Abstract

Drosophila Ctf4 is essential for efficient DNA replication and normal cell cycle progression.

Justin A. Gosnell

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Director: Dr. Tim Christensen

Department of Biology

Proper DNA replication and well-timed cell cycle progression are vital to the normal functioning of a cell. Precise coordination between these mechanisms' constituent proteins ensures their processivity while safeguarding against DNA damage. The Ctf4 protein is a central member of the replication fork and links the replicative MCM helicase and polymerase α-primase. In addition, it has been implicated as a member of a complex that promotes replication fork stability, the Fork Protection Complex (FPC).

This investigation represents the first phenotypic analysis of the function of the Ctf4 protein within a multicellular organism model. We show that Ctf4 interacts with Polymerase α, MCM2, Psf1, and Psf2. We also demonstrate that knockdown of this central replication fork component via a GAL4-UAS RNAi system results in a lower frequency of mitosis due to an S-phase delay, endoreplication defects, as well as mitotic bridging in early embryonic development.

Drosophila Ctf4 is essential for genome stability and normal cell cycle progression.

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By Justin A. Gosnell

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Justin A. Gosnell

Drosophila Ctf4 is essential for efficient DNA replication and normal cell cycle progression.

By Justin A Gosnell

Approved by:

Thesis Director: ______________________________

Tim W. Christensen, Ph.D

Committee Member: ______________________________

Paul Hager, Ph.D

Committee Member: ______________________________

R. Martin Roop, II, Ph.D

Committee Member: ______________________________

Jean-luc Scemama, Ph.D

Chair, Department of Biology: ______________________________

Jeff McKinnon, Ph.D

Dean of Graduate School: ______________________________

Paul J. Gemperline, Ph.D

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Table of Contents

Chapter 1: Introduction....1

I. Background. 1

a. Cell Cycle and DNA Replication. 1

b. Ctf4 and the Fork Protection Complex....3

c. RNAi Knockdown in *Drosophila melanogaster*. 5

d. The Yeast 2-hybrid system....8

II. Specific Aims. 10

III. Materials and Methods....11

Chapter 2: Outcomes. 17

IV. Results....17

V. Discussion. 26

VI. Conclusion. 28

VII. References....30

VIII. Appendix I. 35

IX. Appendix II. 37

X. Appendix III. 38

XI. Appendix IV. 40

XII. Appendix V. 43

List of Figures

Figure 1. Schematic representation of eukaryotic cell cycle. 1

Figure 2. Pre-initiation complex forms in preparation for S-phase. 2

Figure 3. Alignment of yeast and metazoan Ctf4 protein sequences...3

Figure 4. Partial representation of the RPC. 4

Figure 5. Eukaryotic siRNA mechanism. 7

Figure 6. Transformation of budding yeast for yeast 2-hybrid system.....................................9

Figure 7. GAL4/UAS-driven RNAi system in *Drosophila*..12

Figure 8. Chart showing survival at the third-instar larval stage and the eclosion stage...17

Figure 9. Boxplot of mitotic indices determined from larva brain squashes. 19

Figure 10. Boxplot of S-phase indices of larval brains. 20

Figure 11. Confocal micrographs of DNA stained with DAPI in *Drosophila* early embryos. 21

Figure 12. Confocal micrographs of egg chambers...22

Figure 13. (A) Confocal micrographs of Ctf4 knockdown polytene chromosomes. 23

Figure 13. (B) Boxplot of average polytene chromosome arm widths. 24

Figure 14. Chart showing survival of larvae when grown with hydroxyurea..........................25

Figure 15. Yeast 2-hybrid analysis of Ctf4 interactions. 26

Figure 16. Live imaging of model RFP-expressing embryo (Supplementary File). 36

Figure 17. SEM images of Aberrant wing in Ctf4 knockdown eclosion survivors..................37

Figure 18. (A) Cross scheme to acquire white-eyed flare-3 stock flies......................................41

Figure 18. (B) Cross scheme to acquire white-eyed multiple wing hair stock flies. 42

Figure 19. Diagram of *Drosophila* Ctf4 with upstream region and P-element.........................43

Figure 20. Upstream mutant polytene chromosome spreads..44

Figure 21. Boxplot of mitotic index in Ctf4 upstream mutant larval brains. 44

Figure 22. Confocal micrographs of *Drosophila* early embryos with upstream mutant. 45

Chapter 1: Introduction

Background

a. Cell Cycle and DNA Replication

As central and conserved a mechanism as DNA replication is to life, intensive study continues to reveal the existence of more of its factors as well as more integrated relationships among those already characterized. Nearly all somatic cells in the human body undergo mitosis toward eventual cytokinesis. This commonplace, yet intricate undertaking results in the production of two complete daughter cells. S-phase is the fraction of mitosis during which faithful replication of all the cell's genomic DNA must take place. Despite the remarkable accuracy with which this mechanism passes information to the next generation of cells, its integrated nature allows for problems on many fronts to contribute to mistakes (1). Over 6 billion nucleotides are synthesized in humans with each cell division (2), and the risk of genome instability that leads to cancer can be initiated by a simple mutation in one of many proteins at the site of replication, namely, the processive Replication Fork (RF).

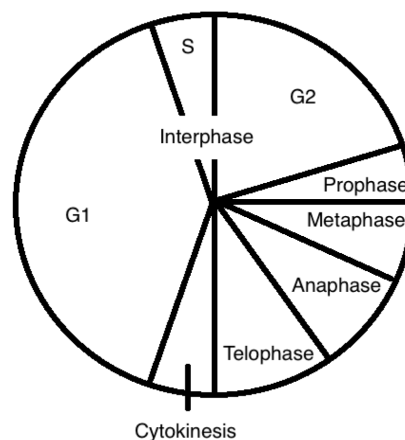


Figure 1. Schematic representation of eukaryotic cell cycle.

The G1 segment of interphase is the period in which the cell spends the largest portion of its life (3). Normal metabolism and cell growth are carried out until the approach of S-phase, when DNA replication begins (Figure 1). At the latter end of G1, the pre-Replication Complex must assemble to license a replication origin (usually an AT-rich nucleotide sequence) (4).

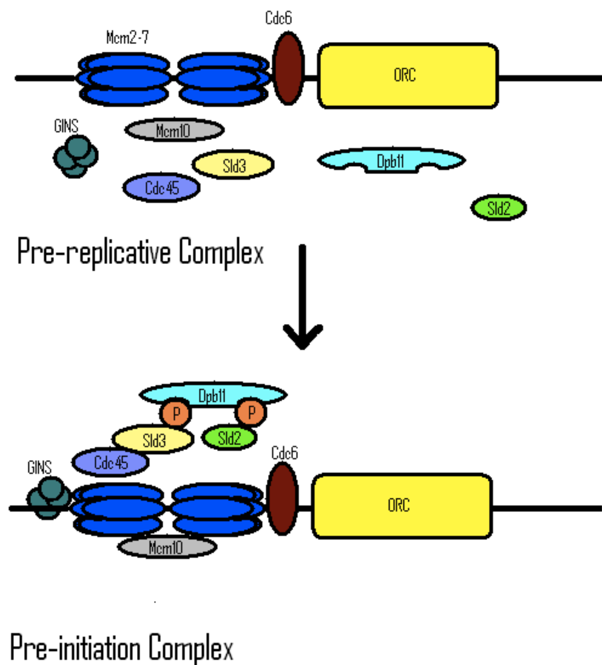


Figure 2. Pre-initiation complex forms in preparation for S-phase. The pre-RC is activated and is termed pre-IC (pre-initiation complex) when additional proteins, including CDC45, SLD2–3, DPB11, the GINS complex (SLD1 and PSF1–3) and MCM10, associate with the fork. The RPC will now assemble. (adapted from (5))

This pre-RC is composed of the Origin Recognition Complex, which recognizes the AT-rich sequence, and the putative helicase, MCM (Mini Chromosome Maintenance) (6). The pre-RC is activated and is termed pre-IC (pre-initiation complex) when additional proteins, including CDC45, SLD2–3, DPB11, the GINS complex, and MCM10, associate with the fork (7) (Figure 2). The helicase separates the two strands, yielding the beginnings of a replication “bubble” with one replication fork unwinding in each direction. Upon phosphorylation of MCM2-7 by Dcd7-Dbf4 kinase, the Replisome Progression Complex (RPC) is assembled with the addition of several polymerases and S-phase is entered (9).

Tight regulation of initiation and progression of S-phase is necessary to ensure that the cell's genome is replicated completely and only once (9).

The RPC must faithfully copy an organism's genome in the context of cell cycle regulation and chromatid segregation while at the same time safeguarding against DNA damage. In response to this highly integrated nature of the RPC, recent studies have generated models at the replication fork that link MCM helicase activity to primase activity and establishment of sister chromatid cohesion throughout S-phase (10).

b. Ctf4 and the Fork Protection Complex

The Ctf4 protein has recently become the subject of several investigations in eukaryotes due to its position as a central component of the RPC. It was initially identified in yeast during a screen for mutants affecting chromosome transmission fidelity, after which it is named (11).

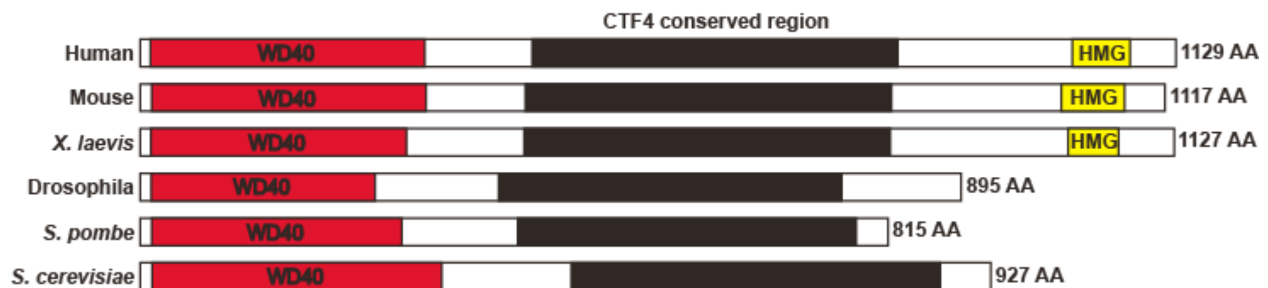


Figure 3. Alignment of yeast and metazoan Ctf4 protein sequences. The variable WD40 repeat region and central region are conserved from fungi to metazoans. However, the DNA-binding HMG box is exclusive to vertebrates. (12) (13)

The Ctf4 gene in *Drosophila* encodes a protein whose structure and function is mostly conserved across lower and higher eukaryotes (12). The layout of this protein (Figure 3) contains a series of variable WD40 repeats at the N-terminus predicted to facilitate protein-protein interactions, a central SepB domain seen as a common feature in scaffolding proteins, and an HMG (High Mobility Group) box domain at the C terminus that has DNA-binding activity. The WD repeats and SepB domain are conserved across all studied species. However, the HMG box domain is only present in vertebrates (12). Ctf4 has been shown to link the MCM helicase activity to lagging-strand polymerase α-primase activity in eukaryotes (14) (Figure 4).

