CHARACTERIZING THE ROLES OF *NEMO* IN TGFβ SIGNALLING

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the

Department of Molecular Biology and Biochemistry

SIMON FRASER UNIVERSITY

November 2004

Approval

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Degree: **Master of Science**

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Date Defended/Approved: November 22nd, 2004

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Abstract

Drosophila nemo encodes a serine threonine MAP kinase that is involved in patterning and cell fate determination. *nemo* participates in crosstalk with several pathways; with studies linking the vertebrate homologue, *nlk* to the TGFβ pathway. TGFβs are structurally related extracellular polypeptides including the Bone Morphogenetic Proteins (BMPs) that are potent regulators of development.

In *Drosophila* the BMP molecules Decapentaplegic (Dpp) and Glass bottom boat (Gbb) promote vein formation while Nemo promotes intervein fates. Genetic studies revealed that Nemo counteracts the effects of components of the BMP pathway; and *nemo* mutant pupal wings show high levels of BMP signalling activity in ectopic veins, supporting an inhibitory role for Nemo on BMP activity.

These studies show that *nemo* may act as a negative regulator of TGFβ signalling; and supports the emerging roles of *nemo* as an important regulator of signalling in different pathways.

Dedication

This work is dedicated to my late father, Winfred Sosu-Sedzorme, who gave me a lot of moral support and encouragement in pursuit of my academic ambitions.

Acknowledgements

I received a lot of support and encouragement from many people without whom this work could not have been completed. Firstly, I express my sincere gratitude to Dr. Esther M. Verheyen, my Senior Supervisor, who gave me the opportunity to study in her lab at Simon Fraser University and guided me in every way to organize my research and studies. I also appreciate her immense support in times of difficulty. Furthermore, I am indebted to members of my Supervisory Committee, Drs Nicholas Harden and Nancy Hawkins of Simon Fraser University. Their contributions and advice helped structure my project at crucial times. I also want to thank Dr. Lynne Quarmby for being the internal examiner during the final oral examination of this work.

My appreciation goes to members of the Verheyen lab, past and present, for their assistance and friendship throughout my studies. I am grateful to Miss Arial Yi Zeng, Mr. Darrell C. Bessette and Miss Wendy Lee (graduate students) for teaching me various techniques in molecular biology. Miss Desiree M. Essen helped me with a lot of genetic crosses and donated a computer for the composition of my thesis; I am also grateful to her for a wonderful friendship. Moreover, I am enormously thankful to Maryam Rahmaje-Chistaz who helped with a number of experiments in molecular biology.

Outside of the Verheyen Lab, Mr. Muneer Esmail (Leroux lab, Simon Fraser University) deserves mention for his wonderful friendship and support in every way. The generosity and friendship I shared with my roommate, Sister Eugenia Amporfu, was irreplaceable, as was the care given to me by my landlady, Ms. Barbara Stewart.

My family provided me with immense support throughout my education. My heartfelt gratitude goes to my mother Madam Vicentia Kpodzi for her sacrifice and financial support towards my entire secondary and university education. I especially wish to thank my fiancée Miss Fafa Addo for her prayers, commitment and sacrifice through the three years that I have been away from home.

Lastly, I would like to thank all who helped me in diverse ways but are not mentioned here.

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Chapter One: Introduction

Multicellular organisms develop from a single cell that differentiates into distinct and specialized tissues. This process requires well organized and coordinated signals between individual cells; a function that is achieved through effective communication between cells. This ensures correct patterning and organization of the embryo into the distinct structures that make up the adult body. Cell-cell communication is also vital in regulated growth, homeostasis and defensive mechanisms in the adult body. Genetic pathways play fundamental roles in development; and interact with each other to ensure that cells receive the appropriate signals at the right time and in the required amounts. The importance of these pathways and their regulation in development is manifest in the several developmental conditions associated with loss or deregulation of their components. This has made the challenge to understand and characterize genetic pathways and their modes of action very crucial in addressing several developmental problems.

The transforming growth factor beta family

Members of the transforming growth factor β (TGFβ) family of growth factors have been shown to play important roles in various biological processes. The family consists of structurally related soluble extracellular polypeptides which have diverse roles in growth and development, homeostasis, and repair of tissues in both vertebrates and invertebrates (Massague, 1998; Raftery and Sutherland, 1999). The importance of this

pathway is demonstrated in the multitude of human disorders that are associated with its dysfunction. Among the effects of TGFβ on target cells is cell cycle arrest at the G1 phase and the loss of this growth inhibitory effect due to defects in TGFβ signalling is one of the possible causes of cancer (Massague, 1998). Abnormal TGFβ activity is also implicated in a number of inflammatory disorders. Excessive TGFβ signalling underlies various disorders of the kidney, lung, liver, and other organs (reviewed in Massague, 1998).

The TGFβ family of growth factors are characterized by the presence of six conserved cysteine residues, and consists of three major subfamilies namely TGFβs, Activins and Bone Morphogenetic Proteins (BMPs). The TGFβ subgroup is involved in early embryonic development and also has roles in late development and in adult tissues. It also functions as a growth inhibitor of most cell types including epithelial cells, endothelial cells, haematopoietic cells and lymphocytes (reviewed in Miyazono, 2000). Activins and BMPs perform vital roles in early embryogenesis and direct patterning of the early embryo. In addition BMPs are further involved in the morphogenesis of most tissues. Signalling by this family of proteins controls the development and homeostasis of almost all cell types, and constitutes a bulk of intercellular signals between cells. The importance of the TGFβ family has led to its characterization in most organisms and it is found to be well conserved in both vertebrates and invertebrates. Table 1 lists some components of the TGFβ pathway in various organisms.

A family of transmembrane protein serine/threonine kinases (Types I and II receptors) transduce the TGFβ signal from the extracellular matrix to the cytoplasm. The receptors activate the Smad family of cytoplasmic proteins which move into the nucleus

to regulate the transcription of target genes. There are three kinds of Smads; receptor regulated Smad (R-Smad) that binds to the receptors and mediate signalling; common mediator Smad (Co-Smad) which takes part in TGFβ signalling by associating with the R-Smads; and inhibitory Smad (I-Smad) that antagonizes ligand-dependent signalling.

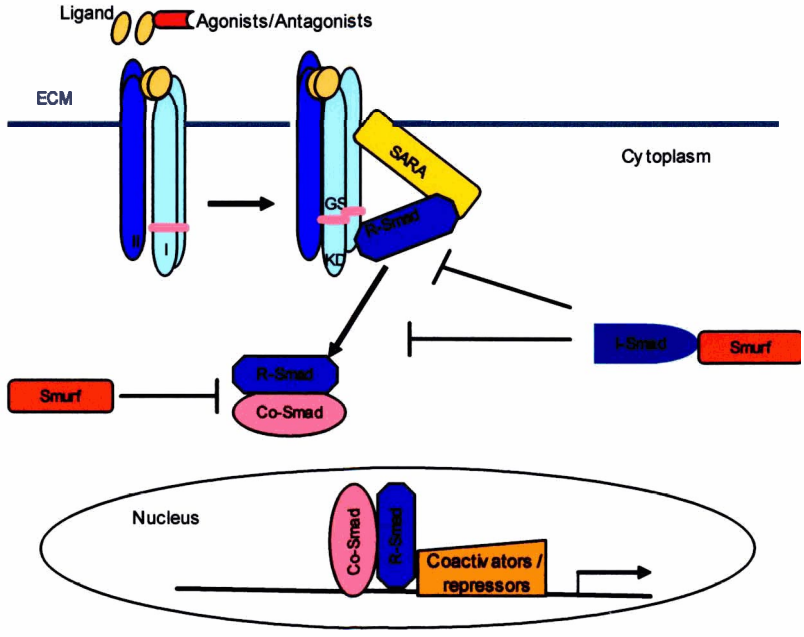
In *Drosophila*, the BMP subclass of TGFβ has been well characterized and includes Glass bottom boat (Gbb), Decapentaplegic (Dpp) and Screw (Scw) (Padgett *et al.*, 1987; Wharton *et al.*, 1991; Arora *et al.*, 1994; Doctor *et al.*, 1992; Khalsa *et al.*, 1998). Dpp has a variety of biological roles including dorsoventral patterning of the embryo, gut formation, as well as outgrowth and patterning of the eye and wing imaginal discs (Sekelsky *et al.*, 1995). Gbb is also involved in larval cuticle patterning, midgut morphogenesis and wing vein patterning (Doctor *et al.*, 1992; Khalsa *et al.*, 1998; Wharton *et al.*, 1999). Dpp and Gbb form heterodimers and act together to regulate development in certain contexts; but also have independent roles, in which case they signal as homodimers. Scw play roles in the patterning of the dorsal epidermis (Ray and Wharton, 2001). These ligands signal through a common Type II receptor called Punt (Put), but utilize different Type I receptors. Dpp and Gbb signals are mediated through Thickveins (Tkv) while Saxophone (Sax) is employed in Scw signalling (Nguyen *et al.*, 1998). Recently another Type II receptor, Wishful thinking (Wit) has been identified and was shown to mediate BMP signalling at neuromuscular junctions (Marques *et al.*, 2002; 2003). The R-Smad Mothers against Dpp (Mad) and the Co-Smad, Medea (Med) mediate signalling downstream of Tkv. Mad and Medea are homologues of vertebrate Smad1 and Smad4 respectively. Among the target genes of Dpp signalling is the inhibitory Smad,

Daughters against Dpp (Dad) which is related to vertebrate Smads 6 and 7 and is involved in a feedback loop to inhibit Dpp signalling (Tsuneizumi *et al.*, 1997).

The Types I and II receptors are glycoproteins that share conserved features, including an N-terminal extracellular region, a transmembrane domain and a C-terminal kinase domain (Shi and Massague, 2003). The major difference between these receptors is the presence of a 30-amino acid region preceding the kinase domain in the Type I receptors. This region consists of a characteristic glycine-serine (TTSGSGSGLP) sequence and is therefore referred to as the GS domain, which contributes to the activation state of the receptors (reviewed in Massague, 1998; Zimmerman and Padgett, 2000). The GS domain exists in a wedge-like conformation within the catalytic domain of the receptor, thus rendering it inactive in the absence of a ligand (Huse *et al.*, 1999). The active TGFβ ligands exist as dimers that are stabilized through hydrophobic interactions and disulphide bonds (Shi and Massague, 2003). In the absence of ligand, the receptors exist as homodimers at the cell surface but the ligand dimers generally recruit them into heterotetrameric complexes (thereby bringing these two receptors in close proximity with each other) (Fig. 1). The formation of these receptor complexes is crucial for signalling by the receptors (reviewed in Massague, 1998; Derynck and Zhang, 2003), as it allows easy access of the Type I receptor to the activated type II receptor. The ligands induce autophosphorylation of the Type II receptor on serine residues in its kinase domain. The activated Type II receptor then phosphorylates the Type I receptor on serine and threonine residues in its GS region, causing the GS region to be dislodged from the catalytic domain. It is important that the Type I receptor remains inactive and only induced through ligand activation of the Type II receptor. There is evidence from yeast

and mammalian cells that indicate that certain proteins bind to the GS domain of the Type I receptor to prevent its phosphorylation in the absence of ligand (Huse *et al.*, 1999).

Figure 1: The TGFβ pathway



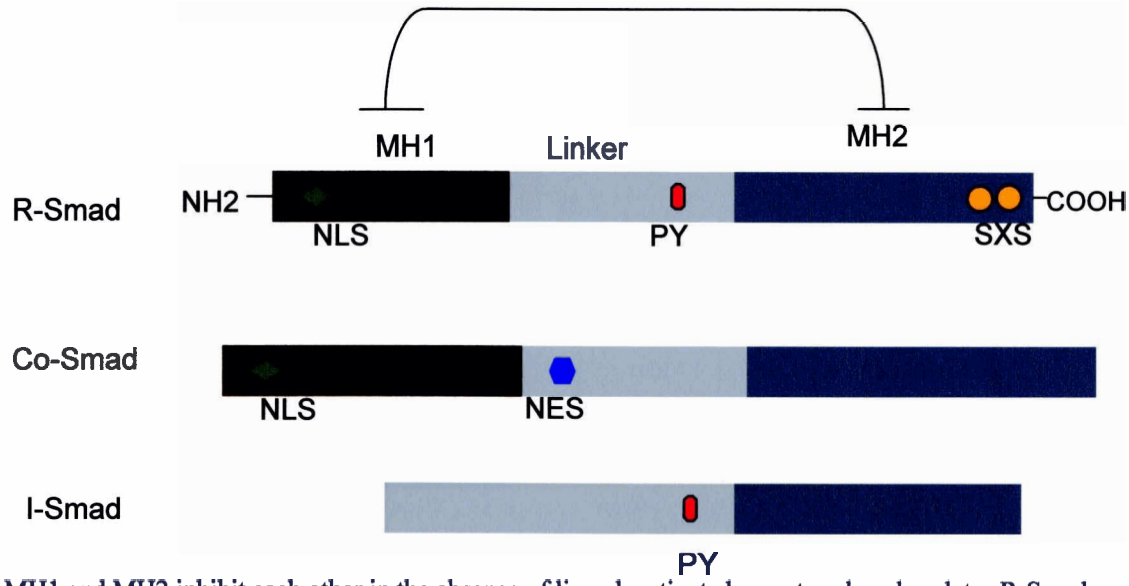
Abbreviations: ECM- Extracellular matrix; GS- GS domain; KD- Kinase domain I- Type I receptor; II- Type II receptor. The TGFβ ligands bind to the receptors as homodimers, and induce receptor hetero-oligomerization and phosphorylation of the Type I receptor in the GS domain by the Type II receptor. SARA presents R-Smad to the activated Type I receptor, and R-Smad is phosphorylated. Phosphorylated R-Smad complexes with Co-Smad and moves into the nucleus to associate with co-activators or repressors to regulate transcription of target genes. I-Smads compete with R-Smads for binding to the receptor, and also block R-Smad/Co-Smad oligomerization. Smurf targets R-Smad/I-Smad for degradation.

An essential component of the pathway is the Smad family of proteins that mediate signalling downstream of the Type I receptors in the cytoplasm. The Smads typically consist of conserved N-terminal Mad homology 1(MH1) and a C-terminal Mad homology 2 (MH2) domains which are separated by a poorly conserved proline-rich

linker region (Christian and Nakayama; 1999) (Fig. 2). Both MH1 and MH2 domains are present in R-Smads and Co-Smads but the I-Smads lack an MH1 domain. The MH2 domain interacts with receptors as well as DNA binding proteins and transcription factors. This domain contains a number of serine residues (SSXS) located at its C terminal end, the distal two of which are necessary for activation by the receptors. The MH1 domain is involved in DNA binding. In the inactivated state, the MH1 and MH2 domains interact and this interaction leads to inhibition of MH2 transcriptional and biological activity. The MH1 domain therefore inhibits MH2 activation in the absence of signalling; hence providing a level of regulation of signalling. The linker region is important in homo-oligomerization of the Smads, and also contains MAPK phosphorylation sites that provide a potential point of crosstalk with other pathways (reviewed in Massague, 1998).

