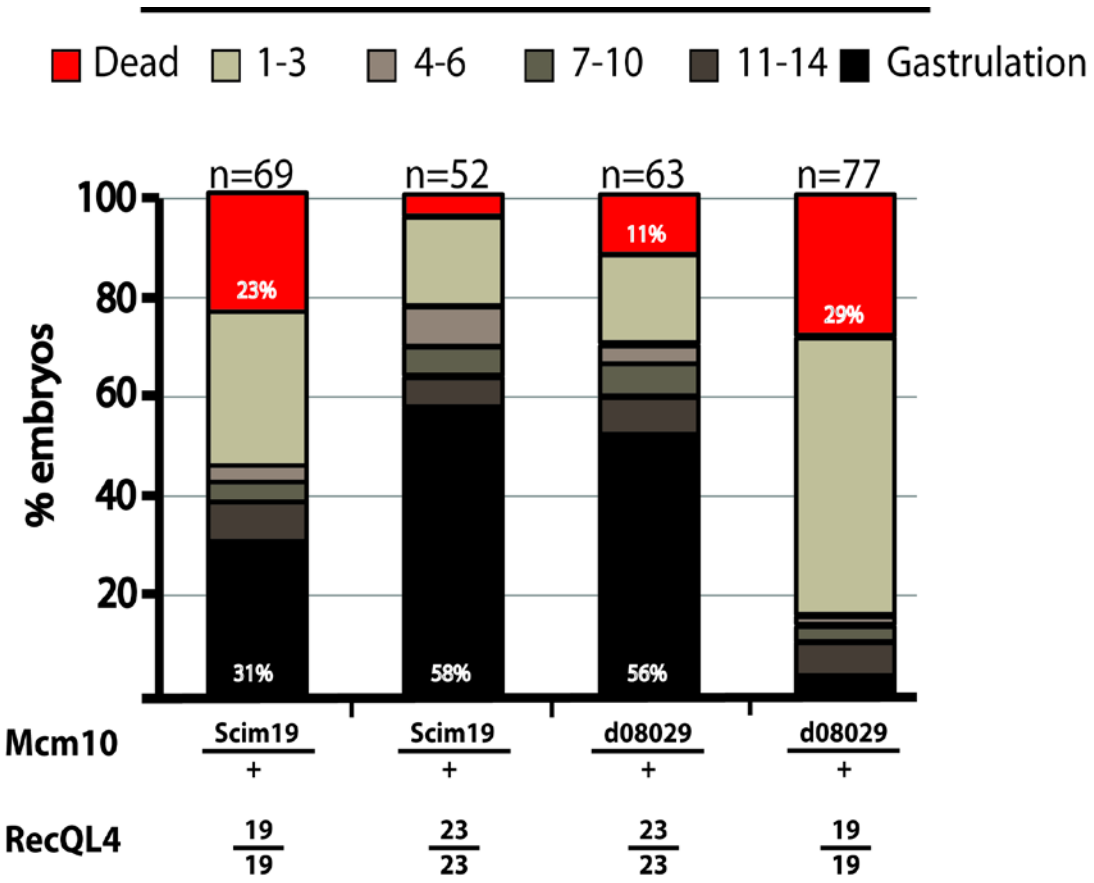


Figure 2-9: Less severe embryonic phenotypes associated with *Mcm10* mutant dosage in double mutant embryos. In all combinations of examined double mutants, except for the *Mcm10*^{d08029}*;recql4*^{19}, more embryos make it to gastrulation with a lower proportion of dead embryos present.

