

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Structural and Functional Analysis of Bone Morphogenetic Proteins: Crystal Structure of  
Bone Morphogenetic Protein-9, Binding Studies with Pro-domain and Receptors, and  
Mutational Studies in *Drosophila decapentaplegic*

A Dissertation submitted in partial satisfaction of the Requirements for the degree Doctor of  
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in

Neurosciences

by

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2008



The Dissertation of Monica Anne Brown is approved, and is acceptable in quality and form for publication on microfilm.

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Chair

University of California, San Diego

2008

For Mary-Catherine Brown

*Whose strength, stubbornness, and support bolstered more than my education*

*“I love fool’s experiments. I am always making them.”*  
*Charles Darwin*

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## LIST OF ABBREVIATIONS

### **General**

**TGF- $\beta$** : Transforming Growth Factor- $\beta$

**GDF**: Growth and Differentiation Factor

**BMP**: Bone Morphogenetic Protein

**RNA**: Ribonucleic Acid

**RMSD**: Root Mean Square Deviation

**MES**: 3-morpholineethanesulfonic acid

**ME**: Malic Enzyme

**SEAP**: Secreted Alkaline Phosphatase

**ECD**: Extra-cellular Domain

**HHT**: Hereditary Hemorrhagic Telangiectasia

**SPR**: Surface Plasmon Resonance

**PCR**: Polymerase chain reaction

**S2**: Schneider-2

**DNA**: Deoxyribonucleic Acid

**RCF**: Relative Centrifugal Force

**TBS(-T)**: Tris-Buffered Saline (Tween)

**PBS(-T)**: Phosphate-Buffered Saline (Tween)

**EDTA**: Ethylenediaminetetraacetic

**UAS**: Upstream Activation Sequence

### **Specific Genes and Proteins**

**dpp**: Decapentaplegic

**scw:** screw

**MAD:** Mothers Against Dpp

**SMAD:** Mothers Against Dpp homologue

**Med:** Media

**Gbb:** Glass Bottom Boat

**Wit:** Wishful Thinking

**Pnt:** Punt

**Sax:** Saxophone

**Tkv:** Thinkveins

**ALK:** Activin-Like Kinase

**BMPR:** Bone Morphogenetic Protein Receptor

**ActR:** Activin Receptor



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## ABSTRACT OF THE DISSERTATION

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Controlling cellular processes from before gastrulation and continuing throughout the life of the animal, Bone Morphogenetic Proteins (BMPs) have been the subjects of intense study for decades, but there is still much to be learned about the function of individual BMPs and the general nature of their signaling. Chapter 1 presents the crystal structure of BMP-9, an orphan ligand with significant therapeutic interest. Chapter 2 establishes some of the nature of the relationship between BMP-9 and its pro-region, and examines binding with some potential

receptors. One of these receptors, Activin-Like Kinase-1 (ALK-1), was an orphan receptor with known biological function and disease pathology. ALK-1 showed strong binding and functional activity with BMP-9 in several cell-based assays, and was proposed as a candidate for a functional receptor for BMP-9. Chapter 3 shows preliminary work on the conservation of binding determinants across species, using structural and mutagenesis studies in human BMPs to direct mutagenesis in *Drosophila* BMPs. These experiments are intended as the first steps towards examination of the fundamental components of the signaling complex, and structural analysis of heterodimer functionality. The further directions for this project are discussed in Chapter 4. The thesis attempts to contribute to the growing understanding of how BMP ligands establish the overlap and specificity with receptors and other binding proteins necessary for highly precise control of signaling that still preserves the safeguards of redundancy.



## **Introduction**

### **Function**

Life as we know it would not be particularly interesting without the Bone Morphogenetic Protein (BMP) signaling family, a particular set of ligands, receptors, their affiliated binding proteins that form a large subset of the Transforming Growth Factor (TGF)- $\beta$  signaling family. In fact, life would never progress past gastrulation (Winnier, Blessing et al. 1995; Beppu, Kawabata et al. 2000), and if one agree with Lewis Wolpert, in that “It is not birth, marriage, or death, but gastrulation that is truly the most important time in your life,” one could argue that BMPs are some of the most important proteins to study. In flies, fish, frogs, mice and humans, BMPs control a tremendous array of developmental, homeostatic, and regulatory processes, beginning with the differentiation of neural and non-neural tissue in the blastoderm, and continuing on to additional types of differentiation, proliferation, organogenesis (including the heart, eye, and kidney) apoptosis, osteogenesis, chondrogenesis, and the establishment of left-right asymmetry (Dudley, Lyons et al. 1995; Hogan 1996; Branford, Essner et al. 2000; Schlange, Andree et al. 2000; Kim, Robertson et al. 2001; Whitman and Mercola 2001; Wozney 2002; ten Dijke, Korchynskyi et al. 2003; Chizhikov and Millen 2005; Gambaro, Aberdam et al. 2006). Recent work has further implicated BMPs in maintaining the integrity of the stem cell niche (Decotto and Spradling 2005; Jones and Wagers 2008). On the clinical side, they have a robust ability to generate *de novo* bone formation (Urist 1965; Wozney 1992) making them appealing targets in the treatment of bone disorders and injury (Brown, Stock et al. 2006; Gautschi, Frey et al. 2007).

### **To Make a Morphogen**

Upon translation, BMPs have three major components. The N-terminal is a short signaling sequence of about 20 amino acids that directs the protein for secretion. The next region, which varies in length, but is often in the range of 240-320 amino acids, is a proregion. In TGF- $\beta$ 1, 2, and 3, and myostatin, the proregion remains non-covalently associated even after cleavage and secretion, and potently inhibits the activity of the protein (Lee and McPherron 2001; Thies, Chen et al. 2001; Annes, Munger et al. 2003; Jiang, Liang et al. 2004), but the function of the general BMP pro-region is poorly understood. It has been implicated in targeting of the protein to the extracellular matrix (Gregory, Ono et al. 2005), and in folding, stabilization and regulation of the ligand (Dick, Hild et al. 2000; Sopory, Nelsen et al. 2006). The C-terminal end forms the active ligand, usually from 100 to 110 amino acids long. The ligands form homodimers or heterodimers and then furin cleaves the pro-region, at which point the proregion is thought to dissociate from the ligand and the mature, dimeric ligand is secreted from the cell (Wozney 1992).

The ligand is now able to induce signaling in other cells by binding the extracellular domains of two type I and two type II receptors. This assembly brings the intracellular domain of both receptor types into close proximity, permitting the constitutively active intracellular kinase domain of the type II receptor to cross-phosphorylate the intracellular GS domain of the type I receptor (Massague 1998). Intermediate proteins called R-Smads (Mothers Against Decapentaplegic, or Mad in *Drosophila*) are phosphorylated by the activated type I receptor kinase. Activated R-Smads associate with other proteins called co-Smads (Medea in *Drosophila*). This complex translocates to the nucleus, interacting with various cofactors to modify gene expression (Wrana, Attisano et al. 1994; Raftery and Sutherland 2003).

## Evolutionary conservation

Evolutionary conservation enables scientists to utilize the many advantages of invertebrate biology (short generation time, small size, low expense, lack of teeth) to study signaling pathways that are critically important to more complex organisms. Representatives of the bone morphogenetic protein (BMP) signaling family are found in flies, frogs, mice, and humans. Across species, they have similar functions in patterning the dorsal-ventral axis (Holley and Ferguson 1997) and directing cells to adopt neuronal or non-neuronal fates (Sasai 2001; De Robertis and Kuroda 2004). Later in development, they also mediate a variety of other functions, including apoptosis, proliferation, and the differentiation of organ tissues in highly conserved ways (Yang 2004; Mehlen, Mille et al. 2005; Aboitiz and Montiel 2007). The homology between the active ligands in a given species is generally around 40%, but can be as high 75%. In flies, the BMPs that will be discussed at length in this thesis are *decapentaplegic* (*dpp*) and *screw* (*scw*). The mature ligand of *dpp* is 75% identical to the vertebrate BMP-4, and another *Drosophila* BMP known as *glass bottom boat* (*gbb*) is 73% identical to BMP-5, -6 and -7. In contrast, the mature ligand of *scw* shares only 49% of its sequence with its closest vertebrate ortholog, BMP-6 (Arora, Levine et al. 1994).

Importantly, BMPs have also remained structurally similar enough to functionally substitute across species. Human BMP-2 can partially rescue a *dpp*-null deficiency in flies (Padgett, Wozney et al. 1993). Noggin is a vertebrate BMP inhibitor, which was shown by co-crystallization studies to bind to BMP ligands and block both the type-I and type-II binding sites, eliminating any possibility of effective contact with a receptor (Groppe, Greenwald et al. 2002; Groppe, Greenwald et al. 2003). *Noggin* has no known fly ortholog; however, expression of *Xenopus noggin* inhibits BMP signaling in flies (Holley, Neul et al. 1996). *Short gastrulation* (*sog*) and *chordin* are orthologs, and it was

shown by injection of mRNA that *sog* and *chordin* can functionally compensate for each other in flies and frogs. BMP-4 normally promotes ventral fates in *Xenopus*, and it was also shown that *dpp* can also cause ventralization in *Xenopus* (Holley, Jackson et al. 1995). These examples suggest that the structure and binding determinants of BMPs and their affiliated proteins are sufficiently well-conserved that these proteins can be functionally interchangeable across species, which leads to the suggestion that information gleaned from structural or mutagenesis studies with BMPs of one species may be applicable to BMPs of another species.

### **BMPs in the Nervous System**

After initially distinguishing neuronal from non-neuronal tissue, BMPs subsequently regulate a variety of neuronal functions, including differentiation of adrenergic, noradrenergic, cholinergic subtypes, specification of cortical and sympathetic neurons, neuronal patterning, axon guidance, dendrite growth, and forebrain development (Varley and Maxwell 1996; Furuta, Piston et al. 1997; Mabie, Mehler et al. 1999; Schneider, Wicht et al. 1999; Lopez-Coviella, Berse et al. 2000; Withers, Higgins et al. 2000; Beck, Drahushuk et al. 2001; Horbinski, Stachowiak et al. 2002; Vogel-Hopker and Rohrer 2002; Butler and Dodd 2003; Chizhikov and Millen 2005; Charron and Tessier-Lavigne 2007; Yamauchi, Phan et al. 2008).

A particularly interesting role for BMPs in synapse formation, stability, and homeostasis has been developing recently. A series of elegant papers has shown the *Drosophila* BMP *gbb*, the type I receptor *saxophone* (*sax*), the type II receptor, *wishful thinking* (*wit*), and the downstream factors *mad* and *media* to be critical for regulation of synaptic growth at the neuromuscular junction (NMJ). *Wit* is required presynaptically, while *gbb* is required postsynaptically, implying an attractive model for retrograde signaling across the synapse (Aberle, Haghighi et al. 2002; Marques, Bao et al. 2002;

McCabe, Marques et al. 2003; Rawson, Lee et al. 2003; McCabe, Hom et al. 2004). Further experiments showed that *wit* and *gbb* were required for synaptic homeostasis, a rapid effect where a presynaptic increase in glutamate release compensates for inadequate postsynaptic responses. This function was distinct from their roles in modulation of synaptic growth, and *gbb* did not serve as a retrograde messenger (Goold and Davis 2007). Rapid synaptic disassembly results from the lack of BMP signaling, and *wit* also appears to control synapse stability through a set of downstream factors separate from those used in synapse growth and homeostasis (Eaton and Davis 2005). Therefore, it appears that a single set of BMP ligands and receptors, through distinct mechanisms, controls at least three separable functions at the NMJ in the fly. As the phenotype of *gbb* is less severe than *wit*, further interesting experiments would involve the possible role of myoglianin, another BMP ligand that may serve a role in providing redundancy or by forming more biologically active heterodimers with GBB (see page 12 for more on BMP heterodimers). It is not yet understood how many of these mechanisms are at play in the central nervous system or in vertebrates.

### **Crystal Clarity**

A number of BMPs have been crystallized and solved previously, including BMP-2 (Scheufler, Sebald et al. 1999) and BMP-7 (Griffith, Keck et al. 1996; Greenwald, Groppe et al. 2003). The characteristic shape of the monomer has been described as similar to a left hand, with a thumb, wrist and two outstretched fingers (Sebald, Nickel et al. 2004). The shape of the “wrist,” or “core” of the protein is driven primarily by seven highly conserved cysteines (Figure 2), six of which pair up and form disulfide bonds. The last cysteine pairs with the remaining free cysteine from another monomer to form the active dimeric ligand. The “fingers,” formed by two parallel  $\beta$ -sheets, extend in opposite directions in the dimer. An  $\alpha$ -helix forms the “thumb.” The cores of the ligands

in this family are highly structurally similar, though the overall sequences tend to be only about 30-40% identical. Much of the structural variation, along with receptor binding, occurs in the “fingers” and in the  $\alpha$ -helix.

It is known from mutational studies and crystal structures that binding to type I receptors occurs near the  $\alpha$ -helix on the concave side at the junction between the two subunits (Kirsch, Sebald et al. 2000), whereas binding to the type II receptors is located on the convex side of the “hand” near the “fingertips” (Greenwald, Groppe et al. 2003; Thompson, Woodruff et al. 2003). It is not understood whether the variations in binding specificities are determined primarily through differences in sequence or through differences in the relative locations of the binding sites between ligands.

Two exceptionally informative structures that were solved are BMP-2 in complex with a type I receptor (Kirsch, Sebald et al. 2000) and BMP-7 in complex with a type II receptor (Greenwald, Groppe et al. 2003). To gain a more complete picture of the full signaling complex, the cystine knot cores of the ligands in each of these models were aligned. From this representation, it was concluded that the receptors probably do not contact each other outside of the membrane, interacting only by means of binding to the ligand (Greenwald, Groppe et al. 2003). This idea was later confirmed when the entire extracellular signaling complex was crystallized and solved (Allendorph, Vale et al. 2006). This leads to the hypothesis that the affinity of the ligand to its receptors is the primary determinate of signaling efficacy, rather than any direct interaction between the extracellular domains of the receptors.

Each of the structures of BMPs, receptors, and binding proteins has provided the clearest view of how these proteins are interacting with each other, which regions are involved in direct contact and perhaps how to interfere with these interactions. The importance of BMP signaling in clinical applications has resulted in tremendous interest

in modifying the binding of these proteins, and it is hoped that crystal structures will lead to some insights in that realm. Additionally, crystal structures have provided guidance to complementary studies, in that they show where researchers should focus their attention when determining which residues on a ligand or receptor are likely to affect binding.

### **Methods of Regulation**

As BMP signaling occurs throughout the lifetime of most organisms, the same ligands and receptors often serve very different if not contradictory functions. This heterogeneity of function mandates exquisitely tight control of signaling, which is achieved in a variety of ways, including cell-specific production and temporally regulated secretion of BMP ligands (Tomizawa, Matsui et al. 1995; Toshiyuki and Ichiro 2004), proteolytic cleavage of precursors into active ligands (Constam and Robertson 1999), sequestering of active ligands by inhibitory molecules, including Noggin, Short Gastrulation (sog)/Chordin, and Follistatin (Rafferty and Sutherland 2003; Yamamoto and Oelgeschlager 2004), inhibition of ligands by various pro-regions (Lee and McPherron 2001; Thies, Chen et al. 2001; Yang, Ratovitski et al. 2001; Haramoto, Tanegashima et al. 2004; Jiang, Liang et al. 2004), and the varied expression, binding efficiencies, and signaling capabilities of receptors (Rafferty and Sutherland 2003). All of these mechanisms of control occur before the signal is induced in the receiving cell. The means by which the receiving cell can modulate control of signaling after the type II receptor phosphorylates the type I receptor, including production of SMADS and co-SMADs, transcription factors, and proteins designed to inhibit the initiation of transcription, is beyond the scope of this thesis and will not be discussed herein.