The R-Smad is recruited to the activated Type I receptor and is phosphorylated on the two distal C-terminal serines SXS. Once activated, R-Smad associates with the Co-Smad. This R-Smad/Co-Smad complex then translocates to the nucleus to regulate the transcription of target genes. The pathway specific Smads (R-Smad and Co-Smad) exist as monomers but are induced to form homo-oligomers and hetero-oligomers in response to receptor activation (Kawabata *et al.*, 1998). Smad oligomerization is mediated through the MH2 domain. The subcellular localization of Smads is controlled to regulate signalling. There is a nuclear localization signal (NLS) made up of a Lys-Lys-Leu-Lys sequence located at the N-terminal region of the Smads that ensure their nuclear import. On the other hand, a Leucine-rich nuclear export signal (NES) in the vertebrate Co-Smad, Smad4 keeps it in the cytoplasm in unstimulated cells (Watanabe *et al.*, 2000).

Figure 2: Schematic diagram showing the structure and domains of Smads



MH1 and MH2 inhibit each other in the absence of ligand; activated receptor phosphorylates R-Smad on the C-terminal distal serine residues. MH1 domain is important for binding to DNA, and is missing in I-Smads which also have a longer linker region. Also shown are various motifs that play essential roles in Smad signalling and interaction. NLS in both R- and Co-Smads enhance their nuclear import, while NES induce export of Co-Smad from the nucleus. The PY motifs in the linker region of R-Smads and I-Smads are important for interacting with the WW motif of Smurf; and could be targeted by other proteins

Ligand-induced complex formation between R- and Co-Smad is thought to be necessary to relieve their nuclear export (reviewed in Itoh *et al.,* 2000). The mechanisms regulating Smad localization are not well understood; however emerging evidence indicate that cytoplasmic and nuclear retention factors may be involved. These factors are presumably selective in interacting with Smads, with cytoplasmic factors binding preferentially to monomeric Smads, while nuclear factors likely bind to complexed Smads (reviewed in ten Dijke and Hill, 2004). For example, the nuclear export protein, chromosome region maintenance 1 (CRM1) was shown to bind to the NES region of Smad4 (Pierreux *et al.*, 2000; Watanabe *et al.*, 2000), while importin-α interacts with the NLS of the same Smad to induce its nuclear import (Pierreux *et al.,* 2000; Watanabe *et al.*, 2000; Xiao *et al.*,

2003). Interaction with the activated receptor is therefore critical in determining the localization of Smads, as it will presumably induce their release from cytoplasmic retention factors and promote their association with nuclear import factors. Hence in the absence of ligand, both R and Co-Smads remain chiefly in the cytoplasm, and move into the nucleus only after phosphorylation of the R-Smads by the activated receptor.

Smads are shown to be presented to the receptor by an anchor protein, Smad anchor for receptor activation (SARA). SARA contains a central Fab1p/YOTP/Vac1p/EEA1 (FYVE) domain, consisting of two zinc-finger motifs. FYVE domains are present in several proteins that mediate endocytic vesicular traffic; and are known to bind phosphatidylinositol-3-phosphate to tether proteins at endosomal membranes (Wurmser *et al.,* 1999). SARA resides at the cell membrane through the lipid-binding FYVE domain; and interacts with Smads through its Smad-binding domain. It also contains a C-terminal domain which binds the Type I receptor. In this way, SARA promotes effective recruitment of R-Smads to the receptor (Fig. 1). The R-Smad-SARA complex is disrupted upon phosphorylation of the R-Smads, thus allowing the Smads to move into the nucleus (Tsukazaki *et al.,* 1998).

Table 1: TGFβ signalling components and homologues in various organisms

	Ligand	Type II Receptor	Type I Receptor	R-Smad	Co-Smad	I-Smad
Vertebrates	TGFβ1, 2, 3	TβR-II	TβR-I	Smad2, Smad3	Smad4 or Smad4β[a]	Smad7
	Activinβ A; B; C; D	ActR-II	ActR-IA			
	BMP 2; 3; 4; 5; 6; 7; 8	BMPR-II	BMPR-IA; IB	Smad1; 5; 8		Smad6; 7
Drosophila	dActivin	Punt	Baboon	Mad (Smad1)	Medea (Smad4)	?
	Dpp; Gbb	Punt Wit[b]	Tkv			Dad
	Scw		Sax			?
C. elegans	DAF-7 (BMP)	DAF-4	DAF-1	DAF-8[c]	DAF-14[c]	?
	DBL-1 (BMP)		SMA-6	SMA-2; 3	SMA-4	?

Abbreviations: ACTR, Activin-like receptor; BMPR, BMP receptor; DAF, Dauer formation; DBL, Dpp, BMP-like; TβR- TGFβ receptor; Wit, Wishful thinking; SMA- Small body size. [a] The Smad4β is found only in *Xenopus*; [b] found in Drosophila neuromuscular junction; [c]DAF-8 and DAF-14 are diverged from the R-Smads in their MH1 domain.

Control of gene expression by Smads

Once in the nucleus the R-Smad/Co-Smad complex interacts with co-activators or co-repressors that determine their transcriptional activity. This final step in TGFβ signalling is important in determining which genes are turned on in response to different TGFβ signals and in varying contexts. Irrespective of the TGFβ ligand that activates them, R- and Co-Smads recognize and bind with low affinity to the sequence CAGAC (also referred to as Smad binding element (SBE)) on DNA (Shi *et al.,* 1998) and thus require DNA-binding co-factors to allow efficient regulation of specific target genes (Massague and Chen, 2000). The cell-specific expression of these co-factors determines which cells express TGFβ-responsive genes both spatially and temporally. As such, these co-factors are selective in the kinds of TGFβ target genes they activate, through differential association with individual Smads, leading to pathway specific activation of various TGFβ target genes (Table 2). For example, the winged-helix/forkhead family member forkhead activin signal transducer-1 (FAST-1) interacts with Smad2 and Smad4 to mediate the expression of the *Mix.2* gene in *Xenopus*, in response to activin-like signals. FAST-1 does not associate with other Smads and hence does not promote BMP signalling for example. On the other hand, the 30 zinc finger nuclear protein Olf-1/EBF associated zinc factor (OAZ), associates with the Smad1-Smad4 complex in *Xenopus* to promote the expression of BMP-induced *Xvent2*. OAZ is limited to the BMP pathway and mediates BMP target gene expression only. In *Drosophila*, the homeobox transcription factor *tinman* cooperates with Mad and Medea to regulate its own transcription in response to BMP signalling (Massague and Wotton 2000; Zimmerman and Padgett, 2000).

Table 2: Various Smad transcriptional regulators and their effects on signalling

Organism	Ligand type	Interacting Smad	Regulator	Target gene	Effect
Mammals	TGFβ	Smad4	MSG1	?	activation
	TGFβ, BMP	Smad1,2,3,4	CBP/p300	?	activation
	TGFβ, Activin	Smad3,4	c-Jun/c-Fos	*c-fos*	activation
	BMP	Smad1	Hoxc-8	*osteopontin*	repression
	TGFβ	Smad2,3,4	Ski/SnoN	?	repression
	TGFβ	Smad2,3	SIP1	?	repression
	TGFβ	Smad2,3	SNIP1	?	repression
	TGFβ	Smad2,3	TGIF	?	repression
Xenopus	Activin	Smad2,3,4	FAST-1	*mix.2, nodal, lefty*	activation
	TGFβ, Activin	Smad2,4	FAST-2	*goosecoid*	activation
	TGFβ, Activin	Smad2	Mixer	*goosecoid*	activation
	BMP		OAZ	*Xvent-2*	activation
Drosophila	BMP	Mad/Medea	Tinman	*tinman*	coactivator
	BMP	Mad?	Brinker	*sal*	repression
	BMP	Mad/Medea	CREB?	*ubx*	coactivator

Abbreviations: Act, Activin; FAST, forkhead activin signal transducer; MSG, melanocyte specific gene; *sal, spalt*; SIP, Smad interacting protein; Ski/SnoN, Sloan-Kettering avian retrovirus/ski-related novel gene.

Smads interact with the cyclic adenosine mono phosphate (cAMP) response binding (CREB) binding protein (CBP/p300) through the MH2 domain, and use them as transcriptional co-activators to induce target gene transcription (Janknecht and Wells, 1998). *Drosophila* Mad is shown to interact with the CBP/p300 homologue Nejire (Nej) to activate transcription of Dpp target genes (reviewed in Torres-Vazquez, 2001). CBP and p300 have histone acetylase transferase (HAT) activity which enables them to modify chromatin structure, and probably expose specific promoter sequences to DNA binding proteins such as the Smads (Massague and Wotton, 2000). The HAT activity of these co-activators on DNA enhances transcriptional activation. Conversely, histone deacytalses (HDACs) repress transcription by causing tighter nucleosomal packing of

DNA and impair the accessibility of promoter sequences to transcription factors. Some transcriptional co-repressors inhibit transcription by recruiting the HDACs. An example is the TGFβ inhibitory factor (TGIF), a homeodomain protein that forms a complex with Smads and recruits HDACs to inhibit Smad transcriptional activity. The structurally related proto-oncogenes Ski and SnoN also repress Smad transcriptional activity by recruiting the HDACs. Another repressor, Smad nuclear interacting protein 1 (SNIP1) is found to interact with Smad4 and CPB/p300 to suppress TGFβ signalling (reviewed in Itoh *et al.*, 2000). As a result of all these interactions Smad transcriptional activity in the nucleus is well regulated both temporally and spatially in response to specific TGFβ signals. TGFβ signalling usually leads to direct activation of target genes; however evidence suggests that signalling may also activate genes indirectly by de-repressing certain target genes from the effects of inhibitors. This view is supported by studies from dauer development in *C. elegans.* In response to unfavourable environmental conditions, *C. elegans* L3 larvae arrest as dauer, a state that allows them to survive and move away from suboptimal conditions. The Co-Smad-related protein, DAF-3 induces dauer formation even under ideal conditions. This activity of DAF-3 is inhibited by BMP signalling to prevent worms from undergoing dauer arrest under optimal conditions (reviewed in Patterson and Padgett, 2000). DAF-3 is similar to Co-Smads but appears to have distinct functions (Patterson and Padgett, 2000). DAF-3 is also shown to recognize and bind to the sequence, GTCTG in the *myo-2* gene to repress its transcription. The *myo-2* gene product is a component of the pharyngeal muscle in *C. elegans* (Thatcher *et al.*, 1999). This inhibitory activity of DAF-3 is also repressed by TGFβ signals (Thatcher *et al.*, 1999). Another example is found in *Drosophila*, where the transcriptional

repressor, Brinker inhibits the transcription of Dpp target genes. Dpp signalling leads to inhibition of Brinker activity, as a result de-repressing and allowing the transcription of these genes (Jazwinska *et al.*, 1999).

Regulators of TGFβ signalling

There are several mechanisms in place to ensure that cells do not receive excessive TGFβ signals. A number of negative regulators (including I-Smads) exist to monitor TGFβ signal levels in the cell. The I-Smads function mainly as negative feedback antagonists of TGFβ signalling. These Smads lack the C-terminal phosphorylation sites (i.e. the SSXS motif, Fig. 2) and as such are able to bind to the receptor without being phosphorylated. It is proposed that these proteins inhibit signalling through competing with the R-Smads for binding to the Type-I receptor as well as competing with Co-Smads for association with activated R-Smads (Itoh *et al.*, 2000). Smad6 and Smad7 are the inhibitory Smads in vertebrates and specifically repress BMP signalling; however Smad7 is able to antagonize other TGFβ signals as well (Nakao *et al.*, 1997). Smad7 is recruited to the Type I receptor by the serine/threonine kinase receptor-associated protein (STRAP) to exert its effect on signalling (Datta and Moses, 2000). In *Drosophila*, Dad, which is induced in response to Dpp signalling, functions in a negative feedback loop to inhibit Dpp. Similar to the scenario in vertebrates, Dad is proposed to interact with Mad to prevent it from being activated by the receptor; and also competes with Mad for binding to the type I receptor, Tkv (Tsuneizumi *et al.*, 1997). The antagonistic function of I-Smads in the feedback circuit ensures that TGFβ signalling is well modulated.

Emerging studies also ascribe positive roles to I-Smads in TGFβ signalling. Smad6 and Smad7 were shown to mediate TGFβ-induced adipocyte differentiation, while Smad7 upregulation by TGFβ is found to be critical in apoptosis of prostate carcinoma cells (Choy *et al.*, 2000; Landstrom *et al.*, 2000). The mechanism of this positive modulation of signalling by I-Smads is however not clear. I-Smads are predominantly nuclear and are exported to the cytoplasm in response to signalling (reviewed in Itoh *et al.*, 2000) probably to effectively modulate signalling. Given the conserved nature of the Smads in various organisms, it is surprising that no I-Smad has yet been discovered in *C. elegans* (Patterson and Padgett, 2000).

There are additional factors that control TGFβ signalling at different levels of the pathway. A number of studies show that certain proteins regulate the accessibility of the ligands to the receptors. Ligands of the TGFβ subfamily are expressed as inactive precursor molecules that are cleaved in the secretory pathway into an amino terminal propeptide and a carboxy-terminal fragment which is the mature growth factor. A number of proteins including thrombospondin-1 (TSP-1), act on the inactive molecules to make them functional (Crawford *et al.*, 1998). Unlike TGFβ, Activins and BMPs are synthesized as active molecules but their activity in the extracellular matrix is regulated by a number of antagonists (Miyazono *et al.*, 2000) that bind and prevent their access to the receptors. Both Activins and BMPs are inhibited by the secreted glycoprotein, Follistatin. Activin induces the release of follicle stimulating hormone (FSH) from the pituitary, an effect inhibited by Follistatin which prevents binding of Activin to the receptors. In a similar way, Follistatin counteracts the inhibitory action of BMPs on *Xenopus* neural fate (Hemmati-Brivanlou *et al.*, 1994). Other BMP antagonists are the

secreted proteins Chordin and Noggin, both of which are expressed in the Spemann's organizer (a signalling centre located at the dorsal lip of the amphibian gastrula blastopore) of the amphibian embryo and inhibit BMP-induced ventral mesoderm (Zimmermann *et al.*, 1996; Piccolo *et al.*, 1996). Both Noggin and Chordin contain cysteine-rich (CR) repeats and prevent BMPs from interacting with the receptors. Short gastrulation (Sog) is the Chordin homologue in *Drosophila* which antagonizes BMP ligands (Holley *et al.*, 1996). Sog is found to inhibit the activity of Screw, and genetic data also show that Sog has inhibitory effects on signalling mediated by Dpp (Yu *et al.*, 1996). These negative regulators ensure that signalling is activated in the right cells; and also help establish concentration gradients of the ligands across the developing embryo (Marques *et al.*, 1997). The latter role is very important due to the morphogenetic nature of the ligands and the varying concentration-dependent roles they perform in directing the specification of different parts of the embryo.

