

2012

## INVESTIGATING THE PED PROTEIN AND ITS EFFECT ON TRANSLATIONAL CONTROL IN DROSOPHILA MELANOGASTER SPERMATOGENESIS

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INVESTIGATING THE PED PROTEIN AND ITS EFFECT ON TRANSLATIONAL  
CONTROL IN DROSOPHILA MELANOGASTER SPERMATOGENESIS

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THESIS

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A thesis submitted in partial fulfillment  
of the requirements for the degree of Master of Science in the  
College of Arts and Sciences  
at the University of Kentucky

By

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2012

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## ABSTRACT OF THESIS

### INVESTIGATING THE PED PROTEIN AND ITS EFFECT ON TRANSLATIONAL CONTROL IN DROSOPHILA MELANOGASTER SPERMATOGENESIS

Inactive mutants of the *ped* gene cause two phenotypes in *Drosophila melanogaster*: male sterility and the early translation of DHODH within spermatogenesis. Investigation of the PED amino acid sequence revealed an OTU domain and an ubiquitin interacting motif, suggesting that it is a member of the otubain sub-family of de-ubiquitinating enzymes. To test this, the putative active cysteine residue was mutated. Results show that this single cysteine residue is required for *ped* to confer male fertility. Purified wild type PED was also used to carry out *in vitro* deubiquitinating assays. These assays failed to show any ability for PED to cut ubiquitin chains of varying length or linkage type. Previously, a translational control element was identified in *dhod* mRNA which is required for its early translation phenotype in *ped* mutants. In an attempt to identify additional transcripts that have their translational timing affected by PED, the *don juan-like* 5' UTR was inserted into a reporter gene and examined in a *ped* mutant background. No delay of this reporter gene was observed suggesting that *don juan-like* mRNA is not under the exact control pathway that *dhod* is.

KEYWORDS: translational control, deubiquitination, spermatogenesis, otubain, proteolysis

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5/1/12

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## CHAPTER ONE: INTRODUCTION

Translational regulation is a form of post-transcriptional regulation that is an important method of controlling the timing and levels of protein available within cells and can be mediated by several elements within a mRNA transcript (reviewed in Gebauer and Hentze 2004). The 5' cap, which is composed of a 7-methylguanylate attached to the most 5' nucleotide of the mRNA, is attached in a 'backwards' fashion that protects the transcript from exonuclease degradation and stabilizes it. This cap is also recognized by translation initiation factors that participate in canonical ribosome assembly which involves addition of the 43S ribosomal subunit near the cap and the subsequent scanning by the 43S subunit along the transcript to find the appropriate start codon (reviewed by Kozak 1978). The poly(A) tail is also an important factor in translational control. It is generally required that the length of a transcript's poly(A) tail be within a certain range for translation to occur. Tails that are either too short or too long can prevent translation of the associated transcript (Kleene 1989). Eukaryotes can also employ internal ribosome entry sites (IRES) which facilitate cap-independent translation. This phenomenon occurs through recruitment of an initiation complex directly to the secondary structure of the IRES element, bypassing the need for any cap binding factor completely (Kullmann, Gopfert *et al.* 2002). Another method of control involves upstream open reading frames (uORFs). These elements are small reading frames that exist upstream of the start codon of a gene's main protein product, coded by its primary open reading frame (ORF). uORFs can compete with a gene's ORF for available translational machinery, thereby reducing the rate of translation of the main ORF (Gopfert, Kullmann *et al.* 2003; Pickering and Willis 2005; Mehta, Trotta *et al.* 2006). In recent years, MicroRNAs have also become more recognized as regulators of translation. They have been shown to regulate translation through a variety of mechanisms including promoting mRNA degradation, inhibition of ribosome assembly, prevention of translation initiation, and inhibition of the elongation phase of translation (reviewed in Fabian, Sonenberg, *et al.* 2010). *Drosophila melanogaster* oogenesis and early embryogenesis are systems in which silencing of translation plays a role in establishing axes-defining gradients (reviewed in Lasko 2011). Furthermore, because a *Drosophila* embryo does not begin to

transcribe its own mRNA until the ninth mitotic division, it must utilize transcripts that are maternal in origin which have been translationally silenced (reviewed in Lasko 2011).

The mechanisms discussed above may work in combination with one another. For example, an IRES located between a uORF and ORF can help increase the rate of translation of the ORF, or an IRES may be located within a uORF (Gerlitz, Jagus *et al.* 2002; Chappell and Mauro 2003; Yaman, Fernandez *et al.* 2003; Bastide, Karaa *et al.* 2008). A sequence within a transcript that affects the timing of the translation of that transcript is often referred to as a translational control element, or TCE.

While the process of translational control is important to all eukaryotic cells, it comes to the forefront during spermatogenesis. In *Drosophila melanogaster*, as the DNA in the forming sperm is packaged for delivery to an egg cell, transcription is largely shut down. About halfway through spermatogenesis, around the point at which the cells begin to undergo meiosis, histones are replaced with protamines and the chromosomes are compacted for delivery (reviewed in Fuller 1993). This phenomenon is prevalent among eukaryotes that reproduce sexually (reviewed in Schafer, Nayernia *et al.* 1995; Siffroi, Pawlak *et al.* 2001; Yang, Medvedev *et al.* 2005). Consequently, organisms which utilize spermatogenesis, including *Drosophila melanogaster*, have very little to almost no genes transcribed after the meiotic step in the process (reviewed in Fuller 1993). Because of this, pre-meiotic spermatogenesis must include the transcription of numerous mRNAs which will have their translation delayed until post-meiotic spermiogenesis, the morphological formation of the spermatids (reviewed in Fuller 1993). At which time, they will presumably be called upon by the cell to be translated in the distinct order necessary to drive the remaining morphological changes and maturation into the final sperm product. This delayed translation model is supported by work in *Drosophila* that demonstrated that spermiogenesis can continue to completion despite the removal of the vast majority of DNA from the spermatids before meiosis occurred (McCloskey 1966; Baldwin and Chovnick 1967; Lindsley and Grell 1969).

Spermatogenesis, therefore, provides us with an ideal model for investigating translational control as numerous transcripts are reproducibly found to be either stored or translated depending on the stage of maturity of the sperm. Furthermore, it provides an

arena wherein groups of transcripts whose translation is coordinated in various ways, whether that be in a simultaneous or step-wise manner, may be studied.

Relatively few translational control elements have been identified within transcripts specific to spermatogenesis (Yanicostas and Lepesant 1990; Kempe, Muhs *et al.* 1993; reviewed in Schafer, Nayernia *et al.* 1995; Fajardo, Haugen *et al.* 1997; Luo 1999; Blumer, Schreiter *et al.* 2002; Hempel, Rathke *et al.* 2006). The *janB* gene of *Drosophila* was demonstrated to have a 5' element which delayed the translation of the mRNA until post-meiotic spermatogenesis in 1990 (Yanicostas and Lepesant 1990). An 18-bp element was next discovered in the *beta 2 tubulin* gene in *Drosophila*, which is only expressed in late stages of spermatogenesis within the fly and is a key component of the flagellum (Michiels, Buttgereit *et al.* 1993). Work done by Schäfer, *et al.* identified a 12bp element in the 5' UTRs of the four members of the *mst(3)CGP* gene family. These elements seemed to be somewhat conserved (all being AU rich) and also provided a translational delay until post-meiotic stages of spermatogenesis (Schafer, Kuhn *et al.* 1990). Furthermore, work on the 5' TCE within the family member *mst87f* showed that a protein bound to it with specificity (Kuhn, Kuhn *et al.* 1991; reviewed in Schafer, Nayernia *et al.* 1995). In mice, the *protamine 1* transcript was determined to have a 3' element which conferred a spermatogenesis-specific delay (Fajardo, Haugen *et al.* 1997) and in 2005, a RBP/binding motif combination was discovered in mouse which seemed to mark transcripts for cytoplasmic storage during spermatogenesis (Yang, Medvedev *et al.* 2005). Most recently, in 2006, the discovery of the 5' translational control elements in the *don juan* and *don juan-like* genes of *Drosophila* were discovered (Hempel, Rathke *et al.* 2006). All of this work has led to relatively little understanding of the mechanisms of the delays these control elements provide, nor how these elements provide for specific timing of translation to occur. In the *Drosophila* examples of delayed expression above, most are translated in the elongation stage, with the exception of *janB*, which is expressed before the elongation stage shortly after meiosis occurs. Other than the conserved AU rich control elements and timing similarities previously mentioned, no other connection has been found amongst the above examples.

In *Drosophila melanogaster*, the *dihydroorotate dehydrogenase* gene (*dhod*) became of interest in the context of delayed translation within spermatogenesis when it

was observed that there was a testis-specific, longer isoform of its mRNA. This isoform had its product's activity delayed until late spermatogenesis. Investigation of the testis-specific mRNA isoform led to the discovery of a 5' translational control element, thereafter called the *dhod* Translational Control Element (*dTCE*) that, when attached to a reporter construct, conferred the same delay upon the product of the reporter gene (Yang, Porter *et al.* 1995; Luo 1999).