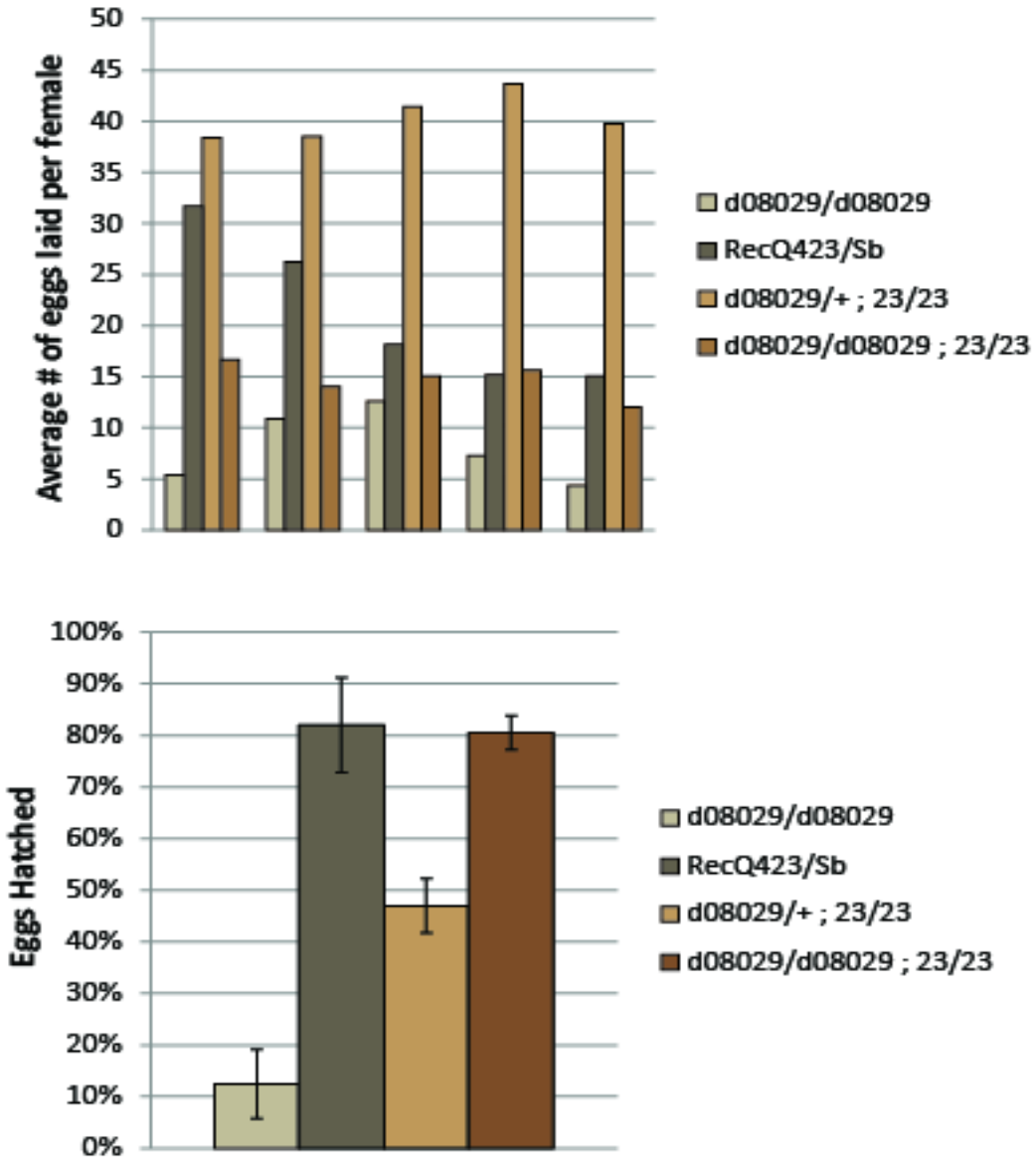


Figure 2-10: Female egg production and hatch frequency shows epistatic relationship between Mcm10 and RecQL4. (A) Average number of eggs laid for each respective genotype. Eggs were collected on even numbered days over the course of ten days. (B) Average hatch frequencies of eggs laid for each respective genotype (n=200). Flies were maintained at 25ºC for the duration of this analysis.

DISCUSSION

During organismal development, alterations in the cell cycle and DNA replication programs are often utilized to ensure proper cell and tissue function. Failure to regulate and adhere to these developmental changes increases the susceptibility of cancer or other detrimental disease states. Here in this chapter, we present a genetic interaction between Mcm10 and RecQL4 through the rescue of lethal and sterile phenotypes associated with mutant *recql4* alleles in a mutant *mcm10* background. Additionally, our results demonstrate that Mcm10 and RecQL4's interaction contributes to several modes of DNA replication, female egg production, and egg hatching in *Drosophila melanogaster*. Taken together, these findings support the notion that Mcm10 and RecQL4 work cooperatively to ensure faithful completion of DNA replication and to promote overall organismal health.

The study of phenotypes of *D. melanogaster* double mutants, also referred to as epistasis analysis, allows one to infer, within limitations, whether the products of the two genes act sequentially in a single pathway, whether they function in two independent pathways, or whether their activity is interdependent (Hereford and Hartwell 1974). In our investigation, we generated flies harboring mutations in *Mcm10* and *recql4* (located on the second and third chromosome, respectively) to dissect their cooperative function in DNA replication during variant modes of cell cycling in *D. melanogaster*. Shockingly, in a heterozygous or homozygous mutant *Mcm10* background, flies possessing homozygous *recql4* mutant alleles were rescued of their severe phenotypes of early larval lethality and adult female sterility (Figure 2-3). This fascinating observation suggests a genetic interaction between Mcm10 and RecQL4, which further supports the results of our yeast-two hybrid analysis (Figure 1-5). Additionally, the nature of this genetic rescue allows one to infer that other compensatory mechanisms are at play when the Mcm10-

RecQL4 interaction is perturbed or abolished. Nonetheless, we and others (Xu *et al* 2009) have now demonstrated that the Mcm10-RecQL4 interaction is conserved in higher eukaryotic species and is critical for the stability of the genome.