Another important and complex aspect of TGFβ signalling is the presence of agonists that inhibit the activities of the extracellular antagonists enumerated above. This action is mediated by secreted metalloproteases which cleave the BMP antagonists to release free and active ligands. These include *Drosophila* Tolloid and Tolloid-related 1 (Tlr-1) and their orthologues in *Xenopus* (Xolloid) and human (BMP1 and hTld1). Xolloid acts on Chordin while Tolloid interacts with Sog in *Xenopus* and *Drosophila* respectively to relieve the repression of the respective BMP ligands (reviewed in Massague and Chen, 2000). Ashe and Levine (1999) observed that the interaction between Sog and Tolloid is needed to establish a gradient of Dpp activity which subdivides the dorsal ectoderm of the *Drosophila* embryo into amnioserosa and dorsal

epidermis. Conley and colleagues (Conley *et al.*, 2000) recently identified another possible Drosophila BMP agonist, Crossveinless-2, which presumably inhibits the action of Sog during crossvein specification. The Twisted gastrulation (Tsg) protein performs a complex role to act as an agonist and antagonist in different contexts. In *Xenopus*, Tsg binds to Chordin-BMP complex to modulate signalling, and help release the active BMP ligand (Oelgelschlager *et al.*, 2000; 2003). Recent studies however suggest inhibitory roles for Tsg in BMP signalling; and implicate it to enhance the activity of both Chordin and Sog (Ross *et al.*, 2001; Chang *et al.*, 2001). The exact role Tsg is playing in BMP signalling is open to debate.

Apart from ligand function, other proteins target the receptors to regulate signalling. Among these is the BMP and activin membrane-bound inhibitor (BAMBI), a transmembrane protein which shares sequence similarity with type I receptors in the extracellular domain (Onichtchouk *et al.*, 1999). BAMBI forms heterodimers with Type I receptors and interferes with their activation. This protein is reported to strongly inhibit both BMP and activin signalling in *Xenopus*; and is also shown to act in a negative feedback loop to suppress BMP signalling during *Xenopus* embryogenesis (Onichtchouk *et al.*, 1999).

Signalling is also regulated by other proteins that control R-Smad availability. Smad ubiquitination regulatory factors (Smurfs) are a group of ubiquitination ligases that bind specifically to R-Smads and target them for proteosome-mediated ubiquitination and subsequent degradation (Ebisawa *et al.*, 2001; Tajima *et al.*, 2003). Smurf1 is an E3 ubiquitin ligase which contains the homologous to E6AP COOH-terminus (HECT) and WW protein-protein interacting domains; the latter of which is involved in interacting

with the PY motif of a proline-rich sequence present in the linker region of Smads (Massague and Chen, 2000). In *Xenopus*, Smurf1 binds Smad1 and induces its degradation; and ectopic expression of Smurf1 inhibits Smad1-induced ventralization in embryos (Zhu *et al.*, 1999). Smurf1 and *Drosophila* Smurf (DSmurf) were also shown to recruit Smad7 and Dad respectively to the Type I receptor to enhance their inhibitory activity (Podos *et al.*, 2001; Suzuki *et al.*, 2002).

Recent evidence also implicates microtubules as regulators of TGFβ signalling in the cytoplasm. Dong *et al* (2000) showed that Smads are bound to microtubules (through β-tubulin); and probably become dissociated only after their phosphorylation by receptors. This interaction is likely to tether Smads in the cytoplasm to prevent their nuclear import and subsequent leaky activation of target genes in the absence of signal.

Crosstalk between TGFβ and other signalling pathways

In addition to the numerous factors that tightly control TGFβ signalling at various levels, the pathway is further regulated through its integration with other signalling networks. Smad proteins provide several points of integration and interaction between signals arising from the TGFβ family and other pathways. The mitogen activated protein kinases (MAPKs) p38 and Jun-N terminal kinase (JNK) enhance TGFβ signalling. On the other hand, Smad7 is activated by signals from a number of sources including tumor necrosis factor alpha (TNFα) through NF-κ B; and Interferon gamma (IFN γ) through the Janus kinases/signal transducers and activators of transcription (JAK/STAT) pathway. In addition, activated Ras under the influence of the Epidermal Growth Factor (EGF) activates the extracellular signal regulated kinases (Erk) to block the R-Smad /Co-Smad complex from translocating into the nucleus (reviewed in Itoh *et al.*, 2000).

Role of Nemo-like kinases in TGFβ signalling

A potential candidate that may be involved in regulating TGFβ signalling is the Nemo-like kinase (Nlk); a mitogen activated protein kinase (MAPK) with important roles in development. Perhaps the strongest link between TGFβ signalling and Nlks is through the TGFβ activated kinase1 (Tak1). Tak1 is a MAPK kinase kinase (MAPKKK) which is activated in response to TGFβ signalling (Yamaguchi *et al.*, 1995). The MAPK pathway mediates signalling downstream of various receptors to produce numerous cellular responses. This pathway is made up of three protein kinases, MAPKKK, MAPKK, and MAPK. MAPKKK phosphorylates and activates MAPKK, which in turn phosphorylates MAPK (Nishida and Gotoh, 1993). Tak1 activates a number of MAP kinases in culture, including JNK, p38 and Nlk (Moriguchi *et al.*, 1996; Shirakabe *et al.*, 1997; Wang *et al.*, 1997; Ishitani *et al.*, 1999). Studies demonstrate that Tak activates Nlk homologues in response to TGFβ signalling in mice and worms (Ishitani et al., 1999; Meneghini et al., 1999; Shin et al., 1999). These findings implicate Nlks as potential modulators of TGFβ signalling. Other targets of Tak1 including JNK and p38 also regulate TGFβ signalling (Derynck and Zhang, 2003) although it is not known if they do so in response to Tak1.

Developmental roles of *nlks*

Nemo-like kinases are proline-directed serine threonine protein kinases that play various roles in cell fate determination and pattern formation. The genes encoding these proteins are well conserved evolutionarily, with homologues in *Drosophila* (*nemo*), *Caenorhabditis elegans* (*lit-1*) and vertebrates (*nemo-like kinases*). Nlk and its homologues regulate various signalling pathways, including Wnt in *C. elegans, Xenopus laevis* and *Drosophila* (Meneghini *et al.*, 1999, Rocheleau *et al.*, 1999, Ishitani *et al.*,

1999, Verheyen *et al.*, 2001; Zeng and Verheyen, 2004). *Nemo (nmo)* was first identified in *Drosophila* as an important component of eye formation. The *Drosophila* compound eye consists of roughly 800 eye units, termed ommatidia. Each of these can be visualized as a secreted hexagonal lens in the adult fly eye. Inside the ommatidium are 20 cells consisting of 8 photoreceptors and 12 accessory and pigment cells. The photoreceptor cells within the eye undergo a precise series of rotations during their development. *nmo* is found to be required for the correct orientation of these photoreceptors; and loss of *nmo* manifests itself in a change from the hexagonal to square shape of the lens (Choi and Benzer, 1994). *nmo* also has roles in cell fate specification during wing development in *Drosophila* with mutations in the gene affecting the specification of veins and the size of the wing (Choi and Benzer, 1994; Verheyen *et al*, 2001). Nemo also regulates embryonic development and apoptosis (Verheyen *et al.*, 2001; Mirkovic *et al.,* 2002) in *Drosophila.*

In *C. elegans, lit-1* plays a central role in the asymmetrical cell division and cell fate specification (Meneghini *et al.*, 1999; Rocheleau *et al.*, 1999). During embryogenesis asymmetrical divisions delineate the anterior cells from their posterior counterparts; with *lit-1* functioning to specify posterior cell fates (Shin *et al.,* 1999). Nlks have been shown to be involved in vertebrate development as well. In *Xenopus xNlk* is essential for neural development during embryogenesis. Kortenjann and colleagues (Kortenjann *et al.,* 2001) demonstrate roles for *mNlk* in embryogenesis, neural development and haematopoiesis in mice. These authors showed that mice lacking *mNlk* die during embryogenesis; or are growth retarded with various neurological disorders. These mice also display aberrant differentiation of bone marrow stromal cells. The importance of Nlks in development has led to efforts to understand and characterize their activity. Emerging studies reveal roles

for Nlks in modulating various signalling pathways; the most characterized of these interactions is in crosstalk with the Wnt pathway.

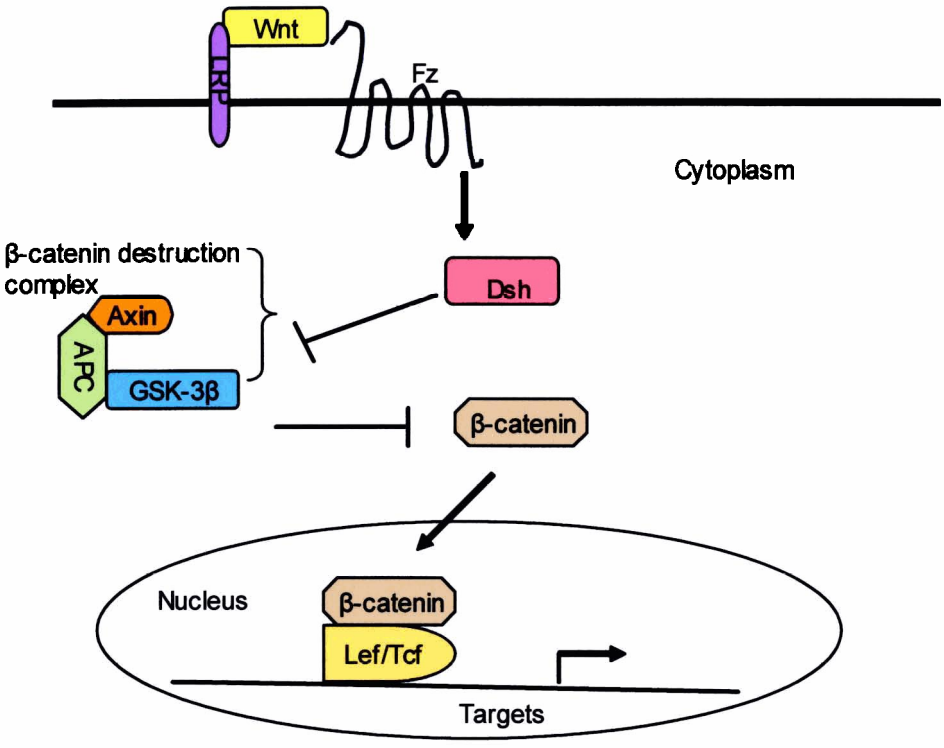
Nlks and regulation of Wnt signalling

The Wnt secretory proteins control many important developmental decisions including embryogenesis, specification of cell fate and polarity, body axis formation and neural development. The Wnt signal proceeds through at least two distinct pathways referred to as canonical (β-catenin-dependent) (Fig. 3) and non-canonical (β-catenin-independent) pathways (Fig. 4). In the canonical pathway, Wnt signalling is mediated by the cytoplasmic transcriptional coactivator, β-catenin. In the absence of Wnt signal, β catenin forms a complex with a group of inhibitors including Glycogen Synthase Kinase 3β (GSK3β), the Adenomatous Polyposis Coli (APC) protein, and Axin. This interaction leads to phosphorylation of β-catenin and its subsequent degradation. The Wnt ligands bind to the Frizzled receptors, leading to the activation of the cytoplasmic protein Dishevelled (Dsh), which consequently inhibits GSK 3β and thereby stabilizes the cytoplasmic pool of β-catenin. β-catenin then translocates to the nucleus and forms a complex with the high-mobility-group (HMG) class of transcription factors, lymphoid enhancer factor 1 (Lef 1) and T-cell factor (TCF) to activate transcription of target genes. There is a deviation from this general scenario in *C. elegans*, where a Lef/TCF-like protein acts as a transcriptional repressor of Wnt signalling. Therefore in *C. elegans*, the β-catenin homologue WRM-1 inhibits the activity of the Lef 1/TCF homologue, POP-1 to allow the transcription of target genes (reviewed in Wodarz and Nusse, 1998).

Wnt signalling is well regulated in development to allow correct specification of body parts. Several studies support a role for Nlks in modulating Wnt signalling both

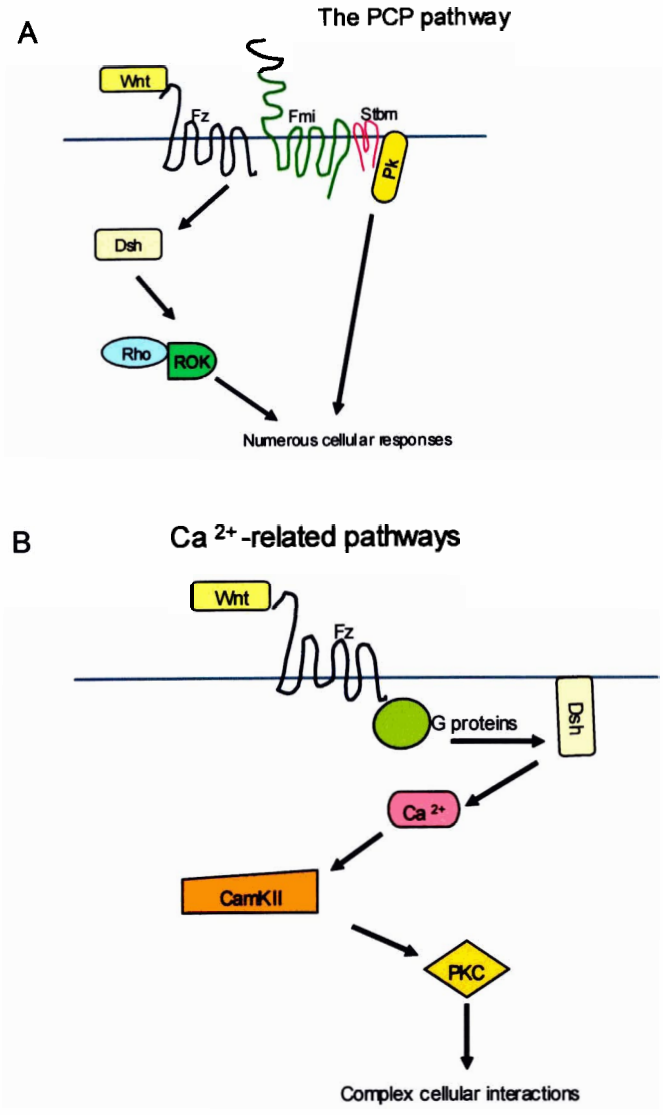
positively and negatively. In *C. elegans*, the Tak1 homologue, MOM-4 (More of MS-4) and the Nlk homologue LIT-1 cooperate with the Wnt signalling pathway to down-regulate POP-1 (Meneghini *et al.,* 1999; Rocheleau *et al.,* 1999). LIT-1 is activated by MOM-4 and forms a complex with WRM-1, this LIT-1/WRM-1 complex then moves into the nucleus to phosphorylate POP-1 and induce its nuclear export to allow the transcription of Wnt target genes (Shin *et al.,* 1999). Recently Thorpe and Moon (2004) reported the finding that Nlk acts with β-catenin to de-repress Wnt target genes in Zebrafish, placing Nlk as a co activator of canonical Wnt signalling.

