# ASSAYING THE FUNCTION OF HUMAN VARIANTS FOUND IN SMAD4 AND BMPR1A USING DROSOPHILA MELANOGASTER

by

Amanda Pisio

### B.Sc., The University of Alberta, 2010

M.Sc., The University of Alberta, 2013

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

# ASSAYING THE FUNCTION OF HUMAN VARIANTS FOUND IN SMAD4 AND BMPR1A USING DROSOPHILA MELANOGASTER

submitted by	Amanda Pisio	in partial fulfillment of the requirements for	
the degree of	Doctor of Philosophy		
in	Cell and Developmental Biology		
Examining Committee:			

Tim O'Connor Supervisor

Doug Allan

Supervisory Committee Member

Michael Underhill

University Examiner

Jane Roskams

University Examiner

### Additional Supervisory Committee Members:

Michael Gordon Supervisory Committee Member

Kurt Haas

Supervisory Committee Member

### Abstract

This thesis describes two projects that examined protein function in neural plasticity and cancer development. The first project created assays that tested the pathogenicity of human variants identified in SMAD4 and BMPR1A, genes that are associated with juvenile polyposis syndrome. As exome sequencing becomes easier and more cost-effective, many human variants are being identified. However, for most variants, their impact on protein function and their ability to cause disease are unknown. Drosophila melanogaster offers an efficient system for testing human variant protein functionality in a panel of assays to screen through many variants. I have used simple overexpression assays in Drosophila to test human SMAD4 and BMPR1A variants. I developed two assays in which wildtype SMAD4, but not loss of function variants, caused either lethality or wing vein defects. I screened through seven human SMAD4 variants implicated in disease to assess their relative function and identified four that exhibit functional differences to wildtype. I also tested human BMPR1A but found that overexpression of this gene in Drosophila had no effect. I postulated this is due to a lack of ligand binding. Therefore, I created reagents for alternative methods to screen BMPR1A variant function. First, I generated mimetic mutations in the orthologous tkv gene. Second, I created a chimeric gene comprising the extracellular domain of Tkv and the intracellular domain of BMPR1A. I postulate that this chimera should bind Drosophila BMP ligands and activate canonical BMP signaling, allowing for assays of BMPR1A variants in the intracellular domain. These reagents and assays are important for experimentally determining ariant activity and for improving our understanding of structure/function relationships for SMAD4 and BMPR1A. Going forward, functionally testing

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large numbers of variants will inform personalized medicine approaches and improve computer models for projecting pathogenicity of human variants.

In the second project, I created *CalpA* and *CalpB* double mutants to test whether a reduction of calpain activity could stimulate *de novo* neurite formation. Also, I overexpressed a proteolytic target of Calpain, Cortactin, and created a Calpain proteolysis resistant version of Cortactin. Surprisingly, I was unable to identify any phenotype in the nervous system.

# Lay Summary

Our ability to sequence genes in humans has created large databases of identified mutations. The problem is determining which mutations lead to disease and which mutations are benign. This thesis created reagents and tests to identify mutations that cause disease in two human genes that are known to cause juvenile polyposis syndrome when mutated. The tests are performed in fruit flies, which can assess relative human protein function cost-effectively and rapidly. The tests created identified four mutations that likely cause disease. These tests will help patients with these mutations and will help us better understand the effect of mutations on other genes.

Similarly, I tested and refuted a hypothesis, arising from *in vitro* work, that neurons have a tendency towards sprouting novel branches that has to be persistently restrained by a specific set of proteins called calpains.

# Preface

Identification and design of this project was done by me in conjunction with my supervisors Dr. O'Connor and Dr. Allan. All experiments were carried out by me. Cloning of SMAD4 and BMPR1A transgenes was done by me. Cloning of *tkv* mimetic and *tkv::BMPR1A* chimeric transgenes was done by Tianshun Lian. All analysis was carried out by me. This project did not require ethics approval.

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# List of Abbreviations

Acv	Anterior cross vein
ACVRI	Activin A receptor, type I
ACVRL1	Activin A receptor Like Type 1
ACVR2A	Activin A receptor type 2A
ACVR2B	Activin A receptor type 2B
ALLN	N-[N-(N-Acetyl-L-leucyl)-leucyl]-L-norleucine
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arp2/3	Actin-related proteins 2/3
ATAD3C	ATPase family AAA-domain containing protein 3A
BMP	Bone morphogenetic proteins
BMP-2	Bone morphogenetic protein 2
BMPR1A	Bone morphogenetic protein receptor type-1A
BMPR1B	Bone Morphogenetic Protein Receptor Type 1B
Bor	Belphegor
CADD	Combined Annotation Dependent Depletion
Calp	Calpain
cDNA	Complementary DNA
CNS	Central nervous system
Co-SMAD	Common partner SMAD
DA	Daughterless
DLX1	Distal-Less Homeobox 1

DNA	Deoxyribonucleic acid
DNM1L	Dynamin 1 Like
Dpp	Decapentaplegic
DRP1	Dynamin related protein 1
DSHB	Developmental Studies Hybridoma Bank
Gbb	Glass bottom boat
GFP	Green fluorescent protein
HGNC	Human genome organization gene nomenclature committee
Hrp	Horseradish peroxidase
Hrs	Hours
IE	Imprecise excision
INHBE	Inhibin subunit beta E
MH1	Mad homology 1
MH2	Mad homology 2
Min	Minute
NMDA	N-methyl-D-aspartate
NMJ	Neuromuscular junction
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pcv	Posterior cross vein
PE	Precise excision
p-Mad	Phosphorylated mothers against dad
Polyphen2	Polymorphism Phenotyping v2

PTEN	Phosphatase and Tensin Homolog
R-SMADs	Receptor-regulated SMADs
Sax	Saxophone
SH3	Scr homology 3
SMAD4	SMAD Family Member 4
Smox	Smad on X
SNAP2	Screening for Non-Acceptable Polymorphisms 2
SOE	Splicing by overhang extension
Sol	Small optic lobe
Spl	Specificity protein 1
c-Src	cellular Src
TGFβ	Transforming Growth Factor beta
Tkv	Thickveins
TM2D3	TM2 Domain Containing 3
UAS	Upstream activating sequence
UTR	Untranslated region
Wnt	Wingless/Integrated
WT	Wildtype
YFP	Yellow fluorescent protein

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# Dedication

To understanding and progress.

### **Chapter 1: Introduction**

The work of this thesis stems from the need to create quick and reliable assays to determine the pathogenicity of gene variants found in patients with specific medical conditions. The decrease in sequencing costs have made it easier to sequence genes believed to cause pathogenicity leading to an increase in gene variants identified. Current computational methods that are used to predict the functional effects of these gene variants often fail to accurately diagnose pathogenicity. Specifically, this thesis examines two genes that are known to be causal for juvenile polyposis syndrome, BMPR1A and SMAD4. These two genes are members of the canonical BMP signaling pathway. Identification of pathogenic mutations in these genes is important for human health, as their pathogenicity is applicable to numerous diseases. The canonical BMP pathway is extremely well conserved from invertebrates to vertebrates. Thus, we aimed to take advantage of Drosophila melanogaster to provide a relatively easy and rapid system to test human *BMPR1A* and *SMAD4* variant function. For example, genetic tools allow for controlled expression of any human variant in specific tissues or the whole organism. Assays can be as general as looking for lethality or can be targeted to specific genetic interactions in discrete tissues where previous studies have created excellent models for BMP signaling. This makes Drosophila a useful system for studying human variants in vivo.

### 1.1 Studying human variants in Drosophila melanogaster

The now routine exome sequencing that occurs in the clinic has created a need for efficient and robust experimental approaches for variant functionalization. Thousands of human exomes have been sequenced and variants are available at databases such as ExAC, gnomAD, COSMIC and ClinVar. However, it is difficult to interpret the functional consequence that may arise from many of these sequence variants (Lek et al. 2016; Karczewski et al. 2019). For example, a study of 2500 human genomes showed that the typical human genome differs from the reference genome at 4 - 5 million sites with about 500,000 sites in protein or gene regulatory regions. Each genome also contains 24 - 30 variants that are implicated in disease and have been listed on ClinVar (Gibbs et al. 2015). Looking at individual genes, 632 single nucleotide variants in *BMPR1A* and 636 single nucleotide variants in SMAD4 are listed on ClinVar (Landrum et al. 2016). This high variability in the human genome makes pinpointing disease-causing variants a daunting but critical challenge.

One way to predict whether a variant is deleterious is to use computational algorithms like PolyPhen2, SNAP2 and CADD. However, when current computational methods are compared to experimental data testing variant function, none are able to surpass 81% accuracy and are strongly biased towards categorizing a variant as pathogenic (Gnad et al. 2013; Stanley et al. 2014). When pathogenic variants identified by computational models were tested *in vivo*, as few as 20% showed a discernible phenotype, while 58% only showed an effect on protein function *in vitro* (Miosge et al. 2015). This discrepancy shows the importance of testing variants experimentally.

Therefore, there is an urgent, unmet need for fast, cost-effective, reproducible assays for human variant functionalization. *Drosophila melanogaster* allows for relatively quick screening *in vivo* of human variants, allowing for an experimentally-derived determination of pathogenicity. With 60% of human genes known to be linked to disease being conserved in flies, the majority of human disease genes and variants can be studied (Rubin et al. 2000). *Drosophila* offers a powerful system for studying human disease in an intact living organism, by virtue of its ease of maintenance, quick generation time, cost-effective use in the lab, capacity for high volume screening, and the many genetic tools accumulated over the past hundred years (Takano-Shimizu-Kouno and Ohsako 2018; Wangler et al. 2017; Wangler, Yamamoto, and Bellen 2015). Moreover, the high conservation of signaling pathways allows the use of *Drosophila* to screen gene variant impact on signaling. Even if the precise phenotype being explored is different; such as using *Drosophila* wing assays to precisely tease out the function of gene variants in the BMP pathway that have relevance to human cancer, the underlying function of a gene within its pathway is typically similar enough in different tissues and contexts for us to discriminate functional from non-functional variants (McGary et al. 2010).

Over the years, many genetic tools and models have been developed in *Drosophila*, two of which have been indispensable for this study. The yeast GAL4/UAS system has been adopted in flies as a binary expression system in which the GAL4 transcription factor is expressed in specific cells or tissues, and binds to UAS sites within a transgene to drive the expression of transcripts of interest (Duffy 2000; Brand and Perrimon 1993). This allows any gene to be expressed in a tissue specific manner, providing tremendous flexibility in the range of assays that can be performed. As a model, the developing wing imaginal discs and the mature wing have become well established systems for studying signaling pathways and genetic interactions (Beira and Paro 2016). Years of studies have identified how changes to different signaling pathways results in specific phenotypes within these discs. The power of using these tools and systems has been recognized by clinicians, resulting in the formation of growing partnerships between

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clinicians and model organism researchers around the world in order to study the pathogenicity of specific gene variants found in their patients (Oriel and Lasko 2018).

These collaborations have led to numerous studies looking at variants of disease linked genes in *Drosophila* to determine pathogenicity. A study looking at two variants in the *DNM1L* gene, involved in mitochondrial fission, showed that only one of the two variants was able to rescue lethality, when expressed in the absence of its *Drosophila* ortholog, *Drp1* (Y.-H. Chao et al. 2016). In another approach, that we term the mimetic approach, a mutation of interest found in a human gene is engineered into the identical amino acid in the orthologous fly gene. For example, variants in the human ATAD3C gene were made in the identical amino acids in the orthologous fly *bor* gene. Upon overexpression, the authors found that human-mimetic *bor* variants acted in a dominant negative manner, interfering with endogenous Bor protein activity to resemble a *bor* loss of function mutant (Harel et al. 2016). This provides evidence for how the human variant may function. Similar studies have been carried out on *TM2D3* and its role in Alzheimer's, as well as *EBF3* as a cause for a neurological disorder (H.-T. Chao et al. 2017; Jakobsdottir et al. 2016). These studies have established *Drosophila* as a useful and versatile model organism for the identification of pathogenic human variants.

### **1.2 BMP pathway in mammals**

BMP signaling ligands are members of the TGFβ superfamily of growth factors and are required for the formation and maintenance of many tissue types (Moustakas and Heldin 2009). As this thesis is primarily focused on BMP signaling, the other partially-overlapping branches of this pathway will not be further discussed here; see section 1.2.3.1 below for more details. The BMP family of ligands bind to specific combinations of type I and type II serine/threonine kinase

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cell surface receptors. Binding of the ligand increases the oligomerization of these two receptors types into hetero-tetramers containing two each of type I and type II receptors (Yamashita et al. 1994). The type II receptor then phosphorylates the GS domain of the type I receptor with its constitutively active kinase domain (Huse et al. 1999). Phosphorylation of the type I receptor activates its kinase domain allowing it to then phosphorylate R-Smads. R-Smads then bind with *SMAD4* and are trafficked to the nucleus where they act as transcription factors to regulate specific target genes (Heldin, Miyazono, and ten Dijke 1997).



**BMP** Pathway

Figure 1.1 Schematic of the BMP pathway in vertebrates.

A BMP ligand dimer (pink) binds to two type I and two type II receptors (green and yellow respectively). The type I receptor phosphorylates an R-Smad (dark blue) which then forms a complex with the co-Smad (light blue) and is trafficked to the nucleus where they regulate transcription. In vertebrates there are multiple genes for each component of the pathway except for SMAD4 which is the only co-Smad.

In vertebrates, there are 15 structurally related BMP ligands, three type II receptors, four type I receptors, three R-Smads and a single co-Smad, *SMAD4* (Katagiri and Watabe 2016) (Figure 1.1). All the components in this pathway have multiple synonyms, but for clarity the approved HGNC identifiers will be exclusively used in this thesis. The three type II receptors are *BMPR2, ACVR2A* and *ACVR2B*. The four type I receptors are *BMPR1A, BMPR1B, ACVRL1* and *ACVR1*. The three R-Smads are *SMAD1*,5 and 9. These each show different expression patterns, but many tissues will express multiple BMPs and multiple receptors leading to a combinatorial expression of ligand and receptor subtypes (Antebi et al. 2017). Therefore, knockout of different components of the BMP pathway in mice results in differing phenotypes in different tissues (Miyazono, Kamiya, and Morikawa 2010). Therefore, mutations in different components of the BMP pathway can result in diverse human diseases.

#### **1.2.1** BMP signaling in human disease

The BMP pathway acts in many tissue types leading to BMP-related human diseases that affect as many tissues. BMPs were first identified for their ability to induce bone formation in rodents (Urist 1965). They have since been linked to many diseases involving bone formation such as Fibrodysplasia ossificans progressiva which results in ectopic bone formation caused by a mutation in the BMP type I receptor, *ACVRI* (Shore et al. 2006). Another disease is

Acromesomelic dysplasia, a form of dwarfism involving the hands and feet and is caused by mutations in another type I receptor, *BMPR1B* (Graul-Neumann et al. 2014). Also, brachydactyly type A2, which involves a shortening of the middle phalanges, is caused by duplications involving a conserved regulatory element downstream of BMP2 (Dathe et al. 2009). Even within bone formation, mutations in different components of the pathway lead to different phenotypes in different areas of the body (Gomez-Puerto et al. 2019).

Bone is not the only tissue affected by mutations in the BMP pathway. BMP signaling plays an important role in cardiovascular function. Mutations in *ACVRL1*, are found in families linked with the autosomal dominant disorder hereditary haemorrhagic telangiectasia, which is characterized by direct connections between arteries and veins with the most common symptom being nosebleeds (Johnson et al. 1996). Interestingly some patients with mutations in *SMAD4* exhibit a combined syndrome of juvenile polyposis syndrome, involving polyps in the gastrointestinal tract, and hereditary haemorrhagic telangiectasia indicating a role for *SMAD4* in both tissues (Gallione et al. 2010). Gain of function mutations in *SMAD4* cause Myhre syndrome, characterized by short stature as well as many cardiovascular phenotypes (Lin et al. 2016). In another example of mutations in the same gene in the BMP pathway affecting different tissues, an antimorphic allele of *ACVR1* causes defects in the formation of the atrioventricular septum in humans (Smith et al. 2009). These examples show not only the importance of BMP signaling in cardiovascular function but also the range of human diseases associated with this one pathway.

Finally, the BMP signaling pathway is linked to numerous cancers. Juvenile polyposis syndrome leads to an increase in colon cancer. *ACVR1* mutations found in patients with

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fibrodysplasia ossificans progressiva have also been found in patients with diffuse intrinsic pontine gliomas (K. R. Taylor et al. 2014). Reduced expression of *BMPR1A* in pancreatic cancers results in increased proliferation and invasion causing a poor prognosis implying its role as a tumor suppressor (Voorneveld et al. 2013). However, deletion of *BMPR1A* impairs tumor formation and metastasis in conditional knockout mice, suggesting it may promote tumorigenesis (Pickup et al. 2015). BMP ligands show a similar phenomenon, where in some cancers they suppress tumor growth and metastasis and in other types of cancer they accelerate tumorigenesis (Bach, Park, and Lee 2018). This means that not only is it important to understand the effect a mutation has on components of the BMP pathway, but also what effect decreasing or increasing BMP signaling has on that specific type of cancer.

#### 1.2.1.1 Juvenile polyposis syndrome

This thesis focuses on establishing assays for two genes, *SMAD4* and *BMPR1A*. Function-altering mutations in these genes are causative in juvenile polyposis syndrome. This syndrome is characterized by the presence of multiple juvenile polyps in the gastrointestinal tract which leads to an increased risk for colon cancer (Brosens et al. 2011). It is an autosomal dominant syndrome with 40 - 60% of patients carrying a heterozygous germline mutation in either *SMAD4* or *BMPR1A*, with most identified mutations being point mutants (Huiying Ma et al. 2018; Calva-Cerqueira et al. 2009). Patients with mutations in *SMAD4* develop a more aggressive gastrointestinal phenotype and have a higher incidence of colonic adenomas and carcinomas than patients with mutations in *BMPR1A* (Friedl et al. 2002). This means that it is important to be able to identify which mutations in *SMAD4* and *BMPR1A* are pathogenic and cause this increased risk of cancer so that patients can be screened early for this disease. As with many cancers, surveillance and preventative measures improves outcome, so early detection is preferred. Experimental assays of variant function can form a valuable part of such detection efforts.

Very little is understood about the cellular mechanisms that cause these juvenile polyps. However, in mice it has been shown that inhibition of BMP signaling leads to the development of polyps phenotypically similar to those seen in juvenile polyposis syndrome (Haramis et al. 2004). Conditional inactivation of *BMPR1A* in mouse intestine causes an expansion of stem and progenitor cell populations due to lack of repression of Wnt signaling (X. C. He et al. 2004). It has also been shown that in patients with mutations in *SMAD4*, there is a loss of the second copy of *SMAD4* in the polyps (Woodford-Richens et al. 2000). However, work in mice showed that mice heterozygous for *SMAD4* developed polyps before the loss of heterozygosity occurred indicating that a decrease in *SMAD4* levels is sufficient for polyps to develop (Xu et al. 2000). Therefore, there is still a lot to be understood about the mechanisms involving *SMAD4* and *BMPR1A* in polyp formation and their contribution to tumor formation specifically in the gastrointestinal system and how levels of BMP signaling contribute to these phenotypes.

### 1.2.2 BMPR1A

*BMPR1A* is a type I serine/threonine kinase cell surface receptor in the BMP pathway. It contains an extracellular domain that binds BMP-2 and BMP-4 ligands: a single-pass transmembrane domain, a GS domain named for its string of glycines and serines, and a kinase domain that phosphorylates *SMAD1*,5,9 (ten Dijke et al. 1994; Keller et al. 2004; Hatta et al. 2000). Upon binding of the BMP ligand to the extracellular domain, *BMPR1A* can oligomerize with another type I receptor and two type II BMP receptors using the E6 loop in the kinase

domain (Huse et al. 1999). The GS domain of *BMPR1A* is then phosphorylated on multiple serine and threonine residues by the type II receptor. This alters the GS domain so that *SMAD1*,5,9 can bind, allowing for their phosphorylation by *BMPR1A*. A region in the kinase domain called the L45 loop, specifies which R-Smads can bind *BMPR1A* (X.-H. Feng and Derynck 1997). Mutations in these various domains can have differing effects on the function of the protein. (Yigong Shi and Massagué 2003) (Figure 1.2; Table 1)





#### Figure 1.2 Structure of BMPR1A.

BMPR1A is a single-pass transmembrane protein with an extracellular domain that binds ligands and an intracellular kinase domain that phosphorylates R-Smads (dark blue). The GS domain (light blue) is phosphorylated by type II receptors and activates BMPR1A's kinase domain. Red loops highlight important domains that are name here. The glycine rich loop is where phosphates from ATP bind. The L45 loop is involved is specifying which R-Smad can bind. The E6 loop is involved in binding with other receptors. Red stars are mutations whose effect on protein function have been assayed (Table 1.1).

