

***Drosophila* models of motor neuron disease**

A Thesis

Submitted in partial fulfillment of the requirements

Of the degree of
Doctor of Philosophy

By

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2015

CERTIFICATE

Certified that the work incorporated in the thesis entitled “***Drosophila*** **models of motor neuron disease**”, submitted by Senthilkumar D. was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or Institution.

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Date:

DECLARATION

I declare that this written submission represents my ideas in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources that have not been properly cited or from whom proper permission has not been taken when needed.

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Synopsis

Drosophila models of motor neuron disease

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INTRODUCTION

Neurodegenerative diseases (NDs) are emerging as a major health problem causing immense suffering to the patients, stress to caregivers and a huge financial burden both to families and society as a whole. With increasing average life span in response to better health-care globally more people are living longer and therefore increasingly diagnosed with a variety of later onset NDs. NDs are characterized by progressive loss of neurons in different regions of the nervous system that result in a range of symptoms. These diseases seem to affect either the peripheral neurons or the neurons of the central nervous system. In some of the cases, patients affected might have familial history while in most cases the disease appears to be sporadic. Even in familial cases where the disease is because of mutation(s) in a single gene, a spectrum of clinical symptoms is seen. For example, a mutation in the *MAPT* gene can give a range of clinical symptoms that can be classified as progressive supra-nuclear palsy or fronto-temporal dementia or primary progressive aphasia (Pievani et al. 2014). This is likely because of background mutations and environmental predispositions. The diseases that affect the central

nervous system include Alzheimer's disease (AD), fronto-temporal dementia (FTD), etc and those that affect the peripheral neurons include Amyotrophic lateral sclerosis (ALS), Charcot-Marie-Tooth disease (CMT), Spinal muscular atrophy (SMA) and others (Carr 2015) (Pochet and Timmermans 2009).

In many NDs a specific subtype of neuronal cells are affected. For example in case of ALS only motor neurons are affected and in case of CMT disease both sensory and motor neurons are affected (Lambrechts et al. 2007). However, it is not clear why there is selective neuronal vulnerability. There is increasing evidence that there is a genetic network operating in the cell and breakdown of this network might lead to disease (Santiago and Potashkin 2014). It is a possibility that the susceptible genetic network varies between different cell types.

Here, I describe our attempts to understand the genetic and mechanistic basis of two peripheral NDs namely, ALS and CMT disease. In the first part, we have under taken a genetic screen to identify interactors of a genetic loci involved in ALS, *VAPB*. We show that modifiers we identified in the screen can modulate the disease model associated phenotypes. In second part we show that CMT disease causative loci *Rab7* and its upstream regulator *Mon1* are involved in regulating glutamate receptor levels at the *Drosophila* neuro-muscular junction (NMJ).

Results and Discussion

A reverse genetic screen identifies interactors of VAPB

Over twenty different loci have been identified to cause familial form of ALS (Andersen and Al-Chalabi 2011, Robberecht and Philips 2013); with ALS8 caused by mutations in VAMP (Vesicle associated membrane protein)-Associated Protein B (VAPB/VAP). VAP performs a number of cellular functions including unfolded protein response, vesicle release, lipid

biosynthesis and regulation of mitochondrial morphology (Skehel et al. 1995, Lev et al. 2008, Han et al. 2012, Kumagai et al. 2014). A missense mutation in the highly conserved proline residue at 56th position of VAP results in aggregation of the protein and leads to range of motor neuron associated clinical symptoms in patients (Nishimura et al. 2004). This mutant form of protein has been shown to act in a dominant negative manner sequestering the wild type protein into aggregates (Teuling et al. 2007, Ratnaparkhi et al. 2008). Studies have shown that VAP levels are also affected in case of sporadic ALS patients while familial ALS and taupathy disease models suggest that it could be a central player in neurodegeneration (Karsten et al. 2006, Teuling et al. 2007, Anagnostou et al. 2010). Hence, knowledge of *VAP* genetic interactors would be useful in understanding *VAP* function and allow us to predict possible consequences of malfunction of *VAP*.

Drosophila is an excellent model system to do genetic screens and we have used RNA interference (RNAi) based screen to identify genetic interactors of *dVAP* (Perrimon et al. 2010). Over-expression of VAP in sensory neurons using *scabrous-Gal4* (*Sca-Gal4*) leads to loss of thoracic bristles and this phenotype can be reversed by over expressing the VAP (P58S) mutant or knocking down VAP (Ratnaparkhi et al. 2008). Publicly available RNAi lines from National Institute of Genetics, Japan was used for the screen. Using the bristle phenotype as a sensitized background we have screened approximately 17% of the fly genome (2635 genes) to identify genes that interact with VAP. In total we identified 103 modifiers of which 45 were enhancers of the phenotype and 58 were suppressors of the bristle phenotype. Many of the genes that were picked up by the screen have functions related to known functions of VAPB including genes involved in regulation of energy metabolism and mitochondrial morphology, lipid biosynthesis, and cellular trafficking. We also identified known physical interactors (protein level interactors)

of VAPB as modifiers in the screen (Deivasigamani et al. 2014).

We have analyzed these interactions using the gene network approach and find that known ALS loci, *superoxide dismutase 1 (sod1)*, *vap*, *alsin* and *tar binding protein homolog (TBPH)* appear to be part of a connected network. This is important considering that there is wealth of data emerging that suggests that many of the loci implicated in ALS might be interacting with each other to regulate cellular processes (Suzuki and Matsuoka 2011, Wang et al. 2011, Stoica et al. 2014). We also find that 35% of the genetic modifiers show protein-protein interaction among themselves and nearly 60% of them can be linked via a common interacting partner. Interestingly, many of the genes we identified in our screen have also been implicated in other neurological disorders leading to the idea that VAPB may be a central player in many neurodegenerative diseases. Orthologs of some of the interactors we have found have been discovered in earlier studies, increasing confidence of our enhancer/suppressor screen to detect VAP interactors (Costanzo et al. 2010, Lin et al. 2010).

A subset of VAPB genetic interactors also interact with VAPB(P58S)

The genetic screen was conducted to identify interactors of wildtype VAP and it is essential to see if these genes interact with disease causing *VAP (P58S)*. We used the *Drosophila* larval NMJ as an assay system to study interactions of modifiers identified in the screen with VAP(P58S). VAP regulates bouton size at the NMJ in a dose dependent manner (Pennetta et al. 2002, Ratnaparkhi et al. 2008). Over-expression of VAP(P58S) in neurons using pan-neuronal *C155-Gal4* driver, leads to enlarged boutons at the larval NMJ (Ratnaparkhi et al. 2008). To test interaction with VAP (P58S), we decided to knockdown a subset (14) of discovered interactors of interactors in the background of neuronal VAP (P58S) expression and test if the reduction in

transcripts of individual interactors, identified in a VAP(wt) screen modified the enlarged bouton phenotypes. Knockdown of *Ada2b*, *CG18110*, *CG6048*, *CG9172*, *NaPi-T*, *Nup75*, *Ssh*, *TBPH* and *Tor* suppressed the bouton phenotype observed in *C155-Gal4>VAP (P58S)* animals while *Ars2*, *Droj2*, *Karyβ-3*, *Prx5*, *Snama* knock down failed to rescue or worsen the bouton size.

One key modifier identified in the bristle screen as a suppressor was the *Drosophila* homolog of mTOR. Target of rapamycin (TOR) integrates upstream nutrient signals, growth factors, ATP and cellular stressors to regulate protein synthesis, mitochondria and lipid metabolism, cytoskeletal organization, metabolism and autophagy (Bove et al. 2011, Betz and Hall 2013). In neurons *tor* knockdown significantly reversed the VAPB (P58S) associated large bouton phenotype. The interaction with the TOR pathway was further confirmed by demonstrating interaction with upstream and downstream members of the TOR signaling pathway. Over-expressing the proteins of the Tuberous Sclerosis Complex (Tsc1,2) that down regulates TOR signaling reverses VAPB(P58S) bouton phenotypes as does reducing activity of S6Kinase. The increase in TOR signaling in neurons seen with VAP(P58S) expression is in contrast with lowered TOR signaling seen in response to expression of VAP (wt), shown by the fact that *Tsc1* knock down and *S6Kinase-CA* can rescue the reduced bouton size. Importantly VAPB (P58S) bouton phenotypes in larvae can be rescued by feeding with the TOR signaling inhibitor Rapamycin (Deivasigamani et al. 2014).

TOR pathway has been implicated in age related neuro-degeneration and has been shown to be affected in other ALS disease models (Swiech et al. 2008, Hoeffler and Klann 2010). In case of SOD1^{G93A} mutant mice TOR pathway has been found to up-regulated and in case of VCP mutants it is down regulated (Ching et al. 2013, Saxena et al. 2013). Thus, the mode of TOR regulation appears to be case dependent and complex. Recent studies have shown role of

VAPB in AKT activation in breast cancer (Rao et al. 2012). The well characterized cross talk between AKT and TOR pathway makes our finding interesting.

Mon1 and Rab7 regulate Glutamate receptor levels at *Drosophila* NMJ

Glutamate is the major neurotransmitter in the mammalian central nervous system. Invertebrates like *Drosophila* and *C. elegans* use it as a neurotransmitter at the NMJ (Keshishian et al. 1996). Glutamate receptors respond to release of presynaptic glutamate and are critical for function and plasticity of the nervous system. The post-synaptic membrane docking of glutamate receptor is activity dependent and on the endocytic machinery with recycling endosomes acting as source of the receptors (Burbea et al. 2002, Schwarz et al. 2010, Lussier et al. 2011). Deregulation of glutamate receptor levels has been observed in case of diseases like Down syndrome and Amyotrophic Lateral Sclerosis (ALS) (Rembach et al. 2004, Wang et al. 2013).

In this study carried out in collaboration with Anuradha Ratnaparkhi, using *Drosophila* NMJ we show that two players in endocytosis namely Mon1 and Rab7 regulate glutamate receptor levels. Mon1 is a highly conserved GEF that is essential for conversion of Rab5 positive early endosomes to Rab7 positive late endosomes (Kinchen and Ravichandran 2010, Poteryaev et al. 2010).. We observed that *Mon1* mutants have NMJ defects, reduced levels of pre-synaptic active zones and increased levels of glutamate receptor subunit IIA (GluRIIA) at the synapse. Strikingly these mutants also show GluRIIA accumulation in the muscle. This increased amount of GluRIIA is not because of increased transcript levels. We think that the regulation of glutamate receptor levels by Mon1 is mediated through a pre-synaptic mechanism and possibly a post synaptic one as well. Importantly, knock down of *Rab7*, a molecule that functions downstream of Mon1 in endocytosis also lead to increased GluRIIA levels at the synapse.

Mutations in *Rab7* are known to cause a sensory neurodegenerative disease called Charcot-Marie-Tooth disease (CMT disease) (Cogli et al. 2009). Recent study from flies indicates that some of the CMT disease associated *Rab7* mutants behave as loss of function alleles (Cherry et al. 2013, Janssens et al. 2014). Our studies indicate a role for late endosomal machinery in regulating glutamate receptor levels at the synapse suggesting that CMT disease associated mutants may show similar defects.

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3. *Drosophila* Mon1 regulates glutamate receptor levels at the larval Neuromuscular Junction. A Ratnaparkhi*, A Basargekar, D Senthilkumar, K Shweta, P Sonawane, GS Ratnaparkhi.

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CHAPTER 1

Introduction

1.1 Neurodegenerative Diseases

Diseases and disorders that affect the nervous system are emerging as a major class of non-communicable diseases. With increasing life span more people are being diagnosed with a variety of neurodegenerative diseases (NDs). It is estimated that nervous system disorders will cost society more than 6 trillion USD per year. NDs are characterized by progressive loss of neurons in different regions of the nervous system with a range of clinical symptoms. Some of the patients have familial history while most cases are sporadic. It is also becoming clear that mutations in a single gene can give rise to a spectrum of clinical symptoms. For example, a mutation in the *MAPT* gene can give a range of symptoms that can be classified as progressive supra-nuclear palsy or fronto-temporal dementia or primary progressive aphasia (Figure 1.1) (Pievani et al. 2014). It is also likely that background mutations in other genetic loci can contribute to the development of these different outcomes.

Most NDs show characteristic inclusions of specific proteins in neurons and glial cells. These aggregates are believed to start off cascade of biochemical changes and eventually structural changes that lead to neuronal death. NDs can affect either the central or peripheral nerves. The clinical symptoms observed in the patients are an outcome of several years of molecular pathology that was not evident in early years (Pievani et al. 2014). The diseases that affect the Central Nervous System (CNS) include Alzheimer's disease (AD), Parkinson's disease (PD) and Frontotemporal dementia (FTD). Diseases affecting the peripheral nerves include

Amyotrophic Lateral Sclerosis (ALS), Hereditary Spastic Paraplegia (HSP), Spinal Muscular Atrophy (SMA) and Charcot-Marie-Tooth (CMT) disease. Many NDs are predicted to affect large-scale brain networks. For example in case of AD, regions like locus coeruleus, entorhinal regions, basal ganglia and hippocampus are preferentially affected. It is also important to note that the causative mutation can dictate regional specificity in patients. This has been seen in case of Alzheimer's caused because of mutation in tau and amyloid-beta. In case of tau, regions like locus coeruleus, entorhinal regions and neocortex are primarily affected while in case of amyloid-beta regions like neocortex, allocortical regions and basal ganglia are affected. Interestingly these are the regions that primarily show deposition of the protein aggregates (Brettschneider et al. 2015). In this chapter, I discuss some of the peripheral NDs, their genetic basis and molecular mechanisms that appear to cause disease.

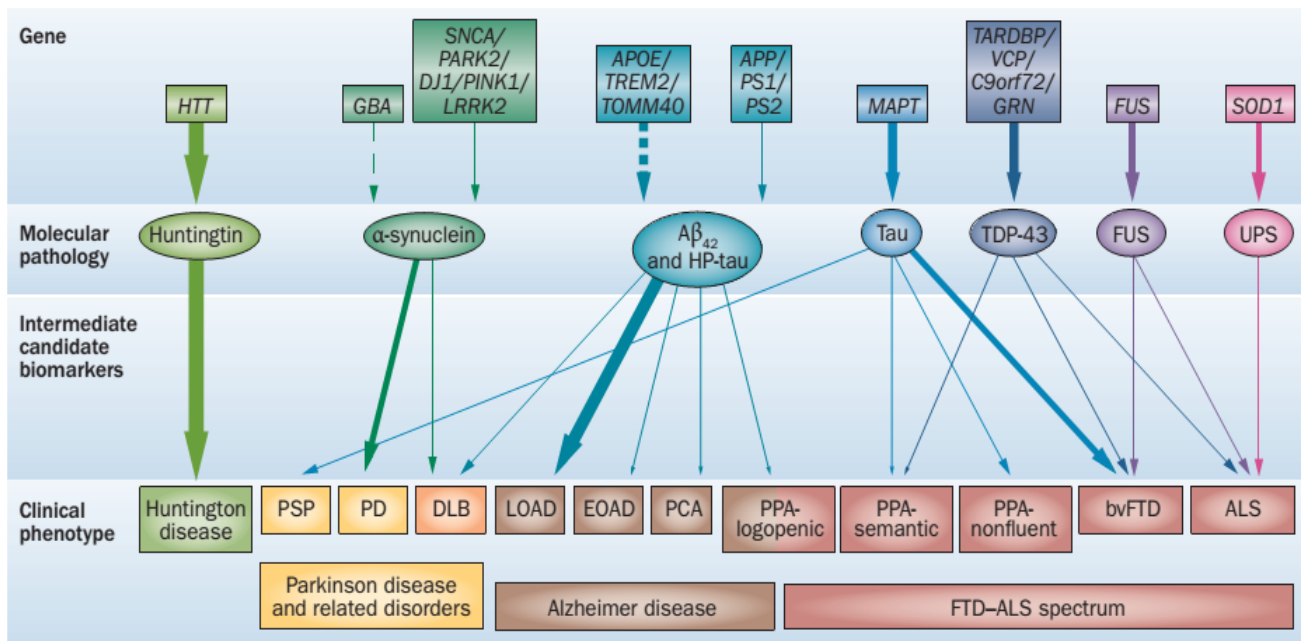


Figure1.1: Molecular determinants and clinical symptoms associated with major neurodegenerative diseases. Causative mutations are shown via continuous arrow and genetic risk factors via dotted arrow. The strength of the arrow indicates the correlation between molecular pathology and clinical phenotype in patients. (Adapted from (Pievani et al. 2014))

1.2 Peripheral neurodegeneration

Over a hundred diseases that affect the peripheral neurons have been identified. These diseases can affect sensory, motor and autonomic nervous system of the body. NDs affecting the peripheral system include Paraneoplastic sensory neuropathy, Sjögren's syndrome (autoimmune disease affecting the peripheral nervous system (PNS)), Marinesco–Sjögren syndrome, CMT, HSP, ALS and SMA. If the motor neurons are affected, it can lead loss of motor function, muscle weakness and impaired balance as in case of ALS. In case of sensory diseases, symptoms include numbness to touch, pain, and, loss of other sensory functions such as vision. In case of CMT, both sensory and motor functions are affected. Diseases affecting the autonomic system lead to a range of symptoms depending on the organ affected. Peripheral NDs are progressive; however, there is considerable difference in the age of onset. Some of these diseases share common symptoms and they are categorized based on the part of the nervous system affected as also based on the affected genetic loci (Lambrechts et al. 2007).

1.3 Amyotrophic lateral sclerosis (ALS)

ALS also known as Charcot disease or Lou Gehrig's disease is characterized by progressive loss of motor neurons leading to disability in voluntary movements of patients. The disease was first described by Jean Martin Charcot in 1869. 'Amyotrophy' refers to weakening of muscles and 'lateral sclerosis' refers to hardening of lateral and anterior spinal tract because of death of motor neurons and gliosis. The prevalence of the disease is about 5 individuals in 100,000. The average age of onset of disease is about 60 years. Some cases of ALS are associated with other neurodegenerative disease like FTD and SMA (Wijesekera and Leigh 2009).

1.3.1 Clinical Symptoms

The symptoms associated with ALS occur as the axonal connections get disrupted leading to denervation of muscles. ALS usually follows ‘spinal’ onset that affects limbs; however, there are known cases of ‘bulbar’ onset, where the disease affects swallowing and speech. Lower motor neurons of the spinal cord and brainstem along with upper motor neurons of the cortex are affected in case of ALS. In the Spinal form patients eventually develop bulbar symptoms that lead to respiratory failure. Patients initially show muscle weakness along with twitching and cramps. Gradually spasticity develops in atrophic limbs, affecting gait and causing difficulty in movement. At later stages, patients develop ‘flexor spasms’ during which the limb moves up towards the body; due to activation of flexor arc in a spastic limb. Patients with bulbar form have difficulty in speech with excessive drooling. Oculomotor function is unaffected in ALS; however, some patients on ventilator support can develop symptoms. In most cases, the sensory functions are spared. Variants of ALS include primary lateral sclerosis (PLS) where only upper motor neurons are affected and Motor neuron disease (MND) where lower motor neurons are affected (Wijesekera and Leigh 2009). Most patients die within 3-5 years of onset of symptoms, typical of rapidly progressing ALS. Also seen is slow progression where patients live more than 10 years. There is no cure available for ALS as of now. The only FDA approved drug Riluzole can extend life span by few months.

1.3.2 Etiology of ALS

ALS is likely to be a multifactorial disease with genes, environment and different cell types contributing to the disease. A number of pathogenic mechanisms like excitotoxicity and protein aggregation are expected to play a role in the disease. Non-neuronal cells including glia and muscle are also being implicated in contribution to development of disease (Boyer et al. 2013,

Dissel et al. 2014, Dadon-Nachum et al. 2015).

1.3.2a Environmental factors

Studies suggest that there might be a link between environmental factors and onset of ALS. Risk factors that are believed to contribute to ALS include smoking, increased physical activity, pesticides and head injuries. In the pacific island of Guam, high incidence of ALS is believed to be because of consumption of bats, which have high levels of b-methylamino-L-alanine (BMAA), an excitotoxin. However, the link between these risk factors and ALS is not consistent (Steele and Guzman 1987, Rao et al. 2006).

1.3.2b Genetics of ALS

Most cases of ALS are sporadic (SALS, 90-95%) with no familial history. However, 5-10% cases have a clear familial link (FALS). In terms of clinical symptoms there is no difference between SALS and FALS cases suggesting a common mechanism underlying the disease. The first gene identified to cause ALS was *SOD1*; identified after a consortium effort in 1993 (Rosen et al. 1993). After this over 20 other genes in which mutations leads to ALS have been identified (Table1)(Andersen and Al-Chalabi 2011). Most of these mutations are dominant but some like in case of *Alsin2* are recessive. Though there is no genetic basis in sporadic cases some patients do show molecular patho-physiological features as in familial cases. For example, oxidized misfolded SOD1 is found in a subset of sporadic patients while other sporadic patients show inclusions of TDP-43, Optineurin and Ubiquilin2, which are loci that have been implicated in ALS (Andersen and Al-Chalabi 2011, Robberecht and Philips 2013).

Table1.1: Genetic loci implicated in ALS and tabulation of their normal function in the cell.

FALS TYPE	GENE/LOCI	INHERITANCE PATTERN	CELLULAR FUNCTION	AGGREGATES	SEEDED AGGREGATES
<i>ALS1</i>	Super Oxide Dismutase1 / 21q22.11	AD, AR	Superoxide metabolism	Yes	Yes
<i>ALS2</i>	Alsin2 / 2q33.1	AR ³	GTPase regulation	No	ND ¹
<i>ALS3</i>	18q21	AD ⁴	NA ²	NA	ND
<i>ALS4</i>	Senataxin / 9q34	AD	DNA repair, RNA binding	No	ND
<i>ALS5</i>	Sptacsin/ 15q15-q21.1	AR	ND	NA	ND
<i>ALS6</i>	Fused in sarcoma / 16p11.2	AD, AR	RNA splicing	Yes	Yes
<i>ALS7</i>	20p13	AD	NA	NA	ND
<i>ALS8</i>	Vesicle Associated Membrane Protein (VAMP) Associated Protein / 20q13.32	AD	Lipid biosynthesis, UPR, cytoskeletal organization	Yes	ND
<i>ALS9</i>	Angiogenin / 14q11.2	AD	Ribonuclease		ND
<i>ALS10</i>	Trans activating Region (TAR) DNA Binding Protein / 1p36.2	AD	Transcriptional repression, pre-mRNA splicing	Yes	Yes
<i>ALS11</i>	Factor induced gene 4 homolog / 6q21	AR	Polyphospho inositide phosphatase	ND	ND
<i>ALS12</i>	Optineurin / 10p13	AD, AR	Vesicle / membrane trafficking	Yes	ND

<i>ALS13</i>	Ataxin2 / 12q24.12	AD	RNA binding	Yes	ND
<i>ALS14</i>	Valosin Containing Protein / 9p13-p12	AD	Protein degradation, Vesicle trafficking	Yes	ND
<i>ALS15</i>	Ubiquilin2 / Xp11.23-p11.1	AD	Protein degradation	Yes	ND
<i>ALS16</i>	Sigma non-opioid intracellular receptor1 / 9p13.3	AR (juvenile)	Lipid transport	No	ND
<i>ALS17</i>	Charged multivesicular body protein 2B/3p11.2	AD	Autophagy, protein sorting	NA	ND
<i>ALS18</i>	Profilin1/ 17p13.2	AD	Cytoskeletal organization	Yes	ND
<i>ALS19</i>	ERBB4/ 2q34	AD	Receptor tyrosine kinase	No	No
<i>ALS20</i>	HNRNPA1/ 12q13.13	AD	RNA processing	Yes	Yes
<i>ALS21</i>	Matrin3/ 5q31.2	AD	Nuclear matrix	Yes	ND
<i>ALS22</i>	Tubulin Alpha 4A/ 2q35		Cytoskeletal organization	Yes (only W407X mutant)	ND
	C9ORF72/ 9p21.2	AD	Endosomal trafficking	Yes	ND
	Sequestosome 1/ 5q35	AD	Protein degradation, Autophagy	Yes	ND
	Dynactin1/ 2p13	AD	Axon transport, cytoskeleton	Yes	ND
	TATA box binding protein (TBP)-associated factor, 68 kDa	AD	RNA polymerase II	Yes	ND
	EWS RNA-binding protein 1	AD	RNA metabolism	Yes	ND

¹ND, Not determined. ²NA, Not applicable. ³AR, Autosomal recessive. ⁴AD, Autosomal dominant.

Data Source: (Andersen and Al-Chalabi 2011, Robberecht and Philips 2013, Johnson et al. 2014, Renton et al. 2014, Smith et al. 2014)

1.3.2c Oligogenic basis of ALS

There is evidence to suggest that mutations in two or more genes can lead to ALS. Screening of ALS patients has revealed that in some sporadic patients along with mutations in *TDP-43* loci mutation in *ANG* and *c9orf72* are also seen. Similarly some sporadic patients have mutation in two other ALS loci, *VAPB* and *c9orf72* (van Blitterswijk et al. 2012a, van Blitterswijk et al. 2012b). These secondary or accompanying mutations have also been found in familial cases. A similar idea has been proposed in case of cancer; namely the Knudson two-hit hypothesis (Knudson 1996). However, as of today, the evidence for this in case of ALS is not strong. One of the ways by which mutations in two genes lead to a disease might be that of an additive effect. The other possibility is that one of mutation is the primary mutation that increases susceptibility while secondary mutation(s) modulates symptoms and disease progression. For example, there are modifiers mutations in *CNTF* which can reduce the risk of ALS due to *SOD1* mutations (Giess et al. 2002). Modifier screens, epistasis studies and large scale Genome wide association studies can help in resolving the idea of oligogenic basis for ALS.

1.3.3 Pathophysiology of ALS

With no treatment available for ALS, there is an increasing need to understand the molecular mechanisms behind the initiation and progression of the disease. Research over the past decade to understand the basis of ALS suggests that there might be complex interplay between a number of factors involving glutamate excitotoxicity, defects in spinal inhibitory circuits, inflammatory events mediated by microglia, axonal transport, oxidative stress and mitochondrial dysfunction, intracellular Ca^{2+} , protein aggregation and misfolding and others (Figure 1.2) (Pasinelli and Brown 2006, Andersen and Al-Chalabi 2011, Robberecht and Philips 2013). Importantly, all

three cell types; neurons, glia and muscle can contribute to the disease. However, nothing is known about degree of contribution of these cells types to cell death. It should also be noted that data has been collected mostly using familial ALS models and not much is known about sporadic cases. Some of the pathophysiological mechanisms discovered have been summarized in Table1.2.

Table1.2: Pathophysiology of ALS.

Molecular mechanism	Damage	Possible origin	ALS model
Oxidative stress	Damage to proteins, lipids and mRNA in cell	1.SOD1- NADPH dependent ROS production, Reduced NRF2/ARE signaling	SOD1, TDP-43, sporadic
Mitochondrial dysfunction	Change in morphology, altered calcium homeostasis, reduced energy production	1.VAPB- reduced MSP secretion and cytoskeletal defects 2. SOD1- mutant aggregation in inner mitochondrial space.	SOD1, VAPB, sporadic
Excitotoxicity	Hyperexcitability of neurons, altered calcium homeostasis	1.SOD1& TDP-43- reduced EAAT2 and GLAST levels 2.SOD1- Altered AMPA receptor subunit levels	SOD1, TDP-43, sporadic
Protein aggregation	Impaired protein degradation and sequestering of other proteins	Most ALS loci mutants show protein aggregation	See table1
Deregulated Endosomal trafficking	Altered endosomal mobility and conversion, increased degradation of glutamate receptors	1. Alsin2 is a GEF of Rab5 and is required in endocytosis. 2.VCP is required for autophagy	Alsin2, VCP, FIG4
Impaired axonal transport	Reduced cargo and organelle transport	1.SOD1- probably MAPK dependent disruption of kinesin function	SOD1, VAPB, TDP-43
Neuroinflammation	Activation of microglia and infiltration by lymphocytes	SOD1- Secretion of inflammation mediators (prostaglandin E2, leukotriene B4, and nitric oxide) by astrocytes	SOD1, sporadic
ER stress	Prolonged activation activates apoptosis	Misfolded proteins VAPB regulates UPR	SOD1, VCP, VAPB and

			others
Dysregulated RNA processing	Altered splicing, defective stress granule formation	Altered binding and mislocalisation of TDP-43 and FUS	TDP-43, FUS, SETX, HNRNPA1
Reduced lactate support	Reduced nutrition to neurons	SOD1- reduced levels of lactate transporters from astrocytes	SOD1
Altered lipid metabolism	Altered membrane fluidity and signaling	SOD1- changes in levels of PUFA, cholesterol and sphingolipids	SOD1, VAPB, sporadic
Microvascular defects	Blood-brain barrier damage, capillary leakage and altered blood flow	SOD1- down regulation of junctional proteins and altered capillary permeability because of reduced pericytes	SOD1, sporadic
RNA toxicity	Stalled transcription and formation of RNA foci.	<i>C9ORF72</i> - Formation of DNA-RNA quadruplex and sequestering of nuclear proteins	<i>C9ORF72</i>

Data Source: (Al-Chalabi et al. 2012, Han et al. 2012, Robberecht and Philips 2013, Schmitt et al. 2014, Walsh et al. 2015)

Of the above mechanisms listed in Figure 1.2 below, a few have been explored for finding treatments. Meta-analysis studies show that antioxidants provide better intervention to the course of disease in SOD1 mouse models (Andersen and Al-Chalabi 2011). However, some preliminary trials in human patients do not agree with this thesis and are not conclusive. Dietary supplements and Olanzapine- a drug which alters metabolism, are under clinical trials to check weight loss in ALS patients (Schmitt et al. 2014). Neuroprotective drugs, namely olexisome and dexamipexole that have been found to reduce mitochondrial damage failed to reduce ALS progression in clinical trials. A Few anti-excitotoxicity drugs (anti-glutamate) like gabapentin, lamotrigine and topiramate have failed in clinical trials (Andersen and Al-Chalabi 2011). As of now Riluzole is the only FDA approved drug available that delays progression of disease.

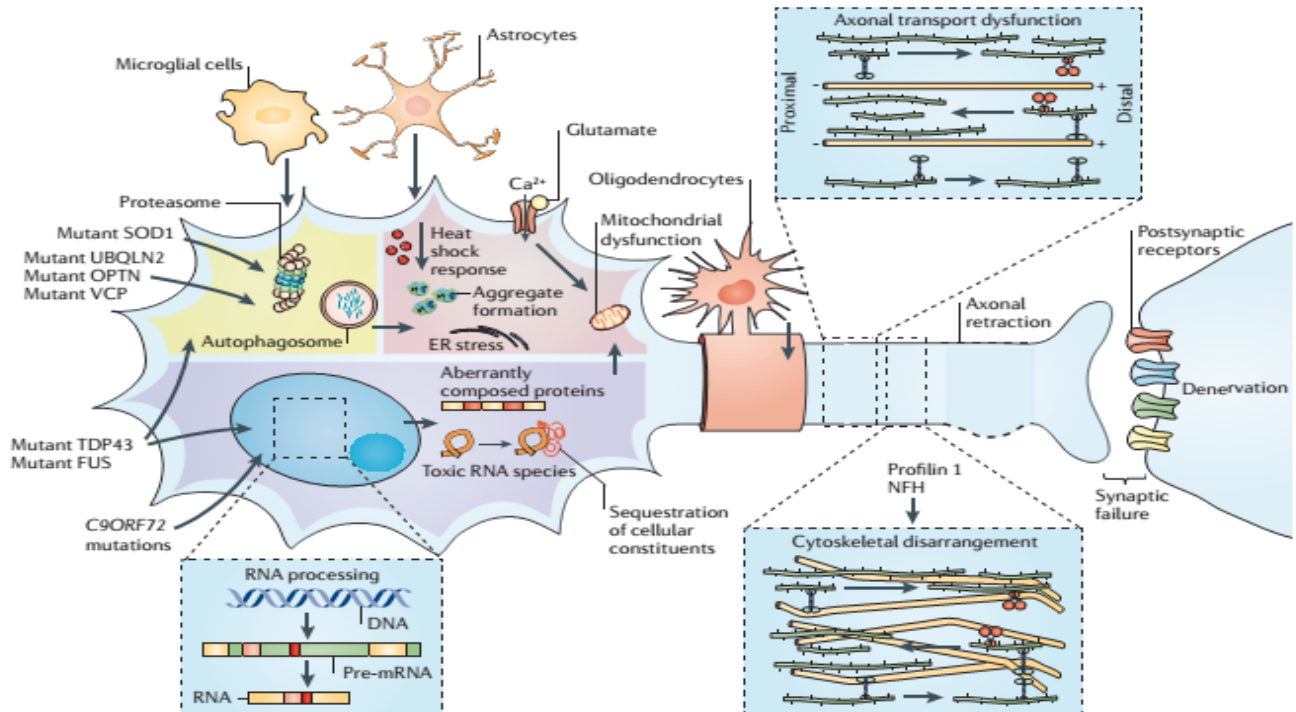


Figure1.2: Mechanisms underlying initiation of ALS. The disease can originate either in motor neurons or associated glial cells like microglia or astrocytes. Microglia on activation can contribute to motor neuron death by secreting neurotoxic factors. Astrocytes contribute to the disease by reduced lactate secretion and reduction in removal of glutamate resulting in excitotoxicity. Other molecular mechanisms like RNA toxicity, aggregation of misfolded proteins, defective proteasome function and mitochondrial dysfunction also trigger the death of the motor neurons. Adapted from (Robberecht and Philips 2013)

1.3.4 Selective motor neuron vulnerability

In ALS sensory, autonomic and cerebellar neurons are either not affected or affected mildly. Only motor neurons are affected and in a few cases fronto-temporal neurons are also affected. Even among motor neurons, the large fast fatigable neurons are more susceptible while neurons controlling oculomotor activity are not affected at all. These fast fatigable motor neurons have large muscle fields and limited sprouting ability. This suggests that an increased functional load may leave these neurons selectively vulnerable to ALS (Lambrechts et al. 2007, Andersen

and Al-Chalabi 2011, Saxena and Caroni 2011).

There are certain features that may make motor neurons more susceptible to ALS.

- A. Large cell size, long axonal compartment that makes cells more reliant on cytoskeletal machinery and increased metabolic demand.
- B. A high reliance on mitochondrial function and thus increased ROS and oxidative stress.
- C. Vulnerability to excitotoxicity because of reduced calcium buffering proteins, high expression of AMPA receptors lacking GluR2 subunit and also a high reliance on mechanisms that recycle secreted glutamate.
- D. Nervous tissue contains transcripts with long introns in them, compared to other tissues. It has been shown that TDP-43 and FUS bind to long introns. It has been proposed that this might make the neurons more susceptible to mutations in these genes than other cells.

1.3.5 Interplay between different ALS loci

There is increasing evidence that there might be a genetic network in the cell that on break down might lead to a disease. It is proposed that several familial ALS loci might be part of a common genetic network. Recent studies suggest that this might be true and interactions have been observed between different ALS loci. For example Alsin2 can accelerate motor defects in SOD1 mice (Hadano et al. 2010). In SOD1 (G93A) mice level of ALS8 protein VAPB is reduced (Teuling et al. 2007). In VAPB (P56S) mice TDP-43 has been shown to mislocalize to cytoplasm (Stoica et al. 2014). In ALS8 patient derived motor neurons and fibroblasts SIGMAR1 localization is affected. Treating ALS8 patient derived fibroblasts with PRE-084, an agonist of SIGMAR1 can reduce ER stress and removes VAPB aggregates (Prause et al. 2013). Fly studies indicate that FUS and TDP-43 might be genetically interacting (Lanson et al. 2011). This has also been supported by studies from yeast (Sun et al. 2011, Couthouis et al. 2012).

