The Role of RNA Binding Protein Pumilio in Regulation of Nociceptive Sensation.

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ABSTRACT

Nociception is the sensation of potentially tissue damaging stimuli, and is necessary for the survival of all animals. Without it, organisms would not be able to navigate their environment safely and efficiently, both avoiding potentially dangerous situations and not wasting energy responding to every stimulus like a possible threat. Many of the underlying nociceptive processes are conserved throughout metazoan systems, and the overlap between *Drosophila melanogaster* and humans is extensive. Characterizing the regulatory processes behind nociceptive sensation is important to provide avenues for treatment of chronic pain in the human population, and one potential point of regulation is the Pumilio (Pum) protein. Flies with decreased and increased expression of *pumilio* were tested for nociceptive defects to mechanical and thermal stimuli, and the effects of changed Pumilio expression on dendrite morphology were also quantified. Reduction of *pumilio* expression using RNA interference (RNAi) led to hypersensitivity to mechanical and thermal stimuli, and a reduced dendrite phenotype. Increase of *pumilio* expression by expression of *pumilio* cDNA in nociceptor neurons led to an insensitive phenotype to mechanical stimuli. To study the possible downstream effectors of Pumilio, a fluorescent live-imaging tool will be created that will allow visualization of *para* mRNA in the nociceptor neurons. These experiments begin to elucidate the role Pumilio plays in the regulation of nociception and the molecular mechanisms by which it regulates nociception.

INTRODUCTION

The sensation of pain in humans and other organisms is a highly studied but not completely understood subject. This research may one day have a significant effect on the world of healthcare: chronic pain leads to a cost of about $17000 per patient annually (Lalonde et al., 2014), not to mention the emotional costs. Also, it affects at least 100 million U.S. adults (Institute of Medicine (US) Committee on Advancing Pain Research, Care, and Education, 2011). One of the main model systems for studying the mechanisms behind the development of pain is *Drosophila melanogaster*, which has a genome that shares many homologs with humans and a similar but simplified structure to its nervous system (St Johnston, 2002). Determining how exactly the regulation of cellular and molecular mechanisms in the nervous system and nociception in particular occurs in *Drosophila* can elucidate the most effective targets of drug regulation in humans would be to treat disorders of these systems. The different ways that noxious thermal and mechanical stimuli are perceived and the many layers of regulation that contribute to differential levels of sensitivity have been studied and partially determined. However, the mechanism through which RNA binding proteins, which regulate gene expression, function in nociception offers another avenue to study the regulation of pain. Many of these proteins, like Pumilio for example, have human homologs and would provide a point of application to the human system from this model organism. Pumilio was identified in a screen (Dyson, 2017) for nociceptive defects in *Drosophila* larvae where the expression of RNA binding proteins was knocked down. The characterization of its effect on nociception through behavioral and molecular assays reveals a novel role for Pumilio in regulating nociceptor sensitivity.

Nociception in *Drosophila melanogaster*

D. melanogaster larvae show a distinctive response when they encounter a noxious stimulus. They curl into a C shape and roll laterally quickly, which is easily distinguishable from their normal peristaltic motion (Tracey et al., 2003). This response, called nocifensive escape locomotion (NEL), was identified while researchers were attempting to design a simple pain assay. It was elicited when the larval body wall was touched with a probe heated above 39˚C, and could be quantified based on the latency between contact with the probe and execution of the response. This latency could be related to the sensitivity of the animal or to the strength of the stimulus; a faster response indicates a more sensitive animal or stronger stimulus. Because the NEL was both obvious and quantifiable, this became the traditional noxious heat assay. This NEL response could also be provoked based on the application of force; in this assay (Tracey et al., 2003; Zhong et al., 2010), researchers used a Von Frey filament to deliver a specific amount of force to the larval midline. Both of these assays gave researchers the tools they needed to study nociception in *Drosophila*. In later studies, it would be shown that *Drosophila* larvae used this behavior to escape from wasps intending to parasitize them (Hwang et al., 2007), giving an evolutionary background for this behavior to develop.

To continue developing a model for nociception in *Drosophila*, the neurons that sensed nociceptive stimuli had to be identified. These neurons in the peripheral nervous system responsible for the sensation of pain are called nociceptors, and they enable animals to swiftly react to the potentially harmful things they encounter throughout their life. There

are two different types of sensory neurons in the peripheral nervous system in *Drosophila* which could possibly be used for nociception: Type I and Type II. Type I neurons have a single dendrite and have been linked to light touch (Kernan, 1994). Type II, or multidendritic (md) neurons have highly branched dendrites, and are much more similar to the vertebrate nociceptor neurons: they have complex dendrite morphology, and project these dendrites without a special sensor cell directly below the skin (Jenkins and Lumpkin, 2017; Karkali and Martin-Blanco, 2017). The similarity in structure between these neurons and vertebrate nociceptors provided evidence that these were the nociceptors. The researchers used a tissue specific driver to reduce the function of protein in these neurons and see how this affected the behavior of *Drosophila* larvae (Gao 1999). In this case, the GAL4 driver was crossed to a strain containing UAS genes upstream of the tetanus toxin light chain to block calcium dependent release of vesicles, which is necessary to pass signals from one neuron to another. The transgenic progeny of the cross of *ppk*-GAL4 and UAS-TnT-E, had a significantly longer latency compared to wild type (Tracey et al., 2003), indicating one of the subclasses of these Type II neurons, Class I, II, III or IV, would most likely be the nociceptor neurons. Another study used multiple different GAL4 drivers to knock out these subclasses alone and in combinations to narrow down which class was most responsible for nociception (Hwang et al., 2007). This led to the identification of the Class IV neurons as the nociceptors. They confirmed the multidendritic class IV (mdIV) neurons were necessary and sufficient for nociception through both the above described behavioral assays and by optogenetically activating the neurons and quantifying the response.

The *Drosophila* Nociceptive Pathway

To begin studying the molecular mechanisms behind nociception, a study was conducted that screened mutants for 1500 mutant lines for an effect on thermal nociception (Tracey et al., 2003). The genes were mutated using randomly inserted transposable elements that disrupted their function, and then a thermal assay was conducted to determine their behavioral phenotype. These researchers were able to identify multiple candidates with an effect on the response to thermal stimuli, and chose to study the *painless* (*pain)* gene. The *painless* mutant larvae showed a more insensitive behavioral phenotype in response to both mechanical and thermal noxious stimuli, which indicated it was important to both pathways. After cloning this gene, it was shown to encode a member of the transient receptor potential (TRP) family of ion channels, which have been implicated in the transduction of nociceptive stimuli in other metazoans (Sokabe et al., 2008; for review, see Ramsey et al., 2006).

Because *Drosophila* larvae respond in a similar way to both mechanically and thermally noxious stimuli (Tracey et al., 2003), and eliminating the function of Class IV neurons removes mechanical and thermal nociception (Hwang et al., 2007), it would be simple to conclude that the pathways are most likely regulated in the same way. However, this is not the case. There is a great diversity in the processing of these two types of stimuli, although the pathways are similar and parallel each other in many ways. The first gene that was found to function specifically in the mechanical nociception pathway was *pickpocket (ppk)* (Zhong et al., 2010). This gene was previously shown to encode a component of a degenerin/epithelial sodium channel (DEG/ENaC), to be expressed specifically in the md neurons and to have some effect on locomotion (Ainsley et al., 2003), all characteristics that

pointed to its possible function in nociception. In the study by Zhong et al., larvae that had a loss of function mutation in *ppk* had a defective behavioral response to mechanical stimuli, but not to thermal stimuli. This result was further confirmed through a procedure called RNA interference (RNAi), where an enzyme complex targeted to breakdown *ppk* or another mRNA of interest is expressed in a tissue of interest. If expressing *ppk-RNAi* in the nociceptor neurons led to the same defective behavioral response, it would indicate that mechanical nociception through these neurons depended on *ppk.* Although the effect was not as severe in the RNAi phenotype as in the genetic null mutation, it was still significant and confirmed that *ppk* was important in the mechanical nociceptive response.

The pathway through which mechanical nociceptive stimuli was transduced throughout the Class IV neurons was further studied using another gene, *piezo* (Kim et al., 2012). The *piezo* gene product is a protein with multiple transmembrane domains, with homologs in mice and other mammals implicated in mechanical activation of sensory neurons. The effect of *piezo* on the nociceptive response of *Drosophila* larvae was characterized using behavioral assays similar to those mentioned above, and continued through studying interactions between RNAi knockdown phenotypes *piezo* and *painless* and *piezo* and *pickpocket.* The first combination produced defects similar to those shown when either gene was knocked down, but knocking down *piezo* and *pickpocket* increased the defect even further, nearly completely eliminating the response to noxious mechanical stimuli. This suggested Piezo and Ppk were important components of two different mechanical transduction pathway that comprised the majority of signaling from mechanical stimuli.

The thermal nociception pathway was characterized in a similar manner to the mechanical nociception pathway, but thermal sensation must be more tightly regulated. Thermal nociceptive neurons have to sense specific ranges that indicate either innocuous or noxious temperatures, and then signal the motor neurons accordingly. One of the first identified genes contributing to specifically thermal nociception was *TrpA1*, another member of the TRP channel family (Neely et al., 2011). This gene was identified through a screen for nociception defects *in vivo* of the whole genome in adult *Drosophila* flies, which identified many genes that had a possible role in thermal nociception. It was then further characterized using the typical method of mutating flies to be deficient for the gene, then assaying the larvaes' nociceptive abilities. This screen (Neely et al., 2011) was one of the first to test many different genes for their effect specifically on thermal nociception, and was very important as it allowed researchers to identify many different candidates for action in nociception. In this case, they found that the *TrpA1* mutants lost their ability to sense noxious thermal stimuli. This was a novel component for the *Drosophila* thermal nociceptive apparatus, and interest in the TRP family for their role in nociception in *Drosophila* swiftly grew.

As *TrpA1* was further studied, some discrepancies between its verified characteristics and its purported function were found. The *TrpA1* channel is activated by temperatures of 27˚C, which is significantly lower than the activation threshold for noxious thermal stimuli. As well, it did not appear to have a site of action in the nociceptive neurons. No reporters had yet been found that demostrated *TrpA1* gene's expression in the Class IV multidendritic neurons. Another study based on these questions found that flies with a mutation in *TrpA1* had thermal and mechanical nociception defects (Zhong et al., 2012). However, the canonical

TrpA1 gene did not have the same thermal sensitivity implicated in thermal nociception; it was activated at 37˚C, not 42˚C. When the experimenters sequenced the *TrpA1* mRNA, they found that upstream exons originally annotated as part of a different gene were indeed a part of the *TrpA1* gene and expressed in the animals. Forcing the expression of this novel isoform rescued the deficient thermosensory phenotype of *TrpA1* mutants, and did not lead to the hypersensitive phenotype which was seen when the canonical isoforms were expressed in the nociceptors. The lack of a hypersensitive phenotype in the rescue animals helped confirm the involvement of this TRPA1 isoform in nociception in *Drosophila,* as it showed they were activated at a temperature close to the original nociceptive temperature range. As well, using a GAL4 reporter and green fluorescent protein (GFP) tag, the expression of this isoform was mapped specifically to the Class IV multidendritic neurons, further supporting the theory that the TRPA1 channels are important for thermal nociception.

A feature of nociceptive neurons is their ability to be sensitized. This can occur due to tissue damage, and can lead to irregular nociceptive phenotypes, like hyperalgesia, an exaggerated response to noxious stimuli, or allodynia, a nocifensive response to a normally innocuous stimuli (Ji and Woolf, 2001). In order to study this phenomenon, a model system had to be established, which was done using UV radiation and *Drosophila* larvae (Babcock et al., 2009). In these assays, the third instar larvae are exposed to UV radiation for a set amount of time that should not detrimentally affect the health of their epidermis and sensory neurons. The larvae are then stimulated with an innocuous thermal stimulus and a noxious thermal stimulus at different time points after irradiation, in order to characterize the development of allodynia and hyperalgesia respectively. In this study by Babcock et al. (2009),

they were able to use this method to tie the development of allodynia to apoptosis in epidermal cells and the development of hyperalgesia to Tumor Necrosis Factor (TNF) signaling. Interestingly, these processes also develop somewhat separately: using RNA interference to knock down the TNF receptor did not block allodynia. A link between these two pathways was found in Hedgehog (Hh) (Babcock et al., 2011). This study suggested Hh acted through sensory ion channels like dTRPA1 and Painless to modulate the sensation of nociception under sensitized conditions. These results could have a great impact on the treatment of chronic pain, as it develops in neurons that become sensitized after injury and remain sensitized after that injury is gone. To further apply these studies to vertebrate systems, researchers set out to use the *Drosophila* system to study how well known sensitizing factors, Substance P and Tachykinin, affect the behavioral phenotype of larvae (Im et al., 2015). These researchers found Tachykinin was required for thermal allodynia, and that this was upstream of the Hedgehog signaling found earlier. This could lead to better treatments for chronic pain, as the earlier in the pathway the treatment acts the more effective it is, although it might also have a stronger off-target effect as well.

