CHARACTERIZATION OF TWO NOVEL MUTANTS OF DNA POLYMERASE DELTA IN *DROSOPHILA MELANOGASTER*

by

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July 15th, 2011

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DNA is the genetic material for all living organisms which is constantly being unpackaged, replicated and repackaged. The replication of this genetic material involves numerous different proteins; however, DNA polymerase delta (pol δ) carries much of the load by replicating a major portion of the genome in both leading and lagging strand synthesis. Using the model organism, *Drosophila melanogaster*, we investigate two novel mutations in two different evolutionary conserved regions. One region corresponds with the polymerase's ability to polymerize new nucleotides onto an existing strand of DNA. The other region corresponds with the polymerase's ability to proofread in the 3' to 5' direction.

These two mutants, both homozygous lethal and recessive, show interesting phenotypes with a delay in S-phase, numerous chromosome aberrations, defects in endoreplication and possible protection form DNA damage. Using these two mutants, these two domains can be further characterized. By understanding how pol δ functions in an *in vivo* setting, we can apply this knowledge to the mechanics of cancer biology in humans, another multicelluar organism, and inform new therapies to treat it.

CHARACTERIZATION OF TWO NOVEL MUTANTS OF DNA POLYMERASE DELTA IN *DROSOPHILA MELANOGASTER*

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by

Chad Michael Hunter

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DEDICATION

I would like to dedicate this thesis to my parents, Michael and Dori Hunter, for their continued support through my academic career. I am in great debt to them for all their support.

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I would like to thank my family first for their support throughout my academic career, my parents, Michael and Dori, and my siblings, Dana and Brett. I would like to thank Dr. Tim W. Christensen for the opportunity to perform research in his lab and all his continued guidance and support through my time at East Carolina. I would like to thank my committee members, Dr. Mary Farwell, Dr. Dave Rudel, and Dr. Maria Ruiz-Echevarria for all their feedback and suggestions about my project. I would like to thank all the members of the Christensen lab (past and present): Jen Apger, Jeff Chmielewski, Divya Devadsan, Laura Henderson, Catherine Gouge, Justin Gosnell, and Michael Reubens [graduate students]; Lena Keller and Wayne Rummings [undergraduate students]. I would like to thank Dr. Bonnie Bolkan for her donation and previous work with DNA Polymerase Delta fly strains. I would like to thank Dr. Tom Fink for his assistance in my microscopy work. I would like to thank Dr. Steve Rogers and Dr. Jean-Luc Scemama for his assistance in all my S2 cell work. I would like to thank Dr. Doug Weidner for his assistance in my flow cytomery work. I would like to thank Dr. Tao Sheih for his donation of the RecQ4 mutant fly strains. I would also like to thank Dr. Jeff McKinnon, Dr. Terry West, the Department of Biology and the Biology Graduate Student Association for their support during my time at ECU.

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CHARACTERIZATION OF TWO NOVEL MUTANTS OF DNA POLYMERASE DELTA IN *DROSOPHILA MELANOGASTER*

LIST OF ABBREVIATIONS

B. taurus ..... *Bos tarus*

BER ..... Base excision repair

bp ..... Base pair

BrdU ..... Bromodeoxyuridine (5-bromo-2'-deoxyuridine)

BSA ..... Bovine serum albumin

Cdk1 ..... Cyclin dependent kinase 1

Cyclin B1 ..... G2/mitotic-specific cyclin-B1

D. melanogaster ..... *Drosophila melanogaster*

DAPI ..... 4',6-diamidino-2-phenylindole

DNA ..... Deoxyribonucleic acid

dNTP ..... Deoxyribonucleotide

DSB ..... Double-strand break

DSBR ..... Double-strand break repair

E. coli ..... *Escherichia coli*

EDTA ..... Ethylenediaminetetraacetic acid

EdU ..... 5-ethynyl-2'-deoxyuridine

EtOH ..... Ethanol

FEN1 ..... Flap structure-specific endonuclease 1

G_0 Phase ..... Gap 0 phase

G_1 Phase ..... Gap 1 phase

G_2 Phase ..... Gap 2 phase

GFP ..... Green fluorescent protein

H. sapiens ..... *Homo sapiens*

H_2O ..... Water

HU ..... Hydroxyurea

kb ..... Kilobases

kDa ..... Kilodalton

M ..... Molar

M Phase ..... Mitosis phase

MCM2-7 ..... Minichromosome Maintenance Proteins 2-7

$MgSO_4$ ..... Magnesium sulfate

ml ..... Milliliter

mM ..... Millimolar

MMR ..... Mismatch repair

MMS ..... Methyl methanesulfonate

µl ..... Microliter

µM ..... Micromolar

Mus musculus ..... *M. musculus*

mut ..... Mutant

NaCl ..... Sodium chloride

NER ..... Nucleotide excision repair

ng ..... Nanograms

Nm ..... Newton meter

nt ..... Nucleotide

P. falciparum ..... *Plasmodium falciparum*

PBS ..... Phosphate buffered saline

PCNA ..... Proliferating cell nuclear antigen

PCR ..... Polymerase chain reaction

PEG ..... Polyethylene glycol

pg ..... Picograms

pol ..... Polymerase

pol α ..... DNA polymerase alpha (α)

pol β ..... DNA polymerase beta (β)

pol γ ..... DNA polymerase gamma (γ)

pol ε ..... DNA polymerase epsilon (ε)

RFC ..... Replication factor c

RNA ..... Ribonucleic acid

RNase H1 ..... Ribonuclease H1

rpm ..... Revolutions per minute

RTS ..... Rothmund-Thomson Syndrome

S Phase ..... Synthesis phase

S. cerevisiae ..... *Saccharomyces cerevisiae*

S. pombe ..... *Schizosaccharomyces pombe*

TAE ..... Tris-acetate-EDTA

X. tropicalis ..... *Xenopus tropicalis*

LIST OF SYMBOLS

˚C.....Degrees Celsius

χ^2.....Chi Square

♀.....Female

∞.....Infinity

☠.....Lethality

♂.....Male

☿.....Virgin Female

+.....Wild Type

CHARACTERIZATION OF TWO NOVEL MUTANTS OF DNA POLYMERASE DELTA IN *DROSOPHILA MELANOGASTER*

1. INTRODUCTION

DNA is the genetic material for all living organisms which is constantly being unpackaged, replicated and repackaged. During these processes, errors can occur which can have dire consequences on the fate of the cell. Some of these consequences include unregulated growth, failure to differentiate and defects in chromosome biology. To understand what is causing the consequences, it is necessary to have a firm understanding of the key players involved in unpackaging, replicating and repackaging the DNA. In this thesis, I investigate one of these key players, DNA Polymerase Delta (pol δ). By understanding the function of this factor, more data can be obtained to understanding how to prevent these dire consequences and developing new therapies for the treatment of these consequences.

To understand DNA replication, it is fundamental to understand where the process takes place in the life of a cell. The cell cycle, or life cycle of the cell, "is the universal process by which cells reproduce, and that it underlies the growth and development of all living organisms" (NURSE 2000). The cell cycle is composed of 4 main phases: Gap 1 (G_1), Synthesis (S), Gap 2 (G_2) and Mitosis (**FIGURE 1.01**). The first three phases (G_1, S, and G_2) are collectively known as Interphase.

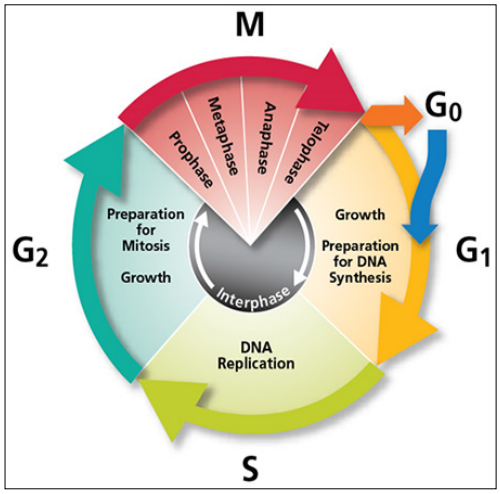

FIGURE 1.01 - Diagram of the Cell Cycle (BD BIOSCIENCES)

When a cell has become dormant, it is referred to being in the Gap 0, G_0, stage, also known as an extended G_1 phase. When cells are actively dividing however, they will progress through the 4 phases mentioned above. G_1 is the phase of a cell's life where it begins to grow and collect nutrients and essentials before it moves onto to S phase. Many different stimuli factors affect a cells progression into S phase, a checkpoint known as the G_1/S checkpoint. If the cell proceeds past the "restriction point" (late G_1), it is making a defined switch from mitogen-dependent growth to largely growth factor-independent progression, a switch necessary to prepare for the rest of the cell cycle (BARTEK and LUKAS 2001). If the cell passes the checkpoints set in place, it will enter the S phase where it will replicate its DNA, or genetic material. Synthesis will be described in detail after recognition, licensing and activation. The cell then proceeds to G_2, another growth stage where the cell prepares for division. The growth in G_2 has been hypothesized as a method to control cell size (MOSELEY *et al.* 2009) which is sometimes lacking in certain cancers. The end of G_2 is marked by another checkpoint, the G_2/M checkpoint. The G_2/M is a checkpoint in order to prevent defects in synthesis (incompletely replicated DNA) or

various DNA damage from being passed onto to the daughter cells (HARTWELL and KASTAN 1994). The cell will either fix these mistakes using appropriate DNA repair machinery entering in cell cycle arrest or undergo programmed cell death or apoptosis if the mistakes are severe enough. However, if the cell has no mistakes, various proteins such as cyclin B1 and CDK1 will trigger the cell to progress to M phase (PORTER and DONOGHUE 2003). Mitosis (**FIGURE 1.02**), a continuous process, is in turn broken down in 5 stages: Prophase, Prometaphase, Metaphase, Anaphase, Telophase.

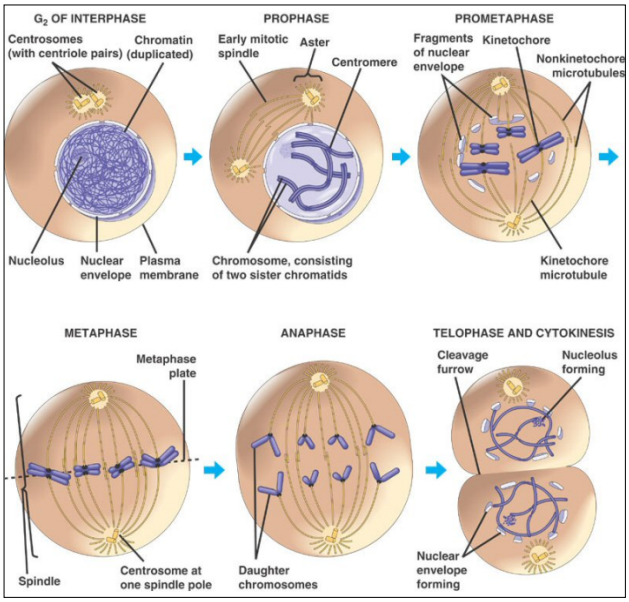

FIGURE 1.02 - The Stages of Mitosis (CAMPBELL *et al.* 2007)

During the transition to M phase, the chromosomes begin to condense. Prophase is characterized by the early formation of the mitotic spindle along with further condensation of the chromosomes and the nuclear envelope beginning to break down. Prometaphase is indicated by attachment of microtubules to the kinetochores of the chromatids followed by attachment of the sister chromatids to the microtubules, allowing the chromosomes to align along the metaphase

plate. Metaphase begins once all chromatids are aligned properly along the metaphase plate and kinetochore microtubules achieve a proper amount of tension across the chromosomes. During anaphase, separation of the sister chromatids occurs allowing the individual chromatids to move along the kinetochore microtubules to opposite poles of the cell. The fully separated chromatids are encompassed by a new nuclear envelope and begin to decondense during telophase. The two daughter cells are physically separated during cytokinesis, which occurs by the formation of a cleavage furrow between the asters of the spindle apparatus. These new cells then enter the G_1 stage and the process begins again. "The most important events of the cell cycle are those concerned with the copying and partitioning of the hereditary material, that is replicating the chromosomal DNA during S phase and separating the replicated chromosomes during mitosis" (NURSE 2000).

With one of the most important events of the cell cycle being concerned with replication of the DNA, what are the driving forces behind this synthesis? Enter the DNA polymerases, more specifically pol α, δ, and ε. Polymerases are various enzymes that function in the replication and repair of DNA by catalyzing the linking of nucleotides together. These are the enzymes responsible in replicating the genome that encode for over 20,000 protein coding genes (in *H. sapiens*). It was soon after the suggestion of the double helix model of DNA (1953 by James Watson and Francis Crick) that the machinery to replicate the DNA was identified. The first polymerase, pol α, was discovered in 1957 followed in the 1970s by the discovery of pol β and γ leading to the idea that pol α was the enzyme responsible for nuclear DNA replication, pol β for DNA repair, and pol γ for mitochondrial DNA replication (HÜBSCHER *et al.* 2000). The 1980s led to the discovery of pol δ and pol ε and arose the notion that a particular polymerase might have multiple functional tasks and that a DNA synthetic events may require more multiple

polymerases (STUCKI *et al.* 2000). Since then, there have been over 10 novel polymerases discovered. However, the main three players in replication still remain to be pol α, δ, and ε. Studies in *S. cerevisiae*, showed that those three main players share the task of replicating the nucelar genome. In addition, DNA repair events repair might require not only pol β but also pol δ or ε, or both (BRIDGES 1999).

The polymerase of interest in the study is pol δ. Although discovered in 1976, it took almost 10 years for this enzyme to be classified as an actual polymerase due its interaction with other factors such as Proliferating Cell Nuclear Antigen (PCNA) (will be described below) (HÜBSCHER 2000). Interestingly, pol δ is the most conserved of all the polymerases, sharing an identity ranging from 93% from humans (*H. sapiens*) to mouse (*M. musculus*), 60.6% from humans to *Drosophila* (*D. melanogaster*) and 35% from human to yeast (*S. cerevisiae*) (**FIGURE 1.03**).

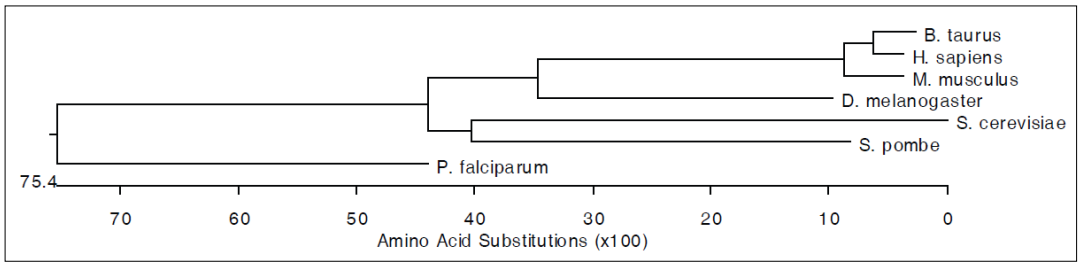

FIGURE 1.03 - Cladogram of pol δ among different species

In addition to an overall identity similarity, there are many different regions of high similarity between species (**FIGURE 1.04**) [Note: the protein structure of *D. melanogaster* is shown due to its direct use in this study]. Pol δ has 15 overall conserved regions: 2 N-Terminal, 4 Exonuclease, 6 Polymerase and 3 C-Terminal.

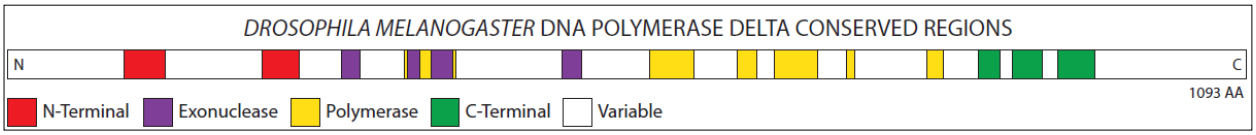

FIGURE 1.04 - Conserved Regions in pol δ

DNA replication is a multifaceted sequence of events. For the sake of clarity in this chapter, I will be discussing replication after recognition, licensing and activation and beginning with loading of pol α/primase after the unwinding of the DNA; however, these previous steps are an essential part of replication. Although polymerases are fundamental to replication, there is still much debate to the exact role of each polymerase and additionally if there are more enzymes involved in this intricate process.

After the two strands of the double helix of DNA have been unwound by various helicases, pol α/primase is loaded onto the DNA, also known as primosome assembly (**FIGURE 1.05**).

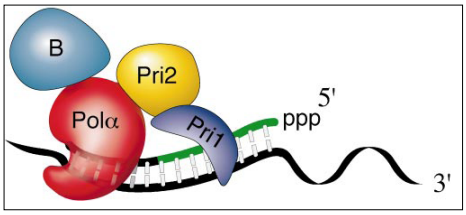

FIGURE 1.05 – Pol α/Primase (FRICK and RICHARDSON 2001)

Primosome assembly normally involves a DNA helicase, more than likely the MCM2-7 helicase complex (GARG and BURGERS 2005) (**FIGURE 1.06**), interacting with the pol α/primase (WAGA and STILLMAN 1998).

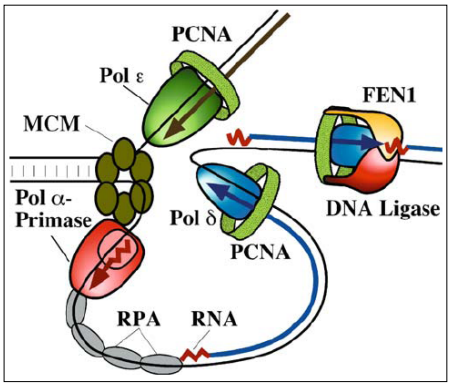

FIGURE 1.06 - The Minimal Set of Proteins Required for DNA Synthesis (GARG and BURGERS 2005)

Pol α/primase is the only enzyme capable of initiating DNA synthesis afresh by first synthesizing an RNA primer and then extending the primer by polymerization to produce a short DNA extension (RNA-DNA primer) (DEPAMPHILIS 1996). Pol α/primase has been shown to be able synthesize a RNA-DNA primer of approximately 40 nucleotides (nt) in length, including about 10 nt of RNA primer (NETHANEL *et al.* 1988). The short RNA-DNA then serves as a primer for extension by another pol for DNA synthesis on either the leading strand or for each Okazaki fragment on the lagging strand (TSURIMOTO and STILLMAN 1968). To continue with synthesis, a polymerase substitution must occur from the initiating pol α/primase to either pol δ or ε. This substitution occurs because pol α/primase is not capable of processive DNA synthesis and detaches from the template DNA following primer synthesis (MURAKAMI and Hurwitz 1993).

This substitution of pol α/primase to the more processive pol δ is known as the pol

switch. By itself, pol δ is not much more processive than pol α; however, its association with PCNA makes this holoenzyme (PCNA and pol δ) the main replication machinery. Pol δ's interaction with PCNA will be discussed later in this chapter. The pol switch has been proposed to be mediated by Replication Factor C (RFC), which once bound ends primer synthesis by pol α/primase of a 30 nt sequence (10 nt of RNA and 20 nt of DNA) (BELL and DUTTA 2002). RFC binding also activates the assembly of the primer recognition which is accomplished through the loading of PCNA followed by its subsequent association with pol δ. This holenzyme then continues synthesis on the leading strand for at least 5-10 kilobases of DNA. There are conflicting theories that RFC is not the sole recruiter for the holenzyme but it is actually a RFC-PCNA complex that then in turns recruits PCNA followed by pol δ (MAGA *et al.*1999). In addition to conflicts about RFC, one of the biggest conflicts remains to be what polymerase is involved in leading strand synthesis. The literature is conflicting with some reports of PCNA-pol δ being the replication machinery while others reporting PCNA-pol ε. This debate is one of the biggest in the field of DNA replication and is often avoided in the literature. In my opinion, it seems that pol δ and pol ε have redundant functions and there might multiple pol switches occurring between these two pols on leading strand synthesis. The abundance of a certain pol at the time of synthesis might dictate which pol is used in leading strand synthesis but more research still needs to be done to clarify the exact polymerase responsible for leading strand synthesis; however, it seems that pol δ is a better candidate. With the leading strand being synthesized by either pol δ and/or ε, it is now time to investigate lagging strand synthesis which is slightly more complicated.

Semidiscontinuous replication (**FIGURE 1.07**) makes the synthesis of the lagging strand a little different than leading strand synthesis.

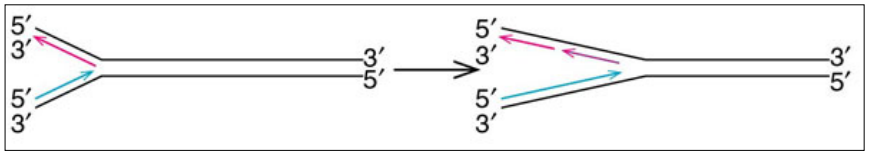

FIGURE 1.07 – Semidiscontinuous Replication (WEAVER 2008)

Lagging strand replication includes several distinct stages: initiation by DNA primase, limited elongation of the RNA primer by Pol α, a switch of the primer terminus from pol α to pol δ, elongation by pol δ similar to leading strand synthesis but also maturation of the completed Okazaki fragment (GARG and BURGERS 2005). Since polymerases can only synthesize in the 5'→3' direction, the lagging strand is synthesized in short pieces of DNA known as Okazaki fragments. These fragments are joined into long, ungapped DNA products which involves removal of the RNA primer, DNA gap synthesis, and sealing together of the two DNA pieces (**FIGURE 1.08**).

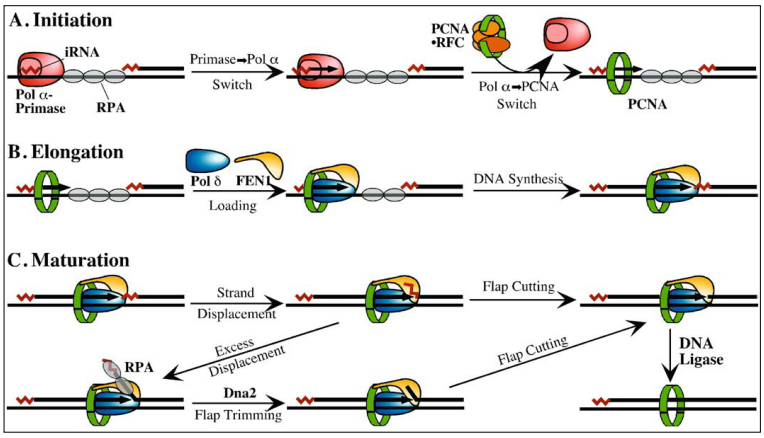

FIGURE 1.08 - Initiation, Elongation and Maturation (GARG and BURGERS 2005)

Two different nucleases, RNase H1 and FEN1, are involved in the complete removal of the RNA primer (WAGA *et al.* 1994). Pol δ and FEN1 act together in order to produce and maintain nicks (discontinuity in a double stranded DNA where there is no phosphodiester bond between adjacent nucleotides of one strand) that can be ligated by DNA ligase 1 (GARG *et al.* 2004). The nick translation process can be terminated by ligase action, as rapidly as a few nucleotides past the RNA-DNA junction of an Okazaki fragment. Because pol ε lacks this strong coordination with FEN1 for producing a ligatable nick, it strengthens the case for pol δ as the lagging strand enzyme. If pol ε was only a leading strand enzyme, it certainly would not need a coordination with FEN1. When a replicating pol δ complex runs into a doublestranded region, it displaces 2 to 3 nt of the downstream RNA or DNA, a process also known as idling. When FEN1 is present in the replicating complex that runs into the double-stranded region, efficient nick translation ensues, and idling caused by pol δ is inhibited (GARG *et al.* 2004). The tight coupling between pol δ and FEN1 results in mostly mononucleotides released during nick translation. Finally, with DNA ligase 1 also present, the nick translation process can be terminated by ligase action, as rapidly as a few nucleotides past the RNA-DNA junction of an Okazaki fragment (AYYAGARI *et al.* 2003).

It is inappropriate to discuss pol δ without also discussing its accessory protein, PCNA, in detail as well. PCNA, first discovered in the late ‘70s and was first associated with pol δ in the mid ‘80s. PCNA plays important roles in nucleic acid metabolism as has been described by some as the “Maestro of the Replication Fork” (MOLDOVAN *et al.* 2007). The protein is essential for DNA replication but has also recently shown to interact with many different proteins whose involvements range from Okazaki fragment processing, DNA repair, translesion DNA synthesis,

DNA methylation, chromatin remodeling and cell cycle regulation (MAGA and HÜBSCHER 2003). It has also been suggested to be involved in chromatin assembly, and in several instances has been shown to be involved in RNA transcription. However, its main function still remains to be as the processivity factor of pol δ and thus is the functional homologue of the processivity factor in *E. coli*, the β subunit of the DNA polymerase III holoenzyme (KELMAN and O'DONNELL 1995). Similarities in the function of these proteins gave the first indication of the structure of PCNA. PCNA has been described as a "sliding clamp" by forming a ring around the DNA (**FIGURE 1.09**).

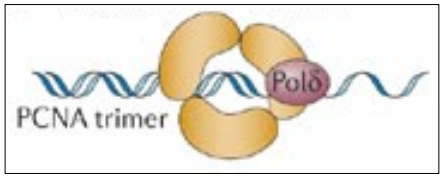

FIGURE 1.09 - PCNA and pol δ (Adapted from HOELLER *et al.* 1996)

The binding to PCNA stimulates the 5' to 3' exonuclease activity of FEN-1, a function which is important for the formation of a continuous lagging strand. Therefore, PCNA plays a role in DNA synthesis not only as part of the polymerase holoenzyme but also in the final steps leading to the formation of a complete DNA duplex.

In addition to being directly involved in DNA synthesis, pol δ and PCNA are involved correcting mistakes during this synthesis. DNA repair is essential part of replication; one misplaced nucleotide can have dire consequences causing an entire protein not to be translated. Three types of excision repair are acting in the cell: base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR) (LINDAHL and WOOD 1999), each of them targeted to specific DNA lesions. The repair system depends on whether the damaged bases are

excised as free entities (BER), as components of oligonucleotide fragments (NER) or are simply mismatched (MMR) (MODRICH 1991; FRIEDBERG *et al.*1995). A common feature of all these repairs is that DNA synthesis is required in order to replace the damaged DNA with a repaired copy. Genetic and biochemical studies are consistent with an involvement of both PCNA/ pols δ and ε in NER and BER (WANG *et al.* 1993). For example, either pol δ or ε can be used to reconstitute NER (ABOUSSEKHRA *et al.* 1995) or long-patch BER (STUCKI *et al.* 1998) *in vitro*. Assays also showed the ability of pol δ to reconstitute MMR in deficient extracts and that PCNA facilitates the interaction of many key MMR proteins (KOLDNER and MARSISCHKY 1999).