There is evidence from *Drosophila* to suggest that VCP and FUS might interact with each other (Azuma et al. 2014). Expression of VCP mutant which cause ALS leads to redistribution of TDP-43, and this can mediate neurodegeneration. Expressing ALS causing FUS mutant proteins in NSC-34 cell lines causes golgi fragmentation and expressing ALS associated Ataxin2 repeats in this background enhances these defects. Interestingly, this enhancement depends on the FUS mutant coexpressed (Farg et al. 2013). In our studies (Deivasigamani et al. 2014) described in Chapters 2 & 3 we discover interactions of four ALS loci in *Drosophila* sensory organ precursor cells.

1.4 Charcot-Marie-Tooth (CMT) disease

CMT disease, also called hereditary sensory and motor neuropathy or peroneal muscular atrophy, is a clinically heterogeneous disorder affecting the peripheral nervous system. The disease was first described by Jean Martin Charcot, Pierre Marie and Howard Henry Tooth in 1886. It is one of the most common inherited neurological disorders and the prevalence is about one in 3300 people in United States. The disease affects both the sensory and motor neurons of the nervous system. The age of onset of disease is between 2nd and 5th decade of life (Auer-Grumbach 2008).

1.4.1 Clinical symptoms

CMT disease mainly affects the sensory neurons. However, there is also varying levels of motor involvement. Severity of the symptoms varies among individuals. A consistent symptom of CMT is loss of sensation in distal regions of the upper and lower limb. Patients fail to differentiate warm and cold stimuli and are insensitive to pain. The motor symptoms in a family could range from being completely absent to severe muscle weakness and wasting of foot extensors. With progression of disease the loss of sensation and muscle weakness spreads from distal regions to

the proximal regions. In rare cases, respiratory muscle weakness has been observed. Other common complications seen in patients are spontaneous fractures, osteomyelitis and neuropathic arthropathy that may necessitate amputations. CMT disease is not considered fatal and most patients have normal life expectancy. Patients show enlargement of the nerves. These enlarged nerves, called hypertrophic nerves are caused because of abnormally thickened myelin sheath around the axons. There is no cure for CMT. Occupational therapy involving muscle strength training and stamina training is used to help the individual cope with the symptoms (Auer-Grumbach 2008, Tazir et al. 2014).

1.4.2 Etiology of CMT disease

Over 40 different loci have been identified that cause familial form of CMT disease (Table 1.3). The disease is classified into different forms based on it being axonal or myelinating or intermediate. These groups are further sub-divided into dominant or recessive subtypes that are further numbered based on the mutation in the gene or loci involved. For example, CMT1 is a demyelinating form (autosomal dominant), whereas CMT4 subtype is an autosomal recessive inherited disease with demyelination. CMT2A is an axonal form with autosomal recessive inheritance pattern while other CMT2 subtypes are axonal forms with autosomal dominant inheritance. The intermediate forms are denoted by CMT-RI (recessive) and CMT-DI (dominant) (Zuchner and Vance 2006, Timmerman et al. 2014).

1.4.4 Pathophysiology of CMT disease

The genes that are mutated in CMT disease perform very different functions, which include maintenance of myelin, transport through myelin, cell signaling, cytoskeleton formation, axonal transport, mitochondrial metabolism and organization, vesicle and endosomal trafficking, and stress response (Figure 1.3) (Juarez and Palau 2012). The function of these genes does not seem

to correlate with the demyelinating and axonal forms of the disease.

Table 1.3: Genes involved in CMT disease and their normal cellular function.

CMT locus	Locus/Gene	Inheritance pattern	Cellular function
<i>CMT1A</i>	17p11.2/ Peripheral myelin protein 22	AD ¹	Myelin formation
<i>CMT1B</i>	1q22/ Myelin protein zero	AD	Myelin formation
<i>CMT1C</i>	16p13.1–p12.3/ SIMPLE	AD	Schwann cell endosomal sorting and signaling
<i>CMT1D</i>	10q21.1–q22.1/ Early growth response protein 2	AD	RNA metabolism
<i>CMT1F</i>	8p21/ Neurofilament triplet L protein	AD	Axonal transport
<i>CMT2A</i>	1p36/ Mitofusin 2	AD	Mitochondrial morphology
<i>CMT2B</i>	3q21/ Ras-related protein Rab-7	AD	Endosome conversion
<i>CMT2B1</i>	1q21.2/ Lamin A/C	AD	Nuclear envelope organisation
<i>CMT2B2</i>	19q13.3/ Unknown	AD	NA
<i>CMT2C</i>	12q23–q24/Unknown	AD	NA
<i>CMT2D</i>	7p15/ Glycyl-tRNA synthetase	AD	tRNA charging and translation
<i>CMT2F</i>	7q11–q21/ Heat-shock protein B1	AD	Stress response
<i>CMT2G</i>	12q12–q13/ Unknown	AD	NA
<i>CMT2H</i>	8q21.3/ Unknown	AD	NA
<i>CMT2K</i>	8q13–q21.1/ Ganglioside-induced differentiation protein 1	AD	Mitochondrial morphology
<i>CMT2L</i>	12q24/ Heat shock protein B8	AD	Stress response
<i>CMT4B2</i>	11p15/ SET binding factor 2	AR ²	Endosomal sorting and signaling
<i>CMT4C</i>	5q32/ SH3 and tetratricopeptide repeat domain 2	AR	Endosomal sorting and signaling
<i>CMT4D</i>	8q24.3/ NDRG1 protein	AR	Endosomal sorting and signaling
<i>CMT4F</i>	19q13.1–q13.2/ Periaxin	AR	Cytoskeletal organization
<i>CMT4G</i>	10q23.3/ Unknown	AR	NA

<i>CMT4H</i>	12p11.21–q13.11/ FRABIN	AR	Cytoskeletal organization
<i>CMT4J</i>	6q21/ FIG4	AR	Inositol metabolism
<i>DI-CMTA</i>	10q24.1–q25.1/ Unknown	AD	NA
<i>DI-CMTB</i>	19p12–13.2/ Dynamin 2	AD	Endosomal sorting and signaling
<i>DI-CMTC</i>	1p35/ Tyrosyl-tRNA synthetase	AD	tRNA charging and translation
<i>CMTX</i>	Xq13.1/ Gap junction -1 protein, connexin 32	X-linked D	Cell-cell contact

¹AD, Autosomal dominant. ²AR, Autosomal recessive.

Data Source: (Zuchner and Vance 2006, Tazir et al. 2014, Timmerman et al. 2014)

Demyelination forms (CMT1&4) are characterized by reduced nerve conduction velocity (NCV) and segmental demyelination and remyelination. Axonal form (CMT2) shows no or mild change in NCV, however there is selective loss of large diameter axons (diameter $\geq 8\mu\text{m}$) (Szigeti and Lupski 2009, Brennan et al. 2015). A number of genetic disease models of CMT disease have been developed. These models have shed some light on the mechanisms of the disease. Many of these mechanisms are similar to those seen in case of ALS. Some of the molecular mechanisms which are basis for CMT disease have been listed in Table1.4.

Table 1.4: Mechanisms underlying CMT disease.

Molecular mechanism	Damage	CMT disease model	Explored drugs
Demyelination	Aggregation of mutant proteins leads to unstable myelin sheath	PMP22, MPZ	Geldanamycin – reduces PMP22 aggregation Onapristone – increases myelination
Mitochondrial dysfunction	Altered mitochondrial morphology	MFN2, GDAP1	-
Axonal transport defects	Disrupted cytoskeleton and neurofilaments which affect cargo transport	Periaxin, NEFL, Frabin	HDAC6 inhibitors - reduces axon transport defects
Endosomal trafficking	Defective trafficking of cargo and endocytosis	SIMPLE. SBF2, SH3TC2, FIG4, RAB7	-

Protein aggregation	Increased stress and UPR	HSPB8, HSPB1	Arimoclomol – induces heat shock proteins
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Data Source: (Szigeti and Lupski 2009, Juarez and Palau 2012, Bouhy and Timmerman 2013, Rossor et al. 2013, Baets et al. 2014, Jerath and Shy 2015).

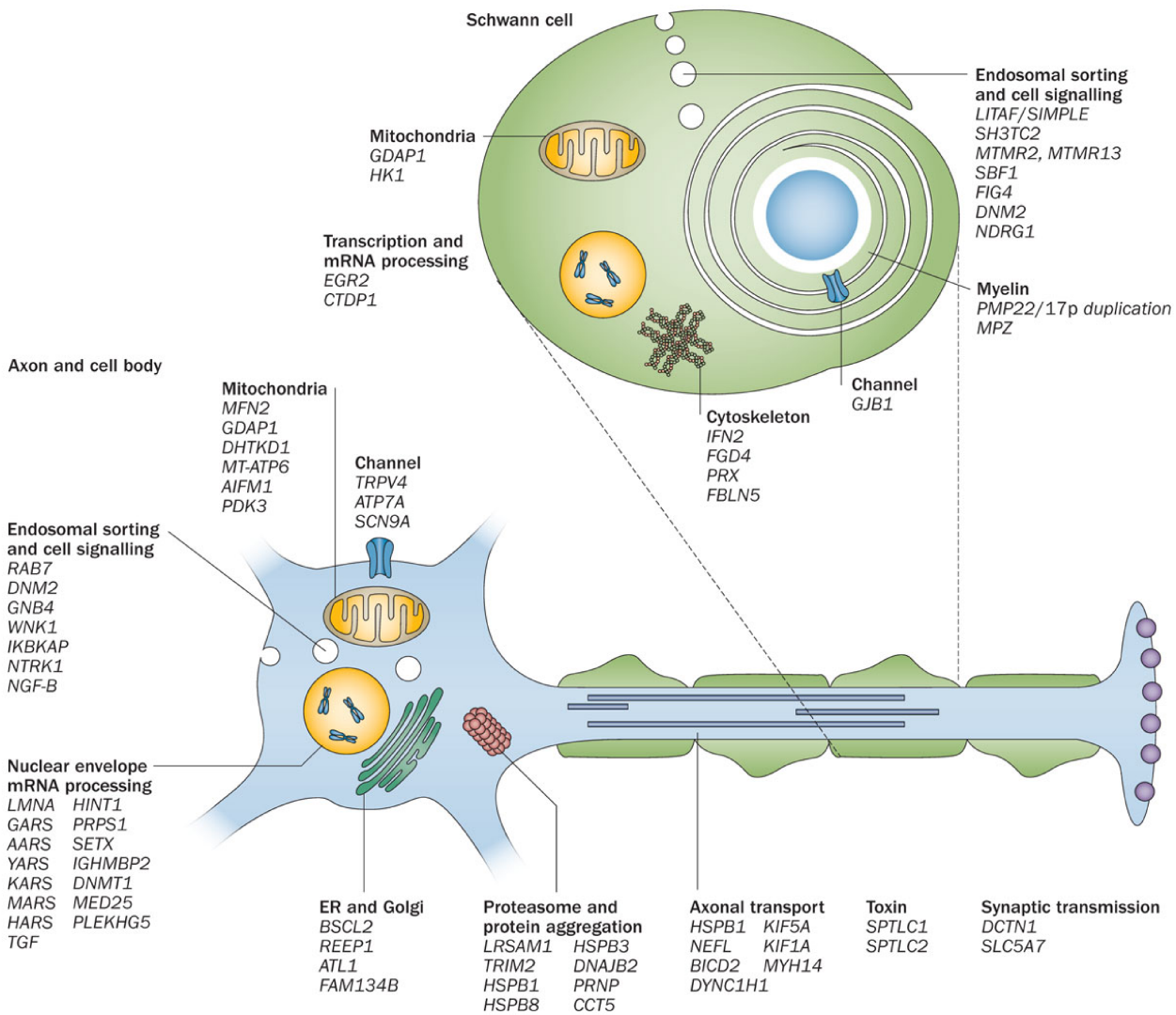


Figure1.3: A schematic showing various genes involved in CMT disease and possible mechanisms underlying the disease. These genes can be grouped into those involved in myelination, which primarily function in Schwann cells and those involved in other function. The genes involved in myelination are PMP22 and MYZ. Other genes can be classified as those involved in RNA processing, mitochondrial function, axon transport, endosomal sorting, synaptic transmission and others. Adapted from (Rossor et al. 2013)

1.5 Spinal Muscular Atrophy (SMA)

SMA is an autosomal recessive neurodegenerative disease marked by progressive loss of alpha motor neurons leading to proximal muscle weakness and paralysis. The incidence of the disease is estimated to be 1 in 6000 to 1 in 10,000 individuals. The disease is classified into four subtypes based on severity of the disease and level of motor function in patients. SMA I is most severe with onset as early as six months; SMA IV is least severe and onset occurs in 2nd or 3rd decade of life. Patients with SMA I subtype show hypotonia, paralysis and loss of head control. Spontaneous movement is lost in these children and they do not show any antigravity movement of limbs. In most severe cases even intrauterine movement is lost. The other symptoms include tongue fasciculation and difficulty in swallowing and feeding. Most of the children die within first two years. The disease is caused because of mutations in *SMN* (Survival of Motor Neuron) gene. SMN is a part of complex of eight ribonucleoproteins called Gemini complex. SMN is involved in mRNA splicing and in motor neurons has roles in mRNA transport (D'Amico et al. 2011). A recent study from *Drosophila* suggests that *SMN* might primarily function in the cholinergic interneurons, and expressing SMN in these neurons could rescue disease associated phenotypes (Imlach et al. 2012). This study further showed that *SMN* is not necessary in motor neurons and muscles. Also, increasing neuronal activity in cholinergic neurons of *SMN* mutant was sufficient to rescue the defects in these animals. There are other forms of SMA which are caused because of mutations in other genes including *VRK1*, *UBA1*, *GARS* and *IGHMBP2*. There is no cure available for SMA. Attempts have been made to increase energy metabolism in patients by administering creatine and albuterol. HDAC inhibitors have shown some promise in alleviating symptoms of SMA in mouse models. Of these valproic acid and phenyl butyrate did not show any efficiency in patients. New classes of HDAC inhibitors namely LBH589 and

SAHA have shown promise as they have been found to increase SMN levels in patient derived cells that were unresponsive to valproic acid (D'Amico et al. 2011).

1.6 *Drosophila* as a model organism to study human neurodegenerative disease.

Drosophila has contributed immensely to understand the genetics of nervous system development and behavior. The first behavioral mutants that govern circadian rhythm like *period*, and learning mutants like *dunce*, were isolated in *Drosophila* in efforts led by Seymour Benzer (Vosshall 2007, Bellen et al. 2010). Similarly the first set of proneural genes like *Enhancer of split* were identified from *Drosophila* ((Knust et al. 1987)Campos-Ortega).

Flies and humans seem very different their size, morphology and size of the genome. However, in terms of number of genes, there the difference is reasonable i.e. ~14,000 in *Drosophila* vs. ~20,000 in humans. Approximately 60% of the *Drosophila* genes show alternate splicing. Above all, more than 74% of human genes implicated in disease have at least one homolog in *Drosophila* (Bonini and Fortini 2003). Apart from this, a number of signaling pathways in flies are well conserved in vertebrates.

Many tools exist for genetic manipulation of *Drosophila*. These range from classical methods to generate mutantions like EMS, X-ray mediated deletions and transposon excision mutagenesis, to modern methods like the CRISPR/cas system. The less number of chromosomes (four pairs) and availability of balancer chromosomes make the generation and maintenance of mutation straightforward and simple. The other tools available include over expression systems like *UAS/ GAL4* system, *QUAS* system and *lexA* based techniques. Also, many sources of genomic mutants, *GAL4* lines, and *UAS/GAL4* system based RNAi collections and over expression lines are available publicly (Bellen et al. 2004, Perrimon et al. 2010, Awasaki and

Lee 2011). These lines are of great use for doing genetic screens. The short life span and easy accessibility of the nervous system make it a suitable genetic system to model neurodegeneration.

Drosophila eye has been used extensively to study neuronal death and neurodegeneration. It provides an easy way to assess age dependent progression of neurodegeneration. *Drosophila* compound eye has a stereotypically arranged 800 ommatidia with each containing 8 photoreceptor neurons. The severity of the phenotype ranges from roughening of the eye to large scale loss of photoreceptors. For example, over expressing *SCA5* mutation in β -III-spectrin leads to roughness in the eye in a dose dependent manner (Lorenzo et al. 2010).

Drosophila can be used to study memory related disorders and quantitative behavioral assays are available (Beharry et al. 2013). Similarly, *Drosophila* has been used to model diseases which involve motor function. Both larvae and adult flies can be used for performing locomotor assays and life span assays. Apart from this *Drosophila* larval neuromuscular junction is stereotypically arranged, easily accessible and is suitable for electrophysiology and looking at anatomical and molecular changes. A number of NDs have been modeled in *Drosophila* and some of these include Alzheimer's disease (*APP* and *A β* based), Parkinson's disease (*parkin*, *LRRK2* and human *α -synuclein* based), tauopathies (human *tau* and *tau* mutants), fragile X-syndrome (*fmr1* nulls and *fmr1* CGG repeat based) and polyglutamine diseases (human *Huntington* and *SCA3-Q78* based) (Bonini and Fortini 2003, Bilen and Bonini 2005, Lu and Vogel 2009). In a reverse approach, recent studies have also used fly mutants which show characteristic neurodegeneration to screen for patients with mutations in those genes (Bayat et al. 2012). The next section describes some of the fly models generated for peripheral NDs which have been described earlier.

1.6.1 *Drosophila* models of ALS

The tripartite synapse of the *Drosophila* neuromuscular junction is well suited to model ALS (Menon et al. 2013). The development of larval NMJ is well studied, electrophysiologically well characterized and a number of pre (brp, syt-1, CSP) and postsynaptic (dlg, GluRs) markers are available to assess the molecular changes. Some of the ALS loci have counterparts in flies. However, most of the models are based on over expression of the disease causing mutants in flies.

Table1.5: Summary of *Drosophila* models of ALS.

ALS Type	Gene name	Phenotype
ALS1	<i>SOD1</i>	Expression of both WT and mutant hSOD1 (A4V or G85R) caused motor defects, reduced synaptic transmission and altered mitochondrial structure. hSOD1 aggregation is seen in motor neurons.
ALS2	<i>Alsin</i>	Homozygous mutants show age dependent reduction of locomotion.
ALS8	<i>VAPB</i>	<i>dVAP(P58S)</i> mutant protein expression in neurons and muscle causes increased bouton size at NMJ and induced the formation of aggregates. <i>dVAP(P85S)</i> mutant protein sequesters Sac1 and OSBP and this leads to increased PtdIns4P levels and ER stress respectively. <i>dVAP(T48I)</i> mutant over expression leads to eye degeneration and increased levels of Hsp70 in neurons.
ALS10	<i>TDP-43</i>	Expressing <i>hTDP-43 A315T</i> , <i>G298S</i> , <i>D169G</i> , <i>N345K</i> and <i>M337V</i> mutants causes eye degeneration and depigmentation. Expressing either WT or A315T mutant TDP-43 results in mislocalization and aggregate formation in axons of eye imaginal discs. In glial cells, expression of A315T, D169G, G298S and N345K mutants mislocalize to the cytoplasm, affected larval motor function, reduce bouton number and increased glutamate receptor levels. Loss and gain of function of TDP-43 in glia alters glutamate transporter levels (dEAAT1 and dEAAT2). Similarly motor neuron expression of these mutants led to decreased bouton number, altered motor function and reduced number of active zones.

ALS6	<i>FUS</i>	Expression of WT <i>hFUS</i> and mutant <i>R518K</i> , <i>R521C</i> , <i>R521H</i> <i>R524S</i> and <i>P525L</i> causes eye degeneration in adult flies. These flies have reduced locomotion, reduced bouton number in larval NMJ and <i>hFUS</i> <i>R521C</i> mutants show decrease in number of presynaptic active zone. <i>R521G</i> mutant expression in motor neuron alters bouton size. Some of these mutants also mislocalize to cytoplasm.
-	<i>C9orf72</i>	Multiple repeat expansion (over 100 repeats) of riboGGGGCC causes eyedegeneration, reduced adult locomotion, and this is likely because of the arginine rich repeats. Similar number of repeats of riboGGGGCC with stop codon does not produce any phenotypes.
ALS20	<i>hnRNPA2</i>	<i>hnRNPA2</i> <i>D290V</i> mutant expression causes degeneration of indirect flight muscles and it mislocalizes to cytoplasmic aggregates.
ALS14	<i>VCP</i>	Expression of <i>R152H</i> and <i>A229E</i> mutants results in degeneration of eye. <i>R152H</i> mutant expression in motor neuron leads to reduced motor function, reduced bouton number, accumulation of ghost boutons and reduced active zone density. Muscle expression leads to muscle degeneration and dropped wing.
	<i>TAF-15</i>	Overexpression of human <i>TAF-15</i> wild type and ALS mutants <i>R408C</i> and <i>G391E</i> in motor neuron reduces life span. However, ALS mutants were stronger than wild type itself.
	<i>EWSR-1</i>	Overexpression of human <i>EWSR-1</i> wild type and ALS mutants <i>G511A</i> and <i>P552L</i> in eye results in degeneration of the eye. Wild type <i>EWSR-1</i> overexpression in neurons resulted in reduced climbing and life span.

Data Source: (Chen et al. 2010, Estes et al. 2011, Han et al. 2012, Estes et al. 2013, Forrest et al. 2013, Kim et al. 2013, Casci and Pandey 2014)

1.6.2 *Drosophila* models of CMT

Drosophila peripheral nerves are ensheathed by wrapping glia which are similar to Schwann cells in vertebrates. Perineurial and subperineurial glia cover the nerves and act as blood-brain barrier. *Drosophila* lacks myelin and salutatory conduction and accordingly it does not express CMT related genes which are associated with myelination (PMP22 and MPZ). Hence it may not be suitable to study demyelinating forms of the disease. However it can be used to model axonal and intermediate form of the disease (Wang and O'Kane 2008). Many of the genes implicated in axonal form of CMT disease have homologs in *Drosophila*. However not many of these genes have been well studied.

Table1.6: Summary of *Drosophila* models of CMT disease.

CMT Type	Gene name	Phenotype
CMT2B	<i>Rab7</i>	L129F mutant expression in sensory neurons reduces larval sensitivity to pain and temperature. When mutant is expressed in motor neurons these animals show crawling defects. Several CMT related mutants L129F, K157N, N161T, V162M show reduced colocalization with endosome markers like Rab5 and Hrs and are inefficiently recruited to endosome. But all of them can rescue lethality seen in null animals.
CMT4A	<i>GDAP1</i>	Overexpression and knock down of <i>gdap1</i> results in eye and muscle degeneration in an age dependent manner. These animals also show reduced levels of ATP and ROS levels.
CMT2C	<i>GARS</i>	G240R and P234KY mutants show reduced eclosion and show reduced climbing ability and and electrophysiology defects.
DI-CMTC	<i>YARS</i>	CMT disease causing G41R and 153–156delVKQV mutants failed to rescue loss of bristle phenotype seen in YARS RNAi while E196K mutants can rescue the same. However, all the three mutants showed reduced climbing ability in an age dependent manner and electrophysiology defects.
CMT2A	<i>Mitofusin</i>	<i>Marf</i> knockdown results in climbing defects. <i>Marf</i> nulls have altered mitochondrial and ER morphology and have ER stress. While <i>hMfn2</i> rescues lethality because of knockdown of <i>Marf</i> in muscles (<i>Mef2-GAL4</i>), the CMT causing <i>hMfn2</i> ^{R94Q} mutant does not rescue lethality. Similarly, it also fails to rescue climbing defects seen in knock down animals (<i>MHC-GAL4</i>). Expression of <i>M393I</i> and <i>R400Q</i> mutants but not wild type <i>Marf</i> resulted in degeneration of eye.

Data Source: (Storkebaum et al. 2009, Cherry et al. 2013, Debattisti et al. 2014, Ermanoska et al. 2014, Janssens et al. 2014, Lopez Del Amo et al. 2015)

1.6.5 Limitations of *Drosophila* as a system to study disease

Most of the studies using flies to study neurodegeneration have relied on over expression, which may not resemble the disease condition. However, the development of new genetic methods like recombineering (Venken and Bellen 2005) and *CRISPR/cas* (Gratz et al. 2013) should help in developing disease related mutations in the endogenous gene. The difference in organization of

the nervous system like absence of myelin sheath and different neurotransmitters used by flies and mammals in CNS and PNS can contribute to discrepancies.

While studying loss-of-function alleles using *Drosophila*, one might overestimate the contribution of that gene, as there is usually only one or two paralogs for every gene in flies, while human genes have multiple paralogs. For example *VAPB* deletion in flies is lethal while in mouse, the animals are viable (Pennetta et al. 2002, Kabashi et al. 2013). There are genes which are involved in neurodegeneration but have no clear homologs in flies. Finally, though there is conservation in coding sequence there is less conservation in non-coding sequence. This is important while studying genes which are involved in splicing or those which are alternately spliced (*TDP-43*, *FUS*).

CHAPTER 2

A reverse genetic screen identifies interactors of VAPB

2.1 Summary

In this chapter, we describe a RNA interference based reverse genetics screen that was conducted to identify interactors of *dVAP* (henceforth referred to as VAP) - the *Drosophila* ortholog of *hVAPB*. Knowledge of *dVAP* genetic interactors would be useful in understanding *dVAP* function and allow us to predict possible consequences of malfunction of *dVAP* on its local gene regulatory network. Over-expression of VAP in sensory neurons using *scabrous-Gal4* (*Sca-Gal4*) leads to loss of thoracic bristles (Ratnaparkhi et al. 2008). Using this phenotype, we have screened approximately 17% of the fly genome to identify genes that interact with VAP. We have analyzed these interactions using the gene network approach and find that known ALS loci, *superoxide dismutase 1 (sod1)*, *vap*, *alsin* and *tar binding protein homolog (TBPH)* appear to be part of a connected regulatory network. Many of the genes that were picked up by the screen have functions related to known functions of VAPB and also include known physical interactors (protein level interactors) of VAPB. We also find that 35% of the genetic modifiers show protein-protein interaction among themselves and nearly 60% of them can be linked via a common interacting partner. Interestingly, many of the genes we identified in our screen have also been implicated in other neurological disorders leading to the idea that VAPB may be a central player in many neurodegenerative diseases. Orthologs of some of the interactors we have found have been discovered in earlier studies, increasing confidence of our enhancer/suppressor screen to detect VAP interactors.

2.2 Introduction

VAMP Associated Protein B (VAPB; ALS8) was first identified in *Aplysia* as an interactor of Vesicle Associated Membrane Protein (Skehel et al. 1995). A mutation in VAPB was later identified as the cause of familial ALS in a large Brazilian family (Figure 2.1) (Nishimura et al. 2004, Nishimura et al. 2005). VAPB is a trans-membrane protein that is highly conserved from yeast to mammals (Nishimura et al. 2004, Lev et al. 2008). The protein contains an N-terminal MSP domain, a central coiled coil domain and a C-terminal trans-membrane domain (Skehel et al. 1995, Nishimura et al. 1999). VAP proteins (Fig. 2.2A,B) perform an array of cellular functions: the MSP domain of VAPB interacts with proteins having FFAT motif to regulate lipid transport (Lev et al. 2008, Peretti et al. 2008, Tsuda et al. 2008, De Vos et al. 2012, Yang et al. 2012). The cleaved MSP domain acts as a ligand for Ephrin, Robo and Clr-1 receptors (Tsuda et al. 2008, Han et al. 2012); its interaction with Nir (N terminal interacting receptor) group of proteins modulates the ER and microtubule architecture (Skehel et al. 2000, Amarilio et al. 2005). VAPB also regulates Unfolded Protein Response (UPR) by interacting with components of two major UPR pathways, Ire1 and ATF6 (Nikawa et al. 1995, Gkogkas et al. 2008). Patients with this mutation show spectrum of symptoms including typical ALS, slow progressive ALS and Spinal Muscular Atrophy (SMA; (Nishimura et al. 2004)). This suggests that other genetic factors might decide the phenotypic outcome of the mutation. Interestingly, VAPB levels are lowered in sporadic ALS patients and also it has been shown to go down in SOD1 G93A mutant mice. VAPB has been shown to increase in taupathy mice and loss of it can alleviate phenotypes in a *Drosophila* model (Karsten et al. 2006). Recently, it was shown that VAPB levels are also up regulated in case of primary and metastatic tumors (Rao et al. 2012).

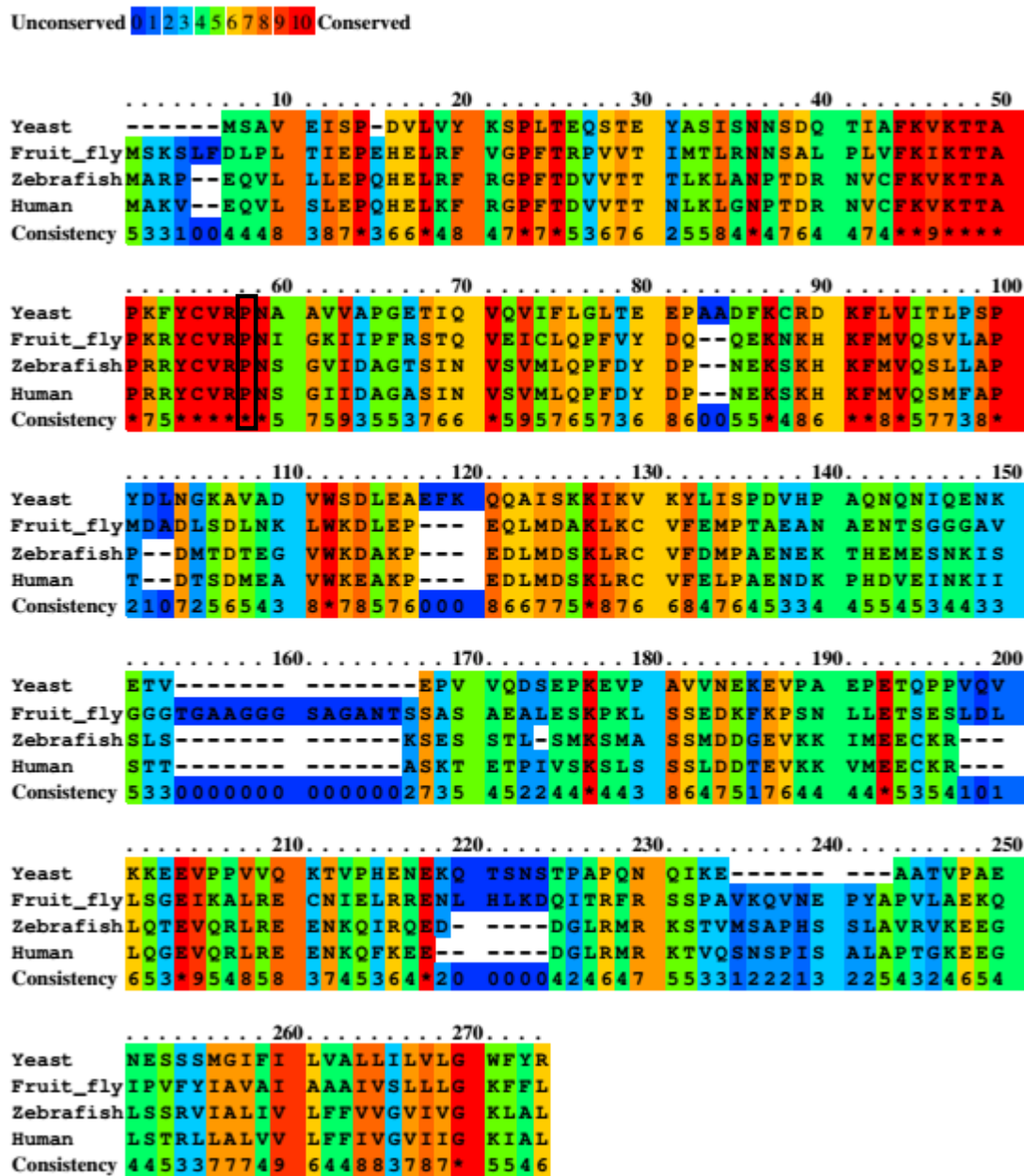


Figure 2.1: Multiple sequence alignment of VAPB protein sequence from different organisms. Please note the highly conserved proline residue at the 58th position which fall in the Major Sperm Protein domain (MSP domain). Mutation of this proline to serine leads to Amyotrophic Lateral Sclerosis and Spinal Muscular Atrophy in humans. Conversion of this proline to any other amino acid leads to aggregation of this protein (Kanekura et al. 2006). The sequence alignment was done using PRALINE.

VAPB has been shown to regulate growth of mammary tumors in part through AKT pathway (Rao et al. 2012). Also, there is suggestive evidence that polymorphisms in 3'UTR of VAPA

might have implications in bipolar disorder (Lohoff et al. 2008). This makes VAPB an interesting candidate to look in context of diseases and in particular neuro-degeneration and ALS. Identification of the genetic network involved in regulation of VAP function can help in the identification of mechanisms involved in pathogenesis of VAPB mediated ALS.

2.2.1 RNAi based genetic screen

In order to understand the biological function of a gene one can perform assays like Yeast two hybrid screens, protein pull down followed by mass spectrometry and genetic screens. Modifier screens (enhancer/ suppressor) have been used extensively in yeast, *C.elegans* and *Drosophila* to understand the biological pathways and function of genes. Much of information on cell cycle has come from genetic screens in yeast and screens in *Drosophila* have shed light on the patterning of organism and development (Jorgensen and Mango 2002, St Johnston 2002). Over years a large number of genetic tool kits have been developed which enable the manipulation of the genome and aid in screens. Such tools available in *Drosophila* include deficiencies, EMS mutants, P-element based excision/ insertion mutants, RNAi lines and over-expression lines. Of these RNA interference is a powerful reverse genetic approach, particularly in organisms where genome information is available (Boutros and Ahringer 2008).

There are three RNAi based, whole genome public collections for *Drosophila* namely NIG, VDRC and TRiP consortium lines. These collections use different strategies to achieve knock down of the target gene and they also vary in the methods used for transgenesis. However, all these collections use UAS/ Gal4 system to express the dsRNA or shRNA. While VDRC and NIG lines use long dsRNAs for knock down; most of TRiP lines use shRNAs. NIG lines have been developed through random P-element insertion and TRiP lines are based on site specific insertion. VDRC collection has both site specific insertions and P-element based line. These

lines also vary in their efficacy of knock down depending on the tissue of interest. The NIG lines have been generated using pUASTR57 vector and UAS-RNAi lines that target over 6000 genes are available publicly (approximately 42% of *Drosophila* genes) (Perrimon et al. 2010). We have chosen to use the NIG collection based on availability and access to lines.