RNA-Binding Proteins in Neurons

One role of RNA-binding proteins is to regulate where mRNAs are translated. Most neurons have a cell body, or soma, from which dendrites extend a relatively large distance. For many organisms, it is more energetically favorable to transport one mRNA to the dendritic terminal for local translation instead of transporting all of the protein that is needed. One function of these RNA-binding proteins is their ability to bind to these mRNAs and prevent

them from being translated until the proteins they encode are needed. RNAs that are localized are also very important in the morphogenesis of dendrites, specifically by allowing the asymmetric formation of structures and body patterning necessary for normal development (Misra et al., 2016).

One gene that was identified as participating in RNA localization (Ephrussi et al., 1991) is *oskar.* This gene is important to regulate organization of germ plasm in development (Xu et al., 2013) and the transport of mRNAs like *nanos* in both developmental stages and after development in neurons (Ephrussi et al., 1991). The Oskar protein may function to activate the *nanos* mRNA when it reaches the neurons, as Oskar and *nanos* are cotransported in neurons and *nanos* is not localized without Oskar (Xu et al., 2013), but the full mechanism has not yet been resolved. The functionality of local translation has been implicated specifically in nociception in mammalian organisms (Jiménez-Díaz et al., 2008). The translation regulator mammalian target of rapamycin (mTOR) was found both to localize with the machinery needed to initiate translation to sensory fibers in rats, and also changed how excitable these nociceptive neurons were independent of any other neurons in the organism, presumably by changing local protein synthesis. The regulation of RNA localization and local protein synthesis is important to the regulation of how sensitive nociceptors are, whether in mammalian or insect model systems.

The alternative splicing of the *TrpA1* gene is another example of one of the many avenues through which the sensitivity of *Drosophila* nociceptors is regulated. Alternative splicing has also been shown to be important in the regulation of other channels important for signal transduction in general, like Paralytic (Para), a voltage gated sodium channel (Lin et

al., 2012; O'Dowd et al., 1995). The expression of different isoforms of ion channels can lead to different levels of activation, and thereby modulate the sensitivity of the neuron. This alternative splicing and the expression of the different isoforms is mediated by RNA binding proteins, which also make up many of the proteins that regulate sensory transmission. This is especially true in the growth of neurons, exemplified in one screen showing 88 genes that encoded RNA binding proteins affected dendrite morphogenesis (Olesnicky et al., 2014). The different methods through which these proteins regulate RNAs include directing alternative splicing, encoding translation initiation, elongation, termination or repression factors, monitoring cytoskeleton formation, or targeting cell death and engulfment. Indeed, some even are implicated in multiple of these pathways (Vicente-Crespo et al., 2008). The screen mentioned above (Olesnicky et al., 2014) was conducted by first compiling a list of various RNA binding proteins encoded in the *Drosophila* genome that would also allow the use of a GAL4 driver to knockdown the genes in the Class IV neurons, as well as the expression of a GFP marker to allow for confocal fluorescence visualization of the dendrites. The dendrites that formed after this knockdown were analyzed to see significant deviations from normal development. Some of these genes had larger effects on the dendrites, and were further analyzed and their effects quantified. The results of this screen pointed to the importance of translation factors especially in dendrite morphogenesis, and possibly in other neuron regulated activity such as nociception.

The initiation of translation requires many proteins, and can be seen as a rate-limiting step for the production of proteins; therefore, it serves as an important point for regulation. Cap dependent translation initiation utilizes the eukaryotic initiation factor 4F (eIF4F)

complex, which binds to the 5' cap of an mRNA transcript and induces the binding of the 40S ribosomal subunit to the mRNA in order to begin translation. There are three components to this complex: cap-binding eIF4E, RNA helicase eIF4A, and scaffolding protein eIF4G. Especially eIF4E has been studied extensively for its role in nociception and neuronal signaling in general (Menon et al., 2004; Sigrist et al., 2000); there is more work to be done with the other two proteins, although there have been studies that indicate 4G could operate independently of 4E to initiate translation (Kaiser et al., 2008; Ohlmann et al., 1996). However, there have been studies that have shown the necessity of factors that activate this translation initiation complex for nociceptive pathways: mTOR, as mentioned above, activates this complex during local translation which leads to heightened sensation in rats (Jiménez-Díaz et al., 2008). Also, cytokine interleukin 6 (IL6) and neurotropin nerve growth factor (NGF) have been shown to induce allodynia via a translation dependent pathway, by upregulating the expression of some subset of proteins in nociceptive neurons (Melemedjian et al., 2010).

Translation factors regulate RNA through multiple pathways. One of these includes modification of the mRNA. This adds another layer of regulation to mRNA translation by allowing the expression of these modifying factors to be changed and have a cascading effect down the pathway. For example, mRNA transcripts may be stabilized by the addition of a polyadenonucleotide (polyA) tail (Du, 2005). mRNAs without a polyA tail are often either degraded quickly or are not recognized for translation, and therefore the protein encoded is not expressed very highly in the cell. Du and Richter (Du, 2005) found polyadenylation was often used to increase the expression of certain proteins needed for synaptic transmission when neurons were stimulated, coining the term activity-dependent polyadenylation. They

did so by first identifying genes that undergo polyadenylation, and treated cultured neurons with glutamate, to stimulate the firing of the neuron. They then extracted the total RNA and used a poly(U) agarose column to identify which mRNAs were polyadenylated after the neurons were excited. The levels of the proteins encoded by these polyadenylated RNAs were found to be elevated after neuronal excitation. This experiment was done in *Xenopus* oocytes, but the implication that polyadenylation is important for the regulation of neuronal activity applies relatively broadly in metazoans. For instance, it has been shown that how well *Drosophila* neuromuscular junctions function is partially regulated by the translation of *eIF-4E*, an initiation factor recruited by the polyadenylation of mRNAs, and *poly adenosine binding protein (*pAbp), which directly binds the poly-A tail of mRNAs (Sigrist et al., 2000). *Drosophila* who had a higher number of *eIF-4E* aggregates were more active than those that had normal levels of aggregates. These neuromuscular junctions are important in nociception as they allow the NEL to occur by transmitting a signal to the muscles in the larval wall to move, so their higher activity would indicate a more sensitive phenotype. This activity-dependent polyadenylation of mRNA transcripts could be happening in mdIV neurons too, and therefore lead to changes in their sensitivity.

Pumilio, an RNA-Binding Protein

A particularly well studied RNA binding protein is Pumilio (Baines, 2005; Parisi and Lin, 2000). This protein is a member of the PUF family of proteins, which occurs in many different metazoan organisms and has a conserved RNA-binding domain with a relatively similar function throughout those organisms (Zamore et al., 1997). This similarity extends to humans,

making the *Drosophila* nervous system a useful model to study how the Pumilio protein functions as could be applied to the treatment of pain in humans. In *Drosophila melanogaster*, *pumilio* has been shown to act in neuronal development of dendrites and the neuromuscular junction, neuronal excitability, the formation of long-term memory, and translation of localized RNAs (Baines, 2005). As mentioned above, localized translation and neuronal excitability are important for nociception in neurons, implying the importance of this gene in nociception and sensation in general. As well, *pumilio* has been shown to associate with over a thousand different mRNAs (Gerber et al., 2006), many of which are related to the function of the nervous system. Thus, *Pumilio* is a very prolific RNA binding protein important in nociception, and the elucidation of the mechanisms through which it regulates these RNA transcripts will illuminate how *Drosophila* and potentially other metazoan nervous systems work.

Pumilio regulates translation by binding mRNAs in the cell with an evolutionarily conserved binding domain (Wharton et al., 1998). This has been most thoroughly studied in the development of *Drosophila* embryos, where Pumilio binds *hunchback (hb)* mRNA(Murata and Wharton, 1995). This allows for downregulation of the expression of *hb* that is necessary for the patterning and body segmentation of the larvae. Expressing just the Pumilio RNA binding domain is sufficient to rescue normal development in knockdown *pumilio* larvae, and the sequence of the RNA-binding domain is conserved across species (Zamore et al., 1997). Further confirming that this binding process is how Pumilio contacts transcripts, this 8-nucleotide binding domain was found among many of the 3' UTRs of associated mRNAs in studies of yeast and *D. melanogaster* (Gerber et al., 2004, 2006). The gene ontologies of

these transcripts were found to be significantly enriched as nucleic acid binding or localized to membranes. One category of these membrane localized transcripts includes ion channels, which provide an important pathway for Pumilio to influence the sensitivity of nociceptive neurons through their importance in sensitivity to these stimuli (Ainsley et al., 2003; Caterina et al., 1997; Kim et al., 2012; Littleton and Ganetzky, 2000; Mee, 2004; Turner et al., 2016). The interactions between mRNAs of ion channels and Pumilio have not been extensively studied in the context of neuronal sensitivity, but the relationships between Pumilio and other mRNA transcripts have been elucidated.

Many experiments that are designed to study interactions between proteins and substrates follow a similar design; the experimenters first characterize the phenotypic effect resulting from changes in genetic expression for both the protein and the substrate, then characterize the changes in the substrate level due to changes in the protein expression, and lastly describe a model for how the protein acts on the substrate. One example of this experimental process is a study by Menon et al. (2004). In this study, the researchers characterized the interactions between eIF4E and Pumilio in the neuromuscular junction (NMJ). eIF4E is an cap-binding protein (Sonenberg and Gingras, 1998), and is categorized as nucleic acid-binding in gene ontology. They show that the changing *pumilio* expression leads to changes in the morphology of the NMJ, and that eIF4E forms aggregates and NMJ morphology is changed when its expression is increased. The interactions they found between the two proteins show that Pumilio represses the accumulation of these aggregates, and maintains the correct morphology. This occurs when both eIF4E and Pumilio are overexpressed, and therefore can be assumed to occur at normal levels of both proteins.

They also investigated the physical interaction between Pumilio and *eIF4E* mRNA by testing the binding strength of fragments of eIF4E mRNA to Pum, and found that the 3' UTR of *eIF4E* bound as tightly as the whole mRNA, and that this binding is specific. This type of experiment exploring the interaction between Pumilio and mRNA transcripts is relatively typical, although it can also be further extended to explain the process by which Pumilio represses the expression of the transcript.

Another study began to evaluate multiple models of repression by *pumilio* (Weidmann et al., 2014). The first model was Pumilio binding to the Argonaute protein, which would then bind an elongation factor, eEF1A. This would prevent the elongation of polypeptides and prevent the production of proteins. They disproved this model as the primary mode of repression by mutating the binding motifs of Argonaute and eEF1A so the complex could not form, and showing that the level of repression by *pumilio* was not changed. Another model stated that the Pumilio RNA-binding domain recruits deadenylases to shorten the poly(A) tail of mRNAs, increasing their instability and the likelihood that they are degraded. This model has been shown to be important in development and in other processes where Pumilio acts (Richter, 1999; Wreden et al., 1997). They found that this was not the primary method of repression, because even though the poly(A) sequence was necessary for repression it did not have to occur at the terminus of the mRNA to be effective. As well, removing the deadenylases that have been shown to act with Pumilio in deadenylation (Goldstrohm et al., 2006, 2007; Van Etten et al., 2012) did not lead to a significant decrease in repression. This leads to deadenylation not being the main model of repression. Another protein that binds to the poly-A sequences is the poly-A binding protein (pAbp). Lack of pAbp

has been shown to decrease mRNA stability in other studies (Bernstein et al., 1989; Coller et al., 1998; Ko et al., 2013; Vazquez-Pianzola et al., 2011; Wang and Kiledjian, 2000), which could lead to repressed expression. They hypothesize that *pumilio* most likely negatively effects the activity of pAbp in the cell, thereby repressing translation of other mRNAs in a broad manner by decreasing their stability and making them more likely to be degraded. This possible mechanism of action was further supported In one study of mRNA decay in *Drosophila* embryo neurons (Burow et al., 2015). This study quantified levels of decay for multiple classes of mRNAs in these neurons, and found interesting differences amongst them. For example, proteins that need to be active in time sensitive scenarios in development, like transcription factors that regulate cell fate decisions, have mRNA transcripts that tend to have a much shorter halflife than those that have longer lasting roles in neurons, like those that maintain neuroblast polarity. Some of the regulatory elements that this study found were enriched among those transcripts that decayed more quickly were Pumilio recognition elements, possibly suggesting that Pumilio could act on these mRNAs to decrease their stability and lead to decay.

One category of the above mentioned membrane-localized transcripts includes ion channels, which provide an important pathway for Pumilio to influence the sensitivity of nociceptive neurons through their importance in sensitivity to these stimuli (Ainsley et al., 2003; Caterina et al., 1997; Kim et al., 2012; Littleton and Ganetzky, 2000; Mee, 2004; Turner et al., 2016). An example of this is the sodium channel coded by the gene *paralytic,* and has been shown to be directly regulated by Pumilio (Muraro et al., 2008). This channel is the major mediator of sodium conductance during action potentials in *Drosophila* neurons,

whether in motoneurons or Class IV multidendritic neurons. In one study, more of the *para* mRNA is produced when the larval NMJ is more active, indicating its dynamic role in the nervous system (Mee, 2004). Also, the mRNA of *para* and *pumilio* were inversely related, with *para* increasing when the larval neurons were more excited and *pumilio* decreasing, specifically in the motoneurons. Not only are the transcript levels correlated, but *pumilio* knockdown mutants do not have reduced *para* mRNA levels when synaptic activity is low. This suggests the larvae are more sensitive because they can conduct more sodium current at lower thresholds of stimulation. The effect the Pumilio protein has on the *para* mRNAs has been found to occur through its RNA binding domain (Muraro et al., 2008). Specifically, this conserved domain binds to certain sequences in the mRNA and, through one or multiple mechanisms, makes it more likely to be degraded and then not detectable in the cell. The way that differential expression of *pumilio* leads to changes in neuronal excitability makes it an interesting candidate for research in nociception. Because both *pumilio* and *para* are active in the Class IV multidendritic neurons, the above described relationship could contribute to the sensitivity of these nociceptors and be a part of the pain sensation pathway. Further exposing the mechanism through which Pumilio binds to and regulates *para* and many other mRNA transcripts will be helpful to completely understand how that happens.