Amino Acids Involved	Description	Reference
M1 – G23	Signal Peptide	("UniProt: A Worldwide Hub of
		Protein Knowledge" 2019)
Q24 – R152	Extracellular Domain	("UniProt: A Worldwide Hub of
		Protein Knowledge" 2019)
I85, E87, T95, F108	Ligand binding sites	(Hatta et al. 2000) (Mahlawat et
		al. 2012)
W153 – Y176	Transmembrane Domain	("UniProt: A Worldwide Hub of
		Protein Knowledge" 2019)
S205 – I234	GS domain: phosphorylated by	(Wieser, Wrana, and Massagué
	type II receptors	1995)
T229V	Cannot be phosphorylated by	(Wieser, Wrana, and Massagué
	type II receptor	1995)
Q235 – V525	Kinase Domain	("UniProt: A Worldwide Hub of
		Protein Knowledge" 2019)
G241 – G246	Glycine rich loop, involved in	(Hubbard et al. 1994)(S. S.
	anchoring phosphates of ATP	Taylor and Radzio-Andzelm
		1994)
G246D	Inactivates kinase activity	(Weis-Garcia and Massague
Wa(1D	<b>T</b>	1996)
K261R	Inactivates kinase activity	(Wieser, Wrana, and Massagué
C200E		1995)
G290E	4-fold less active kinase,	(Weis-Garcia and Massague
<u> </u>	phosphorylated less by type II	1996) (II
G300 – S301	L45 Loop: responsible for R-	(Huse et al. 1999)
T247 D256	Smad specificity	(11 ( 1 1000)
134/-P336	E6 Loop: Oligomerizes with	(Huse et al. 1999)
CONV. CIAID. CIAOD	other receptors	
1082 Y, $124$ K, $130$ K, $1470$ T	Affect receptor function when	(Akiyama, User, and Gibson
1014/01	assayed in the <i>Drosophila</i> wing	2018)
	disc	

Table 1.1	Critical	domains	and	tested	mutations	found	in	BMPR1A.
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Mouse studies have provided a wealth of insights into *BMPR1A*'s role in development and disease. The majority of studies have been performed using conditional knockouts as mice with a homozygous null allele of *BMPR1A* fail to form mesoderm and die by embryonic day 8.0 (Mishina et al. 1995). *BMPR1A* is expressed ubiquitously throughout mouse embryogenesis and in most adult tissues (Dewulf et al. 1995). Consistent with its broad expression, conditional knockouts have shown a role for *BMPR1A* in many different processes. During chondrogenesis, *BMPR1A* is expressed in the limb bud mesenchyme and has an overlapping function with *BMPR1B* in chondrocyte proliferation, survival and differentiation (Yoon et al. 2005). It inhibits intramuscular adipogenesis, differentiation and proliferation of hair follicles, is essential for tooth development and is important for kidney regeneration (P. Huang et al. 2014; Andl et al. 2004; Sugimoto et al. 2012). In summary, BMPR1A is a protein that is critical for the development of numerous human tissues, so it is not surprising that variants in this gene contribute to human disease.

#### 1.2.3 SMAD4

*SMAD4* is the co-Smad that complexes with all R-Smads. It forms a complex with two phosphorylated R-Smad molecules and is trafficked to the nucleus where it binds to regulatory elements of target genes to regulate their transcription (Zhao, Mishra, and Deng 2018; Kawabata et al. 1998). Importantly, Smads bind the genome in a cell type-dependent manner, which helps explain the differences in effect on tumors from different tissues (Morikawa et al. 2013; Budi, Duan, and Derynck 2017). This is largely because Smads bind DNA sequences weakly, and typically require additional, cell type-specific co-factors for high affinity DNA interactions (Schmierer and Hill 2007; Ross and Hill 2008). This also ensures Smads act as part of cell typespecific complexes that coordinate diverse, context-dependent gene expression profiles (Koinuma et al. 2009; X.-H. Feng and Derynck 2005). Mutations that inhibit binding with these transcription factors or decrease SMAD4's stability could alter how SMAD4 regulates gene expression in that cell.



#### Figure 1.3 Structure of SMAD4.

Dark blue boxes represent the MH1 and MH2 domains. The light blue box is a region that contain amino acids that when mutated affect DNA binding. The red loops show critical domains. The DNA binding motif contains amino acids that directly interact with DNA. The nuclear localization and export signals allow for trafficking back and forth from the nucleus. The SMAD4 activation domain is required for downstream transcription. Red stars indicate mutations whose effect on function has been studied. The red triangles are sumoylation sites. The green triangle is a ubiquitination site.

SMAD4 is comprised of two major domains, MH1 and MH2, that have an intervening disordered linker region. This linker region contains sites for modification by multiple pathways, a nuclear export signal, and the SMAD4 activation domain that is required for transcriptional activation through p300 co-activator binding (Massagué, Seoane, and Wotton 2005; de Caestecker et al. 2000). This nuclear export signal allows SMAD4 to be shuttled out of the nucleus but is inactivated upon binding with R-Smads leading to an enrichment of SMAD4 in the nucleus (Watanabe et al. 2000). There is also a nuclear localization signal in the MH1

domain from amino acids 45 – 110 (Xiao, Latek, and Lodish 2003). Both of these signals allows for the independent shuttling of SMAD4 in and out of the nucleus(Pierreux, Nicolás, and Hill 2000). There is also evidence that SMAD4 can be sumoylated at Lys-159 and Lys-113, which increases its stability (Lee et al. 2003). MH2 is responsible for protein interactions with other Smads as well as other transcription factors. As an example, MSG1, a nuclear protein with strong transcriptional activation function but no DNA binding capability, binds to SMAD4's MH2 domain (Shioda et al. 1998). There is a ubiquitination site at Lysine-519 that when ubiquitinated stops SMAD4 from binding with R-Smads regulating its activity (Dupont et al. 2009). Finally, MH1 is responsible for binding DNA, using an 11 residue beta hairpin which binds to the major groove of DNA (Y Shi et al. 1998). SMAD4 acts as a scaffolding protein and its function is dependent on its ability to bind DNA and other proteins therefore, mutations in different sites have the potential to affect different parts of its function. (Figure 1.3; Table 2)

Analysis of *SMAD4* mutants have led to an increased understanding of *SMAD4s* role *in vivo*. Homozygous *SMAD4* mutant mice die before day 7.5 of embryogenesis and fail to undergo gastrulation or form mesoderm, very similar to *BMPR1A* mutants. *SMAD4* is expressed throughout embryogenesis and in all adult tissues (Sirard et al. 1998). Interestingly, mice that are heterozygous for *SMAD4* develop polyps similar to that seen in juvenile polyposis syndrome (Weinstein, Yang, and Deng 2000). Heterozygous or homozygous deletion of *SMAD4* is seen in a variety of human cancers, such as cholangiocarcinoma and colorectal cancer where *SMAD4* expression is lost 30% of the time. In many cases, *SMAD4* contributes to tumorigenesis through loss of regulation of its downstream target genes (Zhao, Mishra, and Deng 2018). Similar results have been shown in *SMAD4* conditional knockout mice where loss of SMAD4 leads to the

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development of carcinomas (Yang and Yang 2010). This shows that loss of SMAD4 plays an important role in tumorigenesis, often through its impact on gene regulation.

Amino Acids Involved	Description	References
S18 - D142	MH1 Domain	("UniProt: A Worldwide Hub
		of Protein Knowledge" 2019)
L43 – R135	DNA binding domain: required to	(Jones and Kern 2000)
	bind DNA	
L43S, R100T	Reduced translocation to the nucleus	(Morén et al. 2000)
K45 - K110	Nuclear localization signal	(Xiao, Latek, and Lodish
		2003)
L78 – F90	DNA binding motif: $\beta$ -hairpin	(Y Shi et al. 1998)
K113, K159	Sumolyation sites: increases stability	(Lee et al. 2003)(Miles et al.
	when sumolylated	2008)
S138 - Q149	Nuclear export signal	(Watanabe et al. 2000)
A274 - E321	SMAD4 activation domain: required	(de Caestecker et al. 1997)
	for expression of downstream genes	
W323 – D552	MH2 Domain	("UniProt: A Worldwide Hub
		of Protein Knowledge" 2019)
D351H	Stops interactions with SMAD3	(Chacko et al. 2001)
D351H, R361C,	Stops binding with SMAD2	(Y Shi et al. 1997)
V370D		
1500T, 1500V, 1500M	Found in Myhre patients: causes a	(Le Goff et al. 2012)
	stabilization of the protein but	
	decreased expression of downstream	
	genes	
G508S, D537E	Stops binding with SMAD2	(Y Shi et al. 1997)
R515S	Reduces interactions with SMAD3	(Chacko et al. 2001)
K519	Ubiquitination Site that when	(Dupont et al. 2009)
	ubiquitinated stops interactions with	
	SMAD2	
D537E	Stops interactions with SMAD3	(Chacko et al. 2001)

Table 1.2 Major domains and functional mutations found in SMAD4.

### 1.2.3.1 SMAD4 and the activin pathway

The TGF $\beta$  pathway has two major branches. One branch is the BMP pathway; the other is the activin pathway. (Figure 1.4) These two branches are primarily characterized by the different R-Smads that are phosphorylated by the type I receptors. BMP pathway results in the phosphorylation of SMAD1,5,9, while the activin pathway phosphorylates SMAD2,3. The pathway is characterized this way because the type I receptors are not specific to each pathway. For example, while TGFBRI normally phosphorylates SMAD2,3, in some cell types it is able to phosphorylate SMAD1 (Wrighton et al. 2009). Both pathways are also able to share type II receptors (F. Huang and Chen 2012). These two branches often have opposing functions in a tissue and the pathway with the stronger signaling is theorized to be the one that can recruit more SMAD4, the limiting factor for downstream transcriptional regulation (Sartori et al. 2013; M.-J. Goumans et al. 2002; Candia et al. 1997). There is also evidence that type I receptors from both pathways can form a complex together and that the type I receptor from the BMP pathway, requires the type I receptor from the activin pathway to function (M. J. Goumans et al. 2003). Taken together, this means that loss or reduction of SMAD4 can affect both signaling pathways however, the connections between these two signaling branches is often not well understood in most tissues.

As with its BMP counterpart, the activin pathway plays a role in many processes such as cell proliferation, differentiation, organogenesis, tissue homeostasis as well as tumor progression (Liu et al. 2016). BMP and activin pathways are antagonistic in the regulation of muscle mass with myostatin, an activin ligand, negatively regulating muscle growth and BMPs acting as a positive regulator (Sartori, Gregorevic, and Sandri 2014). The activin pathway also plays an important role in stem cell maintenance and differentiation along with the BMP pathway (Pauklin and Vallier 2015). These two branches of the TGF $\beta$  family often function antagonistically through many points of interaction with SMAD4 being a common component to both.

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Figure 1.4 The two major branches of the TGF<sup>β</sup> pathway in vertebrates.

The two branches share many components, including SMAD4, with only the RSMADs belonging to a single branch. As with the BMP pathway, there are multiple genes for each component in the activin pathway. There are many points of interaction between these two pathways that are not well understood.

Two SMAD2,3 form a trimeric complex with one SMAD4 molecule. SMAD3 can bind DNA but SMAD2 cannot, so SMAD2/4 complexes are believed to bind DNA solely through SMAD4 (X.-H. Feng and Derynck 2005). Interestingly, SMAD3 is able to compensate for SMAD4 mutants that are unable to bind DNA meaning that these SMAD4 mutants will not act like nulls in the activin pathway (Morén et al. 2000). This makes looking at the phenotypes of SMAD4 mutants complicated because not only is it unclear which pathway is being affected, but also what effect the mutation will have on the function of the protein.

A number of proteins that bind SMAD4 have been identified with relation to the activin pathway. Some examples include an inhibitor of the activin pathway, DLX1, which is able to bind SMAD4 and plays a role in differentiation of hematopoietic cell lines (Chiba et al. 2003). SMAD4 is also able to bind Sp1, a transcription factor involved in cell cycle inhibition. Signaling through Sp1 may be another reason why loss of SMAD4 frequently results in cancer (X. H. Feng, Lin, and Derynck 2000). The MH1 and linker region of SMAD4 can bind ERalpha and act as a co-repressor on estrogen-responsive elements (Wu et al. 2003). Yin Yang 1, a nuclear factor, binds SMAD4's MH1 domain and stops it from binding its DNA sites decreasing signaling from both the BMP and activin pathway (Kurisaki et al. 2003). Mutations that affect these binding partners could have specific effects on SMAD4s downstream signaling in a cellspecific and variant specific manner.

### 1.3 BMP pathway in Drosophila

The BMP pathway is well conserved between humans and *Drosophila* and it functions in much the same way as it does in vertebrates, with ligand binding to serine/threonine kinase transmembrane receptors which phosphorylate an R-Smad protein that couples with the co-Smad and moves to the nucleus to regulate transcription. There are 3 major BMP ligands in *Drosophila*: Decapentaplegic (Dpp), Screw and Glass bottom boat (Gbb). There are 2 type II receptors, Wishful thinking and Punt, as well as 2 type I receptors, Thickveins (Tkv) and Saxophone (Sax). Sax is broadly expressed while Tkv is expressed in restricted dynamic patterns during development, although there is functional overlap between the two (T. J. Brummel et al.
1994). These receptors phosphorylate the single BMP R-Smad, Mothers against dpp (Mad), which forms a complex with the single co-Smad, Medea. (Aidan J Peterson and O'Connor 2014). The smaller number of proteins combined with the similarity in pathway architecture makes *Drosophila* an excellent system for studying how changes to individual components affect signal output (Figure 1.5).







The pathway is conserved between *Drosophila* and vertebrates but with fewer genes in *Drosophila* making it a simpler genetic system to study. Vertebrate protein names are in grey and *Drosophila* protein names are in black.

As in vertebrates, BMPs in *Drosophila* are involved in developmental patterning of the embryo, as well as stem cell function and regulation (Hamaratoglu, Affolter, and Pyrowolakis 2014). There are two classic phenotypes used for studying BMP signaling in *Drosophila*. First, Dpp is required for establishing embryonic dorsal-ventral patterning of the early blastoderm. This model is useful when studying mutants in the pathway that do not survive past this point (Irish and Gelbart 1987). Secondly, both Dpp and Tkv are required for growth and proliferation of the imaginal disc that forms the wing (Burke and Basler 1996). Imaginal disks are easily accessible, genetically tractable systems to study patterning of embryonic lethal genes and the BMP pathway has been extensively studied in the wing disc (Beira and Paro 2016). These two, well characterized, developmental processes allow for the study of new potential genetic interactions in the pathway, as well as characterizing mutations in known components.

#### 1.3.1 Thickveins

Thickveins is the type I receptor that shows the highest sequence identity to BMPR1A (Newfeld, Wisotzkey, and Kumar 1999). Tkv is required for dorsal-ventral patterning, followed by dorsal closure, as well as for patterning of the visceral mesoderm during embryogenesis. This makes *tkv* null mutants embryonic lethal (Affolter et al. 1994). Tkv has functions in many other tissues, one of which is the maintenance of germline stem cells. BMP ligands from cap cells signal to germline stem cells to stop differentiation; however, one cell away from dividing stem cells, daughter cystoblasts begin differentiating due to loss of BMP signaling. This quick loss of signaling is accomplished through phosphorylation of Serine-238 of Tkv by Fused, a serine/threonine kinase, targeting it for degradation through ubiquitination by the E3 ligase Smurf (Xia et al. 2010). Another way that Tkv regulates BMP signaling is through its ability to

inhibit long range Dpp diffusion. Tkv is frequently expressed at low levels near sources of Dpp. This allows Dpp to diffuse further away from its source as Tkv acts to sequester BMP ligands. If Tkv is expressed at high levels, then Dpp cannot diffuse past it, creating a border. In places where Tkv levels have to be low to allow diffusion, BMP signaling can be enhanced through Sax being activated by Gbb (T E Haerry et al. 1998). These studies show that protein levels of Tkv are critical to BMP signaling levels.

#### 1.3.2 Medea

Medea is the *Drosophila* ortholog for SMAD4 and is required for embryonic dorsalventral patterning (Wisotzkey et al. 1998; Das et al. 1998). *Medea* mutants are larval/pupal lethal, lack obvious imaginal discs and have central nervous systems that are reduced in size (Raftery et al. 1995). *SMAD4* mRNA injected into *Medea* mutants is able to rescue dorsal-ventral patterning in the embryo, demonstrating a high degree of conservation of protein functionality from *Drosophila* to humans (Hudson et al. 1998). Medea can also be sumoylated, like SMAD4, causing it to be exported from the nucleus resulting in decreased BMP signaling (Miles et al. 2008). These experiments, along with their high amino acid identity and orthologous function, show that SMAD4 is not only conserved with Medea, but that human SMAD4 is likely functional in *Drosophila*.

The activin pathway is also present in *Drosophila*, and Medea acts as the only co-Smad for this pathway, as in vertebrates. In *Drosophila*, there are four ligands: Activin- $\beta$ , Dawdle, Myoglianin and Maverick. There is only a single activin type I receptor, Baboon. The single R-Smad for this pathway is Smox. The type II receptors are shared between the two pathways, as they are in vertebrates. (Figure 1.6) Similar to that seen in vertebrates, the activin and BMP pathways can work in parallel to regulate disparate processes, such as in the specification of R7 and R8 photoreceptors (Wells et al. 2017). Just as occurs in vertebrates, interactions between these two pathways can make the interpretation of phenotypes difficult to assign to a single pathway.





Both pathways are conserved from *Drosophila* to vertebrates. There are fewer genes in the *Drosophila* pathways but their interactions are very similar to that seen in vertebrates. Vertebrate protein names are in grey and *Drosophila* protein names are in black.

To study the activin pathway, many studies have looked at *baboon* mutants to see which processes are affected. Baboon is not required during embryogenesis as *baboon* mutants die during late larval to early pupal stages due to reduction of cell proliferation within the primordia for adult structures (T. Brummel et al. 1999). Baboon is also involved in regulating neuroblast number and proliferation rates in the developing larval brain (Zhu et al. 2008). It is also required for the ecdysone release that triggers metamorphosis (Gibbens et al. 2011). Baboon is the only type I receptor with the type II receptor, Punt, that can phosphorylate Smox when bound to Activin- $\beta$  or Dawdle but, it can also phosphorylate Mad when bound to any of the four activin ligands, similar to what has been seen in vertebrates (Gesualdi and Haerry 2007; Wrighton et al. 2009). The activin pathway appears to mostly be involved in proliferation, however Baboon's ability to act on the BMP pathway makes it difficult to tease the two pathways apart.

#### 1.4 Drosophila wing development

The *Drosophila* lifecycle starts with embryogenesis, proceeds through three larval stages, then pupariation where it undergoes metamorphosis before finally eclosing as an adult fly. The adult wing is formed from tissue that is put aside during embryogenesis and becomes the wing imaginal disc. It grows and becomes patterned in the developing larvae. The wing disc is a flat disc-like epithelial structure that proliferates during growth of the larvae. As with many epithelial sheets, the wing disc is patterned dorsal to ventral by the Wingless morphogen gradient and anterior to posterior by expression of engrailed (Neumann and Cohen 1997; Zecca et al. 1995). During pupariation, the larval wing disc everts so that the dorsal and ventral edges of the wing pouch zipper together leading to a two-layer flat tissue. The wing pouch then elongates into a flat wing blade and continues to grow in size. As it grows, it undergoes a number of phases where



Figure 1.7 Overview of wing morphogenesis.

