A Pumilio Domain that Forms Heritable Amyloid Aggregates in Yeast Can Regulate Pumillio-Mediated Translational Repression in *Drosophila*

Thesis by

Anna Maria Salazar

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

in Biology

California Institute of Technology

Pasadena, California

2008

(Defended September 24, 2007)

Acknowledgements

I would like to thank my thesis advisor, Dr. Kai Zinn, for a multitude of intellectual conversations, his willingness to allow me to pursue the difficult scientific questions that I found to be incredibly intriguing, and for being a generally kind individual and great lab mate; my thesis committee: Dr. Bruce Hay, Dr. Gilles Laurent, Dr. Angela Stathopolous, and Dr. Kai Zinn for their support and time; Dr. Kaushiki Menon for numerous scientific conversations and advice on numerous aspects of this project, in addition to her friendship; Viola Nesterova for all the help that she has given me through the years and for a very nice friendship; Elena Armand for everything that she did in lab to assist me and for her constant amiable nature; Dr. Nina Tang-Sherwood for her amazing friendship, her too-numerous-to-mention kind acts over the years and for being an all-around amazing person; Ashley Wright for her friendship and constant support and intellectual conversations; all the amazing friends that I have made on campus, especially Dan Gold, Ben Rubin Johannes Grauman, and Juanse Ramirez-Lugo; Lisa Nesterova for being a fabulous assistant throughout the years and a good friend; Dasha Goloub for all of her help in lab and her friendship; Nicki Fox for her advice, constant support, and friendship; Alice Schmid for interesting conversations and for her political fervor; all members of the Zinn lab past and present who have been fabulous friends and have made lab scientifically interesting and fun; all of the administrative and supporting staff

at Caltech, who truly make Caltech a great place to be: Liz Ayala, Gwen Murdock, Sam Westscott, Janie Malone, and Mary Alvarez; my friends on the gardening, custodial, and plumbing staff who work so hard yet are rarely recognized for their efforts, especially Walter Estrada, Patricia Estrada, and Erick Flores who took me into their home as a member of their family when I first came to Caltech as an undergraduate; Rosetta Pillow and Carmen for all of their hard work when we were in the basement of BBB and for their kindness, thereafter.

I would especially like to thank my family for all of their support, especially my mom, who has always given so much to me over the years and never expected anything in return. Finally, I would like to thank Sean Brennan for creating the music that helped me get through many long nights in lab, for being my best friend, and for constant support.

ABSTRACT

Numerous human diseases have been described in which defects in protein folding pathways play a role in the development of a disease state. A subset of these diseases result in a decrease in the level of the active, native conformation of the protein. This could arise from several mechanisms, including an increase in degradation caused by misfolding, alterations in trafficking of the misfolded protein, or aggregation of the protein with a consequent decrease in the soluble, active form of the protein. At least 40 human diseases that fit into this last category, and are associated with the formation of amyloid fibers, deposits, or inclusions, have been characterized. Domains rich in glutamine (Q) and asparagine (N) are one class of sequences that seem to possess an affinity to form amyloids under native conditions. These domains are present in many metazoan proteins, including 472 in *Drosophila* and 143 in *C. elegans*. Q/N domains are found in several yeast prions. It is hypothesized that these domains may have been positively selected during evolution, perhaps in order to allow reversible switching of the functional domain of the protein into an inactive aggregated state. We wondered if this type of selection might also maintain Q/N domains in metazoans. The *Drosophila melanogaster* and *Caenorhabditis elegans* proteomes were searched for predicted proteins that contain nucleic acid binding domains (for RNA, DNA or both), and Q/N-rich sequences, using a threshold of 30 Q/Ns in 80 residues. One of the two strong *Drosophila*

candidates is the translational repressor Pumilio (Pum). Earlier work by our group (Menon *et al.*, *Neuron* 44, 663-676 (2004)) had shown that Pum is localized to the postsynaptic side of the larval neuromuscular junction (NMJ), where it acts as a regulator of local mRNA translation. In *pum* mutants, synaptic morphology is altered and GluRIIa is dramatically upregulated. This study shows that a Q/N-rich domain (denoted NQ1) from Pum is able to form ordered aggregates in budding yeast and is able to recapitulate the activity of a yeast prion, New1p: including visible aggregates, heritable phenotypic switching, and reversibility of an induced yeast prion state, *[Psi+]*, by guanidine hydrochloride. To test whether NQ1 aggregate formation can perturb Pum's function in the nervous system, transgenic fly lines in which NQ1 expression is driven by GAL4 were created. Postsynaptic NQ1 expression generates alterations in the NMJ that phenocopy the *pum* loss-of-function phenotype and interact genetically with *pum* mutations. This is observed through an increase in type 1s and type 1b boutons and an increase in GluRIIa at the NMJ. Postsynaptic Pum overexpression in muscles is lethal, but co-overexpression of NQ1 partially rescues this lethality, resulting in Drosophila that survive until eclosion. Thus, in both the wild-type and overexpression contexts, a domain that forms heritable aggregates acts as a negative regulator of Pum activity.

TABLE OF CONTENTS

Acknowledgements……..iii

Abstract……v

Table of Contents……..vii

List of Figures……...viii

Introduction ……1

References……...14

Chapter 1

A Pumilio Domain Can Form Heritable Ordered Aggregates in Yeast..19

References……...56

Chapter 2

A Pumilio Domain Regulates Pumilio-Mediated Translational Repression In *Drosophila*……....62

References……..97

List of Figures

Chapter 1

Figure 1. (A) *Drosophila* Pumilio Domains

(B) Yeast Plasmid Constructs and Ability to Aggregate or Induce the *[Psi+]* State……51

Figure 2. Visualization of CFP Aggregates in *[Psi-][pin-]* Yeast……52

Figure 3. *Drosophila melanogaster* Pumilio Amino Acid Sequence With Q/Ns Highlighted……..53

Figure 4. Gamma Integration, the Method for Replacing Endogenous Sup35 with NQ1-EF in Yeast Genome……54

Figure 5. Percent of *[Psi+]* Induced Yeast in an NQ1-EF Chimera Strain...55

Chapter 2

Figure 1a. NMJ Boutons……...91

Figure 1b. NMJ Boutons……92

Figure 2a. Type 1s Bouton Counts……93

Figure 2b. Type 1b Bouton Counts……94

Figure 3. (A)Total number of Pupae after 24 hours in a vial

(B)Percent of Flies That Do Not Eclose……95

Figure 4. GluRIIa Expression is increased in a Manner Similar to et7/et9…96

Introduction

Numerous human diseases have been described in which defects in protein folding pathways play a role in the development of a disease state. A subset of these diseases result in a decrease in the level of the active, native conformation of the protein. This could arise from several mechanisms, including an increase in degradation caused by misfolding, alterations in trafficking of the misfolded protein, or aggregation of the protein with a consequent decrease in the soluble, active form of the protein. At least 40 human diseases that fit into this last category and are associated with the formation of amyloid fibers, deposits, or inclusions, have been characterized (Dobson, 2006). Huntington's Disease, Parkinson's Disease, Alzheimer's Disease, Spino-cerebrellar Ataxias, Type II Diabetes, Injection-localized Amyloidosis, Lysozyme Amyloidosis, Spinal and Bulbar Muscular Atrophy, and Spongiform Encephalopathies are some of these diseases, and are caused by the aggregation of these different respective proteins: Huntingtin with an expanded polyQ expansion, α-Synuclein, Amyloid-β, Ataxins with a PolyQ expansion, Amylin, Insulin, Lysozyme, Androgen Receptor with an expanded polyQ expansion, and Prion Protein (Dobson, 2006). It is evident from this subset of human diseases involving protein aggregates that the types of diseases and the proteins that cause the diseases are quite variable. In addition, it is also apparent that some of these diseases are quite common, like Alzheimer's Disease, which affects approximately 14.4 million people in the world (Ferri et al., 2005), or Type II Diabetes, with 150 million people affected

worldwide, including 20% of the population of the United States over the age of 65 (Zimmet et al., 2001). Understanding more about the causes of protein folding diseases, therefore, is imperative. In order to understand more about these diseases, however, it is necessary to understand more about protein folding and misfolding in general.

Recent studies have suggested that ordered aggregates composed of β-pleated sheets, such as amyloid fibers, can possibly form from many, and perhaps even all, polypeptide chains in vitro (reviewed by Dobson, 2006). Protein folding theories have proposed that it is the identity of the side chains of a polypeptide sequence that determine how a protein will fold into its native form. In keeping with this theory, several sequences have been identified that seem to promote aggregation, including glutamine (Q) and asparagine (N) rich domains, and specific short peptide repeats (Chiti et al., 1999; Lopez de la Paz et al., 2002).

It has also been observed, however, that numerous sequences that have not been known to form beta sheets or amyloid fibers in vivo are capable of doing so in vitro, under appropriate conditions. It has, therefore, been proposed that all polypeptides have the capability of forming amyloid fibers under certain conditions and that the formation of amyloid fibers is not dependent on sequence. For example, myoglobin, a protein whose native conformation is composed of mostly alpha helices, can form beta-pleated sheets, and even amyloid fibers in vitro (Fandrich et al., 2001). Myoglobin is a 153 amino acid

16.7kD protein. Its crystal structure, determined in 1958, was shown to be comprised of mostly alpha helices. Partially folded states that have been studied have also revealed alpha helices, although there is some evidence for other structures in the precipitated or aggregated form (Eliezer et al., 1998). There is no evidence that myoglobin is capable of folding into a beta-sheet structure in vivo, and myoglobin amyloid fibers have not been seen in any disease state. However, it has now been shown that myoglobin can form amyloid fibers in vitro, as assayed by electron microsopy (EM), thioflavin-T binding, and Congo red birefringence (Fandrich et al. 2001). These fibers also show a single circular dichroism minimum at 215 nm (diagnostic of beta structure), and an X-ray diffraction pattern that corresponds to beta structure. The amyloid fibers formed from apomyoglobin, (myoglobin lacking its haem group), were created under conditions that destabilized the alpha-helix native state, including high pH and 65°C (Fandrich et al. 2001).

Other examples of proteins that have not previously been shown to be associated with an amyloid disease, yet possess either a domain or an entire protein sequence that is capable of forming amyloid fibers, have also been published. The SH3 domain of the p85α subunit of phophatidylinositol 3-kinase is capable of forming amyloid fibers under acidic conditions in vitro (Guijarro et al., 1998). In addition, human muscle acylphosphatase, a small protein with both alpha and beta secondary structure and consisting of 98 amino acids, has been

shown to be capable of forming amyloid fibers under moderately denaturing conditions that still allow the formation of hydrogen bonds (Chiti et al, 1999).

Furthermore, polyamino acids, or peptides that are composed of multiple copies of one amino acid, are also capable of forming amyloid fibers under specific in vitro conditions. For example, poly-L-lysine, poly-L-glutamic acid, and poly-L-threonine form amyloid fibers that are visible under EM, bind Congo red, bind thioflavin-T, reveal a 215 nm minimum as determined by CD, show a cross-β structure when analyzed by X-ray diffraction, and display long-term amyloid fiber stability at room temperature (Fandrich and Dobson, 2002). Although the group who conducted these experiments attempted to form amyloid fibers from several other amino acids, the other poly amino acids precipitated too readily, thereby preventing the ability to determine whether other polyamino acids could form amyloid fibers as well (Fandrich and Dobson, 2002). Previous studies, however, have shown the ability of various other polyamino acids to form beta-sheet aggregates in vitro (reviewed by Dobson 2006). This data implies that the character of the amino acid side chain is not a determinant of the capability of a polypeptide to form an amyloid fiber in vitro. In fact, it is proposed that it is the polypeptide backbone itself that confers the ability to form crossed beta-sheets and amyloid fibers. The polypeptide backbones of these polyamino acids form hydrogen bonds as they fold back onto themselves. It is, therefore, theorized that the generic conformational state of any peptide is an amyloid formed from

cross beta sheets, and that the native, globular shape of the protein is conferred by the side chains.

Although poly-L-glutamine expansions were one of the first poly amino acid expansions shown to form amyloid fibers and to confer a disease state in vivo because of such expansions, other diseases corresponding to expansions in other amino acids have been discovered. Pathological fibers formed from poly-L-alanine, oculopharyngeal muscle dystrophy (Calado et al., 2000), or poly-L-leucine, Huntington disease-like 2 (Margolis et al., 2001), expansions have also been observed. These examples provide more evidence that extended expansions of amino acids other than glutamine may be capable of forming amyloid fibers in vivo and may be responsible for other pathological states.

Contrary to accepted perceptions, therefore, it seems quite plausible that any polypeptide can form amyloid fibers under the correct conditions. The amyloid and cross-beta-sheet formation, therefore, is a generic form of polypeptides, requiring only the peptide backbone for hydrogen bonds and stabilization. The native, globular forms of proteins, therefore, are acquired through the ability to form alpha helices and other structures that may be more rapidly formed under physiological conditions, thereby disrupting the access of the polypeptide backbone to hydrogen bond with itself and form cross-beta sheets and amyloid fibers. In addition, it is proposed that specific chaperones, including heat shock receptors, are essential for preventing amyloid fiber formation and promoting native globular protein folding. Disulfide bond formation

and specific residues, such as prolines, that disrupt the structure of the peptide background, also contribute to the formation of the globular protein (Chiti and Dobson, 2006).

There have also been recent advances in the elucidation of the steps of protein folding, an understanding of which is necessary for studying diseases caused by protein aggregation. There are numerous proteins in living organisms. Humans are thought to possess over 100,000 different proteins. However, this number still represents an infinitesimally small subset of the total number of sequences that could exist, given the average number of amino acids per protein and the fact that 20 different amino acids make up these proteins. Thus, out of a vast number of choices, a very small specific set of proteins have evolved and been maintained in order to carry out specific tasks. It is the specific sequence of these proteins that encode the tertiary and quaternary structure of proteins that allow them to carry out their required cellular function. It is, therefore, fairly clear that precise protein sequence and folding are important for the daily activity of all organisms, and that these protein sequences have been targets of immense evolutionary pressure in order to successfully maintain many of the numerous complex functions of a cell.

In order for a cell to accomplish such a complex task as specifying precise protein structure and folding, the sequence of a protein is essential. Cells, however, also require chaperone proteins to assist in the final attainment of appropriate secondary and tertiary structure and to prevent incorrect states from

being formed. This is especially essential given the fact that most cells contain a very high concentration of proteins and other molecules, estimated to be between 300-400 mg/ml (Ellis RJ, et al, 2003). In fact, it seems as though the concentration of a particular protein in a cell corresponds well to being just below the concentration under which the protein spontaneously starts to aggregate. An analysis was conducted on known in vitro aggregation rates for a group of 12 proteins that compared in vitro aggregation rate data with the expression-level data obtained from microarray analysis. A clear anti-correlation was observed, with the proteins containing higher aggregation rates having lower expression levels. In fact the rates of the 12 proteins analyzed resulted in a correlation coefficient of .97, quite remarkable (Tartaglia, et al, 2007). This means that evolution has not only created proteins with suitable amino acids sequences to allow appropriate folding, but also the expression levels of each of these proteins may have evolved to be just under the concentration at which that specific protein will start to self-aggregate, given the rate of aggregation. One item to note is that when the protein Pmel17, a human protein involved in premelanosome morphogenesis which is thought to be functional in the amyloid state (Berson et al. 2001), is analyzed, it possesses a much higher aggregation rate than would be expected from where it would fall on the graph, given its expression level (Tartaglia, et al, 2007). This means that it is expressed at a concentration that is conducive to its aggregation, which makes sense because the amyloid form may be required for its normal activity.

Another study has provided more evidence for an evolutionary adaptation against self-aggregation. Given the observation that increases in the local concentration of a protein can result in aggregation, experiments were conducted on proteins that contained repeating domains to analyze whether these proteins might promote aggregation between such domains. At least two amyloid diseases are caused by aggregates involving immunoglobulin domains (Selkoe, 2003). Given the fact that numerous proteins contain tandem repeat domains, and the fact that the immunoglobulin and fibronectin domains are two of the most common tandem repeats, both were analyzed. The 27th immunoglobulin domain of human cardiac titin, which has not been implicated in an amyloid diseased state, was first shown to be capable of forming amyloid fibers in vitro under moderate denaturing conditions. When peptides were created that contained two, three, or eight repeats of this domain, an increase in the rate of fiber formation was observed. In fact, each of these peptides showed an approximate 10-fold increase in aggregation rate in comparison to the monomer itself. Given the lack of differences in the rates as long as the construct contained at least two tandem repeats of the immunoglobulin domain, this result gives credence to the concept that two interacting domains may be a key step in the formation of amyloids, perhaps in the same manner that seeding increases the rate of amyloid formation. Furthermore, when constructs containing two immunoglobulin domains that vary by more than 60-70% are created, no detectable increase in aggregation rate is observed. For constructs containing tandem domains with

more than 70% similarity, however, rates of aggregation seem comparable to using two tandem repeats with exact sequence homology. When the fibronectin and immunoglobulin superfamilies in the human genome are compared, only 10% of the proteins contain adjacent domains with greater than 40% sequence identity. This data may imply that sequence diversity between tandem domain repeats in a protein have evolved to decrease the capacity to form intermolecular aggregates that might lead to amyloid fiber formation. This is also an example in which sequence similarity seems to increase the ability to form amyloid fibers, perhaps because the specific manner in which the side-chains are arrayed do play an important role in the stability of an aggregate, in addition to the main chain. Although it may be the case that any protein can form amyloid fibers, these observations may be one of the reasons why oligomers often seem to seed the formation of amyloid aggregates that consist of the same protein as the seed and the fact that amyloid aggregates most often consist of one dominant protein. An amyloid fiber, therefore, may be stabilized by repeating residues, in a manner similar to crystal structures (Wright, 2005).

Another interesting piece of data involving the study of amyloid formation involves the Congo red binding assay. It has recently been shown that Congo red is not as specific for amyloid species as previously believed and as obvious from the fact that most papers that reference a protein's ability to form amyloid fibers include data about Congo red binding and birefringence. It has long been known that Congo red can bind to collagen fibers and cytoskeleton filaments and

still exhibit yellow-green birefringence (Linder et al., 1979). Congo red has been shown to bind to proteins in their native conformation, regardless of the nature of their secondary structure. For example Congo red is capable of binding to the native conformation of proteins as diverse as insulin (composed mostly of α helices), lysozyme (α and β), concavalin A (β), citrate synthase (α), interleukin-2 (α), malate dehydrogenase (α and β), beta-lactoglobulin, and apomyoglobin (α) and exhibiting yellow-green birefringence (Khurana et al., 2001). Because of this lack of specificity, Congo red does not seem like the ideal diagnostic of amyloid formation. This is important because many papers claim Congo red binding and birefringence as evidence for the amyloid structure, when this is not necessarily the case. Furthermore, thioflavin T is recommended as a better diagnostic tool, as it seems more specific to amyloid fibers than Congo red (Khurana et al., 2001).

Some sequences have a high propensity to form specific secondary structures, such as alpha helices. Other sequences are more prone to form β-sheets. Sequence information, therefore, is important for determining what type of structure a protein may be more prone to form. Even though all proteins may be capable of forming amyloid structures in vitro, some sequences seem to be more highly represented in proteins known to fold into amyloids in vivo, like Q/N rich regions, but these regions are also common protein-protein interaction domains. It has been posited that these Q/N regions interact fairly tightly with other Q/N rich regions, perhaps forming a type of preliminary interaction to the

“zipper” structure deduced from NMR (Chiti and Dobson, 2006). Furthermore, the sequence of a protein determines its native globular state, so sequence is extremely important. Numerous Q/N rich regions have been known to be important in protein-protein interactions. For example, the promoter specific factor 1 (SP1), requires a Q rich region alternating with hydrophobic residues in order to bind to dTAF110, a protein that is part of the TFIID complex that is required for the recruitment of RNA polymerase and transcription (Gill et al., 1994). These Q/N rich regions may be important for protein-protein interaction, which is supported by the presence of numerous proteins that contain these regions at the synapse. In fact, several proteins involved in translation contain Q/N rich domains.

