CHARACTERIZATION OF RanBPM IN DROSOPHILA MELANOGASTER

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Abstract

RanBPM is a conserved putative scaffold protein of unknown function. Loss-of-function in RanBPM leads to pleiotropic phenotypes such as reduced locomotion, decreased size and larval lethality in the Drosophila melanogaster.

dRanBPM mutants have decreased branching and boutons at the neuromuscular junction, which may contribute to their locomotory defect. To investigate if dRanBPM is involved in controlling synaptic architecture at the neuromuscular junction, levels of two cytoskeletal proteins, Futsch and profilin, were assessed in dRanBPM mutants.

Due to time constraints, immunoblots for Futsch were not fully optimized for protein measurement. Immunoblots for profilin, on the other hand, were successfully carried out. However, results from the reproduction of a blot demonstrating the negative regulation of Drosophila FMRP on profilin did not agree with that of the literature. In addition, results from an epistatic experiment demostrated that profilin levels were not affected in FMRP deficient flies when compared to those with additional decrease in dRanBPM function.

Mutant dRanBPM phenotypes can be restored back to wildtype by expressing one of two dRanBPM constructs, each believed to encode for either one of dRanBPM isoforms. However, it was demostrated in this thesis that the long construct expresses both short and long dRanBPM isoforms.

Targeted expression of dRanBPM to neurosecretory cells is able to rescue size and lethality of dRanBPM mutants, suggesting a common pathway through which both phenotypes operate is disrupted in these mutants. Activation of the insulin signaling

pathway was indeed found to be downregulated in dRanBPM mutants. A longevity assay was alternatively carried out to demostrate decreased insulin pathway activation in dRanBPM mutants. Unfortunately, due to inappropriate controls used for this experiment, no conclusive points can be made. Together, these findings contribute to the knowledge of the pleiotropic roles that RanBPM plays and to designing future experiments to test for RanBPM function.

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List of Abbreviations

2D	2-dimensional
BDNF	brain-derived neurotrophic factor
bHLH	basic helix loop helix
cDNA	complementary DNA
CNS	central nervous system
CTLH	C-terminal to LisH
CRA	CT11-RanBPM
da-GAL4	daughterless-GAL4
dsRNA	double stranded RNA
FMR1	gene encoding for FMRP
FMRP	Fragile X Mentral Retardation Protein
FOXO	forkhead box sub-class O
FXS	Fragile X Syndrome
GAL4	transcription factor of yeast
GFP	green fluorescent protein
GSC	germline stem cell niche
HGF	hepatocyte growth factor
HRP	horseradish peroxidase
Hsp70-GAL4	heatshock70-GAL4
IPC	insulin producing cell
IIS	Insulin/IGF (Insulin-like Growth Factor)-like Signalling
IR	insulin receptor
IRS	insulin receptor substrates
KC	Kenyon cell
LisH	Lissencephaly type-1-like homology
MAP	microtubule associated protein
MAPK	mitogen-activated protein kinase
MB	mushroom body
NMJ	neuromuscular junction
NSC	neurosecretory cell
RanBP9	Ran Binding Protein 9
RanBPM	Ran Binding Protein in the Microtubule Centre
PDK1	phosphatidylinositol-dependent protein kinase 1
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol-3,4,5-triphosphate
PKB	protein kinase B, also known as Akt
PlacW	a modified P element containing a white+ marker. It is used as a gene disruptor
PNS	peripheral nervous system
PTM	posttranslational modification
RISC	RNA-induced silencing complex

ssRNA	single stranded RNA
TrkB	tropomyosin-related kinase B
TF	transcription factor
TOR	target of rapamycin
SDS	Sodium dodecyl sulfate
SPRY	repeats in sp1A and RyR
UAS	upstream activating sequence

Introduction

Chapter 1

Biology, the study of living things, has a long history. Although the term biology was not coined until 1800 (Colman, 1971), modern study of this field started much earlier, with records dating back to ancient Greece. Aristotle (384-322 BC) established the foundations of the scientific method, emphasizing the need for accurate and unbiased observations, while Hippocrates (460-377 BC) explored healing and health and taught these topics in his school of medicine (Magner, 1979).

More than 2000 years later, biology has exploded into numerous disciplines. From ecology to biochemistry, the breadth of topics these branches cover has expanded enormously. This is particularly true in the areas of cellular research, where leaps in technological advancements have allowed biologists to further explore the fields of developmental biology, molecular biology and genetics.

Despite the accumulation of knowledge gathered over the years, there is still much to be learned. One must appreciate that all living things, no matter how small, are complex in their own right. The level of complexity, however, varies between organisms. In many biological experiments, the use of simpler animals to develop and define general concepts from is a common approach.

1.0 Models for biological studies

Conservation in behaviour and cellular processes shared between all living things is a key factor in deriving information about ourselves from simpler, less complex organisms. Depending on which questions are addressed, different kinds of model organisms and their systems can be used. For example, the simple yeast, Saccharomyces

cerevisiae, has been instrumental in understanding basic cellular processes in eukaryotic cells; Caenorhabditis elegans has established itself as an excellent model for the study of cell lineages; and finally, the mouse has proven to be a valuable animal in elucidating mammalian development and diseases (reviewed by Muller and Grossniklaus, 2010).

1.1 Drosophila as a model organism

Drosophila melanogaster has long served as an important invertebrate model organism in genetic research. It shares a high degree of genetic conservation with that of vertebrates. For example, sixty percent of genes associated with human diseases have homologs in Drosophila (Fortini et al, 2000; Rubin et al, 2000; Reiter et al, 2001).

Drosophila is also genetically powerful itself— the number of molecular and genetic tools available in Drosophila is as of yet, unmatched by any other multicellular organisms (reviewed by Beckingham et al., 2005). Notable genetic tools and their uses include: visible dominant markers to track the segregation of chromosomes in genetic crosses, deficiency chromosomes to perform genetic mapping and complementation tests, and balancer chromosomes to prevent the loss of a given mutation from homologous recombination (reviewed by Beckingham et al., 2005).

An ingenious addition to the Drosophila genetic toolbox is a naturally occurring transposon called P-element (reviewed by Beckingham et al., 2005). This transposon can be modified to introduce a gene of interest, whether it is cloned from Drosophila or from another species, into the Drosophila genome. Depending on the location of its insertion, P-elements may also serve as a mutagen.

The incorporation of yeast elements into the Drosophila genome through the use of P-elements has given rise to a powerful bipartite system for targeted gene expression called UAS/GAL4 (Brand and Perrimon, 1993). This system is composed of a yeast transcription factor, GAL4, and its target, the upstream activating sequence (UAS). Expression of GAL4 is spatially controlled by a tissue specific promoter. Upon binding to UAS, GAL4 induces transcription of the gene fused downstream to it.

A further refinement of the UAS/GAL4 system allows temporal control on spatial gene expression. This is achieved by incorporating a gene encoding for the temperature sensitive yeast protein called GAL80 (GAL80[TS]) into the fly genome. Activity of GAL80[TS] can be controlled at either the permissive temperature of 18°C, where GAL80[TS] binds to GAL4 to suppress its activity, or at the restrictive temperature of 29°C, where GAL80[TS] is unable to function and the gene downstream of the UAS construct is expressed (McGuire et al., 2003).

1.2 Drosophila as a model for behaviour

The mechanisms regulating neurodegeneration (reviewed by Michno et al., 2005) and neurodevelopment (reviewed by Hidalgo and ffrench-Constant, 2003) are similar between Drosophila and humans. For this reason, many neuronal processes such as olfaction, memory (reviewed by Busto et al., 2010 AND Davis, 2011), circadian rhythms and social behaviours including courtship and aggression (reviewed by Sokolowski, 2011) are studied using the fruit fly.

The Drosophila larva possesses a nervous system less structurally complex than that of the adult and provides another valuable model to understand underlying genetic and physiological contributions to behavioural responses. Larval behaviours to food and light have been extensively characterized. Briefly, once a Drosophila larva emerges from the egg, it starts to feed. The larva avoids light by burying itself in the food (Sawin-McCormack et al., 1995; Scantlebury et al., 2010). The time through which this set of behaviour manifests is called the foraging stage and lasts through three developmental periods called instars. Some time after the third instar (84-90 hr after egg lay), the larva enters another stage called wandering where it stops feeding and becomes indifferent to light. At this time, the larva leaves the food to find a place to pupate.

1.3 Drosophila as a model for synaptogenesis and plasticity

The processes of neuronal innervation and pruning are similar between Drosophila and vertebrates; once again, the fly serves as an excellent organism for study. The Drosophila neuromuscular junction (NMJ), a region where terminals of motoneurons called boutons make synaptic connections onto their muscle targets, has been thoroughly characterized to investigate genes involved in synaptogenesis (reviewed by Brunner and O'Kane, 1997 AND Featherstone and Broadie, 2000) and plasticity (reviewed by Brunner and O'Kane, 1997 AND Schuster, 2006).

1.4 Drosophila as a model for physiological processes

Drosophila has been particularly important in understanding the highly conserved Insulin/IGF (Insulin-like Growth Factor)-like Signalling (IIS) pathway, which is involved

in metabolism and aging (Tissenbaum and Ruvkun, 1997; reviewed by Garofalo, 2002 AND Baker and Thummel, 2007 AND Giannakou and Partridge, 2007). This pathway initiates when insulin binds to the insulin receptor (IR) (reviewed by Taguchi and White, 2008 AND Teleman, 2009). IRs then autophosphorylate and recruit effectors to activate a serine/threonine kinase called Akt (also known as protein kinase B, or PKB) (reviewed by Chan and Tsichlis, 2001 AND Fresno et al., 2004 AND Taguchi and White, 2008 AND Teleman, 2009).

Among the downstream targets of activated Akt is the transcription factor (TF) called forkhead box sub-class O (FOXO). This TF controls the transcription of genes functioning in processes such as cell cycle, apoptosis, DNA repair, metabolism and oxidative stress resistance (reviewed by Taguchi and White, 2008). The activity of FOXO is negatively regulated by IIS pathway. Upon phosphorylation by Akt, FOXO undergoes a conformational change and translocates to the cytoplasm (reviewed by Taguchi and White, 2008 AND Teleman, 2009).

2.0 Functions of scaffold proteins

Within the eukaryotic cell, as many as 50 million proteins may exist (Milo et al., 2010) but only a fraction of this popultaion participate in signaling cascades (reviewed by Good et al., 2011). How does a protein of a particular signaling pathway find its binding partner(s)? How are pathway components organized in a spatial and temporal manner to respond rapidly to signaling cascades?

Discovered more than 15 years ago, scaffold proteins function similarly to protein complexes, where they bind to components of a particular pathway and organize their localization and orientation within a cell (reviewed by Good et al., 2011).

Scaffold proteins are also capable of distributing information from one pathway to one or more different pathways, directing the strength of pathway activation through feedback or through allosteric control of enzymes or substrates and organizing assembly line processes (reviewed by Good et al., 2011). Pathologically, scaffold proteins may provide an easy method to hijack and disrupt normal cellular processes of a host by forcing interactions between proteins which do not bind under normal circumstances (reviewed by Good et al., 2011). Scaffold proteins are regulated by external signals, which allow for rapid changes and coordination of signaling activity (reviewed by Good et al., 2011). With these possibilities, scaffold proteins have enormous impact upon the overall efficiency of cellular responses. Below, I will introduce a putative scaffold protein called RanBPM.

2.1 RanBPM, an introduction

Ran Binding Protein in the Microtubule centre (RanBPM) is a conserved protein first identified in 1995 by Yokoyama and colleagues when they searched for novel binding partners to a Ras-like G protein regulator called Ran (RAs related Nuclear protein) (reviewed by Murrin and Talbot, 2007; Hosono et al., 2010). Ran is part of a classic nucleocytoplasmic transport system responsible for the trafficking of

macromolecules and ribosomal subunits through nuclear pore complexes (reviewed by Stewart, 2007).

RanBPM is the ninth protein found to bind to Ran and is also known as Ran Binding Protein 9 (RanBP9). Although RanBPM belongs to a family of Ran binding proteins, it neither contains the conserved Ran binding domain nor binds to Ran in vivo (reviewed by Murrin and Talbot, 2007). Additionally, it does not participate in nuclear trafficking (reviewed by Murrin and Talbot, 2007).

RanBPM is an ubiquitously expressed protein (Nishitani et al., 2001; Wang et al., 2002; Kramer et al., 2005; Hafizi et al., 2005; Poirier et al., 2006; Yuan et al., 2006), suggesting that it plays vital roles in all cells. Its expression is decreased in certain carcinomas, and it may play a role in cancer development (Denti et al., 2004). RanBPM is alternatively spliced to give rise to short and long isoforms of sizes 55 and 90 kDa respectively (Nishitani et al., 2001). The amino acid sequences of both isoforms are nearly identical, save for a proline and glutamine rich N-terminal stretch present only in the long isoform (Nishitani et al., 2001).

Spatial localizations of RanBPM isoforms have not been clearly defined and may depend upon its phosphroylation to its consensus serine/threonine kinases sites (Denti et al., 2004). Unphosphorylated RanBPM is found in cytoplasmic fractions, while the phosphorylated form is mostly localized to the plasma membrane (Denti et al., 2004). It is unknown at this time whether or not phosphorylation is isoform specific.

The kinase(s) responsible for RanBPM phosophorylation has not been fully investigated. Cells treated with p38 MAPK kinase inhibitor have decreased levels of phosphorylated RanBPM when subjected to UV treatment or osmotic shock (Denti et al., 2004). Wang and colleagues (2002) found that RanBPM increases the activation of the tyrosine kinase MET, which is associated with cell growth, morphogenesis and mobility. In another study, Zou and colleagues (2003) report that RanBPM inhibits the serine/theronine kinase Mirk/Dirk1B (Zou et al., 2003), which mediates cellular growth and differentiation (reviewed by Mercer and Friedman, 2006).

2.2 RanBPM, a putative scaffold protein

In the years since its discovery, RanBPM has been found to bind to more than 40 proteins. It interacts with transmembrane receptor kinases (Wang et al., 2002; Hafizi et al., 2005; Ying et al., 2010) and members of the apoptotic pathway (Ideguchi et al., 2002; Bai et al., 2003; Mikolajczyk et al., 2003; Kramer et al., 2005; Atabakhsh et al., 2009; Suresh et al., 2010;). RanBPM affects transcription of genes through the activity of nuclear receptors (Poirier et al., 2006; Rao et al., 2002), participates in myogenesis (Johnson et al., 2005; Bowman et al., 2008) and regulates the cell cycle (Chang et al., 2010). Finally, RanBPM has links to neurodevelopment (Cheng et al., 2005; Togashi et al., 2006; Yuan et al., 2006) and neurodegeneration (Lakshmana et al., 2009; Lakshmana et al., 2010).

RanBPM binds to its partners through four protein-protein interaction domains (**Fig 1.1;** by Murrin and Talbot, 2007). Closest to the N-terminus is the SPRY (repeats in

sp1A and RyR) domain, a protein interacting domain known to bind to the receptor tyrosine kinase MET (Wang et al., 2002). Adjacent to the SPRY domain is the LisH (Lissencephaly type-1-like homology) motif, a dimerization domain associated with regulating microtubule dynamics, such as cell migration and chromosome segregation (Emes and Ponting, 2001; Kim et al., 2004). The CTLH (C-terminal to LisH) domain is of unknown function (Emes and Ponting, 2001). Finally, closest to the C-terminus is the CRA (CT11-RanBPM) domain.

