Elucidating the multiple functions of POLDIP2 in *Drosophila melanogaster*

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Abstract

DNA polymerases are essential components of DNA repair processes. They depend on a variety of proteins to be recruited to the site of synthesis and to increase their processivity. Polymerase delta interacting protein 2 (POLDIP2) was identified as a novel protein that interacts with both polymerase δ and PCNA in the yeast two-hybrid system. POLDIP2 has also been shown in human cells and other yeast-two hybrid systems to interact with polymerase η, polymerase λ, and PrimPol, which are recruited to bypass lesions induced by external damage. In the case of pol η and λ, POLDIP2 increases their processivity and catalytic activity. POLDIP2 appears to be pleiotropic in nature as it has been implicated in a variety of cellular processes, including tau aggregation in human cells, mitotic spindle rearrangement in rat brain endothelial cells, and upregulating NADPH oxidases. Homozygous POLDIP2 knockout mice incur perinatal lethality and significantly lag in development in comparison to wildtype mice.

We are interested in the potential role POLDIP2 might play regarding polymerase recruitment and switching during DNA repair in Drosophila melanogaster. Complete deletion of the gene was induced via the CRISPR Cas-9 editing system and a DsRed marker was inserted into the POLDIP2 locus via homology-directed repair. Intriguingly, flies homozygous for the deletion incur lethality at the second-instar larval stage. We are currently utilizing several approaches to determine the genetic basis for this lethality. Crosses between knockout mutants and a P-element stock suggest that POLDIP2 might be essential for viability. However, rescue of the homozygotes is impeded by the presence of a second site lethal mutations. Mutagen sensitivity assays will be conducted with these mutants to better characterize the interplay between POLDIP2 and the DNA repair and replication machineries.

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Introduction

DNA replication and repair

All living organisms are comprised of a genome that functions as a repository of all the information needed to make essential proteins for living functions. The production of these proteins begins by replicating the genome to create a second copy to protect the original information from permanent damage. This process, known as DNA replication, is highly regulated to ensure the stability of both the parent and daughter strands. However, our cells are exposed to endogenous and exogenous mutagenic agents that can disrupt the process of replication in the form of lesions, fork stalling, and double-stranded breaks. Cell cycle check points to DNA repair pathways are employed to ensure faithful and efficient replication, even in the presence of these mutagenic agents.

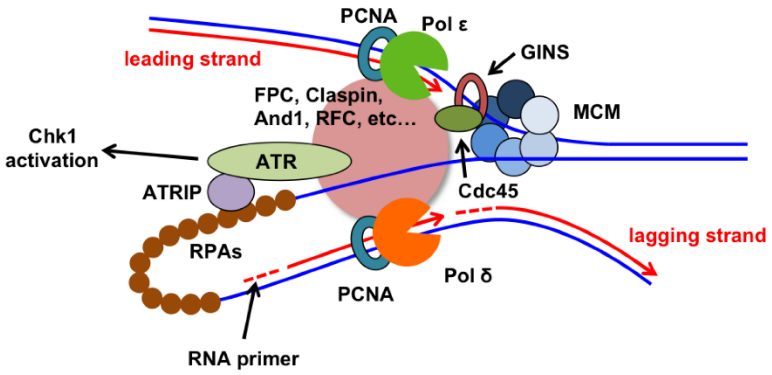

Figure 1. Components of a eukaryotic replication fork. A replication fork with leading and lagging strand synthesis is shown. Pol ε interacts with PCNA to conduct leading strand synthesis, while pol δ associates with PCNA to conduct lagging strand synthesis. Single-stranded DNA is covered by RPA. The MCM helicase complex unwinds DNA to allow for synthesis to occur. Image from Leman A. & Noguchi E. (2013).

DNA replication employs DNA polymerases to create new copies of DNA. DNA polymerases use a pool of free nucleotides (dNTPs) to synthesize new strands using the parent

strand as the template for the daughter strand. Sliding clamps increase the processivity of replicative DNA polymerases (eg. DNA polymerase δ), holding the polymerase to the template, and allowing for efficient synthesis of the daughter strand (Jonsson and Hubscher, 1997) (Figure 1). In the presence of DNA damage, however, these replicative polymerases often have issues replicating past bulky DNA lesions due to their size-specific catalytic sites. In these cases, the cell employs translesion synthesis (TLS) polymerases which can continue synthesizing DNA opposite a region of damaged DNA (Strzalka & Ziemienowicz, 2011). Sliding clamps, or proliferating cell nuclear antigen (PCNA), can assist in the loading or the recruitment of these TLS polymerases (eg. DNA polymerase ζ, η, and κ) (Strzalka & Ziemienowicz, 2011). This switch from replicative DNA synthesis to translesion synthesis is mediated by the monoubiquitination of PCNA at Lys164 (Chen et al., 2011). PCNA can also be polyubiquitinated or SUMOylated to trigger a variety of different DNA damage responses, demonstrating how critical the interaction between a polymerase and PCNA is in the replisome (Chen et al., 2011). Even though TLS polymerases are more error-prone compared to the replicative polymerases, the cell will tolerate these errors in exchange to continue replicating its genome.

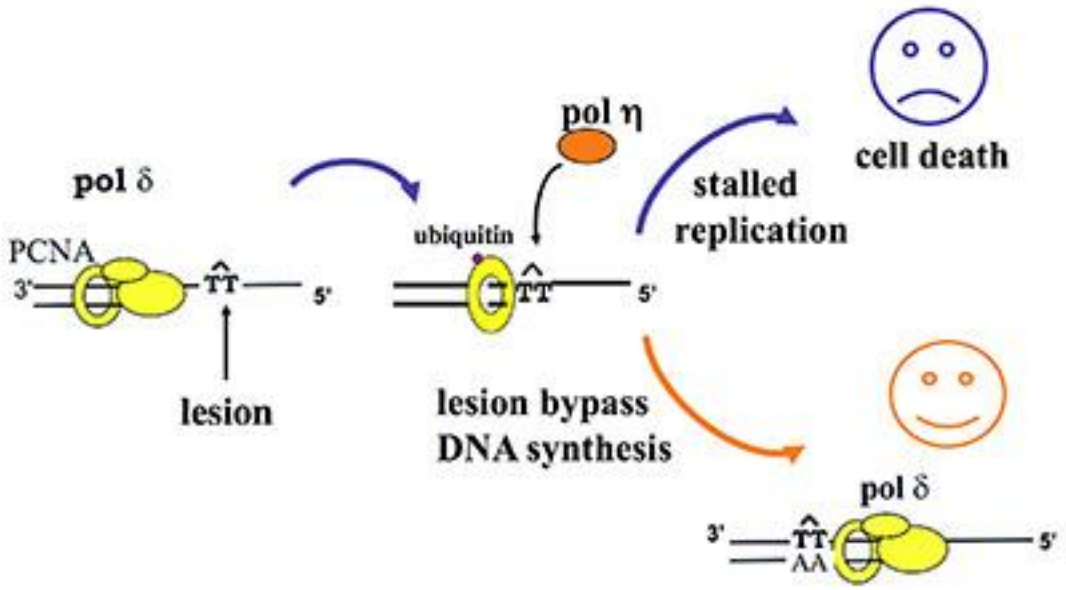

Figure 2. Switch from replicative polymerases to translesion synthesis polymerases. The polymerase-PCNA complex encounters a legion in the form of a thymine dimer and is unable to continue replication. Ubiquitination of PCNA allows for switch to a translesion synthesis polymerase, in this case, polymerase η. This switch allows for the region genome shown to be replicated in an error-prone manner. Failure to recruit a translesion synthesis polymerase can lead to genome instability and eventually, cell death (Yang Lab, NIH).

POLDIP2: A novel protein interactor with polymerase δ

POLDIP2, or polymerase delta interacting protein 2, was identified in a yeast-two hybrid screen, which attempted to characterize novel proteins that associated with polymerase δ (Liu et al., 2003). The 368-aa protein was characterized as PDIP38 due to its molecular weight of 38 kiloDaltons. It contains an N-terminal mitochondrial localization sequence (Xie et al., 2005) and two conserved domains: an ApaG/F-box A domain and a hemimethylated DNA binding domain called YccV (Brown et al., 2014). Polymerase δ is one of the main replicative polymerases, specifically involved with synthesis of the lagging strand during replication (Lee et al., 2017) but also exhibits the ability to also conduct leading strand synthesis in the absence of catalytically functional polymerase ε in yeast (D'Urso and Feng, 2001, Figure 1). POLDIP2 was also found to

interact with PCNA in the same study done by Liu et al, an observation that contextualizes POLDIP2 as being a potential participant in DNA replication and/or repair. POLDIP2 binds to the p50 subunit of polymerase δ, encoded by *pol31* in *S.cerevisiae*, while PCNA binds to polymerase δ via the p68 subunit, encoded by *pol32* (Lee et al., 2017). PCNA can also bind to p50 subunit, however, this interaction is much weaker in comparison to that of p68. Even though they bind to different subunits, the 3D structure of the protein structures would not allow simultaneous binding.

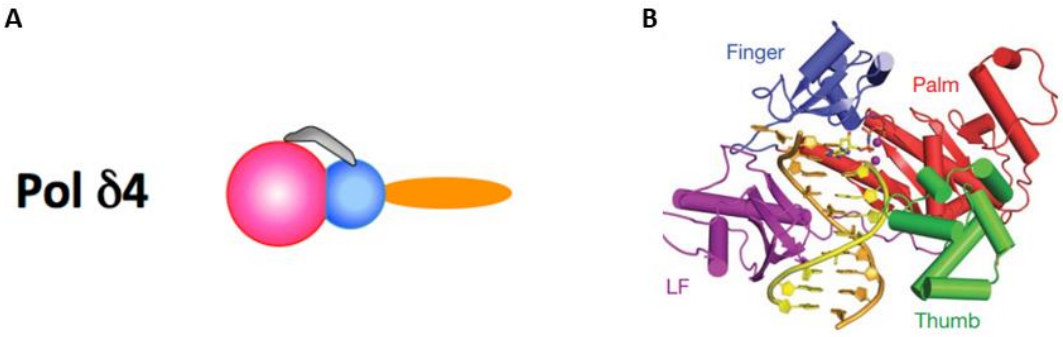

Figure 3. Subunits of polymerases δ and η. A) Polymerase δ comprised of its four subunits. POLDIP2 has been shown to interact with the p50 subunits, shown in blue, while PCNA has been shown to have some activity with all four subunits (Wang et al., 2011). Figure adapted from Lee et al., 2017. B) Polymerase η interacting with a molecule of DNA. POLDIP2 has been reported to interact with the finger domain of this polymerase (Tissier et al., 2010). Figure adapted from Biertumpfel et al., 2010.

POLDIP2 Interacts with a Variety of Polymerases

POLDIP2 is a potential player in DNA replication and/or repair due to its protein interactions with polymerase δ, PCNA, and other polymerases. One of the polymerases that POLDIP2 has been found to interact with is polymerase η. Polymerase η has been shown to localize to lesions sites containing pyrimidine dimers (TT dimers) and allows for synthesis

across the lesion (Kusumoto et al., 2004). However, DNA synthesis via polymerase η is restricted to these sites as its fidelity is extremely low compared to the classical replicative polymerases (Kusumoto et al., 2004). POLDIP2 was shown to physically interact with polymerase η via its ubiquitin-binding zinc finger domain via myc-pull-down assays (Tissier et al., 2010). Another set of yeast-two hybrid experiments demonstrated that POLDIP2 also interacts with Rev7 and Rev1, two polymerases known to be involved in translesion synthesis. The binding is different from that of polymerase η as Rev7 does not contain ubiquitin-binding motifs and Rev1 has two ubiquitin-binding motifs that are structurally different than those of polymerase η (Tissier et al., 2010). Furthermore, POLDIP2 was knocked down in MRC5 cells and subsequently treated with UV radiation. These cells displayed a level of growth similar to growth seen in polymerase η-knock out cells. Thus, POLDIP2 could be an important protein implicated in UV survival, specifically for bypass of lesions created by the damaging agent.

Polymerase η can also insert ribonucleotides (rNMPs) across a variety of DNA lesions such as 8-oxo-G, 8-met-G, cis-PtGG, 3-met-c, and an AP site (Mentegari et al., 2017). This is due to the poor sugar discrimination in some polymerases, which can cause a delay in the removal of the rNMPs or the lesions themselves (Mentegari et al., 2017). RNase H2 has been shown to be involved in the removal of these rNMPs, with PCNA acting as a recruitment protein to localize RNase H2 to the site of misincorporation. (Bubeck et al., 2011). Interestingly, Western blots and enzymatic assays in XP-V patient-derived XP30R0 fibroblasts reveal that POLDIP2 increases the catalytic efficiency of polymerase η and its ability to bypass certain lesions (Mentegari et al., 2017). These results, in conjunction with those found by Tessier et al., suggest that POLDIP2 might have a role in translesion synthesis.

Another study aimed to characterize the role of POLDIP2 in translesion synthesis as it pertains to five different polymerases: Pol δ, Pol η, Pol ι, Pol λ, and Pol β. It was shown *in vitro* that while POLDIP2 can stimulate pol λ and pol η, pol β and pol ι were not stimulated (Maga et al., 2013). Comparisons of DNA products created by POLDIP2-bound and unbound polymerase η and polymerase λ demonstrate that POLDIP2 increases the processivity of the polymerase. Maga et al. also made the distinction that POLDIP2 does not increase fidelity of the polymerases by comparing sequences created by both POLDIP2-bound and unbound polymerases, specifically across 8-oxo-guanine lesions (Maga et al., 2013).