Stress granules have recently been colocalized with protein aggregates in post-ischemic hippocampal CA1 neurons (DeGracia et al., 2007), and may provide a possible expanation for the retention of Q/N rich domains in the genome. TIA-1 and TIAR are two related proteins that are involved in the formation of stress granules. Both of these proteins contain three N-terminus RNA-binding domains and a C-terminus glutamine-rich motif termed the PRD (prion-related domain) because of shared structural similarity to prion proteins. In environmentally stressed cells, TIA-1 and TIAR are involved with removing stalled initiation complexes to cytoplasmically localized foci termed stress granules. These sites are shown to contain mRNA, ribosome subunits, and RNA binding proteins (RBP), and are hypothesized to be involved in sequestration of

mRNA and their ribonucleoprotein partners in times of stress prior to determining whether an mRNA will be translated or degraded. TIA-1 and TIAR have also been proposed to play both a general role and to be directly involved in translational repression (Mamczarz et al., 2006). Under times of stress, TIA-1 and TIAR are hypothesized to interact with eukaryotic translation initiation factors 1 (eIF1), eIF3, and the 40S ribosomal subunit, instead of the eIF1, eIF2, eIF3, eIF5, and 40S ribosomal subunit complex that normally associates with the 5' end of capped mRNAs, thereby inhibiting protein translation (reviewed by Hocik and Sonenberg, 2005). Furthermore, TIAR and TIA-1 mediate the translational repression of specific AU-rich element (ARE) mRNAs, including tumor necrosis factor alpha 1 (TNFα), where cells that either lacked or contained TIA-1 contained the same level of mRNA, but differing levels of translated protein. The ARE seems to confer a rapid degradation signal to mRNAs, which might be related to translational repression (Piecyk et al., 2000).

Overexpression of full-length TIA-1 causes stress granule formation and represses reporter gene expression. In experiments where the PRD of TIA-1 was overexpressed, the PRD was shown to be capable of forming visible cytoplasmic aggregates and sequestering endogenous TIA-1 and TIAR, thereby reducing their ability to repress a reporter gene's expression. Removing the PRD domain of TIA-1 perturbs stress granule formation and promotes mRNA translation (Mamczarz, et al., 2006). Furthermore, replacing the PRD with the aggregate-forming prion domain of SUP35, the NM domain, results in normal

stress granule formation and repression of mRNAs (Gilks et al., 2004). Furthermore, in neurons, fragile X mental retardation protein, FMR1, plays a comparable role in the production of granules that seem to act in a similar manner and possesses a similar structure. FMR1 has also been co-localized with Pum in stress granules (Vessey et al., 2006). Furthermore, Q/N rich proteins such as Pumilio 2, cytoplasmic polyadenylation element binding protein (CPEB), and Poly A Binding Protein (PABP) have also been localized to stress granules (reviewed by Kiebler and Bassel, 2006). This is a very interesting observation because (PABP) was also found in the database search for putatitive prions that we conducted (see Chapter 1), the Aplysia version of the (CPEB) has been implicated in possessing a prion-like activity (Si et al., 2003), and Pumilio is discussed in this thesis.

The Q/N region of Pum may be important for various different functions, such as interacting with protein partners in translational repression or binding to proteins involved in stress granule formation and function. This study, however, only investigates Pum's possible propensity for aggregation, epigenetic interaction with known prions, and epigenetic interaction with endogenous Pumilio.

.

Introduction References

Calado, A., Tome, F. M., Brais, B., Rouleau, G. A., Kuhn, U., Wahle, E. and Carmo-Fonseca, M., 2000. Nuclear inclusions in oculopharyngeal muscular dystrophy consist of poly(A) binding protein 2 aggregates which sequester poly(A) RNA. Hum Mol Genet. 9, 2321-2328.

Chiti, F. and Dobson, C. M., 2006. Protein misfolding, functional amyloid, and human disease. Annu Rev Biochem. 75, 333-366.

Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G. and Dobson, C. M., 1999. Designing conditions for in vitro formation of amyloid protofilaments and fibrils. Proc Natl Acad Sci U S A. 96, 3590-3594.

DeGracia, D. J., Rudolph, J., Roberts, G. G., Rafols, J. A. and Wang, J., 2007. Convergence of stress granules and protein aggregates in hippocampal cornu ammonis 1 at later reperfusion following global brain ischemia. Neuroscience. 146, 562-572.

Eliezer, D., Yao, J., Dyson, H. J. and Wright, P. E., 1998. Structural and dynamic characterization of partially folded states of apomyoglobin and implications for protein folding. Nat Struct Biol. 5, 148-155.

Ellis, R. J. and Minton, A. P., 2003. Cell biology: join the crowd. Nature. 425, 27-28.

Fandrich, M. and Dobson, C. M., 2002. The behaviour of polyamino acids reveals

an inverse side chain effect in amyloid structure formation. EMBO J. 21, 5682-5690.

Fandrich, M., Fletcher, M. A. and Dobson, C. M., 2001. Amyloid fibrils from muscle myoglobin. Nature. 410, 165-166.

Ferri, C. P., Prince, M., Brayne, C., Brodaty, H., Fratiglioni, L., Ganguli, M., Hall, K., Hasegawa, K., Hendrie, H., Huang, Y., Jorm, A., Mathers, C., Menezes, P. R., Rimmer, E. and Scazufca, M., 2005. Global prevalence of dementia: a Delphi consensus study. Lancet. 366, 2112-2117.

Gill, G., Pascal, E., Tseng, Z. H. and Tjian, R., 1994. A Glutamine-Rich Hydrophobic Patch in Transcription Factor Sp1 Contacts the dTAF11110 Component of the Drosophila TFIID Complex and Mediates Transcriptional Activation. Proceedings of the National Academy of Sciences %R 101073/pnas911192. 91, 192-196.

Guijarro, J. I., Sunde, M., Jones, J. A., Campbell, I. D. and Dobson, C. M., 1998. Amyloid fibril formation by an SH3 domain. Proc Natl Acad Sci U S A. 95, 4224-4228.

Harris, D. A. and True, H. L., 2006. New insights into prion structure and toxicity. Neuron. 50, 353-357.

Holcik, M. and Sonenberg, N., 2005. Translational control in stress and apoptosis. Nat Rev Mol Cell Biol. 6, 318-327.

Khurana, R., Uversky, V. N., Nielsen, L. and Fink, A. L., 2001. Is Congo red an amyloid-specific dye? J Biol Chem. 276, 22715-22721.

Kiebler, M. A. and Bassell, G. J., 2006. Neuronal RNA granules: movers and makers. Neuron. 51, 685-690.

Lopez de la Paz, M., Goldie, K., Zurdo, J., Lacroix, E., Dobson, C. M., Hoenger, A. and Serrano, L., 2002. De novo designed peptide-based amyloid fibrils. Proceedings of the National Academy of Sciences %R 101073/pnas252340199. 99, 16052-16057.

Margolis, R. L., O'Hearn, E., Rosenblatt, A., Willour, V., Holmes, S. E., Franz, M. L., Callahan, C., Hwang, H. S., Troncoso, J. C. and Ross, C. A., 2001. A disorder similar to Huntington's disease is associated with a novel CAG repeat expansion. Ann Neurol. 50, 373-380.

Mazan-Mamczarz, K., Lal, A., Martindale, J. L., Kawai, T. and Gorospe, M., 2006. Translational repression by RNA-binding protein TIAR. Mol Cell Biol. 26, 2716-2727.

Petkova, A. T., Leapman, R. D., Guo, Z., Yau, W. M., Mattson, M. P. and Tycko, R., 2005. Self-propagating, molecular-level polymorphism in Alzheimer's beta-amyloid fibrils. Science. 307, 262-265.

Piecyk, M., Wax, S., Beck, A. R., Kedersha, N., Gupta, M., Maritim, B., Chen, S., Gueydan, C., Kruys, V., Streuli, M. and Anderson, P., 2000. TIA-1 is a

translational silencer that selectively regulates the expression of TNF-alpha. Embo J. 19, 4154-4163.

Prusiner, S. B., 1998a. The prion diseases. Brain Pathol. 8, 499-513.

Prusiner, S. B., 1998b. Prions. Proc Natl Acad Sci U S A. 95, 13363-13383.

Selkoe, D. J., 2003a. Aging, amyloid, and Alzheimer's disease: a perspective in honor of Carl Cotman. Neurochem Res. 28, 1705-1713.

Selkoe, D. J., 2003b. Folding proteins in fatal ways. Nature. 426, 900-904.

Stefani, G., Fraser, C. E., Darnell, J. C. and Darnell, R. B., 2004. Fragile X mental retardation protein is associated with translating polyribosomes in neuronal cells. J Neurosci. 24, 7272-7276.

Tartaglia, G. G., Pechmann, S., Dobson, C. M. and Vendruscolo, M., 2007. Life on the edge: a link between gene expression levels and aggregation rates of human proteins. Trends Biochem Sci. 32, 204-206.

Vessey, J. P., Vaccani, A., Xie, Y., Dahm, R., Karra, D., Kiebler, M. A. and Macchi, P., 2006. Dendritic Localization of the Translational Repressor Pumilio 2 and Its Contribution to Dendritic Stress Granules. J Neurosci %R 101523/JNEUROSCI0649-062006. 26, 6496-6508.

Walsh, D. M. and Selkoe, D. J., 2007. A beta oligomers - a decade of discovery. J Neurochem. 101, 1172-1184.

Wickner, R. B., Edskes, H. K., Ross, E. D., Pierce, M. M., Baxa, U., Brachmann, A. and Shewmaker, F., 2004. Prion genetics: new rules for a new kind of gene. Annu Rev Genet. 38, 681-707.

Wright, C. F., Teichmann, S. A., Clarke, J. and Dobson, C. M., 2005. The importance of sequence diversity in the aggregation and evolution of proteins. Nature. 438, 878-881.

Zimmet, P., Alberti, K. G. and Shaw, J., 2001. Global and societal implications of the diabetes epidemic. Nature. 414, 782-787.

Chapter 1

A Pumilio Domain Can Form Heritable Ordered Aggregates in Yeast

Chapter 1 Introduction

The Prion Hypothesis was first proposed by Stanley Prusiner to describe a protein-only method of infectivity that was responsible for spongiform encephalopathies. Spongiform encephalopathies are diseases characterized by a spongiform appearance in the brain. Some of these diseases were genetic, like Creutzfeld-Jacob Disease (CJD), or fatal familial insomnia (FFI), while others seem to have been spontaneous. Bovine Spongiform Encephalopathy (BSE), or mad cow disease and other spontaneous forms of the disease derive from consuming an infected organism, like the ritual cannibalism that led to Kuru, another prion disase. The prion protein, PrP, was eventually isolated and shown to be a protein that was capable of epigenetic infectivity and the cause of the diseased state (Prusiner, 1998). Furthermore, endogenous PrP was shown to be necessary for the ability of an organism to contract the disease. This was shown through studying mouse PrP knockouts, which were incapable of developing the disease (Sailer, 1994). This data implied that the disease state is caused by something occurring with the endogenous protein, and not just some infective or toxic agent. A prion, therefore, would have to possess the ability to infect and the ability to make use of a protein-only method of function, with a protein acting as the genetic agent.

Prions are capable of catalyzing their own propagation in a process whereby the protein serves as a seed or template in which the WT protein becomes folded into the abnormal structure and aggregates. This process has

been observed in various organisms, including yeast, in which a model system has been developed to increase the understanding of prion-like behavior (reviewed by Wickner et al., 2004). Analyses comparing the amino acid sequences of PrP, the protein responsible for the infectivity in transmissible spongiform encephalophathies, and the recently identified yeast prions, reveal motifs that may be involved in the aggregation and infectivity of these proteins. PrP contains a short repeating motif, while the yeast prions seem to share an enrichiment in glutamine/asparagine (Q/N) rich domains. A subset of these putative prions possessed oligopeptide domains that have recently been shown to be necessary and sufficient for infection and maintenance of a prion-like state in yeast (Osherovich, 2004).

Other Eukaryotic proteomes contain many Q/N rich blocks as well. For example, Weissman defined Q/N rich domains as those containing 30 Q/N residues in 80 consecutive amino acids. In *S. cerevisiae*, 107 polypeptides with Q/N rich regions were identified, 143 were found in *Caenorhabditis elegans*, and 472 were found in *Drosophila melanogaster* (Michelitsch and Weissman, 2000). Q/N rich domains are important in normal protein-protein interactions. In fact, the proteins that may be capable of epigenetic switches when overexpressed may still require the Q/N rich domains for other normal functions. Even so, retaining such a high number of such sequences, given the putative beneficial effects of prions in yeast, leads to immediate questions in higher organisms. Do prion-like activities of a subset of these proteins provide a positively functional, and hence

evolutionarily conserved, activity? In other words, might there exist some function that has acted as a positive selection mechanism, thereby retaining seemingly deleterious sequences within the genome?

The concept that all amyloid-forming proteins and, therefore, perhaps all proteins, have the capacity to alter conformation upon seeding is relatively new. Previously, it was believed that this was one of the fundamental characteristics of prion proteins. In fact, many of the original attributes associated with prions may now be a general description of all proteins that are capable of forming amyloid fibers. Prions were believed to be unique amongst proteins capable of aggregating in that they are capable of not only seeding aggregates, but also are able to replicate their form, are interconvertible with the WT form, and act in an infectious manner (Prusiner, 1998). It now seems as though any protein can seed and replicate the amyloid form in vitro. Some proteins may even be considered interconvertible under specific conditions. Other proposed prion traits that no longer seem specific to prions are the observed prion strain differences in various mammalian and yeast prions.

These strain differences were defined as different prion isolates that result in different rates of infectivity and pathology when placed into a host (Prusiner, 1998). When a host organism is inoculated with prions from a different species, a species barrier that either precluded or decreased the propagation of the infective protein in the host was sometimes observed. The species difference was also observed in yeast, where differences in the aggregation state, or normal

activity of the protein were observed (reviewed by Harris and True, 2006). These species barriers could sometimes be caused by a protein derived from the same amino acid sequence, but differ in the manner in which the prion was formed. For example, when the amyloid-forming domains of a yeast prion, Sup35, were purified and allowed to form aggregates in vitro at different temperatures, two different *[Psi+]* phenotypes, the prion state derived from aggregated Sup35, were observed when these amyloid fibers were introduced into yeast. When the two strains of Sup35 were analyzed, they were observed to possess differences in the manner in which the subunits interacted with one another and in the length of their amyloid core. (Tanaka et al., 2005). Similar differences in structures obtained from Amyloid β have also been observed, implying that other proteins may be capable of different amyloid structures as well (Petkova et al., 2005). This data makes sense given the possibility that amyloid formation is a generic capability of all proteins involving the peptide backbone. If this is the case, there may be numerous manners in which an amyloid fiber can form. Amyloid fibers are stabilized by repeating domains, and once a fiber is seeded the rest of the domains, comprising the fiber, may necessarily adopt the same structure as the seed.

One of the most fundamental characteristic of a prion is its ability to infect. This however, may also not be a quality that is unique to proteins previously considered prions. The prion hypothesis describes the protein-only manner of infection by an altered conformation of PrP, PrP scrapie, which was capable of

changing the endogenous PrP, a cell-surface glycoprotein, into the scrapie version (Prusiner, 1998). This was an infection without any nucleic acids and is the fundamental quality of a prion. In yeast, cells have been shown to replicate and pass on the phenotype accompanying the aggregated form of yeast prions, thereby "infecting" the budding yeast. In addition, yeast can be inoculated and transformed with amyloid fibers grown in vitro, which can elicit a prion state. Although this shows that the amyloid fibers are capable of causing a prion state, it does not show that this is actually an infection or that other amyloid fibers cannot do the same thing. It would be especially interesting to examine if amyloid fibers from a protein like myoglobin could elicit a prion state when transformed into yeast.

There is also evidence that shows that amyloid Aβ42 oligomers are capable of "infecting" neurons in culture. Aβ42 is the 42 amino acid peptide that is formed from the proteolysis of APP. It is known to be a major component of amyloid plaques. Low molecular weight oligomers, perhaps containing up to 12 copies of the Aβ42 protein, have been shown to be toxic. Low molecular weight oligomers of Aβ42 have been shown to be sufficient to decrease the number of spines in hippocampal slices grown in the presence of the oligomer, block LTP, and impair short term memory in rats injected with Aβ42 oligomers. Furthermore, larger fibrillar aggregates of Aβ42 do not show similar changes and some of these phenotypes can be specifically blocked by anti-oligomer antibodies, which prevent the production of low molecular weight oligomers but do not bind to or

break up larger Aβ42 fibers, implying that these effects are specific to the low molecular weight oligomers (reviewed by Walsh and Selkoe, 2007). This data may imply that many of the disease-related states associated with Alzheimer's disease, especially loss of memory and morphological changes in the brain, are actually caused by small oligomers of Aβ42 and not the large fibrillar aggregates or amyloid plaques observed in autopsies. It has been observed that small oligomers of Aβ42 are capable of entering into neurons and causing the toxic effects from within the neurons. This also provides a gross example of protein infectivity, in that a protein is able to cause a disease state within the cells that the oligomers were not created within. All of this could mean that Aβ42 is more prion-like than previously believed, or it could mean that definitions of prions need to be re-established.

A major question arises, however, as to what really should be considered a prion and whether the yeast assays used really distinguish between prion-like activity, or mere aggregation. Recent evidence about the folding and misfolding of proteins (reviewed by Dobson 2004; reviewed by Chiti and Dobson 2006), the detailed mechanism about the action and activity of yeast prions (reviewed by Wickner et al., 2004; Wickner et al., 2006), and the ability of other aggregation prone proteins to act in a similar manner to putative yeast prions, might either challenge the prion hypothesis regarding yeast prions, or at least make a prion state a much more general protein ability than previously believed.

There is now data that implies that some mammalian proteins as different as insulin, poly Q proteins, and amyloid beta behave in a manner similar to well-established yeast prions in assays that are similar to the ones that we have conducted in yeast. For example, Aβ42 can replace the N-terminus of Sup35 when an Aβ-Sup35EF chimera is used to replace the endogenous Sup35 in the yeast genome (Bagriantsev and Liebman, 2006). Low molecular weight amyloid fibers derived from this chimera have been shown to be capable of inducing this novel yeast strain into a "prion" state, in a manner comparable to the endogenous function of the prion state of Sup35, *[Psi+]*, which can be observed by loss of nonsense repression of ADE1, resulting in white yeast colonies and growth on –ADE plates. This data, however, shows that this chimera possesses a higher propensity to form aggregates, given the observation that no other prion, such as a *[pin+]*, is required to induce the formation of the Aβ-Sup35EF prion state, *[Aβ+]*. In addition, Western blot analysis shows that mostly small oligomers derive from this chimera, containing mostly dimers, trimers, and tetramers. This is interesting, in that although the prion state seems to be induced readily, larger fibers are not created in vivo. Furthermore, mutations in Aβ42, which block aggregate formation in vitro, decrease the ability of the chimera to form aggregates and prevent the prion state from forming. Interestingly, deletion of Hsp 104 seemed to inhibit the formation of the oligomerization of Aβ-Sup35EF (Bagriantsev and Liebman, 2006). This seems unusual in that HSP 104 is known to be required for maintenance of the prion state, with HSP 104 deletions

normally resulting in a loss of the yeast prion state only because of a decrease of an ability to break down larger aggregates into smaller, more soluble seeds that could be passed on to daughter cells, not necessarily direct chaperone assistance in the actual de novo formation of small oligomers from soluble protein.

The importance of Q/N rich sequences on the ability of amyloids to initiate a *[Psi+]* state in yeast has also been studied. It has been observed that many of the proteins studied thus far that are capable of expressing a prion-like behavior in yeast seem to possess Q/N rich repeats. This is the case for Sup35, New1, Rnq1, Ure2, and several other proteins that are putative yeast prions. In addition, proteins that are not endogenous yeast prions are capable of acting in a manner similar to yeast proteins if they contain polyQ or Q/N rich domains, such as Huntingtin, or Machado Joseph's disease (MJD) proteins. These observations have lead to the hypothesis that these Q/N regions are important for the seeding of the *[Psi+]* state, and that the seeding occurs through an interaction between these Q/N rich regions, which have already been shown to be important protein interaction domains (Osherovich and Weissman, 2001; Derkatch et al., 2004). An alternative hypothesis posits that any aggregated protein may titrate out essential chaperones that normally prevent misfolding, such as Hsp104, and that no seeding takes place.