### **Two Orphan Proteins: BMP-9 and ALK-1**

BMPs face similar challenges to single people seeking a partner—they must be in the right place at the right time, overcome their personal baggage from prior

interactions, and realize that just because a person (or receptor) initially seems right, the chemistry just might not be there. All TGF- $\beta$ /BMP ligands are translated as precursor proteins, consisting of an amino-terminal pro-region and a carboxy-terminal ligand. This precursor forms a disulfide-linked homodimer in the cytoplasm, and the pro-region is then cleaved from the ligand. In most cases, the pro-region disassociates and the mature ligand is secreted from the cell, but in some cases, including GDF-8 (also known as myostatin) and TGF- $\beta$ 1, -2 and -3, the pro-region remains noncovalently associated with the ligand after secretion and inhibits binding of the ligand to its prospective receptors (Gentry, Webb et al. 1987; Wakefield, Smith et al. 1989; Gentry and Nash 1990; Lee and McPherron 2001; Thies, Chen et al. 2001; Jiang, Liang et al. 2004). GDF-8 serves to stop the growth of muscle, and transgenic mice overexpressing the pro-region of GDF-8 are exceptionally muscular due to overgrowth of individual fibers (rather than an overall increase in number of muscle fibers) and show substantial reductions in body fat (Yang, Ratovitski et al. 2001). As pro-regions are dissimilar in sequence, the only apparent commonality for inhibitory pro-regions is their continued association after the ligand is secreted from the cell.

In the BMP family, there are many more ligands than receptors. As a consequence, there is a substantial amount of overlap in specificity, particularly among the reasonably promiscuous type II receptors. Assembling a functional signaling complex can be cooperative, in that the affinity of a receptor for a ligand will be higher once the ligand is already bound to another receptor. In the case of BMPs, it has been shown that type II receptors are generally lower-affinity than type I receptors, and that their affinity for a free ligand is lower than for a ligand already bound to a type I receptor. This is in contrast to TGF- $\beta$  signaling, where the type I receptor has a



relatively low affinity for the ligand until the ligand is bound to a type II receptor (Shi and Massague 2003; Massague 2008).

Thusly, in order for a ligand to induce signaling, it must arrive at a receiving cell unencumbered by inhibitory proregions or any other inhibitory proteins, and the receiving cell must express both an acceptable type I and type II receptor.

This brings us to the case of BMP-9. Of the many human BMPs, BMP-9 distinguished itself primarily due to a superlative ability to induce ectopic bone and cartilage formation. In the adult rat, BMP-9 is expressed predominantly in the liver, and has been shown to induce proliferation of cultured liver cells (Miller, Harvey et al. 2000). BMP-9 mRNA was also found in the septum and spinal cord of E13 mice. *In vitro* and *in vivo*, BMP-9 was found to promote cholinergic differentiation and the synthesis of acetylcholine, and effectively maintained the cholinergic phenotype of differentiated cells (Lopez-Coviella, Berse et al. 2000; Lopez-Coviella, Berse et al. 2002). Other studies indicate that BMP-9 produces ectopic bone growth and potently directs the differentiation of mesenchymal cells into cartilage (Majumdar, Wang et al. 2001; Li, Li et al. 2003; Kang, Sun et al. 2004). It also induces cell proliferation (Song, Celeste et al. 1995; Scharpfenecker, van Dinther et al. 2007). More recently, BMP-9 was identified as a regulator of glucose metabolism, by modulating the transcription of several genes that are involved in glucose and fatty acid metabolism, decreasing glucose production in cultured cells, and reducing glycemia in diabetic mice (Chen, Grzegorzewski et al. 2003). However, a receptor for BMP-9 was unknown.

Similarly, Activin-like kinase-1 (ALK-1) was an orphan receptor that was known for its important biological functions. It has been implicated as an inhibitor of lateral TGF- $\beta$ /ALK-5 signaling (Goumans, Valdimarsdottir et al. 2003), correlated with vasculogenesis and angiogenesis (Roelen, van Rooijen et al. 1997) and have been

shown to cause multiple forms of hereditary hemorrhagic telangiectasia (Berg, Gallione et al. 1997; Kjeldsen, Brusgaard et al. 2001; Abdalla, Cymerman et al. 2003; Harrison, Flanagan et al. 2003; Letteboer, Zewald et al. 2005; Yan, Fan et al. 2006; Assis, Costa et al. 2007), an autosomal dominant disease affecting approximately 1.2 million people worldwide. HHT is characterized by vascular malformations that may affect the skin, nose, gastrointestinal tract, lungs, liver, and brain, caused by structurally weak connections between veins and arteries that are prone to rupture. Frequent and severe nosebleeds are the most noticeable symptom of HHT, but are incompletely penetrant and are often attributed to other causes, leading to the unfortunate reality that HHT is often undiagnosed until life-threatening complications result from vascular malformations in other organs (Sabba, Pasculli et al. 2002; Sabba, Gallitelli et al. 2006).

By utilizing a combination of biochemical and cell-culture assays, this work provided the first evidence that BMP-9 and ALK-1 might be functional binding partners, and that the proregion of BMP-9 remained associated after cellular secretion but does not have an inhibitory function. Subsequent studies have confirmed ALK-1 and BMP-9 as a functional ligand-receptor pair (David, Mallet et al. 2007; Scharpfenecker, van Dinther et al. 2007).

#### Formation of the Dorsal/Ventral Axis in the *Drosophila* Embryo:

It has been postulated for decades that gradients can specify position for differentiating cells. In the fly, the maternally expressed *dorsal* protein induces the production of two BMPs, *decapentaplegic* (*dpp*) and *screw* (*scw*). Levels of dorsal signaling are inversely correlated with BMP signaling, setting up a gradient of BMP signaling that the embryo interprets to initiate dorsal-ventral patterning. The signal is so well regulated that the highest levels of signaling create the aminoserosa, an extraembryonic tissue on the dorsal midline, lower signals create the dorsal ectoderm,

and the absence of BMP signaling create the mesoderm. Other proteins known to be involved in establishing this gradient are Short Gastrulation (Sog, known in mammals as Chordin (Holley, Jackson et al. 1995)), Tolloid (Tld), and Twisted (Tsg). Sog, in conjunction with Tsg, binds to a mature BMP ligand and blocks it from binding to a receptor. Sog remains bound to the Dpp ligand until it is cleaved by Tld, releasing the ligand. Tld is found at the dorsal midline in a developing embryo.

Mutations in *dpp* result in embryos that are progressively more ventralized, with the null mutation differentiating no dorsal tissues at all. *Scw* mutants also show ventralization, but to a much lesser degree than *dpp* mutants. A complete loss-of-function *scw* mutant does not differentiate amniosera, but will differentiate dorsal neuroectoderm. An unexpected result of *sog* mutants was overall dorsalization, but a lack of amnioserosa, indicating a general increase in BMP signaling but an absence of the highest levels of BMP signaling. *Tsg* mutants have a similar phenotype. It is also known that when BMP ligands bind to receptors and induce signaling, they are often internalized and degraded. These observations led to a two-part explanation for the role of Sog in gradient formation, which suggests that inhibition by Sog prevents signaling, but also prevents internalization and degradation of the ligand. Sog is produced in the lateral neuroectoderm and diffuses into the dorsal ectoderm, which allows it to both potently inhibit BMP signaling in the neuroectoderm and transport ligand to the dorsal midline. The high levels of BMP signaling that drive differentiation of amnioserosa at the dorsal midline are a result of Tld cleaving Sog and releasing accumulated ligand specifically in that region (Ross, Shimmi et al. 2001; Ashe 2002; Srinivasan, Rashka et al. 2002; Raftery and Sutherland 2003; Mizutani, Nie et al. 2005).

BMPs are secreted dimeric ligands that may signal as both homodimers and heterodimers. Most research focuses on homodimeric signaling, but there is substantial

evidence for the existence and necessity of heterodimers, in that heterodimers have been shown to form *in vitro* and *in vivo*, and produce a much higher level of signaling output than an equivalent combination of homodimers. Furthermore, it has also been shown in a null mutant that adding physiologic concentrations of homodimers does not rescue an axon guidance phenotype, while adding physiologic concentrations of heterodimers does (Butler and Dodd 2003).

One model in early *Drosophila* development suggests that homodimeric Dpp and Scw produce low to moderate levels of signaling, while heterodimers of Dpp and Scw produce the highest level of signaling, and it is the concentration of heterodimers at the dorsal midline that allows the early embryo to differentiate into aminoserosa. The presence of homodimers therefore defines the dorsal ectoderm, which requires low levels of BMP signaling, and the absence of BMP signaling specifies the mesoderm (Shimmi, Umulis et al. 2005). It was demonstrated that Sog has a much higher binding affinity for a heterodimer of Dpp and Scw, and suggested that the gradient of signaling in the fly embryo is created by a source of Dpp (as Scw is made by every cell in the embryo (Arora, Levine et al. 1994)), which forms heterodimers with Scw. When these heterodimers are preferentially bound by Sog, they are blocked both from activating and from being internalized by receptors. Sog then transports them to the dorsal-most region of the embryo, where this complex finally encounters Tld. Tld cleaves Sog and releases the heterodimer. As the heterodimer is more effective at inducing signaling and is now concentrated at one end of the embryo, this region alone now has the high levels of signaling required to create amnioserosa (Ashe and Levine 1999; Shimmi and O'Connor 2003; Shimmi, Umulis et al. 2005). Therefore, heterodimers may play two roles in an organism: higher levels of activation and preferential binding to other proteins. The molecular basis for either of these activities is not known.

## Summary of Content

Due to the impressive conservation across species and nearly continual necessity over the lifetime of these organisms, they have been the subjects of a tremendous amount of scientific effort. As a result, a great deal is known about this signaling family, from the molecular interactions that govern the binding between ligands and receptors or inhibitory proteins to the details of the signaling pathway and the ways in which the pathway is modulated, to an ever-increasing array of cellular functions governed by these proteins. However, new studies and controversies appear frequently, and it appears that there is much still to be understood.

The first part of this thesis will examine the crystal structure of human BMP-9. BMP-9 was selected for crystallization trials in part because of its particular therapeutic interest, including a superlative ability to induce ectopic bone formation (Dayoub, Dumont et al. 2003; Li, Hankins et al. 2003; Li, Li et al. 2003; Li, Li et al. 2003; Kang, Sun et al. 2004) and chondrogenesis (Majumdar, Wang et al. 2001; Blunk, Sieminski et al. 2003; Hills, Belanger et al. 2005). Additionally, it may regulate glucose homeostasis and metabolism (Chen, Grzegorzewski et al. 2003; Hills, Belanger et al. 2005; Takahashi, Morris et al. 2007). Structural differences between it and known BMPs will be discussed, as well as the possible implications for receptor affinity.

The next section shows evidence that the pro-region of BMP-9 remains tightly associated after secretion, but is functionally inactive. There is no reliable indicator of whether a given pro-region will inhibit its ligand, but all of the proregions known at the time of this study that continue to associate after secretion also inhibit their ligands. The pro-region of BMP-9 also remains tightly associated after secretion from the cell, leading to the prediction that it will be inhibitory. To thoroughly examine its activity, BMP-9 was tested in separate cell-based assays for osteogenesis, proliferation, and

glucoglycogenesis. Results of these assays indicate that both isolated BMP-9 and BMP-9 in complex with its pro-region are equally active in signaling and cell-growth stimulation, whereas the pro-region alone is inactive. Additionally, it presents evidence that Activin-Like Kinase-1 (ALK-1) may be the type-1 receptor for BMP-9. Previous to this work, BMP-9 had no known receptor and ALK-1 had no well-established ligand. ALK-1 is known to be involved in Hereditary Hemorrhagic Telangiectasia, a serious but manageable disease that results in uncontrolled bleeding due to unstable connections between veins and arteries (Berg, Gallione et al. 1997; Kjeldsen, Brusgaard et al. 2001; Abdalla, Cymerman et al. 2003; Harrison, Flanagan et al. 2003; Letteboer, Zewald et al. 2005; Yan, Fan et al. 2006; Assis, Costa et al. 2007). To examine the possibility that ALK-1 was a receptor for BMP-9, the soluble extracellular domain (ECD) of ALK-1 and BMP-9 were added to several cell-based assays to check for functional inhibition. ALK-1 demonstrated the ability to completely inhibit BMP-9 activity to a similar degree as another soluble BMP receptor ECD and ligand that are known to be functionally related. Therefore, based on surface plasmon resonance and functional inhibition in several cell-based assays, ALK-1 is suggested as a functional receptor for BMP-9. This result has been verified by subsequent publications (David, Mallet et al. 2007; Scharpfenecker, van Dinther et al. 2007), and it has been shown that BMP-9 functionally inhibits angiogenesis (David, Mallet et al. 2008).

The third section of the thesis presents preliminary work in creating mutations of a *Drosophila* BMP called decapentaplegic (*dpp*), which are designed to selectively inhibit binding to only one of the two receptor types. This research is based on studies showing functional inactivation of BMP-2 by point mutations affecting binding to only one receptor type (Kirsch, Nickel et al. 2000) and the high degree of conservation of many of these residues between BMP-2 and *dpp*. It is hoped that this preliminary work will

indicate the degree of functional conservation of certain BMP residues in receptor binding across species, and create a foundation for determining the mechanism for the enhanced signaling seen with heterodimeric BMPs.

## **Chapter 1: Crystallization and Structural Analysis of Bone Morphogenetic**

### **Protein-9**

#### **Abstract**

Bone morphogenetic protein-9 (BMP-9) potently induces osteogenesis and chondrogenesis. It has also been implicated in the differentiation of cholinergic neurons, and may help regulate glucose metabolism. In order to understand the structural basis of its molecular interactions, BMP-9 was crystallized and the structure determined to 2.3 Å. To examine the significance of various features of the ligand in determining its receptor specificity, the differences were examined between this model and existing crystal structures of other BMPs. The structural differences between BMP-9 and BMP-2, BMP-7, the BMP-2/BMPR-IA complex and the BMP-7/ActR-IIB complex were analyzed by alignment of the core regions of these structures with BMP-9 and examination of variations in the regions that are likely to determine binding specificity, most notably the pre-helix loops and the tips of the “fingers.” It was determined that BMP-9 differed significantly from these BMPs structurally in regions known to be important for type I receptor binding, but only in sequence in regions important for type II receptor binding. It was concluded that the position of the pre-helix loop may be the most significant determinant of type I receptor binding with this ligand, but that type II receptor binding may be more sensitive to variations in sequence in the finger regions.

#### **Methods**

Protein Expression and Purification – The coding sequence for full length BMP-9 with pro-region was cloned into pC4, a proprietary mammalian expression vector, and



the construct was transfected into DHFR-negative CHO-DG44 using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Typically,  $\sim 1 \times 10^6$  cells were transfected with 5 mL of Lipofectamine 2000, 5 mg of pC4. BMP-9 and 0.5 mg of pSV2.NEO plasmids. The transfected cells were put into selection after  $\sim 36$  hrs in MEM- $\alpha$  medium containing 25 nM MTX and 1 mg/mL Geneticin. After  $\sim 2$ -3 weeks, single clones were isolated and seeded into 24-well plates. When confluent, the supernatant from these clones were screened for BMP-9 activity using cell-based reporter assays. The high-expressing clones were selected and amplified stepwise up to 0.5 mM MTX. For large-scale production of BMP-9, a high-expressing clone was grown in CHO-5 medium (an HGS proprietary serum-free medium without insulin) for 72 hours, and the conditioned medium was harvested.