The precursor of the wing (the wing imaginal disc) contains two different territories that will give rise either to the adult wing blade (wing pouch, green), or the hinge and part of the notum (brown). During larval stages, the monolayer epithelium that forms the precursor of the wing blade exhibits A-P and the D-V compartment boundaries, oriented cell division, and tissue stretching by different division rates. At the beginning of metamorphosis and as a result of eversion of the wing pouch, the wing blade consists of two epithelial layers facing each other at their basal surfaces (apposition). From 7 h APF, the developing wing undergoes expansion, elongation, separation and re-apposition of both epithelial sheets. Contraction of the wing hinge leads to a global force pattern that induces oriented cell division and cell rearrangement to reshape the wing to its 'definitive stage' at 40 h APF. Features such as veins and hairs also form by 40 h APF. Finally, after eclosion of the adult fly, the folded wing spreads out due to fluid filling of the veins and the intervein epidermis is removed resulting in an adult wing composed mostly of cuticle. (Figure from (Diaz de la Loza and Thompson 2017))

the two layers peel apart and come back together, allowing for the formation of veins and the migration of cells. It continues to grow curling in on itself due to the lack of space in the pupal case. After eclosion, fluid fills the wings, allowing them to expand before most cells die off leaving the structure of the fully formed adult wing (Aldaz, Escudero, and Freeman 2010). (Figure 1.7)

One of the most visible features of the wing are the veins, which are ectodermal tubes strengthened with a thicker cuticle. There are at least five signaling pathways that converge to pattern these veins: Hedgehog signaling, BMP signaling, epidermal growth factor receptor signaling, Wnt signaling and Notch signaling. Of the veins important for this study, there are five main longitudinal veins (L1-5) that run proximodistally and two cross veins that bridge anterior-posteriorly between L3-L4 (ACV) and L4-L5 (PCV) (Blair 2007)(Figure 1.8). The pattern of

these veins offers an easy assay to assess how wing patterning has been perturbed by changes in signaling pathways.



Figure 1.8 Annotation of veins in the Drosophila wing.

Image of a female wildtype right wing showing stereotypical vein patterning. L: Longitudinal Vein, ACV: anterior crossvein, PCV: posterior crossvein.

## 1.4.1 BMP signaling during wing development

The wing imaginal disc has become one of the classic models to examine BMP signaling and patterning. *Engrailed* is expressed in posterior cells of the wing disc and governs the expression and release of Hedgehog. Hedgehog is a short-range ligand that activates Dpp in cells that do not express *engrailed*, resulting in a strip of cells at the posterior-anterior boundary that express the long-range ligand Dpp. Dpp creates a morphogen gradient along the posterioranterior axis affecting cells differently depending on whether they express hedgehog or not (Zecca et al. 1995). This morphogen gradient is what determines anterior and posterior domains in the wing disc. Dpp does this by establishing a gradient of BMP signaling across the tissue. Different genes are activated or repressed depending on the level of BMP signaling they respond to. Examples of this are *bifid*, which is expressed across the entire wing blade region as even low levels of BMP signaling can activate its expression. In contrast, *spalt* requires high levels of BMP signaling to be expressed and therefore, is only expressed in a small strip near where *dpp* is expressed (Nellen et al. 1996). The gene *brinker* has the opposite pattern where it is inhibited by high levels of Dpp signaling causing it to be expressed on the anterior-posterior edges of the wing disc (Müller et al. 2003). This means that if the BMP signal gradient is disrupted then the cell boundaries that express these downstream target genes will also change and this will affect wing patterning.

The wing disc contains many opposing gradients. Tkv expression is negatively regulated by Dpp and Hedgehog signaling causing low levels of Tkv where Dpp is expressed and higher levels at the lateral regions of the disc (Tanimoto et al. 2000). Tkv levels influence the distance that Dpp can travel, with higher levels decreasing the distance (Lecuit and Cohen 1998). This implies that Dpp negatively regulates Tkv to keep receptor levels low in areas with high levels of Dpp so that Dpp can migrate further. In areas further away that have lower levels of Dpp there are higher levels of Tkv making it more sensitive to Dpp signaling. It is important for patterning that BMP signaling levels follow a specific gradient and that is why it is tightly regulated. Alterations to this signaling results in wing defects (Ramel and Hill 2012).

BMP signaling in the wing disc occurs through more than a single ligand and receptor. A second BMP ligand, Gbb, works synergistically with Dpp to augment BMP signaling (Ray and Wharton 2001). The other type I receptor, Sax, is expressed ubiquitously in the wing disc (T. J.

Brummel et al. 1994). Gbb has a high affinity for Sax but mutations in Gbb and Sax do not cause the same phenotype, meaning that Gbb may also be able to signal through Tkv (Ray and Wharton 2001). This led to studies that showed that Sax and Tkv can form complexes together and that Tkv must be present in those complexes for downstream BMP signaling activation. This means that Sax can increase BMP signaling but, by itself, Sax may sequester Gbb making it act as an antagonist to the BMP pathway (Bangi and Wharton 2006; Theodor E. Haerry 2010). This dual role of Sax means its function will be affected by levels of both Tkv and Gbb in the wing disc. (Figure 1.9)

After the initial patterning of the wing disc, finer patterning of the veins occurs. Ectopic expression of Dpp in the wing is sufficient to induce ectopic veins showing that BMP signaling is important in vein formation (Celis de 1997). Gbb is also required for vein formation with *gbb* mutants showing a loss of the PCV, distal regions of L4 and L5 and often the ACV (Wharton et al. 1999). *Dpp* mutants show a similar phenotype with the distal region of L4 and L5 as well as the PCV missing. In contrast to this, *tkv* mutants show ectopic vein formation and thicker veins. This could be because *Dpp* and *tkv* negatively regulate each other. There are higher levels of Dpp in the veins so they can differentiate but Tkv expression on the edges of the vein area stops Dpp signaling outside of the vein area (Celis de 1997). To corroborate this, ectopic expression of a constitutively activated version of Tkv also causes ectopic vein formation showing that BMP downstream signaling leads to vein formation (Sotillos and De Celis 2005). Depending on how components of the pathway are perturbed, they can result in a loss or increase in veins in the wing.



Figure 1.9 Location of signaling proteins in the Drosophila wing imaginal disc.

Schematic of late L3 wing disc. The wing pouch is the circle on the bottom of the wing disc. The stripe in the middle is where Dpp is expressed. Red color indicates location of each of the proteins.

Patterning of the crossveins occurs separately from the longitudinal veins. BMP is active in the crossveins, and crossveins are sensitive to reductions in *Dpp* or *gbb* (Conley et al. 2000). Longitudinal vein patterning first begins to appear 4 - 8 hrs after pupariation while the wing blade is elongating, meanwhile the ACV and PCV are not visible until 19 - 22 hrs after pupariation (Conley et al. 2000). This is because Dpp from the longitudinal veins supplies the signal for crossvein formation (Ralston and Blair 2005). If signaling in the longitudinal veins is perturbed than it will also affect the crossveins which is why loss of the crossveins frequently occurs if part of the longitudinal veins is lost.

#### 1.4.2 Activin signaling during wing development

In the wing disc, the activin pathway is required for cell proliferation and is expressed in all wing disc cells (T. Brummel et al. 1999; Hevia and de Celis 2013). As in other tissues, there is crosstalk between the activin and BMP pathways. Smox mutants have drastically widened wing imaginal discs similar to that seen with increases in BMP signaling. This occurs through interactions with the BMP pathway, and does not occur in baboon mutants (A. J. Peterson and O'Connor 2013). Instead, loss of Smox causes Baboon to phosphorylate Mad leading to increased phosphorylated Mad levels in the wing disc and ectopic vein formation (Aidan J Peterson et al. 2012). Interestingly, human SMAD3 overexpression in the Drosophila wing causes a loss of veins. This shows that human Smads can function in Drosophila, and also, that increasing levels of human activin Smads causes loss of BMP signaling phenotypes (Sander et al. 2010). In fact, human and Drosophila orthologs of Smads show similar phenotypes when expressed in Drosophila, while human SMAD2 and SMAD3 showed very distinct phenotypes (Marquez et al. 2001). Punt, the shared type II receptor also appears to be limiting during this process. Baboon decreases BMP signaling through sequestration of this shared receptor (Hevia and de Celis 2013). So, while the activin pathway itself is required for proliferation, its interactions with the BMP pathway can lead to wing vein phenotypes.

#### 1.5 Calpain, Cortactin and axon consolidation

#### **1.5.1** Axon consolidation and human disease

The regulation of neuron morphology during development and throughout the lifetime of the organism is critical to the health of the nervous system. Axon consolidation is the final step of development of the axon. After filopodia have extended the axon and microtubules, vesicles and organelles have entered the filopodia, then the actin filaments depolymerize allowing the cell membrane to shrink around the microtubule frame resulting in consolidation (Dent and Gertler 2003). In order for a new axon branch to form, the Arp2/3 complex branches actin away from the main axon, and that new branch also consolidates (Kalil and Dent 2014). Differences in neuron morphology have been seen in numerous neurological disorders such as autism spectrum disorder, mental retardation, psychosis and aging (Hutsler and Zhang 2010; Kaufmann and Moser 2000; Crayton and Meltzer 1979; Tank, Rodgers, and Kenyon 2011). This shows that regulating the morphology of neurons is critical for the health of the nervous system. Therefore, studying proteins such as Calpain and Cortactin, which are known to regulate the cytoskeleton, can lead to increased understanding of these disorders (Chan and Mattson 1999; Ammer and Weed 2008).

## 1.5.2 Calpain

Calpains are calcium-dependent cysteine proteases that are ubiquitously expressed and can cleave hundreds of different proteins (Goll et al. 2003). Loss of Calpain function causes a reduction in working spatial memory and Calpains have been implicated in neural plasticity (Olson, Ingebretson, and Harmelink 2015). In contrast, Calpain function can be detrimental to

neuronal health; after traumatic brain injury they contribute to behavioral deficits and cell death (Saatman, Creed, and Raghupathi 2010). There are four *Calpain* genes in *Drosophila*, with two resembling typical *Calpains* (Friedrich, Tompa, and Farkas 2004). Calpains in *Drosophila* play a role in dendritic pruning and border cell migration in egg chambers (Kókai et al. 2012; Kanamori et al. 2013). The studies already performed in *Drosophila* and the small number of *Calpain* genes makes *Drosophila* an excellent model for studying the role of Calpain in axon branching and consolidation.

## 1.5.3 Cortactin

One of the substrates of Calpain is Cortactin (C. Huang et al. 1997; Perrin, Amann, and Huttenlocher 2006). Cortactin is a monomeric protein that binds and activates the Arp2/3 complex, promoting actin nucleation and stabilizing actin branch points (Weaver et al. 2001). Cortactin is able to interact with the Arp2/3 complex, actin and numerous other proteins through its SH3 domain (Katsube et al. 1998; Ammer and Weed 2008). In *Drosophila, Cortactin* mutants are viable and fertile but do show defects during oogenesis (Somogyi and Rørth 2004). A role for Cortactin in activity-dependent modification of synapse structure has also been identified (Alicea et al. 2017). Previous work in the lab has shown that loss of Calpain function or an increase in Cortactin protein levels can cause branching along a consolidated axon (Mingorance-Le Meur and O'Connor 2009). The next step is to identify if Calpain and Cortactin function can affect axon branching in *Drosophila*.

#### 1.6 Thesis objectives

There is a clinical need to develop rapid assays that can determine pathogenicity of human variants. While sequencing technologies now provide a wealth of variants, the ability to interpret the function of these variants has not advanced accordingly. In addition, *in silico* models have proven unreliable and have too high a rate of false positives to be relied upon in a clinical setting, they also fail to consider compensatory effects that exist *in vivo*. *Drosophila* offers an excellent system to examine the effect of mutations in the genes of conserved pathways. This thesis looks at two genes in the BMP pathway, *BMPR1A* and *SMAD4*, or *tkv* and *Medea* in *Drosophila*. The BMP pathway has been well characterized in the development of the wing, making it an excellent model for studying mutations in members of the BMP pathway. The goal of this thesis was to create quick assays to determine the impact of human *BMPR1A* and *SMAD4* missense mutations *in vivo*.

In order to create this assay, a system had to be identified that could show activity levels of both the BMPR1A and SMAD4 protein variants. Overexpression assays can be accomplished quicker than rescue experiments because after integration of the UAS human gene into the *Drosophila* genome, it can be crossed immediately to a panel of GAL4 line and assayed for phenotypes in a large number of diverse assays. With overexpression studies, ideally there should be a phenotype when the functional wildtype (WT) human gene is expressed, but one must also calibrate the system with known or predicted loss of function mutations that should have no effect or reduced effect when overexpressed. Using this approach, I established two assays to examine the function of human *SMAD4* gene variants. First, overexpression of *UAS-hSMAD4* using a ubiquitous GAL4 driver caused pupal lethality at 18°C while the loss of

function mutation remained viable. The second assay was overexpression of *UAS-hSMAD4* with *engrailed*-GAL4 (*en-GAL4*) that is expressed in the posterior region of the wing disc. At 18°C, hSMAD4 expression caused a loss of the distal region of veins L4 and L5 as well as loss of the ACV and PCV while the loss of function mutation had no effect on vein patterning. Using both assays, human variants identified from patients with juvenile polyposis syndrome that have unknown pathogenicity were assayed. The *BMPR1A* gene proved more challenging. Overexpression of the human *BMPR1A* gene in *Drosophila* had no effect, when driven with many GAL4 drivers. Therefore, I created orthologous, mimetic, mutations in *tkv* and created a chimeric Tkv<sub>EC</sub>::BMPR1A<sub>IC</sub> protein that will be tested for their utility in the study of human variants in the *BMPR1A* gene in *Drosophila*.

Lastly, to examine the impact of Calpain and Cortactin on axon consolidation I created *Calpain* double mutants and discovered that they had no gross morphological defects and were viable. I also created a *Cortactin* transgene that could not be cleaved by *Calpains*, in order to study whether Calpain regulates Cortactin directly through cleavage. I also studied the effect of the loss of Calpains and the overexpression of Cortactin on axon branching in both the central and peripheral nervous system and did not find they caused additional branching in *Hugin* expressing neurons or at the neuromuscular junction.

## **Chapter 2: Materials and Methods**

#### 2.1 Fly genetics and stocks

Strains used: *w;da-GAL4* (Bloomington stock # 55849), *P(GAL4)A9* (Bloomington # 8761), *w;UAS-Medea* (a gift from Esther Verheyen), *w;en-GAL4,UAS-RFP/Cyo* (Gaumer et al. 2000), *w;apterous-GAL4,UASnlsGFP/Cyo,wg* (O'Keefe, Thor, and Thomas 1998), *w;;dpp-GAL4/TM6B* (Bloomington stock #7007). *w;patched-GAL4/Cyo, Frizzled3-dsRed/TM6B, UAS-Dicer2;nubbin-GAL4* (gifts from Ester Verheyen). and, *UAS-Tkv, w;UAS-Tkv\_DN;UAS-Tkv\_DN* (a gift from Justin Kumar).

## 2.2 Human variant transgenic construction

The *hBMPR1A* and *hSMAD4* cDNA sequences were obtained from Dharmacon (Clone ID 4824378 and 2961238 respectively). cDNA sequences were PCR-amplified from these vectors with primers that add the Cavener sequence to the 5' end of the ATG start site (Cavener 1987) (Table 2.1). An additional TAA stop sequence was added to the 3' end along with an additional G to assist with insertion into the plasmid using the pCR8/GW/TOPO TA Cloning Kit (Thermo Fisher). Site directed mutation of the human variants in the TOPO plasmid was done using the Q5 site-directed mutagenesis kit (NEB) (Table 2.1).WT cDNA and human variant sequences were then transferred to the pUASgAttB plasmid using the Gateway cloning LR reaction (Bischof et al. 2013). These pUASgAttB plasmids were injected into a *Drosophila* line with the *attP2* locus, allowing for integration of the transgenes on the third chromosome by phiC31-integrase (performed by Rainbow Transgenics Inc, CA). Separate lines were isolated and sequenced to verify insertion. attP2 control line is from a line that did not integrate a transgene.

Primer Name	Primer Sequence	
BMPR1AtopoF	CAACATGCCTCAGCTATACATTTACATCA	
BMPR1AtopoRg	GTTATCAGATTTTTACATCTTGGGATTC	
SMAD4topoF	CAACATGGACAATATGTCTATTACGAATACA	
SMAD4topoRg	GTTATCAGTCTAAAGGTTGTGGGTCTG	
BMPK261RmutF	GTGGCGGTGAgAGTATTCTTTAC	
BMPK261RmutR	TTTTTCGCCACGCCATTT	
BMPE502KmutF	GCTAATGTCAaAATGCTGGGC	
BMPE502KmutR	TTCAAAACTGCTCGTAGAC	
SMADRQKmutF	GCTGGTCGGGCAGGATTTCCTCATGTGATC	
SMADRQKmutR	CACCAGAAGCGCCCCATCCAATGTTCTCTG	
SMADOligmutF	CAACTCTCCAATGACCACAGGACAGAAGCCAT	
SMADOligmutR	ACCCAAACAAAAGCAATCTCCTCCAGAAGGGTC	
SMADI525VMutF	ACCTTGCTGGgTTGAAATTCAC	
SMADI525VMutR	GTTTCTTTGATGCTCTGTC	
SMADT59AMutF	TTCTTTAATAgCAGCTATAACTACAAATG	
SMADT59AMutR	TCCAATTCATCTTTTTTCTCC	
SMADI40VMutF	AAAAAGAGCAgTTGAAAGTTTGG	
SMADI40VMutR	GCAAATGTTTCACTCTCTC	
SMADG336RMutF	TGTTCAGGTAcGAGAGACATTTAAG	
SMADG336RMutR	TCCATTTCAAAGTAAGCAATG	

SMADC324RMutF	TGAGTATTGGcGTTCCATTGCTTAC
SMADC324RMutR	GGAGCAGGATGATTGGAAATG
SMADG386DMutF	ATAGGCAAAGaTGTGCAGTTG
SMADG386DMutR	GTGCAACCTTGCTCTCTC
SMADP292LMutF	CAGCACCACCtGCCTATGCCG
SMADP292LMutR	AAGATGGCCGTTTTGGTGGTG

Table 2.1 List of primers used to create transgenes.

Topo primers were used for PCR-amplifying the WT cDNA for BMPR1A and SMAD4. Mut primers were used for site directed mutagenesis to create the human variants.

#### 2.3 Lethality assay

Five virgin females from *w;;da-GAL4* were crossed to three males from each genotype balanced by *TM3*, *Ser*, *Sb*, *GMR-Dfd-EYFP*. Flies were left to mate at 18°C for one day before being flipped into fresh vials. They were then left to lay eggs for two days. The adults were then cleared, and the progeny was left to develop at 18°C. Six vials were prepared for each genotype. When black wing pupae began to form, each pupa was counted using the GFP fluorescence to identify whether the pupae contained the balancer or the transgene. From the first day of eclosion until ten days later, adult flies were counted daily and the Sb marker was used to differentiate flies with the balancer or the transgene. Percentages were calculated using the total number of flies carrying the transgene divided by the total flies in each vial. Counts for a vial were only kept if there was a minimum of 20 balancer carrying flies and a maximum of 100 balancer carrying flies. Statistics were performed using GraphPad Prism. Percentages were compared using one-way ANOVA and *post-hoc* Tukey testing.

#### 2.4 Wing phenotype assay

Adult flies 1-3 days old were soaked in isopropanol. Wings were then removed in isopropanol and placed into Canada balsam (Sigma Aldrich) on a slide. A coverslip was placed on top and the slides were left overnight with weight on top of the coverslips. Wings were then imaged using brightfield imaging on a Zeiss Axioplan 2 with a 5X lens. Ectopic veins were scored by eye. Analysis of vein loss and image stitching was performed in Fiji. Vein loss and vein distance was measured using the measure feature of Fiji (Figure 2.1). Images were stitched together using the Pairwise Stitching plugin. Statistics were performed using GraphPad Prism. Percentages were compared using one-way ANOVA and *post-hoc* Tukey testing.



#### Figure 2.1 Method used to measure vein loss.

(A) Female wing from 1 – 3 day adult expressing UAS-hSMAD4\_WT driven by en-GAL4. Veins are labelled
(L) Longitudinal veins (ACV) Anterior Crossvein (PCV) Posterior Crossvein. (B) Total distance measured as the total vein. (C) Total distance measure as the missing vein. Percentage is then calculated by dividing C / B.

## 2.5 Immunofluorescence

Wing discs were dissected from wandering L3 larvae maintained at 18°C in phosphatebuffered saline (PBS). Discs were fixed in 5.3% Paraformaldehyde for thirty minutes. They were then blocked with 5% donkey serum in PBS + 0.3% Triton X-100 for one hour at room temperature. Discs were then incubated at 4°C overnight with 1:100 dilution of Rabbit  $\alpha$ - pMad (41D10, Cell Signaling Technologies). The secondary antibody donkey anti-rabbit Cy5 (1:750; Jackson ImmunoResearch) was incubated with the discs for 1 hour at room temperature and then were mounted onto slides. Images were taken on a Zeiss Axio Imager.Z2 with a 20X lens using the same settings. Intensity projections were measured using Zen blue software.

