Chapter 1

Introduction

LIGs and BMP Signaling

An enormous array of molecules working through a number of signaling pathways governs the complex and elaborate process of development and patterning of the animal body. Starting with the discovery of inductive signals in the animal-vegetal patterning of sea urchins to the Nobel Prize winning work of E. Wieschaus, E. Lewis and C. Nüsslein-Volhard on the genetic control of animal development in the 1980s to the advent of genomics in present times, new components of the cellular signaling machinery continue to be uncovered (Nusslein-Volhard and Wieschaus, 1980). Characterization of these molecules will be critical as we seek to develop a more accurate and sophisticated systems-level understanding of cellular communication pathways and their contributions to development.

LIGs: A Novel Class of Transmembrane Proteins

One new class of signaling molecules whose role in development is only beginning to be elucidated is the LIG family of transmembrane proteins. LIG is an acronym given to a novel, unique and relatively small class of molecules that contain both Leucine-Rich Repeats (LRR) and Immunoglobulin (Ig) domains. While these two sequence elements are among the most commonly found in the metazoan proteome, their combination in a protein is relatively rare. For example, only nine and thirty six such molecules, LIGs, exist in *Drosophila melanogaster* and *Homo sapiens*, respectively (Pruess et al., 2003). This is in contrast to the large numbers of proteins that contain either domain alone; for example in humans over 375 LRR containing proteins and >750 proteins with the Ig domain (MacLaren et al., 2004; Ng et al., 2010). Moreover, within the family, the number of LRRs and Ig domains is variable among family members. For example, LRIG-1 has fifteen LRRs and three Ig domains, while AMIGO-1 has seven LRRs and one Ig domain.

Structurally, all LIGs are single pass Type I transmembrane proteins oriented with their N-terminal LRR and Ig domains in the extracellular region (in that order) and in most cases with a non-catalytic cytoplasmic tail. The sole exception to this latter point is the Neurotrophin (Trk) receptors, which have a cytoplasmic tyrosine kinase domain (Figure 1.3 and Figure 1.4). In the LIGs, the LRRs are, as with most LRR containing proteins, flanked by cysteine-rich regions.

Given the abundance of LRRs and Ig domains in proteins in the metazoan proteome, it is not surprising to find their participation in a vast array of biological processes. However, the unification of these two domains in one molecule presents itself as an interesting evolutionary event. The true functional nature of such a proteomic union in LIGs is still under investigation, but information can be gleaned from understanding what functions have been attributed to each motif separately. Therefore, background on LRRs and Ig domains is provided in the following sections before LIGs are reviewed in detail.

Leucine-rich repeats (LRRs)

Leucine rich repeat motifs, present in over 4500 proteins (Matsushima et al., 2005) from viruses to eukaryotes were identified as a repeating motif for the first time in leucine-rich α2 glycoprotein (LRG1) found in human serum (Takahashi et al., 1985). LRRs are now one of the most common interaction motifs known and have been shown to associate with a diversity of molecules, including metal ions, DNA and proteins (Buchanan and Gay, 1996). A single LRR typically consists of 20-29 amino

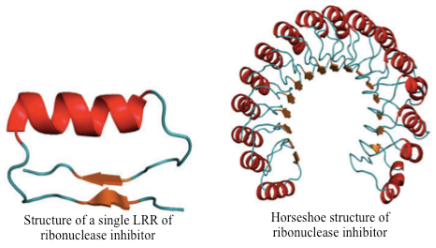

Figure 1.1: Structure of a Leucine rich repeat. Crystal structure of the LRR domain of porcine ribonuclease inhibitor (15 LRRs). This was the first 3D structure of an LRR and revealed the classic horseshoe shape of the motif (de Wit et al., 2011; Kobe and Deisenhofer, 1993).

acids with a conserved 11 amino acid consensus sequence, LxxLxLxxC/NxL, where x can be any amino acid and a variable region (Matsushima et al., 2005). The number of LRRs in a protein can range from two to fifty two and the set is often flanked on its N- and C-termini by cysteine-rich regions. The motif typically forms a horseshoe shape, the concave surface of which contains right handed parallel β-strands connected by loops to α-helices on the convex surface that are also parallel to the β-strands (Enkhbayar et al., 2004) (Figure 1.1).

In proteins that contain them, LRRs provide a structural framework for molecular interactions. Typically, the parallel β-strands and the adjacent loops on the concave surface of the horseshoe domain form the interface for the ligand/binding partner, thereby providing the basis for the role of LRR molecules in signaling. The LRR containing proteins are involved in processes including immune response in plants and animals (Nurnberger et al., 2004; Padmanabhan et al., 2009; Rairdan and Moffett, 2007), early mammalian development (Tong et al., 2000), cytoskeletal organization (Xu et al., 1997) and apoptosis (Inohara et al., 1999); they are also emerging as key players in neuronal development (de Wit et al., 2011; Ko and Kim, 2007; Proenca et al., 2011). In this latter role, the wide range of functions includes axon guidance and myelination, target selection, induction of synaptic contact formation, and stabilizing neuronal circuits. Given the number of important processes LRRs are involved in, it is not surprising to find disease conditions associated with proteins containing this motif. Some examples are presented in Table 1.1.

Table 1.1: LRR proteins and associated human diseases (de Wit et al., 2011; Matsushima et al., 2005; Shmelkov et al., 2010).

Disease	LRR containing protein	Reference
Schizophrenia	**Nogo-66 receptor (NgR)**	(Sinibaldi et al., 2004)
Parkinson's disease	**Leucine-rich repeat kinase 2 (LRRK2)**	(Zimprich et al., 2004)
Crohn's disease	**Nod2**	(Hugot et al., 2003; Ogura et al., 2001)
Autosomal dominant lateral temporal epilepsy (ADLTE)/autosomal-dominant partial epilepsy with auditory features (ADPEAF)	**Leucine-rich glioma-activated protein1 (LGI1)**	(Robinson and Gardiner, 2004)
Congenital insensitivity to pain with anhidrosis (CIPA)	**Neurotrophic tyrosine kinase receptor 1 (Trk-A)**	(Alberti et al., 2003)
Autosomal dominant polycystic kidney disease type 1 (ADPKD)	**Polycystin 1 (PKD1)**	(Stayner and Zhou, 2001)
Congenital stationary night blindness type 1 (CSNB1)/X-linked congenital stationary night blindness (XLCSNB)	**Nyctalopin**	(Bech-Hansen et al., 2000; Pusch et al., 2000)
Ovarian dysgenesis 1 (ODG1)	**Follicle stimulating hormone receptor (FSHr)**	(Aittomaki et al., 1995; Beau et al., 1998)
Leydig cell hypoplasia (LCH)	**Luteinizing hormone/Choriogonadotropic hormone receptor (LH/CGr)**	(de Roux and Milgrom, 2001)
Thyrotropin resistance, hypothyroidism, familial gestational hyperthyroidism, papillary cancer or Graves disease	**Thyrotropin receptor (TSHr)**	(Davies et al., 2005; Hebrant et al., 2011)
Autosomal recessive cornea plana (CNA2)	**Keratocan**	(Khan et al., 2004; Lehmann et al., 2001; Pellegata et al., 2000)
Bernard-Soulier syndrome (BSS) or pseudo-von Willebrand disease (PT-vWD)	**Glycoprotein Ibα (GPIbα)**	(Ramasamy, 2004)
Chronic infantile neurologic cutaneous and articular syndrome (CINCA)/Neonatal-onset multi-system inflammatory disease (NOMID)	**Cold autoinflammatory syndrome 1 protein (CIAS1)**	(Hull et al., 2003)
Tourette's syndrome and Obsessive compulsive disorder	**Slit and Trk-like family member 1 (Slitrks)**	(Abelson et al., 2005; Shmelkov et al., 2010)

As noted earlier, LRRs are found in diverse species, including *Drosophila*, which has a large number of proteins containing LRRs, including the Toll receptor (identified as a key component of the immune response) and Slit (plays a role in axon guidance). The roles of these

particular LRR proteins (and often others) have been conserved from flies to humans. LRR proteins in flies are also involved with diverse biological processes and various aspects of the nervous system and the immune system as can be seen from the selection of *Drosophila* LRR proteins listed in Table 1.2.