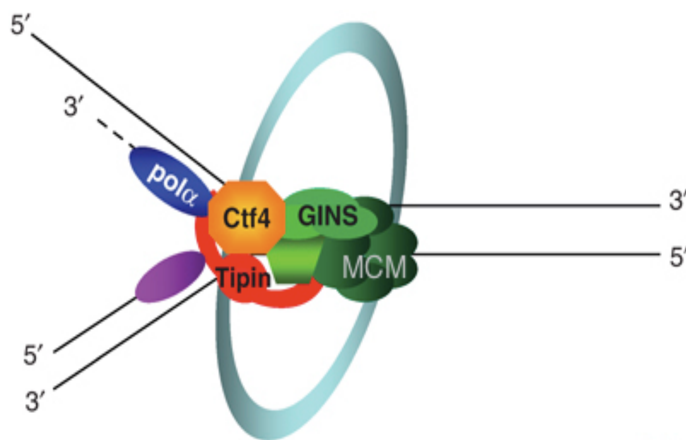


Figure 4. Partial representation of the RPC illustrating the central association of FPC proteins Ctf4 and Tipin with Polymerase alpha and the MCM helicase. (adapted from (15))

While depletion studies in *Xenopus laevis* (15) have suggested its importance in the stabilization of this interaction and thus replication fidelity in general, Ctf4 has also been implicated in proper chromatid segregation and cohesion from its initial establishment in S-phase to separation in anaphase (16). In fact, other members of the FPC have been shown to be essential for proper chromatid cohesion during S-phase, including Tim1/Tipin,

which interact with Cohesin in humans (17). This information suggests that replication fidelity is coordinated with chromatid cohesion.

Defects in cell cycle progression and chromatid segregation have been observed in higher eukaryotic systems isolated *in vitro* (*Xenopus* egg extract, HeLa cell culture) (13, 30). This study is the first to undertake phenotypic analysis of the effects of Ctf4 knockdown on development in a whole-organism, multicellular eukaryote.

c. RNAi Knockdown in *Drosophila melanogaster*

The goal of the investigation is to characterize the Ctf4 knockdown phenotype in *Drosophila melanogaster* in the context of DNA replication. It centers upon the knockdown of Ctf4 mRNA in all prominent stages of the fruit fly's life cycle. Following the introduction of siRNAs (small interfering RNA) targeting Ctf4 mRNA, few transcription products remain to be translated into functional protein. By studying how this whole-organism system behaves in a limiting quantity of Ctf4 protein, we are closer to understanding what functions Ctf4 is responsible for.

Drosophila melanogaster, the common fruit fly, is a higher eukaryote that is generally well suited for the study of genetics and development due to its short life cycle, ease of upkeep, and four chromosomes whose modularity is easily conceptualized on the drawing board and manipulated by simple crosses in the laboratory. The *Drosophila* life cycle also lends itself to the isolation of a large array of tissues by dissection, and each tissue type allows unique phenotypic investigation into spatial (Ex. instances of chromosome aberration) and temporal (Ex. mitotic delays) information (18). For example, using epifluorescence microscopy analysis of early embryos, the investigator is allowed the

detection of asynchrony between nuclei during the rapid cell divisions that take place directly after fertilization. Third-instar "wandering" larvae can be dissected in order to analyze a host of tissues, including the brain (for cell-cycle arrests and chromosomal aberrations) and salivary glands (for endoreplication studies, heterochromatin banding patterns). The adult female fly can also be dissected for ovary analysis (for condensation, endoreplication studies).

In order to study the effects of Ctf4 depletion in whole organisms and tissues, an RNA interference strategy is used to knock down transcripts of the Ctf4 protein's mRNA. The GAL4-UAS system is used to introduce this knockdown to *Drosophila*. The GAL4/UAS system has traditionally been used for targeted gene expression and has been coined "A fly geneticist's Swiss army knife" (19). This is because, in a single genetic cross, two components, one from each parent's genotype, are brought together in the progeny and activate transcription of a gene. The GAL4 protein has a DNA binding domain that binds to a UAS (Upstream Activating Sequence) upstream of the gene of interest. Recently, an exciting new application has become common for use in gene knockdown studies: instead of a protein-coding sequence downstream of the upstream activating sequence, a palindrome sequence designed to form siRNA specific to a target gene can be inserted here, which then uses *Drosophila*'s native RNAi mechanism to knock down the gene, as shown in Figure 5.

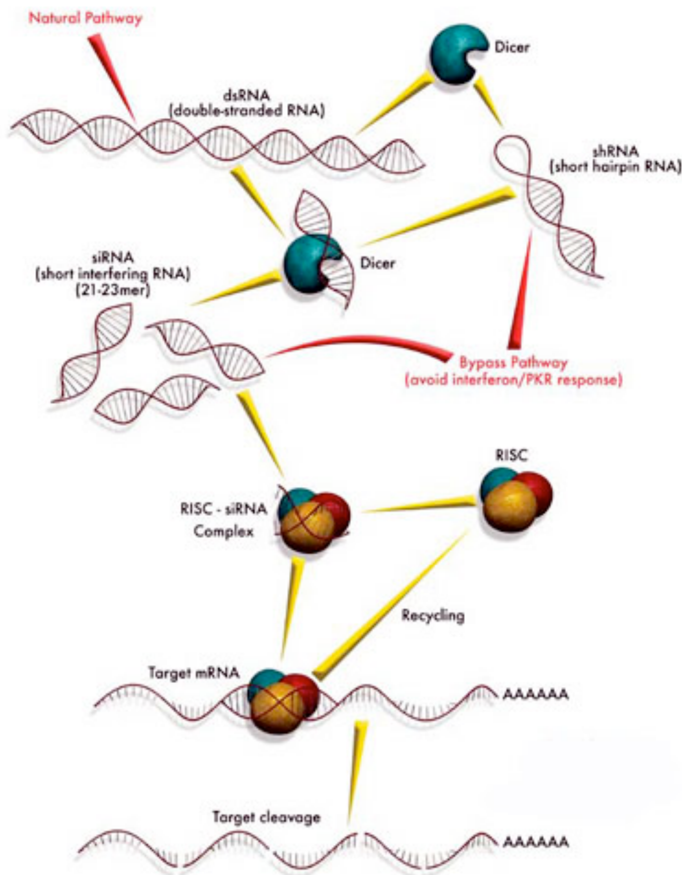


Figure 5. Eukaryotic siRNA mechanism. Dicer cleaves the transcribed palendrome sequence into small interfering RNA (siRNA). RISC complex forms and uses siRNA template to recognize and degrade target mRNA. (adapted from (20))

The transcription of the sequence downstream of UAS can be made tissue-specific by screening for individuals that have insertions of the GAL4 gene downstream of tissue-specific promoters. For global expression of GAL4, and therefore organism-wide knockdown of the target gene, an actin promoter is used. For this study, an RNAi fly line containing UAS followed by the RNAi sequence targeting *Drosophila* Ctf4 is used. An actin-GAL4 line over a GFP Curly balancer was created in order to track the progeny that have both elements of the system. This allows for an experimental group and an internal

negative control for analysis. In addition, the RNAi UAS line possesses a heat-shock promoter upstream of the palindrome sequence (19).

d. The Yeast 2-hybrid System

Saccharomyces cerevisiae, a budding yeast, is also used in this study as a tool to evaluate the interaction of *Drosophila* Ctf4 with known proteins in the replication fork using the yeast 2-hybrid assay. This assay is a relatively easy and inexpensive way to detect interactions by generating fusion proteins which, upon interaction, mediate the transcription of a reporter gene. Study of these interactions allows comparison between previously determined interactions in yeast and newly discovered interactions in *Drosophila* to ultimately help us understand how the two eukaryotic systems compare to one another. In budding yeast, Ctf4p has been shown to interact with Mcm10p physically and genetically and also interacts with Polymerase α (16). Chromatin association of Ctf4p is dependent on Mcm10 in yeast, and its deletion destabilizes Mcm10p and Polymerase α at temperatures above 38 degrees C (16). These insights suggest that Ctf4p plays a facilitating role in the initiation of DNA replication (16).

Another goal of this investigation is to assess the interaction of Ctf4 with Polymerase α and Mcm10 in *Drosophila*, as well as screen for new putative interactors, using the yeast 2-hybrid system. This system is an ideal first step when testing a wide array of possible interactors because it is relatively cheap and relatively easy to perform.