Experiments to address this question involved overexpressing aggregate prone proteins that contained either poly Q/N regions or proteins, such as α-

synuclein or synfilin-1, that aggregate but do not contain these regions. It has previously been shown that the in vitro production of amyloid fibers can induce the in vitro switch from a prion- state into a prion+ state in yeast (Brachmann et al., 2005). Proteins that contained either PolyQ or Q/N rich regions were much more capable of both initiating a *[Psi+]* state in vivo and seeding the formation of Sup35 fibers in vitro. In vitro, however, Sup35 seeds were still much better at seeding their own conversion into the aggregated state than any of the other fibers tested. In addition, both insulin and human Ig light chain were able to significantly seed Sup35 aggregation in vitro (Derkatch et al., 2004). This data actually provides contrary evidence. It shows that in vivo, sequences that contain Q/N rich domains seem to be more capable of seeding Sup35 aggregates, but that various forms of amyloid fibers are capable of seeding Sup35 amyloid formation in vitro, regardless of Q/N levels. This data might imply that although similar sequences might play a role in seeding, the general amyloid form, regardless of sequence specificity, can seed the formation of other amyloids, especially under in vitro conditions.

Yeast studies have also shown that these prions are quite widespread in the genome, fairly conserved, and possibly functional. In fact, some of these yeast prions may be beneficial, which may explain the possible evolutionary conservation of such sequences. There is some controversy, however, about the true beneficial effects of Sup35 or Ure2p. A study that analyzed the native state of numerous strain of yeast throughout the world did not find any in the *[Psi+]*

state, even if the yeast resided in harsh yeast environments like high temperature or high ethanol content. Because the observed rate of yeast in the prion state was significantly different from what might be expected, given spontaneous *[Psi+]* rates, this implied that the *[Psi+]* prion state might actually be a disease, in other words, somewhat toxic to yeast. The same was observed with *[Ure3]* (Nakayashiki et al. 2005). This may contradict the theory that the *[Psi+]* state is beneficial because it confers the ability to read through nonsense codons, thereby allowing for an increase in genetic diversity under times of stress (Serio and Lindquist 2001).

Specific examples, however, support the theory that prions can be benefical. For example, one study showed that the *[Psi+]* state confers an increase in thermotolerance and chemical tolerance in some yeast strains (Eaglestone et al., 1999). In addition, the *[Psi+]* state was found to impart an increase in genetic variation and phenotypic diversity (True and Lindquist, 2000; True and Lindquist, 2004). Furthermore, it is proposed that the lack of presence of *[Psi+]* in the natural environment may be because the prion state need only exist transiently to unlock genetic diversity conferred by the read-through of various nonsense mutations and that the truly important traits will eventually work their way into the genome, negating the need for the *[Psi+]* state (Shorter and Lindquist, 2005; reviewed by von der Haar and Tuite, 2007). Another explanation that has yet to be put forth in the publications on this issue revolves around known beneficial effects of some known diseases. Just because a state is a

disease state, in other words, somewhat toxic, does not mean that there is not an accompanying beneficial impact found in that disease state. The common example of this is sickle cell disease conferring an increase in resistance to malaria.

Evidence for a less controversial conferment of beneficial traits has been found in *Podospora* with the *[Het-S]* prion state. The *[Het-S]* state provides *Podospora* fungi with the ability to fuse with normally incompatible fungi strains (Coutou et al. 1997). In addition, a group proposes that the behavior of the B protease protein from *S. cerevisiae* should be considered to be a prion because of the ability to convert itself into the active state when overexpressed, termed the *[β]* state. The state is self-propagating and involves its ability to act like a protease on itself, removing a C-terminal expansion that inhibits activity, resulting in the activation of the protein. This state is stably inherited. (Roberts and Wickner, 2003; Roberts and Wickner, 2004). In this example there is no aggregation, so this is a large step away from more common prion concepts. The authors, however state that the prion theory of PrP was held before it was found to be an aggregate, therefore the definition of a prion as a protein-only epigenetic switch is in accordance with the observed data in the *[β]* state. It is proposed that the ability of protease B to self-activate in trans may be a much more common type of prion, defined as mere epigenetic inheritance of a trait. To this end, it is proposed that other proteases, and possibly other protein-modifying enzymes such as kinases, methylases, acetylases, or ubiquitin ligases may also

be capable of being called prions if they modify themselves, resulting in a self-propagating state (Wickner et al., 2004).

Going by this definition, it does seem as though many proteins can be termed prions. Also, what is considered maintenance of the prion state? In Podospora and yeast, replication is fast, so the propagation of an epigenetic "prion" state is easily observed. The question arises, however, as to how to define a prion state in post-mitotic cells, like neurons. In other words, how is propagation classified when there are no cellular progeny. This is actually important because in the case of Sup35, the cells are considered "cured" even though the parent yeast cell still retains aggregates. It is only the breakdown of the aggregates into smaller oligomers that can be passed to daughter cells that is impaired (see more details later in this chapter). In addition, it has been proposed that a neuronal Aplysia protein, CPEB, can act like a prion and that the putative prion-like state of this protein could be induced by synaptic activity. The conversion to the prion-like state is proposed to be a mechanism for creating long-term synaptic changes that may be involved in the formation of memory. In this case, the prion state is not propagated to daughter cells. Aggregated proteins are maintained as aggregates. In these types of cases, one could define a prion state as a local change that is locally propagated within a cell type and maintained for a long period of time until a new signal reverses the prion state by some mechanism. In order for this to occur within one cell for a lengthy period of time, however, there must be a tight regulation of the numerous

aggregation-prone proteins in the cell. Whether the evolutionary advantage, in such cases, that is conferred by the prion state is sufficient for the amount of energy necessary to prevent numerous toxic protein aggregates from forming in these cells is yet to be determined. Ultimately, the fact that many proteins, and perhaps even all proteins, are capable of forming amyloid fibers under specific conditions because of a general capability of all peptide backbones to form hydrogen bonds with adjacent peptide backbones, and the propensity of certain proteins to form aggregates, implies that the conservation of these amyloid-prone sequences may have been for an important functional reason.

The background presented in this introduction provides numerous examples where the specific terminology used when discussing aggregating proteins and putative prions must be better universalized in order to provide specific requirements for defining an actual prion. As one learns more about the universality of protein aggregation, attempting to define a subset of this group becomes more difficult. Should the term prion be reserved for only a few very specific proteins, or might a prion state be more common than previously held? Different groups offer different requirements for prion determination. It is proposed by some groups that any transfer of information without the use of nucleic acids may be considered evidence of prions. A further elucidation of this concept is the possibility that any chromosomally derived protein, which is capable of altering its own conformation and is capable of carrying out the

conformation change of its own endogenous, normal form is a prion (Wickner et al., 2004).

RESULTS

Pumilio Is a Candidate Protein Identified in a Database Search to Find Possible Functional Prions in *Drosophila melanogaster* and *Caenorhabditis elegans*

To search for proteins that might act as prion switches, we reasoned two major things: 1) that many switch proteins, including transcriptional and translational regulators, would contain an annotated nucleic acid binding domain; 2) if such a protein contained a glutamine/asparagine (Q/N)-rich sequence, this would be unlikely to be present in its ortholog in a distantly related species, since these Q/N sequences evolve very rapidly. If the Q/N sequence were maintained in the ortholog, it would imply that it had been selected. The argument is based on the observation that Sup35p proteins in distantly related yeast species all contain highly conserved EF functional domains attached to Q/N sequences. The Q/N regions are not conserved at the primary sequence level, but amino acid composition is maintained. These data imply that the presence of the Q/N region confers a selective advantage. This was hypothesized to be due to selection for the ability of the protein to convert to a prion state.

The *Drosophila melanogaster* and *Caenorhabditis elegans* proteomes were searched for predicted proteins that contain nucleic acid binding domains

(for RNA, DNA, or both), and Q/N-rich sequences, using a threshold of 30 Q/Ns in 80 residues. This is the same threshold used in a search for new, putative yeast candidates (Michelitsch and Weissman, 2000). As described above, we specified that the ortholog in these two very distantly related species must contain both the Q/N rich domain and the nucleic acid binding domain in order to show that Q/N rich region is important for function, and has, therefore, been maintained. To this extent, candidate genes obtained by the search of these two species databases, would be blasted and orthologs analyzed for the maintenance of a Q/N rich region and the functional nucleic acid binding domain. Of the 472 *Drosophila* and *143 C. elegans* Q/N- rich proteins previously shown to exist, (Michelitsch and Weissman, 2000), only two orthologous pairs satisfied all of the above parameters: Drosophila pumilio (Pum) and its worm ortholog Puf9, and a pair of large chromodomain proteins.

Pum was already being studied in the lab, after being obtained in a *Drosophila* larval neuromuscular junction (NMJ) overexpression/misexpression screen (Kraut et al., 2001). This screen involved taking the set of 2293 GAL4 driven EP element lines available at the time and crossing them to a pan-neuronal GAL4 driver and a UAS-green fluorescent protein (GFP) line, then looking at the third instar larvae NMJ for deviations from wild type. The neuronal overexpression of Pum was found to produce an NMJ phenotype with numerous tiny boutons. Subsequently, Pum has been shown to be involved in translational repression at larval (NMJ) synapses and to play a role in both growth and

morphology of the neuromuscular junction (NMJ) synaptic boutons (Menon et al., 2004). Hypomorphic pum mutants were reported to have learning and memory phenotypes in adult *Drosophila* (Dubnau et al. 2003). A search through the genomic databases reveal that Pum and its orthologs in other organisms, including other arthropods such as members of the bee family and mosquitoes also have Q/N rich regions. Human and mouse Pum2 proteins also have Q/N rich sequences, although these do not surpass the threshold used in our search. However, some Pum orthologs, including human and mouse Pum1, Pum2 from bulls and dogs, or the more distantly related Arabidopsis, do not contain sequences with significant Q/N enrichment. If Q/N enrichment is really important for normal protein function in fly and worm Pum proteins, perhaps other amino acid sequences in these orthologs are playing the same role. Interestingly, a gene that had a lower Q/N count, but was also noted in the screen was Poly-A Binding Protein, (PABP), which is another gene expressed at the synapse, is involved in translation initiation, and is found in stress granules (see Introduction).

Different Constructs of Pumilio Form Visible Aggregates in Yeast

Various plasmids were made from *Drosophila* Pum, *C. elegans* Puf9, and various controls (Figure 1B) in order to visualize the overexpression of these peptides in yeast. In order to accomplish this, constructs were created that used a copper-inducible promoter in conjunction with a cyan fluorescent protein (CFP)

tag and a URA+ gene, which allows selection of transfomants in ura- strains. The full-length Pumilio without the RNA-binding domain, the first Q/N repeat of Pum (NQ1), the second Q/N repeat of Pum (NQ2), the first Q/N repeat of Pum lacking the longest run of Qs (ΔNQ1), CFP alone, New1PrD (the N-terminal aggregate-forming domain of a well-studied putative yeast prion), Sup35NM (the aggregate-forming domain of another well-studied putative yeast prion that confers the *[Psi+]* prion phenotype) fused to YFP, and the C. elegans Puf9 protein were analyzed for the ability to form aggregates when overexpressed individually and with co-expression of Sup35 in specific yeast strains.

These constructs were transformed individually into [*Psi+][pin+*], [*Psi][pin+*], and [*Psi-][pin-*] strains and visualized through confocal microscopy for individual aggregates of the various Pum domains, as evidenced through the presence of CFP foci, an established method for determining a protein's ability to aggregate in vivo in yeast. All Pumilio constructs revealed obvious foci after 8 hours of overexpression (Figure 2 A-H). CFP did not show any significant aggregates at this time point (Figure 2A). In addition, Sup 35NM seemed mostly diffuse in the yeast cells (Figure 2B). After 48 hours, however, all constructs, including CFP, showed obvious aggregates (Figure 2I). This data shows that after a short period of induction only certain constructs are capable of forming visible aggregates, however at later time points all overexpressed proteins may be capable of aggregating under these experimental conditions.

These constructs were also cotransformed with Sup35NM-YFP in order to ascertain their ability to cause the aggregation of Sup35. All constructs resulted in Sup35-YFP aggregates. Given the fact that Sup35 was capable of forming aggregates when overexpressed on its own, however, this data is not surprising. The various constructs, however, may be responsible for catalyzing the Sup35 aggregated state at an earlier time point. Moreover, the aggregates seemed to contain larger, brighter foci, implying an increase in aggregate formation, (data not shown).

Pumilio Can Recapitulate the *[pin+]* Phenotype in Yeast

It is known that Sup35 requires the presence of another putative yeast prion, *[pin+]*, derived from RNQ1 in its aggregated form, in order to form the prion *[Psi+]* state (Sondheimer et al., 2000). Because another aggregated protein is a prerequisite for Sup35's ability to go from the *[Psi-]* state to the *[Psi+]* state, overexpression of the various constructs were tested for their ability to reduce Sup35 function, implying a *[Psi+]* state. This is assayed by suppression of a nonsense mutation of ADE1, an enzyme necessary for adenine production in the yeast. The presence of the nonsense allele normally results in the absence of functional ADE1, so that the yeast fail to grow in the absence of adenine. When Sup35 is not functional and, therefore, not capable of performing its role as a termination factor, causing polypeptide chains to be released from the ribosome when a stop codon is reached, the early stop codon mutation is read through and

a functional protein is produced. This phenotype can be analyzed by counting colonies that appear on –ADE plates when transformed strains are plated 48 hours after the initiation of overexpression of the cotransformed constructs.

In these experiments, the various constructs delineated in Figure 1 were cotransformed with Sup35NM-YFP into a *[Psi-][pin-]* strain and grown for 48 hours after inducing overexpression with Cu+. These were then plated on –Ade plates and –Ura –Leu plates, and colonies were counted in order to determine the percentage of *[Psi+]*, prion state induction. This percentage was compared with the levels of the positive control, New1Prd-CFP. A yes on the table means that the percentage of *[Psi+]* induction was comparable to those observed for New1Prd-CFP (Figure 1B). Co-overexpression of NQ1-CFP and Sup35NM-YFP is observed to cause a decrease in nonsense suppression of ADE1, presumably through the aggregation of Sup35 into a nonactive conformation, characterized as the *[Psi+]* state, at the same or better rate as NEW1, a previously characterized yeast prion (Osherovich and Weissman, 2001) (Figure 1B and data not shown). New1 has already been shown to act in a manner comparable to the *[pin+]*, prion state, which is required for the efficient induction of the Sup35 prion state (Derkatch et al., 2001) . FLPum, Puf9, and NQ2 seem to be able to induce the Sup35 state, but may do so at lower efficiency. ΔNQ1 cannot induce the *[Psi+]* state at the same efficiency as the other constructs. Neither Sup35 nor CFP seem to be capable of inducing the *[Psi+]* state to a significant extent (Figure 1B and data not shown).

A Pumilio-Chimera Diploid Can Result in a *[Psi+]* State in Yeast When the NQ1 Region of Pumilio Is Overexpressed

In order to test if the NQ1 region of Pum was capable of replacing the aggregating domain of Sup35, a chimera was made consisting of the Pum NQ domain and the RNA Binding domain (EF) of Sup35, and integrated into a diploid genome, replacing one copy of the endogenous Sup35 (Figure 3). Resulting transformed lines, as determined by presence of a His tag, were then sporulated and dissected in order to obtain haploid yeast lines. PumNQ1-Sup35-EF, however, did not seem viable in the haploid state because only two yeast colonies were consistently obtained after tetrad dissections, perhaps indicating that yeast expressing only the chimera fail to survive. Of the very few putative haploid colonies that actually resulted from surviving tetrads, only two lines seem to contain NQ1 by PCR analysis. This result has been observed in the production of similar chimeras that seem to aggregate very strongly, thereby reducing the availability of the necessary element Sup35 to such a degree that the line is lethal (Cameron and Weissman, unpublished data). Complete Sup35 knockouts are known to be lethal.

Both of the isolated lines still contained the N-terminus of Sup35, which should have been excised by the integration method (Figure 3). This implied that the NQ1-SupEF hybrid gene either integrated elsewhere into the genome, and the endogenous Sup35 was retained, or the lines were diploid. Both lines seem

capable of sporulating and forming tetrads, implying both were diploids, but mating tests seemed to show the ability of one of these lines to mate to one mating type of yeast, implying that this line might possess haploid qualities and actually be a triploid.

In order to generate a *[Psi-][pin-]* version of this putative chimera line for use as a recipient for Pum constructs, this line and the parent *[Psi+]* diploid strain were treated with Gu-HCl (see below for details) and derivative lines that formed red colonies on a YPD plate were chosen for use in subsequent experiments. When the lines bearing NQ1-Sup35EF hybrid genes were transformed with the same constructs as used in the previous set of experiments (Figure 1B) at the same time as the similarly transformed diploid *[Psi-]* strain as a control, the NQ1-Sup35EF lines were capable of significantly increasing (more than 10 fold) the induction of *[Psi+]* above the control diploid line when both Sup35NM-YFP and NQ1-Cfp, or Sup35NM-YFP and New1-CFP were overexpressed in these lines (Figure 4). Furthermore, the double overexpression of Sup35NM with ΔNQ1-CFP did not result in a similar increase of induction. Although this line is not well characterized, this data provides more supporting evidence that NQ1 can act in a manner similar to New1 as far as inducing the *[Psi+]* state. Even if the data from the putative chimera is disregarded, the data from the cotransformation of either NQ1 or New with Sup35NM into the diploid strain reveals similar rates of *[Psi+]* induction as each other and as the results that were observed in haploid lines,

again supporting the concept that NQ1 is capable of inducing the Sup35 prion state to the same extent as the N-terminal of New1 (Figure4).

The *[Psi+]* state induced by Pumilio is reversible in the presence of Guanidine HCL

The *[Psi+]* state, characterized by growth of yeast on –ADE plates and white yeast on rich medium plates is reversible in the presence of Guanidine-HCl (Gu-HCl). Low levels of Gu-HCl, a known chaotropic agent that can unfold proteins, is a common method used in yeast for showing that the prion state is interconvertible with the native state and that the prion state is reversible (review, Jones and Tuite, 2005).

Gu-HCl is capable of reversing the prion state of the putative chimera and the *[Psi+]* state induced by Sup35NM and NQ1 overexpression. This data from the putative chimera line implies that perhaps NQ1 is capable of being cured, at least to the extent of not maintaining the Sup35 prion state. The data presented here shows that NQ1 is capable of acting in a manner similar to the N-terminal of New1, which has been shown to be a “stronger” prion in several assays than RNQ1 (Derkatch et al., 2004), and is capable of acting like *[pin+]* (Osherovich et al. 2001). In addition, one may hypothesize that if the putative chimera NQ1-EF line was not cured by Gu-HCl, then the [*Psi+]* prion state could possibly be maintained. It is possible, however, that the lack of HSP 104 is sufficient to cure only the Sup35 prion and does not impact NQ1-EF.

Discussion

The first NQ-rich domain of Pum (NQ1), the second NQ-rich domain (NQ2) of Pum, the full-length region of Pum, lacking the RNA-binding domain, as well as the pumilio ortholog, Puf9, in *C. elegans* can act in a manner similar to putative yeast prions in vivo. The domains aggregate when overexpressed in different strains of yeast, can act in a manner similar to *[New+]* in nonsense suppression assays with Sup35, and the putative NQ1-EF prion state may be reversible when grown on Guandine-HCl (Gu-HCl). In the assays analyzed, these domains of *Drosophila* Pum and the *C. elegans* ortholog of Pum seem to act in a prion-like manner to the same extent as the previously characterized putative yeast prion New1.

In the yeast aggregation assay, specific Pumilio domains aggregate in vivo as observed through confocal microscopy and result in CFP foci that are visually similar to those observed in well-studied putative yeast prions. Longer incubation times result in more localized, larger foci, which is also consistent with previous observations of yeast prions, polyQ proteins, and other known amyloid forming proteins overexpressed in similar strains of yeast. Introducing another prion state, *[pin+], [Psi+],* or both, results in larger aggregates. This is also consistent with previous observations. As a negative control, data was also conducted on a vector containing only CFP. At early time points a lack of CFP foci are observed when overexpressed in *[Psi-] [pin-]* lines, with CFP expression

diffuse throughout the yeast cell. Longer incubations, however, result in yeast overexpressing only CFP starting to show aggregates. This data is consistent with the observation that overexpressed proteins in general are capable of forming aggregates, such as numerous overexpressed proteins in E. coli and other organisms. This is also consistent with the hypothesis that endogenous proteins are expressed at a level comparable to their ability to form aggregates (Tartaglia et al., 2007), implying that the overexpression of many proteins could lead to in vivo aggregation.

It is well known that overexpression of many proteins in *E. coli* result in aggregation and the formation of inclusion bodies, which may support this concept. *E. coli*, however are prokaryotes with different cellular attributes, so the comparison between the two organisms is not necessarily true. Even though it may be possible for all proteins to form aggregates in yeast when overexpressed, there is still an obvious rate difference, with some proteins capable of forming visible aggregates at a much earlier time point when overexpressed. In addition, the yeast prion state can form spontaneously, albeit at a very low frequency for most putative yeast prions. Even so, this is not necessarily catalyzed by overexpression of the protein, although it might be. Ultimately these yeast assays may be sufficient to distinguish between proteins that might be more capable of readily aggregating in vivo, versus proteins that may take longer to aggregate or might require a higher expression level in order to start aggregating.