The Sodium Ion Channel Paralytic

As mentioned above, Paralytic (Para) is a voltage gated sodium ion channel, and is essential for most action potential propagation along the axon of *Drosophila* neurons (Loughney et al., 1989). The function and structure of the protein coded for by this gene was

found by first mapping where the mutations that caused temperature-sensitive paralysis occurred (Suzuki et al., 1971). Once this mutation was mapped and characterized, the next step was to apply this information to define the gene at the molecular level (Siddiqi and Benzer, 1976). By comparing multiple different types of mutations causing temperature-sensitive paralysis, the researchers in this study were able to pinpoint which components of the mutations derived from differences in the protein structure. Another mutation that caused paralysis in animals with excitable muscles was named *no action potential* or *nap* (Wu et al., 1978). This study characterized the effects on the conduction of nerve impulses in *Drosophila* nerves, and found that at the higher temperature there were significantly fewer complete action potentials conducted through the nerve. Because of the similarities between the behavior and physiology in *nap* and *para* mutants, this experiment was replicated with *para* (Wu and Ganetzky, 1980). The same result was found, and it was concluded that both *nap* and *para* coded for voltage gated sodium channels on the *Drosophila* axon that were essential for action potential propagation.

Considering Para is essential for the propagation of action potentials in *Drosophila* neurons, it would follow that it is important for many different types of processes that require neuronal firing, including nociception. Indeed, this was seen, as knocking down the expression of *para* in nociceptor neurons using RNA interference led to a very insensitive phenotype to thermal and mechanical stimuli (Dyson, 2017). Indeed, the phenotype is so robust that it can be used (and is) used as a positive control for experiments testing defective nociception phenotypes. However, the molecular mechanisms of how *paralytic* expression is regulated have not been fully elucidated in sensory neurons. At the neuromuscular junction,

more work has been done. One study (Xiao et al., 2017) shows that knocking down the expression of Para leads to fewer end plate potentials, as would be expected, but that this effect is amplified by the ER-associated chaperone protein Calnexin (Cnx). As well, as mentioned above, Pumilio and *para* mRNA have been found to bind and affect neuronal excitability in the motoneurons and at the NMJ (Mee, 2004; Muraro et al., 2008).

The sensation of pain is as complicated a pathway as any particular type of sensation. There are many components of the pathway that have been identified in the *Drosophila* nervous system, particularly in the Class IV multidendritic neurons. As perception of distinct types of noxious stimuli, like mechanical and thermal, requires distinct pathways, there are many genes required to code for different ion channels to conduct those signals. This separate perception of noxious thermal and mechanical stimuli requires many different genes to code for channels, like *TrpA1* and *pickpocket.* This calls for another method to regulate the expression of these genes, and RNA binding proteins are perfect for the job. There are many different levels where RNA binding proteins can regulate the expression of genes, whether co- or post-transcriptionally. Particularly in translational control, how often and where an mRNA is translated determines how much of the protein is in the cell and how much energy needs to be expended to allow it to reach its full potential, and RNA binding proteins have been shown to provide that layer of regulation in many processes.

The interaction between Pumilio and *paralytic*, a sodium channel needed in nociceptive neurons, provides an interesting avenue of study about the mechanism through which *pumilio* regulates nociception. If Pumilio decreases or increases the amount of the *para* transcript that can be translated into protein in the sensory neurons, this could decrease or

increase the ability of the sensory neuron to relay the presence of noxious stimuli and thereby decrease or increase the sensitivity of the animal to noxious stimuli. In a similar way, it could alter the expression of other proteins important for nociceptive sensation and modulate the sensory abilities of a neuron. I have endeavored to characterize this relationship, as well as investigate some other potential avenues through which Pumilio could regulate nociceptor neuron sensitivity. These downstream targets could possibly offer new targets for drug treatment, like to combat the increase in sensitivity due to injury. Our hypothesis is Pumilio is repressing the translation of genes necessary for nociception, therefore regulating sensitivity in nociception.

METHODS

Crosses

Five to six virgins and three males are placed in vials with about two centimeters of Nutrifly food (Gennessee Scientific) and yeast. After two days, they are flipped to another vial, and for two days they are flipped daily for a total of four rows of vials. The genotype of the virgins is selected based on the driver needed for the experiment. The tissue-specific driver for the nociceptor neurons is *ppk*, and this can be with either just a GAL4 driver for overexpression or a gene encoding the Dicer enzyme for knockdown of expression. The genotype of the males is selected based on the experimental design. In RNAi knockdown, the males have transgenic inserts, called UAS-RNAi transgenes, to express RNA sequences that will target the RISC complex to the mRNA of interest and thereby lower expression of the gene of interest (Perrimon et al., 2010). In overexpression, the flies have UAS sequences preceding the gene of interest that will allow GAL4 to bind and express a cDNA copy of the gene (St Johnston, 2002). Cross vials were kept in an incubator at 25 ˚C at about 50% humidity. Only wandering 3rd Instar larvae were used for behavioral assays.

For knockdown experiments, the female virgins used were *ppk-GAL4; UAS-dicer 2,* which contain the appropriate driver to activate the RNA interference system. The *pickpocket (ppk)* gene codes for a channel that is expressed only in the nociceptive neurons (Ainsley et al., 2003; Hwang et al., 2007), so the *ppk-GAL4* driver forces expression in these neurons. The males used in these experiments contain UAS-RNAi, which directs the activation of the RNA interference system to one specific gene. There were three separate crosses for this type of experiment, each with the ppk-GAL4; UAS-dicer 2 virgins: one with the UAS-RNAi males, one

with males without an UAS-RNAi transgene as a negative control, and one with *paralytic* UAS-RNAi males as a positive control. The negative control is chosen based on the background of the RNAi line. The positive control is chosen because knocking down the *paralytic* gene through RNA interference leads to a very insensitive phenotype to most types of nociceptive stimuli, and that allows confirmation that the RNAi is functioning as expected.

For experiments with overexpression, the female virgins are either *ppk-GAL4* or *w1118*, with the GAL4 driver increasing expression by targeting UAS sequences at a target gene. This leads to overexpression as both the genetic copy and a cDNA copy of the gene of interest are expressed in the targeted tissue. The males used are either *w1118* or contain the UAS sequences preceding the gene of interest. There were three separate crosses for this type of experiment: one with *ppk-GAL4* virgins and males of the UAS-gene of interest, one with *ppk-GAL4* virgins and *w1118* males, and one with *w1118* virgins and males of the UAS gene of interest, which is *UAS-pumilio*in this work.

Table 1. Fly Stocks

Stock Name	Genotype	Source
TRiP HMs01564 Valium 20 attP2	Y[1] sc[*] v[1]; P{y[+t7.7]v [+t1.8]=TRiP.HMs01564}attP2	Gavis Lab
UAS-pum RNAi (on 3) Zinn	UAS-pum RNAi (on 3)	Gavis Lab
VDRC 101399 KK pum RNAi	P{KK109048}VIE-260B	Gavis Lab
VDRC 45815 pum RNAi (on 3)	W[1118];P {GD14303}v45815/Tm3	Gavis Lab
Para RNAi		Tracey Lab
w1118	w[1118]	Tracey Lab
isoW	isogenized w[1118]	Tracey Lab
yw; attP	y w1118; P{attP,y[+],w[3']}	Tracey lab
36303	y[1] v[1]; P{y[+t77]=CaryP}attP2	Bloomington
ppk-GAL4; UAS-dicer 2	w{1118]; ppk-GAL4; UAS Dicer 2	Tracey Lab
ppk-GAL4	w[*]; P{w[+mC]=ppk-GAL4.G}2	Tracey Lab
6907-2-5; UAS-pum**		
eIF4AIII	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00442}attP2	Bloomington
eIF4G2	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00762}attP2	Bloomington
eIF3S4	w[1118]; P{GD13992}v28937/TM3	Gavis Lab
BOB		
Ppk-GAL4-GFP	W; ppk-GAL4 UAS MC D89GFP; UAS Dicer 2	

** this genotype was verified through Taq PCR amplification of the cDNA copy of the gene inserted to cause overexpression.

Nociceptive Assays

The thermal and mechanical nociception assays are used to test the effect of RNAi knockdown or overexpression has on the mechanical and thermal nociceptive sensitivity of *D. melanogaster* larvae. The thermal assays measure the latency between the stimulus and the NEL reflex, and the mechanical assays measure the presence or absence of the NEL reflex.

The thermal assays were set up using a digital camera connected to a dissecting microscope to film the assay, and larvae were placed in a glass petri dish with water and yeast. Just enough water was added to allow larvae to crawl across the dish, but not so they were floating, and yeast was added to disturb the surface tension. To apply the heat, a soldering iron with a copper tip was chiseled to have a tapered edge and the voltage was controlled with a Variac, and the temperature was monitored using a fine thermocouple (Tracey et al., 2003). The soldering iron was heated to 42˚C to test the *pumilio* RNAi knockdown and negative control, and to 46˚C for all other tests. This heated iron was brought into contact with the lateral wall of the animal near the middle until either a nociceptive response occurred or 11 s had elapsed. The entirety of the testing was recorded using a video camera, and then analyzed using Adobe Premiere Pro, which allowed more precise recording of the latency. A marker is placed when the probe touches the larval body wall, and when the larva completes a full roll, and the time in between is calculated and rounded to the nearest tenth. Times over 10 s were noted as 11 s (Tracey et al., 2003).

The mechanical assays were set up under a light microscope and larvae were placed in a plastic disposable petri dish to reduce slip. Water and yeast were added to achieve the same effect as previously described. The force was applied using a 10 nm length Von Frey filament. There were three trials per larvae and they were scored as either 0 = no nocifensive escape locomotion or 1 = executed nocifensive escape locomotion (Hwang et al., 2007).

Statistics

For analysis of thermal nociceptive assays, a Mann Whitney rank sum test was performed. The mean latency and standard error was calculated and used for figures. For analysis of mechanical nociceptive assays, a Chi Square test was performed. The minimum number of trials for thermal tests was 50, and for mechanical tests was 80. The proportional response and the standard error of the proportion were calculated and used for figures. The statistical tests compared the specific gene knockdown or overexpression and the wildtype background or negative control. A Bonferonni correction was done for tests that required multiple comparisons; i.e., multiple mutant genotypes were compared to the same wildtype genotype.

Molecular Biology

Genomic DNA analyses: These tests were done to verify the presence of the *pumilio* cDNA in the overexpression line. Flies for the DNA extraction were taken from the stock vials of the genotype of interest. About 20 flies were homogenized using a 1000 µL pipette tip in a microcentrifuge tube with 1000 µL of phenol chloroform. A protocol (Appendix A) was followed. The purity and concentration of DNA was quantified on a NanoDrop spectrophotometer and then the sample was used in TaqPCR with NEB Standard Taq Buffer, dNTPs and Taq Polymerase. The primers for these reactions were designed using Vector NTI and downloaded sequence information from FlyBase and NCBI Gene (Actin 42AF and R, pumilio primers, Table 2).

Creating the *para*-MS2 insertion: In order to understand how the *paralytic* gene mRNA transcript localizes in neurons, we decided to create a reporting system that would utilize the MS2-MCP system (Bertrand et al., 1998). In this system, we would insert MS2 stem loops in the 3' UTR of the *para* gene. We based parts of this experiment on previous insertions in *Drosophila* (JayaNandanan et al., 2011).

All plasmid minipreps, maxipreps, gel purifications and PCR product concentrations were done using Zyppy kits. The cloning utilized restriction enzymes from New England Biolabs and T4 DNA ligase and T4 DNA ligase buffer also from New England Biolabs.

CRISPR method: For the generation of pRKS1 and pRKS2, CRISPR guide RNAs (*para* oligos 1 and 2) were subcloned into pU6-*BbsI*-chiRNA using the *BbsI* restriction site. This was confirmed using a double digest of *BbsI* and *XhoI* and Sanger sequencing from Eton Bioscience. For the generation of pHD-DsRed-attP-*para*-MS2, *Taq* polymersase chain reaction (*Taq*-PCR, NEB) was performed from genomic DNA extracted from *w1118* flies to replicate homology arms. These homology arms were subcloned into the pHD-DsRed-attP plasmid using *EcoRI* and *NotI* for the upstream arms, creating pRKS3, and *BglII* and *XhoI* for the downstream arms, creating pRKS4. The upstream insertion was confirmed using *NotI*-HF and *EcoRI*-HF digestion, and the downstream insertion by *BglII* and *XhoI*. The MS2 stem loops were digested from the plasmid pCR4-24XMS2L-stable using *BglII* and *BamHI*, gel purified, and ligated to a gel purified *BglII* digest of the pHD-DsRed-attP-UD plasmid. This would place the MS2 loops just upstream of the downstream homology arm.