To purify the BMP-9/pro-region complex, 2-5 L of BMP-9 culture supernatant was adjusted to pH 6.4 with pH 5.0, 1 M MES and diluted to a conductivity of  $< 7$ -8 ms. The sample was loaded on a 50 mL POROS 50 HS (Perseptive Biosystems) column which had been pre-equilibrated with pH 6.4, 20 mM MES, 50 mM NaCl. BMP-9 complex was eluted with a 20 CV pH gradient from pH 6.4 to 8.0 with pH 8.0 buffer containing 20 mM Tris-HCl and 50 mM NaCl. The fractions of partially purified BMP-9/pro-region complex were pooled and adjusted to pH 7.4 with pH 9.0, 1 M Tris and diluted to the conductivity  $< 5$  ms. The sample was loaded onto an 8 mL MonoQ column (Amersham Biosciences) equilibrated with pH 7.4, 20 mM Tris. BMP-9/pro-region complex was eluted by a 15 CV salt gradient of 0-0.5 M NaCl.

Crystallization, Data Collection and Refinement - Dimeric BMP-9 was crystallized from a solution containing BMP-9 dimer, BMP-9 monomer, pro-region, and other secreted proteins in a condition identified from sparse matrix (Hampton) screening. Total protein concentrations ranged from 3.8 to 9 mg/mL, with BMP-9 dimer constituting

approximately 15-20% of the total protein. Crystals of BMP-9 were obtained by hanging drop at 23°C with a well solution of 1-1.2M NaCl, 7-10 mM Hexadecyltrimethylammonium Bromide, and 10mM MgCl<sub>2</sub>.

Drops containing crystals of BMP-9 were flooded with mother liquor supplemented with 12-15% glycerol as a cryoprotectant. Crystals were flash frozen in liquid nitrogen and diffraction data was obtained at Stanford Synchrotron Radiation Laboratory on beam line 9.2. All integration and scaling were performed with HKL2000. The space group was found to be I4<sub>1</sub>22, with unit cell dimensions a = b = 71.23, c = 144.90, α = β = γ = 90°. A molecular replacement solution was found using MolRep (1994) with the structure of BMP-2 monomer as the search model. The asymmetric unit contains one copy of the BMP-9 monomer, leaving a solvent content of 67.6%. The protein was manually rebuilt in O (Jones, Zou et al. 1991) and the structure was refined using Refmac5 (CCP4) and CNS (Brunger, Adams et al. 1998). Four TLS (translation/liberation/screw) groups were used, dividing the protein into regions from A4 to A34, Y35 to H54, A55 to K78, and L79 to G108, and treating each of these sections as rigid bodies that could be moved independently.

## Results

Determining crystallization conditions for a given protein is a completely stochastic process, and requires a broad search over various combinations of precipitants, salts, and buffer conditions. The initial conditions for the crystallization of BMP-9 were determined this way, and three of these conditions resulted in the formation of crystals. Only one condition yielded crystals reproducibly, and these crystals were too small and unstable to produce high-quality data. Subsequent modifications to these conditions ultimately resulted in sufficiently large and stable crystals. Data was collected

once at the Berkeley National Laboratory and on several occasions at the Stanford Synchrotron Radiation Laboratory. Multiple trips were required, as the data proved extremely difficult to refine to acceptable error levels.

BMP-9 was solved with molecular replacement using BMP-2 as the search model, manually rebuilt with O, and refined using a combination of EPMP and CNS. The final resolution was determined to be 2.32 Å (Table 1). BMP-9 shows the characteristic cystine-knot scaffold and overall butterfly-like conformation, with an  $\alpha$ -helix (“knuckle”,  $\alpha 3$ ) epitope and two  $\beta$ -stranded sheets (“fingers” F1 and F2) extending from the core of the molecule (Figure 1A).

**Table 1**

*Data collection and refinement statistics*

Beamline	SSRL 9-2
Wavelength (Å)	1.0000
R <sub>merge</sub> (%)	7.7 (35.7) <sup>b</sup>
Number of unique reflections	8,346
Average redundancy	13.5 (11.3)
Average I/ $\sigma$ I	26.6 (6.9)
Completeness (%)	99.5 (97.4)
<b>Refinement</b>	
Resolution Range (Å)	50.00-2.32
R <sub>cryst</sub> (%)	24.9
R <sub>free</sub> (%) <sup>a</sup>	27.6
Average B (Å <sup>2</sup> )	60.2
<b>Root mean square deviation</b>	
Bonds (Å)	0.018
Angles (Å)	1.958
<b>Number of atoms</b>	
Protein	871
Water	40
<b>Ramachandran non-Gly non Pro residues in:</b>	
Most favored	77 (85.6%)
Additional allowed	12 (13.3%)
Generously allowed	1 (1.1%)

<sup>a</sup> Calculated from 5% of data not used in refinement

<sup>b</sup> Numbers in parentheses correspond to highest resolution shell (2.32-2.40 Å)

One of the primary goals of this project was to evaluate likely determinants of receptor binding between BMP-9 and other BMPs. To this end, the structure of BMP-9 was compared to existing crystal structures of other BMPs, unbound and in complex with the extracellular domains of either a type I or a type II receptor. Previous structural studies of BMP-2 in complex with the BMPR-IA extracellular domain suggested residues likely to be involved in determining binding affinity and specificity. These are defined by a maximum distance of 4.0 Å between the C $\alpha$  of any amino acid in the ligand from the C $\alpha$  of any amino acid on the receptor (on the receptor, these residues are colored green in Figure 1B) (Kirsch, Sebald et al. 2000). To examine the structural differences between BMP-2 and BMP-9 that might influence receptor binding, BMP-2/BMPR-IA (PDB entry 1ES7) was aligned with BMP-9 using least squares minimization. This method provides the best alignment by translating and rotating one of the structures relative to the other until the sum of the squared distances between corresponding residues is at a minimum. This comparison reveals the regions of the ligands that are likely to be significantly different, particularly the pre-helix loop in BMP-9 (Table 2B, Figure 1A).

In order to determine where the structures were significantly different from each other, it was first necessary to quantify the variability between BMP-9 and BMP-2. To this end, C $\alpha$  root mean square deviations (RMSDs) between BMP-9 and free (uncomplexed) BMP-2 (PDB entry 3BMP) were calculated. The RMSD is defined as:

$$RMSD = \sqrt{\frac{1}{N} \sum_{i=1}^{i=N} \delta_i^2}$$

where  $N$  is the number of atoms and  $\delta$  is the distance between two analogous atoms. The RMSD between two sets of points  $v$  and  $w$  (in this case, the location  $(x, y, z)$  of  $C\alpha$  for each aligned protein in a Cartesian space) is defined as:

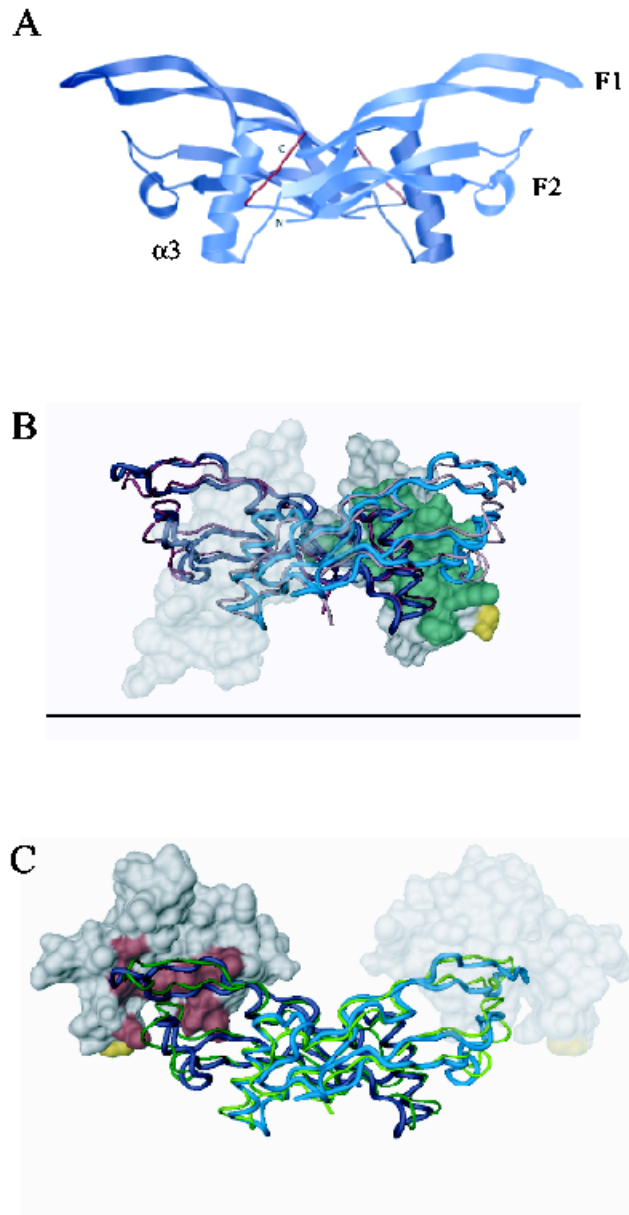
$$RMSD(v, w) = \sqrt{\frac{1}{n} \sum_{i=0}^n \|(v_i - w_i)\|^2}$$

$$RMSD(v, w) = \sqrt{\frac{1}{n} \sum_{i=0}^n (v_{ix} - w_{ix})^2 + (v_{iy} - w_{iy})^2 + (v_{iz} - w_{iz})^2}$$

The RMSD for the two monomers of BMP-9 and BMP-2 is 1.50 Å. Of the amino acids likely to be involved in binding a Type I receptor,  $C\alpha$  distances greater than one standard deviation above the mean occur between residues G27 in BMP-2 and G21 in BMP-9 and the segments F49-D53 in BMP-2 and F43-D47 in BMP-9 (Table 2A). One residue pair (P50 in BMP-2 and P44 in BMP-9) at the type I interface has a  $C\alpha$  distance greater than 1.5 standard deviations from the RMSD. All of these residues are located in the pre-helix loop, or “wrist” epitope (colored red in Figure 1A). As the residues in this region are highly conserved (Table 2A), this data suggests differences in type I receptor binding between BMP-9 and BMP-2 would be due to structural deviations in this region rather than primary sequence.

The crystal structure of BMP-7 in complex with Activin Receptor IIA (ActR-IIA, PDB entry 1LX5) has previously been solved, and the residues of the receptor likely to be involved in binding (as defined by 4.0 Å maximum distance between the  $C\alpha$  of an

amino acid in the receptor from any  $C\alpha$  in the ligand) are colored red in Figure 1C (Greenwald, Groppe et al. 2003). The RMSD was also calculated between BMP-9 and free BMP-7 (PDB entry 1LXI, Table 2B). One position at the type-II binding interface, A58 in BMP-7 and A28 in BMP-9, was found to have a distance greater than one standard deviation of RMSD (1.76 Å).



**Figure 1** Structure of BMP-9. (A) Ribbon diagram of BMP-9, showing “finger” regions 1 and 2 (F1 and F2) and  $\alpha$ -helix 3 ( $\alpha$ 3). The pre-helix loop, which likely encodes specificity determinants, is colored red. (B) Overlay of BMP-9 (blue) and BMP-2 (pink) with BMPR-IA ectodomain. Residues of BMPR-IA within 4.0 Å of BMP-2 are shaded green (Kirsch, Sebald et al. 2000). The C-terminal residue of BMPR-IA ectodomain is shaded yellow. (C) Overlay of BMP-9 (blue) and BMP-7 (green) with ActR-II ectodomain. Residues of ActR-II within 4.0 Å of BMP-2 are shaded red (Greenwald, Groppe et al. 2003). The C-terminal residues of ActR-II ectodomain are shaded yellow. Figures 1B and 1C were produced with DINO (<http://www.dino3d.org>).

**Table 2A***BMP-9 vs. BMP-2, Type I Receptor Binding*

BMP-2	BMP-9	Type of change	RMSD >1 $\sigma$ ?	Receptor Interface (BRIA)
S24	E18	Hydroxyl to Acidic		Basic
D25	D19			Basic
V26	I20	Aliphatic to Aliphatic		Aromatic/cyclic
G27	G21		X	Cyclic
W28	W22			Acidic/Basic/Aromatic
W31	W25			Basic
F49	F43		X	Aliphatic/Acidic
P50	P44		X	Acidic/Aromatic/Aliphatic
L51	L45		X	Acidic
A52	A46		X	Sulfur
D53	D47		X	Sulfur/Hydroxyl/basic
H54	D48	Basic to Acidic		Cyclic/Basic/Sulfur
N56	T50	Acidic to Hydroxyl		Basic
N59	K53	Acidic to Basic		Aliphatic/Acidic
I62	I56			Aliphatic/Acidic
L66	L60			Basic/Acidic/Hydroxyl
S69	L63	Hydroxyl to Aliphatic		Acidic/Basic/Aliphatic
V70	K64	Aliphatic to Basic		Acidic/Basic/Hydroxyl
Y103	Y99			Aromatic/Acidic

**Table 2** Comparison of likely receptor binding regions of BMP-9 with BMP-2 and BMP-7. For all amino acids likely to be directly involved in receptor binding as determined by 4 ang or less distance from the the receptor, the corresponding binding sequence and difference in main chain position for BMP-9 are shown. If there is a difference in sequence between BMP-9 and the reference BMP, the type of change in amino acid is shown. If there is a difference in main chain position between BMP-9 and the reference BMP, it is indicated with an X. For all positions, the type of residue the amino acid contacts in the receptor is shown. Multiple types indicate that the amino acid from the ligand contacts more than one amino acid in the receptor.