The effects of Gu-HCl on reversing the prion state in yeast are known to act through HSP104, the chaperone required for the formation of oligomers and for the creation and maintenance of prion state (Ferriera et al., 2001). In a HSP104 deletion strain, the prion state of any putative yeast prion cannot be induced or maintained (Chernoff et al., 1995). Furthermore, it has been shown that Hsp104 is actually the protein that is affected by Gu-HCl, and that it is the inactivation of HSP104 by Gu-HCl that leads to the reversibility of the prion state (Ness et al., 2002). It has also recently been shown that cell division is required for the elimination of the *[Psi+]* prion state with Gu-HCl (Byrne et al., 2007). These details about the function of Gu-HCl in "curing" yeast actually call into question the ability of this assay to be a real measure of prion reversibility in the sense that it is not the Sup35 aggregated state that is reversed, but the ability to pass the aggregated state on to daughter cells. This data implies that Gu-Hcl does not actually cause a switch in the protein conformation of aggregated proteins in the prion conformation, but rather affects the ability to seed the aggregation in progeny. If one considers the fact that PrP has not yet been shown to be reversible, however, perhaps the requirement for the reversibility of the prion state needs to be redefined or eliminated. This is especially the case if one considers a putative prion that resides in postmitotic cells, because the ability to pass on the prion state to progeny cells is irrelevant. In addition, diseases that form seemingly nonreversible aggregate clumps may be more similar to proteins that are capable of being cured by Gu-HCl than previously

believed. This also implies that assays that induce the overexpression of HSP 104 may actually be a better diagnosis of the reversibility of the prion. The overexpression of HSP 104, however, seemed to only cause the loss of the prion state in a very small set of prions tested (Weissman lab, unpublished data), yet still termed yeast prions.

It also seems as though NQ1 is capable of inducing Sup35 into the prion state at a rate comparable to New1. This is very interesting in that this specific assay was originally used by Weissman to prescreen through the numerous N/Q rich yeast proteins for yeast that could act like prions in the other typical prion assessment assays. This assay seemed to only work with proteins that were later considered yeast prions (Osherovich, 2001). In addition, NQ1 consistently showed similar *[Psi+]* induction rates as New1Prd, showing that it may possess similar properties to New1. It has been proposed that the N/Q rich sequences are capable of directly seeding Sup35 aggregate formation (Derkatch, 2004). The Q rich domain of NQ1, therefore, may be responsible for its ability to induce the *[Psi+]* state, which is substantiated by the fact that the removal of the longest run of Qs results in a decrease in Sup35 induction into the prion state.

The fact that many proteins, and perhaps even all proteins, are capable of forming amyloid fibers under specific conditions because of a general capability of all peptide backbones to form hydrogen bonds with adjacent peptide backbones, implies that the conservation of these Q/N rich, amyloid-prone sequences may have been for an important functional reason. Given the latest

knowledge about general protein folding, misfolding, and aggregation, it seems as though the initial search paradigm used to find putative beneficial prions may have overlooked numerous proteins that still maintain a strong propensity towards aggregation, yet lack large Q/N repeats, such as PrP itself. Furthermore, the precise cutoff number of 30 residues in 80 may be an overestimation of the number of Q/Ns really required to form a transmissible aggregate. In addition, confining the search to domains that are involved in nucleotide binding also possibly reduced the number of hits. It now seems that numerous functional types of proteins can be considered prions as long as they show an epigenetic propagation (see Chapter 1 introduction). This could include proteases, ubiquitin ligases, acetylases, methylases, etc. (Wickner et al., 2004). Even so, the database search for proteins that might be involved in an epigenetic switch still produced a very interesting candidate, Pum, and another interesting protein, PABP, which may actually interact with Pum at NMJ synapses.

Experimental Procedures

Yeast Strains and Plasmids

Yeast plasmids were obtained from Jonathan Weissman (Osherovich and Weissman, 2001). The n-terminal prion domain of New1, amino acids 1-153, was tagged with eCFP and under control of the CUP1 promoter within the pRS426 (URA3) plasmid. Sal I and Bgl II sites in this vector were used to remove the New1 sequence and the specific regions of Pum and Puf9 were obtained through PCR of cDNA clones, subcloned into topo vectors, then digested and ligated into

the PRS426 vector. A plasmid containing exon 1 of Huntingtin with 46 Qs was obtained from Pamela Bjorkman and PCR was conducted to insert appropriate restriction sites. This was also subcloned into a topo vector, then digested and ligated into the pRS426. Sup35NM was obtained in the pRS425 (LEU2) plasmid tagged with YFP. *[Psi-] [pin-], [Psi-][pin+],* and *[Psi+][pin+]* yeast strains derived from 74D-694 MATa adel-l4(UGA) trpl-289(UAG) hzs3A-200 ura3-52 leu2-3,112, and W303 strains containing ade1-14 (Osherovich and Weissman, 2001) were used in these studies. In addition, a *[Psi+][pin+]* diploid with the same background was also obtained for genomic integration experiments.

In Vivo Aggregate Visualization Under Confocal Microscopy

The Zeiss LSM 510 inverted microscope was used for obtaining images and visualizing foci. Appropriate lasers were used, possessing wavelengths to excite CFP and YFP. Random fields were scanned and images represent the majority of yeast observed. Z slices were taken using .25 μm intervals.

Chimera Creation and Chromosomal Integration

A chromosomally integrated gene fusion of the NQ1-Sup35EF chimera was generated by homologous recombination of PCR-amplified sequences containing a His tag. These were transformed into diploid yeast strains, and grown up on –His plates. Resulting colonies were PCR screened for the presence of NQ1 and the N-terminus of Sup35, which should have been excised,

and appropriate strains were sporulated using standard procedures, dissected, and grown up on YPD plates.

Guanidine-HCl treatment

Yeast were grown on minimal media plates containing 5mM Guanidine-HCl for 5-7 days at 30°C. Colonies were then streaked out on –ADE plates in order to show loss of *[Psi+]* state and on YPD plates in order to better visualize color reversion.

Acknowledgements

We would like to thank Erich Schwartz for the database search; Kaushiki Menon, Ashley Wright, and Nina Sherwood for useful discussions; Violana Nesterova for administrative assistance, assistance with figures, and experimental assistance; Lisa Nesterova for general experimental assistance; Lakshmi Bugga for yeast reagents and assistance; Sean Brennan for graphic assistance; Johannes Graumann for yeast protocols; Deshaies Lab for yeast plates; Lev Osherovich, Dale Cameron, and Jonathan Weissman for yeast plasmids, protocols, yeast strains, and antibody, and for kindly allowing me to come to the lab to learn the gamma integration technique; Susan Lindquist for yeast plasmids and yeast strains; Robin Wharton for yeast strains and plasmids; Susan Liebman for α-Sup35 antibody; Pamela Bjorkman for the Huntingtin plasmid; and Chris Waters for confocal microscopy maintenance.

Figure Legends

Figure 1 (A) *Drosophila* Pumilio Domains. After analyzed the sequence of Pum, two large Q/N rich tracts were observed: the first Q/N-rich tract (NQ1) and the second (NQ2) region, as well as the RNA binding and Puf domain regions. (B) Because Pum is a very large protein and endogenous yeast prions are significantly smaller, we decided to construct several yeast-expressing plasmids, in case the bulky nature of FL-pum was difficult to efficiently express. The Sup35NM region and the New1 constructs were used as positive controls for some assays and the same yeast plasmid containing only CFP was used as a negative control. The ability for each of the constructs to form aggregates in *[Psi-][pin-]* yeast strain is shown at 6 hour and 48 hour timepoints. The ability of the construct to induce the *[Psi+]* state when overexpressed in *[Psi-][pin-]* yeast strains along with Sup35NM-YFP is also shown. Yeast were induced at OD 6 and grown at 30°C in both experiments. The yeast were plated after 48 hours of growth onto –ADE plates to analyze the prion state.

Figure 2. Visualization of CFP aggregates in *[Psi-][pin-]* Yeast. Yeast were analyzed on an inverted 510 Zeiss confocal microscope for the ability to form aggregates (A-H) *[Psi-][pin-]* yeast strain transformed with the one of the constructs described in Figure 1., and analyzed after 6 hours of induction by copper, and (I-L) analyzed after 48 hours of growth at 30 degrees in the presence of copper.

Figure 3. *Drosophila melanogaster* Pumilio amino acid sequence with Q/Ns highlighted

Figure 4. A schematic diagram describing gamma integration, the method used to attempt to replace one copy of genomic Sup35 with an NQ1-Sup35-EF chimera.

Figure 5. Percent of *[Psi+]* induction in yeast in an NQ1-EF chimera strain. The X-axis contains the yeast strain used for these experiments, and the plasmid or plasmids transformed into the strain and induced by copper for 48 days prior to plating on –ADE plates, and diluted and plated on either –Ura, -Ura –Leu, or –Leu, depending on the plasmid(s) used. The percent of *[Psi+]* induction was determined by dividing the number of yeast that grew on –ADE plates by the total number of transformed yeast and multiplying by 100. The Gd-HCl cured NQ1 diploid strain is the putative NQ1-EF chimera line diploid. The control diploid strain is the original parent strain used in the gamma integration procedure, but cured with GdHCl to eliminate the [Psi+] state.

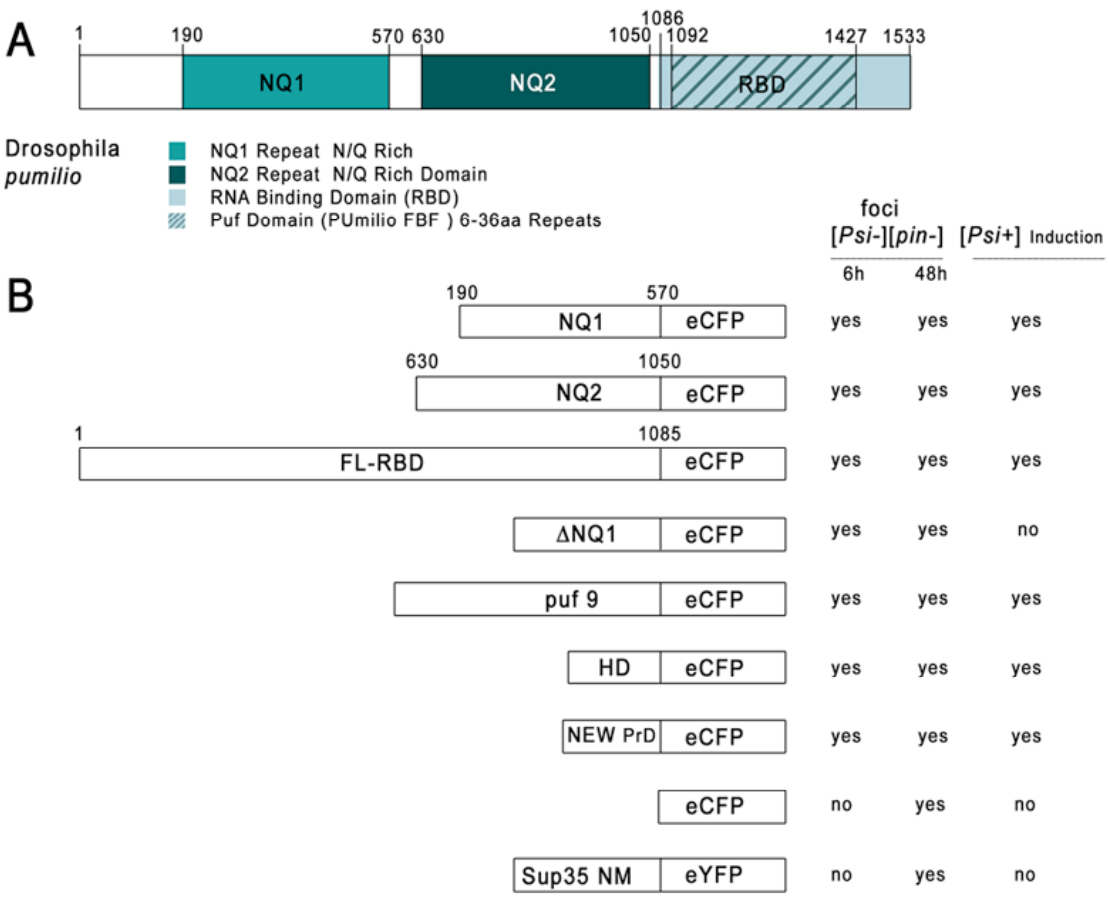

Figure 1. (A) *Drosophila* Pumilio Domains

(B) Yeast Plasmid Constructs and Ability to Aggregate or Induce the *[Psi+]* state

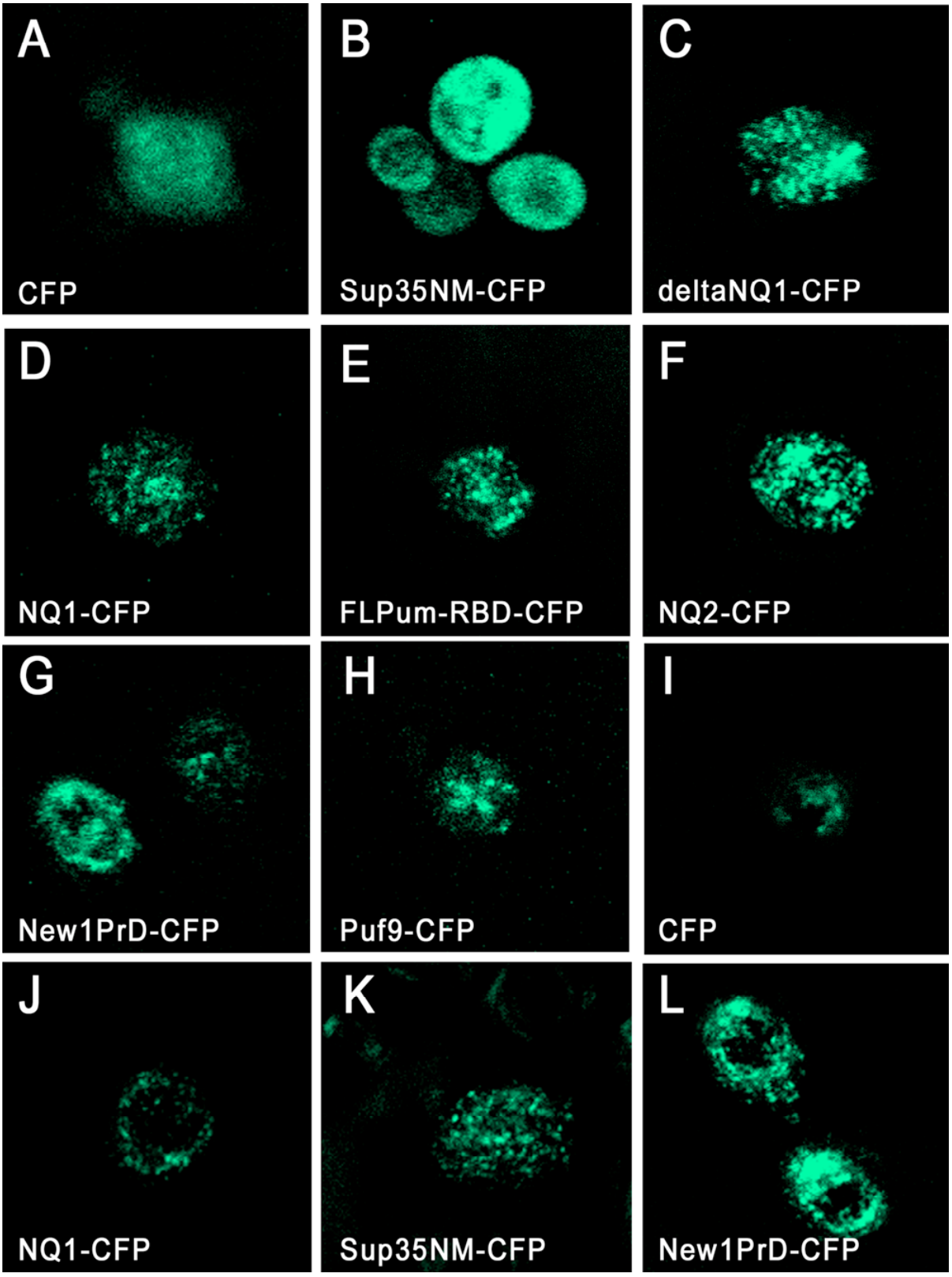

Figure 2. Visualization of CFP aggregates in *[Psi-][pin-]* Yeast

MKFLGGNDDRNGRGGVGVGTDAIVGSRGGVSQDAADAAGAAAAAAVGYVF
QQRPSPGGVGVGVGGVGGGVPGVGAVGSTLHEAAAAEYAAHFAQKQQQTR
WACGDDGHGIDNPDKWKYNPPMNPANAAPGGPPGNGSNGGPGAIGTIGMGSGL
GGGGGGGAGGGNNGGSGTNGGLHHQSMAAAAAANMAAMQQAAALAKHNHMI
SQAAAAVAAQQQHQHPHQQHPQQQQQQQQAQNQGHPHHLMGGGNGLGNG
NGLGIQHPGQQQQQQQQQQQQQHPGQYNANLLNHAAALGHMSSYAQSGGS
MYDHHGGAMHPGMNGGMPKQQPLGPPGAGGPQDYVYMGGQTTVPMGAAM
MPPQNQYMNSAAVAAANRNAAITTSTAKKLWEKSDGKGVSSSTPGGPLHPLQI
PGIGDPSSVWKDHTWSTQGENILVPPPSRAYAHGGASDTSNSGNAGILSPRDSTC
AKVVEYVFSGSPTNKDSSLSGLEPHLRNLKFDDNDKSRDDKEKANSPFDTNGLK
KDDQVTNSNGVVNGIDDDKGFNRTPGSRQPSPAEESQPRPPNLLFPPLPFNHML
MDHGQGMGGGLGGVVGSGNGVGGGSGGGGAGGAYAAHQQMAAQMSQLQP
PMMNGVGGGMPMAAQSPMLNHQAAGPNHMESPGNLLQQQNFDVQQLFRSQ
NPGLAAVATNAAAAAAAAAAATSAASAAAAVGAPPVPNGSLQQSQQQQQQQ
QQQQQQQQMHMAAASQQFLAAQQQAQNAAYAAQQATSYVINPGQEAAPY
MGMIAAAQMPYYGVAPWGMYPGNLIPQQGTQPRRPLTPSQQGAENQPYQVIP
AFLDHTGSLLMGGPRTGTPMRLVSPAPVLVPPGATRAGPPPPQGPQLYQPQPQT
AQQNLYSQQNGSSVGGLALNTSSLTGRRDSFDRSTSAFSPSTMDYTSSGVAAAA
NAVNSTVAQAAAAAAAAAAARGKWPGAMSGAASGAYGALGAGNASASPLGA
PITPPPSAQSCLLGSRAPGAESRQRQQQQQQLAAVGLPATAAAAQAAVAAAAN
NMFGSNSSIFSNPLAIPGTAAVAAAAAAAAAANSRQVAATAAAAAAVAAAAGG
VGGAPQPGRSRLLEDFRNQRYPNLQLRDLANHIVEFSQDQHGSRFIQQKLERAT
AAEKQMVFSEILAAAYSLMTDVFGNYVIQKFFEFGTPEQKNTLGMQVKGHVLQ
LALQMYGCRVIQKALESISPEQQQEIVHELDGHVLKCVKDQNGNHVVQKCIEC
VDPVALQFIINAFKGQVYSLSTHPYGCRVIQRILEHCTAEQTTPILDELHEHTEQLI
QDQYGNYVIQHVLEHGKQEDKSILINSVRGKVLVLSQHKFASNVVEKCVTHAT
RGERTGLIDEVCTFNDNALHVMMKDQYANYVVQKMIDVSEPTQLKKLMTKIRK
NMAALRKYTYGKHINAKLEKYYMKITNPITVGTGAGGVPAASSAAAVSSGATS
ASVTACTSGSSTTTTSTTNSLASPTICSVQENGSAMVVEPSSPDASESSSSVVSGA
VNSSLGPIGPPTNGNVVL

Figure 3 *Drosophila melanogaster* Pumilio Amino Acid Sequence With Q/Ns Highlighted