MiMIC method: Vectors were ordered from the Drosophila Genome Resource Center (1313, 1032, 1297, 1305, 1322) in order to utilize the methods described by Venken et al (Venken et al., 2011). A *XhoI* site was inserted over the *BamHI* site in vector 1297 using site directed mutagenesis, creating pRKS5 (Appendix B). This was confirmed using a double digest of the plasmid with *XhoI* and *HindIII*-HF, and Sanger sequencing from Eton Bioscience. The MS2 stem loops were replicated as described above, then *XhoI* sites were added at either end to allow insertion into the vector.

Table 2. Primers

Primer	Sequence
Para antisense 1 oligo	AAACTATGCCTACATCTGCCTAGC
Para antisense 2 oligo	AAACACATTAGTTGCTTGACACGC
Para sense 1 oligo	CTTCGCTAGGCAGATGTAGGCATA
Para sense 2 oligo	CTTCGCGTGTCAAGCAACTAATGT
Pumilio antisense 1	GAGCTATCTTTGTTGGTGGG
Pumilio antisense 2	CAATCCGGAAAGCGAGCTATC
Pumilio sense 1	CCACATCCACTGCCAAGAAATTG
Pumilio sense 2	GGGAGAAATCCGATGGCAAG
Actin 42A F	GCGTCGGTCAATTCAATCTT
Actin 42A R	AAGCTGCAACCTCTTCGTCA
SDM BamHI to XhoI F	GTGGAAGCGGAGGTAGCGGCCTCGAGGGAGGTAGCGGTGGAAGCGG
SDM BamHI to XhoI R	CTCCTTCGCCTCCATCGCCGGAGCTCCCTCCATCGCCACCTTCGCC
NotI MS2F-oligo	TAGCGCGGCCGCGCCGCGAATTCGCCTTGGATCC
NotI MS2R-oligo	TAGCGCGGCCGCAGATCTGATGAACCCTGG
MS2F (no NotI site)	TGAATTGTAATACGACTCACTATAG

Confocal Imaging

Pumilio Neuronal Morphogenesis: In order to visualize the neurons in both *pumilio* RNAi and overexpression larvae and determine if they had normal or abnormal development, we crossed *ppk*-GAL4-GFP virgins to *pumilio*-RNAi and *W1118* males as described above. The third instar larvae of these crosses were ligated using a hair tied just posterior to their ventral nerve chord, and placed in glycerol between two cover slides. These larvae were imaged on a Zeiss 880 LSM with a 488 nm laser line, and the images were imported into ImageJ and analyzed using the NeuronJ plugin.

Bioinformatics Analysis

Lists of mRNAs associated with Pumillio in previous studies (Gerber et al., 2006; Zhang et al., 2017) were analyzed for significant GO annotations through GO Stat, with the p value cut off being set to $1x10^{-5}$, only overrepresented annotations listed, and clusters set to 3. The clusters were grouped under names listed in figures.

A few studies were selected that could identify mRNA transcripts that could be important for nociception (Honjo et al., 2016; Misra et al., 2016; Neely et al., 2012), and the results of these studies were compared to the results of Gerber et al. to find possible targets for nociceptive regulation by Pumilio.

RESULTS

Behavioral Analyses of pumilio *RNAi lines shows a hypersensitive nociception phenotype*

We chose to knock down transcript levels of our genes of interest using RNA interference lines. In these lines, the mRNA transcripts of the gene are targeted using short interfering RNAs to the RISC complex for destruction, thereby lowering the expression of that gene. In order to activate this system, the RNAi lines must be crossed to another line that includes the Dicer enzyme and a tissue specific driver for nociceptive neurons, which in this case is *ppk-GAL4*; *UAS-dicer2*. These flies then have lowered expression of the gene of interest only in the Class IV multidendritic nociceptive neurons. In order to ensure there truly is an effect with the knockdown of the gene of interest, a negative control is set up that uses the same genetic background as the RNAi line without the RNAi itself.

Specifically, we applied this approach to knockdown expression of the *pumilio* gene. We tested multiple *pum*-RNAi lines with a thermal probe at 42˚C to allow us to view the full extent of any hypersensitization. We used multiple backgrounds in order to increase the robustness of our results, and saw that each was significantly more sensitive than wildtype (Fig. 1). Because RNAi depends on the effectiveness of the Dicer enzyme complex, it is possible for it to not be as effective in one background as in another. If so, this can be controlled for by testing in multiple backgrounds and comparing the behavioral phenotypes for each. For the KK *pum-RNAi* line, the average latency was 6.3s, and for the *yw; attP* negative control the average latency was 9.04s. For the *TRIP pumilio* line, the average latency was 6.9s, and for the *yw; attP2* negative control the average latency was 9.3s. For the *UAS-pumilio* RNAi line, the average latency was 6.5s, and for the w1118 negative control the

average latency was 9.8s. For each of these, the difference between the latencies of the RNAi knockdown line and the latencies of the wild type was statistically significantly different (Mann Whitney U Test, $p<0.05$). From these data, we could conclude that decreased expression of Pumilio in the nociceptor neurons lead to the development of hypersensitivity to noxious thermal stimuli.

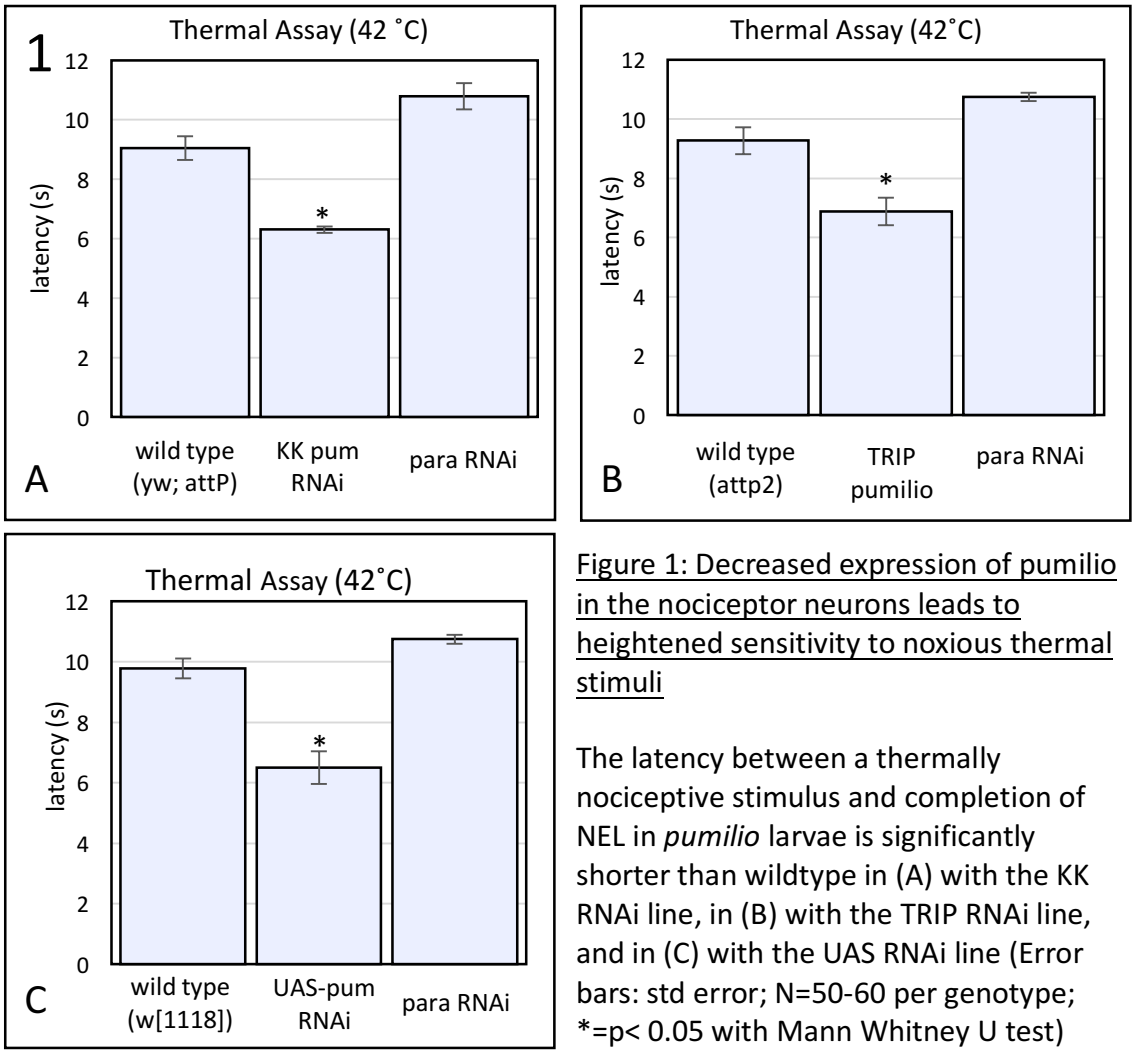

Figure 1: Decreased expression of pumilio in the nociceptor neurons leads to heightened sensitivity to noxious thermal stimuli

The latency between a thermally nociceptive stimulus and completion of NEL in *pumilio* larvae is significantly shorter than wildtype in (A) with the KK RNAi line, in (B) with the TRIP RNAi line, and in (C) with the UAS RNAi line (Error bars: std error; N=50-60 per genotype; *=p< 0.05 with Mann Whitney U test)

We then continued to explore the effect that decreased Pumilio function had on the sensitivity of *pumilio* knockdown larvae using mechanical stimuli. We found that *pumilio* RNAi knockdown larvae were hypersensitive to mechanical stimuli (Fig 2). This score is based on the number of larvae that execute a nocifensive roll when they are poked on the dorsal midline. The proportion of larvae that responded on the first poke in the KK *pum-RNAi* line was 46.2%, and the proportion of the *yw;attP* negative control was 18.7%. The proportion of larvae that responded on the first poke in the *TRIP pumilio* line was 42.7%, and the proportion of the *yw;attP2* negative control was 25.9%. The proportion of larvae that responded on the first poke in the UAS *pum-RNAi* line was 44.1%, and the proportion of the *w1118* negative control was 25.9%. The proportion of larvae responding to the first poke were all statistically significantly different from their respective wildtype backgrounds (Chi square test, $p< 0.05$). This data, combined with the data from Figure 1, indicated that decreased expression of Pumilio in nociception led to the development of hypersensitivity to noxious mechanical and thermal stimuli, and that there was most likely a role for Pumilio-dependent regulation in nociceptor sensitivity.

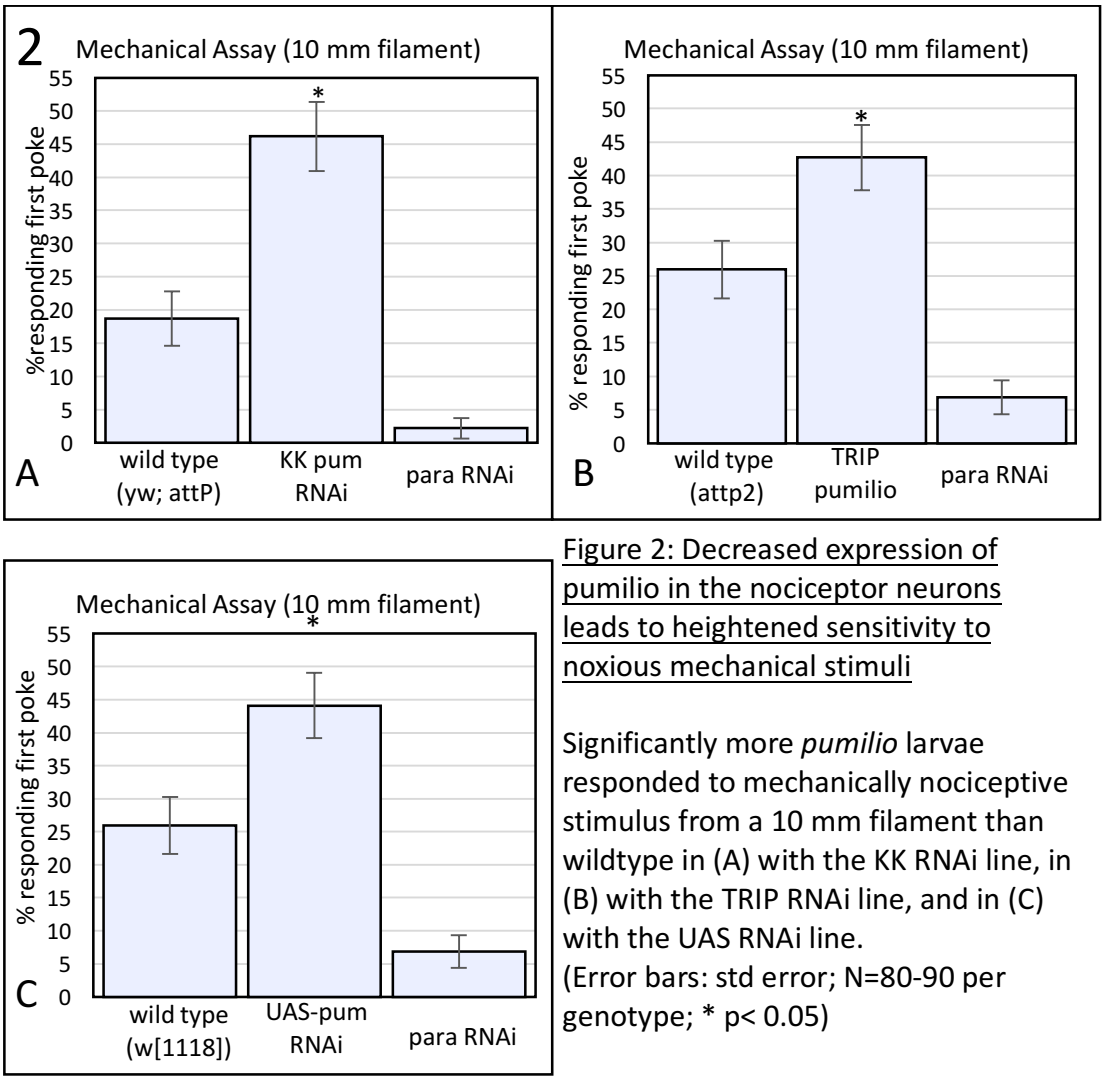

Figure 2: Decreased expression of pumilio in the nociceptor neurons leads to heightened sensitivity to noxious mechanical stimuli