Figure 3: The canonical Wnt pathway



The Wnt ligands interact with the Fz receptors leading to the phosphorylation of Dsh. Dsh in turn inhibits the destruction complex made up of APC, Axin and GSK-3β; to stabilize β-catenin in the cytoplasm. Free β-catenin moves into the nucleus where it interacts with the Lef/TCF transcription factors to regulate the transcription of target genes.

Figure 4: The non-canonical Wnt pathway



Abbreviations: CamKII- Calmodulin-kinase II; Fmi- Flamingo; Pk- Prickle; PKC- Protein kinase C; ROK- Stbm-strabismus. Signalling through the non-canonical Wnt pathway is not fully understood; the figures presented above represent simplified versions of the two characterized lineages. (A) The PCP pathway: Wnt ligands interact with the Fz, and Fmi transmembrane proteins leading to the activation of Dsh; and subsequently Rho and ROK which influence target gene transcription in the nucleus. Another lineage involves the membrane proteins Stbm and Pk downstream of Fmi to regulate target genes. This latter part of the pathway is less understood. (B) The Ca^{2+}-dependent pathway is activated when G proteins downstream of Fz activate membrane-tethered Dsh. Dsh then activates intracellular calcium ion which signals through one of two mediators; PKC or CamKII to influence targets.

This is in contrast to what is seen in mammalian cells, where the Tak1/Nlk pathway phosphorylates and down-regulates Lef-1/Tcf, preventing the β-catenin-Tcf complex from binding to DNA and thus inhibiting transcriptional activity downstream of Wnt signalling (Ishitani *et al.*, 1999; 2003). This result is supported by studies in *Xenopus* where Nlk prevents double axis formation induced by overexpression of β-catenin (Ishitani *et al.*, 1999). Studies in *Drosophila* also indicate that *nmo* is an inhibitor of Wingless (Wg) signalling. Zeng and Verheyen (2004) showed that *Nemo* suppresses Wg-dependent gene expression in the wing disc, and affects the stability of the β-catenin homologue, Armadillo (Arm). Nemo is also shown to bind Arm (Bessette, Zeng and Verheyen, Unpublished data). However the effect of this binding is not known.

In most instances signalling pathways employ negative feedback circuits to monitor their activity in a precise manner. Nlks have been demonstrated in recent studies to act in feedback loops to monitor Wnt signalling. Smit and colleagues (Smit *et al.*, 2003) showed that the Tak1/NLK pathway (which inhibits Wnt signalling) is activated in response to Wnt signalling in vertebrates. In the Drosophila wing imaginal disc, high Wg signalling induces the transcription of *nemo* which acts to inhibit Wg (Zeng and Verheyen, 2004).

In addition to the canonical pathway, new studies reveal a positive role for Nlks in the non-canonical Wnt pathway. The non-canonical Wnt pathway uses other mediators in the place of β-catenin to achieve signalling downstream of Wnt ligands and receptors. This pathway is divided into two separate branches, the planar cell polarity (PCP) and calcium ion (Ca^{2+})-dependent lineages. The PCP pathway controls planar tissue polarity in *Drosophila* and convergent extension (CE) movements during gastrulation in

vertebrates. A number of proteins including the membrane protein Strabismus (Stbm), the LIM domain protein Prickle (Pk), the seven-pass trans-membrane cadherin Flamingo (Fmi), and the small guanosine triphosphate (GTPases) Rho, and Rho kinase (ROK) make up this pathway. Certain Wnt ligands (e.g. Wnt5a and Wnt11) also induce the release of intracellular calcium which activates downstream kinases including the Calmodulin protein kinase II (CamKII) and protein kinase C (PKC) (reviewed in Veeman *et al.*, 2003). There is evidence that non-canonical Wnt signalling may antagonize the canonical pathway (Torres *et al.*, 1996; Park and Moon, 2002; Topol *et al.*, 2003; Veeman *et al.*, 2003).

The first evidence for involvement of Nlk proteins in the non-canonical Wnt pathway came from studies in *Drosophila*. Two studies by Choi and Benzer (1994) and Verheyen *et al.*, (2001) show that *nmo* mutants exhibit defects in PCP signalling. This finding is buttressed by recent evidence from vertebrates. In mammalian culture, Nlk functions as a downstream effector of the non-canonical Wnt ligand, Wnt-5a to inhibit β - catenin signalling (Ishitani *et al.*, 2003a); while Zebrafish *nlk* interacts genetically with the non-canonical *wnt11* homologue, *silberblick* (*slb*) to regulate gastrulation movements (Thorpe and Moon, 2004).

Role of Nlk in signalling crosstalk

Apart from the Wnt pathway, emerging evidence also implicates Nlks in crosstalk with other signalling pathways. This is through a new role of the Nlks as repressors of transcriptional co-activators such as the CREB binding protein (CBP/p300), thus repressing the transcriptional activity of several transcription factors including Nuclear Factor kappa B (NFkB), Smads and p53, all of which utilize CBP/p300 to activate transcription (Yasuda *et al.,* 2004). As a result Nlk is likely to inhibit a wide range of signalling pathways that employ these transcription factors.

Although Nlks are linked to the TGFβ pathway through Tak1, the potential involvement of these kinases in modulating TGFβ signalling is not well characterized. Given the numerous roles Nlks play in regulating signalling in other pathways, the major aim of this thesis is to investigate any possible interaction between *nmo* and the TGFβ pathway in *Drosophila*.

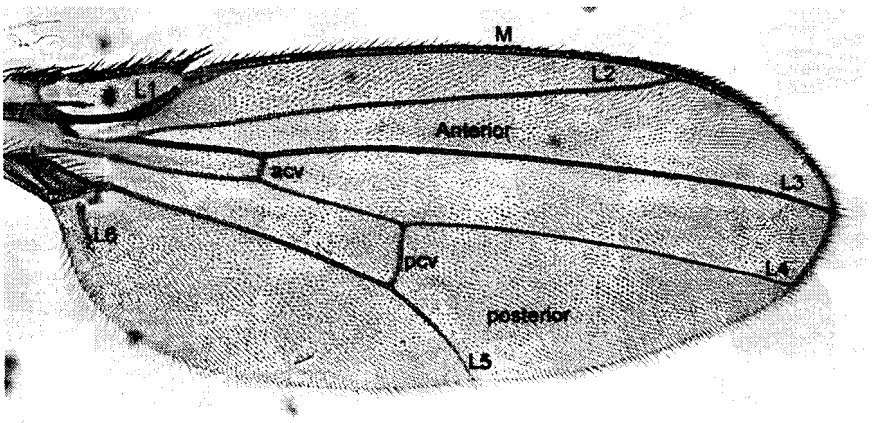
TGFβ signalling and wing development in *Drosophila*

The wing of the fruitfly, *Drosophila melanogaster* presents an excellent model for uncovering the intricate genetic interactions that govern development in multicellular organisms. *Drosophila* possesses a pair of wings for flight that are attached to the second thoracic segment of the body. The adult wing consists of a wing blade in which linear cuticular structures known as veins are distributed in a characteristic pattern among groups of intervein cells. Vein cells are more compact and differentiate dark pigmented cuticle, hence are easily distinguished from intervein cells (de Celis, 2003, Milan *et al.,*

1997) (Fig. 5). The veins provide structural rigidity to the wing and also carry haemolymph, axons and in some cases trachea (transverse veins do not carry trachea) (reviewed in de Celis and Diaz-Benjumea, 2003).

In *Drosophila* there are four longitudinal veins (L2-L5) that span the length of the wing, and two transverse veins (anterior crossvein, acv, and posterior crossvein, pcv) that connect the longitudinal veins L3 and L4, as well as L4 and L5, respectively. There are two incomplete longitudinal veins L1 and L6 which are located in the anterior and posterior compartments, respectively, of the wing. In addition, there is a marginal vein (M) that spans the length of the anterior wing margin (de Celis, 2003) (Fig. 5). The differentiation and positioning of these veins is maintained in the wildtype *Drosophila* wing through the activities of many genes. Subtle wing defects in *Drosophila* are readily identified, and many of the genes controlling wing vein patterning in the fly have been characterized (de Celis, 2003; Sturtevant and Bier, 1995) and have been found in most cases to play similar roles to direct development in other organisms. These features make *Drosophila* wing development a good model for studying genetic interactions that govern pattern formation during development.

Figure 5: Structure of the adult *Drosophila* wing.



Abbreviations: L1-L6- longitudinal veins; acv- anterior crossvein; pcv-posterior crossvein; M- marginal vein. The adult wing has longitudinal (L1-L6) and transverse (acv and pcv) veins arranged in a species-specific pattern among intervein cells. The marginal vein spans the anterior wing margin, ending at the tip of L3. Specification of all these veins and intervein cells is controlled by specific genetic programs. Vein cells are densely packed and more pigmented than intervein cells.

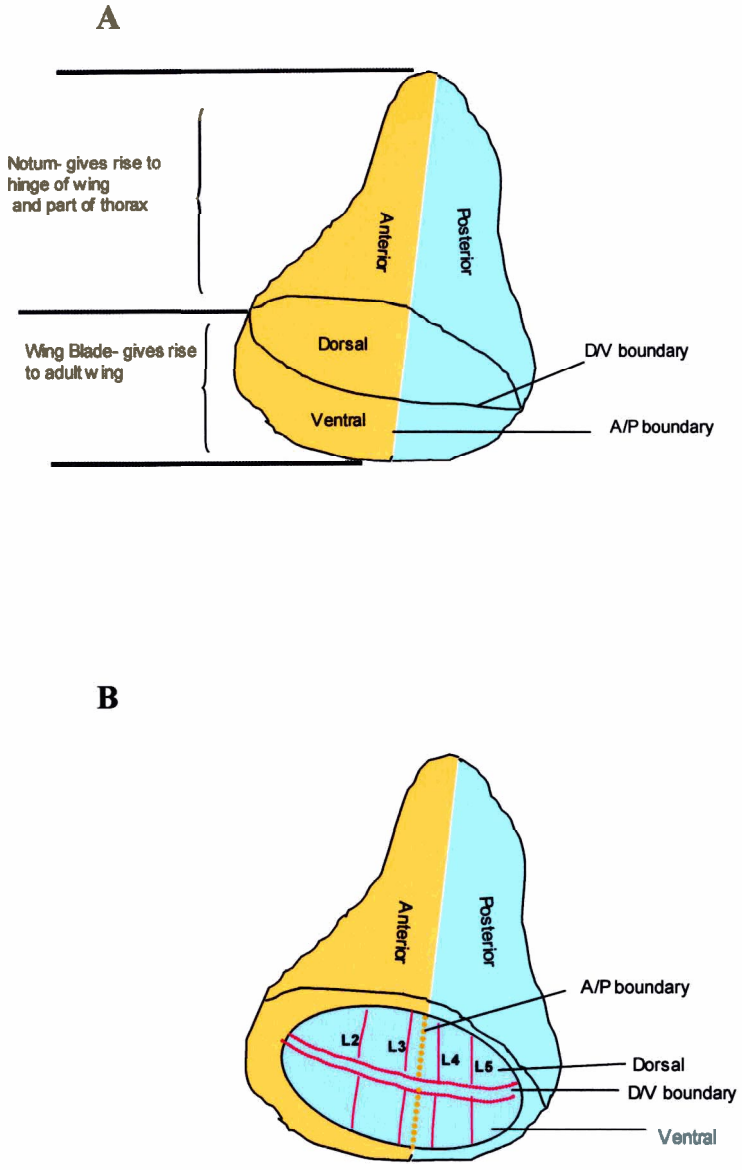
Vein development in the imaginal disc

Drosophila undergoes complete metamorphosis (i.e. distinct stages with specialized body patterns adapting to the needs of each stage). The fertilized egg hatches into a mobile larva, which undergoes three successive molts (referred to as instars) to form an immobile pupa from which the adult fly emerges. The structures that give rise to the adult body are formed in the embryo and proliferate separately during larval development (Snodgrass, 1954; Anderson, 1963a, b, 1972b.: in Cohen,1993). Epidermal structures of the adult head, thorax (including the wings and legs), and external genitalia are formed from sac-like structures called imaginal discs; while the adult abdominal epidermis derives from cells known as histoblast nests (Cohen, 1993).

Vein specification occurs in three successive stages, starting with the 3rd instar wing disc and involves interaction between various gene products. A number of genes regulated by Hedgehog (Hh), Decapentaplegic (Dpp), Notch and epidermal growth factor receptor (EGFR) pathways play significant roles in specifying vein and intervein regions in the wing.

The precursor cells that form the wing disc are subdivided into anterior and posterior, as well as dorsal and ventral halves through the differential expression of the *engrailed (en)* and *apterous (ap)* genes respectively (Fig. 6A). *en* is expressed in posterior cells and repressed in anterior cells; while *ap* is expressed specifically in dorsal cells. These genes also influence the expression of other genes to maintain distinct anterior-posterior (A-P), as well as dorsal-ventral (D-V) compartments. The A-P boundary is set up during embryogenesis but the D-V boundary only appears in the second larval instar (Diaz-Benjumea and Cohen, 1993). Both *en* and *ap* activate various genes that contribute to growth and patterning of the disc. These subdivisions are maintained into the pupal stage. Vein specification is initiated in mid third instar along the A-P axis of the wing disc. *en* encodes a homeobox transcription factor and regulates the expression of other genes, which direct vein specification among other functions. Proteins encoded by the *hedgehog (hh)* and *dpp* genes play essential roles in the growth and specification of vein and intervein territories within the disc.

Figure 6: Subdivisions of the 3rd instar wing imaginal disc



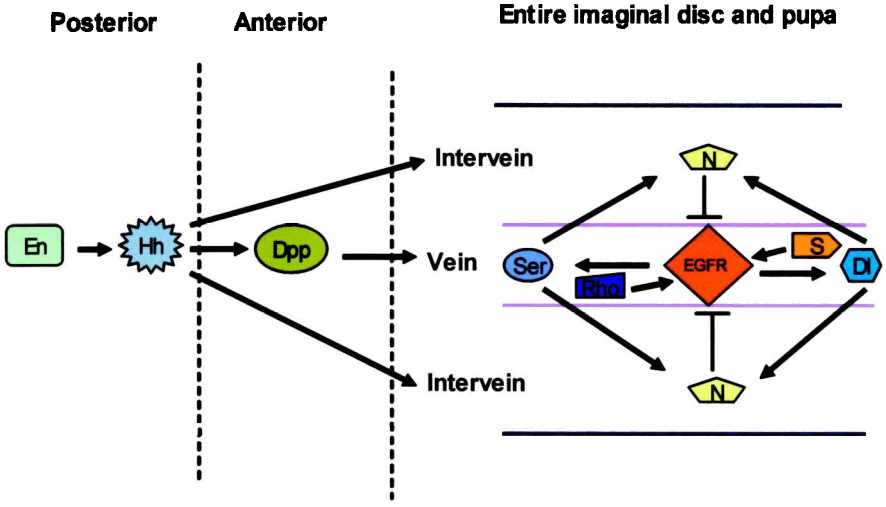
Abbreviations: A/P, antero-posterior; D/V, dorso-ventral. (A)The wing disc is divided along two planes: anterior and posterior; dorsal and ventral. These divisions arise, and are maintained through differential gene expressions. Posterior cells express *en*, which is repressed in anterior cells. Likewise, *ap* is expressed in the dorsal half, but repressed in the ventral domain. The adult wing arises from the wing blade region of the disc. This region folds during pupal development to bring dorsal and ventral halves in contact with each other. The D/V boundary becomes the future wing margin. (B) The interaction between various genes directs the specification of veins along the A/P boundary of the wing blade, on either surface i.e. ventral and dorsal.

hh is a direct target of En and as a result is activated in the posterior compartment (where the *en* gene is expressed); however it encodes a secretory protein that diffuses to the anterior region to activate target genes, including the TGFβ gene, *dpp*. Other targets of Hh include specific transcription factors that direct the specification of different longitudinal veins as well as intervein tissue. Dpp protein has long range effects and diffuses to reach most cells in the disc. The major mediators of Dpp signalling in the disc are the genes of the *spalt* complex, *spalt (sal)* and *spalt-related (salr)*. These genes encode zinc finger domain transcription factors and control the positioning of provein territories (de Celis, 1996; de Celis *et al.*, 2000, Milan *et al.*, 2000). The combined effects of Hh and Dpp signalling therefore divides the third instar wing disc into individual proveins and interveins along the proximo-distal axis (mediated by specific transcription factors), accompanied by a general increase in the size of the disc.