# Chapter 3: Creation of assays to test pathogenicity of SMAD4 human variants

## 3.1 Synopsis

One of the goals of this thesis was to create assays that would determine the function of human SMAD4 variants in a fly in vivo model. To this end, I created a series of human SMAD4 variants by site-directed mutagenesis and placed these into a transgenic vector for genomic integration into attP sites and inducible expression in flies under the control of GAL4/UAS. First, I created variants with the highest likelihood of being biochemically inactive, to serve as loss of function controls. Second, I selected human variants from ClinVar that had unknown pathogenicity, but were linked to juvenile polyposis syndrome, that would be tested using the assays I developed. I developed two assays in which UAS-hSMAD4 WT generated a robust phenotype, but the loss of function mutation did not. The first assay overexpresses hSMAD4 variants ubiquitously, and I found that UAS-hSMAD4 WT results in lethality during larval stages, but the engineered loss of function variants were fully viable. The second assay expresses hSMAD4 variants in the posterior domain of the developing wing, and I found that UAShSMAD4 WT caused vein defects, but one of the engineered loss of function variants did not. With these two assays established, the next step was to test the selected variants of unknown pathogenicity.

I demonstrate the relative function of a set of variants of unknown pathogenicity, finding that four out of the seven variants tested were functionally distinct from wildtype *hSMAD4*. In the ubiquitous expression assay, two variants of unknown pathogenicity showed increased

lethality compared to wildtype. In the wing development assay, two variants generated a reduced L4, L5, ACV and PCV vein phenotype compared to wild type *hSMAD4*, while two other variants caused ectopic vein formation between L4 and L5 veins that resembled wild type *hSMAD4*. These results confirm that the assays I created are capable of functionally discriminating human *SMAD4* variants that show a difference in function compared to wildtype hSMAD4 *in vivo*.

#### 3.2 Human SMAD4 and Drosophila Medea show high sequence identity

SMAD4 was selected as a candidate gene for testing human variants in *Drosophila* because it is highly conserved to *Drosophila* Medea, and they share strikingly similar functions with the same interacting partners in pathways that are critical to a wide variety of cells. They share 59% identity along the complete length of the protein, with 76% identity in the MH1 domain and 80% identity in the MH2 domain (Wisotzkey et al. 1998) (Figure 3.1). One difference between the two proteins is that Medea has a longer linker region and a notable polyglutamine region. This high level of conservation, and their similar function across species, is reflected in the ability of hSMAD4 to rescue *Medea* null mutant embryos; these nulls have dorso-ventral patterning defects, and *hSMAD4* injected mRNA was shown to rescue this defect (Hudson et al. 1998). These findings led me to test whether *Drosophila* could be used to test the relative function of hSMAD4 variants.



Figure 3.1 There is high identity between human SMAD4 and Medea.

This is an alignment of hSMAD4 amino acid sequence and the *Drosophila* Medea-PB isoform amino acid sequence. Alignment was done in MView using its P1 colour map. Highlighted colors indicate shared identity. Coloured letters indicate shared chemical properties. Missense mutations are shown above the altered amino acid; green stars and green letters indicate the position and change of amino acid used as loss of function controls. Also, blue stars and the blue letter below them indicate the position and change of amino acid of the human variants of unknown pathogenicity used in this study. P1 colour map: bright-green = hydrophobic, dark-green = large hydrophobic, yellow = cysteine, bright-blue = negative charge, bright-red = positive charge, purple = polar, dull blue = small alcohol.

## 3.3 Design and selection of human variants

The first step to creating assays, was to select mutations that would predictably act as loss of function mutations, to assist in calibrating the assays, and also to determine whether any phenotype we observed resulted from appropriate and specific hSMAD4 function or resulted as a non-specific artifact of overexpressing non-functional hSMAD4 protein. In order to act as a true control for overexpression studies, I preferred missense mutations over nonsense mutations as it was important that a non-functional protein exist inside the cell to control for artifacts of overexpression studies, for example, protein aggregation and toxicity effects. I created two predicted loss of function constructs with mutations that are predicted to disrupt two separate functions of SMAD4; R-Smad interaction and DNA-binding. The MH1 domain contains the DNA binding site, so I created three mutations that altered the amino acids (each one to alanine) that directly bind DNA, in order to abolish the protein's ability to bind DNA (Y Shi et al. 1998). The three amino acid changes in hSMAD4 are R81A, Q83L, K88A; hereafter referred to as the *hSMAD4\_RQK* variant (Figure 3.2). Blocking SMAD4's ability to bind DNA would predictably inhibit SMAD4's ability to regulate downstream genes, but the phenotype arising from this could be amorphic or may dominantly interfere with BMP signaling by sequestering R-Smads and preventing downstream gene expression.

The MH2 domain is critical for binding to R-Smads. Yigong Shi et al. (1997) demonstrated that a number of mutations in hSMAD4, found in human tumours, disrupt oligomerization with SMAD2. They examined the crystal structures of hSMAD4 and hSMAD2 binding that indicated binding occurs at amino acids in SMAD2 that are conserved in all R-Smads and therefore, the mutations they identified in hSMAD4 likely disrupt oligomerization with all R-Smads. I selected two of these mutations to mutate in combination in order to remove SMAD4's ability to bind R-Smads. These mutations are V370D and R361C, hereafter referred to as the hSMAD4\_Olig variant (Figure 3.2). SMAD4 cannot regulate downstream genes without

binding R-Smads nor can it sequester R-Smads; therefore, it is predicted that these mutations will act as amorphs. Both *hSMAD4\_RQK* and *hSMAD4\_Olig* were used as loss of function mutations.

After designing variants that we predict would severely disrupt hSMAD4 function, I selected seven variants with unknown pathogenicity (Table 3.1). To select seven from the hundreds of available variants for hSMAD4 on ClinVar, I selected variants that were associated with juvenile polyposis syndrome, that were in amino acids that were conserved to Drosophila and were spaced throughout the protein. I postulated this would provide a suitable diversity of variants for testing while retaining clinical relevance. By selecting variants in amino acids that are conserved to Drosophila, it increases the likelihood that the amino acid is important to the protein's function. I also chose variants that ranged from likely benign to likely pathogenic based on association with the general population or patients with juvenile polyposis syndrome respectively. The hSMAD4 G386D variant is noteworthy because it has been found in many cancers and is thought to be pathogenic, though this has never been functionally tested. Also, this mutation is near the mutations that were selected to create one of the loss of function constructs (Olig) that should be unable to bind other SMADs (Figure 3.2). In contrast, while the I525V mutation has been identified in patients with a number of diseases, many of the submissions to ClinVar classify it as benign and, it is one of the most frequent alleles of hSMAD4 found in the ExAc database. The seven variants I selected for this study are spaced throughout the protein with two in the MH1 domain, one in the linker region and the remaining four in the MH2 domain (Figure 3.2).

Mutation	Amino Acid Change	RS#	Associated Conditions listed on ClinVar	Allele identification
A118G	I40V	rs878854764	Juvenile polyposis syndrome	1 submission of uncertain significance to ClinVar
A175G	T59A	rs587781977	Juvenile polyposis syndrome, Hereditary cancer-predisposing syndrome	3 submissions of uncertain significance to ClinVar
C875T	P292L	rs786201404	Juvenile polyposis syndrome, Hereditary cancer-predisposing syndrome, Wilms tumour	4 submission of unknown significance to ClinVar, gnomAD frequency: 0.00007
T970C	C324R	rs377767339	Juvenile polyposis syndrome	2 submission of unknown significance to ClinVar
G1006C	G336R	rs878854763	Juvenile polyposis syndrome	1 submission of unknown significance to ClinVar
G1157A	G386D	rs121912580	Juvenile polyposis syndrome, Lung adenocarcinoma, Pancreatic adenocarcinoma, neoplasm of the large intestine, Carcinoma of esophagus, Colorectal Neoplasms, Adenocarcinoma of the stomach, Adenocarcinoma of the prostate	8 submissions of pathogenic or likely pathogenic to ClinVar
A1573G	I525V	rs149755320	Myhre Syndrome, Juvenile polyposis syndrome, Osler Hemorrhagic telangiectasia, Gastrointestinal polyposis, Cardiovascular phenotype	11 submission of likely benign to ClinVar, 2 submission of uncertain significance to ClinVar, ExAc frequency: 0.0007

Table 3.1 List of variants selected to be tested.

Selected variants are associated with juvenile polyposis syndrome, are conserved to *Drosophila* and are spaced throughout the protein. Numbering for the single nucleotide mutations and amino acid changes refer to human SMAD4 sequences. RS# is a common reference number used in many databases.



Figure 3.2 Location in SMAD4 of human variants selected for this study.

Blue boxes represent both major domains MH1 and MH2. Light blue box is the DNA binding domain. Red loops indicate structures with determined function. Red triangles are known sumoylation sites. Green triangles are known ubiquitination sites. Red stars are human variants that have been previously functionally characterized. Green stars are amino acid changes used to create loss of function constructs. Blue stars are the human variants chosen to be studied using these assays.

#### 3.4 Ubiquitous overexpression of human SMAD4 in *Drosophila* causes lethality

The first assay that I employed involved looking for early lethality from overexpression of the *hSMAD4* variant proteins. I explored this phenotype at a number of rearing temperatures,

due to the sensitivity of the GAL4/UAS system. *UAS-gene* expression can be decreased by lowering the temperature of the flies (Duffy 2000). At 25°C, the ubiquitous GAL4, *daughterless-GAL4* (*da-GAL4*), driving expression of either *UAS-hSMAD4\_WT* or *UAS-Medea* caused lethality before the L2 larval stage. However, expression of *UAS-hSMAD4\_Olig* at 25°C also resulted in larval lethality. Thus, I reduced GAL4/UAS activity by rearing animals at 18°C; *da-GAL4* driving *UAS-hSMAD4\_WT* resulted in reduced pupariation but very few flies were able to make it to adulthood (Figure 3.3). When *UAS-hSMAD4\_Olig* was ubiquitously expressed at 18°C, there was no lethality. Going forward, this assay was performed at 18°C to increase the dynamic range of the assay and to eliminate the toxicity of the non-functional protein, hSMAD4\_Olig.

The assay was further designed to internally control for variation in egg laying. Homozygous *da-GAL4* was crossed to heterozygous *UAS-SMAD4* variants over a yellow fluorescent protein (YFP) marker balancer chromosome. This allowed YFP+ progeny to act as an internal genetic control for all *UAS-SMAD4* variants tested. An empty *attP2* site was used as a negative control. Parental flies were allowed to mate for one day at 18°C before being flipped into new vials and left to lay eggs for two days. This was necessary to provide sufficient number of eggs, as fewer eggs are laid at 18°C. Six vials were set up as replicates for each genotype, each time the experiment was performed. Only vials with between 20 – 100 balancer carrying pupae were included in the results in order to confirm that the vials were healthy. Progeny either *hSMAD4* variant or the balancer chromosome were counted after pupariation and after they eclosed. The percentage of total pupae or adults from the vial with the *UAS-hSMAD4* construct were then compared to identify variants that changed the function of hSMAD4.



Figure 3.3 Two human variants have increased lethality compared to hSMAD4\_WT. Graphs of each of the *UAS-variants*, when driven by DA-Gal, showing the percentage of variant flies compared to the total number of flies in each vial (internal genetic control + variant). (A) Numbers of flies counted after pupariation but before eclosion. (B) Number of adult flies that eclosed in each vial. Each dot represents an individual vial. Mean ± SEM, \*\*\*\*P<0.0001, \*\*P<0.01, One-way ANOVA with Tukey test. A minimum of 3 vials were analyzed for each genotype.

Using this assay, on average only 14% of progeny expressed UAS-hSMAD4\_WT compared to its internal control at pupal stages and only 3% of adults (Figure 3.3). While, *attP2* control flies pupariated and eclosed at the same levels as balancer flies showing that only the expression of the *hSMAD4* constructs caused the lethality. There were similar numbers of UAS-*hSMAD4\_Olig* expressing progeny compared to their internal control for both pupae (on average 51% of progeny) and adults (on average 48% of progeny), which indicates that hSMAD4's ability to bind R-Smads is required for its ability to cause lethality (Figure 3.3). 54% of pupae expressed UAS-hSMAD4\_RQK, which is unable to bind DNA, compared to internal controls but

only 34% of adults expressed *UAS-hSMAD4\_RQK* compared to its internal control (Figure 3.3). This modest lethality may reflect the likelihood that this variant is acting to reduce transcriptional output from the BMP pathway, much like the antimorphic *Mad* mutant, *Mad<sup>1</sup>*, which also cannot bind DNA (Takaesu et al. 2005). Once it was determined that control flies were acting as designed, the assay was then performed with the human variants.

Performing this assay with the selected human variants, identified two variants that differed from  $hSMAD4_WT$ . UAS- $hSMAD4_G386D$  or UAS- $hSMAD4_G336R$  expression resulted in no pupae expressing the variant compared to 14% with expression of UAS- $hSMAD4_WT$  (Figure 3.3A). This was an unexpected result, as the loss of function mutants showed decreased lethality while expression of these variants resulted in increased lethality. This result suggests two possible explanations. The first, is that both variants are hypermorphs and the increased activity results in the increased lethality. The other possibility is the variants have altered the function of hSMAD4 in a way that increases its lethality perhaps through increased or decreased interactions with the TGF $\beta$  signaling pathways. Importantly though, this assay shows that variants with a range of functionality can be distinguished within this assay. In the future, it will be useful to count numbers of larvae at earlier time points, to provide a higher resolution quantitative assessment of the precise stage of lethality.

#### 3.5 Posterior wing overexpression of human SMAD4 results in vein phenotypes

The second assay I developed looks at the impact of variants on the development of the adult wing. As discussed in Chapter 1.4.1, the wing is an excellent model for examining BMP and TGF $\beta$  gene function. The first step in establishing this assay was to identify a suitable *GAL4* driver for wing phenotype generation and assessment. I examined phenotypes caused by

expression of UAS- $hSMAD4_WT$  and UAS- $hSMAD4_Olig$  driven by several developing wing expressing GAL4s at both 18°C and 25°C; these include: apterous-GAL4, dpp-GAL4, patched-GAL4, nubbin-GAL4, and  $GAL4^{A9}$ . When crossed with UAS- $hSMAD4_WT$  or UAS $hSMAD4_Olig$ , all these GAL4 drivers resulted in strong wing phenotypes or lethality. In contrast, at 18°C, *engrailed*-GAL4 (*en*-GAL4) generated a robust wing vein phenotype when crossed to UAS- $hSMAD4_WT$ , but no phenotype when crossed to UAS- $hSMAD4_Olig$ . *en*-GAL4is expressed in the posterior region of the wing disc and adult wing. In order to analyze the degree of severity of the vein loss, I measured and combined the distances between the longitudinal veins where the ACV and PCV cross, as well as the length of the L4 and L5 vein distally to where the PCV intersects both of these veins (Figure 2.1). I then measured the length of the missing vein and calculated the percentage of missing vein from the total distance and length. I refer to this as the percentage of vein missing. Therefore, I established this assay using *en*-GAL4 at 18°C to study hSMAD4 variants.

Expression of *UAS-hSMAD4\_WT* driven by *en-GAL4* caused loss of part of the ACV, PCV and parts of veins L4 and L5 distally of the PCV (Figure 3.4B). On average, 35% of the veins are missing with the ACV and PCV always being affected, the L4 vein is affected in 92% of wing, but the L5 vein is partially missing in only 8% of wings. This is nearly identical to what is seen with the overexpression of *hSMAD3* implying that *hSMAD4* may be acting through the same mechanism (Sander et al. 2010). In the activin pathway, *hSMAD3* forms a complex with *hSMAD4* to regulate gene expression, therefore if increased levels of *hSMAD3* or *hSMAD4* causes loss of wing veins then it is probable that the loss of veins is due to increased activin pathway activity. Expression of *UAS-hSMAD4\_Olig* driven by *en-GAL4* never causes any



Figure 3.4 Wings expressing two human SMAD4 variants posteriorly showed reduced vein loss compared to wings expressing *hSMAD4 WT*.

Wings are right wings from 1 – 3 day old female progeny resulting from crossing *w;en-GAL4,UAS-RFP/Cyo* virgins to *w;;UAS-hSMAD4\_variant.* (A – K) Representative image from each genotype. Variable amounts of the ACV, PCV and distal regions of L4 and L5 are missing. (A) Contains an attP2 site instead of a *UAS-hSMAD4\_variant.* (L) Graph showing the amount of vein missing from the wing as a percentage of the total distance the vein covers under normal conditions including the ACV, PCV, as well as L4 and L5 distally from where the PCV intersects them. Each dot represents an individual wing. Means ± SEM, \*\*\*\*P<0.0001, One-way ANOVA with Tukey test. A minimum of 10 wings were analyzed for each genotype.

missing veins, as is seen with the *attP2* control (Figure 3.4A, C). UAS-hSMAD4 ROK had interesting results when driven by en-GAL4 and resembled the phenotype seen with UAShSMAD4 WT with an average of 29% of the vein missing (Figure 3.4D). Wings expressing UAShSMAD4 RQK standout from wings expressing UAS-hSMAD4 WT because there is only partial loss of the PCV 15% of the time compared to 100% of the time with UAS-hSMAD4 WT. This indicates that hSMAD4 RQK has reduced function compared to hSMAD4 WT, but not as great a reduction as was detected in the lethality assay; thus, the wing assay seems less responsive to the loss of DNA-binding. The exception is the PCV vein, which is almost wildtype in the presence of hSMAD4 ROK, suggesting an important role for SMAD4 DNA-binding in disrupting normal development of this crossvein. The crossveins receive Dpp signaling from the longitudinal veins, because of the distance, they need to be very sensitive to Dpp signaling therefore, there are several factors that augment BMP signaling in the crossveins (Ralston and Blair 2005). If hSMAD4 RQK acts as a hypomorph because other R-Smads are able to bind DNA to compensate, then the upregulation of BMP signaling in the PCV, may be enough to counteract the decrease in hSMAD4 RQK activity.

This assay was then used to study the selected human SMAD4 variants. Two of them showed a significant decrease in vein loss when expressed compared to expression of UAShSMAD4\_WT. Similar to the previous assay UAS- hSMAD4\_G386D showed a significant difference from UAS-hSMAD4\_WT. In this assay it showed a decrease in vein loss with part of L4 or L5 missing in only 30% of wings compared to 92% of wings when UAS-hSMAD4\_WT was expressed (Figure 3.4E). The other variant that showed a significant difference from wings expressing UAS-hSMAD4\_WT was UAS-hSMAD4\_I525V (Figure 3.4G). This contradicts the ClinVar submissions calling hSMAD4\_I525V benign. It is interesting that the lethality and wing assays do not show the same variants as being different from hSMAD4\_WT and indicates the importance to running more than a single assay in order to determine pathogenicity.

## 3.6 SMAD4 overexpression in the wings also triggers the formation of ectopic veins

Overexpression of *UAS-Medea* in the wing, driven by *en-GAL4*, results in ectopic vein formation (Figure 3.5). The PCV, instead of connecting the L4 and L5 vein, is ectopically positioned parallel to the L4 and L5 vein. Also, there is partial loss of the ACV and very occasionally loss of the distal end of the L5 vein. These phenotypes are very similar to what is observed with overexpression of *UAS-hSMAD4\_WT*, however the severity of phenotype cannot be compared, as *UAS-Medea* and *UAS-hSMAD4\_WT* are in different positions in the genome and therefore will have different expression levels. Looking specifically for veins positioned parallel to the L4 and L5 veins highlighted two of the selected human variants that more frequently displayed this phenotype than with expression of *UAS-hSMAD4\_WT*.



Figure 3.5 Two human SMAD4 variants showed an increased penetrance for an ectopic vein phenotype. Representative wings are right wings from 1 – 3 day old female progeny resulting from crossing *w;en-GAL4,UAS-RFP/Cyo* virgins to (A) *w;UAS-Medea* showing loss of part of the ACV and ectopic vein formation of the PCV running parallel to the L4 and L5 vein. (B) *w;;UAS-hSMAD4\_WT* also shows partial loss of ACV and L4 vein, with vein formation parallel to the L4 and L5 vein coming from the PCV. (C – D) *w;;UAS-hSMAD4\_I40V* and *w;;UAS-hSMAD4\_G336R* both showed ectopic vein formation parallel to vein L4 and L5. Black arrows point to ectopic veins.