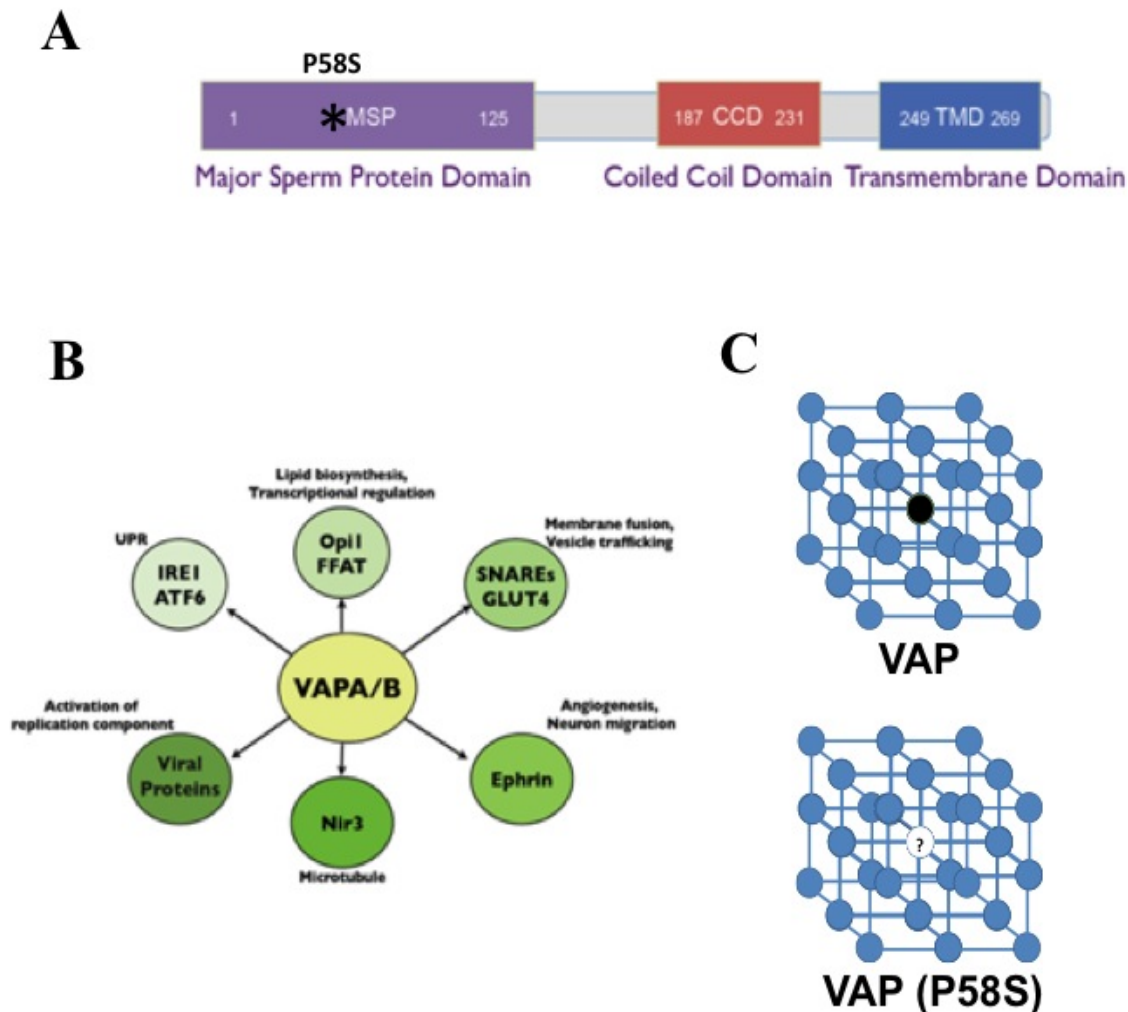


Figure 2.2: VAP, VAP functions, VAP network and categorization of modifiers.

(A) *Drosophila* VAP (dVAP) is a single pass trans-membrane protein (TMD) containing a coiled coil domain and an N-terminal major sperm protein (MSP) domain. A P56S mutation in the human ortholog of dVAP is linked to Amyotrophic Lateral sclerosis (ALS), a motor neuron disease.

(B) VAP, with its cytoplasmic/ER localization, with the TMD inserted in the ER membrane interacts physically with a large number of proteins and has been demonstrated to have roles in diverse cellular function.

(C) We hypothesize that a subset of genetic interactors, when discovered for VAP, may modify dVAP related phenotypes.

2.2.2 Network hypothesis

Availability of large scale libraries and several disease models have led to identification of a number of modifiers of neurodegeneration. This combined with gene expression studies have led to the emergence of ‘network hypothesis.’ The network hypothesis is based on the idea that complex diseases such as neurodegeneration are not because of single mutations; but because of the failure of large scale interaction networks existing in a cell. These networks have helped in the identification of previously unknown association between two different diseases (Santiago and Potashkin 2014). A number of tools Cytoscape, STRING are available to identify and build interaction networks. Network analysis has been used in past to extend/built upon available network of modifiers of taupathies (Ambegaokar and Jackson 2011). In case of Alzheimer’s disease, the interactome mapping has led to the identification of previously unknown candidate genes involved in Late Onset Alzheimer’s disease (LOAD)(Santiago and Potashkin 2014). Our goal was to define a network of genes (Figure 2.2C) that interact with VAPB in *Drosophila*. We hypothesize that these interactors identified in wild type VAPB background might interact with disease causing mutant VAPB (P58S). Identifying these central nodes in the genetic network can help mitigation of ALS.

2.3 Materials and Methods

2.3.1 Stocks and Genetic screening

Fly lines were maintained at 25 °C on standard cornmeal agar medium. UAS-GAL4 system was used for over-expression of transgenes. All RNAi lines used in screen were from NIG, Japan. Given the large number of candidates involved, the efficacy of knockdown couldn’t be determined for individual lines. However, many of these lines have been tested for functionality using an *actin-Gal4* driver. This information can be found at NIG-FLY website

(<http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp>). *UAS-VAP* wild type and *UAS-VAP (P58S)* lines used in this study have been described earlier (Ratnaparkhi et al. 2008). Canton S flies were used as a wild type control.

2.3.2 Primary and Secondary screen

A recombinant fly line over-expressing wild type *VAP* using *sca* driver was used for the screening. For screening, males from RNAi stocks were crossed to virgins of *sca>VAP* over-expression line. The primary screening was performed at 25 °C and 28 °C. The secondary screen was performed only at 25 °C. The Dorso-central, Scutellar and Anterior Postalar thoracic macrochaetae on F1 progeny females were considered for the analysis. The statistical significance was analyzed using Student's t-test.

2.3.3 Data Analysis

The Gene Ontology (GO) information was obtained based on Flybase (<http://flybase.org/>) entries and GOTOolbox (<http://genome.crg.es/GOTOolBox/>) online application and was grouped manually. For identifying the physical interactors of the modifiers we queried all the target genes against the GeneMANIA (<http://www.genemania.org/>) and STRING (<http://string-db.org/>) databases. To obtain all possible interaction we included low confidence interactions. Only known and predicted protein-protein interaction data based on other organisms was considered for identifying interactions. To identify physical interaction within genetic interaction, the information from these databases was curated manually. The network map was constructed using Cytoscape V2.8.3. Ortholog prediction was performed using DIOPT and DIOPT-DIST tools (<http://www.flyrnai.org/diopt>). Score of 1 on a scale of 10 was set as threshold to consider a gene as an ortholog. DIOPT combines information from different prediction tools to suggest a possible ortholog.

2.4 Results

2.4.1 A genetic screen uncovers interactors of *VAP*

Presence of a sensitized background and a visible phenotype will aid in discovering the genes that modulate the function of VAPB. Stable expression of VAP in the *sca* domain leads to a dose dependent decrease in the number of macro chaetae in the dorsal thoracic region of the adult fly (Ratnaparkhi et al. 2008); Fig. 2.4A). The penetrance of this phenotype is 100% with all the flies showing loss of bristles. At 25 °C, the average number of macro chaetae observed in the stable recombinant lines (*Sca-Gal4>UAS-VAP/Cyo*) generated in our laboratory was between 5-6 macro chaetae and this number was seen to reduce further to 0-1 at 28 °C. This phenotype was suppressed by co-expression of double stranded RNA (dsRNA) specific to VAP (VAP-RNAi, Fig. 2.3 A). The loss of bristle phenotype was not significantly affected by the presence of an extra copy of UAS over-expressing RFP, eliminating the possibility of Gal4 dilution. The *Sca-Gal4>UAS-VAP/Cyo* is viable only as a heterozygote, and was used as a sensitized background to identify modifiers of VAP function through an RNAi based screen.

We utilized the publicly available RNAi collection ((Kambris et al. 2006); <http://www.shigen.nig.ac.jp/fly/nigfly/about/aboutRnai.jsp>) from National Institute of genetics (NIG, Japan) to screen for the modifiers. 4600 individual lines, representing 2635 genes were crossed to *Sca-Gal4>UAS-VAP* females and grown at 25 °C & 28 °C to screen for enhancers and suppressors, respectively (Fig. 2.3B). 5-10 F1 females were screened from each cross; animals with <4 bristles at 25 °C were scored as enhancers and those with >2 bristles at 28 °C were scored as suppressors (Fig. 2.3B). Using these criteria, 930 genes were identified as modifiers of the phenotype. Of the 2635 genes screened, 2095 had orthologs in humans, based on DRSC Integrative Ortholog Prediction Tool (DIOPT; (Hu et al. 2011)).

Next, in order to eliminate any effects of RNAi itself and to quantify the interaction, the 930 RNAi lines were rescreened at 25 °C by crossing to *sca-Gal4* and *Sca-Gal4>UAS-VAP* (Fig. 2.3B). Bristle numbers of ten F1 females from both control and experimental crosses were counted and analyzed in two steps. In the first step, using 5.5 macro chaetae as the baseline (*sca-Gal4>UAS-VAP/+*), F1 females that showed significant rescue in macro chaetae number (average macro chaetae >7.5, $p < 0.01$) were grouped as suppressors (Fig. 2.3B). Lines/genes with macro chaetae number 5.5-7.4 and $p > 0.01$ were not considered significant hits. In the second step, control RNAi crosses for individual genes were taken into consideration. The effect of individual RNAi lines (*Sca-Gal4>gene-i*) on bristle number was compared to corresponding experimental value (*Sca-Gal4>UAS-VAP/gene-i*) in order to negate the effects of knockdown of the gene alone. Any line that did not have a strong effect on its own but led to a further loss of macro chaetae in a VAP over expression background was scored as an enhancer (average macro chaetae <4.0, $p < 0.01$).

Using this strategy, we identified 45 enhancers and 58 suppressors of VAP phenotype (Fig. 2.3B; Table 2.1). Of these, 89 genes (86%) have known human orthologs predicted using DIOPT (Hu et al. 2011). Using information from Flybase and DAVID (Huang da et al. 2009, Marygold et al. 2013), we classified all modifiers into different categories (Figure 2.4A) based on their known or predicted functions. These included mitochondrial proteins, RNA binding proteins (*Arsenic resistance protein 2*, *CG7564* –a component of the spliceosome commitment complex), proteins associated with the cytoskeleton (*tropomodulin*, *dynactin subunit p-25*, *slingshot*, *zye*) and proteins associated with the Unfolded protein response (*Droj2*, *Hsp83*, *l(2)35Cc*).

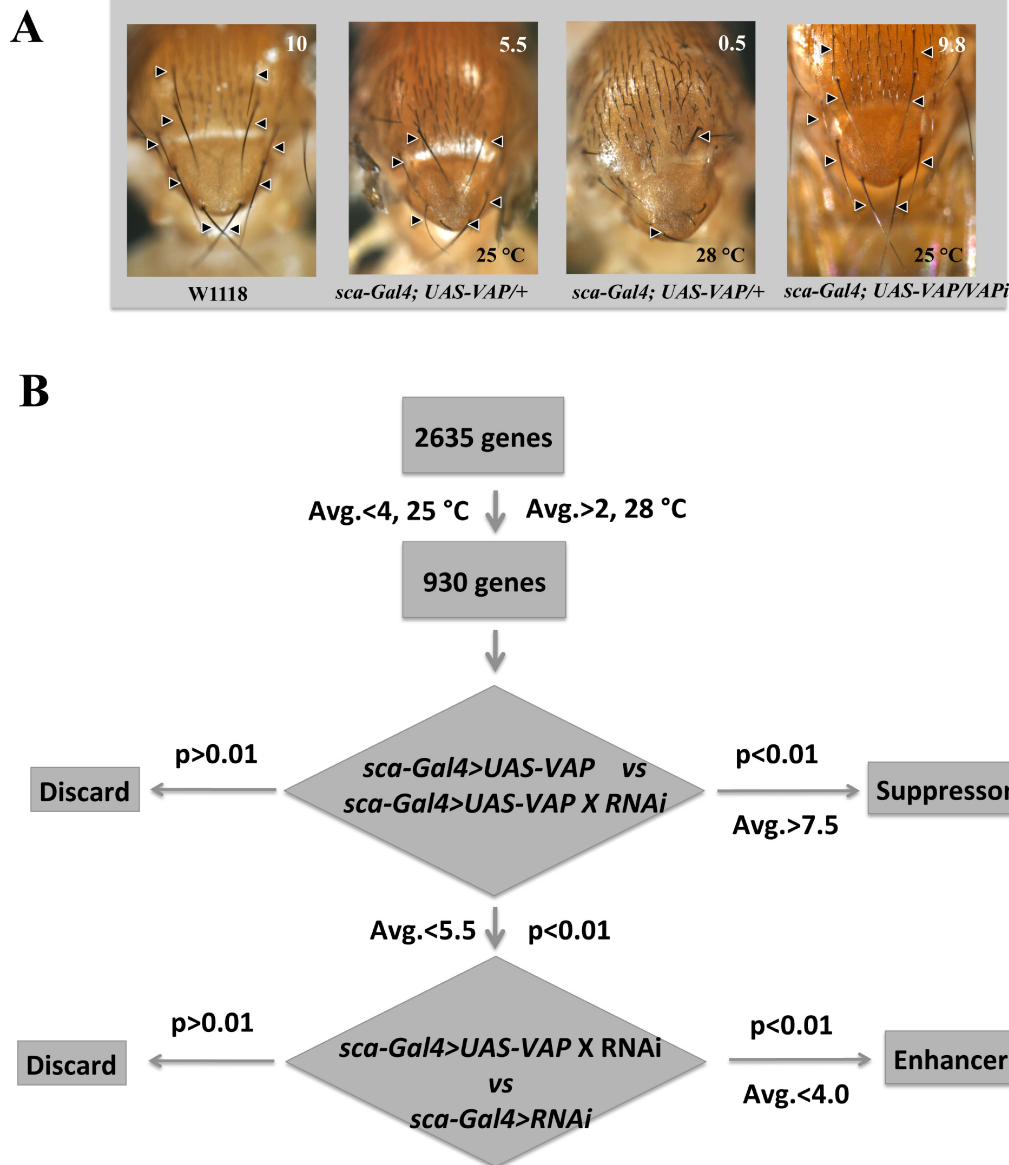


Figure 2.3: Scheme for the enhancer/ suppressor screen.

(A) A sensitized genetic background (*sca-Gal4,UAS-dVAP*) was used for a screen to identify interactors of dVAP. A recombinant stable line, expressing *VAP* in the *sca* domain was found to reduce the number of 10 macro chaetae (marked by arrowheads in *w1118*) to about 5-6 at 25 °C. At an increased VAP dosage, at 28 °C, the macro chaetae reduced to 0-1. Expressing dsRNA for *VAP*, in a dVAP over-expression background, led to a reversal of the phenotype with macro chaetae reverting to wild type levels both at 25 & 28 °C. Numbers at the top right hand corner of each picture are average macro chaetae, counted for ten females of the corresponding genotype.

(B) Primary screening was done at both 25 °C and 28 °C. A *sca-Gal4, UAS-VAP/Cyo* recombinant, stable line was generated and females from that line were crossed to males with different transgenic RNAi inserts. Genes that lead to a further decrease of macro chaetae (from 5-6) at 25 °C were deemed enhancers and genes that increased number of macro chaetae (from 0) at 28 °C were considered to be suppressors. 2635 genes, encompassing 4600 individual lines were used for the primary screen. 930 genes showed change in the phenotype and were categorized as modifiers.

2635 genes, encompassing 4600 individual lines were used for the primary screen. 930 genes showed change in the phenotype and were categorized as modifiers. 930 genes identified in the primary screen were used for a thorough, quantitative screening, with controls, at 25 °C. Macrochaetae from ten F1 females were counted and compared to the base line 5.5 macro chaetae in the master control (*sca-Gal4, UAS-VAP/+*). Student's t-test was used to select lines that had significantly greater macro chaetae and these were considered *bona-fide* suppressors. Lines that did not meet our threshold for significance ($p > 0.01$, Average macro chaetae < 7.5) were discarded.

F1 females with average macro chaetae < 5.5 were compared to the related RNAi control (*sca-Gal4/+; UAS-RNAi/+*) at 25 °C. Again, a student's t-test was used to select lines above our threshold for significance ($p < 0.01$, average macro chaetae < 4). Starting with 2635 genes in the primary screen, the final numbers for enhancers and suppressors after comparison with controls and rigorous statistical analyses was 45 and 58, respectively. These genes were shortlisted for the validation process.

Adapted from (Deivasigamani et al. 2014).

The largest class was a group of 10 genes, predicted to have roles in energy homeostasis or mitochondrial function (Fig. 2.4C). For example, *Prx5*, a gene involved in redox homeostasis was identified as a strong suppressor while *Tor* was identified as a strong enhancer. Another interesting group of interacting genes were those involved in nuclear import-export. *Embargoed*, a gene involved in nuclear export was identified as a strong enhancer in this while other nuclear import-export components like *Nup75*, *CG8219* and *karyopherin- β 3* were also picked up as enhancers.

2.4.2 A genetic network for *dVAP*

In order to identify components of the extended VAP network, we built an interaction network between the modifiers identified in our screen and physical interactors of VAP using interactions from the databases STRING and GeneMANIA (Mostafavi et al. 2008, Franceschini et al. 2013) for *Drosophila* proteins. We considered only direct interactions and those separated by single node for building the integrated network. Our extended network contains 406 proteins, connected by 953 edges. Of the 103 modifiers identified in our screen, we found that 36 (35 %) physically interact among themselves, connected by 53 edges (Fig 2.5A). Of these, 13 physical interactions involving 12 proteins are ranked as medium confidence interactions (Combined

score or weight of ≥ 0.4) by STRING or GeneMANIA.

We also found that 61 (59%) of the genetic interactors can be linked via a common physical interactor. Based on predictions by DIOPT-DIST and literature mining, 23 of the 103 genes (22 %) identified have been implicated to be involved in or regulating a human nervous system disease (Table 2.2). This is significant considering that there is increasing evidence pointing to the idea of common network of genes/ processes that are affected in several NDs (Shulman and De Jager 2009, Chen and Burgoyne 2012). Our analysis leads us to believe that we have identified a core network of genes and proteins that interact with VAP and, thus, have the ability to modulate and be modulated by VAP function.

Table 2.1: List of the 103 modifiers (58 Suppressors, 45 Enhancers) of VAP function identified in our genetic screen. The modifiers are listed in alphabetical order of their Flybase symbol.

Symbol	Name	Modifier class	Flybase Gene Number	Function
<i>Ars2</i>	-	Enhancer	FBgn0033062	Response to arsenic, catalytic step2 spliceosome Negative regulation of viral genome replication Conversion of ds sirna to ss sirna involved in rna interference Gene silencing by mirna Nuclear mrna splicing, via spliceosome
<i>Bmcp</i>	<i>Bmcp</i>	Suppressor	FBgn0036199	Regulation of metabolic process,mitochondrial transmembrane transport
<i>Bx</i>	<i>Beadex</i>	Enhancer	FBgn0000242	Imaginal disc-derived leg segmentation Leg disc development Locomotor rhythm Response to cocaine Phagocytosis, engulfment Inter-male aggressive behavior
<i>Cdc6</i>	<i>Cdc6</i>	Enhancer	FBgn0035918	Pre-replicative complex assembly

<i>CG10089</i>	-	Suppressor	FBgn0036369	Protein dephosphorylation
<i>CG11560</i>	-	Suppressor	FBgn0036249	-
<i>CG11811</i>	-	Suppressor	FBgn0036099	Purine nucleotide metabolic process
<i>CG12743</i>	<i>ovarian tumor</i>	Enhancer	FBgn0003023	Female germ-line sex determination, Germ cell development Germ cell development Germ-line cyst formation Germ-line sex determination Oogenesis Chromosome organization Positive regulation of dna endoreduplication
<i>CG12822</i>	-	Suppressor	FBgn0033229	-
<i>CG13048</i>	-	Enhancer	FBgn0036593	-
<i>CG13063</i>	-	Suppressor	FBgn0036601	-
<i>CG13075</i>	-	Suppressor	FBgn0036563	Chitin metabolic process
<i>CG13296</i>	-	Enhancer	FBgn0035687	Nucleic acid binding
<i>CG13387</i>	<i>embargoed</i>	Enhancer	FBgn0020497	Protein export from nucleus, Multicellular organismal development Protein export from nucleus Protein transport Protein export from nucleus Nuclear export Centriole replication
<i>CG13994</i>	-	Enhancer	FBgn0031772	-
<i>CG14043</i>	-	Suppressor	FBgn0031659	-
<i>CG14125</i>	-	Suppressor	FBgn0036232	Chitin metabolic process
<i>CG14222</i>		Enhancer	FBgn0031043	Metabolic process
<i>CG14326</i>	-	Suppressor	FBgn0038528	-
<i>CG14435</i>	<i>smallminded</i>	Enhancer	FBgn0029911	-
<i>CG14606</i>	-	Suppressor	FBgn0037485	Phagocytosis, engulfment, hexose transmembrane transport
<i>CG14837</i>	-	Suppressor	FBgn0035797	-
<i>CG15160</i>	-	Enhancer	FBgn0032688	-
<i>CG17327</i>	-	Suppressor	FBgn0038107	Translation
<i>CG17760</i>	-	Suppressor	FBgn0033756	G-protein coupled receptor protein signaling pathway, protein adp-ribosylation
<i>CG17985</i>	-	Suppressor	FBgn0033199	Cell wall macromolecule catabolic process
<i>CG18110</i>	-	Suppressor	FBgn0039677	Sodium ion transport

CG30043	-	Suppressor	FBgn0050043	Proteolysis
CG30060	-	Suppressor	FBgn0050060	-
CG30161	-	Enhancer	FBgn0050161	Neurogenesis
CG31702	-	Enhancer	FBgn0051702	Transcription initiation, dna-dependent
CG31769	-	Suppressor	FBgn0051769	Biological_process
CG32437	-	Suppressor	FBgn0052437	-
CG32685	-	Suppressor	FBgn0052685	-
CG32703	-	Suppressor	FBgn0052703	Protein phosphorylation
CG33090	-	Suppressor	FBgn0028916	Bile acid metabolic process, O-glycoside catabolic process Glucosylceramide catabolic process
CG34113	-	Enhancer	FBgn0083949	-
CG3476	-	Suppressor	FBgn0031881	Acyl carnitine transport, Carnitine transport Mitochondrial transport Transmembrane transport
CG3500	-	Enhancer	FBgn0034849	-
CG3884	-	Enhancer	FBgn0033786	-
CG3975	-	Suppressor	FBgn0027559	Dna replication
CG4169	-	Suppressor	FBgn0250814	Mitochondrial electron transport, ubiquinol to cytochrome c, Proteolysis Lipid particle associated Microtubule associated complex
CG42255	-	Suppressor	FBgn0259140	Microtubule associated complex, calcium binding, metalloendopeptidase
CG4627	-	Suppressor	FBgn0033808	-
CG4646	-	Suppressor	FBgn0033810	Biological_process
CG5325	-	Suppressor	FBgn0032407	Nervous system development , peroxisome
CG5733	Nucleoporin 75	Enhancer	FBgn0034310	Smad protein nuclear translocation,nls-bearing substrate import into nucleus
CG5953	-	Suppressor	FBgn0032587	-
CG6048	-	Suppressor	FBgn0029827	Proteolysis
CG6220	Enhancer of Polycomb	Enhancer	FBgn0033865	-
CG6345	-	Suppressor	FBgn0037816	Regulation of cyclin-dependent protein kinase activity,trna modification
CG6502	Enhancer of zeste	Enhancer	FBgn0000629	Histone methylation,

				Chromatin silencing Syncytial blastoderm mitotic cell cycle Axon guidance Muscle organ development Dendrite morphogenesis Neurogenesis Histone h3-k27 methylation Histone h3-k27 methylation Histone h3-k9 methylation Histone methylation Cuticle hydrocarbon biosynthetic process
CG6674	-	Enhancer	FBgn0036063	-
CG6885	-	Suppressor	FBgn0036810	-
CG6950	-	Suppressor	FBgn0037955	Biosynthetic process
CG7306	<i>obstructor-F</i>	Enhancer	FBgn0036947	Chitin metabolic process
CG7564	-	Enhancer	FBgn0036734	Nuclear mrna splicing, via spliceosome
CG7564 (<i>Alsin2</i>)	-	Suppressor	FBgn0037116	Rab guanyl-nucleotide exchange factor activity,regulator of chromosome condensation, rcc1; vacuolar sorting protein 9
CG7776	-	Enhancer	FBgn0000581	Chromatin organization
CG8219	-	Enhancer	FBgn0035693	Protein import into nucleus, nuclear pore
CG8571		Enhancer	FBgn0016983	-
CG8863	<i>DnaJ-like-2</i>	Enhancer	FBgn0038145	Protein folding, response to heat
CG9172	-	Enhancer	FBgn0030718	Mitochondrial electron transport, nadh to ubiquinone, Determination of adult lifespan Mitochondrial respiratory chain complex i assembly Response to reactive oxygen species
CG9391		Enhancer	FBgn0037063	Dephosphorylation
CG9638	<i>Ada2b</i>	Enhancer	FBgn0037555	Positive regulation of histone acetylation, Regulation of transcription , saga complex, polytene chromosome Chromatin remodeling Cell proliferation Histone h3 acetylation Eye pigment metabolic process
CG9953	-	Enhancer	FBgn0035726	Proteolysis
<i>Cyp4ac1</i>	<i>Cyp4ac1</i>	Suppressor	FBgn0031693	Hormone metabolic process, insecticide catabolic process

<i>Cyp6a19</i>	<i>Cyp6a19</i>	Suppressor	FBgn0033979	Oxidation-reduction process
<i>Cyp6a2</i>	<i>Cytochrome P450-6a2</i>	Suppressor	FBgn0000473	Response to insecticide, response to caffeine, oxidation-reduction process
<i>Cyp9b2</i>	<i>Cytochrome P450-9b2</i>	Suppressor	FBgn0015039	Microsome
<i>dila</i>	<i>dilatory</i>	Suppressor	FBgn0033447	Adult locomotory behavior, cilium axoneme assembly, spermatogenesis, peripheral nervous system neuron development
<i>dyn-p25</i>	<i>dynactin-subunit-p25</i>	Enhancer	FBgn0040228	Microtubule-based movement
<i>ear</i>	<i>ENL/AF9-related</i>	Suppressor	FBgn0026441	Transcription initiation from rna polymerase ii promoter, regulation of transcription, dna-dependent
<i>Ef1beta</i>	<i>Elongation factor 1 beta</i>	Enhancer	FBgn0028737	Translational elongation
<i>eIF2B-delta</i>	<i>eIF2B-delta</i>	Enhancer	FBgn0034858	Translational initiation, neurogenesis
<i>epsilonCOP</i>	<i>epsilonCOP</i>	Suppressor	FBgn0027496	Retrograde vesicle-mediated transport, golgi to er
<i>GlyP</i>	<i>Glycogen phosphorylase</i>	Suppressor	FBgn0004507	Glycogen catabolic process, flight
<i>Hsp83</i>	<i>Heat shock protein 83</i>	Suppressor	FBgn0001233	Spermatogenesis,
<i>icln</i>	<i>Icln</i>	Enhancer	FBgn0029079	Cell volume homeostasis , chloride transport , volume sensitive anion channel
<i>Irp-1B</i>	<i>Iron regulatory protein 1B</i>	Suppressor	FBgn0024957	Regulation of translational initiation by iron
<i>Karybeta3</i>	<i>Karyopherin beta 3</i>	Enhancer	FBgn0087013	Protein import into nucleus
<i>l(1)G0222</i>	<i>lethal (1) G0222</i>	Suppressor	FBgn0028343	-
<i>l(2)35Cc</i>	<i>lethal (2) 35Cc</i>	Enhancer	FBgn0259982	Unfolded protein binding, protein folding
<i>mRpS34</i>	<i>mitochondrial ribosomal protein S34</i>	Enhancer	FBgn0260460	Mitochondrial small ribosomal subunit
<i>mys</i>	<i>mysospheroid</i>	Enhancer	FBgn0004657	Central nervous system development, cell migration Midgut development Muscle attachment Calcium-dependent cell-cell adhesion Calcium-dependent cell-matrix adhesion Cell-matrix adhesion Heterophilic cell-cell adhesion Apposition of dorsal and ventral imaginal disc-

				derived wing surfaces Cell adhesion Regulation of cell shape Axon guidance Dorsal closure Epithelial cell migration, open tracheal system Tracheal outgrowth, open tracheal system Maintenance of epithelial integrity, open tracheal system Hemocyte migration Substrate-dependent cell migration, cell extension, Determination of adult lifespan Germ-line stem cell maintenance Sensory perception of smell Imaginal disc-derived male genitalia morphogenesis Cell-substrate adhesion Integrin-mediated signaling pathway Cell-matrix adhesion
<i>NaPi-T</i>	<i>Na[+]-dependent inorganic phosphate cotransporter</i>	Suppressor	FBgn0016684	Phosphate transport
<i>Nlp</i>	<i>Nucleoplasmin</i>	Enhancer	FBgn0016685	Nucleosome positioning
<i>Osi20</i>	<i>Osiris 20</i>	Suppressor	FBgn0037430	Biological_process unknown
<i>pallidin</i>	<i>pallidin</i>	Enhancer	FBgn0036192	-
<i>porin</i>	<i>porin</i>	Enhancer	FBgn0004363	Mitochondrial transport , Ion transport Phototransduction Sperm individualization Mitochondrion organization Sperm mitochondrion organization Transmembrane transport Enhancer regulation of anion transport
<i>PrBP</i>	<i>Prenyl-binding protein</i>	Enhancer	FBgn0032059	Mushroom body development,visual perception , 3',5'-cyclic-gmp phosphodiesterase activity
<i>Prx5</i>	<i>Peroxiredoxin 5</i>	Suppressor	FBgn0038570	Response to oxidative stress, peroxisome, mitochondrion, cell redox homeostasis
<i>sname</i>	<i>something that</i>	Suppressor	FBgn0086129	-

	<i>sticks like glue</i>			
<i>SOD1</i>	<i>Superoxide dismutase</i>	Suppressor	FBgn0003462	Superoxide dismutase (cu/zn) / chaperones; superoxide dismutase, copper/zinc binding domain; superoxide dismutase, copper/zinc, binding site, response to oxidative stress
<i>ssh</i>	<i>slingshot</i>	Enhancer	FBgn0029157	Protein dephosphorylation, Actin cytoskeleton organization Regulation of actin polymerization or depolymerization Mushroom body development Regulation of axonogenesis Regulation of lamellipodium assembly Mitotic cell cycle Protein dephosphorylation
<i>Su(P)</i>	<i>Suppressor of ref(2)P sterility</i>	Suppressor	FBgn0004465	Cell redox homeostasis, protein disulfide oxidoreductase
<i>TBPH</i>	<i>TBPH</i>	Suppressor	FBgn0025790	Neuromuscular junction development,adult locomotory behavior, rna metabolism
<i>tectonic</i>	<i>tectonic</i>	Suppressor	FBgn0261697	Regulation of smoothened signaling pathway
<i>tmod</i>	<i>tropomodulin</i>	Suppressor	FBgn0082582	Cytoskeleton organization
<i>Tor</i>	<i>Target of rapamycin</i>	Enhancer	FBgn0021796	Growth, Response to nutrient Phosphorylation Detection of nutrient Positive regulation of cell size Positive regulation of organ growth Positive regulation of cell growth Positive regulation of multicellular organism growth Determination of adult lifespan Autophagy Endocytic recycling Regulation of cell growth Positive regulation of cell size Positive regulation of ribosome biogenesis Response to dna damage stimulus Dendrite morphogenesis
<i>torp4a</i>	<i>torp4a</i>	Suppressor	FBgn0025615	Protein folding, endoplasmic reticulum lumen
<i>Tsp2A</i>	<i>Tetraspanin 2A</i>	Enhancer	FBgn0024361	Integral to membrane

<i>Use1</i>	<i>Use1</i>	Suppressor	FBgn0035965	Golgi organization, neurogenesis, protein secretion
<i>Zif</i>	<i>Zinc-finger protein</i>	Enhancer	FBgn0037446	Establishment or maintenance of neuroblast polarity, neuroblast proliferation
<i>zye</i>	<i>zye</i>	Suppressor	FBgn0036985	Regulation of embryonic cell shape, filamentous actin Cell-matrix adhesion Actin filament organization

2.4.3 Known ALS loci and physical interactors of VAP act as modifiers

Recent studies have shown hVAPB levels to be affected in patients with sporadic ALS. SOD1 transgenic mice and VAP mutant mice show TDP-43 pathology suggesting that many of these pathogenic pathways may be linked. We therefore sought to see if any of the loci involved in ALS act as modifiers of VAP phenotype. Indeed, knockdown of *SOD1*, *Alsin2* and TBPH suppressed the loss of bristle phenotype (Fig 2.5B) in *sca-Gal4>VAP* animals. The above results support the idea that causative genetic loci for ALS interact genetically with each other.

A subset of RNAi lines in our primary screen represented genes whose protein products are known to physically interact with VAP. Of the known thirty-five physical interactors that were tested in our screen, a significant fraction (8 genes; 22%), were genetic modifiers in our screen (Fig 2.5C). Of these *SNAMA*, *Tropomodulin*, *lethal (1) G0222*, *epsilon-COP* and *Pex-19 (CG5325)*, were identified as suppressors and *Droj-2*, *Karyopherin-β3*, *porin* as enhancers. A physical interaction with a protein may or may not manifest in the phenotype being used to score for genetic interactions. Moreover, many of these interactions have been identified in S2 cells (Guruharsha et al. 2011) or tissues other than neurons. It is possible that some of these interactions are not represented in neurons, which is our tissue of interest.

2.5 Discussion

2.5.1 A genetic screen that enriches VAP modifiers

VAP was originally identified as an interactor of VAMP involved in vesicle release (Skehel et al. 1995). In the last few years many mutations in VAP leading to ALS have been identified (Chen et al. 2010, van Blitterswijk et al. 2012c, Han et al. 2013a, Kabashi et al. 2013). It has also been observed that levels of VAP increase in case of some tumors (Rao et al. 2012). Recent studies have identified a number of roles for VAP and how these functions might be affected in case of a mutation (Chen et al. 2010, van Blitterswijk et al. 2012c, Han et al. 2013a, Kabashi et al. 2013).

We report the first screen to identify modifiers of ALS8 gene VAP using a *Drosophila* over-expression model. The outcome of the screen, which is a list of genetic interactors, is evaluated with two primary concerns; one regarding the modifiers being related to a general cellular phenomenon such as proteostasis rather than VAP function and the second, the possibility of identifying large number of interactors, indicating false positives. In order to negate the first possibility, we compared our modifiers with genes involved in protein homeostasis (Silva et al. 2011) as well as the ERAD network (Christianson et al. 2012).

None of the modifiers from our screen were a part of the nine genes identified as regulators of proteostasis network and although thirteen of the ERAD network proteins were represented in our screen, we identified only *Hsp83* as an interactor. The second concern was related to the high number of interactors identified, which was about 3.9 % of the total genes screened. This would, in a normal scenario, indicate that our screen is identifying non-specific genetic interactions. This finding has to be balanced by the fact that VAP has been linked to a large repertoire of functions within and outside the cell. In a genome wide screen performed by radiation hybrid method in cell lines, larger numbers, namely 2500 genetic interactors of VAP

and VAPA have been identified. 19 of the 103 (18.4%) interactors in our screen have been identified in this and other genome wide screens in other organisms ((Costanzo et al. 2010, Lin et al. 2010); Table 2.3). One hit in our screen is *Tor*, which itself interacts with many housekeeping and homeostatic cellular components, leading to a possible increase in hits in our screen.

2.5.2 VAP modifiers and ALS

The discovery of *TBPH*, *alsin2* and *sod1* as modifiers in our screen implies that even in a wild type scenario, these genes are part of the VAP genetic network. There is increasing evidence to suggest that interplay exists between different ALS causing loci and that ALS causing loci may be part of a core network involved in determining the progression of neuro-degeneration. Further evidence for this thesis come from earlier data that indicates that VAP (P58S) mutation affects localization of sigma receptor 1 (Prause et al. 2013), another ALS locus. Localization of TDP-43 – another gene associated with ALS, is also affected because of the P58S mutation (Tudor et al. 2010). Supporting this, we show that loss of *Drosophila* TDP-43 is able to alter the VAP (P58S) mediated bouton size (Fig. 3.3E,G).

Additional evidence for interactions between VAP and other ALS loci comes from the finding that VAP levels are lowered in sporadic ALS patients and mice expressing mutant SOD1 (Teuling et al. 2007). A recent study in *smn* based spinal muscular atrophy (SMA) shows that *SOD1* can alter the bouton morphology caused because of *smn* knock down (Sen et al. 2013). Our screen identifies *SOD1* as an interactor of VAP. Our observation together with other studies implies that many if not all ALS causing loci interact with each other and a core network exists in cells, which is sensitive to perturbation of its individual members and has a tendency to breakdown in motor neurons late in life.