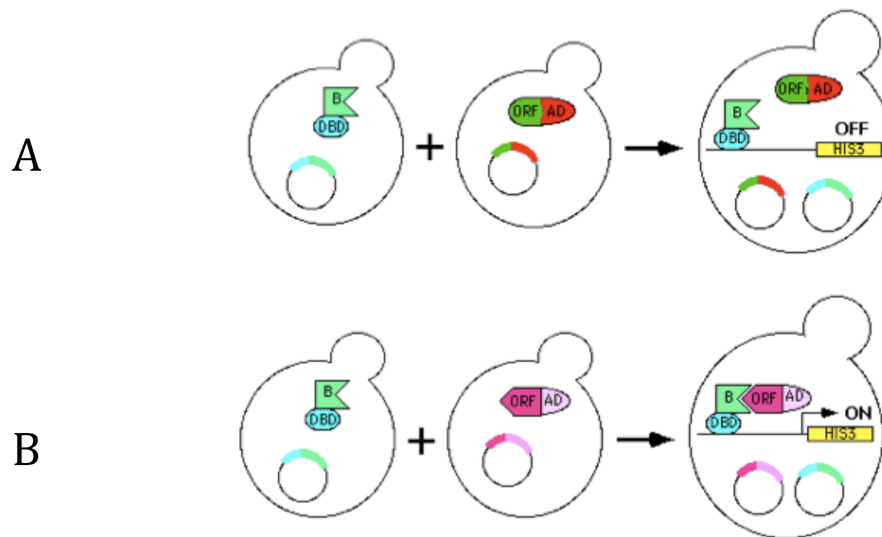


Figure 6. Transformation of budding yeast for yeast 2-hybrid system. (A) The two fusion proteins are not brought together with an interaction, therefore GAL4's activating domain and DNA-binding domain stay separate, yielding no transcription of the reporter gene. (B) "B" and "ORF" are interactors in this case and so bring the two GAL4 domains together, enabling transcription of the HIS3 reporter gene. (adapted from (21))

This system consists of a downstream reporter gene that is activated by a transcription factor that binds to a UAS (Upstream Activating Sequence) (Figure 6). In this study, we used GAL4 as the transcription factor and a HIS3 reporter gene; if the activating domain of GAL4 is brought into close proximity of the sequence, the reporter gene will be transcribed. In the technique, two fusion proteins are created: one consists of a protein of interest fused to the DNA-binding Domain (DBD) of GAL4. The other fusion protein consists of a second protein of interest fused to the Activating Domain of GAL4 (AD). If the two proteins of interest interact, they will bring the two separate GAL4 domains together,

creating a complex that will be able to bind to the UAS and activate the reporter gene. The fusion protein that contains the DNA-binding Domain is labeled the bait, and the fusion protein that contains the Activating Domain is labeled the prey. (21)

II. Specific Aims

The risk of genome instability that leads to cancer can be increased by a simple mutation in one of many proteins that function at the site of DNA replication. A better understanding of how DNA replication is regulated and maintained throughout the cell cycle may lead to the development of better treatments that target this genome instability. Phenotypic investigation into the importance of Ctf4 in *Drosophila* will provide a better understanding of the tie between MCM2-7 helicase and polymerase activity in the RPC.

Drosophila melanogaster was used as a model multicellular eukaryotic organism to address the following hypothesis:

Drosophila Ctf4 is important for DNA replication and normal cell cycle progression.

The following specific aims were used to test this hypothesis:

Specific Aim 1: Characterize phenotypic effects of Ctf4 knockdown in whole-organism *Drosophila*.

Aberrant initiation and progression of DNA replication observed in yeast Ctf4p mutants (16) prompted investigation of the effects of Ctf4 knockdown on the cell cycle in Drosophila. Following RNAi knockdown of Ctf4, the following phenotypic analyses were performed to achieve this aim: Larval CNS tissue was dissected and initially analyzed for M-phase delay (downstream of replication in the cell cycle); larval CNS tissue was then used in an EdU incorporation procedure to assess S-phase delay; larval salivary glands were

dissected and surveyed for endoreplication defects, and band areas were quantified; adult ovaries were dissected and surveyed for endoreplication defects; early embryos were harvested and surveyed for defects in syncytical nuclear division due to incomplete replication.

Specific Aim 2: Assess the importance of Ctf4 in replication pausing.

Hydroxyurea was incorporated into fly medium to deplete dNTPs. Ctf4 knockdown cross was performed and larvae were scored for survival to observe impact of replication pausing on these knockdowns.

Specific Aim 3: Identify protein interactions between Ctf4 and other members of the replication fork complexes pre-RC, pre-IC, and RPC.

Because Ctf4 has been implicated in *in vitro* studies as a recruiter of Polymerase alpha primase to the replication fork as well as a stabilizing element for the link between MCM2-7, GINS, and polymerase activities, this study uses the yeast 2-hybrid system to explore how the RPC preserves genome integrity.

III. Materials and Methods

Fly Husbandry / Stocks

The *Drosophila* lines used in our analyses were acquired from the Bloomington Fly stock center, the Exelixis *Drosophila* Stock Collection at Harvard Medical School, and the Vienna *Drosophila* RNAi Center. These strains consisted of: wt (Flybase ID: FBst0006326), w [1118]; P{GD4433}v44474 (GAL4 / heat-shock inducible Ctf4 RNAi, FlyBase ID: FBst0465598), P{Act-GAL4.U} (GAL4 reporter/driver, Flybase ID: FBtp0039579), P{Act-

GAL4.U}/CyO,GFP derived from FBtp0039579 and FBba0000585. All stocks were kept over *Drosophila* K12 media (US Biological # D9600-07B) at room temperature.

RNAi Fly Cross Scheme

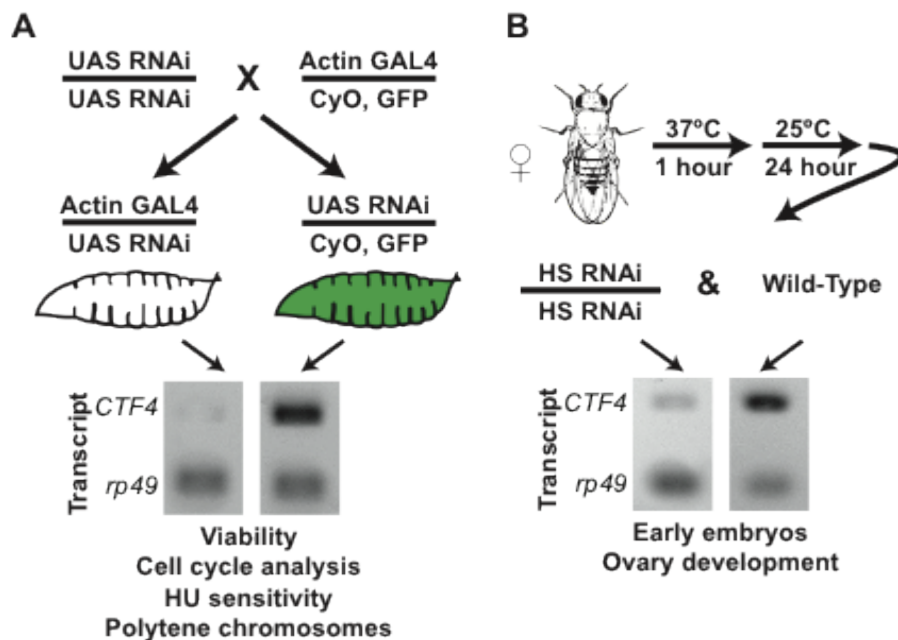


Figure 7. GAL4/UAS-driven RNAi system in Drosophila. (A) A GFP marker allows differentiation of the Ctf4 knockdown group (non-glowing) and sibling control group (glowing). (B) A heat-shock promotor upstream of the RNAi sequence is activated by 1 hour heat shock at 37 degrees Celcius followed by 24 hour recovery/knockdown period. RT-PCR verification of the knockdown of transcripts was performed using rp49 as a loading control.

For this study, an RNAi fly line containing UAS followed by the RNAi sequence targeting *Drosophila* Ctf4 is used (Figure 7). An actin-GAL4 line over a GFP Curly balancer was created in order to track the progeny that have both elements of the system. This allows for an experimental group and an internal negative control for analysis.

Yeast 2-hybrid

The Clonetech Matchmaker^{tm} Yeast 2-hybrid system was used to detect interactors. Budding yeast strain PJ69α (Clonetech) was transformed using the protocol in (25) with Gateway-modified Clonetech plasmids pGBKT7 (bait) and pGADT7 (prey). Gateway entry vectors used in the construction of 2-hybrid plasmids were made from amplified *Drosophila* cDNA and were sequence-verified. The final plasmid constructs used in figure 15 were also verified to establish that the sequence was in-frame and that no mutations were introduced in the process. Dilution series were plated to quantify strength of interaction.

Early Embryos

Heat-shock-inducible RNAi adult females (3-7 days post-eclosion) were incubated above yeast paste for 24 hours before the heat-shock procedure. They were then acclimated at 30°C for 30 minutes in a water bath, heat-shocked at 37°C for 1 hour, and finally incubated over yeast paste a second time for 24 hours. Females were then allowed to lay eggs over grape agar with a thin film of yeast paste for 8 hours. Embryos were collected and analyzed according to (25) with an Olympus IX81 Motorized Inverted Microscope with Spinning Disk Confocal controlled by the SlideBook^{tm} software.

Polytene Chromosomes

Third-instar wandering larvae were dissected in 1XPBS pH 7.2 with 1% PEG 8000 and salivary glands isolated and fixed with 50% acetic acid, 2-3% lactic acid, 3.7% formaldehyde. Glands were transferred to slides with siliconized cover slips and spread using periodic compression with the tip of a pencil. Spreads were then compressed with

15Nm using a precision vise. Squashes were then frozen in liquid N_2 and the cover slips were afterward removed. After rinsing with ethanol and subsequent drying, squashes were mounted with Vectashield containing DAPI and imaged using an epi-fluorescence microscope. Images were analyzed for average area of chromosome arms using quantitation software included in the Adobe Photoshop CS5 suite. Three 15.6μm lengths per image (n=10) were quantitated for area in square μm. A boxplot graph was generated using the average area per fixed length for each image using the Minitab^{tm} software package.

Adult Ovary Dissection and Analysis

Adult female *Drosophila* were heat-shocked using the procedure outlined above and ovaries were isolated by dissection following the second 24-hour yeast paste incubation. Ovarioles were teased apart and fixed in 4% Formaldehyde PBX (PBS + 0.1% Triton X-100) for 20 min. Ovaries were then stained for 5 min with 1ug/mL DAPI in PBS. Ovaries were then washed 3X for 5 min in PBX, followed by a 1 hour PBX wash and finally 3 10 minute PBX washes. Ovaries were mounted using Vectashield^{tm} and imaged using confocal optical sectioning microscopy.

Larval Brain Squashes / Mitotic Indices

Glowing and non-glowing third-instar larvae were separated and dissected in PBS/PEG solution. Brains were isolated and incubated at room-temperature for 10 minutes in a hypotonic solution consisting of 0.5% sodium citrate, then incubated in a 11:11:2 mixture of acetic acid:methanol:water for 20 seconds. The squashes were then prepared by placing

the brains on individual slides, applying a siliconized coverslip to each, and sandwiching the coverslip under a second slide. A precision vise was then used to apply 15Nm of force to the sandwiches for 2 minutes. The squash preparations were then frozen with liquid nitrogen, rinsed with ethanol, and dried before finally receiving a new coverslip over Vectashield with DAPI.

The brain squashes were analyzed by capturing 10 random fields under 600X magnification that are moderately populated (between 100 and 300 cells in view) for each of 10 slides, making for a total of 100 pictures. The Minitab™ software package was used to generate a box plot graph of the mean indices from each slide.