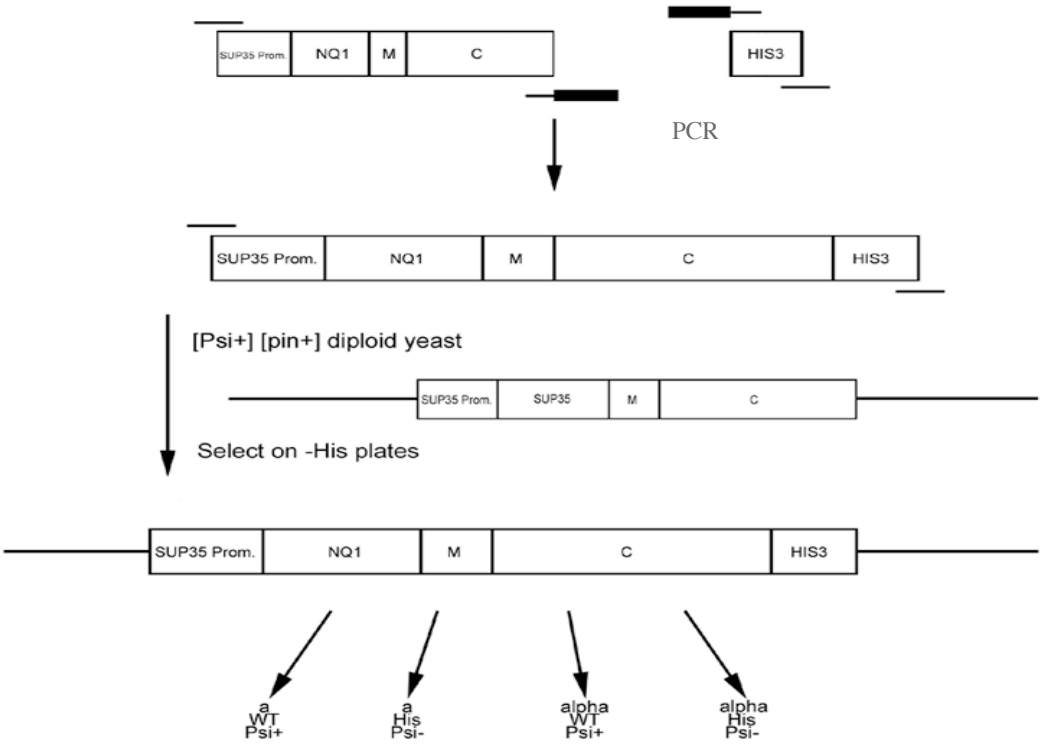

Figure 4. Gamma Integration, the Method for Replacing Endogenous Sup35 with NQ1-EF in the Yeast Genome

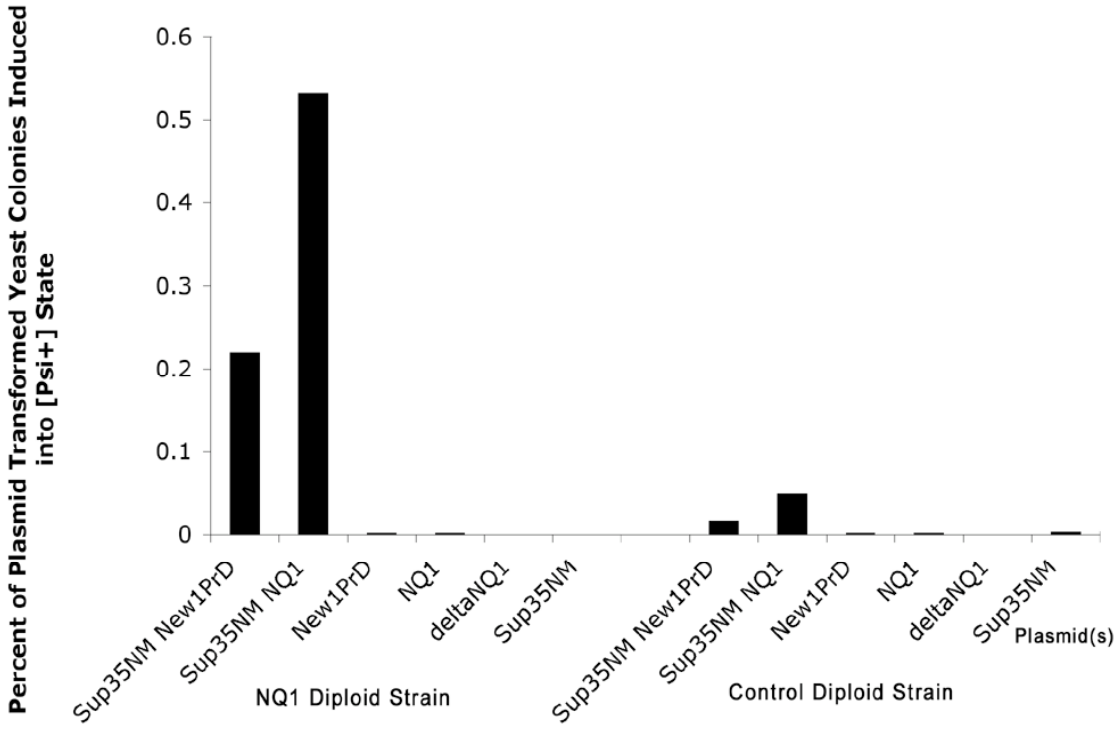

Figure 5. Percent of *[Psi+]* Induced Yeast in an NQ1-EF Chimera Strain

Chapter 1 References

Bagriantsev, S. and Liebman, S., 2006. Modulation of Abeta42 low-n oligomerization using a novel yeast reporter system. BMC Biol. 4, 32.

Brachmann, A., Baxa, U. and Wickner, R. B., 2005. Prion generation in vitro: amyloid of Ure2p is infectious. EMBO J. 24, 3082-3092.

Byrne, L. J., Cox, B. S., Cole, D. J., Ridout, M. S., Morgan, B. J. and Tuite, M. F., 2007. Cell division is essential for elimination of the yeast [PSI+] prion by guanidine hydrochloride. Proc Natl Acad Sci U S A. 104, 11688-11693.

Chernoff, Y. O., Lindquist, S. L., Ono, B., Inge-Vechtomov, S. G. and Liebman, S. W., 1995. Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]. Science. 268, 880-884.

Chiti, F. and Dobson, C. M., 2006. Protein misfolding, functional amyloid, and human disease. Annu Rev Biochem. 75, 333-366.

Coustou, V., Deleu, C., Saupe, S. and Begueret, J., 1997. The protein product of the het-s heterokaryon incompatibility gene of the fungus Podospora anserina behaves as a prion analog. Proc Natl Acad Sci U S A. 94, 9773-9778.

Derkatch, I. L., Bradley, M. E., Hong, J. Y. and Liebman, S. W., 2001. Prions affect the appearance of other prions: the story of [PIN(+)]. Cell. 106, 171-182.

Derkatch, I. L., Uptain, S. M., Outeiro, T. F., Krishnan, R., Lindquist, S. L. and Liebman, S. W., 2004. Effects of Q/N-rich, polyQ, and non-polyQ amyloids on the de novo formation of the [PSI+] prion in yeast and aggregation of Sup35 in vitro. PNAS. 101, 12934-12939.

Dobson, C. M., 2004. Principles of protein folding, misfolding and aggregation. Seminars in Cell & Developmental Biology. 15, 3-16.

Dubnau, J., Chiang, A. S., Grady, L., Barditch, J., Gossweiler, S., McNeil, J., Smith, P., Buldoc, F., Scott, R., Certa, U., Broger, C. and Tully, T., 2003a. The staufen/pumilio pathway is involved in Drosophila long-term memory. Curr Biol. 13, 286-296.

Dubnau, J., Chiang, A. S. and Tully, T., 2003b. Neural substrates of memory: from synapse to system. J Neurobiol. 54, 238-253.

Eaglestone, S. S., Cox, B. S. and Tuite, M. F., 1999. Translation termination efficiency can be regulated in Saccharomyces cerevisiae by environmental stress through a prion-mediated mechanism. Embo J. 18, 1974-1981.

Edskes, H. K., Naglieri, B. M. and Wickner, R. B., 2006. Nitrogen source and the retrograde signalling pathway affect detection, not generation, of the [URE3] prion. Yeast. 23, 833-840.

Ferreira, P. C., Ness, F., Edwards, S. R., Cox, B. S. and Tuite, M. F., 2001. The

elimination of the yeast [PSI+] prion by guanidine hydrochloride is the result of Hsp104 inactivation. Mol Microbiol. 40, 1357-1369.

Gary W. Jones, M. F. T., 2005. Chaperoning prions: the cellular machinery for propagating an infectious protein? Bioessays. 27, 823-832.

Jones, G. W. and Tuite, M. F., 2005. Chaperoning prions: the cellular machinery for propagating an infectious protein? Bioessays. 27, 823-832.

Kraut, R., Menon, K. and Zinn, K., 2001. A gain-of-function screen for genes controlling motor axon guidance and synaptogenesis in Drosophila. Curr Biol. 11, 417-430.

Menon, K. P., Sanyal, S., Habara, Y., Sanchez, R., Wharton, R. P., Ramaswami, M. and Zinn, K., 2004. The translational repressor Pumilio regulates presynaptic morphology and controls postsynaptic accumulation of translation factor eIF-4E. Neuron. 44, 663-676.

Michelitsch, M. D. and Weissman, J. S., 2000. A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions. Proc Natl Acad Sci U S A. 97, 11910-11915.

Nakayashiki, T., Kurtzman, C. P., Edskes, H. K. and Wickner, R. B., 2005. Yeast prions [URE3] and [PSI+] are diseases. Proc Natl Acad Sci U S A. 102, 10575-10580.

Ness, F., Ferreira, P., Cox, B. S. and Tuite, M. F., 2002. Guanidine hydrochloride inhibits the generation of prion "seeds" but not prion protein aggregation in yeast. Mol Cell Biol. 22, 5593-5605.

Osherovich, L. Z., Cox, B. S., Tuite, M. F. and Weissman, J. S., 2004. Dissection and design of yeast prions. PLoS Biol. 2, E86.

Osherovich, L. Z. and Weissman, J. S., 2001. Multiple Gln/Asn-rich prion domains confer susceptibility to induction of the yeast [PSI(+)] prion. Cell. 106, 183-194.

Prusiner, S. B., 1998a. The prion diseases. Brain Pathol. 8, 499-513.

Prusiner, S. B., 1998b. Prions. Proc Natl Acad Sci U S A. 95, 13363-13383.

Roberts, B. T. and Wickner, R. B., 2003. Heritable activity: a prion that propagates by covalent autoactivation. Genes Dev. 17, 2083-2087.

Roberts, B. T. and Wickner, R. B., 2004. A new kind of prion: a modified protein necessary for its own modification. Cell Cycle. 3, 100-103.

Sailer, A., Bueler, H., Fischer, M., Aguzzi, A. and Weissmann, C., 1994. No propagation of prions in mice devoid of PrP. Cell. 77, 967-968.

Santoso, A., Chien, P., Osherovich, L. Z. and Weissman, J. S., 2000. Molecular basis of a yeast prion species barrier. Cell. 100, 277-288.

Serio, T. R. and Lindquist, S. L., 2001. The yeast prion [PSI+]: molecular insights

and functional consequences. Adv Protein Chem. 59, 391-412.

Shorter, J. and Lindquist, S., 2005. Prions as adaptive conduits of memory and inheritance. Nat Rev Genet. 6, 435-450.

Sondheimer, N. and Lindquist, S., 2000. Rnq1: an epigenetic modifier of protein function in yeast. Mol Cell. 5, 163-172.

Tanaka, M., Chien, P., Naber, N., Cooke, R. and Weissman, J. S., 2004. Conformational variations in an infectious protein determine prion strain differences. Nature. 428, 323-328.

Tanaka, M., Chien, P., Yonekura, K. and Weissman, J. S., 2005. Mechanism of cross-species prion transmission: an infectious conformation compatible with two highly divergent yeast prion proteins. Cell. 121, 49-62.

Tartaglia, G. G., Pechmann, S., Dobson, C. M. and Vendruscolo, M., 2007. Life on the edge: a link between gene expression levels and aggregation rates of human proteins. Trends Biochem Sci. 32, 204-206.

True, H. L., Berlin, I. and Lindquist, S. L., 2004. Epigenetic regulation of translation reveals hidden genetic variation to produce complex traits. Nature. 431, 184-187.

True, H. L. and Lindquist, S. L., 2000. A yeast prion provides a mechanism for genetic variation and phenotypic diversity. Nature. 407, 477-483.

von der Haar, T., Josse, L., Wright, P., Zenthon, J. and Tuite, M. F., 2007. Development of a novel yeast cell-based system for studying the aggregation of Alzheimer's disease-associated Abeta peptides in vivo. Neurodegener Dis. 4, 136-147.

Walsh, D. M. and Selkoe, D. J., 2007. A beta oligomers - a decade of discovery. J Neurochem. 101, 1172-1184.

Weissmann, C., Fischer, M., Raeber, A., Bueler, H., Sailer, A., Shmerling, D., Rulicke, T., Brandner, S. and Aguzzi, A., 1998. The use of transgenic mice in the investigation of transmissible spongiform encephalopathies. Rev Sci Tech. 17, 278-290.

Wickner, R. B., Edskes, H. K., Ross, E. D., Pierce, M. M., Baxa, U., Brachmann, A. and Shewmaker, F., 2004. PRION GENETICS: New Rules for a New Kind of Gene. Annual Review of Genetics. 38, 681-707.

Wickner, R. B., Edskes, H. K. and Shewmaker, F., 2006. How to find a prion: [URE3], [PSI+] and [beta]. Methods. 39, 3-8.

Wickner, R. B., Edskes, H. K., Shewmaker, F. and Nakayashiki, T., 2007. Prions of fungi: inherited structures and biological roles. Nat Rev Microbiol. 5, 611-618.

Chapter 2

A Pumilio Domain Regulates Pumilio-Mediated Translational Repression In *Drosophila*

Chapter 2 Introduction

Drosophila melanogaster is one of the best species for investigating many basic science questions and also for investigating human diseases. It is a small invertebrate that is easy to maintain and propagate, contains an enormous array of molecular and genetic tools, is well-characterized, and at least 50% of *Drosophila* genes are similar to those found in humans (Rubin et al., 2000), with at least 75% of genes associated with human diseases containing a *Drosophila* ortholog (Reiter et al., 2001). Specifically, the *Drosophila* neuromuscular junction (NMJ) is an ideal model system for studying neuronal connections. One can make use of the attributes of the fruit fly delineated above, while having the ability to observe single cell synapses and conduct electrophysiological measurements (Keshishian et al., 1996). The *Drosophila* NMJ contains glutamatergic synapses that are homologous to mammalian AMPA receptors and can be used as a model for glutamatergic synapses in the mammalian CNS because of the molecular and developmental similarities between the two. At the *Drosophila* NMJ, synapses occur at axonal swellings, termed boutons.

Synapses are the primary location where the transfer of information between neurons, or from neurons to muscles, takes place. Synapses contain a presynaptic density, which is associated with synaptic vesicles and termed the active zone, and a postsynaptic density (PSD) directly across a cleft from the active zone. Synapses are quite remarkable in their dynamic nature, with an

ability to maintain stable connections for long periods of time, or the ability to rapidly disassemble or form (Eaton and Davis, 2003). Although the assembly and disassembly of synapses is especially common during development, it has been shown that synapses retain the ability to change throughout the existence of an organism (Purves and Lichtman, 1980).

Many recent studies have led to a detailed description of the proteins involved at the synapse and their regulation. It is now commonly believed that mRNAs are targeted to the synapse and are translated in response to neuronal activity, which was first postulated when polyribosmes were discovered at the base of dendritic spines (Steward and Levy, 1982). Various mRNAs have been discovered at the synapse. These include mRNAs encoding microtubule-associated protein 2 (MAP2), the α-subunit of Ca2+/calmodulin-dependent protein kinase II (CamKIIα), and different neurotransmitter receptor subunits (reviewed by Martin and Zukin, 2006). It has also been discovered that local protein synthesis in dendrites seems to be involved in moment-to-moment regulation of synapses, hypothesized from the observation that the spontaneous release of neurotransmitter has a repressing effect on local protein translation (Sutton et al., 2004; Sutton et al., 2006). Although polyribosome-containing mRNAs are constantly localized at synapses, the mRNAs do not seem to be translated until they receive a signal, one of which can be initiated through neurotransmitter binding to receptor. In addition, postsynaptically induced neurotransmitter cascades seem to be involved in local protein synthesis and

may cause long-term changes in synaptic function (reviewed by Schuman et al., 2006). Given the observation that local protein translation can be repressed in response to activity, the proteins involved in mRNA translational repression are important to study in order to further elucidate how synaptic connections are formed and maintained.

An mRNA binding protein that is involved in translational repression (Wharton et. al., 1998) and is found at synapses of the Drosophila NMJ, is pumilio (Pum) (Menon et al, 2004). Pum is a 1533 amino acid protein containing 13 exons that spans greater than 200kb of DNA (Macdonald, 1992). Several Pum isoforms have been observed. There are a 156 kD protein (Barker et al., 1992). There are also a 130 kD isoform (Parisi and Lin, 1999) and a 95 kD isoform (Schweers et al., 2002). Full length Pum contains a Q/N rich N-terminal domain and an RNA binding C-terminal domain containing several puf motifs. The C-terminus has been shown to be involved in binding to Nanos (Nos) and Brain Tumor (Brat), as well as to RNA targets (Edwards, 2001).

Pum is a well-characterized protein that was first described in its role as a regulator of *Drosophila* embryonic development. Maternally synthesized pumilio, along with Nos, another translational repressor, has been shown to bind to nanos response elements (NREs) at the 3' untranslated region (utr) of *hunchback* mRNA in order to attain correct abdominal pattern formation (Barker et al., 1992). The repression of *hunchback* mRNA at the posterior of the embryo occurs when Pum recruits Nos and another protein, Brat, to the *hunchback* mRNA, allowing

for *hunchback* expression only at the anterior end of the embryo (Sonada and Wharton, 1999, 2001). This is because Nos is localized in a gradient at the posterior end, which provides a spatial repression mechanism, even though the *hunchback* mRNA is distributed throughout the embryo (Gavis and Lehmann, 1994).

Although there is much information about the role of Pum in the embryo, only a few more recent papers have contributed more information about the role of Pum at later stages. Pum was shown to localize to granules in the spines of dopaminergic neurons and may be comparable to stress granules seen in mammalian dendrites, (see thesis Introduction). These granules, however, may be a protein repression complex containing Nos, Pum, and other proteins necessary for repression at the synapse (Ye et al., 2004). In addition, Pum hypomorphs possess associative memory phenotypes (Dubnau et al., 2003). Pum has been shown to be involved in regulating the morphology and growth of synapses at the fly NMJ (Menon et al., 2004). Pum is primarily localized to the postsynaptic side of the larval NMJ and seems to be a regulator of local mRNA translation. In *pum* mutants, synaptic morphology is altered, an increase in local translation of the eIF-4E translation initiation factor is observed, and an increase of the GluRIIa glutamate receptor subunit at the neuromuscular junction is also evident. This data provides an interesting new role for Pum, and perhaps some of the other proteins that are also involved in germline development.

Given the experimental data that shows that Pum is involved in synaptic growth and changes in dendrite and NMJ synapse morphology, in addition to the possible role of Pum in associative learning, it is intriguing to hypothesize about overexpressed Pum's ability to aggregate in vivo in yeast. Learning and memory require synapses to be able to maintain their characteristics for longer periods than the lifetimes of any proteins within the synapse. Since prion aggregates can be inherited, a prion within a synapse should be maintained indefinitely after it is induced. In addition, any new prion proteins synthesized at the synapse would become incorporated into the old aggregates. If such a protein regulated synaptic local translation, it would then be possible for a translational state to be maintained for long periods of time by an activity-regulated conversion of the protein to a prion form. This could represent a form of synaptic long-term memory. In this model, the prion state does not have to be infectious in the sense of passing on the trait to a daughter cell, given the lack of division of these neurons. The putative prion, however, must possess a metastable state that enables the aggregated form to avoid normal protein degradation and continue to recruit newly created proteins. Chaperones may be required to maintain or degrade such an aggregated state. In a recent paper, Eric Kandel and his colleagues described some evidence that such a prion might exist at *Aplysia* synapses, whose strength can be regulated by local translation (Si et al. 2003).