Wings expressing UAS-hSMAD4\_I40V or UAS-hSMAD4\_G336R both showed a higher frequency of ectopic veins than wings expressing UAS-hSMAD4\_WT. When UAS-hSMAD4\_WT was expressed, only two wings (8%, n=25) showed these ectopic veins. Meanwhile expressing UAS-hSMAD4\_I40V in the wings showed ectopic veins in 24% (n = 29) of wings observed and wings expressing UAS-hSMAD4\_G336R showed ectopic veins in 25% (n = 20) of wings observed. Larvae expressing UAS-hSMAD4\_G336R also showed increased lethality compared to larvae expressing UAS-hSMAD4\_WT making this the second instance where the variant acts hypermorphic indicating that this variant is likely pathogenic. In contrast, hSMAD4\_I40V seems
to be functionally different from hSMAD4\_WT only in the wing assay. Of additional interest is that these two variants have mutations in two different domains of SMAD4 indicating that this ectopic growth does not rely on a single domain of SMAD4 and instead my act through increased signaling compared to hSMAD4\_WT.

### 3.7 Expression of UAS-hSMAD4 WT causes an increase in phosphorylation of Mad

I next wanted to confirm that hSMAD4 was acting on the BMP pathway. Overexpression of *Mad* is typically characterized by ectopic vein formation, this differs from the loss of veins as is seen with the overexpression of *UAS-hSMAD4\_WT* (Sander et al. 2010). This indicated that hSMAD4 is not solely working through Mad, but did it have an effect on Mad at all? Therefore, using the *en-GAL4* driver that gave the wing phenotypes, I looked at levels of Mad phosphorylation in the late L3 larval wing disc as an indicator of BMP signaling and Mad activation (Tanimoto et al. 2000). Wings with decreased p-Mad staining have a loss of veins and this could be an explanation of the vein loss with the expression of *UAS-hSMAD4\_WT* (Gui et al. 2018).

Interestingly, there was an increase in p-Mad staining with the overexpression of *UAS-hSMAD4\_WT* driven by *en-GAL4* (Figure 3.6C). This increase was not seen with overexpression of *UAS-Medea* though that may be due to different expression levels caused by being in different genomic locations or this may indicate that hSMAD4 has an increased ability to stabilize p-Mad (Figure 3.6B). Unexpectedly, expression of the loss of function variants had differing effects on p-Mad levels. Expression of *UAS-hSMAD4\_Olig* appeared to decrease levels of p-Mad compared to attP2 controls (Figure 3.6D). One explanation for this is that high levels of hSMAD4 that



Figure 3.6 UAS-hSMAD4\_WT expression increases levels of p-Mad.

Wing discs from late L3 larvae maintained at 18°C with expression driven by en-GAL4. Red is *UAS-RFP* indicating the region of *en-GAL4* expression. Blue is p-Mad antibody staining. Red arrow indicates line used for intensity profile. (A) attP2 site control. Shows typical p-Mad staining with a peak near the center of the wing disc. (B) Expression of *UAS-Medea* shows similar p-Mad intensity as control. (C) Expression of *UAS-*

*hSMAD4\_WT* shows an increase in p-Mad staining intensity in the center peak where *en-GAL4* is also expressed. (D) Expression of *UAS-hSMAD4\_Olig* shows a decrease in p-Mad staining intensity. (E) Expression of *UAS-hSMAD4\_RQK* shows similar intensity levels as seen with *UAS-hSMAD4\_WT* but with a wider peak of intensity.

Mad, as it is less likely to encounter Medea over hSMAD4. This would be the opposite of how overexpression of *Medea* is capable of rescuing hypomorphic alleles of *mad* by increasing the probability that p-Mad and Medea will come in contact to bind (Das et al. 1998). In contrast, expression of UAS-hSMAD4 ROK shows the same increase in p-Mad levels seen with expression of UAS-hSMAD4 WT as well as an expansion of the region with increased p-Mad staining (Figure 3.6E). Both hSMAD4 WT and hSMAD4 RQK may be increasing p-Mad staining through a stabilized interaction with Mad. Also, because there is increased p-Mad staining with the expression of UAS-hSMAD4 RQK, the increase may be due to a lack of downstream upregulation of negative feedback regulators of the BMP pathway, such as Dad (Hamaratoglu, Affolter, and Pyrowolakis 2014). Mad's inability to regulate downstream genes could also explain the unexpected result of increased p-Mad staining leading to a loss of veins in the adult wing. These hypotheses can be addressed by determining how expression of UAShSMAD4 WT affects Mad interaction with Medea and expression of genes regulated by Mad. It will also be important to test the affinities of hSMAD4 for Mad and its DNA binding sequences compared to Medea, which could explain the slight differences in phenotypes between the two proteins.

### 3.8 Four out of seven variants tested differed in function from hSMAD4 WT

Each assay identified different human variants that showed a change in severity of phenotype compared to *UAS-hSMAD4\_WT* expression. This is interesting because it was expected that pathogenic variants would disrupt the function of SMAD4 in all assays instead of only in particular assays. Two of the four variants that showed a phenotypic difference did have differences in effect in both assays but the other two were only identified in a single assay. This points to a potential problem of identifying variants as either completely pathogenic or completely benign when it may depend on a particular biological process as to whether there is an effect. Each assay is likely testing a different function of SMAD4. The next step is identifying these functions. Currently the assays are able to identify human variants that function differently from hSMAD4\_WT; but, without further testing, is unable to tell us the nature of this difference.

There are many computer models for predicting the effect of single nucleotide variants on protein function. SNAP2 is a common model that uses evolutionary conservation, predicted secondary structures and available annotated residues or regions in order to predict whether the variant will have a functional effect on the protein (Hecht, Bromberg, and Rost 2015). Another model is MutPred2 which has been found to be one of the most accurate computer models when looking at variants that had been functionally characterized (Seifi and Walter 2018). MutPred2 uses machine learning and is trained using information on variants from the Human Gene Mutation Database, SwissVar, dbSNP as well as using inter-species pairwise alignment in order to determine the probability of pathogenicity for amino acid substitutions (Pejaver et al. 2017). REVEL is a computational method for predicting pathogenicity that combines the scores from thirteen individual tools as well as being trained with new pathogenic or benign missense

mutations (Ioannidis et al. 2016). A limitation of all 3 computer models is that they can only indicate that the variant affects the function of the protein but gives no indication of the nature of this difference. MutPred2 and REVEL do not even make predications of pathogenic or benign and give only increased likelihoods of the variant affecting function. Scores from these three computer models were determined for the selected human variants used in this study (Table 3.2). All three models show similar scores for the variants which can be expected as all three models use the same variant databases to create their models.

C324R, G336R and G386D have high scores using all three computer models indicating that they are likely pathogenic. This agrees with the results found in this study for two of the three variants. Both G336R and G386D showed a difference from hSMAD4\_WT in both assays meaning that they are most likely pathogenic. This matches the information from ClinVar which implicated G386D in several disorders and cancers. On the other hand, C324R, while showing a high score using computer models, did not differ from hSMAD4\_WT in either assay. One possibility is that this is an instance where the computer models are inaccurate. Another possibility is that C324R is important for a biological process that has not yet been assayed.

For the remaining variants, the scores from the computer models are low indicating a prediction of benign. However, two of these variants did show a difference in severity of phenotype compared to hSMAD4\_WT indicating that the variant does affect the function of the protein. In both cases, the variants only acted differently in one of the two assays. This could indicate that while they do affect function of the protein, they do so to a lesser degree compared to G386D or G336R. Another possible explanation is that the effect they have on the function of

the protein is only required in specific biological contexts. That I40V and I525V affect SMAD4 function is a novel finding that had not been predicted using computer models.

Variant	Lethality	Wing Vein Loss	Ectopic veins	SNAP2	MutPred2	REVEL
I40V	-	-	1	-56 (78%) neutral	0.504	0.305
Т59А	-	-	-	-33 (66%) neutral	0.586	0.467
P292L	-	-	-	-24 (61%) neutral	0.619	0.454
C324R	-	-	-	79 (85%) Effect	0.932	0.969
G336R	1	-	1	82 (91%) Effect	0.959	0.948
G386D	1	Ļ	-	79 (85%) Effect	0.845	0.986
I525V	-	Ļ	-	-71 (87%) neutral	0.475	0.458

 Table 3.2 Comparison of functional effects found in this study compared to predicted effect from computer models.

(↑) indicates that an increase in effect was found between the variant and hSMAD4\_WT. (↓) indicates that a decrease in effect was found between the variant and hSMAD4\_WT. (-) indicates no difference from hSMAD4\_WT. Lethality refers to Figure 3.3. Wing vein loss refers to Figure 3.4. Ectopic veins refer to Figure 3.5. SNAP2 scores range from -100 to 100 with positive numbers indicating likely to have an effect. The percentage indicates the expected accuracy of the score. Neutral and Effect indicates the prediction given by

SNAP2. MutPred2 scores range between 0 to 1 with scores over 0.5 suggesting pathogenicity but higher scores indicating a higher likelihood. REVEL scores are also from 0 to 1 with higher scores indicating a greater likelihood of the variant being pathogenic.

In conclusion, this study identified four variants that showed a difference from hSMAD4\_WT using two newly established assays (Table 3.2). Both assays have been validated using a loss of function missense variant to show that the effect seen with the overexpression of *UAS-hSMAD4\_WT* requires the biological functions of the SMAD4 protein. They have also been validated by their ability to identify human variants that affect the function of hSMAD4. Two of the variants identified as pathogenic were predicted using computer models or association with several diseases and cancers. Therefore, these two assays can be used to test the function of human variants in a *Drosophila* model.

## Chapter 4: Development of tools to test BMPR1A human variant function

## 4.1 Synopsis

*BMPR1A* is also associated with juvenile polyposis syndrome. Creating assays to determine the relative function of potential pathogenicity of human *BMPR1A* variants will provide experimentally-derived information that clinicians may use to assist with diagnosis or to propose surveillance or early intervention. To develop appropriate assays, I started by selecting missense mutations that would result in expression of a loss of function. These loss of function constructs along with *UAS-BMPR1A\_WT*, were integrated into the *Drosophila* genome. Using several GAL4 drivers, I examined phenotypes resulting from overexpression of *UAS-BMPR1A\_WT*, as well as *UAS-Tkv*, the *Drosophila* ortholog. While numerous GAL4 drivers generated robust phenotypes when expressing *UAS-Tkv*, there was no discernable phenotype when *UAS-BMPR1A\_WT* was expressed. Therefore, I concluded that *BMPR1A* does not function in *Drosophila*.

In order to use *Drosophila* as a model to study human variants in BMPR1A, a different system for expressing variants had to be developed. Between BMPR1A and Tkv, there is high conservation in the kinase domain but lower conservation in the extracellular domain where the ligand binding sites are located. Therefore, I reasoned that the *Drosophila* ligands may be unable to bind and activate human BMPR1A, because if it could bind the ligand then it would sequester the ligand and cause a phenotype associated with decreased BMP signaling. So, I designed a chimeric protein with the Tkv extracellular domain fused with the kinase domain of BMPR1A. If

expression of this gene resulted in phenotypes similar to what is seen with expression of *UAS-Tkv* then human variants found in the intracellular domain of BMPR1A could still be assayed in the BMPR1A context. Another solution would be to use *Drosophila* mimetics that replicate the amino acid change of the human variant. This can only be used to study variants that are in residues that are identical or similar (Tkv and BMPR1A have 51% identity and 63% similarity) but guarantees that Tkv\_WT will be functional in *Drosophila*. This is a common method to study human variants in a non-human model (Harel et al. 2016). Together, these two systems will allow for examination of relative variant function and pathogenicity of human BMPR1A variants using *Drosophila* as a model.

#### 4.2 Selection of BMPR1A human variants

Tkv clusters closest to BMPR1A and BMPR1B in phylogenetic trees, making *tkv* the closest ortholog in fly to human *BMPR1A* (Newfeld, Wisotzkey, and Kumar 1999). BMPR1A and Tkv show 45% identity within the GS domain and 62% identity within the kinase domain. However, the extracellular domain only shares 22% identity (Figure 4.1). This is higher identity compared to other type I receptors in flies, Sax and Baboon share 39% identity with BMPR1A. Although the *BMPR1A* gene had not been expressed in *Drosophila* to test for function, the BMP4 ligand was able to rescue loss of Dpp during embryonic dorsal-ventral patterning (Padgett, Wozney, and Gelbart 1993). As Tkv is the receptor for Dpp, it was reasoned that if BMP4 can activate Tkv, Dpp may be able to activate BMPR1A in *Drosophila*.

The first step to testing BMPR1A variants involved selecting loss of function mutations to use as negative controls. First, I generated the BMPR1A\_K261R variant, as several biochemical studies found this mutation abolished kinase function (Penheiter et al. 2002;

Ungefroren et al. 2005). BMPR1A\_K261R was shown, in cell culture, to be unable to phosphorylate downstream targets because it is a critical amino acid for binding ATP (Wieser, Wrana, and Massagué 1995). I also generated a loss of function mutation, BMPR1A\_E502K, that was designed to mimic the *tkv<sup>7</sup>* anti-morph allele isolated in *Drosophila* (Penton et al. 1994). These two loss of function variants, as well as BMPR1A\_WT, were integrated into the *Drosophila* genome to develop assays for testing other human BMPR1A variants.



Figure 4.1 Sequence alignment of BMPR1A and Tkv.

Alignment of BMPR1A amino acid sequence and the *Drosophila* Tkv-PA isoform amino acid sequence. There is lower sequence identity in the extracellular domain at the N-terminal of the protein. There is high sequence identity in the GS and kinase domain. Alignment was done in MView using its P1 colour map. Highlighted colors indicate shared identity. Coloured letters indicate shared chemical properties. Missense mutations are shown above the altered amino acid; green stars and green letters indicate the position and change of amino acid used as loss of function controls. Also, blue stars and the blue letter indicate the position and change of amino acid in the sequence below, of the unknown pathogenicity human variants used in this study. P1 colour

map: bright-green = hydrophobic, dark-green = large hydrophobic, yellow = cysteine, bright-blue = negative charge, bright-red = positive charge, purple = polar, dull blue = small alcohol.

Amino Acid Change	RS#	Associated Conditions listed on ClinVar	Allele identification
C63T			From sequence in a patient from clinician Kasmintan Schrader, Dept. Medical Genetics, UBC
N190S	rs574229174	Juvenile polyposis syndrome, Hereditary cancer- predisposing syndrome	3 submissions of uncertain significance to ClinVar, gnomAD frequency: 0.00001
R238Q	rs191742018	Juvenile polyposis syndrome, Hereditary cancer- predisposing syndrome	4 submissions of uncertain significance and 1 submission of likely benign to ClinVar, gnomAD frequency: 0.00006
R406C	rs587781332	Juvenile polyposis syndrome, Hereditary cancer- predisposing syndrome	4 submissions of uncertain significance to ClinVar, gnomAD frequency: 0.00003
E411K	rs786202611	Juvenile polyposis syndrome, Hereditary cancer- predisposing syndrome	2 submissions of uncertain significance to ClinVar
R443C	rs35619497	Juvenile polyposis syndrome, Hereditary cancer- predisposing syndrome	4 submissions of uncertain significance, 4 submissions of likely benign, 2 submissions of benign to ClinVar, ExAc frequency: 0.00061
V474L	rs567733221	Juvenile polyposis syndrome, Hereditary cancer- predisposing syndrome	4 submissions of uncertain significance to ClinVar, gnomAD frequency: 0.00003

 Table 4.1 List of human BMPR1A variants selected to develop functional assays.

Variants were selected based on their association with juvenile polyposis syndrome, showed conservation with

Drosophila and are predominantly in the kinase domain. Numbering for the amino acid changes refer to

human SMAD4 protein sequence. RS# is a common reference number used in many databases.

Seven human variants in BMPR1A were selected to test in *Drosophila* assays (Table 4.1). Similar to the selections made for SMAD4, I chose variants that were associated with juvenile polyposis syndrome, where amino acids were conserved to *Drosophila*, and were biased towards being in the kinase domain which has the highest sequence identity. All the mutations in the variants, except one, are located in the cytoplasmic domain of the protein, with all but two of those in the kinase domain.

#### 4.3 Overexpressed BMPR1A is non-functional in Drosophila

*UAS-BMPR1A\_WT* was integrated into the *Drosophila* genome in order to identify phenotypes caused by its expression. In conjunction with expressing *UAS-BMPR1A\_WT*, I also studied the effect of expressing *UAS-tkv* and *UAS-Tkv\_DN*, a dominant negative allele of *tkv* (Kumar and Moses 2001). I first examined the effect of ubiquitously expressing these constructs at 25°C. Expression of *UAS-tkv* driven by *da-GAL4* resulted in lethality during pupal stages. Expression of *UAS-tkv\_DN* driven by *da-GAL4* resulted in adult flies with several defects in their thorax, bristles and wings (not shown)(Kumar and Moses 2001). Expression of *UAS-BMPR1A\_WT* driven by da-GAL4 resulted in no discernable phenotype. As there was no obvious phenotype, I then looked for more minor phenotypes, specifically in the wing.

There are a number of widely used *GAL4* lines that are expressed in the wing. The wing GAL4 driver, *en-GAL4* was used previously for the SMAD4 assays and therefore, was a likely useful candidate for generating a phenotype upon driving *UAS-BMPR1A\_WT*. Expressing *UAS-tkv* with *en-GAL4* resulted in small wings that were crumpled (Figure 4.2A). Expression of *UAS-tkv* DN driven by *en-GAL4* resulted in loss of the ACV and PCV and the distal end of L5 vein



Figure 4.2 Wing phenotypes caused by expression of *UAS-tkv*, *UAS-tkv\_DN* and *UAS-BMPR1A\_WT*. Wings are right wings from 1 – 3 day old females maintained at 25°C. None of the GAL4s led to a phenotype when expressed with *UAS-BMPR1A*. (A)Expression driven by *en-GAL4*. *UAS-tkv* caused small crumpled wings while *UAS-tkv\_DN* results in loss of veins. (B) Expression driven by *GAL4<sup>49</sup>*. *UAS-tkv* again caused small crumpled wings while again expression of *UAS-tkv\_DN* leads to loss of veins. (C) Expression driven by *Dpp-GAL4*. *UAS-tkv* expression caused a thickening of the L3 vein.

(Figure 4.2A). *UAS-BMPR1A\_WT* expression driven by en-Gal4 or an attP2 control showed no discernable phenotype. As an alternative, I employed *GAL4<sup>49</sup>*, which is a driver that expresses in the dorsal compartment of the wing pouch (A. J. Peterson and O'Connor 2013). Expression of *UAS-tkv* driven by *GAL4<sup>49</sup>* also resulted in a small crumpled wing (Figure 4.2B). Expression of *UAS-tkv\_DN* driven by *GAL4<sup>49</sup>* caused a small wing as well as loss of part of the L4 vein, the ACV and PCV (Figure 4.2B). Expression of *UAS-BMPR1A\_WT* driven by *GAL4<sup>49</sup>* once again had no discernable phenotype (Figure 4.2B). Because both drivers resulted in a very severe phenotype when expressed with *UAS-tkv*, I then looked at *dpp-GAL4* which is expressed in a stripe along the anterior/posterior regions of both the wing disc and wing, which is a smaller region than the other GAL4 drivers used above. Expression of *UAS-tkv\_DN* driven by *Dpp-GAL4* causes a thickening of the L3 vein (Figure 4.2C). Expression of *UAS-tkv\_DN* driven by *Dpp-GAL4* caused lethality. However, once again expression of *UAS-BMPR1A\_WT* caused no defects to the wing. From this I concluded that BMPR1A is not functional in flies and a different approach would be required.

### 4.4 An alternative approach to creating an assay to study BMPR1A human variants

Based on the above results, a different approach was required for creating an assay for determining the relative function and potential pathogenicity of human BMPR1A variants.

Therefore, I developed tools for two additional approaches (Figure 4.3). The first was to create Tkv mimetics of the human BMPR1A variants. This has the advantage of guaranteeing phenotypes when overexpressed as I have already shown that the overexpression of UAS-Tkv causes a range of phenotypes depending on the driver used. The disadvantage is that the variants are being removed from the human BMPR1A context. However, especially in the kinase domain, Tkv and BMPR1A have a high degree of identity, so it is likely that amino acids will have the same functional roles in both proteins, and therefore can be directly compared. A limitation is that the only variants that can be assayed are those that are in conserved amino acids; although as stated above the proteins are highly conserved. The second method is to create a chimeric protein with the Tkv extracellular domain and the BMPR1A kinase domain. This has the advantage of allowing for native ligand engagement, while being able to study BMPR1A intracellular domain variants in their native protein context and can include amino acids that are not conserved. The disadvantage is that there is a possibility that this protein will be equally non-functional as was seen with BMPR1A, and potentially act as a dominant negative by sequestering ligand. Another limitation is that variants in the extracellular domain cannot be assayed. Between these two methods, I propose that it will be possible to create assays to determine the pathogenicity of BMPR1A variants.