EdU Incorporation

To determine the percentage of cells in S-phase for each group, the Click-It® reaction kit from Invitrogen (Cat. # C10337) was used to detect EdU incorporation within larval brains (22). These were dissected in fresh Grace's un-supplemented cell culture medium. An equal volume of 200mM EdU solution in DMSO was added to the well and brains from each strain were incubated for 30 minutes in the dark at room temperature. Following incubation the liquid was removed from each well and the brains were rinsed three times with 1X PBS. They were then treated with a hypotonic solution of 0.5% sodium citrate for 10 minutes to expand the cells. Brains were then incubated in a 11:11:2 mixture of acetic acid:methanol:water for 30 seconds. Brains were squashed and frozen as per the above procedure, and squashes were rinsed with 3% BSA in 1X PBS. Squashes were then treated with 0.1% TritonX in PBS for 15 minutes at room temperature in the dark. The liquid was removed and the slides were rinsed twice with 1X PBS. Squashes were then incubated in

the Click-It® reaction cocktail as per the manufacturer's instructions for 30 minutes. The squashes were rinsed twice with the reaction rinse buffer provided by the manufacturer. After removing the rinse buffer, the counterstain Hoescht 33342 was prepared as per the manufacturer's instructions for nuclear visualization and applied for 10 minutes. The Hoescht solution was then removed and the squashes were washed four times with 1X PBS. Squashes were mounted in Vectashield^{tm} for fluorescence and imaged using confocal microscopy. Analysis was carried out by capturing 5 random fields under 600X magnification for each of 5 slides for each group. Incidence of S-phase was detected by scoring EdU-positive cells. The Minitab^{tm} software package was used to generate a box plot graph of the mean indices from each slide.

Deoxyribonucleotide Depletion by Hydroxyurea in Ctf4 Knockdown Larvae

Hydroxyurea (HU) (MP Biomedicals, LLC) was added to *Drosophila* K12 media at a final concentration of 10mM. A non-mutagenic cross vial was prepared and adult *Drosophila* allowed to mate and incubate for 2 days before transfer to the HU-containing vial. Eggs were laid for 2 days and parents were transferred to successive new HU-containing vials to obtain statistically reliable numbers. Third-instar wandering larvae were scored and a ratio determined between Ctf4 knockdown survivors and sibling control survivors.

Chapter 2: Outcomes

IV. Results

Ctf4 Knockdown has a severe impact on viability at pupation and adult stages.

Investigation into the life-cycle of *Drosophila* in the context of Ctf4 knockdown demonstrated the importance of Ctf4 to survival. Maternal loading was sufficient for viability from the egg stage to pupation. However, only 41.2% of Ctf4 knockdown flies succeeded in eclosing (Figure 8). Flies surviving this stage exhibited 100% lethality after 3 days.

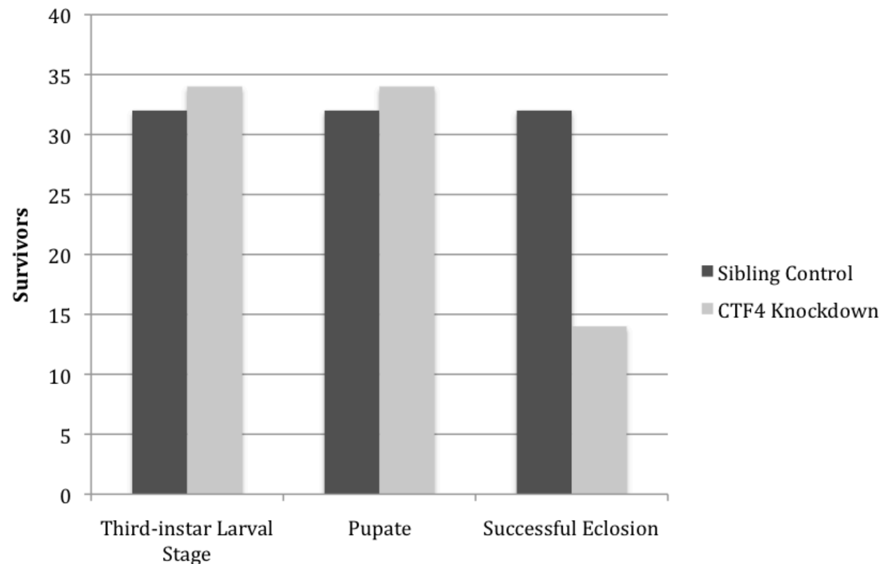


Figure 8. Chart showing survival at the third-instar larval stage and the eclosion stage. Maternal loading is no longer sufficient to augment knockdown of Ctf4 at pupation. A 1:1 Mendelian ratio existed between larvae with genotypes RNAi-Ctf4/Actin-GAL4 (Ctf4 knockdown) and RNAi-Ctf4/CyO,GFP (sibling control) (n=66). Though all individuals proceeded to pupation, only 41.2% of Ctf4 knockdown flies survived the dark pupa stage to eclosion (n=34). Lifespan of these adult survivors varied from 1-3 days (data not shown).

These findings demonstrate the importance of Ctf4 to survival. Maternal loading was sufficient for viability from the egg stage to pupation. However, eventual 100% lethality is seen within 3 days, some specimens having died upon pupation, others surviving to adulthood appearing emaciated and with wings un-inflated. The expression profile of Ctf4 indicates a global minimum in larvae just prior to pupation (24), which is to be expected due to the majority of larval somatic cells growing in size without dividing. The expression profile then indicates a relative maximum of expression needed for the cell divisions in metamorphosis during the 12-hour pupa stage. In this stage, maternally loaded Ctf4 is no longer present in sufficient quantities to supplement knockdown of Ctf4 mRNA.

Cell Cycle Delay in Third-instar Wandering Larvae

The interface between maternal loading and subsequent turnover of Ctf4 was investigated by assessing effects of Ctf4 knockdown on the cell cycle in third-instar larval tissues. The *Drosophila* CNS exhibits the highest expression of Ctf4 of any larval tissue and is historically chosen for cell cycle analysis due to its highly regular cell divisions. The importance of Ctf4 in S-phase entry and progression has been documented in budding yeast (16), and observation of single-cell layer tissue expanded with hypotonic solution allowed for mitotic indices to be calculated (Figure 9A). The low incidence of cells in mitosis within Ctf4 knockdown cells compared to sibling control gave reason to suspect an arrest point upstream of metaphase.

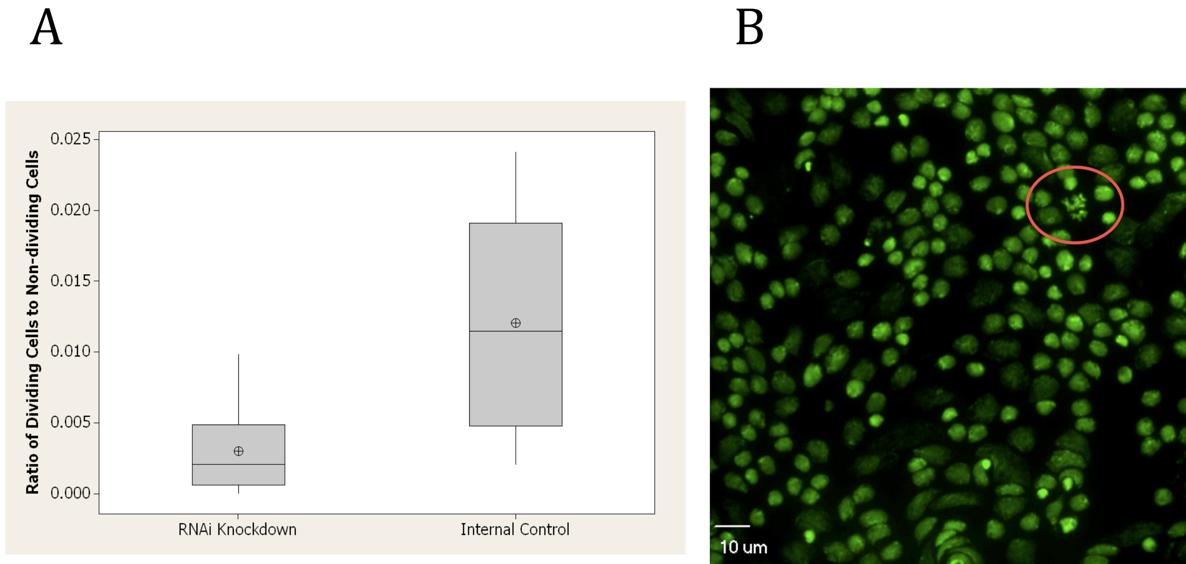


Figure 9. (A) Boxplot of mitotic indices determined from 3rd-instar wandering larva brain squashes. Larvae with the genotype RNAi-Ctf4/Actin-GAL4 (Ctf4 knockdown) yielded a significantly lower ratio of cells progressing through M-phase compared to sibling control. 10 brains were analyzed for each group, and 10 fields of view were acquired for each brain (P = 0.004). (B) Example control field of view with circled mitotic figure.

Severe S-phase delay observed in Ctf4 knockdown brains

S-phase occurs before M-phase. Due to the low representation of mitotic cells in Ctf4 knockdown larval brain squashes, and because of the suspected importance of Ctf4 to DNA replication, aberrant progression through S-phase was suspected. S-phase delay was evaluated using EdU (5-ethynyl-2'-deoxyuridine) incorporation and detection by fluorescence. EdU is a thymidine analog incorporated during DNA replication whose reactive alkyne group is detected by forming a triazole ring (34). Quantitation of EdU incorporation in Ctf4 knockdown larval brains showed a severe S-phase delay (Figure 10), indicating progression of the RPC depends upon the availability of Ctf4.