Using a *lacZ* reporter construct containing the *dTCE*, approximately 200 existing male-sterile mutant lines were screened (Rawls unpublished work). If the sterility of one of these lines was caused by a faulty pathway that was also responsible for the translational delay conferred by the *dTCE*, then premature translation of the reporter gene mRNA should occur. This proved to be true in one of the lines screened. Subsequent mapping revealed the mutation to be in the *CG6091* gene, thereafter called *ped* (precocious expression of *dhod*) (Rawls unpublished work). Microarray data from other labs conducting high-throughput experiments has shown an 8-fold enrichment of *ped* mRNA signal within the testes over that found in the whole fly (Chintapalli, Wang *et al.* 2007).

Expressed sequence tag (EST) data suggests the existence of four *ped* transcripts (figure 1.2). Two transcripts representing the most abundant ESTs have been studied to date. These being *CG6091-RE* and *CG6091-RD* which are the full length transcript and the transcript with the alternate start site which eliminates a large N-terminal portion of the protein, respectively. GFP tagged alleles have been created which allow only the expression of either one or the other isoform, along with GFP and TAP tagged versions of the gene which allow expression of all isoforms of the transcripts. The GFP-tagged versions' expression patterns were investigated. The long form of the mRNA is expressed early in pre-meiotic stages of spermatogenesis while transcription is still occurring. It is seen throughout the testis however, and may be expressed throughout spermatogenesis or simply may be only expressed early and persist into later stages. The short form, however, is translated only after meiosis and expressed in a discrete subset of elongated spermatids. The long form of GFP tagged PED rescues the male sterility and the precocious expression phenotypes, while the GFP tagged short form rescues neither (Rawls unpublished work).

The mutant *ped*<sup>l</sup> allele that was identified through the screening of male sterile lines is a nonsense mutation within an ovarian tumor (OTU) domain toward the middle of the full-length PED protein. The C-terminal end of the protein contains a ubiquitin interacting motif. The N-terminal-extended portion of the PED isoforms encoded by the longer transcripts contains no known conserved domains (figure 1.4) and is highly conserved only among *Drosophila* species. Additional mutant *ped* alleles have been isolated, all of which result in precocious expression of *dhod* as well as spermatogenesis arrest during later stages of the process.

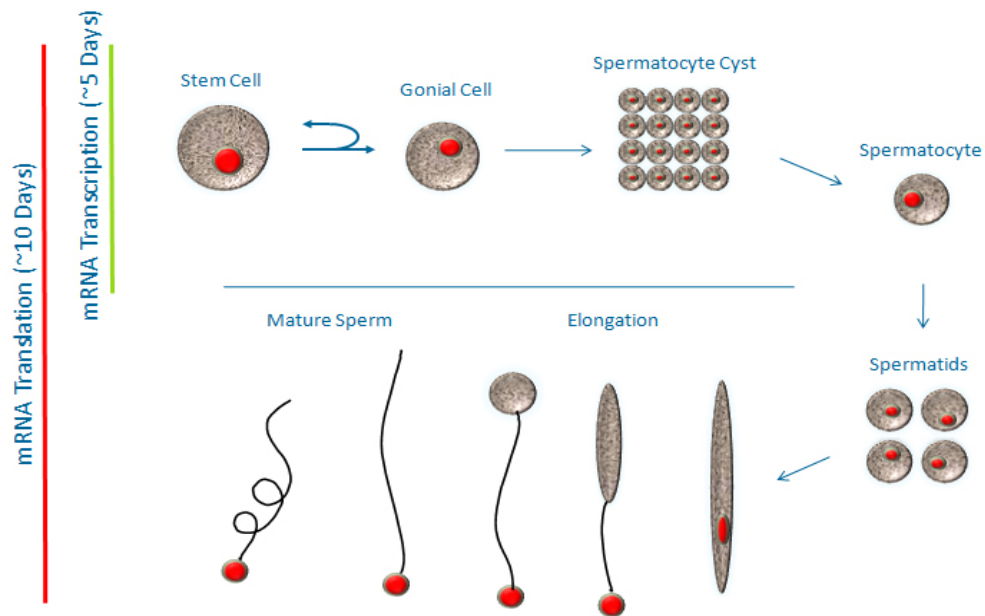
Having an OTU domain and a ubiquitin interacting motif, PED is identified as a member of the otubain-like cysteine protease sub-family of deubiquitinating (DUB) enzymes (Hunter, Apweiler *et al.* 2009). Within the OTU conserved domain, there is relatively poor conservation of the sequence itself between PED and other members of the sub-family. Despite this, the I-Tasser protein folding prediction software has shown that the PED OTU region can be folded in a strikingly similar manner to the active site of otubain 1, the defining sub-family member. The predicted structure includes a beta sheet similar to the single beta sheet found within otubain 1 (figure 1.5) (Zhang 2008).

Translational control pathways might affect groups of mRNAs on a global scale or regulate only a small specific set of mRNAs. Several pieces of preliminary evidence suggest that the PED pathway affects additional transcripts in spermatogenesis than just *dhod*. For example, *dhod* mutants are fertile while *ped* mutants are sterile (Rawls 2000-2006). This suggests that transcripts required for fertility have their expression affected by PED as well. Also, the *mst87F* TCE competed with the *dhod* TCE in EMSA experiments (Luo 1999), suggesting that other essential transcripts are controlled by the PED pathway.

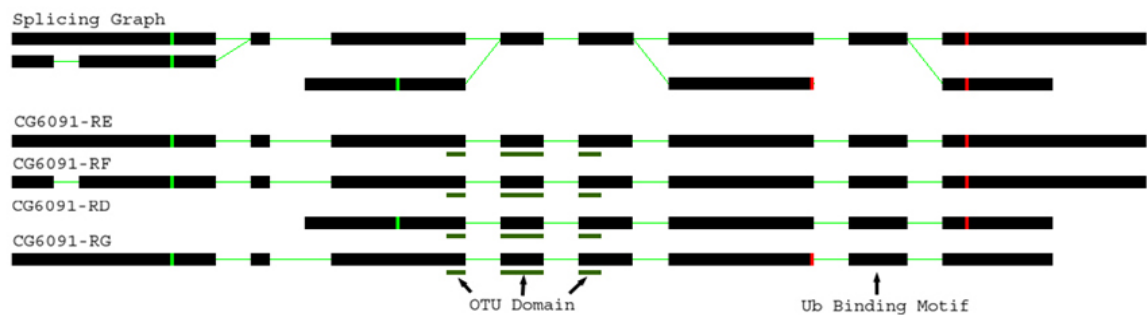
In this study I investigated different aspects of the PED protein and its role in delayed translation. To test the hypothesis that the putative catalytic cysteine residue is required for PED functionality, the cysteine was mutated to an alanine residue. This mutant transgene, within a *ped* mutant background, did not rescue the male sterility or early translation phenotypes. Additionally, I investigated whether PED has the ability to cut ubiquitin chains. To test this, PED was expressed and purified from *E. coli* and that purified product was incubated with ubiquitin chains of varying length and linkage type.

No cutting of any of the ubiquitin chains was seen using this approach. Finally, to investigate the idea that other transcripts require PED for proper translational timing during spermatogenesis I investigated the *don juan-like (djl)* gene. Like DHODH, translation of the *djl* protein is delayed within spermatogenesis until the late tail elongation stage due to a control element in its 5' UTR (Santel, Winhauer *et al.* 1997). To test whether the *djl* protein was translated prematurely in a *ped* mutant background, the expression pattern of a reporter construct containing the full *djl* gene 5' UTR coupled with a GFP open reading frame was observed in fly testes which lacked functional copies of *ped*.