Table 1.2: Selection of *Drosophila* LRR proteins – (de Wit et al., 2011)

LRR protein	Localization	Function	Binding partner
Slit (Sli)	Secreted	Axon guidance, dendrite arborization, target selection;	Robo receptors
Capricious	TM	Neuronal target selection	Unknown
Tartan	TM	Neuronal target selection	Unknown
Connectin	GPI-anchored	Neuronal target selection	Self
Toll	TM	Neuronal target selection and innate immunity	Späetzle
Chaoptin (Krantz and Zipursky, 1990)	TM	Photoreceptor cell morphogenesis	Self
Signed wing (swi)/Halfway (hwy) (Schwartz et al., 2004)	EC	Ecdysone pathway	Fem-1
Larval translucida (ltl)	EC	BMP signaling	Dally-like
Protein flightless-1 (fli1) (Liu and Yin, 1998)	CY	Myogenesis	Actin and Fli LRR associated protein (FLAP)
Tehao (Luo et al., 2001)	TM	Innate immunity and male aggression behavior	Pelle protein kinase
Leucine-rich repeat kinase (lrrk2)	TM	Synapse organization	-
Rickets (rk) (Baker and Truman, 2002)	GPCR	Post ecdysis processes	-
Tollo (Aoki et al., 2007; Kim et al., 2006)	TM	Innate immunity, regulation of protein glycosylation and inhibition of BMP signaling.	-
Mapmodulin	CY	Microtubule binding	-
Lambik		Bristle morphogenesis	
Windpipe (wdp) (Huff et al., 2002)	TM	Tracheal development	-
18 wheeler (18w)	TM	Immune response	-

*Note: Information taken from Flybase unless otherwise noted. * TM - Transmembrane; CY - Cytoplasmic; EC - Extracellular; GPCR- G-Protein Coupled Receptor*

It is clear from Table 1.1 and 1.2 that the LRRs function in diverse processes involving cellular communication reinforcing the emerging notion that LRR containing proteins are likely to function as components of the signal transduction machinery.

Immunoglobulin (Ig) domains

Along with the LRRs, the Ig domain is also found in abundance in the metazoan proteome and represents a structure for molecular interactions. This fact becomes apparent, particularly in humans when considering the hundreds of leukocyte surface proteins that contain the Ig-domain (Barclay et al., 1992). Crystal structure of the first immunoglobulin revealed a modular configuration with barrel shaped β-strands (Richardson, 1981). Subsequent analysis uncovered a number of proteins with a related architecture and together these form the immunoglobulin superfamily of proteins. Most of these are membrane proteins, but there are some cytoplasmic proteins as well. Included in the family are cell surface receptors like CD2, CD4, CD8 and growth hormone (de Vos et al., 1992; Driscoll et al., 1991; Main et al., 1992; Wang et al., 1990), matrix proteins like Fibronectin (Main et al., 1992), DNA-binding proteins like NFκβ and p53 (Rudolph and Gergen, 2001), adhesion proteins like the yeast α-Agglutinin (de Nobel et al., 1996), and muscle proteins like Titin, Palladin and Myomesins (Otey et al., 2009).

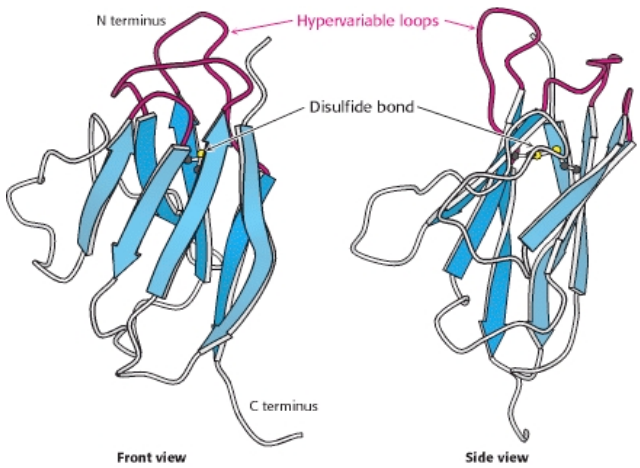

Figure 1.2 Structure of the immunoglobulin domain. Anti–parallel (blue arrows) β-strands are arranged in two sheets that are linked by disulfide bonds (Berg et al., 2002).

The Ig-domain is composed of 7 – 9 anti-parallel β-strands forming a barrel shaped architecture where two β-pleated sheets are linked together by disulfide bonds and hydrophobic interactions (Figure 1.2). This forms a hydrophobic core for the immunoglobulin domain that is surrounded by the β-strands. With respect to function of the Ig-domain, it lacks any enzymatic activity but has important binding properties, particularly when one considers the mode of action of antibodies (Barclay, 2003).

Ig-domain containing proteins have a number of roles, ranging from their classically known function in the immune system to muscle development to neural development. As expected from these roles, many Ig-domain containing proteins are associated with human disorders (Table 1.3).

Table 1.3: Ig Proteins and Disease

Ig-domain containing protein	*Associated human disorders*
Down syndrome cell adhesion molecule (DSCAM)	Associated with mental retardation
Dystroglycan (DAG1)	Muscular dystrophy
Roundabout (ROBO)	Implicated in small cell lung cancer, breast cancer (ROBO1) and multiple congenital abnormalities (ROBO2)
Receptor for advanced glycosylation end products (RAGE)	Alzheimer's disease
Ankyrin-1	Spherocytosis type 1
Filamin	Periventricular nodular heterotopia, cardiac valvular dysplasia X-linked, Melnick-Needles syndrome and many more
Nephrin	Finnish congenital nephrosis
Titin	Many muscle related defects
Kalirin	Coronary heart disease type 5
Anosmin	Kallmann syndrome type 1
Contactin (CONT)	Compton-North congenital myopathy

Selected human *Ig proteins associated with specific diseases*

A number of the Ig-domain containing proteins present in humans are also conserved in *Drosophila*, including Dystroglycan, Down syndrome cell adhesion molecule (Dscam) and Roundabout (Robo) (Table 1.4).

Table 1.4: *Drosophila* Ig containing proteins

Ig-domain containing protein	*Function*
Down syndrome cell adhesion molecule 2 (Dscam2)	Homophilic cell adhesion and axon pathfinding (Liu et al., 2009)
Dystroglycan (Dg)	Establishment of cell polarity (Deng et al., 2003)
Roundabout (Robo)	
Fasciclin 2 (Fas2)	Homophilic cell adhesion
Contactin (Cont)	Septate junction biology and paracellular barrier function (Faivre-Sarrailh et al., 2004)
Sevenless (sev)	Differentiation of cells to R7 photoreceptors
Neuroglian (Ngr)	Neural and glial cell adhesion
Lachesin (Lac)	Required for tracheal development and maintenance of trans-epithelial diffusion barrier
Papilin (Ppn)	Essential extracellular matrix protein
Titin (Sls)	Required in the biology of striated muscles and tendons
Turtle (Tutl)	Essential for establishment of motor control
Peroxidasin (Pxn)	Functions in the immune system through phagocytosis
Echinoid (Ed)	Cell adhesion molecule; functions upstream of Hippo signaling (Yue et al.)