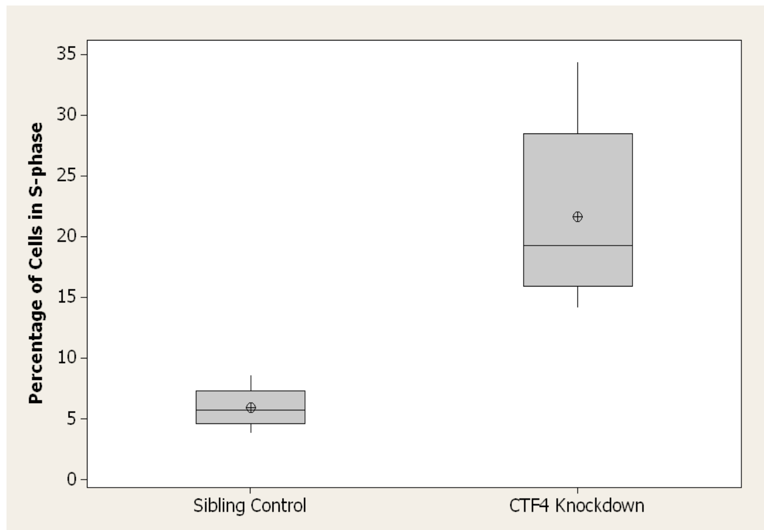
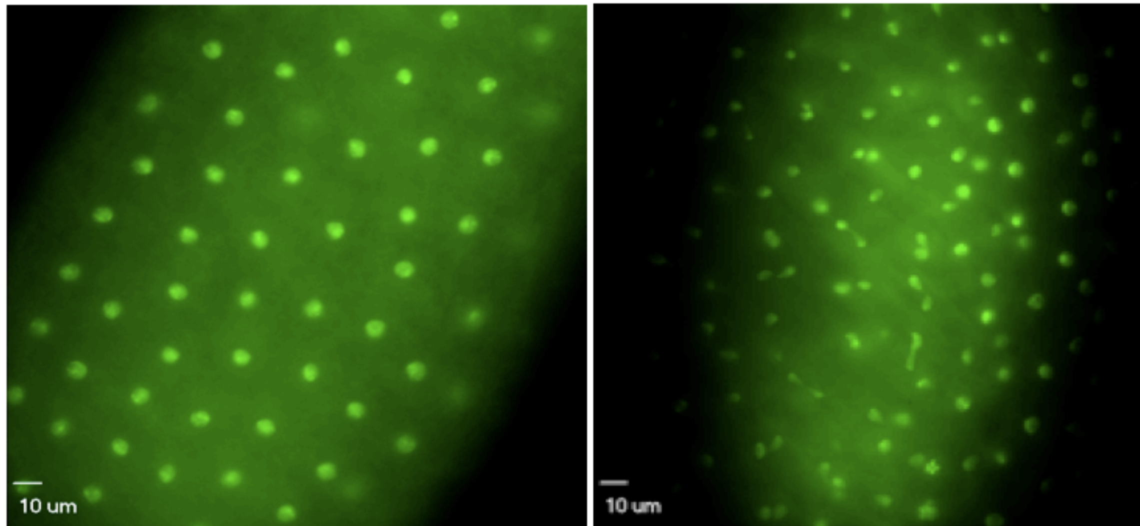


Figure 10. Boxplot of S-phase indices of larval brains. 5 brains were dissected for each group, and 5 random fields of view were analyzed for each brain.) (P = 0.009)

Consistent nuclear bridging observed in heat-shock-induced Ctf4 knockdown embryos.

Analysis of early *Drosophila* embryos gives additional insight into aberrant cell cycle events. The syncytical nuclei divide rapidly with no gap phase, but remain synchronous with limited check point control (23). In order to bypass the problem of maternal loading in knockdown studies of early development, a heat shock-inducible RNAi line was used. Adult females were acclimated and heat shocked as described previously, and early embryos were collected. Nuclear bridging was observed in 69% of heat shock-induced Ctf4 knockdown embryos between cell cycles 4-10 (Figure 11). The likely cause of bridging is incomplete S-phase progression upon entry into mitosis. This data is consistent with S-phase delays observed in larval brains.



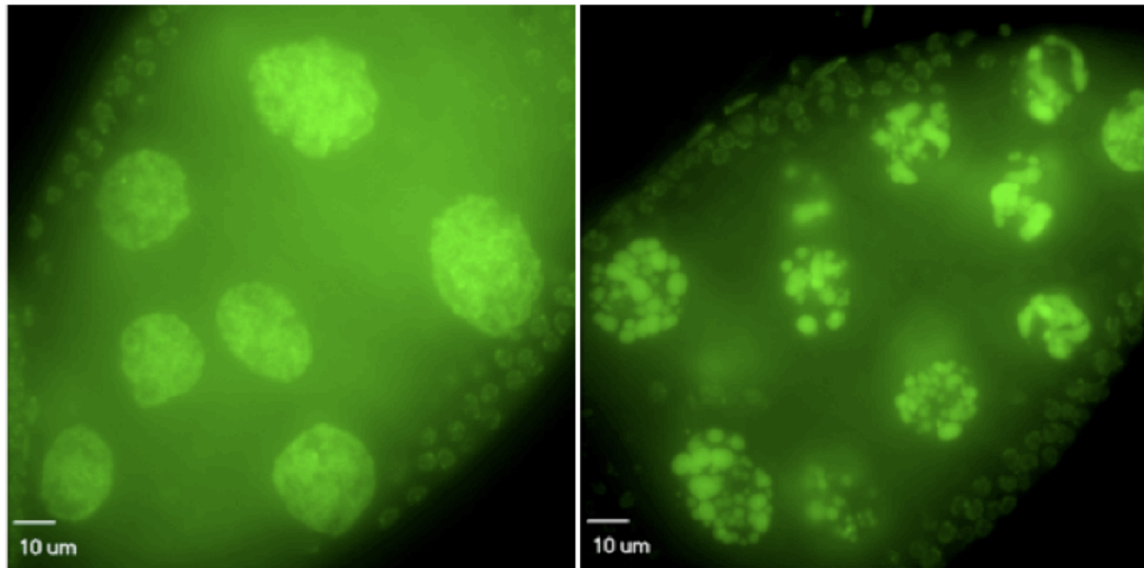
Sibling Control Ctf4 Knockdown

Figure 11. Confocal micrographs of DNA stained with DAPI in Drosophila early embryos. Mitotic bridging can be seen in the Ctf4 knockdown group with a frequency of 69% among those in cell cycles 4-10 (n=26).

Dropout of nurse cell nuclei observed in heat-shock-induced Ctf4 knockdown adult ovaries.

The expression profile of Ctf4 in adult *Drosophila* indicates it is most actively transcribed in mature ovaries (24). These ovaries are subdivided into individual ovarioles that themselves consist of multiple egg chambers. Egg chambers are progressively more mature in the posterior direction and contain nurse cells that decondense to allow abundant production of RNA and organelles, which nurture the oocyte (25). During this decondensation, a mitosis-like phase of endoreplication occurs during endocycle 5 that promotes the dissociation of sister chromatids (26, 27), making ovaries an important tissue in our study of the effect of Ctf4 knockdown. Abnormal chromosome fragmentation in nurse cell nuclei upon decondensation at stage 7 was observed exclusively in 17.4% of

ovarioles (Figure 12). This dropout event suggests the importance of Ctf4 in replication events in which completion of mitosis is not required (endoreplication), thus isolating the role of Ctf4 in S-phase.



Wild-type CTF4 knockdown

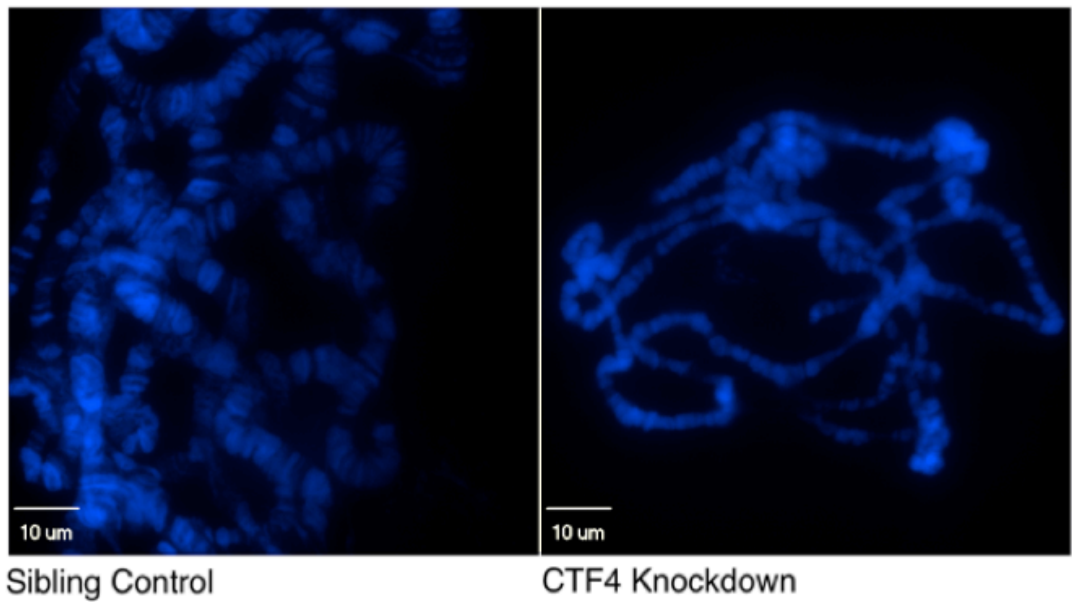
Figure 12. Confocal micrographs of egg chambers upon stage 7 decondensation of nurse cells. Nurse cells were observed undergoing premature apoptosis in 17.4% of Ctf4 knockdown ovarioles scored (n=23).

Under-replication observed in Ctf4 knockdown larval salivary glands.

To further investigate the impact of Ctf4 knockdown on endoreplication, *Drosophila* salivary glands were dissected and spread. The chromosomes within *Drosophila* larval salivary glands undergo DNA replication without mitosis, resulting in large polytene chromosomes from which abundant salivary enzymes are produced. Under-replication of polytene chromosomes has been documented in RNAi depletions of the DNA replication factor dup/cdt1 (29) as well as in mutant studies of a documented Ctf4 interactor, Mcm10

(25). A survey of polytene micrographs revealed a consistent thinning of polytene chromosomal arms (Figure 13A), suggesting under-replication defects. Images were acquired with confocal microscopy and analyzed to reveal a 43.4% reduction band area within Ctf4 knockdown larvae compared to sibling controls (Figure 13B).