Once provein and intervein territories are specified, other factors ensure their integrity, width and continual maintenance of their respective fates. The EGFR and Notch pathways maintain vein and intervein fates respectively within the disc, and regulate each other's activity to maintain the position and width of the proveins. EGFR signalling is maintained in the centre while *Notch* signalling is restricted to the borders of each provein. In the middle of the third larval instar expression of the *rhomboid (rho)* gene is initiated in all vein primordia (Bier, 2000) probably due to Dpp activity (Yu *et al*, 1996). Rho and the EGFR ligand Star, activate the EGFR pathway in the centre of the proveins. As a result of EGFR signalling, the Notch ligand Delta (Dl) is expressed in the proveins. Dl and another ligand Serrate (Ser) are expressed in the centre, and activate Notch

signalling at the edges of the proveins. The major function of Notch signalling is to maintain the width of the proveins by restricting EGFR signalling to the centre of the vein territory. This activity is mediated by the expression of a member of the *Enhancer of split* genes, *E(spl)mb*, which inhibits Rho and Star on the border of the proveins and prevents vein formation in these regions (de Celis *et al.,* 1996; de Celis *et al.,* 1997).

Figure 7: A simplified schematic of genetic interactions guiding wing development.



Abbreviations: Dl, Delta; N, Notch; Rho, Rhomboid; Ser, Serrate; S, Star. Wing vein patterning is initiated from the posterior region of the wing disc, where En activates *hh*. Hh activates several genes (including *dpp*) that mediate vein and intervein fates. Dpp and Hh activities lead to the specification of veins among intervein cells. In both the imaginal disc and pupa, EGFR is activated by Rho and Star in the centre of the veins. EGFR in turn activates the Notch ligands Ser and Dl, which activate N in intervein cells. Notch acts to limit the width of the vein cells by inhibiting EGFR on the vein borders. EGFR activity is thought to be indirectly regulated by Dpp.

Vein development in the pupa

By the end of the third larval instar, the effects of Hh, Dpp, EGFR and Notch pathways create distinct regions where the longitudinal veins will later differentiate among intervein spaces. The actual differentiation of provein regions into vein cells occurs during pupal development, and also requires different sets of genes. For example, the expression of the *blistered* (*bs)* gene is maintained in the intervein cells, whereas expression of two POU-domain containing genes, *ventral veinless (vvl)* and *nubbin (nb)* are restricted to the proveins (de Celis *et al.*, 1995; *de Celis*, 1998). The activities of these genes help maintain the subdivision of the wing blade into pro-vein and intervein territories during early pupal development.

One major consequence of EGFR signalling is the activation of *dpp* expression in the provein cells where it drives the differentiation of veins (Yu *et al*, 1996; de Celis, 1997). Dpp in turn contributes to maintain the expression of *Dl* and *rho* in the vein cells, hence reinforcing vein differentiation.

Shortly after pupariation, the wing disc everts and folds back onto itself, allowing the corresponding dorsal and ventral surfaces to be in contact (Garcia-Bellido and de Celis, 1992). Vein and intervein territories are specified (separately) in both the ventral and dorsal surfaces of the wing; however during disc eversion, there are inductive signals from either surface that control correct vein specification on both surfaces (Milan *et al*, 1997). These dorso-ventral inductive signals define the final vein differentiation pattern in the wing (Garcia-Bellido and de Celis, 1992).

Unlike their longitudinal counterparts which are specified in third larval instar, crossveins only appear at late pupal stages (Conley *et al.*, 2000). The acv is formed from

components of both dorsal and ventral epithelia, while the pcv is formed entirely from the ventral epithelium, but depends on inductive signals arising from the dorsal epithelium (reviewed in Marcus, 2001). Although longitudinal vein development is well characterized, the development of the crossveins is not clearly understood. Genetic studies reveal roles for genes belonging to the *crossveinless (cv)* family, namely *cv, cv-2, cv-c, cv-d,* in the specification of the crossvein fate. Mutants of these genes lack one or both crossveins (reviewed in Diaz-Benjumea and de Celis, 2003), confirming the importance of these loci in crossvein development. In addition, various studies link BMPs as possible mediators in cross vein formation; as reductions in BMP genes have led to losses of the acv and pcv (Yu *et al.*, 1996; de Celis, 1997; Haerry *et al.*, 1998; Khalsa *et al.*, 1998; Nguyen *et al.*, 1998; Wharton *et al.*, 1999; Ray and Wharton, 2001). The similarities in the effects of mutations in the *cv* and BMP genes support a possible collaboration between these genes to promote crossvein formation. In fact, the two *cv* genes that have been molecularly characterised so far (*cv* and *cv-2*) are found to encode proteins that may mediate BMP signalling.

Crossveinless 2 (Cv-2) (Conley *et al.*, 2002) and its vertebrate homologues (Coffinier *et al.*, 2002; Moser, *et al.*, 2003 Binnerts *et al.*, 2004) contain the characteristic cysteine-rich (CR) domains similar to that found in the BMP antagonists, Sog and Chordin. The CR domains in Sog and Chordin are important for binding to the BMPs (Larrain *et al.*, 2000); which suggest that Cv-2 may bind BMPs through the CR domain. Moser and colleagues (Moser *et al.*, 2003) demonstrate that mammalian Cv-2 binds BMPs in vitro, in support of this hypothesis. Unlike the BMP antagonists however, Cv-2 appears to have a positive effect on BMP signalling, probably through competition with

the antagonists to bind the ligands and enhance their activity (Conley *et al.*, 2000). This positive effect on BMP signalling is likely to be mediated through the presence of VWF domains in Cv-2, which makes it distinct from the antagonist, Sog (Conley *et al.*, 2000). Conley *et al* demonstrated the need for Cv-2 for efficient BMP signalling during the development of the crossveins, and proposed that it may be necessary to protect the ligands from cleavage by Sog, or it may be needed to activate the ligands themselves (Conley *et al.*, 2000). The other gene *crossveinless (cv)*, is found to encode a protein that is similar to the BMP modulator Twisted gastrulation (Tsg) (Ross *et al.*, 2001; Vilmos *et al.*, 2001). Recall that Tsg forms complexes with BMPs and their antagonists to modulate signalling (Oelgeschlager *et al.*, 2000, 2003; Ross *et al.*, 2001; Shimmi and O'Connor, 2003). However, the exact effect of Cv on BMP signalling is not well understood.

The importance of BMPs in crossvein development is further revealed through the overexpression of the BMP antagonist Sog which leads to loss of the acv and pcv (Yu *et al.*, 1996). Interestingly, misexpressed *Nmo* also leads to a variable loss of the pcv; suggesting a role for *nemo* in crossvein development. The sensitivity of the pcv to both BMPs and Nemo therefore provided a perfect assay to investigate the involvement of *nmo* in TGFβ signalling. Several studies from our lab indicate that *nmo* is involved in wing patterning where it probably promotes the specification of intervein fates and inhibits vein fates in intervein regions (Verheyen *et al.*, 2001; Mirkovic *et al.*, 2002). This role is supported by two facts: *nmo* mutant wings exhibit ectopic veins emerging from the pcv between L4 and L5 (indicative of a role for *nemo* in the correct formation of the pcv); beneath L5 and above L2. Furthermore, *nmo* transcript is localized in the intervein regions and excluded from all vein primordia in the pupal wing (Verheyen *et al.*, 2001).

Various gene products are strategically distributed to regulate specific fates during wing disc and pupal development. An example is the *bs* gene which is expressed in the intervein cells to suppress the vein fate. The localization of *nmo* transcript coupled with the mutant wing phenotypes, suggest that *nmo* probably acts to promote intervein fates during wing development. Other studies in our lab revealed that *nmo* exhibits genetic interactions between other intervein-promoting genes such as *net* and *px*; and seem to inhibit EGFR signalling (K. Charish, unpublished results).

nmo's role as an intervein-determining gene places it as a possible regulator of BMP signalling, given that BMPs promote vein-fates. This interaction is characterized at various levels of the BMP pathway in this study.

Chapter Two: Materials and Method

Drosophila stocks and handling

Flies were kept on standard media made of cornmeal, molasses, yeast and agar. The following strains were obtained from Bloomington stock centre:

cv[1]	*cv-2[1]*
cv-c[1]	*cv-d[1]*
y1 w1118; P{lacW}Dadj1E4/TM3	*Sb1Dpps[11]/cyo*
net[1]	*69B-Gal4*
ptc-Gal4	*A9-Gal4*

gbb^{4} was a kind gift from Kristi Wharton. nmo^{adk1}; and *UAS-nmo* $^{c5\text{-}1e}$ are described in Mirkovic *et al.*, 2002. *nmo* ^{DB24} was described in D. Bessette (MSc Thesis, Simon Fraser University, 2003). *UAS-tkv* and *UAS-Sog* were obtained from Ethan Bier (University of California, San Diego). *en-Gal4* was kindly provided by Norbert Perrimon (Harvard University).

ptc-Gal4 is expressed along the A/P domain of the wing disc, while *en-Gal4* is expressed in the posterior margin of the wing. Also, *A9-Gal4* is expressed in the dorsal wing pouch of the imaginal disc, whereas *69B-Gal4* is ubiquitously expressed in the wing.

Dissection and mounting of wings

Wings were dissected from adult flies and washed in 100% ethanol, then mounted in Aquamount (BDH). Wings were handled at the hinge region to prevent damage.

Aging, fixation and dissection of pupae

White prepupae were picked from bottles and placed on a moist kimwipe in a petridish, and aged at 25°C for 19, 26 or 28 hours. The aged pupae were cut at the head and tail ends, and then fixed in 4% formaldehyde in PBS at 4 °C overnight. Pupal wings were dissected away from the body in Phosphate Buffered Saline (PBS) and fixed for 5 minutes in methanol.

Antibody Staining

Fixed pupal wings were washed in PBS and incubated with primary antibodies at 4°C overnight. The following primary antibodies were used at the concentrations indicated: rabbit anti-pMad, (from Tetsuya Tabata and P. ten Dijke), 1:10,000 in PBT (0.1% Tween 20 in PBS); and rabbit anti-β-galactosidase, (Promega), 1:5,000 in PBT. Wings were later washed in PBT containing 0.2% bovine serum antigen (BSA); and incubated with secondary antibody. Biotinylated goat-anti-rabbit (Jackson ImmunoResearch laboratories Inc.) was used as secondary antibody at a concentration of 1:200 in PBT; and detected with Streptavidin Texas Red (Jackson Immunoresearch) at a concentration of 1:1,000 in PBT, in all cases.

In situ hybridization

Digoxigenin (DIG)-labelled RNA probes were made using the DIG labelling kit (Roche) which allows the synthesis of RNA from promoter sequences by polymerase enzyme, using linear DNA (as a template) and ribonucleotides. The synthesis of new RNA strand involves the incorporation of a DIG-labelled uracil every 20-25 nucleotides. The synthesized RNA strand (probe) is able to pair with its complementary strand of RNA in tissues and is detected through staining with an antibody that specifically binds to DIG. Anti-sense probe is complementary to, and binds to the sense RNA; while sense probe does not bind to the RNA that is made from the sense strand of DNA. The sense probe is therefore used as a control to confirm specific localization of RNA transcript.

cv-2 cDNA was provided by Amy Ralston (Blair lab, University of Winsconsin, Madison), as an insert in the pGEM vector with the promoters T7 and SP6 flanking the insert at the 5' and 3' ends respectively. The restriction enzymes Xho I (at the 5' end) and Hind III (at the 3' end) were used to clone the insert into the vector. Sog cDNA was obtained from Invitrogen Inc. in the pBluescript SK+ vector. The insert was cloned between T7 (at the 5' end) and T3 (at the 3' end) promoters of the pBluescript vector. The restriction enzymes Cla I (located at the 5' end) and Pst I (located at the 3' end) were used to clone the cDNA in to the vector.

The cDNAs were linearized by digestion with specific restriction enzymes, and were used to generate sense or antisense probes. Linearized DNA was run on agarose gel to confirm the size, and later gel purified to extract the DNA. Riboprobes were generated through in vitro transcription. The linear DNA (from *cv-2* or *sog* insert respectively) served as templates in a transcription reaction in which ribonucleotides were used to

synthesize the probe. *cv-2* cDNA was digested with Hind III and transcribed from the T7 promoter, using T7 polymerase to generate a sense probe, while DNA digested with Xho I was used with SP6 polymerase to make antisense probe from the SP6 promoter. *sog* antisense probe was synthesized from the T3 promoter using the T3 polymerase and DNA digested with Pst I. On the other hand, the sense strand was made using T7 polymerase to initiate transcription from the T7 promoter, using DNA digested with Cla I as template. The ribonucleotides used in these transcription reactions are contained in a DIG labelling mix made up of 10 mM adenosine triphosphate (ATP), 10 mM cytosine triphosphate (CTP), 10 mM guanosine triphosphate (GTP), 6.5 mM uridine triphosphate (UTP), 3.5 mM DIG-labelled UTP. Other components of the transcription reaction are transcription buffer (Roche): 400 mM Tris-HCl, pH 8.0; 60mM MgCl2, 100 mM dithioerythritol (DTE), 20 mM spermidine, 100 mM NaCl, 1µl unit/ml RNase inhibitor. 1µg of linear DNA was added to 2µl 10X DIG RNA labelling mix; 2µl 10X transcription buffer; 2µl of appropriate RNA polymerase and sterile distilled water as needed to make up 20µl final volume; in an eppendorf tube on ice. The tube was centrifuged at 13,000 rpm briefly to mix the components and was later incubated at 37C for 2 hours. The transcription reaction was stopped by adding 2µl of 0.2 M EDTA (pH 8.0).