Results

PUASP Derived NQ1 Transgenic Fly Lines Show Normal Embryonic Segmentation

In order to analyze the ability of the first NQ-rich region (NQ1) of Pum's ability to cause the aggregation of endogenous, full-length Pum, I created a construct that contained the NQ1 region of Pum tagged with GFP under control of the UAS Gal4 system. I used a pUASP contruct that contains changes in the 3'utr of the pUAST expression vector, a common *Drosophila* vector used for chromosomal insertion containing 5 copies of the GAL4 binding sites, UAS, surrounded by transposable P elements, for insertion into the fly genome, that enable maternal expression during oogenesis, something that the pUAST construct can not do very well. The use of pUASp has allowed assessment of gain-of-function maternal phenotypes (Rorth P, 1998). This vector was inserted into flies and transgenic fly lines were obtained.

About 200 independent lines containing the NQ1 region of Pumilio tagged with CFP under the control of UASp were generated. About 100 of these lines were mapped to the appropriate chromosome and balanced, with 11 X-chromosome lines, 41 2nd-chromosome lines, and 61 3rd-chromosome lines. I obtained 10 different Gal4 drivers inserted on different chromosomes in order to further analyze early embryonic expression of these lines. All balanced lines were analyzed for CFP expression by crossing to several ovary drivers and looking at early stage embryos. No obvious aggregates of CFP were observed.

All lines were categorized according to CFP expression levels on a scale from 1-10 with 10 being the brightest. All lines were also examined for decreases in abdominal segmentation as observed in loss of function Pum (Barker, 1992) through a cuticle preparation on stage 12 embryos by crossing to various ovary drivers. All 100 lines were analyzed with at least one driver. The lines that seemed to reveal the strongest CFP expression were analyzed with all of the drivers. There were no observed alterations in number of abdominal segments or gross morphology of the embryos.

Creation and Characterization of PUAST-NQ1 PUAST-ΔNQ1 Transgenic Lines

Because the pUASP construct has been reported to show lower levels of expression in larvae and adults than the pUAST vector, I created two new constructs, one containing NQ1 in a pUAST vector and another construct with a deletion of NQ1's longest Q run, as described in the previous chapter (ΔNQ1) and (deltaNQ1). About 500 independent lines containing the NQ1 region of Pumilio or the ΔNQ1 region of Pumilio tagged with CFP under the control of UAS were generated. About 200 of these lines (100 of NQ1 and 100 of delta NQ1) were mapped to the appropriate chromosomes and have been balanced. Some lines seem to have multi-colored eyes, suggesting multiple insertions of the plasmid. All balanced lines were analyzed for CFP expression by crossing to elav, which drives expression in all postmitotic neurons, and to elav-GAL4;24B-

GAL4, a double driver that includes both a post mitotic neuronal driver and a muscle driver, and looking at early stage embryos under a fluorescent microscope. They were categorized on a scale from 1-10, with 10 being the brightest. Adults from these 200 balanced lines were analyzed in order to observe eye color phenotypes. All lines were also crossed to GMR-GAL4, a driver that expresses in all cells of the eye, including neurons and supporting cells (Ellis et al., 1993), or Tub-GAL4 (Lee and Luo, 1999), a ubiquitous GAL4 driver, to look for possible rough eye phenotypes. Only very slight rough eye phenotypes were observed. A subset of lines (some with the brightest cfp, darkest eye color, or rough eyes) were analyzed further by crossing to c155-GAl4;24B-GAL4, c155-GAL4;elav-GAL4, a strong postmitotic neuronal double driver, and 24BGAL4, and dissecting third instar larvae. NMJ larval phenotypes of promising lines were analyzed.

A Domain of Pumilio Shows an Increase in 1s and 1b Boutons When Overexpressed in Muscles in a Pum Heterozygote Mutant Background

Various different mutant alleles of Pum were used to determine if decreasing the expression of full-length Pum might have an impact on results observed in these experiments. Msc, et7, and et9 are 3 different mutant alleles of *pum* that were used. et7 and et9 are both mutations caused by ethane methane sulfonate (EMS) induction that results in premature stops in the RBD region of Pum. Msc, on the other hand, is an inversion containing a breakpoint in

the eighth intron of *pum*. Strong phenotypes of each of these lines have been observed in the early embryo, including a decrease in abdominal segmentation (Forbes and Lehmann, 1998). Msc revealed a weaker phenotype at the larval NMJ (Menon et al., 2004). Furthermore, both et7 and et9 mutant alleles still produced protein that stained with anti-PumN, a rabbit antibody made against the N-terminal region of Pum from amino acids 408-883 (Forbes and Lehmann, 1998) and anti-PumRBD, a mouse antibody made against the RBD region of Pum from amino acids 1093-1533 (Sonoda and Wharton, 1999). Although there was a visible decrease in antibody staining in et7/et9 lines when stained with anti-PumN, this decrease was less strong when anti-PumRBD was used (Menon et al, 2004). In addition, in Western blot analysis, the two larger wild-type Pumilio isoforms are decreased in lysates produced from et7/et9 larvae body walls; however, the third and smallest, 93kD isoform, is increased (Menon et al., 2004). This data implies that the et7 and et9 lines are still producing a protein

Two types of boutons were analyzed in this study. Type 1b boutons are larger boutons that stain much more strongly with a specific post-synaptic antibody α-Discs-large (dlg). Type 1s boutons are smaller and have a weaker dlg staining pattern (Figure 1a and 1b). It has been shown that a significant increase in type 1s boutons occurs in et7/et9 lines and that this increase can be rescued only by expression of Pum in muscle, with a weak muscle driver to avoid lethality. In contrast, a decrease in 1b boutons was observed in et7/et9 lines, and this phenotype could only be rescued by driving neuronal expression of Pum

with elav. This data implies that the neurons and muscles have different impacts on the bouton numbers. Different UAS-NQ1 lines were crossed to 24B-GAL4 and grown at 29 degrees. Live third instar larvae were dissected, fixed, and stained with anti-Discs-large (dlg), a mostly post-synaptic marker that recognizes a protein located under each bouton at the subsynaptic reticulum, and a Tetramethyl Rhodamine Isothiocyanate (TRITC), a derivative of rhodamine) conjugated anti-HRP antibody, that recognizes a neural specific carbohydrate antigen in *Drosophila* and other insects. Boutons on muscle 4 were counted. Control lines which contained w118 x 24B-GAL4 and w118 x UAS-NQ1 were also analyzed, as well as UAS-ΔNQ1 x 24B-GAL4. There was no significant difference in 1s or 1b bouton number among any of the lines (Figures 2a and 2b).

UAS-NQ1 lines, w118, and UAS-ΔNQ1 lines were crossed to et9,24B-Gal4/tm6b recombinant lines (Menon et al., 2004), stained as above, and boutons were counted. A highly significant increase in both type 1s and type 1b boutons was observed (Figures 1, 2A, and 2B, and data not shown). An increase in both type 1a and type 1b were also observed when UAS-NQ1 lines were crossed to msc,24B-Gal4/tm6b (data not shown). UAS-ΔNQ1 lines showed no significant change in bouton counts for either type 1s or type 1b boutons and were comparable to controls (Figures 1, 2A, and 2B and data not shown).

Lethality Caused by Full-Length Pumilio Overexpression in Muscle Is Rescued by Overexpression of the NQ1 Region of Pumilio

A UAS-NQ1 line was recombined with UAS-full-length Pum (FLPum) containing either the 3' untranslated region (utr) of *pum* or the 3'utr of tubulin (a strong ubiquitous promoter). Approximately 50 different lines were obtained. Several of these recombinant lines, and a line created from multiple crosses containing UAS-NQ1 and UAS-FLPum on different chromosomes, were crossed to 24B-GAL4 to check for any changes in phenotype. UAS-FLPum containing the 3'utr of *pum* or the the 3'utr of tubulin are lethal when crossed to 24B and grown at 29°C, but escapers are present with UAS-FLPum containing the *pum* 3'utr at lower temperatures (Menon et al., 2004). UAS-NQ1 was able to rescue the observed lethal phenotype at 29°C, with larvae surviving until eclosion. An interesting eclosion phenotype was observed only with the UAS-NQ1, UAS-FLPum lines crossed to 24B. No adult flies could fully eclose from the pupae, yet most flies appeared to survive until the point of eclosion, and some even partially eclosed (Figure 3). This showed a very significant partial rescue, up to the state of eclosion) of the complete lethality (no first instar larvae) observed with the overexpression of Pum in the muscles.

Overexpression of A Domain of Pumilio in Muscles Results in an Increase of GluRIIa

In et7/et9 mutants, GluRIIa levels have been shown to increase, and eIF-4E levels have also been shown to increase. Pumilio binds directly to eIF-4E, a translation factor, mRNA and represses its expression (Menon et al., 2004).

Observed increases in GluRIIa could derive from a direct de-repression of GluRIIa mRNA by Pum, or from the observed increase in eIF-4E because eIF-4E is a limiting agent during translation (Sonenberg and Grings, 1998). We wanted to test to see if this putative loss of function phenotype could be recapitulated by overexpressing NQ1. Overexpression of NQ1 in muscles by crossing UAS NQ1 to 24B-GAL4 showed no significant increase in GluRIIa levels. UAS NQ1 crossed to et9, 24B-GAL4, however, did show an increase in GluRIIa levels comparable to that observed in in et7/et9 (Figure 3). This implies that the overexpression of UAS NQ1 is able to act in a manner similar to *pum* mutant alleles.

Discussion

The overexpression of a domain of Pumilio that is capable of aggregating in vivo in yeast, and recapitulating some of the “prion” phenotypes associated with well-characterized yeast “prions,” is capable of regulating Pum-mediated activities at the *Drosophila* neuromuscular junction synapse, including altering bouton numbers and increasing levels of GluRIIa. Interestingly, these phenotypes are only observed under heterozygote Pum mutant alleles, putative Pum loss of function backgrounds, which have no phenotypes on their own. This implies that there is some type of interaction, not necessarily direct, between endogenous Pum and the overexpression of an aggregate-prone domain of Pum.

It is tempting to hypothesize that the observed data might be accounted for if the overexpression of the NQ1 region of pumilio was aggregating in vivo and slowly seeding the aggregation of endogenous Pum, such that under conditions where there is already a decrease of full-length Pum, phenotypes occur that represent Pum loss of function.

No Abdominal Segmentation Phenotype Observed

The lack of any abdominal segmentation phenotype or other gross morphological phenotype in lines that were clearly overexpressing NQ1 as visualized through CFP expression can be explained in several ways. This may be because NQ1 overexpression was not capable of causing either NQ1 aggregation, or the ability of NQ1 aggregates to catalyze the aggregation of endogenous Pum in Drosophila embryonic cells. The lack of a phenotype could also be attributed to sufficient levels of molecular chaperones to ensure lack of aggregation of a Q/N rich, aggregation-prone protein at such a critical stage for survival. In addition, it could be that there was still sufficient endogenous Pum to carry out hunchback repression, resulting in a normal segmentation pattern. This explanation makes the most sense given the larval phenotypes, and it might be a good idea to revisit this line and look for any abdominal segmentation alterations in an et9 heterozygote background.

NQ1 Overexpression in the Muscles Leads to a Pum Loss of Function Phenotype Resulting in an Increase of Type 1s and Type 1s Boutons

Specifically, type 1s boutons increase when NQ1 is overexpressed in muscles, phenocopying the observed increase in *pum* mutant lines. Given the data that shows that this increase in 1s boutons is only rescued by expressing full-length Pum in muscles and not in neurons, yet observed decreases in type 1b boutons in *pum* mutant lines are only rescued through full-length Pum overexpression in neurons, implies that Pum possesses distinct roles presynaptically when it comes to regulating synaptic growth (Menon et al., 2004). The increase in type 1s boutons that were observed, therefore, may be through a decrease in endogenous FLPum caused by the aggregation seeded by aggregates of overexpressed NQ1 in the muscle. This would mean that the loss of function in this case is only on the muscle side, in contrast to the et7/et9 cross, where there is putatively no wild type Pumilio in any tissue, except for maternal contributions in the embryo. Given the fact that the increase in 1s boutons in et7/et9 is only rescued in the muscles, however, this implies that this observed increase in 1s boutons is due to loss of function from the muscle side. This is not to say that loss of function of Pum at the neuronal level might also lead to an increase in 1s, but that a loss of function, or whatever phenotype conveyed by a lack of wild-type *pum* allele, on the muscle side is sufficient to cause the increase in 1s boutons. We do not yet know whether abnormal Pum expression on the muscle side is necessary for an increase in 1s, but it does not seem to be the

case from preliminary experiments which show that neuronal-only overexpression of NQ1 leads to an increase of type 1s (Salazar, unpublished data). In these experiments, normal levels of Pum are expressed in the muscle because NQ1 is driven only in the neurons.

NQ1 Overexpression in the Muscles Leads to a Pum Loss of Function Phenotype Resulting in an Increase of Type 1b Boutons

Furthermore, it was observed that *Drosophila* larval NMJ type1b boutons decreased under the different *pum* mutant backgrounds studied (Menon et al., 2004). This phenotype, however, seemed to be regulated on the neuronal side. because this phenotype was only rescued when full-length Pum was driven in neurons and not when Pum was driven in muscles. This means that the observed et7/et9 effect on 1b boutons is caused from the neuronal side of the synapse. At first glance, the data presented here might seem to contradict the et7/et9 data observed by Menon, but in the case of the data presented here, there are no large forced genetic changes at the neuronal side of the synapse because everything is being driven in the muscle. There is the heterozygote effect of having et9 instead of one wild-type copy of *pum* on both the neuronal and muscle side, but there are no observed changes in boutons with this line. It is only when NQ1 is overexpressed, in this case on the muscle side, that a phenotype occurs. The increase in 1b, therefore, could be explained by the possibility that in the case where two copies of mutant *pum* are not present in the

neuron, and a putative loss of function is driven only in the muscles, numbers of type 1b boutons increase.

The observed increase in type 1b boutons also makes sense if one considers eIF-4e data. It has been observed by two groups that an overexpression of eIF-4e in the muscle results in an increase in type 1b boutons (Sigrist et al, 2000, Menon et al., 2004). Since it has also been observed that overexpression of Pum in the muscle can rescue this phenotype, this data implies that muscle expression of Pum does impact levels of 1b. Since an increase in eIF-4e levels are observed in et7/et9 crosses, and Pum directly represses eIF-4e levels (Menon et al., 2004), this implies that a loss of function of Pum on the muscle side may be responsible for the increase in type 1b boutons. Since more eIF-4e arises when there is less Pum available and more type 1b boutons occur when there is an increase of eIF-4e, observed increases in type 1b presented here make sense if NQ1 overexpression in the muscle is causing a decrease in Pum repression of eIF-4e.

A caveat to these hypotheses is the fact that we do not know the exact nature of the proteins being made by these pum mutants. For example, we know that both et7 and et9 produce a protein, albeit more of the smaller isoform of Pum normally observed on Western blots, but we do not yet know the exact nature of the protein. This is especially true considering that when using the α-PumN antibody, a greater decrease of Pum is observed with the et7/et9 larvae than when using the α-PumRBD antibody. Given the locations of the EMS

mutation, this data is slightly confusing, unless the variance is caused by antibody quality, a reasonable possibility. If, however, the proteins being made are just not completely functional but still retain the various Q regions, one might expect that these proteins may be capable of forming aggregates themselves, although overexpression may be necessary to initiate the formation of aggregates. Furthermore, it could be that the putative loss of function phenotypes are actually a gain-of-function caused by the presence of the proteins made from these alleles. This hypothesis, however, is not backed up by the observation that any of these alleles over +, in other words, heterozygote expression in the presence of one good copy of Pumilio does not show any changes in 1b or 1s, while the Pum mutant alleles expressed as a heterozygote over deficiency do have a phenotype. If the observed phenotypes were due only to a gain of function of the Pum mutant allele, this observation would not make sense because both crosses have only one mutant allele, yet phenotypes differ depending on whether the flies contain one other copy of *pum*. The only other explanation for this observation is that there is another gene in the various deficiency lines used that genetically interacts with Pum, or is involved in decreasing its ability to act in a gain of function manner. This seems fairly unlikely, however. It therefore seems, from this argument as well as from unpublished RNAi data, that the observed phenotypes are caused by a loss of function of Pum and that loss of function in either the neuron, the muscle, or both may result in different phenotypes.

NQ1 Overexpression in the Muscles Leads to a Pum Loss of Function Phenotype Resulting in an Increase of GluRIIa

GluRIIa levels are increased in a manner that is similar to et7/et9, when one wild-type copy of *pum* is replaced with et9, and NQ1 is driven in the muscles. This data can also be explained by a loss of function of Pum in muscles caused by the overexpression of NQ1. This also phenocopies previous data showing that GluRIIa levels increase in a Pum loss of function background (Menon et al. 2004) because Pum may either directly regulate GluRIIa levels, or eIF-4e could regulate GluRIIa levels. Under conditions when there is an increase in eIF-4e, it is hypothesized that more transcripts, possibly including GluRIIa, can be translated because eIF-4e is one of the limiting factors for translation (Sonenberg and Gringras, 1998). Again, this data can be explained by the possibility that the overexpression of NQ1 in muscles results in the aggregation of the NQ1 domain of Pum, which then is capable of seeding the aggregation of endogenous Pum, resulting in a loss of Pum function phenotype that causes a decrease in Pum repression of its targets, resulting in an increase of GluRIIa.

Overexpression of Both NQ1 and FL-Pumilio in Muscles Partially Rescues Overexpression of FL-Pumilio Lethality

Overexpression of both NQ1 and FL-Pum in muscles results in a phenotype where the embryos not only hatch and form larvae, in contrast to

overexpression of Pum with 24B, but they survive until the moment of eclosion, but cannot make it out of their pupae. This observation also implies that an interaction exists between NQ1 and Pum, with NQ1 ameliorating the deleterious effect of overexpressing Pum. The exact cause of the lethality when overexpressing Pum is not known. Given Pum's crucial role in early embryonic development, however, too much Pum may result in the over-repression of genes that are necessary for survival.

Another interesting hypothesis is that overexpressing Pum actually initiates self-aggregation and the resulting lethality is actually caused by aggregates of some type or the loss of function of Pum, which is believed to be toxic. Furthermore, overexpressing the RBD domain of Pum is capable of rescuing early embryonic phenotypes (Barker, 1992), perhaps implying that the RBD alone is sufficient for the translational repression activity of Pum. Also, overexpressing the RBD region alone may not be as lethal as overexpressing the FL-Pum. Even so, overexpressing the RBD region in neurons is not capable of rescuing the same the decrease in type 1b boutons observed in et7/et9, while overexpressing FL-Pum could rescue the phenotype (Menon et al., 2004). This means that the RBD is not capable of recapitulating Pum overexpression phenotypes in neurons, implying that the N-terminal part of Pum does play a role in the function of Pum. This means that the differing level of lethality observed when overexpressing either Pum or the RBD domain are most likely caused by differences in function and not differences in the ability to aggregate.

Furthermore, if Pum toxicity was a result of its ability to aggregate, thereby leading to a loss of function of Pum, or leading to the toxic effects of unnatural aggregate formation, then one might expect that NQ1 overexpression would be even more lethal if it could further seed the aggregation of Pum. Instead, it partially rescues the phenotype, perhaps because it is involved in decreasing the availability of Pum, with a normal conformation produced by the overexpression of FL-Pum, for repression. In other words, NQ1 decreases levels of functional Pum to a level that is less toxic than when FL-Pum is overexpressed. This could imply that the overexpression of FL-Pum does not aggregate on its own and that a seed is required for this to occur, or it could imply that the rate of FL-Pum aggregation is much slower with being seeded by the overexpression of NQ1 and that the embryos die prior to aggregation causing a sufficient decrease in overexpressed FL-Pum. Although this data does not show that NQ1 is aggregating, or that NQ1 aggregates result in a decrease of FL-Pum in its natural conformation, and, therefore, a decrease in the abnormal repression possibly caused by overexpressing FLPum, the data does show that an interaction between Pum and NQ1 is occurring. It is not necessarily direct, however.