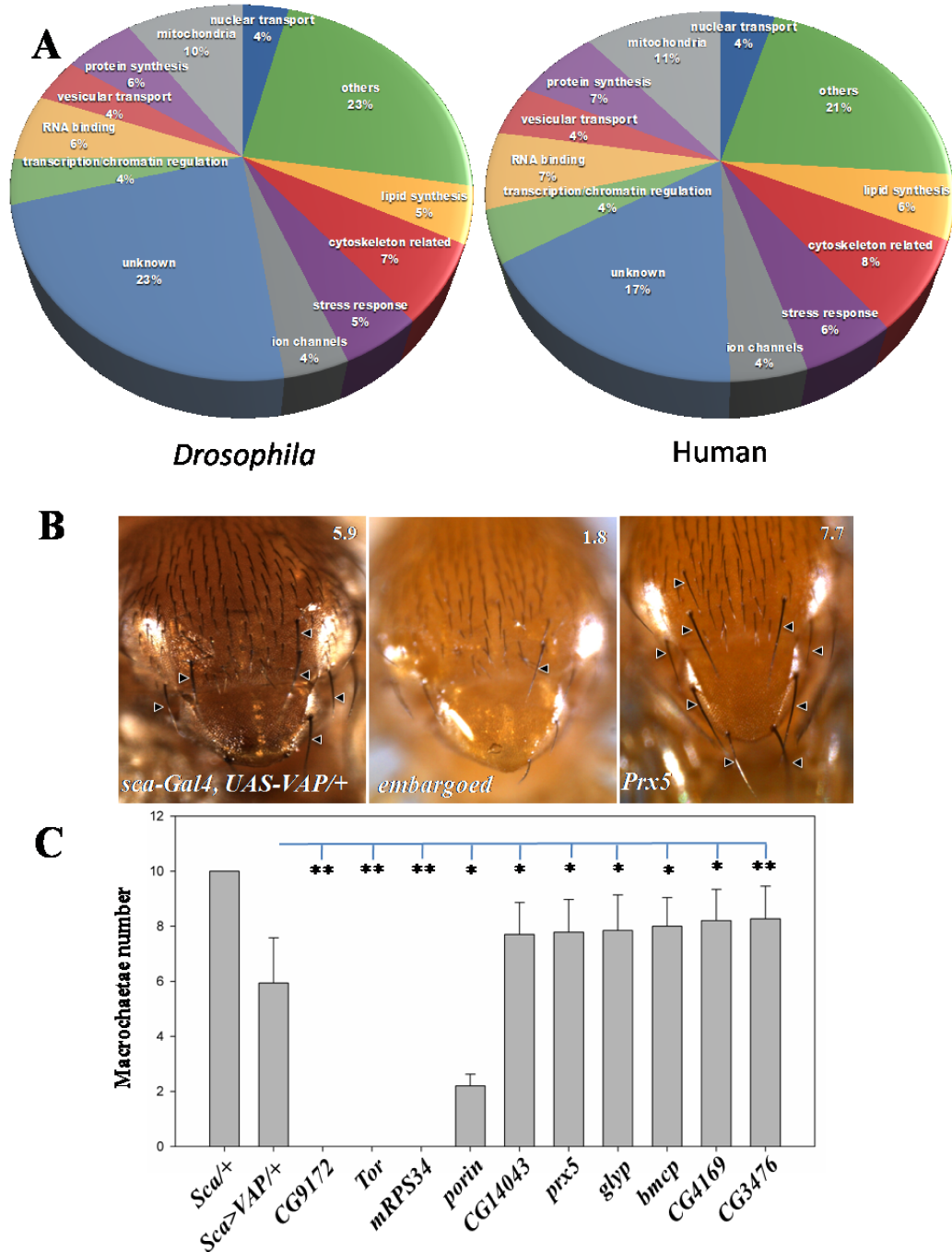


Figure 2.4: Gene Ontology of modifiers identified in the screen.

(A) Graphical, GO representation for *Drosophila* 103 VAP interactors and also their human homologs.

(B) Representative examples of thoracic macro chaetae for modifiers along with control, *sca-Gal4,UAS-VAP/+*. *Embargoed*, a nuclear pore protein is an enhancer of VAP function while *Prx5* involved in oxidative stress is a suppressor.

(C) Average Macrochaetae number in ten genes involved in energy & mitochondrial metabolism. A full list of the 103 interactors can be found in Table 1. “*” indicates a p-value<0.01 (but > 0.001), while “**” indicates a p-value of <0.001. Adapted from (Deivasigamani et al. 2014).

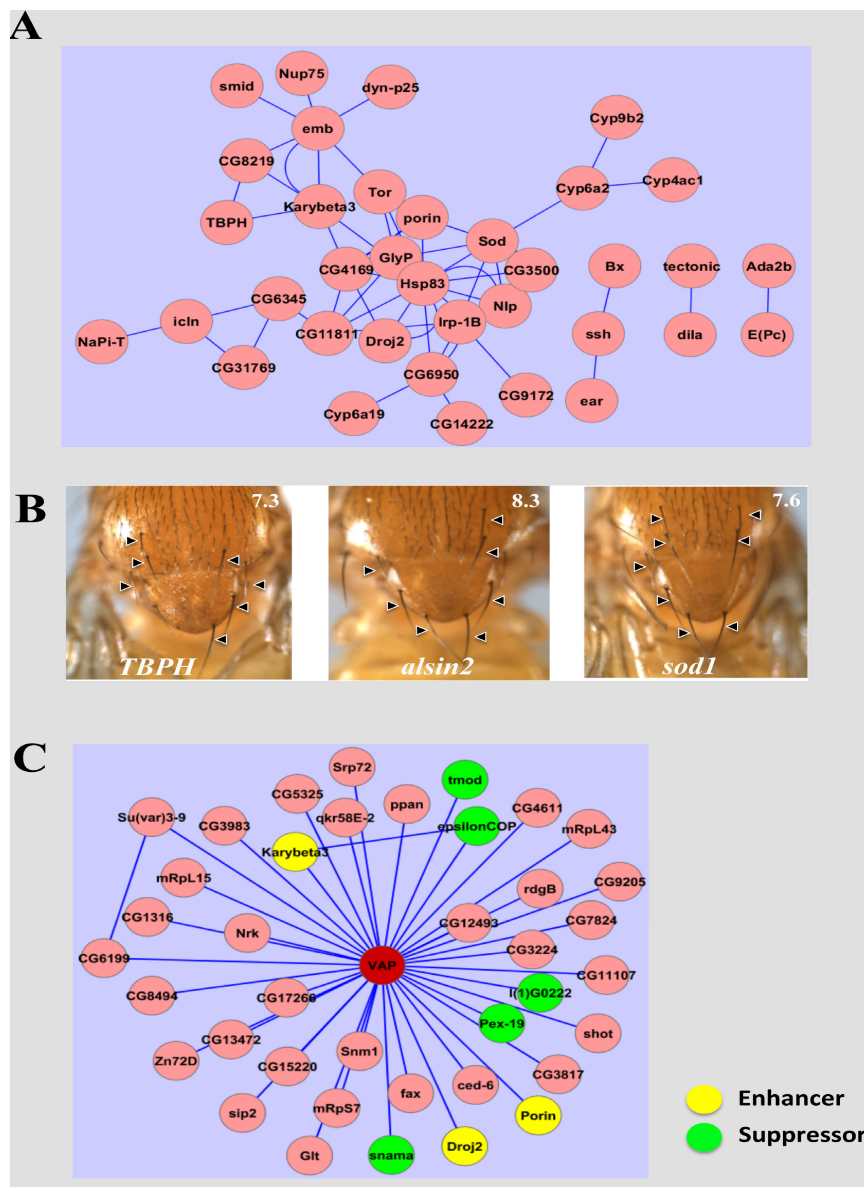


Figure 2.5: An integrated network of dVAP genetic interactors.

An extended network of dVAP network was built by integrating VAP interaction data from our screen (103 genes) with known physical interactors of dVAP. The extended network (not shown) includes 406 genes and 953 edges.

(A) Analysis indicated that 36 modifiers (displayed as circles), which are a subset of the 103 modifiers discovered, interact physically among themselves. Blue connecting lines (edges) indicate physical interaction.

(B) *Drosophila* homologs of known ALS causing loci, *TBPH*, *Alsin* & *sod1* were suppressors of dVAP function and are part of the genetic network. The genotype for each cross is *Sca-Gal4/+; UAS-VAP/UAS-Gene-RNAi*. For each figure, average macro chaetae values from ten females are represented on the top right hand corner.

(C) Thirty-five known physical interactors of VAP are a subset of the 2635 genes screened in this study. Of these, eight genes were found to be *genetic* interactors of VAP. *SNAMA*, *tmod*, *lethal (1) G0222*, *epsilon-COP* and *Pex-19* are suppressors while *Droj2*, *karyopherin beta3* and *Porin* are enhancers. Adapted from (Deivasigamani et al. 2014).

Table 2.2: 22 of the 103 modifiers identified in our screen have been implicated in several neuronal disorders. Known ALS loci (*TBPH*, *alsin2*, *sod1*) identified in our screen are not a part of this list.

FLY GENE	MAMMALIAN ORTHOLOG	MOLECULAR FUNCTION (FLIES AND HUMANS)	ASSOCIATED NERVOUS SYSTEM DISORDER	REFERENCE/ SOURCE
<i>Karyopherin beta-3</i>	<i>IPO5</i>	Nuclear export/import	Huntington's Disease	(Zhang et al. 2010)
<i>Target of Rapamycin</i>	<i>MTOR</i>	Nutrient sensing, energy homeostasis, protein synthesis	Huntington's Disease	(Ravikumar et al. 2004)
<i>Ada2b</i>	<i>TADA2B</i>	Chromatin remodelling	Huntington's Disease	(Zhang et al. 2010)
<i>Ars2</i>	<i>SRRT</i>	mRNA binding	Huntington's Disease	(Zhang et al. 2010)
<i>CG6950</i>	<i>CCBL2</i>	Kynurenine-Oxoglutarate Transaminase	Parkinson's Disease	(Hartai et al. 2005)
<i>CG9172</i>	<i>NDUFS7</i>	NADH dehydrogenase I (ubiquinone) Fe-s	Huntington's Disease, Leigh syndrome due to cytochrome c oxidase deficiency	(Kaltenbach et al. 2007)
<i>Hsp83</i>	<i>HSP90AA1</i>	Chaperone	Huntington's Disease, Alzheimer's Disease	(Zhang et al. 2010), (van Ham et al. 2009)
<i>CG8219/Transportin</i>	<i>TNPO1</i>	Nuclear export/import	Amyotrophic Lateral Sclerosis/FUS	(Dormann et al. 2010)
<i>icln</i>	<i>CLNS1A</i>	Chloride channel, Methylosome	Spinal Muscular Atrophy	DIOPT-DIST/OMIM
<i>E(Pc)</i>	<i>EPC2</i>	Transcription factor	Alzheimer's disease (biomarkers)	DIOPT-DIST/OMIM
<i>CG5325</i>	<i>PEX19</i>	Peroxisome biogenesis	Peroxisome biogenesis disorder 12A	DIOPT-DIST/OMIM
<i>CG14043</i>	<i>KIAA1279</i>	Unknown	Goldberg-Shprintzen megacolon syndrome	DIOPT-DIST/OMIM
<i>CG3476</i>	<i>SLC25A20</i>	Acyl carnitine transporter	Carnitine-acylcarnitine translocase deficiency	DIOPT-DIST/OMIM
<i>CG9391</i>	<i>IMPA2</i>	Inositol metabolism	Stroke	DIOPT-DIST/OMIM

<i>eIF2B-delta</i>	<i>EIF2B4</i>	Translation initiation	Leukoencephalopathy with vanishing white matter	DIOPT-DIST/OMIM
<i>smid</i>	<i>NVL</i>	Valosin containing protein	Taupathy	(Ambegaokar and Jackson 2011)
<i>dyn-p25</i>	<i>DCTN5</i>	microtubule-based movement	Bipolar Disorder	DIOPT-DIST/OMIM
<i>ear</i>	<i>AP5Z1</i>	Transcription factor	Spastic Paraplegia	DIOPT-DIST/OMIM
<i>Irp-1B</i>	<i>ACO2</i>	Translation and metabolism	Infantile cerebellar-retinal degeneration	DIOPT-DIST/OMIM
<i>NaPi-T</i>	<i>SLC17A5</i>	Ion channel	Salla Disease	DIOPT-DIST/OMIM
<i>tectonic</i>	<i>BD92/TCTN3</i>	Smoothened signaling	Joubert syndrome, Meckel Syndrome	DIOPT-DIST/OMIM
<i>CG32685</i>	<i>ZAP</i>	Unknown	Spastic Paraplegia	DIOPT-DIST/OMIM

2.5.3 VAP and cellular homeostasis

A GO analysis of the 103 modifiers as well as their human counterparts identifies clusters of genes related to protein trafficking, lipid biosynthesis, protein biogenesis, stress and cellular energetics. These categories include some of the possible cellular functions that researchers increasingly believe to be affected in NDs (Table 2.2) in general and ALS in particular. The role of VAP in lipid biosynthesis is best understood with its association with ceramide transfer protein (CERT) (Perry and Ridgway 2006, Peretti et al. 2008). In our screen, we identify novel genes implicated in lipid and inositol metabolism, namely CG33090, CG14222 and CG9391 whose characterization may lead to a better understanding of VAP function. Secreted MSP domain of VAP regulates mitochondrial morphology (Han et al. 2012) and VAP(P56S) mutant is known to affect anterograde transport of mitochondria along the axons (Morotz et al. 2012). VAP interacts

with PTPIP51 and helps in maintaining calcium homeostasis (De Vos et al. 2012).

Neuronal loss of VAPB affects reduces ATP levels, altered fat metabolism in muscles and this is mediated via DAF-16 (Han et al. 2013b). In our screen, *Bmcp*, *CG4169*, *CG3476*, *CG9172*, *porin*, *Prx5* and *mRpS34* (Fig 2.3C) are mitochondrial proteins that may further link VAP to mitochondrial function. VAP misfolding and aggregation causes ER stress (Gkogkas et al. 2008) (Moustaqim-Barrette et al. 2014), a phenomenon that may involve *Droj*, *Hsp83* & *l(2)35Cc*. We also identified nucleoporins as interactors of VAP. siRNA mediated knock down of VAP results in cytoplasmic retention of Nup-214 and emerin; and VAP (56S) mutant leads to nuclear envelope defects (Tran et al. 2012). TOR, which is the focus of our study, is a major player in cellular homeostasis & disease (Laplane and Sabatini 2012). Thus, many of the genes identified in our screen, though novel, fall in the same categories of known VAP functions and an understanding of their function in relation to VAP and VAP (P58S) may lead to a better understanding of the disease mechanisms.

Table 2.3: List of conserved interactors of VAP that has been identified in other studies.

Gene	Function	Conserved in
<i>ssh</i>	cytoskeleton organisation	Human
<i>tmod</i>	cytoskeleton organisation	Human
<i>GlyP</i>	glycogen metabolism	Human
<i>CG17760</i>	GPCR	Human
<i>Cyp4ac1</i>	hormone metabolism	Human
<i>Bx</i>	locomotor rhythm	Human
<i>Cyp6a19</i>	monooxygenase	Human
<i>Zif</i>	neuroblast polarity	Human
<i>Pex19</i>	Peroxisome biogenesis	Yeast
<i>Cyp9b2</i>	smoothened signaling	Human
<i>Eflbeta</i>	translation elongation	Human
<i>Use1</i>	Ubiquitin ligase	Yeast
<i>CG13296</i>	unknown	Human
<i>smid</i>	unknown	Human
<i>CG32703</i>	unknown	Human
<i>CG34113</i>	unknown	Human
<i>Tsp2A</i>	unknown	Human
<i>Nlp</i>	nuclear chaperone	Human
<i>TBPH</i>	RNA splicing	Human

CHAPTER 3

A subset of VAPB genetic interactors also interact with VAPB(P58S)

3.1 Summary

In this chapter we show that a subset of the modifiers identified from the VAP (wt) bristle screen can modulate the large bouton phenotype seen in larvae over expressing VAPB (P58S) neuronally. One key modifier identified in the bristle screen as a suppressor was the *Drosophila* homolog of mTOR. In neurons *tor* knockdown significantly reversed the VAPB(P58S) associated large bouton phenotype. The interaction with the TOR pathway was further confirmed by demonstrating interaction with upstream and downstream members of the TOR signaling pathway. Over-expressing the proteins of the Tuberous Sclerosis Complex (Tsc1,2) that down regulates TOR signaling reverses VAPB(P58S) bouton phenotypes as does reducing activity of S6Kinase. The increase in TOR signaling in neurons seen with VAP(P58S) expression is in contrast with lowered TOR signaling seen in response to expression of VAP (wt), shown by the fact that *Tsc1* knock down and *S6Kinase-CA* can rescue the reduced bouton size. Importantly, VAPB(P58S) bouton phenotypes in larvae can be rescued by feeding with the TOR signaling inhibitor Rapamycin.

TOR pathway has been implicated in age related neuro-degeneration and recent studies have shown role of VAPB in AKT activation in breast cancer. The well known cross talk between AKT and TOR pathway makes our finding interesting. The VAPB network and further mechanistic understanding of interactions with key pathways, such as the TOR cassette, will

pave the way for a better understanding of the mechanisms of onset and progression of motor neuron disease.

3.2 Introduction

Synaptic growth and plasticity are essential mechanisms underlying the memory, learning and coordinated movement. Similarly, synaptic degeneration is a feature of neurological diseases. Hence studying synaptic development in *Drosophila* might shed light on these aspects. *Drosophila* neuromuscular junction has provided valuable information about the molecular mechanisms that might be involved in the development and function of synapse. Fly models of ALS have been shown to have defects in locomotion and studies using larval NMJ have provided insights into possible synaptic defects in these models (Charng et al. 2014). Studies from our and other groups have shown that the VAPB null mutants show reduced bouton number and increased size. Expression of VAP(P58S) mutant shows increased bouton size, while VAP(wt) over-expression shows reduced bouton size and increased bouton number (Pennetta et al. 2002, Chai et al. 2008, Ratnaparkhi et al. 2008). We have also shown that there are retrograde BMP signaling defects in VAPB (P58S) mutants (Ratnaparkhi et al. 2008). Over-expression of ALS causing FUS mutants in motor neuron of the larvae reduces the levels of presynaptic brp and postsynaptic dlg and increase glutamate receptor IIA. Electrophysiological studies in these animals have shown that the Evoked junction potential and mini EJPs are altered (Machamer et al. 2014). It has also been shown that the FUS and TDP-43 act in a common pathway and regulate *Drosophila* NMJ development (Lanson et al. 2011, Wang et al. 2011).

3.2.1 *Drosophila* NMJ

The motor system has a stereotyped arrangement of 30 muscles within each hemi-segment of the body wall. The body wall muscles can be classified into internal and external muscles. External

muscles are arranged along the dorso-ventral axis while the internal muscles are arranged longitudinally. Coordinated contraction of these muscles helps in the peristaltic movement of the larvae. These muscles receive input from a total of 32 motor neurons which exit the ventral nerve cord via three major nerve roots namely the inter-segmental nerve (ISN), segmental nerve (SN) and transverse nerve (TN). The ISN motor neurons innervate the internal muscles and the SN and TN neurons innervate the external muscles (Figure 3.1) (Landgraf et al. 2003, Kohsaka et al. 2012). In our studies, we have looked at muscle 4 that is innervated by a single neuron and has Type I boutons.

Drosophila NMJ is a tripartite synapse with a muscle, neuron and glia as partners. The NMJ of *Drosophila* has majorly glutamatergic synapses, unlike those in mammals, which are cholinergic. Glutamatergic synapses are the major excitatory neurotransmitter in the human brain. Hence, the NMJ has been used extensively to study the molecular mechanisms and pathways involved in development of glutamatergic synapses. The neurons innervating each of the muscle are well characterized. The *Drosophila* NMJ synapse has a characteristic structure including neuronal branches and synaptic boutons. From hatching of embryo to third instar larvae, the muscle grows by over 100 fold in volume and this is adequately compensated by increase in number of boutons. There are three classes of boutons namely, Type I, II and III. The classification of boutons is based on their size, shape and the kind of neurotransmitter released by them. Type I boutons are glutamatergic in nature and are further divided into Ib (large) and Is (small). Type II and III boutons are neuro-modulatory and use octapamine, insulin like peptides and leukocinin1 (Kohsaka et al. 2012, Menon et al. 2013). A number of presynaptic and post synaptic markers like Bruchpilot, Discs large, Synaptotagmins and glutamate receptors are available to understand the mechanisms and defects in synapse formation.

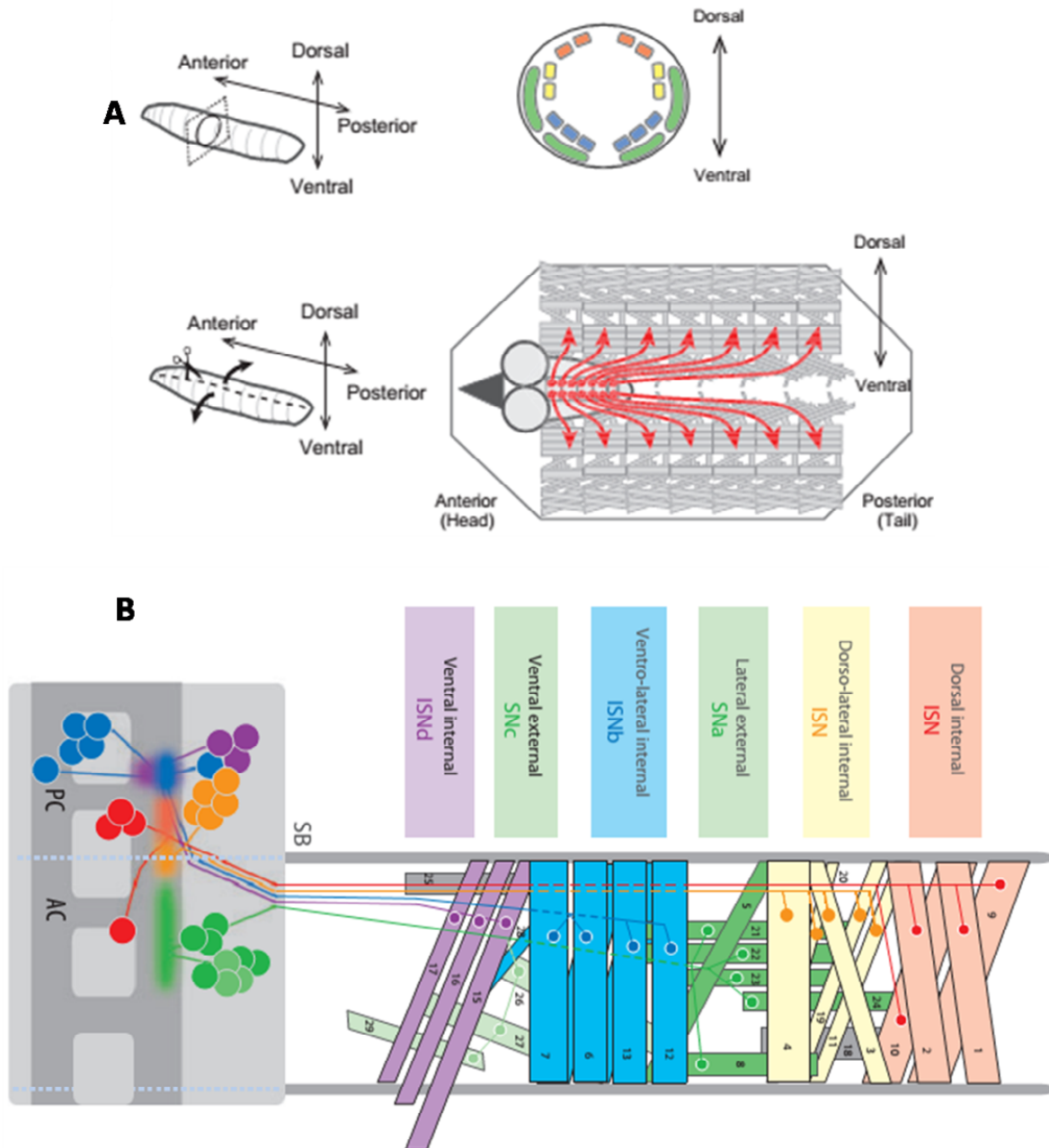


Figure 3.1: Organization of *Drosophila* larval body muscles and neuro-muscular junction. (A) Schematic showing the organization of different muscles. In our experiments larvae were dissected along the dorsal line and flattened to expose the muscles. (B) Different nerve fibres originating from ventral nerve cord innervate muscles. The muscles have been color coded according to the nerve fibre innervating them. Adapted from (Kohsaka et al. 2012).

3.2.2 TOR pathway

Tor was first identified from yeast in a screen for identifying targets of Rapamycin. *Tor* is Phosphatidylinositol Kinase related Kinase and phosphorylates target proteins on serine and threonine residues. In yeast, flies and mammals *Tor* exists in two functional complexes (TORC1 and TORC2 complex). These two complexes differ in the interacting proteins and their targets. TORC1 complex contains LST8, Raptor and PRAS40 apart from other components and its effectors include S6k, 4EBP1, Atg1 and SREBP1 (Betz and Hall 2013). TORC2 complex has LST8, Rictor and Sin1 among others. Torc2 complex is involved in regulating Akt, SGK and others. These two complexes respond to different stimuli. TORC1 activity is inhibited by Tsc1&2 complex via Rheb and this inhibition can be relieved by stimulating with glucose or amino acids. Torc2 activity is regulated by PI3K and is activated by growth factors. Tor pathway can be inhibited by addition of Rapamycin (Figure 3.2). Though rapamycin is more selective for TORC1 complex, chronic addition can inhibit TORC2 complex as well (Inoki et al. 2012, Betz and Hall 2013).

In mammalian neurons, TORC1 activity has been shown to increase with calcium influx and neuronal activity. TORC1 has been shown to have roles in late phase Long Term Potentiation, which require de novo protein synthesis. The role of TORC1 in regulating proteins synthesis has also been implicated in memory formation as well (Swiech et al. 2008, Hoeffler and Klann 2010). Not much is known about the role of TORC2 complex in nervous system. Recent studies suggest that TORC2 complex contributes to long term memory formation in mammals and *Drosophila* by regulating actin polymerization and spine formation (Huang et al. 2013). Also, altered TORC1 signaling has been observed in case of Parkinson's disease, Alzheimer's disease and Huntington's disease (Caccamo et al. 2010, Graber et al. 2013).

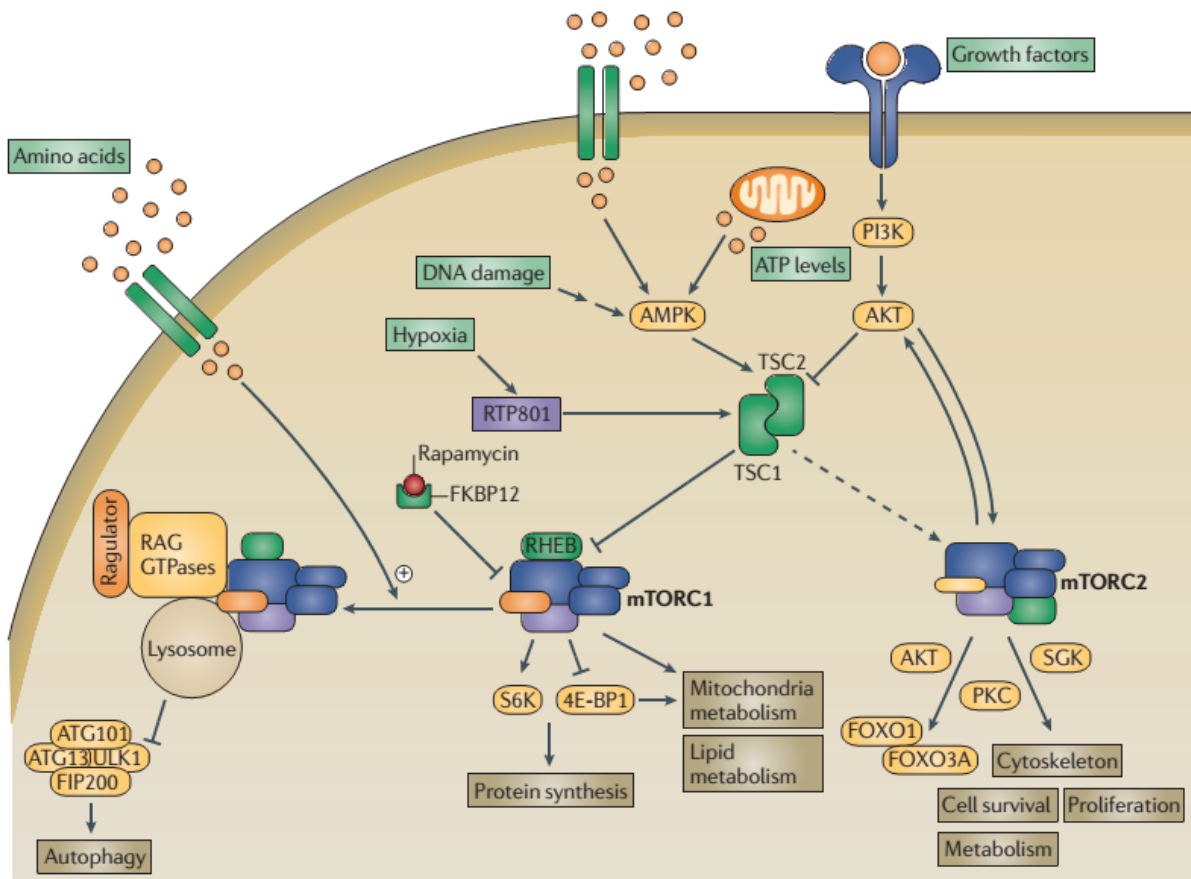


Figure 3.2: Components and function of TOR pathway.

Target of rapamycin complex 1 (mTORC1) integrates upstream nutrient signals (amino acids), growth factors (insulin), ATP and cellular stressors to regulate protein synthesis, mitochondria and lipid metabolism and autophagy. mTORC2 is activated in response to growth factors and regulates cytoskeletal organization, metabolism and cell survival. Adapted from (Bove et al. 2011)

3.3 Materials and Methods

3.3.1 Stocks

All stocks were reared on regular corn flour medium. *UAS-VAP* wild type and *UAS-VAP (P58S)* lines used in this study have been described earlier (Ratnaparkhi et al. 2008). Canton S flies were used as a wild type control. *UAS-Atg1* line was kindly provided by Dr. Chen, Academia Sinica. *UAS-Tsc1*, *UAS-Tsc2* line was provided by Prof.L.S.Shashidhara (Donor's source: (Tapon et al.

2001)). The following lines were obtained from Bloomington stock center: BL7013 (*Tor-DN*), BL6911 (*S6K-DN*), BL6914 (*S6K-CA*), BL33951 (*Tor*-TRIP RNAi), BL31314 (*Tsc1* RNAi), BL24854 (*Thor CA /ThorLL*), BL9689 (*UAS-Rheb*), BL33966 (*UAS-Rheb* RNAi). The *elav*^{C155} - *Gal4* line has been described earlier (Lin et al. 1994).

3.3.2 Immunohistochemistry and imaging

For NMJ analysis, animals were raised in standard cornmeal agar medium at 28 °C. Wandering third instar female larvae were dissected in cold PBS and fixed in Bouin's fixative for 20 minutes, except for p-S6K staining where larvae were fixed with 4% PFA in PBS. The tissues were washed, blocked in PBS containing 0.3% TritonX-100 and 2% BSA and incubated with primary antibody at 4 °C overnight. Samples were washed and incubated with secondary antibody for overnight at 4 °C. Then samples were washed and mounted using 70% glycerol with n-propyl gallate. The following antibodies were used for immuno-staining, Anti-HRP (1:500 dilution, Sigma) and anti-dlg (1:100 dilution, DSHB), anti-p-S6K (1:100, Cell signaling). Alex flour 488 and 568 secondary conjugate antibodies (1:1000) were obtained from Molecular Probes, Invitrogen. Synapse of muscle 4 in A2 and A3 segments were imaged for bouton size measurement. Only dlg positive type1b boutons were considered for analysis. The largest diameter of the every bouton in NMJ of muscle 4, excluding junctional boutons was measured. Confocal imaging was carried out using a Zeiss LSM 710 microscope. The bouton measurements were compared for significance using ANOVA.

3.3.3 Rapamycin feeding

20 virgin females were allowed to mate for 24 hours and transferred to vials containing standard cornmeal media mixed with either DMSO or rapamycin (200nM, Invitrogen). rapamycin at doses ranging from 200-1000 nM was tested, with 200 nM chosen as the dose for our

experiments, based on increasing lethality at doses >800 nM. Larvae were continuously raised on Rapamycin/DMSO containing food and dissected at third larval instar stage for bouton analysis.

3.3.4 Western Blotting

10-15 third instar larval brains were collected, crushed and immediately lysed in Laemmli loading buffer (2x stock) with Phosphatase inhibitor cocktail (sigma) and Sodium Vanadate. Extracts were cleared by centrifugation and were run on 10% poly-acrylamide gel and transferred to PVDF membrane. Primary antibodies used were, Rabbit anti-p-S6K (1:1000 dilution, Cell Signaling), Rabbit p-4EBP1 (1:1000, Cell Signaling) and Mouse anti-tubulin DM1A (1:20,000, Sigma). HRP conjugated secondary antibodies were used at 1:10000 dilution (Jackson Immunoresearch). Blots were developed using Immobilon luminal reagent (Millipore) using a LAS imaging system. The p-S6K levels were normalized to tubulin and quantitation was performed using ImageJ software (US-NIH).

3.4 Results

3.4.1 Modifiers identified in screen alter VAP (P58S) induced bouton size

The genetic screen was conducted to identify interactors of wildtype VAP. In order to evaluate the interaction of these genes with disease causing VAP (P58S), we used the *Drosophila* larval NMJ as an assay system. VAP regulates bouton size at the NMJ in a dose dependent manner (Pennetta et al. 2002, Ratnaparkhi et al. 2008). Over-expression of VAP(P58S) in neurons using pan-neuronal *C155-Gal4* driver, leads to enlarged boutons at the larval NMJ (Ratnaparkhi et al. 2008). To test interaction with VAP (P58S), we decided to knockdown a small subset (~14%) of discovered interactors in the background of neuronal VAP(P58S) expression and test if the reduction in transcripts of individual interactors, identified in a VAP(wt) screen modified the enlarged bouton phenotypes. Fourteen of the 103 interactors

were knocked down using RNAi in *C155-Gal4>UAS-VAP (P58S)* animals. The choice of interactors was made so as to include suppressors (*CG18110*, *CG6048*, *NapiT*, *Prx5* & *TBPH*), enhancers (*Ada2b*, *Ars2*, *CG9172*, *Droj2*, *Karβ3*, *Nup75*, *ssh* & *tor*) and known physical interactors of VAP (*Droj2*, *Karyβ3* & *Snama*) that were identified as interactors in the genetic screen. Some of the interactors we chose also have roles in human disease.

In control *C155-Gal4* /+ animals, the average bouton size was $3.98 \pm 0.09 \mu\text{m}$ (n=17); in *C155-Gal4>VAP (P58S)* animals, boutons are large with an average size of $4.84 \pm 0.25 \mu\text{m}$ (n=16) (Fig. 3.3A and 3.3B respectively, p-value=0.0016) without any significant change in the bouton number. Knockdown of *Ada2b*, *CG18110*, *CG6048*, *CG9172*, *NaPi-T*, *Nup75*, *Ssh*, *TBPH* and *Tor* suppressed the bouton phenotype observed in *C155-Gal4>VAP (P58S)* animals (Fig. 3.3C-3.3F) while *Ars2*, *Droj2*, *Karyβ-3*, *Prx5*, *Snama* knock down failed to rescue or worsen the bouton size. We have interpreted the rescue of bouton size as a scaling back of the perturbation effect of VAP (P58S) on the pan neuron-glia-muscle network. Rescue of the bouton size did not seem to be additive since knockdown of these genes on their own (Fig. 3.3G, bottom panel) did not give rise to smaller boutons. On the contrary, the boutons were found to be marginally bigger as compared to the controls though many of them were not statistically significant except in the case of *Snama* and *Karyβ-3* wherein, bouton size increased to $4.45 \pm 0.13 \mu\text{m}$ and $4.80 \pm 0.14 \mu\text{m}$, respectively (Fig. 3.3G). None of the genes we tested, by themselves, showed any significant increase in bouton size compared to *C155-Gal4>VAP (P58S)*.