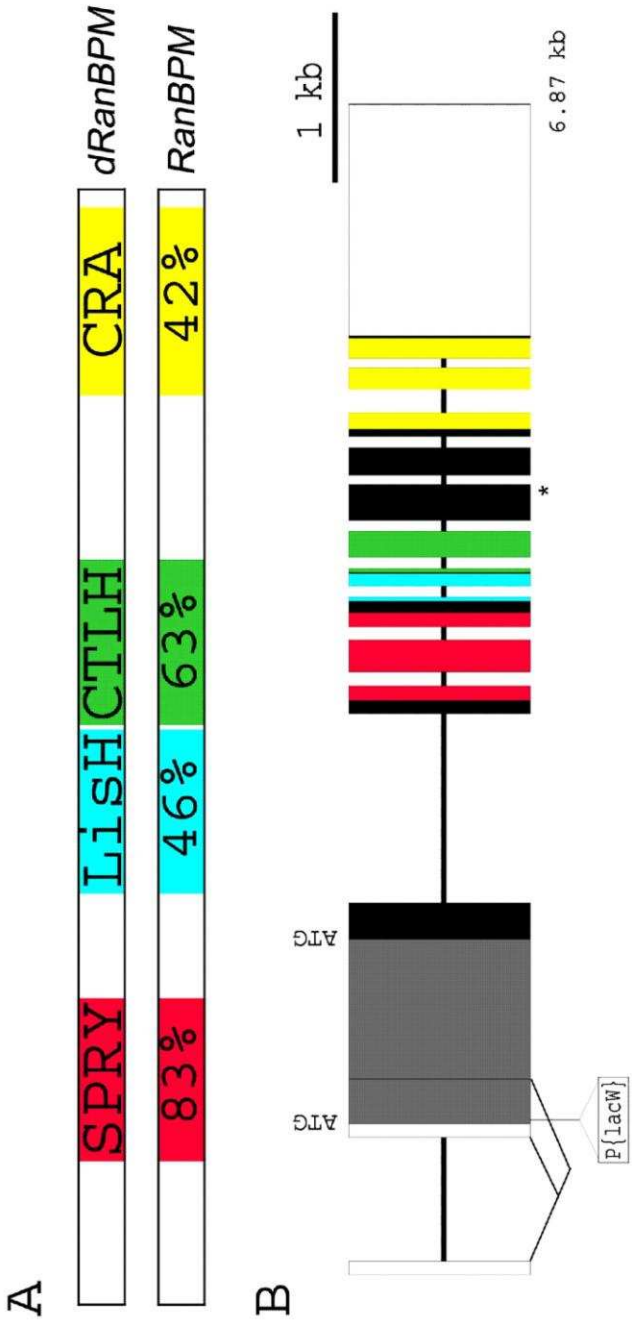
Additional domains exist at the proline and glutamine rich N-terminus of the long RanBPM isoform. This region contains putative SH3-binding domains, which are believed to bind to two upstream activators of the mitogen activated protein kinase (MAPK) pathway called Src and Grb2 (reviewed by Murrin and Talbot, 2007).

Despite that RanBPM binds to numerous RanBPM protein partners, the exact role(s) that RanBPM plays is unclear. For nearly 15 years since its discovery, no RanBPM alleles were generated. As such, consequences of disrupting RanBPM function were largely unknown.

2.3 Characterization of Drosophila RanBPM

In 2008, the first RanBPM mutations were recovered in Drosophila melanogaster (Dansereau et al., 2008). Drosophila and human RanBPM (dRanBPM and RanBPM respectively) share an overall amino acid identity of 40% (Hubbard et al., 2007), with higher conservation observed in the four individual domains: SPRY (83%), LisH (46%),

Fig 1.1: Characterization of Drosophila RanBPM. (A) Percentage of amino acid identity between Drosophila RanBPM (dRanBPM) and human RanBPM protein-protein interaction domains. (B) Alternative splicing of dRanBPM gives rise to two transcripts containing 12 exons. Transcript A encodes for a long isoform, while transcript C encodes for a short isoform. Protein coding regions are shown as black boxes, while the alternative coding region of the long isoform in the second exon is coloured grey. UTRs are coloured white. Colours corresponding to the four RanBPM protein-protein interaction domains are as follows: red-SPRY domain, blue-LisH motif, green-CTLH motif, yellow-CRA domain. The loss-of-function dRanBPM allele, RanBPM[k05201], was created from a P{lacW} insertion into the second exon. Region of where the immunogenic fragment used to raise anti-RanBPM for Western Blots is denoted by *.


A
dRanBPM
SPRY
LisH
CTLH
CRA
RanBPM
83%
46%
63%
42%
B
1 kb
6.87 kb
*
ATG
ATG
P{lacW}

CLTH (62%), and CRA (42%) **(Fig 1.1;** NCBI, 2002; Tweedie et al., 2009). dRanBPM encodes for a 6.78kb transcript containing 12 exons **(Fig 1.1)**. Like that of the vertebrate RanBPM, the Drosophila homologue undergoes alternative splicing to give rise to a short and long isoform of sizes 67kDa and 140 kDa respectively (Dansereau and Lasko, 2008). The long dRanBPM isoform contains an unconserved N-terminal region that is believed to act similarly to the proline and glutamine rich region of the vertebrate isoform (Dansereau and Lasko, 2008).

2.4 dRanBPM has pleiotropic roles

The function of dRanBPM was first demonstrated in the Drosophila germline stem cell (GSC) niche of the ovary by Dansereau and Lasko (2008a). This niche establishes a microenvironment where GSCs maintain their fates by physically attaching themselves to somatic cap cells (reviewed by Fuller and Spradling, 2007). The surface area of cap cells available for GSC attachment is limited, and a cluster of 4-7 cap cells can normally support 2-3 GSCs (reviewed by Fuller and Spradling, 2007).

Dansereau and Lasko (2008a) found that while dRanBPM is expressed in the nucleus and cytoplasm of both somatic and germline cells in the ovary, only the long dRanBPM isoform is specifically enriched in the niche. Loss of the long isoform expression led to an increase in both cap cell size and the number of GSCs they can support (Dansereau and Lasko, 2008a). From this observation, the long dRanBPM isoform is necessary for the organization of a wildtype GSC niche (Dansereau and Lasko, 2008a).

A change in adhesion between GSCs to cap cells via adherens junctions (reviewed by Dansereau and Lasko, 2008b) in dRanBPM mutants was also found (Dansereau and Lasko, 2008a). From a loss of the long dRanBPM isoform expression alone, the average number of adherens junctions increased by more than three times the amount when compared to wildtype controls (Dansereau and Lasko, 2008a). This evidence suggests that the short isoform suppresses cell adhesion between GSCs and cap cells (Dansereau and Lasko, 2008a).

The effects from loss-of-function of dRanBPM have been investigated in whole organisms. dRanBPM mutant larvae exhibit a decrease in locomotion and response to light (Scantlebury et al., 2010). In the neuromuscular junction (NMJ), a well characterized model for the study of plasticity (reviewed by Brunner and O'Kane, 1997 AND Schuster, 2006), mutants have decreased axonal branching and decreased bouton number when compared to wildtypes (Xiao Li Zhao, unpublished results). In addition, mutant larvae are not attracted to food and feed less than wildtype larvae (Scantlebury et al., 2010). All dRanBPM mutants die shortly after the third larval instar, presumably from starvation (Scantlebury et al., 2010).

To understand how the lack of dRanBPM function gives rise to these phenotypes, the NMJ phenotype in dRanBPM mutants was selected for further investigation. This choice was influenced by another factor: the Fragile X Mental Retardation Protein (FMRP) contributes to the development of the NMJ (Zhang et al., 2001) and is a known binding partner to RanBPM (Menon et al., 2004). Both proteins may interact to regulate normal NMJ growth.

3.0 Fragile X Mental Retardation Protein, an introduction

Fragile X Mental Retardation Protein (FMRP) is a widely expressed RNA-binding protein encoded by Fmr1 (reviewed by Bagni and Greenough, 2005). In humans, Fmr1 has two autosomal fragile X-related protein (FXRP) paralogues called FXR1P and FXR2P. Both paralogues share more than 60% amino sequence identity to FMRP (reviewed by Bagni and Greenough, 2005). For the purposes of this thesis, I will focus on FMRP.

3.1 Roles of FMRP

FMRP is a negative translational regulator that contains multiple RNA binding regions: two ribonucleoprotein K homology (KH) domains and a stretch of arginine and glycine residues called the RGG box (reviewed by Bagni and Greenough, 2005). FMRP binds to RNA by recognizing specific mRNA structures and sequences such as the G quartet motif and the poly(U) stretch (reviewed by Bagni and Greenough, 2005).

FMRP regulates mRNA stability and translation through the RNA interference (RNAi) pathway. This conserved pathway utilizes two types of non-coding single stranded RNAs (ssRNAs) called microRNA (miRNA) and short interfering RNA (siRNA). These ssRNAs are produced from cleaving double stranded RNAs (dsRNAs) by the ribonuclease enzyme Dicer (reviewed by Novina and Sharp, 2004 AND Siomi et al., 2004).

siRNA and miRNA inhibit translation of mRNAs through slightly different methods. In the case for siRNAs, they form heteroduplexes with mRNAs which are then recognized and cleaved for degradation by the endonuclease called RNA-induced silencing complex (RISC) (reviewed by Novina and Sharp, 2004). On the other hand, miRNAs, together with RISC, bind to mRNAs undergoing translation to hinder the ribosome machinery (reviewed by He and Hannon, 2004 AND Novina and Sharp, 2004 AND Bagni and Greenough, 2005).

FMRP activity may be required in the RNAi pathway involved in chromatin remodeling (reviewed by Grewal and Moazed, 2003). In yeast, the RNAi pathway initiates the formation and maintanence of heterochromatin at the centromeric region (Volpe et al., 2002). Mutants with loss-of-function in Dicer or a component of the RISC called argonaute lose centromeric silencing (Volpe et al., 2002).

A recent study demonstrated that FMRP is capable of stabilizing the mRNA of a scaffold protein called PSD-95 (Zalfa et al., 2007). PSD-95 is required for the function and plasticity of excitatory synapses (reviewed by Han and Kim, 2008). This protein is abundant in an electron-dense region of neuronal dendrites called postsynaptic density (reviewed by Nimchinsky et al., 2002 AND Han and Kim, 2008). In the presence of FMRP, levels of PSD-95 were higher by as much as 20% compared to levels without FMRP (Zalfa et al., 2007).

3.2 Fragile X Mental Retardation Syndrome phenotypes in the brain

Loss or reduction of FMRP function is responsible for the most common form of inherited mental retardation (reviewed by Oostra and Willemsen, 2003 AND Bagni and Greenough, 2005). In most cases, hypermethylation to upstream CpG islands and expanded CGG trinucleotide repeats of Fmr1 leads to its transcriptional silencing (reviewed by Bardoni et al., 2000 AND Jin et al., 2004b; Bagni and Greenough, 2005). Loss of FMRP function can also occur from point mutations (De Boulle et al., 1993; Mila et al., 2000) and deletions (Gedeon et al., 1992; Meijer et al., 1994).

Individuals with Fragile X Mental Retardation Syndrome (FXS) have cognitive defects in areas such as attention and memory (reviewed by Oostra and Willemsen, 2003 AND Bagni and Greenough, 2005). Microscopically, the most obvious abnormalities in the brains of these patients are the morphology and density of dynamic protrusions on the dendrites of neurons called spines (reviewed by Bagni and Greenough, 2005).

3.3 Dendritic spines in FXR patients

Dendritic spines are structures important for neurotransmission in both vertebrates and invertebrates (reviewed by Nimchinsky et al., 2002). They receive presynaptic signals and modulate synaptic strength (reviewed by Coss and Perkel, 1985). In the vertebrate brain, around 90% of all excitatory glutamatergic synapses occur on dendritic spines (reviewed by Nimchinsky et al., 2002 AND Shen and Cowan, 2010).

The developmental process that these spines undergo is highly conserved and regulated. Briefly, in early development of the brain, spines appear in high densities and reach out to their synaptic partners. During the process of maturation, spines which have

made inappropriate contacts are pruned back while those which have made correct contacts morphologically mature from being long and thin to short and stubby (reviewed by Nimchinsky et al., 2002). Maturing spines also undergo ultrastructural reorganization to support for synaptic transmission (reviewed by Nimchinsky et al., 2002).

Dendritic spines are able to respond to changes in synaptic activity even after maturation. They are capable of synaptic plasticity through altering their cytoskeleton (reviewed by Nimchinsky et al., 2002 AND Shen and Cowan, 2010 AND Dent et al., 2011). Decreases in sensory, learning and/or memory activity result in reduction in spine number, modifications to the ultrastrucutral architecture and morphology of spines (reviewed by Nimchinsky et al., 2002; Matsuzaki et al., 2004; Zhou et al., 2004).

It is believed that dendritic spines of FXS patients are unable to undergo synaptic plasticity. Even in adults, the spines are present in high densities and their morphology remain long and thin (reviewed by Irwin et al., 2000 AND Bagni and Greenough, 2005).

3.4 The Drosophila model for FXS

Drosophila models for FXS have been developed. The Drosophila FMRP homolog (dFMRP) (Wan et al., 2000) shares an overall identity of 34% to human FMRP (Hubbard et al., 2007). Its RNA binding regions, particularly the KH domains, are more highly conserved, with greater than 70% amino acid similarity (Wan et al., 2000). Like the vertebrate FMRP, dFMRP is widely expressed (Wan et al., 2000; Zhang et al., 2001) and associates with miRNA, siRNA, Dicer and RISC to perform similar regulatory gene functions (reviewed by Siomi et al., 2004). Drosophila dFmr1 mutants exhibit defects in

attention span, circadian rhythm and hyperactivity, similar to behaviours observed from FXS patients (reviewed by Siomi et al., 2004).

At the cellular level, mutant dFmr1 mutant flies possess several neuronal phenotypes. In the mushroom body (MB), a bilateral, lobular structure of the brain associated with learning and memory (reviewed by Busto et al., 2010), axon growth is poorly regulated, with increased projections and supernumerary branches (Pan et al., 2004). Particularly, the beta lobes of the MB do not terminate at the midline boundary which separates the MB of either hemispheres from each other (Pan et al., 2004). In more severe cases, beta lobes appear fused from the amount of crossed fibers (Michel et al., 2004).

At the NMJ, loss of dFmr1 function leads to an increase in both size and number of synaptic terminals (Zhang et al., 2001). These boutons contain a high density of synaptic vesicles (reviewed by Rodesch and Broadie, 2000; Pan et al., 2004), which may explain the increase in evoked synaptic transmissions (Zhang et al., 2001). Branching patterns of the NMJ arbors are also more complex, reminiscent of early synaptic development (Zhang et al., 2001).

3.5 Molecular phenotypes of FXS

A defect in the cytoskeleton of dendritic spines is a likely reason for the abnormal neuronal phenotypes observed in FXS individuals and the Drosophila FXS model. In the brain, FMRP is transported to the base of dendritic spines where it negatively regulates the translation of local proteins involved in structural stabilization of the cytoskeleton

(reviewed by Jin et al., 2004a AND Bagni and Greenough, 2005). FMRP has been found to negulate the levels of two cytoskeletal proteins: profilin (Reeve et al., 2005) and MAP1B (Zhang et al., 2001).

3.6 Profilin, an actin-binding protein

Dendritic spines are able to respond to changes in synaptic activity due to filamentous actin, which is the major component of their cytoskeleton (reviewed by Birbach, 2008; Honkura et al., 2008). The property for actin to polymerize in a dynamic manner is regulated by a conserved protein called profilin (reviewed by Gieni and Hendzel, 2009). Mutations in the profilin gene lead to disruptions in motility, cell cycle and embryonic lethality (reviewed by Yarmola and Bubb, 2009). In mammals, several profilin paralogs exist, while only one homolog, encoded by chickadee, is present in Drosophila (reviewed by Birbach, 2008).