A



B

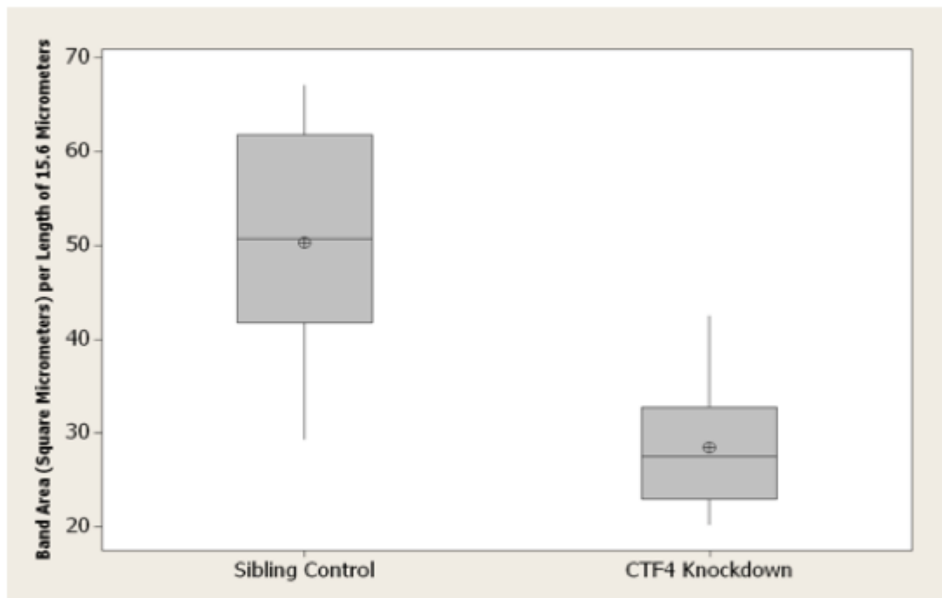


Figure 13. (A) Confocal micrographs of Ctf4 knockdown polytene chromosome compared to sibling control at 1000X magnification. (B) Boxplot of average polytene chromosome arm widths over a length of 15.6μm. Knockdown of Ctf4 results in overall thinning of chromosome arms by an average of 43.4% (n=10 polytene chromosomes).

dNTP depletion decreases larval viability in Ctf4 knockdown.

An important feature of the Ctf4 protein is its role in the Fork Protection Complex. This complex is thought to assist in the stabilization of paused replication forks during low nucleotide availability that occurs during replication of repetitive sequences, as well as during repair events that lead to stalling (16). An investigation of the importance of Ctf4 in replication pausing was carried out by a nucleotide-depletion hydroxyurea (HU) assay. Arrest of the replication fork is accomplished through the depletion of dNTPs, which is thought to occur as a result of the inhibition of ribonucleotide reductase (35). A slight decrease in survivability was detected in Ctf4 knockdown larvae compared to sibling control (Figure 14), indicating that Ctf4 is important in the context of replication fork pausing. This data is consistent with models showing involvement of Ctf4 in the Fork Protection Complex.

Survival		
(+) hydroxyurea	(-) hydroxyurea	Relative Survival
0.56 (172)	1.06 (66)	0.53

Figure 14. Table showing relative survival of larvae when grown with 10mM dNTP-depleting hydroxyurea. Ctf4 knockdown larvae exhibit a decrease in relative survival compared to sibling control. Survival ratios represent the percentage of glowing to non-glowing larvae. Parentheses indicate total number of larvae scored. Relative survival is the ratio of (+) hydroxyurea survival to (-) hydroxyurea survival, and shows the effect of hydroxyurea treatment on Ctf4 knockdown larvae. (Note: deviation from a 1:1 Mendelian ratio in (-) hydroxyurea group is not significant).

***Drosophila* Ctf4 interacts with members of MCM helicase and GINS, as well as Polymerase alpha.**

Studies in yeast and *Xenopus* have shown a conserved role for Ctf4 in tying the activity of Polymerase α primase to the MCM helicase complex (16, 30, 31), and that Mcm10 recruits Ctf4 for this role (16). Yeast 2-hybrid analysis was performed to investigate this role in *Drosophila* and to identify novel protein interactions with Ctf4. As expected, *Drosophila* Ctf4 was found to interact with Polymerase α primase. Additionally, consistent interaction with Psf1, Psf2, and Mcm2 was also detected (Figure 15). GAD fusion proteins tested showed no one-hybrid activity (data not shown).

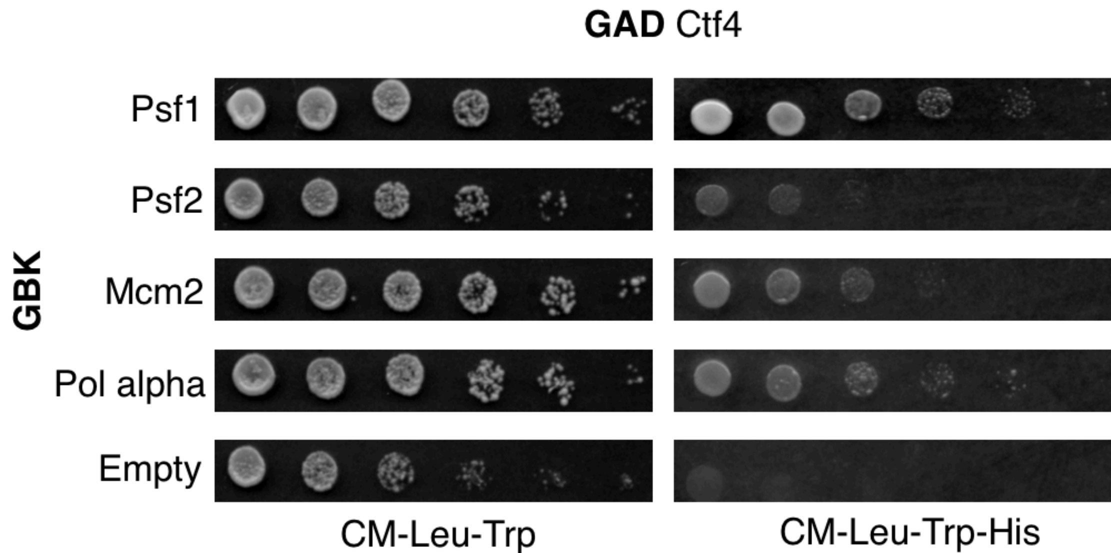


Figure 15. Yeast 2-hybrid analysis of Ctf4 interactions. Ctf4 in GAD interacts with Psf1, Psf2, Mcm2, and Pol alpha. Growth controls on left are suplimented with histidine, while only successful interactors grow on experimental medium lacking histidine. Serial dilution allows strength of interaction to be assessed as in the case of the weaker interaction of Ctf4 with Psf2.

V. Discussion

Intricate processes take place at the replication fork to ensure the faithful duplication of genetic information by simultaneously stabilizing and regulating helicase-polymerase association. Among those protein complexes that move with the replication fork are GINS and the Fork Protection Complex. In this study, we report that Ctf4, a member of the FPC, interacts physically with Psf1 and Psf2, both members of GINS, which has been studied in the past due to its role in stabilizing Mcm2-7 helicase activity (36). A physical interaction between Ctf4 and Mcm2, a component of the helicase itself, was also observed. These data further illuminate the association of Ctf4 with GINS and the helicase

observed in past studies (14). We have also confirmed a physical interaction between Ctf4 and Polymerase alpha in Drosophila, analogous to the event described in yeast (16).

Our study demonstrates that Ctf4 is vital for normal S-phase progression in Drosophila. Although this role has been suggested by previous studies in eukaryotes (15, 16), we present new data revealing its importance in multiple contexts within a whole-organism, metazoan model. The newly discovered importance of human Ctf4 to the association between Cdc45, Mcm2-7, and GINS (37) paved the way for another study to demonstrate an S-phase arrest in HeLa cells upon RNAi knockdown of human Ctf4 (38). Neither in our study is Ctf4 knockdown in whole-organism Drosophila, with S-phase delays observed in the larval stage, ultimately viable.

The involvement of Ctf4 in endoreplication evidenced by thinning of salivary gland polytene chromosomes and degeneration of ovariole nuclei shows that it functions outside of normal pre-mitosis replication in which S-phase progression is coordinated with eventual cytokinesis (39). This is important because the S-phase delay observed in our analysis of larval brain tissue, as well as in previous studies (15, 16), occurred in cells that would eventually undergo mitosis, thus demonstrating the involvement of Ctf4 in a more modular replication fork with multiple contexts.

Another piece of evidence suggesting involvement of Ctf4 in the core process of DNA replication comes from analysis of Drosophila early embryos. Factors that impede or otherwise aberrantly retard S-phase progression historically correlate with mitotic bridges, which may result from cellular or nuclear division amidst incomplete DNA replication (40). Drosophila early embryos display these defects upon Ctf4 depletion, which means that,

even in the context of a less-regulated cell cycle within syncytical nuclei (41), the function of Ctf4 in DNA replication is crucial.

Previous studies have suggested that the crucial link between members of the FPC, polymerases, and helicase components provides a flexible tie that provides stability (15). The increased susceptibility to replication fork pausing afforded by the incorporation of hydroxyurea in Drosophila medium allowed a sensitive way to determine the importance of adequate levels of Ctf4 in survival during these pauses. A significant decrease in survivability of Ctf4 knockdown larvae in this assay suggests that the stabilizing role of the aforementioned link was compromised. Ctf4 shares in this stabilizing role with FPC members Tim and Tipin, the co-depletion with Ctf4 of which magnifies the deleterious effects seen in S-phase progression (15).

VI. Conclusion

Past *in vitro* studies of Ctf4 have suggested an important role in DNA replication and cell cycle progression. This is the first whole-organism *in vivo* knockdown study of the Ctf4 protein in a higher eukaryote. On multiple fronts, this investigation shows the effects of the resulting S-phase delay in *Drosophila* development, as well as defects in endoreplication. *Drosophila* Ctf4 was also shown to interact with a member of the MCM helicase, namely Mcm2, members of the helicase-stabilizing GINS complex, namely Psf1 and Psf2, as well as Polymerase α.

Future investigation into the suggested interaction of Ctf4 and Mcm10 would help to further clarify the role of Ctf4 in the recruitment of polymerase activity to the replication fork. Embryonic studies would also benefit from live imaging due to the suggested role of

Ctf4 in sister chromatid cohesion. DNA damage assays will further clarify the effect of Ctf4 knockdown on situations that require stabilization during replication pausing. Finally, investigations into the dropout of *Drosophila* ovarioles in response to Ctf4 knockdown can be corroborated with apoptosis studies. The replication pausing complex, a central component of the DNA replication machinery, will prove to be a subject of intensive study over the next several years, and the discoveries from these studies could provide us with greater insight into disease.

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VIII. Appendix I.