Fixed pupal wings were washed in PBT and digested with 0.2% proteinase K in PBT. The wings were then re-fixed in 4% formaldehyde in PBT at room temperature for 25 min. *In situ* hybridization was performed according to Sturtevant and Bier (1996). Pupal wings were incubated with probe at 55 °C overnight; and incubated with anti-DIG antibody (Roche, at a concentration of 1 in 2,000 in PBT) overnight at 4 °C. mRNA localization was detected through alkaline phosphatase reaction, using 20µl Nitro-Blue

Tetrazolium chloride/5-Bromo-4-Chloro-3-indolylphosphate p-Toluidine salt (NBT/BCIP) in 1ml of alkaline phosphatase buffer. Pupal wings were mounted in 80% glycerol and observed under the light microscope.

Chapter Three: Results

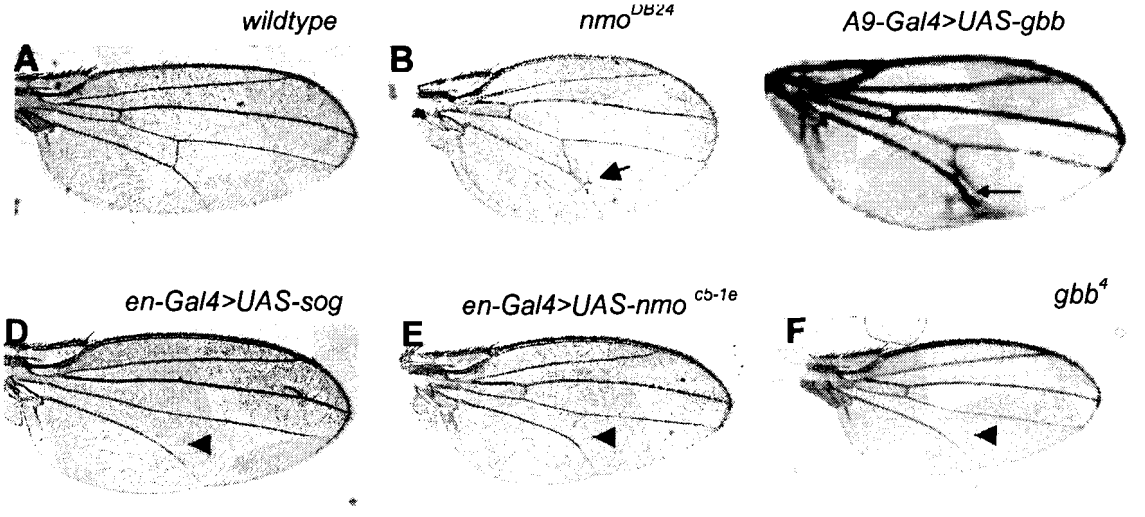
Genetic interaction between BMP components and *nemo*

The roles of *nmo* and the BMPs, *dpp* and *gbb,* in wing development implicate *nmo* as a potential regulator of BMP signalling. Phenotypic analyses of the BMP genes confirm that they promote vein formation in the wing. Losses of either *dpp* (not shown) or *gbb* generally leads to loss of veins (Fig. 8F), and produce ectopic vein phenotypes when overexpressed (Fig. 8C, see loss of pcv). In contrast, loss of *nmo* produces extra veins in the wing (Fig. 8B), while its overexpression produces loss of veins (Fig. 8E). Interestingly, the phenotype induced by misexpression of *Nmo* resembles that of the misexpressed BMP antagonist Sog (Fig. 8D); and is very similar to *gbb* mutants (Fig. 8F). These wing phenotypes support a possible role for *nmo* in TGFβ signalling, and like *sog,* it probably functions to inhibit the pathway.

The possible interaction between these genes was tested through genetic studies, using *nemo* and components of the BMP pathway. As described above, the BMP proteins Dpp and Gbb signal through the Type I receptor Tkv; and overexpression of Tkv leads to increased activation of the BMP pathway (de Celis, 1997). Thus, the BMP pathway was activated ectopically through the overexpression of the Tkv protein, using the UAS- Gal4 system (Brand and Perrmimon, 1993). In this system, the Gal4 gene is inserted in front of a genomic enhancer that drives Gal4 expression in specific tissues. Gal4 binding sites (Upstream activating sequences, UAS) are inserted within the promoter region of the

gene to be misexpressed, allowing this gene of interest to be activated in those cells in which Gal4 is produced, when flies bearing these two separate lines are crossed together.

Figure 8: *nmo* and BMPs exhibit opposing phenotypes.

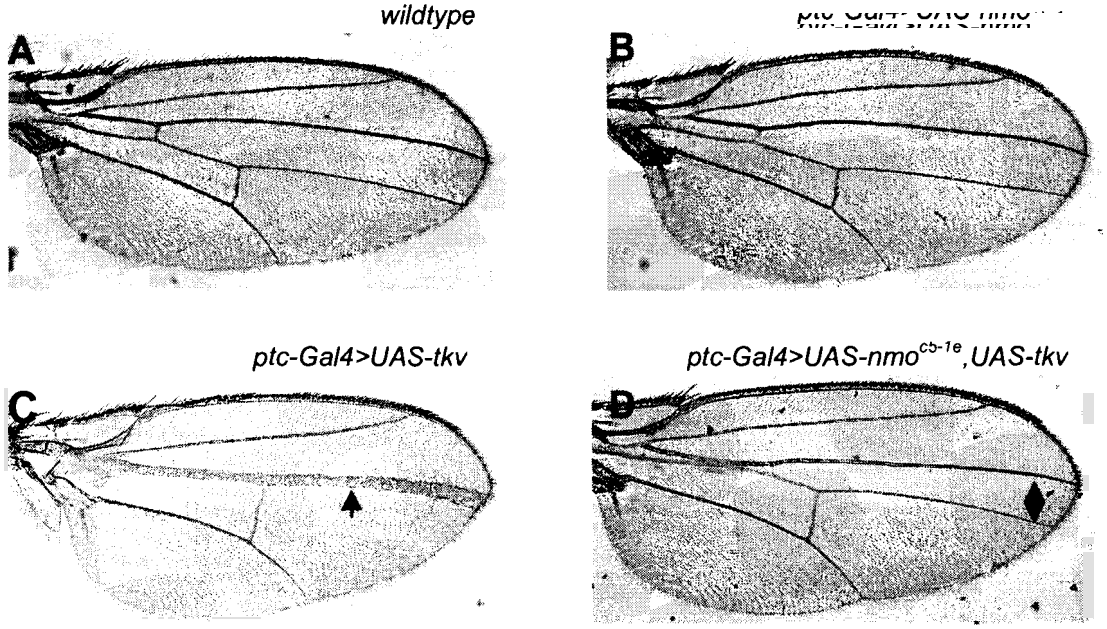


Loss of function *nmo* mutants exhibit ectopic veins (significantly) between L4 and L5 (B, arrow); and are similar to wings overexpressing the BMP gene, *gbb* (C, arrow). Ectopically expressed *nmo* leads to loss of the pcv (E, arrowhead) and resembles wings ectopically expressing the BMP antagonist Sog (D). A similar phenotype is also seen in the *gbb* loss of function mutant (F, arrowhead); demonstrating opposing roles of *nmo* and BMPs in crossvein development.

The *patched (ptc)* gene is expressed along the A/P boundary of the wing disc; along the same domain that Dpp is expressed, so the *ptc-Gal4* epidermal driver was used to overexpress *tkv* in the wing. *ptc Gal4>UAS-tkv* flies develop wings that are slightly smaller than wildtype, and also show a fusion of veins L3 and L4 (Fig. 9C). In contrast wings from flies overexpressing *nmo* under the *ptc-Gal4* driver do not show any severe phenotype (Fig. 9B). Overexpression of these two genes in the same fly resulted in a dramatic rescue of the *UAS-tkv* phenotype, with almost complete separation of veins L3 and L4 (Fig. 9D). In a separate study, A. Zeng (Verheyen Lab) found that expressing a

constituvely active Tkv receptor results in flies with bifurcated wings, and this phenotype is rescued by the expression of *UAS-nmo* (A. Zeng, unpublished results). Our lab also has found a similar effect of *nmo* on the BMP cytoplasmic mediator, Mad. Overexpression of *UAS-mad* produced wings that are broader than wildtype and show defects in vein patterning; again this phenotype is suppressed by ectopically expressing *nmo* in the same background (Zeng A., 2004, unpublished data).

Figure 9: *Nmo* antagonizes BMP signalling



Overexpression of *nmo* under the *ptc-Gal4* driver produces a phenotype (B) that is similar to wildtype (A). However, *ptc-Gal4>UAS-tkv* results in the fusion of the veins L3 and L4 (C); but these veins are separated to almost wildtype positions by *ptc-Gal4>UAS nmo*$^{c5\text{-}1e}$ (D).

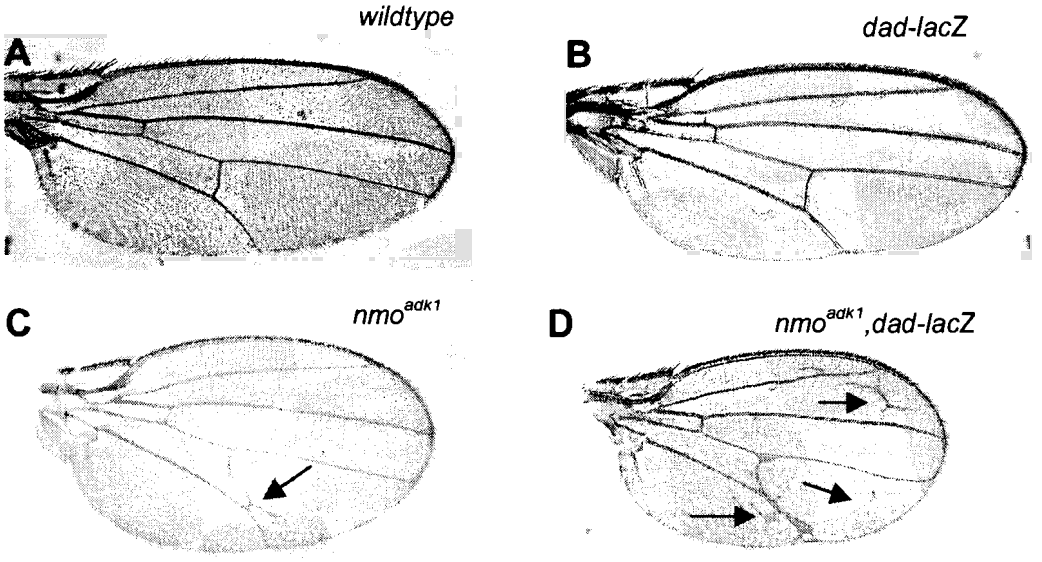
The above results strongly support a possible antagonistic interaction between *nmo* and BMPs. This fact was pursued further by examining possible genetic interaction between *nmo* and the Dpp antagonist *dad.* Tsuneizumi and colleagues (Tsuneizumi *et al.*, 1997) demonstrated that Dad specifically inhibits Dpp signalling; so in a bid to clarify *nmo*'s role in the pathway, mutational analysis was carried out between *nmo* and *dad.* A P element insertion into the *dad* gene (used to monitor *dad* transcription) produces no visible wing phenotype (Fig. 10B), however *dad-lacZ,nmo* double mutants produce wings with ectopic vein phenotypes more severe than is seen in *nmo* mutants alone (Fig. 10C and D). This may indicate a synergistic relationship between *dad* and *nmo*, and further supports the view that *nmo* may be exerting an inhibitory effect on the BMP pathway.

Nemo's effect on BMP signalling

The Smad1 homologue, Mad is a central component of BMP signalling in Drosophila, and is phosphorylated by activated Tkv in response to ligand stimulation. Phosphorylation of Mad is therefore often used as a measure of BMP signalling (Tanimoto *et al.*, 2000), and an antibody that is directed against the phosphorylated Mad (pMad) protein is employed to detect signalling levels. BMP signalling is very crucial for vein development and is needed in the pupal wings for the correct formation of the veins (de Celis, 2003). I carried out pMad staining in pupal wings either mutant for *nmo* or overexpressing *nmo* to determine whether *nmo* has any effect on the signalling level of the BMP ligands. As can be seen in Fig. 11 pMad staining is present along all the veins

but is missing from intervein territories in the wildtype wing (Fig. 11A), supporting the need for BMP signalling in vein specification.

Figure 10: loss of *dad* enhances *nmo* ectopic vein phenotype

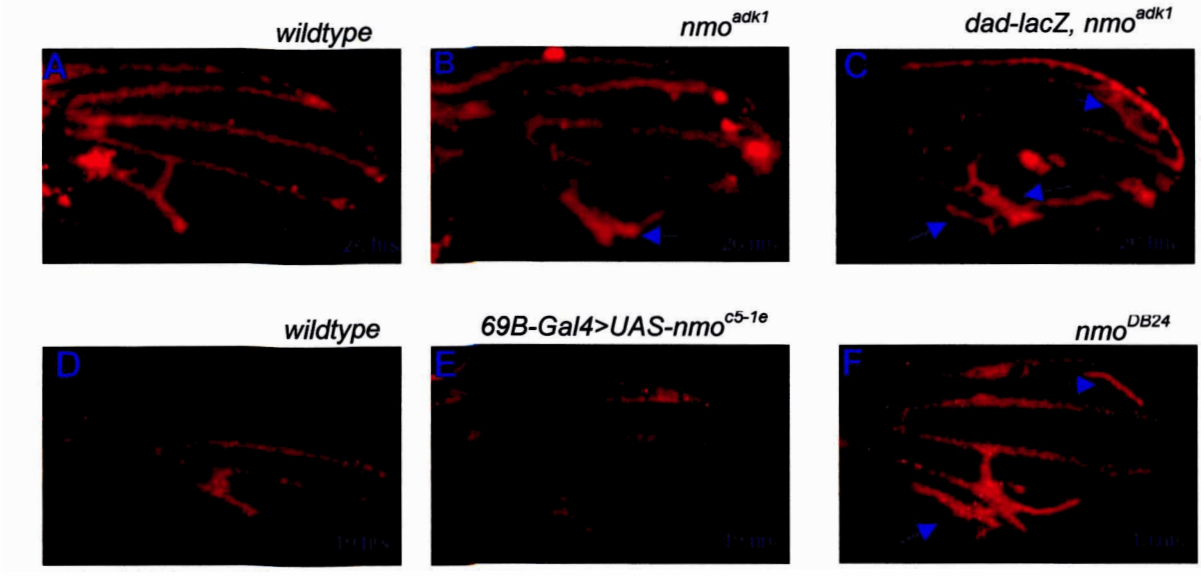


dad-lacZ wings (B) do not show any obvious phenotype and look like wildtype (A). However, these wings display ectopic vein phenotypes when combined with nmo^{adk1} in the same background; and are more severe than the *nmo* phenotype alone (compare arrows in D and C).