PrimPol is a relatively new polymerase that has been characterized in a variety of eukaryotic species. It is primarily involved in translesion synthesis in both nuclear and mitochondrial DNA, with the ability to re-prime and synthesizing across a lesion (Guilliam et al., 2016). PrimPol is unique in comparison to the classical translesion polymerases because it has been shown to have no activity with PCNA, an important protein in the switch from canonical synthesis to TLS. Instead, pull-down assays demonstrate that PrimPol interacts with RPA70, single-stranded DNA binding protein, and mitochondrial single-stranded binding protein (Guilliam et al., 2016), but little is known about how PrimPol is regulated *in vivo*. PrimPol can synthesize across two types of lesions, 6-4 pyrimidone photoproducts (6-4 PPs) and 8-oxo-guanines (Bianchi et al., 2013). PrimPol has also been shown to be error-prone, favoring insertions and deletions, begging the questions as to how PrimPol is regulated. It was found that when increasing levels of POLDIP2 are presented to catalytically active PrimPol, primer extension increases in dose-dependent manner (Guilliam et al, 2016).

PrimPol is known to have a low binding affinity for DNA. The paper also demonstrates that POLDIP2 increases PrimPol's ability to bind DNA. The researchers removed the zinc finger

ssDNA binding domain to remove all ability for PrimPol to bind DNA. The electrophoretic gel shift assay demonstrates that increasing levels of titrated POLDIP2 results in a stronger signal for PrimPol that is bound to DNA in a dose-dependent manner. POLDIP2's ability to bind DNA was also analyzed and showed no ability to bind the DNA substrate, in accordance with previous reports (Guilliam et al., 2016). Interestingly, POLDIP2 can increase the processivity and fidelity of PrimPol over the sites of specific lesions, much like in the case of pol λ. POLDIP2 siRNA cells demonstrate slowed replication fork rates upon exposure to UV damage. These results are mirrored by cells that are PrimPol$^{-/-}$, but depletion of POLDIP2 in these cells do not lead to more lag in the replication forks. These data support the hypothesis that POLDIP2 and PrimPol are functioning epistatically in the same pathway (Guilliam et al., 2016).

Other Functions of POLDIP2 in the Nucleus

Aside from its role in DNA repair, POLDIP2 has been shown to have other functions in the nucleus. One of those processes is known as chromosomal segregation, an essential process to obtain daughter cells with the proper ploidy. Failures in this process lead to aneuploidy in mitotic or meiotic cells. Depending on the organism, aneuploidy might not be tolerated and can lead problems at the organismal level. The mechanism of chromosomal segregation is therefore tightly regulated to ensure two daughter cells of the correct ploidy. During the prometaphase, mitotic spindles to bind the centromere of the chromosomes via kinetochores. Once all the chromosomes are correctly lined up during metaphase, the spindles can segregate the sister chromatids to their respective cells in the anaphase (Figure 3).

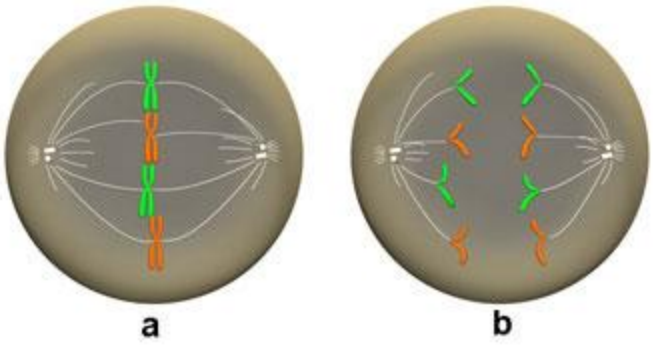

Figure 4. Alignment and segregation of chromosomes during replication. A) Chromosomes are lined up at the metaphase plate in preparation for segregation. Microtubule spindles extend from the centrosomes and are tethered to the centromere of the chromosome. B) The cell has entered anaphase and the spindles pull the chromosomes apart. Successful segregation of chromosomes will result in daughter cells with the correct ploidy. Figure adapted from Nature Education.

POLDIP2 was found to associate with the α-tubulin monomer that helps for the microtubules that aid in chromosomal segregation in mitotic cells (Klaile et al., 2008). This interaction was seen through prophase to telophase. The study also reports that POLDIP2 is found in high concentration in the cytoplasm and a much lower concentration in the nucleus, agreeing with the observations of other work on POLDIP2 (Paredes et al., 2018, Lyle et al., 2009, and Klaile et al. 2007). The researchers demonstrated that inhibition of POLDIP2 via an anti-rabbit-POLDIP2 IgG antibody in rabbit brain endothelial cells results in defects in spindle organization and chromosomal segregation during metaphase and anaphase (Klaile et al., 2008). Confocal images demonstrate multiple spindle poles, monopolar spindles, unaligned chromosomes, and chromatin bridges between daughter cells (Klaile et al., 2008). These phenotypes are mimicked in RNAi-depleted-POLDIP2 cell lines, reinforcing the idea that POLDIP2 co-localization with microtubules is important to ensure correct spindle formation and successful chromosomal segregation.

POLDIP2 in the context of oxidative stress and vascularization

Aside from its involvement in DNA repair and mitotic spindle arragnement, POLDIP2 has been cited to have a role in a variety of non-nuclear cellular processes. POLDIP2 was shown to interact with Nox1 and Nox4, both NADPH oxidases, via a yeast-two hybrid screen, leading to an increase in reactive oxygen species (ROSs) *in vivo* (Lyle et al., 2009). ROSs are important have been implicated in the pathogenesis of a variety of cardiovascular diseases and Nox NADPH oxidases are a family of enzymes that are one of the biggest sources of ROS generation (Lyle et al., 2009). While Nox1 is involved in vascularization, Nox4 can be found in the nucleus but not much is understood about its function in the nucleus as it tends to be a membrane bound protein (Bedard and Krause, 2007). Lyle et al. characterized POLDIP2's interaction with both Nox4 and p22phox, a secondary protein essential to Nox4 catalytic activity, via a GST-pulldown assays. Overexpression of POLDIP2 in rat vascular smooth muscle cells (VSMC) demonstrated an increase in O_2^- and H_2O_2 concentrations (Lyle et al., 2009). The increase in ROSs could be significant at an organismal level. Overexpression of POLDIP2 was found to increase Tau aggregation in human cell lines (Kim et al., 2015). An important observation was that the Tau aggregation was a result of increased ROSs in the environment. Silencing of POLDIP2 was found to alleviate ROS-induced Tau aggregation and knockdown of the gene in *Drosophila* expressing human Tau in the retina rescued the phenotype of loss of photoreceptor neurons (Kim et al., 2015).

Silencing of POLDIP2 reduced levels of ROSs generated by Nox4, but also led to an unexpected change in the morphology of VSMCs, with cells appearing elongated, spindly, and having fewer points of contact with the dish they were plated on (Lyle et al., 2009). The researchers noted that these exact morphology changes can be seen in cells that are siNox4

treated. VSMCs that were siPOLDIP2 treated demonstrated less localization of Nox4/p22phox localization to focal adhesions, or large macromolecular complexes that connect to the extracellular matrix (Lyle et al., 2009). The researchers hypothesize POLDIP2 interactions with the Nox4/p22phox complex might be responsible for focal adhesion integrity and/or stress fiber formation (Lyle et al., 2009). Increased concentration of ROSs is known to activate RhoA, a small GTPase associated with skeletal regulation. siPOLDIP2 treated VSMCs were transduced with constitutively active RhoA, resulting in a rescuing of the cell phenotype observed in the depletion of POLDIP2 (Lyle et al., 2009). Altogether, the data contextualizes POLDIP2 as an up regulator of reactive oxygen species, which can lead to dynamic changes in cellular morphology, vascularization, and even tau aggregation in *Drosophila* retinal cells.

The role of POLDIP2 in vascularization has been revisited by a variety of papers that corroborate the findings from 2009. Knockdown of POLDIP2 in rat aorta cells result in reduced H_2O_2 levels and phenotypic changes in the cells such as increased stiffness, impaired potassium-chloride contractions, and excessive extracellular matrix deposition (Sutliff et al., 2013). Manickman et al. characterize further the interplay between POLDIP2 and RhoA, demonstrating that recue of focal adhesion is seen when RhoA is presented to siPOLDIP2 treated VSMCs (Manicman et al., 2014). The effects of this depletion as it pertains to vascularization is observed at the organismal level in postischemic mouse limps. Reduced levels of POLDIP2 in heterozygous mice lead to reduced blood flow and reduced angiogenesis in comparison to the wildtype mice (Amanso et al., 2014). POLDIP2 knockdown was shown to decrease permeability of the blood brain barrier in mice ischemic brains (Hernandes et al., 2018). Heterozygous mice for POLDIP2 had reduced expression of TNF-α, IL-6, MCP-1, VEGF, and MMPs. The

protective depletion of POLDIP2 with regards to blood brain barrier permeability could be reversed by administration of TNF-α (Hernandes et al., 2018).

An important observation from the studies presented above is that the models used were either heterozygous organisms or those in which POLDIP2 was knocked down via RNAi, because homozygous mutation of POLDIP2 in cells is lethal. This lethality is confirmed in mice as progeny homozygous for a deletion of POLDIP2 incur perinatal lethality (Brown et al., 2014). The mice were smaller compared to their heterozygous siblings and POLDIP2 -/-mouse embryonic fibroblasts (MEFs) were shown to have reduced growth.

POLDIP2 in the context of the mitochondria

POLDIP2's properties as they relate to DNA do not seem to be restricted to the nucleus. Mitochondrial DNA codes for essential ribosomal proteins and has been identified in mammalian and yeast systems. (Cheng et al., 2005). Mitochondrial transcription factor A (TFAM) is a major component of the nucleoid complex. Cheng et al. identified POLDIP2 as a novel interacting partner with this protein. Importantly, this characterized POLDIP2 as a potential mitochondrial protein. Bioinformatics and immunohistochemistry demonstrated that POLDIP2 contains a mitochondrial targeting sequence (Cheng et al., 2005). Interestingly, immunoprecipitation with anti-POLDIP2 antibodies yields TFAM and mitochondrial single-stranded binding protein (mtSSB), but immunoprecipitation with anti-TFAM antibodies does not always co-precipitate POLDIP2 protein (Cheng et al., 2015). The researchers believe that only a fraction of the POLDIP2 in the cell interacts with TFAM. Polymerase γ, the main replicative polymerase in the mitochondria, was not co-immunoprecipitated with POLDIP2 (Cheng et al., 2015). Cross-linking via formaldehyde treatment was used to determine more interacting partners of POLDIP2.

mtSSB was found to co-immunoprecipitate with POLDIP2, but not TFAM (Cheng et al., 2005). Additionally, POLDIP2 was found to coimmunoprecipitate with mitochondrial 60 kDa heat shock protein (CH60). The yeast ortholog of CH60 is known to bind to DNA, while the human ortholog is known to bind to ssDNA (Cheng et al., 2005).

The interaction between POLDIP2 and mtSSB was further characterized in mice with juvenile visceral steatosis. A defining phenotype in these mice is an increased number of mitochondria and abnormal mitochondrial morphology in cardiac cells (Arakaki et al., 2006). A screen designed to understand gene expression in these mice identified POLDIP2 (Mitogenin I in this paper) and mtSSB as genes that were upregulated and played an important role in mitochondrial fusion and fission, respectively (Arakaki et al., 2006). The paper demonstrates the same bioinformatics and cellular localization data as Cheng et al., reinforcing the idea that POLDIP2 is targeted to the mitochondria in mice and human cells. Overexpression of Mitogenin I (POLDIP2) leads to the elongation of mitochondria in both mice and human cells, while silencing leads to fragmented mitochondria (Arakaki et al., 2006).

The same group also reports to observe differential localization of Mitogenin I based on the stage of the cell cycle. They report localization of Mitogenin I at the mitochondria during all stages of the cell cycle in in mouse C2C12 myoblast cells, but distribution to both the nucleus and mitochondria during G_2/M phase to late G_1 phase. A similar situation is observed in proliferating and quiescent NBT-II cells (Nara Bladder Tumor No. 2). POLDIP2 was characterized as an interacting partner of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a protein that acts as a regulator of contact-dependent cell survival, differentiation, and growth. Researchers designed various antibodies to demonstrate interactions between POLDIP2 and both the long and short isoform of CEACAM1 (Klaile et al., 2007). They

observed no localization of POLDIP2 in confluent, non-proliferating cells, but clustering of CEACAM1 via antibody perturbation lead POLDIP2 translocation to the nucleus in 66% of cells (Klaile et al., 2007). This lab previously demonstrated that CEACAM1 clustering can induce cell cycle entrance and DNA synthesis in quiescent cells. This entrance into the cell cycle is achieved by activation of Erk1/2 pathway (Klaile et al., 2007). However, POLDIP2 is not translocated to the nucleus upon Erk1/2 activation, demonstrating that its movement is dictated by CEACAM1.

Current Questions about POLDIP2

POLDIP2 is an enigmatic protein that appears to be pleiotropic in nature. There are still many questions that must be addressed about this protein *in vivo*. At the forefront of these questions is if POLDIP2 is a main regulator of one or two specific pathways or processes in the cell, or is its role more decentralized to a variety of processes? This question is highlighted by the perinatal lethality observed in homozygous knockout mice (Brown et al., 2014). Work on POLDIP2 -/- mouse embryonic fibroblasts demonstrated increased autophagy, reduced cellular growth, and arrests at different stages of the cell cycle (Brown et al., 2014). It is necessary to elucidate the involvement of POLDIP2 in these various phenotypes. These results also raise the question as to whether POLDIP2 is essential in metazoans or only essential in higher eukaryotes like mice and humans. There has been little work done to characterize the domains of POLDIP2 and their functional relevance for different cellular processes.