LIGs – Functional Insights

As discussed above, LIGs are a novel, unique and relatively small class of molecules that have both LRRs and Ig domains. While an enormous body of work exists for LRR and Ig domain proteins, there is a paucity of work on LIGs and the functional significance of the union of these two protein modules. Work currently being done points to functions in neurogenesis, as a number of LIGs have surfaced in mutant screens for neural development (Figure 1.3). For example, LRR and Ig domain containing axon extension protein or Linx (5 LRRs and 1 Ig domain) was identified in a microarray screen for proteins involved in axonal projection in mice (Mandai et al., 2009). Linx was subsequently shown to interact with Receptor Tyrosine kinases

and thus help contribute to modulating axonal guidance, extension and branching. Another LIG, LINGO-1 was identified in a search for Slit-related molecules expressed in the brain. Functional analysis of Lingo-1 has revealed that it was the missing link in neurite outgrowth through Nogo 66 receptor/p75/RhoA signaling (Mi et al., 2004). The LIG NGL-1, a synaptogenic molecule, was found as an interactor of Netrin-G1 (a cell adhesion molecule). This interaction appears to be mediated by binding of Netrin-G1 to the LRR domain of NGL-1 (Klein et al., 1996; Lin et al., 2003).

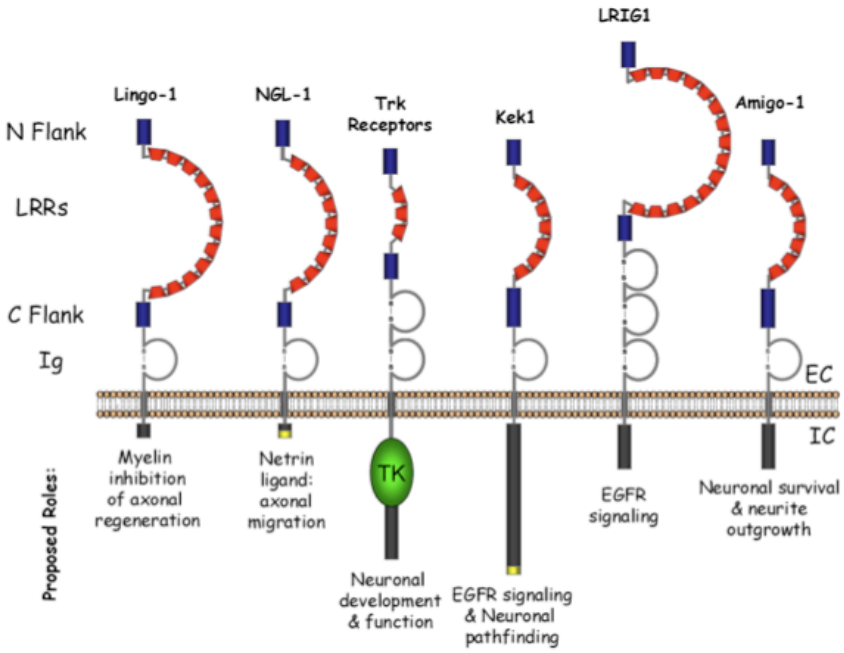

Figure 1.3 Schematic representations of various LIG proteins in vertebrates and *Drosophila*. LIG proteins vary in the number of LRR repeats and Ig domain they carry. They are enriched in the nervous system and are found to be mediators and modulators of various signaling pathways (Evans and Duffy, 2006).

Given the emerging links between members of the LIG family and neural development, it is not surprising they are increasingly being associated with various human neurological disorders (Table 1.5). As such, LIGs might represent new therapeutic targets for these conditions. For example, LINGO-1 antagonists have been shown to promote re-myelination in models of Multiple Sclerosis (Rudick et al., 2008).

Table 1.5: LIG proteins and associated human disorders.

LIG protein	*Disease/Medical condition*	*Notes*	*Reference*
Neurotropic tyrosine kinase receptor type 1 (Trk-A)	Congenital insensitivity to pain with anhidrosis (CIPA)	Mutations associated with CIPA have been seen in signal peptide, the LRRs and the IGs.	(Mardy et al., 2001; Miura et al., 2000)
Adhesion molecule with Ig-like domain (AMIGO1)	Parkinson's	-	(Karic, 2011; Vilarino-Guell et al., 2010)
Leucine rich repeat and Ig domain containing 1 (LINGO1) and LINGO2	Parkinson's disease and essential tremor, Multiple sclerosis	-	(Satoh et al., 2007; Wider et al., 2010)
Leucine-rich repeat and Immunoglobulin-like domains proteins (LRIG1-3)	Several cancers	Regulates EGFR signaling.	(Hedman and Henriksson, 2007; Krig et al., 2011)
Netrin-G ligand (NGL-1)	Schizophrenia, bipolar disorder, and Rett syndrome	Post-synaptic adhesion molecules required for development of axon, dendrites and synapses.	(Woo et al., 2009)

In *Drosophila,* two LIGs were identified in an enhancer trap screen looking for genes expressed in the embryonic nervous system. Kekkon1 (Kek1) and Kekkon2 (Kek2), the founding members of the Kekkon family of LIG proteins, were identified in this screen (Musacchio and Perrimon, 1996). Kekkon is Japanese for marriage, in this case marriage of the LRRs and the Ig

domain. Subsequent sequence analysis has revealed the presence of eight additional LIG molecules in *Drosophila*, five of which are very similar to Kekkon1 and thus belong to the Kekkon family (Kek1-Kek6) (Figure 1.5). The non-Kekkon family members include Peroxidasin (Pxn; six LRRs, four Ig domains and a peroxidase domain) (Nelson et al., 1994), Lambik, the fly LRIG1 (Lbk; fifteen LRRs and three Ig domains) and a predicted protein CG16974 (twelve LRRs and one Ig domain).

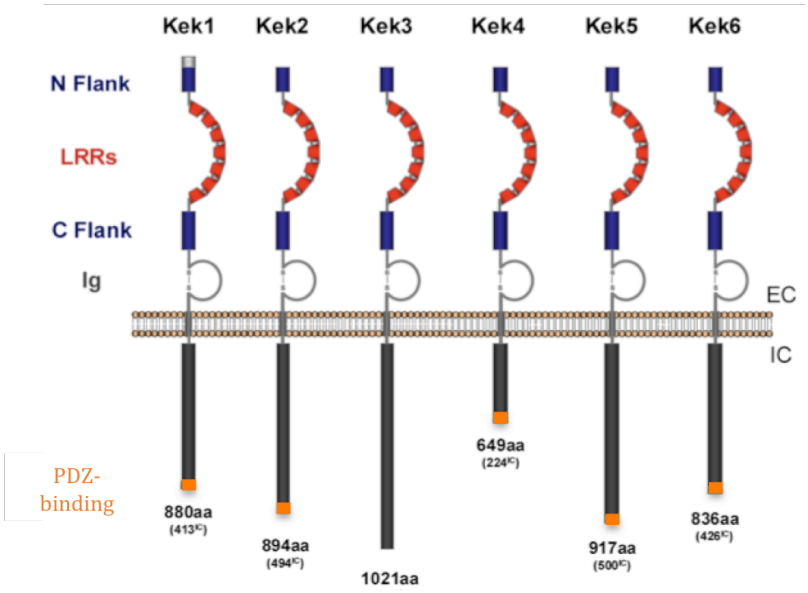

Figure 1.4 Kekkon family of proteins in *Drosophila*.