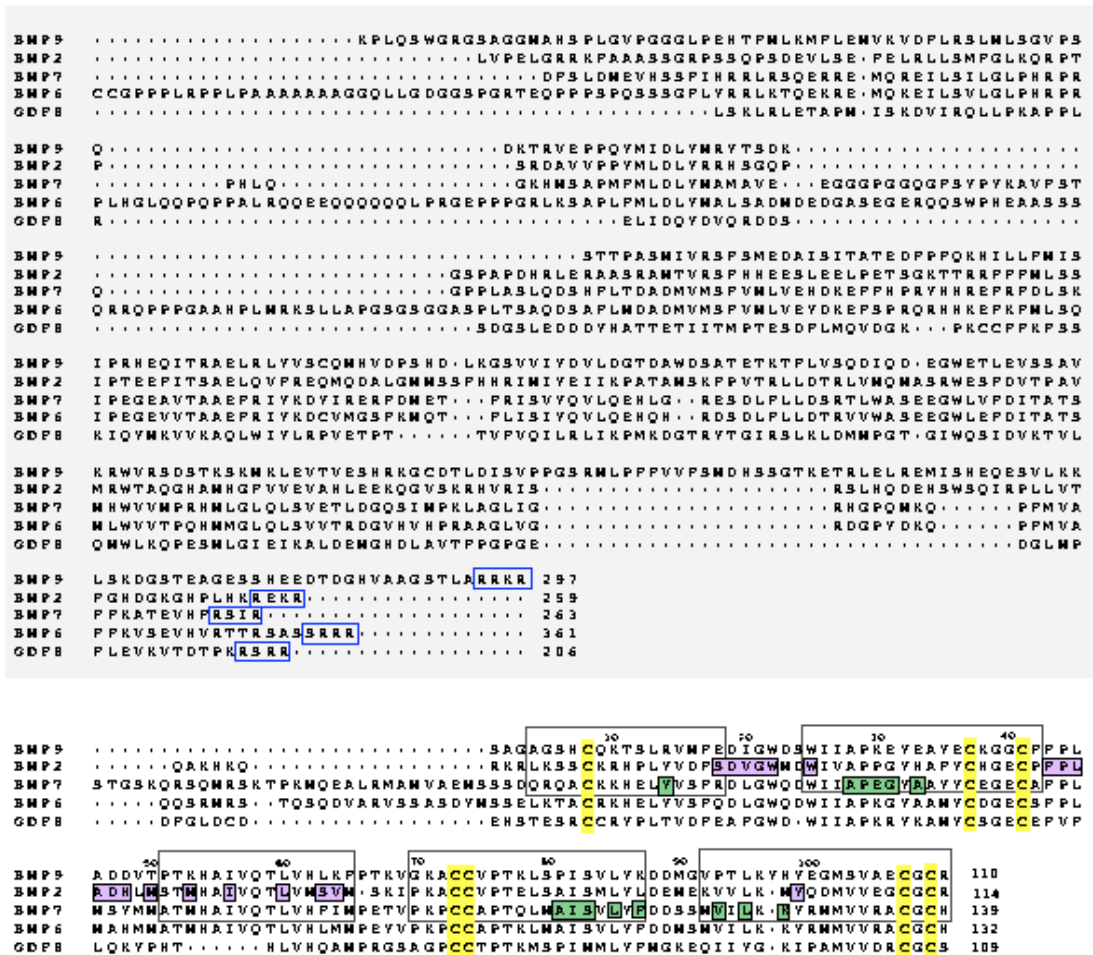


**Table 2B***BMP-9 vs. BMP-7, Type II Receptor Binding*

<b>BMP-7</b>	<b>BMP-9</b>	<b>Type of change</b>	<b>RMSD &gt;1<math>\sigma</math>?</b>	<b>Receptor Interface (ActRII)</b>
<b>Y44</b>	<b>R14</b>	<b>Aromatic to Basic</b>		<b>Acidic</b>
<b>A58</b>	<b>A28</b>		<b>X</b>	<b>Aromatic</b>
<b>P59</b>	<b>P29</b>			<b>Acidic</b>
<b>E60</b>	<b>K30</b>	<b>Acidic to Basic</b>		<b>Basic</b>
<b>G61</b>	<b>E31</b>	<b>Aliphatic to Acidic</b>		<b>Acidic</b>
<b>A63</b>	<b>E33</b>	<b>Aliphatic to Acidic</b>		<b>Aliphatic</b>
<b>A111</b>	<b>P81</b>	<b>Aliphatic to Cyclic</b>		<b>Aliphatic</b>
<b>I112</b>	<b>I82</b>			<b>Aliphatic</b>
<b>S113</b>	<b>S83</b>			<b>Aromatic/Aliphatic</b>
<b>L115</b>	<b>L85</b>			<b>Aromatic</b>
<b>F117</b>	<b>K87</b>	<b>Aromatic to Basic</b>		<b>Aliphatic</b>
<b>V123</b>	<b>P93</b>	<b>Aliphatic to Cyclic</b>		<b>Aliphatic</b>
<b>L125</b>	<b>L95</b>			<b>Aromatic</b>
<b>K127</b>	<b>H97</b>	<b>Basic to Basic</b>		<b>Aliphatic</b>

(Table 2 continued)

In a sequence alignment of BMP-9 and BMP-2 (Figure 2), sequence differences in binding regions identified by structural and mutational (Kirsch, Sebald et al. 2000) studies are most notable in residues N59 in BMP-2 and K53 in BMP-9 (acidic to basic) and in residues H54 in BMP-2 and D58 in BMP-9 (basic to acidic, see Table 2A). A comparison between the amino acid sequences of BMP-9 and BMP-7 (Figure 2) also reveals significant changes, including Y44 in BMP-7 to R14 in BMP-9 (aromatic to basic), E60 in BMP-7 to K30 in BMP-9 (acidic to basic), and G61 in BMP-7 to E31 in BMP-9 (aliphatic to acidic) at the type II binding interface (see Table 2B). Based on the BMP-7/ActR-IIIB structure (Greenwald, Groppe et al. 2003), Y44 and G61 both interact with the acidic N65, and E60 interacts with basic K76. These data indicate that differences between BMP-7 and BMP-9 in binding affinity for type II receptors are most likely due to amino acid changes at the binding interface, though type II receptors are generally quite promiscuous (Sebald, Nickel et al. 2004).



**Figure 2** Sequence alignment of BMP-9, BMP-2, BMP-7, BMP-6, and GDF-8. Pro-regions are boxed and shaded gray, furin cleavage sites at the end of each pro-region are boxed in blue. Regions of type II receptor binding are boxed and shaded green (Greenwald, Groppe et al. 2003), regions of type I receptor binding are boxed and shaded purple (Kirsch, Sebald et al. 2000), and conserved cysteines are shaded yellow. Profiles of structural similarity determined by STAMP (Russell and Barton 1992) are boxed in gray. N-terminal signal sequences of each protein were omitted from the alignment. Ligands and pro-regions were aligned separately with CLUSTALW (Thompson, Higgins et al. 1994).

The PDB entry for BMP-9 is 1ZKZ.

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M., Tsareva T., Parice Y., Mahoney A., Roschke V., Sanyal I., Choe S. (2005). "Crystal structure of BMP-9 and functional interactions with pro-region and receptors." J Biol Chem **280**(26): 25111-8. The author of this dissertation was the primary researcher of this work.

## Chapter 2: Functional Studies of BMP-9

### Abstract

Bone Morphogenetic Proteins (BMPs) are a subset of the Transforming Growth Factor (TGF)- $\beta$  superfamily of ligands and receptors. All of the ligands are translated as precursors, with pro-regions that generally dissociate after cleavage from the ligand, but in some cases, including GDF-8 and TGF- $\beta$ 1, -2 and -3, the pro-region remains associated after secretion from the cell and inhibits binding of the ligand to its receptor. Though the pro-region of BMP-9 remains tightly associated after secretion, we find in several cell-based assays that the activities of BMP-9 and BMP-9/pro-region complex were equivalent. Activin receptor-like kinase 1 (ALK-1), an orphan receptor in the TGF- $\beta$  family, was also identified as potential receptor for BMP-9, based on surface plasmon resonance studies (SPR) and the ability of soluble ALK-1 to block the activity of BMP-9/pro-region complex in cell-based assays.

### Methods

Materials -All recombinant receptor:Fc chimeria were purchased from R & D Systems, and were reconstituted as instructed by the manufacturer. BIAcore instrumentation, software and CM5 sensor chips were purchased from BIACOREä (Uppsala, Sweden). Fetal bovine serum and all other cell culture media were purchased from Invitrogen Corporation unless otherwise stated.

Cell Lines - Rat hepatoma cell line H4Ile, mouse myoblast cell line C2C12 and mouse preadipocyte cell line 3T3-L1 were all obtained from American Type Culture Collection (ATCC). These cell lines were propagated in media recommended by ATCC. The dihydrofolate reductase (DHFR)-deficient Chinese Hamster Ovary cell line DG44 (CHO-DG44) (Urlaub, Kas et al. 1983) was propagated in MEM-a<sup>+</sup> medium, 5% dialyzed

fetal bovine serum and 4mM L-Glutamine. Selections were performed in MEM- $\alpha$  medium lacking ribonucleotide and deoxy-ribonucleotide supplements, supplemented with 5% dialyzed fetal bovine serum and 4 mM glutamine. Methotrexate (Sigma) was used at the concentrations indicated.

For all cell-based assays, BMP-9 dimer was separated from its pro-region using 50% cold ethanol precipitation. After cold precipitation, pro-region and remaining BMP-9/pro-region complex were spun down, leaving isolated, dimeric BMP-9 in the supernatant. The precipitation was repeated once after dissolving the pro-region and BMP-9/pro-region complex in pH 7.4, 20 mM Tris, 200 mM NaCl. The pro-region was further separated from BMP-9/pro-region complex on a MonoQ column using the same procedure as used to purify the complex. Soluble BMP-9 in the supernatant was concentrated using a CentriPrep (Amicon) and the buffer was changed to pH 7.4, 20 mM Tris, 200 mM NaCl, 30% ethanol.

Size exclusion and reverse phase chromatography – Approximately 100 mg of total protein from CHO cell media was run in 10 mM Tris, 100 mM NaCl over an S-200 size exclusion chromatography column. For native and SDS gels, purified BMP-9 dimer and pro-region were obtained by reverse phase chromatography, using a C4 high-pressure liquid chromatography column (Vydac) and eluted with a 27-42% acetonitrile (ACN) gradient. BMP-9 dimer eluted at 31% ACN and the pro-region eluted at 35% ACN. Fractions were frozen in liquid nitrogen, lyophilized, and reconstituted in water.

Native Gels and SDS-PAGE - Native gels for BMP-9/pro-region were 6% polyacrylamide, pH 8.8, and run at 27mA. Proteins separated by C-4 columns, lyophilized, reconstituted in water, were mixed to final concentrations of 1-3 mg/mL and allowed to equilibrate for at least 15 min at room temperature or overnight at 4°C before loading. Native gels for ligand-receptor binding were 6-12% polyacrylamide and run at

27mA. BMP-9 directly from cell culture purification was used, without separation from the other components of that mixture by C-4 or other means. SDS gels were 12% polyacrylamide and run at 200mV.

Protein Sequencing - N-terminal protein sequencing was performed by the Salk Institute for Biological Sciences in-house protein sequencing facility.

Alkaline Phosphatase Assay - Mouse pluripotent C2C12 cells were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 100 IU/mL penicillin, and 100 mg/mL streptomycin. For the alkaline phosphatase assay, C2C12 were seeded in 96-well tissue culture plates at 1,000 cells/well in 100 mL media. The following day, medium was removed and replaced with treatments in DMEM media containing 0.1% fetal bovine serum. Cells were cultured for 4 days and conditioned media were collected for measurement of alkaline phosphatase activity. In inhibitor studies, soluble receptors were added at the indicated concentrations with BMP-9 complex (5 nM) or BMP-4 (4.7 nM). Alkaline phosphatase activity was measured using the Phospha-Lightä System (Applied Biosystems) according to manufacturer's directions. Briefly, cells were rinsed with PBS, lysed in buffer containing 0.2% Triton-X-100, incubated 5 minutes in assay buffer, and 20 minutes in reaction buffer containing CSPD (disodium 3-(4-methoxyspiro[1,2-dioxetane- 3,2'-(5'-chloro)tricyclo [3.3.1.1(3,7)]decan-4-yl)phenyl phosphate) substrate. CSPD substrate produces a luminescent signal when dephosphorylated by alkaline phosphatase in an alkaline, hydrophobic environment, resulting in anion production, which emits light upon decomposition. Luminescent signal was read using a luminescent plate reader (Applied Biosystems). All treatments were performed in triplicate. The average and standard deviation were determined and data plotted using Prism software (GraphPad Software, Inc., San Diego, CA).

Cell Proliferation Assay - Mouse pre-adipocyte 3T3-L1 cells were plated at 4,000 cells/well in white 96 well tissue culture plates (Costar) in 100 mL DMEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin. Cells were cultured overnight. To arrest cell growth, cells were washed once with low serum media (0.1% fetal bovine serum), 100 mL low serum media was added, and cells were cultured overnight. BMP-9, BMP-9/pro-region complex, pro-region, or controls were added to cells at indicated concentrations. In inhibitor studies, soluble receptors were also added at the indicated concentrations with BMP-9 proteins (4 nM) or BMP-4 (3.7 nM). Cells were incubated for 4 days, and cell number assayed using Cell Titer Glo assay (Promega, Madison, WI) according to manufacturer's instructions. In brief, 100 mL of assay reagent was added to cells at room temperature, mixed and incubated for approximately 30 minutes. Luminescent signal was read using a luminescent plate reader. All treatments were performed in triplicate and analyzed with Prism software.

Malic Enzyme-SEAP Reporter Assay - Promoter region (base pairs -1183 to -77) of rat malic enzyme (ME) was cloned into the promoterless pSEAP2-neo vector and fused in-frame with the SEAP gene. The construct was transfected into rat hepatoma cell line H4Ile and stable clones were selected for neomycin resistance. To perform the reporter assay, ~75,000 cells per well were plated in 96 well plates. Growth medium was DMEM supplemented with 10% FBS, 15 mM HEPES, 100 IU/ml Penicillin, and 100 mg/ml Streptomycin. Cells were cultured for 24 hours. Reporter cells were then serum-deprived for 18-24 hours before being treated with BMP-9, BMP-9/pro-region complex, pro-region, or controls. For the experiments using soluble BMP receptors, cells were treated with various concentrations of soluble receptors prior to addition of BMP-9 complex (2 nM) or BMP-4 (0.4 nM). After a 48-hour incubation period, conditioned media were removed and SEAP activity was determined using Phospha-Lightä System

according manufacturer's recommended protocol. Light emission from the wells was measured in a MLX Microtiter Plate Luminometer (Dynex Technologies). All treatments were performed in triplicate and analyzed with Prism software.

Biosensor Surface Preparation for Surface Plasmon Resonance Analysis - To determine receptor specificity for BMP-9, three high-density CM5 chips were prepared. Each chip contained four flowcells, with one flowcell per chip immobilized with an unrelated receptor:Fc used as a negative Fc control (Trail-Receptor:Fc). The remaining flowcells contained one of the following BMP receptors (each fused to Fc): BMPR-IA, BMPR-IB, BMPR-II, ALK-1, ALK-7, Activin Receptor IA (ActR-IA), ActR-IB, ActR-IIA, and ActR-IIB. The immobilization level, defined in relative units (R.U.), ranged from 7,110-17,300. Purified BMP-9/pro-region complex was applied at 50 mg/mL (1 mM) to each individual flow cell for two minutes. Measured on-rates and off-rates after global fits to binding curves were used to derive binding affinity.