Significantly more *pumilio* larvae responded to mechanically nociceptive stimulus from a 10 mm filament than wildtype in (A) with the KK RNAi line, in (B) with the TRIP RNAi line, and in (C) with the UAS RNAi line.
(Error bars: std error; N=80-90 per genotype; * $p < 0.05$)

Mechanical nocicpetion analyses of a molecularly confirmed pumilio *overexpression line show an insensitive phenotype*

To assess the extent of Pumilio's ability to negatively regulate translation, thermal and mechanical assays of the Pumilio overexpression line were performed. First, the genotype of this line was confirmed. In this line, the ppk-GAL4 driver is used to express a cDNA copy of the *pumilio* gene in the nociceptive neurons. To confirm the presence of this cDNA in the genome of the UAS-*pumilio* line, the DNA from the larvae is replicated via *Taq* PCR to show both a copy of the full *pumilio* transcript and the spliced cDNA copy. Fig 3 A., showing two bands, indicates the presence of the cDNA in the stock line. In lane 1, the template is *W1118* genomic DNA, and the product is of a control gene that is present in all genotypes, and is about 800 base pairs. In lane 2, the template is UAS-*pumilio* genomic DNA, and the primers are designed to replicate over an exon-intron junction and produce fragments of 800 base pairs (without intron) and 1000 base pairs (with intron), which are showin in Fig 3A. In lane 3, the template is *W1118* genomic DNA, and the product is from the same primers used in lane 2, but there is no copy with intron because there is no cDNA copy of the UAS-*pumilio* gene and the band is only 800 base pairs.

With the genotype of the larvae confirmed, we could move on to characterizing the behavioral phenotype based on this change in expression. The mechanical nociception assay revealed an insensitive phenotype in the larvae with overexpressed *pumilio*, shown in Fig 3 B., as compared to one of the controls (Chi square test, $p<0.05$). The thermal nociception

assay showed no significant differences in the behavioral phenotype of the different genotypes of larvae, shown in Fig 3 C (Mann Whitney U Test, p>0.05).

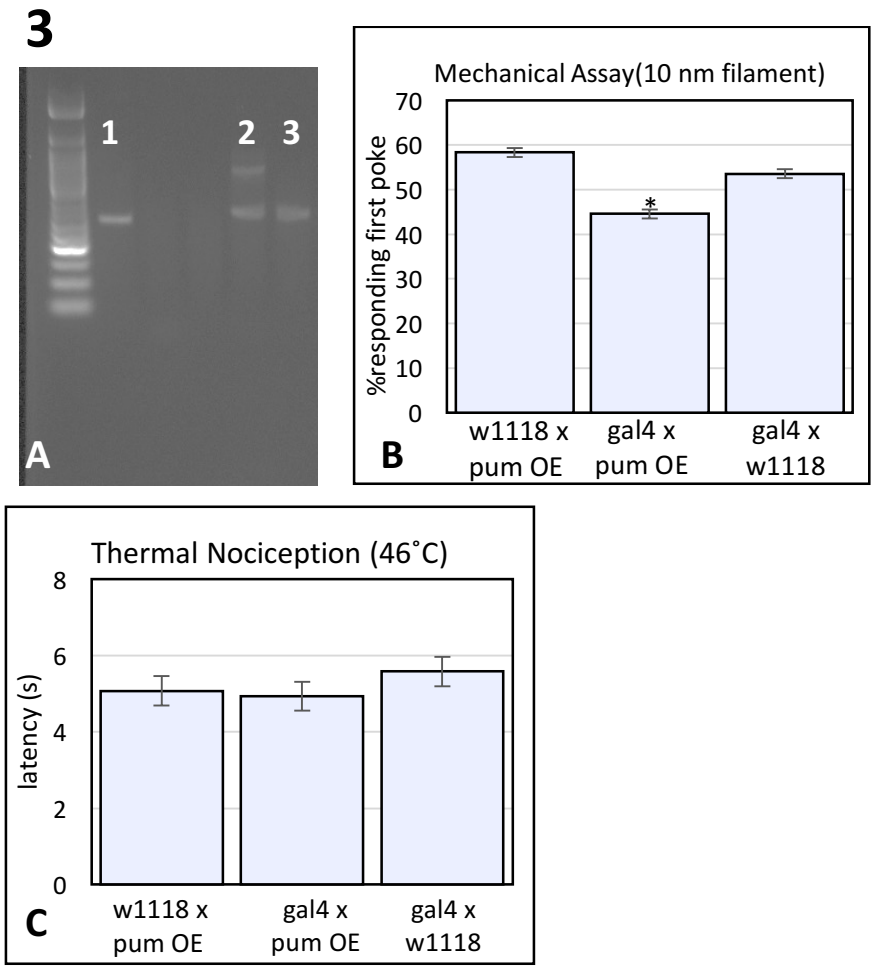

Figure 3: Increased expression of pumilio in the nociceptor neurons leads to lessened sensitivity to noxious mechanical stimuli but no change in sensitivity to noxious thermal stimuli.

(A) shows the PCR fragments that confirm there was overexpression in the stock line. "1" is a control to show the PCR reaction is replicating correctly, "2" is the Pumilio PCR fragments and "3" is the w1118 PCR fragments.

(B) Significantly more gal4 x *pumilio* overexpression (Pumilio OE) larvae responded to mechanically nociceptive stimulus from a 10 mm filament than w1118 x Pumilio OE. (Error bars: std error; N=80-90 per genotype; * p< 0.05 with Chi Square test)

(C) The latency of response to thermally nociceptive stimuli at 46˚C was not significantly different among the three genotypes. (Error bars: std error; N=50-80 per genotype, Mann Whitney U test)

Mechanical nociception of eukaryotic Initiation Factors (eIFs) generates diverse phenotypes

Other interesting candidates for study found in the Dyson screen included many components of the eIF4F complex and other eIFs. Lines in which *eIF4AIII, eIF4G* and *eIF3S4* were knocked down using RNAi were all found to be insensitive to thermal stimuli compared to wildtype. However, this is no guarantee of the response to mechanical stimuli. We decided to investigate whether the mechanical nociceptive phenotype would reflect the defect in thermal nociception. Only *eIF4G* was found to respond in a significantly different proportion than wild type, and in that the proportion was larger. Both *eIF4AIII* and *eIF3S4* were not significantly different from controls (Fig. 4). The proportion of larvae that responded on the first poke in the *eIF4AIII-RNAi* line was 19.8%, the proportion for *eIF4G2-RNAi* line was 50% and the proportion of the *yw;attP2* negative control was 32.3%.The proportion of larvae that responded on the first poke in the *eIF3S4*-RNAi line was 44%, and the proportion of the *yw;attP2* negative control was 49% in this cross. The proportion of *eIF4G2 RNAi* larvae responding on the first poke was statistically significantly different from the wildtype background (Chi square test, Bonferonni corrected $p < 0.025$).

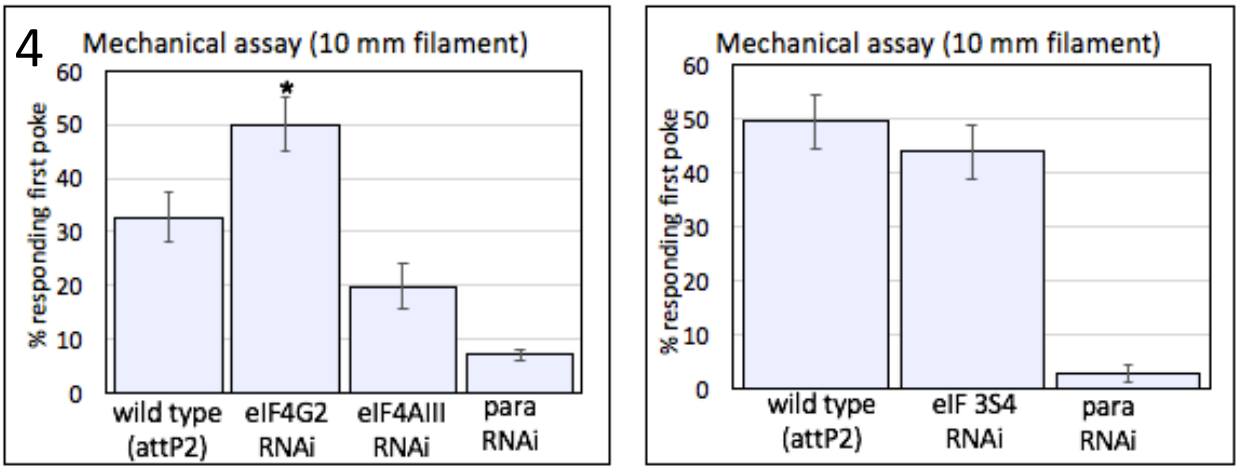

Figure 4: Thermally insensitive knockdowns of certain eukaryotic initiation factors are not necessarily mechanically insensitive.
The proportion of *eIF4G2* larvae responding to a mechanically noxious stimulus from a 10 mm filament is significantly larger than the proportion of wildtype larvae responding. The proportions of *eIF4AIII* and *eIF3S4* larvae were not significantly different. (Error bars: std error; N=60-80 per genotype; *: Bonferonni corrected p< 0.025)

Analysis of Class IV neuronal dendrites for defects in pumilio *RNAi larvae show*

In order to determine whether the hypersensitive phenotype seen in knockdown larvae is due to changes in dendritic morphology, Class IV neurons were imaged using confocal microscopy. These neurons were made to fluoresce by expressing a *ppk*-GAL4-driven UAS-mcD8::GFP molecule in *pumilio* knockdown and control larvae. This cross leads to the expression of GFP tethered to the cell membrane, and easily allows the nociceptive neurons to be visualized. Once the images of the neurons have been obtained, multiple characteristics describing the structure and morphology can be measured. The images I analyzed are shown in figure 5 A and B, and the total dendrite length and branch points for each image are shown in figure 5 C and D respectively. There appears to be a trend toward lower branching and dendrite length in the *pumilio* KD larvae, but more samples are needed before any claims can be made.

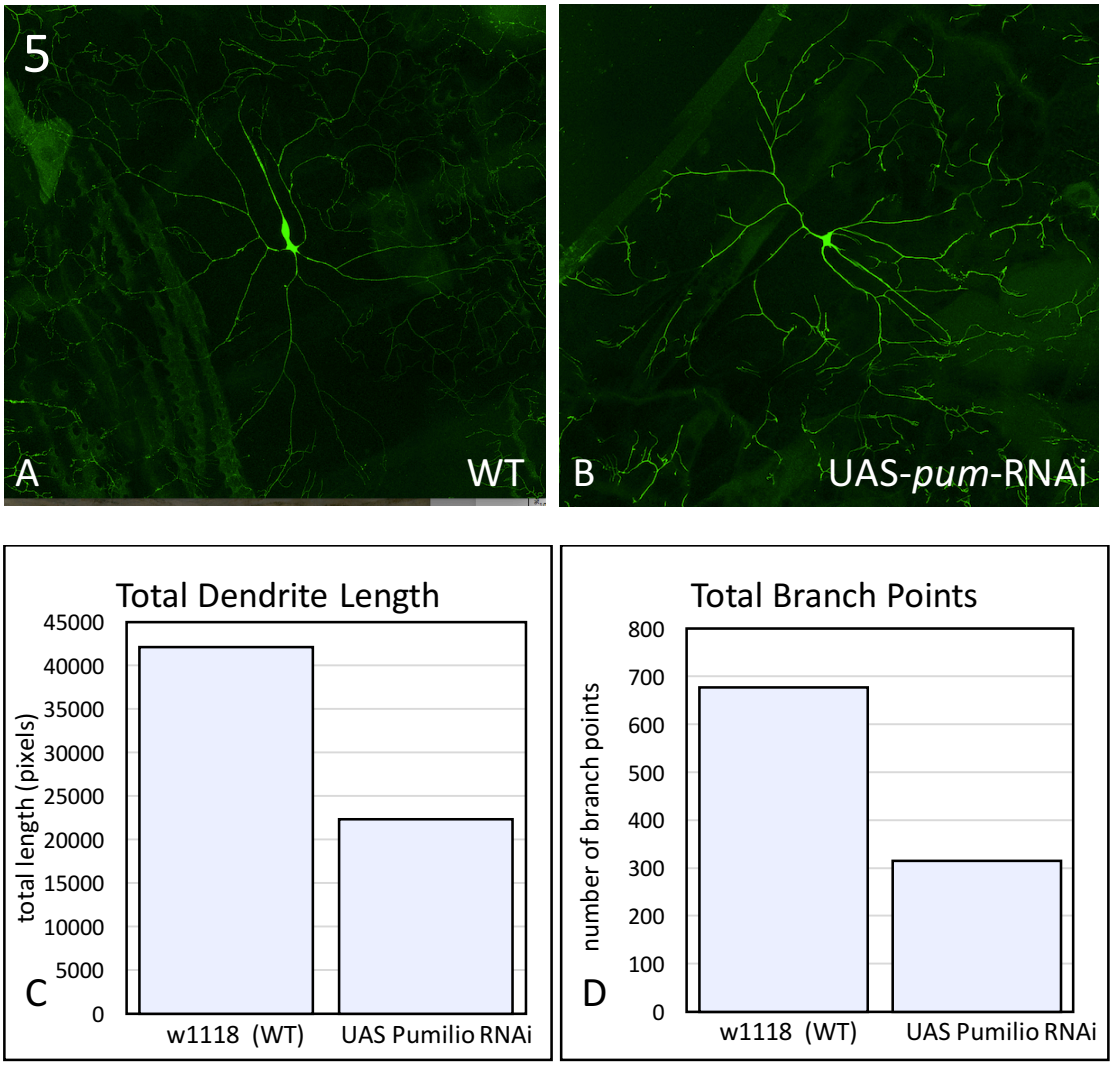

Figure 5: Preliminary Analysis of Dendrite Morphology reveals some differences
(A) and (B) are images taken of a Class IV neuron in larvae of the genotypes annotated on the images. These images were imported in Neuron J, where the total dendrite length (C) and total number of branch points (D) were measured. A larger sample size is needed to analyze the significance of this data.