In contrast, all Kekkon family members have seven LRRs and one Ig domain. Other than a C-terminal PDZ-domain binding site (PDZ – Post synaptic density protein, Discs large and Zonula occudens), Kek family members have variable cytoplasmic regions with no known catalytic activity (MacLaren et al., 2004). The cytoplasmic tails do, however, contain short sequences that have been conserved over millions of years, but for which no biochemical functions are currently known.

While the Keks don't have vertebrate orthologs, they have been shown to be expressed in and function in the nervous system, including in synaptic growth (Evans et al., 2009; Guan et al., 2005; Musacchio and Perrimon, 1996). Despite the fact that the Keks are evolutionarily related,

distinct functions have been associated with some family members. They are emerging as mediators and modulators of various signal transduction pathways. For example, Kek1 has been shown to inhibit the Epidermal Growth Factor Receptor (EGFR) pathway by directly binding to EGFR through the LRR domain and juxta/transmembrane region (Alvarado et al., 2004a, b; Derheimer et al., 2004). Kek5 on the other hand, has been shown to be a modulator of the Bone Morphogenetic Pathway (BMP) through an as yet unknown mechanism (Evans et al., 2009). In addition to roles in cellular signaling, Keks also appear to participate in cellular junction biology. Kek6, for example, is excluded from tricellular junctions and may function in the formation of epithelial barriers (Arata, 2011). Additional work on the Keks promises to provide important insight into the roles of LIGs in cellular communication (like BMP signaling) and the functional significance of the union of LRRs and Ig domains.

Bone Morphogenetic Protein Signaling

Bone Morphogenetic Protein or BMP signaling is one of the most complex evolutionarily conserved signal transduction pathways and is involved in a plethora of processes during animal development. The diverse range of biological processes controlled by BMP signaling is overseen by a core set of components in both vertebrates and invertebrates. These core components include the ligands, receptors and downstream transcription factors Smads.

BMP signaling is crucial for normal embryonic development, as well as for the development of the skeletal, nervous, vascular and renal systems, among other processes (Bayat et al., 2011; Cain et al., 2008; Hoffmann and Gross, 2001; Katsuno et al., 2011; ten Dijke and Arthur, 2007). BMP signaling is also a key regulator in stem cell biology, playing a role in renewal, maintenance and differentiation of neural, hematopoetic, mesenchymal and epithelial

cell lineages (Kitisin et al., 2007; Larsson and Karlsson, 2005). Given the involvement of BMP signaling in a myriad of biological processes, it's not surprising that its dysfunction contributes to a wide array of diseases, including Cancer, Alzheimer's, Fibrodysplasia Ossificans Progressiva or FOP, and hypertension (Caraci et al., 2012; Katagiri et al., 2010; Mishra et al., 2005; ten Dijke and Arthur, 2007; Wharton and Derynck, 2009). Understanding the various aspects of BMP pathway regulation will be crucial for the development of diagnostic markers and new therapeutics for treatment of BMP-related pathological conditions (Park et al., 2009; Steinert et al., 2008).

The Core BMP Pathway and Morphogenetic signaling

BMP ligands bind a receptor complex, thereby leading to phosphorylation and nuclear translocation of transcription factors, Smads that trigger regulatory effects on target genes. A number of representatives for each step of the pathway are known in vertebrates, while *Drosophila* represents a simplified version. Table 1.6 lists the BMP pathway components in *Drosophila* and their known vertebrate counterparts.

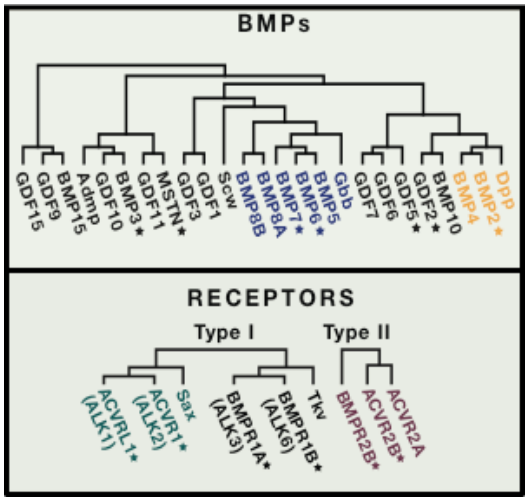

Figure 1.5: Ligands and Receptors of the BMP signaling pathway. (Dutko and Mullins, 2011)

The BMP ligands in *Drosophila* are Decapentaplegic (Dpp), Glass bottom boat (Gbb) and Screw (Scw). While Dpp is a functional ortholog of vertebrate BMP2/4, Gbb and Scw are related to BMP5/6/7/8 (Figure 1.5) (Arora et al., 1994; Spencer et al., 1982; Wharton et al., 1999).

Table 1.6: BMP pathway components in *Drosophila* and their vertebrate counterparts

Function	*Drosophila*	*Vertebrate*
Ligand	Dpp (Decapentaplegic)	BMP2/4
	Gbb (Glass bottom boat)	BMP5/6/7/8
	Screw (Scw)	BMP5/6/7/8
Receptor	Tkv (Thickveins)	BMPR-1A/1B
	Sax (Saxophone)	Alk1/2
	Put (Punt)	ActR-1B
	Wit (Wishful Thinking)	BMPR-II
Intracellular mediators	Mad	Smad
	Medea	co-Smad
Extracellular regulators	Sog	Chordin
	Tsg (Twisted gastrulation) and Tsg2/Cv	
	Tlr (Tolloid related)	Xolloid
	Cv-2 (Crossveinless-2)	CV2, BMPER
	Pentagone (also Magu)	
	Ltl (Larval translucida)	
	Dally (Division abnormally delayed)	
	Dlp (Dally-like)	GPC1-6 (Williams et al.)
Intracellular regulators	dSmurf	Smurf1/2
	Sara (Smad anchor for receptor activation)	SARA

Mature BMP ligands result from N-terminal cleavage of the precursor protein and are secreted as disulfide linked dimers. This mature ligand dimer can exist either as a homodimer or a heterodimer that activates signal transduction pathway by binding to the heterotetrameric receptor complex (Figure 1.6).

The receptor complex is composed of two Type I and Type II transmembrane serine-threonine kinases that are distantly related. Type I receptors are Thickvein (Tkv, BMPR-IA/IB homolog), Saxophone (Sax, Alk1/2 homolog) and Baboon (Babo, ActR-IB homolog, receptor for Activin) (Brummel et al., 1994 and 1999 ; Nellen et al., 1994; Penton et al., 1994; Ruberte et al., 1995: Terracol and Lengyel, 1994; Xie et al., 1994). Type II receptors are Punt (Put, ActR-II homolog) and Wishful thinking (Wit, BMPR-II homolog) (Aberle et al., 2002: Letsou et al., 1995; Ruberte et al., 1995) (Figure 1.5). The union of two Type I and two Type II receptors form the active heterotetrameric complex. Upon activation of the pathway, the constitutively active Type II receptors phosphorylate the Type I receptors. Tkv is the essential type I receptor for Dpp, while Sax is important for signaling through Gbb and Scw. Specificity of Type II receptor is determined by the Type I receptor that it associates with. During embryogenesis signaling requires both Dpp and Scw while signaling activities in the fly wing requires Dpp and Gbb.