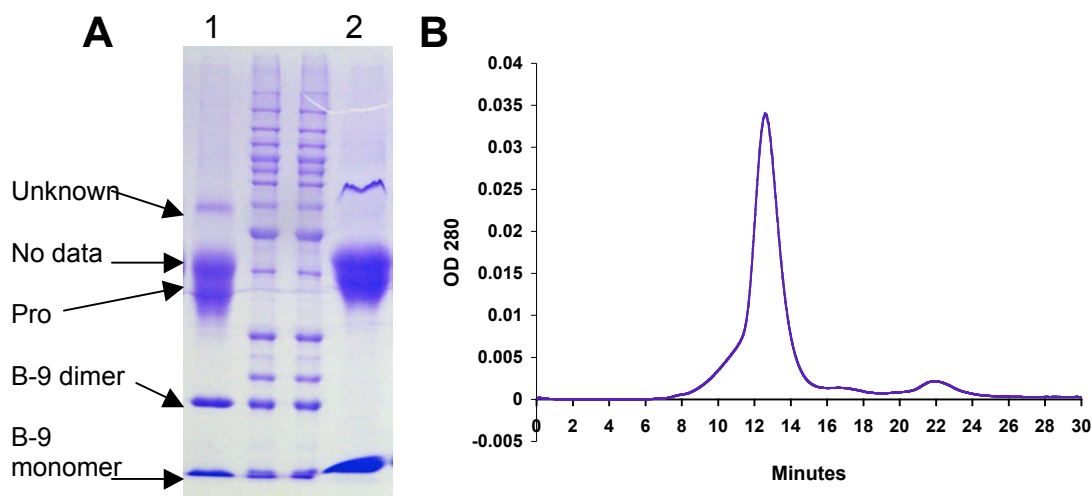
## **Results**

BMP-9 was crystallized from a heterogeneous mixture of proteins that contains BMP-9 dimer and monomer, as well as the pro-region and other proteins (Figure 3A). This mixture runs as a single peak by size exclusion chromatography, with a retention volume consistent with a complex of one BMP-9 dimer and two pro-regions (Figure 3B), implying that BMP-9, when purified from cell media, remains noncovalently associated with its pro-region. This complex was then separated using reverse-phase chromatography, which uses an acetonitrile gradient to cause non-covalently linked proteins to elute based on their unique hydrophobicities. Several peaks can be identified from the chromatogram (Figure 3C). When the protein corresponding to these peaks is

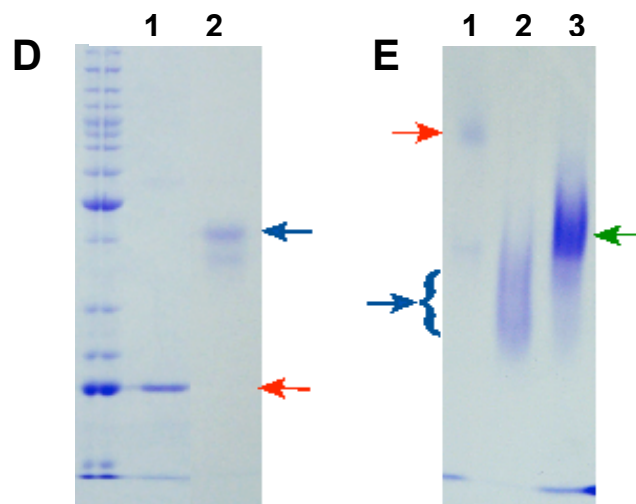
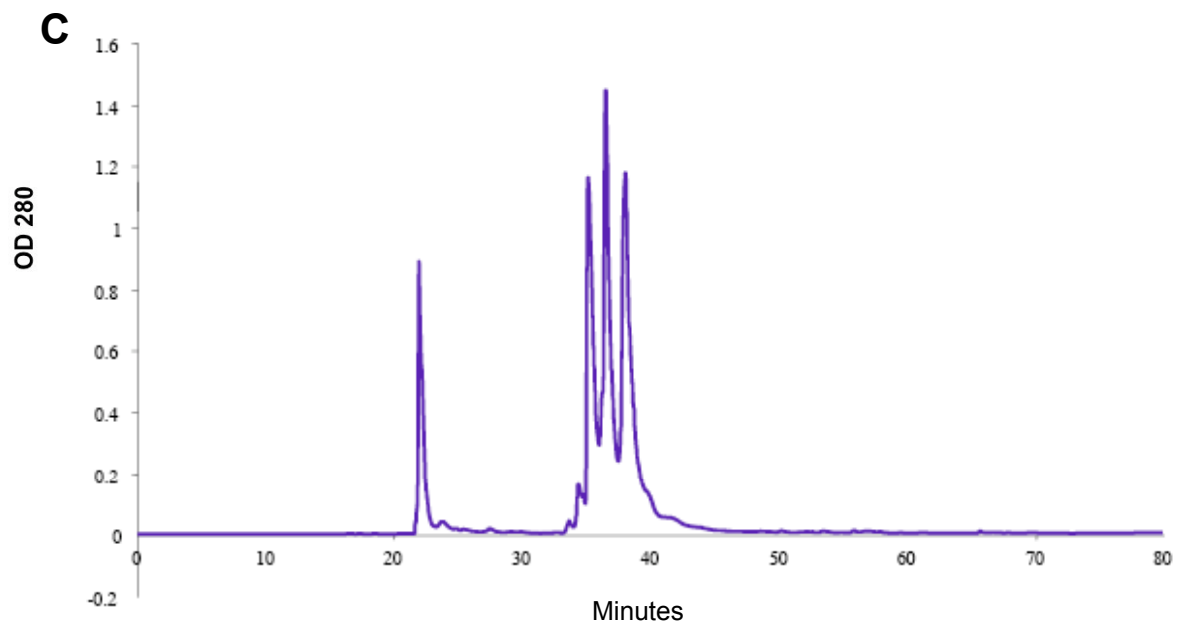


concentrated and run on an SDS gel, it is clear that BMP-9 and the pro-region are cleanly separated (Figure 3D).

When the isolated BMP-9 and pro-region from the reverse-phase chromatography are recombined in equimolar quantities and run on a native gel, a band similar to what is seen in the original protein solution appears. Neither the isolated dimeric BMP-9 band, nor the isolated pro-region band is observed, indicating that the complex is sufficiently high-affinity that it can be fully reformed without any additional proteins in an aqueous environment (Figure 3E).



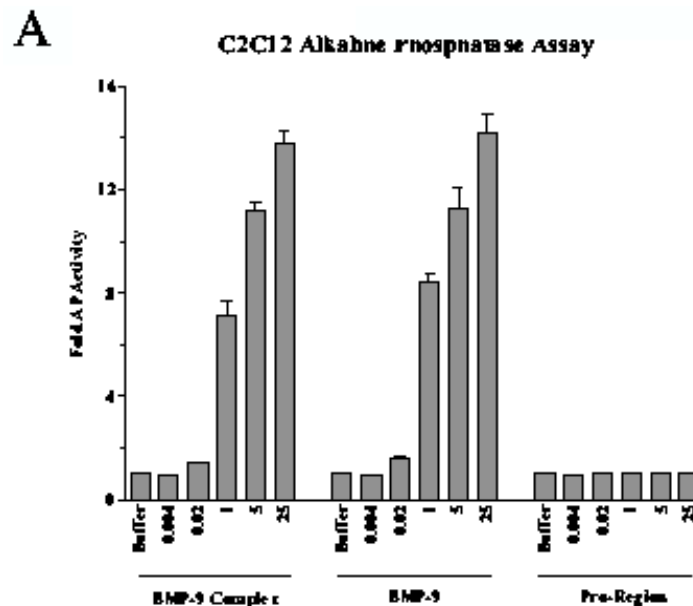
**Figure 3** Size of the BMP-9/pro-region complex and separation and reformation of BMP-9/pro-region complex. (A) Coomassie stain of SDS-PAGE gel showing BMP-9 total protein solution under non-reducing (lane 1) and reducing (lane 2) conditions. N-terminal sequencing identified the bands as BMP-9 and pro-region. Other bands yielded no data (ND) or insufficient data for identification (Unk). (B) Size exclusion chromatogram from S-200 column showing that the retention volume (12.5 ml) of the BMP-9/pro-region complex is consistent with a complex of one BMP-9 dimer associated with two pro-regions. (C) Reverse-phase chromatogram of BMP-9 over a 27-42% acetonitrile gradient. Fractions were frozen, lyophilized, reconstituted in water for further analysis. (D) SDS-Page gel showing components from reverse phase separation. Lanes are 1) BMP-9 dimer (blue arrow) 2) BMP-9 pro-region (red arrow). (E) Native gel showing components after reverse phase separation. Lanes are 1) BMP-9 dimer (blue arrow) 2) pro-region (red arrow) 3) BMP-9/pro-region complex (green arrow).



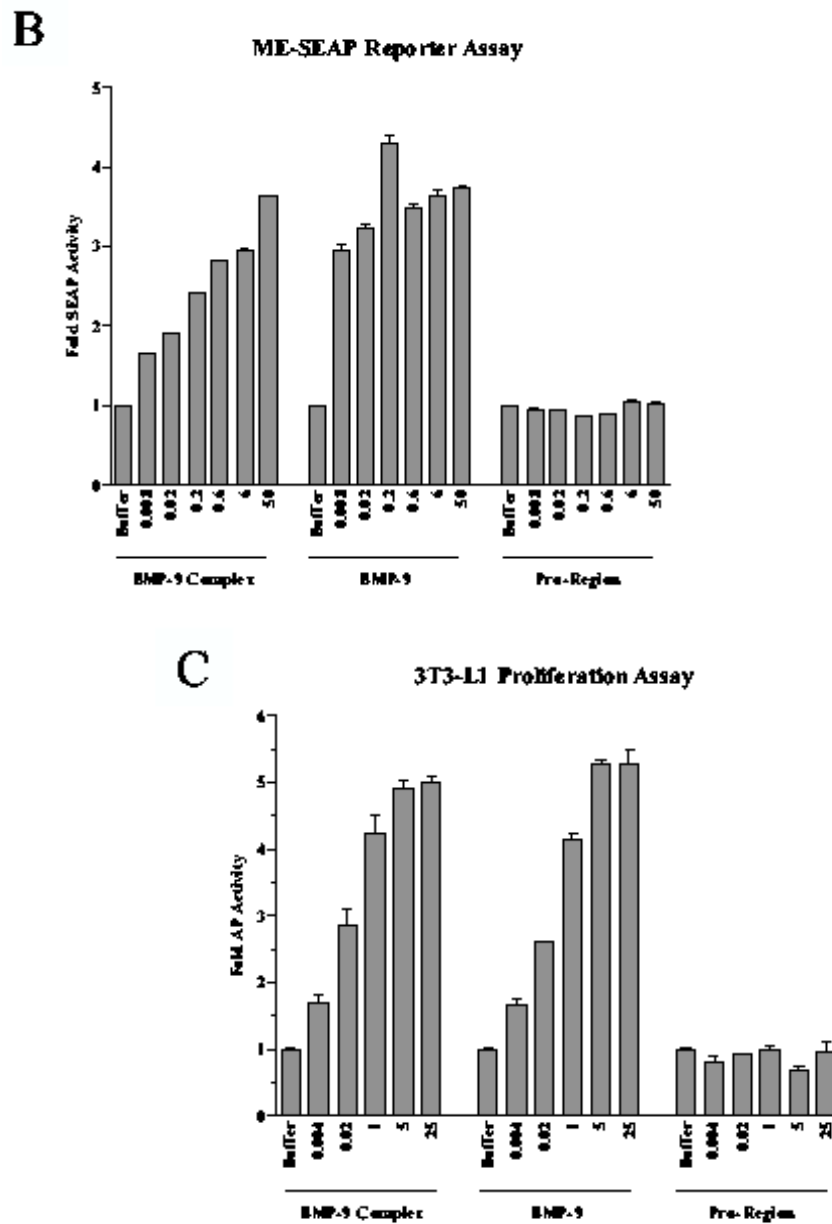
(Figure 3 continued)

Biological activities of isolated BMP-9, BMP-9/pro-region complex, and pro-region alone were tested by 3 different *in vitro* assays using distinct cell types. Isolated BMP-9/pro-region complex, BMP-9, and pro-region were tested for the ability to

stimulate secretion of alkaline phosphatase from C2C12 cells (Figure 4A), to activate transcription of H4Ile/ME-SEAP gene reporter (Figure 4B), and to induce proliferation of 3T3-L1 pre-adipocyte cell line (Figure 4C). Both purified BMP-9 and BMP-9/pro-region complex were highly active in all three assays, and the degree of activation was dose-dependent. The presence of the pro-region does not alter the activity of BMP-9, as the activities of BMP-9 and BMP-9/pro-region complex are not significantly different. The pro-region alone is devoid of biological activity in these assays.



**Figure 4** Activity of BMP-9 and BMP-9/pro-region complex is similar in cell-based functional assays, while pro-region alone has no effect. (A) C2C12 alkaline phosphatase assay: C2C12 cells were growth arrested for 24 hours in low serum media and treated with either buffer, or BMP-9, BMP-9/pro-region complex, or pro-region at indicated concentrations (nM). After 4 days, cells were lysed and alkaline phosphatase activity determined. (B) H4Ile/ME-SEAP reporter assay: Serum-deprived reporter cells were treated with either buffer or BMP-9, BMP-9/pro-region complex, or pro-region at indicated concentrations. SEAP activity in the conditioned media was measured after a 48-hour incubation period. (C) 3T3-L1 cell proliferation assay: 3T3-L1 cells were growth arrested for 24 hours in low serum media and treated with either buffer or BMP-9, BMP-9/pro-region complex, or pro-region at indicated concentrations. After 4 days, cell numbers were determined by Cell-Titer Glo assay. For all assays, cells were treated in duplicate or triplicate wells and average  $\pm$  SD for each treatment is shown. Data is normalized to vehicle-treated samples, and presented as: (signal from BMP-9 treated sample / signal from vehicle-treated sample).

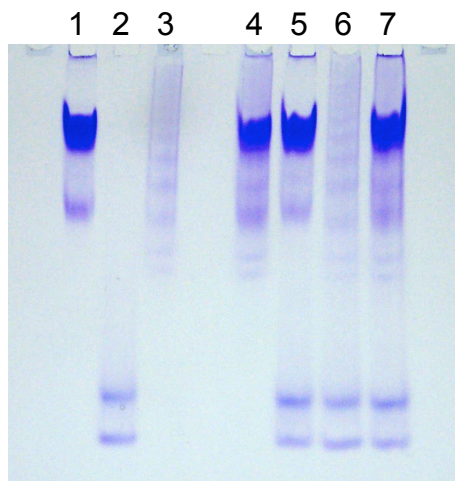


(Figure 4 continued)

In order to identify potential receptors for BMP-9, several types of binding studies were conducted using recombinant extracellular domains for ALK-3 (a type I receptor)

and ActRIIb (a type II receptor). Some binding was predicted between BMP-9 and ActRIIb, due to the promiscuous nature of type II receptors. One of the complexities of using recombinant ActRIIb is that only 10-25% of the protein purified from *E. coli* is active. In order to isolate the active ActRIIb, the construct was tagged with His and a combination of active and inactive ActRIIb was eluted from a nickel column. This mixture was then run over an ion-exchange column, and the active component was isolated. Only the active component is used in these studies.

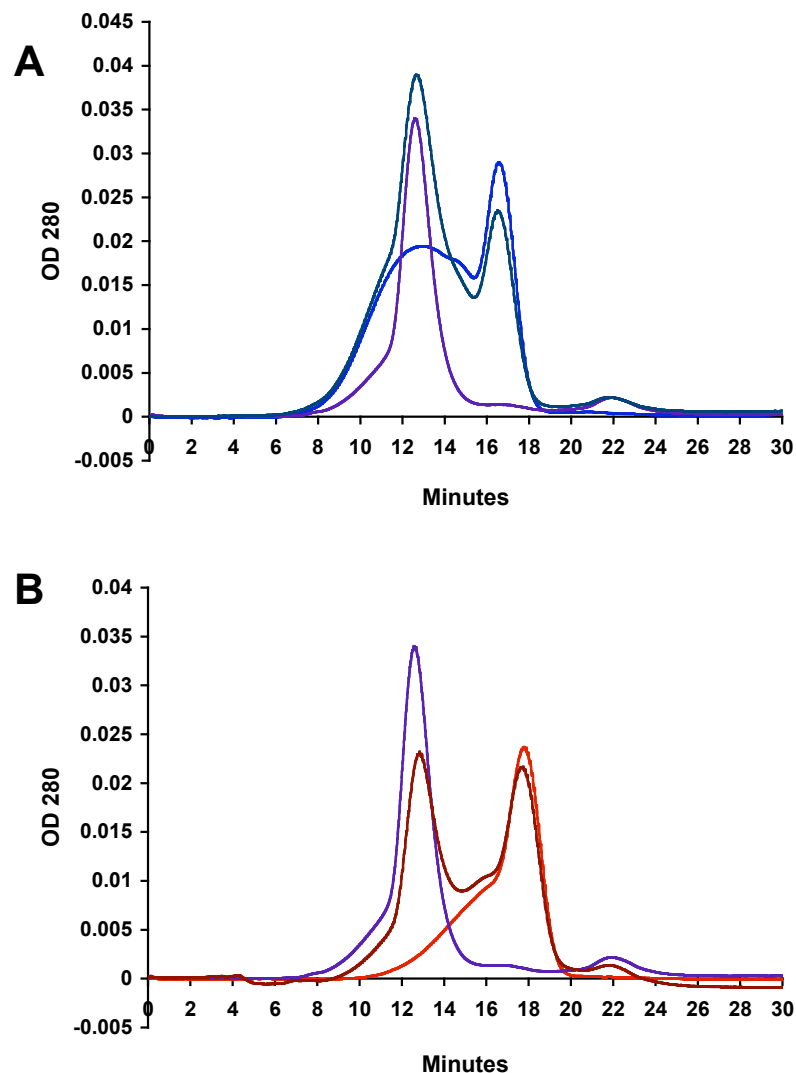
One method that was attempted was the use of native gels, which are similar to standard SDS protein gels, but do not use SDS, which is intended to separate non-covalently associate proteins, or any kind of reducing agent, which is intended to break covalent linkage between proteins. As a result, they preserve covalent and strong non-covalent interactions, as in the case of BMP-9 and its pro-region (Figure 5).