A particular kind of DNA repair is double-strand break repair (DSBR) which occurs through a recombination-type mechanism (**FIGURE 1.10**) using information on the undamaged sister chromatid or homologous chromosome (KANAAR *et al.* 1998)**.**

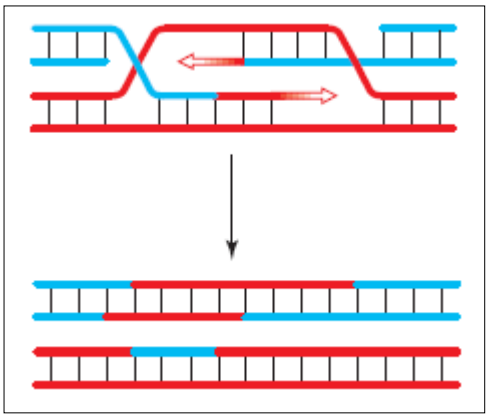

FIGURE 1.10 - Homologous Recombination (Adapted from KANAAR *et al.* 1998)

Genetic studies of yeast with temperature-sensitive pol δ mutants suggested that pol δ might be involved in DSBR (HOLMES and HABER 1999). The repair of DSBs can be accomplished through a homologous recombination pathway termed break-induced replication, involving DNA synthesis initiated by the free end of the chromosomal fragment (KRAUS *et al.* 1999). The

recipient chromosome has both strands newly synthesized, with the generation of structures like a replication fork, requiring coordinated leading and lagging strand synthesis which in turn leads to indications for a requirement of a replicative pol.

Pol δ could be argued as the most important polymerase; however, studies about pol δ in the context of a multicellular organism are far and few in between. There have been some notable studies however. One study involving mice with a frameshift mutation in the second conserved exonuclease domain showed that 50% of mice homozygous for this mutation developed cancer within 2 months of age showing that the proofreading ability of pol δ is necessary to prevent increased spontaneous mutations or increased cancer susceptibility (GOLDSBY *et al.* 2001; GOLDSBY *et al.* 2002). Another study involving mice with a framesift mutation in the sixth conservered polymerase region showed a phenotype of homozygous lethality. In addition, the heterozygous mice showed a reduced life span, increases in genomic instability, and accelerated tumorgenesis (VENKATESAN *et al.* 2007). Studies in the human model are even rarer to find. Of those done, one showed that pol δ is severely mutated in human colon cancer cells (FLOHR *et al.* 1999) giving support to the mutator hypothesis, which states that normal human cells increase their rate of genetic change as a mechanism for speeding up the transformation to cancer cells (LOEB 2001).

To investigate pol δ, the obvious multicellular organism of choice is *Drosophila. melanogaster*, or the common fruit fly. From the early work of Charles W. Woodsworth and Thomas Hunt Morgan, the fruit fly was molded into a model genetic organism. Early experiments by Morgan in 1900s in a crowded fly room helped to define the basis of modern genetics and also helped to push *Drosophila* as a genetic tool. Since 2000, the *Drosophila* genome has been fully sequenced and the database, http://www.flybase.org, is constantly

expanding with more and more detailed information not only with sequence information but also developmental and expression data. Around 50% of *Drosophila* protein sequences have a mammalian homolog while over 75% of genes related to human disease have a match in the *Drosophila* genome. *Drosophila* are ideal for a lab setting with a short generation time (around 10-14 days), very small cost to maintain large cultures and high fecundity of females. In addition, setting up genetic crosses are very easy since virgin females are easily isolatable, males do not undergo meiotic recombination and there are "balancer chromosomes" to keep lethal stocks. Various tissues undergo different cell cycles (which will be discussed later) which in turn makes it ideal to analyze the function of replication in an *in vivo* setting.

To say that pol δ is an essential polymerase would be an understatement. Ranging from its direct role in DNA synthesis of both the leading and lagging strands to its involvement in DNA repair pathways, pol δ has multiple functions that vital for every cell to survive. Interestingly enough, the polymerase has been poorly studied in the context of a multicellular organism. In this thesis using the model genetic organism, *D. melanogaster*, I investigate two novel mutants. These mutants, disrupting two different conserved regions, show different phenotypes and will help elucidate more information about this enigmatic enzyme and its role in DNA synthesis and genome stability. By understanding how pol δ functions in an *in vivo* setting, we can apply this knowledge to the mechanics of cancer biology in humans, another multicelluar organism, and inform new therapies to treat it.

2. MATERIALS AND METHODS

DNA PURIFICATION:

Plasmid DNA was isolated using Wizard® Plus SV Minipreps DNA Purification System by Promega (Cat. #A1460) per manufacturer instructions.

E. COLI TRANSFORMATION

DNA was transformed into α-Select Chemically Competent Cells from Bioline (Cat. # BIO85027) per manufacture instructions.

EdU INCORPORATION ASSAYS:

S-phase detection in *Drosophila* neural tissue was ascertained using the Click-It® reaction kit from Invitrogen (Cat. # C10337). 3rd instar wandering larva were harvested in age and density matched bottles. In fresh HyQ® Grace's Unsupplemented Insect Cell Culture Medium from HyClone (Cat. # SH30610.01), the larvae were dissected, pulling the larvae apart from holding onto the mouth hooks located on the anterior portion of the larvae and pulling from the posterior end. An equal volume of 200 mM EdU solution in Grace's was added to the well and brains from each strain were incubated for 30 minutes in the dark at room temperature. Following the incubation, the liquid was removed from each well and the brains were rinsed two times with 3% BSA. The brains were transferred to hypotonic solution (0.5% Sodium Citrate in 1X PBS) and incubated for 10 minutes. Brains were then fixed in an Acetic acid : Methanol : Water 11:11:2 solution for 30 seconds. Brains were then transferred to cleaned microscope slide, mounted in the fixative and overlaid with a siliconized coverslip. The microscope slide and coverslip were sandwiched between filter paper and an additional microscope slide. This was then placed in a machinist vise and pressure was applied using a torque wrench to 15 Nm. Following a 2 minute incubation at this pressure, slide and coverslip were removed and lowered into liquid nitrogen for

one minute. Once equilibrated, slide and coverslip were removed and the coverslip was popped off via a razor blade. Brains, now adhered to the slide, were then permeabilized using 0.1% Triton-X in 1X PBS for 20 minutes at room temperature in the dark. The liquid was removed and the brains were rinsed two times with 3% BSA. Brains were then incubated in the Click-It® reaction cocktail as per the manufacturer's instructions for 30 minutes. The brains were rinsed two times with 3% BSA. After removing the 3% BSA, Hoescht 33342 was prepared as per the manufacturer's instructions for nuclear visualization for 20 minutes. The Hoescht solution was then removed and the brains were washed twice with 1X PBS. Brains were then mounted on Vaseline lined Poly-lysine coated slides with Vectashield^{tm} and imaged.

FIGURE DESIGN:

All figures were designed using either Adobe Illustrator CS4^{tm} or Adobe Photoshop CS4^{tm}**.**

FLY HUSBANDRY/ STOCKS:

All fly stocks were maintained on *Drosophila* K12 media (US Biological # D9600-07B) at room temperature.

Wild Type: Wild Type flies were obtained from Bloomington *Drosophila* Stock Center - w^{1118} (Stock 6326, FlyBase ID FBst0006326).

Deficiency: 3 deficiency lines were obtained from Bloomington *Drosophila* Stock Center. The lines were as follows : Df(3L)brm11/TM6C,cu,Sb,ca (Stock 3640, FlyBase ID FBst0003640); Df(3L)th102mh,kni,e/TM6C,cu,Sb,ca (Stock 3641, FlyBase ID FBst0003641) and w^{1118}; Df(3L)BSC443/TM6C,Sb,cu (Stock 24947, FlyBase ID FBst0024947).

DNA Polymerase Alpha: DNA Polymerase Alpha flies were obtained from Bloomington *Drosophila* Stock Center - w^{1118}; PBac{WH}DNApol-α50f02992/TM6B, Tb (Stock 18605, FlyBase ID FBst0018605).

DNA Polymerase Delta: Fly Stocks (DNAPolDelt^{C496Y}) and (DNAPolDelt^{G694N}) were kindly provided by Dr. Bonnie Bolkan. They were both balanced over TM6B, Tb, Hu. DNAPolDelt^{G694N} is also an existing stock available from Bloomington *Drosophila* Stock Center (with different balancer and markers however) - w^{1118}; l(3)72Ac^{I10} P{white-un4}71F/TM3, Sb (Stock 4110, FlyBase ID FBst0004110).

GFP: For GFP visualization, a GFP line from Bloomington *Drosophila* Stock Center was obtained. Its geneotype is w^{1118}; Df(3L)Ly,sens/TM6B,P{w[=mW.hs]=Ubi-GFP.S65T}PAD2, Tb (Stock 4887, FlyBase ID FBst0004887).

PCNA: PCNA flies were obtained from Bloomington *Drosophila* Stock Center – cn, P{PZ}mus209^{02448}/CyO; ry^{506} (Stock 11192, FlyBase ID FBst0011192)

Transgenic: DNA Polymerase Delta transgene-containing flies were generated through germline transformation (BestGene Inc.). The transgenic construct was based on the Murphy vector pTWH where genomic DNA Polymerase Delta was cloned into the Gateway system (Invitrogen, Carlsbad, CA). 4 transgenic fly lines containing a nonlethal insertion into the second chromosome were identified.

Other Lines: An additional line used for crosses was w*; T(2;3)ap^{Xa}, ap^{Xa}/CyO; TM3, Sb (Stock 02475, FlyBase ID FBst0002475) which was also obtained from Bloomington *Drosophila* Stock Center.

GATEWAY® CLONING:

Gateway Cloning was achived by using *E. coli* Expression System with Gateway® Technology Kit by Invitrogen (Cat. # 11824-026) per manufacturer instructions.

GEL ELECTROPHORESIS:

DNA products were run on a 0.5% agrose in 1X TAE gel at ~130 volts until bands seperated

approximate distances based on colored marker. A 1kb Plus ladder was used for size determination. Gels were imaged using BioRad ChemiDoc™ XRS machine.

GENOMIC DNA PREPARATION:

30 flies (unless otherwise noted) were anesthetized in a 1.5 ml mircocentrifuge tube and placed on ice. The flies were grinded in 200 µl of Buffer A (100 mM Tris-Cl pH 7.5, 100 mM EDTA, 100 mM NaCl, 0.5% SDS) with a disposable tissue grinder. Another 200 µl of Buffer A was added and the flies were grinded until only cuticles remained. The mixture was incubated at 65˚C for 30 minutes. After the incubation, 800 µl of Buffer B (200 ml of 5 M potassium acetate, 500 ml of 6 M lithium chloride) was added and mixed well by inverting followed by incubation on ice for 3 hours. The mixture was then centrifuged at 12,000 rpm for 15 minutes. 1 ml of supernatant was transferred to a new microcentrifuge tube and 600 µl of isopropanol was added and mixed well by inversion. The mixture was once again centrifuged at 12,000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with 70% EtOH, air-dryed and resuspended in 50 µl of nuclease free H_2O.

LARVAL BRAIN SQUASHES:

3rd instar wandering larva were harvested in age and density matched bottles. The larvae were dissected, pulling the larvae apart from holding onto the mouth hooks located on the anterior portion of the larvae and pulling from the posterior end in 1X PBS pH 7.2 with 1% PEG 8000. Removed larval brains were transferred to hypotonic solution (0.5% Sodium Citrate in 1X PBS) and incubated for 10 minutes. Brains were then fixed in an Acetic acid : Methanol : Water 11:11:2 solution for 30 seconds. Brains were then transferred to cleaned microscope slide, mounted in and overlaid with a siliconized coverslip. The microscope slide and coverslip were sandwiched between filter paper and an additional microscope slide. This was then placed in a

machinist vise and pressure was applied using a torque wrench to 15 Nm. Following a 2 minute incubation at this pressure, slide and coverslip were removed and lowered into liquid nitrogen for one minute. Once equilibrated, slide and coverslip were removed and the coverslip was popped off via a razor blade and the slide was washed gently with 100% EtOH, allowed to air dry and mounted with 7 µl of Vectashield^{tm} with DAPI. The slides were then imaged.

MICROSCOPY:

Microscopy was performed using an Olympus IX81 Motorized Inverted Microscope with Spinning Disk Confocal. Images were analyzed using Slidebook™ software.

MITOTIC INDICES:

Mitotic index determination were performed on larval brain squashes preparations by selecting 10 random well populated fields of view for each brain squash using a 60X objective. Total mitotic figures were counted for each field and this was divided by the total number of cell observed in each field to generate the fraction of cells in mitosis. Statistical analysis was performed using Minitab^{tm}.

PCR (POLYMERASE CHAIN REACTION):

Platinum® *Pfx* DNA Polymerase from Invitrogen (Cat. # C11708) was used for all PCRs. Manufacture instructions were followed including component mixture (**TABLE 2.01**) and three-step cycling programming (**TABLE 2.02**).

Component	Volume	Final Concentration
10X *Pfx* Amplification Buffer	10 µl	2X
10 mM dNTP mixture	1.5 µl	0.3 mM each
50 mM $MgSO_4$	1 µl	1 mM
Forward Primer (10 µM)	1 µl	10 µM
Reverse Primer (10 µM)	1 µl	10 µM
Template DNA (10 pg-200 ng)	≥ 1 µl	As required
Platinum® *Pfx* DNA Polymerase	0.4 µl	
Nuclease Free H_2O	to 50 µl	

TABLE 2.01 – PCR COMPONENTS

		Temp.	Time
1.	Denature	94˚C	2 mins.
2.	Denature	94˚C	15 secs.
3.	Anneal	55˚C	30 secs.
4.	Extend	68˚C	1 min. per kb
5.	Repeat steps 2-4 for 23-35 X		
6.	Hold	4˚C	∞

TABLE 2.02 – THERMOCYCLER PROGRAM FOR PCR

PCR PURIFICATION:

PCR products were purified using MinElute® PCR Purification Kit by Qiagen (Cat. # 28006) per manufacturer instructions.

POLYTENE CHROMOSOMES (SPREAD):

3rd instar wandering larva were harvested in age and density matched bottles. The larvae were dissected, pulling the larvae apart from holding onto the mouth hooks located on the anterior portion of the larvae and pulling from the posterior end in 1X PBS pH 7.2 with 1% PEG 8000. The salivary glands were then transferred to a solution of 50% acetic acid, 2-3% lactic acid, 3.7% formaldehyde by the use of a shortened 20 µl pipette tip and fixed for 2 min. Glands were then transferred to the center of clean microscope slide and overlaid with a siliconized coverslip. The slide was covered in a plastic sheet and the polytene chromosomes were spread using a spiral tapping method with dull pencil tip. Spreading was monitored using phase-contrast microscopy. Once spread, the microscope slide and coverslip were sandwiched between filter

paper and an additional microscope slide. This was then placed in a machinist vise and pressure was applied using a torque wrench to 15 Nm. Following a 2 minute incubation at this pressure, slide and coverslip were removed and lowered into liquid nitrogen for one minute. Once equilibrated, slide and coverslip were removed and the coverslip was popped off via a razor blade and the slide was washed gently with 100% EtOH, allowed to air dry and mounted with 7 µl of Vectashield^{tm} with DAPI. The slides were then imaged.

POLYTENE CHROMOSOMES (WHOLE):

3rd instar wandering larva were harvested in age and density matched bottles. The larvae were dissected, pulling the larvae apart from holding onto the mouth hooks located on the anterior portion of the larvae and pulling from the posterior end in 1X PBS pH 7.2 with 1% PEG 8000. The salivary glands were then transferred to a solution of 4% Formaldehyde in PBX (1X PBS + 0.1% Triton X-100) for 20 minutes. After fixing, the salivary glands were stained for 5 minutes with 1ug/mL DAPI in in 1X PBS. The glands were then washed 3X for 5 minutes in PBX, followed by a 1 hour PBX wash, and 3X 10 minute PBX washes. Finally, salivary glands were mounted using Vectashield^{tm} and a Vaseline lined slide. The slides were then imaged.

PRIMER DESIGN:

Primers were designed using DNAStar Lasergene PrimerSelect^{tm}.

PROTEIN STRUCTURE:

Cn3D 4.3 from NCBI was used to design 3-dimensional structures and edited using Adobe Illustrator CS4^{tm}**.**

SALIVARY GLAND (WHOLE) GENOMIC PREPS:

3rd instar wandering larva were harvested in age and density matched bottles. The larvae were dissected, pulling the larvae apart from holding onto the mouth hooks located on the anterior

portion of the larvae and pulling from the posterior end in 1X PBS pH 7.2 with 1% PEG 8000. The salivary glands were then transferred to a solution of 25 µl of squishing buffer composed of 10 mM Tris pH 8.2, 25 mM NaCl, 1 mM EDTA and 200 µg/ml Proteinase K in a 200 µl PCR tube. The glands were then incubated in a thermocycler at 37˚C for 30 minutes followed by 85˚C for 10 minutes. The tubes were then spun down for 1 minute at maximum speed. The DNA content was analyzed using a Thermo Scientfic NanoDrop 2000c machine.

S-PHASE INDICES:

S-Phase index determination was performed on the EdU incorporated brain squash preparations by selecting 10 random well populated fields of view for each brain squash using a 100X objective. Total number of EdU incorporated nuclei (GFP filter) were counted for each field and this was divided by the total nuclei (DAPI filter) observed in each field to generate the fraction of cells in S-Phase. Statistical analysis (two-sample t-tests) was performed using Minitab^{tm}.

SEQUENCE ANALYSIS:

Pol δ sequences from *D. melanogaster* were obtained from http://www.flybase.org. Pol δ sequences from *H. sapiens, M. Musculus, P. falciparum, S. cervisiae, S. pombe, and X. tropicalis* were obtained from http://www.ncbi.nlm.nih.gov/. Sequences were analyzed using DNAStar Lasergene EditSeq Pro^{tm} and DNAStar Lasergene MegAlign Pro$^{tm.}$ Additional sequences from genomic preps were also analyzed using EditSeq Pro^{tm} and DNAStar Lasergene SeqMan Pro^{tm}

TOPO® CLONING:

TOPO® Cloning was achieved using pENTR Directional TOPO® Cloning by Invitrogen (Cat. # K2400-20) per manufacture instructions.

VIABILITY ASSAYS:

DNAPolDelt^{C496Y} / TM6B, Tb, GFP and DNAPolDelt^{G694N} / TM6B, Tb, GFP were both

separately crossed to wild type flies (+/ +). 15 males (DNAPolDelt^{mut}) were placed in the same vial as 15 virgin females (WT). At the first sign of 3rd instar larvae, the adults were removed and the F1 progeny were separated based on the GFP marker and placed in separate vials and monitored for pupation and eclosion. Flies were counted for 10 days after the first eclosion. In addition, to measure the viability of the balancer chromosome, +/ TM6B, Tb, GFP were also crossed to WT flies.

VIABILITY ASSAYS (WITH MUTAGENS):

Viability assays with mutagens were carried out the same way as the viability assays except with the addition of either 6.4 mM HU, 0.1% MMS, or 10mM paraquat.

3. RESULTS

ALIGNMENT OF POL δ

Using amino acid sequences from several model organisms (*D. melanogaster*, *H. sapiens*, *M. musculus*, *X. tropicalis*, *S. cerevisiae*, *S. pombe* and *P. falciparum*), the protein sequences were aligned using ClustalW method. The results showed that pol δ is highly conserved among different species (**TABLE 3.01**). For example, *Drosophila* and *H. sapiens* are 60.2% similar.

Genetic Interaction of pol α and pol δ

Pol δ relies heavily on pol α for the initiation and priming of DNA replication (GARG and BURGERS 2005). To check for unlinked non-complementation, we crossed an existing uncharacterized allele of pol α (DNAPolAlpha$^{[F02992]}$). Much to our surprise, flies harboring both of these mutations were viable. Further characterization of these double mutants is still needed.

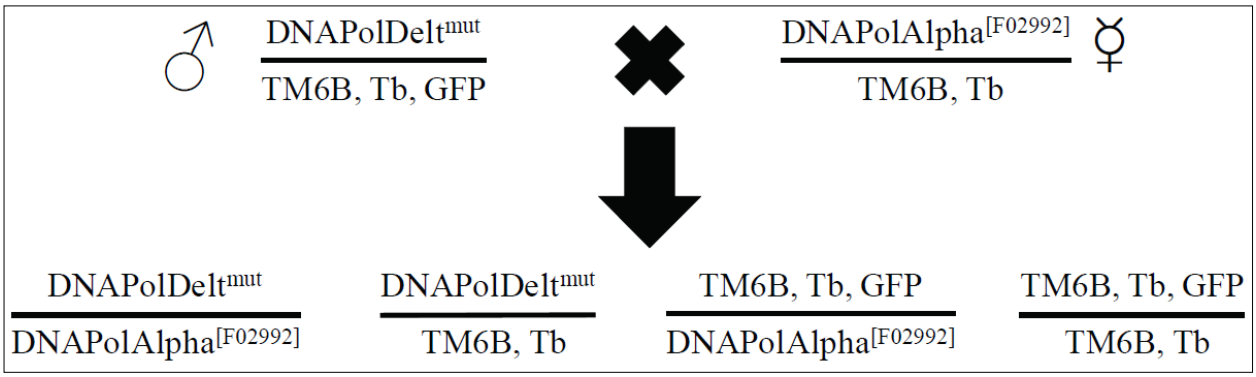

FIGURE 3.25 - POL α CROSS SCHEME – Cross scheme used to generate flies with pol $\alpha^{mut.}$ and pol $\delta^{mut.}$. All alleles are on the third chromosome.

Genetic Interaction of pol δ and PCNA

PCNA has been shown in to be the replication clamp required for DNA replication and for various other DNA repair processes, most importantly being the processivity factor pol δ (MOLDOVAN *et al.* 2007). To check for possible unlinked non- complementation, we crossed an existing allele of PCNA (P[PCNA]^{02448}) to both of the pol δ^{muts}. Some phenotypes of the P[PCNA]^{02448} include reduced BrdU incorporation in third larval instar brains in homozygotes compared to heterozygotes. Additionally, there is a 3.3 fold increase in the percentage of mitotic cells in the mutant larval brain compared to wild type. 91% of mitotic figures appear arrested in a metaphase-like state in which a highly condensed chromosome mass is present (PFLUMM and BOTCHAN 2001; JACKSON *et al.* 2005). These phentypes led us to the hypothesis that flies harboring both of these mutations would more than likely be lethal; however, progeny with both of these mutations came out in a relative Mendenlian ratio. More investigation is needed into the possible phenotypes that arise from these two mutations in unison.

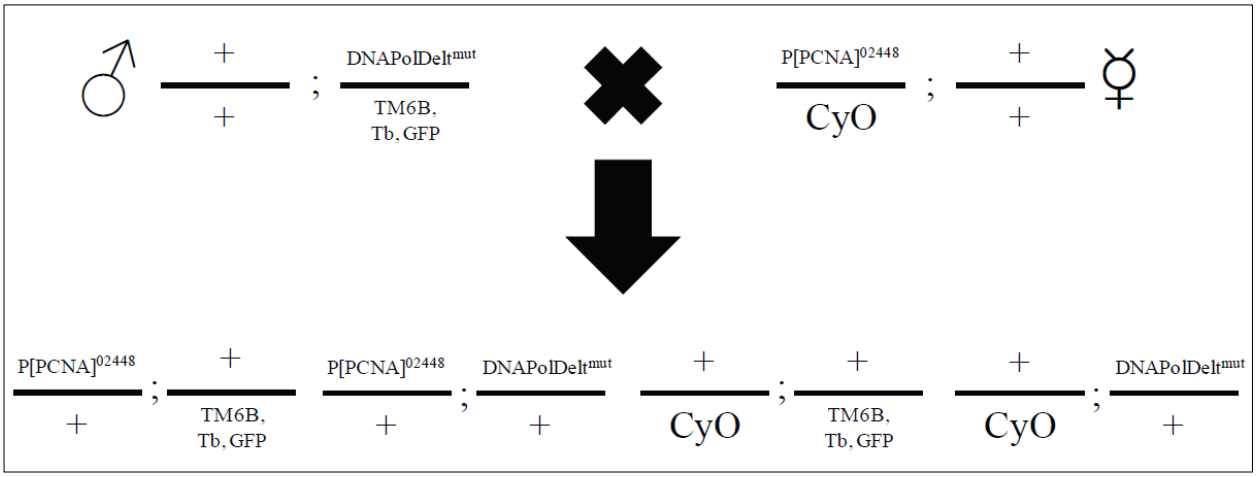

FIGURE 3.26 - PCNA CROSS SCHEME – Cross scheme used to generate flies with PCNA$^{mut.}$ and pol δ$^{mut.}$. Alleles are on the second and third chromosomes.

	D. melanogaster	*H. sapiens*	*M. musculus*	*X. tropicalis*	*S. cerevisiae*	*S. pombe*	*P. falciparum*	
D. melanogaster		60.2	59.8	63.6	50	54.2	44.7	*D. melanogaster*
H. sapiens	56.2		89.4	71.4	47.2	53.3	41.9	*H. sapiens*
M. musculus	56.9	11.4		71.4	47.4	52.8	42.2	*M. musculus*
X. tropicalis	49.6	35.9	35.9		83.7	65.8	96.7	*X. tropicalis*
S. cerevisiae	80	87.7	87.1	48.6		53.1	103.2	*S. cerevisiae*
S. pombe	69.3	71.6	72.7	55.7	72.1		100.7	*S. pombe*
P. falciparum	95.3	104.6	103.7	44.2	42.3	43		*P. falciparum*
	D. melanogaster	*H. sapiens*	*M. musculus*	*X. tropicalis*	*S. cerevisiae*	*S. pombe*	*P. falciparum*	

TABLE 3.01 - ALIGNMENT OF POL δ – Table showing percent similarity (in upper triangle) and percent divergence (in lower triangle)..

DISCOVERY OF MUTANTS

Previous work by Dr. Bonnie Bolkan discovered a mutant that had mitotic defects and arrested as a 3rd instar homozygote. This mutant was labeled as MA41. Deficiency crosses were used to pinpoint the location of this mutant (**FIGURE 3.01**). The crosses narrowed the gene region to a 540 kDa region containing 19 genes (71F1-72D10). Of the 19 genes in this region, it was proposed that the most likely candidate for the resulting phenotypes was pol δ. An existing lethal, l(3l)72Ac ^{I10}, failed to complement the MA41 mutant along with several deletion lines. The lethal, l(3l)72Ac ^{I10}, had yet to be characterized.