## *Drosophila* Spermatogenesis



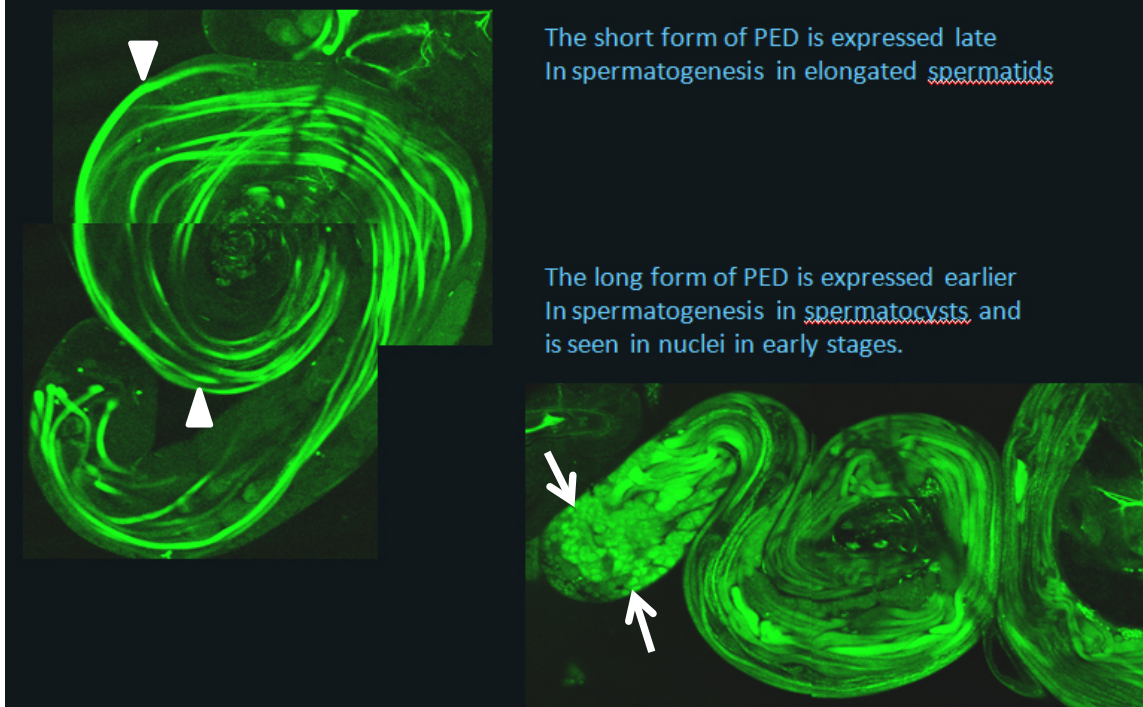
**Figure 1.1: *Drosophila* spermatogenesis.** In *Drosophila melanogaster*, spermatogenesis lasts for approximately ten days. During the first five days, (upper panel), a single gonial cell undergoes four mitotic divisions to produce 16 spermatocytes. Each of these then undergoes meiosis and each of the 64 resulting spermatids elongates and continues to develop into a mature sperm cell during the last five days (lower panel). Only during the first five days, before meiosis, are transcripts made. This requires that proteins that must be translated during the last five days which are required for final spermatid maturation, to be translated from mRNA which was made during the pre-meiotic stages and stored until needed (figure modified from Rawls, unpublished data).



**Figure 1.2: *ped* transcript isoforms.** All *ped* transcript isoforms are supported by at least 46 cDNA clones and all are categorized as strongly supported by FlyBase (McQuilton, *et al.*). Black and green horizontal bars represent exons and introns, respectively. Green and red vertical stripes represent start and stop codons, respectively.



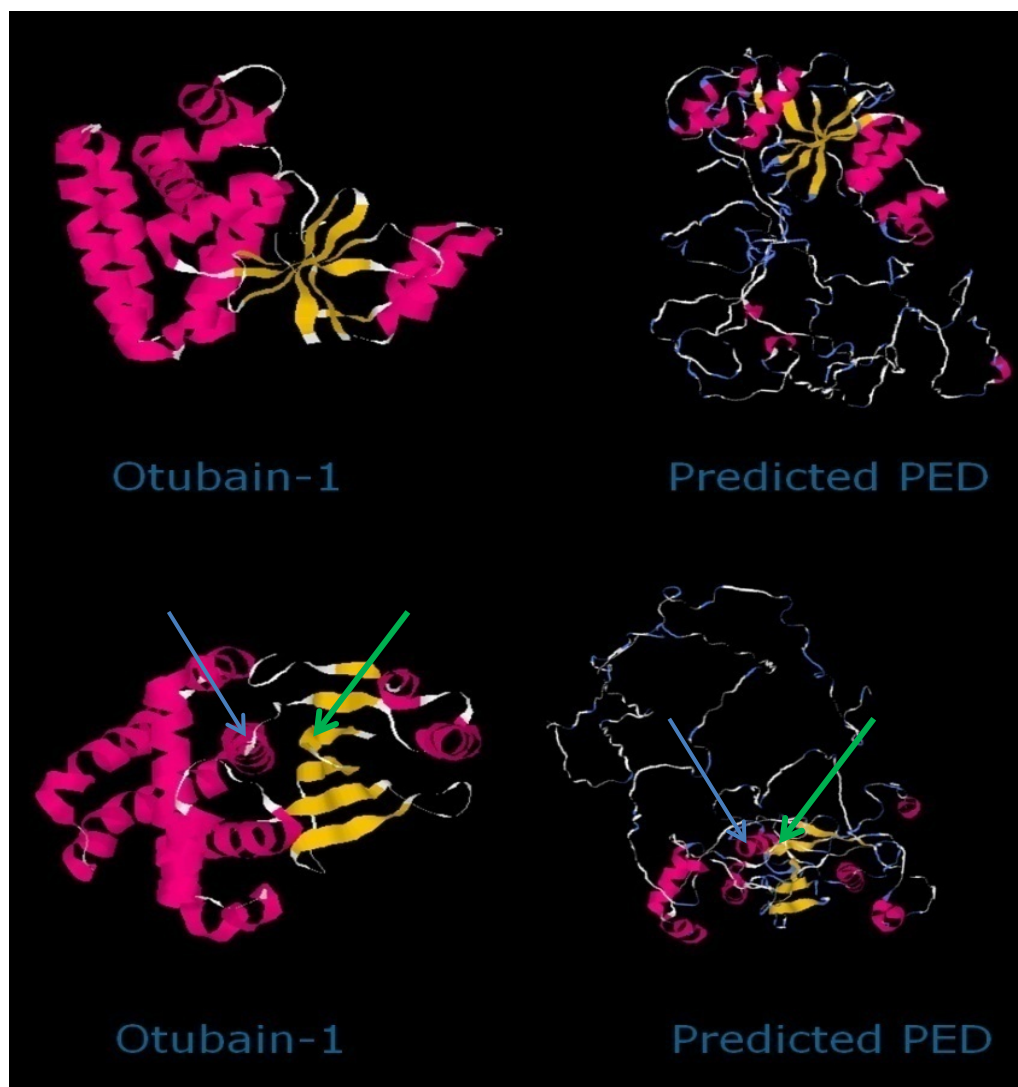
## Expression of the Two Isoforms



**Figure 1.3: PED isoform expression patterns.** The expression patterns of the short and long forms of PED with C-terminal GFP fusions. The upper-left image represents late, post-meiotic expression of the short form within spermatid tails (arrowheads). The left-most portion of the lower image shows earlier expression of the long form in round spermatocytes of pre-meiotic cysts (arrows) (Rawls, unpublished data).

OTU Domain	→ long form (spermatocytes)		
	MTIKPVSAPPSAKRVAGNVDNKEAQVVVVEQSVVSQTHNHAHNHRNVV	50	
	IDGQSPQRRGEVYDELARTHRCSPHKSTRSKRREHHEAHAHLKYRDLER	100	▼ K→stop ( <i>ped<sup>ds7</sup></i> )
	EKLVHPTAAAGSGAPGVSGSKCNSPPSTSTSGSSSPSAVGRNSPEHLGLG	150	
	CTTVPTAQVQMSTSTAAANLLKTVEETFSGYNSGDEHHQPKERLIPVEEW	200	→ short form (spermatids)
	QRRDLEFAKCMERQGYELKPVVEEDGACLFRSISLQIYGDEEMHDHENREY	250	
	FGQFVTEDINSYIQRKRARDAHGNHIEIQAISEIYSRTVEVYCYQSNPIN	300	▼ Q→stop ( <i>ped<sup>d1</sup></i> )
	IFNSEQSQAGYPPLRLSYQRGSHYNAILDPYNATVGVLGLAGYKPEIQT	350	
	KEAVRLSEQLEIEQTMFEDKLKTTDWEATNEAIEEQIARESYLQWCRENT	400	
	QSRNSNTAAGSATSSVTSAEALTDSDASPSKYSACGGTGSSGNPPVAL	450	
	TSGSGGDGAGPAFSLSPKTLNQFSHKLPQEVNELGGYDSDATDMSSTSSV	500	
	GHSGGSSSPSTAASQRSGKSKSQRRTALRKKRRHETRETTLASKSAEA	550	
	LEAPLRKSPKRDSAPSVARADTPEAEQRPSTSKQSSHSPQKNVDSKSPTE	600	Δ <i>ds11</i>
	QSYSSFYQELLEASYANEGANESEMLQQAIQMSTRDYMEDQKRKFLFGP	649	
			Cysteine Protease Catalytic Triad
			Ubiquitin Interacting Motif

**Figure 1.4: Annotated sequence of PED.** Different isoforms of PED and the currently available mutant alleles are indicated. Both isoforms contain the OTU domain and ubiquitin-interacting motif, both indicated by bold sequence. The conserved residues within a cysteine catalytic triad are shown, with the putative chemically active residues (the triad) underlined (figure modified from Rawls, unpublished data)



**Figure 1.5: Predicted folding of PED.** Two views of the I-Tasser folding prediction results (Zhang 2008). Of the catalytic residues in the cysteine protease domains (figure 1.4), two are found in the beta sheet (green arrows) and one is found in an adjacent helix (blue arrows). Note the similar orientation of the nearest helices to the beta sheet in each image.

## CHAPTER TWO: MATERIALS AND METHODS

### Fly lines

All transgenic fly lines were created by micro-injecting the respective pCasper-containing construct into a fly line homozygous for a P transposase (Robertson, Preston *et al.* 1988). The P transposase was subsequently crossed out of flies which expressed the  $w^+$  gene until flies stably expressing the  $w^+$  gene and having markers which indicated the absence of the transposase-containing chromosomes were obtained.