**Figure 5** Native Gel of BMP-9 and Receptor ECDs. Lanes 1-3 show each protein alone. 1) BMP-9, 2) ActRIIb, 3) ALK-3. Lanes 4-7 show various combinations of ligand and receptors. 4) BMP-9 + ALK-1. 5) BMP-9 + ActRIIb. 6) ALK-3 + ActRIIb. 7) BMP-9 + ALK-3 + ActRIIb. None of these cases show any interaction between the proteins, as would be shown with a novel band and a decrease in intensity of the individual protein bands. Standard protein benchmarks are unreliable under native gel conditions, which is why they are not used here.

The native gel did not yield any useful results, indicating that the association between BMP-9 and these receptors, if it existed, was not strong enough to withstand the relatively mild conditions of a native gel.

BMP-9 and each receptor ECD were run over a sizing column separately, then combined to check for binding (Figure 6). If binding were to occur, it would be expected that the peaks associated with each of the individual proteins would decrease in size as a result of those proteins becoming part of a larger complex, and that a novel peak from the complex would appear. In the case of ALK-3, it was clear that there was no decrease in the size of the individual peaks and no novel peak appeared on the sizing column (Figure 6A). In fact, the spectra resulting from the combination of BMP-9 and ALK-3 could easily be explained by added the individual spectra together, which is what would be expected if either there was no binding, or the association was not strong enough to be visible or maintained during the run on the sizing column. In the experiment with ActRIIb, it was clear that the peak from BMP-9 decreased in size, and possible that a novel peak appeared between the two peaks (Figure 6B). It would be possible in each of these cases that binding did occur, but that the dissociation constant was high enough that the complex would separate and reform when at a high concentration in solution, but in the substantially more dilute environment of a sizing column, once the complex dissociated, it could not reform. Overall, this method provided less robust results than desired, so other methods were tried.



**Figure 6** Overlays of S-200 sizing column chromatograms from BMP-9 and type I and type II receptor ECDs. A) BMP-9 (purple), ALK-3 (blue) and BMP-9 + ALK-3 (green). The trace from the combination of the two proteins appears to be a simple addition of the peaks from the individual proteins. B) BMP-9 (purple), ActRIIb (red), and BMP-9 + ActRIIb (brown). The peak from BMP-9 appears to decrease in size, though the peak from ActRIIb does not change and no convincing novel peak is seen.

The next method employed was surface plasmon resonance (SPR). The principle behind this technique is that when a beam of light is directed at a partially transparent and partially reflective surface, the angle that creates total internal reflection (TIR) changes based on the amount of mass associated with that surface. To test for

protein-protein interactions, one protein can be bound to the surface, and a buffer containing the second protein flows over that surface-bound protein. If the two interact, the mass on the surface of the chip will increase, the angle of TIR changes and provides a real-time readout of binding kinetics and equilibrium ([www.biacore.com](http://www.biacore.com)).

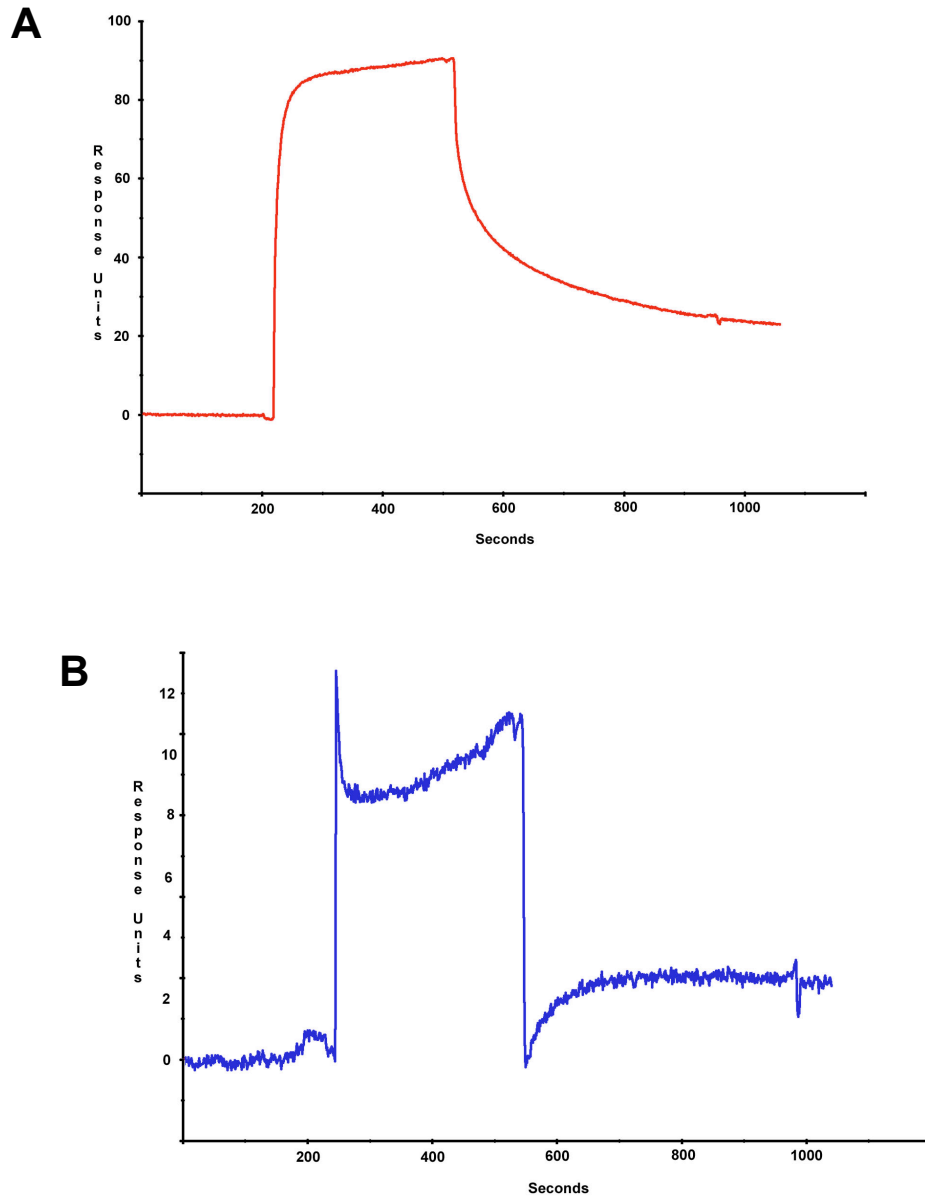
The benefits of this technique include the microgram or less quantities of protein required to determine binding, affinity, association and dissociation kinetics, and equilibrium constants (Berggard, Linse et al. 2007; Tanious, Nguyen et al. 2008). However, there are limitations of this technique with respect to BMP signaling. The receptors in a cell membrane have a specific orientation. The amide-coupling chemistry that dictates binding to the chip in these studies is non-specific, leading to a random orientation of the bound proteins, many of which may not be conducive to binding, leading to an artificially low estimate of affinity. Additionally, BMP ligand-receptor assembly can be cooperative, with type II receptors having a lower affinity to the free ligand than to the ligand once it is bound to a type I receptor (Liu, Ventura et al. 1995; Nohno, Ishikawa et al. 1995). Using Biacore to estimate binding using only a free ligand and a type II receptor therefore might also lead to low estimates of affinity.

However, the time efficiency and low protein requirements of this technique seemed to outweigh the limitations. As the previous studies had indicated that the pro-region of BMP-9 did not impede its ability to signal, a selection of commercially available receptors was screened, with each receptor was bound to the chip and BMP-9 in complex with the pro-region flowed over the surface to determine binding ability. It was shown that activin receptor-like kinase-1 (ALK-1) showed the highest affinity of all the type I receptors tested.

Some initial SPR studies were conducted to identify possible receptors for BMP-9. In these studies, recombinant activin receptor type IIb (ActRIIb), and ALK-3 were



bound to the SPR chip and BMP-9/proregion flowed over. ActRIIb was identified as a potential type II receptor, while ALK-3 showed no binding (Figure 7).



**Figure 7** Sample traces of SPR data. In each case, the receptor ECD is bound to the chip and BMP-9 is flowing over. A) BMP-9 and ActRIIb show some binding and gradual dissociation. B) BMP-9 and ALK-3 show no binding.

SPR studies were limited by the lab's ability to produce these receptor ECDs, so further tests were performed by our collaborators at Human Genome Sciences, Incorporated. To measure relative affinities between BMP-9/pro-region complex and BMP/activin receptors, SPR analysis using purified BMP-9/pro-region complex and commercially available receptor:Fc chimera immobilized on the chip surface (Table 2, column 1). BMP-9/pro-region complex did not bind the control flow cell. However, it did bind strongly to ALK-1 and BMPR-II. In addition, BMP-9 also binds weakly to ActR-IA, ActR-IIA, and ActR-IIB at different relative levels. Binding constants cannot be accurately derived from this study as only one ligand concentration was applied to a high-density chip, and it has been shown in other studies that dissociation constants vary based on the density of the fixed receptor and whether the ligand or the receptor was immobilized due to cooperative binding by the receptors (Kirsch et al, 2000; Sebald et al, 2004). However, by comparative analysis, it is clear that BMPR-II and ALK-1 have the highest affinity.

In an effort to identify the receptors that may mediate the effects of BMP-9, 5 type I and 4 type II commercially available soluble BMP or Activin receptor:Fc chimera were tested for the ability to block activity of BMP-9/pro-region complex in the C2C12 alkaline phosphatase assay. BMP-4, a known ligand of BMPR-IA (ten Dijke, Yamashita et al. 1994), was used as the control (Table 3, columns 2 and 3). In this experiment, BMP-9/pro-region complex (5 nM) or BMP-4 (4.7 nM) was added to C2C12 cells either alone or in the presence of 10-fold molar excess of soluble receptor. After an incubation period, conditioned medium was collected and alkaline phosphatase activity was measured as described above. As presented in Table 2, ALK-1:Fc completely inhibited BMP-9/pro-region complex activity, but had no effect on BMP-4. In contrast, BMPR-IA:Fc completely abolished BMP-4 activity, but had no effect on BMP-9/pro-region

complex. Of the other soluble receptor:Fc chimeras tested, only BMPR-II:Fc partially blocked BMP-9/pro-region complex activity, while the rest did not exhibit any inhibitory activity.

**Table 3**

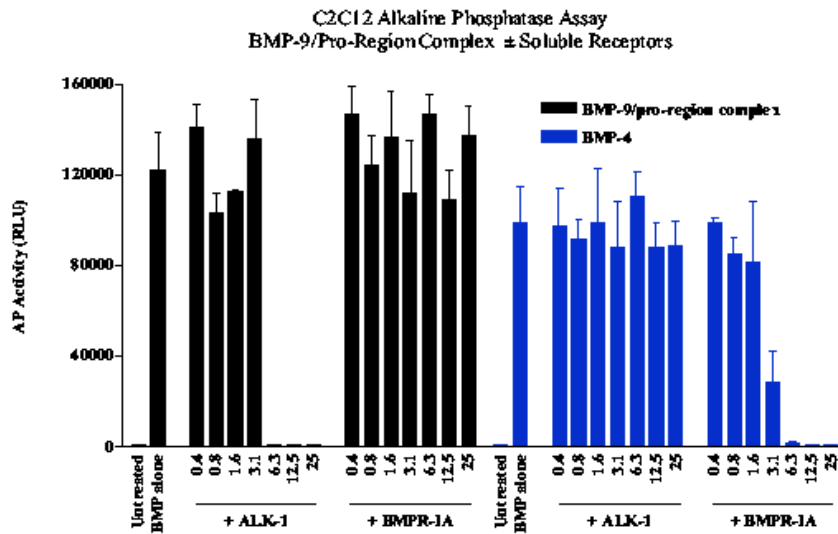
*Summary of inhibition studies with soluble receptors in C2c12 alkaline phosphatase assay and BIAcore analysis on binding of BMP-9 pro-region to soluble receptors*

Receptor	Relative affinity of BMP-9 pro-region complex to receptor (SPR)	Inhibition of BMP-9 activity in AP assay	Inhibition of BMP-4 activity in AP assay
ALK-1	1801.8	+++	-
BMPR-II	1336.9	+	-
Activin RIIA	384.8	-	-
Activin RIIB	99.3	-	-
Activin RIA	39.9	-	-
ALK-7	7.6	-	-
BMPR-IB	4.7	-	-
BMPR-IA	-3.9	-	+++
Activin RIB	-10.1	-	-

To further examine interactions between BMP-9 and ALK-1, BMP-9/pro-region complex was used in the absence or presence of various concentrations of soluble ALK-1 or BMPR-IA receptors in the C2C12 alkaline phosphatase and H4Ile/ME-SEAP reporter assays (Figure 5). BMP-4 was included in the experiment as a control. ALK-1:Fc completely inhibited BMP-9/pro-region complex activity in both assays when given at greater than 6-fold molar excess, but showed no effect on BMP-4. As expected, BMPRIA:Fc inhibited BMP-4 activity in a dose-dependent manner, ranging from 1- to 6-fold molar excess, but did not block activity of BMP-9/pro-region complex. Combining

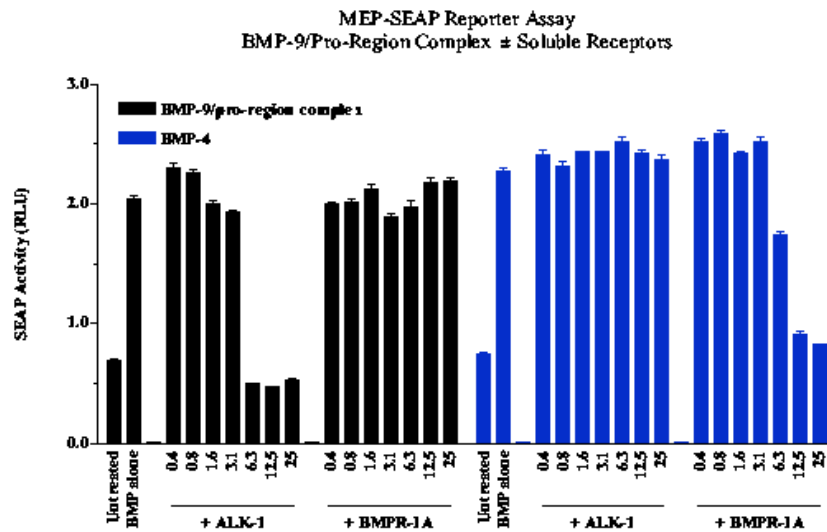
the results, our data demonstrate that ALK-1 can bind to purified BMP-9/pro-region complex and neutralize its biological activity in cell-based assays.