The use of mitotic indices as a means of assessing cell cycle progression, chromosome morphology, and as a diagnostic test the presence of certain cancerous cell types, has been a technique utilized in a variety of model systems ranging from cell culture to canines (Reubens *et al* 2014). In *Drosophila melanogaster*, the central nervous system, or brain, of third instar wandering larvae has been used extensively to evaluate mutants for effects on cell cycle progression and chromatin morphology through the generation of mitotic indices. Before investigating the importance of the Mcm10-RecQL4 interaction during the developmentally-regulated variant cell cycles of *D. melanogaster*, we first were prompted to inspect the impact of our *Mcm10* and *recql4* mutant alleles on the cell types undergoing canonical cell cycles in the *D. melanogaster* brain. Intriguingly, in the respective genotypes analyzed, we did not observe any delay in cell cycle progression (Figure 2-4). In contrast, our group previously reported a significant reduction in mitotic indices generated for our two *Mcm10* alleles (Apger *et al* 2010). The discrepancies between our results and the findings previously made could be explained by differing technical approaches to generate mitotic indices. In our approach, we incorporated a phospho-histone H3 antibody to obtain mitotic indices amendable to parametric testing after performing square root transformations on raw data sets (Reubens *et al* 2014). Even with the added sensitivity, our results suggest that the Mcm10-RecQL4 interaction is not essential for normal cell cycle progression during the more canonical cell cycles of the *D. melanogaster* central nervous system. In addition, no abnormal chromosomal phenotypes were observed in any genotypes examined. Potentially, given the established roles for RecQL4 and Mcm10 in other

DNA metabolic process, including DNA damage repair, the cooperative function of Mcm10-RecQL4 could be reserved for microenvironments of higher stress such as those found in *D. melanogaster* salivary glands and the developing embryo.

Endoreplicating tissues provide a unique system to study defects in DNA replication. These tissues possess nuclei that undergo a common cell cycle variant in which cells successively duplicate genomic DNA without segregating their chromosomes during mitosis (Edgar *et al* 2014). Analysis of nuclear volume (Figure 2-5), DNA content per nuclei (Figure 2-6), and polytene chromosome condensation (Figure 2-7) in *D. melanogaster* larval salivary gland nuclei has suggested an epistatic relationship between our two proteins of interest. Our findings indicate that Mcm10 may sequentially function upstream of RecQL4 in single pathway during this alternative mode of cell cycling. Previously, we have shown that normal levels of full length Mcm10 protein are not required for proper endoreplication; however, $Mcm10^{d08029}$, lacking 71 amino acids from the carboxy-terminus, resulted in underreplication of polytene chromosomes in a dominant manner (Apger *et al* 2010). Likewise, Wu and colleagues (2008) reported endoreplication initiation defects in $recql4^{23}$ homozygotes and attributed this to threshold requirements of RecQL4. Our genetic epistasis experiments showed that double mutants ($Mcm10^{d08209};recql4^{23}$) had comparable salivary gland nuclear volume, total DNA content per nuclei, and chromosome compaction to that of $Mcm10^{d08029}$ homozygous single mutants. Additionally, our results from the adult female egg production and hatch frequency analysis was consistent with our upstream and *cis*-acting Mcm10 hypothesis (Figure 2-10). Taken together, it seems plausible that the Mcm10-RecQL4 interaction is reserved for alternate modes of DNA replication where cells are exposed to a high magnitude of intra- and extracellular stress. Further analysis will be required to decipher the importance of the Mcm10-RecQL4 interaction in the

endoreplicating nuclei of *D. melanogaster* salivary glands and other cell types where endocycles are found.

It is important to note that endocycling is but one of the various forms of cell cycles found across organisms. In the early developing *D. melanogaster* embryo, the first 13 cell cycles consist of alternating M- and S-phases with no intervening gap phases. Knowing this, we categorized the nuclear cycles, based primarily on the number of DAPI stained nuclei within individual syncytium, of embryos laid by flies harboring *Mcm10* and *recql4* mutant alleles to dissect their impact on early development. Intriguingly, we observed delayed developmental progression with fewer gastrulating individuals in *Mcm10* mutant embryos (Figure 2-8). The evidence of our results suggest that there may be a threshold of *Mcm10* required for the faithful completion of embryogenesis, as $Mcm10^{Scim19}$ homozygous individuals have an increased proportion of delayed or dead embryos (Figure 2-8). Similarly, in embryos laid by double mutant flies, there is a consistent trend of delayed development and increased rate of early embryonic death (Figure 2-8). The mechanistic capabilities of the Mcm10-RecQL4 interaction in the early embryo will require detailed biochemical analysis but our findings highlight the essential nature of these two proteins in these rapid nuclear divisions.

The genetic manipulations of *Mcm10* and *recql4* has been beneficial in beginning to understand the importance of their conserved interaction in alternate modes of replication. Through the *in vivo* approaches taken in this report, we have shown a bona-fide genetic interaction through the rescue of severe phenotypes. Additionally, our double mutant analysis suggests an epistatic relationship, with Mcm10 functioning upstream of RecQL4, and indicates that a cooperative function exists during the rapid nuclear divisions of *D. melanogaster* early embryogenesis. Understanding how these two proteins work in-complex with each other will

further shed light onto the ways in which DNA replication and packaging of DNA into chromatin states are intertwined to maintain genome stability.

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