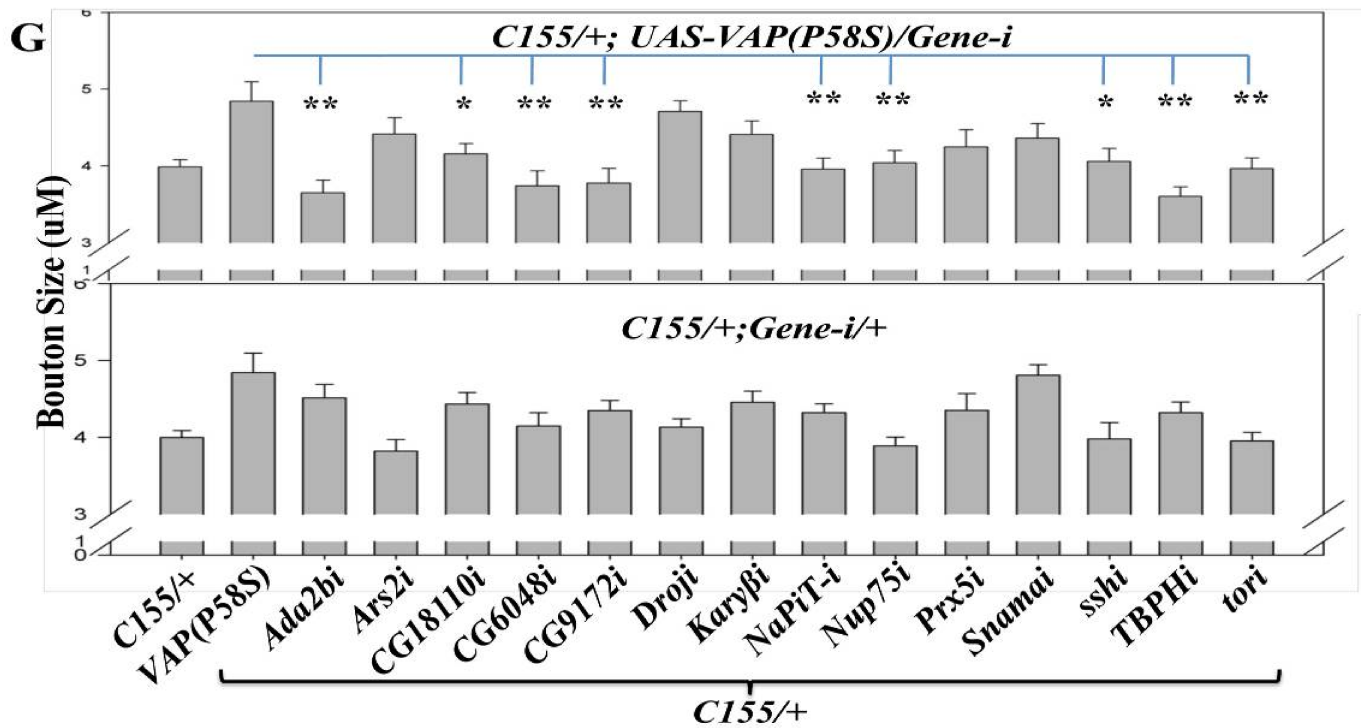
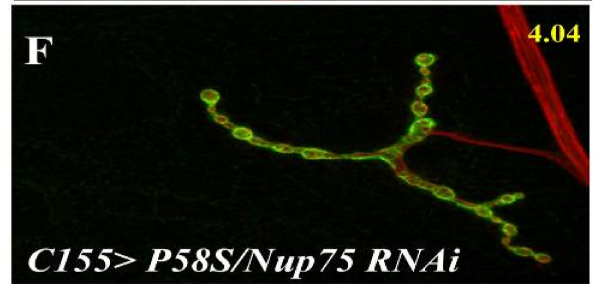
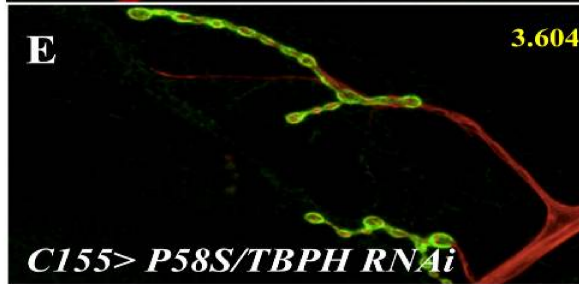
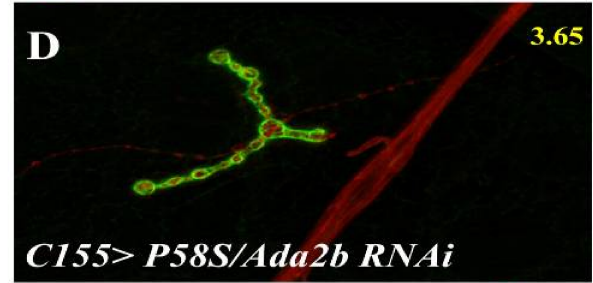
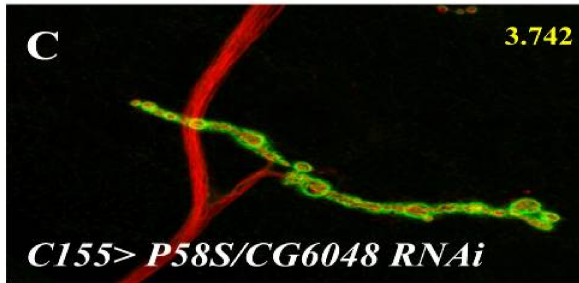
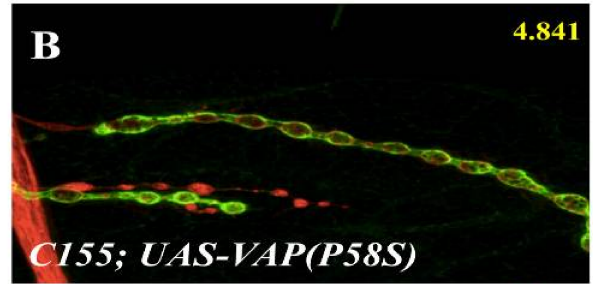
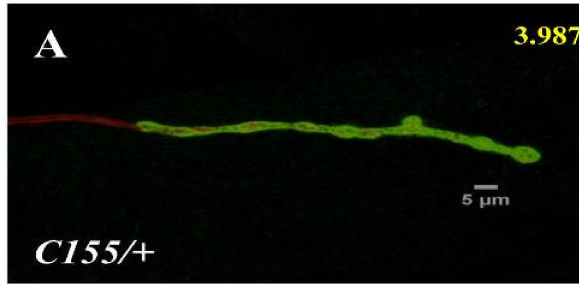


Figure 3.3: The *Drosophila* NMJ is used to screen for interaction of modifiers with VAP (P58S).

Thirteen of the 103 modifiers discovered in our macro chaetae screen were tested in the larval muscle-4 NMJ for interaction with VAP (P58S). For this and subsequent figures, approximately 15 NMJs were dissected, stained (anti-HRP, red; anti-dlg, green), imaged and measured for the average size of boutons (displayed in yellow at the top RHS of each figure).

(A) A Wild type (*C155-Gal4/+*) NMJ. The average bouton size is 3.987 (± 0.03). Shown here and below is Z-series of a synapse rendered as maximum intensity projection.

(B) Expression of *UAS-VAP (P58S)*, using the *C155-Gal4* driver increases the size of the boutons.

(C-F) Knockdown of *CG6048* (C), *CG9172* (D), *TBPH* (E) & *Nup75* (F) in a *C155/UAS-VAP (P58S)* background reverses the effect of the VAP(P58S) over expression and rescues bouton size to wild-type levels.

(G) Quantitation of bouton size (in micrometer) for the RNAi knockdown of each gene tested in a VAP (P58S) background (top panel) and in a wild type background (bottom panel).

For this and subsequent NMJ figures, error bars represent standard errors of the mean (SEM). Scale bar= 5 μ m. “*” indicates a p-value<0.01 (but > 0.001), while “***” indicates a p-value of <0.001. Adapted from (Deivasigamani et al. 2014).

3.4.2 *Tor* may be up-regulated in cells expressing VAP(P58S)

One of the enhancers we identified in the bristle-based screen was *Tor*. Knockdown of *Tor* suppressed the large bouton phenotype of VAP (P58S) (Figure 3.4A,B), to wild type levels ($4.75 \pm 0.08 \mu$ m to $3.96 \pm 0.09 \mu$ m, p-value=0.0001). Knockdown of *Tor* by itself did not change the bouton size in comparison to *C155-Gal4* ($3.98 \pm 0.03 \mu$ m vs. $3.95 \pm 0.03 \mu$ m, p-value=0.8115, Figure 3.4C). This suggests that *VAP(P58S)* interacts with *Tor* and that these neurons may have increased TOR signaling.

To confirm this result, we tested if the dominant negative form of *TOR* (*TOR-TED*) affects NMJ phenotype in the *C155>VAP (P58S)* background. We found that TOR-TED when expressed in P58S background reduced the bouton size significantly compared to P58S over expression ($4.75 \pm 0.08 \mu$ m vs. $3.09 \pm 0.07 \mu$ m, p-value=0.00001, Figure 3.4D). The decrease in bouton size was also significantly lower than the *Gal4* control ($3.98 \pm 0.09 \mu$ m vs. $3.09 \pm 0.07 \mu$ m, p-value=0.001). In contrast, expressing only TOR-TED using *C155-Gal4* alone increased bouton size marginally ($3.98 \pm 0.09 \mu$ m vs. $4.50 \pm 0.10 \mu$ m, p-value=0.0005, Figure 3.4E).

In order to further validate the genetic interaction between VAP and TOR we tested both downstream components (Figure 3.5), namely S6K, *Autophagy 1* (*Atg1*) and *Thor* (*4EBP1*) of the TOR signaling cassette. TOR activates S6K by phosphorylating it and this promotes protein translation. Co-expressing constitutively active (CA) form of S6K (*UAS-S6K^{STDETE}*) with VAP(P58S) did not rescue the bouton size (Figure 3.5A,B), though there was a marginal increase that was not statistically significant ($4.67 \pm 0.15 \mu\text{m}$ vs. $5.13 \pm 0.19 \mu\text{m}$, $p\text{-value}=0.0848$, Figure 3.5B).

Over-expressing *S6K^{STDETE}* with *C155-Gal4* did not result in any change in bouton size consistent with earlier results ($3.72 \pm 0.14 \mu\text{m}$ vs. $3.94 \pm 0.25 \mu\text{m}$, $p\text{-value}=0.4651$, Figure 3.5A and (Cheng et al. 2011)). However, when we co-expressed kinase-dead/ dominant negative form (DN) of S6K using *UAS-S6K^{KQ}* along with P58S form of VAP we observed a significant decrease in the bouton size ($4.67 \pm 0.15 \mu\text{m}$ vs. $3.55 \pm 0.12 \mu\text{m}$, $p\text{-value}=0.0001$, Figure 3.5D). By itself, reduction of S6K activity via *UAS-S6K^{KQ}* did not significantly decrease bouton size ($3.72 \pm 0.14 \mu\text{m}$ vs. $3.48 \pm 0.11 \mu\text{m}$, $p\text{-value}=0.3654$, Figure 3.5C). In comparison, *S6K* null mutants show decreased bouton size without affecting the bouton number (Cheng et al. 2011). Co-expressing constitutively active *Thor* (*Thor CA*) form with VAP(P58S) did not rescue the large bouton phenotype (Figure 3.6D,E,F). Overexpression of *Atg1* in VAP(P58S) background leads to a decrease in bouton size (Fig. 3.6B,C,F), a result negated by the control experiment (*C155>Atg1*).

The above results indicate that *Tor* may be up-regulated in VAP(P58S) mutants. Knocking-down or over-expressing *Rheb* in VAP(P58S) background did not affect the bouton size (Figure 3.7 A-D) but co-expression of *Tsc1* & *Tsc2*, rescued the bouton size back to wild type levels ($4.67 \pm 0.15 \mu\text{m}$ vs. $3.79 \pm 0.16 \mu\text{m}$, $p\text{-value}=0.00067$, Figure 3.7E, F). As expected,

knock down of *Tsc1* in VAP(P58S) background did not alter the bouton size ($4.67 \pm 0.15 \mu\text{m}$ vs. $4.29 \pm 0.19 \mu\text{m}$ p-value=0.1621, Figure 3.7G, H).

3.4.3 *Tor* may be down regulated in cells expressing VAP(wt)

Since *Tor* interacts with *VAP(P58S)*, we conducted equivalent experiments in a VAP(wt) overexpression (*C155>VAP*) background in order to compare and contrast the genetic interactions of VAP(P58S) and VAP(wt) with *Tor*.

In a VAP(wt) background, co-expressing *Tsc1,2* in did not rescue the small bouton phenotype (Figure 3.8 A,B,I). But, down-regulation of *Tsc1* rescued the phenotype dramatically (3.39 ± 0.13 vs. 4.75 ± 0.16 , p-value<0.0001, Figure 3.8 A, C, I) suggesting lowered TOR signaling when VAP(wt) is expressed in neurons. *TOR-TED*, when expressed, did not modify the small bouton size ($3.69 \pm 0.08 \mu\text{m}$ vs. $3.39 \pm 0.13 \mu\text{m}$, p-value=0.055; Figure 3.8 A,D,I). *Thor CA*, when expressed in a VAP(wt) background, rescued the small bouton phenotype (3.39 ± 0.12 vs. $4.41 \pm 0.15 \mu\text{m}$, p-value=0.0002, Figure 3.8 G,I); but *Thor CA* by itself gave large boutons (Fig. 3.4 E). *Atg1* expression over VAP(wt) led to smaller boutons, a phenotype negated by the fact that *Atg1* over expression by itself led to small boutons. *S6K-DN* in a VAP(wt) expression background did not change the small bouton phenotype but *S6K-CA* expression rescued the phenotype ($3.39 \pm 0.12 \mu\text{m}$ vs. $4.15 \pm 0.13 \mu\text{m}$, p-value=0.0003, Figure 3.8 E,I).

3.4.4 Rapamycin feeding mitigates VAP (P58S) phenotype

The above genetic data clearly suggests an up regulation of TOR signaling in neurons expressing VAP(P58S). If this is true, one would predict that treatment with Rapamycin, by inhibiting TOR signaling would reverse the large bouton morphology of VAP(P58S) expressing neurons. One would also expect an increase in phosphorylation of kinases downstream of the TOR pathway in neurons expressing VAP(P58S).

200 nM Rapamycin was fed to larvae of the appropriate genotype (*C155-Gal4/+* and *C155-Gal4/+; UAS-VAP(P58S)*). A dose dependent study for Rapamycin was carried out (See Materials & Methods) to determine the correct dosage. Control *C155-Gal4* larvae showed a moderate increase in bouton size compared with their respective control ($3.96 \pm 0.14 \mu\text{m}$ vs $4.43 \pm 0.11 \mu\text{m}$, $p\text{-value}=0.0156$, Figure 3.9A, B). VAP (P58S) expressing larvae, when fed with Rapamycin, showed a significant decrease in bouton size, compared to DMSO fed controls ($4.88 \pm 0.19 \mu\text{m}$ vs $3.99 \pm 0.17 \mu\text{m}$, $p\text{-value}=0.0021$, Figure 3.9C, D), confirming that inhibiting TOR pathway can reverse the VAP (P58S) mediated bouton phenotype. We also tried to measure the levels of p-S6K in larval brains lysates using western blotting using an antibody raised against mammalian p-S6K and 4EBP1. However, we did not observe any significant change in the levels of p-S6K with western blots, carried out using brain lysates from larvae expressing VAP(P58S), based on four different biological replicates (Fig. 3.10).

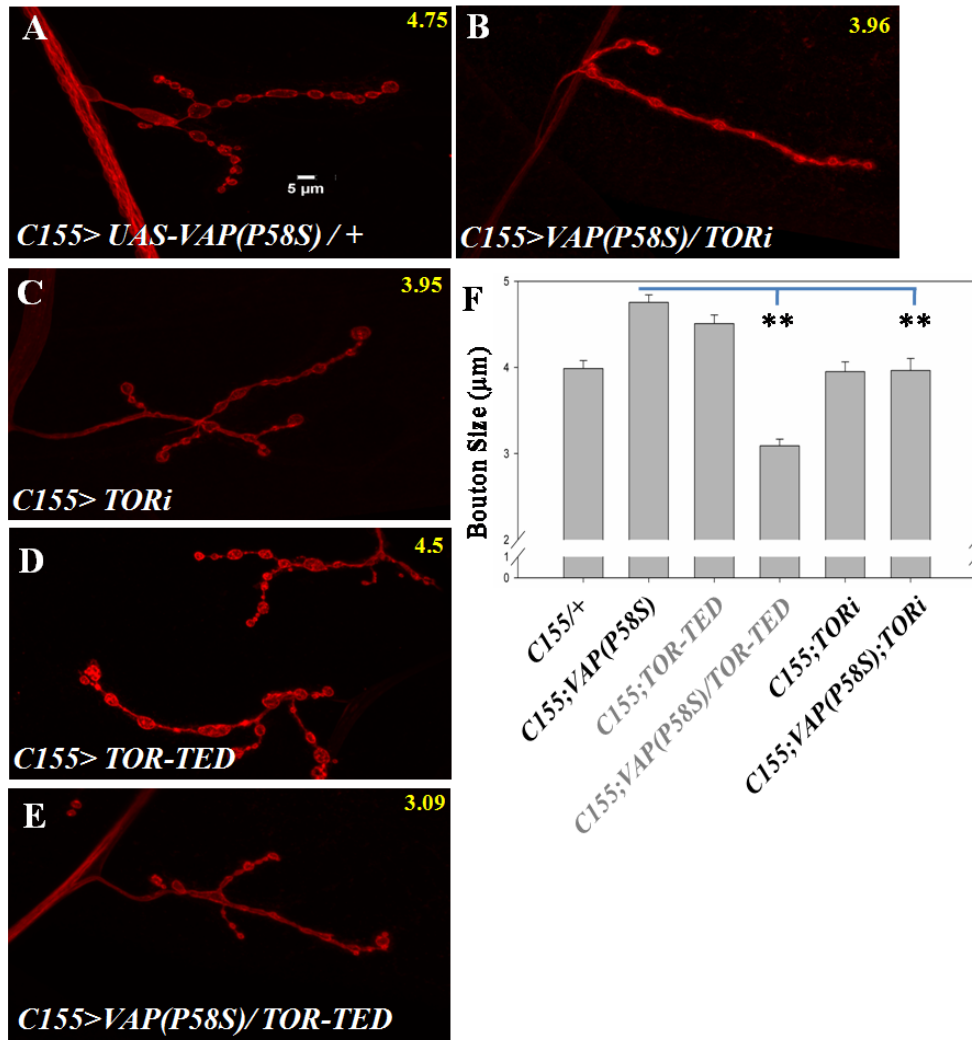


Figure 3.4: dTOR, the fly homolog of mTOR modifies the VAP (P58S) over expression phenotype at the NMJ.

(A) VAP (P58S) over expression using *C155-Gal4* leads to larger boutons at the NMJ.

(B) Knockdown of *Tor* by RNAi in VAP(P58S) background restores the bouton size to wild type levels.

(C) Knocking down *Tor* using RNAi did not have any effect on bouton size.

(D) Over expression of *TOR-TED* alone using *C155-Gal4* resulted in increased bouton size.

(E) Reduction of TOR activity by expressing a dominant negative form of TOR, *TOR-TED* reversed the increase in bouton size caused by *UAS-VAP (P58S)*.

(F) Quantitation of rescue in bouton size in VAP(P58S) background in response to reduced TOR.

Adapted from (Deivasigamani et al. 2014).

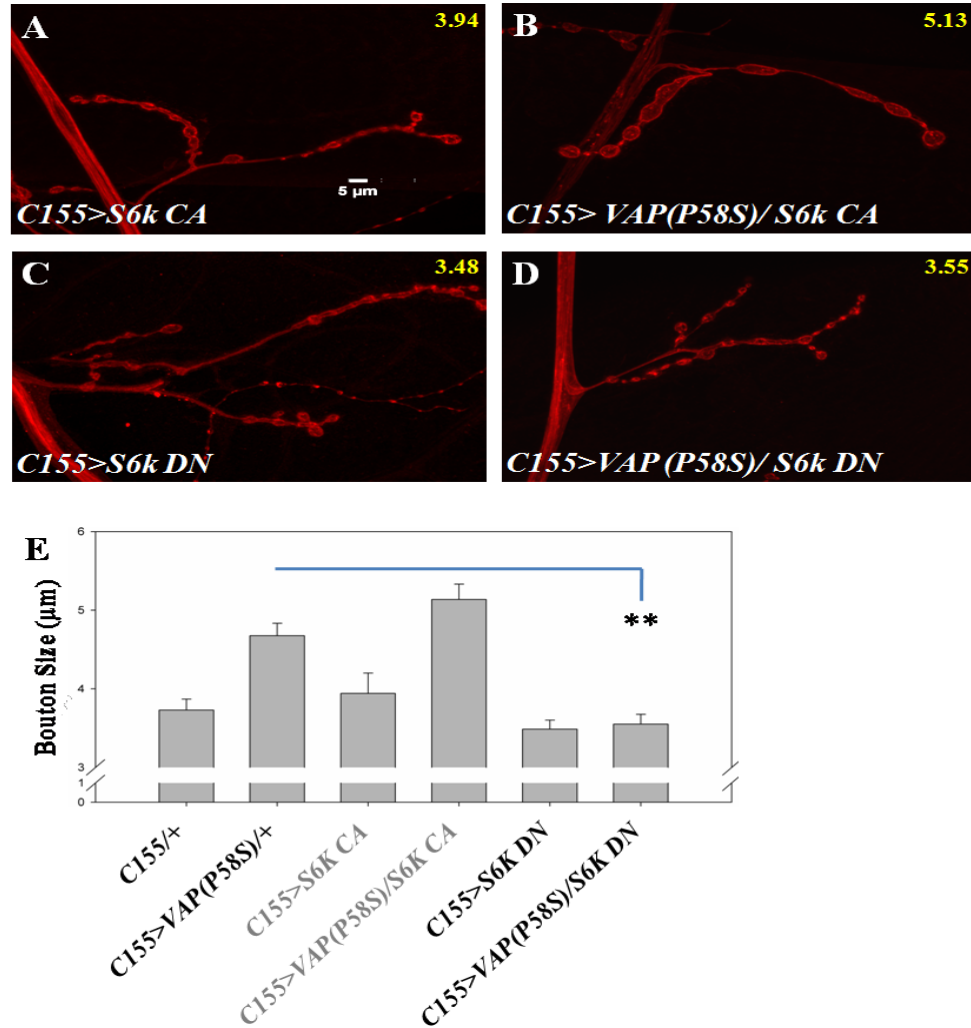


Figure 3.5: Increased TOR signaling activates its downstream component S6K.

(A) Over expression of constitutively active S6K using *C155-Gal4* in a wild type background did not affect the bouton size.

(B) Over expression of constitutively active S6K using *C155-Gal4* in a VAP (P58S) over-expression background did not reduce the bouton size.

(C) Reducing S6K activity using dominant negative form did not have significant effect on bouton size.

(D) Reducing S6K activity using dominant negative form rescued the increased bouton size in a VAP (P58S) background. Average size of boutons from about 15 NMJs is displayed in yellow at the top right of each figure.

(E) Quantitation of effect of S6K on the bouton size in presence and absence of VAP(P58S).

Adapted from (Deivasigamani et al. 2014).

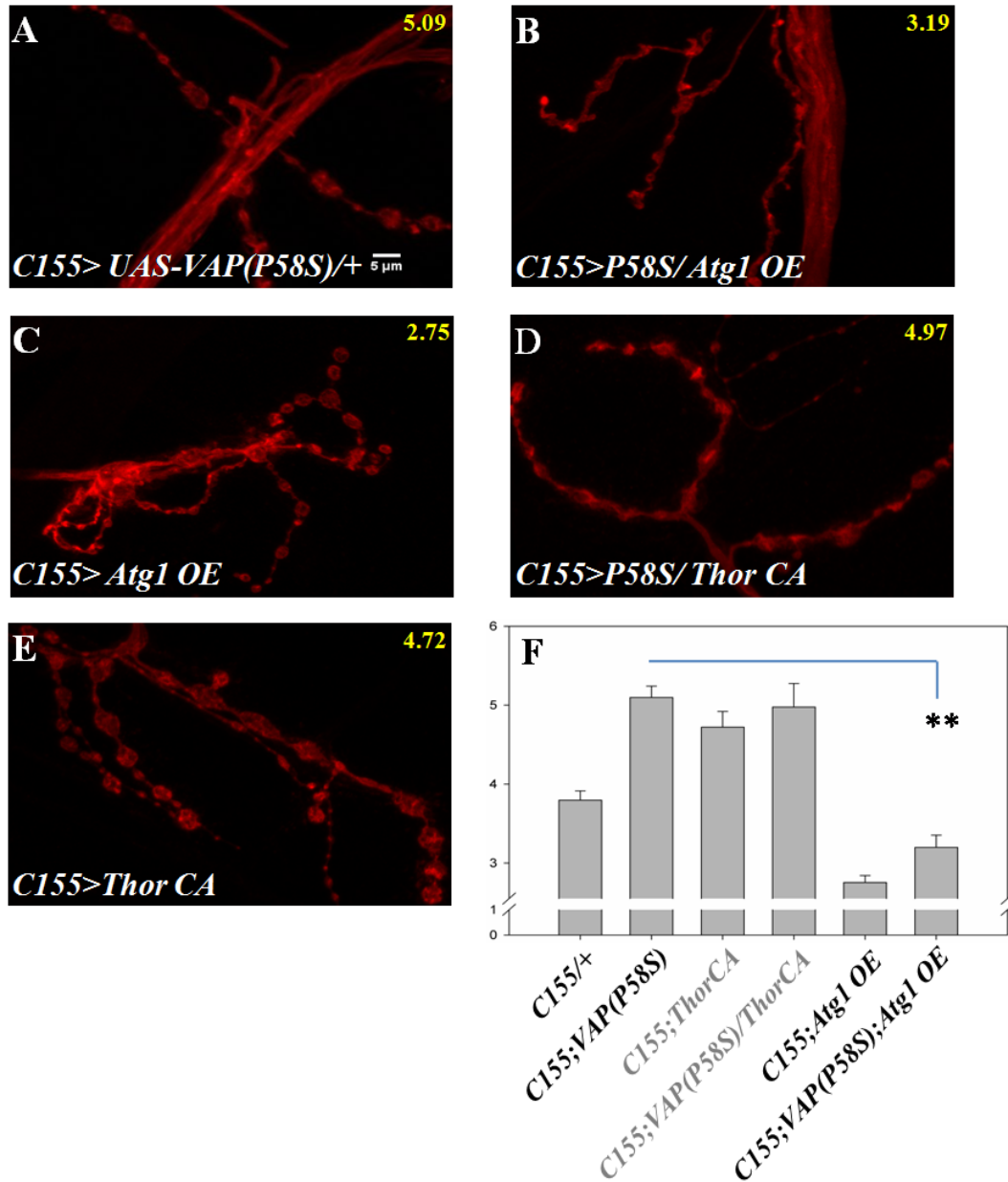


Figure 3.6: TOR downstream components ATG1 and 4EBP1 (Thor) modulate the VAP (P58S) bouton phenotype.

(A) VAP (P58S) over expression using *C155-Gal4* leads to larger boutons at the NMJ.

(B) Over-expression of *Atg1* in VAP(P58S) background reduces the bouton size, to levels lower than wild type.

(C) Over expression of *Atg1* alone using *C155-Gal4* resulted in reduced bouton size.

(D) Over-expression of a constitutively active form 4EBP1 (Thor) does not rescue the bouton size.

(E) Over-expression of constitutively active 4EBP1 leads to increased bouton size.

Average size of boutons from about 15 NMJs is displayed in yellow at the top right of each figure.

(F) Quantitation of effect of TOR downstream effectors in VAP (P58S) mediated bouton size.

Adapted from (Deivasigamani et al. 2014).

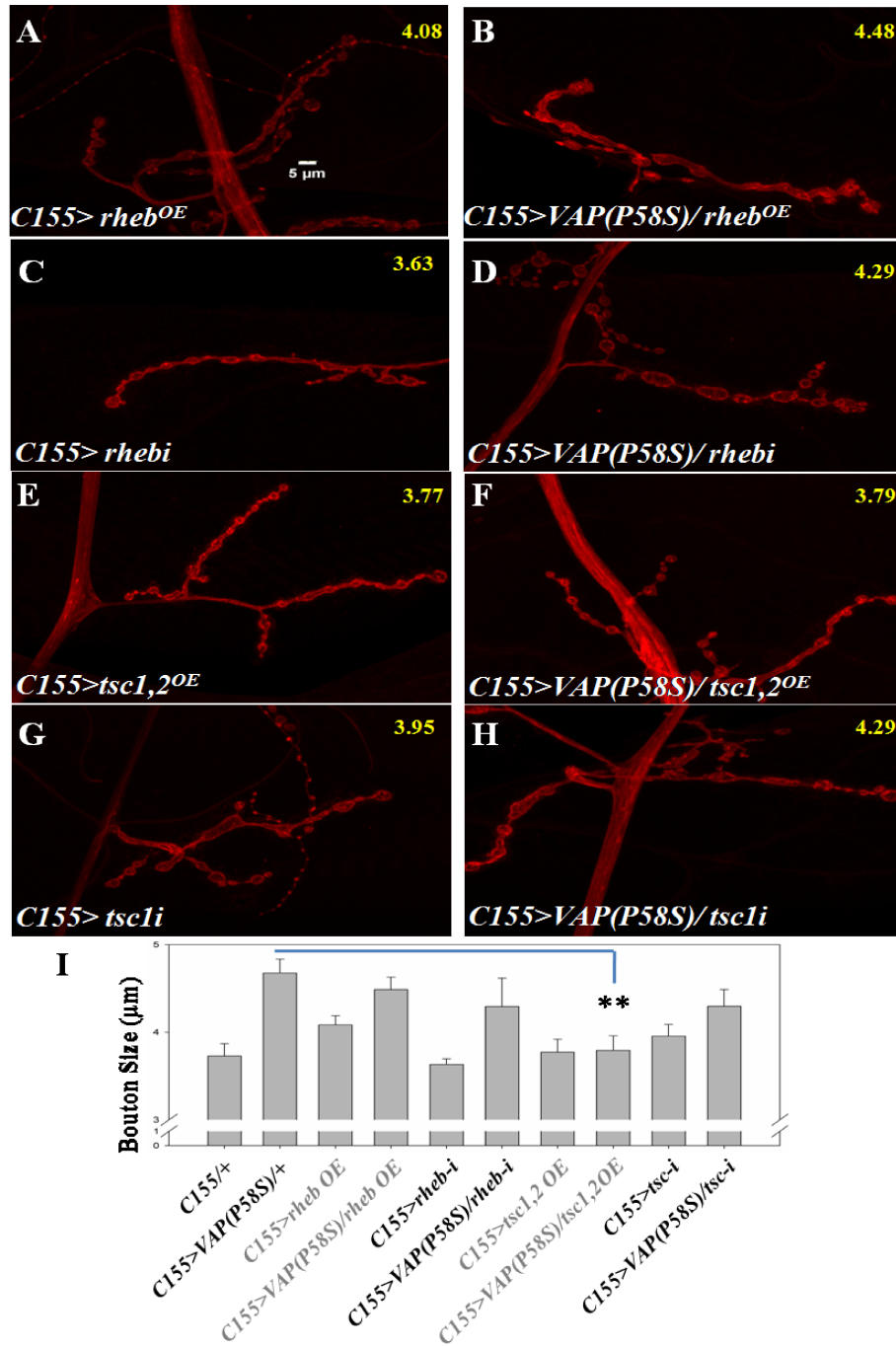


Figure 3.7: Increased TSC activity reverses the VAP(P58S) bouton phenotype.

(A-D) Over expression and knocking down of *Rheb* had no effect on bouton size in the VAP (P58S) background. (E-F) Increasing *Tsc* activity by co-expressing *Tsc1,2* rescued the bouton size in VAP (P58S) background (F) while *Tsc1,2* over expression by itself did not have any effect on bouton size (E).

(G-H) Knocking down *Tsc1* in VAP (P58S) background did not rescue the bouton size. (bar= 5 μ m). Average size of boutons from about 15 NMJs is displayed in yellow at the top right of each figure.

(I) Quantitation of effect of perturbations in *Tsc* & *Rheb* levels on bouton size. Adapted from (Deivasigamani et al. 2014).

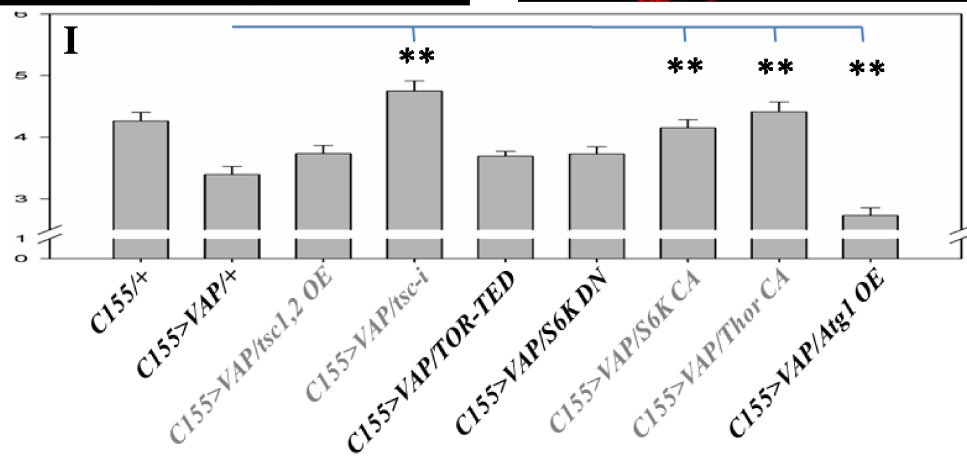
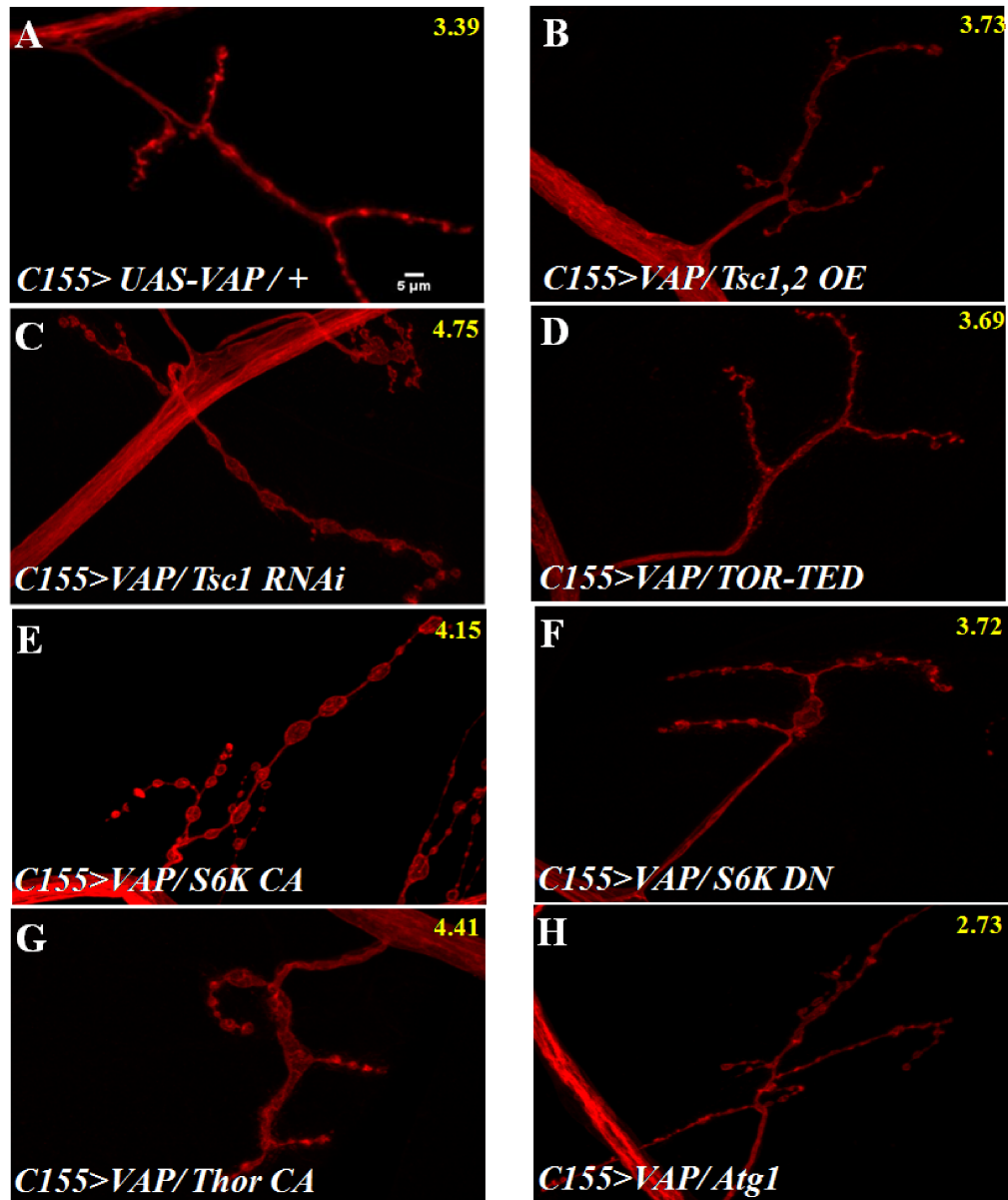


Figure 3.8: Decreased TSC activity and increased S6K activity reverses the VAP(wt) small bouton phenotype.