Interactions with DNA polymerases	Association with polymerase δ and PCNA; no data on functional relevance of interaction (Liu et al., 2003).
	Association with polymerase η, increasing its processivity (Mentegari et al., 2017).
	Association with polymerase λ, increasing its processivity (Maga et al., 2013).
	Association with PrimPol, increasing its processivity and increasing PrimPol's affinity for DNA (Guilliam et al., 2015).
Involvement in mitotic spindle arrangement	Co-localization with α-tubulin monomer during mitosis (Klaile et al., 2008).
Upregulation of oxidative stress	Association with Nox4/p22phox enzyme complex, upregulating its catalytic activity and producing reactive oxygen species and altering cellular morphology (Lyle et al., 2009).
Vascularization	Depletion causes reduced angiogenesis and blood flow (Amanso et al., 2014).
Mitochondrial DNA and morphology	POLDIP2 is co-immunoprecipitated with mitochondrial transcription factor A and mitochondrial single-stranded DNA binding protein (Cheng et al., 2005).
	Overexpression in mice embryonic cells lead to elongated mitochondrial formation, while depletion causes mitochondrial fragmentation. (Arakaki et al., 2006).

Table 1. Summary of POLDIP2 in various cellular processes. The table describes some of the protein interactors of POLDIP2 and its function is relation to different cellular processes.

Drosophila melanogaster as a model organism

Drosophila melanogaster is a convenient model system to study DNA repair as its developmental and genetic properties have been thoroughly studied. There are a wide variety of tools that can be used to manipulate fly genetics create mutants and keep track of the mutation *in vivo*. The life cycle of fruit flies consists of four main stages: egg, larva, pupa, and adult (Figure 5). There are a variety of phenotypes that can be observed across these stages of development

that can be used to track mutations, infer lethality, and understand the effects of DNA damaging agents. Fruit flies are also inexpensive compared to other model organisms, making them an ideal candidate for large-scale assays and screens.

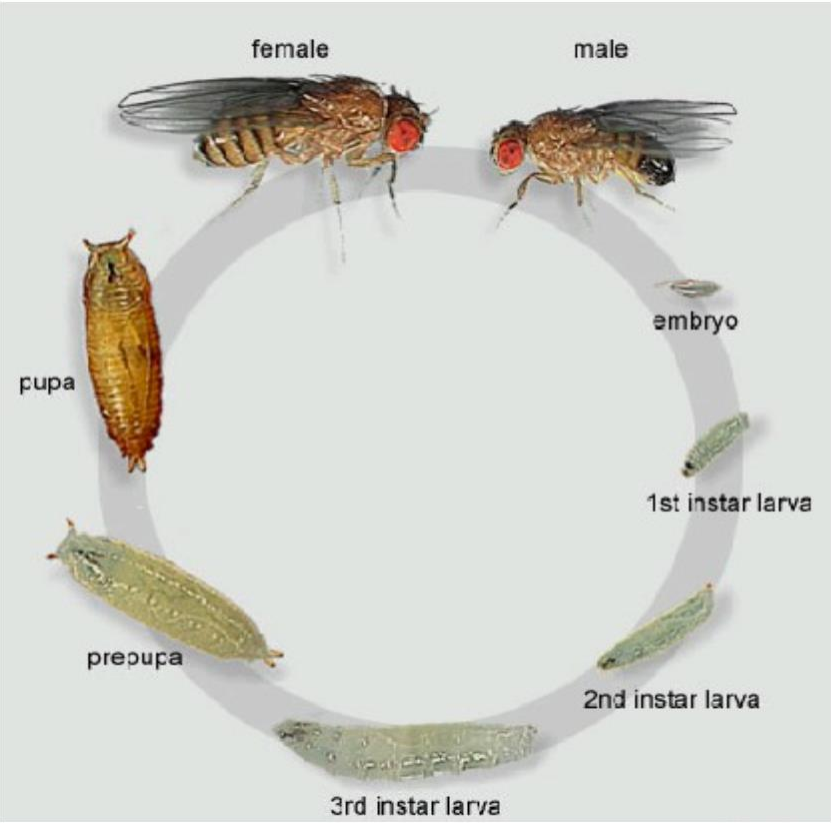

Figure 5. Life cycle of Drosophila melanogaster. Males and females are mated in bottles or vials containing standard cornmeal recipe. Eggs are deposited on the food over the next days, giving rise to 1st instar larvae around day three. Larvae ingest food over the next four to six days, in which they pass onto being 2nd and 3rd instar larvae. 3rd instar larvae typically crawl up the side of the container in preparation for pupation. During pupation, the fly undergoes metamorphosis to reach its final adult form. The developmental cycle of a fruit fly takes ten days in a relatively healthy stock.

The goal of this thesis aims to understand the role of POLDIP2 in DNA repair in fruit flies. POLDIP2 is severely understudied, with many papers focusing on the physical interactions between POLDIP2 and a variety of proteins, instead of characterizing its functions *in vivo*.

Additionally, most of the work conducted on POLDIP2 has taken place in mice and human cell lines. Only one study has looked at POLDIP2 in the context of *Drosophila*, however, it was only a knockdown localized to the eyes of the flies (Kim et al., 2015). It is important to observe any recapitalization of phenotypes observed in mice and human cells in the fruit fly system. *Drosophila melanogaster* is also an ideal system to study POLDIP2 because of the in-depth understanding of DNA repair in the model organism and our laboratory, specifically translesion polymerases.

Materials and Methods

Fly Maintenance

Flies were kept in a standard cornmeal medium at 25˚C for development. Stocks were routinely flipped every three to five days based on.

Deletion of POLDIP2 and Insertion of dsRed Marker

The POLDIP2 gene found on the third chromosome of *Drosophila melanogaster* was deleted via CRISPR Cas-9. Guide RNAs (Supplementary Figure 1) were created to excise POLDIP2 and homology arms designed to replace POLDIP2 with a dsRed insert (678bp) via homology directed repair. Best Gene injected flies with the constructs above and isolated one dsRed positive fly. This fly was crossed to virgin wildtype females to create 7 individual stocks, all derived from the same male.

Cross Scheme for POLDIP2Δ/TM3Ser Flies

The cross scheme in Supplementary Figure 2 was used to place the POLDIP2 mutation in trans to a balancer chromosome for visible phenotypic markers and to avoid recombination of the deletion. Flies arrived with the *vasa Cas-9-GFP* gene. RFP^+/GFP^- were selected for the beginning of the cross (not shown). PCR was used to confirm the insertion of the dsRed marker (Supplementary Figure 2). Mutant A (MA), Mutant C (MC) and Mutant D (MD) stocks were used for subsequent protocols.

Fly Preparations

DNA was extracted from individual male flies by sacrificing them in a 0.5 mL micro centrifμge tube. 49 μL of Squishing Buffer and 1 μL of Proteinase K was added to the tube. Flies

were squished with a pipette tip for about five minutes. Tube was incubated at 37˚C for one hour and at 65˚C for five minutes to inhibit Proteinase K.

Developmental Assay for Larvae

Flies were given active yeast pellets 24 hours before setting up cages for breeding. Grape plates with yeast paste were used for flies to lay eggs. Grape plates were switched every day for 3-4 days. Development of larvae was tracked via fluorescent microscopy to determine homozygous viability. GFP markers with the TM3, Ser, P{Act-GFP, w+} balancer chromosome was used to differentiate heterozygous larvae from homozygous larvae.

RNA Isolation from via TRIzol

Thirty to fifity mutant and wildtype 2nd instar larvae were collected from the grape plates and stored at -20˚C. Fifteen to thirty flies can be collected if the RNA is being extracted from adμLt flies. Flies were homogenized in TRIzol using a pestle. Chloroform was used to create two phases and isopropanol was used for precipitation. The RNA pellet was washed with 75% ethanol and resuspended in 40 μL of RNase-free H_2O. 8 μL of RNA was aliquoted and treated with 5 μL of DNase to reduce genomic contamination.

cDNA Synthesis

1 μg of RNA was used to create cDNA using the Protoscript II First Strand cDNA Synthesis Kit (SE6560S). Avian oligo dT was used to prime the RNA strand. The reaction was conducted at 42˚C for 1 hour and reverse transcriptase enzyme was inactivated at 80˚C for 5 minutes.

Semi-quantitative PCR

Semi quantitative PCR was done by removing PCR tubes at cycles 25, 30, and 35, to visualize bands under UV light. Primer sequences can be found in Supplementary Table X.

Genomic DNA Preparation

Thirty male flies were collected from the stock and kept at -20˚C for 20 minutes. Flies were squished for in Buffer A and incubated on ice with LiCl/KAc solution. Isopropanol and ethanol washed DNA and resuspended in ppH_2O. Genomic preparations are cleaner and yield higher amounts of DNA in comparison to fly preparations.

Creation of pJC_attB-POLDIP2 Rescue Construct

POLDIP2 gene locus was isolated from a genomic preparation via PCR using Q5 High Fidelity Polymerase. The forward primer was designed with an overhang containing the EcoRI recognition sequence and the reverse primer was designed with an overhang containing the XbaI recognition sequence. Both recognition sequences were followed by 6 random nucleotides to ensure proper binding of the restriction enzymes to the substrate. These two restriction enzymes were chosen for their compatibility in NEB Buffer 3.1. PCR product was purified via agarose gel extraction using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nachel, REF 740609.250)**.** The total length of the gene product was 2,641 nucleotides. The pattB plasmid was digested for 1 hour at 37˚C with EcoRI and XbaI. Ratio of insert to vector was determined using the Duesseldorf ligation calculator. DNA for both insert and vector were quantified via nanodrop. Ratios of 1:1 and 1:3 of insert to vector concentration were used for ligation. Digested plasmid was purified using NucleoSpin Gel and PCR Clean-up kit. Insert and plasmid were

incubated overnight with T4 DNA ligase at 4˚C. The products were subsequently digested with NotI to reduce levels of religated vector or inefficiently digested vector.

Competent DH5α-*E.coli* were transformed with the treated DNA. Cells were thawed for 10 minutes and 2 μL of DNA were added to 50 μL of cells. Cells remained on ice for 30 minutes and heat shocked for 45 seconds at 42˚C. Cells were incubated on ice for 2 minutes before adding 200 μL of LB agar for rescue on spinner for 1 hour. Cells were plated on ampicillin plates that were broμght to room temperature. Cells were grown overnight at 37˚C. A no-insert and puc19 control were used to determine transformation efficiency. Individual colonies were selected and grown overnight in 2 μL LB agar with 200 mL ampicillin. Cells were mini prepped using the NucleoSpin Gel and PCR Clean-up kit. DNA quantity and quality were determined via nanodrop. Individual double diagnostic digests were set up to determine successful cloning.

Incorporation of Rescue Construct in Flies

5 μg of pJC_attB-POLDIP2 were shipped to BestGene for Midi prep and subsequent injections into PhiC31 fly stocks. The site of recombination is 3L32B, on the left arm of the 3rd chromosome. BestGene returned larvae from a F1 generation screened for w+ marker (red-eyes). Three of the five mutant strains were screened for red eyes and mated with CRISPR knockout flies.

P{GSV7} Stock

A stock of flies containing a 4.942 kilo base pair P-element insertion in the second exon of POLDIP2 was obtained from Kyoto Stock Center (Stock #202312). This stock is referred to as the P{GSV7} stock in this paper. Flies were used for sensitivity assays and crosses.

Mutagen Sensitvitiy Assays

6 μL of 100% concentrated MMS was diluted in 594 μL of pico pure H_2O. X μL of the diluted MMS were added to x μL of pico pure H_2O to obtain 0.08% MMS concentration. P{GSV7} homozygous males were mated to P{GSV7}/TM3 Ser, w^+ males in a total of eight to ten vials. Flies were allowed to lay eggs for three days before being flipped into a new set of vials. On day four, larvae in the first set of vials were dosed with 250 μL of 0.08% MMS. The second set of vials were dosed after the flies had three days to lay eggs. These control flies were dosed with 250 μL of pico pure H_2O. Ten days after the flies layed eggs, each vial was counted daily under a microscope. Flies were tallied based on the phenotype of serrated wings or lack thereof. These numbers are used to determine the relative homozygote survival, which is used as a benchmark to infer sensitivity. 4-Nitroquinilone-1-oxide (4-NQO) sensitivity assay were conducted with 1mM and 10mM solution, with 10% and 20% DMSO in pico pure H_2O for controls, respectively. H_2O_2 sensitivity assays were conducts with a 50% solution.

RNAi-POLDIP2 Stock

A stock containing POLDIP2 under RNAi control was received from Bloomington Stock Center (Stock #51514). Homozygous males were mated to virgin females homozygous for Gal-4 under a UAS promoter.

Calculating Hatching Frequencies

Grape cages were set up for P{GSV7} homozygotes, {PGSV7} heterozygotes, and wildtype flies.

Results

Previous literature demonstrates that POLDIP2 is essential for viability in higher eukaryotes (Brown et al., 2014). The first aim of the project was to observe and characterize any lethality in POLDIP2 knockout flies. Guide RNAs were designed to create a knockout via the CRISPR Cas 9 system (Figure 6). A DsRed marker was inserted via homology directed repair to allow tracking of the mutation in subsequent stocks (Figure 7). To validate that the POLDIP2 gene was disrupted in our stocks, we isolated POLDIP2Δ/TM3 Ser, P{Act-GFP, w+} males flies and recovered DNA via fly squishes for PCR. All stocks contained the dsRed insertion (Figure 8). Furthermore, we can conclude that the fly stocks contain the POLDIP2 deletion as the products are smaller in size compared to the wildtype product.