### Active site amino acid substitution

To test whether the putative catalytic cysteine residue within PED is required for PED functionality, the cysteine residue was mutated to a non-functional alanine. Efforts to mutate the existing pedGFP construct (p6091 $\Delta$ ApaGFP) (Rawls, unpublished work) as a whole using linear amplification with a non-strand displacing polymerase proved unsuccessful. Therefore, the smaller SalI-XhoI fragment from this construct, which contained the codons for the active site residues, was inserted into the pBSKSII+ vector and named pBSWT (wild type). Mutations were introduced into the pBSWT construct using the non-strand displacing Pfx Accuprime system (Invitrogen) with the CA+, CA- primer set (table 2.1). The mutated product was transformed into DH5 $\alpha$  cells and verified through sequencing. The mutated SalI-XhoI fragment was then excised from pBSWT and re-inserted into pedGFP. This new vector was called PCAG. After verification of proper ligation, the presence of the mutation was verified by confirming the absence of the NarI site which was destroyed by the CA+, CA- primer set.

The mutated, GFP-fused *ped* of PCAG was ligated into a pCasper4 vector using the unique KpnI and EcoRI sites. This construct was introduced into a fly line as described above. To ensure the insertion site of PCAG was not a factor, the element was re-mobilized and two additional stable lines were isolated.

### $\beta$ -galactosidase histochemical staining

Testes expressing  $\beta$ -galactosidase were dissected in fixative (0.25% glutaraldehyde, 10 mM NaPO<sub>4</sub> - pH 7.5, 150 mM NaCl, 1 mM MgCl<sub>2</sub>) and incubated for 5-15 minutes at room temperature. They were washed twice with Buffer A (10 mM NaPO<sub>4</sub> - pH 7.5, 150 mM NaCl, 1 mM MgCl<sub>2</sub>). 50  $\mu$ l of 8% X-gal was added to 2 ml staining solution (10 mM PO<sub>4</sub> buffer – pH 7.2, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 3 mM

K<sub>4</sub>[FeII(CN)<sub>6</sub>], 3 mM K<sub>3</sub>[FeIII(CN)<sub>6</sub>], 0.3% Triton X-100) which was pre-warmed to 37°C. This stain mixture was applied to the testes and incubated at 37°C from one hour to overnight. Once the desired level of development had been reached testes were washed in distilled water and mounted on a slide in 50% glycerol. Pictures were immediately taken using incidental light.

### **PED expression**

A cDNA fragment representing the whole of the *ped* gene region (RE19005) was used as a template to amplify cDNA fragments which would allow either the expression of the long or short isoforms of the PED protein. Primers used to create the 'long form,' or P1, cDNA fragment were pET 5' P1a and pET 3' ped. Primers used to create the 'short form,' or P2, cDNA fragment were pET 5' P2a and pET 3' ped. The resultant PCR products were cut with BclI and NdeI and the cut products (2 kb for P1 and 1.5 kb for P2) were gel purified. These fragments were ligated into the pET16b vector using the compatible and unique BamHI and NdeI sites present in the vector.

Plasmids were transformed into BL21(DE3) *E. coli* cells using heat shock. Transformed colonies were heavily inoculated onto a new plate and incubated at 37°C overnight. A loop-full of cells from each plate (P1 and P2) were then introduced into 220 ml each of pre-warmed LB with ampicillin. These were shaken at 200 rpm at 37°C until an OD<sub>600</sub> between 0.6 and 0.8 were obtained for each of the flasks. To induce expression of the PED isoforms, the flasks were chilled on ice for 60 minutes then 1.7 ml of 50 mM IPTG was added to each and then each was shaken again overnight at 200 rpm at 17°C. The induced strains were then aliquoted into 50 ml conical vials. Each of these was spun in an SH3000 rotor at 3000 rpm for 40 min at 4°C. The supernatant was discarded and pellets were stored at -70°C.

To purify the expressed protein, a 50 ml aliquot was thawed on ice. To it, 2.5 ml Buffer A (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 10% sucrose) and 12 µl of 50 mg/ml lysozyme was added (a low-salt modified form of Buffer A was also used in some experiments, where noted, which used only 50 mM NaCl). This was shaken for 40 minutes on ice. 50 µl 5% Triton X-100 was added and shaking resumed for another 15 minutes. The resultant mixture was sonicated on ice for six ten-second pulses with 30 seconds recovery time between each pulse. It was then centrifuged in an SS34 rotor at

13k rpm for 30 minutes. The supernatant was combined with 1 ml Ni-NTA Superflow slurry in a 6 ml tube and slowly rotated for 60 minutes at 4°C. All subsequent steps were carried out at 4°C. The resin was centrifuged in a microfuge at 4.5k rpm for five minutes. The pellet was washed three times in buffer E25 [50 ml 2X Buffer E (100 mM Tris-HCl pH 7.4, 500 mM NaCl, 20% glycerol), 2.5 ml 1 M imidazole pH 7.5, 47.5 ml H<sub>2</sub>O] and resuspended in buffer E25. This mixture was loaded onto a gravity column and allowed to flow through. The resin was washed three more times with buffer E25. The PED protein was eluted by first adding 0.4 ml (approximate volume of resin bed) buffer E500 (50 ml 2X buffer E, 50 ml 1 M imidazole pH 7.5). Flowthrough was discarded. Then, after addition of 0.5 ml buffer E500, the eluate was captured. Another 0.5 ml buffer E500 was added and the resulting eluate added to the previous. The combined eluate was dialyzed overnight against Buffer D (50 mM Tris-HCl pH 7.4, 2 mM DTT, 1 mM EDTA, 10% glycerol) with two early changes of Buffer D after 15 and 30 minutes. Protein was quantified using the Bradford assay. Protein purity was checked by SDS-PAGE analysis.

#### **Ubiquitin cutting assay**

0.1 µg of either purified P1 or P2 PED protein was incubated with 0.5 µg ubiquitin substrate [Tetra-ubiquitin (Ub<sub>4</sub>), (K63-linked), Tetra-ubiquitin (Ub<sub>4</sub>), (K48-linked) from Boston Biochem or poly-ubiquitin chains (Ub<sub>2-7</sub>) (K63-linked) and poly-ubiquitin chains (Ub<sub>2-7</sub>) (K48-linked) from Enzo Life Sciences] and 10 µl 2x Assay Buffer (100 mM HEPES-KOH pH 7.4, 300 mM KCl, 10% glycerol, 0.2% Triton X-100, 20 mM DTT) in a 20 µl volume at 30°C for 60 minutes. The reaction was stopped by adding 20 µl 2x Laemmli Buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl – pH 6.8, 0.02% bromphenol blue) and heating at 95°C for five minutes. Results were visualized using western blot with anti-ubiquitin primary and alkaline phosphatase secondary antibodies.

#### ***Don Juan-like* reporter construct**

The *don juan-like* reporter construct (*djlGFP*) was assembled using the pCasper-AUG-βgal construct and the *don juan-like* 5' UTR region defined by the primers *djl* -555 and *djl* +43 (table 2.1). The PCR product was ligated into the backbone at unique EcoRI and BamHI sites present in each constituent piece. Fly lines were created as described above.

Primer Sequences	
CA+	CGAGGAGGATGGCGCAGCCCTGTTTCGCTCTATATCCC
CA-	GGGATATAGAGCGAAACAGGGCTGCGCCATCCTCCTCG
djl +43	GCGGATCCGCTAAGCAAAGGAAATAACCAAACCTTCACA
djl -555	GCGAATTCATACAATACGGCATTTTACCGAAAAATTAG
pET 5' P1a	GCCGCCGCCATATGACAATCAAGCCAGTG
pET 5' P2a	GGCGGCGGCATATGTCTACGTCTACGGCG
pET 3' ped	GGTGATCACATGCGGCACCGTCTACGGTC

**Table 2.1: Primer sequences.**

## CHAPTER THREE: RESULTS

### PCAG rescue of fertility

The existing *pedGFP* fusion transgene that was used as a template to create the alanine-substituted *PCAG* transgene was selected because it had previously demonstrated the ability to rescue the male sterility and early expression phenotypes when in a *ped* mutant background. The *PCAG* transgene was introduced into flies as described in Methods and Materials. GFP expression of the *PCAG* transgene within testes indicated that the GFP portion of the PED-GFP open reading frame was in-frame and being translated (figure 3.1). This GFP expression pattern of *PCAG* was compared to the GFP expression pattern of the original *pedGFP* transgene in order to get a semi-quantitative comparison of the expression levels and patterns of the two transgenes. This comparison revealed that the expression patterns and levels of the two transgenes were indistinguishable (figure 3.2). In order to reduce concerns that position effects skew the results of the rescue experiments, the stably inserted *PCAG* transgene was re-mobilized and two additional stable insertion lines were isolated for testing. These new insertions were referred to as [*PCAG*]/2 and [*PCAG*]/4.