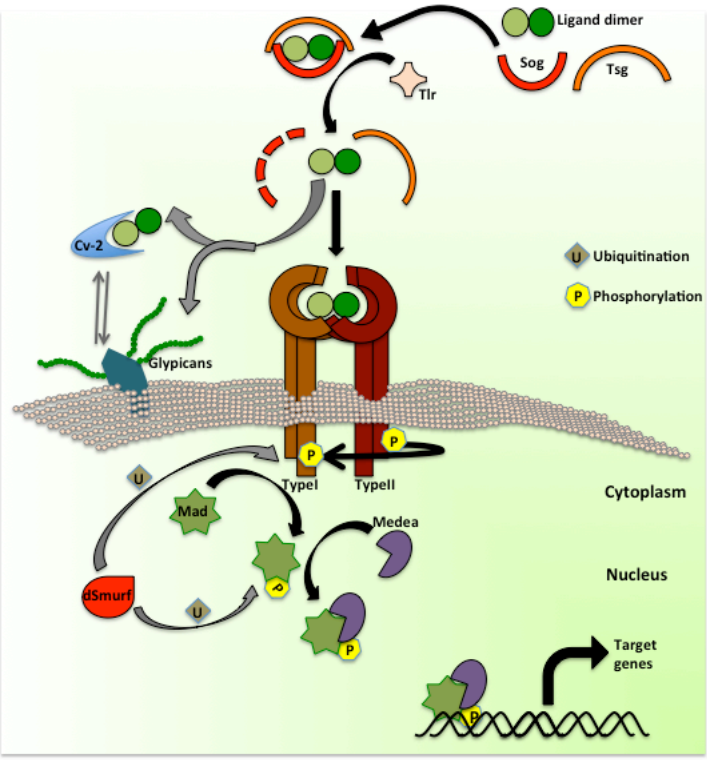

Figure 1.6: Schematic representation of the BMP signaling pathway. See Text for details. The core pathway is represented in black arrows while gray arrows indicate regulatory steps.

Binding of ligands to the receptor complex results in the phosphorylation of the Type I receptor by the Type II receptor thereby conferring a structural change in the Type I receptor.

This change creates a docking site for the intracellular mediator of signaling, Mad (Mothers against Dpp), which then gets phosphorylated at the carboxy-terminus by the Type I receptor (Shi and Massague, 2003). An endosomal protein Sara (Smad anchor for receptor activation) assists in presentation of Mad to the activated receptor complex for phosphorylation (Bokel et al., 2006). Phosphorylated Mad (pMad) is a readout of BMP signaling. pMad then associates with the co-Smad Medea and gets translocated to the nucleus to bring about transcriptional activation or repression of target genes by cooperating with sequence specific transcription factors (Figure 1.6).

Arguably the most notable BMP ligand, Dpp, is a morphogen with a multi-faceted role in development (Affolter and Basler, 2007). As a morphogen Dpp is capable of specifying cell fates in a concentration dependent manner by triggering the expression of respective target genes. For example in the *Drosophila* wing disc, Dpp is produced along the antero-posterior (A/P) boundary, generating a BMP signaling gradient along this axis. The Dpp target gene *spalt* requires high levels of Dpp and is therefore expressed in a region closer to the A/P boundary. In contrast, the gene *optomotor-blind,* which does not require as high levels of pMad, is expressed in a broader area around the A/P boundary (Nellen et al., 1996). The formation and maintenance of this activity gradient is regulated by secreted and membrane bound molecules, as discussed in the following sections.

Regulation of BMP signaling

BMP signaling is regulated extracellularly through ligand processing and activation, ligand dimerization (homo or hetero), spatial control of ligand and receptor expression, and intracellularly at multiple points as well (Moustakas and Heldin, 2009).

Extracellular regulation of BMP signaling

BMP ligands are secreted and act as either homo or hetero dimers: Dpp can function either as homodimers, or heterodimers with Scw (in the embryo) or Gbb (in the wing), while Scw cannot form homodimers and Gbb can (Parker et al., 2004). Once secreted, the availability of the ligand to the receptor is controlled by a number of disparate extracellular molecules. This process is well documented in the *Drosophila* embryo (specifying dorso-ventral fates) and in larval and pupal wings (cross-vein specification) (O'Connor et al., 2006; Parker et al., 2004). These extracellular modulators include among others, cysteine-rich motif (CR) containing proteins, metalloproteases and heparan sulfate proteoglycans (HSPGs) (Table 1.6) (Parker et al., 2004; Raftery and Umulis, 2012).

Sog and Tsg bind the ligand dimer, thereby preventing it from interacting with the receptor complex. This allows for the BMP signal to diffuse long distances, where it can activate signaling when the metalloprotease Tlr cleaves Sog. Upon release the ligands are then able to bind the receptors. The heparan sulfate proteoglycans Dally and Dally-like regulate ligand movement, signaling and intracellular trafficking (reviewed in (Yan and Lin, 2009). Dally has been shown to physically interact with a number of proteins to regulate Dpp distribution, including Cv-2, Ltl and Pent (Serpe et al., 2008; Szuperak et al., 2011; Vuilleumier et al., 2010). Cv-2, in addition to physically binding Dally, has also been shown to physically bind the Type I BMP receptor Thickveins thereby exerting its role in fine tuning BMP signaling (Serpe et al., 2008). Larval translucida (Ltl), a secreted LRR-containing protein, physically interacts with Dally-like in concert with Cv-2 to modulate signaling (Szuperak et al., 2011). Pentagone (Pent), a transcriptional target of BMP signaling, is a secreted protein that has also been shown to physically interact with Dally in controlling Dpp distribution (Vuilleumier et al., 2010).

Regulation of receptor expression is another mode of regulation of BMP signaling. Master of thickveins (Mtv) has been shown to down-regulate the expression of Tkv, thus controlling the Dpp morphogen gradient (Funakoshi et al., 2001). Type I receptors have also been shown to be regulated by a posttranslational modification, SUMOylation, which facilitates Smad recruitment and phosphorylation (Kang et al., 2008).

Intracellular regulation of BMP signaling

A significant focal point of BMP regulation is Mad, the *Drosophila* Smad (Feng and Derynck, 2005). Subsequent to activation of BMP pathway, Mad gets phosphorylated at the C-terminus. This C-terminal phosphorylation can be followed by phosphorylation of the linker region of Mad by MAPK, a BMP signaling inhibiting/reducing event, shown in *Xenopus* embryos to be important for neural induction by Fibroblast Growth Factor (FGF) and Insulin Growth Factor (IGF) signaling (Pera et al., 2003). MAPK phosphorylation of Mad is followed by additional phosphorylation of Mad by Glycogen Synthase 3 (GSK3, which is inhibited by Wingless or Wnt signaling) making it destined for proteosomal degradation after polyubiquitination (Fuentealba et al., 2007). This polyubiquitination is one of the important approaches in termination of BMP signaling and illustrates the integration of BMP signaling with other signaling pathways (Eivers et al., 2011). In addition to ubiquitination induced by GSK3 phosphorylation of activated Mad, a putative ubiquitin E3 ligase, dSmurf also targets phosphorylated Mad for ubiquitination-dependent proteosomal degradation (Liang et al., 2003). Another regulatory mechanism is SUMOylation of the MAD partner, Medea, which is required for nuclear export thereby ensuring unwanted signaling events (Miles et al., 2008).