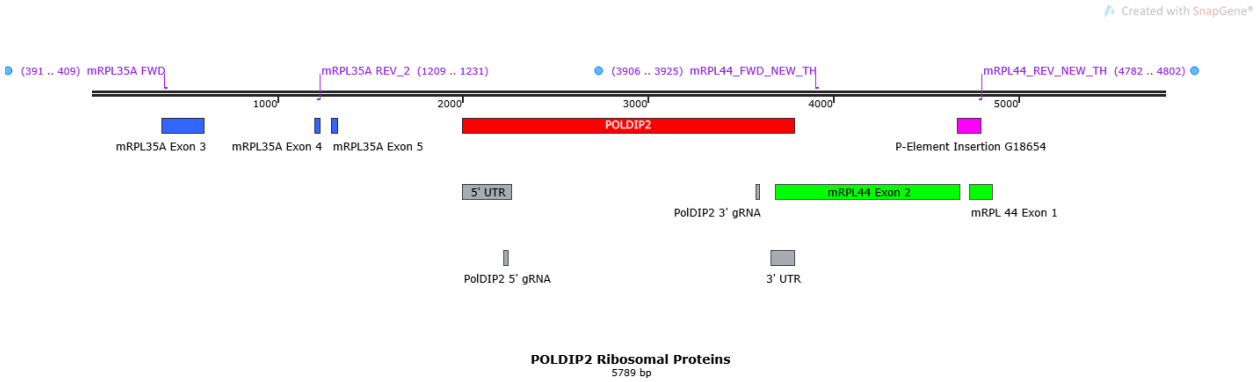

Figure 6. Genomic map for POLDIP2. Open reading frame for POLDIP2 is shown. Exons 3, 4, and 5 are shown for mRpL 35A, while exons 1 and 2 are shown for mRpL44. A P-element insertion in mRpL44 is also represented. This insertion is located across the first and second intron of mRpL 44. Ribosomal proteins are essential for the development and viability of *Drosophila melanogaster*. Primers for semi-qPCR amplification are shown in purple for each ribosomal proteins. mRpL 35A lies 676 base pairs from the 5' gRNA for POLDIP2, while mRpL 44 lies 86 base pairs away from the 3' gRNA in POLDIP2.

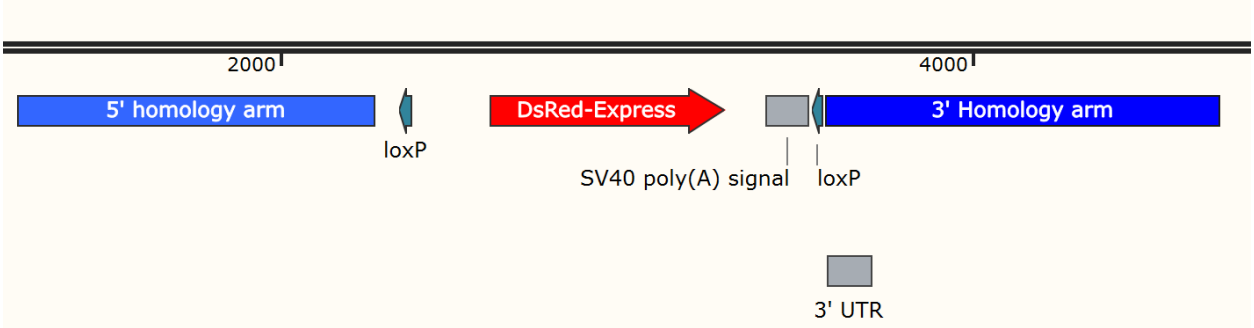

Figure 7. DsRed construct integrated into third chromosome. The locus of POLDIP2 replaced by DsRed marker. Protein was integrated via homology-directed repair using the homology arms highlighted in blue.

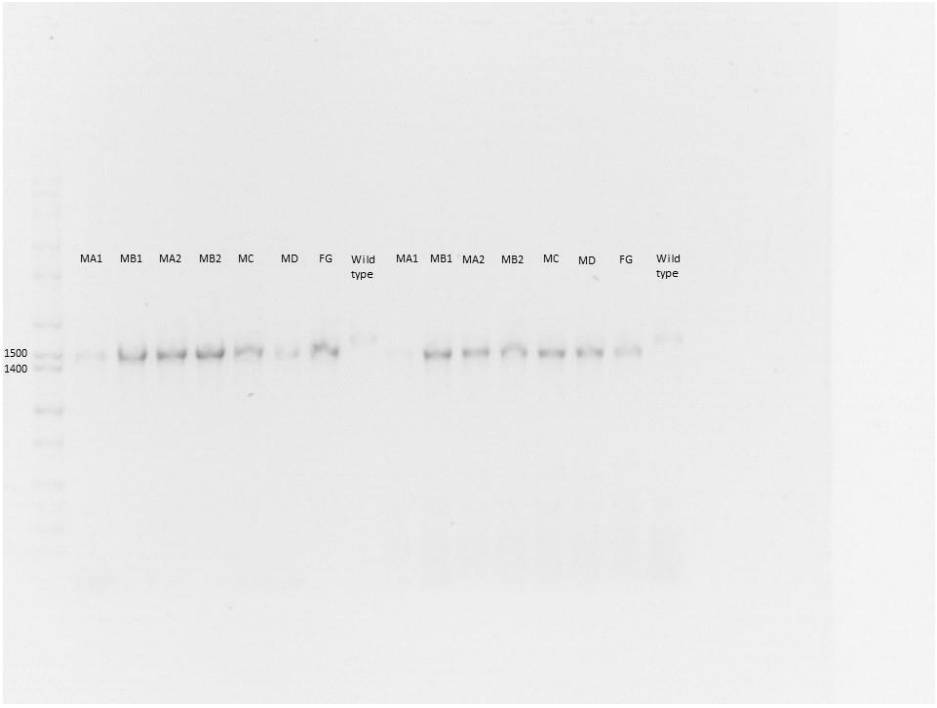

Figure 8. DsRed insertion in POLDIP2 locus confirmed. PCR was conducted on all 7 stocks to confirm deletion of POLDIP and insertion of dsRed marker. Stocks MA, MB, MC, MD, FG, and wildtype are represented. All mutant stocks were derived from one fly. 2 isolates were used for stocks MA and MB as the first DNA isolation done for both stocks were older. Expected product size was 1470 base pairs for mutant flies, while 1625 base pairs were expected for wildtype flies.

After establishing a balanced stock, only heterozygotes were observed, indicating that the stock was homozygous inviable. A developmental assay was conducted to track the development of the flies and understand at what point homozygotes incurred lethality. Figure 9 depicts two homozygous larvae and one heterozygous larvae at seven days after flies were mated in the grape cages. The homozygous larvae were developmentally lagged compared to the heterozygous larvae, unable to make it paste 2nd instar larval stage. Heterozygous larvae had reached pupation or were transitioning to pupation, while homozygous larvae were lethargic on the food and thin in comparison. This lethality is an uncommon phenotype for DNA repair/replication mutants. DNA repair mutants tend to incur lethality during the later stages of development, such as 3rd

instar larval stage or pupation. These stages of development exhibit heightened levels of proliferation in imaginal wing disc tissues that require functional DNA repair to ensure viability.

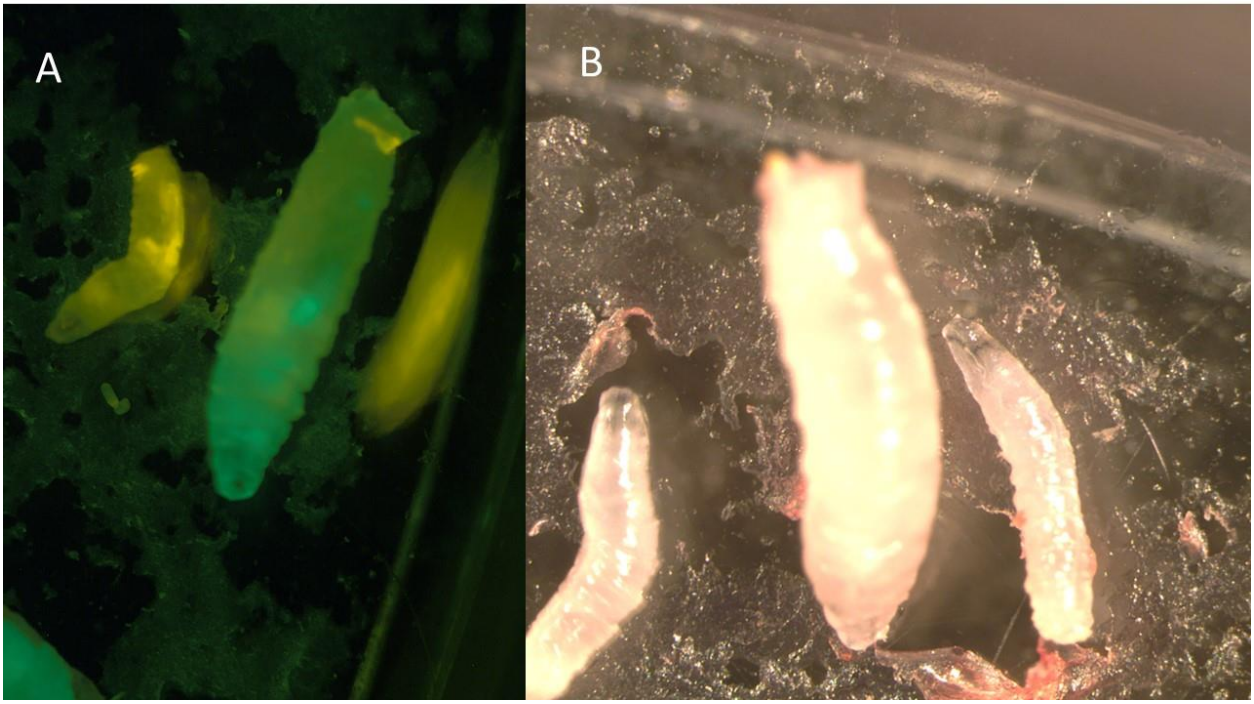

Figure 9. POLDIP2 mutant homozygotes incur lethality at second instar larval stage. A developmental assay was used to determine the stage of development at which homozygotes died. Figure 3 depicts two larvae under fluorescent (A) and bright light (B). Heterozygotes were identified using a GFP marker associated with TM3 Ser balancer chromosome, while homozygotes only expressed dsRed. In Figure 9A dsRed flies are represented by the yellow light due to the filter that is applied. Homozygote larvae did not make it past the 2nd instar larval stage. Clear phenotypic differences can be seen in Figure 9B as homozygotes are visibly shorter and thinner. Only heterozygote pupae were identified past 7-8 days post fertilization.

The timing of the lethality led us to believe that the deletion of POLDIP2 was not responsible for the lethality if it was indeed a DNA repair protein. Deficiency chromosomes are typically used in *Drosophila* to investigate the function of a specific gene or genes by having a region of the chromosome containing the gene locus completely removed. Unfortunately, no deficiency chromosome exists for the genomic region of POLDIP2 as it is flanked by two essential genes, mRpL 44 and RpL 35A (Figure 6). This observation was important because

there was a possibility that the CRISPR knockout affected expression of these essential proteins. CRISPR Cas-9 had been previously shown to have off-target effects due to potential promiscuity in guide RNAs (Fu et al., 2014). RT semi-qPCR demonstrated that expression of both the proteins were the same in both mutant and wildtype flies (Figures 10 and 11). There were some reservations about the functionality of the expressed mRpL 44 in the mutant flies. Figure 6 demonstrates that POLDIP2 and mRpL44 share a 3' UTR. The 3' gRNA for the CRISPR deletion targeted a sequence 44 base pairs upstream the 3' UTR of mRpL 44. The proximity of these two elements made it possible for an off-target effect to disrupt faithful termination of mRpL 44, therefore, producing a semi-functional protein. Figure 12 depicts a cross used to test the functionality of mRpL 44 in the mutant flies. The stock containing a P-element within mRpL 44 was not able to homozygoze, demonstrating that one functional copy of mRpL 44 is required for viability. The cross successfully generated transheterozygote flies carrying both the P-element and the deletion of POLDIP2, therefore, we conclude that mRpL 44 expression and function was unaffected in these flies.

Figure 10. mRpL35A is expressed at wildtype levels in POLDIP2-knockout mutants. The gel demonstrates results for semi-qPCR for mRpL35A, an essential gene in Drosophila melanogaster. Tubes were removed at cycles 28, 30, and 36. Each cycle contained 5 samples. The samples used were cDNA from POLDIP2 Mutant A and wildtype flies, as well as genomic DNA from the mutant fly. The remaining two wells are RP49 controls for both mutant and wildtype flies. The third lane in every cycle is labeled Mutant cDNA*. This sample was meant to be genomic DNA but only cDNA is used. The cDNA product is expected to be 220 base pairs, while the genomic product is expected to be 818 base pairs. RP49 controls are very dim but can be observed starting at 30 cycles.

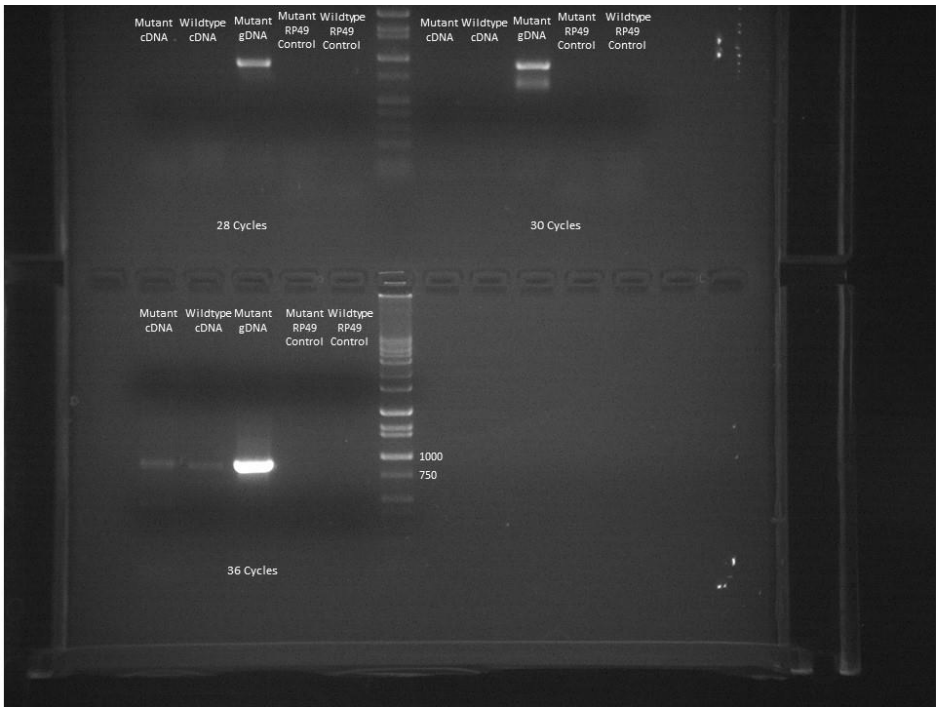

Figure 11. mRpL is expressed at wildtype levels in POLDIP2-knockout mutants. The gel demonstrates results for semi-qPCR for mRpL 44, an essential gene in Drosophila melanogaster. Tubes were removed at cycles 28, 30, and 36. Each cycle contained 5 samples. The samples used were cDNA fromPOLDIP2 Mutant A and wildtype flies, as well as genomic DNA from the mutant fly. The remaining two wells are RP49 controls for both mutant and wildtype flies. Bands for RP49 controls are not visible at any cycle. Expected band size for cDNA is 845 base pairs, while genomic DNA is expected to yield 897 base pairs. cDNA bands can only be seen at 36 cycles.