Profilin controls actin dynamics by catalyzing the exchange of ADP to ATP in monomeric actin after its depolymerization from filamentous actin (reviewed by Bugyi and Carlier, 2010). In addition, it maintains the pool of monomeric actin-ATP by preventing ATP hydrolysis (reviewed by Bugyi and Carlier, 2010).

Genetic interaction experiments have been conducted to investigate the relationship between dFMRP and profilin. Elevated levels of profilin observed in dFmr1 mutants can be reduced by partially restoring dFmr1 function (Reeve et al., 2005). The increased length of circadian neuronal branches in dFmr1 mutants are shortened by decreasing the levels of profilin expressed (Reeve et al., 2005). Finally, the MB midline

gap narrows when dFmr1 is ectopically expressed, while ectopic co-expression of dFmr1 and chickadee increases gap distance (Reeve et al., 2005).

3.7 MAP1B/Futsch, a microtubule binding protein

Microtubule associated protein 1B (MAP1B) is a structural protein belonging to the MAP1 family (reviewed by Halpain and Dehmelt, 2006). It localizes to developing dendrites and axons (Hummel et al., 2000; reviewed by Riederer, 2007) where it interacts with microtubules to aid in their organization (reviewed by Riederer, 2007 AND Conde and Caceres, 2009).

In Drosophila, Futsch is the MAP1B homolog and shares 60% sequence identity to the vertebrate protein (reviewed by Halpain and Dehmelt, 2006). Futsch is found in the cytoskeleton of axons, dendrites and neuromuscular synaptic terminals (Roos et al., 2000).

MAP1B is initially expressed in high amounts during development but gradually decreases as development progresses (Tucker and Matus, 1987; reviewed by Riederer, 2007). This regulation is dependent upon FMRP function (Menon et al., 2008) to bind directly to MAP1B through its RGG box (Roos et al., 2000). Loss-of-function in FMRP delays the decline of MAP1B expression (Lu et al., 2004). Elevated levels of Futsch in dFmr1 mutants are restored to that of wildtype in flies double mutant for dFmr1 and futsch (Roos et al., 2000; Zhang et al., 2001).

3.8 Interaction between RanBPM and FMRP

FMRP was first found to bind to RanBPM from a yeast-2-hybrid screen (Menon et al., 2004). This interaction occurs between the CRA domain of RanBPM and the C terminal region of FMRP, within which contains a RNA-binding RGG box (Menon et al., 2004). In vitro, presence but not absence of RanBPM inhibits FMRP ability to bind to RNA homopolymers and MAP1B (Menon et al., 2004).

3.9 dRanBPM and dFMRP influence NMJ branching pattern

Since the NMJ of dRanBPM mutants is characterized by decreased boutons and branches (XL Zhao, unpublished results), it suggests that dRanBPM may be involved in synaptic formation. Another possibility to this simplified NMJ architecture, however, may arise from a reduction in the mutant size (Stewart and McLean, 2004). To begin investigating on the cause of this phenotype, several pieces of information were gathered: 1) dRanBPM mutants have a simplified NMJ architecture, 2) this NMJ undergrowth can be phenocopied by overexpressing dFMRP, a mRNA-binding protein that negatively regulates Futsch (Zhang et al., 2001) and profilin (Reeve et al., 2005) to control synaptic structure and, 3) interaction between FMRP and RanBPM reduces the mRNA binding ability of FMRP (Menon et al., 2004). By considering these pieces of information together, a possible role for dRanBPM in modulating synaptic structure through FMRP may exist.

A genetic epistasis experiment was designed to observe for changes in NMJ architecture in larvae carrying loss-of-function for both dRanBPM and dFmr1 (Scantlebury et al., 2010). Since a loss of negative regulation of dRanBPM on the

mRNA-binding function of dFMRP (Menon et al., 2004) may suppress the complex synaptic structure observed in dFmr1 mutants (Zhang et al., 2001), NMJs in double homozygous dRanBPM;dFmr1 mutants were expected to possess a reduced level of synaptic overbranching when compared to dFmr1 mutants.

Double homozygous dRanBPM; dFmr1 mutants do not survive, however, as they die at the embryo stage. For this reason, homozygous dFmr1 mutant larvae carrying one copy of loss-of-function dRanBPM were used. NMJs of these mutant larvae were compared to those homozygous for a hypomorphic or an amorphic dFmr1 allele (dFmr[EP3517] and dFmr1[Delta50M] respectively).

As described in the literature, mutant larvae with a loss of dFMRP function displayed an over-elaborated NMJ structure, with marked increases in both boutons and branchings (Scantlebury et al., 2010). With additional reduction of dRanBPM function, this overgrowth phenotype was partially suppressed and was sufficient to revert bouton and branching numbers back to those similar in wildtype larvae (Scantlebury et al., 2010). These results suggest that dRanBPM acts in a dose-dependent manner to regulate NMJ growth through a dFMRP-dependent process.

3.10 Rationale and first aim of thesis

To demostate that dRanBPM is dependent upon dFMRP function to regulate NMJ growth at the molecular level, the first objective of my thesis was to ask whether dRanBPM affects the levels of profilin and Futsch. This was done by measuring

Table 1.1: List of publications and their findings. Brief **s**ummary of those publications relating FMRP function to the regulation of NMJ structure in Drosophila or to RanBPM interaction**.**

Table 1.1: List of publications and their findings.

	Observations and findings	**Reference**
1	dRanBPM mutants demonstrate decreased number of boutons and branching at the NMJ	XL Zhao, unpublished results
2	dFmr1 mutants have increased levels of Futsch in the brain. dFMRP negatively regulates expression of Futsch	Zhang et al., 2001; Reeve et al., 2005
	dFmr1 mutants have increased levels of boutons and branchings at the NMJ	Zhang et al., 2001
	Normal levels of Futsch are required for proper NMJ synaptic architecture	Roos et al., 2000; Zhang et al., 2001
3	dFmr1 mutants have increased level of profilin in the brain. dFMRP negatively regulates expression of profilin	Reeve et al., 2005
4	RanBPM directly interaction between RanBPM and FMRP decreases RNA binding affinity of FMRP	Menon et al., 2004

levels of the two cytoskeletal proteins between dFmr1 mutants expressing normal levels of dRanBPM to those with a decreased level of dRanBPM function. I expected to find decreased levels of either proteins in dFmr1 mutants with reduced dRanBPM function to flies with only a loss-of-function to dFmr1.

4.0 Size and feeding phenotypes of dRanBPM mutants

Aside from locomotory defects, dRanBPM mutants are about half the size smaller than control larvae at the same developmental stage (Scantlebury et al., 2010). These mutants are unattracted to food and display early wandering behaviour at the start of the third instar (Scantlebury et al., 2010). Their bodies become more translucent, suggesting that fat reserves are consumed despite the presence of food (XL Zhao, personal communication). Finally, shortly after the third instar, dRanBPM mutants die (Scantlebury et al., 2010).

It is possible for the phenotypes listed above to be connected in some way. Decreased feeding has enormous impact on growth and survival of a larva: a reduction in nutrient intake leads to fewer resources available to spend on development (Ikeya et al., 2002; reviewed by Nijhout, 2003 AND Edgar, 2006). Eventually, without adequate energy to support for basic cellular functions, dRanBPM larvae die.

4.1 dRanBPM function is required in neurosecretory cells

To explore the idea that reduction in feeding in dRanBPM mutants is a primary cause of death, dRanBPM was spatially targeted for expression in these mutants through

the GAL4/UAS system (Xiao Li, unpublished results; Harris, 2007). Ectopic source of dRanBPM was supplied from either a short or a long dRanBPM construct, each encoding for a different isoform.This experiment allowed insights into which tissue(s) requires dRanBPM function to restore normal feeding. Success of this rescue was based on the percentage of dRanBPM mutants surviving to adulthood.

Expressing the long dRanBPM construct in neurons (elav-GAL4) or in both neurons and muscles (Dmef-GAL4) rescued dRanBPM mutants from lethality, but with limited success **(Table 1.2)**. Ubiquitous (tub-GAL4) expression of either dRanBPM constructs was able to rescue around 50% of dRanBPM mutants **(Table 1.2)**. Surprisingly, expression of only the short dRanBPM construct in neurosecretory cells (c929-GAL4) was able to rescue all mutant larvae from lethality **(Table 1.2)**.

4.2 DIMMED, a transcription factor expressed in neurosecretory cells

The c929-GAL4 driver used to target expression of dRanBPM in neurosecretory cells (NSCs) reflects the expression of a basic helix loop helix (bHLH) transcription factor (TF) called DIMMED (Park et al., 2008). This pro-secretory factor is required for neurosecretory cell fate and those NSCs with loss-of-function in DIMMED are unable to produce, store and release the high levels of neuropeptides (Hewes et al., 2003).

In the Drosophila larva, DIMMED is expressed in 306 cells of the central nervous system (CNS) and 52 cells in the periphery (Park et al., 2008). It should be noted that while all DIMMED expressing cells are neurosecretory, not all neurosecretory cells

Table 1.2: Percentages of rescue from lethality in dRanBPM mutants from targeted expression of dRanBPM in various tissues. dRanBPM was ectopically expressed in dRanBPM mutants using one of two dRanBPM constructs: the short construct (UAS-dRanBPM.S) or the long construct (UAS-dRanBPM.long). Amount of rescues differ between targeted tissues and constructs.

Table 1.2: Percentages of rescue from lethality in dRanBPM mutants from targeted ectopic expression of dRanBPM in various tissues.

Targeted tissue and driver	**UAS-dRanBPM.S (short construct)**	**UAS-dRanBPM.long (long construct)**	**Reference**
All neurons (elav-GAL4)	0%	14%	Scantlebury et al., 2010
Neurons and muscles (Dmef2-GAL4)	0%	3%	Scantlebury et al., 2010
All cells (tub-GAL4)	45%	55%	L Harris, 2007, research thesis
Neurosecretory cells (c929-GAL4)	100%	0%	XL Zhao, unpublished results

express DIMMED (Park et al., 2008).

4.3 Drosophila insulin like peptides and the insulin signaling pathway

The neuropeptides governing growth and metabolism are the Drosophila insulin-like peptides (DILPs), homologs to the vertebrate insulin (reviewed by Altstein and Nassel, 2010). Seven DILPs have been identified and are expressed in various regions of the Drosophila larva: dilp1, -2-3,-5 from the medial neurosecretory cells (Brogiolo et al., 2001; Ikeya et al., 2002; Rulifson et al., 2002), dilp4 from larval midgut (Brogiolo et al., 2001), dilp6 from the fat body (Slaidina et al., 2009) and dilp7 from the ventral nerve cord (Brogiolo et al., 2001). Collectively, these DILP producing cells are referred to as insulin producing cells (IPCs).

DILPs activate the conserved Insulin/IGF (Insulin-like Growth Factor)-like Signalling (IIS) pathway, which regulates growth and size through nutrient intake (reviewed by Goberdhan and Wilson, 2003a, 2003b AND Hietakangas and Cohen, 2009). Decrease in pathway activation reduces organism size (Ikeya et al., 2002; So et al., 2011).

4.4 Rational and second aim of thesis

To find if loss of dRanBPM function interferes with expression of DILPs and consequently, the activation of the IIS pathway, the second objective of my thesis was to investigate whether or not IIS pathway activation is altered in dRanBPM mutants. This was done by quantifying the levels of a downstream target called dAkt, a serine/threonine kinase which becomes phosphorylated upon IIS pathway activation (reviewed by Chan

and Tsichlis, 2001 AND Fresno et al., 2004 AND Taguchi and White, 2008 AND Teleman, 2009). I expected to see a decreased level of phosphorylated dAkt in dRanBPM mutants compared to that of wildtype larvae or to dRanBPM larvae with rescued feeding behaviour from targeted expression of dRanBPM to NSCs. Total dAkt levels, which include both phosphorylated and unphosphorylated pools of dAkt, should not differ between groups.

5.0 The IIS pathway and longevity

Aside from growth, the IIS pathway affects lifespan by acting upon a transcription factor (TF) called Forkhead Box sub-class O (FOXO) (reviewed by Taguchi and White, 2008 AND Teleman, 2009). FOXO transcribes those genes involved in protective functions such as oxidative damage, stress response and immunity (reviewed by Partridge and Bruning, 2008 AND Van der Vos and Coffer, 2011). As a result, increased FOXO activity contributes to the extension of lifespan.

The activity of FOXO is regulated by posttranslational modifications (PTM) (reviewed by Zhao et al., 2011). Upon its phosphorylation by Akt, FOXO undergoes a conformational change (reviewed by Greer and Brunet, 2005) and translocates to the cytoplasm (Biggs et al., 1999; Nakae et al., 1999; Giannakou et al., 2004; Hwangbo et al., 2004; reviewed by Zhao et al., 2011).

5.1 Rational and third aim of thesis

To find whether dRanBPM affects the activation of IIS pathway signaling, I measured the lifespans of flies with and without loss-of-function dRanBPM. I expected that mutant dRanBPM flies to live longer than those with normal dRanBPM function.

6.0 Overall goals of thesis

To summarize, the goals of this thesis are to investigate (1) is dRanBPM regulates the levels of two cytoskeletal proteins known to alter synaptic architecture and (2) if dRanBPM is involved in activation of the IIS pathway

Materials and methods

Chapter 2

1.0 Fly maintenance

Fly stocks were housed at 20-23°C in either polystyrene vials or in glass pairwise tubes. Both tubes and vials were plugged with cotton. Food medium was made by first separating food ingredients into two flasks. The first flask contained a mixture of sucrose, agar, potassium phosphate, potassium sodium tartarate, sodium chloride and ferric sulphate, while the second flask held yeast dissolved in water. Ingredients were autoclaved and contents from both flasks were combined shortly afterwards. The mixture was allowed to cool to 60°C before adding tegosept and acid mix. Fly food was ready to be poured at 55°C.

To obtain a higher proportion of progenies carrying mutant homozygous copies of Fmr1 from Fmr1(Delta113) and Fmr1(Delta50) stocks, flies were raised on food medium containing nutritional yeast flakes, corn flour and D-glucose (recipe kindly provided by Linda L. Restifo). Preparation method was similar to that described above.

All stocks were kept as follows: adults were allowed to lay eggs on the food medium for one week, after which they were removed. Progenies were transferred to a fresh food vial or tube on the second week.

1.1 Genetic stocks

All fly stocks and their genotypes used to carry out experiments in this thesis **(Table 2.1)**.

RanBPM[k05201] is a loss-of-function RanBPM allele created from a forward genetic screen mobilizing a P{lacW} transposon carrying a mini w+ gene. The P{lacW} insertion has been mapped to the second exon of RanBPM **(Fig 1.1)**.

Table 2.1: a complete list of all Drosophila stocks used for this thesis.