(A) VAP wild type over expression using *C155-Gal4* leads to reduced bouton size.

(B-D) Reducing TOR pathway activity by over-expressing *Tsc1,2* (B) or *TOR* dominant negative (D) does not affect the bouton size in the VAP wild type over-expression background. However, increasing TOR pathway activity using *Tsc1* knock down increased the bouton size to wild types levels (C).

(E-H) TOR downstream components can alter the bouton size in VAP wild type over-expression. Over-expression of a constitutively active form of S6K (E) and constitutively active Thor (G) rescues the bouton size, while the dominant negative form does not (F). Over-expression of Atg1 along VAP wild type reduced the bouton size further (H).

(I) Quantitation of effect of TOR pathway and its downstream components in VAP wild type over-expression mediated bouton size. Adapted from (Deivasigamani et al. 2014).

3.5. Discussion

3.5.1 VAP (P58S) and VAP(wt) overexpression have opposing effects on *Tor*.

Mammalian TOR (mTOR), an atypical serine-threonine kinase is an anabolic promoter activated by insulin receptor that increases protein synthesis while inhibiting autophagy. In humans, TOR signaling appears to occur via two independent complexes, TORC1 and TORC2. The TOR complexes along with FKBP12 were identified in yeast as targets of the fungal immunosuppressant drug Rapamycin (Heitman et al. 1991). mTOR accepts signals, integrates the information and regulates downstream cellular functions, such as autophagy, cytoskeleton rearrangements and protein synthesis (Johnson et al. 2013). Also, S6K and TSC complex have recently been shown to play a role in regulating *Drosophila* NMJ development (Cheng et al. 2011, Natarajan et al. 2013).

Reducing TOR activity by either RNAi interference, expressing Dominant negative (DN) constructs or by reducing activity of its downstream effector S6K, rescued the bouton size in VAP (P58S) background suggesting that the TOR pathway may be up-regulated by VAP (P58S). Overexpression of TSC complex components as well as feeding larvae with Rapamycin rescued the bouton size. The effect of Rapamycin is consistent with its historical role as a TOR signaling inhibitor.

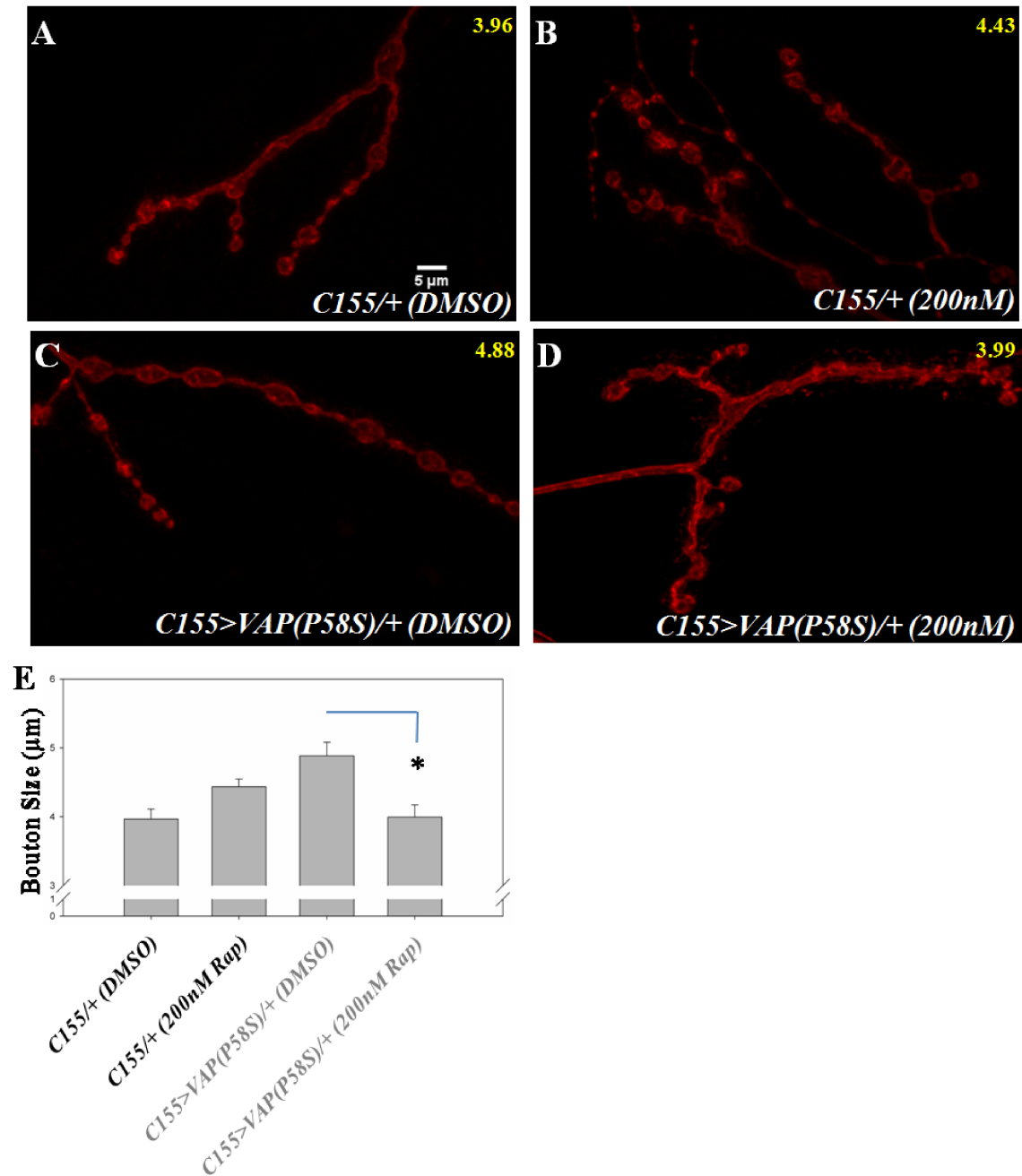


Figure 3.9: Rapamycin feeding mitigates VAP(P58S) bouton phenotype.

(A-B) *C155-Gal4* larvae fed with DMSO (A) and 200nM Rapamycin (B)

(C-D) *C155>VAP(P58S)* larvae fed with DMSO (C) and 200nM Rapamycin (D). Rapamycin, a chemical inhibitor of TOR effectively restores bouton size to control levels in VAP (P58S) animals.

(E) Quantitation of effects of Rapamycin on VAP (P58S) expressing animals. Adapted from (Deivasigamani et

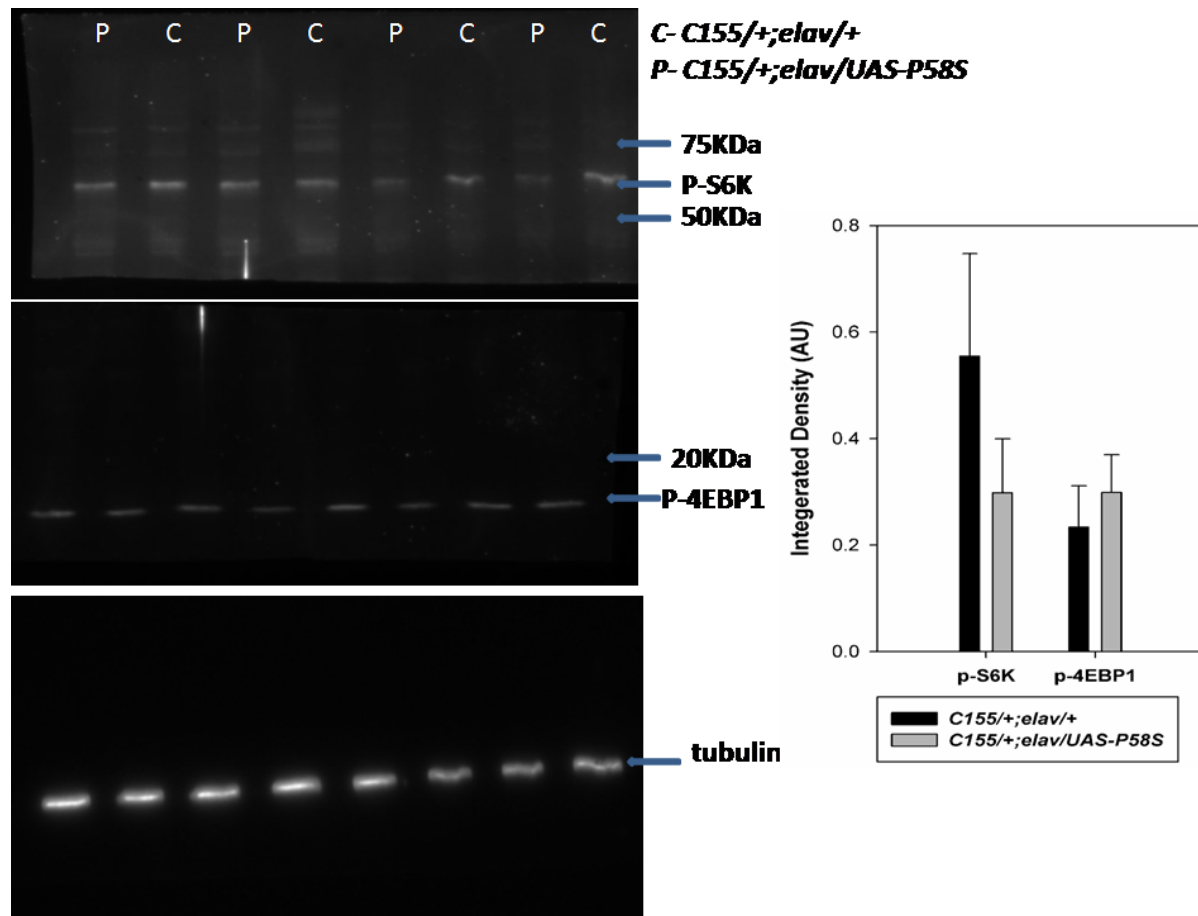


Figure 3.10: Western blots of downstream effectors of TOR signaling.

(A-D) Western blots of larval brains from *C155/+;elav/+* (lanes marked as C) and *C155/+;elav/UAS-VAP(P58S)* (lanes marked as P). Phospho-S6K (A) and Phospho-4EBP1/Thor (B) levels do not change significantly ($p > 0.01$), when normalized to tubulin (C). Quantitation of band intensity (D) is also displayed. The data includes four biological replicates (Labeled 1,2,3 & 4). Adapted from (Deivasigamani et al. 2014).

Expression of a constitutively active form of *Thor* did not modulate the effects of VAP(P58S) expression, and we could not elucidate the effects on Atg1 activity because it had a strong effect on bouton size in control experiments. In addition to biochemical experiments that related to inhibiting TOR signaling using Rapamycin, we also attempted to visualize changes in Phosphorylation for S6K and Thor by measuring levels of phosphorylated antibody within the cell in conditions of VAP(P58S) over expression (See Results). We could not detect any increase in phosphorylation and this may be because of technical limitations of our experiment - namely

that a large background of p-S6K from other cells may not allow detection of the change in phosphorylated states. The antibody used is a mammalian p-S6K antibody that cross-reacts with *Drosophila* p-S6K, and an antibody that recognizes total S6K is not available.

In a background of VAP(wt) over expression, TOR signaling appeared to be decreased, in contrast to its possible up-regulation in VAP(P58S) background. The strongest evidence for this is the rescue of the small bouton phenotype of VAP(wt) when TSC1 levels are reduced or when S6K-CA are expressed in the same neurons.

3.5.2 Rapamycin, TOR inhibition and human neurodegenerative disease

Up-regulation of TOR signaling appears to be a common feature of the progression of NDs such as Alzheimer's, Parkinson's and Huntington's, in animal models, with inhibition of signaling by rapamycin reversing cognitive and motor deficits (Ravikumar et al. 2004, Spilman et al. 2010), attenuating development of posttraumatic epilepsy, reducing aggregation (Caccamo et al. 2010), promoting autophagy (Ravikumar et al. 2004, Floto et al. 2007) and protecting against neuronal cell death (Wang et al. 2009, Malagelada et al. 2010).

TOR pathway has been shown to be involved in regulating aging and a number of diseases. Reduced TOR signaling has been shown in case of VCP mediated ALS (Ching et al. 2013). Feeding SOD1^{G93A} mutant mice with rapamycin, an inhibitor of TORC1 shortens life span (Zhang et al. 2011). Treating N2A cells expressing mutant *TDP-43* with rapamycin has been shown to alter the localization of protein (Caccamo et al. 2009). Increased TOR activity has been observed in case of Fragile X syndrome, Huntington's, PINK1 based Parkinson's disease models (Liu and Lu 2010). Increased TOR activity may lead to reduced autophagy or increased global translation in these disease models. Deregulated protein translation is believed to cause energy imbalance in the cell and lead to tissue degeneration. Supporting this idea, it has been

shown that ATF4 and CHOP, the two ER stress responsive transcription factors, up-regulate protein synthesis genes (Han et al. 2013a). The increased protein translation results in depletion of cellular ATP and induces ROS production, which eventually activates apoptotic pathways in cell. Interestingly, recent studied in VAP (P56S) transgenic mice models show increased nuclear levels of ATF4 and CHOP (Aliaga et al. 2013). In *apoe/ldlr* based atherosclerotic model and Polyglycoprotein based Marie-Charcot Tooth disease model, it has been observed that reducing ATF4 and CHOP activity mitigates the disease pathogenesis (Pennuto et al. 2008, Thorp et al. 2009). Pharmacological interventions targeting components of the TOR pathway might help in alleviating the progression of ALS.

In summary, we demonstrate that some members of the network can reverse NMJ phenotypes of VAP (P58S) expression in neurons, indicating that these modifiers are both possible targets to understand the mechanism of VAP mediated ALS and potential drug targets. *Tor*, an important regulator of cellular function interacts with both *VAP (P58S)* and *VAP(wt)*, but in an opposing manner. The TOR inhibitor rapamycin reverses the architectural effects on VAP (P58S) expression on the *Drosophila* NMJ.

CHAPTER 4

Mon1 and Rab7 regulate Glutamate receptor levels at *Drosophila* neuro-muscular junction

4.1 Summary

Glutamate is the major neurotransmitter in the mammalian central nervous system. Invertebrates like *Drosophila* and *C. elegans* use it as the neurotransmitter at neuro-muscular junction (NMJ). Glutamate receptors respond to release of presynaptic glutamate and are critical for function and plasticity of the nervous system. The post-synaptic membrane docking of glutamate receptor is dependent on activity and on the endocytic machinery where the recycling endosomes act as a source of the receptors. Deregulation of glutamate receptor levels has been observed in case of diseases like Down syndrome and Amyotrophic Lateral Sclerosis (ALS). Here, using *Drosophila* NMJ we show that two players in endocytosis, namely Mon1 and Rab7, regulate glutamate receptor levels. Mon1 is a highly conserved GEF that is essential for conversion of Rab5 positive early endosomes to Rab7 positive late endosomes. In this study, we show that *Mon1* mutants have NMJ defects, reduced levels of pre-synaptic active zones and increased levels of glutamate receptor subunit IIA at the synapse. Strikingly, these mutants also show GluRIIA accumulation in the muscle. We think that there may be a pre-synaptic mechanism, and possibly a post synaptic one as well through which *Mon1* is involved in regulation of glutamate receptor levels. Importantly, knock down of *Rab7*, which functions downstream of Mon1 in endocytosis, also leads to increased GluRIIA levels at the synapse. Mutations in *Rab7* are known to cause a sensory neurodegenerative disease called Charcot-

Marie-Tooth disease (CMT disease). Our studies indicate a role for late endosomal machinery in regulating glutamate receptor levels at the synapse and suggest exploration of these players in CMT disease.

Data displayed in this chapter has been carried out jointly with Anuradha Ratnaparkhi as a part of collaborative research project. A Research paper titled “*Drosophila* Mon1 regulates synaptic development at the larval neuromuscular junction” Ratnaparkhi et. al., 2015 has been communicated and is under review.

4.2 Introduction

4.2.1 Rab Conversion

Within the cell, vesicles transport cargo between organelles. Coat proteins help in cargo sequestration and vesicle budding, and the vesicles are propelled by motor proteins. The fusion of vesicles is mediated by SNAREs ((Soluble NSF Attachment Protein) Receptor) (Sudhof 2004). Rab GTPases, tethering factors and the SNARE machinery localize to specific organelles and cooperate to promote fusion of membranes. Rabs are small GTP/GDP-binding proteins with C-terminal prenyl anchors and function as molecular switches. They bind in their GTP-form to tethering factors to bring membranes into proximity before SNAREs present in each membrane form four-helix bundles and, thus, drive bilayer mixing. There are 11 Rab GTPases in yeast, 29 in *C. elegans*, 30 in *Drosophila* and 60 in humans. Removal of Rabs from vesicles is mediated by cytoplasmic guanine nucleotide dissociation inhibitory (GDI) proteins which bind to prenylated moiety in Rab. GDFs (GDI displacement factors) help in the redelivery of Rabs to vesicles from the GDI-Rab complex (Stenmark 2009, Guichard et al. 2014). Rabs are active in their GTP bound form and inactive in their GDP bound form. Group of proteins called GEFs (guanine nucleotide exchange factors) and GAPs (GTPase activating proteins) are involved in

this regulation. GEFs are involved in GDP dissociation, thus, activating Rabs while GAPs promote GTP hydrolysis, leading to inactivation. Early endosome serves as a choice point where the cargo is directed for degradation or recycling. During transition from early to late endosome, the vesicle loses Rab5 GTPase and gains Rab7. The fast recycling endosomes are marked by presence of Rab4 and slow recycling endosomes are marked by the presence of Rab11. Mutations in Rab GTPases and associated proteins lead to number of diseases like albinism (Rab27A), choroideremia (Rep1), Martsolf syndrome (Rab3) and neurodegeneration (Charcot-Marie-Tooth disease, Rab7A) (Stenmark 2009).

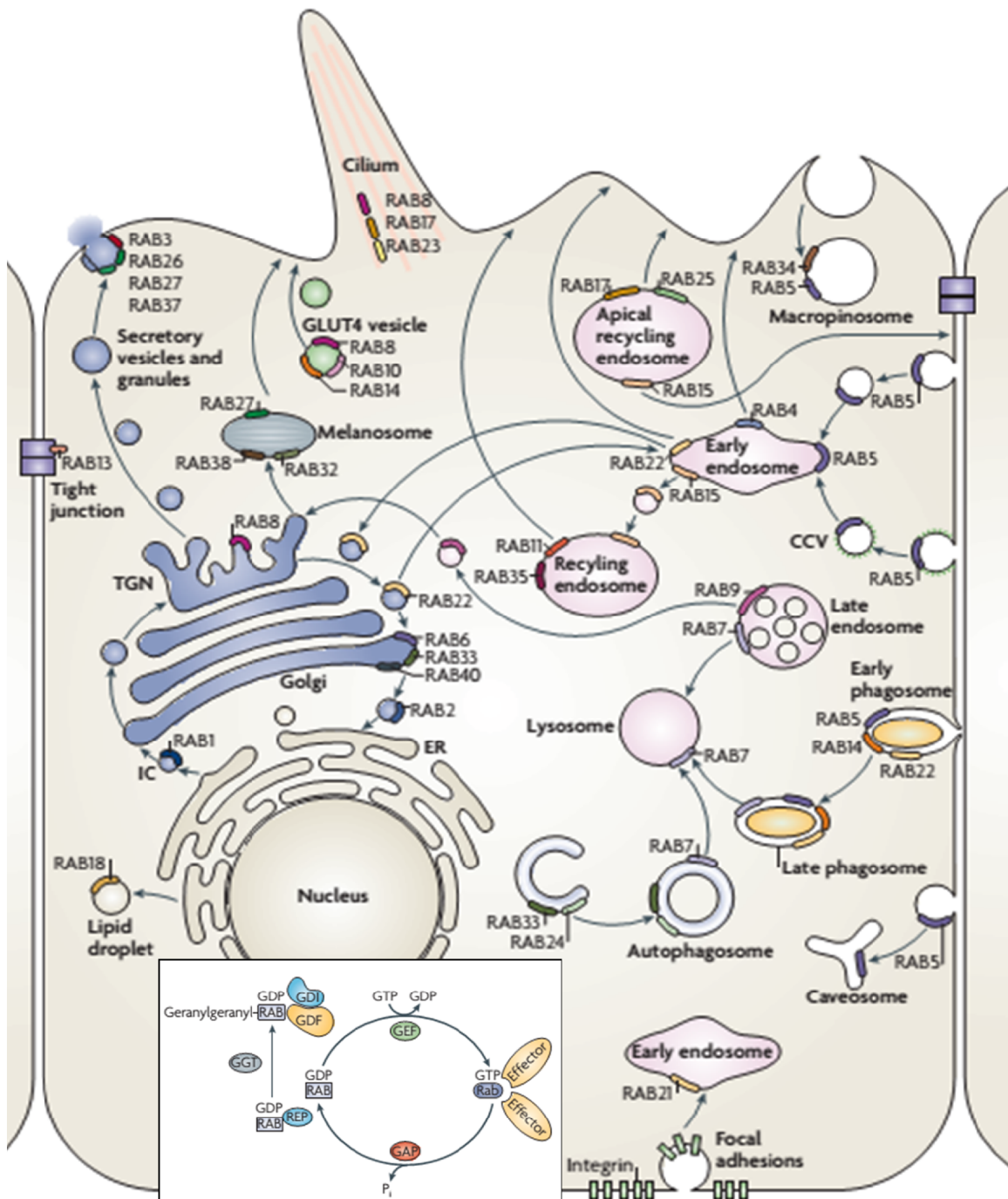


Figure 4.1: Rab switch, localization and function of Rab GTPases.

An epithelial cell showing various vesicular pathways and localization of several Rabs to different organelles. The inset shows the general Rab switching and circuitry involving GEFs, GAPs, GDIs and GDFs. Adapted from (Stenmark 2009)

4.2.2 Mon1

Mon1 (Monensin sensitive 1) is a highly conserved gene with a longin domain (Figure 4.2) and has been shown to serve as a GEF for Rab7 (Ypt7 in yeast). Longin domains have been found in several GEFs like TRAPP, DENN and in case of Mon1-Ccz1 and BLOC-3. In TRAPP, they serve as a platform for interaction with a GTPase. Yeast, *Drosophila* and *C. elegans* have one copy of Mon1 while humans have two related genes. Role of Mon1 in membrane trafficking was identified in a screen for mutants defective in the vacuolar import of aminopeptidase I via the cytoplasm to vacuole targeting pathway (Wang et al. 2002). The same study showed that *mon1* and *ccz1* mutants have defects in autophagy, pexophagy and degradative pathways.

Work so far has established an important role for SAND-1/Mon1 during Rab conversion, although its precise function and regulation is not clear. It is proposed that Rab5GTP recruits Mon1, which in turn recruits Rab7 to the early endosomes. Mon1 binds to Rab7 only in complex with its binding partner Ccz1. Studies in *C. elegans* show that Mon1-Ccz complexes are necessary for removal of Rabex5 from the early endosomes. Rabex5 helps in maintaining Rab5 in the active form. The same study also showed that Mon1 is recruited to early endosomes along with Rab7. Mon1 has been shown to interact with HOPS and helps in Vps39 mediated Rab7 activation (Poteryaev et al. 2010). Also, in yeast, Mon1 requires PIP3 for interaction with the vacuoles and its release from the vacuoles is regulated by its phosphorylation by type 1 casein kinase Yck3 (Lawrence et al. 2014). In *Drosophila*, loss of function of Dmon1 results in an enlargement of maturing endosomes and loss of their association with Rab7. The enlarged endosomes contain Notch and other trans-membrane proteins as cargo (Yousefian et al. 2013).

Mon1

Unconserved 0 1 2 3 4 5 6 7 8 9 10 Conserved

	 10	20	30	40	50
yeast	-----	-----	-----	-----	MNLN
Human	MHPGGGSPRA	ERLELGLGRE	RPAKAIFLHR	RPGEGGGRER	CLRCGHVCVR
Fruit_fly	-----	-----	-----	-----	-----
Consistency	0000000000	0000000000	0000000000	0000000000	0000001021
	 60	70	80	90	100
yeast	ESYLD-AEIP	KGQLKHSKSG	NFEGIPIVAT	TS-----EPT	TSVNLDETFF
Human	RGPGPREAVP	SGRPRPDTLT	PPWVRQRAVT	GTFCASWTPL	RNRRRAQRMAT
Fruit_fly	-----	-----	-----	-----	MEV
Consistency	1100001123	1310201100	0000010113	0100000131	1101111523
	 110	120	130	140	150
yeast	KKAPIAMPIC	DDHSVSKSTS	VN---SLNT	TYLASRRSPL	QTKKLQAKNN
Human	DMQRKRSSEC	LDGTLTSPDG	QSMERAESPT	PGMAQGMEPG	AGQEGANFVH
Fruit_fly	EQTSSVRSDTN	STCEYLDABG	DP-----ES	PNLYQEADPD	QEAEQQNHISI
Consistency	5432455323	2514433746	2300001037	52846235*1	5247152232
	 160	170	180	190	200
yeast	LLSADLAKSN	DDTTRALNSP	KKDFGPLYDS	ENDIRSRLAE	SIYSMETTSIR
Human	ARSYEDLTES	EDGAASGDSH	KEGTRGPPPL	PTDMRQISQD	FSELSTQLTG
Fruit_fly	ISELRDGLGT	MRDNSALSPE	PGQENKGLAA	SVESLALSTS	TSAKTEDSIG
Consistency	5263432335	3423473552	5332321424	3373443535	3523453554
	 210	220	230	240	250
yeast	GSELQRRPYV	-----	-----SNEIP	NVFKFSKFNS	NCKLNESTQTF
Human	VARDLQEEML	PGSSEDWLEP	PGAVGRPATE	PPREGTTEGD	EEDATEAWRL
Fruit_fly	-----	-----	---GGLSEY	DYQHDLSWQG	-----
Consistency	0210011112	0000000000	0000010111	0003434432	5142464441
	 260	270	280	290	300
yeast	CDKNFFIFTS	AGKPIYCMHG	KDEQIMSYTG	LVNTVISYFQ	VNGPSELKTI
Human	HQKHVFLVSE	AGKPVYSRYG	SEEALSSTMG	VMVALVSFLE	A-DKNAIRSI
Fruit_fly	QKKHIFILSE	AGKPIFSLHG	NEDKLATLFG	VIQALVSFVQ	M-GQDAITSI
Consistency	14*65*9676	***97546*	477484723*	762679*747	405355847*
	 310	320	330	340	350
yeast	STLTSGKRLT	FLDKSPILLM	AQSERGESEN	ELNLQIDFLY	SYILSSSESER
Human	HAD--GYKVV	FVRRSPVLV	AVARTRQSAQ	ELAQELLYIY	YQILSLTLTG
Fruit_fly	HAGG--IKFA	FMQRSSILV	AASRSNMSVQ	QQLQLGQDVY	NQILSLITYS
Consistency	5610032744	*637*587*7	*376434*46	7*327*127*	35***4*714
	 360	370	380	390	400
yeast	QLLRLFSKRE	NFDLRNYLET	TDFENLDEIC	SLI-----CN	RMEPDLLLNS
Human	QLSHIFQOKQ	NYDLRRLLSG	SER-ITDNLL	QLM-----AR	D--PSFLMGA
Fruit_fly	HMTKIFERRK	NFDLRRLLSG	SER-LFYNNL	ANDSSSAKVS	NNIFTFLTNS
Consistency	58348*5575	*7***65*64	7740333685	4420000034	301346*467

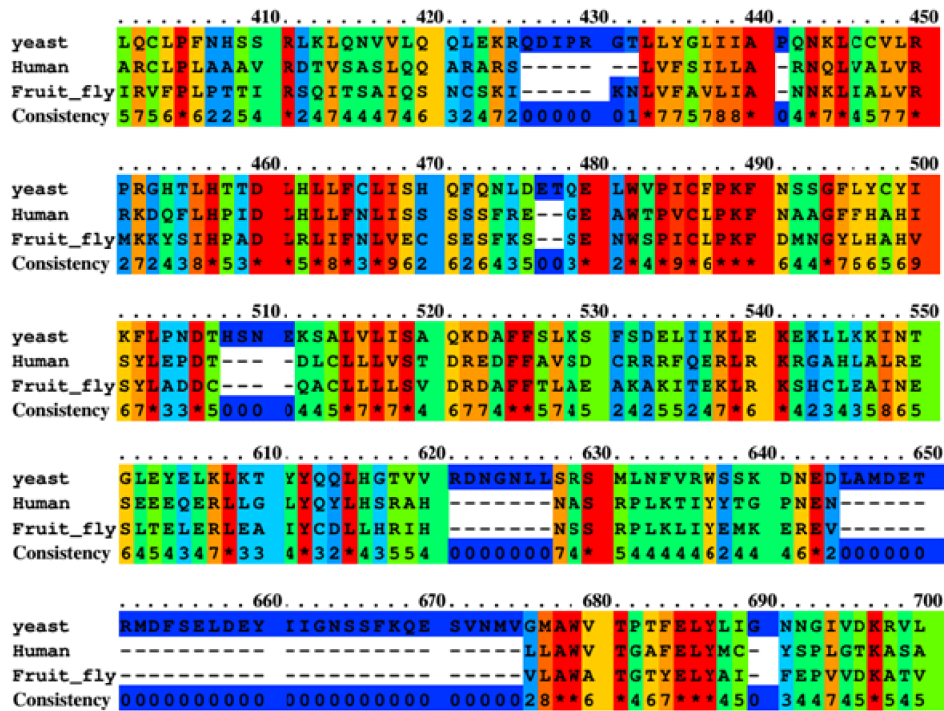


Figure 4.2: Sequence alignment of Mon1 from yeast, human and fruit fly.

Mon1 is a highly conserved protein with a longin domain, a typical signature of the GEF family of proteins. In *Drosophila* the longin domain extends from 255-370 amino acids (amino acid numbers as in alignment).

4.2.3 *Drosophila* Glutamate receptors

Neurotransmitter secreted by the pre-synaptic neuron binds to post-synaptic ionotropic receptor and results in depolarization of the membrane. The prominent neurotransmitter in the vertebrate central nervous system is glutamate, while in *Drosophila* it is acetylcholine. The major neurotransmitter of *Drosophila* neuromuscular junction is glutamate while in vertebrates it is acetylcholine. Glutamate receptors can be either metabotropic or ionotropic. Ionotropic glutamate receptors can be classified into AMPA, NMDA and Kainate sensitive. The number of ionotropic glutamate receptors subunit genes in flies are about fifteen (Rytz et al. 2013). Of these Dnr1 & 2 are NMDA receptors, GluR1 is AMPA subtype (Xia et al. 2005). Five of the subunits are expressed in the muscles; GluRIIA, GluRIIB, GluRIII/GluRIIC, GluRIID and GluRIIE. The functional receptor is a tetramer and has one subunit each of GluRIIC, GluRIID and GluRIIE and

one subunit of either GluRIIA or GluRIIB. Of these GluRIIA and GluRIIB can substitute each other. This is supported by the fact that null alleles of GluRIIC, GluRIID and GluRIIE are lethal while GluRIIA or GluRIIB are viable. These subunit sequences vary at the N-terminal region which is involved in ligand binding and receptor assembly (Marrus et al. 2004, Schmid et al. 2006). It is not clear how each of these subunits contribute to receptor assembly; however, the GluRIIC with PDZ (PSD-95/DLG/zona occludens-1)-binding domain is believed to have an anchoring role. *Drosophila* glutamate receptors are activated by millimolar concentrations of glutamate unlike the mammalian counterparts which are activated by micromolar concentrations (Chen et al. 2009).

In *Drosophila*, spontaneous activity has been shown to be necessary for the assembly of glutamate receptors at the sub-synaptic reticulum (Saitoe et al. 1997, Saitoe et al. 2001, Schmid et al. 2008). The levels of the glutamate receptors are maintained by activity (Ljaschenko et al. 2013). Live imaging of glutamate receptors has provided insights into their assembly. Immature Post synaptic densities (PSDs) typically have more of GluRIIA and less amount of GluRIIB. During subsequent PSD maturation, the GluRIIA/GluRIIB composition changes and becomes more balanced. Reducing presynaptic glutamate release increases GluRIIA, but decreases GluRIIB incorporation (Schmid et al. 2008). Treating the NMJ preparations with high levels of glutamate for prolonged time reduces the levels of receptor at the synapse, however the levels of protein in the cell does not change (Chen et al. 2009).

The mechanism involved in the sorting and assembly of these receptors is not clear. In mammals, internalization of AMPA receptors is mediated via Rab5 (van der Sluijs and Hoogenraad 2011) and recycling endosomes are the source of AMPA receptor during long term potentiation and sorting nexin 27 (SNX27) is involved in the recycling of GluR1 containing

AMPA receptors (Loo et al. 2014). Interestingly, reduced levels of SNX27 and its transcription factor CCAAT/enhancer binding protein β (C/EBP β) has been observed in Down syndrome patient brains (Wang et al. 2013). Recently, it has been shown that scribble1/Arf6 and scribble1/AP2 complex is involved in NMDAR trafficking. Scribble activity is regulated in an activity dependent manner and prevents GluN2A subunits from undergoing lysosomal trafficking, and thereby increasing its recycling (Piguel et al. 2014). Also, studies from *C. elegans* have shown that the GluA1 containing AMPA receptors are ubiquitinated by Nedd4 and are targeted to lysosomal degradation in a clathrin dependent manner (Burbea et al. 2002, Schwarz et al. 2010). Interestingly, the level of ubiquitination increases as the neurons mature and is also activity dependent (Schwarz et al. 2010). Here, we show that *Drosophila* Mon1 and Rab7 that are involved in conversion of early endosomes to late endosomes regulate glutamate receptor levels at NMJ.