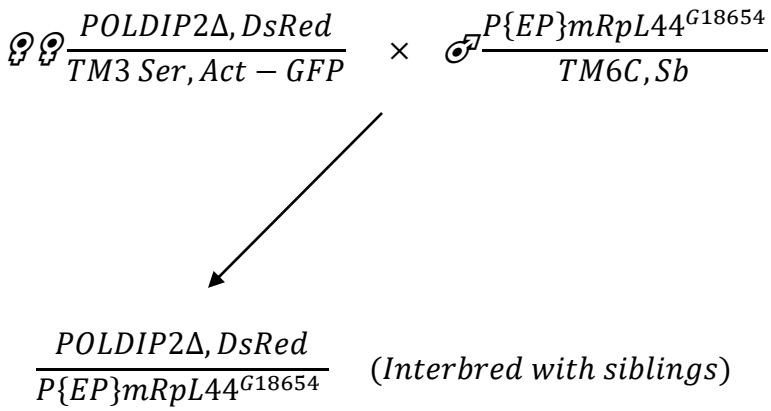

Figure 12. mRpL 44 P-element cross scheme. A cross was designed to corroborate the results of the semi-qPCR for mRpL44. Virgin females, denoted by two female symbols, were crossed to heterozygote flies containing a P-element insertion in mRpL44. Progeny of trans-heterozygotes were recovered. The viability of these flies support the conclusion that mRpL 44 is not disrupted in POLDIP2-deficient flies as the P-element insertion is hypothesized to disrupt mRpL 44 function as it is located within the first intron, but also spans across the first and second exon. The copy on the POLDIP2Δ chromosome must be functional to allows trans-heterozygotes to be viable.

Although no mutations were identified in the neighboring ribosomal gene for POLDIP2, there was a possibility that a mutation may have occurred elsewhere in the third chromosome. To test this, we attempted to separate the putative second-site mutations from the poldip2 deletion by meiotic recombination. Knockout mutants were mated to wildtype flies to allow for recombination in the female germline in the subsequent generation (Figure 13). Ninety single male crosses were conducted in the second generation of the cross to determine single cross-off events. None of the vials produced viable homozygotes upon completing sibling crosses. This data suggested two possibilities: either POLDIP2 is essential in *Drosophila melanogaster*, or the mutation was very tightly linked to the POLDIP2 locus, and therefore, the mutations is difficult to separate from the DsRed marker.

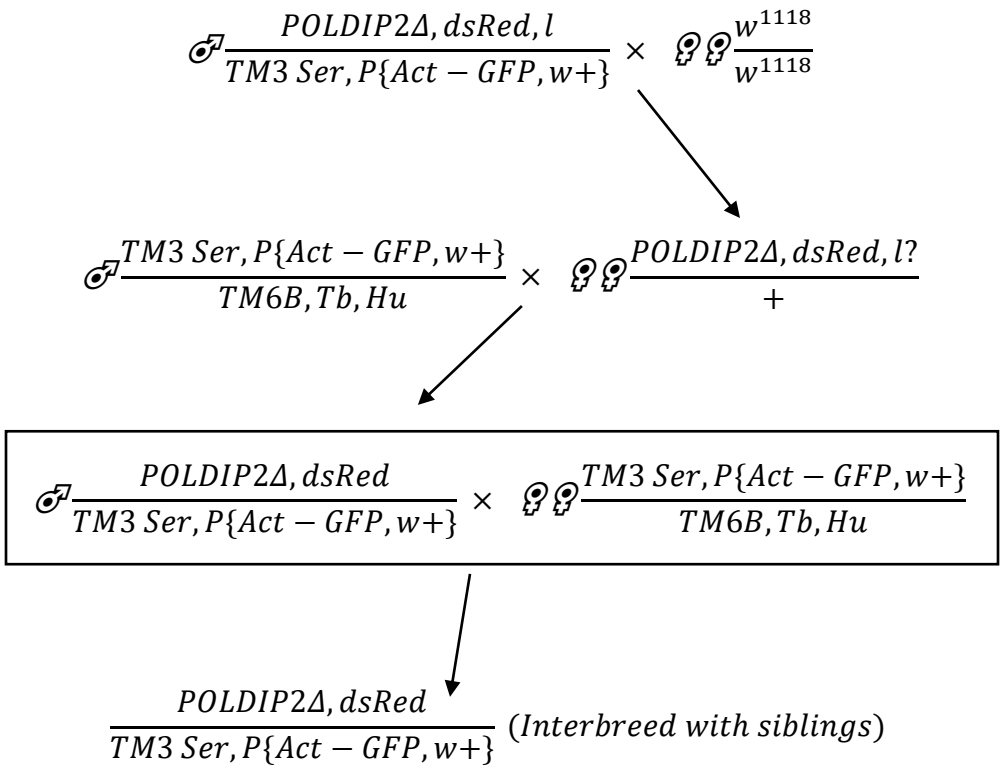

Figure 13. Second-site mutation cross-off cross scheme. A cross was designed to cross off second site mutations found on the 3rd chromosome. Virgin wildtype females were crossed to single males containing the dsRed insertion. Virgin females expressing dsRed and no other phenotypic marker were recovered in the next generation and mated to double balancer males. Single males containing the dsRed marker and serrated wing were recovered and mated to virgin female double balancers. Virgin females and males were recovered to interbreed and observe potential homozygotes in the next generation. The black box indicates single male crosses to identify single cross-off events.

To test the hypothesis that POLDIP2 is an essential gene in flies, a rescue construct bearing the genomic locus of POLDIP2 was created to test if insertion and expression of the gene could rescue homozygotes. The genomic locus, along with 623 base pairs upstream and 182 base pairs downstream of POLDIP2, were cloned into a pattB plasmid. Ample distance was conserved for the insert to ensure proper transcription initiation and termination. The rescue construct (Figure 14) was sent to Best Gene for injections into PhiC31 integrase-expressing flies.

Integration occurred at the 3L62B locus to allow ample recombination to occur between the transgene and the DsRed insert (Figure 15). Transgenic flies containing both the transgene and the fluorescent marker were established over a TM6B, Hu, Ubx balancer chromosomes (Figure 16). The final generation of the previous cross was mated to POLDIP2Δ mutants. This cross produced four phenotypically distinct offspring that could be scored. Figure 17 demonstrates the possibility from this cross. The genotype in the red box was not observed in the adult population when the offspring eclosed. Flies containing two copies of the POLDIP2 deletion and a copy of the transgene were not viable. This result was surprising as it was expected that POLDIP2 was essential for viability, therefore, introduction of a transgene should theoretically rescue the homozygotes. This data would support the idea that POLDIP2 is not essential in *Drosophila*, therefore, it was possible that the lethality was being caused by a second site lethal mutation elsewhere on the third chromosome.

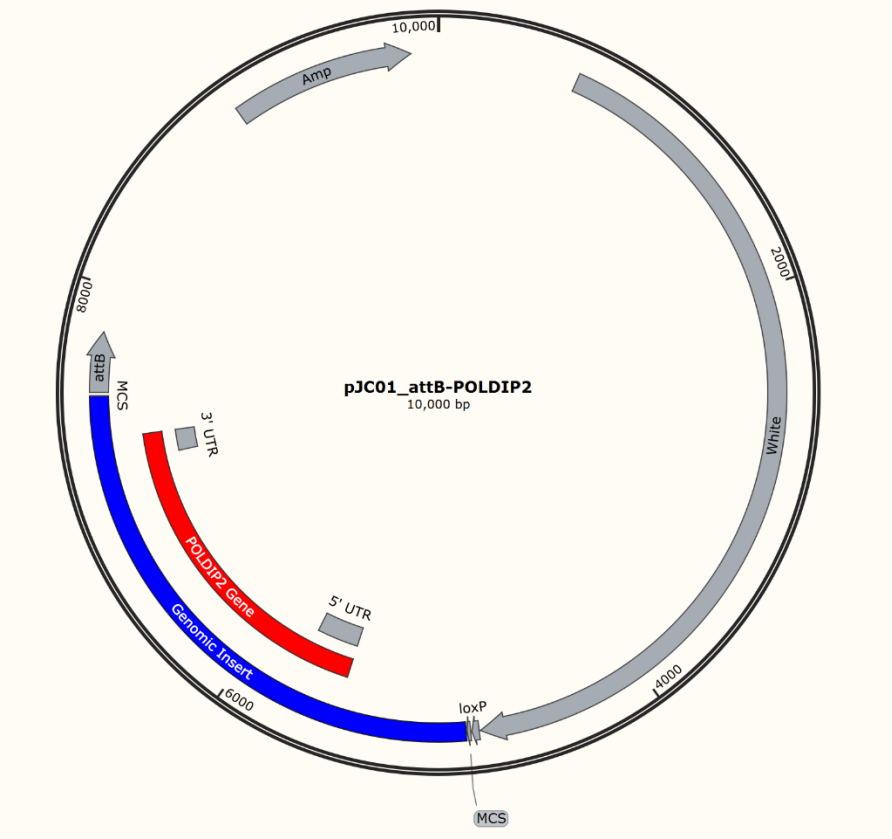

Figure 14. pJC01-attB-POLDIP2 rescue construct. POLDIP2 genomic locus was amplified and cloned into the pattB plasmid backbone. The red element describes the open reading from for POLPDIP2, with untranslated regions labeled. The blue element describes the entire fragment that was cloned in, which includes 623 base pairs upstream the POLDIP2 locus and 182 base pairs downstream, to ensure proper transcription initiation and transcription.

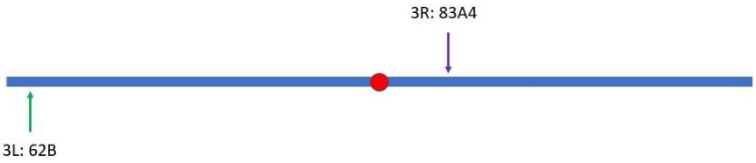

Figure 15. Site for recombination on third chromosome. The sites demonstrated above were used for recombination in PhiC31 flies. The green arrow labeled “3L: 62B” represents the site of integration for the rescue construct described in Figure #. The purple arrow labeled “3R:83A4” represents the cytological location of POLDIP2 in fruit flies. The red circle in between the two arms is representative of the centromere.

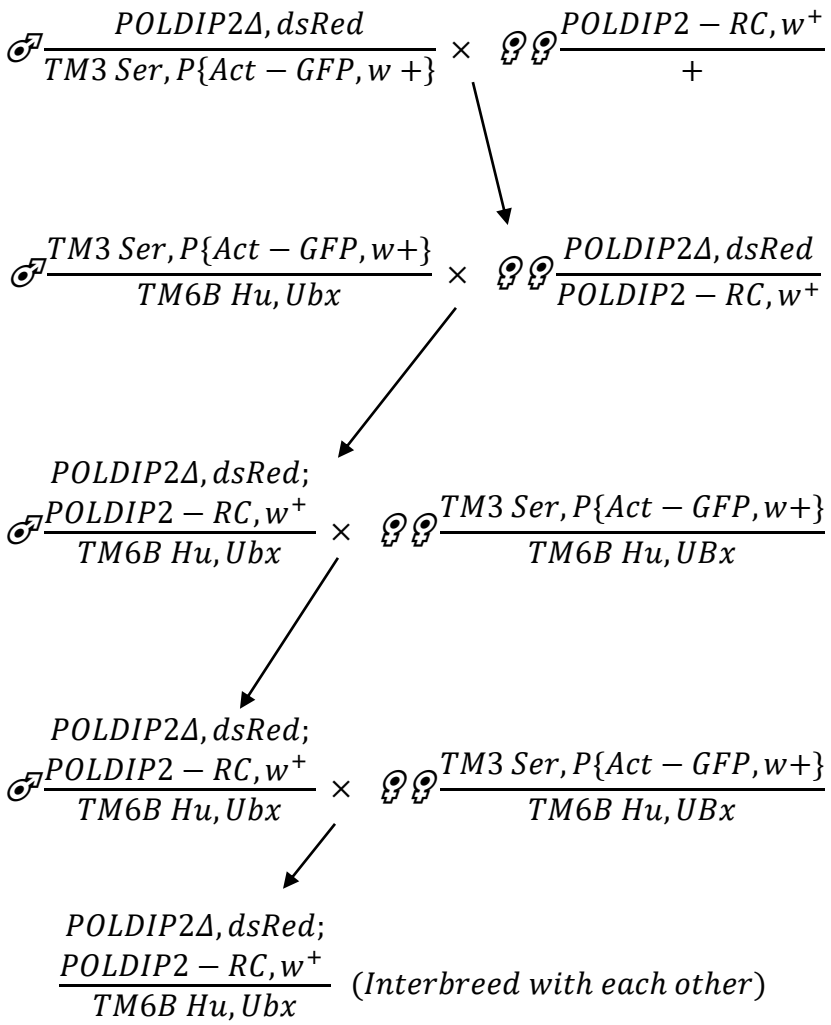

Figure 16. Cross to establish recue construct in mutant flies. Flies with transgene were received from Best Gene and scored based on the w^+ marker. Virgin females were mated to males carrying the POLDIP2 deletion. Virgin females were mated to double balancer in the next generation. The next two generations consisted of crosses to double balancer, males were selected for crosses based on the presence of both the transgene and dsRed marker. The cross was a sibling cross to establish a stock of transgenic flies with both the deletion and transgene.