Genotype	**Source and comments**
yw; S/CyO(y+); UAS-RanBPM.S/TM3,Sb	This thesis
yw; S/CyO(y+); UAS-RanBPM.long H^{1}_{1}/TM3,Sb Tb	This thesis
+; P{GAL4-Hsp70.PB}/CyO	Campos central stocks
+;+;P{da-GAL4.w(-)}	Bloomington #8641
Oregon-R (OR)	Campos central stocks
yw	Campos central stocks
yw;; Fmr1(Delta113M)/TM6,Sb Tb	Campos central stocks
yw; P{w(+mC)=lacW}RanBPM[k05201]; Fmr1(Delta113M)/T(2;3)TSTL; CyO: TM6B, Tb	Campos lab
yw;; Fmr1[Delta50M]/TM6,Sb Tb	Campos central stocks
yw; P{w(+mC)=lacW}RanBPM[k05201]; Fmr1[Delta50M]/T(2;3)TSTL; CyO: TM6B, Tb	Campos lab
w; P{w(+mC)=EP}Fmr1[EP3517]	Campos central stocks
yw; P{w(+mC)=lacW}RanBPM[k05201];P{w(+mC)=EP}Fmr1[EP3517]/ T(2;3)TSTL; CyO: TM6B, Tb	Campos lab
yw; P{w(+mC)=lacW}RanBPM[k05201]/CyO(y+); tub-GAL4/TM6,Sb. Tb	Campos central stocks
yw; P{w(+mC)=lacW}RanBPM[k05201], c929-GAL4/CyO(y+)	Campos central stocks

P{w(+mC)=tubP-GAL80[TS]}9, w(*)/FM7c, P{w(+mC)=GAL4-Kr.C}DC1, P{w(+mC)=UAS-GFP.S65T}DC5, sn(+); P{w(+mC)=lacW}RanBPM[k05201]/CyO{Act GFP}; UAS-RanBPM.S/TM3,Sb	This thesis
yw;RanBPM[k05201]/CyO(y+)	Campos central stocks
P{w(+mC)=tubP-GAL80[TS]}9, w(*)/FM7c, P{w(+mC)=GAL4-Kr.C}DC1, P{w(+mC)=UAS-GFP.S65T}DC5, sn(+);;D/TM3,Sb	This thesis
+;+;P{UAS-Kir2.1}	Campos central stocks
yw;Pin/CyO;UAS-GFP	Campos central stocks
yw; RanBPM[k05201]/CyO(y+); P{tub-GAL4}/TM6, Sb Tb	Campos central stocks
yw; P{w(+mC)=lacW}RanBPM[k05201], c929	Campos central stocks
P{w(+mC)=tubP-GAL80[TS]}9, w(*)/FM7c, P{w(+mC)=GAL4-Kr.C}DC1, P{w(+mC)=UAS-GFP.S65T}DC5, sn(+); P{w(+mC)=lacW}RanBPM[k05201]/CyO{Act GFP}; UAS-RanBPM.long H11/TM3,Sb	This thesis
P{w(+mC)=tubP-GAL80[TS]}9, w(*)/FM7c, P{w(+mC)=GAL4-Kr.C}DC1, P{w(+mC)=UAS-GFP.S65T}DC5, sn(+); P{w(+mC)=lacW}RanBPM[k05201]/CyO{Act GFP}; UAS-RanBPM.long H213/TM3,Sb	This thesis
P{w(+mC)=tubP-GAL80[TS]}9, w(*)/FM7c, P{w(+mC)=GAL4-Kr.C}DC1, P{w(+mC)=UAS-GFP.S65T}DC5, sn(+); P{w(+mC)=lacW}RanBPM[k05201]/CyO{Act GFP}; UAS-RanBPM.S/TM3,Sb	This thesis
Yw; P{w(+mC)=lacW}RanBPM[k05201]/CyO(y+); UAS-RanBPM.S/TM3,Sb	Campos central stocks
yw; P{w(+mC)=lacW}RanBPM[k05201]/CyO(y+); UAS-RanBPM.long H11/TM6,Sb Tb	Campos central stocks
yw; P{w(+mC)=lacW}RanBPM[k05201]/CyO(y+); UAS-RanBPM.long H213/TM6,SbTb	Campos central stocks

+; P{w(+mC)=lacW}RanBPM[k05201]/CyO{Act GFP}; elav-GAL4/TM6,Sb. Tb	This thesis
+; P{w(+mC)=lacW}RanBPM[k05201]/CyO{Act GFP}; tub-GAL4/TM6,Sb. Tb	This thesis
+; P{w(+mC)=lacW}RanBPM[k05201], c929-GAL4/CyO{Act GFP}; D/TM3,Sb	This thesis
yw; P{w(+mC)=lacW}RanBPM[k05201]/CyO(y+); elav-GAL4/TM6,Sb. Tb	Campos central stocks
yw; P{w(+mC)=lacW}RanBPM[k05201]/CyO(y+); tub-GAL4/TM6,Sb. Tb	Campos central stocks
yw; P{w(+mC)=lacW}RanBPM[k05201], c929-GAL4/CyO(y+)	Campos central stocks

2.0 Western blotting

2.1 Sample preparation and loading

Cryogenic tubes containing adult flies were briefly immersed into liquid nitrogen then vortexed. Severed fly heads were separated and approximately 30 heads were transferred to a new microcentrifuge tube. Approximately 30 heads were lysed in 120 ul of SDS sample buffer, boiled for three mins and centrifuged at maximum speed (13 000 rpm) for 20 mins.

For mid-late second instar whole larvae preparations, larvae were collected and lysed in 24x volume of sodium dodecyl sulfate (SDS) sample buffer. Larvae homozygous for RanBPM[k05201] mutation were selected from the presence of brown mouth hooks. Samples were boiled for three mins and centrifuged at maximum speed for 45 mins.

After centrifugation, supernatant was transferred to a new microcentrifuge tube and Bradford assay was carried out. Approximately 100 ug of protein was loaded into each lane of a sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel.

For the detection of Futsch, protein lysate was prepared using O'Farrell buffer to account for the greater risk of protein degradation due to its large molecular weight. First, approximately 200 adult heads were homogenized in 200 ul of O'Farrell buffer. Samples were spun down at 4°C at maximum speed for 20 mins. Supernatant was transferred into a new microcentrifuge tube. With approximately 50ul of supernatant in each tube, 130 ul of ddH_2O and 8x volume of 80% acetone was then added and mixed. Protein was allowed to precipitate at -20°C for 1 hour. Sample tubes were centrifuged in the coldroom for 20 mins. Afterwards, supernatant was removed and protein pellets were allowed to dry. Pellets were then suspended in SDS sample buffer. Lysate was then boiled and centrifuged. Supernatant was collected and quantified using Bradford Assay.

2.2 Gel run and transfer

SDS-PAGE gels were allowed to run overnight at 75 V. On the following morning, proteins were blotted onto nitrocellulose membrane (Whatman Protran, Germany) at 60 V for 3.5 hours at 4°C. To confirm whether or not transfer was successful, the blot was stained for protein bands with PonceauS (Sigma, St. Louis, MO). This stain was then washed off and the blot was blocked for one hour on a shaker at room temperature.

2.3 Incubation, antibodies and band visualization

Primary antibodies used in this thesis were α-RanBPM (1:100) (New England Peptides), α-chic (1:10) and α-22C10 (1:500) (both from Developmental Studies Hybridoma Bank), α-actin (1:10 000) (Millipore, Temecula, CA), α-phospho Akt (Ser505) (1:1000) and α-Akt (1:1000) (both from Cell Signaling, #4054 and #4091 respectively). Actin was used as loading control.

The α-RanBPM was ordered to be made to recognize both RanBPM isoforms. This antibody was raised using an immunogenic fragment (AVIKPQGGDRPDIKN) located at the end of the 8th exon **(Fig 1.1)**.

All nitrocellulose blots were incubated with appropriate primary antibody dilutions at 4°C overnight on a rotator. Afterwards, blots were washed once in TBS, three times in TBST and once again in TBS. Blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibody for one hour at 4C. Excess secondary antibodies were washed off blots in TBST. Protein bands were visualized with enhanced chemiluinescence (ECL) solution (Amersham).

3.0 Longevity assay

Flies were put into a house and allowed to lay for one day at 25°C. Hatched larvae fed on food plates at 29°C until early to late second instar. These larvae were then transferred to food vials (70 larvae/vial) and allowed to continue development at 29°C. Upon eclosure, female adults were selected and sorted according to their genotypes. Approximately ten virgin females (low population density) of each genotype were placed into a glass pairwise tube containing approximately 2 ml of fly food. To control for

temperature effect on the activity of TubGAL80[TS], flies were placed at either 18°C (TubGAL80[TS] restrictive temperature) or 29°C (TubGAL80[TS] permissive temperature). Flies in each tube were switched to a new food tube every four days until all flies have died. Population in each tube was also recorded at the same time. Flies trapped in wet food but were still alive were recorded as part of the population count for that day. In the next population count, these flies were recorded as deceased.

4.0 Statistical analysis

Protein bands from exposed hyperfilm are scanned and quantified using ImageJ software. Protein levels were then graphed and statistical analyses was done to assess for significance. Pairwise comparisons between protein levels were analyzed using Student's t-test. Level of significance was established at $p<0.05$. All statistical calculations were done using Minitab student release 14 software (Minitab Inc, State College, PA).

Median ages of death from all genotypes of longevity assay were statistically analyzed using the Kruskal-Wallis test for significance. Since multiple pairwise comparisons were done within the same dataset, p value of significance was recalculated using the Bonferroni correction. As a result, pairwise comparisons were considered significant only when $p<0.00833$. All calculations were done using Comprehensive R Archive Network (R Foundation for Statistical Computing, Vienna, Austria)

Results

Chapter 3

1.0 Detection of profilin in dFmr1 and dRanBPM mutant flies

Loss-of-function of dRanBPM causes locomotory, behavioural and physiological defects in the dRanBPM larvae (Scantlebury et al., 2010). To begin understanding how lack of dRanBPM function gives rise to these mutant phenotypes, the locomotory defect was first selected for examination. At the NMJ, dRanBPM mutants have decreased branching and bouton number (XL Zhao, unpublished results).

FMRP regulates NMJ architecture (Tessier and Broadie, 2008) by controlling the levels of two known cytoskeletal proteins: profilin (Reeve et al., 2005) and Futsch (Roos et al., 2000; Zhang et al., 2001; Reeve et al., 2005). FMRP is also a binding partner of RanBPM (Menon et al., 2004), suggesting that RanBPM may be involved in establishing the structure of NMJs.

A previous experiment demonstrated that dRanBPM acts through dFMRP in a dose-dependent manner to regulate the synaptic architecture seen at the NMJ of dRanBPM mutants at the cellular level (Scantlebury et al., 2010). To gain further support that dRanBPM modulates a dFMRP-dependent process, I sought to determine whether or not dRanBPM is able to regulate the levels of profilin and Futsch in dFmr1 mutant flies.

I reproduced an experiment by Reeve et al. (2005) where they demonstrated a decrease in dFMRP function lead to increased levels of profilin. I used flies carrying either one of the two amorphic dFmr1 alleles, dFmr1[Delta50M] or dFmr1[Delta113M]. In addition, I crossed the two amorphic dFmr1 lines to obtain heteroallelic progenies (dFmr1[Delta50M]/ dFmr1[Delta113M]). Flies carrying yw, or only one copy of loss-of-

function dFmr1 allele were used as controls. After quantifying and analyzing the levels of profilin of these flies, no significant differences were found between individuals with or without dFmr1 expression (**Fig 3.1**).

To investigate whether or not reducing dRanBPM function in dFmr1 mutant flies led to a decrease in levels of profilin, three different dFmr1 alleles were used: a hypomorphic allele (dFmr1[EP3517]) and two loss-of-function alleles (dFmr1[Delta50M] and dFmr1[Delta113M]). Both dFmr1[EP3517] and dFmr1[Delta50M] alleles were previously used by Scantlebury and colleagues (2010) to show that synaptic overgrowth at the NMJ of dFmr1 mutants decreased upon reduction of dRanBPM function.

No significant differences in the levels of profilin were detected between flies homozygous and heterozygous for dFmr1[EP3517] or between dFmr1 mutants with and without reduction in dRanBPM function **(Fig 3.2A,B).**

Similar blots probing for profilin were performed using flies carrying dFmr1[Delta50M] **(Fig 3.2C,D)** or dFmr1[Delta113M] **(Fig 3.2E,F)** with no significant differences in levels of profilin were found between the genotypes. In summary, these results demonstrate that (1) dFMRP does not negatively regulate levels of profilin and (2) a reduction in dRanBPM function does not decrease levels of profilin.

1.1 Detection of Futsch in wildtype flies

To investigate if levels of Futsch decreased from a reduction in dRanBPM

Fig 3.1: dFMRP function does not significantly affect levels of profilin. (A) Fly head lysate (30ug) were immunobloted and probed for profilin. Actin served as loading control. (B) No significant differences were found between flies with and without dFmr1 expression after quantification. Three blots were used for quantitative analysis (N=3). All error bars represent ±S.E.M. Level of significance was established at $p<0.05$.