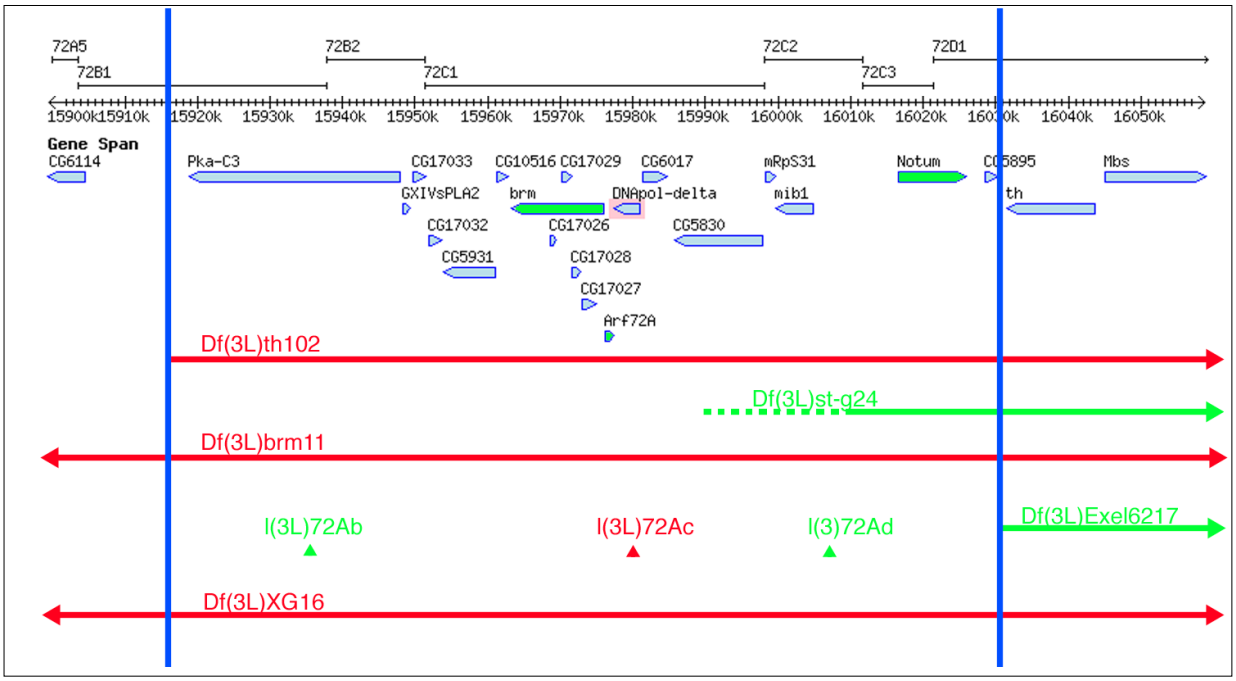

FIGURE 3.01 - MA41 MAP - A gene map of the cytological region 72A5 to 72D1 of chromosome 3, the region containing the pol δ gene. Deficiencies and genes in green represent deletions that complimented the MA41 mutant, while ones in red represent deletions that failed to compliment. The blue vertical lines delineate the region to where the mutation was narrowed down. Pol δ is highlighted in pink. (Modified from Flybase 2007, Courtesy of B. BOLKAN)

IDENTIFICATION OF MUTATIONS

Genomic preps were performed on MA41 and l(3l)72Ac^{I10}. Due to the most likely suspect of these phenotypes being pol δ, it was decided to analyze this region first. PCR amplification of the pol δ region was obtained using forward primer 5'-CAC CTT CGC TCC TAT CCA AA-3' and reverse primer 5'-CGA ACC GAA AGA AAC TTT GTA A-3' using standard procedures. The product was purified and was sequenced with step wise primers (**TABLE 3.02**) spanning around 500-600 base pairs to give an accurate reading of upstream, including and downstream of the pol δ gene region. The resulting sequences were analyzed with SeqMan^{tm}. MA41 had a point mutation of AAT to ACT at bp 1488-1490 resulting in a change of amino acid 496 from a Cysteine to a Tyrosine (**FIGURE 3.02**, **FIGURE 3.03**). Strain l(3l)72Ac^{I10} had a point mutation of GGC to GAC at bp 2082-2084 resulting in a change of amino acid 649 from a Glycine to an Asparagine (**FIGURE 3.02**, **FIGURE 3.04**). For clarification, from here forward, the MA41 mutant will be known as DNAPolDelt^{C496Y} or C496Y and the l(3l)72Ac^{I10} mutant will be known as DNAPolDelt^{G694N} or G694N.

Start of Primer in Relation to First bp (+1) of pol δ Sequence	Primer Sequence
-153	5'-TTT TGA AAT ATT TGG AGG ACT-3'
+116	5'-CTG ATG ACG ATG AGG AAA TGG-3
+597	5'-CAA TGG AGA CAA GAA GCA GAG GTA-3'
+1026	5'-GGT GAT AAG GCA GGG AGA ACG AGA-3'
+1452	5'-GCA GGA GCA AAA GGA GGA TGT G-3'
+1915	5'-CTG CAG GAC GAT CAA GTG GAA CG-3'
+2349	5'-CGA GGC TGC CGA ACT GGT CA-3'
+2793	5'-TGC GGC AGC CAA AAA CAC A-3'
+3210	5'-CTT GCA CGA GGA GGT CAT CTG-3'
+3594	5'-CCT TGG TGG CCG ACG TTT TGA ATA-3'

TABLE 3.02 - STEPWISE SEQUENCING PRIMERS – Primers used to sequence pol δ. For reference, +1 refers to the start of the sequence (ATG) and the sequence contains bp from +1 to +3279.

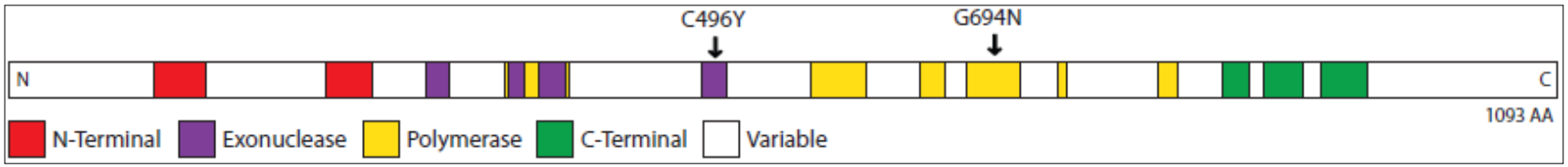

FIGURE 3.02 - *DROSOPHILA* DNA POL δ CONSERVED DOMAINS – Amino acid visualization of Drosophila DNA Polymerase Delta highlighting conserved domains and also location of the C496Y and G694 mutations.

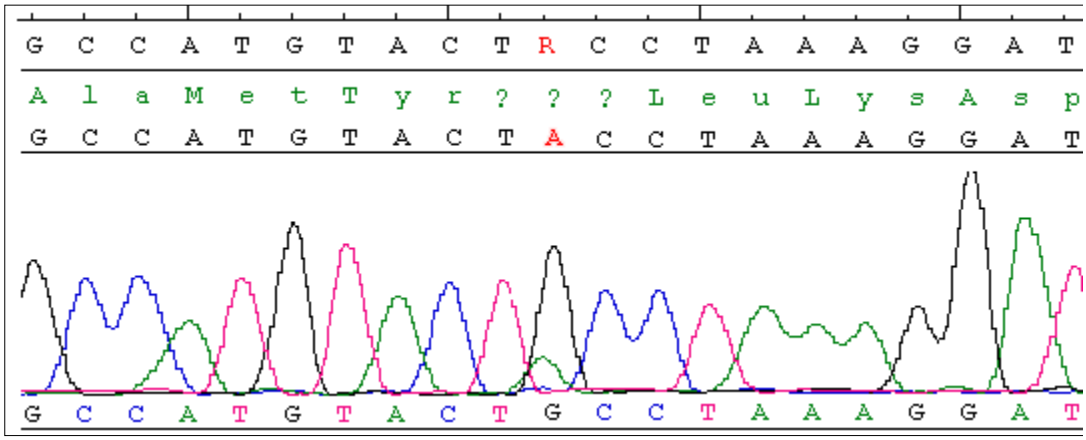

FIGURE 3.03 - C496Y SEQUENCE ANALYSIS – Sequences showing bases 1479 to 1499 or amino acids 493 t

C496Y flies.

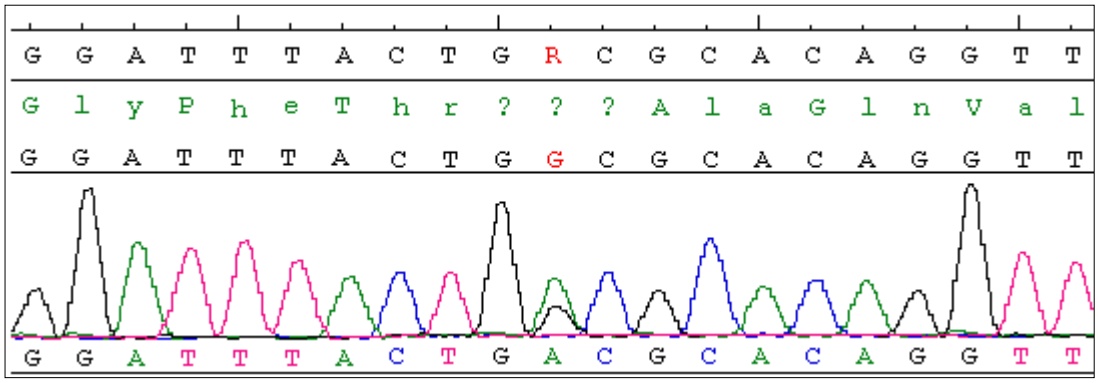

FIGURE 3.04 - G694N SEQUENCE ANALYSIS – Sequences showing bases 2073 to 2093 or amino acids 691 to 697 of G694N flies.

LOCATION OF MUTANTS

Interestingly, the two mutants C496Y and G694N are both in highly conserved residues. C496Y is in the Exonuclease III conserved region (**FIGURE 3.02**, **FIGURE 3.05**) and changes a residue that is conserved all the way to simple eukaryotes, *S. cervisiae*, *S. pombe* and *P. failciparum*. G694N also mapped to a highly conserved region, this time in the Polymerase III conserved region (**FIGURE 3.02**, **FIGURE 3.06**). This residue is also conserved to simple eukaryotes.

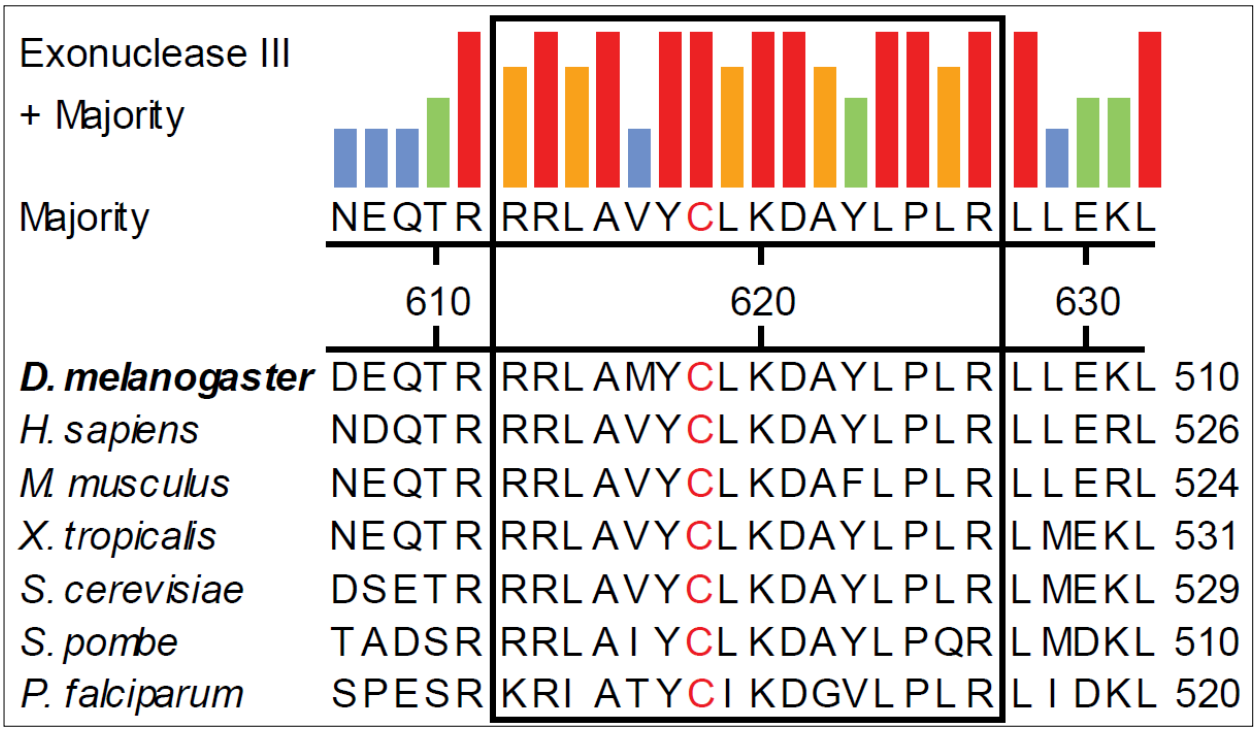

FIGURE 3.05 - EXONUCLEASE III ALIGNMENT – Alignment of conserved Exonuclease III region of several model organisms. Note that the *Drosophila* Cys496 residue is conserved through all species.

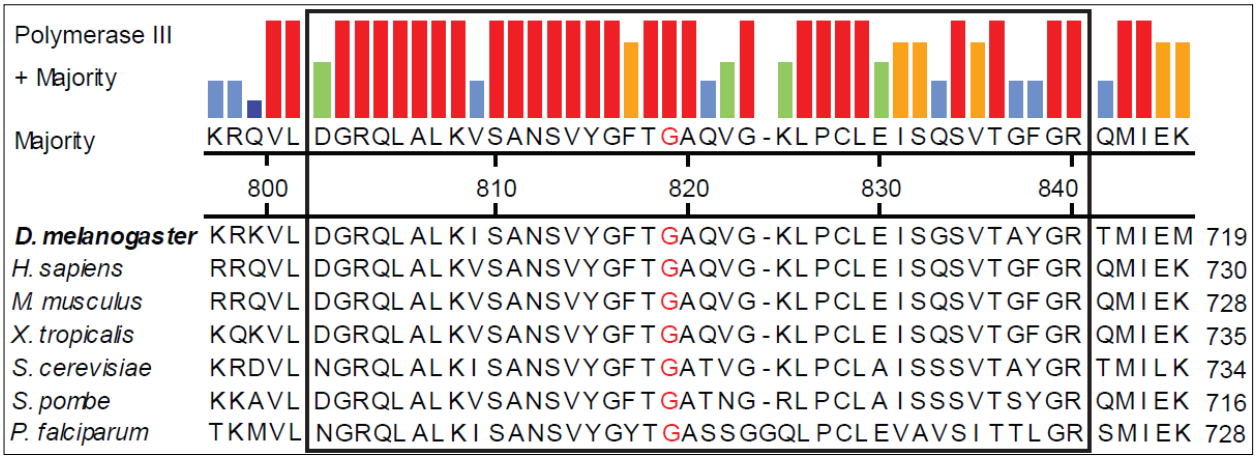

FIGURE 3.06 - POLYMERASE III ALIGNMENT – Alignment of conserved Exonuclease III region of several model organisms. Note that the *Drosophila* Gly694 residue is conserved through all species.

Protein Structure Analysis

Unfortunately, the crystal structure of *Drosophila* pol δ has not been resolved; however, it has been resolved in yeast (*S. cerevisiae*) (SWAN *et al.* 2009). Due to the very high conservation between *S. cerevisiae* and *Drosophila* pol δ, we can infer positions of the mutated residues against the *S. cerevisiae* structure. **FIGURE 3.07** shows the entire resolved crystal structure highlighting major components such as the exonuclease and polymerase active sites. **FIGURE 3.08** maps the mutated residues to against the conserved domains in yeast. The C496Y residue is in the exonuclease active site while the G694N residue is in the polymerase active site.

Additionally, looking at the amino acid structure for both mutations, it is clear that there are major changes.

In the C496Y flies, there is a cysteine to a tyrosine change (**FIGURE 3.09)**. Cysteine ($C_3H_7NO_2S$) contains a thiol as a side chain which is nonpolar and is also hydrophilic. Tyrosine ($C_9H_{11}NO_3$) contains a much larger side chain, a phenol. This leads to a polar amino acid but it also partially hydrophobic.

In the G694N flies, there is a glycine to an asparagine change (**FIGURE 3.10)**. Glycine ($C_2H_5NO_2$) contains simply two hydrogen atoms as its side chain, making it the smallest amino acid. It is also slightly polar and hydrophobic. Asparagine ($C_4H_8N_2O_3$) contains a carboxamide as its functional group. It is polar and hydrophilic.

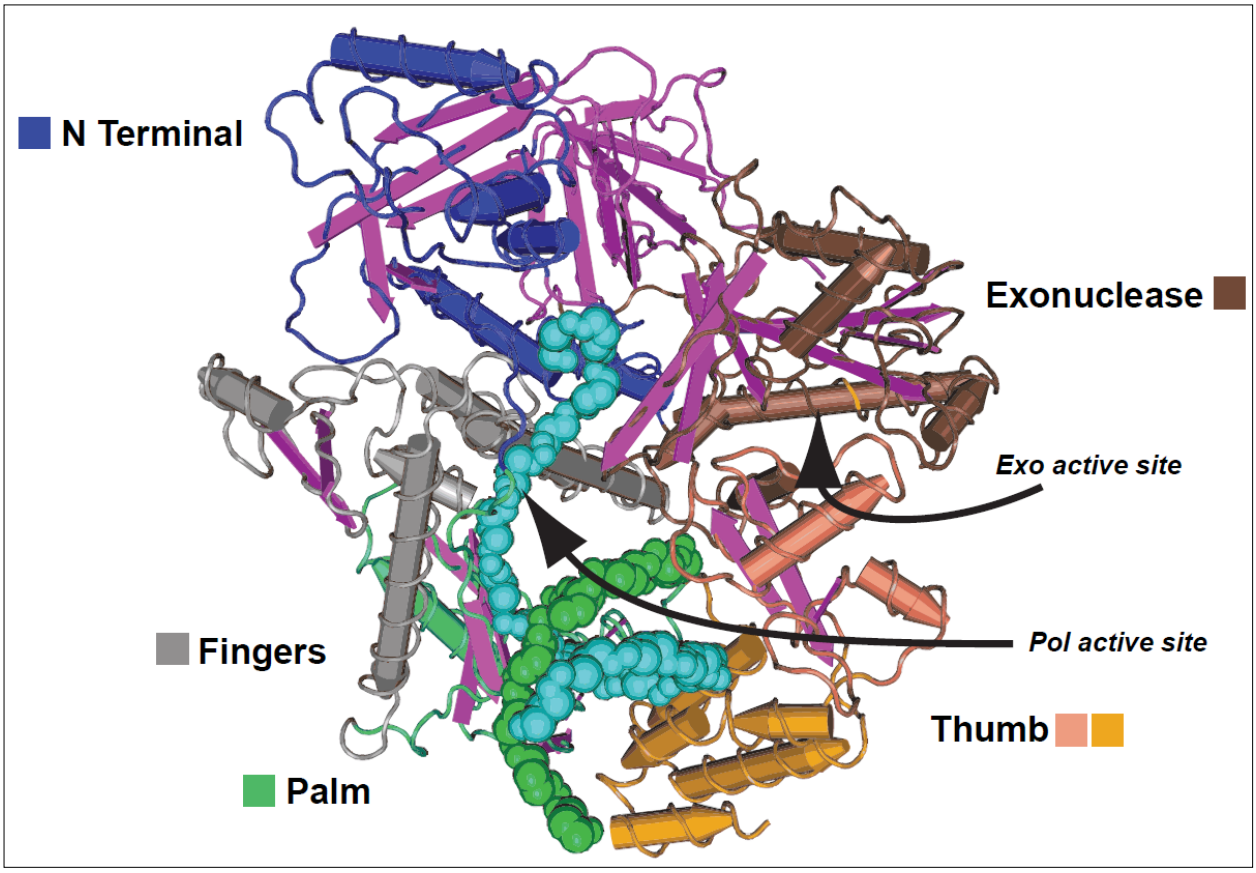

FIGURE 3.07 - CRYSTAL STRUCTURE OF YEAST POL δ – The C496Y and G694N mutations both lie in active site for their respective domains.

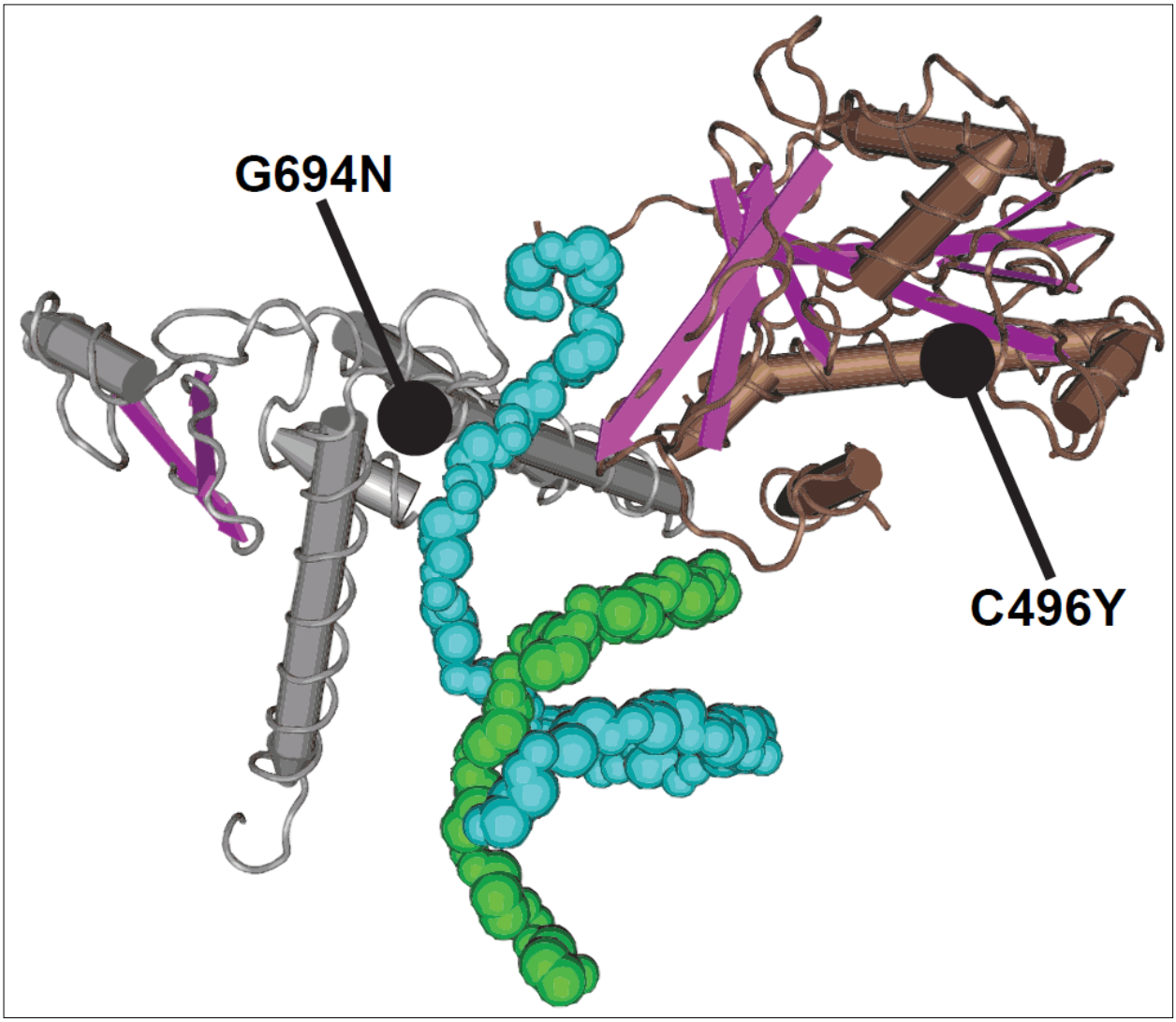

FIGURE 3.08 - POINT MUTANTS MAPPED AGAINST CRYSTAL STRUCTURE OF YEAST POL δ – The C496Y and G694N locations mapped against yeast pol δ.

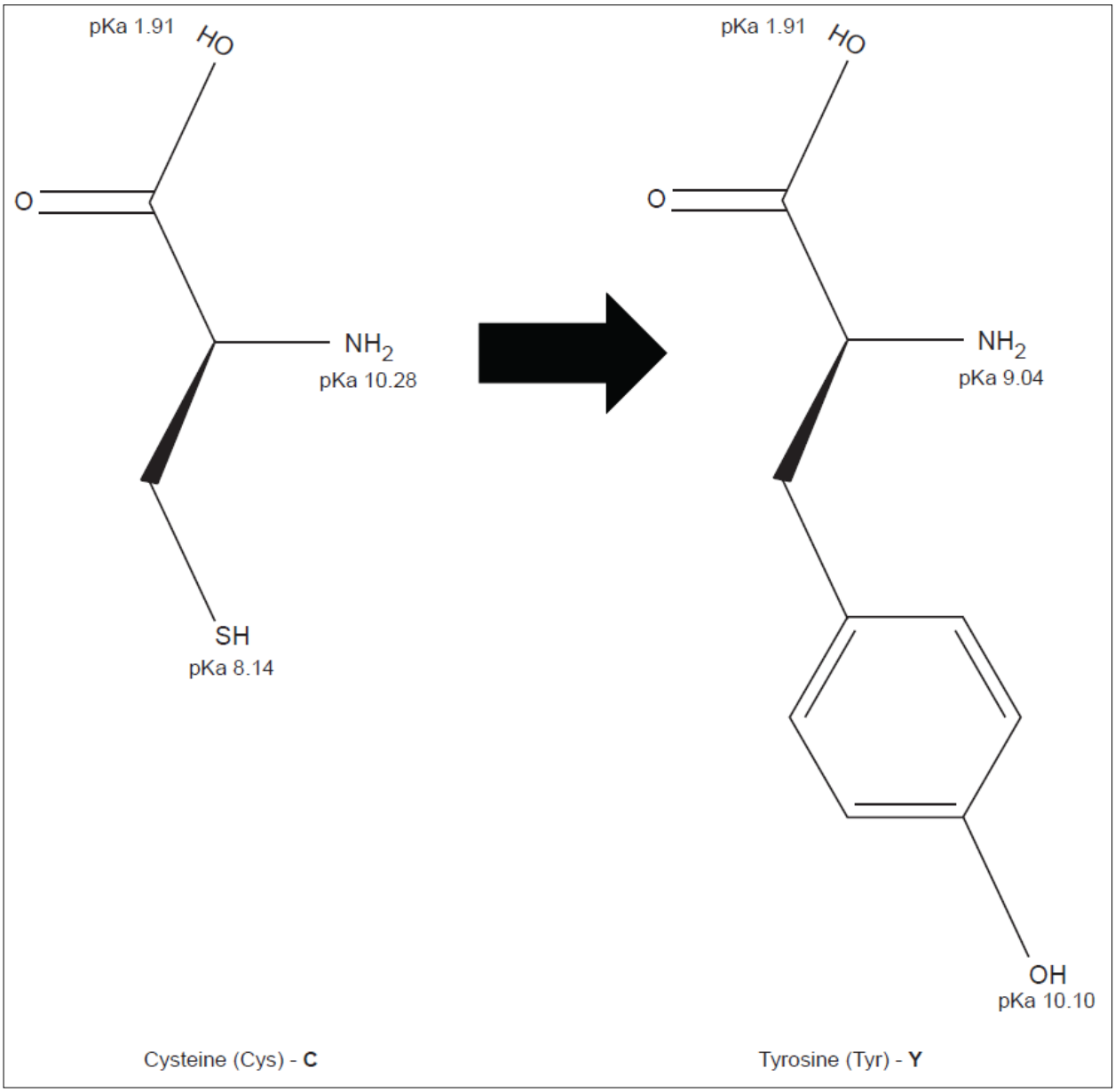

FIGURE 3.09 - C496Y AMINO ACID CHANGE – Amino acid structure change in the C496Y flies.