This staining pattern is also observed in the overexpressed *nmo* background (Fig. 11E); however *nmo* mutant pupal wings show ectopic pMad staining in the extra veins (Fig. 11B and F). In addition to BMP signalling, other pathways such as the EGFR pathway promote vein formation during the pupal stage so high pMad levels may be the result of an indirect effect of *nmo* on one of these pathways. BMP signalling however is found to be required for the specification of the crossveins between 18-20 hours after puparium formation (apf) (Conley *et al.*, 2000). Most of the extra veins in *nmo* mutant wings are around the region of the posterior crossvein, raising the possibility that they are of the same fate as the pcv. The pMad staining was therefore repeated at 19 hours apf to

confirm that the results observed earlier are due to changes in BMP signalling levels. Interestingly, the same ectopic staining was observed at 19 hours apf (Fig. 11E). This experiment was performed on the more severe *dad-lacZ,nmo* double mutant pupal wings and high pMad levels were observed in the extra veins (Fig. 11C). This increases the possibility that Nemo is targeting the BMP pathway and the high pMad levels in *nmo* wings is due to the loss of the inhibitory activity of Nemo on the pathway. *nmo*'s effect on BMP signalling was also observed in the wing imaginal disc by A. Zeng (Verheyen Lab), who noticed comparatively high pMad levels in clones of cells in the imaginal disc that lose *nmo* activity (unpublished results). Together these results suggest that Nemo negatively regulates BMP signalling and its loss may lead to high levels of signalling from these ligands.

Figure 11: pMad is elevated in *nmo* ectopic veins



Anti-pMad staining of 26 hr old (A-C), and 19 hr old (D-F) pupal wings. pMad is present along the veins; with no detectable difference in the levels in wildtype and ectopically expressed *69B-Gal4>UAS nmo*$^{c5\text{-}1e}$ (compare D to E). However mutant *nmo* wings; *nmo*^{adk1} and *nmo*^{DB24} (B and F respectively); as well as *dad-lacZ,nmo*^{adk1} (C) wing show pMad staining in ectopic veins (arrows in B; C, and F).

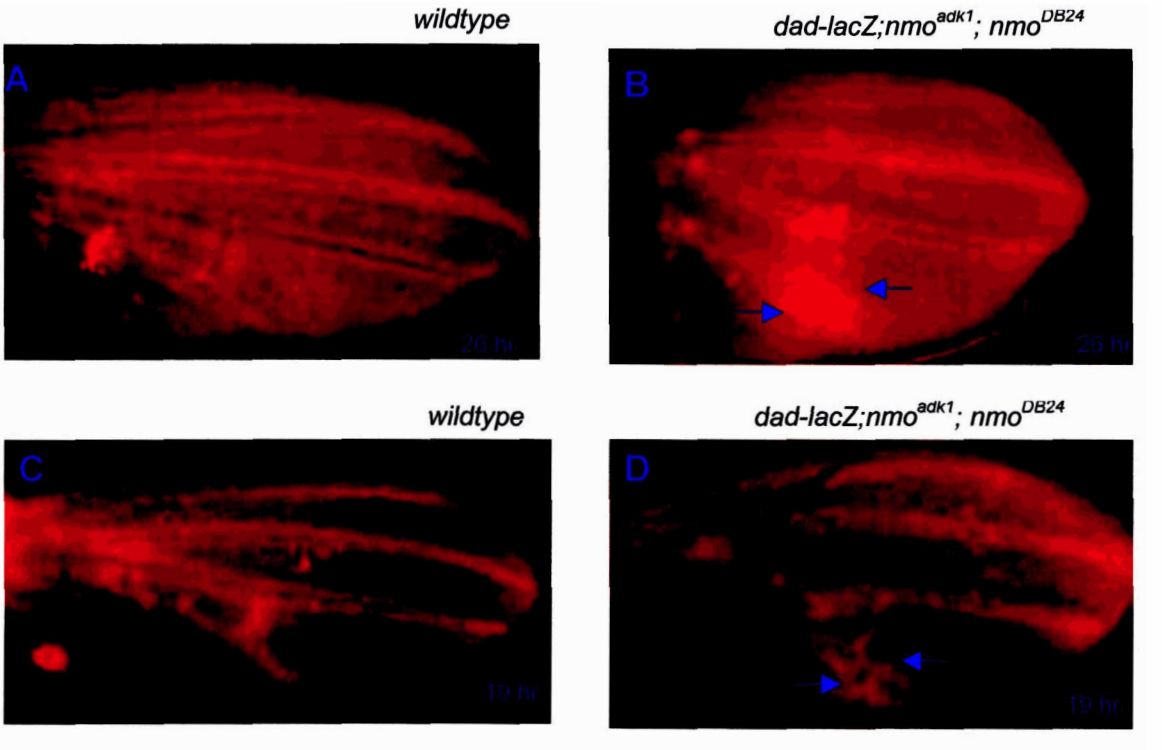
Although the genetic interaction and pMad levels reveal that *nmo* may have antagonistic effects on BMP signalling this effect will be irrelevant without any influence on target gene expression. The significance of Nemo's activity on BMP signalling was examined by monitoring the transcription levels of the Dpp target gene, *dad.* Recall that *dad* is expressed in response to Dpp signalling but acts in a negative feedback loop to suppress Dpp activity (Tsuneizumi *et al.,* 1997). One way of measuring transcriptional activity of *Drosophila* genes is through enhancer trap insertion of the *E. coli lacZ* gene under the control of the enhancer of a target gene. The *lacZ* gene activity is detected by staining for the β-galactosidase protein (a product of *lacZ*) levels to determine the transcriptional activity of the target gene (O'Kane and Gehring, 1987). This technique was used to determine the transcription of *dad* in *dad-lacZ,nmo*^{adk1}*/nmo*^{DB24} pupal wings, through anti - β-galactosidase staining of pupal wings. *dad* is expressed in the intervein regions and appears to be elevated on the borders of the veins in *dad-lacZ* pupal wings (Fig. 12A and C) probably to regulate the activity of Dpp and limit it to the veins. In the *nmo* background expression is slightly elevated around the ectopic veins (Fig. 12 B and D); indicating that *nmo* may be required to suppress Dpp signalling in these regions. This effect was also seen in imaginal wing discs, where *dad* expression is suppressed by ectopic *nmo* (A. Zeng, 2004, unpublished results).

Effect of Nemo on BMP pathway components

Since the evidence seen so far strongly indicates that *nmo* is having a negative impact on TGFβ signalling; it is important to identify the exact way it is interacting with the pathway as well as how it is exerting its effect on the pathway. In an effort to

characterize the interaction, I looked at the effects of Nemo on other components of the BMP pathway.

Figure 12: ***nmo*** **inhibits ectopic** ***dad*** **transcription**

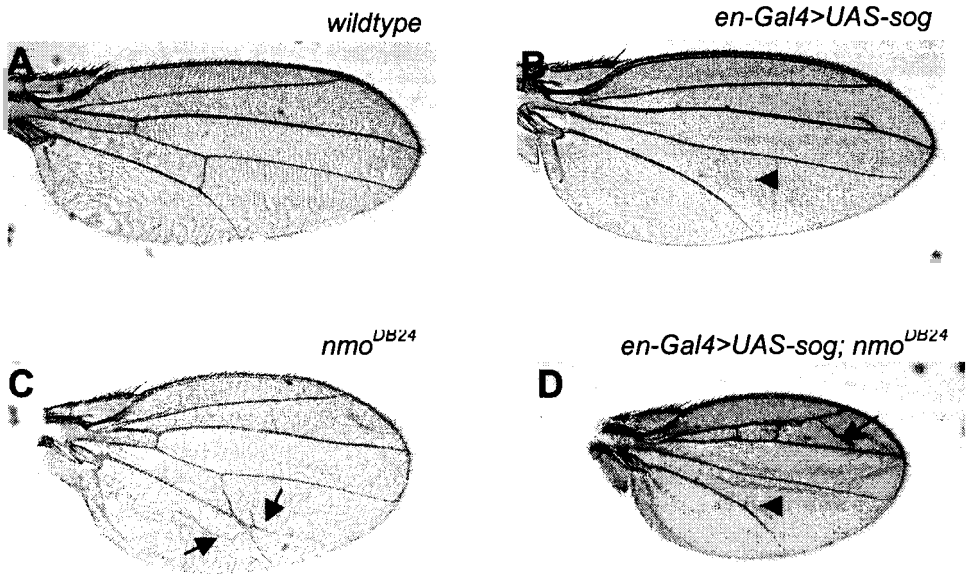


Anti-β-galactosidase staining of 19 hr (C, D); and 26 hr pupal wings. Expression is localized in the intervein cells and is enriched along the vein borders (probably to inhibit Dpp-induced ectopic vein formation); but is excluded from the vein cells in *dad-lacZ*. Ectopic veins in *nmo* mutants however show *dad* expression (arrows in B and D); indicating that *nmo* is affecting BMP target gene expression in regions that correspond to ectopic veins.

As discussed above, Sog has been reported to inhibit the BMP pathway probably through binding to, and cleaving the ligands. Evidence for the inhibitory action of Sog in this pathway in wing development is demonstrated through the opposite phenotype induced by the overexpressed protein compared to the mutant phenotype of the BMP

genes (compare Fig. 8D to F) which is characterized by loss of vein tissue in the crossveins. In support of this view, Yu and colleagues reported that Sog is a Dpp antagonist and inhibits Dpp activity in intervein tissues (Yu *et al.*, 1996). I explored the possibility of *sog* and *nmo* acting through a similar pathway to inhibit BMP signalling. This was done by misexpressing Sog in a *nmo* background. Overexpression of *sog* under the *en-Gal4* driver leads to the loss of both crossveins (Fig. 13B). As discussed above, BMPs play significant roles in crossvein specification and their loss leads to the absence of these veins (Fig. 8F) (de Celis, 1997; Haerry *et al.*, 1998; Khalsa *et al.*, 1998; Nguyen *et al.*, 1998; Wharton *et al.*, 1999; Ray and Wharton, 2001), therefore the loss of crossvein phenotype displayed by flies overexpressing Sog indicates that Sog might be interfering with BMP-mediated development of the crossveins (Yu *et al.*, 1996; Conley *et al.*, 2000). The specific loss of the crossveins could be due to the fact that the crossveins are formed in tissues that are originally specified as intervein early in wing development (Conley *et al.*, 2000), the same tissues where *sog* normally expressed (Yu *et al.*, 1996). As discussed above, the crossveins appear late in pupal development at a stage when longitudinal veins and intervein tissues are already specified, so the crossveins are formed within designated intervein tissue (Conley *et al.*, 2000). Recall that loss of *nmo* leads to the formation of ectopic veins some of which are located near the pcv (Fig. 13C). The overexpression of *sog* in this *nmo* mutant background led to the rescue of *nmo* ectopic vein phenotype; however the UAS-*sog* phenotype (i.e. loss of both crossveins) is unchanged (Fig. 13D). This interaction is complicated by the presence of ectopic veins between L2 and L3 (Fig. 13D, arrows) which are difficult to explain.

Figure 13: *nmo* shows a complex interaction with *sog*.



nmo ectopic veins (arrows in C) are rescued through misexpression of *sog* in the posterior compartment of the wing; however there is no change in the phenotype caused by ectopic expression of *sog*; i.e. loss of the crossveins (arrowheads B and D). The interaction is complicated by the presence of ectopic veins between L3 and L4 (arrow in D).

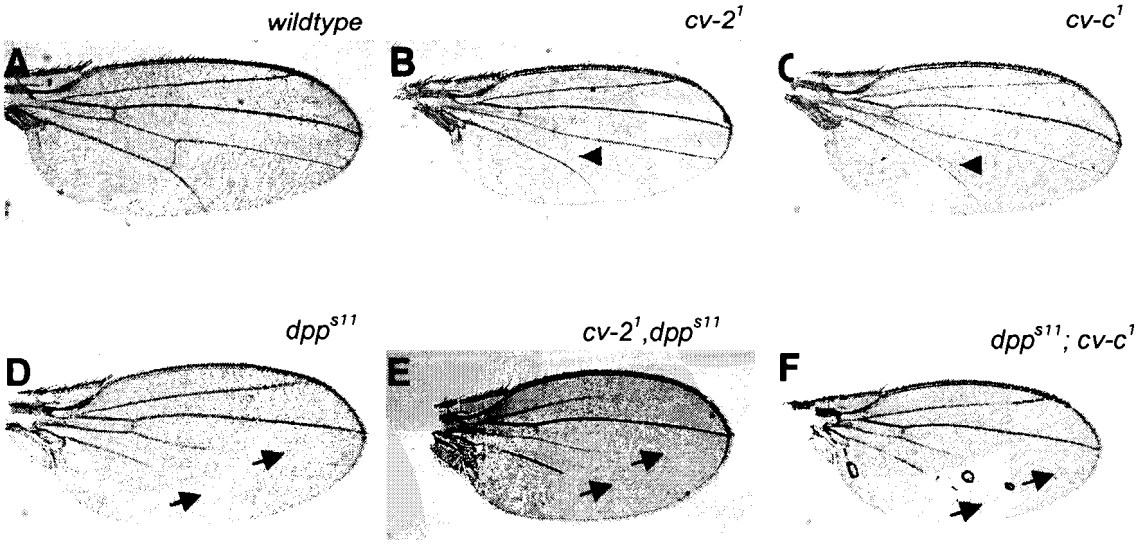
Although this result does not confirm any interaction between *sog* and *nmo*, it consolidates *nmo*'s antagonistic role on the BMP pathway, since the ectopic veins induced by loss of *nmo* in the posterior region of the wing are rescued by overexpressing *sog* (Fig. 13D). The high levels of Sog may thus be preventing the formation of ectopic veins (a role that *nmo* is likely to perform in wildtype wings) in the absence of *nmo* function. If this assumption is true, then *nmo* may also be acting in a parallel pathway to *sog* but they both ultimately inhibit BMP activity.

There is no conclusive evidence that *nmo* interacts with *sog* from the experiment above, hence the investigation of interaction between *nmo* and the BMP components was extended to the *cv* genes. As noted above, these genes are critical to the formation of the crossveins, and their mutants exhibit similarities to mutations in the BMP genes. One

would suspect a possible collaboration between BMPs and Cv proteins in crossvein specification, as proposed by Conley *et al.,* (2000), who showed that Cv-2 may be required to potentiate BMP signalling in the crossvein. The first effort then was to determine if the BMPs interact with the *cv* genes. I therefore generated double mutants between *dpp* and the *cv* genes, *cv-2* and *cv-c* to examine genetic interactions between them. These double mutants display only the *dpp* phenotype (Fig. 14E and F), suggesting a genetic interaction, and also placing BMPs downstream of these *cv* genes. This is not surprising, in light of the proposed role of the Cv-2 protein for instance, in activating BMP ligands (Conley *et al.*, 2000). It is possible then that the other *crossveinless* genes may perform similar roles in modulating BMP signalling in the crossvein.