Dephosphorylation of Mad by pyruvate dehydrogenase phosphatase (PDP) provides yet another mechanism for pathway regulation (Chen et al., 2006).

LRR Proteins implicated in BMP Signaling

Adding further complexity to BMP signaling, LRR proteins belonging to different cellular classes are part of the array of proteins participating in BMP signaling (Table 1.7). One such class of molecules is the Small Leucine Rich Repeat Protein (SLRP) sub-family of the LRR super-family. These include Decorin (Dcn), Biglycan (Bgn), Fibromodulin (Fmod), Asporin (or PLAP-1) and Tsukushi (Tsk). All of these molecules appear to inhibit BMP signaling through interactions with extracellular components including the ligands (Hildebrand et al., 1994) (Ikegawa, 2008; Yamada et al., 2007; Zheng et al., 2011). For Asporin this is accomplished by binding BMP-2 through its LRR5 (Tomoeda et al., 2008). Yet another LRR protein inhibiting BMP signaling is Tsukushi (Tsk), which forms a ternary complex with BMP and Chordin (Ohta et al., 2004). Finally, the secreted protein Larval translucida (Ltl) has recently been implicated in BMP signaling at or above the level of the BMP receptors (Szuperak et al., 2011).

Table 1.7: LRR proteins involved in BMP signaling.

LRR Protein	Number of LRRs	Class	Mechanism/Notes
Decorin (hDcn)	12	SLRP	Leu155-Val260 fragment (between LRR3-5) of Decorin binds TGF-β and blocks signaling.
Biglycan (hBgn)	12	SLRP	Binds BMP4 and inhibits signaling.
Asporin (hAspn)	11	SLRP	LRR5 of Asporin inhibits signaling by binding BMPR-1B
Fibromodulin (hFmod)	11	SLRP	Inhibits TGF-β signaling
Tsukushi (hTsk)	10	SLRP	Inhibits BMP signaling
*Larval translucida (Ltl)	14	Secreted	Interacts with Cv-2 and glypican Dally-like.

** Drosophila proteins; SLRPs- Small Leucine-Rich Repeat proteins; LIG – LRR and Immunoglobulin*
Note: Number of LRR domains for the human proteins was noted from Uniprot (www.uniprot.org) for the aforementioned proteins while Interpro was used for the Drosophila proteins.

Kek5 has been shown to be a modulator of BMP signaling and loss and gain of function phenotypes are seen in the adult wing and the scutellum (Evans et al., 2009). Consistent with this, BMP signaling is also required for wing and scutellar bristle patterning. The following section provides background on *Drosophila* development, specifically wing and scutellar bristle.

Drosophila development

Our understanding of the complexity of signaling pathways, such as the BMP pathway, and their roles in development have benefitted tremendously from work on *Drosophila melanogaster*. For over a century *Drosophila* has served as a great model system for investigating various biological questions ranging from understanding basic biology to gaining insights about various human disorders through disease models. With its genome sequenced and the availability of a number of genetic, biochemical and molecular tools the tiny fruit fly remains one of the best model systems for investigating cellular and developmental events that are common to higher organisms (Adams et al., 2000).

Investigators have used distinct aspects of *Drosophila* development for exploring biological questions. For example, wing development has been extensively used to study signaling pathways. Wings are dispensable for adult viability enabling researchers to study this tissue without compromising recovery of individuals with the respective perturbation. Through such studies, a number of signaling pathways like BMP, EGFR, Hedgehog, Notch and Wingless have been shown to play a crucial role in wing development. Such studies have been helpful in dissecting out all the mechanistic details and leading to a greater understanding of these evolutionarily conserved signal transduction pathways. The development and patterning of thoracic and scutellar bristles derives from the same larval structure as the wing and has been a similarly fruitful system for the analyses of these signaling pathways.

Wing Development

The *Drosophila* wing is an ectodermal tissue composed of two apposed dorso-ventral epithelial sheets that secrete a cuticular exoskeleton. The adult wing is segmented into two

regions: intervein and vein. The intervein region forming the wing blade is composed of dead cells that leave behind a transparent thin cuticle with the two layers (dorsal and ventral) tightly adhered together. In contrast, vein tissue is composed of live cells that secrete a thicker cuticle, providing structural support to the wings and serving as conduits for trachea, nerves and hemolymph. There are 2 types of veins: longitudinal veins (L1-L5) and crossveins (anterior and posterior crossveins) as seen in lower panel of Figure 1.7.

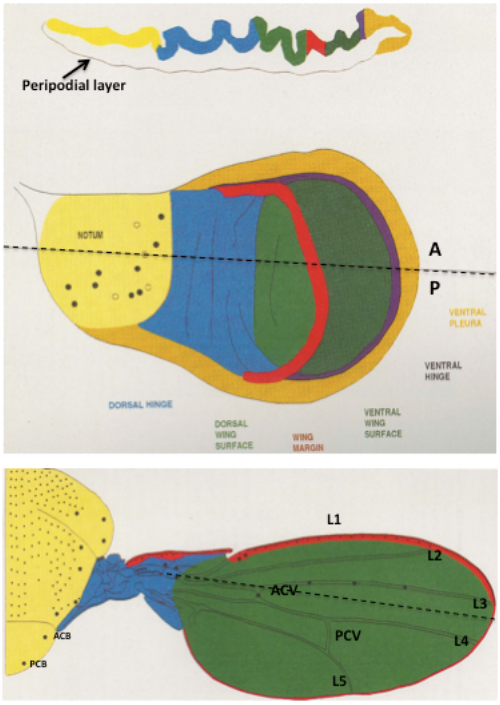

Figure 1.7: Fate map of wing imaginal disc. Color scheme in the larval wing disc (top) indicates the corresponding structures adult wing (bottom). At the top of the top panel is a cross section of the wing disc showing its convoluted morphology. Red line in the wing disc demarcates the dorso-ventral boundary. Dashed line indicates the A/P boundary. A, anterior; P, posterior; L1-L5, longitudinal veins; ACV, anterior cross vein; PCV, posterior cross vein; ASB, Anterior scutellar bristle; PSB, posterior scutellar bristle (Cohen 1993).

The adult wing develops from the wing imaginal disc, an invaginated epithelial sac that increases in size tremendously by cell division during larval development. The disc starts off as

20-30 cells that are set aside during embryogenesis, which increases to about 50,000 cells during larval and pupal development. The cells in this flattened sac are composed of columnar epithelium that is contiguous with the thin squamous epithelium of the peripodial layer (Figure 1.8). The central region of the wing disc (light and dark green, Fig.1.7) is called the 'wing pouch or disc proper' and it is this part of the wing disc that gives rise to the wing blades (Figure 1.8) (Cohen, 1993). The proximal half or the dorsal side of the disc proper forms the dorsal side of the adult wing while the distal or the ventral half forms the ventral side of the adult wing.