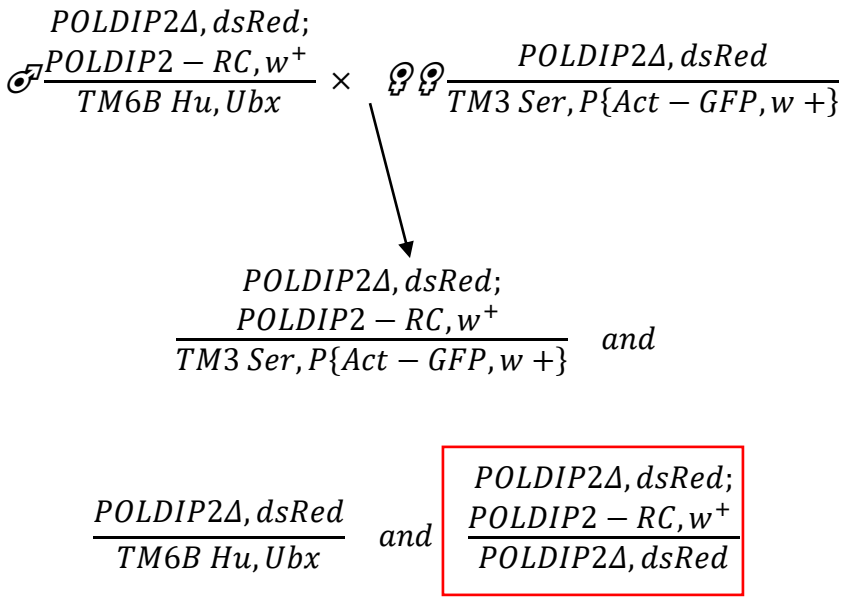

Figure 17. Homozygotes are not rescued by POLDIP2 transgene. Flies containing both the transgene and dsRed marker were mated to the virgin females from the POLDIP2Δ stock. The figure demonstrates the four genotypes expected to eclose in the next generation. All four offsprings are phenotypically distinguishable. The red box represents the genotype/phenotype not observed in the next generation.

Failure to rescue homozygotes with a POLDIP2 transgene was not only surprising but also left us without a full knockout mutant. Fortunately, a stock of flies containing a P-element within POLDIP2 was received from Kyoto Stock Center (Stock # 202312). This stock provided the potential to create a mutant that could be studied in parallel to the CRISPR knockouts. This P-element is 4.8 kilo base pairs in length and is inserted into the third exon of POLDIP2 (Figure 18). PCR was used to demonstrate that the P-element inserted was P{GSV7} (Figure 19). Expression of POLDIP2 in the P-element and wildtype stock appear to be the same based on RT semi-qPCR (Figure 20 and 21). Figures 22 and 23 demonstrates crosses with the original POLDIP2 deletion and the transgenic strain containing both the deletion and the POLDIP2 transgene. Progeny containing both P{GSV7} and the POLDIP2 deletion were not observed in

the subsequent generation, while P{GSV7}/transgene and DsRed flies were observed. The results from both crosses were surprising because we expected the P{GSV7}-POLDIP2 allele to function as a wildtype allele because the flies can homozygoze in stock. The cause of the inviability is most likely due to disruption of protein activity of POLDIP2 due to the P-element insertion. We hypothesize that this allele is functioning to some degree because we could establish it over the transgenic third chromosome, which differs from what is seen in with the knockout over the same transgenic chromosome. Although we do not have data establishing the amount of transgene expression, this cross suggests there is some level of expression that is enough to rescue a genotype that would otherwise be inviable. These data support the hypothesis that POLDIP2 is essential for *Drosophila melanogaster*, however, the rescue is masked by a second site lethal mutation that is associated with the same chromosome as the CRISPR knockout.

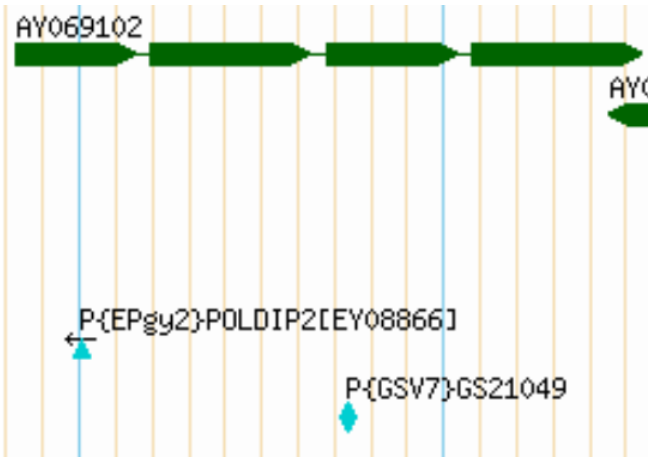

Figure 18. Stock # 202312 contains a 4.8 kbp P-element in the third exon. Image from FlyBase demonstrated the POLDIP2 transcript in green (AY069102). Two P-elements are shown by the blue shapes and demonstrate their insertion site within the POLDIP2 transcript. The stock obtained in the lab contains the P{GSV7} P-element.

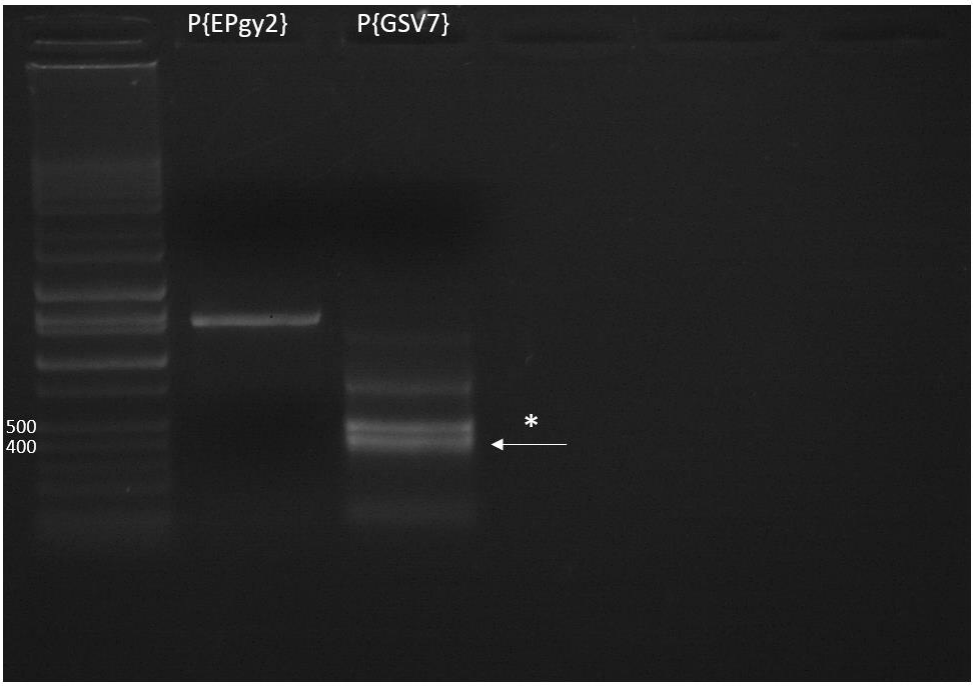

Figure 19. Characterization of P{GSV7} in P-element stock. Genomic DNA was isolated from a single fly from a stock carrying a P-element. The arrow demonstrates the 430 base pair band corresponding to the P{GSV7} P-element. The band in the first lane does not correlate with a positive signal for another P-element, P{EPgy2}, therefore, it is not present in our stock. These sizes were compared to the initial characterization done by a previous member of the lab (Yee et al., unpublished).

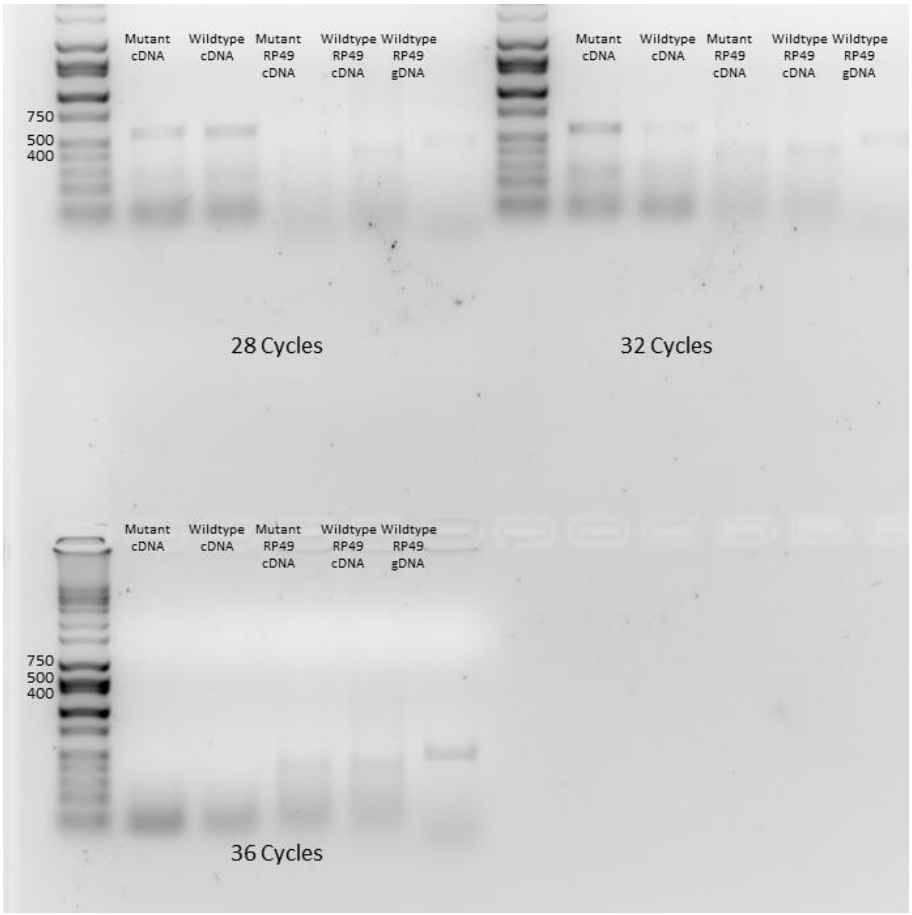

Figure 20. POLDIP2 is expressed at wildtype levels upstream of P{GSV7}. The gel demonstrates semi q-PCR for a primer set upstream the P{GSV7} P-element. Tubes were removed at 28, 32, and 36 cycles. There is product present in both the mutant and wildtype cDNA, with similar intensity. Products with expected product size are also visible in all RP 49 control lanes. Loss of signal for cDNA well at 36 cycles might be due to DNA degradation.

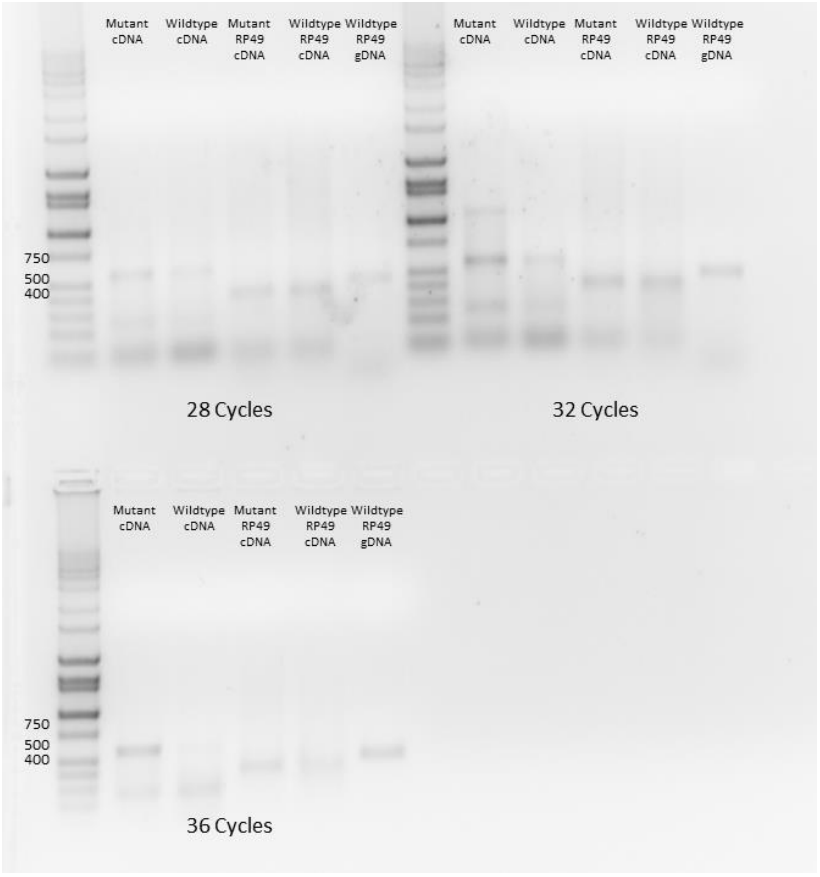

Figure 21. POLDIP2 is expressed at wildtype levels downstream of P{GSV7}TMB. The gel demonstrates semi q-PCR for a primer set downstream the P{GSV7} P-element. Tubes were removed at 28, 32, and 36 cycles. There is product of expected size present in both the mutant and wildtype cDNA, with similar intensity. Products with expected product size are also visible in all RP 49 control lanes.