A



**Figure 8** Inhibition of BMP-9 complex activity by soluble ALK-1:Fc. (A) C2C12 alkaline phosphatase assay: C2C12 cells were growth arrested for 24 hours in low serum media. Cells were treated with either BMP-9/pro-region complex (5 nM) or BMP-4 (4.7 nM,) alone or in the presence of indicated fold excess molar concentration of soluble ALK-1:Fc or BMP-RIA:Fc proteins. After 4 days, cells were lysed and alkaline phosphatase activity determined. (B) H4IIE/ME-SEAP reporter assay: Serum-deprived reporter cells were treated with either BMP-9/pro-region complex (2 nM) or BMP-4 (0.1 nM,) alone or in the presence of indicated fold excess molar concentration of soluble ALK-1:Fc or BMP-RIA:Fc proteins. SEAP activity in the conditioned media was measured after a 48-hour incubation period. All assays used chemiluminescent detection methods, and data were presented in relative light units (RLU). Experiments were performed with duplicate or triplicate wells, and average  $\pm$  SD for each treatment is shown.

B



(Figure 8 continued)

I would like to acknowledge Kent Baker for technical advice on all things biochemical, Wei Zhou for showing me how to troubleshoot HPLC, Senyon Choe for lab space and financial support, and the collaborators at Human Genome Sciences, Incorporated: Qinghai Zhao, Chethana Naik, Cecil Chen, Laurie Pukac, Mallika Singh, Tatiana Tsareva, Yanick Parice, Angela Mahoney, Viktor Roschke, and Indra Sanyal for supplying BMP-9 protein SPR, and cell culture data. This data was published in part, in Brown M. A., Zhao Q., Baker K., Naik C., Chen C., Pukac L., Singh M., Tsareva T., Parice Y., Mahoney A., Roschke V., Sanyal I., Choe S. (2005). "Crystal structure of BMP-9 and functional interactions with pro-region and receptors." *J Biol Chem* **280**(26): 25111-8. The author of this dissertation was the primary researcher of this work.

### Chapter 3: Point Mutations in *Drosophila* BMPs

#### Abstract

This section details the initial stages of a project to determine the extent of conservation of important binding determinants across species and to utilize these mutants to establish the minimum components of the signaling complex and whether a specific topography was required. The basis for this project is the fundamental lack of knowledge about what constitutes a functional signaling complex for BMP ligands and receptors. Point mutations in certain residues significantly affected either type I or type II receptor binding for BMP-2, and it was observed that these residues were often conserved in *dpp* and other fly BMPs. The hypothesis is that at least some of these residues may also affect binding of Dpp or Scw to type I or type II receptors, and that this could be exploited to study the conservation of binding motifs, and the stoichiometry and minimal components of a functional signaling complex. The first objective is to make the selected mutations and test them in *Drosophila* Schneider-2 (S2) cells. These cells are appealing for this type of study because they endogenously express all of the receptor and downstream components required for phosphorylation of Mothers Against DPP (Mad) by Dpp. A variety of other studies have utilized them for this purpose (Marques, Musacchio et al. 1997; Ross, Shimmi et al. 2001; Shimmi, Umulis et al. 2005). It was found that some of the chosen mutations expressed well. The next step is to add these proteins to S2 cells and examine their ability to induce the phosphorylation of Mad, which would give some indication of the conservation of binding determinates across species. Once mutants that affected either type I or type II binding were found, various combinations of heterodimers could be produced to reduce or eliminate binding of receptors in the signaling complex, indicating whether the model of one ligand and two

type I and two type II receptors is required for full signaling capabilities, or if a partial complex can be as effective. Ultimately, these proteins would be expressed in flies and their signaling ability would be tested in an intact system.

## Methods

Constructs - Expression constructs for pRMHA-dpp-HA and mad-FLAG were obtained from Michael O'Connor. Their construction and use has been described elsewhere (Marques, Musacchio et al. 1997; Shimmi, Umulis et al. 2005)

Selection and generation of point mutants - Point mutations in DPP were generated using the Quikchange Site-directed Mutagenesis kit (Stratagene) according to manufacturer's instructions. Briefly, primers were designed such that they matched the dpp sequence except for the desired mutation. PCR was used to amplify the desired construct according to manufacturer's instructions, and the original vector was digested with DPNI, a restriction enzyme that cuts methylated DNA. The remaining PCR product was transformed into XL-Blue or XL-Gold competent cells. Correct transformants were selected based on ampicillin resistance and blue/white screening. Primers were designed using the Stratagene online tool.

Primer sequences are as follows, denoted by the residue in BMP-2 that was mutated. Bases that are changed from the *dpp* sequence in order to produce the mutations underlined.

W31C FORWARD	GCTGGGACGACTG <u>C</u> ATTGTGGCGCCTC
W31C REVERSE	GAGGCGCCACAAT <u>G</u> CAGTCGTCCCAGC
A34D FORWARD	CGACTGGATTGTGG <u>A</u> TCCTCTGGGCTACG

A34D REVERSE CGTAGCCCAGAGGATCCACAATCCAGTCG  
 F49A FORWARD CGGGAAGTGCCCCGCCCCGCTGGCCGAC  
 F49A REVERSE GTCGGCCAGCGGGGCGGGGCACTTCCCG  
 P50A FORWARD GTGCCCTTCGCCCTGGCCGACC  
 P50A REVERSE GGTCCGCCAGGGCGAAGGGGCAC  
 A52R FORWARD GCCCCTCCCGCTGCGCGACCACTTCAACT  
 A52R REVERSE AGTTGAAGTGGTCGCGCGAGCGGGAAGGGGC  
 D53A FORWARD CTTCCCGCTGGCCGCCCACTTCAACTCGAC  
 D53A REVERSE GTCGAGTTGAAGTGGGCGGCCAGCGGGAAG  
 H54E FORWARD CTTCCCGCTGGCCGACGAGTTCAACTCGACCAATC  
 H54E REVERSE GATTGGTCGAGTTGAACTCGTCGGCCAGCGGGAAG  
 L90A FORWARD GGACAGCGTGGCCATGGCCTATCTCAACGACCAA  
 L90A REVERSE TTGGTCGTTGAGATAGCCATGGCCACGCTGTCC  
 L100A FORWARD CGACCAAAGTACGGTGGTGGCCAAGAACTACCAGGAGATGA  
 L100A REVERSE TCATCTCCTGGTAGTTCTTGGCCACCACCGTACTTTGGTCC

Transfection of S2 cells - S2 cells were obtained from Drosophila Genomics Resource Center. Cells were grown in 25 cm<sup>3</sup> flasks in Schnieder's Drosophila media (Gibco) with 5% FBS (Invitrogen) and split into 6 well plates 1 day prior to transfection. Transfection was performed with Effectene (Qiagen) according to the manufacturer's instructions. Media was harvested 48 hours post transfection and cleared by centrifugation at 3-5 minutes at 1000 relative centrifugal force (RCF).

Immunodetection of Dpp proteins - Samples were run on 12% NuPage SDS gels (Invitrogen) at 180 mV and transferred to PVDF membranes at 34 mV for 1-3 hours (Millipore). The membranes were blocked with 1X Phosphate Buffered Saline, pH 7.5



with 3% skim milk. The membranes were immunoblotted with high-affinity rat primary antibody (Roche) at 1:500 and anti-rat secondary antibody at 1:10,000. Membranes were developed using West Pico chemiluminescence substrate (Pierce) according to manufacturer's instructions.

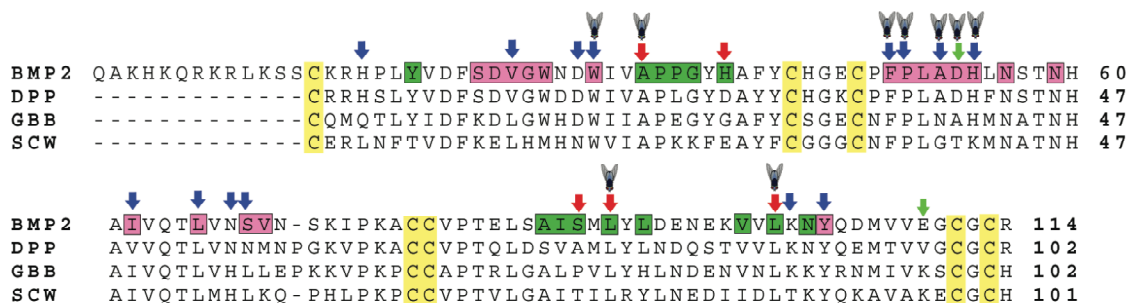
Activity assay - S2 cells were split into 6 well plates 1 day prior to transfection. On the day of transfection, cells at 40-80% confluence were transfected with *mad*-FLAG using Effectene transfection reagent (Qiagen) according to manufacturer's instructions. 48 hours later, transfected cells were pooled and split 1:5. 750  $\mu$ l of cleared media from each *dpp* or mutant *dpp* transfection was added to each well of transfected *mad*-FLAG cells. 3 hours later, cells were harvested by 3-5 minutes of centrifugation at 1000 RCF, lysed with 1X TBS with 0.5% Tween-20 (TBST) and 1mM ethylenediaminetetraacetic acid (EDTA) and two freeze-thaw cycles. Cells were spun at 15,000XG for 10 minutes, and the supernatant was used for Western blot analysis as above. Membranes were probed with anti-phosphorylated Mad at 1:1000 (courtesy of P. ten Dijke) and anti-Rabbit at 1:10,000 or anti-FLAG M2 (Roche) and anti-mouse at 1:10,000.

## Results

A variety of human BMPs, receptors, and accessory proteins have been crystallized and their structures have been solved, including some BMPs in complex with receptors or other binding partners, including inhibitory proteins (Scheufler, Sebald et al. 1999; Kirsch, Sebald et al. 2000; Nickel, Dreyer et al. 2001; Groppe, Greenwald et al. 2002; Greenwald, Groppe et al. 2003; Schreuder, Liesum et al. 2005; Thompson, Lerch et al. 2005; Allendorph, Vale et al. 2006; Mace, Cutfield et al. 2006; Allendorph, Isaacs et al. 2007). These data have both provided specific details about how these proteins

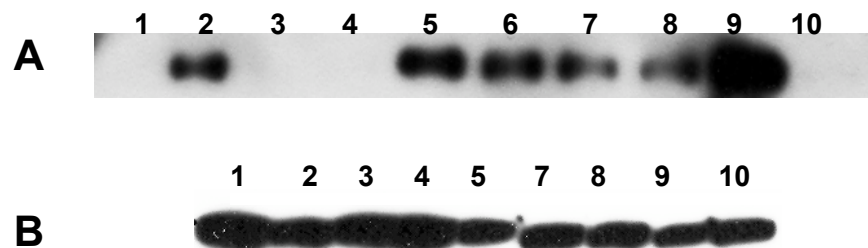
physically interact and directed further molecular experiments aimed at disrupting this interaction.

A series of point mutations was made in human BMP-2, to which *Drosophila dpp* is closely related. Several of these mutations significantly reduced or eliminated downstream signaling in a cell-based assay, while a few mutations caused increased levels of signaling. Of those that reduced or eliminated signaling, most appeared to do so by specifically disrupting binding to either the type I or the type II receptor by means of a single point mutation in the dimeric ligand (Kirsch, Nickel et al. 2000). Upon examination of sequence similarities with fly BMPs, it was apparent that many of the residues that showed strong effects in the experiments with BMP-2 were conserved in flies (Figure 9).



**Figure 9** Alignment of BMP-2 and Drosophila BMPs. Conserved cysteines are highlighted in yellow. Residues in BMP-2 that are within 4.0 Å of the type I receptor (as indicated from the structural data of BMP-2 in complex with BMPRIA (Kirsch, Sebald et al. 2000)) are shaded in pink. Residues that correspond to the residues that are within 4.0 Å of the type II receptor (as indicated from the structural data of BMP-7 in complex with ActRIIb (Greenwald, Groppe et al. 2003)) are shaded in green. Residues that caused a decrease in type I binding and signaling efficacy are marked with blue arrows. Residues that caused a decrease in type II binding and signaling efficacy are marked with red arrows. Residues that caused an increase in signaling efficacy are marked with green arrows (Kirsch, Nickel et al. 2000). Residues likely to affect receptor binding that were selected for mutation in *dpp* are marked with flies. It is expected that the mutations would affect the same receptor type as in BMP-2. Alignment was performed by ClustalW.

These observations led to the hypothesis that these residues might also be important for binding between fly BMPs and their receptors and that if the same mutations were made in *dpp*, they would have similar effects. A further prediction is that, of the mutated residues, those are also conserved in other fly BMPs (Figure 9), would not be likely to determine to which receptors the ligand will have the highest affinity, while the ones that differ are more likely to influence specificity.



**Figure 10** Expression of *dpp* variants and *mad*-FLAG. A) Dpp-HA variants taken from the media of transfected S2 cells. Lanes are: 1) Untransfected, 2) Dpp, 3) Dpp W31C, 4) Dpp A34D, 5) Dpp F49A, 6) Dpp P50A, 7) Dpp A52R, 8) Dpp D53A, 9) Dpp H54E, 10) Dpp L90A. B) Mad-FLAG expression in S2 cells. Cells were transfected with *mad*-FLAG and split into wells after 48 hours. Media from the transfections in A was added to each well, and 3 hours later the cells were lysed. Numbering as in A, with P50A omitted.

The mutations chosen for *dpp* were W31C, A34D, F49A, P50A, A52R, D53A, H54E, and L90A. L100A was attempted but proved exceptionally difficult to clone. Each of these point mutations was made with PCR using overlapping primers that contained the desired mutation. Each construct was sequenced to verify the presence of the intended mutation. S2 cells were transfected, induced with copper sulfate, and media was harvested 48 hours later. The mutations W31C, A34D, and L90A did not express as well as the other mutations.

Dpp-HA binds well to a HA-affinity column, but unfortunately does not elute, making clean purification and precise quantification impossible in the limited time frame

available for this project. The next steps would be inserting another tag and using an affinity column to purify the protein. As there is already an interrupted myc-tag in this construct, the easiest method would be using PCR to mutate or insert the necessary amino acids to complete the tag. Also, HPLC purification would also be an extremely attractive method for purification, but the lab does not have the necessary resources. Owing to these limitations, the cleared media was added directly to cells transfected with Mad-Flag. After 3 hours, the cells were harvested and lysed. Western blot analysis, probing with either anti-FLAG or anti-phospho-Mad, was used to determine the extent of phosphorylated Mad relative to baseline. It was found that Mad-Flag was expressed well, but it is not clear at this time to what extent Mad is activated by these methods. Further experiments are in progress to establish this information.

I would like to acknowledge Margery Smelkinson for solid technical advice on Western blotting and much-appreciated enthusiasm for this project, Par Towb for technical advice on S2 cells and transfection, and Peter ten-Djike for supplying the anti-phosphorylated Mad antibody.