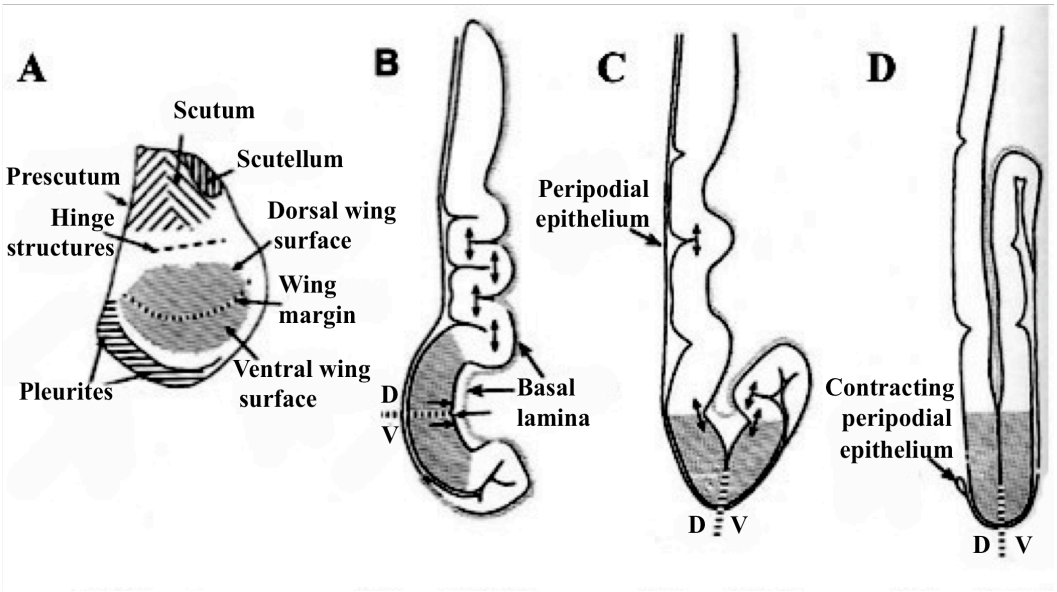

Figure 1.8: Wing development from larval to pupal stage. (A) Wing disc from a third instar larva indicating the various regions and the structures derived from the wing disc. Longitudinal sections (B-D) represent 0-4 hours of pupal development. Images are oriented with the proximal side up and distal side down (Fristrom and Fristrom, 1993).

During pupariation, the wing disc everts and elongates with the peripodial membrane stretching, flattening and then eventually contracting. The side of the wing disc facing the peripodial membrane is the apical side while the opposite side is basal. During the process of eversion the basal surface of both dorsal and ventral sides become apposed to each other leaving

gaps where the wing veins would form (provein) (Figure 1.9). The pattern of this provein is slightly different from the adult wing. Around 16 hours after puparium formation (APF), the wing inflates leaving a large gap between the dorsal and the ventral surface, disrupting the provein morphology in the process. The adult venation pattern is restored around 18-30 hrs APF when the two wing surfaces reappose followed by the secretion of the adult cuticle (Figure 1.9) (Blair, 2007).

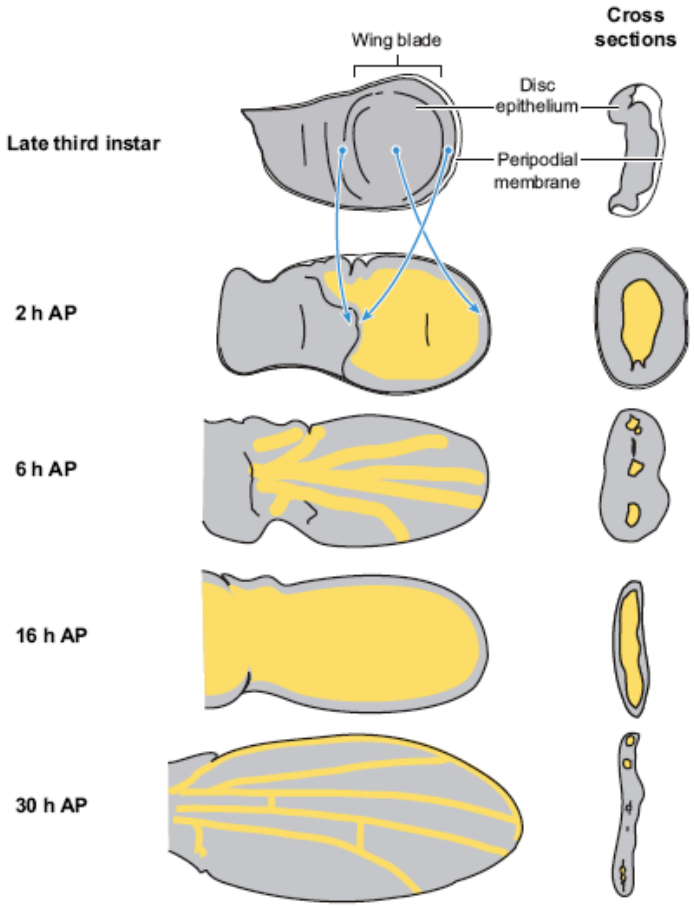

Figure 1.9: Morphogenesis of wing and its veins. Yellow - gaps between the dorsal and ventral wing surfaces; Blue arrows indicate where a certain part of the wing disc tissue is in the pupal wing subsequent to eversion (Blair, 2007)

One important and widely studied aspect of wing development is the patterning of the veins. Formation of veins is achieved by the specification of cells in the wing disc itself and not by cell migration (Bryant, 1970; Garcia-Bellido et al., 1976). However, during early stages of wing disc development compartment boundaries are formed. These boundaries, the anterior-posterior (A/P) and the dorso-ventral (D/V), provide specification cues and limit cells from crossing over. This is accomplished by the concerted efforts of BMP, EGFR, Hedgehog and Notch signaling pathways. EGFR and BMP signaling also play a crucial

role during early stages of vein development. Specification of the provein is initiated and achieved by heightened EGFR signaling (Gabay et al., 1997). BMP signaling is also heightened in the L3 and L4 proveins and plays an instructive role in the specification of the crossveins along the A/P axis (Tanimoto et al., 2000) (Figure 1.10). Later during development, signals from these pathways helps in the refinement and narrowing of the veins.

BMP signaling in the wing vein specification

BMP signaling plays a key role in establishment of the vein pattern starting with specification of the proveins in the wing disc. A gradient of BMP signaling activity visualized as pMad is generated by two ligands, Dpp and Gbb. Dpp is expressed in cells (blue in Figure 1.10) at the A/P boundary while Gbb is more broadly expressed. The type I receptor Tkv on the other hand has a pattern reciprocal to that of Dpp and shows a reduction in levels where there are peak levels of Dpp. As a result of this reciprocal expression pattern of Dpp and Tkv, Mad activity (seen as pMad) has two crests, as seen in Figure 1.10 with the posterior peak being steeper than the anterior one, specifying proveins L4 and L3 respectively (Figure 1.10) {Blair, 2007; Dutko, 2011).

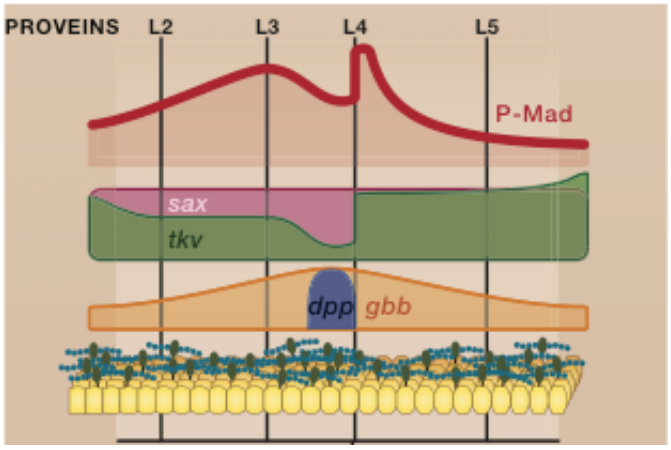

Figure 1.10: Patterning of the *Drosophila* wing disc. The ligand Dpp and its Type I receptor Tkv have a reciprocal expression pattern resulting in heightened BMP signaling seen as two peaks of pMad expression corresponding to the L3 and L4 proveins. Anterior is to left (Dutko and Mullins, 2011).