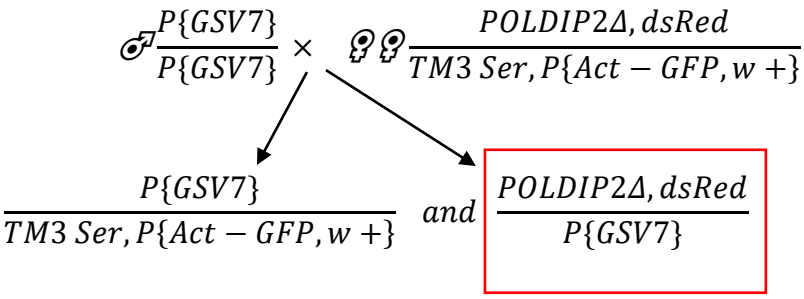

Figure 22. Crossing POLDIP2 deletion to P-Element stock. A cross between the P{GSV7} P-element stock and the POLDIP-CIRSPR knockout. The red box outlines the genotype of inviable progeny.

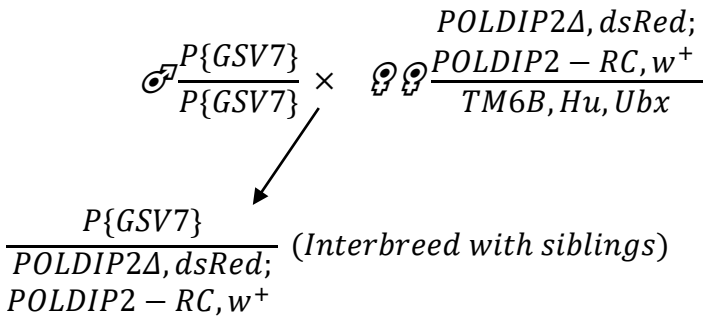

Figure 23. Crossing transgenic chromosomes to P-element stock. A cross between the P-element P{GSV7} stock and transgenic flies created in Figure 18. Siblings were mated in the subsequent generation to establish a stock.

Although the P{GSV7} alleles appears to be hypomorphic, there were no phenotypes associated with the physical appearance of flies. However, both the homozygous and heterozygous (established over a TM3 Ser balancer chromosome) were consistently sick and produced few offspring in bottles. Hatching frequencies were calculated for homozygote and heterozygote P{GSV7} flies, as well as wildtype controls. Percentages are displayed for each day of counting. P{GSV7} homozygotes had a lower hatching frequency compared to its heterozygote and wildtype counterparts. Interestingly, heterozygotes had lower rates compared to the wild type controls (Figure 24). These results support that the P{GSV7} allele had reduced function that lead to delays in hatching. This partial loss of function would explain why DsRed/P{GSV7} flies are not viable, but those with the recue construct are.

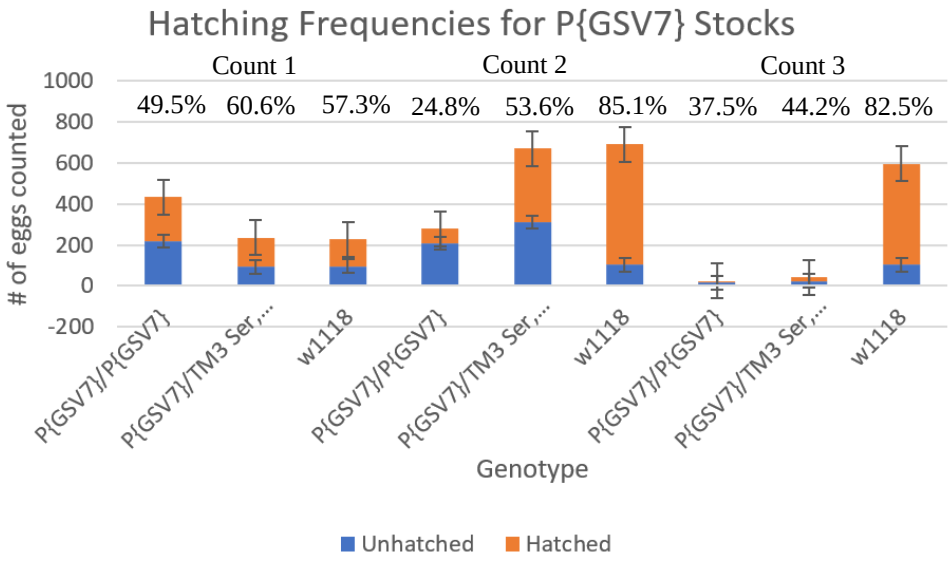

Figure 24. Hatching frequencies for P{GSV7} homozygotes and heterozygotes. Larvae were counted 48 hours after the grape plate was replaced. Three isolated were used and each cage was counted three times. Percentages of hatched eggs versus total eggs laid are shown above each count. Error bars demonstrate percent error.

Since POLDIP2 has been shown to interact with a variety of translesion synthesis polymerases (Maga et al., 2013) (Mentegari et al., 2017) we were interested in studying the effect of DNA mutagens in flies deficient for the protein. Since homozygous knockouts could not be generated, the P-element stock was used to conduct the mutagen sensitivity assay. Previous results demonstrate that these flies have an uncharacterized partial loss of function in POLDIP2, which makes them a good candidate for mutagen sensitivity assay. POLDIP2 interacts with translesion synthesis polymerases so methyl methanesulfonate (MMS) was the first mutagen tested. MMS is an alkylating agent that creates bulky lesions that the replicative polymerases cannot transcribe across. This impasse can be solved by the recruitment of TLS polymerases. However, treatment with 0.08% MMS did not result in any sensitivity when looking at percent homozygous survival between both treatment and control groups (Figure 25).

0.08% is considered a high dose, therefore, lack of sensitivity supports the idea that POLDIP2 is not involved in the bypass of bulky lesions.

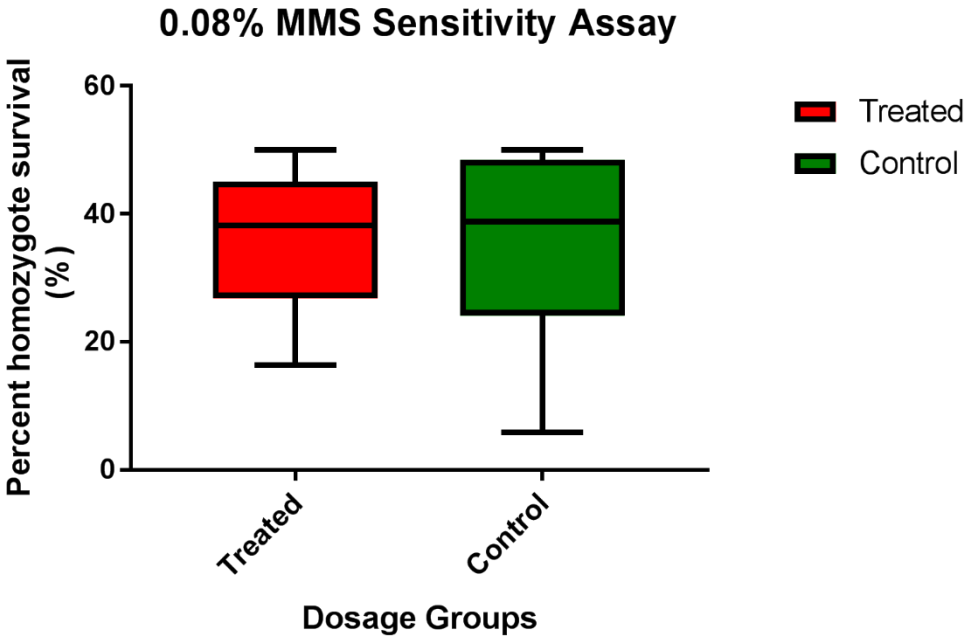

Figure 25. P{GSV7} flies show no sensitivity to 0.08% MMS. Flies were treated with 250µL 0.08% MMS while control flies were treated with 250µL of water. Flies were counted for six days after eclosure and percent homozygote survival was calculated for each dosage group.

The literature has demonstrated that POLDIP2 contains a mitochondrial targeting sequence within its first thirty five amino acids and has a few functions related to the mitochondria (Cheng et al., 2005) (Arakaki et al., 2006) (Klaile et al., 2007). Bioinformatics data was done for human POLDIP2. We were interested if *Drosophila* also has a mitochondrial targeting sequence, which would help elucidate some of its functions Human and fruit fly orthologs for POLDIP2 were tested via online mitochondrial targeting sequence (MTS). The first site used was the TargetP Server 1.1. The tool reports the likelihood of a sequence containing an MTS with a reliability class score. Figures 26A and 26B demonstrate the results for human and

fruit fly protein sequences, respectively, from TargetP 1.1 Server. The human ortholog received an RC of 1, while the fruit fly ortholog received an RC of 3. Figure 26C and 26D demonstrate the results for human and fruit fly protein, respectively, from iPSORT WWW Service. The human ortholog's first thirty five amino acids have a match to a mitochondria targeting sequence, while the first thirty five amino acids for the fruit fly ortholog do not. Due to this sequence difference, it is not expected for POLDIP2 to localize to the mitochondria in *Drosophila*.

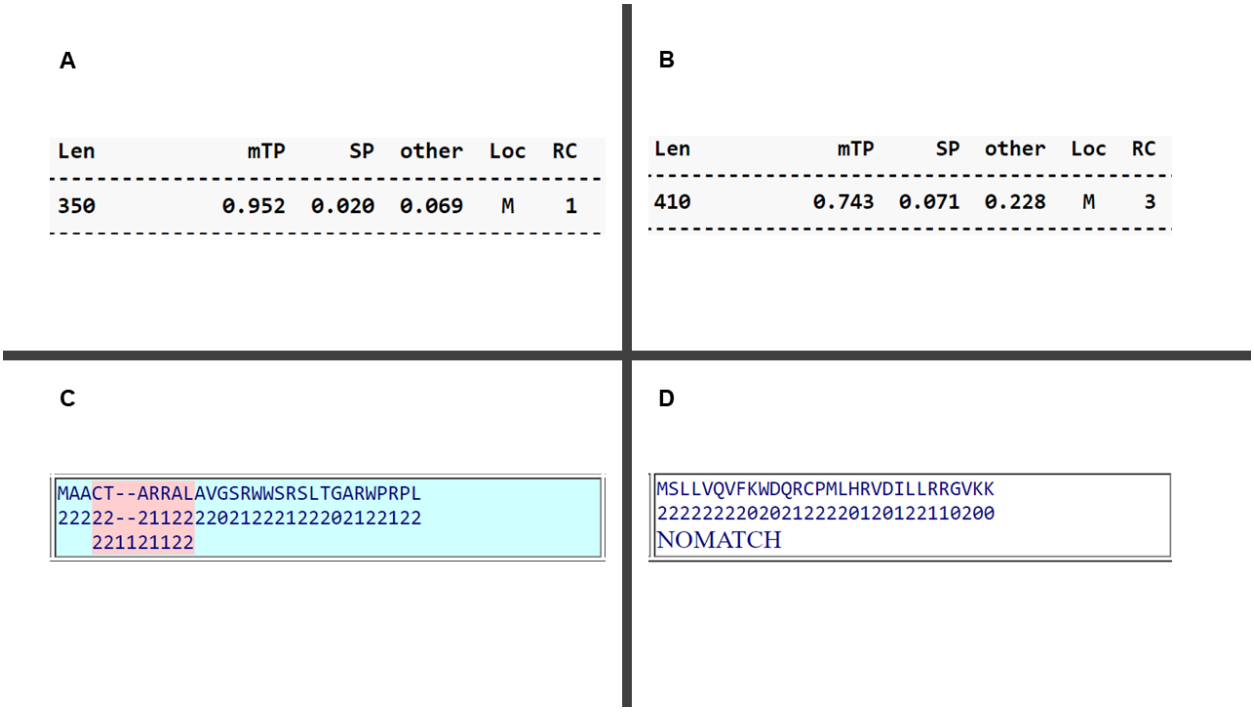

Figure 26. Mitochondrial targeting sequence prediction. A) Results from TargetP 1.1 Server for the human ortholog of POLDIP2. The amino acid sequence received a reliability class (RC) of 1, signifying a high probability of a mitochondrial targeting sequence. B) Results from TargetP 1.1 Server for the fruit fly ortholog of POLDIP2. The amino acid sequence received a reliability class of 3, signifying a middle to low probability of a mitochondrial targeting sequence. C) Results from iPSORT WWW Service for the human ortholog of POLDIP2. The box demonstrates the first thirty amino acids of the protein and amino acids highlighted in red are indicative of a mitochondrial sequence. D) Results from iPSORT WWW Service for the fruit fly ortholog of POLDIP2. The box demonstrates the first thirty amino acids of the protein. There was no match for amino acids involved in a mitochondrial targeting sequence.

Discussion

Summary of findings

POLDIP2 has been previously implicated in DNA repair and replication processes. Complete knockouts were created via CRISPR Cas-9 deletion. After establishing a stock of flies with a near-complete deletion of the POLDIP2 locus, no homozygous mutants were observed. Semi-RT-qPCR demonstrated that nearby essential ribosomal proteins, RpL35A and mRpL44, were expressed at wildtype levels in the mutant flies. Studies demonstrate that POLDIP2 double knockout mice incur perinatal lethality (Brown et al., 2014) and the need to use mouse cells heterozygous for POLDIP2 or siRNA to study the function of the protein *in vivo* (Amanso et al, 2014 and Hernandes et al., 2018). Unfortunately, only one study involving POLDIP2 has been conducted in *Drosophila melanogaster*. However, the stock used carried a P-element within POLDIP2 (Bloomington stock No.17500, CG12162EY08866), which caused reduced expression of mRNA but not a complete knockdown (Kim et al., 2015). The knockdown was also localized to the eye. The homozygous lethality in both human and rat cells led us to believe a similar lethality was being observed in our mutants.