In order to create Tkv mimetics of the human BMPR1A variants, I aligned the human BMPR1A sequence with the Tkv-PA sequence using Clustal Omega in order to identify conserved amino acids between the two sequences (Chojnacki et al. 2017). Once the conserved amino acid changes were identified, those mutations were made in the *Tkv* cDNA sequence. To identify the Tkv\_variant protein separately from the endogenous Tkv, an HA tag was added on

the C-terminal. These UAS constructs, along with *UAS-tkv\_WT*, were then integrated into the same attP2 site in the *Drosophila* genome. Once separate lines are established, assays to determine their pathogenicity can be further developed.



Figure 4.3 Approach for creating assays for determining pathogenicity of human BMPR1A variants. (A) Position of human BMPR1A variants on both BMPR1A as well as their conserved amino acid in Tkv. Top amino acid change relates to the BMPR1A sequence. Bottom amino acid change relates to the Tkv-PA

sequence. Blue boxes represent both major domains MH1 and MH2. Light blue box is the DNA binding domain. Red loops indicate structures with known functions. Red triangles are known sumoylation sites. Green triangles are known ubiquitination sites. Red stars are human variants that have been previously functionally characterized. Green stars are amino acid changes used to create loss of function constructs. Blue stars are the human variants chosen to be studied using these assays. (B) Chimera with the extracellular domain of Tkv and the remainder of the protein is the BMPR1A sequence. It contains the first 179 amino acids of the Tkv-PA sequence and then has the BMPR1A sequence starting at the 153 amino acid relating to the BMPR1A sequence.

The purpose in creating a chimera protein is to express a functional protein in *Drosophila* that can still test human variants in their native protein context. There is low identity between the extracellular domains of BMPR1A and Tkv. If BMPR1A\_WT is unable to be bound by the BMP ligand then BMPR1A\_WT is unable to bind the other type I and type II receptors or phosphorylate its downstream targets. Therefore, its expression would not cause a phenotype as it would be unable to interact with other proteins. The chimera contains the signal peptide and extracellular domain from Tkv but the transmembrane domain, and entire intracellular region including the critical GS domain and kinase domain are from the BMPR1A protein. This chimeric gene has now been placed in an inducible UAS transgene and is currently being integrated into the *attP2* site in *Drosophila*. Once overexpressed in fly tissue, I would expect that the resulting phenotype would match the overexpression of *UAS-tkv*. This would confirm that it was the extracellular domain of BMPR1A that was non-functional in *Drosophila*.

These UAS constructs are currently being integrated into the *Drosophila* genome. The next step will be to establish assays that show a phenotype with the overexpression of *UAS*-*Tkv\_WT* and does not show a phenotype when the loss of function variants are overexpressed. I have already identified several wing GAL4 drivers that generated a phenotype with the

expression of *UAS-Tkv*, so now the effect of expressing the loss of function variants needs to be determined. If the chimera Tkv::BMPR1A protein results in a phenotype, then the next step will be adding the human variants that have mutations in the cytoplasmic domain. Between these two methods, the creation of assays to determine the pathogenicity of human BMPR1A variants will be possible.

# Chapter 5: Effects of Calpains and Cortactin on neuron structure

### 5.1 Synopsis

Calpains and Cortactin have been implicated in regulating neural morphology (Kalil and Dent 2014). Cortactin promotes actin filament branching and can promote neuron branching and growth (Y. He et al. 2015). Calpains are proteases and their mis-regulation is associated with neurodegenerative disorders (Baudry and Bi 2016). Previous work in our lab showed that loss of Calpain or overexpression of Cortactin resulted in increased sprouting along the axons of hippocampal neurons in cell culture (Mingorance-Le Meur and O'Connor 2009). Therefore, I examined whether Cortactin and Calpain has a similar role in vivo. Drosophila melanogaster has four Calpains, though only two are similar to typical Calpains. Null alleles were generated for both of these typical Calpains and double mutant flies were obtained. Surprisingly, analysis of neurons in both the central and peripheral nervous system failed to show any architectural differences from wildtype. The double mutant flies appeared healthy, though the males had decreased fertility. A likely possibility was that other proteases were able to compensate for the loss of calpains. Therefore, a Calpain inhibitor was applied to larvae over a period of 40min but again no change to neural architecture was observed. Overexpression of *Cortactin* also failed to show any changes to neural architecture in both the peripheral and central nervous system. A Cortactin construct that cannot be cleaved by Calpain was generated and overexpressed in motor neurons, but no notable changes occurred. In conclusion, although Calpains and Cortactin are able to affect neuron branching in vitro, I did not observe similar phenotypes in vivo.

## 5.2 Background

#### 5.2.1 Neuron architecture in disease

A hallmark of many neuronal diseases is a change in synapse number, axon loss and degeneration. During development, it is important to eliminate extraneous axons and dendrites and to remove unnecessary synapses; however when similar mechanisms occur in the mature nervous system, this can lead to disease (Luo and O'Leary 2005). Both Huntingtin's and Alzheimer's disease show a loss of dendrites and axons at early stages of the disease (Li et al. 2001; Tsai et al. 2004). There are also many neurodevelopmental disorders that show an increase or reduction in synapses and dendritic spines. Spine densities can be greater in patients with autism spectrum disorder (Hutsler and Zhang 2010). There are dendritic abnormalities found with mental retardation (Kaufmann and Moser 2000). There is also increased branching of motor neurons in psychotic patients (Crayton and Meltzer 1979). And finally, spontaneous neurite branching has been observed during aging (Tank, Rodgers, and Kenyon 2011). Clearly, the mechanisms that modulate neuronal sprouting and retraction represent a point of vulnerability for many neurological disorders. Therefore, in order to understand the mechanism that leads to these varied diseases and the accompanying anatomical changes, it is important to understand the underlying proteins that establish and maintain the structure of neurons.

### 5.2.2 Axon development and consolidation

Axons develop through three steps; the first step is protrusion of filopodia and lamella composed of actin bundles; the second step is called engorgement when microtubules enter the filopodia and lamella along with vesicles and organelles; the final step is consolidation when the actin filaments depolymerizes so that the membrane can shrink back around the microtubule

frame (Dent and Gertler 2003). Branches on axons form in a similar manner. Actin begins to accumulate at the wall of the axon and the Arp2/3 complex allows the actin to branch off and protrude away from the main axon, microtubules then follow and consolidation occurs (Kalil and Dent 2014). In order to generate the force required to create these protrusions, molecules that bind actin and connect it to the cell membrane are required. A complex of *Cortactin* and *Shootin1* allows for the tethering of actin to the cell membrane; this allows the polymerization of actin filaments to apply pressure to the leading edge of the axon as opposed to the entire filament sliding back towards the cell body (Kubo et al. 2015). This makes levels of *Cortactin* an important regulator of axon growth and branching.

#### 5.2.3 Cortactin

There are five main domains in the *Cortactin* protein: a N-terminal acidic domain, a repeat domain, an alpha-helix domain, a proline-rich region and a Src homology 3 (SH3) domain (Schnoor, Stradal, and Rottner 2018a) (Figure 5.1). The N-terminal acidic domain is critical for interactions with the Arp2/3 complex. In the repeat domain in humans there are 6.5 tandem repeats of 37 amino acids, while *Drosophila* only has 4 repeats (Katsube et al. 1998). These repeats allow *Cortactin* to bind to actin (Ammer and Weed 2008). The SH3 domain is critical for protein-protein interactions. *Cortactin* acts as a monomeric protein that binds and activates the Arp2/3 complex in order to promote actin nucleation and then stabilizes actin branching points thereby promoting the actin polymerization required for axon branching (Weaver et al. 2001; Uruno et al. 2001). The SH3 domain is also important to target proteins to specialized actin assemblies such as recruiting *Shank* to post-synaptic densities and *dynamin 2* during receptor-mediated endocytosis (Daly 2004).

Cortactin activity is regulated in a variety of ways. For example, it can be regulated by reversible post-translational modification (phosphorylation, acetylation and ubiquitination) at numerous sites throughout its structure (Schnoor, Stradal, and Rottner 2018b). Cortactin can also be cleaved by *Calpain*, proteases (C. Huang et al. 1997). Cleavage of *Cortactin* by *Calpain* occurs between the repeat domain and alpha helix domain and limits membrane protrusion and fibroblast migration showing that *Calpain* is a negative regulator of *Cortactin* (Perrin, Amann, and Huttenlocher 2006).



#### Figure 5.1 Protein structure of human and Drosophila Cortactin.

Schematic showing similarity between human and *Drosophila Cortactin*. Green box represents 37 amino acid repeats. Yellow box represents an alpha-helical domain. Purple box represents a proline-rich region. Red box represents SH3 domain. Percentages between human and *Drosophila* schematics show percentage of identities between the N-terminal domain, the 37 amino acid repeat region, the alpha-helix domain and the SH3 domain respectively. The total number of amino acid residues in each protein is indicated on the right. (Katsube et al. 1998)

The *Drosophila Cortactin* ortholog is functionally similar to its human counterpart. In *Drosophila, Cortactin* is not an essential gene. *Cortactin* mutants are viable and fertile; however, they do show defects during oogenesis with smaller ring channels and impaired border cell migration (Somogyi and Rørth 2004). *Cortactin* has also been shown to be involved in activity-dependent modification of the synapse structure with increased levels of *Cortactin* observed at

stimulated synaptic terminals leading to an increase in synapses (Alicea et al. 2017). These observations suggest that *Cortactin* in *Drosophila* is involved in cell migration and neuron synaptic plasticity similar to its role in vertebrates.

### 5.2.4 Calpains

*Calpains* are calcium-dependent cysteine proteases that are ubiquitously expressed throughout every cell type that has been carefully examined and can cleave over a hundred different proteins (Goll et al. 2003). In the nervous system, *Calpains* have been shown to be involved in long-term potentiation. As NMDA receptors are stimulated, intracellular calcium increases and activates *Calpains*, which cleave proteins involved in microtubule stability and also regulators of receptor trafficking. This results in destabilization of the synapse and promotes mobilization of AMPA receptors to the postsynaptic membrane (Baudry et al. 2014). In the hippocampus, inhibition of *Calpain* causes a reduction in spatial working memory and inhibits the decreased levels of *Cortactin* normally seen following maze training in rats (Olson, Ingebretson, and Harmelink 2015). This suggests that *Calpains* destabilize the cytoskeleton, which is an important step for neural plasticity.

In contrast, overactivation of *Calpains* has deleterious effects on neurons, and abnormal *Calpain* activation triggers progression of neurodegenerative diseases and causes neuronal death (Yildiz-Unal, Korulu, and Karabay 2015). Calpains have been targeted for therapeutic use in traumatic brain injury. Use of Calpain inhibitors after brain trauma can attenuate functional and behavioral deficits as well as reduce cell death (Saatman, Creed, and Raghupathi 2010). Also, the inhibition of Calpains after stroke can increase proliferation and migration of neural stem cells (Machado et al. 2015). Studies in *Drosophila* have implicated Calpains in the cleavage of Tau

leading to neurotoxicity *in vivo* (Reinecke et al. 2011; Rao et al. 2014). Inhibition of Calpains also protects against the aggregation and toxicity of mutant huntingtin (Menzies et al. 2015). In vertebrates there is a specific endogenous inhibitor of Calpains, Calpastatin, which competes with Calpains's substrates for binding to Calpain's protease domain (Hong Ma et al. 1993). While *Calpains* play an important role in the nervous system, tight regulation of their protease function is critical to maintain normal neuron function.



#### 5.2.4.1 Calpains in *Drosophila*

Figure 5.2 Protein Structures of the four *Calpain* like proteins in *Drosophila*.

Domain numbers are listed at the top. Blue box represents the protease domain with the three active site residues indicated. *CalpC* lacks the three active resides as indicated by the Xs. *CalpA* has an insertion at the beginning of domain IV making it slightly longer. *CalpD* only shares homology with the protease domain. The orange box represents a domain with zing finger like motifs. The pink box represents the SOL-homology domain. (Friedrich, Tompa, and Farkas 2004)

There are four *Calpains* in *Drosophila melanogaster*. Only *CalpA* and *CalpB* resemble the mammalian typical *Calpains*. *CalpC* lacks a functional protease domain and has been hypothesized to act as a Calpain inhibitor, in place of Calpastatin which has not been found in *Drosophila*. *CalpD* shares little homology with the other *Calpains* and was originally identified in *Drosophila* for its function in the eye and was given the name *small optic lobes*. (Friedrich, Tompa, and Farkas 2004). The C-terminal of *CalpD* has 36% identity to the protease domain of human calpain but the N-terminal has no homology and instead has six motifs that are similar to zinc fingers typical of DNA binding proteins (Goll et al. 2003). *CalpA* and *CalpB* have four domains: N-terminal domain I, the catalytic domain II, domain III, and domain IV. The catalytic domain contains three active site residues that are critical to the protease function. Domain III contains a  $Ca^{2+}$  and lipid-binding entity. Domain IV has 5 EF-hand motifs for binding  $Ca^{2+}$ . (Figure 5.2)

Typical *Calpains* in *Drosophila* are involved in similar processes as those observed in vertebrates. Downregulation of *CalpB* delays migration of border cells in egg chambers showing that *Drosophila Calpains* are also involved in cell migration, as seen in mammalian systems (Kókai et al. 2012). *Calpains* also play roles in pruning neurons during development in *Drosophila*. Sensory neuron dendrites show an increase in calcium levels followed by *Calpain* activity hours before the dendrite is pruned (Kanamori et al. 2013). Due to the similarity in function, we postulate that *Drosophila* would be an excellent system for studying the function of *Calpains* in neuron sprouting *in vivo*.

### 5.2.5 Proteolysis of *Cortactin* by *Calpains* represses branching *in vitro*

Previous work in our lab established a model of active repression of protrusions along axons *in vitro* (Mingorance-Le Meur and O'Connor 2009). In neurons isolated from mouse embryo hippocampus, it was found that Calpain localizes to consolidated axons where little or no branching or growth normally occurs. Upon inhibition of *Calpain* using a pharmacological inhibitor, sprouting of new neurites was observed along the previously consolidated axon. Looking at downstream targets of *Calpain*, we found that *Cortactin* localizes primarily to growth cones, the active site of growth. Inhibiting *Calpain* led to an increase in *Cortactin* levels throughout the axon and increased branching along the axon. Finally, after treating the neurons with physiological branching factors, it was observed that there was a decrease in *Calpain* activity that corresponded to an increase in *Cortactin* levels and new branch formation. Together this points to a model where *Calpain* proteolysis of *Cortactin* inhibits branching along the consolidated axon. In order to further explore this mechanism of axonal branching and plasticity as well as to test this in an *in vivo* system such as *Drosophila melanogaster*.

## 5.2.6 Neural plasticity models in Drosophila

### 5.2.6.1 Central nervous system model: *Eh* expressing neurons

To test this model of plasticity, it was important to find identifiable neurons in the central nervous system that could be imaged and had long enough axons that significant branching could be detected. In *Drosophila*, there are two eclosion hormone (Eh) expressing neurons whose cell bodies reside in the ventromedial brain and each sends a single non-branching projection down the ventral nerve cord. Loss of these neurons results in 70% lethality during larval stages

(McNabb et al. 1997a). Therefore, if increased branching results in aberrant neuron function this may result in death at the larval or pupal stages. As the axons of these neurons do not typically exhibit any branching and therefore may be resistant to plasticity, a second set of neurons in the central nervous system, *Hugin* expressing neurons, were selected to increase the odds of observing an increased branching phenotype.

### 5.2.6.2 Central nervous system model: *Hugin* expressing neurons

Hugin expressing neurons were selected as a model because they have single axons that descend into the ventral nerve cord with many small branches that comes off this axon. There are 20 Hugin expressing neurons that are involved in taste-mediated feeding behavior and are innervated by gustatory receptor neurons with axons that innervate pharyngeal muscles as well as the central neuroendocrine organ in higher brain centers (Melcher and Pankratz 2005). Of these Hugin expressing neurons there are a set of two neurons that send projections to the ring gland and down the ventral nerve cord (Bader et al. 2007; Schlegel et al. 2016). These projections in the ventral nerve cord provide a single axon that can be assayed for increased branching. These specific projections have also been implicated in connecting the circadian clock to locomotor activity by receiving signals from neuropeptide diuretic hormone 44 expressing neurons in the subesophageal zone and transmitting them to motor neurons in the ventral nerve cord (King et al. 2017). As these neurons signal to motor neurons, this allows for behavioral assays that test the function of these specific neurons. Thus, Hugin expressing neurons provide a clear model for studying axon branching with the potential to study the effect of structure on function.

### 5.2.6.3 Peripheral nervous system model: NMJ 6/7 and NMJ 4

The *Drosophila* larvae has thirty stereotypically patterned muscle fibers in each of its six abdominal hemisegments, which are innervated by thirty-two motor neurons (Hoang and Chiba 2001). As in vertebrates, these motor neurons begin forming neuromuscular junctions during embryogenesis and the cytoskeleton is a key element in this process (Long and Van Vactor 2013). The most commonly studied neuromuscular junctions (NMJs) are NMJ 6/7 and NMJ 4 (Figure 5.3). NMJ 6/7 spans across two muscle fibers and is large with many branches forming synapses along the muscles, allowing for relative assessment of gross morphological changes and changes to the size of the NMJ. NMJ 4 innervates muscle fiber four and is smaller in size making it simpler for analyzing branch number and length. Together these two NMJs provide slightly different models for peripheral axon branching.



Figure 5.3 Schematic of the location of NMJ 6/7 and NMJ 4.

Depiction of a fillet dissection of a wandering L3 larvae showing the muscle fibers along the body wall. Thirty muscle fibers repeat at each segment and are mirrored on the other side. Close up of the muscle fibers are depicted in the inset. Landmark muscle fibers are numbered. NMJ 4 is depicted in red on muscle fiber four. NMJ 6/7 is depicted in green and spans across muscle fiber six and seven.

#### 5.2.7 Objective

Based on previous work showing that axon branching is actively inhibited by Calpain cleavage and inactivation of Cortactin, it was important to further elucidate this pathway in an *in vivo* system. *Drosophila melanogaster* is a genetically tractable system with only two typical *Calpains, CalpA* and *CalpB*, and a single *Cortactin* gene. Using the Gal4/UAS system, *Cortactin* can be overexpressed in various neurons and its effect on branching can be analyzed. Based on previous work it would be expected that an overexpression of *Cortactin* would cause an increase in branching. As there are only two *Calpain* genes in *Drosophila*, double mutants can be created to study the effect of the loss of *Calpain* on axon branching in the central nervous system as well as at NMJs. Once phenotypes have been established for *Calpain* and *Cortactin* separately they can be studied in combination to see if they are in the same genetic pathway. To this end, a *Cortactin* construct that is missing the *Calpain* cleavage site can be used to see if *Calpain* negatively regulates *Cortactin* by directly cleaving it. This will establish a model system for studying the role of *Calpain* and *Cortactin*, in neuronal plasticity in the developing and mature nervous system.

## 5.3 Materials and Methods

#### 5.3.1 Fly genetics and stocks

Strains used: w;  $P\{RS5\}CalpA^{5-SZ-3979}$  (Ryder et al. 2004). y,w; CyO,  $PBac\{\Delta 2-3\}Exel\}^{2/amos^{Tft}}$  (Bloomington stock 8201). y,w;  $P[EPgy2\}CalpB^{EY08042}$  (Kókai et al. 2012). st,Blm,Sb, $P\{\Delta 2-3\}99B/TM6B$ , Tb (Bloomington stock 8657). y,w;  $P\{UAS-CD4-tdGFP\}8M2$ , w;  $PBac\{UAS-CD4-tdGFP\}VK00033$  (Han, Jan, and Jan 2011). w;  $P\{GAL4-Eh.2.4\}C21$ (McNabb et al. 1997b). y,w;  $P\{UAS-Cortactin.HA3\}2$  (Bloomington stock 9368). w;;  $P\{Hug-GAL4.S3\}3$  (Melcher and Pankratz 2005). w; Df(2R)ED3716/CyO, wglacZ (Ryder et al. 2007). w;  $P\{GawB\}VGlut^{OK371}$  referred to as OK371-GAL4 (Mahr and Aberle 2006).