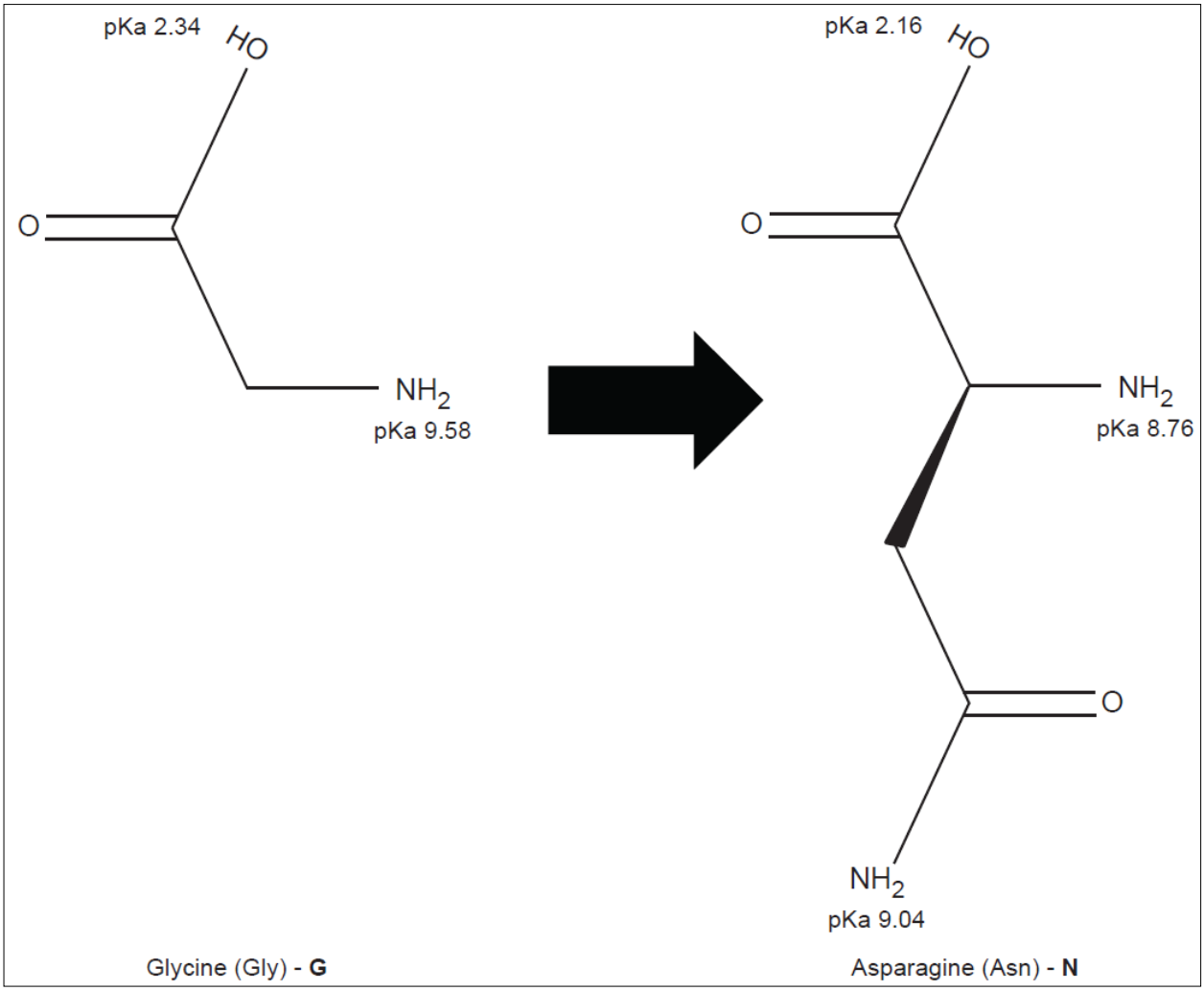

FIGURE 3.10 - G694N AMINO ACID CHANGE – Amino acid structure change in the G694N flies.

BALANCING OF MUTANTS

The two mutants were originally balanced over TM6B, Tb, a common balancer chromosome for the 3rd chromosome (ASHBURN *et al.* 2005). It should be noted that pol δ is located on the left arm of the 3rd chromosome (3L). The original fly lines were crossed to the same balancer fused with GFP (w^{1118}; df(3L)Ly, sens/TM6B, P{w[=mW.hs]=Ubi-GFP.S65T}PAD2, Tb) (**FIGURE 3.11**). This allowed for easier and more efficient visualization of homozygous mutants. It should be noted as well that being homozygous for the balancer chromosome (TM6B, Tb, GFP) is lethal leaving the only possible progeny either homozygous or heterozygous for pol δ^{mut}.

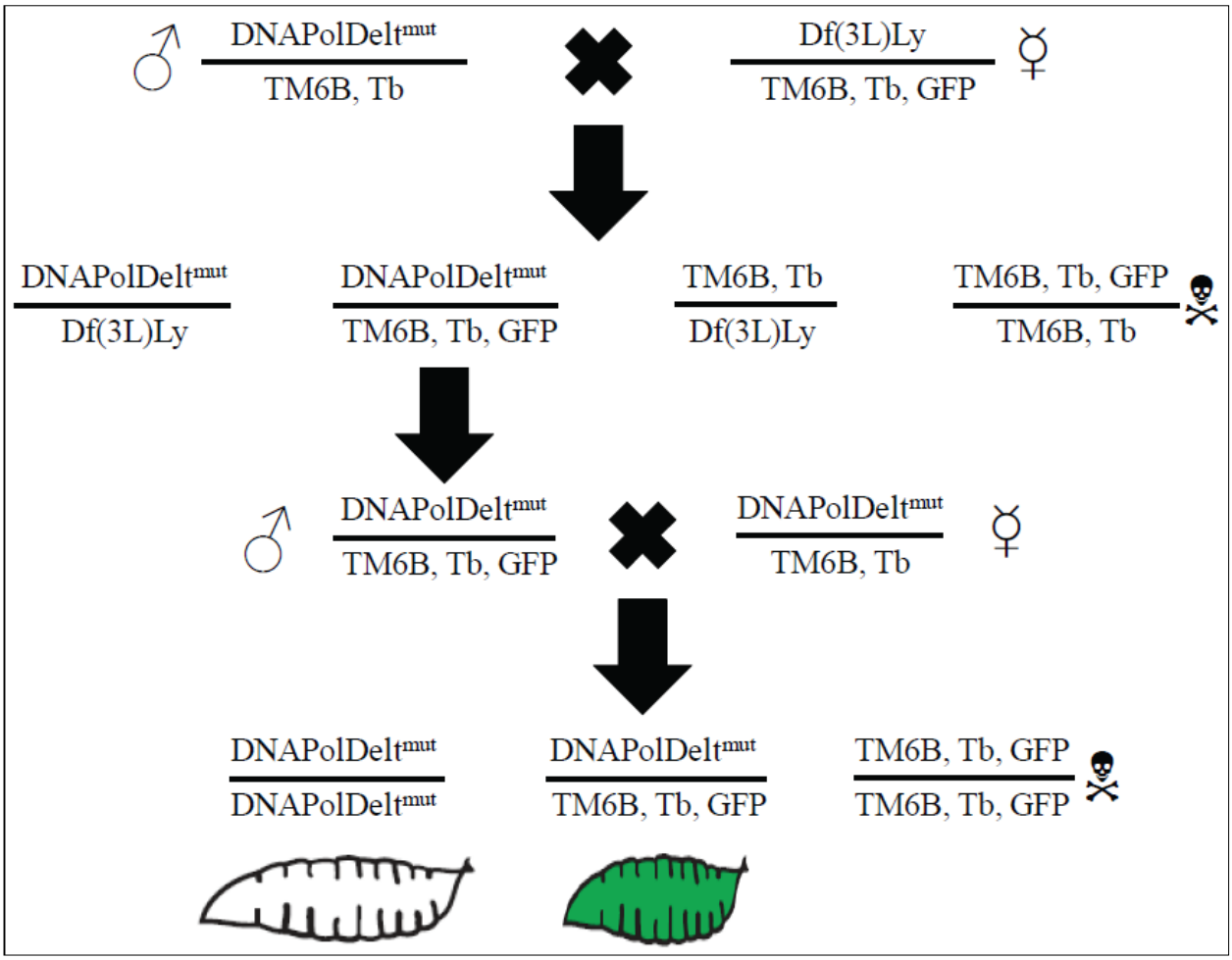

FIGURE 3.11 - BALANCING OF MUTANTS CROSS SCHEME – Cross scheme used to generate balanced mutants. All alleles are on the third chromosome.

COMPLEMENTATION CROSS

The two mutant strains were crossed to each other to see if there was complementation of the two alleles of pol δ (**FIGURE 3.12**). Heteroallelic larvae and flies (C496Y/G694N) were not observed.

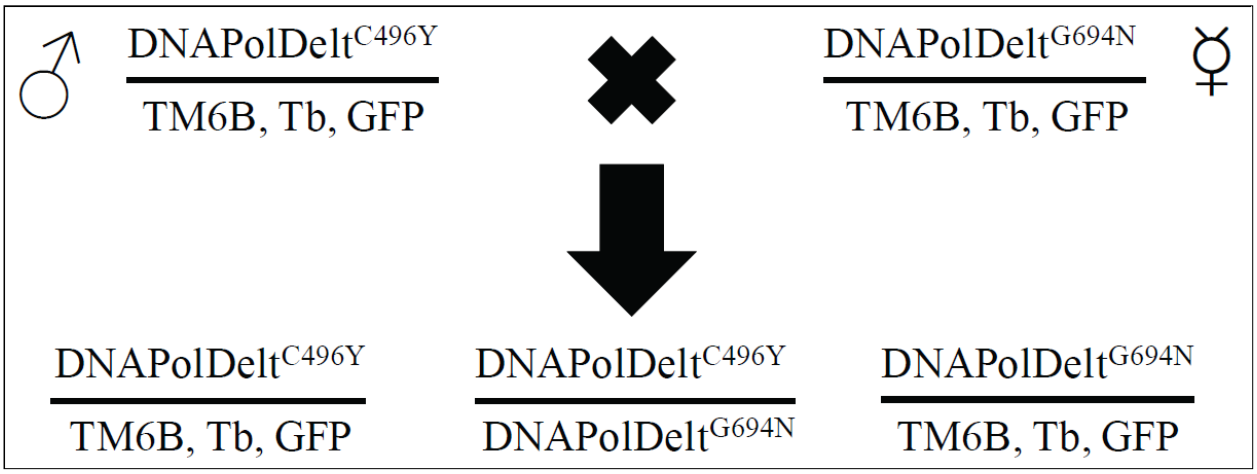

FIGURE 3.12 - COMPLEMENTATION CROSS SCHEME – Cross scheme used to generate heteroallelic mutants. All alleles are on the third chromosome.

DEFICIENCY LINE CROSSES

To verify the work of Dr. Bonnie Bolkan, the pol δ mutants were crossed to two of the same deficiency lines used in the original screen, df(3L)brm11 (**FIGURE 3.13**) and df(3L)th102 (**FIGURE 3.14**) and a new deficiency line previously untested, df(3l)BSC443 (**FIGURE 3.15**). Df(3L)brm11 includes a chromosomal deletion from cytogenetic map location 72A3 to 72D5. Df(3L)th102 includes a chromosomal deletion from cytogenetic map location 72A2 to 72D10. Df(3l)BSC443 includes a chromosomal deletion from cytogenetic map location 72B1 to 72E4. Pol δ is located in cytogenetic map location 72C1. All three deficiency lines failed to produce any progeny harboring both the pol δ mutation and deficiency (deletion of pol δ).

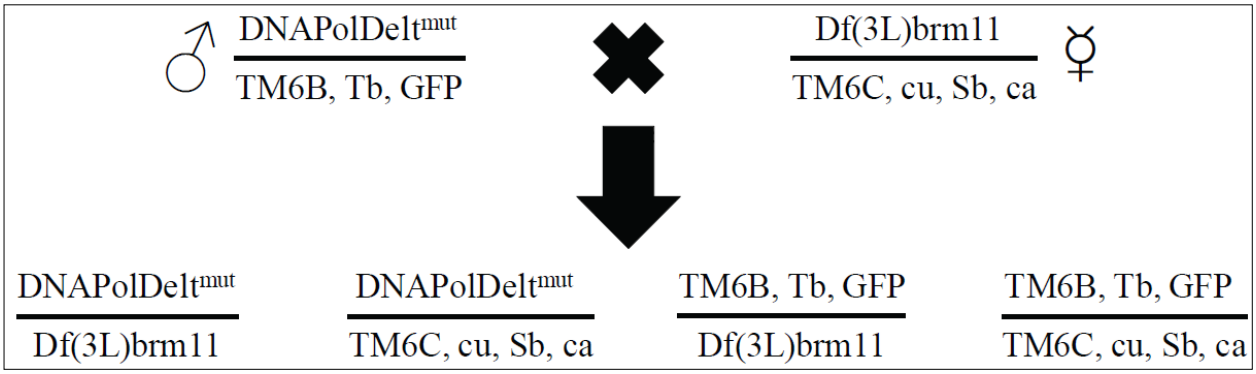

FIGURE 3.13 - Df(3L)brm11 CROSS SCHEME – Cross scheme used to generate flies with pol δ^{mut} and deficiency. All alleles are on the third chromosome.

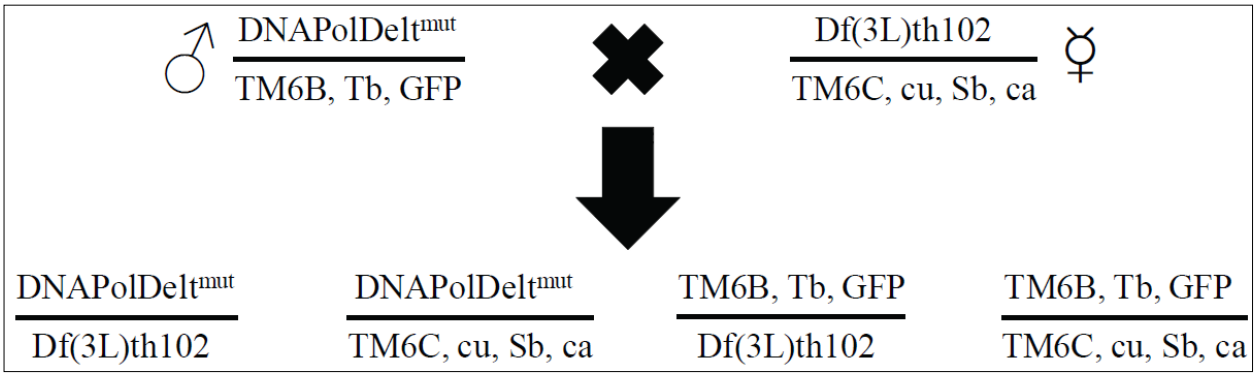

FIGURE 3.14 - Df(3L)th102 CROSS SCHEME – Cross scheme used to generate flies with pol δ^{mut} and deficiency. All alleles are on the third chromosome.

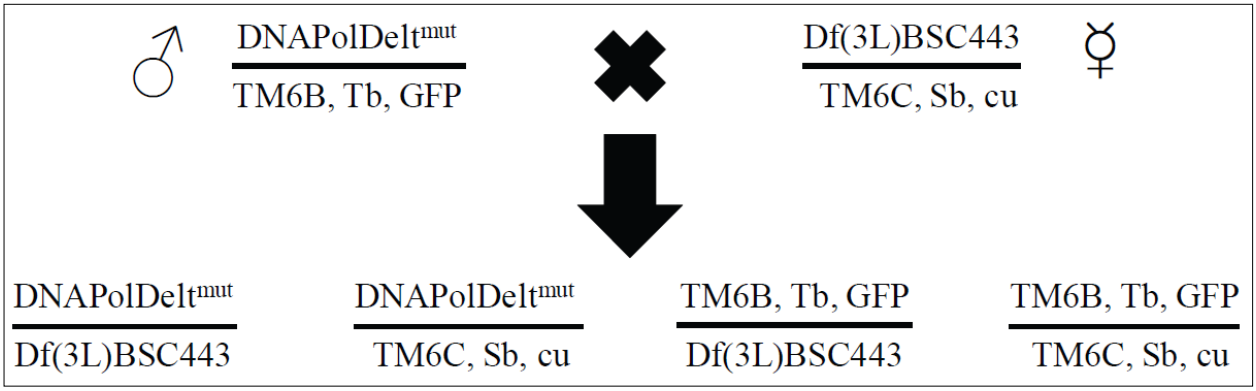

FIGURE 3.15 - Df(3L)BSC443 CROSS SCHEME – Cross scheme used to generate flies with pol δ^{mut} and deficiency. All alleles are on the third chromosome.

GENERATION OF TRANSGENIC FLY

Using genomic DNA from wild type flies, pol δ was amplified using forward primer 5'-CAC CTT CGC TCC TAT CCA AA-3' and reverse primer 5'-CGA ACC GAA AGA AAC TTT GTA A-3' using standard PCR procedures. The forward primer included the CACC necessary for cloning into the pENTR™ TOPO® vector. The product included 1900 bp before the start codon and also included 85 bp after the stop codon. This PCR product was verified by gel electrophoresis (data not included). The product was TOPO® cloned into the pENTR™ vector and transformed into competent cells. Minipreps from individual colonies grown up overnight in Terrific Broth media were performed and the DNA was eluted. The plasmid DNA (pENTR+WT pol δ) was added to a Gateway® LR recombination reaction including the destination vector, pTWH. pTWH is a vector developed as part of the Drosophila Gateway[a] Vector Collection by the Murphy lab group at the Carnegie Institution of Washington. The LR product was then transformed again using competent cells and the DNA was again eluted and verified by sequencing. This product was sent to BestGene Inc. and *Drosophila* embryos were injected with the construct. Flies harboring the transgene on the second chromosome were delivered back. Flies harboring the transgenic pol δ (noted as p[Δ]) had to be crossed to produce a fly that had the genotype ap^{Xa}/p[Δ]; Sb (**FIGURE 3.16**). The balancer ap^{Xa} is a fusion of the 2nd and 3rd chromosomes (ASHBURN *et al.* 2005). This allowed for the easiest way to track the incorporation of the transgenic p[Δ] when introduced with pol δ^{mut}.

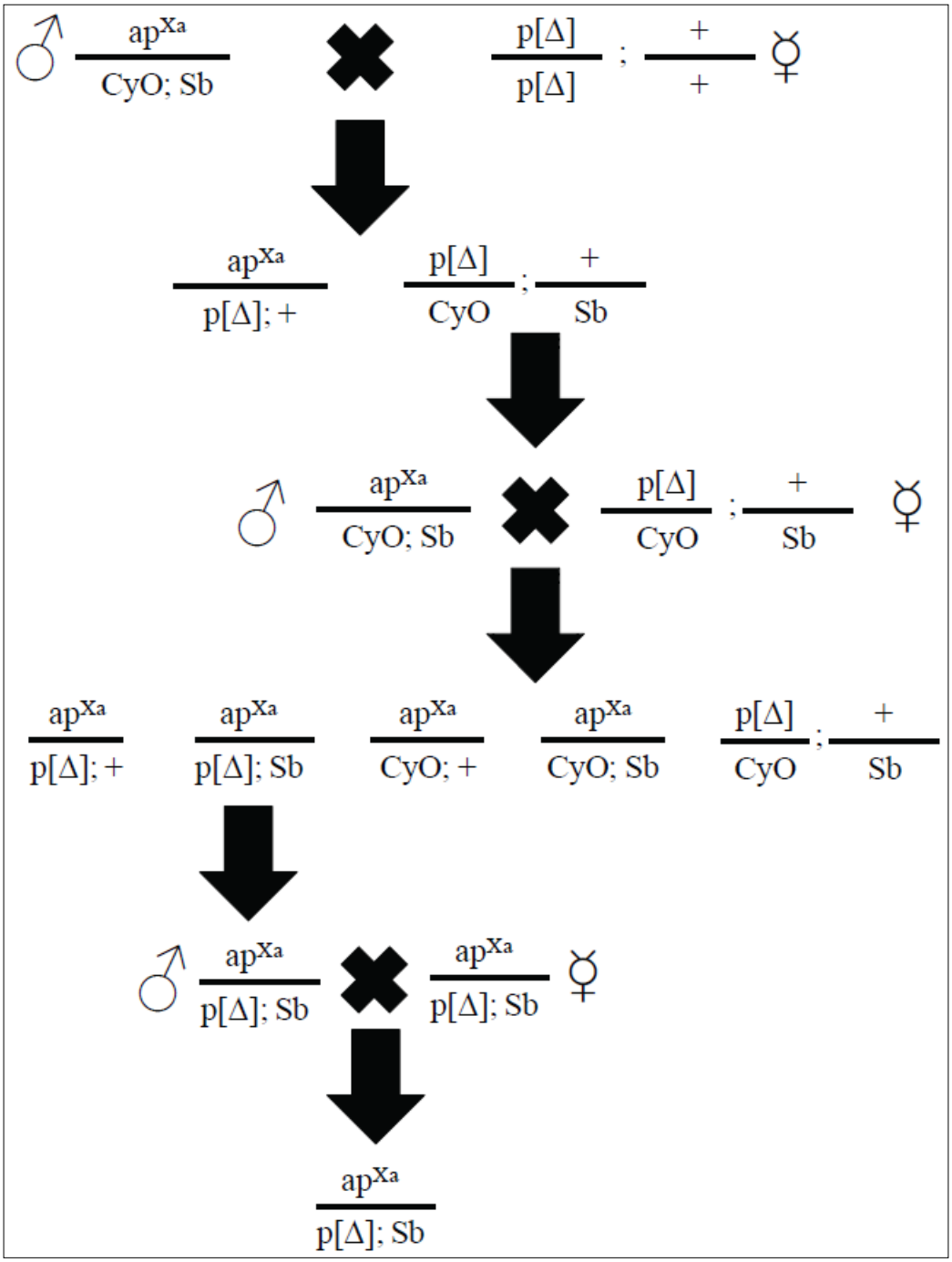

FIGURE 3.16 - TRANSGENIC POL δ CROSS SCHEME – Cross scheme used to generate transgenic fly for pol δ complementation. (ap^{Xa} is a fusion of the 2nd and 3rd chromosome,**;** denotes the separation of the 2nd and 3rd chromosome.)

TRANSGENIC FLY COMPLEMENTATION

The transgenic fly (ap^{Xa}/p[Δ]; Sb) was crossed to both pol δ^{mut} (**FIGURE 3.17**). The transgenic copy of pol δ rescued the lethality of the pol δ^{mut}. This confirms that the lethality of the mutants is due to the changed residues of pol δ and not to a second site lethal. This also confirms that any phenotype that we see is due to mutated pol δ and not another factor.

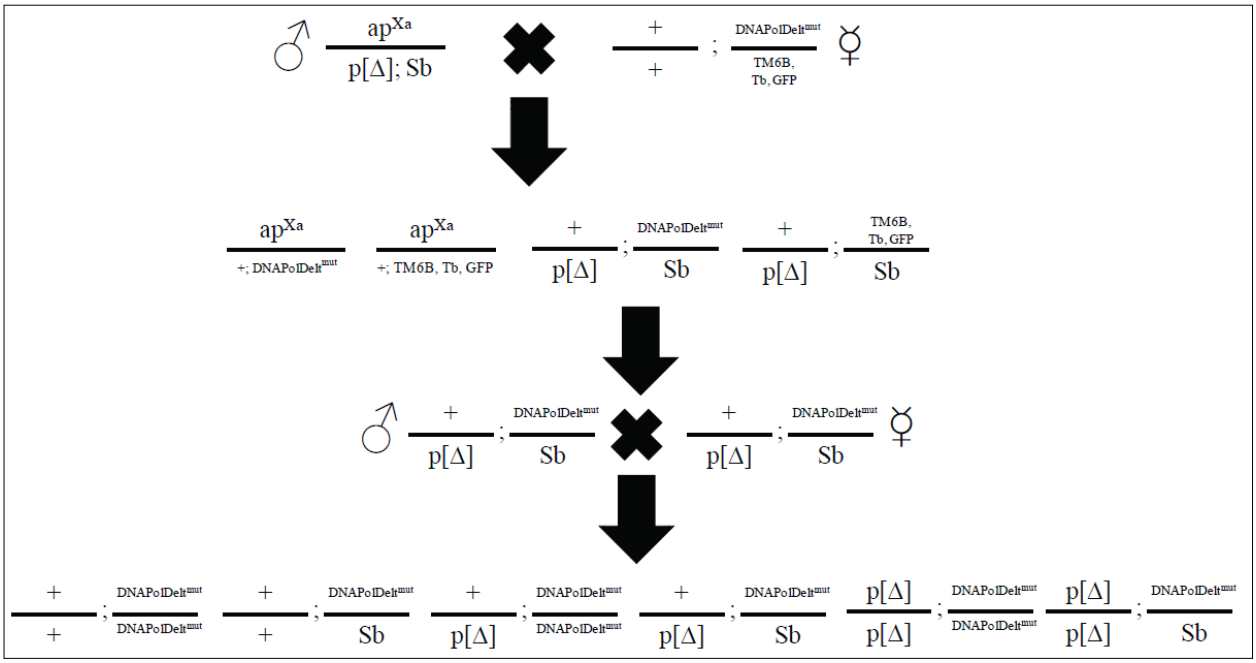

FIGURE 3.17 - TRANSGENIC FLY COMPLEMENTATION CROSS SCHEME – Cross scheme used to generate fly harboring a transgenic copy of pol δ along with two copies of mutated pol δ . (ap^{Xa} is a fusion of the 2nd and 3rd chromosome, **;** denotes the separation of the 2nd and 3rd chromosome.)