## Chapter 4: Concluding Remarks

In the BMP signaling family, which consists of many more ligands than receptors, ligands and receptors often have overlapping specificities. Though the overall sequence similarity between ligands is comparatively low, their common structural scaffold and the common scaffold of their receptors (Greenwald, Vega et al. 2004) allows ligands to signal across various combinations of receptors. This system allows for some functional redundancy, which can be quite beneficial to the organism by enabling compensation. However, the disparate functions of BMP signaling necessitate extremely tight control over signaling, which in turn mandates specificity. Neither crystallography nor molecular biology can independently determine which features of the ligand or receptor control specificity, but the combination has proven extremely powerful.

Crystallization of BMP-2 in complex with BMPR-IA helped to identify the points of contact between a BMP ligand and type I receptor, and further studies identified the hydrogen bonds that determined binding affinity (Keller, Nickel et al. 2004). In BMP-2, these are the amide and carbonyl of L51 and the amide of D53, and these residues are conserved in BMP-9. BMP-9, however, shows no affinity for BMPR-IA in cell-based or Surface Plasmon Resonance (SPR) studies (see Chapter III). Significant C $\alpha$  RMSDs do occur between these residues, however, suggesting that main chain position in the pre-helix loop may be an important determinant of binding affinity for type I receptors. It was determined from the crystal structure of BMP-9 that there are few significant conformational differences between BMP-9 and BMP-7 at the binding sites for the type II receptors. These results are consistent with known promiscuity among type II receptors and SPR data indicating some binding affinity between BMP-9 and ActR-IIA.

The pro-regions of TGF- $\beta$ s and GDF-8 remain associated with their ligands after secretion from the cell and have been found to be functionally inhibitory *in vitro* and *in vivo* (Lee and McPherron 2001; Thies, Chen et al. 2001; Yang, Ratovitski et al. 2001; Jiang, Liang et al. 2004). The mechanism for this inhibition is not known, though particular regions of the pro-region relevant for inhibition have been identified (Jiang, Liang et al. 2004). The pro-region of BMP-9 also remains associated after secretion, but we have shown that both BMP-9 and BMP-9/pro-region complex were equally active in three cell-based assays covering a range of reported BMP-9 activities, including osteoinduction, proliferation and gluconeogenesis (Chen, Grzegorzewski et al. 2003; Li, Li et al. 2003; Kang, Sun et al. 2004). These assays demonstrated that the pro-region of BMP-9, unlike those of TGF- $\beta$ s and GDF-8, does not appear to functionally inhibit BMP-9. It is not clear whether the BMP-9 pro-region does not block the binding sites for either receptor subtype, or whether the pro-region is effectively competed off by one or both of the receptor subtypes.

It is possible that the pro-region of BMP-9 may act to protect and stabilize BMP-9 *in vivo*. Additional studies have shown other regulatory functions for the prodomain, including its requirement for proper folding for BMP-4, and exit from the endoplasmic reticulum (Sopory, Nelsen et al. 2006). For BMP-7, the prodomain also remained associated after secretion, but served to direct the ligand to the extracellular matrix (Gregory, Ono et al. 2005). Work in zebrafish showed that the prodomain of BMP-7 was critical for secretion or stability of the secreted ligand (Dick, Hild et al. 2000). It seems that the proregion may have an array of functions that are only poorly understood at this time. It would be particularly interesting to see if the continued association of the proregion of BMP-9 has biological effects on secretion, stabilization, or targeting of the ligand. The extremely strong affinity of the complex and its ability to reform after

separation strongly tempt one to speculate that the complex does have a biological function.

BMP-9 shows a tissue expression profile largely restricted to the liver and has been shown to stimulate proliferation in hepatic cells (Song, Celeste et al. 1995), but its receptor had not been identified. ALK-1 is highly expressed in endothelial cells, and inactivating mutations of ALK-1 have been associated with hereditary haemorrhagic telangiectasia (Johnson, Berg et al. 1996), an uncommon vascular pathology affecting multiple organs. Transfection of constitutively active forms of ALK-1 has been shown to stimulate Smad-1 (Chen and Massague 1999) and induce alkaline phosphatase expression in C2C12 cells (Fujii, Takeda et al. 1999). However, a functional ligand for ALK1 receptor had not been clearly identified. This work provided the first evidence that ALK-1 is a strong candidate for a functional receptor for BMP-9. In cell-based assays employing two distinct cell types, a soluble ALK-1 protein was able to potently and selectively block BMP-9/pro-region complex activity. This result has been independently confirmed in endothelial cells (David, Mallet et al. 2007; Scharpfenecker, van Dinther et al. 2007), and BMP-9 was also shown to inhibit angiogenesis, which could indicate significance in the development of HHT (David, Mallet et al. 2008). SPR analysis indicates that BMP-9/pro-region complex binds to additional receptors to some degree, including BMPR-II, ActR-IIA and ActR-IIB. Of these, only BMPR-II inhibited BMP-9/pro-region complex activity to any significant extent. The exact nature of the interactions of BMP-9 with ALK-1 and other receptors in physiological functions and potential roles in diseases remain to be understood.

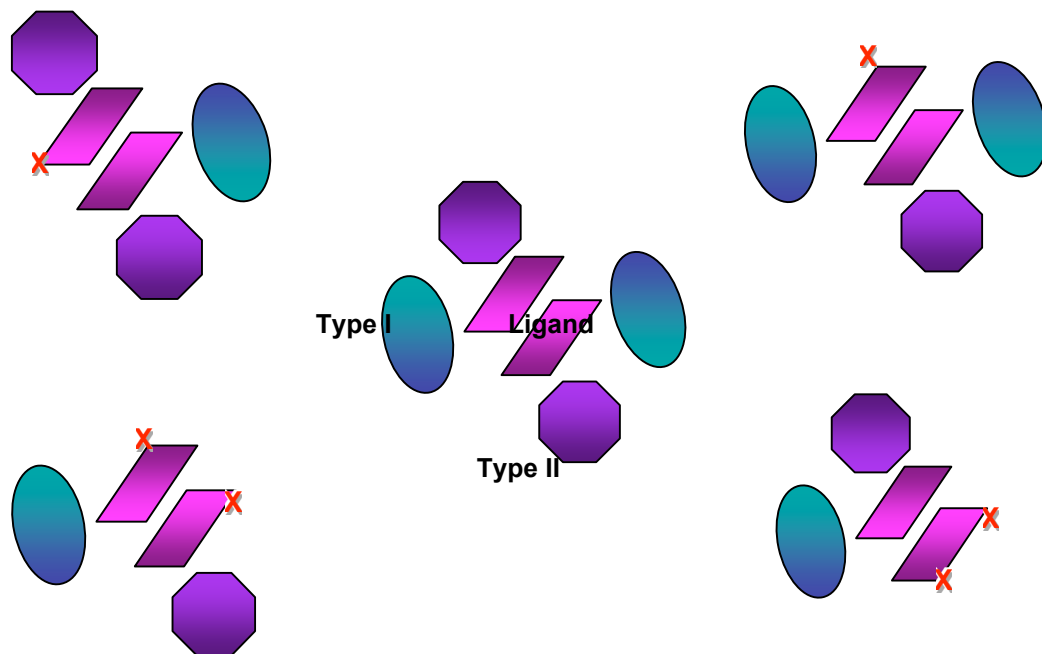
This work solved the structure of BMP-9, and showed that the pro-region of BMP-9 remains associated with the mature ligand, but lacks inhibitory effects. It has also shown, via collaboration, that ALK-1 is a likely candidate for a receptor for BMP-9,

data which have since been verified by independent publications (David, Mallet et al. 2007; Scharpfenecker, van Dinther et al. 2007).

The later work on point mutations in *Drosophila* BMPs, while still in infancy stages, hopefully sets the stage for examining the details of structural similarity between ligands across species. Given time, this approach could be used in conjunction with the many benefits of invertebrate genetics to settle much more fundamental questions in BMP signaling: what are the minimum components of the signaling complex, which receptors affect each other inside the membrane, and what is the molecular basis for improved heterodimer signaling efficacy and improved heterodimer binding to Sog?

Ideally, the mutations of *dpp* described above could be tested for affinity to type I (*thickveins* would be an appropriate choice) and type II receptors (*punt*) via SPR, as described in Chapter III for BMP-9. Assuming that decreases in activity correlated with decreased affinity for either one receptor type or the other, but not both, mutations could be selected for cotransfection into S2 cells. Using Flag-tagged *dpp* variant 1 and myc-tagged *dpp* variant 2, purification of the heterodimers could readily occur. Indeed, a similar purification has also been performed with *scw/dpp* heterodimers (Shimmi, Umulis et al. 2005). Examples of potential mutational combinations are shown in Figure 10.

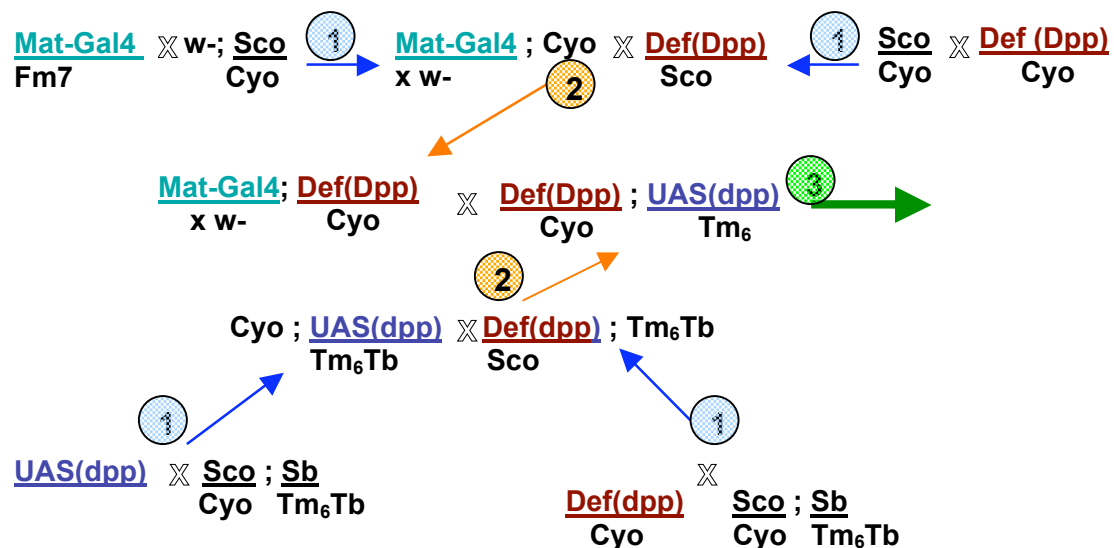




**Figure 11** Diagram of possible mutational combinations. Dimeric ligands are shown as pink trapezoids, generic type I receptors are shown as blue ovals, and generic type II receptors are shown as purple octagons. Center: simplified top-down view of complete signaling complex.

At this stage, the purified *dpp* heterodimers could be added to a phosphorylated-Mad assay to evaluate activity. Based on the combination in question, basic questions about the signaling complex could be addressed. Initially, one could ascertain if any of these heterodimeric combinations induced signaling. If not, the simplest conclusion is that the complex requires all components to affect downstream targets, a question that remains unanswered. If any of the complexes were functional, however, one could determine if signaling occurred in *cis* or *trans* across the complex. For example, if the lower right complex in Figure 10 is functional but the lower left is not, it would indicate that signaling can occur in *cis* between a type I and type II receptor, but not in *trans*.

The next stage of examination of these proteins is expression in flies. To this end, transformation of flies using a GAL-4/UAS expression system would be utilized. As the lab has *dpp* deficient flies, an appropriate cross to test the ability of the *dpp* transgene on the second chromosome to rescue a *dpp*-null phenotype is represented in Figure 11. The driver, Maternal-GAL4, is expressed by the mother, and thereby likely to be early enough to rescue the embryonic lethality of *dpp*. The possible caveat to this cross is that there is a potential for position effects, where the location of insertion of the transgene may substantially affect its expression level. Creating multiple lines would minimize the risk of mistaking position effects for signaling efficacy.



**Figure 12** Example of cross to rescue a homozygous lethal *dpp* deficiency located on the second chromosome. First round crosses are indicated by the number 1, and second round crosses by the number 2. The necessary driver is Maternal-GAL4, as the *dpp* deficiency is embryonic lethal. The progeny resulting from this scheme will have the UAS(*dpp*) driven by Maternal GAL4 as the only functioning copy of *dpp*. Abbreviations are: def: deficiency; Tb: Tubby; Sco: Scotoid; Sb: Stubble; Cyo: Curly; Fm7 is a balancer for the X chromosome; Tm<sub>6</sub> is a chromosomal inversion.

I would like to see an further phase of this project be the induction of point mutations into *scw*, the testing of these mutations in assays as described in Chapter IV,

and the selection of the most potent mutations for further analysis. Heterodimers of *scw* and *dpp* have been purified from transfected S2 cells and from *Drosophila* embryos (Shimmi, Umulis et al. 2005). As with the mutations of *dpp*, *scw* mutations could be tested for affinity to type I (*saxophone* for *scw*) and type II receptors (*punt*) via SPR.

These proposals are representative of the kind of integrated approach to neuroscience I would like to continue to pursue. I believe a tremendous and relatively untapped opportunity exists for structural biology and neuroscience to benefit from each other. Just as it is impossible to know to the same level of precision and certainty which components determine the binding of two proteins without crystallography, it is equally valuable for crystallography to continue to expand into the functional realm. As crystallography becomes more automated, and robots set up trays, load crystals onto synchrotrons, and collect data, it seems clear that structure for its own sake is a limited prospect, and studies that are directed at solving structures and then using that data to design further experiments to decipher function are much more likely to succeed.

Similarly, rapid gains have been made in the study of channels and other proteins by means of their crystallization, in some cases showing that years of inference from molecular studies had been misleading. As these studies have shown that vastly different channels can share similar modes of assembly, the crystallization of a few proteins can guide further studies in a much wider realm (Tsuruda, Julius et al. 2006).

Structural biology lends the clearest view of how proteins interact with one another. Though it has its share of woefully misguided conclusions, it is prone to less interpretation than many other alternatives, and often a single structure can settle extended debates on what determines the function of a protein or its interaction with other proteins (Van Petegem, Chatelain et al. 2005). As the complexity of BMP signaling becomes further elucidated, it seems that solving individual and co-crystal

structures is an important step in determining the mechanism by which heterodimers signal more effectively than homodimers, and Sog binds heterodimers with higher affinity than homodimers. Crystallography is limited in that it cannot independently determine the aspects of the protein that dictate function (just because a residue is conserved, is located in a binding region, and appears to make the right connections does not mean it is significant), but it can direct the researcher towards the most likely candidates.

Figure 9 shows how predictive structural models can be. Of the 20 residues mutated and successfully expressed throughout BMP-2, only one was outside the postulated binding regions based on crystallography. With small proteins, this is less significant, but with larger proteins this means of filtering could mean a substantial savings in time. After establishing the regions most likely to determine binding, further studies, like site-direct mutagenesis and functional studies, are required to tease out the critical residues. Then the researcher can try to predict other interactions of related proteins.

The benefits of structural biology for medicine and drug design are apparent, but the rewards for neuroscience seem to be in their early stages. The very detailed molecular interactions that drive specificity, affinity, and rates of association and dissociation of proteins can determine a tremendous number of cell fate decisions, including whether and to what to differentiate, whether to make or keep a synapse, and whether to live or die. Often these details are overlooked, despite the fact that mild perturbations in any of these factors can result in dramatic effects on the organism in question. Point mutations cause debilitating diseases by changing the affinity of a ligand for its receptor, and the possibility exists for creating small molecules that will bind receptors or ligands with high specificity, treating ailments and causing minimal side effects. It is my personal hope that the structural biology and neuroscience will see

more overlap, and that both fields will benefit enormously.

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