Bioinformatics studies illustrate the potentially wide range of Pumilio effectors

In order to fully elucidate the mechanism *pumilio* acts through to regulate nociception, its downstream effectors must be found and described. I conducted a literature search to find what mRNAs could be bound by Pumilio and also potentially play a role in nociception. There are already studies that show Pumilio binds mRNAs specific to both the

Drosophila genome in general (Gerber et al., 2006) and, in *Mus musculus,* the brain specifically (Zhang et al., 2017). The GO annotations of these results were analyzed to show their similar significance to neuronal function, and are presented in figure 6 A and B. To identify Pum's potential downstream affecters, the mRNA transcripts implicated as being enriched in *Drosophila* nociceptors (Honjo et al., 2016), localized in nociceptors (Misra et al., 2016), or as part of the *Drosophila* pain system (Neely et al., 2012) were compared to those found to bind Pum. These are listed in Table 1 and represent the overlap between the Pumilio binding partners and the various lists of transcripts enriched in nociceptors and nociception related processes.

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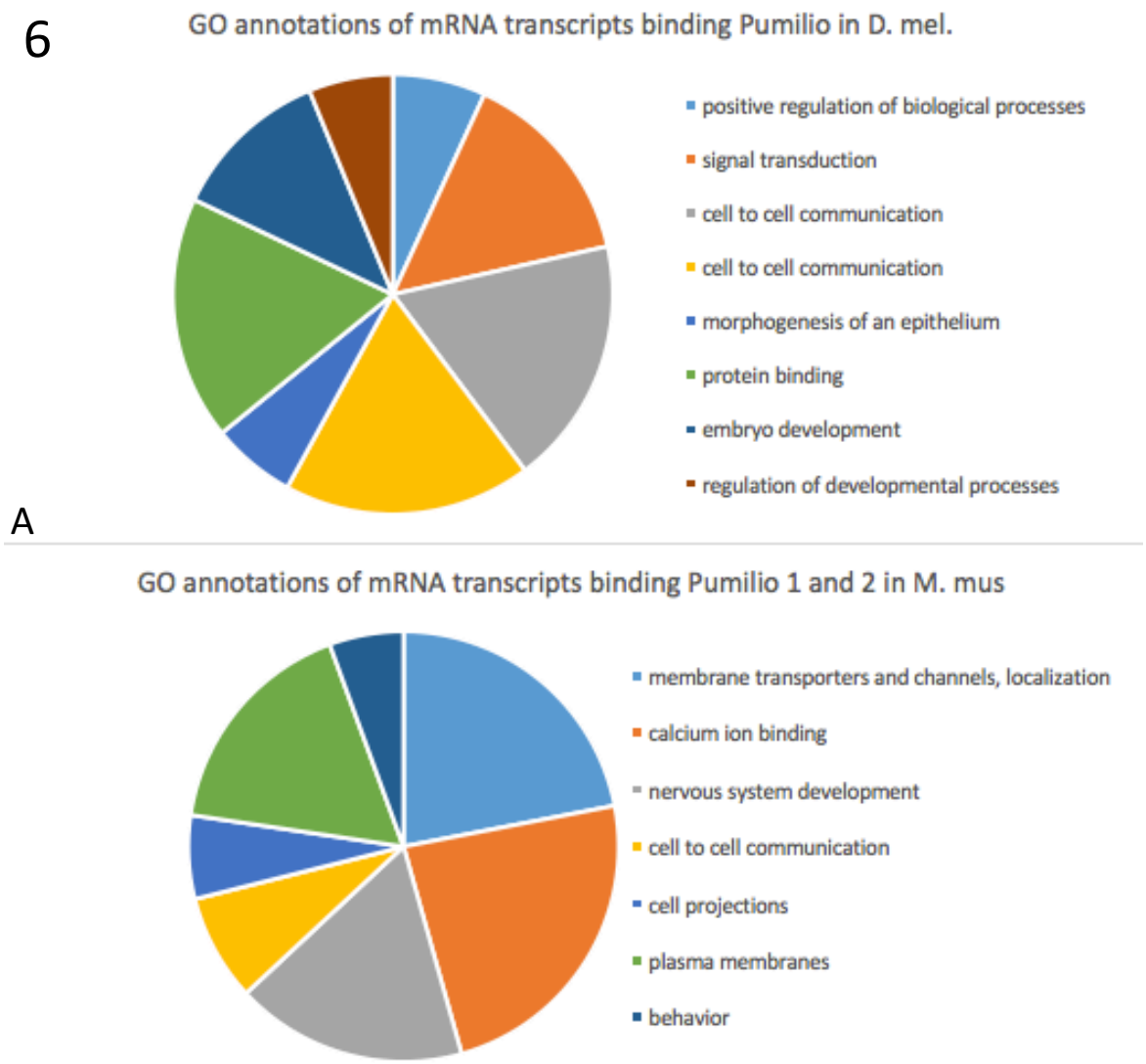

Figure 6: GO annotations of mRNA transcripts that have been shown to bind Pumilio in *D. melanogaster* (A) and *M. musculus* (B) reveals enrichment in categories related to nociceptor sensitivity

Table 1: potential downstream affecters of Pumilio

Genes Enriched in Nociceptors (Honjo et al, 2016)		Genes Localized in Nociceptors (Misra et al, 2016)		Genes Implicated as Part of the *Drosophila* Pain System (Neely et al 2016)			
CG	gene symbol	CG	gene symbol	CG	gene symbol	CG	gene symbol
CG10255	Lap1	CG6319	bru-2	CG1031	alpha-Est1	CG5330	Nap1
CG13603	CG13603	CG9922	CG9922	CG10746	fok	CG5725	fbl
CG13605	CG13605	CG7693	fray	CG11419	CG11419	CG5940	CycA
CG3832	Phm	CG16785	fz3	CG13777	milt	CG9650	CG9650
CG3832	Phm	CG10367	Hmgcr	CG14214	CG14214	CG5969	CG5969
CG3874	frc	CG4311	Hmgs	CG3269	Rab2	CG6721	Gap1
CG4593	CG4593	CG4026	IP3K1	CG3619	Dl	CG7007	VhaPPA1-1
CG6073	CG6073	CG7734	shn	CG11878	CG11878	CG7042	CG7042
CG6207	GlcAT-P	CG3161	Vha16	CG3733	Chd1	CG7175	CG7175
CG8073	Pmm45A			CG3735	CG3735	CG7556	CG7556
CG8798	CG8798			CG3943	kraken	CG7693	fray
				CG4351	CG4351	CG7800	CG7800
				CG4946	CG4946	CG8029	CG8029
				CG5012	mRpL12	CG8114	pbl

Generation of a fluorescently tagged paralytic *mRNA transcript to visualize localization in nociceptors*

I have identified *paralytic* as a possible candidate through which Pumilio may act on nociceptor sensitivity, as it encodes a voltage gated sodium ion channel essential for the propagation of action potentials in *Drosophila* neurons, and modulating its expression could lead to changes in the effective firing of these neurons. To study how *para* mRNA functions in nociceptor neurons, I have begun introducing hairpin sequences specifically to the *para* 3' UTR that will be expressed when the *para* mRNA is transcribed and allow it to be fluorescently tagged. I have set about accomplishing this through two avenues, one using the CRISPR/Cas9

system (Gratz et al., 2013) and one through the *Minos*-mediated insertion cassette (MiMIC) system (Venken et al., 2011).

The first step to begin utilizing the Crispr/Cas9 system was to create a guide RNA plasmid (Fig 7A). This guide RNA will direct the Cas9 enzyme to cleave the genome in the specific place in the *para* 3'UTR, and allow the hairpin sequence to be inserted. The successful cloning of this plasmid was confirmed using a double digest test and sequencing, shown in Fig. 7 B and C respectively. The guide sequence is a 20 base pair sequence in the *para* 3'UTR that is near a three nucleotide sequence recognizable by the Cas9 enzyme, and this area of the plasmid was sequence to be sure the sequence was inserted correctly.

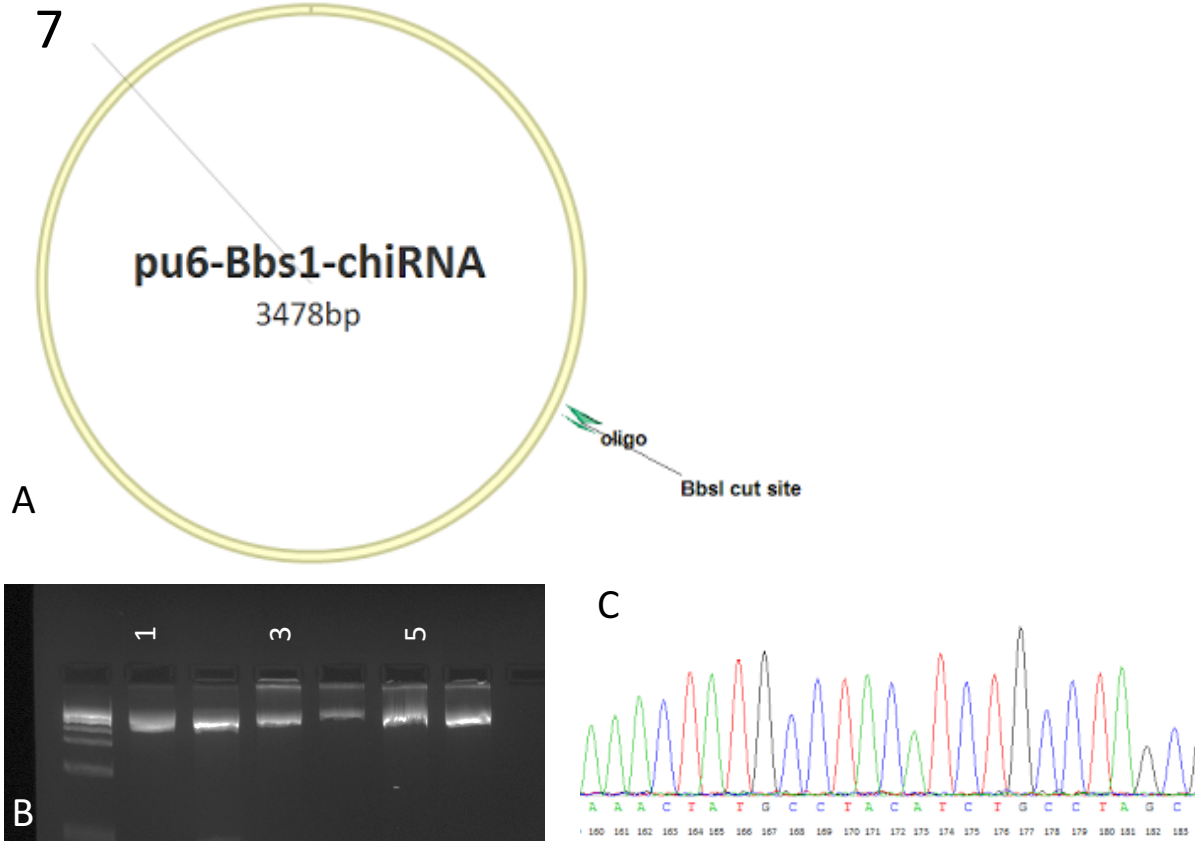

Figure 7: Creating the guide RNA plasmid.
(A) shows a schematic of the guide plasmid, with "oligo" indicating the location of the subcloned guide oligo sequence. (B) shows a double digest test of the created plasmid, with "1" indicating a control of the backbone plasmid, "3" indicating the first target plasmid, and "5" indicating the second, all digested by *BbsI* and *HindIII*-HF. Two bands indicate successful insertion of the guide RNA site, with the larger band being 2500 bps and the smaller being 1000 bp. (C) shows the sequencing results, shortened to just the

Next, the hairpins sequence had to be cloned into a plasmid with attP sequences and genomic homology arms to allow homology directed repair (Fig. 8A). There were three different sequences that had to be inserted in this plasmid: an upstream homology arm, consisting of 1000 base pairs of sequence upstream of the target insertion site, a downstream homology arm, and 24 stem loops and some intervening sequence, which would allow the

binding of the fluorescent molecule. The homology arms were replicated using PCR from the *Drosophila* genome, and the replicated fragments are shown in Figure 8B. The insertion of the homology arms was confirmed using a double digest test, showing an increase in the total size of the plasmid by about 1000 base pairs each time the cloning was completed, shown in Fig 8C, where the upper band is about 4500 base pairs, compared to D, where the upper band is about 5500 base pairs. The insertion of the stem loops has yet to be completed.