To test the ability of the transgenes to rescue male sterility of *ped* mutants, *w*;[*PCAG*]/1; *ped*<sup>1</sup>*st*/TM3 flies were crossed with *w*;[*BHX5*]/1; *Df*(3*L*)/III19/TM3, *Ser* [*w*<sup>+</sup>*GFP*] flies. The *Df*(3*L*)/III19 deleted region includes the *ped* gene. From this cross, male flies exhibiting a double orange eye phenotype and lacking markers from either parental TM3 balancer were selected for testing. Selected flies were assumed to be *w*;[*PCAG*]/1/[*BHX5*]/1; *ped*<sup>1</sup>*st* / *Df*(3*L*)/III19. A few of these flies were sacrificed to verify GFP expression within the testes. All flies examined thusly showed GFP expression in the testes with no exceptions. Siblings of these flies containing a TM3 balancer were used as controls. Fly lines containing the re-insertions [*PCAG*]/2 or [*PCAG*]/4, or the original template transgene *pedGFP*, instead of [*PCAG*]/1 were tested using the same method. All F1 males of the appropriate genotype examined from these control crosses also showed GFP expression in the testes, indicating that they, too, were expressing their respective transgenes.

The fertility of the male flies from the above crosses was examined by crossing them with wild type females. Single male flies were crossed with single wild type



females and allowed to reproduce for five days. Mating pairs were cleared from the vials at the end of the 5<sup>th</sup> day. F1 adult flies produced from each cross were counted ten days after first eclosure was observed. No crosses using male flies which contained no functional copies of endogenous *ped* and only the alanine substituted transgenes (*w*;[PCAG]1/[BHX5]1; *ped*<sup>1st</sup>/Df(3L)III19, *w*;[PCAG]2/[BHX5]1; *ped*<sup>1st</sup>/Df(3L)III19 and *w*;[PCAG]4/[BHX5]1; *ped*<sup>1st</sup>/Df(3L)III19) ever produced any offspring. Males with any of these mutated transgenes and a single copy of endogenous wild type *ped* (*w*;[PCAG]*x*/[BHX5]1; *ped*<sup>1st</sup>/TM3, *Ser* [*w*<sup>+</sup> GFP] or *w*;[PCAG]*x*/[BHX5]1; Df(3L)III19/TM3) were fertile (table 3.1). The number of offspring varied widely in this case, due to female flies getting stuck in the food and dying at different times during the five day course of egg laying. This technical artifact did not detract from my main conclusion that the putative catalytic cysteine is required for PED to function properly for male fertility.

### **PCAG rescue of early expression**

The ability of PCAG to rescue the early expression phenotype seen in *ped* mutants was examined using β-galactosidase histochemical staining. Stains of testes of newly eclosed males of the genotype *w*;[PCAG]1/[BHX5]1; *ped*<sup>1st</sup>/Df(3L)III19 were compared to the stains of either *w*;[PCAG]1/[BHX5]1; *ped*<sup>1st</sup>/TM3, *Ser* [*w*<sup>+</sup> GFP] or *w*;[PCAG]1/[BHX5]1; Df(3L)III19/TM3 males (figure 3.3). Although the process of spermatogenesis was severely crippled in the *ped* mutant background, it appears that β-galactosidase expression occurs at a much earlier stage than in control flies having a functional copy of *ped* on the TM3 balancer chromosome. It should be noted, however, that spermatogenesis in the *ped* mutant flies is compromised and that the earlier expression is likely within necrotic cysts and may only be due to an eventual failure of repression. Without greater magnification, the possibility also certainly exists that the darkest of the stained structures seen in the mutant background are in fact waste bags. However, this would mean that the number and morphology of waste bags would be comparable to that seen in the wild type testis. Yet the lack of a comparable amount of steady-state elongated spermatid tails, which always accompany waste bags, within the mutant testis makes this explanation less likely. Overall, these results also support the

hypothesis that the cysteine residue is required for PED function and consistent with it being a part of a functional OTU domain.

### **Ubiquitin cutting by PED**

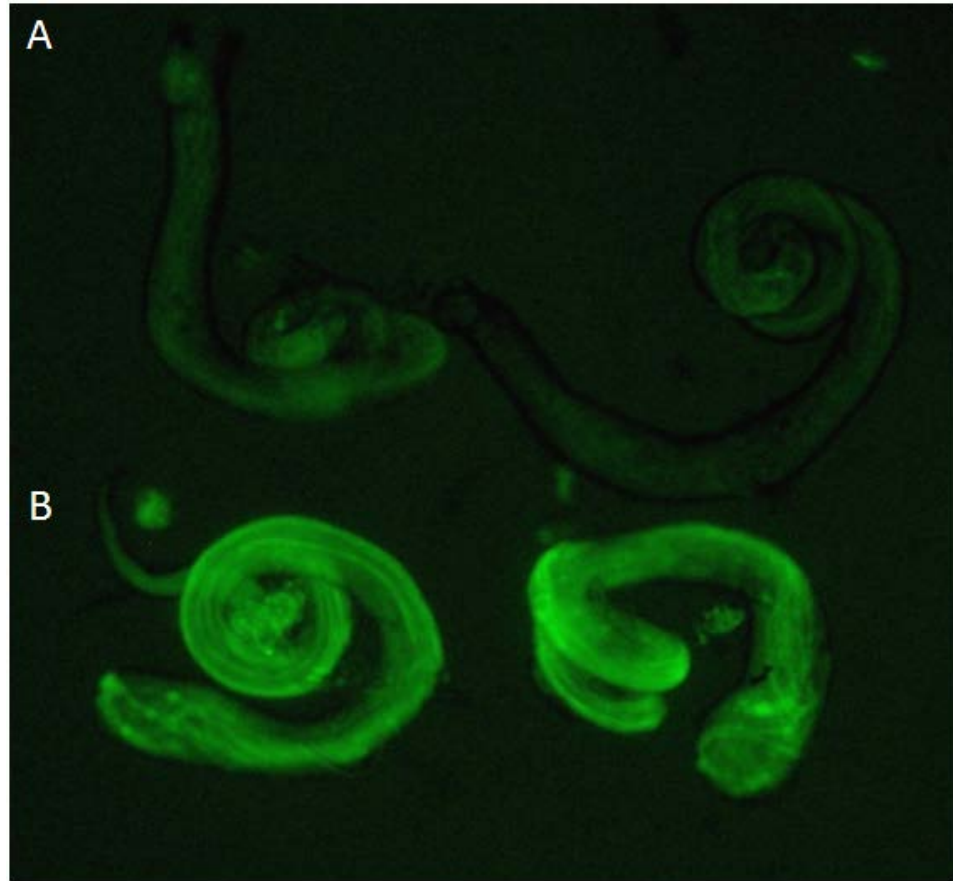
Several different substrates were used in an attempt to cleave ubiquitin chains. Although the expression and purification of PED from bacteria yielded very pure protein of sizes consistent with those expected from the two HIS-tagged PED isoforms (figure 3.4), no cutting of any kind was ever observed by them *in vitro*. There was no cutting observed of tetra-ubiquitin of either K48 or K63 linkage types (data not shown). Next, I wanted to test whether the purified PED isoforms would show activity in the presence of less salt. To test for activity in lower salt, buffer A was made with 50 mM NaCl substituted for 250 mM NaCl. No cutting of either linkage type was observed using this method of protein preparation (data not shown). The next step was to check for PED activity without using dialysis in the purification. The *in vitro* cleavage assay was carried out using PED as prepared in the first experiment and PED which did not undergo dialysis. Again, no cutting of any linkage type of the tetra-ubiquitin was observed (figure 3.5). As a final follow-up, I wanted to investigate whether one of the isoforms of PED may instead prefer to cut ubiquitin chains of lengths not represented within tetra-ubiquitin. Chain lengths varying from Ub<sub>2-7</sub> of both linkage types were used as substrates and PED was purified using 250 mM NaCl and normal dialysis. No obvious cutting was observed in this case either (figure 3.6). These results do not support the hypothesis that PED is a deubiquitinating enzyme. It is possible that ubiquitin chains utilizing other linkage types may be more susceptible to cutting by PED.