The original purpose of this project was to attempt to discover a conserved protein found in both *Drosophila* and *C. elegans*, and, hopefully, higher organisms that could act like a genetic switch. We have shown that Pumilio is capable of aggregating in yeast prion assays and can induce the formation of the aggregated state of Sup35 to the same degree as the New1PrD yeast prion in

the assays. In addition, the overexpression of a domain of Pum has been shown to phenocopy muscle loss of function of Pum in an et9 background. This is observed through an increase in type 1s and type 1b boutons at the larval NMJ, as well as an increase in GluRIIa expression at the NMJ. In addition, the aggregation-prone domain of Pum has been shown to rescue the embryonic lethality caused by overexpression of Pum in the muscles. This data shows that there is a genetic interaction between endogenous Pum and the overexpressed NQ1 domain. This could imply an epigenetic activity if this observed interaction is caused only through the overexpression of NQ1. Whether the observed data corresponds to a direct interaction and aggregation in vivo in Drosophila, is yet to be determined. In addition, even if this domain does cause the aggregation of endogenous Pum, and, hence, the loss of function phenotypes observed in this study, we have yet to address whether Pum actually makes use of this ability to form aggregates normally. Even if Pum does not make use of this ability to form ordered aggregates in order to carry out its function, it is intriguing to hypothesize that other proteins that aggregate and cause diseases, such as Aβ, may either seed the abnormal aggregation of Pum or of other proteins located at the synapse that possess either Q/N rich regions or a proclivity to form aggregates; this might be responsible for the observed toxicity associated with Aβ42 oliogmers in cultured cells and in vivo in the rat. Neurotoxic aggregates do not even have to actually seed the aggregation of other proteins. These toxic Aβ oligomers might, instead, just bind to these Q/N rich regions and perturb normal

function. Whether endogenous Pum turns out to make use of an epigenetic switch in order to mark synapses for long term memory perhaps through directly increasing GluRIIa expression therefore, is not the only intriguing scientific question to come out of this work, but also whether Pum and other putative aggregate prone proteins located at the synapse might play a role in Aβ42 toxicity. In addition, this work might not just be important in the study of Alzheimer's Disease, but it may be able to spread light on neurodegenerative disorders that involve protein aggregation in general, by providing a possible model system for the screening for modifiers of some of the phenotypes observed with the overexpression of NQ1.

Experimental Procedures

Genetics

Wild-type controls were created when w1118 was crossed to UAS-NQ1, UAS-ΔNQ1, 24B, et9,24B/TM6B or msc,24B/TM6B, depending on the experiment. In addition, GMR-Gal4 was crossed to w118 as a control for rough eye phenotypes. UAS-NQ1 (from pUASP), UAS-NQ1 (from pUAST), and UAS-ΔNQ1 were created by standard techniques. Maternal drivers were obtained from Bloomington Stock Center. UAS-NQ1 was recombined with both UAS-*pum-pum* 3'ut*r* and UAS-*pum-tubulin* 3'utr. Most crosses were conducted at 29°C, except for et7/et9 because GAL4 expression is strongest at 29°C, but et7/et9 defects are strongest at 18°C. UAS-NQ1 crosses to maternal drivers were first

conducted at 3 temperatures, 18°C, 25°C, and 29°C to determine which temperature might show the strongest phenotypes. Subsequent crosses were conducted at 29°C.

Cuticle preparations

Cuticle preparations were conducted by standard procedures. Embryos were grown until full development, then removed from the chorions and membranes and mounted under coverslips in a mixture of lactic acid/Hoyer's medium and incubated overnight at 60°C. These slides were then analyzed by microscopy.

Antibodies and Imunocytochemistry

The following antibodies were used in these experiments: rat anti-Pum RBD (Sonada and Wharton, 1999), mouse anti-Dlg (from Corey Goodman), mouse anti-GluRIIa (from Developmental Studies Hybridoma Bank), and TRITC conjugated HRP (from Jackson Labs). Secondary antibodies used were: Alexa Fluor 488 anti-mouse (from molecular Probes) and Alexa Fluor 568 anti-rat (from Molecular Probes).

Third instar larvae were dissected in PBS and fixed in 4% paraformaldehyde or Bouin's fix (Sigma).

Confocal Microscopy

The Zeiss LSM 510 inverted microscope was used for the creation of final images. Appropriate lasers were used to excite the fluorescently tagged antibodies at either 488 or 568. A 63x oil immersion objective was used, and projected z-stacks with intervals of .5 μm were obtained for final images. Muscle 4 from the second and third segments of each larva was imaged and counted, resulting in four hemi-segments per larva.

Acknowledgements

We would like to thank Kaushiki Menon for fly lines, antibodies, protocols, technical assistance, and intellectual discussions and input; Elena Armand for assistance with fly maintenance and larval dissections; Violana Nesterova for administrative assistance, lab management, fly maintenance, assistance with figures, and much more; Lisa Nesterova for fly maintenance and general experimental assistance; Carlos Diaz-Balzac for assistance with pUASP fly lines; Robin Wharton for anti-Pum antibody; Yoshiaki Kidokoro for GluRIIa antibodies; Chris Waters for confocal maintenance; Zinn lab members both current and past; Sean Brennan for graphic assistance and good music; Ashley Wright, Nina Tang-Sherwood, Alice Schmid, and Nicki Fox for intellectual conversations and general experimental assistance.

Figure Legends

Figure 1a. *Drosophila* NMJ boutons on muscle 4 of the second or third abdominal segments of third instar larvae. Different UAS-NQ1 lines were crossed to 24B-GAL4 and grown at 29°C. Live third instar larvae were dissected, fixed for 20 minutes and stained with anti-dlg (green), a post-synaptic marker, and TRITC-conjugated anti-HRP (red), a neuronal marker. (A-I) Control lines containing w1118/24B-GAL4 and w118/+; ET9, 24B/+. They have fewer type 1s boutons (arrowhead) and sometimes 0 type 1s boutons (See D-I). They also have fewer type 1b boutons (arrows). (J-L) UAS-NQ1/+; ET9, 24B/+. A dramatic increase in both type 1s (arrowheads) and type 1b (arrows) is observed.

Figure 1b. (A-C) A different UAS NQ1 line than the one shown in Figure 1a, but containing the same drivers and background: UAS-NQ1/+; ET9, 24B/+. (D-I) Two different lines of delta-NQ1: UAS-NQ1/+; et9, 24B/+. No type 1s boutons (arrowheads) are observed in these segments and few type 1b (arrows). (J-L) A higher magnification view of the same NMJ in (J-L) from Figure 1a.

Figure 2a. Type 1s Bouton Counts are shown. The bars correspond to the average number of boutons per hemi-segment. Only muscle 4 from segments 2 and 3 were used. Error bars show standard error. The only significant difference between amounts of 1s boutons and the control are for the two tallest bars, which are highly significant with p-values < .0001 (student's t test). Those bars

correspond to UAS-NQ1/+; et9,24B-GAL4/+. The genotypes and n are as follows: UAS-NQ1/w1118 (n=9), w1118/24B-GAL4 (n=34), UAS-NQ1/24B-GAL4 (n=13), UAS-NQ1/24B-GAL4 (n=9), UAS-deltaNQ1/24B-GAL4 (n=24), UAS-deltaNQ1/24B-GAL4 (n=16), w1118/+; et9, 24B-GAL4/+ (n=34), UAS-NQ1/+; et9,24B-GAL4/+ (n=39), UAS-NQ1/+; et9,24B-GAL4/+ (n=15), UAS-deltaNQ1/+; et9,24B-GAL4/+ (n=17).

Figure 2b. Type 1b Bouton Counts are shown. The bars correspond to the average number of boutons per hemi-segment. Only muscle 4 from segments 2 and 3 were used. Error bars show standard error. The only significant difference between amounts of type1b boutons and the control are for the two tallest bars, which are highly significant with p-values < .0001 (student's t-test). Those bars correspond to UAS-NQ1/+; et9,24B-GAL4/+. The genotypes and n are as follows: UAS-NQ1/w1118 (n=9), w1118/24B-GAL4 (n=34), UAS-NQ1/24B-GAL4 (n=13), UAS-NQ1/24B-GAL4 (n=9), UAS-deltaNQ1/24B-GAL4 (n=24), UAS-deltaNQ1/24B-GAL4 (n=16), w1118/+; et9, 24B-GAL4/+ (n=34), UAS-NQ1/+; et9,24B-GAL4/+ (n=39), UAS-NQ1/+; et9,24B-GAL4/+ (n=15), UAS-deltaNQ1/+; et9,24B-GAL4/+ (n=17).

Figure 3. (A) Average number of *Drosophila* that survive until the pupal stage after 24 hour embryo collections. Different recombinant lines and balanced lines that drive the overexpression of NQ1 in the muscle were analyzed for their ability to rescue embryonic lethality caused by the overexpression of Pum in the

muscles. Two different UAS-Pum lines were used, one that contained the 3'utr of Pum and one that contained the 3'utr of Tubulin, which is a stronger line. The Pum 3'utr line has escapers when grown at 18 or 25 degrees, while the Tub 3'utr line does not. A dramatic rescue was observed, from 0 larvae and pupae in the UAS-Pum crosses, to at least 15 per vial per 24 hour collection, comparable to w1118/24B. 8 one-week-old virgin 24B-GAL4 females were crossed to 5 males and allowed to grow for 28 hours on green vial food prior to initiating the collection period. The embryos were collected by transferring the adults to new vials and keeping the vials at 29 degrees for 9 days to analyze the phenotype. Lines were transferred over 10 consecutive days at noon. The following lines were used: UAS-NQ1/+; UAS-Pum-Pum3'utr/24B-GAL4, UAS-NQ1,UAS-Pum.Pum3'utr/24B-GAL4, UAS-NQ1,UAS-Pum-Tub3'utr/24B-GAL4, UAS-NQ1,UAS-Pum-Tub3'utr/24B-GAL4, UAS-Pum-Pum3'utr/24B-GAL4, UAS-Pum-Tub3'utr/24B-GAL4, w1118/24B-GAL4. No larvae were consistently found alive in the numerous crosses made with UAS-Pum and 24B-GAL4 when this cross was grown at 29°C.

(B) Percent of *Drosophila* that did not eclose from their pupae. A partial rescue was observed when NQ1 was overexpressed at the same time as UAS-Pum in muscles. Numbers of larvae were almost comparable to the w1118/24B-GAL4 control. Virtually none of these larvae, however, were able to fully develop into adult flies and eclose from their pupae. It did appear that the flies fully form, but

that they could not climb out of their pupae. Many of progeny of this cross could not fully emerge from their pupal cases and died, resulting in a large number of partially eclosed flies. This lack of pupal emergence phenotype was highly significant with p-values << .0001 (student's t-test). Error bars are above the data in the table. Data for the UAS-Pum lines crossed to 24B is not shown because 0 larvae and therefore 0 pupae existed. This phenotype was almost 100% penetrant in all lines analyzed.

Figure 4. GluRIIa expression is increased in a manner similar to et7/et9. Control lines used in this experiment include w1118/24BGAL4 or w1118/+; et9,24B/+. No significant increase in GluRIIa staining was observed in either of these lines. Panels (A-C) show the anti-GluRIIa staining (green), anti-HRP staining (red), and merge of one of the controls. Lines were grown at 29°C. Panels (D-F) show an increase above wild-type of GluRIIa staining in crosses overexpressing NQ1: UAS-NQ1/+; et9,24B-GAL4/+ . (G-I) et7/et9 was used as a positive control.