4.3 Methods

4.3.1 Stocks

All stocks and crosses were reared on regular corn flour medium. The following fly stocks were used: *Df(2L)9062*, *Df(2L)6010* and *Rab7 RNAi (BL27051)* were from the Bloomington Stock Centre, USA. *Dmon1* RNAi line (CG11926 GD40338) was obtained from the VDRC stock centre, Vienna, Austria; and *Sp/Cyo,wglacZ; UAS-mon1::HA* was a kind gift from T. Klein, University of Dusseldorf. Except where stated, all were carried out at 25 degrees. *Mhc-GAL4*, *Mef-GAL4*, *C155-GAL4* and *OK6-GAL4* have been described earlier (Lin et al. 1994, Schuster et al. 1996, Ranganayakulu et al. 1998, Aberle et al. 2002). *W1118* was used as wild type control

4.3.2 Immunohistochemistry and Image Analysis

Wandering third instar larvae were dissected in PBS and fixed with Bouins fixative (15minutes). The following antibodies were used: mAbnc82 (1:100, DSHB, Iowa); anti-GluRIIA/mAb8B4D2 (1:200, ascites DSHB, Iowa); anti-HRP (1:1000, Sigma-Aldrich, USA.). Confocal imaging was carried out on a Zeiss 710 imaging system. All confocal images were taken using a 63X objective (N.A = 1.4).

Image analysis and fluorescence intensity measurements were carried out using Image J software (NIH, Bethesda). Quantitation of GluRIIA levels was carried out using the method described previously in *Menon et al., 2009*. The average gray value per synapse was determined by measuring the intensity of 3 individual boutons. In each case, the measured values were normalized to the background intensity for the same area. Since the mutants show significant GluRIIA staining in muscles, a region of the nucleus or regions free of GluRIIA staining was chosen for background normalization.

Analysis of the size and intensity of Brp punctae was carried as described in (Dickman and Davis 2009). Briefly, 3 times the background was subtracted from the image and the resulting image duplicated. One duplicate image was converted to binary and segmented using watershed algorithm. Puncta size was measured from the segmented binary image using ‘Analyze particles’ tool in ImageJ and intensity using Redirect option referring to the non-binary image in the ‘Set Measurements’ option. Control and experimental larvae used for quantitation were processed simultaneously for immunostaining. A master-mix of the antibodies at appropriate dilutions was made prior to addition to individual tubes. Imaging was carried out under identical confocal settings.

4.3.3 Real Time PCR

Third instar larvae were dissected in PBS and RNA was isolated from body wall muscles of the larvae using Trizol reagent (Invitrogen) according to manufacturer's instructions. Equal amount of RNA from *W1118* (control) and $\Delta 181$ larvae was used for cDNA synthesis (Promega). Real time PCR was performed for GluRIIA with SYBR green mix (Kappa Biosystem) on an Eppendorf RealPlex2. Rp49 was used as internal control. The fold change was calculated using $2^{-\Delta(\Delta CT)}$.

The following primer pairs (IDT oligos) were used for real time PCR,

>RP49_Foward	GACGCTTCAAGGGACAGTATC
>RP49_Reverse	AAACGCGGTTCTGCATGAG
>GluRIIA_Foward	CGCACCTTCACTCTGATCTATG
>GluRIIA_Reverse	CTGTCTCCTTCCACAATATCCG

4.4Results

4.4.1 Rab7 activity regulates GluRIIA levels at the synapse.

Studies in *C. elegans* have shown that Rabs like Rab5, Rab6.2 and Rab10 are involved in regulating glutamate receptor levels (Glodowski et al. 2007, Zhang et al. 2012). Mutation in *Drosophila* Rab7 GTPase activating protein (GAP) *tbc1D15-17* leads to an increase in synaptic GluRIIA levels (Lee et al. 2013c). We show that a gene involved in endosomal pathway (*Mon1*) might be involved in regulating glutamate receptor levels (described below). In order to see if other components of the endosomal machinery might be involved in regulating glutamate receptor levels, we knocked down Rab7 in larval muscles by driving expression of dsRab7 RNA using *C57-GAL4* that expresses in muscles. Compared to control animals,(Fig.4.3A-C), the synapse of *C57-GAL4>UAS-Rab7RNAi* animals showed a

strong increase in the intensity of GluRIIA puncta at the NMJ along with greater number of GluRIIA positive puncta in muscles (Fig.4.3 C-D). A similar phenotype was observed in animals where the knockdown was carried out using Mhc-GAL4. In both cases, a near three-fold increase in synaptic GluRIIA levels was observed (Fig. 4.3G).

4.4.2 *Dmon1*^{Δ181} mutants show reduced levels of Brp

Mon1 mutants (henceforth *Dmon1*^{Δ181}) generated in the Ratnaparkhi lab at ARI, were found to exhibit locomotor defects. The mutants were therefore examined for expression of synaptic proteins involved in neurotransmission. Mutations in trafficking genes are known to affect synaptic growth and neurotransmission (Littleton and Bellen 1995, Sanyal and Ramaswami 2002, Sweeney and Davis 2002, Dermaut et al. 2005). Bruchpilot (Brp; mAb nc82), is one of the core proteins involved in the assembly of active zones at the pre-synapse (Wagh et al. 2006). Wild type synapses showed strong punctate expression of Brp (Fig. 4.4A-C, inset in C). In homozygous *Dmon1*^{Δ181} mutants a decrease in size (17.6%) and intensity (23%) of these puncta was observed (Fig. 4.4D-F, G, and H).

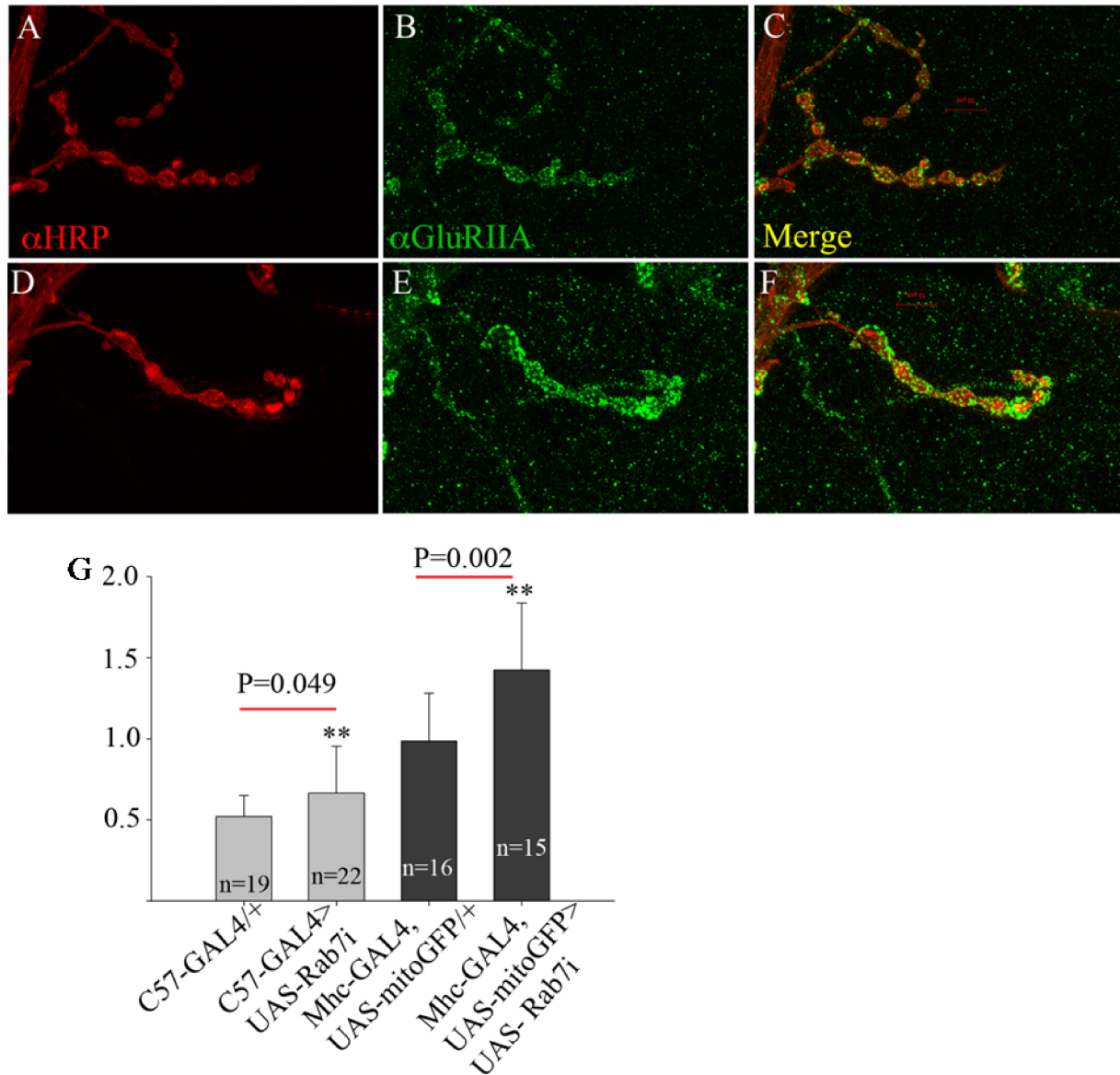


Figure 4.3: Downregulation of Rab7 expression in muscles using RNAi leads to an accumulation of GluRIIA at the post-synaptic site and in muscles.

(A-C) *C57-GAL4/+* animals stained with anti-HRP (red) and GluRIIA (green).

(D-F) Downregulation of Rab7 using RNAi leads to an increase in GluRIIA staining at the post synaptic site and muscles.

(G) Quantitation of GluRIIA levels in Rab7 knockdown animals. Adapted from (Ratnaparkhi et al.,)

4.4.3 *DmonI*^{Δ181} mutants have increased levels of GluRIIA

The larval NMJ synapses in *Drosophila* are glutamatergic. Two classes of glutamate receptor clusters are present at the synapse: those containing GluRIIA and others with GluRIIB (Marrus and DiAntonio 2004, Marrus et al. 2004). We carried out immunostaining to determine whether GluRIIA levels are altered in *DmonI*^{Δ181} mutants. In wildtype synapses, GluRIIA expression is observed as puncta on the post-synaptic side (Fig. 4.5A-C, inset in C). *DmonI*^{Δ181} mutants showed an increase in the intensity of GluRIIA staining (Fig. 4.5D-F, inset in F). Interestingly, GluRIIA positive puncta were also seen in the muscle (Fig. 4.5E). We measured the increase in intensity by calculating the average gray value of GluRIIA per synapse normalized to HRP (Menon et al., 2004). In homozygous *DmonI*^{Δ181} and *DmonI*^{Δ181}/*Df(2L)6010* mutants, the increase in intensity was, approximately 76% (0.83±0.13 (mutant) versus 0.47±0.12 (wildtype)) and 56% (0.83±0.22 (mutant) versus 0.53±0.14 (wildtype)) respectively, while *DmonI*^{Δ181}/*Df(2L) 9062* animals showed a 98% increase in GluRIIA intensity (1.05±0.34 (mutant) versus 0.53±0.14 (wildtype)) (Fig. 4.5G). On the other hand, the levels of GluRIIB were found to be reduced in *DmonI*^{Δ181} mutants, consistent with the idea that the levels of GluRIIA and GluRIIB are inversely proportional (From (Ratnaparkhi et al.)).

4.4.4 Increased GluRIIA levels in *DmonI*^{Δ181} mutants is not because of transcriptional up-regulation

The increase in the levels of GluRIIA could be because of transcriptional up-regulation, translational regulation or trafficking defects (Karr et al. 2009, Menon et al. 2009, Lee et al. 2013a). To check if the increase in GluRIIA levels is due to transcriptional up-regulation of the gene, we carried out

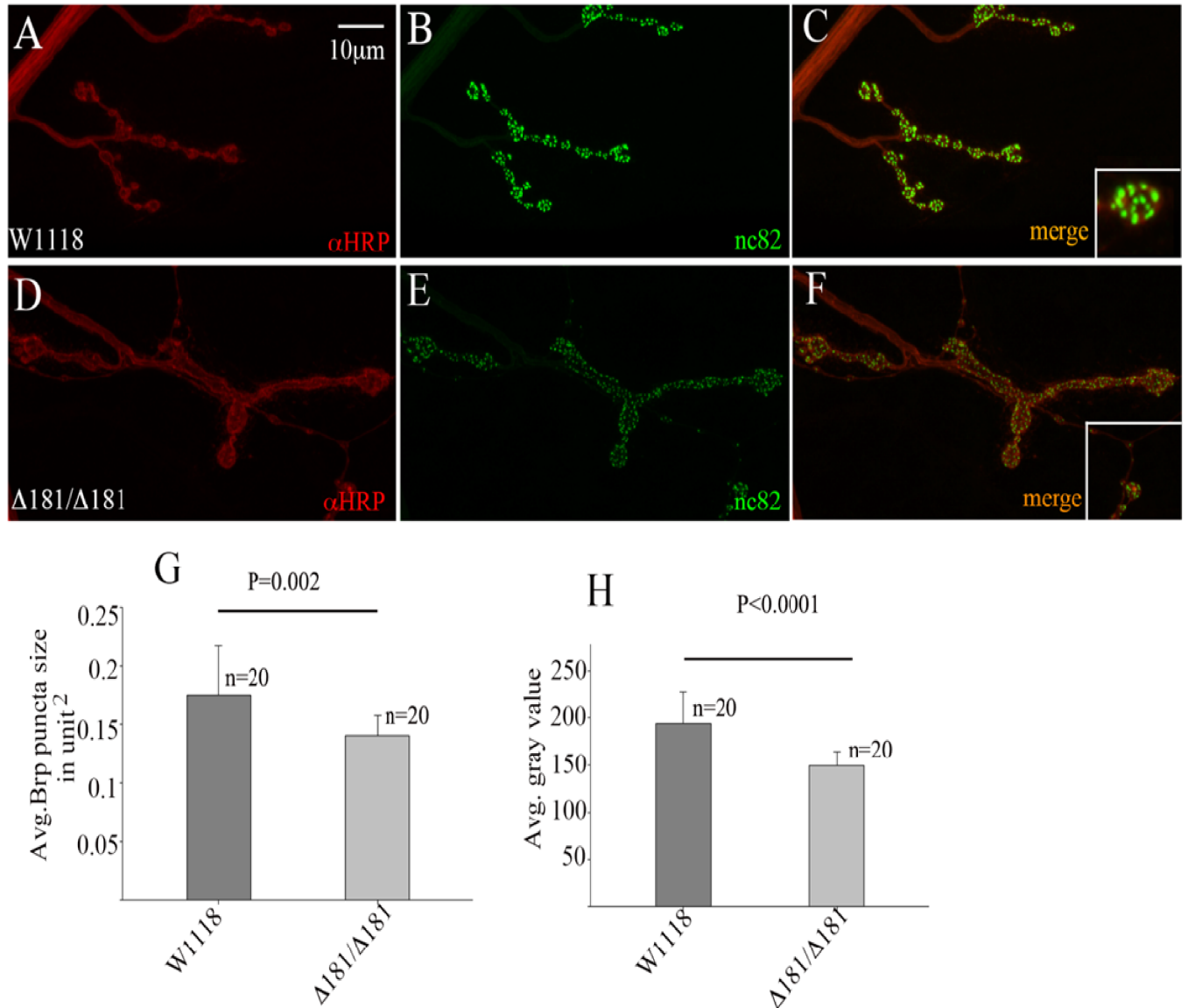


Figure 4.4: Expression level of Bruchpilot is altered in *Dmon1*Δ181 mutants.

(A-F) Neuromuscular junction at muscle 4 stained with anti-HRP (red) and mAbnc82 (anti-bruchpilot, green).

(A-C) A strong and robust staining of Bruchpilot (green) is seen in *w1118* (wildtype) animals (inset in C).

(D-F) In homozygous *Dmon1*Δ181 mutants, the intensity of bruchpilot staining is low (inset in F)

(G&H) Quantitation of the brp puncta size and intensity in wildtype and *Dmon1*Δ181.

Adapted from (Ratnaparkhi et al.)

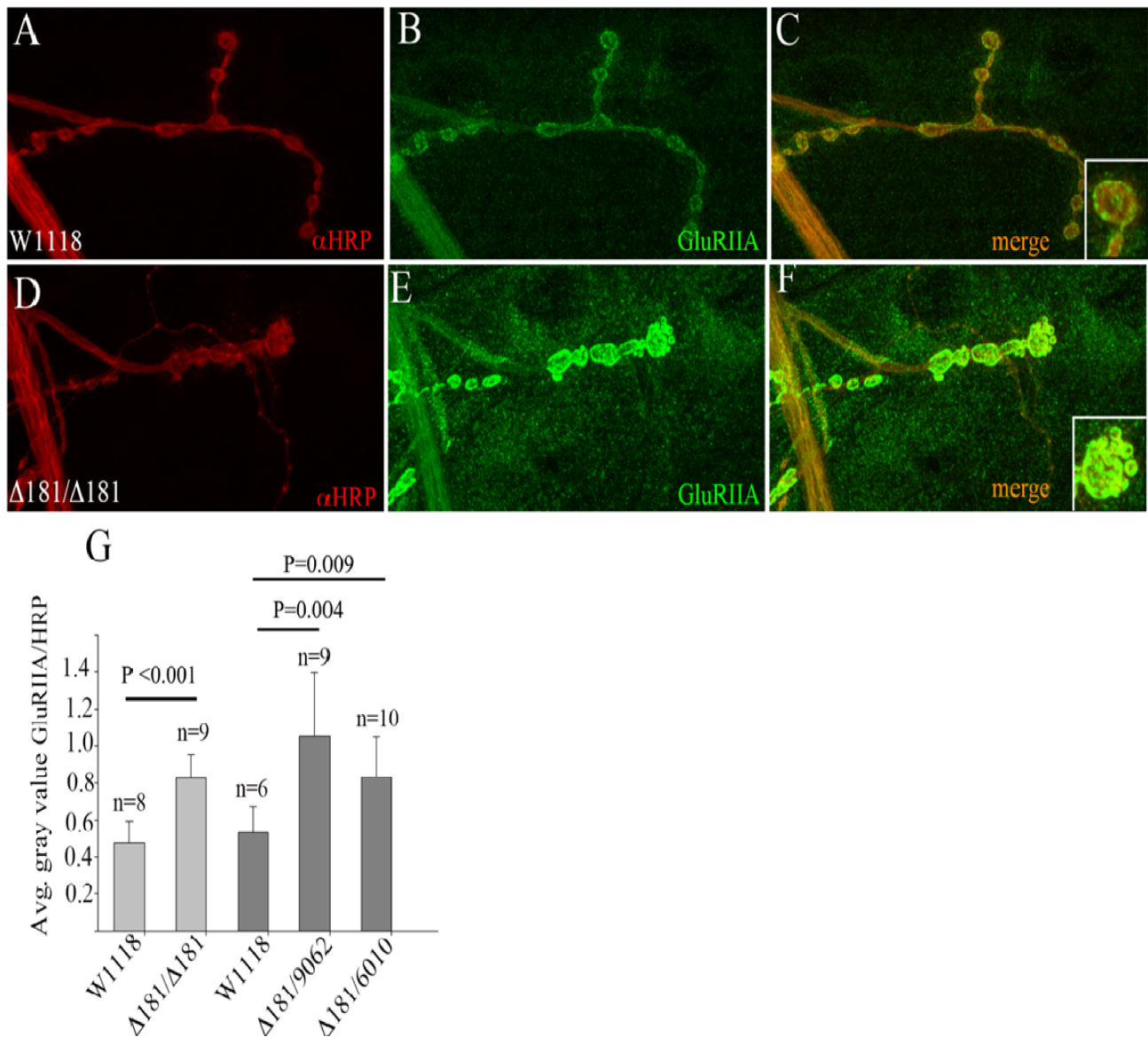


Figure 4.5: Expression level of GluRIIA is altered in *Dmon1* $\Delta 181$ mutants.

(A-F) Neuromuscular junction at muscle 4 stained with anti-HRP (red) and anti-GluRIIA (green). (A-C) Clear punctuate staining of GluRIIA (green) is seen in *w1118* (wildtype) animals (inset in C) and the muscle background is clear.

(D-F) In homozygous *Dmon1* $\Delta 181$ mutants, the intensity of GluRIIA staining is almost two folds higher than wildtype (inset in F). Also there was considerable increase in the muscle background.

(G) Quantitation of GluRIIA intensity in wildtype and *Dmon1* $\Delta 181$. Adapted from (Ratnaparkhi et al.)

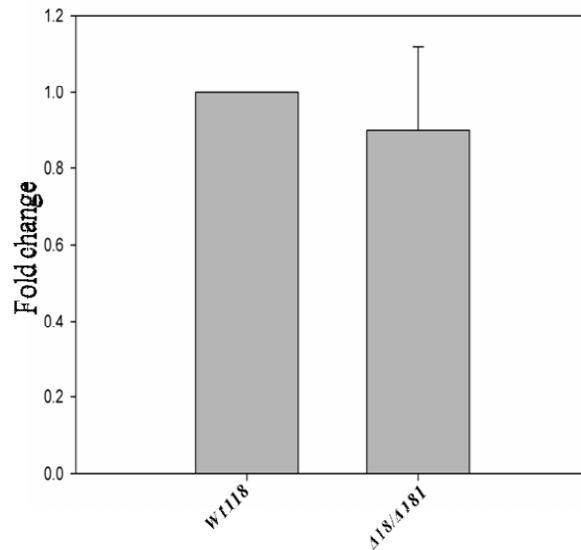


Figure 4.6: Levels of GluRIIA transcript in *Dmon1*^{Δ181}.

Levels of GluRIIA transcript from the muscles of *W1118* and *Dmon1*^{Δ181} were measured using quantitative real time PCR. There was not any significant change in levels of the transcript between control and *Dmon1*^{Δ181}. Adapted from (Ratnaparkhi et al.)

quantitative reverse transcriptase PCR to measure *GluRIIA* transcripts in wildtype and *Dmon1*^{Δ181} animals. Interestingly, we did not observe any significant difference in transcript levels between wildtype and mutant animals. The calculated average fold difference was 1 and 0.89 ± 0.22 ($P=0.47$), respectively. This suggests that the increase in GluRIIA is due to loss of post-transcriptional regulation (Fig. 4.6).

4.4.5 Loss of neuronal *Dmon1* is sufficient to alter the level of GluRIIA.

Given the role of DMon1 in vesicular trafficking, we wondered if the increase in GluRIIA levels is due to a pre-synaptic defect caused by impaired signaling, or defects in post-synaptic trafficking, or both. To determine the contribution of pre and post-synaptic *Dmon1* to the increase in GluRIIA, we independently knocked down *Dmon1* in neurons and muscle using RNAi and examined its effect on GluRIIA. Neuronal knock-down of *Dmon1* was carried out

using two independent GAL4 lines: *C155*-GAL4; *elav*-GAL4 line carrying two copies of the pan-neuronal GAL4, and *OK6*-GAL4. With *C155*-GAL4; *elav*-GAL4 knockdown 70% increase in GluRIIA levels was observed compared to controls (GluRIIA:HRP ratio, 0.85 ± 0.22 vs 0.503 ± 0.13 ; Fig. 4.7A). Pre-synaptic knock-down of *Dmon1* using the motor neuron driver *OK6*-GAL4 led to a 33% increase in the GluRIIA levels (0.36 ± 0.09 vs 0.27 ± 0.08 in controls (Fig. 4.7 B)). In both experiments, an increase in extrasynaptic or muscle punctae was observed. These results suggest that loss of *Dmon1* in the presynaptic compartment is sufficient to trigger post-synaptic increase in GluRIIA.

Next, to evaluate the effect of post-synaptic loss of *Dmon1* on GluRIIA levels, we expressed *UAS-dsDmon1* in muscles. Three independent GAL4 lines: *mhc*-GAL4, *Mef2*-GAL4 and *G14*-GAL4 were used. Upon quantification of GluRIIA levels, we observed variation in our results. However, in all the cases there was a consistent increase in levels of GluRIIA. In case of *Mef2-Gal4* knock down, 33% increase in levels of GluRIIA was observed, and with *G14-Gal4*, 46% increase was seen (Fig. 4.7 C,D). Even in experiments where the change was not statistically significant, 20% increase in levels of GluRIIA was observed (*G14-Gal4*). Secondly, post-synaptic overexpression of *Dmon1* led to a decrease in synaptic GluRIIA levels with both *Mef2-Gal4* and *G14-Gal4*. Over expressing *Mon1* in muscle with *G14-Gal4* led to 84% reduction of GluRIIA and with *Mef2-Gal4* led to 35% reduction (Fig. 4.7E, F). This suggests that receptor levels can be modulated pre and post-synaptically by *DMon1* though loss of neuronal *Dmon1* is sufficient to phenocopy the GluRIIA phenotype observed in the mutants.

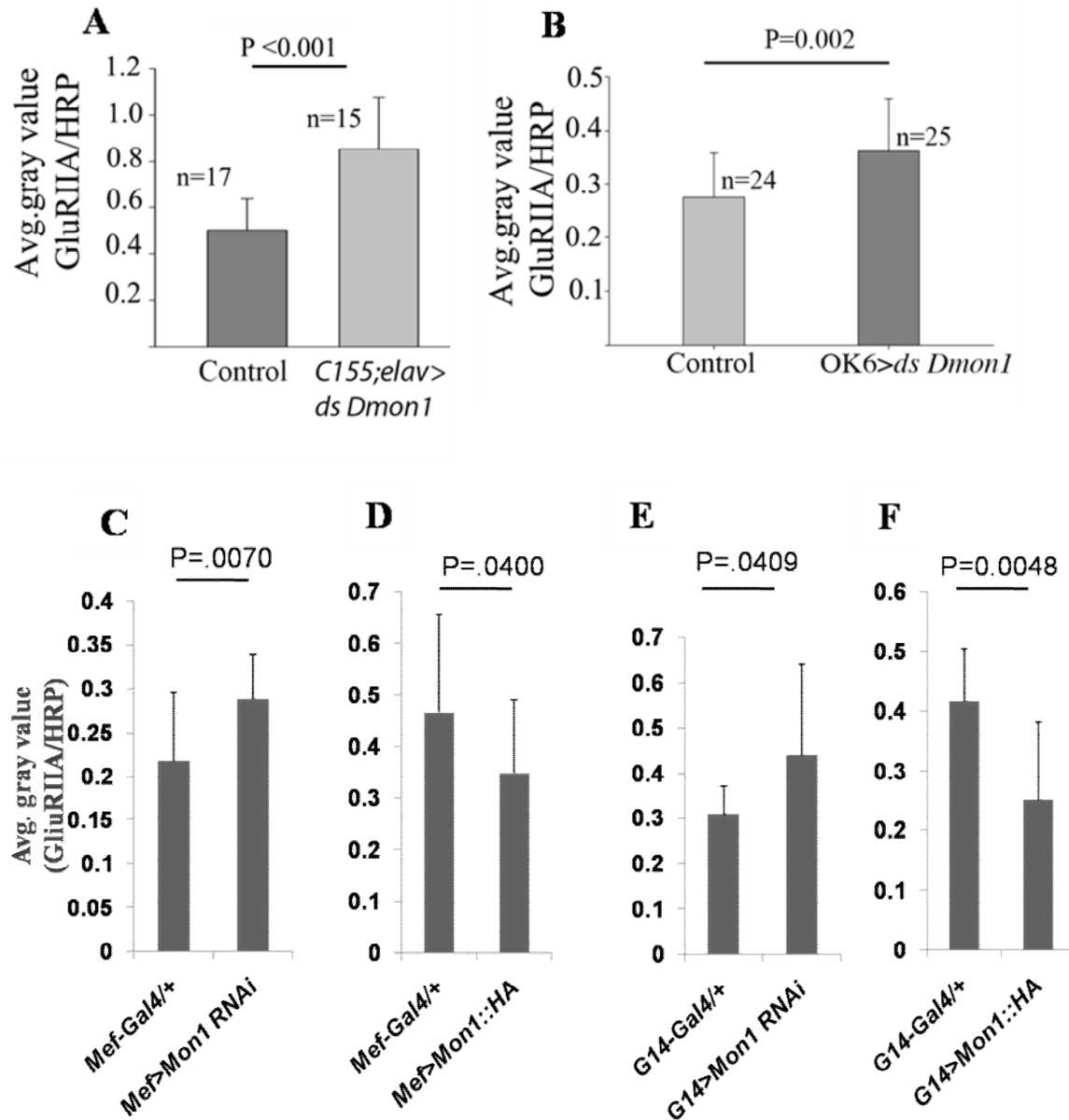


Figure 4.7: Mon1 can regulate GluRIIA levels pre and post-synaptically.

(A,B) Quantitation of GluRIIA levels on neuronal knock down of *Mon1*. Neuronal knock down of *Mon1* using *OK6-Gal4* and *C155-GAL4; elav-Gal4* leads to increase in synaptic GluRIIA.

(C&E) Post-synaptic knockdown of *Dmon1* using *Mef2-Gal4* and *G14-Gal4* leads to an increase in the intensity of synaptic GluRIIA staining.

(E&F) Post-synaptic over expression of *Dmon1* causes an reduction of synaptic GluRIIA staining. All the values are ratio of GluRIIA:HRP. Adapted from (Ratnaparkhi et al.)

4.4.6 Post-synaptic expression of *Dmon1* can rescue the phenotype in *Dmon1* mutants.

To further confirm that the increase in GluRIIA levels is indeed due to loss of *Dmon1*, we checked if expression of the gene rescues the phenotype in the mutant. We expressed *UAS-Dmon1::HA* presynaptically in homozygous *Dmon1*^{Δ181} and *Dmon1*^{Δ181}/*Df(2L) 9062* larvae and examined GluRIIA expression using immunostaining. We found that presynaptic expression of *Mon1* in null background reduced the GluRIIA levels consistently (data not shown). Next, we expressed *UAS-Dmon1::HA* post-synaptically in homozygous *Dmon1*^{Δ181} mutants to check for rescue of the GluRIIA phenotype. Expression of GluRIIA was significantly lowered in the ‘muscle-rescue’ animals (Fig.4.8D, E&F’). Nearly, 70% decrease in the synaptic levels of GluRIIA was observed in these animals (0.12±0.05 in ‘rescue’ versus 0.39±0.15 in mutant control.) (Fig.4.8G). Interestingly, muscle expression of *Dmon1::HA* did not rescue the lethality of *Dmon1*^{Δ181}/*Dmon1*^{Δ181} while pan-neuronal expression of *Dmon1::HA* with C155-GAL4 rescued lethality though not to the same robust extent seen in *Dmon1*^{Δ181}/*Df(2L)9062* animals. Interestingly, both pre-synaptic as well as post-synaptic expression of *Dmon1* rescued the satellite bouton phenotype seen in the mutants (data not shown). Together, our results suggest that while, pre-synaptic and post-synaptic expression of *Dmon1* can rescue the morphology and GluRIIA phenotype in the mutants, neuronal expression is functionally more relevant to the animal.

4.4.7 *Mon1* is essential for regulating GluRIIA levels

We expressed constitutively active form (Q67L) of Rab7 post-synaptically in homozygous *Dmon1*^{Δ181} to test if it could lower GluRIIA levels independent of *Dmon1*. Interestingly, we did not observe any rescue of the GluRIIA phenotype. In contrast, an increase in synaptic GluRIIA was observed with little or no change in the level of

extrasynaptic GluRIIA puncta, (Fig. 4.9 D-F) suggesting that expression of activated Rab7 alone is not sufficient to rescue the GluRIIA phenotype.

4.5 Discussion

4.5.1 Regulation of GluRIIA levels by Mon1

Mon1 is a highly conserved protein whose function across eukaryotic model systems such as yeast, *C. elegans*, *Drosophila* and even plants (*Arabidopsis thaliana*), is to recruit Rab7 onto late endosomes (Kinchen and Ravichandran 2010, Poteryaev et al. 2010, Cui et al. 2014). We

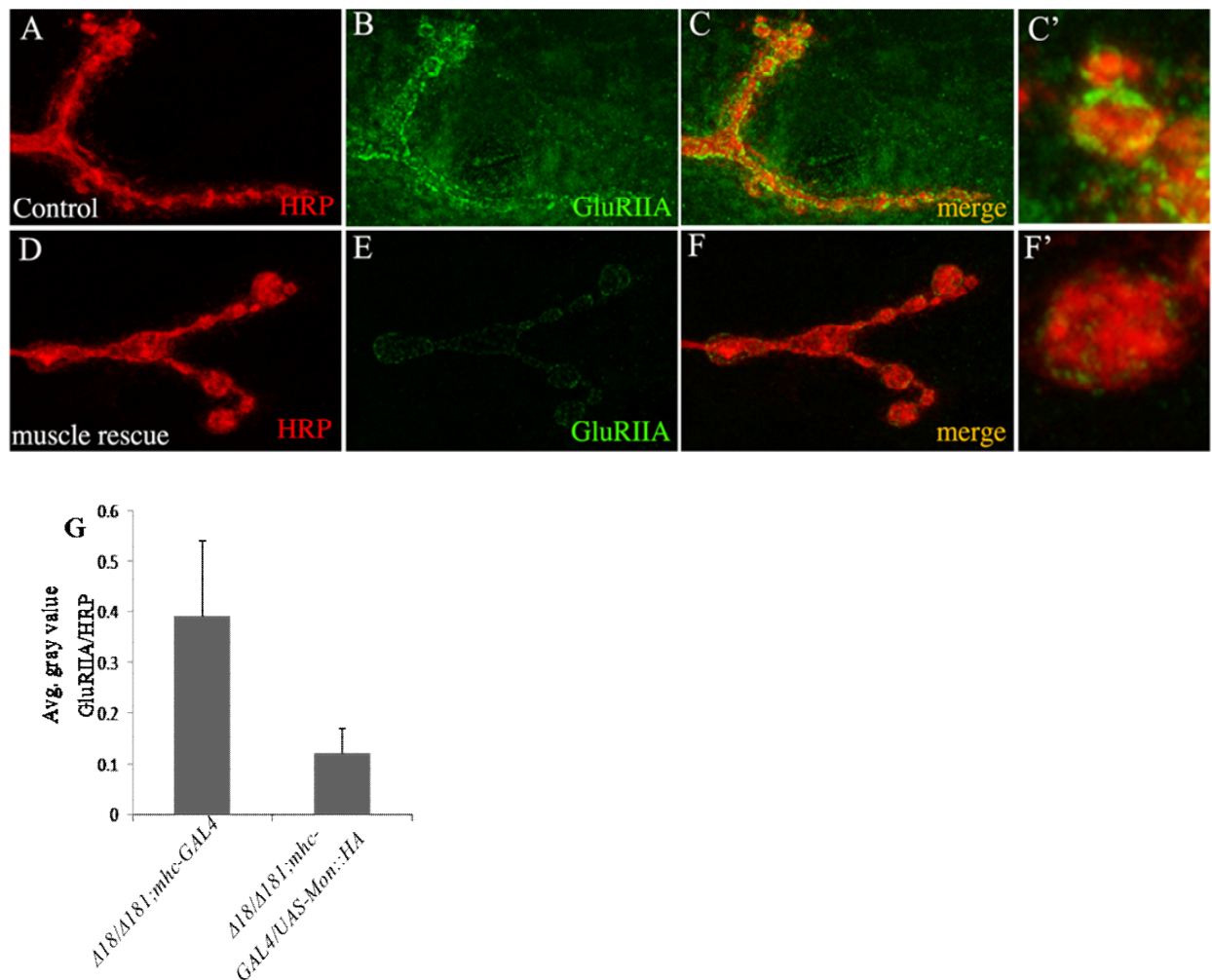


Figure 4.8: Postsynaptic expression of Mon1 rescues the GluRIIA levels in *Dmon1* $\Delta 181$ mutants.

Control animals of genotype *Dmon1* $\Delta 181/\Delta 181$; *mhc-GAL4*/+; high levels of GluRIIA is observed at the synapse and in the muscle (B and C"). Expression of DMon1 in homozygous mutants rescues the GluRIIA phenotype at the synapse and muscle (E, F").