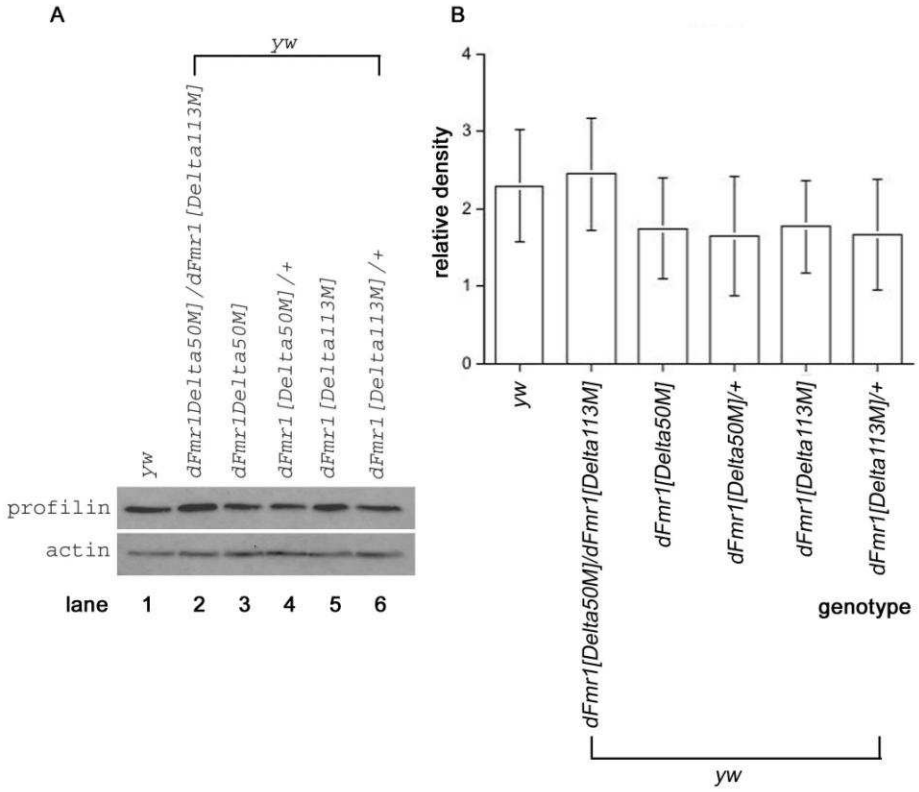
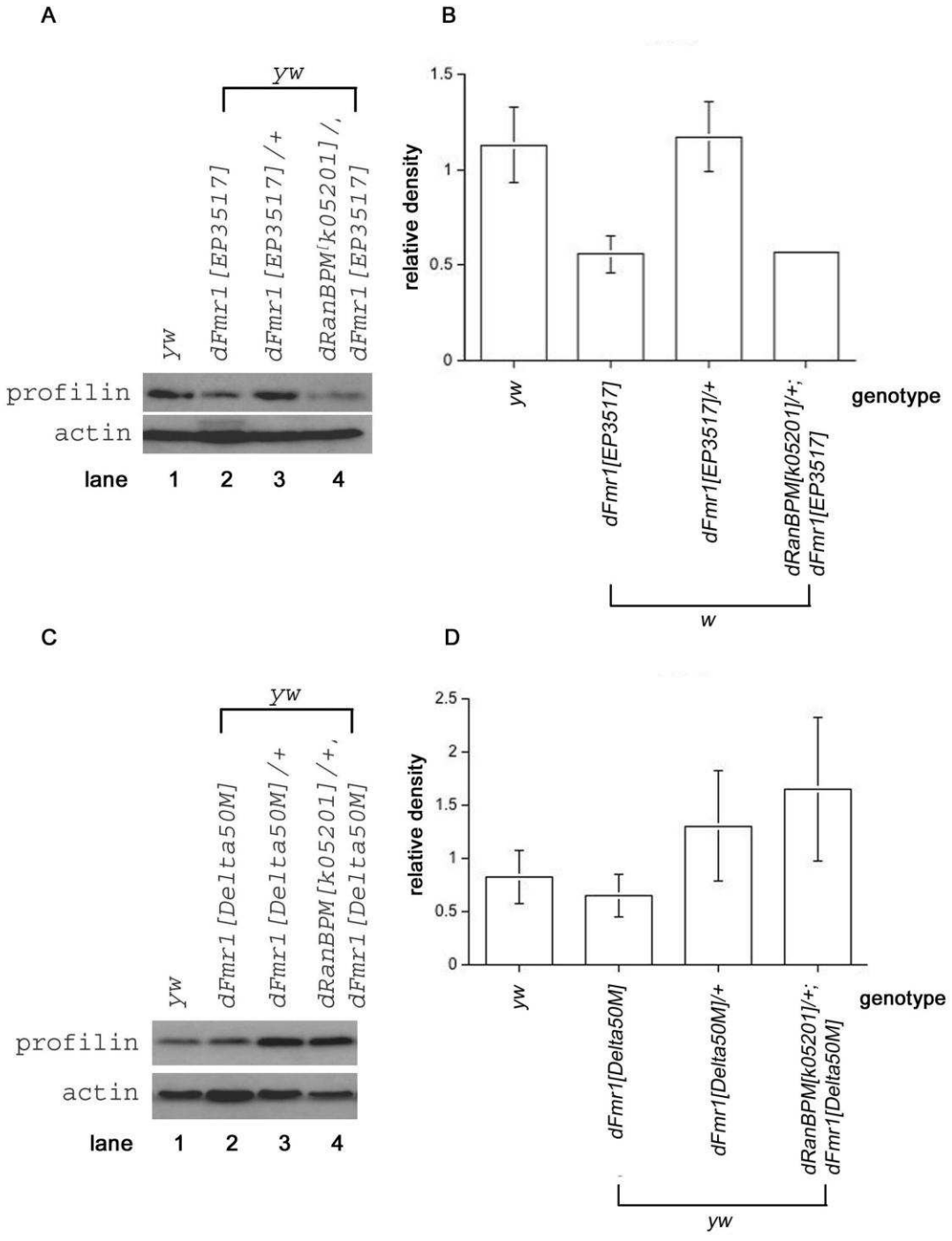
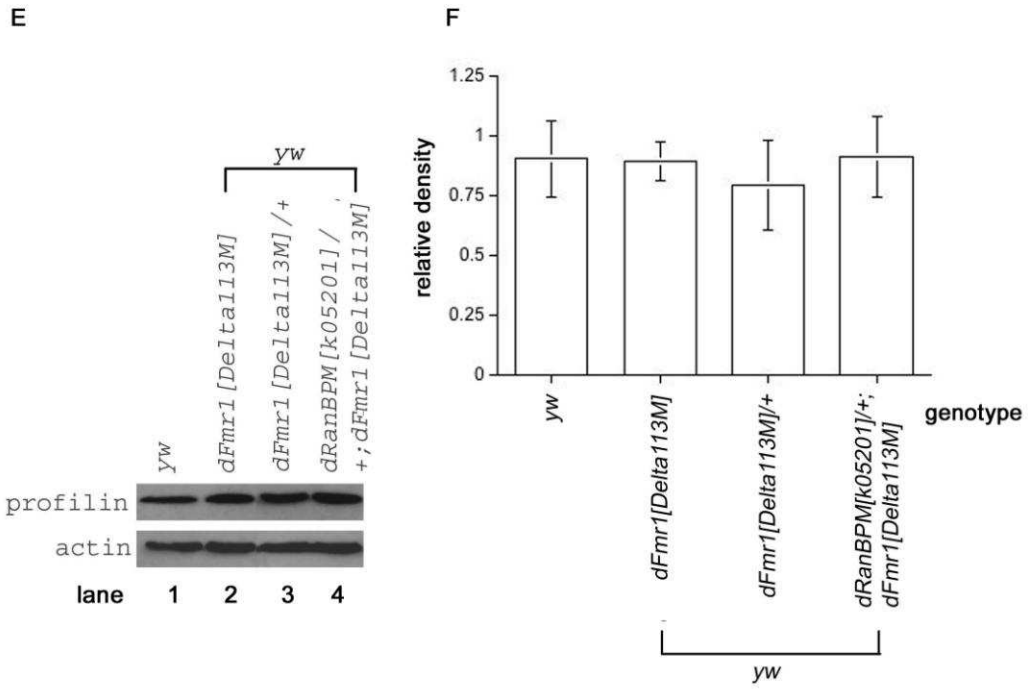

A
yw
dFmr1Delta50M]/dFmr1[Delta113M]
dFmr1Delta50M]
dFmr1[Delta50M]/+
dFmr1[Delta113M]
dFmr1[Delta113M]/+
profilin
actin
lane 1 2 3 4 5 6
B
relative density
0
1
2
3
4
yw
dFmr1[Delta50M]/dFmr1[Delta113M]
dFmr1[Delta50M]
dFmr1[Delta50M]/+
dFmr1[Delta113M]
dFmr1[Delta113M]/+
genotype
yw

Fig 3.2: Decrease in dRanBPM function does not significantly change the levels of profilin in dFmr1 mutants. Fly heads (30ug) carrying hypomorphic allele dFmr1[EP3517] (A,B), amorphic dFmr1[Delta50M] (C,D) or amorphic dFmr1[Delta113M] (E,F) were run on a SDS-PAGE gel, probed for profilin and quantified. Actin served as loading control. No significant differences were found between flies with or without dFmr1 expression, as well as between dFmr1 mutant flies with or without an additional decrease in dRanBPM function. At least two blots were quantified for each dFmr1 allele ($N \geq 2$). All error bars represent +S.E.M. Level of significance was established at $p<0.05$.


A
yw
yw
dFmr1[EP3517]
dFmr1[EP3517]/+
dRanBPM[k05201]/, dFmr1[EP3517]
profilin
actin
lane 1 2 3 4
B
relative density
1.5
1
0.5
0
yw
dFmr1[EP3517]
dFmr1[EP3517]/+
dRanBPM[k05201]/+; dFmr1[EP3517]
genotype
w
C
yw
yw
dFmr1[Delta50M]
dFmr1[Delta50M]/+
dRanBPM[k05201]/+, dFmr1[Delta50M]
profilin
actin
lane 1 2 3 4
D
relative density
2.5
2
1.5
1
0.5
0
yw
dFmr1[Delta50M]
dFmr1[Delta50M]/+
dRanBPM[k05201]/+; dFmr1[Delta50M]
genotype
yw


E
yw
dFmr1[Delta113M]
dFmr1[Delta113M]/+
dRanBPM[k05201]/+;dFmr1[Delta113M]
profilin
actin
lane 1 2 3 4
F
relative density
1.25
1
0.75
0.5
0.25
0
yw
dFmr1[Delta113M]
dFmr1[Delta113M]/+
dRanBPM[k05201]/+; dFmr1[Delta113M]
genotype
yw

function in dFmr1 mutant flies, I began probing for this cytoskeletal protein in wildtype OR flies using heads and body samples. Futsch is molecularly heavy (Hummel et al., 2000) and did not migrate well down a 7% gel despite extending the gel run period **(Fig 3.3A).** Authors of one paper reported that Futsch is able to migrate successfully down a 4-12% gradient gel (Reeve et al., 2005). From my attempts, however, this protein did not migrate past the stacking gel **(Fig 3.3B)**. Due to time constraints, further optimizations for the detection of Futsch were not carried out.

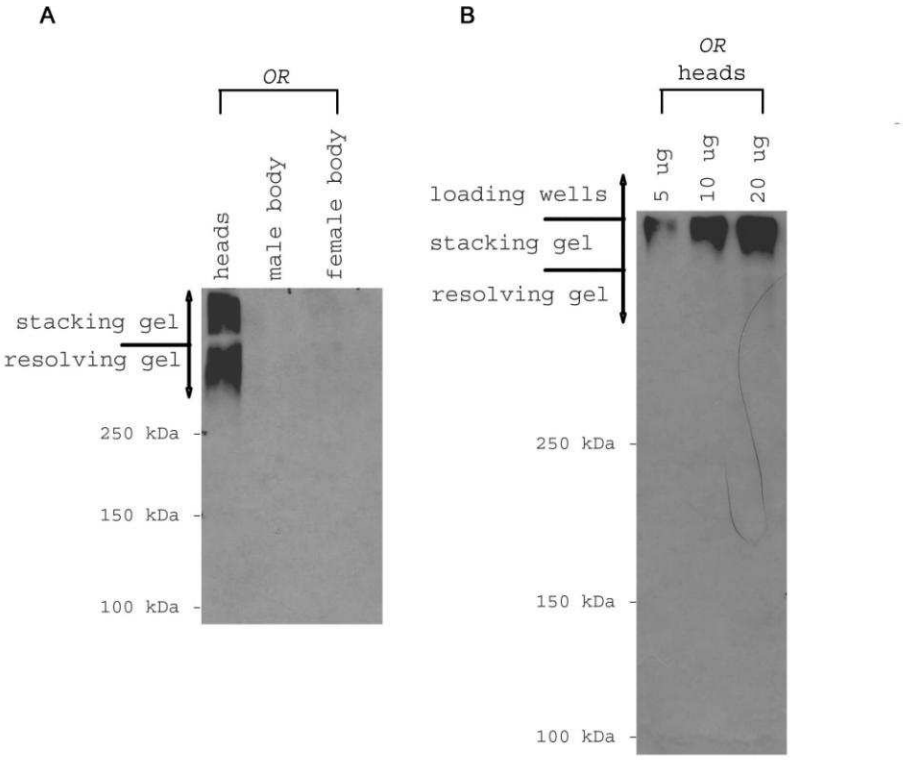
2.0 Products encoded by the long dRanBPM construct

Lethality exhibited by all dRanBPM mutants at the 3rd instar (Scantlebury et al., 2010) can be rescued by spatially targeting dRanBPM expression using either two dRanBPM constructs and a tissue specific GAL4 driver through the UAS/GAL4 system **(Table 1.2;** Brand and Perrimon, 1993**)**.

The two dRanBPM constructs are believed to each express for a short or a long isoform. Both constructs were made by cloning the cDNA of each dRanBPM isoform into separate P-elements. The cDNA of the long isoform, however, contains the ATG start site of the short isoform within its 2nd exon **(Fig 1.1;** Tweedie et al., 2009). To investigate if both isoforms were possibly expressed from the long dRanBPM construct, I overexpresed either dRanBPM constructs in wildtype flies and compared construct products from western blots **(Fig 3.4)**.

Constructs were expressed using either one of two ubiquitous drivers, the heat

Fig 3.3: Futsch cannot travel completely into the resolving gel even after extending gel run period. Heads and bodies of wildtype OR flies were immunobloted and probed for Futsch using two different gel percentages. (A) In a 7% gel, 50 ug of sample lysate was loaded. Futsch was enriched in the heads and the amount of lysate loaded was too high for the sensitivity of western blot detection. In addition, a large proportion of Futsch remained in the stacking gel. (B) A reduction in the amount of head lysate when loaded into a 4-12% gradient gel did not improve migration of Futsch.


A
OR
heads
male body
female body
stacking gel
resolving gel
250 kDa
150 kDa
100 kDa
B
OR
heads
5 ug
10 ug
20 ug
loading wells
stacking gel
resolving gel
250 kDa
150 kDa
100 kDa

shock protein (hsp70-GAL4) or daughterless (da-GAL4). Flies carrying hsp-GAL4 and a dRanBPM construct expressed dRanBPM only after heat shock treatment at 37ºC. The α-dRanBPM used was raised to recognize both dRanBPM isoforms **(Fig 1.1)**.

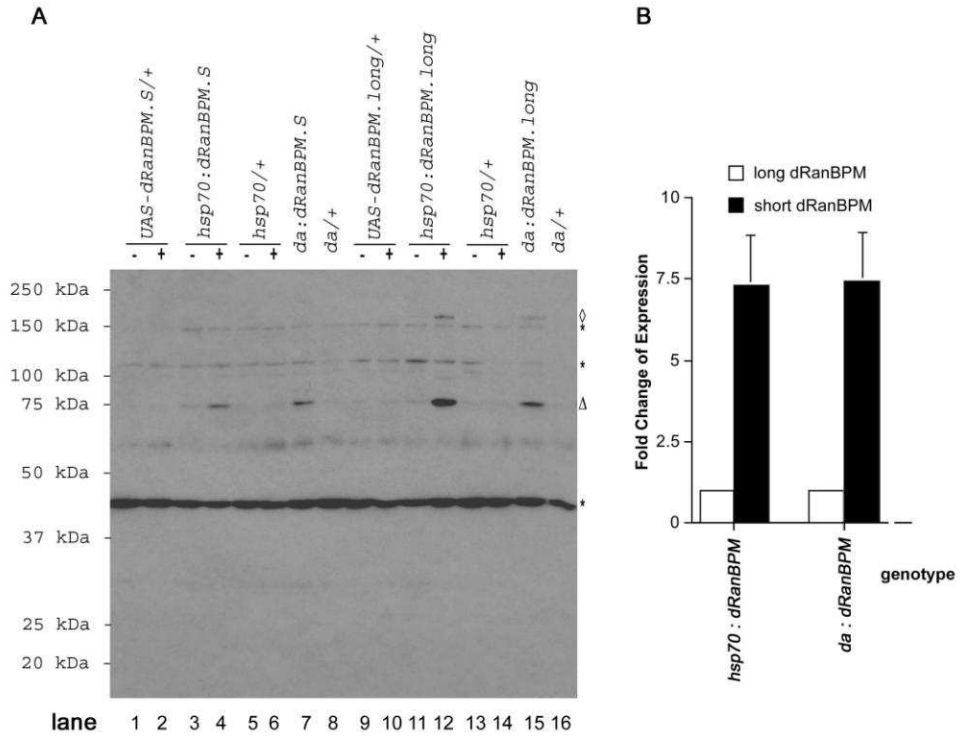
Two unique bands were detected from lanes of those flies expressing the long dRanBPM construct, whereas only one unique band was detected from samples expressing the short dRanBPM construct when compared to controls without construct expression **(Fig 3.4A, bands marked by (Δ) and (◊) respectively)**. The distance migrated by the band observed from expressing the short dRanBPM construct corresponded to the lower migrating band of samples expressing the long dRanBPM construct. These unique bands are believed to represent the short and the long isoforms. From this evidence, the long dRanBPM construct did indeed express both isoforms.

Levels of isoform expression by the long dRanBPM construct were noticeably different. The intensities of band signal, however, were not significantly different from each other **(Fig 3.4B, hsp70-GAL4: p=0.053; da-GAL4: p=0.051)**.