## 5.3.2 Creating CalpA and CalpB imprecise excisions

In order to create null mutants of both *Calpain* genes, imprecise excisions of a P-element were created. An imprecise excision occurs when a P-element is mobilized by a transposase and causes small to large deletions on either side of the site of the P-element insertion. By selecting for P-elements inserted in the 5'UTR of a gene, these small deletions are likely to remove the start codon of the gene and create a null mutation. Precise excisions can also be created during this process. They lack the small deletions and have instead a small footprint where the Pelement had been inserted. The P-elements are marked with  $w^{+mW.hs}$  that gives a red eye phenotype and can be used to track flies that carry or no longer carry the P-element. *CalpA* and *CalpB* imprecise excisions were created by mating w; *P*{*RS5*}*CalpA*<sup>5-SZ-3979</sup> or *yw*; *P*[*EPgy2*]*CalpB*<sup>*EY08042*</sup> to lines carrying a transposase and isolating lines that have an excised Pelement, by selecting for flies with white eyes (Carney et al. 2004). These flies were then

screened using PCR to identify deletions in these *Calpain* genes. Gene deletions were then sequenced to identify the exact nature of the deletion. Simultaneously, in flies without deletions, we sequenced flies to identify those with so-called precise excisions which leaves the gene intact except for a small footprint.

### 5.3.3 Creating wildtype and non-cleavable UAS-Cortactin constructs

We obtained the cDNA of *Cortactin* (Clone stock: LD29964) was obtained from the *Drosophila* Genomics Resource Center. Wildtype *Cortactin* was cloned directly from this cDNA and inserted into pUASTattB vector. The deletion in the *Cortactin* cDNA was created using SOE PCR and then also placed in the pUASTattB vector. These pUASTattB-Cortactin plasmids were injected into a *Drosophila* line with the *attP40* locus, allowing for integration of the transgenes on the second chromosome by phiC31-integrase (performed by Rainbow Transgenics Inc, CA) (Groth et al. 2004). Independently-transformed lines were isolated and sequenced to verify insertion.

Primer Name	Primer Sequence
CortactinRF_1F	agggaattgggaattcatgtggaaggcaagtgccg
CortactinRF_1R	ggcctcacggagacggtccacctggctggcgtg
CortactinRF_2F	gccagccaggtggaccgtctccgtgaggccaagg
CortactinHAEcoRI	aattgggaattettatacccatacgatgtteetgactatgegtgagttetgteecaccacetge

Table 5.1 List of primers used to create non-cleavable Cortactin construct.

Primers were used for the SOE PCR reaction to create the deletion in the *Cortactin* cDNA. Top and bottom primers were used to PCR the *Cortactin* cDNA to create the wildtype construct.

### 5.3.4 Dissections, immunostaining and analysis

The central nervous system (CNS) was dissected in phosphate-buffered saline (PBS), from wandering L3 larvae maintained at 25°C. CNS were fixed in 4% paraformaldehyde for thirty minutes and then mounted on slides. Whole larval fillet dissections, for viewing the NMJs, was also done in PBS on wandering L3 larvae maintained at 25°C from vials that had 60 L1 larvae placed in them (Parton et al. 2010). They were fixed using 4% paraformaldehyde for thirty minutes. They were then blocked with 5% goat serum in PBS + 0.3% Triton X-100 for 20min at room temperature. Discs were then incubated at 4°C overnight with 1:20 anti-Brp (nc82, DSHB), 1:50 anti-HRP conjugated to Cy5 (Jackson ImmunoResearch) and 1:1000 Alexa Fluor 488 phalloidin. The secondary antibody, donkey anti-mouse Cy3 (1:500; Jackson ImmunoResearch) was incubated with the fillets for 1 hour at room temperature and then were mounted onto slides. Images were taken on an Olympus FV1000 confocal. Image analysis was performed using Simple neurite tracer in ImageJ. Statistical analysis was performed in GraphPad Prism.

## 5.3.5 Calpain inhibitor bathing assay

Wandering L3 larvae maintained at 25°C were filleted in HL3.1-Ca<sup>2+</sup> buffer. Pins were only placed in the anterior and posterior ends, so they were not completely pinned open. The media was then replaced with HL3.1+Ca<sup>2+</sup> media with 25uM or 100uM ALLN (Calbiochem) or DMSO control and incubated for 40min (Zhang and Bhavnani 2006). 2ml of the media was removed and replaced with fresh media every 10min. After the incubation, the media was drained and replaced with HL3.1-Ca<sup>2+</sup> and the fillets were pinned completely open. They were then fixed and immunostained as described above.

# 5.4 Results





Figure 5.4 Calpain null mutations and Cortactin constructs created.

(A) *CalpA\_IE57* deletion created using imprecise excision of a P-element. Image is from GBrowse in Flybase showing the genomic location of *CalpA* on chromosome two. The red box covers the deleted region of the gene. (B) *CalpB\_IE67* deletion created using imprecise excision of a P-element. Image is from GBrowse in Flybase showing the genomic location of *CalpB* on chromosome three. The red box covers the deleted region of the gene. (C) Description of non-cleavable and wildtype *Cortactin* constructs inserted into the *Drosophila* genome. cDNA was inserted into the pUASAttB plasmid depicted. 10kb size includes *Cortactin* insert. Schematic of *Cortactin* protein structure with the green box representing the region with the amino acid repeats and the yellow box representing the alpha-helix domain. Deletion is in the connecting region between those two domains. Amino acids deleted are listed at the bottom.

In order to study *Calpain* and *Cortactin* in *Drosophila*, several genetic tools needed to be created. The first was *CalpA* and *CalpB* null mutants as there were none available. P-element imprecise excision was used as there were available lines for both *CalpA* and *CalpB* genes with P-elements inserted in the 5'UTR. These were selected because they had the greatest opportunity to remove the start codon. By using a transposase, the P-element was hopped out of the 5'UTR of both genes. A hundred lines of individual P-element hops were created for each gene. These were then scanned through using PCR to search for instances where the removal of the P-element resulted in the removal of the surrounding genomic DNA. Two excisions that were selected for *CalpB* both had a deletion that includes the start codon and half of the protease domain including the cysteine in the active site (Figure 5.4B). This large deletion would not be expected to generate a functional protein, making this an amorphic allele. Unfortunately, there was no available antibody for either protein to confirm that no part of the protein was produced.

A single line for *CalpA* was isolated. Similar to *CalpB* excisions, this *CalpA* line has a deletion that covers the start codon and most of the protease domain, removing all three of the

active site amino acids (Figure 5.4A). To create controls for these imprecise excision lines, I also screened for excisions that left only a footprint where the p-element excised. These lines were the same genetic background as the imprecise excisions, making them the most appropriate non-mutant controls. These were isolated for both *CalpA* and *CalpB*. Once these lines were created, a double mutant line for both typical *Calpains* in *Drosophila* were generated and analyzed.

Surprisingly, double mutants of both typical *Calpains* in *Drosophila* were viable and fertile. Indeed, double mutants were easily maintained as a stable double homozygous stock. There were no gross morphological differences that could be detected. While the stock could be maintained, there was a decrease in progeny number when the male *Calpain* double mutants were mated to wildtype females. A quick investigation showed that it appeared to be a problem with mating, but this avenue was not pursued further. The lack of obvious phenotypes was surprising because these flies lack all typical *Calpains*. However, there was still the possibility of more subtle phenotypes such as changes to branch number in neurons which may not be sufficient to disrupt neuron function.

Another genetic tool that was required for this project was a version of *Cortactin* that was missing the region that can be cleaved by *Calpain*. Work done by Perrin, Amann, and Huttenlocher 2006 showed that *Calpain* can cleave *Cortactin* at multiple positions in a region between the amino acid repeats and the alpha-helical domain. They created a deletion of this region and showed that *Cortactin* was still biochemically active and could not be cleaved by calpains. The homologous region in the *Drosophila Cortactin* was deleted to create a non-cleavable version of *Cortactin* under control of the UAS enhancer and inserted into the *Drosophila* genome at the attP40 site using phiC31-integrase (Figure 5.4C). To compare to

wildtype *Cortactin* a wildtype version of *Cortactin* was created in the same manner and inserted into the same genomic location to control expression levels. If *Cortactin* is negatively regulated by *Calpain* through direct cleavage of *Cortactin*, then it would be expected that the noncleavable version of *Cortactin* should be more active than the wildtype version. With these new genetic tools created, it was now possible to study the effect of the loss of *Calpain* in *Drosophila*, as well as *Calpains* role in regulating *Cortactin*. The next step was to identify a model of axon branching.

## 5.4.2 Overexpression of *Cortactin* did not cause additional branching in *Eh* neurons



UAS-CD4GFP ; Eh-GAL4

Eh-GAL4

Figure 5.5 Overexpression of *Cortactin* in *Eh* expressing neurons did not cause additional branching.
(A) View of all *Eh* expressing neurons within the central nervous system of the wandering L3. The central nervous system is outlined in white. Neurons are visualized using *UAS-CD4GFP*. White rectangle is inset region shown in B,C. (B) *Eh* expressing neuron's axon in wildtype shows no branching. (C) *Eh* expressing neuron overexpressing *Cortactin* does not show additional branching.
Several *GAL4* drivers were tested for suitability in visualizing single axons that could be assayed for additional branching. *Eh-GAL4* was selected because it sends two axons down the ventral nerve cord of wandering L3s and can be easily visualized. Also, preliminary work done previously in the lab had indicated that the overexpression of *Cortactin* may cause an increase in the length of the axon. However, upon additional testing it was found that overexpressing *UAS-Cortactin* with *Eh-GAL4* did not increase the amount of branching along the axons that run down the ventral nerve cord and did not increase the length of the axons (Figure 5.5). It was possible that because in wildtype larvae there is not any branching along this axon, there might be additional inhibitory signals that stop the overexpression of *Cortactin* from creating branches along the axon. Therefore, I looked at a different set of neurons in the ventral nerve cord that shows branching under wildtype conditions.

# 5.4.3 Loss of *Calpain* or overexpression of *Cortactin* did not increase branching of *Hugin* expressing neurons in the ventral nerve cord

*Hugin* expressing neurons were selected as a model because they send single projections down the ventral nerve cord and under wildtype conditions exhibit a number of small branches off the main axon. Because most of the branches are smaller than 20um, a small section of the axon, 100um, just above where the axon begins to curl inwards, was imaged at high magnification to resolve all branching that existed (Figure 5.6A). In wildtype flies, there was an average of thirty branches that occurred with a branch being any neurite that branches off the axon and its length was measured from where it branches off the axon to where it terminates. Along these projections there was a number of synapses that may synapse onto motor neurons (King et al. 2017). These neurons have been used in locomotion behavioral assays which would allow structural changes to be tested for whether they have an effect on neuron function.

Several genotypes were analyzed. All of the lines carry a copy of *UAS-CD4GFP*, which is a membrane bound GFP that allows for the visualization of small projections due to the membrane association, as well as *Hugin-GAL4* (Han, Jan, and Jan 2011). The precise excisions (PE) made for both *CalpA* and *CalpB* were combined together and mated to *Calpain* double mutant flies making the precise excision neurons heterozygous for both *Calpain* genes. The precise excision was compared to flies expressing only *Hugin-GAL4* and *UAS-CD4GFP* and there was no statistically significant difference in branch number between them (Figure 5.6B). For the *Calpain* double mutants, there is a single imprecise excision (IE) that was created therefore, a deficiency that covers *CalpA* was used as the other allele. For *CalpB* there were two imprecise excisions created and therefore, the double mutant carries an allele of each of them. For flies that are *Calpain* double mutants and overexpress *Cortactin*, *UAS-Cortactin* was recombined into the double mutant background. These lines were used for the analysis of branching and branch length in *Hugin* expressing axons.

*Hugin* expressing neurons were analyzed in double *Calpain* mutants as well as in *Cortactin* overexpressing lines and as combinations of both genotypes. Branch number was counted, and no significant difference was found between these conditions (Figure 5.6B). It was noted though that there was a large variation of branching between experiments even though the experiment was performed under the same conditions each time. While all neurons analyzed have now been grouped in the data, for determination of statistical significance only neurons analyzed from the same experiment were compared. During some experiments there were

statistically significant differences between genotypes however, these differences were not observed when the experiment was repeated. Therefore, *Calpain* mutants or the overexpression of *Cortactin* may play a role in branching, but variation from experiment to experiment is too large for these differences to appear significant.

Additional parameters were analyzed to see if the loss of Calpain or the overexpression of Cortactin affected neuron architecture in a more unexpected way. To see if there was an increase in the length of projections, the sum of the length of all branches within a 100um length of the axon was compared. Neither *Calpain* double mutants nor the overexpression of Cortactin increased the total length of branches compared to the precise excision control (Figure 5.6C). Because there are many tiny branches but only a few longer branches, the number of branches over 20um was counted with the idea that only some of the branches were increased in length and that this difference may be lost by summing all the branches. However, there was no difference in the number of branches over 20um in length (Figure 5.6D). Finally, it was observed that there is typically a single long projection that branches off of the region that was being analyzed. However, looking at the length of that single long projection did not show an increase in length in *Calpain* double mutants or with the overexpression of Cortactin (Figure 5.6E). In conclusion, Hugin expressing neurons show too much variation between experiments to accurately rely on the analysis done and there were no statistically significant differences in branch architecture found between wildtype flies and Calpain double mutants or with the overexpression of Cortactin.













Figure 5.6 *Calpain* double mutants or the overexpression of *Cortactin* does not affect branching of *Hugin* expressing neurons.

(A) Image of w; UAS-CD4GFP/+; Hugin-GAL4/+ flies showing the entirety of neurons that express Hugin. White outline around neurons is the central nervous system. There are two axons that descend into the ventral nerve cord. The white box indicates the region of the axon that was analyzed for branching. (A') A higher magnification image of the region inside the white box. The axon shows a number of projections coming off of it under wildtype conditions. (B - E) Each dot represents an axon of a wandering L3 pooled from multiple experiments. Mean ± SEM. Graphs show pooled data from many separate experiments. Unpaired t-tests were performed within each individual experiment and p values were all greater than 0.05. Genotypes used: w<sup>1118</sup> = w;UAS-CD4GFP/+;Hugin-GAL4/+, precise excision control = w;CalpA PE52/Df(2R)ED3716; UAS-CD4GFP,CalpB PE35/Hugin-GAL4,CalpB IE2, Calpain double mutants = w;CalpA IE57/Df(2R)ED3716; UAS-CD4GFP,CalpB IE67/Hugin-GAL4,CalpB IE2, Cortactin overexpression = w;UAS-CD4GFP,UAS-Cortactin/+;Hugin-GAL4/+, Calpain double mutants with Cortactin overexpression = w;CalpA IE57, UAS-Cortactin/Df(2R)ED3716; UAS-CD4GFP,CalpB IE67/Hugin-GAL4, CalpB IE2. (B) In the 100um region analyzed there is no increase in the number of branches in any of the genotypes. (C) Looking at the sum of the length of all the branches there is no difference in Calpain double mutants or with the overexpression of Cortactin compared to controls. (D) In Calpain double mutants and neurons overexpressing Cortactin there is not an increase in the number of branches that are over 20um in length. (E) Looking at the length of the longest branch shows that there is not a difference in length with loss of Calpain or with the overexpression of Cortactin.

# 5.4.4 Loss of *Calpain* or the overexpression of *Cortactin* did not increase the size or number of branches of NMJ 6/7 or NMJ 4

The peripheral nervous system was also assayed for differences caused by the loss of *Calpain* or the overexpression of *Cortactin*. Looking at a number of parameters there was no differences in NMJ architecture at either NMJ 6/7 or NMJ 4 with the loss of *Calpain* or the

overexpression of Cortactin. Wandering L3 larvae were filleted open and stained for anti-HRP, which stains neuron membranes. The *OK371-GAL4* was used to express *UAS-Cortactin*. *OK371-GAL4* is expressed in glutamatergic neurons that express *Vesicular glutamate transporter*, which includes motor neurons (Mahr and Aberle 2006). This allows for Cortactin to be overexpressed in motor neurons, and then the NMJs can be visualized.

Analysis of branch number and size of NMJ 6/7 showed that overexpression of Cortactin did not increase the size of the NMJ (Figure 5.7 B-C). NMJ 6/7 enters between two muscle fibers and then spans across them producing synapses on both muscles (Figure 5.7A). The overexpression of Cortactin did not increase the number of branches at the NMJ (Figure 5.7B) Also the overexpression of Cortactin did not increase the overall size of the NMJ, which indicates that there were no changes in the lengths of the branches (Figure 5.7C). Therefore, increased levels of *Cortactin* does not affect the size or branching of NMJ 6/7.

I also examined NMJ 4 (Figure 5.7D). Neither the loss of *Calpain* nor the overexpression of Cortactin increased the size or number of branches of NMJ 4. The total area of NMJ 4 was calculated for motor neurons overexpressing Cortactin and compared to wildtype NMJ 4's; however, there was no difference in size (Figure 5.7E). Next, the number of branches were counted for both the *Calpain* double mutants and motor neurons overexpressing Cortactin, but no statistically significant difference was found (Figure 5.7F). In conclusion, the loss of *Calpain* and the overexpression of *Cortactin* was unable to affect the overall size and branching of NMJ 4.



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Figure 5.7 Loss of *Calpain* or the overexpression of *Cortactin* does not affect the overall architecture of NMJ 6/7 or NMJ 4.

(A) Representative image of a NMJ 6/7. (B – J) Each dot represents an NMJ of a wandering L3. Mean ± SEM. Unpaired t-tests were performed for graphs comparing two genotypes, no graph showed a p-value less than 0.05. One-way ANOVA was performed with graphs with more than two genotypes, with no graph having a p-value less than 0.05. Genotypes used:  $w^{1118} = w; UAS-CD4GFP/+; OK371-GAL4/+$ , precise excision control = w;CalpA PE52/Df(2R)ED3716; CalpB PE35/CalpB IE2, Calpain double mutants = w;CalpA IE57/Df(2R)ED3716; CalpB IE67/CalpB IE2, Cortactin overexpression = w;UAS-CD4GFP,UAS-Cortactin/+;OK371-GAL4/+. (B) The number of branches at NMJ6/7 was counted. Overexpression of Cortactin did not affect branch number. (C) The total area covered by NMJ6/7 was calculated. Overexpression of Cortactin did not affect the overall size of NMJ6/7. (D) Representative image of NMJ4. (E) The total area covered by NMJ4 was calculated. Overexpression of *Cortactin* did not change the overall size of NMJ4. (F) The number of branches at NMJ4 was counted. Neither the loss of Calpain nor the overexpression of *Cortactin* affected the number of branches. (G) Representative image of two branches coming off the main axon for NMJ4. (H) The number of branches that come off the main axon for each NMJ4 was counted. Loss of Calpain does not affect the number of branches from the main axon at NMJ4. (I) Representative image of satellite boutons. White arrows point to instances of satellite boutons. (J) The number of satellite boutons at NMJ4 was counted. Neither loss of Calpain nor the overexpression of Cortactin increased the number of satellite boutons.

However, while analyzing NMJ 4s of *Calpain* double mutants, it appeared that *Calpain* double mutants occasionally showed a second branch coming off the main nerve that aberrantly innervated muscle four (Figure 5.7G). However, upon comparing the precise excision control, there was no statistical significance in the frequency of this branching event, and it was seen to occasionally occur in wildtype NMJ 4s (Figure 5.7H). A second feature that was noticed was an increase in satellite boutons (Figure 5.7I). Satellite boutons tend to occur in NMJ overgrowth phenotypes and involves small boutons forming on larger parent boutons (Menon, Carrillo, and

Zinn 2013). Each satellite bouton at NMJ 4 was counted for both *Calpain* double mutants and motor neurons overexpressing *Cortactin*, neither showed a statistically significant increase in satellite boutons (Figure 5.7J). Therefore, though visually there appeared to be an increase in branching and satellite boutons, statistically there was no increase.

#### 5.4.5 A Calpain inhibitor did not affect branching of NMJ 6/7 or NMJ 4

One possibility for a lack of phenotype with the double *Calpain* mutants is that other proteases such as caspases have time to compensate for the lifelong loss of *Calpain*. The original cell culture experiments were performed using a Calpain inhibitor therefore, a Calpain inhibitor may have an effect at the NMJs (Mingorance-Le Meur and O'Connor 2009). Wandering L3s were filleted and the *Calpain* inhibitor, ALLN, was applied. A number of timepoints were tested to obtain the longest timepoint before the tissue began to die or deteriorate. The final timepoint used for the experiment was 40min.

Wildtype wandering L3s were filleted and treated with two different concentrations of ALLN for 40min then fixed, stained and imaged. Looking at both concentrations, and at both NMJ 6/7 and NMJ 4, there were no changes to the structure of the NMJ. The muscle fibers of these larvae were still completely intact and before the fix was added the larvae were checked to confirm they still had muscle contractions occurring. In conclusion, neither the chronic nor acute loss of *Drosophila* typical *Calpain* activity appeared to have any effect on branching or size of NMJ 6/7 or NMJ 4.