VIABILITY OF C496Y

C496Y is a homozygous lethal mutation with progeny making it to the 3rd instar stage of maturation before dying. The heterozygous and homozygous should come out in 2:1 Mendenlian ratio in which they do; however, there is a lot of death occurring with a very smaller ratio of larvae making it to the 3rd instar stage (**TABLE 3.03**). Chi-square analysis was performed on the resulting numbers and indicates a significant change in the number of 3rd instar progeny. [For reference – Critical values for all remaining crosses (2 degrees of freedom) are as follows $p=0.05 \rightarrow \chi^2 = 3.84$; $p=0.01 \rightarrow \chi^2 = 6.64$; and $p=0.001 \rightarrow \chi^2 = 10.83$.] This leads to the conclusion that the homozygous mutants are having a very difficult time in development.

The heterozygous mutants have no defects in viability and the C496Y allele is a recessive allele. This was determined by crossing 15 male C496Y flies to 15 virgin WT flies (**FIGURE 3.18**). This cross scheme allows for a Mendenlian ratio of 1:1 for progeny DNAPolDelt^{C496Y}/+ and +/TM6B, Tb, GFP. Larvae were sorted based on the Tb marker as well as presence of GFP allowing for two methods of selecting the same larvae. No larvae was observed that was Tb and not GFP + or non-Tb and GFP +. 3rd instar larvae, males, females and total flies were all counted. Chi-square analysis was performed on the resulting numbers and showed no significant deviation from expected numbers compared to observed numbers (**TABLE 3.04**).

3rd Instar Larvae					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{ExoIII}$ / $DNAPolDelt^{ExoIII}$		11	84		63.440476
$DNAPolDelt^{ExoIII}$ / TM6B, Tb, GFP		241	168		31.720238
	Total	252	252	χ^2 Value =	95.160714

TABLE 3.03 - VIABILITY OF C496Y – Table showing 3rd instar of homozygous and heterozygous progeny.

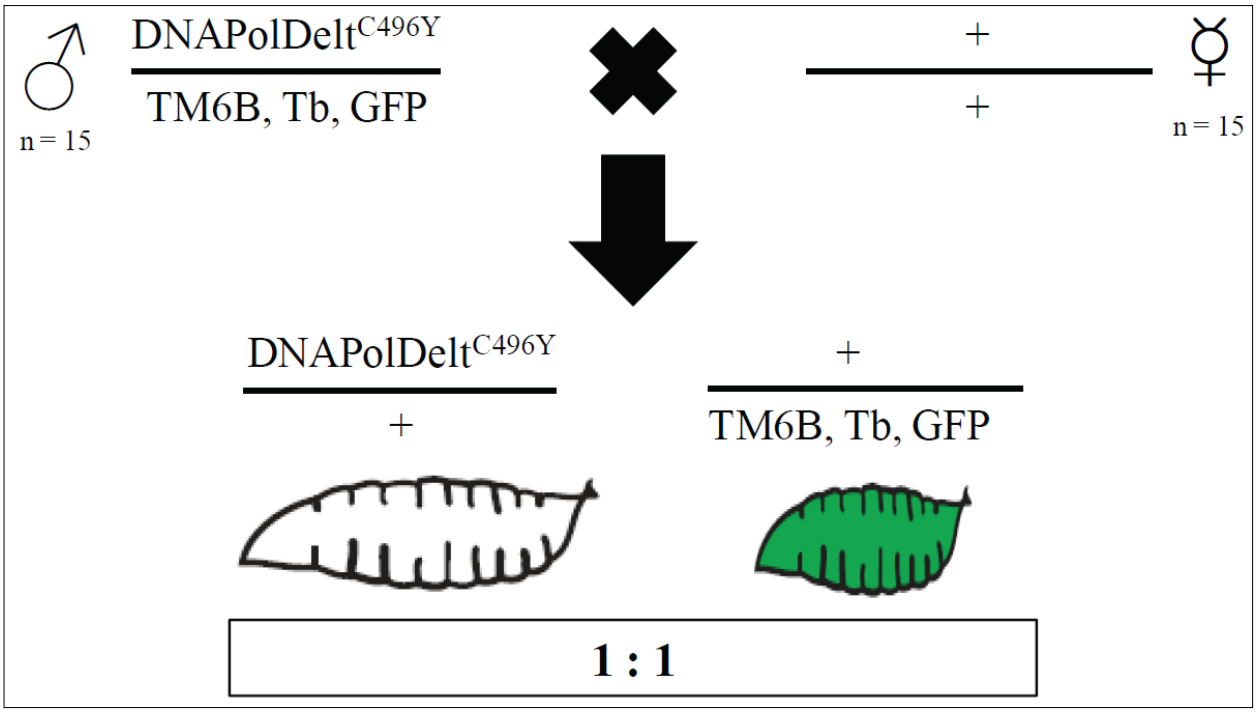

FIGURE 3.18 - C496Y VIABILITY CROSS SCHEME – Cross scheme used to generate heterozygous flies for C496Y and wild type siblings to analyze the effect of the mutation on viability.

3rd Instar Larvae					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		169	164.5		0.1231
+ / TM6B, Tb, GFP		160	164.5		0.1231
	Total	329	329	χ^2 Value =	0.246201
Males					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		51	48.5		0.128866
+ / TM6B, Tb, GFP		46	48.5		0.128866
	Total	97	97	χ^2 Value =	0.257732
Females					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		57	49.5		1.136364
+ / TM6B, Tb, GFP		42	49.5		1.136364
	Total	99	99	χ^2 Value =	2.272727
Adults					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		108	98		1.020408
+ / TM6B, Tb, GFP		88	98		1.020408
	Total	196	196	χ^2 Value =	2.040817

TABLE 3.04 - VIABILITY OF HETEROZYGOUS C496Y – Table showing 3rd instar larvae, males, females and total adult progeny.

VIABILITY OF G694N

G694N is also a homozygous lethal mutation with progeny making it to the only to the 1st instar stage of maturation before dying. Once again, the heterozygous and homozygous should come out in 2:1 Mendenlian ratio in which they do (**TABLE 3.05**); however, homozygous larvae die before the 2nd instar stage. Chi-square analysis was performed on the resulting numbers and showed no deviation from expected numbers compared to observed numbers for 1st instar numbers. This leads to the conclusion that the homozygous G694N mutation is not sufficient for development past the 1st instar stage.

The heterozygous mutants have no defects in viability and the G694N allele is a recessive allele. This was determined by crossing 15 male G694N flies to 15 virgin WT flies (**FIGURE 3.19**). This cross scheme allows for a Mendenlian ratio of 1:1 for progeny DNAPolDelt^{G694N}/+ and +/TM6B, Tb, GFP. Larvae were sorted based on the Tb marker as well as presence of GFP allowing for two methods of selecting the same larvae. No larvae was observed that was Tb and not GFP + or non-Tb and GFP +. 3rd instar larvae, males, females and total flies were all counted. Chi-square analysis was performed on the resulting numbers and showed no significant deviation from expected numbers compared to observed numbers (**TABLE 3.06**).

1st Instar Larvae

		Observed	Expected		$(O-E)^2/E$
$\frac{DNAPolDelt^{G694N}}{DNAPolDelt^{G694N}}$		44	44		0
$\frac{DNAPolDelt^{G694N}}{TM6B, Tb, GFP}$		88	88		0
	Total	132	132	χ^2 Value =	0

TABLE 3.05 - VIABILITY OF G694N – Table showing 1st instar of homozygous and heterozygous progeny.

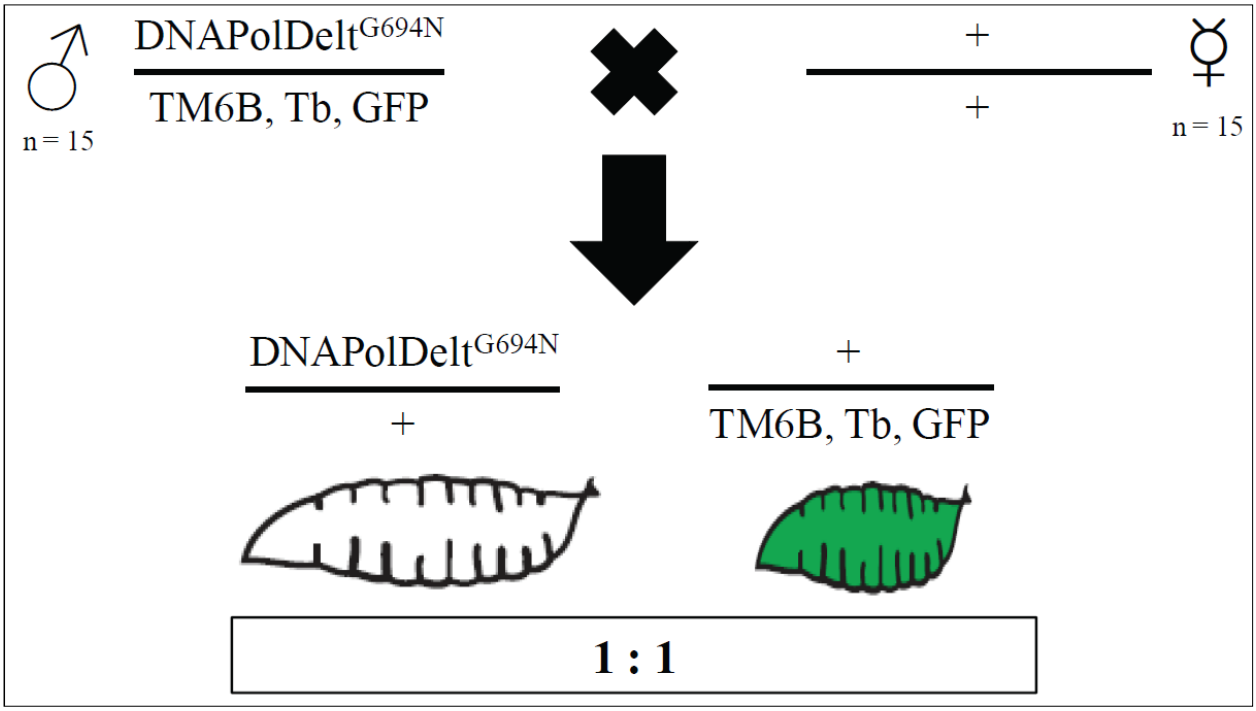

FIGURE 3.19 - G694N VIABILITY CROSS SCHEME – Cross scheme used to generate heterozygous flies for G694N and wild type siblings to analyze the effect of the mutation on viability.

3rd Instar Larvae					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		195	185		0.540541
+ / TM6B, Tb, GFP		175	185		0.540541
	Total	370	370	χ^2 Value =	1.081081
Males					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		70	63		0.777778
+ / TM6B, Tb, GFP		56	63		0.777778
	Total	126	126	χ^2 Value =	1.555556
Females					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		75	69		0.521739
+ / TM6B, Tb, GFP		63	69		0.521739
	Total	138	138	χ^2 Value =	1.043478
Adults					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		145	132		1.280303
+ / TM6B, Tb, GFP		119	132		1.280303
	Total	264	264	χ^2 Value =	2.560606

TABLE 3.06 - VIABILITY OF HETEROZYGOUS G694N – Table showing 3rd instar larvae, males, females and total adult progeny.

CHROMOSOME ABERRATIONS/ MITOTIC INDICES:

In the developing larvae of Drosophila, there are two different types of cell cycles occurring. One is a normal cell cycle, present in the most tissues, the other is slightly different known as endoreplication with a cell cycle moving straight from G1 to S without any division and continuous replication of DNA. To analyze the normal cell cycle, we analyzed larval brains which undergo a normal cell cycle.

Larval brain squashes were performed and the resulting slides were analyzed. When analyzing different populations of cells, it was apparent that the mitotic figures present in the pol δ mutants were extremely abnormal. A majority of the mitotic figures showed anueploidy, under-condensed chromosomes, and also a high frequency of chromosomes with broken arms (**FIGURE 3.20**). Additionally, C496Y/+, C496Y/C496Y and G694N/+ all showed less mitotic figures compared to WT (**FIGURE 3.21**). The average mitotic index for WT was 0.016 while C496Y/+, C496Y/C496Y and G694N/+ was 0.004, 0.006, 0.003 respectively. A 2-sample t-test was performed via MiniTab^{TM} and showed that all three mutants were significantly lower (WT and C496Y/ + → p-value= 0.000; WT and C496Y/C496Y → p-value=0.002; WT and G694N/+ → p-value= 0.000).

These results suggest an S-phase delay in that the cells are taking longer to progress through S-phase to enter M-phase.

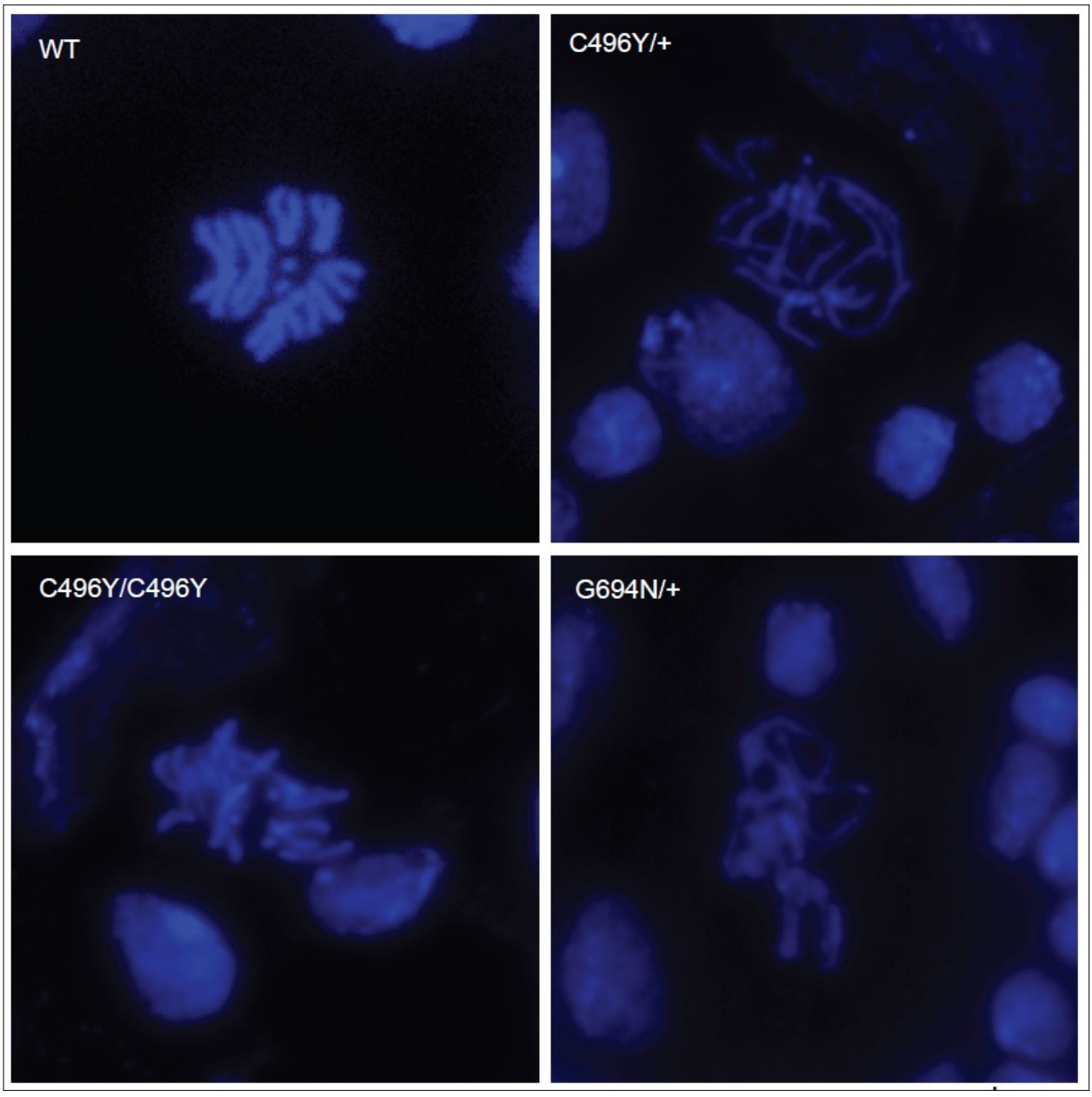

FIGURE 3.20 – EXAMPLES OF MITOTIC FIGURES FROM POL δ MUTANTS – The pol δ mutants show a high frequency of anueploidy, under-condensed chromosomes, and a high frequency of chromosomes with broken arms.

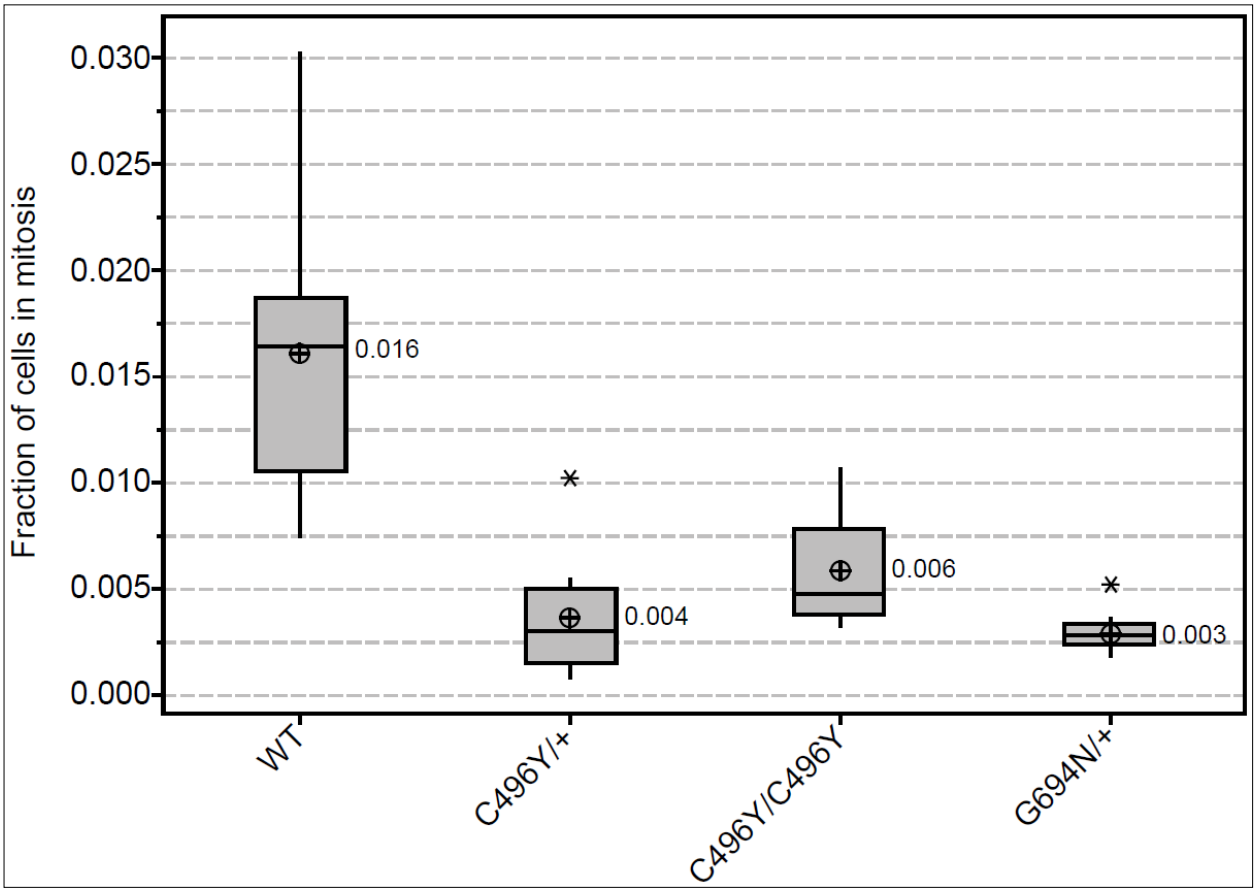

FIGURE 3.21 – MITOTIC INDICES FROM POL δ MUTANTS – The pol δ mutants all have significantly less cells in mitosis compared to wild type.

EdU INCORPORATION/ S-PHASE INDICES:

With such a low mitotic index for all three pol δ mutants, it is imperative to understand what these cells are doing through S-phase especially since such a key component to synthesis (a polymerase) is mutated. To analyze cells in neural tissues that are actively synthesizing, we use a new technique known as EdU incorporation. EdU, or 5-ethynyl-2′-deoxyuridine, is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. Detection is based on a click reaction, a copper (Cu^{+1}) catalyzed covalent reaction between an azide and an alkyne. In this application, the EdU contains the alkyne and the Alexa Fluor® dye (part of the click reaction) contains the azide. This method is much accuracte and less harsh to the cells compared to other methods such as BrdU. After the EdU incorporation, the brains were analyzed. The heterozygous mutants (C496Y/+ and G694N/+) displayed many more cells with EdU incorporation compared to WT while the homozygous mutant (C496Y/C496Y) displayed no cells with EdU incorporation (**FIGURE 3.22**). S-phase indices showed that WT had 0.076 cells in S-phase compared to 0.131, 0.000, 0.152 for C496Y/+, C496Y/C496Y and G694N/+ respectively (**FIGURE 3.23**). A 2-sample t-test was performed via MiniTab™ and showed that all C496Y/+ and G694N/+ were significantly higher (WT and C496Y/ + → p-value= 0.044 and WT and G694N/+ → p-value= 0.008). It was also significant that C496Y/C496Y displayed no cells in S-phase.

These results suggest that the heterozygous mutants have an S-phase delay, confirming assumptions from a lower mitotic index. They also show that the homozygous mutant has a severe S-phase delay.

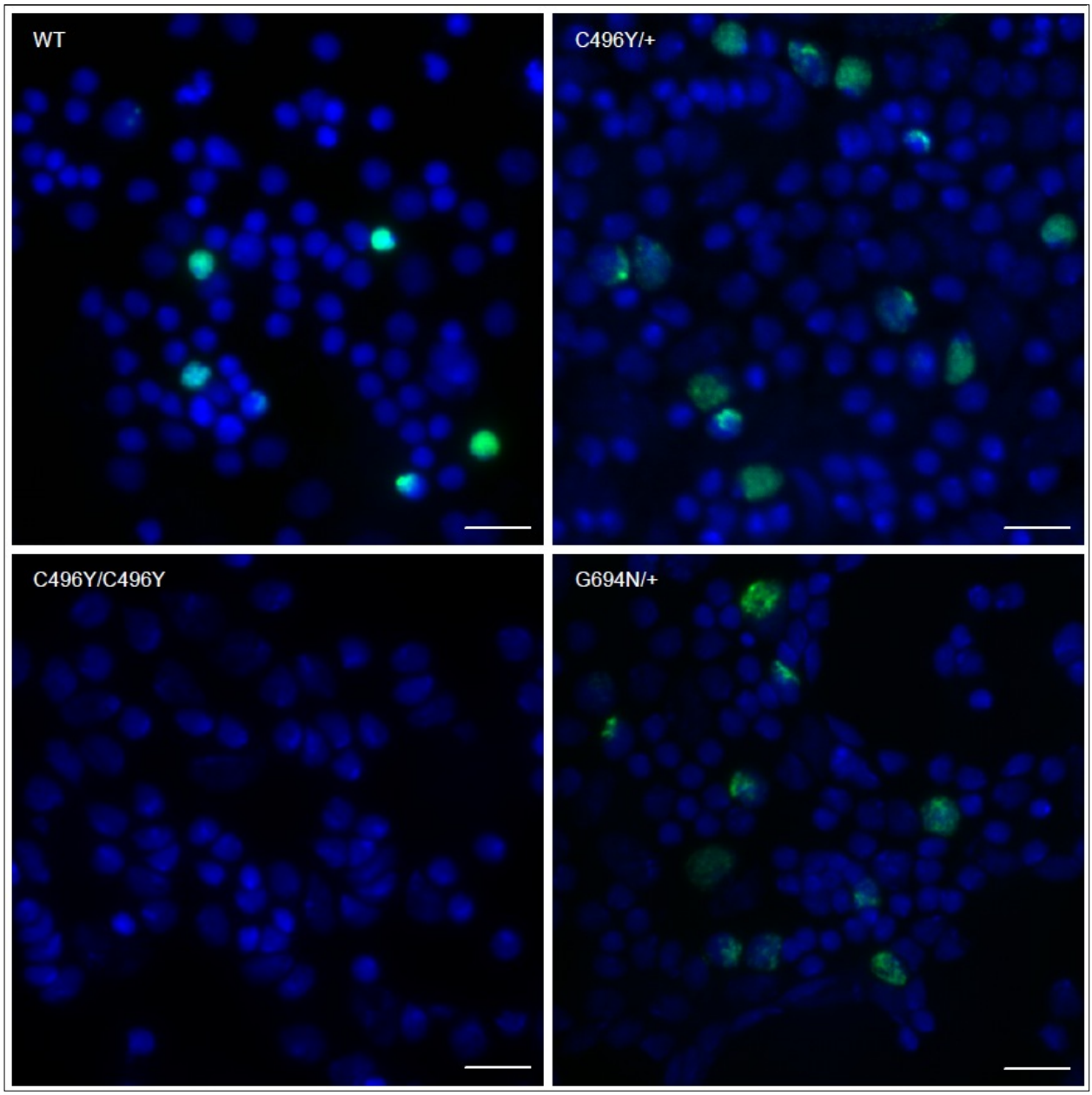

FIGURE 3.22 – EXAMPLES OF EdU FIELDS OF VIEW FIGURES FROM POL δ MUTANTS – The heterozygous pol δ mutants show a more cells with EdU incorporation while the homozygous mutant shows no EdU incorporation.