3.0 dRanBPM is involved in IIS pathway activation

As mentioned previously, dRanBPM mutants can be rescued from lethality by targeting expression of dRanBPM in specific tissues. The percentages of rescue, however, differ in which tissues are targeted **(Table 1.2)**. Only the expression of the short dRanBPM construct in NSCs is capable of rescuing all dRanBPM mutants from lethality. **(Table 1.2)**. dRanBPM larvae also show a decreased in size (Scantlebury et al., 2010),

Fig 3.4: The long dRanBPM construct encodes for both the short and long isoforms. Fly heads (100ug) were lysed and probed with anti-dRanBPM. (A) Endogenous levels of dRanBPM were too low for detection. Ectopic expression of either the short or the long dRanBPM construct was driven either the heat shock (hsp70-GAL4) or daughterless (da-GAL4). α-dRanBPM recognizes both short (Δ) and long (◊) dRanBPM isoforms of sizes around 75 and 160 kDa respectively. (+) and (-) denotes whether flies underwent heat shock treatment at 37°C before they were sacrificed. Cross reactivity bands are marked by asterisks (*). (B) The long dRanBPM construct encodes for both isoforms but at different levels of expression. The short isoform was expressed nearly 7 times higher than that of the long isoform when driven by either ubiquitous drivers. Levels of expression between the isoforms were not significant. Three blots were used for quantitative analysis (N=3). Protein levels were graphed as mean + S.E.M. Level of significance was established at $p<0.05$


A
UAS-dRanBPM.S/+
hsp70:dRanBPM.S
hsp70/+
da:dRanBPM.S
da/+
UAS-dRanBPM.long/+
hsp70:dRanBPM.long
hsp70/+
da:dRanBPM.long
da/+
- + - + - + - + - + - + - +
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
B
long dRanBPM
short dRanBPM
Fold Change of Expression
10
7.5
5
2.5
0
hsp70 : dRanBPM
da : dRanBPM
genotype

but this can be restored to wildtype from targeted dRanBPM expression to NSCs (XL Zhao, unpublished results). The knowledge that organism size is regulated by the IIS pathway (reviewed by Goberdhan and Wilson, 2003a, 2003b AND Hietakangas and Cohen, 2009) and that this pathway is activated by neuropeptides called DILPs (Tissenbaum and Ruvkun, 1997; reviewed by Garofalo, 2002 AND Baker and Thummel, 2007 AND Giannakou and Partridge, 2007), provided suitable clues that dRanBPM mutants may have reduced IIS pathway activation.

To compare IIS pathway activation between dRanBPM mutants and those with normal or rescued dRanBPM expression, I measured the levels of a major downstream IIS pathway effector called dAkt (reviewed by Fresno et al., 2004 AND Chan and Tsichlis, 2001) using 2nd instar larvae from wildtype OR, dRanBPM mutants and rescued dRanBPM mutants with targeted dRanBPM expression to neurosecretory cells (c929:dRanBPM.S).

Levels of total dAkt, which included both activated and unactivated populations of dAkt, as well as levels of phosphorylated dAkt (phospho dAkt) of the three sample groups were statistically analyzed **(Fig 3.5).** Amount of total dAkt were not significantly different between larval groups. Levels of phosphorylated dAkt, however, were significantly decreased in dRanBPM mutants when compared to wildtype OR **(Fig 3.5B, p=0.035)** but not between dRanBPM mutants and rescued dRanBPM larvae. Nonetheless, this result demonstrates that dRanBPM mutants have reduced IIS pathway activation.

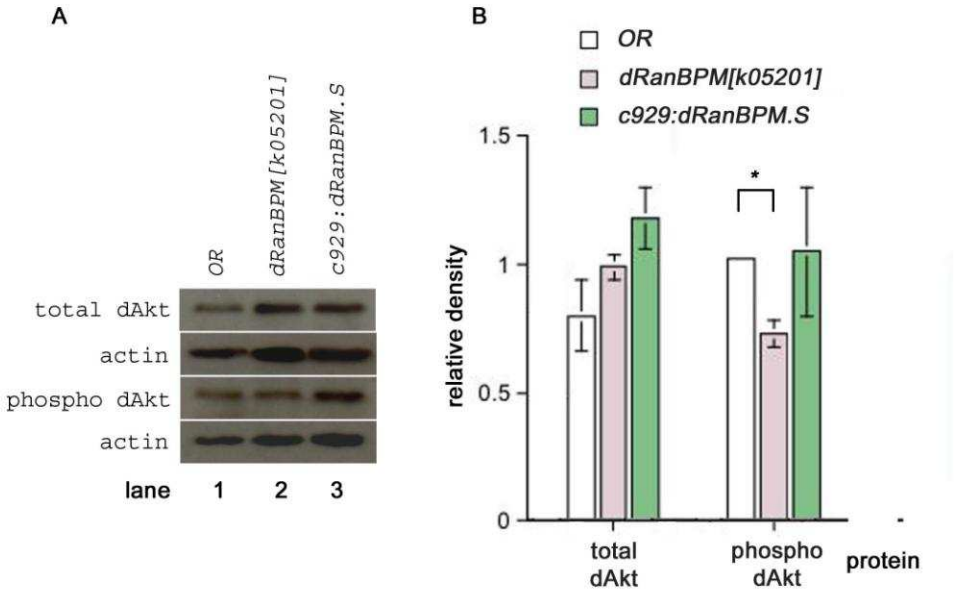
3.1 Involvement of dRanBPM in longevity

Activation of the IIS pathway negatively impacts longevity by ultimately causing the translocation of a transcription factor called FOXO out to the nucleus (Biggs et al., 1999; Nakae et al. 1999; Giannakou et al., 2004; Hwangbo et al., 2004; reviewed by Zhao et al., 2011). FOXO transcribes genes that play in roles such as DNA repair, metabolism and oxidative stress resistance, all which contribute to extending lifespan (reviewed by Taguchi and White, 2008 AND Partridge and Bruning, 2008 AND Van der Vos and Coffer, 2011). The activity of FOXO is regulated through posttranslational modifications (PTMs). Upon phosphorylation by Akt, FOXO travels out to the cytoplasm and becomes inactivated (Biggs et al., 1999; Nakae et al., 1999; Giannakou et al., 2004; Hwangbo et al., 2004; reviewed by Zhao et al., 2011).

Since the previous experiment demonstrated that dRanBPM is involved in IIS pathway activation, lack of dRanBPM function may extend the lifespans of dRanBPM mutants. To conduct a longevity experiment on dRanBPM mutants, these mutants must first bypass the requirement for dRanBPM function during development (Scantlebury et al., 2010). This was done by providing dRanBPM mutants with either ubiquitous or neurosecretory expression of the short dRanBPM construct via the UAS/GAL4 system **(Table 1.2)**.

Once dRanBPM mutants reach adulthood, expression of dRanBPM was shut off using a temperature sensitive yeast element Tub-GAL80[TS] by transferring flies from 18ºC to 29ºC. Expression of Tub-GAL80[TS] was driven by the ubiquitous tubulin promoter.

Fig 3.5: Loss of dRanBPM function significantly decreases the levels of activated dAkt. (A) Levels of total dAkt and phospho dAkt were used to assess IIS pathway activation in 2nd instar dRanBPM mutant larvae (100 ug). Control groups were wildtype larvae (OR) and rescued dRanBPM mutants with targeted expression of the short dRanBPM construct to neurosecretory cells (c929:dRanBPM.S). Actin served as loading control. (B) After quantification and statistical analysis, total dAkt levels did not differ between genotypes. Phospho dAkt levels in dRanBPM mutants were significantly reduced compared to OR larvae ($p=0.035$) but not to rescued larvae. Three blots were used for quantitative analysis (N=3). Protein levels were graphed as mean ± S.E.M. Level of significance was determined as $p<0.05$.


A
OR
dRanBPM[k05201]
c929:dRanBPM.S
total dAkt
actin
phospho dAkt
actin
lane 1 2 3
B
OR
dRanBPM[k05201]
c929:dRanBPM.S
relative density
1.5
1
0.5
0
*
total dAkt
phospho dAkt
protein

To obtain dRanBPM mutant flies carrying Tub-GAL80[TS], a dRanBPM construct and a GAL4 driver, I constructed three fly stocks, which I refer to as A, B and C. Fly stock A carried one copy of functional dRanBPM, a Tub-GAL80[TS] and a dRanBPM construct (stock A genotype: Tub-GAL80[TS]; dRanBPM[k05201]/CyO; UAS-dRanBPM.S). Fly stocks B and C carried one copy of functional dRanBPM as well as either a ubiquitous (stock B: tub-GAL4) or a neurosecretory (stock C: c929-GAL4) specific driver respectively (stock B and C genotype: yw; dRanBPM[k05201]/CyO; GAL4 driver).

Fly stock A was crossed to both B and C stocks. All progenies were raised at 29ºC to allow for dRanBPM expression. Upon eclosion, progenies homozygous for nonfunctional dRanBPM carrying a copy of the Tub-GAL80[TS], the short dRanBPM construct and either the ubiquitous or a neurosecretory specific driver (genotype: Tub-GAL80[TS]; dRanBPM[k05201]; UAS-dRanBPM.S/GAL4 driver) were selected and placed at 29ºC or 18ºC. I refer to these progenies as rescued dRanBPM experimental flies.

The lifespans of flies placed at 18ºC or 29ºC cannot be compared to each other since temperature affects longevity (Miquel et al., 1974). For this reason, lifespans must be compared within the same temperature group. To provide suitable controls to evaluate the lifespans of rescued dRanBPM experimental flies, I included three groups of flies which expressed dRanBPM regardless of temperature. The first two groups were wildtype flies (genotypes: OR and yw), while the last group was constructed to carry homozygous copies of nonfunction dRanBPM, the short dRanBPM construct and either the ubiquitous (tub-GAL4) or neurosecretory (c929-GAL4) specific driver (genotype:

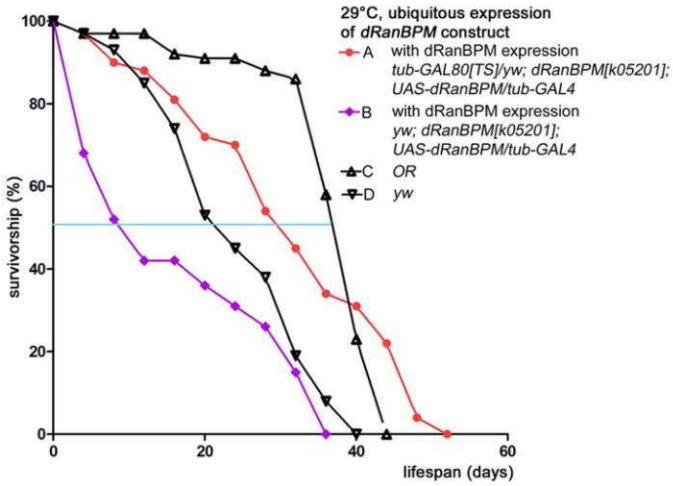
dRanBPM[k05201]; UAS-dRanBPM.S/GAL4 driver). The last groups of flies I refer to as rescued dRanBPM controls.

Survivorship curves of the fly groups were graphed **(Fig 3.6)** and data of each genotype were summarized **(Table 3.1)**. At 29ºC, all flies should express dRanBPM, and therefore, possess normal activation of the IIS pathway. I would not expect the median lifespans of the genotypes to statistically differ from each other. However, significant differences were detected between rescued dRanBPM experimental and rescued dRanBPM control flies **(Fig 3.7A, genotypes A and B, p=0.0003)**, between wildtype OR and yw **(Fig 3.7A, genotypes C and D, $p=2.2*10^{-16}$),** between rescued dRanBPM experimentals and wildtype yw **(Fig 3.7A, genotypes A and D, p=0.001)** and between rescued dRanBPM controls and wildtype OR **(Fig 3.7A, genotypes B and D, $p=6.9*10^{-9}$)**.

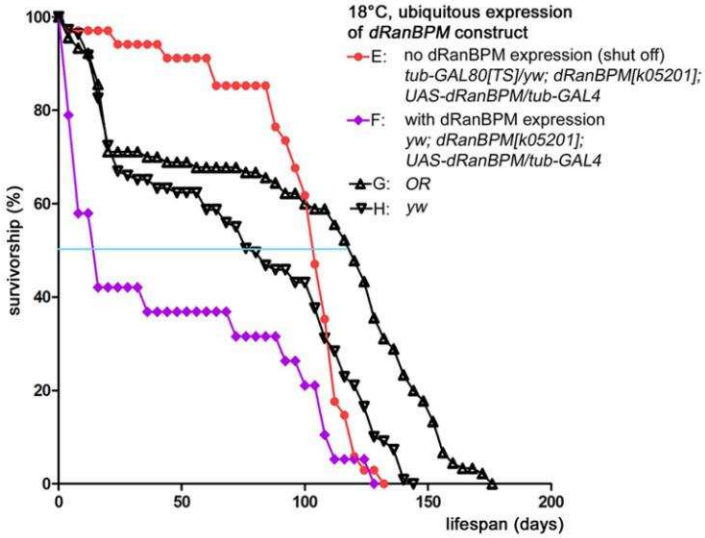
When ubiquitous expression of dRanBPM was shut off in rescued dRanBPM experimental flies at 18ºC, I expected this group to have a significantly greater median lifespan than that of other genotypes. However, the median lifespan of rescued dRanBPM experimental flies was significantly higher to rescued dRanBPM controls only **(Fig 3.7B, genotype E and F, p=0.004)**. dRanBPM expression was not shut off in control groups and I expected for their median lifespans to be similar to each other. Comparisons between controls were statistically different: between rescued dRanBPM controls and wildtype OR and yw **(Fig 3.7B, genotypes F to G and H, $p= 5.05 * 10^{-5}$, genotypes F to H, p= 0.002, respectively)**, and between wildtypes OR and yw **(Fig 3.7B, genotypes G**

Fig 3.6: Survivorship curves of virgin females. Lifespans of rescued dRanBPM experimental flies and their controls were graphed at two different temperatures. The separate temperatures were necessary to control GAL4 activity on dRanBPM expression through a temperature sensitive Tub-GAL80[TS]. At 18ºC, Tub-GAL80[TS] suppressed GAL4 activity, while at 29ºC, Tub-GAL80[TS] was inactive. Expression of the short dRanBPM construct in dRanBPM mutants was driven by either one of two tissue specific drivers: the ubiquitous driver (tub-GAL4) at (A) at 29ºC and (B) at 18ºC, or by the neurosecretory cell driver (c929-GAL4) at (C) at 29ºC and (D) at 18ºC. The blue line denotes 50% survivorship, or median lifespan.