(G) Comparison of synaptic GluRIIA intensity normalized to HRP is shown. Adapted from (Ratnaparkhi et al.)

generated a mutation in *Dmon1* through an imprecise P-element excision, which deletes the 3' end of the gene. Here, we have characterized this mutant (*Dmon1*^{Δ181}) and describe a role for DMon1 in regulating GluRIIA receptor levels at the larval NMJ. In *Dmon1*^{Δ181} mutant, the 3' end of the gene is deleted. Homozygous and *Dmon1*^{Δ181} / *Deficiency* animals showed comparable phenotypes with respect to motor defects, synaptic morphology and GluRIIA expression indicating that these phenotypes arise due to loss of the gene. Moreover, neuronal loss of *Dmon1* also led to an increase in GluRIIA expression at the NMJ confirming that the phenotype is indeed associated with loss of *Dmon1*.

The mechanism regulating GluRIIA expression, localization and trafficking are poorly understood. *Dmon1*^{Δ181} mutants show a dramatic increase in GluRIIA levels at the post-synaptic densities and in extrasynaptic regions. The latter phenotype, though consistent exhibits some variability in intensity. For example, in some experiments we observed intense extrasynaptic GluRIIA punctae while in some others it was less dramatic. However, neuronal as well as muscle rescue of *Dmon1* mutants led to a clear decrease in extrasynaptic expression. Moreover, neuronal knock-down of *Dmon1* led to an increase in extra-synaptic GluRIIA expression confirming that the phenotype is associated with loss of *Dmon1*. The fact that there is an increase in receptor levels at the synapse and muscle, makes it unlikely that this is a part of some homeostatic regulation.

How might DMon1 regulate GluRIIA? In the absence of transcriptional up-regulation, increase in synaptic GluRIIA can occur due to altered translational control or defects in receptor trafficking and turnover. Given the role of DMon1 in intracellular trafficking, it is possible that the increase in synaptic GluRIIA is due to defects in protein turn over and that the muscle punctae represent trapped endosomal intermediates. Muscle rescue of the *Dmon1*^{Δ181} phenotype

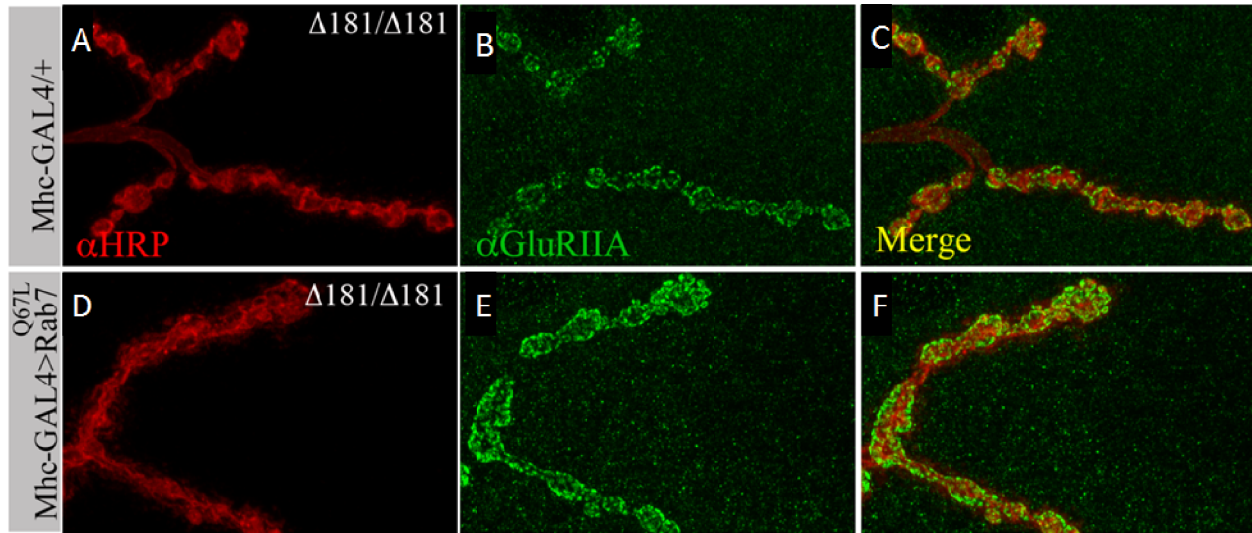


Figure 4.9: Mon1 is essential for regulating GluRIIA levels.

Over-expression of activated Rab7 in Mon1 null background (E&F) does not reduce GluRIIA accumulation at the synapse seen in Mon1 mutants (B&C). Adapted from (Ratnaparkhi et al.)

leading to a decrease in GluRIIA levels seems to support this. The GluRIIA do not seem to be trapped in Rab5 endosomes because the Rab5 endosomes are large in Mon1 mutants (data not shown) and the GluRIIA punctae we observed are small. We attempted to see if the GluRIIA punctae co-localize with recycling endosomes using Rab11 antibody. However, this did not materialize because of incompatibility in fixation conditions for these antibodies.

GluRIIA expression is post-transcriptionally regulated directly and indirectly by RNA binding proteins Pumilio and Nanos, respectively (Menon et al. 2004, Menon et al. 2009). However, neither loss of *pumilio* or *nanos* has been described to lead to accumulation of extrasynaptic GluRIIA as seen in *Dmon1* mutants. Therefore, it seems unlikely that the GluRIIA phenotype is mediated through dysregulation of these RNA binding proteins. Accumulation of GluRIIA in muscles has been observed in *akt* mutants (Lee et al. 2013b). But, unlike the punctate distribution in *Dmon1* mutants, the localization of the receptor in these mutants appears to be membrane associated indicating mis-targeting of the protein.

4.5.2 Regulation of Glutamate receptor levels by endosome machinery and relevance to disease

Role of endosomal machinery in regulating glutamate receptor levels is still not clear. Studies have consistently shown that the recycling endosomes are source of AMPA receptors during long-term potentiation. The mechanisms governing the internalization of these receptors and what decides the fate of these receptors is poorly understood. While most mammalian and *C. elegans* studies have focused on endosomal recycling of receptors, not much is known how these receptors are degraded. It is also important to note that posttranslational modifications of glutamate receptors decide their fate of recycling and degradation. A study from *C. elegans* suggests that ubiquitination of the GIR1 results in clathrin dependent endocytosis (Burbea et al. 2002). In mammals, the GluA1 subunit is ubiquitinated in a Nedd4 dependent manner and is targeted to lysosomes (Schwarz et al. 2010). Both these cases show that reduction in ubiquitination leads to increase in accumulation of glutamate receptors at the synapse. In worms, increased levels of GIR1 are seen in mutants of gene (*vps-4*) involved in formation of multi vesicular bodies/ lysosome pathway (Kowalski et al. 2011). This is similar to the increased levels of GluR seen in *Mon1* mutants and *Rab7* RNAi. Also, increasing the ubiquitination by over-expressing Nedd4 resulted in increased accumulation of GluA1 in the cytoplasm. Similar accumulation of GluRIIA is seen in our studies as well. It would be interesting to see if the muscle GluRIIA punctae has post translational modifications like ubiquitination. It would also be worth seeing whether these punctae localize with specific endosomal markers. Recent study in *Drosophila* showed that some of the CMT disease related mutations in Rab7 might be acting as loss-of-function alleles (Cherry et al. 2013). It would be interesting to see if these mutant proteins show similar increase in GluRIIA levels.

CHAPTER 5

Future perspectives

Mechanisms underlying neurodegenerative diseases are poorly understood. Although a number of molecular pathways have been implied to be involved the disease, it is not clear what makes a few subtypes of neurons susceptible to a particular disease. It is proposed that cellular factors like endoplasmic reticulum (ER) stress, calcium homeostasis, mitochondrial dysfunction, defective axonal transport, altered calcium buffering might predispose neurons selectively to a disease (Pun et al. 2006, Saxena and Caroni 2011). However, this does not explain why in case of a particular mutation only one neuronal type is affected but not all susceptible neurons. In this context it is tempting to propose that a genetic network might be operating in each cell, and each cell might have its own susceptible network. Environmental and cellular factors along with mutation(s) that lead to break down of this particular genetic network can push the neurons towards degeneration. The idea of NDs being caused because of breakdown of genetic networks is gaining momentum (Limviphuvadh et al. 2007, Santiago and Potashkin 2014).

In our study we have identified genes that interact with VAP and have found that other loci that cause ALS are a part of this genetic network. Our future studies will focus on how these genes interact among themselves. Some of these interactions have been identified in other studies (discussed in Section 1.3.5). Currently, we are investigating interactions between different loci involved in ALS and also different mutations in these loci. We are looking for interactions in muscle, neurons and glia (Tendulkar, Dhiman and Ratnaparkhi, ongoing work). We are also performing experiments to see if other loci can influence aggregation of VAP (P58S) using a Schneider cell based model (described below, Appendix I) (Pimpale, Chaplot and Ratnaparkhi,

ongoing work).

Another key finding was that the TOR pathway differentially interacts with VAP(wt) and VAP(P58S). The mechanism behind these interaction(s) as well as the epistatic nature of this interaction is not clear. The downstream effect of up-regulation of TOR also remains to be elucidated. Considering the role of VAP in regulation of GLUT4 trafficking and reduced levels of ATP in VAP mutants, AMPK mediated regulation of TOR pathway seems to be an attractive possibility (Foster et al. 2000, Han et al. 2012). It also needs to be seen if the TOR pathway components can rescue or worsen the motor defects, reduced life span and aggregation seen in VAP(P58S) mutant (Moustaqim-Barrette et al. 2014).

Regulation of glutamate receptor levels has important consequences for normal physiology of the neurons and malfunction can lead to disease. Glutamate receptor regulation has been implicated in a number of neurological diseases like Down syndrome, ALS and Alzheimer's disease. The glutamate receptor at the synapse is regulated by proteosomal and endosomal machinery. In mammals, the GluA1 are ubiquitinated in an age dependent manner and their levels are maintained through endocytosis. Role of endocytosis components in regulating glutamate receptors have been reported in worms also suggesting a conserved mechanism.

We find that reduced levels of *Rab7* and its upstream regulator *Mon1* leads to accumulation of Glutamate receptor IIA subunit at *Drosophila* larval NMJ. Mutations in *Rab7* lead to CMT2B and patients show slow degeneration of both sensory and motor neurons. In a study by Cherry et al. (2013) over expression of these mutants using a *Rab7*-Gal4 knock-in line did not result in any synaptic defects at the larval NMJ. However, in another study over expression of the L129F mutant resulted in both sensory and motor defects (Janssens et al.

2014). It would be worth testing if these CMT associated mutants also show glutamate receptor accumulation. Also, the mechanism behind the GluRIIA accumulation we have observed in *Mon1* and *Rab7* mutants is not clear. Considering the role of these proteins in trafficking it would be interesting to see if other genes involved in endocytosis give similar phenotypes and if neurons are in general more sensitive to defects in endosomal trafficking.

APPENDIX I

An aggregation system for ALS8

This chapter describes the development of VAP (P58S) aggregation systems in both Schneider cells as well as in the larval brain. Aggregation is a key feature of many NDs including ALS. Mutant proteins, implicated in disease, misfold and lead to cellular inclusions/ aggregates. These aggregates may have direct roles in disease induction and progression or may be used as markers for the progression of the disease. In humans, the hVAP (P56S) mutant forms cellular inclusions that co-localize with ubiquitin. Similarly, over expression of the VAP (P58S) *Drosophila* counterpart also results in ubiquitin positive aggregates. In order to study genes that can modulate aggregation we generated stable S2 cell lines expressing VAP wild type and VAP (P58S), fused to fluorescent proteins, under an inducible promoter. This allows kinetic measurements of the increase in aggregation over time using epifluorescence microscopy. To complement the cell culture system we have also developed a fly based aggregation system. Expression of untagged VAP (P58S) mutant in larval brain and body wall muscle leads to robust VAP (P58S) aggregates that can be visualized with an anti-VAP antibody developed in our laboratory.

A1.1 Cloning

The CDS of VAP and VAP (P58S) mutant were cloned into *EcoRI* site of *pRM-GFP* plasmid under a metal inducible metallothionin promoter. The *pRM-GFP* vector has GFP cloned into *pRM-HA3* vector at the *BamHI* site. This resulted in a C-terminally GFP tagged VAP construct. The expression of transgenes can be induced by addition of copper sulphate to the cells.

A1.2 Cell culture and generation of stable lines

Drosophila S2R+ cells were cultured at 22°C in Schneider's media (GIBCO/Sigma) with 10% heat inactivated fetal bovine serum (FBS; GIBCO). Each of the VAP constructs in *pRM-GFP* vector was transfected into Schneider cells along with *pCO-HYGRO* as selection vector in a 20:1 ratio using Effectene reagent (QIAGEN). The cells were selected with 0.2-0.3mg/ml of Hygromycin (Sigma) consecutively for 10-15 generations to establish stably transformed lines. The lines were frozen based on the protocol in the Invitrogen manual and stored in liquid nitrogen. The frozen stable cell lines were thawed for experiments and always maintained in Hygromycin-containing medium.

A1.3 Protein purification and generation of antibodies

For generation of anti-VAP antibody we chose coiled-coiled region of VAP (cc-VAP), as there are other proteins in fly genome that have Major sperm protein-like domains. CDS region spanning amino acids 150 to 243 was cloned into *BamHI* and *Sall* site of *p-GEX4T1* vector. The protein was expressed in *E.coli* (*BL21DE3*) cells by induction with 1mM isopropyl-D-thiogalactoside (IPTG). The cells were lysed by sonication in lysis buffer (50 mM Tris (pH 7.5), 500 mM NaCl, 0.5% Triton X-100) with protease inhibitor cocktail (Roche). 10mg of the recombinant protein was purified by affinity chromatography using glutathione beads (GE healthcare). The GST tag was cleaved off from the recombinant protein using thrombin and purified protein was used for antibody generation from Rabbits (developed Abexome Biosciences). The antibody (VAP-Rb-446) was validated using western blot against purified protein, S2 cell lysates and also by knock down in S2 cells (Figure A1.2A, Image courtesy:Amarendranath Soory).

A1.4 S2 cell based aggregation model

Drosophila S2R⁺ cell based whole genome RNAi screens are simplistic because of the relative ease with which knock down can be achieved in these cells. The protocol for knocking down genes is simple and efficient. Such a system has been used for identifying modifiers of aggregation of Huntington's protein (Zhang et al. 2010). In an effort to establish similar system to understand aggregation of VAP (P58S) we created independent Hygromycin resistant stable cell lines containing VAP wild type and VAP (P58S) mutant tagged with GFP. On induction with copper sulphate cells over expressing VAP wild type tagged with GFP showed uniform GFP distribution throughout the cell (FigureA1.1 B, C). On the contrary stable cell lines expressing VAP (P58S) mutant showed GFP positive cellular inclusions (FigureA1.1 D, E). The number of cells showing cellular inclusion increased with concentration of copper sulphate and induction time (Pimpale *et. al.*, Unpublished). The fraction of cells showing aggregates at 24 hrs with 500uM copper sulphate was around 20%. Also, treating the cells with dsRNA against either GFP or VAP could reduce these aggregates.

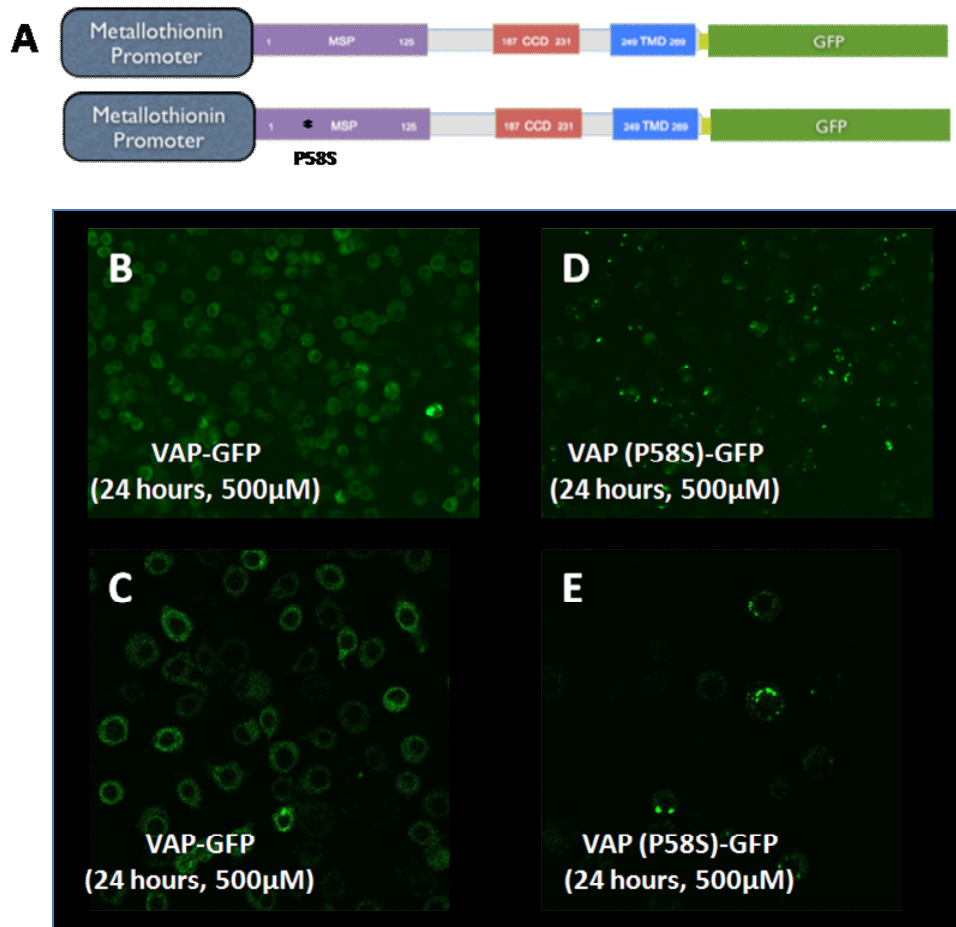


Figure A1.1: Development of S2 cell based aggregation system.

(A) Schematic of the constructs used for generating the stable lines.

(B,C) VAP (wt)-GFP stable lines induced with copper sulphate show uniform distribution of VAP-GFP throughout the cell.

(D,E) VAP (P58S)-GFP stable lines induced with copper sulphate show cellular inclusions/ aggregates that are positive for GFP.

A1.5 Fly based aggregation model

A1.5a Immunostaining and image analysis

All the muscle aggregation experiments were done at 25° C. The fly lines used were *UAS-VAP (P58S)*, *Mhc-Gal4*, *C155^{elav}-Gal4* (Lin et al. 1994, Schuster et al. 1996, Ratnaparkhi et al. 2008). For immunostaining, the animals were dissected in PBS and fixed with para-formaldehyde (4%) in PBS with TritonX-100 (0.3%) for 40 mins. The tissues were washed thoroughly, blocked with BSA (2%) in PBS with Triton X-100 (0.3%) and incubated with Rabbit anti-VAP antibody (1:500) overnight. The tissues were washed and incubated with secondary antibody (Molecular probes, 1:1000) for 2 hrs at room temperature. After washing the samples were mounted in mounting medium. The tissues were imaged using LSM 710 confocal microscope at 63X magnification. The aggregate size and number was quantified using ‘Particle analysis’ plug-in in ImageJ.

To complement the cell culture model of aggregation with an *in vivo* tissue based system, we generated stable fly lines based on UAS/Gal4 system. The stable lines were generated by combining either pan-neuronal *elav-Gal4* driver or muscle *MHC-Gal4* with *UAS-VAP (P58S)*. In both the tissues robust aggregation was seen when they were stained with anti-VAP antibody (muscle, Figure A1.2B). As in case of S2R+ cells these aggregates could be reduced by co-expressing a VAP RNAi line (Figure A1.2C).

Both these aggregation models can help in understanding the genes that modulate aggregation. We have initiated a whole genome based RNAi screen in S2 cells to identify genes that modulate aggregation (Lokesh, Kriti, Senthil and Girish, unpublished). The genes identified in this screen could shed light on the role of aggregates and how they modulate disease progression.

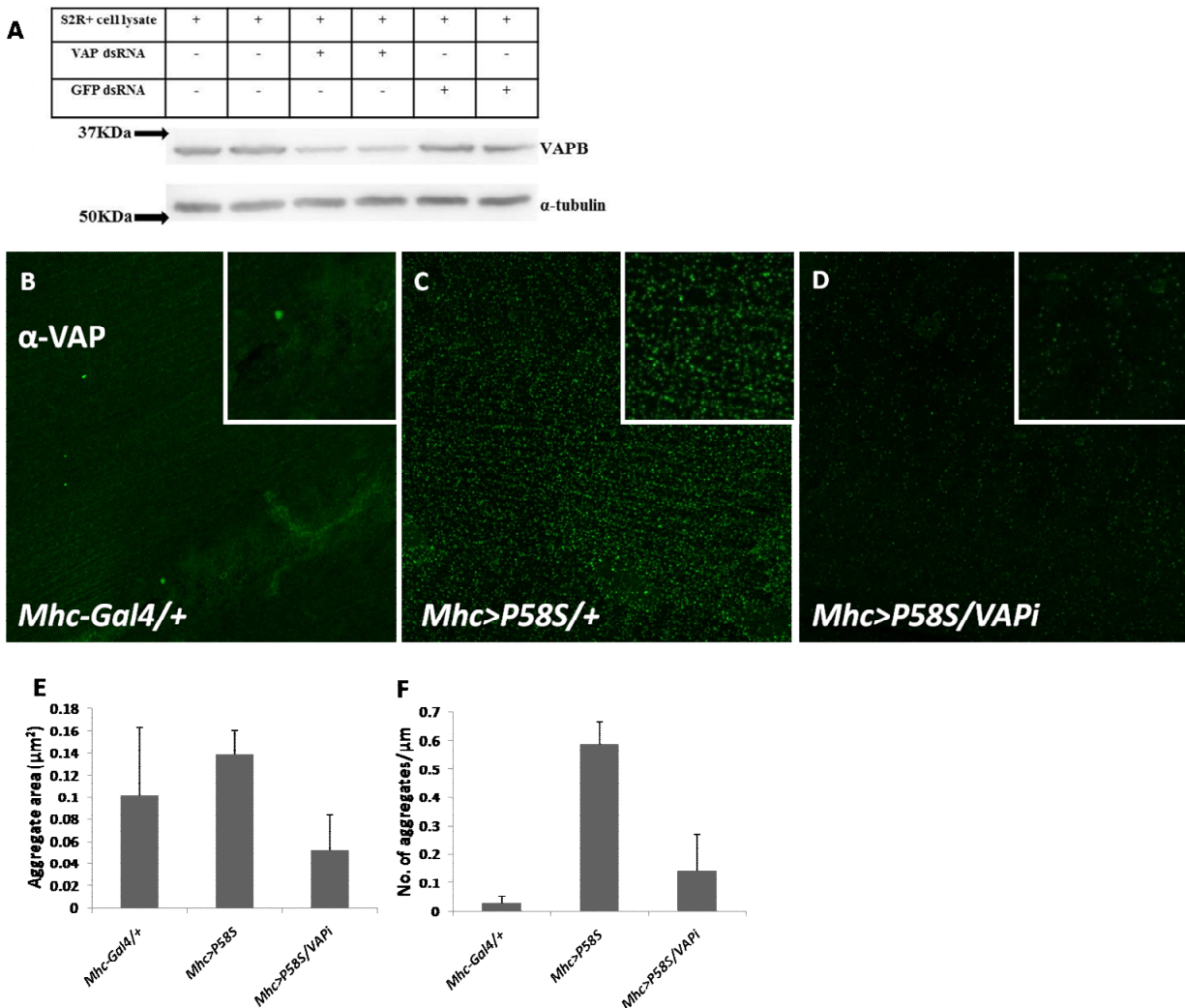


Figure A1.2: Development of fly based aggregation model.

(A) A western blot showing the specificity of anti-VAP antibody using knock down of VAP in S2R+ cells. 30-40% reduction in VAP protein level was observed on treating cells with VAP dsRNA.

(B) Muscle from *MHC-Gal4/+* control showing uniform distribution of VAP.

(C) In a muscle over expressing VAP (P58S), VAP positive aggregates can be seen.

(D) The aggregates seen in *MHC>P58S* line can be cleared by knocking down VAP in muscles.

(E, F) Quantitation of average aggregate area and number of aggregates in unit area from different genotypes.

Please note that the large aggregate area seen in *MHC-Gal4* is because of patches where there is intense staining.

APPENDIX II

Transcriptome changes in a VAP(P58S) aggregation model

Transcriptome profiling using microarray technology has shed light on pathways operating in degenerating tissues. Studies have examined expression patterns in tissues from patient with familial and sporadic ALS (Kirby et al. 2005, Ferraiuolo et al. 2007, Heath et al. 2013). Attempts have been made to examine the expression profiling underlying selective neuronal vulnerability of motor neurons in ALS (Ringer et al. 2012). While the quality of the data from patients derived samples might be of concern with reference to RNA quality, samples derived from model system have filled in those gaps (Heath et al. 2013). In this study we have undertaken transcriptome analysis of the Schneider cell based aggregation system developed in our laboratory. The data obtained from this analysis might shed light on aggregation induced changes to transcript profile in cells and may lead to further insight into initiation of the disease.

A2.1 Cell culture and Microarrays

The S2R⁺ cell and S2R⁺ stable cell lines expressing VAP (wt)-GFP and VAP (P58S) (Appendix I) were grown in Schneider's medium and were induced with copper sulphate for 24 hours. Uninduced stable lines were used as controls. Cells were lysed in TRIzol reagent (Invitrogen) and stored at -80°C till further processing. RNA isolation and quality control of isolated RNA, labeling of RNA probes with cyanine 3-dUTP (Cy3) using Agilent One-Color RNA spike-in kit, hybridization to one Drosophila 8*60 K array AMADID: G4102A_039596 (Agilent Technologies Inc., USA) consisting of 8 replicate 60K microarrays and scanning of the arrays for signal intensities and analysis and normalization of signal intensities were carried out by Genotypic Technology Pvt. Ltd., Bangalore, India. Of the 8 arrays utilized, two were hybridized

with RNA from VAP-GFP (induced), two with VAP (P58S)-GFP (induced), two with S2R+ (induced, control) and one each with VAP-GFP & VAP (P58S)-GFP (both uninduced). The total number of probes included in the array numbered to 34217 (excluding the control probes) and they could be matched to 13789 unique genes.

A2.2 Data Analysis

Data analysis was performed using GeneSpring GX software (Silicon Genetics, USA). Differentially regulated genes were ranked on the basis of signal intensity and normalized ratio. The normalized signal intensity was used for calculating the log₂ of fold change. Only those genes that showed significant change ($P < 0.01$) were considered for further analysis. The Gene ontology analysis was performed using DAVID, PANTHER and tools available in Flybase (Huang et al. 2007, Mi et al. 2013, Dos Santos et al. 2015).

A2.3 Results

Here, we present results from preliminary analysis of the transcriptome data. In order to identify genes that were differentially regulated between VAP-GFP and VAP (P58S)-GFP we compared the expression levels of genes between these two samples and only those that showed a difference in level was considered for analysis. A total of 1887 genes showed differential expression compared to control S2R+ cells and also were differentially expressed between VAP and VAP (P58S). On VAP-GFP over expression 110 genes were found to be up regulated and 42 were down regulated. With over expression of VAP (P58S)-GFP 636 genes were up regulated and 1099 genes were down regulated. Of these, 46 genes showed up-regulation in VAP-GFP, while they were down-regulated in VAP (P58S)-GFP and 11 genes were down-regulated in VAP-GFP while they were up-regulated in VAP (P58S)-GFP. Except for one of the modifiers identified in the bristle screen (CG6950), none of the other modifiers showed differential

expression. This could be because of the difference in cell type and also possibly the modifiers are not regulated at transcript levels, but by other means.

Table A2.2: Partial list of genes that show inverse expression pattern in VAP-GFP and VAP (P58S)-GFP. Only genes that have been characterized are listed here.

Gene	Function	Up/ down regulated in VAP(P58S)-GFP
<i>apterous</i>	Axon guidance, pattern formation	-
<i>Calx</i>	Calcium ion transport	-
<i>CG1607</i>	Amino acid transport	-
<i>Cg25C</i>	Extracellular matrix protein (collagen related)	-
<i>CG42602</i>	Endopeptidase inhibitor	-
<i>CG5665</i>	Lipid metabolism	-
<i>Epac</i>	Protein kinase	-
<i>vkg</i>	Extracellular matrix protein (collagen related)	-
<i>CG10834</i>	Dynein complex	+
<i>RpL37b</i>	Protein translation	+
<i>Rfabg</i>	Vesicle transport	+
<i>niki</i>	Protein kinase	+
<i>Nfl</i>	Transcriptional regulation	+
<i>HP1Lcsd</i>	Chromatin organization	+
<i>CG5250</i>	Ion transport	+
<i>CG17560</i>	fatty-acyl-CoA reductase	+
<i>CG9975</i>	Neurogenesis	+

<i>RhoGAP102A</i>	Rho GTPase activator	+
<i>Caps</i>	Neurotransmitter secretion	+
<i>dpr7</i>	Sensory perception	+

(-) Down regulated. (+) Up regulated.

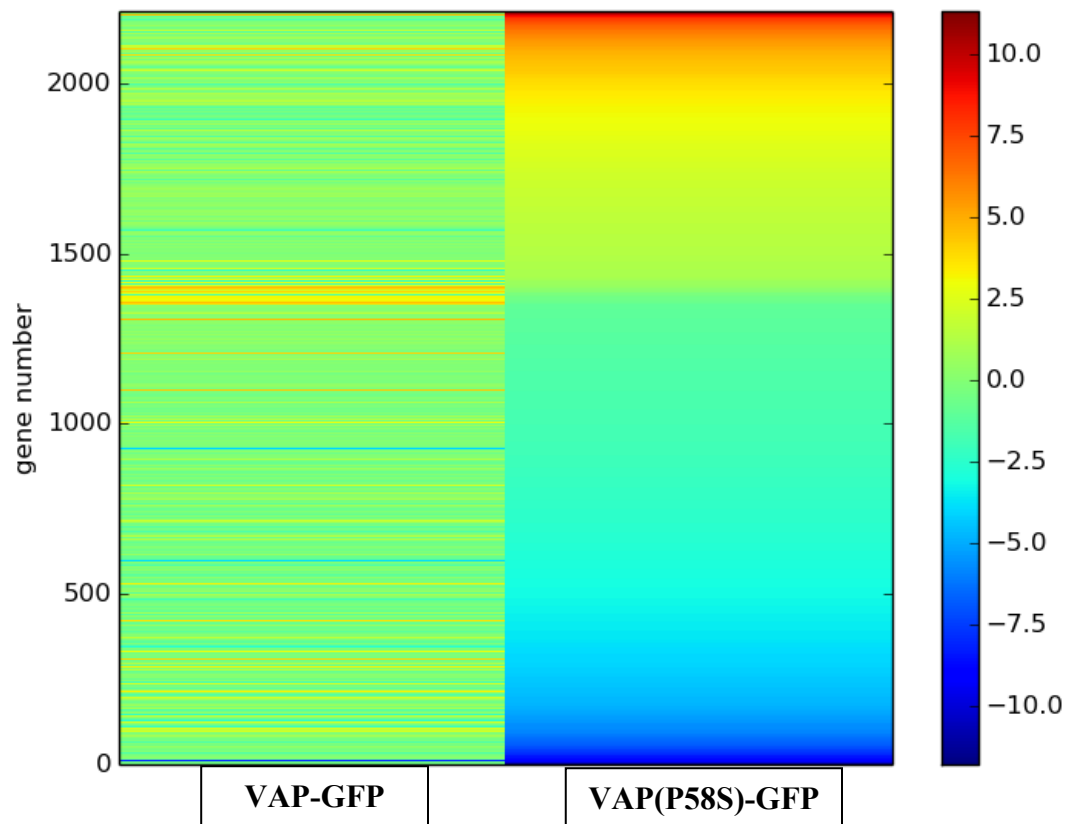


Figure A2.1: Heat map showing expression levels of differentially regulated genes.

The heat map shows expression levels of all genes that are differentially expressed in both VAP-GFP and VAP (P58S)-GFP. Heat map has been plotted by sorting the expression levels of these genes in VAP (P58S)-GFP and their corresponding expression levels in VAP-GFP is shown in the adjacent column.

The genes that were differentially expressed in VAP-GFP could be categorized into those involved in RNA metabolism, transcriptional regulation, cellular communication etc. However, no major categories could be recognized, probably because of the smaller number of genes that show change in levels. Interestingly, in case of VAP (P58S)-GFP over expression

certain categories of genes were found to be showing change in transcript levels. For example, about 12.5% of the total genes involved in G-protein signaling, 13% of genes involved in Ras signaling, 18% of genes involved in torso signaling were found to be differentially expressed. Another class of genes which were differentially expressed in VAP (P58S)-GFP line were involved in immune response (28 out of 221 genes showed change in levels). This is interesting considering that there is emerging role for the immune system in case of ALS (Chiu et al. 2009, Noh et al. 2014). It would worth to look if any of the genes that show differential expression in our experiments have shown similar trends in other studies. Further analysis of the data is in progress.

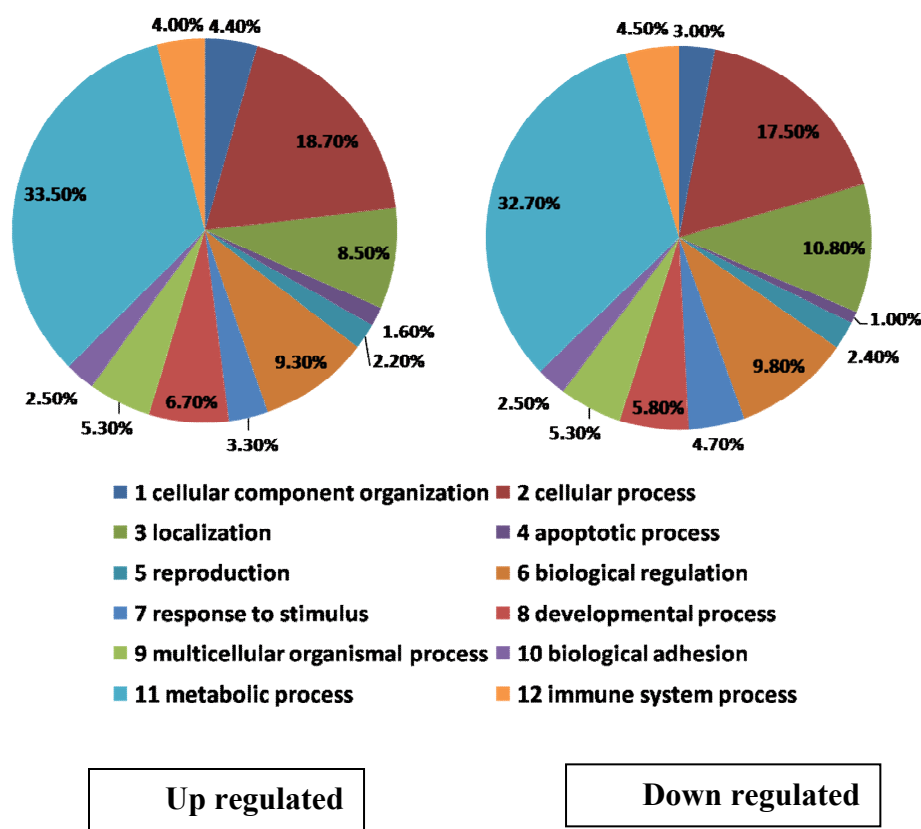


Figure A2.2: GO analysis of differentially regulated genes in VAP (P58S)-GFP. Genes that show differential expression on VAP (P58S)-GFP over expression can be categorized mainly as those involved in metabolism, immune response and apoptosis. Genes whose function is not known are not a part of this chart.

APPENDIX III

The *Hydra* Small Ubiquitin-like modifier

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