Live imaging of *Drosophila* embryos

For future investigation into the relationship between DNA replication and sister chromatid cohesion/segregation, a protocol was developed for the acquisition of confocal video of the rapid nuclear divisions in syncytium within early *Drosophila* embryos. A model fly line expressing a histone binding red florescent protein was used to visualize chromatin under a Texas Red filter. Females were incubated over yeast paste for 24 hours, then incubated over a light film of yeast paste on grape agar for 5 hours for the deposition of embryos. Adults were removed. Embryos were collected by adding water to the plate with gentle stirring using a paintbrush to loosen specimens from the agar. A tube with nylon mesh was used like a sieve to separate embryos from the water. Dechorionation of embryos was then monitored while gently immersing the sieve in 50% bleach for 1 minute. The sieve was then twice rinsed with distilled water. Heptane glue (prepared by immersing double-sided tape into heptane and stirring overnight) was applied to a microscope slide and allowed to dry. After blotting the excess water underneath the sieve with a kimwipe, embryos were individually transferred with a paintbrush to the slide. Immediately after transfer, oxygenated halocarbon 700 oil was pipetted onto the specimens and a lifterslip applied. Embryos were imaged at 200X, 600X, and 1000X magnification using an Olympus IX81 Motorized Inverted Microscope with Spinning Disk Confocal. Real-time analysis of cell-cycle delays is conducted using the timestamp feature of Slidebook to document temporal progression of visible landmark events in mitosis. With the added dimension of time, further investigation into chromatid cohesion defects and other chromosomal aberrations

is possible. An early embryo (wt) undergoing two nuclear divisions can be seen in Figure 16. Video speed has been increased by a factor of 100X for clarity.

Figure 16. (Supplementary file). Live imaging of model RFP-expressing embryo undergoing two nuclear divisions. Video speed has been increased by a factor of 100X for clarity. Images were captured at 600X magnification using z-series florescence microscopy.

IX. Appendix II.

Aberrant phenotype seen in Ctf4 knockdown eclosion survivors

Survivors of Ctf4 knockdown past eclosion showed lethality in all cases no later than 3 days after eclosion. During this time, it was observed that pigment, especially on the dorsal thorax and abdomen, was darker, more pronounced, and less organized. Wings were un-inflated in all cases as seen in the Figure 17. In some cases, specimens lacked sufficient strength to fully emerge from the pupa casing.

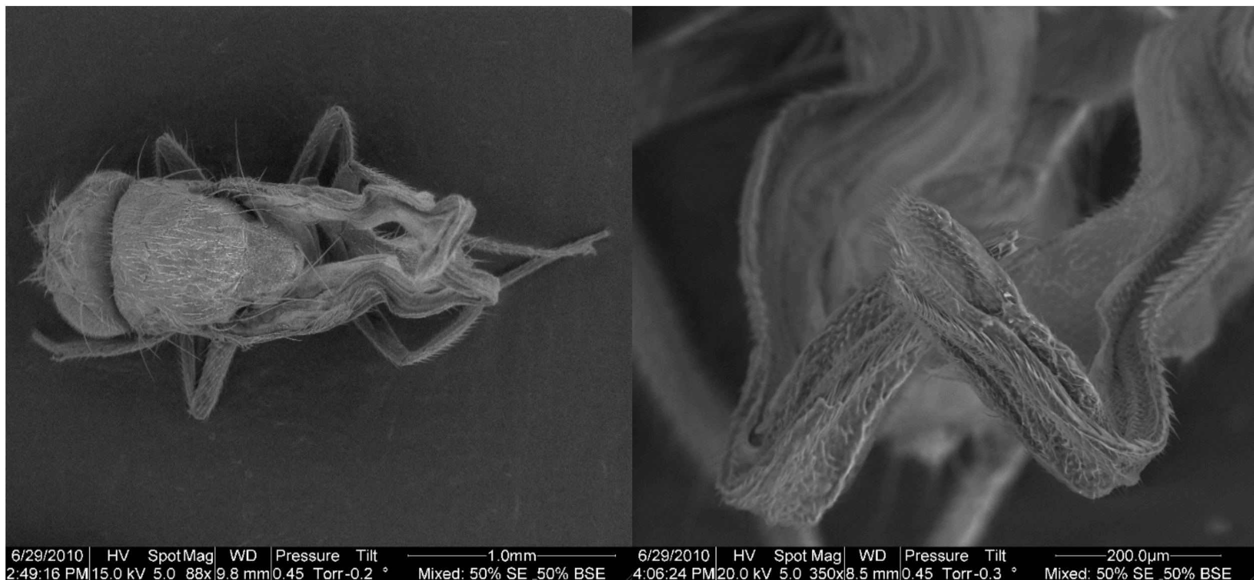


Figure 17.—SEM images of Aberrant wing phenotype in Ctf4 knockdown eclosion survivors.

X. Appendix III. Construction of transgenic fly for future studies

It is important to accompany mutant studies with phenotype rescue assays. If an extra wild-type copy of the gene is inserted into the genome of the mutant fly and can successfully restore the wild-type phenotype, the phenotype is said to be rescued, and the gene of interest is confirmed as the singular cause of any phenomena observed for that mutant. A transgenic fly containing an extra wild-type copy of Ctf4 was prepared using the scheme below.

Genomic Ctf4 prep

50 adult wild-type flies were knocked out with CO_2 and emulsified with a sterile disposable mortar and pestle in 500µL of solution A (0.1M Tris HCl, pH 9.0; 0.1M EDTA; 1% SDS). The tube was incubated at 70 degrees C for 30 minutes to lyse cells and denature proteins. The tube was then incubated on ice in 70 µL of 8M Potassium Acetate to precipitate denatured protein, which was then pelleted and removed from the supernatant. The supernatant was removed to a fresh tube and spun down for 5 minutes in 285µL of isopropanol. The pellet was rinsed with ethanol and dried at room temperature. Distilled water (100µL) was used to resuspend the pellet. Sodium acetate (3M) was added with two volumes of 100% ethanol and the pellet allowed to dry. The purified genomic DNA was then resuspended in 100µL of distilled water. Genomic Ctf4 was amplified from genomic DNA with the primer set 'Ctf4 genomic+2000 Topo Forward' + 'Ctf4 minus stop Reverse' (Integrated DNA Technologies™) with Accuprime Taq (Invitrogen™). PCR product was verified with agarose gel electrophoresis.

Cloning Ctf4 into vectors

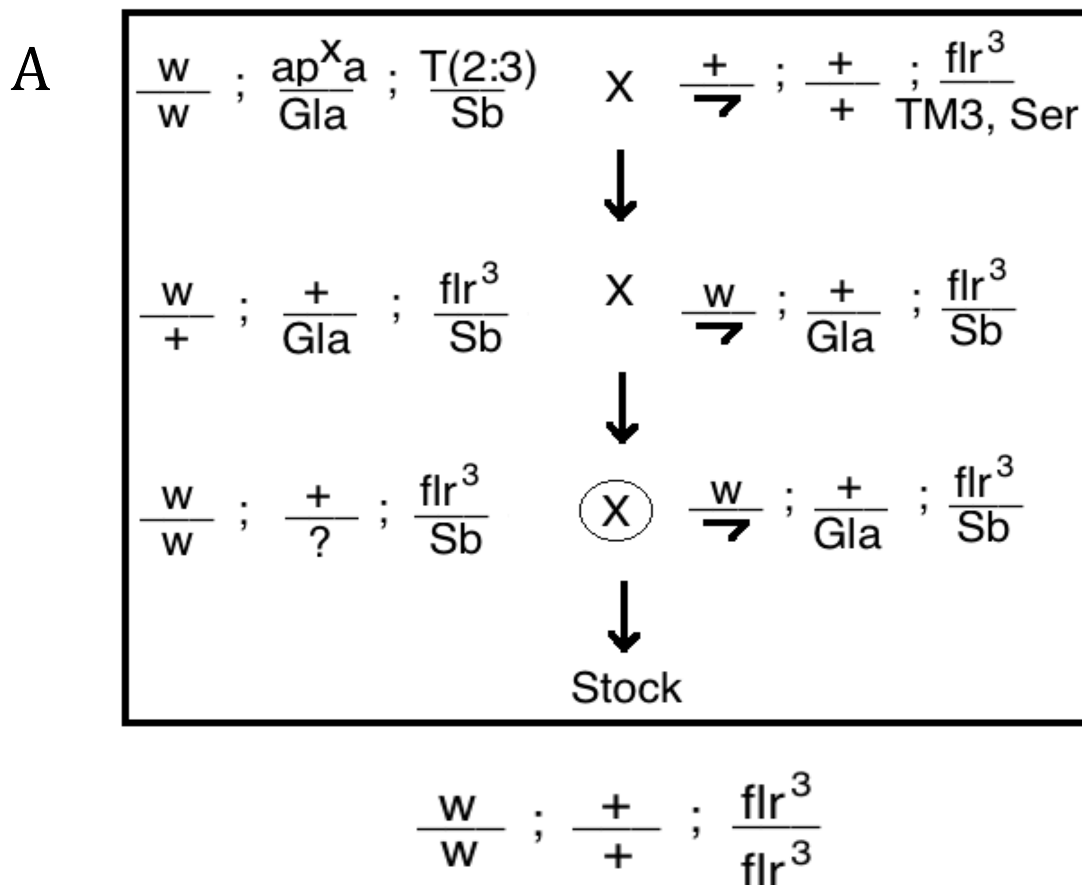
The purified genomic Ctf4 was then integrated into the Gateway cloning system. A TOPO® reaction was used to clone the genomic sequence into the pENTR-d-TOPO® entry vector using the reaction cocktail prescribed in the Invitrogen pENTR™ Directional TOPO® Cloning Kits User Manual. This cocktail was then incubated for 5 minutes at room temperature. Chemically competent E. coli were transformed with the cocktail with a 42 degree heat shock for 30 seconds followed by an incubation on ice for 5 minutes. The bacteria were then allowed to recover on a rotor at 37 degrees for 30 minutes before they were plated on media selective for the kanamycin resistance gene in the pENTR plasmid. Colonies were miniprepped (Promega™) and stock solutions of pENTR Ctf4 sequence-verified using the East Carolina University Core Genomics Facility. Ctf4 was then cloned using an LR clonase reaction™ into the Gateway-compatible destination vector pTWH. The LR recombination reaction cocktail was prepared as prescribed by the Invitrogen User Manual. The cocktail was incubated at room temperature for 5 hours. Transformation of and miniprep from One Shot® competent E. coli was performed as described above with the exception of ampicillin resistance selection to recover Ctf4 pTWH. Sequence verification followed to ensure proper insertion. Finally, an aliquot of Ctf4 pTWH construct was mailed to Best Gene Inc. for the generation of the transgenic fly. Flies were received and stored over *Drosophila* K12 media (US Bio- logical # D9600-07B) at room temperature.