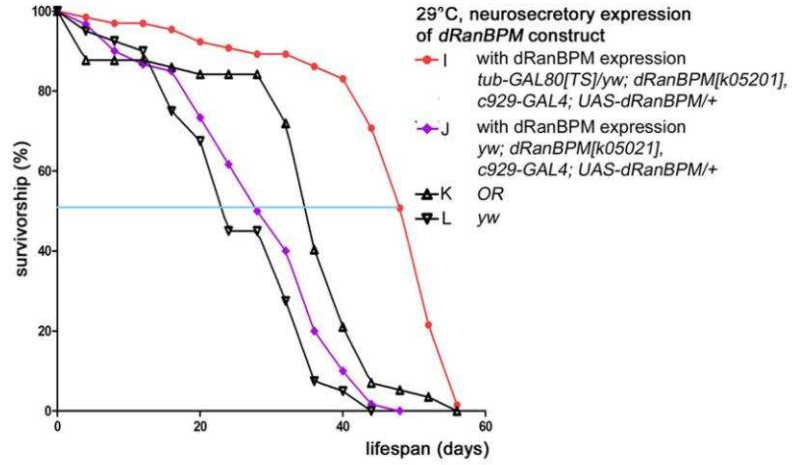
A


29°C, ubiquitous expression
of dRanBPM construct
A with dRanBPM expression
tub-GAL80[TS]/yw; dRanBPM[k05201];
UAS-dRanBPM/tub-GAL4
B with dRanBPM expression
yw; dRanBPM[k05201];
UAS-dRanBPM/tub-GAL4
C OR
D yw
survivorship (%)
lifespan (days)
0
20
40
60
80
100

B


18°C, ubiquitous expression
of dRanBPM construct
E: no dRanBPM expression (shut off)
tub-GAL80[TS]/yw; dRanBPM[k05201];
UAS-dRanBPM/tub-GAL4
F: with dRanBPM expression
yw; dRanBPM[k05201];
UAS-dRanBPM/tub-GAL4
G: OR
H: yw
survivorship (%)
lifespan (days)
0
20
40
60
80
100
0
50
100
150
200

A


29°C, neurosecretory expression
of dRanBPM construct
I with dRanBPM expression
tub-GAL80[TS]/yw; dRanBPM[k05201],
c929-GAL4; UAS-dRanBPM/+
J with dRanBPM expression
yw; dRanBPM[k05021],
c929-GAL4; UAS-dRanBPM/+
K OR
L yw
survivorship (%)
lifespan (days)
0
20
40
60
80
100

B

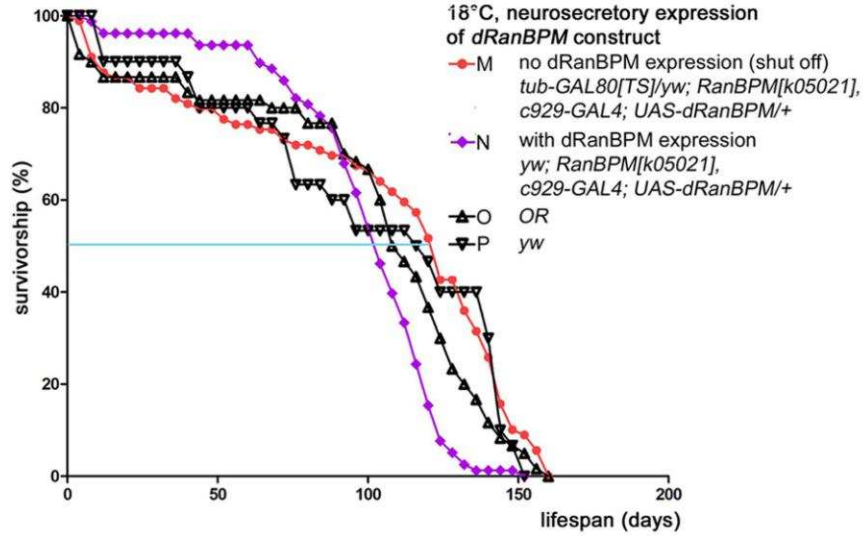

18°C, neurosecretory expression
of dRanBPM construct
M no dRanBPM expression (shut off)
tub-GAL80[TS]/yw; RanBPM[k05021],
c929-GAL4; UAS-dRanBPM/+
N with dRanBPM expression
yw; RanBPM[k05021],
c929-GAL4; UAS-dRanBPM/+
O OR
P yw
survivorship (%)
lifespan (days)
0
50
100
150
200

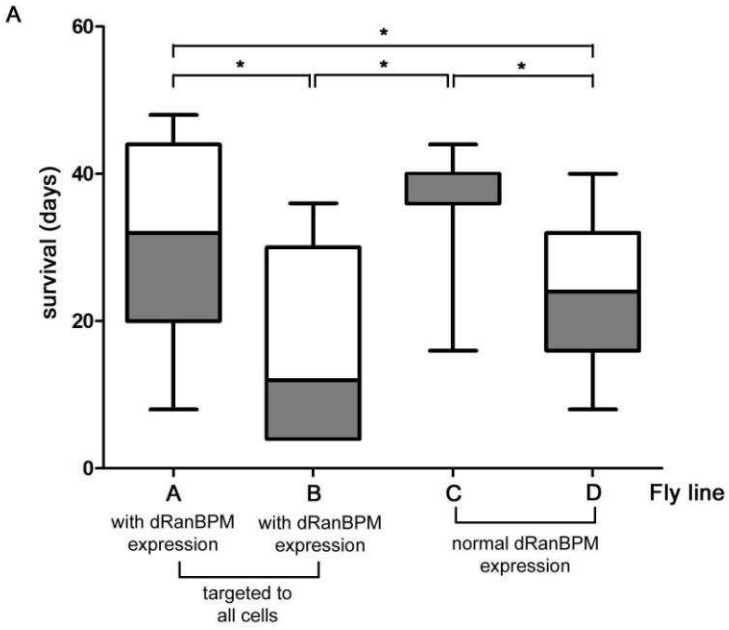
Table 3.1: Data summary of virgin female lifespans. Expression of the short dRanBPM construct was expressed either ubiquitously (tub-GAL4) or specifically in neurosecretory cells (c929-GAL4). N denotes number of individuals used for each genotype. Median lifespans were obtained from calculating 50% survivorship of each genotype.

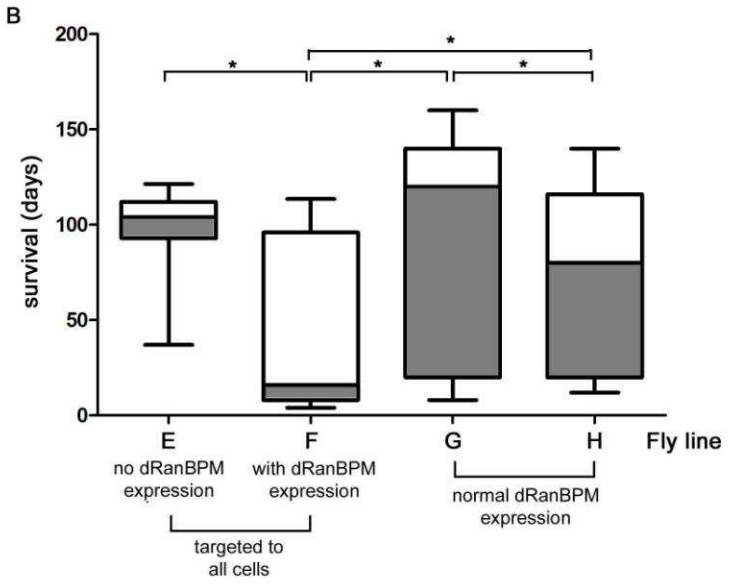
Table 3.1: Summary of data on the lifespans of *Drosophila* virgin females.

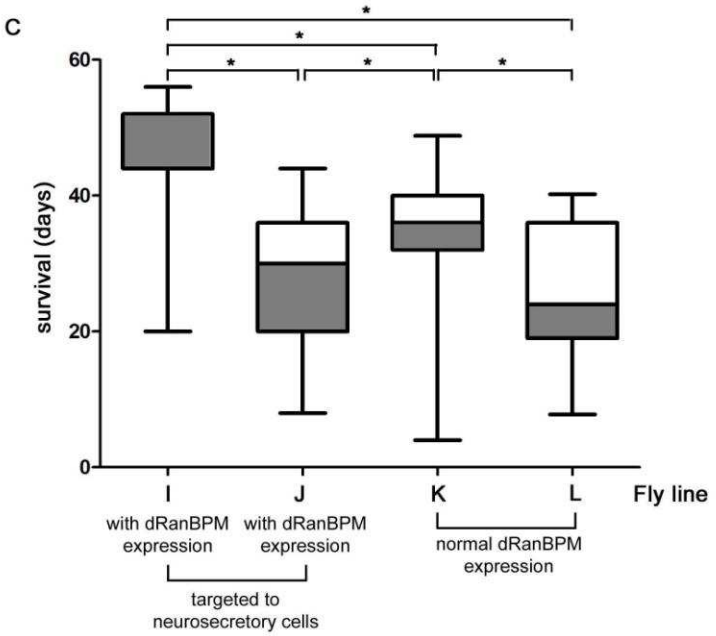
	Genotype	dRanBPM expression	expression targeted to	N	Median lifespan (days)	Maximum lifespan (days)
29°C, ubiquitous expression of *dRanBPM* construct						
A	*tub-GAL80[TS]/yw; dRanBPM[KO5701]/UAS-dRanBPMS;tub-GAL4* (rescued *dRanBPM* experimental flies)	+	All cells	40	32	52
B	*yw; dRanBPM[KO5701]/UAS-dRanBPMS;tub-GAL4* (rescued *dRanBPM* control flies)	+	All cells	19	12	36
C	*OR*	+	-	80	40	44
D	*Yw*	+	-	100	24	40
18°C, ubiquitous expression of *dRanBPM* construct						
E	*tub-GAL80[TS]/yw; dRanBPM[KO5701]/UAS-RanBPMS;tub-GAL4* (rescued dRanBPM experimental flies)	-	All cells	44	104	132
F	*yw; dRanBPM[KO5701]/UAS-dRanBPMS;tub-GAL4* (rescued *dRanBPM* control flies)	+	All cells	19	16	128
G	*OR*	+	-	90	120	176
H	*Yw*	+	-	109	80	144

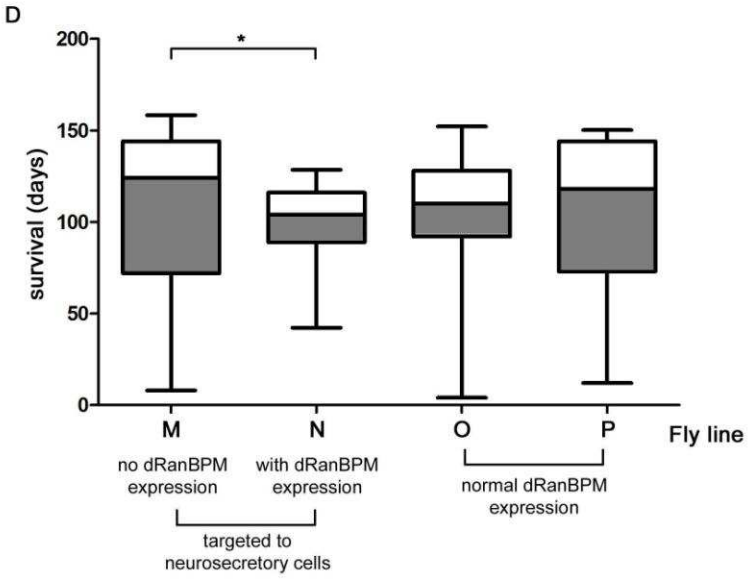
29°C, neurosecretory expression of *dRanBPM* construct							
I	*tub-GAL80[TS]/yw; dRanBPM[k05201],c929-GAL4; UAS-dRanBPMS/+* (rescued dRanBPM experimental flies)	+	Neurosecretory cells	65	52		60
J	*yw; dRanBPM[k05201],c929-GAL4; UAS-dRanBPMS/+* (rescued *dRanBPM* control flies)	+	Neurosecretory cells	60	30		48
K	*OR*	+	-	57	36		56
L	*Yw*	+	-	40	24		44
18°C, neurosecretory expression of *dRanBPM* construct							
M	*tub-GAL80[TS]/yw;d RanBPM[k05201],c929-GAL4; UAS-dRanBPMS/+* (rescued dRanBPM experimental flies)	-	Neurosecretory cells	89	124		160
N	*yw;d RanBPM[k05201],c929-GAL4; UAS-dRanBPMS/+* (rescued *dRanBPM* control flies)	+	Neurosecretory cells	78	104		152
O	*OR*	+	-	60	110		160
P	*Yw*	+	-	30	118		152

Fig 3.7: Box plot analysis of virgin female lifespans. Median lifespans obtained from survivorship curves of rescued dRanBPM experimental flies and their controls were graphed according to the driver used to express the short dRanBPM construct and temperature the flies were placed in: the ubiquitous driver (tub-GAL4) (A) at 29ºC and (B) at 18ºC or the neurosecretory cell driver (c929-GAL4) at (C) at 29ºC and (D) at 18ºC. In each box plot, the solid line separating white and gray portions is the median lifespan. The line at the top of the white box represents 25% of dataset, while the line at the bottom of the grey box represents 75% of dataset. The whiskers represent 5 (top whisker) and 95 (bottom whisker) percentiles of data. Median lifespans were calculated for significant differences using the Kruskal-Wallis test. Level of significance was established at $p<0.00833$.


A
60
40
20
0
survival (days)
*
*
*
*
A
B
C
D
Fly line
with dRanBPM expression
with dRanBPM expression
normal dRanBPM expression
targeted to all cells


B
200
150
100
50
0
survival (days)
*
*
*
*
E
F
G
H
Fly line
no dRanBPM expression
with dRanBPM expression
normal dRanBPM expression
targeted to all cells


C
*
*
*
*
*
*
60
40
20
0
survival (days)
I
J
K
L
Fly line
with dRanBPM expression
with dRanBPM expression
normal dRanBPM expression
targeted to neurosecretory cells


D
*
200
150
100
50
0
survival (days)
M
N
O
P
Fly line
no dRanBPM expression
with dRanBPM expression
normal dRanBPM expression
targeted to neurosecretory cells

and H, p=0.0008).

Similar to what I had expected of the median lifespans 29ºC with mutant flies ubiquitously expressing dRanBPM, the median lifespan of rescued dRanBPM experimental flies with targeted dRanBPM expression to neurosecretory cells should not differ from those of control flies. However, median lifespan of rescued dRanBPM experimental flies was significantly different compared to all controls **(Fig 3.7C, genotypes I to J: p=3.4 * 10-14, genotypes I to K: p=1.5 * 10^{-10}, genotypes I to L: p=1.4*10^{-12})**. Within control groups, statistical differences were also found between median lifespans of wildtype OR flies and rescued dRanBPM controls **(Fig 3.7C, line J and K, p=6.97*10^{-9})** and between wildtype OR and wildtype yw **(Fig 3.7C, line K and L, p=2.2*10^{-16})**.

Finally, when targeted expression of dRanBPM to neurosecretory cells was shut off at 18ºC in rescued dRanBPM experimental flies, I expected this genotype to have a significantly higher median lifespan than all control groups. The only significant comparison was between rescued dRanBPM experimental and rescued dRanBPM control flies **(Fig 3.7D, line M and N, p=0.006)**. All other pairwise comparisons were insignificant.

Discussion

Chapter 4

RanBPM is a conserved and ubiquitous putative scaffold protein first discovered by Yokoyama et al. in 1995 from a yeast-2-hybrid screen (reviewed by Murrin and Talbot, 2007; Hosono et al., 2010). This protein is capable of binding to numerous protein partners, suggesting that RanBPM functions in many cellular processes. However, for all the information known about RanBPM, its exact roles remain unclear (reviewed by Murrin and Talbot, 2007).

The first RanBPM mutations were recovered in Drosophila melanogaster (Dansereau and Lasko, 2008). Loss-of-function in dRanBPM gives rise to pleiotropic phenotypes, such as defects in larval response to light, locomotion, feeding and size (Scantelbury et al., 2010). These mutant larvae die before reaching adulthood (Scantlebury et al., 2010).

To begin understanding the underlying mechanisms that give rise to dRanBPM phenotypes, the larval locomotory defect was examined (Scantlebury et al., 2010). At the NMJ, dRanBPM mutants have reduced branching and bouton number (XL Zhao, unpublished results). Synaptic architecture of the NMJ is controlled by a RanBPM interacting partner called FMRP (Menon et al., 2004). FMRP regulates neuromuscular structure by controlling the levels of two cytoskeletal proteins: Futsch (Zhang et al., 2001; Reeve et al., 2005) and profilin (Reeve et al., 2005).