#### 5.5 Conclusions

It was a very unexpected result that the double *Calpain* mutants exhibited no obvious phenotype and were fully viable. Even use of a *Calpain* inhibitor, which should also have inhibited anything that functioned like a *Calpain*, exhibited no effect. There are a few possible explanations for this result. First, *Caspases* are also cysteine proteases that are involved in many of the same functions as *Calpain*, therefore it is possible that *Caspases* compensate for the loss of Calpain (Unsain and Barker 2015). There is also CalpD, which even though it only shares homology with the protease domain, may be able to cleave some of the downstream targets of the typical *Calpains*. One possible way to confirm if compensation by another protease is occurring, is to identify a downstream target of *Calpain* and use a western blot to detect changes in protein levels and perhaps even see cleaved products. Dendrite pruning has been shown to rely on Calpains in Drosophila and this system could be screened for downstream targets of Calpains (Kanamori et al. 2013). While the bathing experiment was not enough time for compensation to occur, it was a short window of time for the NMJ to change size or create new branches and therefore, this could be why no phenotype was observed (Zito et al. 1999). While Calpains are a family of proteases, the number of downstream targets that they cleave is quite broad, thus it is unlikely that they are the only proteins that can cleave them, this increases the odds of compensation phenotypes.

Another possibility is that unlike in cell culture where there are few external signals and no physical wrapping of glial cells, *in vivo* there are potentially many be additional external signals stopping the axons from growing, as well as associated glia that may constrain process formation. However, I examined motor neurons that can grow in size in response to increased movement of the larvae, which makes this model ideal to examine mechanisms of additional branching (Menon, Carrillo, and Zinn 2013). One possibility is that *Calpains* do not play a role in inhibiting branching at the NMJ, which are different in structure from a long axon due to the number of synapses onto the muscle that occur along its entire length. However, *Calpains* have been shown to have a role at the *Drosophila* NMJ where they negatively regulates glutamate receptors, although this may not directly affect the size of the NMJ (Metwally et al. 2019). Therefore, there remains the possibility that *Calpains* regulate branching in certain neurons, however these neurons were not identified during this study.

There are two important aspects that was achieved with this project. The first was that *Calpain* double mutants lack obvious phenotypes, a surprising result for the field. The second is the number of genetic tools that were created for this project, and would be of use to other researchers. The first tools generated were the null alleles of *CalpA* and *CalpB*. While there were no gross phenotypes observed in this study, this does not preclude more tissue specific phenotypes that have yet to be discovered. It has been found that *Calpains* play a role in migration of border cells in egg chambers (Kókai et al. 2012). There are most likely many other roles for *Calpains* including a role in aging neurons, which has been shown in vertebrates (Nixon 2003). Secondly, I also created a *Calpain* non-cleavable version of *Cortactin*. This tool will be helpful in tying the regulation of *Cortactin* to *Calpain's* protease function. Migration during oogenesis also relies on *Cortactin*, this genetic tool will allow the function of these two proteins to be analyzed together (Somogyi and Rørth 2004). Therefore, this study has resulted in genetic tools that can be used for future studies that can go forward with the knowledge that studies of *Calpain* will require a search for more subtle phenotypes.

# **Chapter 6: Discussion**

The work in this thesis describes the creation of assays to test variant function towards determining pathogenicity of human SMAD4 variants, as well as the initial development of assays and genetic reagents to determine the pathogenicity of human BMPR1A variants. This is the first step towards the development of high-volume testing of human SMAD4 and BMPR1A variants in a robust genetic model. Determining the pathogenicity of large numbers of variants will not only aid clinicians with diagnoses but can also improve our understanding of SMAD4 and BMPR1A's molecular function, as well as improve computer models that predict effect of variants on protein function.

This thesis does not describe the first assays in *Drosophila* to examine juvenile polyposis syndrome. Akiyama, User, and Gibson (2018) created a system to integrate conserved human variant mutations into the *tkv* gene sequence and then integrate them into the *tkv* locus under activation by a heatshock promoter. This has the benefit of examining the function of these variants in a *tkv* mutant background and at endogenous promoter levels. However, it makes it challenging to screen through a large number of variants due to the time taken to generate each variant in the fly. Also, the use of somatic clones requires many replicates due to the random nature of generating these clones. The role of BMP signaling in the *Drosophila* gut has also been studied and compared to the symptoms seen with juvenile polyposis syndrome. Guo, Driver, and Ohlstein (2013) showed that BMP signaling was required in the midgut of *Drosophila* to limit proliferation of stem cells and that loss of BMP signaling led to phenotypes similar to what is

seen with juvenile polyposis syndrome. Therefore, while it is possible to create assays that would directly look at these phenotypes in the midgut, its involvement of dissection and antibody labeling runs contrary to the desired use of *Drosophila* as a simple and fast tool for variant functionalization.

The assays that I have created have the benefit of being quick to integrate novel variants into the genome and isolate fly lines. Also, they are rapid and inexpensive because they do not require laborious dissection, tissue processing for immunoreactivity and imaging. Thus, each assay allows for the rapid accumulation of data in order to determine pathogenicity. I propose that the assays I developed, and follow up assays I will discuss below, are capable of being scaled up for efficient testing all 300 – 400 SMAD4 variants listed on ClinVar. Follow up work using assays such as those mentioned above using the inducible human gene transgenes in specific tissues would then provide a more detailed picture of variant function.

## 6.1 Advantages and limitations of an overexpression approach in a genetic organism

### 6.1.1 Testing the human gene directly, versus testing fly mimetics

A common approach taken by many investigators using *Drosophila* is to generate mimetic mutations in the orthologous fly gene, either for overexpression within an inducible transgene or editing into the genome by CRISPR-Cas9. This has the advantage of testing mutations in the native fly gene locus that allows detailed phenotypic analysis of variant function, including precise determination of whether the allele is hypomorphic, hypermorphic etc. For highly conserved genes, this offers a powerful approach. However, the major caveat is that it is not the human gene itself, which comes with substantial drawbacks. It limits the variant selection to those amino acids that are identical or have similar properties, with the assumption that these amino acids are required in exactly the same way in the orthologous fly gene. This may be demonstrably true for conserved amino acids that directly bind DNA or other proteins, or are critical for enzymatic activity. However, for many other amino acids, this may not necessarily be true, even if conserved.

For these reasons, we wish to test the human proteins directly. Studying human genes in a non-human context presents the possibility that the effect of variants will be different between humans and *Drosophila*. Testing the human gene requires that we take steps to ensure that the human gene is acting appropriately in the fly system, and that variants affecting the gene's activity in humans can actually be screened out in the fly. To do this, we endeavor to establish assays in which the activity of the human protein reflects as closely as possible the activity of the orthologous fly gene. This is best determined by developing assays in which the orthologous fly gene has an important role with clear phenotypes. For example, the role of BMP signaling has been well studied in the formation of the Drosophila adult wing. Also, it is important to compare the effects of the human gene with comparable manipulation with the orthologous fly gene. This could include overexpression of the fly gene in the same assay and comparing phenotypes. As was performed in Ch3.6, overexpression of hSMAD4 and Medea both caused ectopic vein formation. Alternatively, the ability of the human gene to rescue the loss of function in the orthologous fly gene offers a powerful way to be sure that the human gene is acting appropriately.

Finally, it is important to compare functional studies to clinical work; at least for a set of variants in which genetic or clinical data have established variant activity. Such variants can help

validate the fly assays. Should several variants greatly differ from what has been found clinically then there may be a difference in how the variants function in humans compared to *Drosophila*. In my assays, hSMAD4\_G386D was determined to be pathogenic, it is also associated with several cancers as well as juvenile polyposis syndrome according to ClinVar. These results show similar conclusions between both methods. The work in this thesis not only demonstrates the functionality of assays for determining the pathogenicity of human SMAD4 variants but the importance of multiple in vivo assays to understand the nature of each human variant.

## 6.1.2 Overexpression assays, versus other approaches to variant analysis

The assays I am developing involve comparing phenotypes arising from the simple overexpression of human variants. The primary advantage of this overexpression approach is speed and direct testing of the human protein, which allows for assessment of variant function within two months of cloning the variant in one simple experiment. Several approaches that are commonly taken are less direct or considerably slower. An assay that requires the building of the transgene into a complex genetic background adds time, an important consideration for a highvolume screening system. Genome editing to replace the orthologous fly gene locus so that it codes for the human gene is also a powerful approach but would have to be repeated for every variant to test. This would be massively time consuming and would negate any speed advantage of using the fly model.

However, overexpression has a number of important caveats that must be considered, and limited as much as possible. First, the same amount of each variant must be expressed in order to compare phenotypic effects. This is controlled for by using the integrase transgenesis system, which integrates a single copy of the inducible variant transgene into the same genomic site (Bischof et al. 2007). Second, overexpression of human genes can lead to artifacts arising from the high quantity of protein. This is in part controlled for by testing the overexpression of a predicted loss of function variant which would lack the functional ability of the WT protein. As demonstrated in figures 3.3, 3.4 and 4.2, I tested a number of GAL4 drivers and temperatures to gauge the assays so that the WT generated an easily scored phenotype while a predicted loss of function would not. In this way, we know that the normal function of the WT protein is causing the phenotype.

Another concern is that the results of each assay differed in which human variants showed a different effect from *hSMAD4\_WT*, however, each assay was able to identify human variants that were different. Instead of being a weakness of the system, this instead highlights the importance of multiple assays. Each biological process relies on different functions of SMAD4 and so, each assay becomes a test of certain functions of SMAD4. Wings expressing *UAS-hSMAD4\_RQK* show the same phenotype as wings expressing *UAS-hSMAD4\_WT*, this indicates that SMAD4's ability to bind DNA is less important for vein formation. Therefore, human SMAD4 variants that affect DNA binding will not be detected using this assay. Typically, a single assay is used to determine pathogenicity of human variants, but the work of this thesis indicates that differences in variant function are being missed using such a simple strategy. One advantage of the *Drosophila* system is that we can easily test variant function in a wide array of tissues simply by changing the GAL4 driver and potential genetic interactors. Over time, a panel of assays can be established to screen out a diversity of human gene variant activities.

#### 6.2 Drosophila as a quick and cost-effective model in a whole organism

There are many other models for studying human variants that range from cell-based assays, *in vitro* biochemical assays, to *in vivo* mouse genetic analysis. However, there are a number of advantages to employing *Drosophila* as a model system for variant screening. *Drosophila* studies are not as time consuming or expensive as work carried out in rodents, as their generation time is only ten days and they can be maintained at room temperature in vials. *Drosophila* is a whole organism and therefore, does not have the artifacts and limitations that cell culture and *in vitro* systems carry. Transgenes can be stably integrated into single sites in the *Drosophila* genome, allowing for consistent gene expression levels. Cell culture relies on transfections which is not effective in each cell and is done before each assay causing problems with reproducibility and variance between assays. This makes *Drosophila* one of the most accurate, rapid and cost-effective system available to study the functional impact of human variants.

In vitro biochemical studies of human variants allow for the clear determination of whether that variant affects a specific function of that protein; however, it can only test a single specific function at a time and cannot factor in compensation that occurs in an *in vivo* setting. For example, Yigong Shi et al. (1997) identified several mutations in SMAD4 that inhibited binding between SMAD4 and SMAD2 and were found in a number of cancers. However, this fails to tell us what effect it has on TGF $\beta$  signaling in any cell type, and if it inhibits binding of other SMADs or proteins. It could also only be used on variants with mutations found in regions previously determined to affect SMAD4 binding, limiting the number of variants that can be

studied. In summary, *in vitro* assays give very clear details about extremely specific activities of a protein but fail to determine the effect on a whole system.

Human variants have also been studied in cell culture. Cell culture allows for the rapid testing of large numbers of variants but the artifacts in cell culture, due to having cells outside their native environment, can make these results questionable. To get around this, Raraigh et al. (2018) used dozens of cell lines to study the impact of variants in the *cystic fibrosis transmembrane conductance regulator* gene associated with cystic fibrosis. Using this method improved the reproducibility and consistency of their results but increased the cost and time, lessening the advantage of using cell culture. Also, it does not change the fact that the cells being studied are not receiving all the signals, at physiological levels, they would receive in their native environment and that only a single cell type is being studied. Therefore, in order to obtain consistent results when studying human variants in cell culture, the number of lines that have to be used removes the advantage of speed and cost which is the main reason for using this system.

Another model system that has been used to study variant function is mice. Moving the study of human variants into mice has typically been used as a confirmation that studies performed in cell culture are applicable to whole animals. Rastall et al. (2017) used mice to confirm previous work done in cell culture looking at the *endoplasmic reticulum-associated aminopeptidase 1* gene associated with ankylosing spondylitis, arthritis affecting the spine. This disease involves the immune system, which cannot be completely replicated in *Drosophila*. They only studied two human variants and showed that they had an impact on survival as well as the immune response, which added to the understanding of this gene's function *in vivo*. However, due to the cost and time required to perform studies in mice, they are not ideal models for

studying large numbers of variants. While it is important to confirm that functional testing of variants in other systems is applicable to vertebrates, it would be a challenge to test every variant in mice. The more important information that can be gained from vertebrate systems, will be identifying cases where functional variant testing in other model organisms, such as *Drosophila*, is not applicable to vertebrates. This can then improve models, like *Drosophila*, studying the functionality of human variants in the future.

There are several other systems that are currently used to study the functional impact of human variants and each system comes with their own set of strengths and can determine specific properties of variants. However, Drosophila is one of the best systems for the rapid testing of 100s of human variants because it is a cost-effective, reproducible in vivo system. Unpublished work from our lab has created an assay for the functional testing of *Phosphatase* and Tensin Homolog (PTEN) variants (Ganguly, personal communication). Using an eclosion assay, over 100 PTEN variants have been studied. Also, Drosophila is a model that allows for the study of variants in several tissue types, resulting in a more thorough understanding of the effect a variant has on protein function. My work demonstrates the importance of having more than a single assay as not every variant shows the same effect in each assay, indicating that each assay discriminates a specific activity or function of the protein. Currently variants are classified as either pathogenic or benign. Work in this thesis shows that a more nuanced description is required, as variants may affect only certain cell types or perhaps only in combination with certain other variants. Another benefit of the assays that I have created is that they can test the relative functional impact of human variants, in other words an allelic series, in as little as two

months which is a fraction of the time required to perform these studies in mice. Therefore, *Drosophila* is an ideal model for large scale analysis of human variants *in vivo*.

#### 6.3 Creation of genetic tools for future research

The creation of genetic tools allows not only the initial biological question to be answered but provides resources for other researchers to further their inquiries. The creation of an assay for testing human SMAD4 and BMPR1A variants allows for all human variants found in those genes to be easily tested. It also allows for more detailed analysis on how each variant studied in this thesis affects its binding partners or biochemical function. The creation of Calpain null alleles is a resource that was previously lacking and can further studies of the function of Calpain in *Drosophila*. The creation of a Cortactin construct that cannot be cleaved by Calpains will further research into the signaling pathway between Calpain and Cortactin in various biological processes. Together these genetic tools provide an opportunity for researchers to perform experiments that were not possible before.

That *Calpain* double mutants were viable and showed no obvious defects was a surprising discovery. The loss of both proteins that resemble the typical Calpains found in mammals was expected to result in lethality due to the fact that Calpains in *Drosophila* are ubiquitously expressed and that loss of the two major Calpains in mice is lethal at embryonic stages (Arthur et al. 2000). This surprising finding now allows future researchers, using *Drosophila* as a model to study Calpains, to design their experiments to look for more subtle phenotypes. There is a role for Calpains in dendritic pruning, border cell migration in egg chambers and regulation of glutamate receptors at NMJs (Kanamori et al. 2013; Kókai et al.

2012; Metwally et al. 2019). These processes can now be studied in a *Calpain* double mutant background making these studies easier to perform.

#### 6.4 Human variants can increase our understanding of biological systems

Historically, studies in model organisms such as *Drosophila* studied mutations that were randomly created in forward genetic screens, in order to identify genes involved in a specific process. This approach identifies extremely detrimental mutations, but it was difficult to identify mutations that had more subtle effects. However, humans offer a larger variety of mutations ranging from very detrimental to mild in effect. Human variants identified by association with a disease gives an indication that they will affect protein function and, in some cases, can indicate how the protein will be affected. Therefore, human variants can be used as a massive highresolution toolbox for more detailed studies of biological systems.

#### 6.4.1 Using hSMAD4 to improve our understanding of Medea

Looking at the effect of expressing *UAS-hSMAD4\_WT* compared to *UAS-Medea* in the wings shows that hSMAD4 causes increased vein loss compared to Medea, which instead causes increased ectopic vein formation. The similarity in phenotypes demonstrates that they are functionally conserved, but the differences indicate that hSMAD4, either through a difference in stability or strength of interactions with other proteins, slightly alters that function. By comparing these differences in phenotypes as well as the differences in amino acid sequences between the two proteins can increase our understanding of how Medea functions during vein development and which regions of SMAD4 are important for its different functions.

Different assays identified different human variants as having an altered function from WT. This implies that different biological processes utilize SMAD4/Medea differently. Therefore, the question becomes which of SMAD4's protein activity is being tested in each assay? One possibility is that protein levels are important for some processes but not others. Protein expression levels are controlled by each transgene being inserted into the same genomic locus, therefore changes in protein stability or protein degradation specific to each variant could alter protein levels. This can be determined using western blots probing for the SMAD4 protein. Variants of SMAD4's affinity for DNA binding can be determined using electrophoretic mobility shift assays. Genetic interaction studies are one of the advantages of the *Drosophila* model system that cannot be easily done in many other systems. SMAD4's interaction with components of the TGFβ family can be determined in the assays established in this thesis. These experiments, in combination, will not only tell us how the variants affect SMAD4's protein activity, potentially identifying new functional domains, but will also add to our knowledge of Medea's role in various biological processes.

#### 6.4.2 Computer models are only as accurate as the data used to create them

There are dozens of commonly used computer models for predicting the effect of amino acid changes on a protein's function. Each new model claims to be more accurate than the previous model but when comparing the predictions for a single variant using different models, the predictions are often the same. This is because the computer models are all created using the same databases. Benign variants are selected from databases that have sequenced the exomes of healthy individuals, but that does not guarantee that they will not develop a disease later in life, this means that in the database, variants considered benign are not always benign. Another problem with the databases is that it lacks training on the genetic diversity found in the global population, this can result in common alleles appearing rare and being classified as pathogenic (Lappalainen et al. 2019). Pathogenic variants are taken from databases such as ClinVar. The variants in these databases are collected from patients with a specific disease who are sequenced for genes that are commonly associated with that disease. This allows for variants to be detected and classified as pathogenic because they happen to be present is diseased individuals. In conclusion, computer models can only be as accurate as the data that creates them and currently the data being used is not as accurate as it needs to be.

This problem creates the need for large scale functional testing of variants. This would remove the problem of miscalled variants and provide higher quality data for the machine learning algorithms, increasing the accuracy of their predictions. Assays like the ones in this thesis will need to be created for several diverse genes and used to test 100s of variants in order to provide a large enough database for the computer models to use. The assays in this thesis are a step towards achieving this.

# 6.5 Future directions

The next step for this project involves creating assays for determining the pathogenicity of human BMPR1A variants, increasing the number of human SMAD4 variants tested using the established assays and looking at the molecular mechanism causing the lethality, vein loss and ectopic vein formation seen with expression of hSMAD4\_WT. After the BMPR1A mimetics are integrated into the *Drosophila* genome, similar assays as were created for SMAD4 will be established. Expression of *UAS-tkv* causes lethality when ubiquitously expressed and wing phenotypes when expressed in the wing. Therefore, these phenotypes can be assayed once it is

confirmed that the loss of function variants lack phenotypes when expressed ubiquitously or in the wing.

So far, seven human SMAD4 variants have been tested using the established assays; however, increasing these numbers will not only tell us about the specific variants tested but will allow for the study of trends and categories of variants. Each assay may be testing different functions of the SMAD4 protein and that is why they identify different variants. Going forward it will be helpful to biochemically test the variants that are different from hSMAD4\_WT looking at their affinity for binding Mad or their DNA binding sequences, as well as their stability. Finally using these categories of variants, identified from different assays, can allow for the study of how these variants affect the function of SMAD4. Which TGF $\beta$  pathway is responsible for the lethality or for the vein loss? How do these variants affect expression of genes regulated by the BMP pathway? The development of these assays creates the possibility of many downstream studies.

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