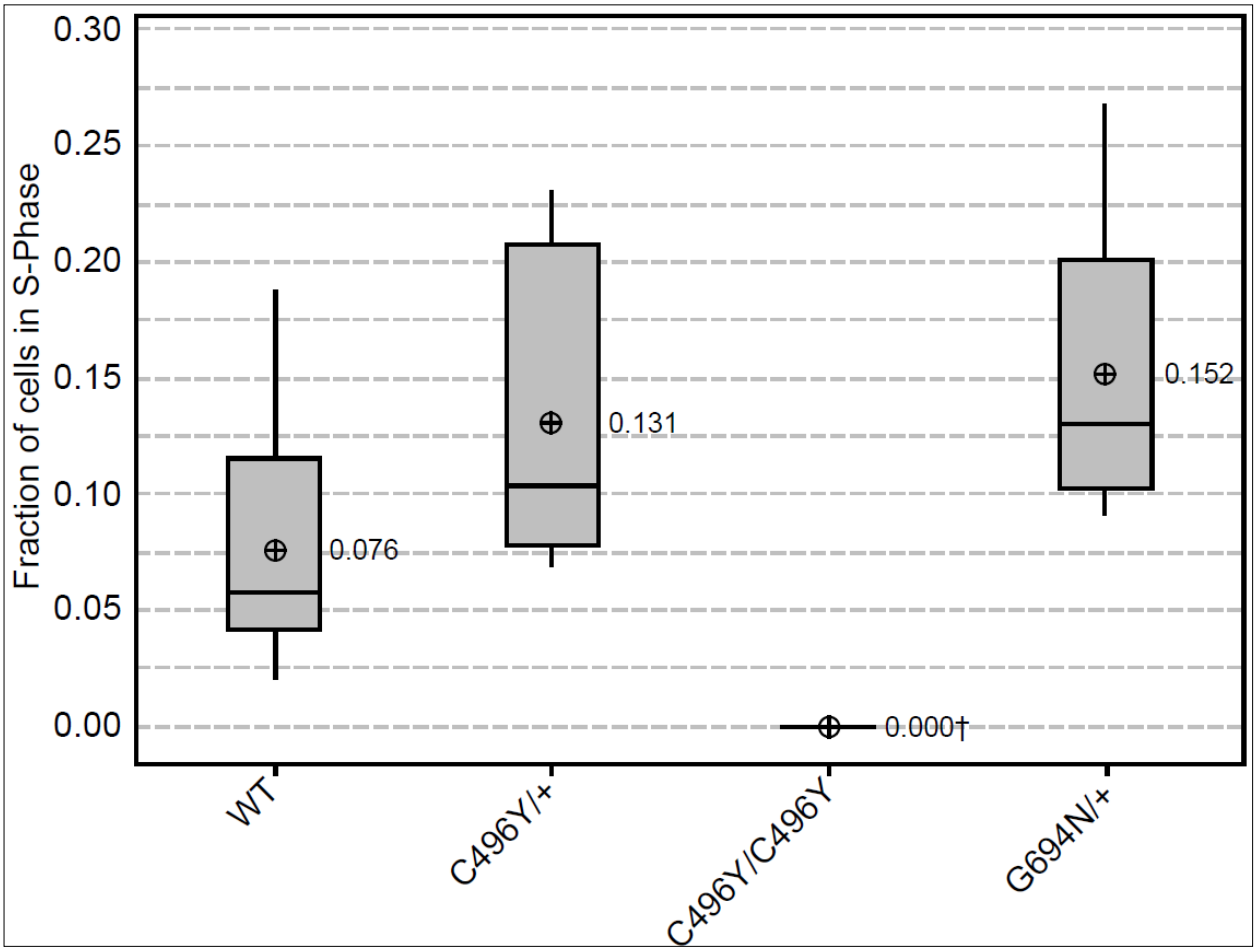

FIGURE 3.23 – S-PHASE INDICES FROM POL δ MUTANTS - Heterozygous mutants show an increase in cells going through S-Phase while the homozygous mutant shows no cells with EdU incorporation (†).

Endoreplication

One reason why *Drosophila* has been embraced as a model organism is because of its possession of a unique cell cycle known as endoreplication. Cells in tissues like the salivary glands and ovaries undergo this cell cycle that does not have a mitosis. In the case of salivary glands, the cells continue to replicate forming giant chromosomes known as polytene chromosomes. Since the cell cycle relies so heavily on S-phase, it becomes an excellent vessel to study the effects of defective a polymerase.

One question that arose is whether or not there were the same number of cells present in the salivary glands. The number of cells in WT and heterozygotes were very close while there was a decrase in cells in the homozyogous C496Y larvae (**FIGURE 3.23**).

After the cell number was determined, we analyzed the genomic DNA content by performing genomic preps on whole salivary glands. There was a decrease of about 27.5% in genomic content in the heterozygous while a decrease of about 64.5% in genomic content in the homozygous C496Y. This was normalized to DNA content per cell.

Finally, we examined spread polytene chromosomes. The chromosomes seemed to exhibit a very similar phenotype as compared to their genomic DNA content with WT being the largest, the heterozygous mutants having around a 30% decrease and the homozygous mutant having a very large decrease in size and very anemic.

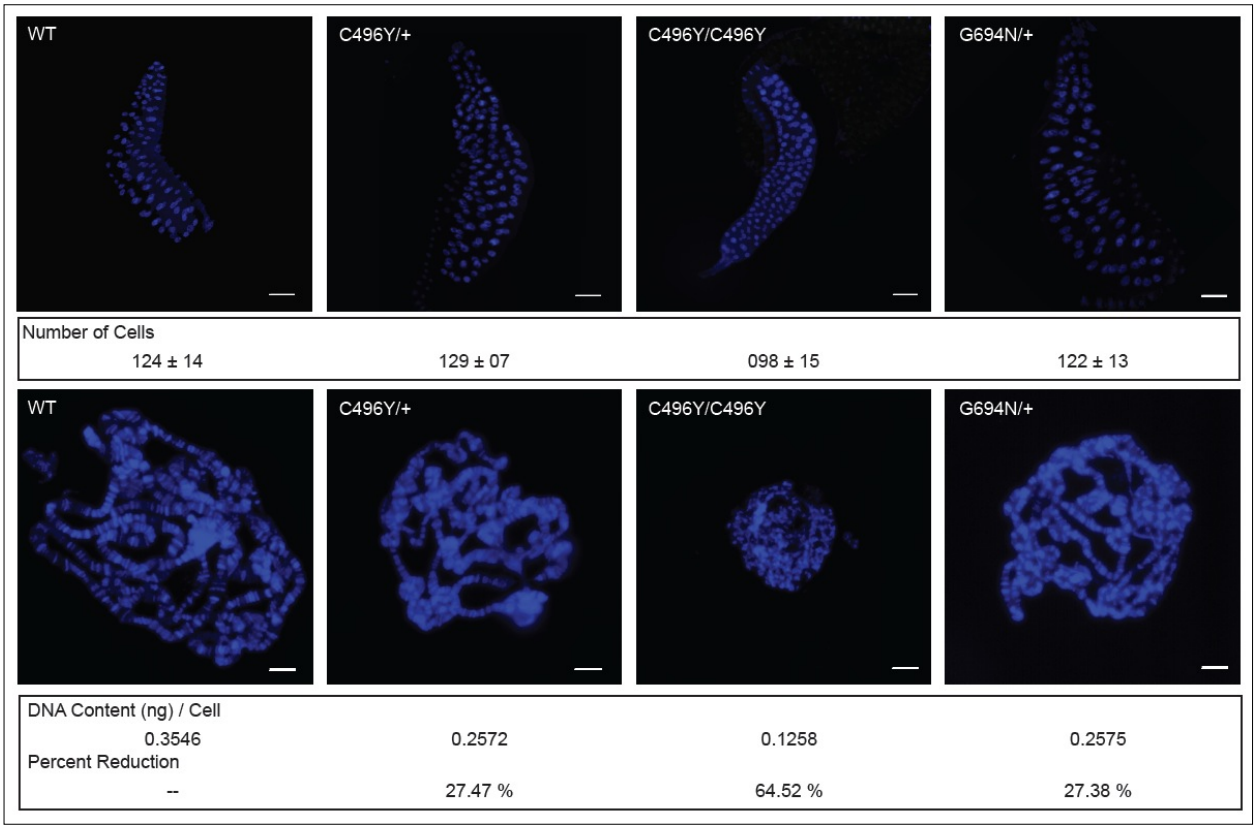

FIGURE 3.23 – ENDOREPLICATION – Pol δ has an effect on endoreplication with a decrease in DNA content and also decrease.

Mutagen Viability

Due to the activity of pol δ in DNA repair, we examined the ability of the pol δ's response to different DNA damaging agents. We examined 3 different mutagens. HU which reduces ribonucleotide reductase impairs replication by decreasing the nucleotide pool and causing stalled forks (MICHEL *et al.* 2004). MMS methylates DNA on N^7-deoxyguanine and N^3-deoxyadenine. Originally, this action was believed to directly cause double-stranded DNA breaks; however, it is now believed that MMS stalls replication forks, and cells that are homologous recombination-deficient have difficulty repairing the damaged replication forks (Lundin *et al.* 2005). Paraquat causes single-base damage which is corrected through base excision repair pathway (Xu *et al.* 2009).

From our crosses(**FIGURE 3.24**) (**TABLES 3.06-3.12**), we can see that the pol δ^{muts} are not sensitive to mutagens (except in the case of G694N with MMS; however, the n is only 4). They actually have more progeny in most cases than their wild type siblings.

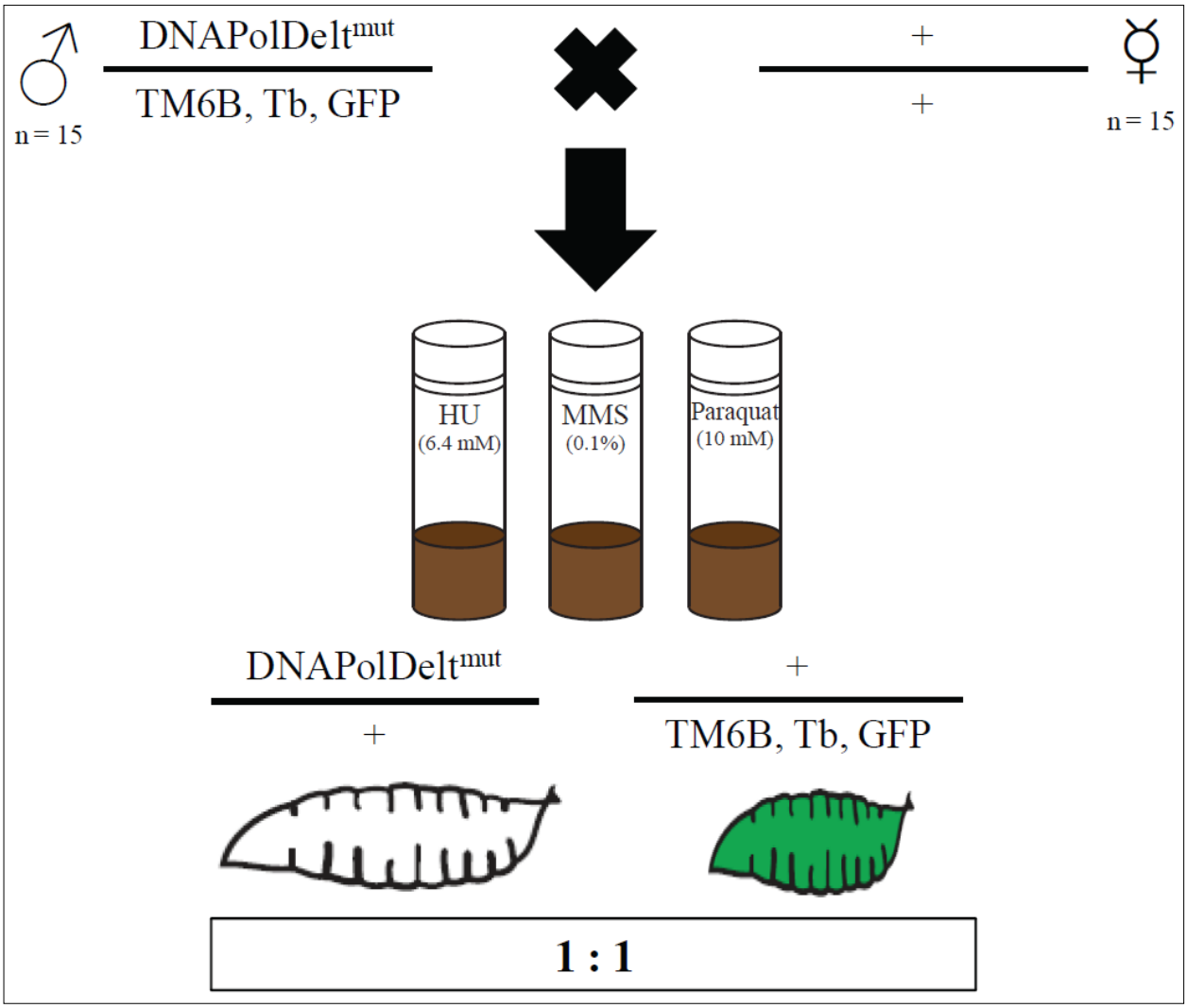

FIGURE 3.24 – MUTAGEN VIABILITY CROSS SCHEME – Cross scheme used for heterozygous mutagen viability.

3rd Instar Larvae

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		172	136		9.52941
+ / TM6B, Tb, GFP		100	136		9.52941
	Total	272	272	χ^2 Value =	19.05882

Males

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		25	21.5		.5697675
+ / TM6B, Tb, GFP		18	21.5		.5697675
	Total	43	43	χ^2 Value =	1.139535

Females

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		7	12.5		2.42
+ / TM6B, Tb, GFP		18	12.5		2.42
	Total	25	25	χ^2 Value =	4.84

Adults

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		43	34		2.382353
+ / TM6B, Tb, GFP		25	34		2.382353
	Total	68	68	χ^2 Value =	4.764706

TABLE 3.06 - VIABILITY OF HETEROZYGOUS C496Y IN THE PRESENCE OF HU IS NOT ALTERED – Table showing 3rd instar larvae, males, females and total adult progeny.

3rd Instar Larvae					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		43	26.5		10.27358
+ / TM6B, Tb, GFP		10	26.5		10.27358
	Total	53	53	χ^2 Value =	20.54717
Males					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		0	1		0.5
+ / TM6B, Tb, GFP		2	1		0.5
	Total	2	2	χ^2 Value =	1
Females					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		0	1		5.142857
+ / TM6B, Tb, GFP		2	1		5.142857
	Total	2	2	χ^2 Value =	10.28571
Adults					
		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		19	11.5		4.891304
+ / TM6B, Tb, GFP		4	11.5		4.891304
	Total	23	23	χ^2 Value =	9.782609

TABLE 3.07 - VIABILITY OF HETEROZYGOUS C496Y IN THE PRESENCE OF MMS IS NOT ALTERED – Table showing 3rd instar larvae, males, females and total adult progeny.

3rd Instar Larvae

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		81	47.5		23.62632
+ / TM6B, Tb, GFP		14	47.5		23.62632
	Total	95	95	χ^2 Value =	47.25263

Males

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		17	8.5		8.5
+ / TM6B, Tb, GFP		0	8.5		8.5
	Total	17	17	χ^2 Value =	17

Females

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		52	32		12.5
+ / TM6B, Tb, GFP		12	32		12.5
	Total	64	64	χ^2 Value =	25

Adults

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$ / +		69	40.5		20.05556
+ / TM6B, Tb, GFP		12	40.5		20.05556
	Total	81	81	χ^2 Value =	40.11111

TABLE 3.08 - VIABILITY OF HETEROZYGOUS C496Y IN THE PRESENCE OF PARAQUAT IS NOT ALTERED – Table showing 3rd instar larvae, males, females and total adult progeny.

3rd Instar Larvae

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		161	125		10.368
+ / TM6B, Tb, GFP		89	125		10.368
	Total	250	250	χ^2 Value =	20.736

Males

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		34	24.5		3.683673
+ / TM6B, Tb, GFP		15	24.5		3.683673
	Total	49	49	χ^2 Value =	7.367347

Females

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		37	29		2.206897
+ / TM6B, Tb, GFP		21	29		2.206897
	Total	28	58	χ^2 Value =	4.413793

Adults

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		71	53.5		5.724299
+ / TM6B, Tb, GFP		36	53.5		5.724299
	Total	107	107	χ^2 Value =	11.4486

TABLE 3.09 - VIABILITY OF HETEROZYGOUS G694N IN THE PRESENCE OF HU IS NOT ALTERED – Table showing 3rd instar larvae, males, females and total adult progeny.

3rd Instar Larvae

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		22	27		0.925926
+ / TM6B, Tb, GFP		32	27		0.925926
	Total	54	54	χ^2 Value =	1.851852

Males

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		0	1		1
+ / TM6B, Tb, GFP		2	1		1
	Total	2	2	χ^2 Value =	2

Females

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		0	1		1
+ / TM6B, Tb, GFP		2	1		1
	Total	2	2	χ^2 Value =	2

Adults

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		0	2		2
+ / TM6B, Tb, GFP		4	2		2
	Total	4	4	χ^2 Value =	4

Y/TABLE 3.10 - VIABILITY OF HETEROZYGOUS G694N IN THE PRESENCE OF MMS IS ALTERED – Table showing 3rd instar larvae, males, females and total adult progeny.

3rd Instar Larvae

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		94	63.5		14.64961
+ / TM6B, Tb, GFP		33	63.5		14.64961
	Total	127	127	χ^2 Value =	29.29921

Males

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		18	9		9
+ / TM6B, Tb, GFP		0	9		9
	Total	18	18	χ^2 Value =	18

Females

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		48	33		6.818182
+ / TM6B, Tb, GFP		16	33		6.818182
	Total	66	66	χ^2 Value =	13.63636

Adults

		Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$ / +		66	42		13.71429
+ / TM6B, Tb, GFP		18	42		13.71429
	Total	84	84	χ^2 Value =	27.42857

TABLE 3.11 - VIABILITY OF HETEROZYGOUS G694N IN THE PRESENCE OF PARAQUAT IS NOT ALTERED – Table showing 3rd instar larvae, males, females and total adult progeny.

Adults - HU	Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$	43	34		2.3823529
+				
+	25	34		2.3823529
TM6B, Tb, GFP				
Total	68	68	χ^2 Value =	4.7647059

Adults - HU	Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$	71	53.5		5.7242991
+				
+	36	53.5		5.7242991
TM6B, Tb, GFP				
Total	107	107	χ^2 Value =	11.448598

Adults - MMS	Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$	19	11.5		4.8913043
+				
+	4	11.5		4.8913043
TM6B, Tb, GFP				
Total	23	23	χ^2 Value =	9.7826087

Adults - MMS	Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$	0	2		2
+				
+	4	2		2
TM6B, Tb, GFP				
Total	4	4	χ^2 Value =	4

Adults - Paraquat	Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{C496Y}$	69	40.5		20.055556
+				
+	12	40.5		20.055556
TM6B, Tb, GFP				
Total	81	81	χ^2 Value =	40.111111

Adults - Paraquat	Observed	Expected		$(O-E)^2/E$
$DNAPolDelt^{G694N}$	66	42		13.714286
+				
+	18	42		13.714286
TM6B, Tb, GFP				
Total	84	84	χ^2 Value =	27.428571

TABLE 3.12 – COMBINED VIABILITY OF HETEROZYGOUS C496Y/G694N ADULTS IN THE PRESENCE OF MUTAGENS – Table showing adult progeny in the presence of mutagens.

Genetic Interaction of pol α and pol δ

Pol δ relies heavily on pol α for the initiation and priming of DNA replication (GARG and BURGERS 2005). To check for unlinked non-complementation, we crossed an existing uncharacterized allele of pol α (DNAPolAlpha$^{[F02992]}$). Much to our surprise, flies harboring both of these mutations were viable. Further characterization of these double mutants is still needed.

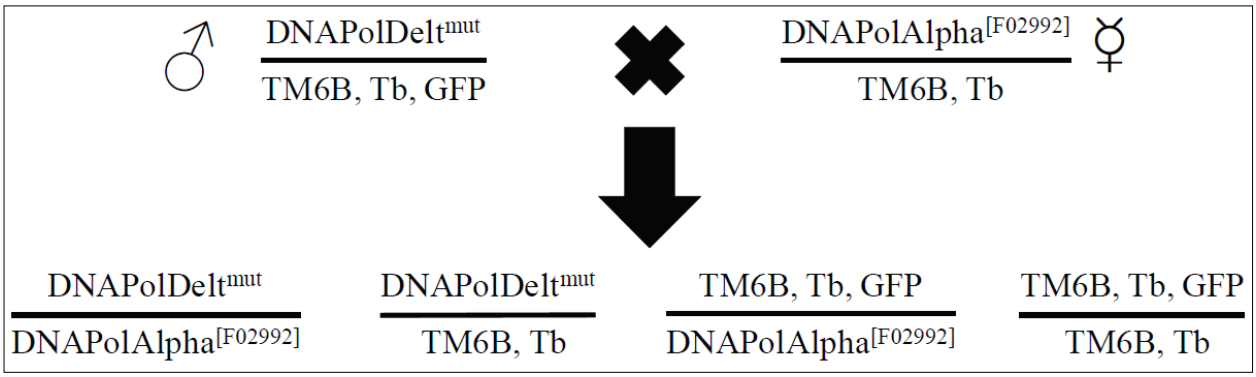

FIGURE 3.25 - POL α CROSS SCHEME – Cross scheme used to generate flies with pol $\alpha^{mut.}$ and pol $\delta^{mut.}$. All alleles are on the third chromosome.

Genetic Interaction of pol δ and PCNA

PCNA has been shown in to be the replication clamp required for DNA replication and for various other DNA repair processes, most importantly being the processivity factor pol δ (MOLDOVAN *et al.* 2007). To check for possible unlinked non- complementation, we crossed an existing allele of PCNA (P[PCNA]^{02448}) to both of the pol δ^{muts}. Some phenotypes of the P[PCNA]^{02448} include reduced BrdU incorporation in third larval instar brains in homozygotes compared to heterozygotes. Additionally, there is a 3.3 fold increase in the percentage of mitotic cells in the mutant larval brain compared to wild type. 91% of mitotic figures appear arrested in a metaphase-like state in which a highly condensed chromosome mass is present (PFLUMM and BOTCHAN 2001; JACKSON *et al.* 2005). These phentypes led us to the hypothesis that flies harboring both of these mutations would more than likely be lethal; however, progeny with both of these mutations came out in a relative Mendenlian ratio. More investigation is needed into the possible phenotypes that arise from these two mutations in unison.

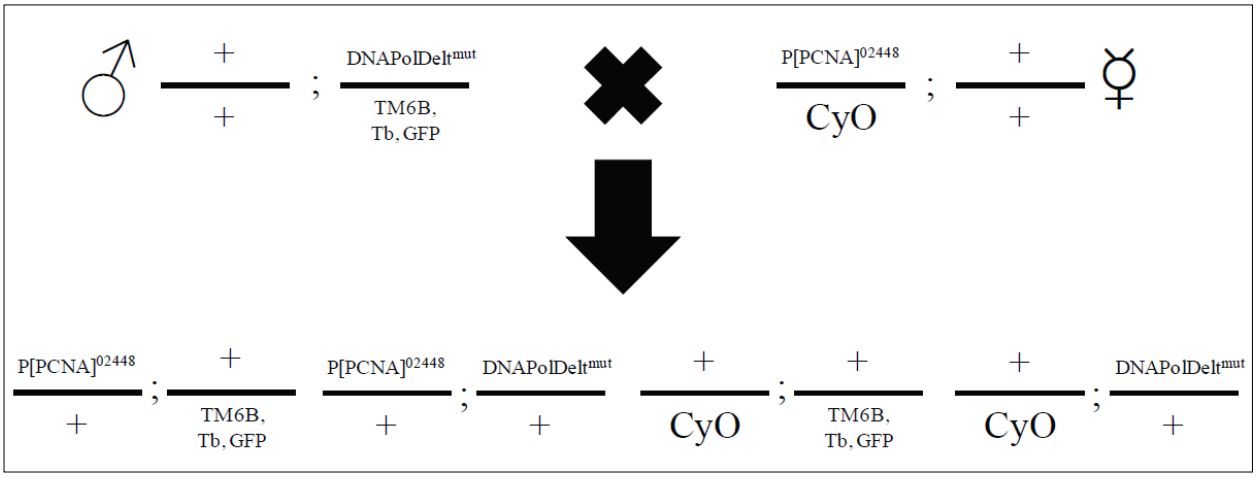

FIGURE 3.26 - PCNA CROSS SCHEME – Cross scheme used to generate flies with PCNA$^{mut.}$ and pol δ$^{mut.}$. Alleles are on the second and third chromosomes.

4. **DISCUSSION**

Much work has been done to elucidate the mechanisms of DNA replication since the initial discovery of the first polymerase (pol α) in 1957; however, the specifics of some of the polymerase have still yet to be unraveled. Arguably, the most important polymerases are the classical and accurate pols (α, β, γ, δ, and ε) (HÜBSCHER *et al.* 2000). Of these five, pols α, δ, and ε are absolutely necessary for DNA replication with pol α initiating synthesis *de novo* and pols δ and ε being the main replicative forces. For our study, we investigate on possibly the most important of these polymerases, pol δ, which is responsible for replicating a major portion of the genome in both leading and lagging strand synthesis.

To begin analyzing, it was important to analyze the structure of pol δ amongst different species. Pol δ is the most conserved of all the polymerases when referring to the largest subunit (in the case of *Drosophila*, this is 120 kD) (HINDGES and HÜBSCHER 1997). Because of this high similarity between species, inferences about certain residues and domains in one species (such as in *Drosophila*) can be more easily transferred to other species (such as *H. sapiens*). Although the majority of this polymerase is highly conserved overall, there are regions of even greater evolutionary conservation. These domains (**FIGURE 3.02**) (N-terminal, Exonuclease, Polymerase, C-terminal) are essential in the function of the protein hence their high conservation (CHIANG and LEHMAN 1995).

When discussing pol δ, the main functions of the polymerase are naturally its ability to polymerize nucleotides and additionally, its ability to proofread in the 3' to 5' direction. Ideally, we have identified a mutant in each of those domains to help elucidate the functions of these domains at a very closer magnitude in the context of a multicellular organism.