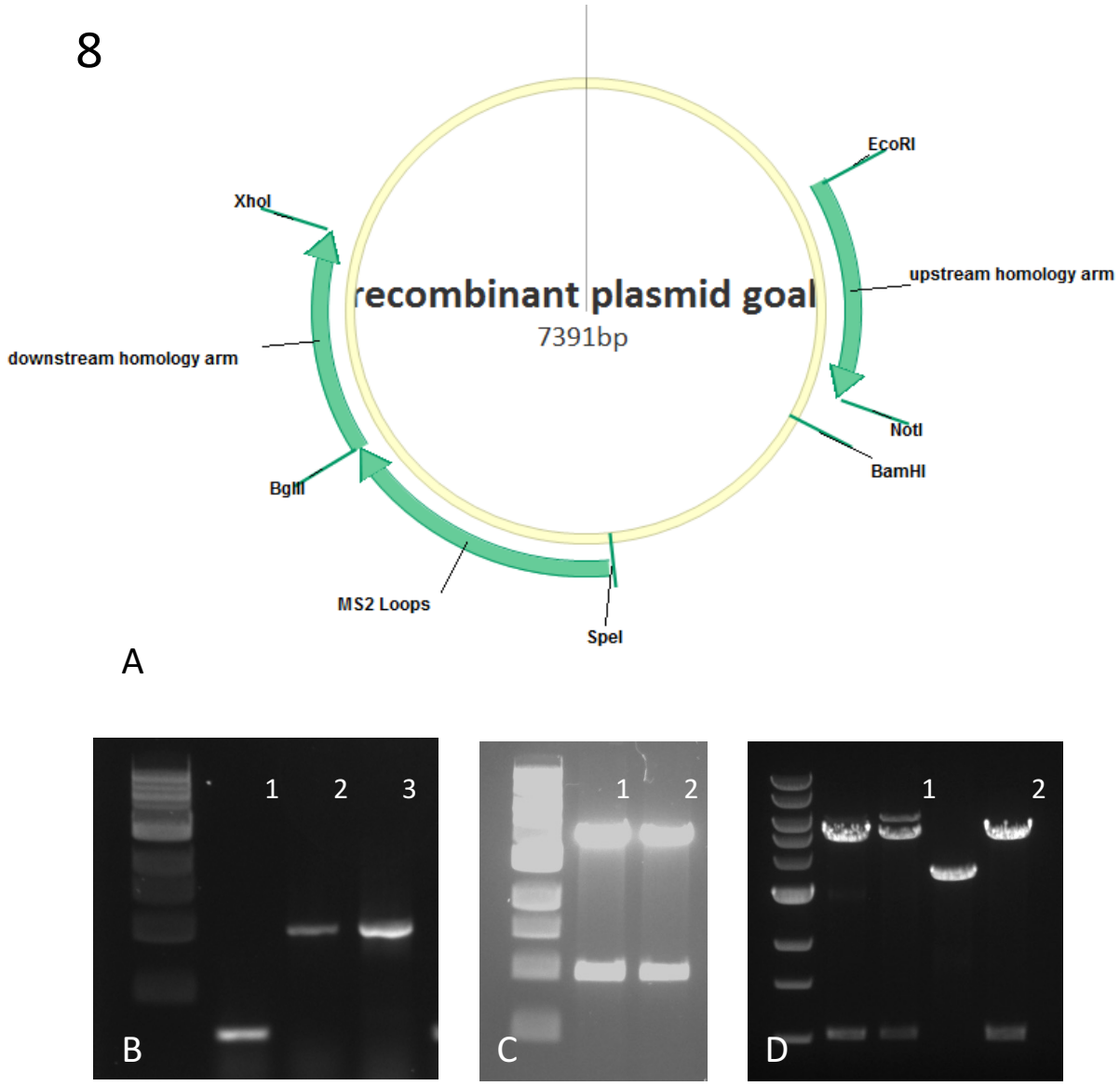

Figure 8: Creation of the insertion sequence plasmid.
(A) shows the schematic of the completed plasmid. (B) shows a gel of the *Taq* PCR product homology arms, with "1" indicating the control and "2" and "3" indicating the homology upstream and downstream homology arms respectively. (C) shows the double digest test of the dsRed-attP backbone plasmid and the upstream homology subclone, with "1" and "2" indicating two copies of the plasmid. (D) shows the double digest test of the dsRedattP backbone with both homology arms inserted, with "1" and "2" indicating two successful inserts.

The first step in using the MiMIC system was to mutate the cloning site in the plasmids provided by Venken, et al, as a *BamHI* site was at the upstream edge of the hairpin sequence we were inserting into the plasmid (Fig 9A). This was done using site directed mutagenesis, and the conversion to an *XhoI* site was confirmed using a double digest and sequencing, shown in Fig 9 B and C. The MS2 stem loops will be replicated in PCR using primers that would add *XhoI* sites to the ends so they can be easily subcloned into the plasmid, and this will be confirmed using a double digest test.

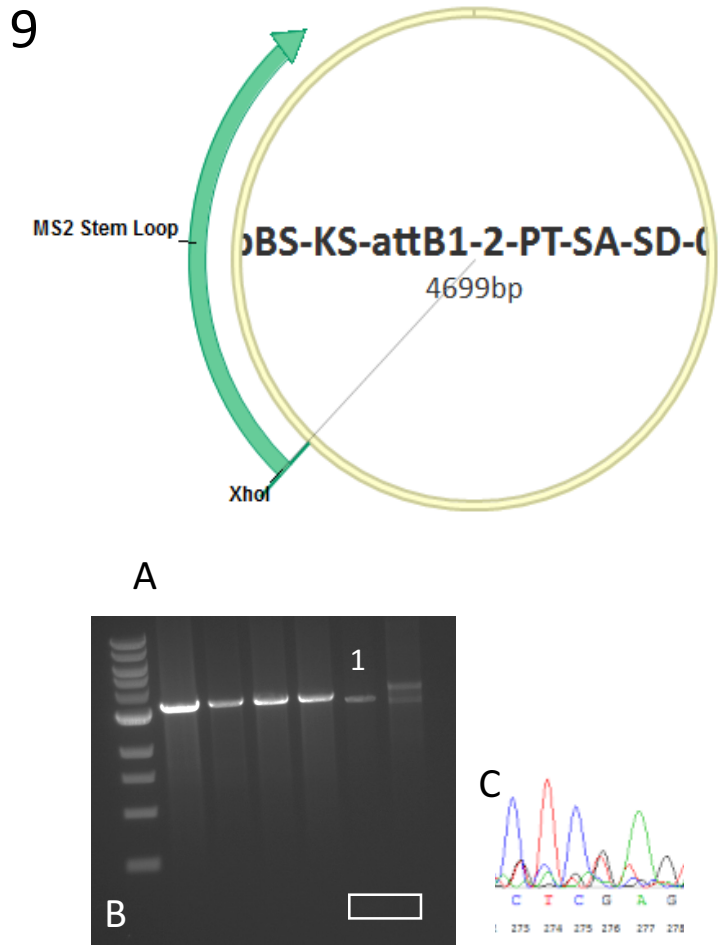

Figure 9: Creation of the insert plasmid for use with MiMIC system.
(A) shows a schematic of the plasmid when completed. (B) shows a double digest of the mutagenized plasmid, with a box indicating the lower fragment from a digest of *XhoI* and *HindIII*-HF. (C) This is the sequencing result of the *XhoI* cut site.

Once these plasmids have been completed, they will be sent for injection into specific background that will allow their insertion into the *para* gene. We will then use live confocal imaging to visualize the localization of the *para* mRNA. On a molecular level, the stem loop sequence will be transcribed into the mRNA and will take the form of the hairpins in the single stranded mRNA molecule. The MS2 capping protein (MCP) bound to a red fluorescent protein (RFP) molecule, encoded by the transgenic background of the embryos these plasmids will be injected into, will then bind the stem loops and fluoresce. This will allow the visualization of the mRNA only in its RNA form.

DISCUSSION

Hypersensitive responses to mechanical and thermal nociceptive stimuli seen in pumilio *knockdown larvae suggests general role in regulation of nociceptive sensation*

In thermal and mechanical assays repeated across multiple backgrounds of *pumilio* RNAi knockdown, larvae were more sensitive than wildtype to nociceptive stimuli. These defects in nociceptive phenotype imply that Pumilio regulates expression of proteins important for nociception. These results were suggested from the canonical effect of *pumilio* at the molecular level, as it has been shown to act in translational repression (Van Etten et al., 2012; Wharton et al., 1998; Zhang et al., 2017; for review see Parisi and Lin, 2000;). When this repression is removed, there are more proteins available to signal the sensation of nociceptive stimuli and to enact a response. However, these results indicate this translational repression is needed to maintain a normal response to nociceptive stimuli in particular.

Insensitive behavioral phenotype to mechanically nociceptive stimuli and insignificant difference to thermally nociceptive stimuli seen in pumilio *overexpression larvae qualifies role in regulation of nociceptive sensation.*

Unlike in the knockdown of *pumilio*, there are not a variety of stocks in which to test the effect of overexpression of Pumilio on nociceptive behavior. In order to determine the exact phenotype of the overexpression line, molecular analyses were conducted to confirm the presence of *pumilio* cDNA in the genomic DNA of the stock line, and they successfully indicated the presence of the cDNA copy of *pumilio*. The mechanical assays of this line indicate an insensitive phenotype, which would mesh with the idea of increasing the amount of repression on the translation of nociceptive stimuli. This further implies the role Pumilio

may play in regulating nociceptive response. However, the insignificant difference in behavioral response to thermally noxious stimuli qualifies this statement. This could have occurred because there is not a successful overexpression of *oum* in these larvae, or because more Pumilio within the nociceptors cannot cause a large effect specifically in thermal nociception.

Diverse phenotypes of translation initiation factors in thermal vs. mechanical nociception suggest different pathways for regulation of different types of nociception

In the Dyson screen (Dyson, 2017), *eIF4AIII, eIF4G and eIF3S4* RNAi larvae were all found to be more insensitive to thermal stimuli than wildtype, while in this study they were not all insensitive to mechanical stimuli. Indeed, neither *eIF4AIII* nor *eIF3S4* RNAi knockdown presented a significantly different phenotype to nociceptive stimuli than wildtype, and *eIF4G2* RNAi larvae were hypersensitive compared to wildtype. The difference between responses depending on types of nociceptive stimulation is not unheard of in nociception studies, however. Some ion channels essential for thermal sensation have no effect on mechanical sensation, while ion channels like *ppk* are essential for only mechanical sensation (for review see Im and Galko, 2012). These diverse responses indicate these initiation factors may be essential for thermal nociception but not for mechanical nociception. In general, these initiation factors may be important to begin the expression of proteins necessary for nociception, and increased or decreased activity on their part could lead to diverse effects on the sensitivity of the neuron to nociceptive stimuli.

Decreased branching and length of dendrites of Class IV neurons and pumilio *expression*

If the dendrites of the Class IV neurons in *pumilio* knockdown larvae are significantly different than wildtype larvae, the hypersensitive phenotype could be due to that instead of regulation of the sensation of nociception itself. This could be possible because *pumilio* is essential for asymmetric gene expression in development, and has been shown to also regulate asymmetric gene expression in neuronal development (Ye et al., 2004). This would still indicate an important role for *pumilio* in nociceptive sensation, but not in the regulation of a pathway that could lead to sensitization. However, it is also difficult to directly relate the neuronal innervation with the behavioral phenotype seen in a *pumilio* knockdown. Because there is less coverage by dendrites of the sensory epithelium as indicated by the decreased length and branch points, one would expect there to be less ability to sense the noxious stimuli. But thinking at a more molecular level, in this Pumilio knockdown larvae, the same amount or more of the sensory machinery were potentially being translated into protein and exported to the sensory neuron dendrites. With a smaller surface area in dendrites, this would give the neurons a more concentrated ability to sense and respond to noxious stimuli, and allow them to respond to stimuli at a lower level. This illustrates that (1) more samples are needed to confirm the effect changes in *pumilio* expression has on dendrite morphogenesis and (2) the relationship between dendritic phenotype and nociception is not straightforward and merits more study.