### **The effect of PED on *don juan-like* translation**

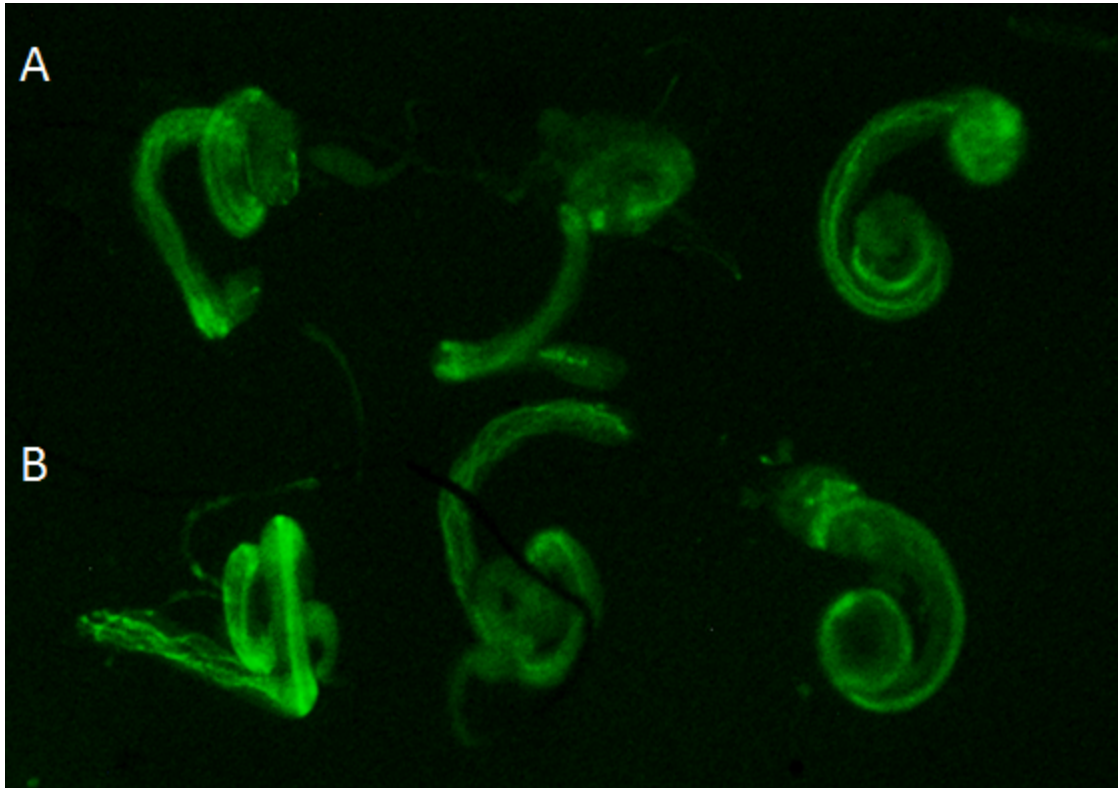
The *djlGFP* construct was assembled and introduced into a fly line as described in materials and methods. Expression patterns of the product of the *djlGFP* construct in a mutant *ped* background were unlike the expression of *dhod* or reporter genes containing the *dhod* 5' UTR. The translation of the latter could clearly be seen in pre-meiotic cysts of round spermatids close to the distal ends of testes, whereas GFP from the *djlGFP* construct was not seen in any structures near the distal ends of the testes, similar to the control flies with a functional copy of PED.

Male Parent Genotype:		F1 Adults Produced:
<i>[PCAG]/+; ped<sup>1</sup>/+</i>	<i>n=25</i>	67.24; stdev=20.88
<i>[PCAG]/+; ped<sup>1</sup>/pedΔ</i>	<i>n=25</i>	0
<i>[PCAG]<sub>2</sub>/+; ped<sup>1</sup>/+</i>	<i>n=10</i>	59.2; stdev=23.35
<i>[PCAG]<sub>2</sub>/+; ped<sup>1</sup>/pedΔ</i>	<i>n=10</i>	0
<i>[PCAG]<sub>4</sub>/+; ped<sup>1</sup>/+</i>	<i>n=10</i>	66.3; stdev=21.47
<i>[PCAG]<sub>4</sub>/+; ped<sup>1</sup>/pedΔ</i>	<i>n=10</i>	0
		Larva observed:
<i>[pedGFP]/+; ped<sup>1</sup>/pedΔ</i>	<i>n=4</i>	>20
<i>[PCAG]/+; ped<sup>1</sup>/pedΔ</i>	<i>n=4</i>	0

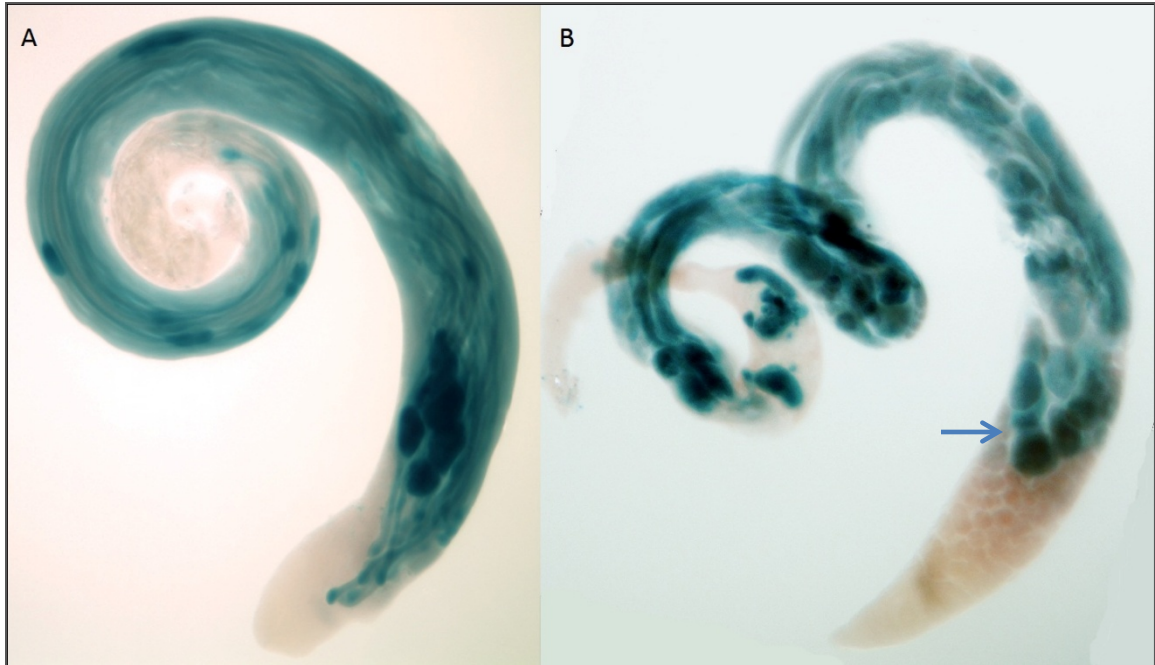
**Table 3.1: Transgene fertility test results.** None of the transgene insertions was able to rescue the male sterility phenotype of the *ped* null background. The original *pedGFP* transgene was able to rescue the phenotype in these specific crosses. Genotypically identical siblings of all tested males showed GFP expression in the testes.



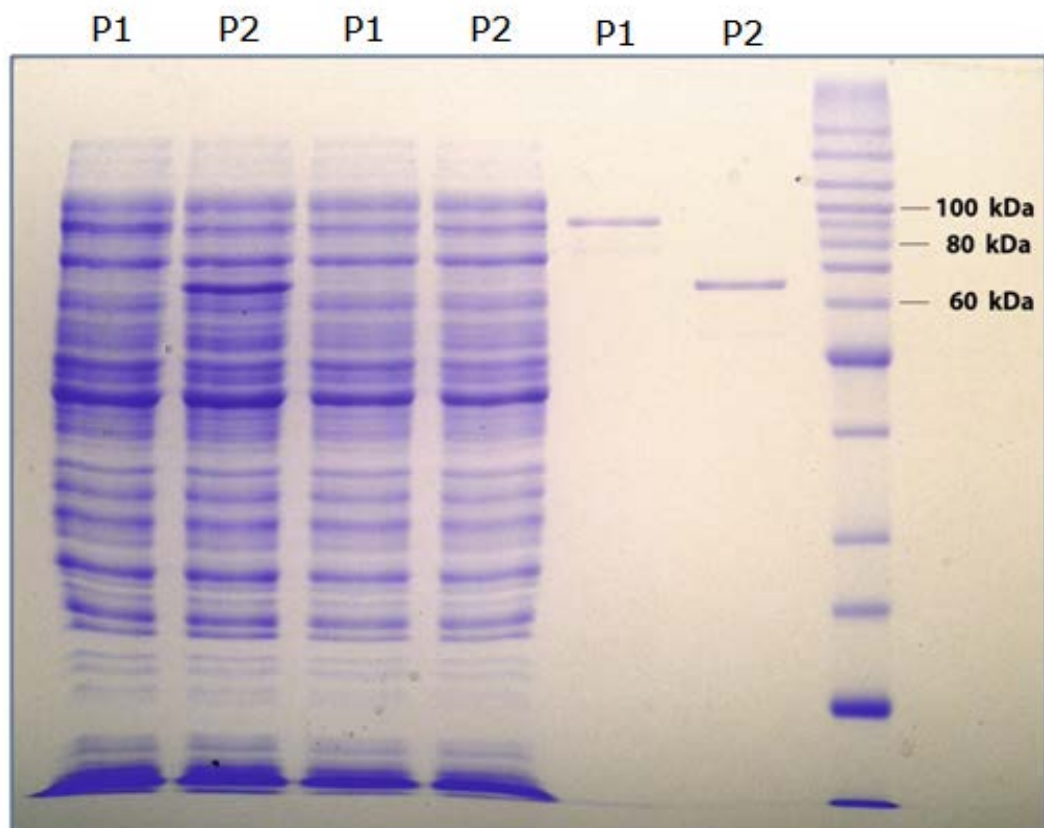
**Figure 3.1: PCAG expression in testes.** A. The top row shows two examples of wild type testes with no GFP expression. B. The bottom row shows two examples of GFP expression in the testes of flies hemizygous for the PCAG transgene.