Since mutations to dRanBPM (Scantlebury et al., 2010) or dFMRP (Zhang et al., 2001) produce defects in the synaptic architecture of the NMJ, an experiment was previously carried out to find whether the two proteins genetically interact to regulate the

neuromuscular structure (Scantlebury et al., 2010). Results from this experiment showed that both proteins indeed share an epistatic relationship at the NMJ.

1.0 Levels of profilin and Futsch in dFmr1 mutant flies

To find if dRanBPM is capable of regulating Futsch and profilin through dFMRP, my first goal was to measure their levels from dFmr1 mutant flies either with or without reduced dRanBPM function.

Due to time constraints, I was unable to optimize the conditions necessary to detect Futsch. This protein is molecularly heavy (Hummel et al., 2000) and is more susceptible to degradation. To minimize the amount of degradation, I used the O'Farrell buffer, a more powerful denaturant than SDS sample buffer, to lyse samples (A Bedard, personal communication; O'Farrell, 1975). The large size of Futsch also presented difficulties in gel migration, where decreasing gel percentage (7% or 4-12% gradient gel) did not improve migration conditions, despite increasing the period of the gel run (up to 18 hours) **(Fig 3.3)**. If another attempt were to be made to detect for levels of Futsch, a lower percentage gel with larger pore size should be prepared. Pore size can be enlarged by increasing the ratio of acrylamide to bis-arcylamide, a cross linking agent.

Immunoblots for the second cytoskeletal protein of interest, profilin, were successfully performed. Blots comparing levels of profilin between flies homozygous and heterozygous for loss-of-function dFmr1 were recreated similarly as that by Reeve and colleagues (2005) to confirm the negative regulation of FMRP on profilin expression **(Fig 3.1)**. Despite what was reported in the literature (Reeve et al., 2005), no significant

differences in levels of profilin were found between dFmr1 mutants and wildtype controls. Not surprisingly, levels of profilin between dFmr1 mutant flies also did not significantly differ between dFmr1 mutant flies with an additional decrease in dRanBPM function **(Fig 3.2)**.

An explaination to why my findings and those from Reeve and colleagues (2005) differ might be because dFMRP does not negatively regulate the levels of profilin at all; since the publication of the paper by Reeve and colleagues (2005), no other literature has reported similar results.

2.0 The long dRanBPM construct encodes for two products

All dRanBPM mutants die at the 3rd instar foraging stage, however, they can be rescued from lethality by ectopically restoring the expression of dRanBPM via dRanBPM constructs **(Table 1.2)**. Two dRanBPM constructs were made by cloning the cDNA of the long and short isoforms. The coding sequence of the long isoform, however, also contains the ATG start site of the short isoform **(Fig 1.1).** For this reason, the long dRanBPM construct could potentially express both isoforms.

To determine whether the long dRanBPM construct is capable of expressing both the dRanBPM isoforms, immunoblots of flies ubiquitously expressing dRanBPM constructs were conducted. I confirmed that the long construct indeed encoded for both dRanBPM isoforms. Levels of isoform expression were not statistically significant **(Fig 3.4).**

The long dRanBPM construct has been used in experiments to rescue dRanBPM mutant phenotypes such as response to light and locomotion (Scantlebury et al., 2010). In light of my findings, results from previously published experiments should be interpreted with caution: it is uncertain whether the mutant phenotypes were rescued from expression of the long dRanBPM isoform alone or a combination of both isoforms.

The molecular sizes of dRanBPM short and long isoforms have been reported to be 67 kDa and 140 kDa respectively (Dansereau and Lasko, 2008a). These sizes differ from those I found from my blots, which were around 75 and 160 kDa for the short and long isoforms respectively **(Fig 3.4).**

Discrepancies in isoform sizes may arise from different Drosophila samples used to carry out immunoblots. Dansereau and Lasko (2008) used larvae while I used fly heads. In addition, RanBPM may be modified posttranslationally (Nakamura et al., 1998; Nishitani et al., 2001) in a spatially and temporally dependent manner (Araki et al., 1998; reviewed by May and Hill, 2008).

3.0 Decreased IIS pathway activation in dRanBPM mutants

Targeted expression of the short dRanBPM construct to NSCs fully rescues size (XL Zhao, unpublished results) and lethality **(Table 1.2)** of all dRanBPM mutants**.** The fact that both phenotypes are restored to wildtype suggests that they share a common cellular pathway which requires the normal function of NSCs.

Activation of the IIS pathway increases organism size (reviewed by Goberdhan and Wilson, 2003a, 2003b AND Hietakangas and Cohen, 2009) as well as sustaining feeding behaviour (XL Zhao, unpublished results). This pathway is activated by Drosophila insulin homologs called DILPs, which are produced by NSCs (reviewed by Nassel, 2002 AND Teleman, 2009).

Levels of IIS pathway activation were indeed found to be significantly lower in dRanBPM mutants when compared to a wildtype control **(Fig 3.5)**. However, I used dRanBPM mutants from the 2nd instar rather than the 3rd instar larvae for immunoblot samples, despite that mutant phenotypes appear most noticeably at the 3rd larval instar (Scantlebury et al., 2010). If this experiment were to be repeared again using 3rd larval dRanBPM mutants, a more pronounced difference in IIS pathway activation levels should be seen between mutant and wildtype samples. Regardless of this fault, my results show that a molecular phenotype can already be detected in 2nd instar dRanBPM mutants.

Studies from the literature have already alluded to the idea that RanBPM functions in IIS pathway. Hafizi and colleagues (2005) found that RanBPM interacts with Axl, a transmembrane tyrosine kinase receptor. Axl, together with a secreted factor called Gas6, activate Akt and PI3K, which are both IIS pathway effectors (Goruppi et al., 1997; Lee et al., 2002). In addition, Yin and colleagues (2010) found that RanBPM interacts with a tyrosine kinase called tropomyosin-related kinase B (TrkB). This kinase is activated by a neurotrophin called brain-derived neurotrophic factor (BDNF), which is involved in nervous system development for processes such as dendritic arborization, neuronal plasticity and differentiation (reviewed by Nagahara and Yuszynski, 2011). When both

TrkB and RanBPM are co-overexpressed in the presence of BDNF in murine cell lines, Akt activation is increased (Yin et al., 2010).

3.1 dRanBPM and longevity

Besides increasing size and feeding, activation of the IIS pathway decreases longevity (reviewed by Taguchi and White, 2008 AND Teleman, 2009) by inhibiting the activity of a TF called FOXO (reviewed by Taguchi and White, 2008 AND Teleman, 2009). With this knowledge, the lifespans of dRanBPM mutants were measured and compared to controls with targeted for wildtype dRanBPM expression **(Fig 3.6-3.7)**. Unfortunately, my hypothesis where dRanBPM mutants would live longer than wildtype groups did not agree with the data from this experiment.

A reason for the discrepancy may be due to different genetic backgrounds of the control groups used to compare median lifespans of dRanBPM mutants against. Rescued dRanBPM experimental flies with Tub-GAL80[TS] possess a wildtype yellow (y). Wildtype OR flies have no known mutations, while wildtype yw and rescued dRanBPM control flies carry mutations for both y and white (w) genes.

y is widely used as a genetic marker. Loss-of-function to y gives rise to a yellow pigmentation seen in larval mouthhooks and adult cuticle and wings (Wittkopp et al., 2002). The molecular function of y is unknown, although it is involved in the distribution of black melanin (Wittkopp et al., 2002) and in male courtship behaviour (Drapeau et al., 2003). Surprisingly, flies carrying wildtype y have a survival advantage and their lifespan

can be increased by up to 10% when compared to those with the null allele (private communication, Gabrielle Boulianne).

Because inappropriate controls were used for this experiment, no conclusive results can be made on whether dRanBPM affects IIS pathway activation. If another longevity experiment were to be conducted, however, the flies used should all have the same genetic background.

Studies in the literature on Drosophila lifespan report using an alternative method call GeneSwitch that works alongside the UAS/GAL4 system to shut off expression of a construct in a temporal manner (Roman et al., 2001). The tissue specific GAL4 is hormone inducible. Upon hormone administration through food, expression of the transgene occurs (Roman et al., 2001).

In conclusion, results from my experiments demonstrate that although dRanBPM function affects synaptic architecture at the NMJ, it does not regulate the levels of the cytoskeletal protein profilin. Additionally, the long dRanBPM construct used to rescue lethality and behavioural phenotypes in dRanBPM mutants does not only express the long isoform, but also the short isoform. Finally, dRanBPM is involved in IIS pathway activation, although its biological function in lifespan could not be evaluated at this time due to unsuitable controls used in the longevity assay.

My work contributes to the further understanding in how dRanBPM partipates in multiple cellular processes. The discovery that dRanBPM is involved in IIS pathway activation presents new and exciting directions to the importance in uncovering the

functions of this protein. Because Drosophila and human RanBPM are highly conserved, the knowledge gained from studying RanBPM in the Drosophila system can have enormous medical impact in what is currently known about the IIS pathway.

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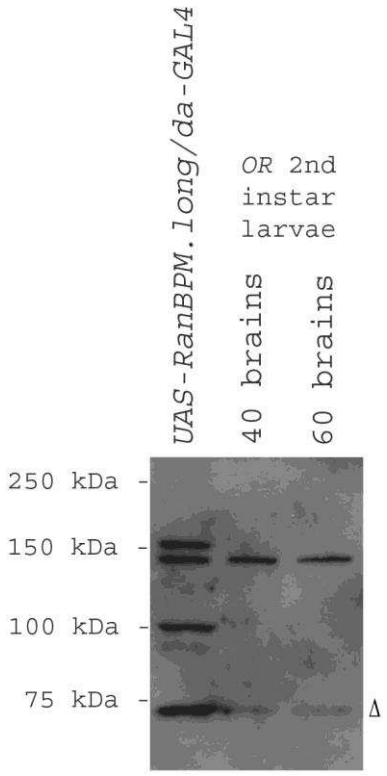
Appendix A

Fig A: The dRanBPM short isoform is expressed in 2nd instar wildtype larval brains. Because endogenus levels of dRanBPM isoforms are too low for their detection, lysates from fly heads overexpressing the long dRanBPM construct under the control of a ubiquitous driver (da-GAL4) (lane 1) was used as a reference to compare for dRanBPM bands detected from 2nd instar larval brains (lane 2,3). Delta (Δ) denotes the band identifying the short dRanBPM isoform. From both larval lanes, a faint band of the same size as the short isoform can be seen. Actin control was not blotted for.


UAS-RanBPM.long/da-GAL4
OR 2nd instar larvae
40 brains
60 brains
250 kDa
150 kDa
100 kDa
75 kDa
Δ

Appendix B

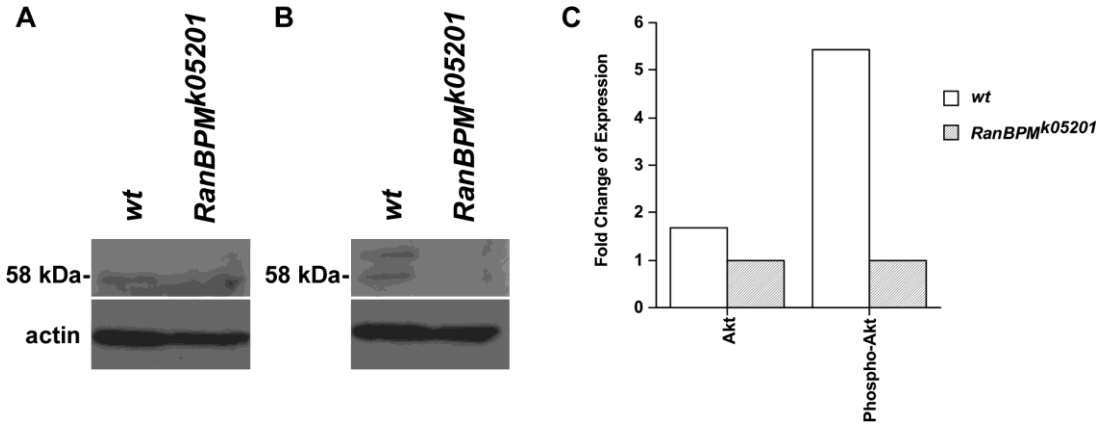
Table B: Targeted expression of dRanBPM constructs rescues lethality. Percent of rescue of flies carrying homozygous copies of RanBPM[k05201], a UAS-RanBPM construct and a tissue specific driver. N denotes number of scored progenies. Expression of the RanBPM construct was controlled by placing flies either at 18°C (Tub-GAL80[TS] permissive temperature) or 29°C (Tub-GAL80[TS] restrictive temperature). All scored flies were females.

Table B: Targeted expression of dRanBPM constructs rescues lethality

		With tub-GAL80[TS]		Without tub-GAL80[TS]	
		18C	**29C**	**18C**	**29C**
UAS-RanPM.S	**elav-GAL4**	0%, n=124	0%, n=69	0%, n=247	0%, n=194
	tub-GAL4	0%, n=57	85.7%, n=70	0%, n=133	88.9%, n=52
	c929-GAL4	82.35% n=406	107.09%, n=195	97.8%, n=551	129.23%, n=214
UAS-RanBPM.long H^{1}_{1}	**elav-GAL4**	0%, n=173	0%, n=111	0%, n=85	0%, n=34
	tub-GAL4	0%, n=52	8.7%, n=96	0 n=74	0%, n=16
	c929-GAL4	0%, n=64	0%, n=229	0%, n=241	0%, n=327
UAS-RanBPM.long H^{2}_{13}	**elav-GAL4**	0%, n=81	0%, n=103	0%, n=141	0%, n=12
	tub-GAL4	0%, n=99	0%, n=9	0 n=33	0%, n=6
	c929-GAL4	0%, n=95	0%, n=126	0%, n=186	0%, n=171

Appendix C

Fig C: Loss of dRanBPM function reduces the level of IIS pathway activation. Samples were collected using wildtype OR (wt) and RanBPM larval mutants from both 2^{nd} and 3^{rd} larval instars. (A) Total Akt levels between the samples did not greatly differ from each other. (B) Phosphorylated Akt level from wildtype larvae was 5.5 times higher than that of RanBPM mutants. Antibody used to detect phosphorylated Akt was specific for phosphorylation to Ser473 (Cell Signaling). Actin was used as loading control.


A
wt
RanBPMk05201
58 kDa-
actin
B
wt
RanBPMk05201
58 kDa-
C
Fold Change of Expression
0
1
2
3
4
5
6
Akt
Phospho-Akt
wt
RanBPMk05201

Appendix D

Table D: Assessing Tub-GAL80[TS] activity on GAL4 suppression. Tub-GAL80[TS] was able to fully suppress the activity of two tissue specific GAL4 drivers expressing either UAS GFP or Kir2.1 constructs. N denotes the number of individuals used in each calculation. Activity of Tub-GAL80[TS] was controlled by placing flies in either 18°C (Tub-GAL80[TS] permissive temperature) or 29°C (Tub-GAL80[TS] restrictive temperature). Only females carried tub-GAL80[TS]. Males acted as internal controls.

Table D: Assessing Tub-GAL80[TS] activity on GAL4 suppression

		Percent of flies exhibiting glowing phenotype (UAS-GFP)		**Percent of flies exhibiting lethality (UAS-Kir2.1)**	
		18°C	**29°C**	**18°C**	**29°C**
tub-GAL4 (ubiquitous expression)	**Female**	0%, n=224	91%, n=116	99.9%, n=176	100%, n=44
	Male	95.7%, n=213	84%, n=83	100%, n=133	100%, n=41
c929-GAL4 (neurosecretory cell expression)	**Female**	0.74%, n=272	79.4%, n=68	0%, n=131	100%, n=111
	male	85%, n=235	113%, n=58	100%, n=48	100%, n=116