XI. Appendix IV. Development of fly stocks for the Wing Spot assay

The Wing Spot assay can be used to study the mutagenic and recombinogenic properties of a system or compound (33). Among its advantages are speed, sensitivity, cost-effectiveness, and reliability to the researcher interested in detecting both somatic mutation and mitotic recombination. These events are represented in imaginal disk cells during embryonic development, and are visible on the wings of the adult fly. The assay uses two wing cell markers: *flare-3* (*flr3*) and *multiple wing hairs* (*mwh*). Flare-3 is a recessive mutation that produces malformed wing hairs with the shape of a flare. This allele is kept over a balancer chromosome carrying multiple inversions and a dominant Ser marker (*flr3/TM3,BdS: Third Multiple 3, Beaded-Serrate*). Given their zygotic lethality, flare alleles must be maintained in stocks over balancer chromosomes carrying multiple inversions and a dominant marker that is homozygous lethal (*TM3, Bds*). *Multiple wing hairs* is a homozygous viable recessive mutation that produces multiple trichomes per cell instead of one trichome. A cross between parents, each with one of these alleles, produces two types of progeny: marker-heterozygous (*mwh+/+flr3*) and balancer-heterozygous (*mwh+/TM3,BdS*). The former progeny are phenotypically wild-type, and the latter are phenotypically serrate. Upon this single cross, progeny are anesthetized and their wings removed and mounted in Polyvinyl-lactophenol or Faure's solution. In marker-heterozygous wings, the researcher scores mwh single spots, which result from point mutations, deletions, and mitotic recombinations between the two markers; *flr* single spots, which also result from point mutations, deletions, and mitotic recombinations between the two markers; and twin spots with adjacent *mwh* and *flr* areas, which are produced exclusively from mitotic recombination between the proximal marker *flr* and the centromere of chromosome 3. Spot sizes are

measured as well because they correlate to the time of damage induction. In balancer-heterozygous wings, the observation of *mwh* single spots excludes recombination because multiple inversions are present. All *mwh* single spots are produced by non-crossover type mutational events.

Not only have dozens of mutagenic compounds been evaluated with this test, but the inherent mutagenic environment potentially caused by a problem with DNA replication can also be assessed. This particularly lends itself to DNA replication protein mutation studies. To initiate the implementation of this assay for mutation studies, white-eyed lines of *mwh* flies and *flr3* flies were developed according to the scheme in Figure 18.



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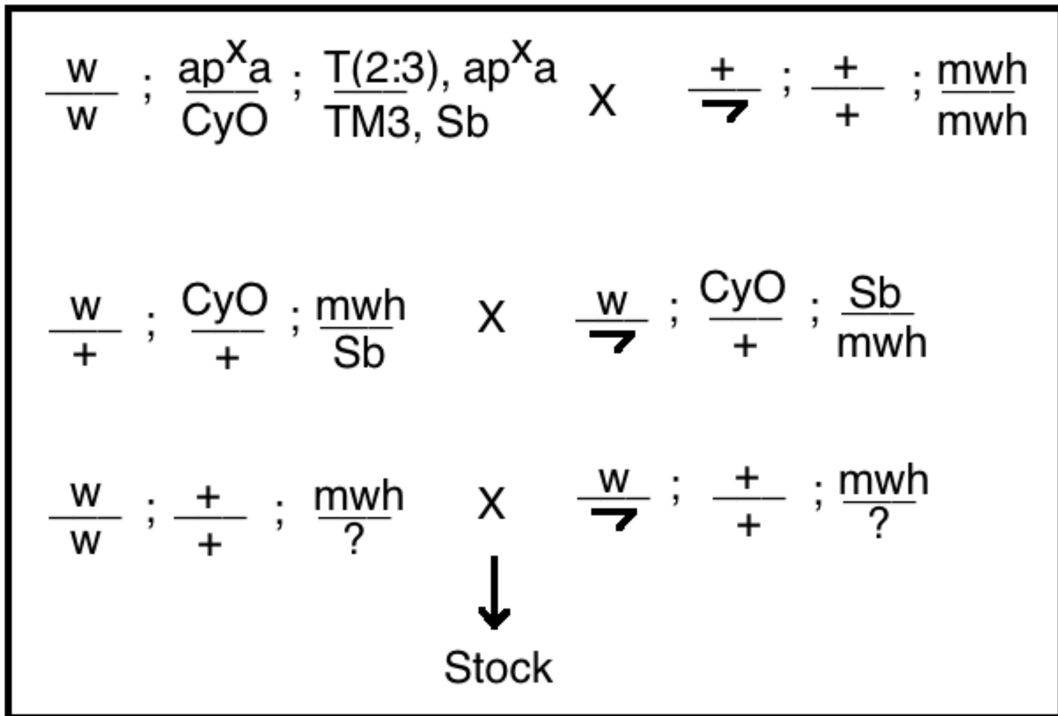

$\frac{w}{w}\ ;\ \frac{+}{+}\ ;\ \frac{mwh}{mwh}$

Figure 18. (A) Cross scheme to acquire white-eyed flare-3 stock flies. (B) Cross scheme to acquire white-eyed multiple wing hair stock flies.

XII. Appendix V. Phenotypic investigation of Ctf4 upstream mutant

A fly strain containing a p-element insertion (Figure 19) in the upstream region of Ctf4 was acquired from Szeged Stock Center (Stock center number CB-0870-3, FBst0106400) and analyzed for defects in replication.

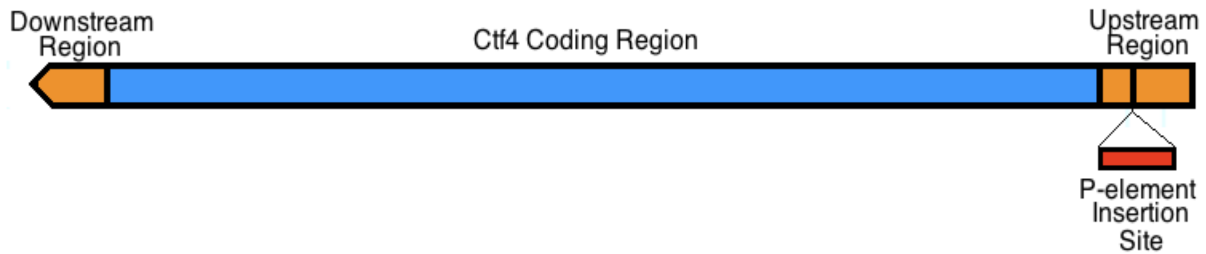

Figure 19. Diagram of Drosophila Ctf4 showing non-coding upstream region with P-element insertion site.

The strain is homozygous viable, and phenotypic investigation of this Ctf4 upstream mutant *Drosophila* line revealed no detectable aberrant properties. A survey of polytene chromosome spreads was indistinguishable from wild-type (Figure 20), and mitotic indices calculated from mutant larval brains were not significantly different from those of wild-type brains (Figure 21). Early embryos were also observed for signs of mitotic asynchrony and bridging (Figure 22); however, no aberrant phenotypes were discovered. Assays were carried out as described in Materials and Methods.

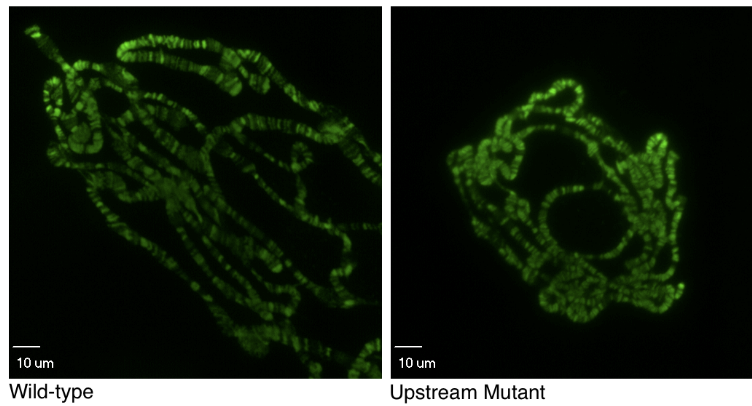

Figure 20. Upstream mutant polytene chromosome spreads viewed at 600X magnification. No significant difference in chromosome arm width or length was detected.

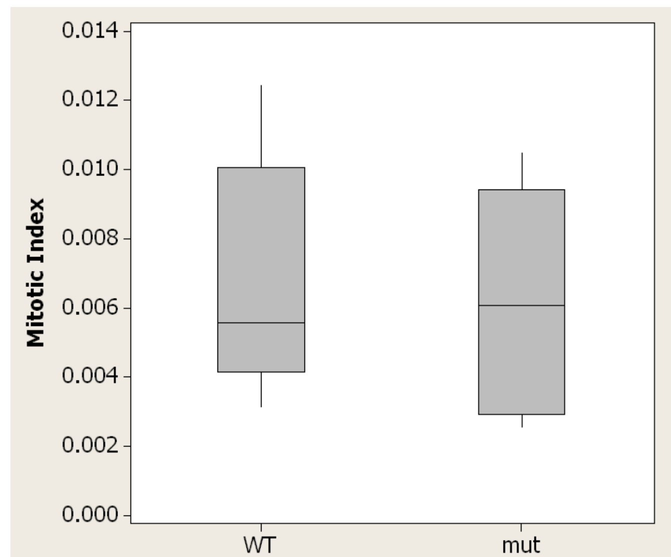

Figure 21. Boxplot of mitotic index in Ctf4 upstream mutant larval brains. Larval CNS of upstream mutants showed no significant difference in ratio of cells in M-phase to cells not in M-phase.

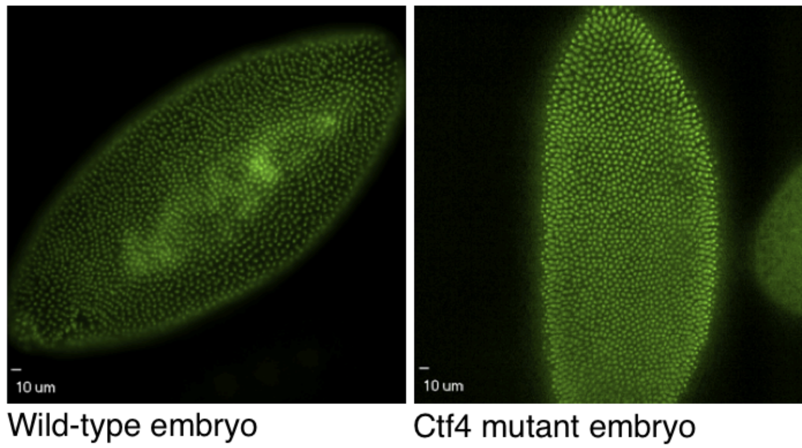

Figure 22. Confocal micrographs of Drosophila early embryos with upstream mutant (stage 10-12). No aberrant phenotype was detected in these specimens. Images acquired at 200X magnification.