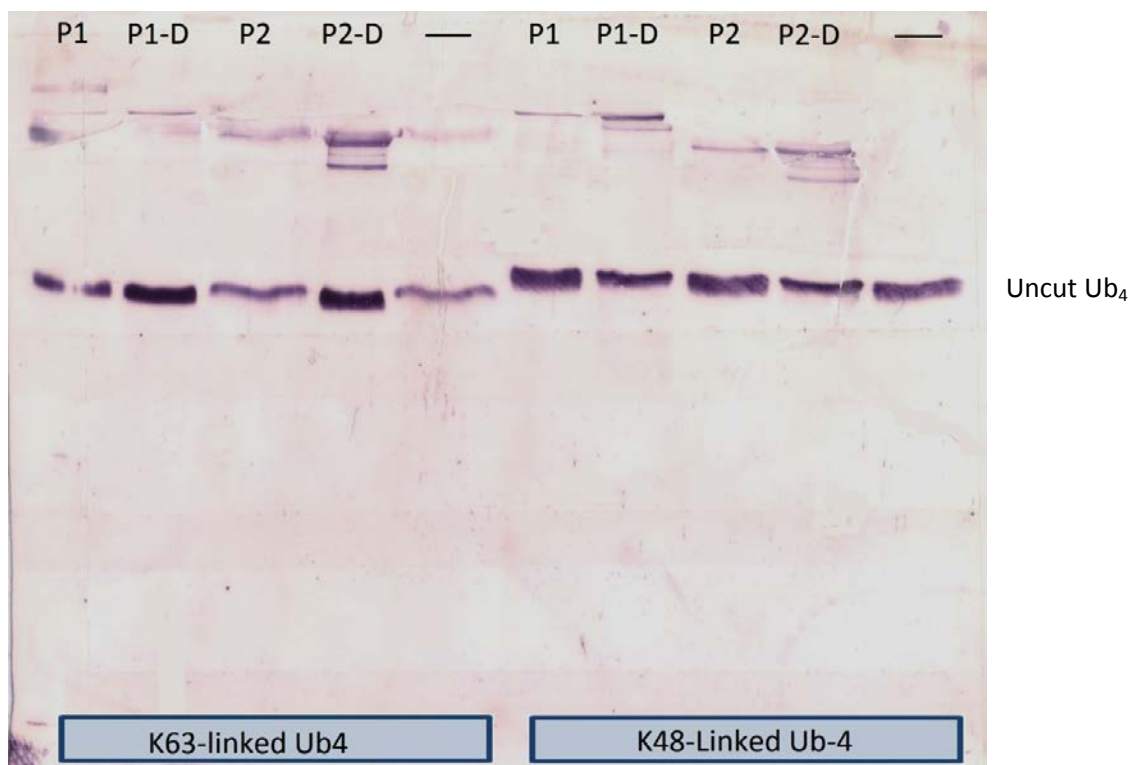
**Figure 3.2: PCAG and PEDGFP expression patterns.** Comparison of the expression patterns of *pedGFP* and PCAG. A. The top row shows three examples of homozygous *pedGFP* expression in testes. B. The bottom row shows three examples of homozygous PCAG expression in testes. Results were typical.



**Figure 3.3: PCAG fails to delay expression of reporter gene.** A. BHX5 expression in testis of *w;[PCAG]1/[BHX5]1; ped<sup>1</sup>/TM3* male. Expression is only seen in very late stages of spermatogenesis in spermatids with elongated tails and corresponding waste bags. B. BHX5 expression in testis of a *w;[PCAG]1/[BHX5]1; ped<sup>1st</sup>/Df(3L)III19* male. Expression is seen in earlier stage cysts with round spermatids closer to the distal end (blue arrow). This earlier expression, however, may only be in necrotic cysts resulting from a failure of spermatogenesis in which the translational repression system has failed as a result. Results were typical.

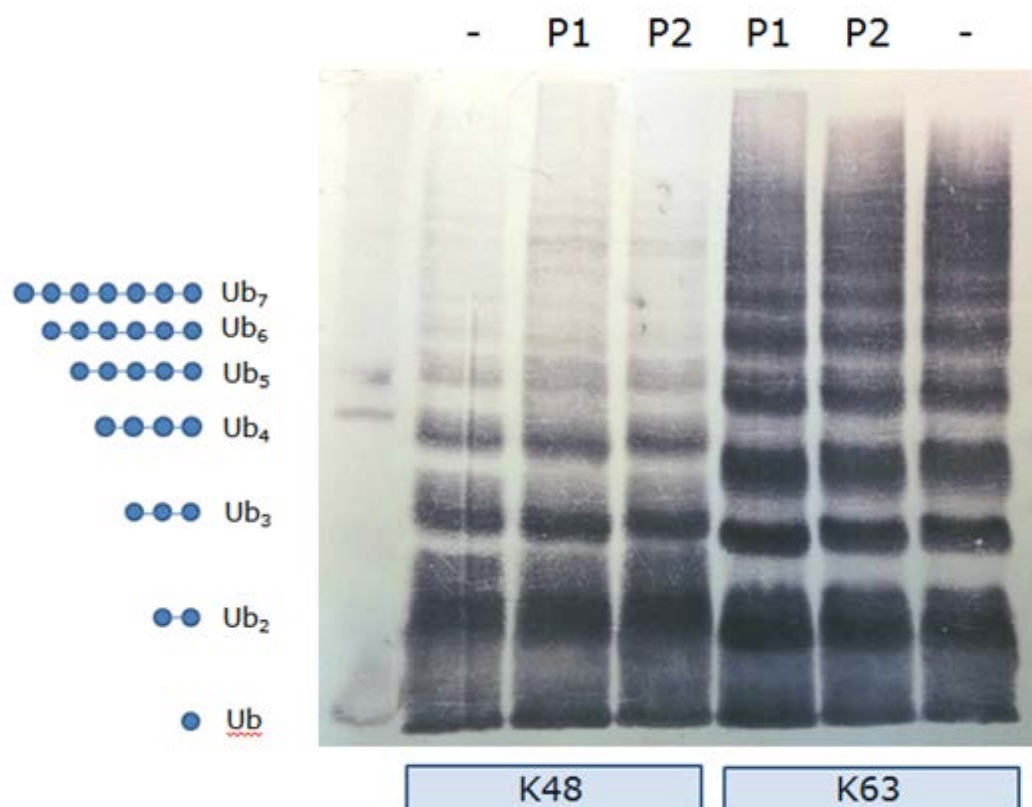


**Figure 3.4: PED purification results.** Lane 1-2 show total soluble protein retrieved from cells for the P1 and P2 isoforms, respectively. Lanes 3-4 show protein not bound by the Ni-NTA during each of the isoforms preps. Lanes 5-6 show protein bound to, and then released from, the Ni-NTA for each of the isoforms. Predicted sizes of his-tagged P1 and P2 were 75 kDa and 58 kDa, respectively.