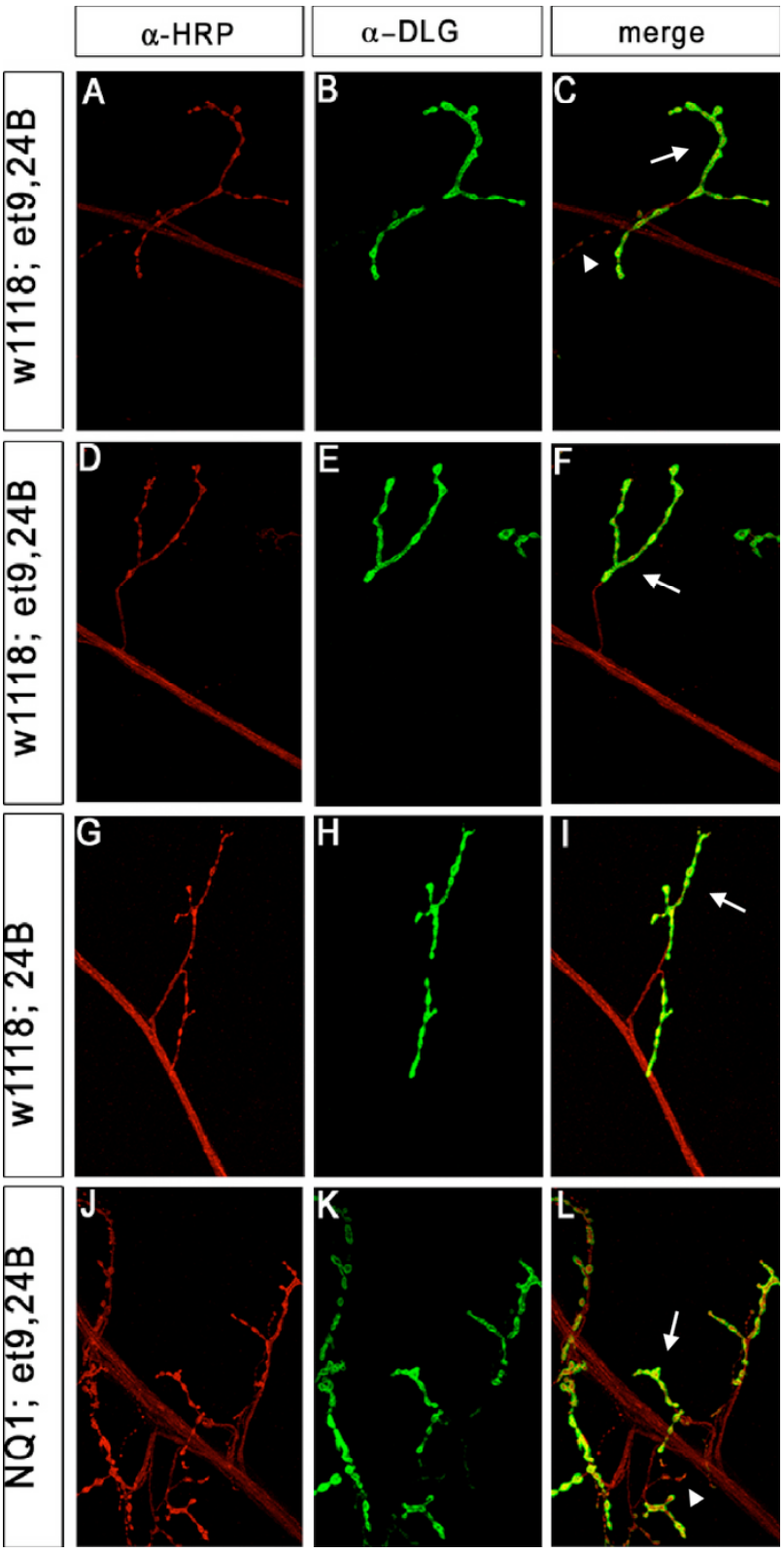

Figure 1a. NMJ Boutons

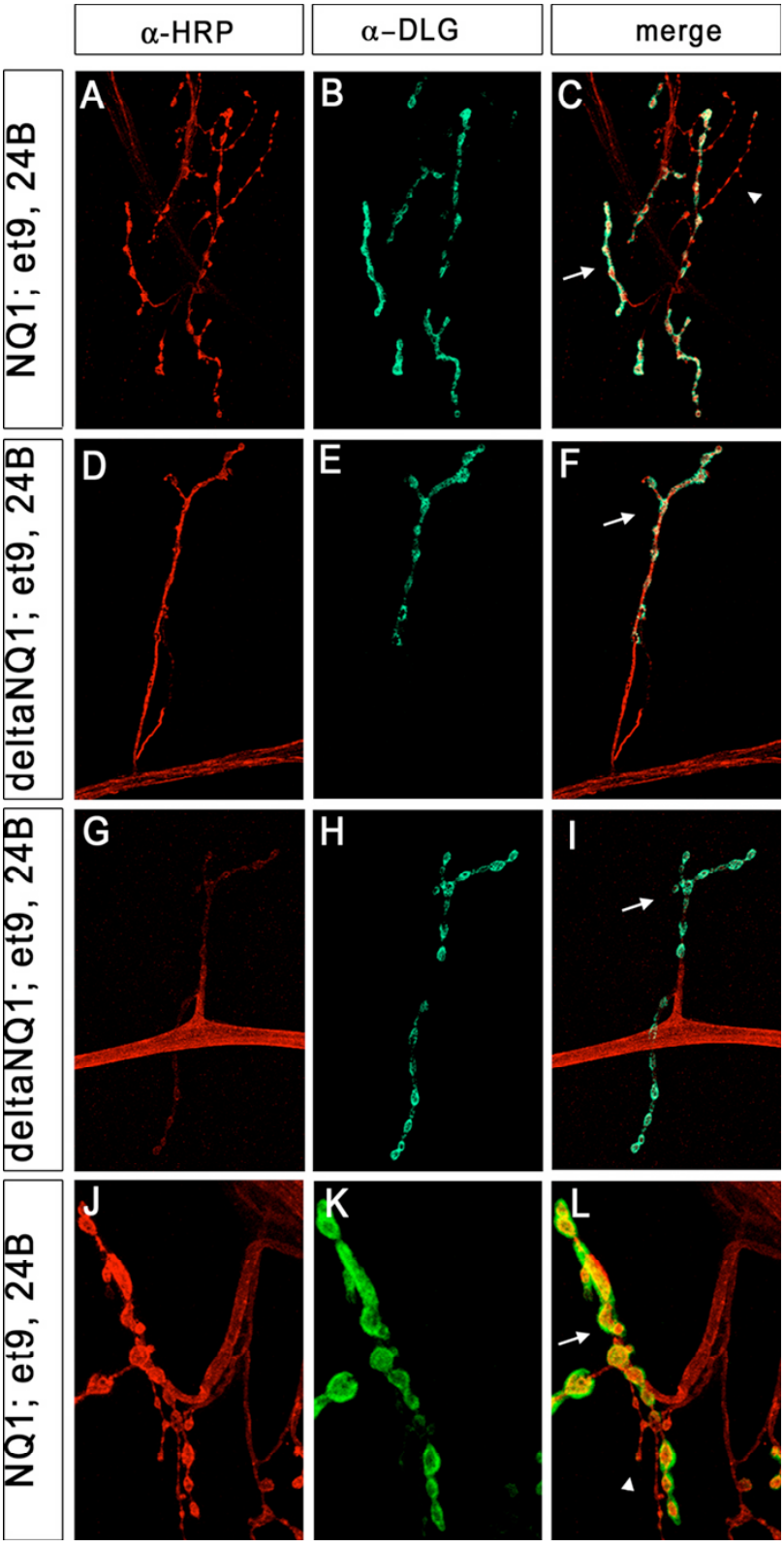

Figure 1b. NMJ boutons

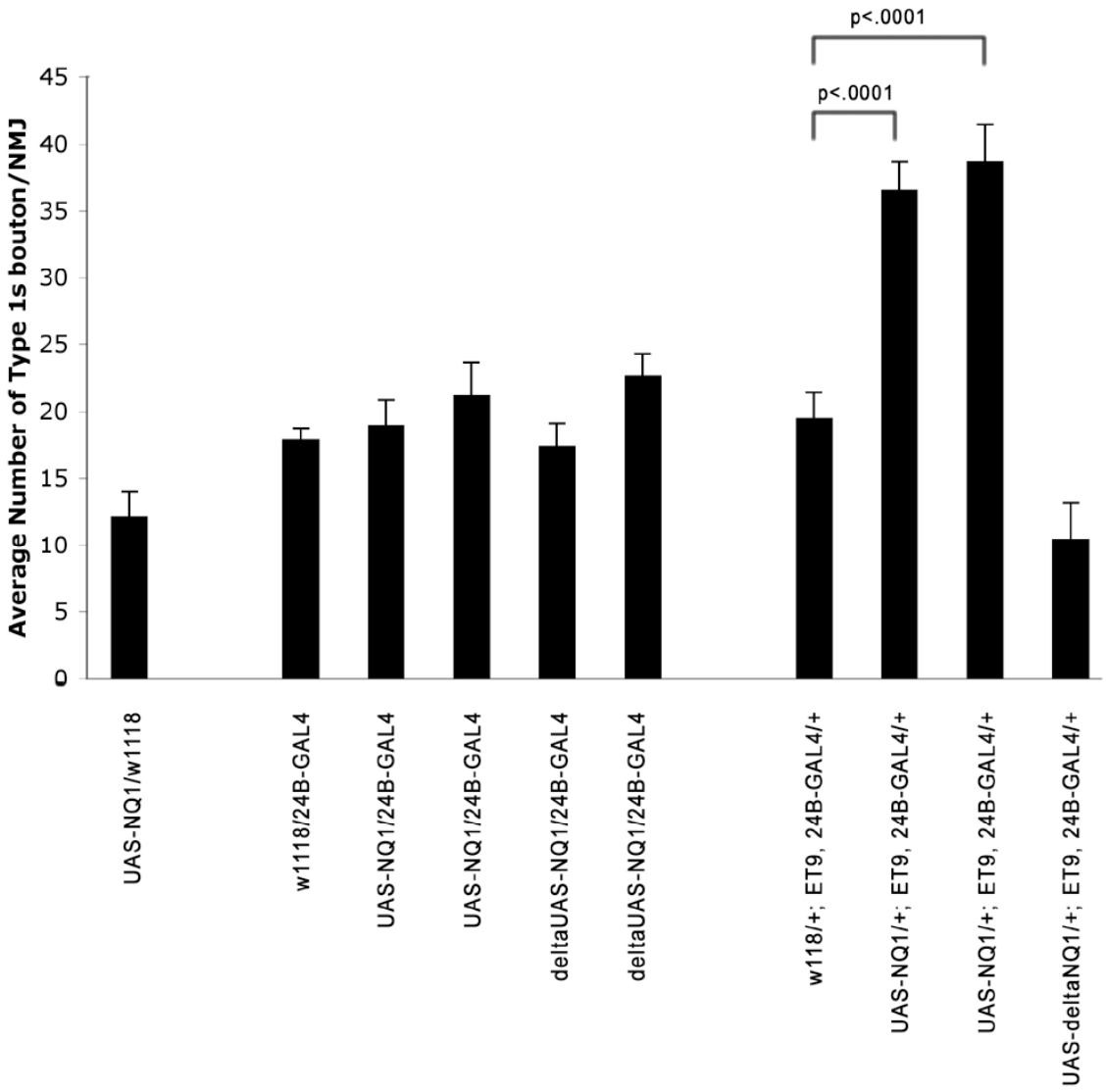

Figure 2a. Type 1s Bouton Counts

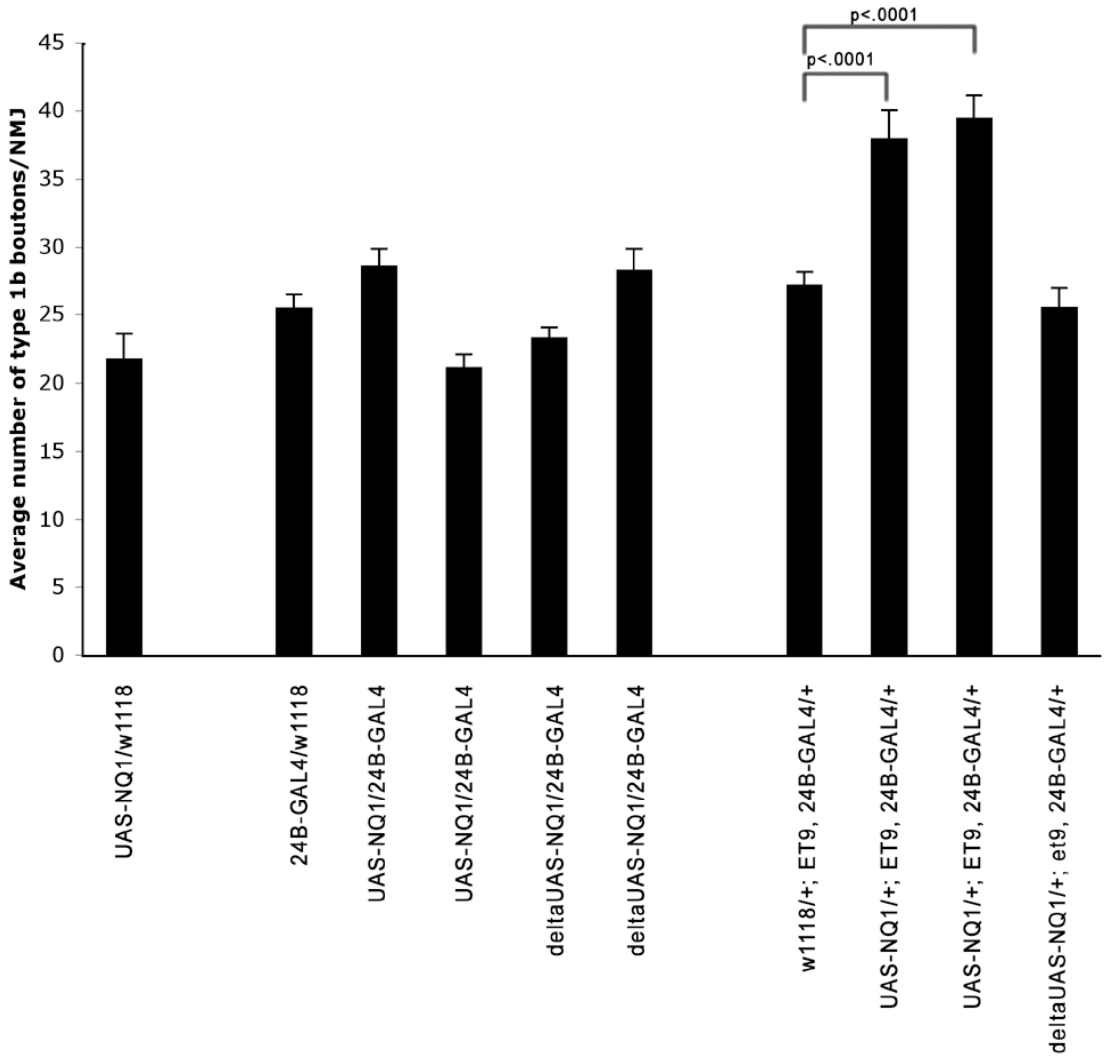

Figure 2b. Type 1b Bouton Counts

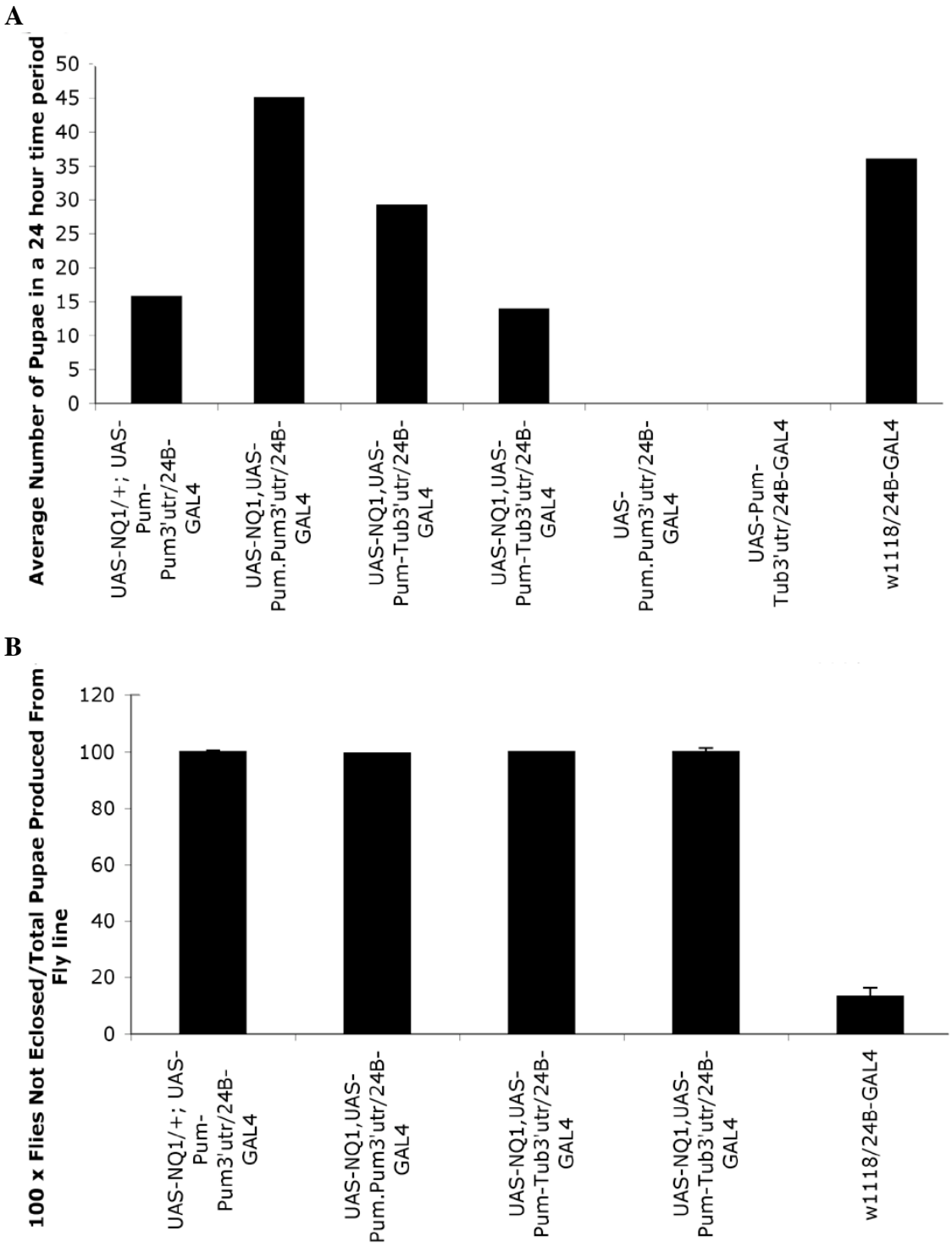

Figure 3. (A) Total Number of Pupae After 24 hours in a Vial

(B) Percent of Flies That Do Not Eclose

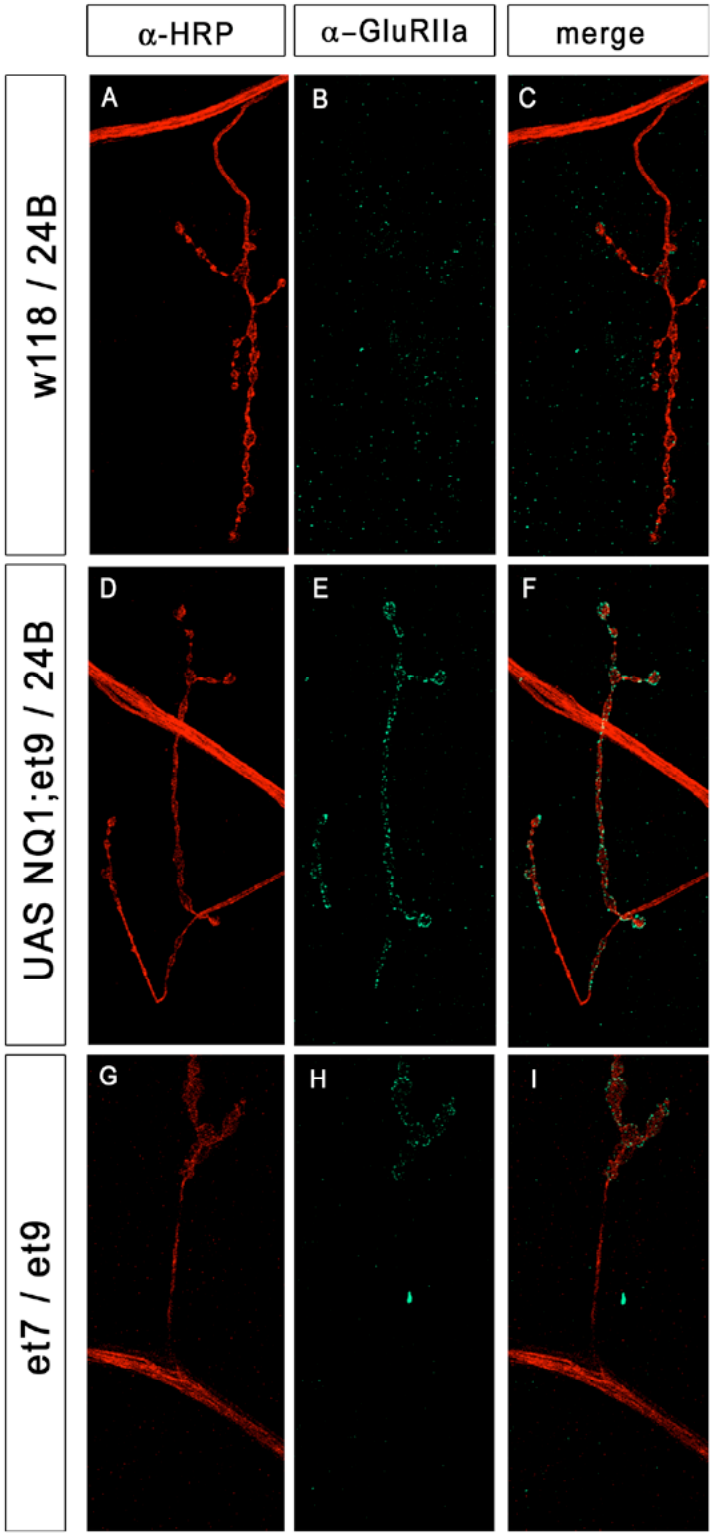

Figure 4. GluRIIa expression is increased in a manner similar to et7/et9

References

Barker, D. D., Wang, C., Moore, J., Dickinson, L. K. and Lehmann, R., 1992. Pumilio is essential for function but not for distribution of the Drosophila abdominal determinant Nanos. Genes Dev. 6, 2312-2326.

Dubnau, J., Chiang, A. S., Grady, L., Barditch, J., Gossweiler, S., McNeil, J., Smith, P., Buldoc, F., Scott, R., Certa, U., Broger, C. and Tully, T., 2003a. The staufen/pumilio pathway is involved in Drosophila long-term memory. Curr Biol. 13, 286-296.

Dubnau, J., Chiang, A. S. and Tully, T., 2003b. Neural substrates of memory: from synapse to system. J Neurobiol. 54, 238-253.

Eaton, B. A. and Davis, G. W., 2003. Synapse disassembly. Genes Dev. 17, 2075-2082.

Edwards, T. A., Pyle, S. E., Wharton, R. P. and Aggarwal, A. K., 2001. Structure of Pumilio reveals similarity between RNA and peptide binding motifs. Cell. 105, 281-289.

Ellis, M. C., O'Neill, E. M. and Rubin, G. M., 1993. Expression of Drosophila glass protein and evidence for negative regulation of its activity in non-neuronal cells by another DNA-binding protein. Development. 119, 855-865.

Forbes, A. and Lehmann, R., 1998. Nanos and Pumilio have critical roles in the development and function of Drosophila germline stem cells. Development. 125, 679-690.

Gavis, E. R. and Lehmann, R., 1994. Translational regulation of nanos by RNA localization. Nature. 369, 315-318.

Keshishian, H., Broadie, K., Chiba, A. and Bate, M., 1996. The drosophila neuromuscular junction: a model system for studying synaptic development and function. Annu Rev Neurosci. 19, 545-575.

Kraut, R., Menon, K. and Zinn, K., 2001. A gain-of-function screen for genes controlling motor axon guidance and synaptogenesis in Drosophila. Curr Biol. 11, 417-430.

Lee, T., Lee, A. and Luo, L., 1999. Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. Development. 126, 4065-4076.

Lehmann, R. and Nusslein-Volhard, C., 1991. The maternal gene nanos has a central role in posterior pattern formation of the Drosophila embryo. Development. 112, 679-691.

Macdonald, P. M., 1992. The Drosophila pumilio gene: an unusually long transcription unit and an unusual protein. Development. 114, 221-232.

Martin, K. C. and Zukin, R. S., 2006. RNA Trafficking and Local Protein

Synthesis in Dendrites: An Overview. J Neurosci %R 101523/JNEUROSCI1801-062006. 26, 7131-7134.

Menon, K. P., Sanyal, S., Habara, Y., Sanchez, R., Wharton, R. P., Ramaswami, M. and Zinn, K., 2004. The translational repressor Pumilio regulates presynaptic morphology and controls postsynaptic accumulation of translation factor eIF-4E. Neuron. 44, 663-676.

Parisi, M. and Lin, H., 1999. The Drosophila pumilio gene encodes two functional protein isoforms that play multiple roles in germline development, gonadogenesis, oogenesis and embryogenesis. Genetics. 153, 235-250.

Purves, D. and Lichtman, J. W., 1980. Elimination of synapses in the developing nervous system. Science %R 101126/science7414326. 210, 153-157.

Reiter, L. T., Potocki, L., Chien, S., Gribskov, M. and Bier, E., 2001. A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. Genome Res. 11, 1114-1125.

Rorth, P., 1998. Gal4 in the Drosophila female germline. Mech Dev. 78, 113-118.

Rorth, P., Szabo, K., Bailey, A., Laverty, T., Rehm, J., Rubin, G. M., Weigmann, K., Milan, M., Benes, V., Ansorge, W. and Cohen, S. M., 1998. Systematic gain-of-function genetics in Drosophila. Development. 125, 1049-1057.

Rubin, G. M., Yandell, M. D., Wortman, J. R., Gabor Miklos, G. L., Nelson, C. R., Hariharan, I. K., Fortini, M. E., Li, P. W., Apweiler, R., Fleischmann, W.,

Cherry, J. M., Henikoff, S., Skupski, M. P., Misra, S., Ashburner, M., Birney, E., Boguski, M. S., Brody, T., Brokstein, P., Celniker, S. E., Chervitz, S. A., Coates, D., Cravchik, A., Gabrielian, A., Galle, R. F., Gelbart, W. M., George, R. A., Goldstein, L. S., Gong, F., Guan, P., Harris, N. L., Hay, B. A., Hoskins, R. A., Li, J., Li, Z., Hynes, R. O., Jones, S. J., Kuehl, P. M., Lemaitre, B., Littleton, J. T., Morrison, D. K., Mungall, C., O'Farrell, P. H., Pickeral, O. K., Shue, C., Vosshall, L. B., Zhang, J., Zhao, Q., Zheng, X. H. and Lewis, S., 2000. Comparative genomics of the eukaryotes. Science. 287, 2204-2215.

Schuman, E. M., Dynes, J. L. and Steward, O., 2006. Synaptic Regulation of Translation of Dendritic mRNAs. J Neurosci %R 101523/JNEUROSCI1796-062006. 26, 7143-7146.

Schweers, B. A., Walters, K. J. and Stern, M., 2002. The Drosophila melanogaster translational repressor pumilio regulates neuronal excitability. Genetics. 161, 1177-1185.

Si, K., Giustetto, M., Etkin, A., Hsu, R., Janisiewicz, A. M., Miniaci, M. C., Kim, J. H., Zhu, H. and Kandel, E. R., 2003a. A neuronal isoform of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in aplysia. Cell. 115, 893-904.

Si, K., Lindquist, S. and Kandel, E. R., 2003b. A neuronal isoform of the aplysia CPEB has prion-like properties. Cell. 115, 879-891.

Sonoda, J. and Wharton, R. P., 1999. Recruitment of Nanos to hunchback mRNA by Pumilio. Genes Dev. 13, 2704-2712.

Sonoda, J. and Wharton, R. P., 2001. Drosophila Brain Tumor is a translational repressor. Genes Dev. 15, 762-773.

Steward, O. and Levy, W. B., 1982. Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. J Neurosci. 2, 284-291.

Sutton, M. A., Ito, H. T., Cressy, P., Kempf, C., Woo, J. C. and Schuman, E. M., 2006. Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. Cell. 125, 785-799.

Sutton, M. A., Wall, N. R., Aakalu, G. N. and Schuman, E. M., 2004. Regulation of Dendritic Protein Synthesis by Miniature Synaptic Events. Science %R 101126/science1096202. 304, 1979-1983.

Vessey, J. P., Vaccani, A., Xie, Y., Dahm, R., Karra, D., Kiebler, M. A. and Macchi, P., 2006. Dendritic Localization of the Translational Repressor Pumilio 2 and Its Contribution to Dendritic Stress Granules. J Neurosci %R 101523/JNEUROSCI0649-062006. 26, 6496-6508.

Wharton, R. P., Sonoda, J., Lee, T., Patterson, M. and Murata, Y., 1998. The Pumilio RNA-binding domain is also a translational regulator. Mol Cell. 1, 863-872.

Ye, B., Petritsch, C., Clark, I. E., Gavis, E. R., Jan, L. Y. and Jan, Y. N., 2004. Nanos and Pumilio are essential for dendrite morphogenesis in Drosophila peripheral neurons. Curr Biol. 14, 314-321.

Zamore, P. D., Bartel, D. P., Lehmann, R. and Williamson, J. R., 1999. The PUMILIO-RNA interaction: a single RNA-binding domain monomer recognizes a bipartite target sequence. Biochemistry. 38, 596-604.