The first mutant, C496Y, disrupts the 16 aa Exonuclease III conserved region by the substitution of a tyrosine (Tyr or Y) in place of a cysteine (Cys or C). This amino acid change is rather unique in the fact that tyr is a much bulkier amino acid with phenol as its side chain compared to a thiol in C. This, more than likely, affects the overall structure and stability of the protein. When mapped to the crystal structure of yeast, this mutation is exposed and far away from the exonuclease active site and more than likely disrupts interactions with accessory proteins needed for correct formation of the pol δ holoenzyme that may contribute to the fidelity of pol δ. Even more interesting with this mutation is the fact that in a screen of human colon cancers cell lines, 15 mutations were found in the pol δ region and 5 of those mapped to some sort of amino acid change in this exonuclease III conserved region (FLOHR *et al.* 1999). This implies that this region, even in higher eukaryotes, is necessary for genomic stability.

The second mutant, G694N, disrupts the 39-40 aa Polymerase III conserved region by the substitution of a asparagine (Asn or N) in place of a glycine (Gly or G). This amino acid change, again, is very unique. Gly is the smallest of the amino acids and with the change to asparagiune, there is the addition of a carboxamide. With the addition of this side chain, it is hard to imagine a instance where the protein structure would not be affected, even if at a minute level. In reference to the colon cancer screen mentioned above, a mutation in this region was also found. Even more interesting is the fact that this individual residue when mapped to yeast is Gly709. This amino acid along with yeast residues Asn705, Ser706, Tyr708 in the fingers domain and Tyr613 from the palm domain shape the binding pocket which is responsible for the high fidelity of Pol δ accommodating the nascent Watson-Crick base pairs (SWAN *et al.* 2009). This implies that the Polymerase III conserved region and more specifically Gly694 in *Drosophila* is essential for the fidelity of pol δ.

These two mutants, C496Y and G694N, pose a great opportunity to study the effects of different domains of pol δ.

Both of these mutations are homozygous lethal; however, they seem to have no effect on viability on regards to being heterozygous suggesting that they are recessive in regards to viability. Mice with mutations in the Polymerase II conserved region (L604G, L604K in Mm; 591 in Dm) are homozygous lethal. The heterozygous mice were viable and displayed no overall increase in disease very similar to the G694 mutants (VENKATESAN *et al.* 2007). Mice with mutations in the Exonuclease II conserved region (D400A in Mm; 386 in Dm) are homozygous viable with a high probability (94%) of developing cancer while only 3-4% of heterozygous animals developed any sort of cancer (GOLDSBY *et al.* 2001; GOLDSBY *et al.* 2002). This is interesting since homozygous mice are viable although with a very high probabity of cancer. The homozygous C496Y are able to make it much later in development as compared to the G694N mutants yet still die before pupation. This might suggest that the change from D to A disrupts the protein structure much less than a C to Y change, allowing for a more normal structure.

Drosophila poses as a great model system to investigate the cell cycle. The developing larvae undergo two distinct cell cycles, the normal mitotic cell cycle and also the endoreplciating cell cycle. The normal mitotic cell cycle follows the natural progression of G1-S-G2-M while endoreplication continues without the M phase and subsequent cytokinesis generating polyploidy tissues.

We first investigated the normal cell cycle by analyzing nueral tissues of 3rd instar larvae. Utilizing EdU incorporation, the heterozygous mutants showed a much higher percentage of cells going through S-phase. The number, almost completely double as compared to WT, is

indicative of almost half of the available polymerases lacking function. Another interesting result was the lack of incorporation by the homozygous exonuclease mutants. These mutants are developmentally slowed as compared to their heterozygous siblings and on the verge of death due to the fact of their lethality at the 3rd instar – prepupae transition. The lack of incorporation can be due to the fact that synthesis is not occurring at all or the fact that synthesis is occurring at such a slow pace that the 30 minute incubation is not long enough for incorporation of the EdU; however, the more likely explanation is the former. The results suggest an S-phase delay for the three mutants which would be expected for a mutant with a defective polymerase, the main player in progression through S-phase. The results combined suggest that dosage of polymerase is important and that pol δ is essential for progression of S-phase. Complex mechanisms more than likely compensate for the lack of polymerase in the heterozygous mutants while the complete removal in the homozygous lead to a failure in synthesis. It is possible that pol ε is aiding in this compensation, and although not as efficient is still able to help the cell proceed with S-phase. However, without the presence of any pol δ, pol ε presence is negligible. A future study involves investigation of the current mutants combined with mutants defective for pol ε.

To further investigate cell cycle progression, we analyzed mitotic indices for these mutants. The three mutants all displayed a much lower mitotic index. In addition, there was also a high frequency of anueploid cells, under-condensed chromosomes, and a high frequency of chromosomes with broken arms. The results together suggest that the mutants are able to progress through a normal cell cycle but at a much slower rate with an extreme S-phase delay. Additionally, the mutant polymerases have dire consequences on chromosome formation which would be expected with improper defects in replication that would in turn cause defects in chromosome biology.

To investigate endoreplication, we analyzed the salivary glands of 3rd instar larvae which produce giant polyploid tissues known as polytene chromosomes. This tissues go through rapid rounds of replication without cell division, an ideal location to study the effects of a polymerase who is responsible for the majority of replication. The genomic DNA content was reduced is the heterozygous mutants and more severely reduced in the homozygous mutants; however, the number of cells between the heterozygous and wild type was not significantly different. There were slightly fewer cells in the homozygous larvae which should be expected as these larvae are lethal at this stage. More emphasis has been put on the proteins that help regulate endoreplication

When investigating the rol of pol δ in response to mutagen treatments and its role in DNA damage repair, we obtained non-conclusive results. We hypothesized that the mutants would have a much harder time repairing DNA damage due to their defective polymerase; however, we saw an increase of these flies compared to wild type sibling controls. It is possible that the mutagens are causing more stalled forks which creates less work for the polymerases. On hypothesis on the results we obtained in which we are still investigating is that the possession of a balancer chromosome reduces mutagen viability hence for the decrease in wild type sibling controls. These results show much more investigation is needed in the area of DNA repair in regards to pol δ.

To investigate pol δ's role with other replication proteins, we crossed the mutant strains to mutant strains of pol δ-PCNA and pol δ-pol α. All four of these fly lines were viable. This brings in the question again of the recessive nature of this allele. This once again plays into the idea that the cell has developed a complex mechanism of compensation to prepare itself for any damages to its most precious replication machinery. This initial characterization arises many

questions and presents great opportunities to study double mutants with pol δ such as PCNA, pol α, pol ε and even others such as RFC and FEN1. However, time constraints prevented further analysis into these new avenues.

Overall, we have shown that pol δ is essential to viability and that dosage is important not only for the canonical cell cycle but also endoreplication. This pioneering study opens up many doors to continue investigating the enginmatic enzyme in the context of a multicellular organism and how this polymerase operates in regards to replication.

REFERENCES

ABOUSSEKHRA A., M. BIGGERSTAFF, M. K. K. SHIVJI, J. A. VILPO, V. MONCOLLIN, V. N. PODUST, M. PROTID, U. HÜBSCHER, J.-M. EGLY and R. D. WOOD. 1995. Mammalian DNA Nucleotide Excision Repair Reconstituted with Purified Protein Components. Cell **80**: 859-868.

APGER, J. A., M. REUBENS, L. HENDERSON, C. A. GOUGE, N. ILIC, H. H. ZHOU and T. W. CHRISTENSEN. 2010. Multiple Functions for *Drosophila* Mcm10 Suggested Through Analysis of Two Mcm10 Mutant Alleles. Genetics **185**: 1151–1165.

ASHBURNER, M., K. G. GOLIC, and R. S. HAWLEY. 2005. Drosophila: A Laboratory Handbook, 2nd Edition. Cold Spring Harbor Labatory Press, Cold Spring Harbor, NY.

AYYAGARI, R., X. V. GOMES, D. A. GORDENIN, and P. M. J. BURGERS. 2003. Okazaki Fragment Maturation in Yeast I. Distribution of Functions between FEN1 and DNA2. The Journal of Biological Chemistry **278**: 1618–1625.

BARTEK, J. and J. LUKAS. 2001. Pathways Governing G1/S transition and their Response to DNA damage. FEBS Letters **490**: 117-122.

BELL, S. P. and A. DUTTA. 2002. DNA Replication in Eukaryotic Cells. Annual Reviews in Biochemistry **71**: 333-74.

BRIDGES, B. 1999. DNA repair: Polymerases for Passing Lesions. Current Biology 1999, **9:** R475-R477.

BROSH, R. M. and V. A. BOHR. 2007. Human Premature Aging, DNA and RecQ Helicases. Nucleic Acid Research 35: 7527-7544.

CAMPBELL, N. B. and J. A. REECE. 2002. Biology, 7th Ed. Benjamin Cummings, San Fransico, CA.

CHATTOPADHYAY, S., and A. K. BIELINSKY. 2007. Human Mcm10 regulates the catalytic subunit of DNA polymerase-alpha and prevents DNA damage during replication. Molecular Biology of the Cell **18**: 4085–4095.

CHIANG, C.S. and I. R. LEHMAN. 1995. Isolation and sequence determination of the cDNA encoding DNA polymerase from Drosophila melanogaster. Gene **166**: 237-242.

CHRISTENSEN, T. W., and B. K. TYE. 2003. *Drosophila* MCM10 interacts with members of the prereplication complex and is required for proper chromosome condensation. Molecular Biology of the Cell **14**: 2206–2215.

DAS-BRADOO, S., R. M. RICKE and A. K. BIELINSKY. 2006. Interaction between PCNA and diubiquitinated Mcm10 is essential for cell growth in budding yeast. Molecular Cell Biology **26**: 4806–4817.

DEPAMPHILIS, M.L. 1996. Origins of DNA Replication. In: DNA Replication in Eukaryotic Cells (DEPAMPHILIS, M.L., Ed.), pp. 45-86. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

DOUGLAS, N. L., S. K. DOZIER and J. J. DONATO. 2005. Dual roles for Mcm10 in DNA replication initiation and silencing at the mating type loci. Molecular Biology Rep. **32**: 197–204.

FLOHR T., J.-C. DAI, J. BÜTTNER, O. POPANDA, E. HAGMÜLLER and H. W. THIELMANN. 1999. Detection of Mutations in the DNA polymerase δ Gene of Human Sporadic Colorectal Cancers and Colon Cancer Cell Lines. Int. J. Cancer **80**: 919-929.

FRIEDBERG, E. C., G. C. WALKER and W. SIEDE. 1995. DNA Repair and Mutagenesis. American Society of Microbiology, Washington, D.C.

FRICK, D. N. and C. C. RICHARDSON. 2001. DNA Primases. Annual Reviews in Biochemistry

7**0**: 39-80.

GARG, P. and P. M. J. BURGERS. 2005. DNA Polymerases that Propagate the Eukaryotic DNA Replication Fork. Critical Reviews in Biochemistry and Molecular Biology **40**:115–128.

GARG, P., C. M. STITH, N. SABOURI, E. JOHANSSON, and P. M. BURGERS. 1994. Idling by DNA polymerase δ maintains a ligatable nick during lagging-strand DNA replication. Genes & Development **18**: 2764-2773.

GOLDSBY, R. E., L. E. HAYS, X. CHEN, E. A. OLMSTED, W. B. SLAYTON, G. J. SPANGRUDE and B. D. PRESTON. 2002. High Incidence of Epithelial Cancers in Mice Deficient for DNA polymerase δ Proofreading. PNAS **99**: 15560-15565.

GOLDSBY, R. E., N. A. LAWRENCE, L. E. HAYS, E. A. OLMSTED, X. CHEN, MALLIKA SINGH, and B. D. PRESTON. 2001. Defective DNA Polymerase-δ Proofreading causes Cancer Susceptibility in Mice. Nature Medicine **7**: 638-639.

HANADA, K. and I. D. HICKSON. 2007. Molecular Genetics of RecQ Helicases. Cell Molecular Life Sciences **64**:2306-2322.

HARTWELL, L. H. and M. B. KASTAN. 1994. Cell Cycle Control and Cancer. Science **266**: 1821-1828.

HINDGES, R. and U. HÜBSCHER. 1997. DNA polymerase delta, an essential enzyme for DNA transactions. Biological Chemistry **378**: 345-362.

HOELLER, D., C.-M. HECKER and I. DIKIC. Ubiquitin and Ubiquitin-like Proteins in Cancer Pathogenesis. Nature Cancer Reviews **6**:776-778.

HOKI, Y., R. ARAKI, A. FUJIMORI, T. OHHATA, H. KOSEKI, R. FUKUMURA, M. NAKAMURA, H. TAKAHASHI, Y. NODA, S. KITO, M. ABE. 2003. Growth retardation and skin

abnormalities of the RecQL4-deficient mouse. Human Molecular Genetics **12**:2293-2299.

HOLMES, A. M. and J. E. HABER. 1999. Double-Strand Break Repair in Yeast Requires Both Leading and Lagging Strand DNA Polymerases. Cell **96**: 415-424.

HÜBSCHER, U., G. MAGA, and S. SPADARI. 2000. Eukaryotic DNA Polymerases. Annual Reviews in Biochemistry **71**: 133-63

JACKSON S. M., A. J. WHITWORTH, J. C. GREENE, R. T. LIBBY, S. L. BACCAM, L. J. PALLANCK and A. R. LA SPAD. 2005. A SCA7 CAG/CTG repeat expansion is stable in *Drosophila melanogaster* despite modulation of genomic context and gene dosage. Gene **347**: 35-41.

KANAAR R., J. H. J. HOEIJMAKERS and D. C. VAN GENT. 1998. Molecular Mechanisms of DNA Doublestrand Break Repair. Trends in Cell Biology **8**: 483-489.

KAWASAKI, Y., S. HIRAGA and A. SUGINO. 2000. Interactions between Mcm10p and other replication factors are required for proper initiation and elongation of chromosomal DNA replication in *Saccharomyces cerevisiae*. Genes Cells **5**: 975–989.

KELMAN, Z. and M. O'DONNELL. 1995. DNA Polymerase III Holoenzyme: Structure and Function of a Chromosomal Replicating Machine. Annual Reviews in Biochemistry **64**:171-200.

KOLODNER, R. D. and G. T. MARSISCHKY. 1999. Eukaryotic DNA Mismatch Repair. Current Opinion in Genetics & Development **9**:89–96.

KRAUS E., W.-Y. LEUNG and J. E. HABER. 2001. Break-Induced Replication: A Review and an Example in Budding Yeast. PNAS **98**: 8255-8262.

LIACHKO, I., and B. K. TYE. 2005. Mcm10 is required for the maintenance of transcriptional silencing in *Saccharomyces cerevisiae*. Genetics **171**: 503–515.

LIACHKO, I., and B. K. TYE. 2009. Mcm10 mediates the interaction between DNA replication and silencing machineries. Genetics **181**: 379–391.

LINDAHL, T. and R. D. WOOD. 1999. Quality Control by DNA Repair. Science **286**: 1987-1905.

LOEB, L. A. 2001. A Mutator Phenotype in Cancer. Cancer Research **61**: 3230-3239.

LUNDIN, C., M. NORTH, K. ERIXON, K. WALTERS, D. JENSSEN, A. S. H. GOLDMAN, and T. HELLEDAY. 2005. Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable in vivo DNA double-strand breaks. Nucleic Acids Research **33**: 3799-3811.

MAGA, G., M. STUCKI, S. SPADARI and U. HÜBSCHER. DNA Polymerase Switching: I. Replication Factor C Displaces DNA Polymerase a Prior to PCNA Loading. Journal of Molecular Biology **295**: 791-801.

MAGA, G. and U. HÜBSCHER. 2003. Proliferating Cell Nuclear Antigen (PCNA): A Dancer with Many Partners. Journal of Cell Science **116**: 3051-3060.

MANN, M. B., C. A. HODGES, E. BARNES, H. VOGEL, T. J. HASSOLD and G. LUO. 2005. Defective sister-chromatid cohesion, aneuploidy and cancer predisposition in a mouse model of type II Rothmund–Thomson syndrome. Human Molecular Genetics **14**:813-825.

MERCHANT, A. M., Y. KAWASAKI, Y. CHEN, M. LEI and B. K. TYE. 1997. A lesion in the DNA replication initiation factor Mcm10 induces pausing of elongation forks through

chromosomal replication origins in *Saccharomyces cerevisiae*. Molecular Cell Biology **17**: 3261–3271.

MICHEL, B., G. GROMPONE, M.-J. FLORÈS and V. BIDNENKO. 2004. Multiple pathways process stalled replication forks. PNAS 101: 12783-12788.

MODRICH, P. 1991. Mechanisms and Biological Effects of Mismatch Repair. Annual Reviews in Genetics **25**: 229-253.

MOLDOVAN, G.-L., B. PFANDER and S. JENTSCH. 2007. PCNA, the Maestro of the Replication Fork. Cell **129**: 665-679.

MOSELEY, J. B., A. MAYEUX, A. PAOLETTI and P. NURSE. 2009. A Spatial Gradient Coordinates Cell Size and Mitotic Entry in Fission Yeast. Nature **459**: 857-861.

MURAKAMI, Y. and J. HURWITZ. 1993. Functional Interactions between SV40 T Antigen and Other Replication Proteins at the Replication Fork. The Journal of Biological Chemistry **15**: 11008-11017.

NETHANEL, T., S. REISFELD, G. DINTER-GOTTLIEB, and G. KAUFMANN. 1988. An Okazaki Piece of Simian Virus 40 May Be Synthesized by Ligation of Shorter Precursor Chains. Journal of Virology **62**: 2867-2873

NURSE, P. 2000. A Long Twentieth Century of the Cell Cycle and Beyond. Cell, **100**: 71–78.

PFLUMM, M.F. and M. R. BOTCHAN. 2001. Orc mutants arrest in metaphase with abnormally condensed chromosomes. Development **128**: 1697-1707.

PORTER, L. A. and D. J. DONOGHUE. 2003. Cyclin B1 and CDK1: Nuclear Localization and Upstream Regulators. Progress in Cell Cycle Research **5**: 335-347.

STUCKI, M., I. STAGLJAR, Z. O. JONSSON, and U. HÜBSCHER. 2000. A Coordinated Interplay: Proteins with Multiple Functions in DNA Replication, DNA Repair, Cell

Cycle/Checkpoint Control, and Transcription. Progress in Nucleic Acid Research and Molecular Biology **65**: 261-298.

STUCKI, M., B. PASCUCCI, E. PARLANTI, P. FORTINI, S. H. WILSON, U. HÜBSCHER and E. DOGLIOTTI. 1998. Mammalian Base Excision Repair by DNA Polymerases δ and ε. Oncogene **17**: 835-843.

SWAN, M. K., R. E. JOHNSON, L. PRAKASH, S. PRAKASH and A. K. AGGARWAL. 2009. Structural basis of high-fidelity DNA synthesis by yeast DNA polymerase δ. Nature Structural & Molecular Biology **16**: 979-987.

TSURIMOTO, T. and B. STILLMAN. 1968. Replication Factors Required for SV40 DNA Replication in Vitro (II. Switching Of DNA Polymerase α and δ During Initiation of Leading and Lagging Strand Synthesis). The Journal of Biological Chemistry 3: 1961-1968.

TYE, B. K. 1999. MCM proteins in DNA replication. Annual. Reviews in Biochemistry **68**: 649–686.

VENKATESAN, R. N., P. M. TREUTING, E. D. FULLER, R. E. GOLDSBY, T. H. NORWOOD, T. A. GOOLEY, W. C. LADIGES, B. D. PRESTON, and L. A. LOEB. 2007. Mutation at the Polymerase Active Site of Mouse DNA Polymerase δ Increases Genomic Instability and Accelerates Tumorigenesis. Molecular and Cellular Biology **27**: 7669-7682.

WAGA, S., G. BAUER and B. STILLMAN. 1994. Reconstitution of Complete SV40 DNA Replication with Purified Replication Factor. Journal of Biological Chemistry **269**: 10923-10934.

WAGA, S. and B. STILLMAN. 1998. The DNA Replication Fork in Eukaryotic Cells. Annual Reviews in Biochemistry **67**: 721-751.

WANG, Z., X. WU and E. C. FRIEDBERG. 1993. DNA Repair Synthesis during Base Excision Repair *In Vitro* Is Catalyzed by DNA Polymerase ε and Is Influenced by DNA Polymerases α and δ in *Saccharomyces cerevisiae*. Molecular and Cellular Biology **13**: 1051-1058.

WEAVER, R. F. 2008. Molecular Biology. McGraw Hill, New York, NY.

WU, J., C. CAPP, L. FENG, T.-S. HSIEH. 2008. *Drosophila* homologue of the Rothmund-Thomson syndrome gene: Essential function in DNA replication during development. Development Biology **323**:130-142.

XU, Y., Z. LEI, H. HUANG, W. DUI, X. LIANG, J. MA, R. JIAO. 2009. dRecQ4 Is Required for DNA Synthesis and Essential for Cell Proliferation in Drosophila. PLoS ONE **4**: 1-13.

XU, X., P. J. ROCHETTE, E. A. FEYISSA, T. V. SU, and Y. LIU. 2009. MCM10 mediates RECQ4 association with MCM2-7 helicase complex during DNA replication. EMBO Journal **28**: 3005-3014.

APPENDIX A: UNDERSTANDING THE SIGNIFICANCE OF THE MCM10-RECQ4 INTERACTION IN *DROSOPHILA MELANOGASTER*

Rothmund-Thompson syndrome (RTS) is characterized by premature aging, skeletal abnormalities, and a pre-disposition to cancer. RTS is the result of a mutation in the DNA helicase RECQ4. Work in human tissue culture and mouse models has suggested that RTS results from defects in DNA replication and maintenance of genomic integrity. Mcm10 is the proposed molecule that links RecQ4 to these processes. Genetic and phenotypic analysis of MCM10/RECQ4 double mutants in *Drosophila* promises to shed light on the importance of RecQ4 and its interaction with Mcm10 in DNA replication and maintenance of genomic integrity and ultimately inform treatment of RTS in humans.

1. INTRODUCTION

RecQ4 is a DNA helicase that is a member of the RecQ family. This family of helicases plays an important role in maintaining genomic integrity. Mutations of this helicase in humans have been linked to three rare syndromes: Rothmund-Thomson syndrome, RAPADILNO syndrome, and Baller-Gerold syndrome. These syndromes have a predisposition to cancer in addition to premature aging.

Of particular interest is RTS which is characterized by premature aging, skeletal abnormalities, and a pre-disposition to cancer (HANADA and HICKSON 2007). RTS symptoms have been recapitulated in mouse models where RecQ4 has been deleted. Curiously, defects in RecQ4 result in phenotypes unlike those observed in other RecQ family members (BROSH and BOHR 2007). Mutations in other family members result in mice that are defective in DNA repair pathways whereas mice deficient for RecQ4 do not appear to be significantly defective in DNA repair pathways. This discrepancy demands an alternative explanation for the RTS like symptoms observed in RecQ4 deficient mice (HOKI *et al.* 2003; MANN *et al.* 2005). It is likely that the RTS-like growth retardation and genomic instability observed in the mouse model may be a function of RecQ4 playing a role in cell cycle progression through involvement in DNA replication. Recent biochemical work using human cell extracts has demonstrated that RecQ4 associates with MCM2-7 replicative helicase complex in an MCM10 dependent manner (XU *et al.* 2009). This observation suggests that Mcm10 may provide the critical link between RecQ4 and its role in DNA replication.

Mcm10 is a highly conserved protein that was identified in *S. cerevisiae* in the same mini-chromosome maintenance assay that yielded the well-studied Mcm2-7 proteins (MERCHANT *et al.* 1997; TYE 1999). Temperature sensitive *mcm10* mutants in yeast arrest in S

phase with a 2C DNA content. At permissive temperatures these mutants are characterized by excessive pausing of replication forks at unfired origins of replication (MERCHANT *et al.* 1997). Further studies have firmly established a role for Mcm10 in replication. It has been shown to interact with members of the pre-replication complex and elongation complex (CHATTOPADHYAY and BIELINSKY 2007; CHRISTENSEN and TYE 2003; DAS-BRADOO *et al.* 2006). Curiously, like the Mcm2-7 proteins, Mcm10 is exceptionally abundant in eukaryotic cells with nearly 40,000 molecules per haploid yeast cell (KAWASAKI *et al.* 2000). A number of studies have suggested the only a subset of the Mcm10 present in the cell may be utilized in DNA replication processes. In *S. cerevisiae*, a portion of the Mcm10 protein pool is diubiquitinated. This modified form of Mcm10 participates in an interaction with PCNA that is essential for cell proliferation (DAS-BRADOO *et al.* 2006). Also suggesting that the majority of Mcm10 does not participate in essential processes is the observation that *Drosophila* tissue culture cells that are depleted of Mcm10 by RNAi continue to proliferate even with very low levels of Mcm10 (CHRISTENSEN and TYE 2003). Additionally, recent evidence has been uncovered which points to an involvement of Mcm10 in chromatin structure. Work using *S. cerevisiae* has demonstrated that Mcm10 is involved in transcriptional repression of the mating type loci and links DNA replication proteins to heterochromatin formation (DOUGLAS *et al.* 2005; LIACHKO and TYE 2005; LIACHKO and TYE 2009). Also pointing to a possible role in chromatin structure and chromosome condensation is that the depletion of Mcm10 in *Drosophila* tissue culture cells results in under-condensed metaphase chromosomes (CHRISTENSEN and TYE 2003).

This new interaction between Mcm10 and RecQ4 (uncharacterized in the context of an *in vivo* multicellular organism) is very intriguing. Using a combination of novel mutants for these

proteins, two mutants defective for RecQ4 and two mutants defective for Mcm10, this interaction will be further investigated in *Drosophila melanogaster*.

RecQ4 fly strains were kindly donated by Dr. Tao-shih Hsieh. $RecQ4^{19}$ is a null mutant while $RecQ4^{23}$ is a hypomorph (Figure A), both which are homozygous lethal. RecQ4 in *Drosophila* was shown to have an expression peak during S-phase and also be required for efficient endoreplication. In addition, it was also shown to be essential for viability, larval development and cell proliferation. However, more advanced functions such as its cellular and biochemical functions have yet to be elucidated (WU *et al.* 2008).

$Mcm10^{Scim19}$ is a hypomorph while $Mcm10^{d08029}$ is a truncation of the last 85 amino acids. Both of these alleles are homozygous viable; however, they are semi-lethal with decreased viability of the homozygous flies. In addition, the c-terminal end of Mcm10 has been shown to interact with the Mcm2-7 helicase complex. Since defects in endoreplication were shown in only the $Mcm10^{d08029}$ mutant, it is proposed that this last 85 aa are important not only for the interaction with Mcm2-7 but also for endoreplication and these might be linked. Multiple other results have shown that Mcm10 mutants have defects in progression from S-phase and also involved in chromosome condensation as evident from problems in ovariole development (APGER *et al.* 2010).