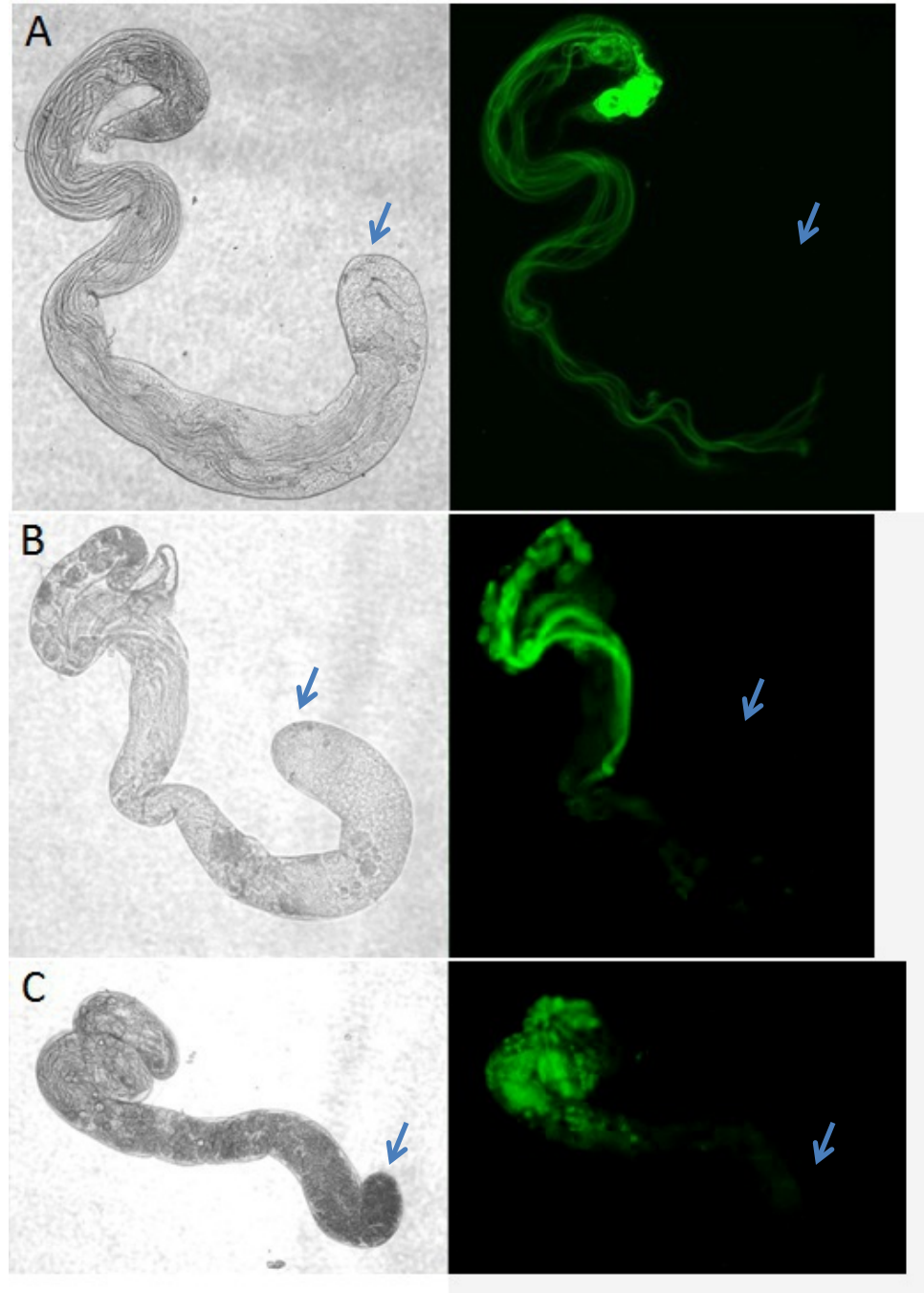


**Figure 3.5: Ub<sub>4</sub> cleavage assay.** Results of cutting K63 and K48-linked tetra ubiquitin using P1 and P2 isoforms of purified PED. Both dialyzed (P1-D, P2-D) and non-dialyzed (P1, P2) PED were used. No evidence of any chains smaller than Ub<sub>4</sub> was seen. It is possible that Ub-associated P1 and P2 comprise the bands seen above the uncut Ub<sub>4</sub>. Though neither isoform produced any ubiquitin fragments smaller than Ub<sub>4</sub>, indicating that neither isoform was able to cut tetra-ubiquitin under these conditions.





**Figure 3.6: Ub<sub>2-7</sub> cleavage assay.** Results of *in vitro* cutting of Ub<sub>2-7</sub> using purified and dialyzed PED. Neither isoform showed the ability to cut either K48 or K63 linked ubiquitin chains of any length. Small amounts of activity may not be observable in this assay.



**Figure 3.7: *djlGFP* expression in *ped* mutant background.** Expression of the *djlGFP* reporter construct. A. Expression pattern of DJLGFP in wild type background, brightfield and fluorescence. B,C. Two examples of DJLGFP expression in mutant *ped* background. Some diffuse low-level expression can be seen in the distal ends (blue arrows) of the testes in B and C. No localization to cysts or other structures near the distal ends of the testes was apparent. Results were typical.

## CHAPTER FOUR: DISCUSSION

PCAG appeared to be inactive in spermatogenesis without the putative catalytic cysteine. The alanine substituted form of PED never provided any fertility to male flies lacking functional endogenous PED. Sequencing data of the *PCAG* construct and observation of the fluorescing of the GFP fused to the C-terminus of this construct indicated that the PCAG protein was otherwise identical to the *pedGFP* construct from which it was made and which has the ability to rescue fertility. Also unlike the original *pedGFP* gene, PCAG failed to delay translation of the *BHX5* reporter gene. This indicates that the cysteine 227 is required for PED to function. This supports the hypothesis this cysteine is a part of an OTU domain and that PED is part of the otubain deubiquitinating enzyme family. More work is necessary to show that this cysteine has a chemical function in proteolysis.

I attempted to take the next step in confirming that PED is indeed an otubain DUB with the *in vitro* ubiquitin cutting experiments. There are many potential reasons why this experiment was unable to show any cutting, of which PED not actually cutting ubiquitin *in vivo* is but just one. With the purification of ubiquitin working very well, it seems that each of the isoforms was at least produced to full length and soluble. I believe there are three main reasons-other than the one in support of the null hypothesis-why the cutting would not have worked. The first possibility is that, while PED was produced in the prokaryotic system, it may have been either folded incorrectly or incorrectly post-translationally modified. The second possibility involves PED not have been presented with its preferred substrate in these experiments. For example, PED may prefer to cut lesser used ubiquitin linkage types like K11, or it may not cut ubiquitin chains at all, instead preferring to remove them from other target proteins (specifically deubiquitination versus ubiquitin cutting). The third reason is that PED may require another protein partner *in vivo* in order to function properly. There may be a partner of PED that may help it specify a target and/or be another component of the ubiquitin pathway. Existing data on DUBs supports these possibilities, suggesting that there is likely to be a partner to assist in targeting the activity of PED to its substrate (Ventii and Wilkinson 2008). Previous work within the otubain family also suggests that it may have a binding partner with E3 ligase capability; both the otubain 1/GRAIL complex and A20

OTU domain DUB, both being members of the otubain family, have been shown to have both DUB and E3 ligase capabilities (Soares, Seroogy *et al.* 2004; Komander and Barford 2008). While otubain 1 does not have its own ligase activity, the complex it has been found in does. A20, on the other hand, does have both DUB and ligase activity. PED could either require a complex for it to act upon another substrate or its activity could be restricted to members within its own hypothetical complex. Possible interacting protein partners are numerous. Standard approaches could be used to identify interacting proteins beginning with tandem affinity purification (TAP) and following up those results with confirmatory yeast two-hybrid assays.

In looking at the other end of the PED translational control pathway, the 5' UTR of the translationally delayed *djl* transcript, unlike the 5' UTR of *dhod*, does not convey an early expression phenotype to a *GFP* reporter gene when placed in *ped* mutant background. While these results show that PED is not required for early translation of this gene product *in vivo*, they also indicate that PED is not required to initiate the delay. It must be noted, however, that testes without functional PED have arrested spermatogenesis. It is also conceivable that transcripts under the control of the PED pathway may be translated at different times. So although the early expression phenotype for the *dTCE* is observable at the times of these arrests, the time points of the arrests might be occurring before the time in the process where *djl* might hypothetically be translated if it were also under the control of the PED pathway. However, if this was the case, then because there is variability in when the arrests occur between testes and even between cysts in the same testis, it might be expected that some expression of *djl* would be seen at some point. This was never seen, though, supporting the notion that *djl* is indeed under a different control system. It would also follow, then, that the early expression afforded by the *dTCE* in *ped* mutants is not simply due to an upstream failure of spermatogenesis in general caused by the absence of PED. The early translation effect appears to be specific to a translational control system and not a side effect of spermatogenesis failure. It is still possible though that PED is involved in the translation of both *dhod* and *djl* and that other components of the respective pathways or a slight difference in the timing allows *dhod* to translate early while *djl* is somehow still able to escape this fate. To get a better idea of the scope of transcripts whose translation PED

can affect, further experiments need to be done. One possible approach is to analyze transcripts whose translational profiles change between wild type and *ped* mutant flies using sucrose gradients and microarrays.

The mechanism and role of PED in delayed translation is unknown. Considering the ubiquitous nature of ubiquitin, the possibilities of where PED fits in this pathway or what it is doing are endless. PED, through DUB activity, could conceivably be regulating levels of a syncytial signal/receptor within the spermatocyte cyst. It might also be regulating a specific translation initiation factor or transcription factor (Kim, Park *et al.* 2003; Soares, Seroogy *et al.* 2004; Komander and Barford 2008; Ventii and Wilkinson 2008). PED could also be helping to regulate the levels of a RNA-binding protein (RBP) which could be inhibiting scanning by the 43S ribosomal subunit or preventing IRES binding by another RBP (Coleman and Miskimins 2009). It may also be regulating the binding of a RBP through activation or inactivation of a RNA helicase which could remove secondary structure from the transcript which could either be required for an RBP to bind or to be removed (Pisareva, Pisarev *et al.* 2008).

This study has shown that PED does not globally affect translational delay in spermatogenesis. Even among the transcripts delayed specifically until the tail elongation stages, the first two translational control elements studied show opposite outcomes. The *dhod* 5' UTR is affected by PED while the *djl* 5' UTR is not. It is also possible that PED exists *in vivo* as part of a complex or complexes. Because PED cannot globally affect translational delay, however, any complex of which PED is a part must also not be able to globally affect translational delay. The existence of more than one isoform of PED, the different expression patterns of these isoforms within the testis and the suggestion that some isoforms may contain an auto-inhibitory region further complicate the problem of specifically identifying the nature and timing of the pathway through which PED is able to affect translational delay.

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