Literature review indicates possible downstream binding partners for Pumilio regulation of nociception

As has been shown above, Pumilio has a very well described function in development and neuronal function in *Drosophila melanogaster* (Baines, 2005; Weston and Baines, 2007; Wharton et al., 1998; Zamore et al., 1997). However, its role in nociception has not been explored yet. It is possible Pumilio could bind mRNA transcripts important for nociception and either (1) decrease their translation so the correct amount of excitation of neurons occurs or (2) prevent their translation until they have been localized to the correct section of the nociceptors. The first option would not be extremely different from how Pumilio functions with *paralytic* in motor neurons (Mee, 2004; Muraro et al., 2008) and with *eIF4E* on the postsynaptic side of the neuromuscular junction (Menon et al., 2004; Sigrist et al., 2000). It has been suggested that Pumilio does this by preventing Poly-A-binding protein (PABP) from recruiting the translation initiation complex (Weidmann et al., 2014). In the model in figure 10A, I suggest this occurs to allow Pumilio to directly decrease the expression of the proteins directly necessary for nociception; these could include sensory ion channels, proteins important for activating these proteins, or proteins important for transducing the sensory stimuli into an electrical signal. Pumilio could also prevent the translation of proteins more distant from directly affecting sensory sensititvity. This model, shown in figure 10B, would suggest that Pumilio would act more similarly to how Rapamycin inhibits Mammalian Target of Rapamycin *(*mTOR*)* in local translation in neurons in rat skin (Jiménez-Díaz et al., 2008). If Pumilio could inhibit the translation of a protein like mTOR, it could have a broad effect on a signaling pathway important for sensory neuron function.

Lastly, a third way that Pumilio could affect mRNA translation is by preventing localization of mRNA transcripts. An example of this process is shown in figure 10C, where Oskar and Rumplestiltskin (Rump) form a a complex to transport *nanos* mRNA to the dendrites of the neurons. It is possible that Pumilio could bind to *nanos* or *oskar* mRNA to prevent the binding of Rump, and therefore prevent the localization, which could change the ability of the neuron to dynamically respond to noxious stimuli. Indeed, Pumilio has been shown to bind both *nanos* and *oskar* mRNA in *Drosophila*, (Gerber et al., 2006) so it is possible this could be way that Pumilio regulates nociceptor sensitivity in nociceptor neurons. Through these three models, Pumilio could either have a very targeted and non-spatially regulated effect by binding specific mRNAs encoding proteins important for sensation and preventing translation, or have a more diffuse effect by repressing the translation of proteins important for different signaling pathways in general, or prevent the localization of mRNAs and therefore affect that area of the neuron specifically. Considering the broad categories in which Pumilio-binding mRNA transcripts are annotated within, there are many avenues through which *pumilio* could regulate nociceptive sensation.

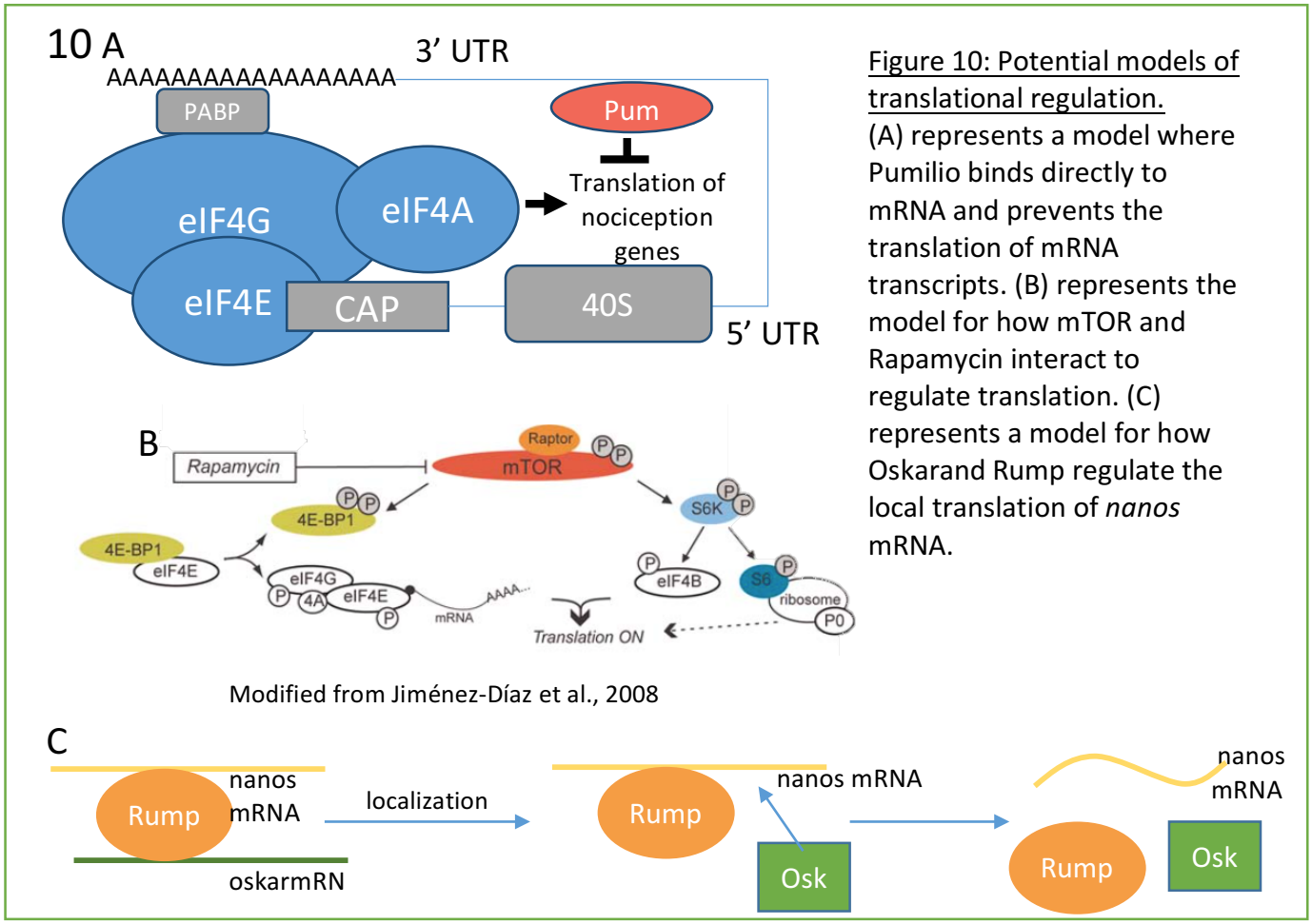

Figure 10: Potential models of translational regulation. (A) represents a model where Pumilio binds directly to mRNA and prevents the translation of mRNA transcripts. (B) represents the model for how mTOR and Rapamycin interact to regulate translation. (C) represents a model for how Oskarand Rump regulate the local translation of *nanos* mRNA.

In order to find a potential binding partner for Pumilio in regulating nociception, a literature review of established binding relationships between Pumilio and mRNA transcripts was conducted. There were quite a few transcripts indicated in this review, and comparing the lists to transcripts implicated in nociception related functions only narrowed the scope somewhat. However, the relative importance *para* in nociception in general and the already established relationship between Pumilio and the transcript in motoneurons implicated it as a likely candidate.

By comparing Pum-binding transcripts (Gerber et al., 2006) and transcripts that could play a large role in nociception (Honjo et al., 2016; Misra et al., 2016; Neely et al., 2012), I was

able to identify some potential downstream effectors. A few of these are proteins that are important for sensory conduction, including a GTPase activating protein (Gap1), a Rho guanine nucleotide exchange factor (Pbl), and a vacuolar ATPase (VhaPPa1-1). Pumilio regulating the expression of these proteins could modulate the strength of the presynaptic potential generated in response to a nociceptive stimuli or the strength of the response in the sensory receptor. There was also a Wnt- signaling receptor (fz3) listed, which could lead in many directions for investigation because of the importance of Wnt-signaling in cancer, development and other processes. In nociception, a study showed that Wnt signaling is upregulated in a neuropathic pain model and blocking Wnt signaling prevented the development of neuropathic pain (Zhang et al., 2013). Pumilio regulating the expression of *fz3* mRNA could lead to less effective Wnt signaling, and therefore less sensation of pain than without Pum.

Fluorescent tagging of para *mRNA transcript could show differences correlated to changes in Pumilio expression*

Although the creation of the fluorescently tagged *para* mRNA transcript has not been completed, there are a few expectations for how it will behave depending on how Pumilio and *para* interact and the success of the cloning. Once we can express the MS2 tagged transcript in the nociceptor neurons, it would be relatively easy to measure the amount of red fluorescence per neuron and correlate that to the amount of mRNA, as well as visualize the localization of the mRNA with live imaging, and compare these values within a wildtype, *pumilio* RNAi and overexpressed background. If there is no significant interaction between Pumilio and *para,* the level and location of fluorescence will not vary significantly between a

pumilio knockdown and *pumilio* overexpression Class IV neuron. If there is no localization of fluorescence to the axon, this could indicate multiple problems with the insertion of the stem loops. For one, the stem loops may not have successfully recombined into the *para* 3' UTR. Another outcome could be the addition of the stem loops leads to problems with transcribing or translating *para* mRNA, as the three-dimensional structure of the sequence could slow down the transcription and translation machinery significantly. However, previous studies using insertions of the MS2 stem loops have not noted this as a problem (Zhang et al., 2013). It could also indicate that *para* mRNA is translated within the cell body, and therefore is localized to the axon as the sodium channel.

If there is a significant relationship between Pumilio and *para* mRNA leading to some inhibition of the translation of this mRNA in normal function, there should be more fluorescence when *pumilio* is knocked down in the nociceptor neurons and less fluorescence when *pumilio* is overexpressed in the nociceptor neurons. If the binding of Pumilio and *para* mRNA has some effect on the localized translation of the mRNA, this would not be as simple to observe. If Pumilio binding leads to inhibition of translation until *para* mRNA has migrated to the axon of the neuron, under live imaging in *pumilio* knockdown larvae we should see more fluorescence disappearing before it reaches the axon, and in overexpression larvae we could see more fluorescence localizing to the axon and remaining fluorescent for longer. However, this is all hypothetical until we can image the insertion line.

Pumilio contains a large capacity for affecting neuronal sensitivity, since it has so many potential binding effectors and many different models for translational repression that could lead to different effects. Finding which mRNA transcripts Pumilio is binding to in order to

cause these effects is key, as they could potentially provide tragets for treatment for chronic pain, but also because they could indicate Pumilio plays an important role in the regulation of nociceptive sensation. Although my part of this project is done, there is still much work to be done, and those experiments will be able to verify the relationships Pumilio has with *para* and other proteins and elucidate how Pumilio acts in the pathway for the regulation of nociceptor sensitivity.

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Appendix A:

Good quality Drosophila genomic DNA extraction

Solutions:

Solution A: Tris HCl 0.1 M (pH 9.0)
EDTA 0.1 M
SDS 1%

Phenol-Cloroform: 1:1
shake
spin for 10 min at 4.000 rpm

KAc 8 M
Isopropanol
EtOH 70%
TE

Procedure:

1. 25 flies per tube □ keep on ice
2. add 250 µl of solution A
3. homogenize the flies □ put back on ice
4. incubate for 30 min at 70 °C
5. add 35µl of KAc □ shake (no vortexing)
6. incubate for 30 min on ice
7. spin for 15 min at 13.000 rpm
8. move supernatant to a new tube (leave back any precipitate or interphase)
9. add 1 vol of Phenol-Chloroform to (8.) (ca. 250µl) □ shake thoroughly (no vortexing)
10. spin for 5 min at 13.000 rpm
11. repeat steps 8 to 10
12. move supernatant to a new tube
13. add 150µl of Isopropanol □ shake
14. spin for 5 min at 10.000 rpm
15. suck off supernatant (don't lose pellet!)
16. wash the pellet with 1 ml 70 % EtOH
17. spin for 5 min at 13.000 rpm
18. dry the pellet 10 min under vacuum
19. resuspend the pellet in 100µl of TE

Appendix B:

Site Directed Mutagenesis Protocol

Modified form of:

Site-Directed Mutagenesis

(Adapted from a combination of Stratagene's QuikChange® Site-Directed Mutagenesis Kit, catalog # 200518, and Wang, W.;Malcolm, B.A. 1999. Biotechniques. 26:680-682.)

Step 1: Synthesize coding and non coding strands

Reaction I: 50 ng plasmid, 2.5 µL forward primer, 1 µL dNTPs, 10 µL Q5 reaction buffer, 0.5 µL Q5 polymerase, ddH_2O to 50 µL

Reaction II: same as above but 2.5 µL reverse primer instead of forward primer

- Thermocycler conditions same for both:

  Initial Denaturation: 98˚C for 30 s,

  30 cycles of: Denaturation at 98˚C for 10 s

  Annealing at 72˚C for 30 s

  Extension at 72˚C for 1 min per kb
- Stop reaction, mix 25 µL of each reaction in fresh PCR tubes and add extra 0.75 µL Q5 polymerase to both
- Thermocycle repeated

Step 2: DpnI Digest

Added 5 µL cutsmart buffer and 1 µL DpnI to each 50µL reaction

- Centrifuged briefly
- Incubated at 37˚C overnight

Step 3: Transformation

Transformed into chemically competent *E. coli*, plated on warmed ampicillin positive plates and incubated at 37˚C overnight

Started liquid cultures using ampicillin positive LB, incubated overnight shaking at 200 RPM at 37˚C

Miniprepped using Zyppy Miniprep kit

Step 4: Digest test with enzymes of choice

XhoI chosen because the site was only present in successfully mutated plasmids, HindIII used because it would allow identification of correct band