These characterized mutants for RecQ4 and Mcm10 provide an excellent opportunity to study the effects of the double mutants. Interestingly, it is possible that there might be some type of genetic suppression associated with these two proteins; however, much more investigation is still needed.

2. MATERIALS AND METHODS

FLY HUSBANDRY/ STOCKS:

All fly stocks were maintained on *Drosophila* K12 media (US Biological # D9600-07B) at room temperature.

Mcm10: Fly stock $Mcm10^{Scim19}$ (Stock 0233, Flybase ID: FBst0013070) y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}Mcm10[KG00233] was obtained from the Bloomington *Drosophila* Stock Center. $Mcm10^{d08029}$ (Flybase ID: FBst1011557) P{XP}Mcm10[d08029] was obtained from Exelixis *Drosophila* Stock Collection at Harvard Medical School. Previous work in the Christensen Lab verified the *Mcm10* P element insertions by PCR. The *Mcm10* lines were both backcrossed >7 times to w; Df(2L), b[82-2] / CyO to remove unwanted second site mutations.

RecQ4: Fly stocks $RecQ4^{19}$/TM3,Sb and $RecQ4^{23}$/TM3,Sb were kindly provided by Dr. Tao-shih Hsieh (Duke University).

OVARY DISSECTION:

Flies 3-7 days post eclosion were fed on yeast paste for 2 days. Ovaries were extracted from female wild-type and mutant flies in PBS. Ovarioles were teased apart, then fixed in 4% Formaldehyde PBX (PBS + 0.1% Triton X-100) for 20 min. After fixing, ovaries were stained for 5 min with 1ug/mL DAPI in PBS. Ovaries were then washed 3 times for 5 minutes in PBX, followed by a 1 hour PBX wash, and 3 10 minute PBX washes. Finally, ovaries were mounted using Vectashield™ and imaged using confocal optical sectioning microscopy.

For all other **MATERIALS AND METHODS**, please refer to pages 15-23.

3. RESULTS

COMPLEMENTATION CROSS WITH RECQ4 MUTANTS

RecQ4^{19} (null allele) and RecQ4^{23} (hypomorphic allele) were crossed together to determine if flies could harbor both mutations (**FIGURE A3.01**). As expected from the lethality of the homozygous null and the lethality of the homozygous hypomorph, we obtained no progeny harboring both of the mutations.

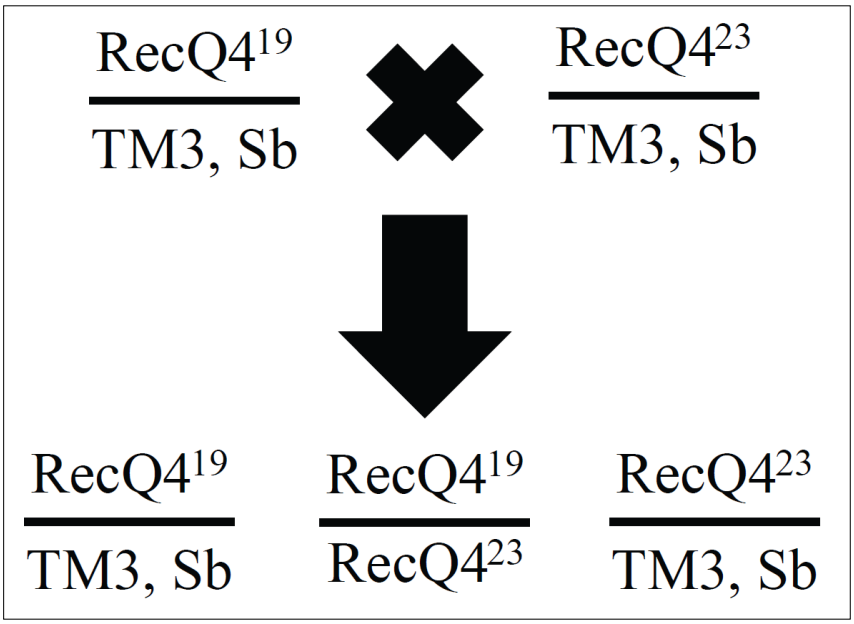

FIGURE A3.01 - RECQ4 COMPLEMENTATION CROSS – Flies were not viable with a copy of each mutated copy of RecQ4.

GENERATION OF BALANCED RECQ4 STRAINS

RecQ4 mutant fly strains ($RecQ4^{19}$ and $RecQ4^{23}$) were originally balanced over TM3, Sb, which is a third chromosome balancer. In order to cross to the Mcm10 mutant fly strains, strains had to be created which had visible markers on the second chromosome (**FIGURE A3.02**). The final progeny (ap^{Xa}/CyO; $RecQ4^{mut}$) were self crossed and kept as a stock.

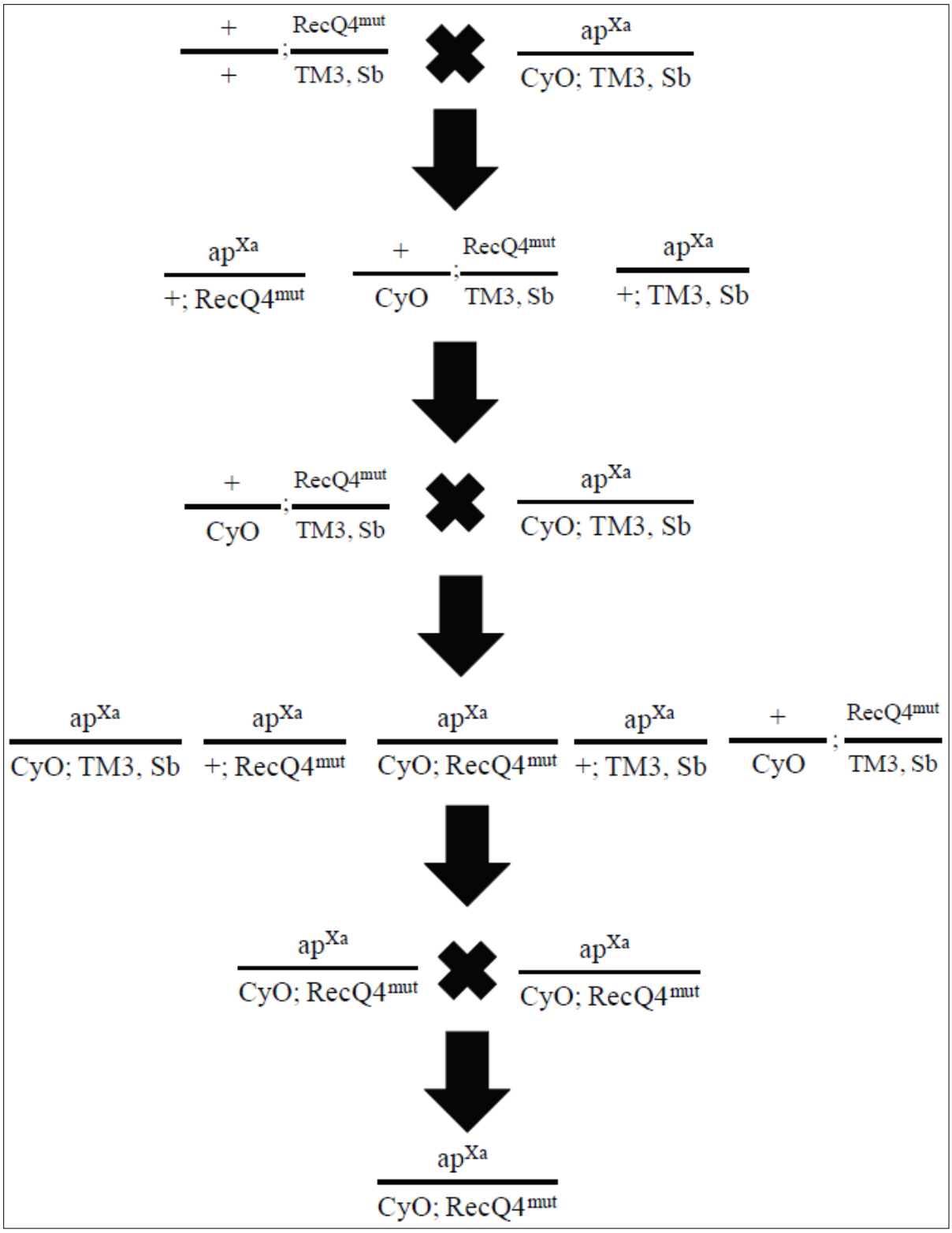

FIGURE A3.02 - NEW BALANCED RECQ4 CROSS – Cross scheme to generate differently balanced RecQ4 flies for use with subsequent crosses.

GENERATION OF DOUBLE MUTANTS

Flies with the $Mcm10^{d08029}$ mutation were crossed to flies with the $RecQ4^{mut}$ (either $RecQ4^{19}$ or $RecQ4^{23}$) to generate flies with genotype $Mcm10^{d08029}$; $RecQ4^{mut}$/CyO; TM3, Sb (**FIGURE A3.03**). Another cross with the $Mcm10^{Scim19}$ allele and RecQ4muts was performed to generate flies similar in nature to the $Mcm10^{d08029}$-$RecQ4^{mut}$ double mutant. Their genotype was $Mcm10S^{Scim19}$; $RecQ4^{mut}$ / CyO; TM3, Sb (**FIGURE A3.04**). This generated a total of 4 different heterozygous flies – 1. $Mcm10^{d08029}$; $RecQ4^{19}$/CyO; TM3, Sb, 2. $Mcm10^{d08029}$; $RecQ4^{23}$/CyO; TM3, Sb, 3. $Mcm10^{Scim19}$; $RecQ4^{19}$/CyO; TM3, Sb, and 4. $Mcm10^{Scim19}$; $RecQ4^{23}$/CyO; TM3, Sb. These four different fly strains were then self crossed with their respective sibling with same genotypes (**FIGURE A3.05**). Interestingly, in all four self crosses, the lethality of the $RecQ4^{mut}$ was suppressed and all four genotypes were present in the F1 progeny at amazingly almost Mendenlian ratios.

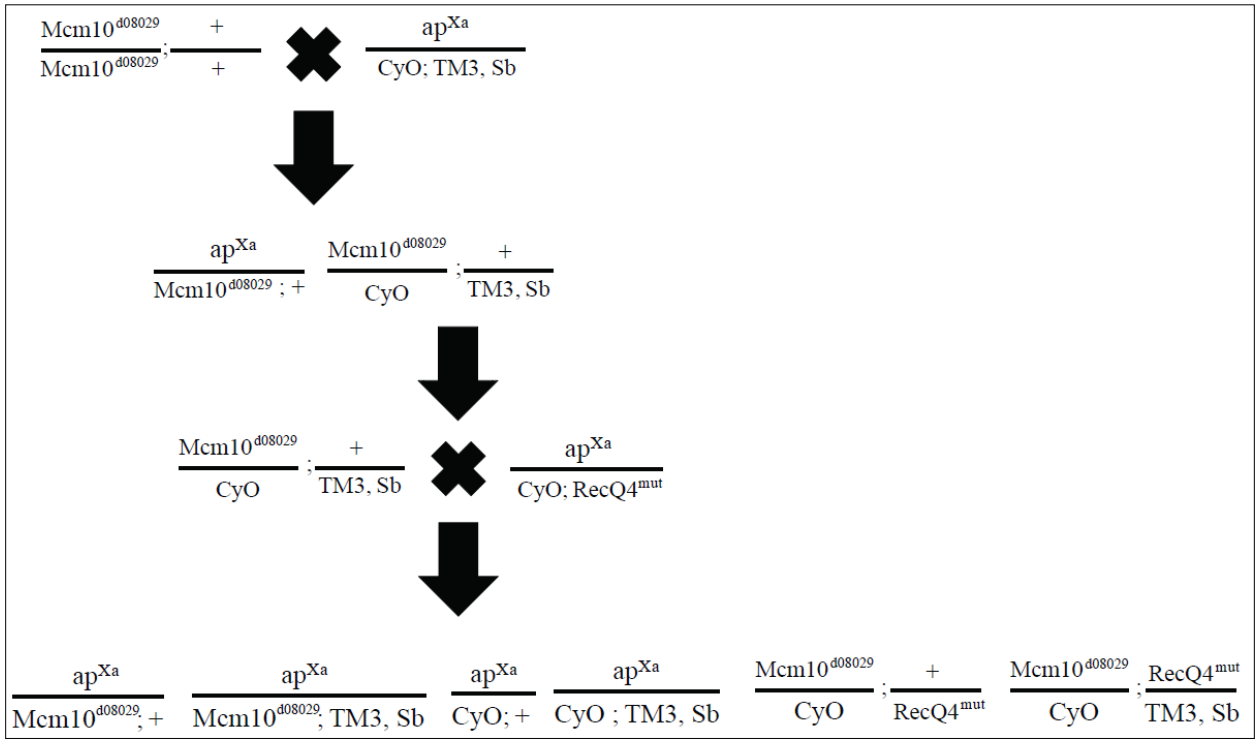

FIGURE A3.03 – GENERATION OF MCM10^{D08029} AND RECQ4^{MUT} DOUBLE MUTANT CROSS - Cross scheme generating Mcm10^{d08029} and RecQ4^{mut} which would later be self crossed.

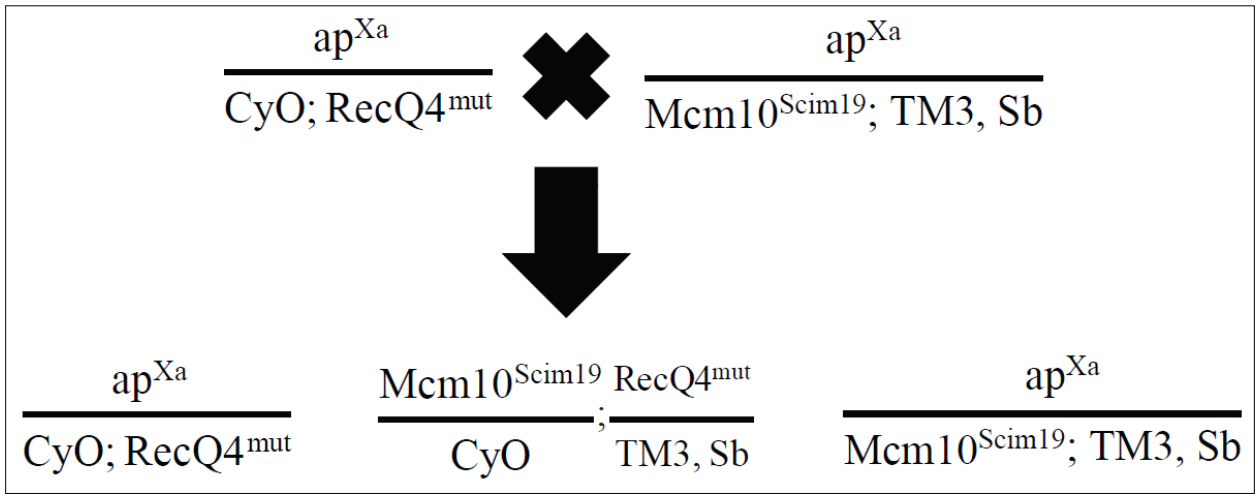

FIGURE A3.04 – GENERATION OF MCM10^{SCIM19} AND RECQ4^{MUT} DOUBLE MUTANT CROSS – Cross scheme generating Mcm10^{Scim19} and RecQ4^{mut} which would later be self crossed.

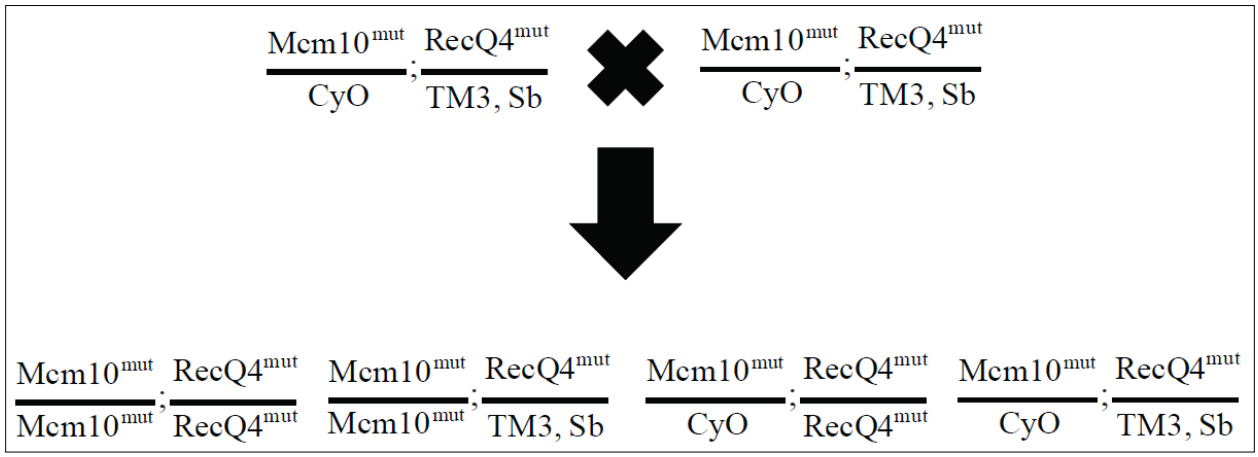

FIGURE A3.05 – DOUBLE MUTANT SELF CROSS – As expected mutants heterozygous and homozygous for Mcm10^{mut} and heterozygous for RecQ4^{mut} eclosed as well as mutants also homozygous for RecQ4.

MITOTIC INDICES

Previous work showed a mitotic delay in the Mcm10^{muts}. However, the mitotic index was never published for the RecQ4^{muts}. Here, I confirm the mitotic indices from the Mcm10^{muts} (heterozygous) and also show that RecQ4^{muts} (heterozygous) both have less mitotic figures as compared to wild type (**FIGURE A3.06**).

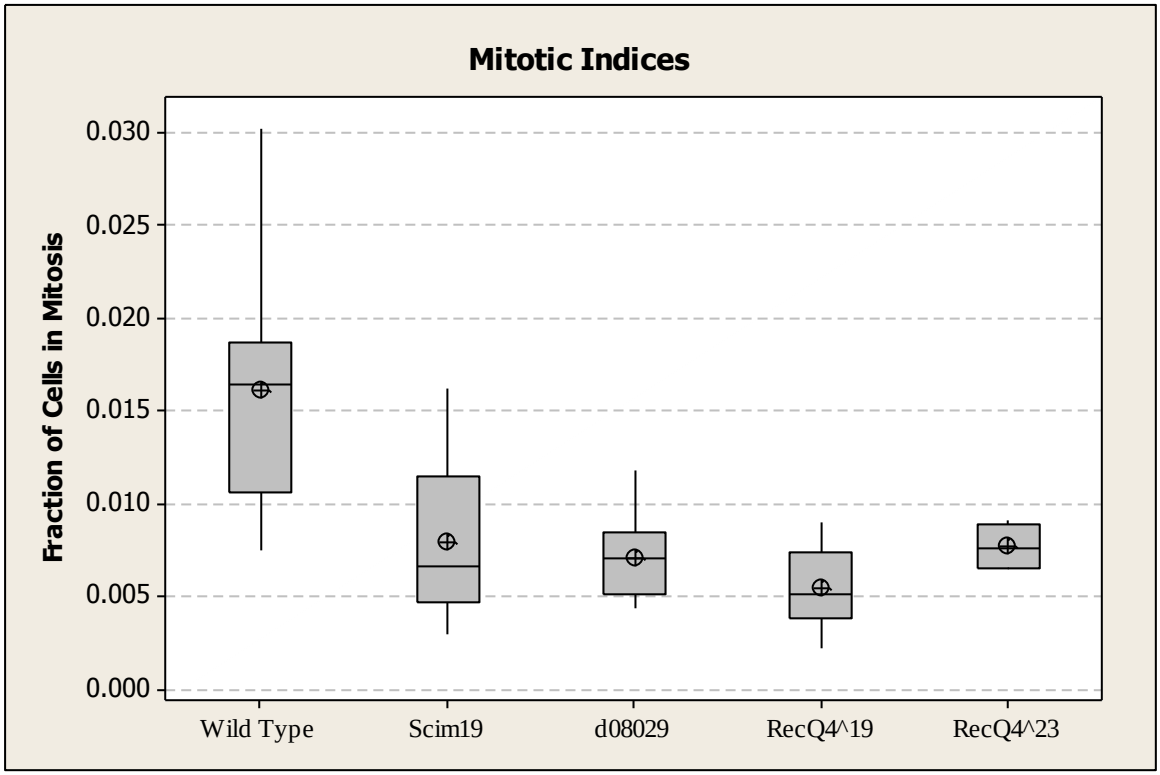

FIGURE A3.06 – MITOTIC INDICES FROM SINGLE MUTANTS – All single mutants (heterozygous) show less mitotic figures compared to wild type.

MISCELLANEOUS RESULTS

Due to the unexpected progeny arising from the self crosses, it was difficult to perform any analysis of any of the double mutants. Future work includes balancing these mutants over balancer chromosomes linked with different fluorescent markers in order to sort appropriate larvae before other markers (in the adult fly) are present. However, during analysis of female adult ovaries, it was apparent that there was a high frequency of abnormal chromosomes in the ovarioles (**FIGURE A3.07**). This result still requires more attention.

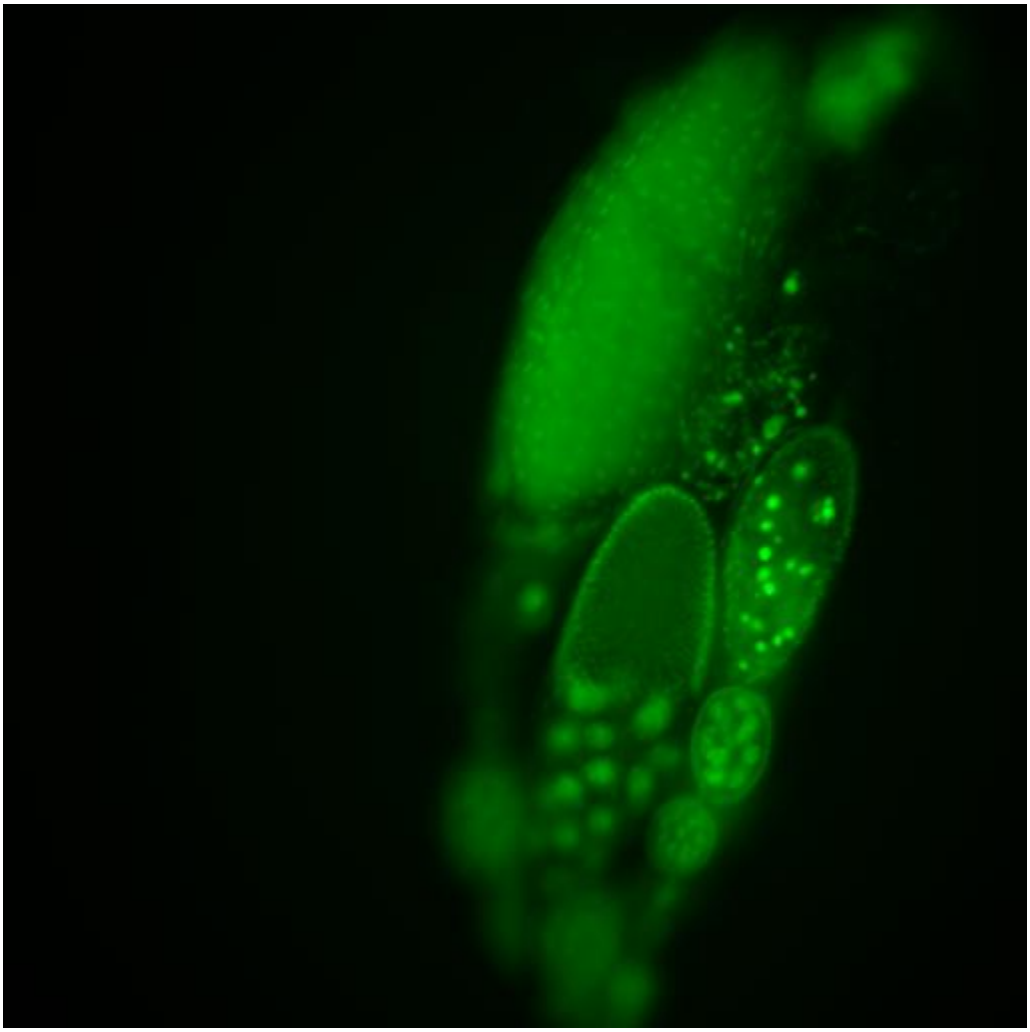

FIGURE A3.07 – OVARY – Homozygous RecQ4^{19} and Homozygous Mcm10^{Scim19} (along with other mutants) display some type of malformation in the ovaries.

4. DISCUSSION

Obviously, the striking revelation that a lower levels of Mcm10 or a truncation of the c-terminal end of Mcm10 suppresses the lethality of a hypomorphic or null allele of RecQ4 is amazing. However, the preliminary results still have yet to be verified by PCR or western blotting but with four separate crosses (repeated in multiples) displaying similar genotypes, it is fairly certain that there is some type of genetic suppression occurring.

When levels of RecQ4 are decreased, there is a severe increase of genomic instability. Various mutations in RecQ4 in mice ranged from embryonic lethality to severe grow retardation; none of these mutations had positive outcomes (HOKI *et al.* 2003; MANN *et al.* 2005). When levels of Mcm10 are depleted in *Drosophila* tissue culture, cells continue to proliferate suggesting that Mcm10 may not be essential for viability but have secondary functions other DNA replication (CHRISTENSEN and TYE 2003).

In human tissue cultures, this Mcm10-RecQ4 interaction has begun to be investigated. Some interesting results from this study include that the most co-purified polypeptides were MCM10, followed by the MCM2-7 helicase complex, CDC45, and the GINS complex (composed of SLD5, PSF1, PFS2, and PSF3). Additionally, it was shown the RecQ4's interaction with these proteins is mediated by cell cycle progression and by Mcm10. Mcm10 additionally regulates RecQ4's helicase activity (XU *et al.* 2009).

With the results present from the generation of mutants harboring homozygous lethal mutation of RecQ4 with decreased levels of Mcm10, it suggests that the level of Mcm10 is important for the role of RecQ4 in the cell cycle. Additionally, without Mcm10 mediation, the role of RecQ4 is possibly dispensable or replaceable. Obviously, much more work needs to be

done to elucidate the function of this interaction but holds promising results for treatment of RTS in humans by altering the interaction of these two proteins.