In the pupal wings BMP signaling aids in refinement of the vein area and helps in the establishment of the crossveins. Since Dpp is produced at the A/P boundary, thereby intersecting the anterior crossvein (ACV) region, the signal does not have to spread in order to specify ACV. However, the signal must diffuse in between L4 and L5 in order to specify the posterior crossvein (PCV) (Ralston and Blair, 2005; Ray and Wharton, 2001) (Figure 1.11). Sog and Tsg2 (or crossveinless, Cv) sequester the BMP ligand dimers produced in the longitudinal veins, enabling the ligands to diffuse to the presumptive PCV region (Serpe et al., 2005; Shimmi et al., 2005). Here a metalloprotease, Tolloid-related, cleaves Sog thereby releasing the ligand dimer for receptor binding (Serpe et al., 2005) (Figure 1.11).

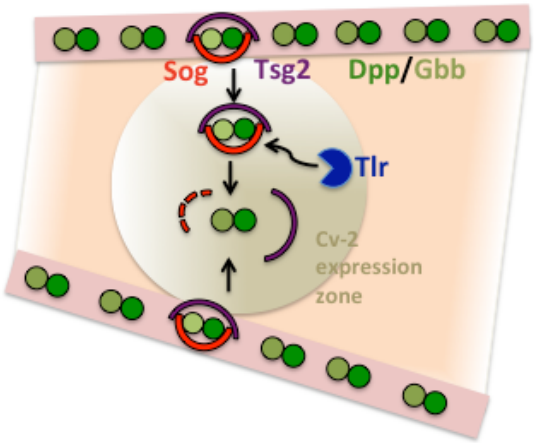

Figure 1.11: Model of BMP signaling in the posterior crossvein. Dpp/Gbb heterodimers diffuse from L4 and L5 with the aid of Sog and Tsg2 (or Cv). Ligand dimers are released to bind receptor upon cleavage of Sog by metalloprotease Tlr in the region of PCV. Cv-2 another protein present in the PCV region modulates BMP signaling by binding the ligands and or exchanging them with Tkv.

In addition to the transport of the ligand dimer to the presumptive PCV region, what gets transferred is also crucial for specification of the PCV. Mosaic analysis revealed that PCV is disrupted when Gbb is eliminated from the adjacent longitudinal veins, where Dpp is still present, suggesting the requirement for a Dpp/Gbb heterodimer and a requirement for Gbb in the process (Conley et al., 2000).

Another level of interesting regulation involves the Sog related protein, Crossveinless-2 (Cv-2) (Conley et al., 2000). Cv-2 promotes BMP signaling in some cellular contexts while

inhibiting it in others. It has been shown to bind Dpp and Gbb, as well as HSPGs and Tkv and appears to act as a short-range modulator of BMP signaling (Serpe et al., 2008).

While its role in crossvein specification has been well documented, BMP signaling also plays a role in scutellar bristle specification. However, there is a lack of mechanistic understanding of the role of BMP signaling in bristle specification and the following section aims to provide background on the development and specification of scutellar bristles.

Scutellar bristle development

In addition to the wing, the wing imaginal disc gives rise to the scutellar bristles, which are part of the sensory system of *Drosophila* and are specified through the action of signaling pathways (Figure 1.7). The sensory system of *Drosophila* is composed of external sensory organs (detects mechanical and chemical signals), chordotonal organs (internal sensory organs) and multiple dendritic neurons (also internal sensory organs) (Jan and Jan, 1993). Bristles belong to the external sensory organ system and are of two types: microchaetae and macrochaetae (Figure 1.12).

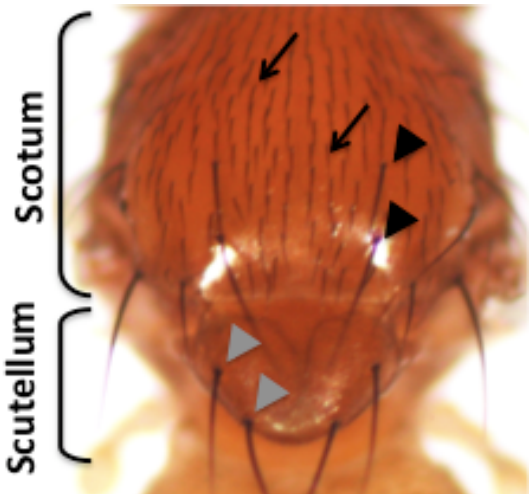

Figure 1.12: Bristle patterning in the adult fly. Thoracic region of *Drosophila* can be divided into scotum and scutellum which contain micro (black arrows) and macrochaetae (black arrowheads). The Scutellar region contains 4 bristles (gray arrowheads), two anterior and two posterior.

Scutellar bristles belong to the macrochaetae class and are a set of 4 mechanosensory bristles on the scutellum of *Drosophila*, two anteriors and two posteriors (Latter and Scowcroft,

1970). These form an external sensory organ as a part of the peripheral nervous system of *Drosophila*. Each sensory organ generally develops from a single precursor cell that is derived from the epidermal layer. This process occurs both during embryogenesis and pupal stages for the development of the larval and adult sensory organs, respectively (Jan and Jan 1993).

Once specified the sensory precursor cell (called the sensory mother cell) undergoes 2 rounds of cell division producing 4 cells: the trichogen (shaft), tormogen (socket), thecogen (sheath) and a neuron. Trichogen forms the bristle that is held in a socket formed by the tormogen, while the thecogen forms the sheath. The first division of the precursor cells occurs at a plane perpendicular to the plane of the epithelium producing an outer and an inner cell. The outer cells gives rise to the trichogen and tormogen while the inner cell produces thecogen and the neuron.

All four cells (called the proneural cluster) have the potential for a bristle fate, which is under the control of the proneural cluster genes, *achaete-scute* complex (*achaetae,scute, asense* and *lethal of scute*), *daughterless, hairy* and *extramacrochatae* (Huang et al., 1991; Jan and Jan, 1993). However, once a cell is specified to form the bristle, it prevents other cells of the proneural cluster from attaining bristle fate by lateral inhibition, a process controlled by the Notch signaling pathway (Hartenstein and Posakony, 1990; Simpson, 1990).

Bristle patterning requires additional signaling inputs, including the EGFR pathway which plays a role in promoting bristle patterning by antagonizing the Notch pathway (Culi et al., 2001). BMP signaling has also been implicated, as a loss of BMP signaling results in ectopic scutellar bristles (Wharton et al., 1999). In addition, the Ig domain protein Echinoid (Ed) plays an enhancing role in bristle patterning either by synergizing with components of Notch signaling or by inhibiting the EGFR pathway (Escudero et al., 2003).

While significant progress has been made deciphering the mechanisms involved in signaling pathways and their contributions to development, many questions remain to be answered. In particular, the contributions of the LIG family to both are largely unknown. Characterization of a few family members has revealed them to be integral players in key signaling pathways, confirming the importance of this unique family. Further studies will be necessary to address mechanistic details, the many uncharacterized family members, and ultimately their broader functions in signal transduction and development.

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