We attempted to rescue the homozygous poldip2 mutants by recombining on a POLDIP2 transgene (Figure 16). After successful integration of the transgene and crossing to our knockout stock, homozygotes were not rescued (Figure 17). Further characterization of the lethality in this stock was done via developmental assay and an additional cross to the original mutant for POLDIP2. RT-PCR data was not collected due to homozygote larvae being phenotypically indistinguishable from those carrying a copy of the deletion and the TM6B balancer, confounding any potential expression data. The transgenic stock is currently being crossed to TM3 Ser/TM6B double balancer to establish the cross over the TM3 Ser balancer, as it can also

be scored for GFP or the loss thereof. The cross to the original mutant was done with the intention of demonstrating that the lethality might not be due to the deletion in POLDIP2 but because of a second-site mutation found on the same chromosome as the deletion of the gene. No homozygotes were observed upon the completion of the sibling cross. This data supported a model in which POLDIP2 is not essential for viability, instead, a second site lethal mutation elsewhere on the third chromosome is responsible for the lethality. RT-PCR must be conducted on homozygotes bearing the transgenic chromosomes to determine if the rescue construct is expressed at levels similar to wildtype flies.

A stock containing a P-element within the second exon of POLDIP2 was used as an additional "mutant" to study the gene. Figures 20 and 21 demonstrate that even though the P-element is 4.8 kilobases in length, there is wildtype expression of the protein, both upstream and downstream of the P-element. Both sites were analyzed as it is known that P-elements can function as secondary transcription start sites, often leading to the expression of hypomorphic alleles (LaFave & Sekelsky et al., 2011). Even though expression does not appear affected, one cannot ignore the presence of such a lengthy sequence. The folding and function of the final protein product could be affected by the additional of the P-element. Notably, the homozygote and heterozygote stocks are very unhealthy compared to the wildtype stocks. Hatching frequencies demonstrate that homozygote flies have a reduced hatching rate compared to heterozygotes, and heterozygotes have a reduced hatching rate compared to wildtype flies (Figure 24). Although the function of POLDIP2 in *Drosophila* is largely unknown, there could be many explanations for reduced hatching frequencies in these stocks. Firstly, it has been shown that in mice loss of POLDIP2 causes reduced growth and perinatal lethality (Brown et al., 2014). Since it is expected that the P{GSV7} allele retains some functionality, we can get adults, but the

stock is not healthy compared to heterozygotes and wildtypes. No notable difference in terms of size has been observed. Slower development, which is observed both in this stock and the knockout mutants, could be explained by problems in the nucleus. POLDIP2 can interact with the α-tubulin monomer during mitotic spindle arrangement (Klaile et al., 2008). Loss of POLDIP2 causes aberrant chromosomal segregation and aneuploidy in the daughter cells. Loss of POLDIP2 could delay any checkpoints for segregation and if it negatively affects the separation of chromosomes, it would explain why only a few of the eggs hatch 48 hours after laying.

The P-element stock allows us shed further light on the cause of the lethality in the knockout mutants. Since the P-element stock displayed a sick phenotype in the form of low hatching frequencies, flies were crossed to the POLDIP2 mutants (Figure 22 and 23). No adults carrying the DsRed marker were observed, demonstrating that the copy of POLDIP2 with the P{GSV7} P-element was not enough to produce a viable organism. However, {PGSV7} was successfully established over the transgenic chromosome carrying both the deletion and the POLDIP2 transgene. This data demonstrates that the transgene is being expressed at a level that allows for the adults to reach adulthood. This level of expression should theoretically rescue the homozygotes that carry the transgene.

Current model for POLDIP2-knockout lethality and future characterization

After failing to rescue homozygotes via the rescue construct, the "second site lethal mutation" hypothesis was the one with the most support from the data. While transgene expression levels must be verified, the results of the crosses to the P-element stock support that POLDIP2 is essential, however, homozygotes cannot be rescued due to the presence of a second

site lethal mutation. The data also suggests that this mutation is tightly linked to the DsRed locus as it has not been recombined off in previous crosses.

Another round of second-site mutation cross-offs have been initiated to recombine off this site of lethality. The goal is to set up a larger number of crosses to increase the chances of getting viable homozygotes. However, if POLDIP2 is essential and the mutation is successfully crossed it would not be possible to recover viable mutants. One way of addressing this problem would be to conduct the second-site mutation cross off in flies carrying the transgene and the POLDIP2 deletion. Since this cross requires the screening of DsRed and w^+ in the second generation, the assay would require a double cross over event to conserve both the rescue construct and the fluorescent marker (Figure 27). This would require us to create mosaic clones to be able to analyze knockout-cells in a viable organism. These systems allow lethal genotypes to exist on a viable organism. Since the fly would contain both knockout and wildtype cells, it would be easy to detect any phenotypes. An additional experiment would be to knockdown POLDIP2 via RNAi in specific fly tissue cell lines. This would give greater insight into the potential roles of the protein while being a point of comparison to the literature in which RNAi used in mammalian systems. Preliminary results with siRNA-POLDIP2 flies demonstrate a similar lag in development as the knockout mutants. This experiment can be complemented by attempting another CRISPR knockout with different gRNAs to create another mutant.

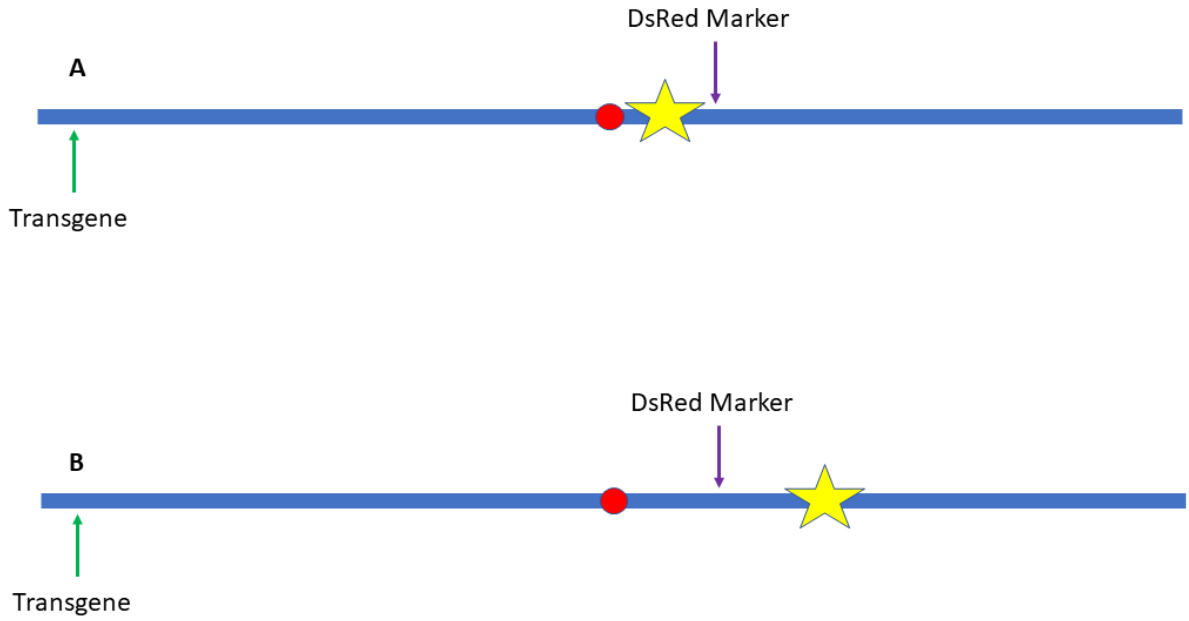

Figure 27. Possibilities for second site cross off with transgenic flies. A) The second site mutation, represented by the yellow star, is located upstream of the DsRed marker. A double cross over event would be required to lose the mutation but conserve the transgene and the fluorescent marker. B) The second site mutation is located downstream of the DsRed marker. Only a single cross over event would be required to recombine the mutation off.

Future directions

POLDIP2 is not only an enigmatic protein because of its multiple functions but also because of its dual localization in the nucleus and the cytoplasm. The literature reports that POLDIP2 is mostly found in the cytoplasm (Klaile et al., 2007), which places a spotlight on its function there versus the nucleus. Figure 26 demonstrates that the *Drosophila* ortholog does not contain any mitochondrial targeting sequences. This is an interesting find as there is a plethora of data indicating that POLDIP2 is regularly found at the mitochondria or in the cytoplasm. Many of the functions POLDIP2 has been implicated in a specifically localized in the nucleus. We attempted to identity a nuclear localization sequence for both the human and fruit fly protein but did not get any matches (results not shown). This result suggests that POLDIP2 would most

likely need an additional protein to shuttle it between the cytoplasm and the nucleus. One paper characterizes the interaction between CEACAM1 and POLDIP2, demonstrating that CEACAM1 aids POLDIP2 in localizing to the nucleus (Klaile et al., 2007). CEACAM1 is a cell-cell adhesion receptor that is a regulator of contact-dependent survival, differentiation, and growth. It is expressed in a short and long isoform, both of which POLDIP2 has been shown to interact with (Klaile et al., 2007). If POLDIP2 can shuttle between the cytoplasm and the nucleus depending on the stage of the cell cycle, this would help understand how it coordinates its multiple functions. For example, it would further support the hypothesis that POLDIP2's DNA repair capabilities are enlisted to guard against the ROSs it has helped create. Interestingly, other papers have also demonstrated that a large fraction of POLDIP2 is maintained in the cytoplasm, and only a small fraction is found in the nucleus (Lyle et al., 2008) (Paredes et al., 2018). This supports the model in which POLDIP2 spends most of its time functioning in the cytoplasm but is recruited to the nucleus during replication. Klaile et al. specifcy that POLDIP2 located in the nucleus primarily during late G2/M-phase, which makes sense as POLDIP2 has been shown to be involved in chromosomal segregation.

Sequence alignment of genomic CEACAM1 to the *Drosophila melanogaster* genome yielded no significant similarity (results not shown), suggesting that CEACAM1 might only be present in higher mammals. Since CEACAM1 has been the only protein reported in aiding POLDIP2 transportation, it is curious that fruit flies do not contain an ortholog as we do not predict POLDIP2 localizing to the mitochondria. While many pulldowns have been done in the literature to identify interactors of POLDIP2, they have all been in done in mice or human cells. A key experiment would be to conduct a pulldown for the Drosophila ortholog to identify protein interactors that could elucidate how POLDIP2 functions and is localized in the cell. Additionally,

tagging POLDIP2 with a fluorescent marker would allow cells to be isolated at different stages of the cell cycle to better map where POLDIP2 localizes. Cells at different stages of the cell cycle would allow us to compare localization with what has been reported in the literature.

Flies with a P-transposable element in POLDIP2 were treated with 0.08% MMS and water for control (Figure 22). No significant sensitivity to the reagent indicated POLDIP2 is not a key player in translesion synthesis across bulky, alkylated lesions. However, the literature has demonstrated that POLDIP2 can be important for processivity across 8-oxo-guanines, a DNA lesion resulting from oxidative damage (Maga et al., 2013) (Mentegari et al., 2017). Some of the chemicals that can induce this type of lesion are 4-nitroquinoline, hydrogen peroxide, and paraquat. If POLDIP2 does play a role in bypassing oxidative damage, it is expected that sensitivity will be observed for some or all these reagents. It is also important to reconcile POLDIP2's DNA repair function with its cytosolic functions. POLDIP2 has been reported as an upregulation of NADPH oxidases, which leads to an increase of reactive oxygen species in the cell (Lyle et al., 2009). ROSs are a common source for oxidative DNA damage. One hypothesis for the multiple functions of POLDIP2 could be that its DNA repair functions exist to alleviate the danger of ROSs produced by NADPH oxidase upregulation in the cytosol.

The POLDIP2 protein is severely understudied and there is not much conclusive data about its properties. A pBLAST shows that POLDIP2 contains an ApaG protein interaction domain. This makes sense as POLDIP2 appears to interact with a variety of proteins and might serve as a potential mediator, especially in the context of DNA repair. It has also been shown that POLDIP2 contains an MTS in the N-terminal domain. Future experiments should aim to create mutants that introduce earlier stop codons or have entire parts of the gene excised out. This could provide information on the functional relevance of different parts of the protein.

Conclusion

POLDIP2 knockouts were created via CRISPR Cas 9. The stock did not yield any homozygotes, and they lag in comparison to their heterozygote counterparts. Nearby essential ribosomal protein genes exhibited wildtype expression. Homozygotes were not rescued by a POLDIP2 transgene inserted on the third chromosome. Crosses knockout mutants and P{GSV7} stock indicate that POLDIP2 is essential in *Drosophila melanogaster*, however, rescue is not possible due to a second site lethal mutation. Additional work must be done to isolate the second site mutation to test if the transgene is able to rescue homozygotes. Mutagen sensitivity assays will be employed to further characterize the role of POLDIP2 as it pertains to DNA repair.

Supplementary Figures

5’ gRNA	5’-GGTGCCCAGAGGCTCGAAAGCCT-3’
3’ gRNA	5’- TCTCGTTCGGCCTGCTGGATCCC-3’

Supplementary Figure 1. gRNA Sequences. Guide RNA sequences for CRIPSR Cas-9 deletion are shown above.

dsRed -114 Forward	5’-TGATCGCCGAAAGAAGCTGC-3’
dsRed +111 Reverse	5’-TGGCTCTGCGTCCTTGTTG-3’
mRpL44 Forward	5’-CATCTTCACCGAATCCCTTG-3’
mRpL44 Reverse	5’-GATGTCATTCTTACGCAGTGC-3’
mRpL35A Forward	5’-GTTCTCGTGCTGGTTCCTC-3’
mRpL35A Reverse	5’-CGGTGTTAATACATTCATCGAAC-3’

Supplementary Figure 2. Primer Sequences for PCR and Semi-qPCR. Primer sequences for ribosomal protein amplification and identification of dsRed insert. Primers were obtained from Eton Bioscience and IDTDNA and resuspended in pico-pure H_2O for a 50mM concentration.

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