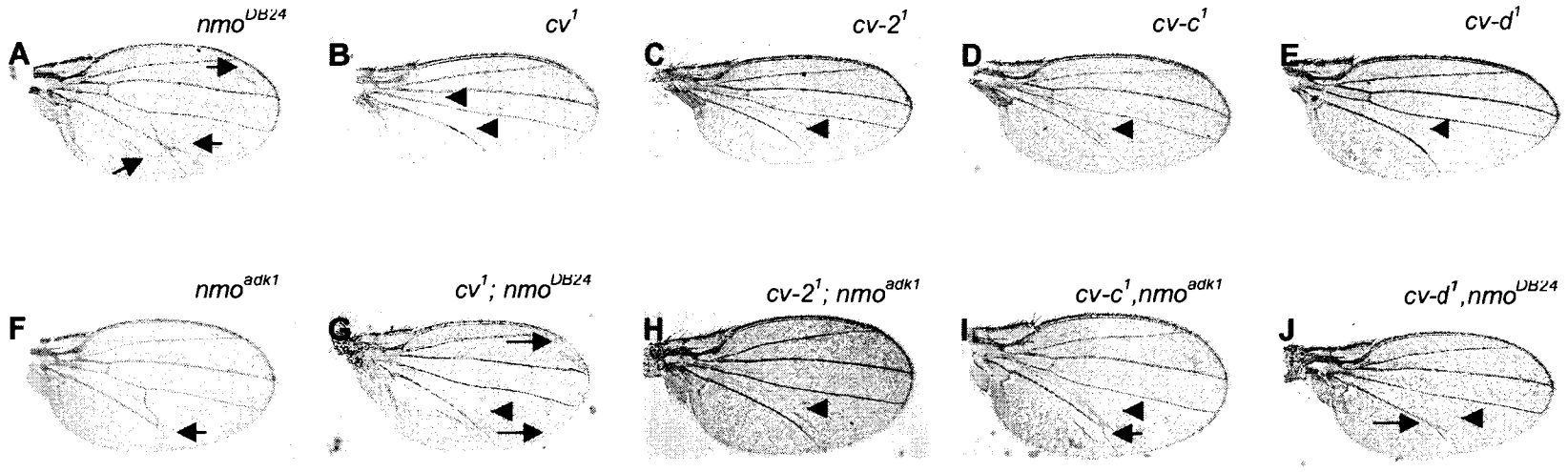
With the evidence of this possible interaction, I then proceeded to find out whether *nmo* interacts with these genes. This was done by generating double mutants between *nmo* and each of the *cv* genes, *cv, cv-2, cv-c,* and *cv-d.* Mutants for the cv genes produce wings that lack the pcv ($cv\text{-}2^{1}$, $cv\text{-}c^{1}$, and $cv\text{-}d^{1}$; Fig. 15 C, D and E) or both the acv and pcv (cv^{1}, Fig. 15B). The *nmo* mutant alleles used $nmo^{DB24}$and nmo^{adk1} both show ectopic veins near the pcv (Fig. 15 A and F respectively) and the nmo^{DB24} allele also show ectopic veins beneath L5 (Fig. 15A). Double mutants generally displayed an additive phenotype which is characterized by the *cv* loss of crossveins, and *nmo* ectopic veins (Fig. 15G, I, and J), except in the case of *cv-2; nmo* mutants where the *nmo* ectopic vein phenotype is lost completely; although the wings retain the round shape of *nmo* mutant wings (Fig. 15 H). This finding demonstrates that *cv-2* suppresses the *nmo* ectopic vein phenotype; and it is possible that these two genes interact at some level.

Figure 14: *cv* genes show genetic interaction with *dpp*.



Loss of *dpp* leads to truncations of the tip of veins L4 and L5 (D, arrows) and loss of the *cv* genes *cv-2* and *cv-c* results in loss of the pcv (arrowheads in B and C respectively). Double mutants of both *dpp* and either of the *cv* genes resulted in the display of the *dpp* phenotype (compare arrows in E and F to D).

Figure 15: ***nmo*** **shows some interaction with the** ***cv*** **genes**



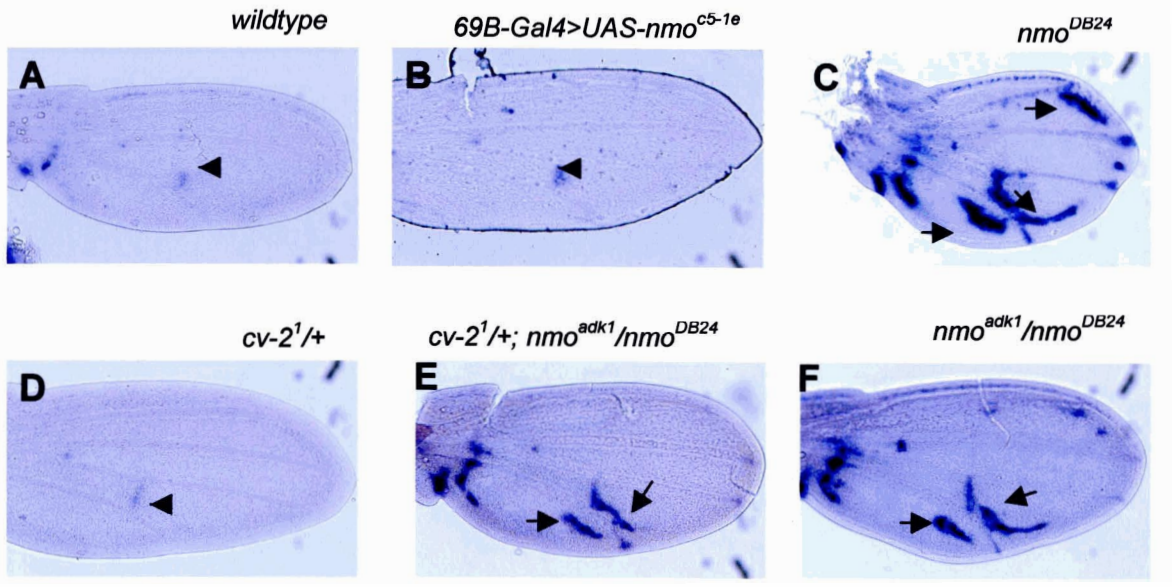
Loss of *nmo* leads to the formation of ectopic veins above L2, between L4 and L5 and beneath L5 (A, arrows) or close to the pcv (F, arrows). cv mutants show loss of both crossveins (B, arrowheads), or loss of the pcv (arrowheads in C, D, E). Both *cv* loss of crossvein phenotypes (arrowheads) and *nmo* ectopic vein phenotypes (arrows) phenotypes are present in most of the double mutants (G; I; and J); except in the case of *cv-2*^{1} where the *nmo* ectopic veins are totally missing (compare H to F).

Any possible effect of *nmo* on the BMP pathway through *cv-2* is likely to be intracellular, since Nlk is shown to be localised in the cytoplasm or the nucleus (Brott *et al.,* 1997); and the structure of Cv-2 suggests it is secretory and mainly extracellular (Conley *et al.*, 2000). Since *nmo* and vertebrate *nlks* are found to influence the effect of transcription factors (e.g. Lef/Tcf and CBP/p300) as reviewed above; a possible means of interaction might be at the level of transcription of the *cv-2* gene. I therefore monitored the effect of *nmo* on the transcription of the *cv-2* through *in situ* hybridization, a method that allows transcript levels to be detected within tissues.

Using this method, cv-*2* transcript levels were monitored in overexpressed Nemo and *nmo* mutant backgrounds in 28 hour pupal wings. Previous studies show that *cv-2* expression is highest at 28 hours apf (Conley *et al.*, 2000). *cv-2* transcript is located mainly in the two pcv in wildtype wings (Fig. 16A), and my results agree with published results (Conley *et al.*, 2000). I used the *69B-Gal4* epidermal driver to ectopically express *nmo* in the wing; and did not observe any clear difference in the *cv-2* transcript levels in the pupal wings of this genotype compared to wildtype (Fig. 16B). In this genotype the pcv is generally reduced or absent so this result is not surprising. However, in *nmo* mutant pupal wings *cv-2* transcript is present in high amounts in the ectopic veins (Fig. 16C) suggesting that Nemo may be required under wildtype conditions to inhibit *cv-2* transcription in the regions corresponding to extra veins. This will explain the similar levels of expression in wildtype and ectopically expressed *nmo* wings since any transcript in these cases is limited to the regular crossveins.

Although the *in situ* hybridization supports the assumption that *nmo* may be repressing *cv-2* transcription; it does not link it directly to this activity.

Figure 16: *nmo* regulates *cv-2* transcription



cv-2 in situ hybridization of 28 hr old pupal wings. The *cv-2* transcript is localized in the crossveins and appears to be unchanged in wildtype and misexpressed *69B-Gal4>UAS-nmo$^{c5\text{-}1e}$* (arrowheads in A and B respectively). It is however very high in *nmo^{DB24}* ectopic veins (arrows in C). Expression is slightly reduced in wings losing one copy of *cv-2* (D), but there is no noticeable change in expression in the *nmo* mutant background, whether the wings have one (E) or both (F) copies of *cv-2* (arrows in E and F).

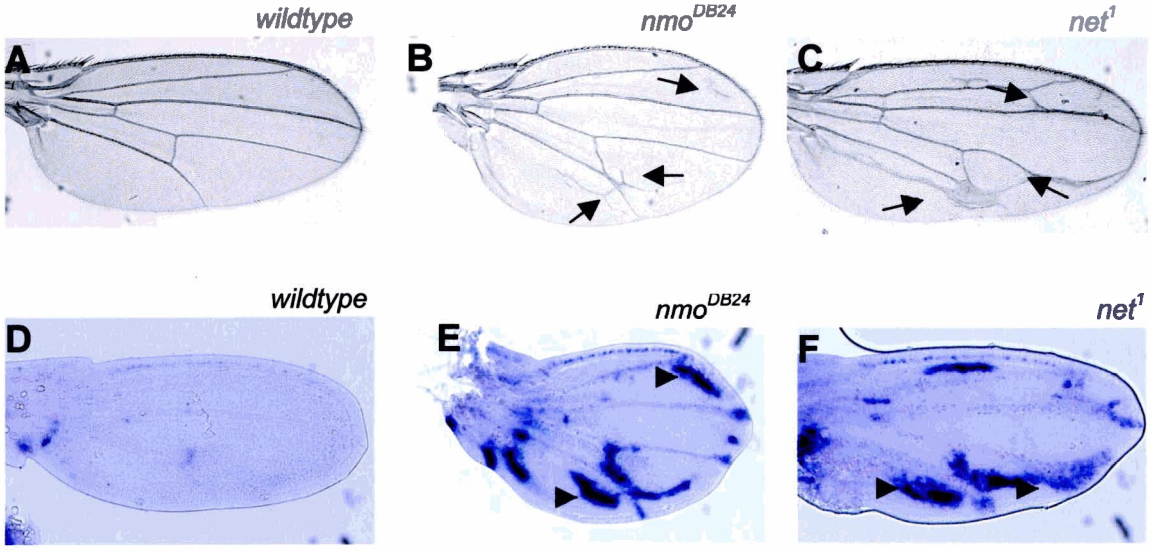
I attempted to see whether reduced levels of *cv-2* activity will result in a corresponding reduction of transcript levels in *nmo* mutant background. The *cv-2^{1}* allele is a hypomorph and shows reduced transcript levels as a heterozygote compared to wildtype levels (Conley *et al.*, 2000). Surprisingly, there was no difference in *cv-2* message levels in either heterozygous or homozygous *cv-2* in *nmo* mutant backgrounds (Fig. 16E or F). I conclude that loss of *nmo* may override any changes in *cv-2* transcription in the heterozygous wing, thus leading to the high *cv-2* transcript levels. Also since the ectopic veins are present in these wings, *cv-2* transcription is likely to go up; whether it is present in wildtype or heterozygous amounts.

One common feature of most genes that promote intervein fate (including *nmo*) is that their mutants exhibit ectopic veins in the wing. Since the high transcript levels are located within these extra veins in *nmo* wings, it is possible that all genes that promote the intervein fate may control *cv-2* transcription in a manner similar to *nmo*. If this is true then one will assume that other intervein-determining genes will likely have a similar effect on *cv-2* transcription. I monitored the expression in mutants of another gene, *net,* that like *nmo* promotes intervein fates, and shows ectopic veins in similar regions of the wing as *nmo* (compare Fig. 17B and C). Consistently, *cv-2* expression is detected within the extra veins in *net* mutants (Fig.17F, arrowheads), and more importantly appear to be of similar level compared to *nmo* mutants (compare Fig. 17E to F).

The above results indicate that *cv-2* levels must be well regulated to ensure correct vein specification; and support roles for *nmo* and other intervein-promoting genes in this regulation. Given the fact that *cv-2* is implicated in BMP signalling, *nmo*'s effect on the pathway may be at the level of *cv-2* transcription.

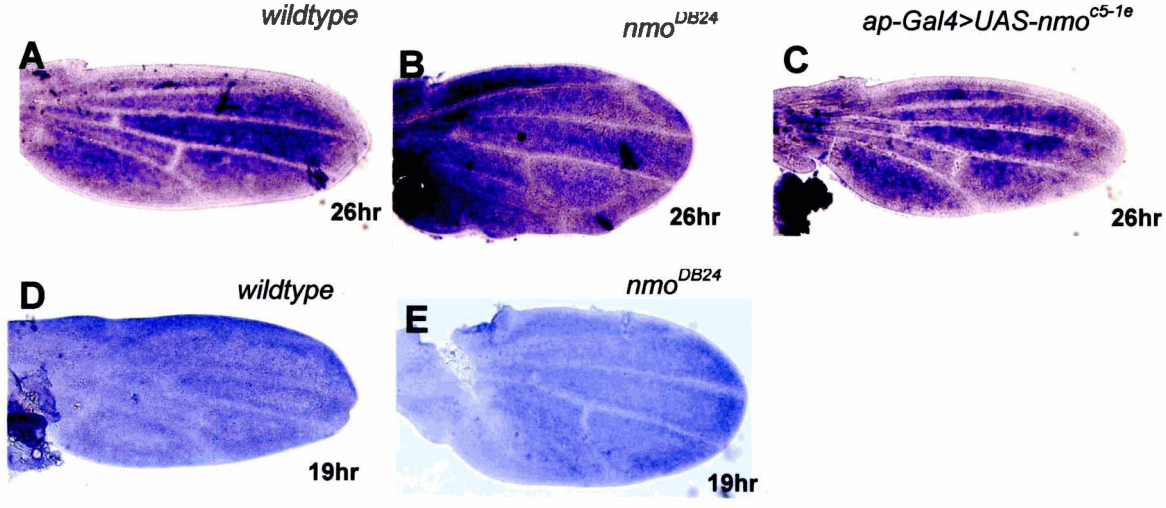
There is also a possibility of *nmo* positively controlling the transcription of the *sog* gene as a means of inhibiting the pathway, so I used *in situ* hybridization to follow the expression of *sog* at 19 and 26 hours apf in the pupal wings. However there is no change in the level of *sog* expression in wildtype , ectopic, or mutant *nmo* wings (Fig. 18A-E).

Figure 17: *nmo* and *net* produce similar effects on *cv-2* transcription



A-C shows adult wings and D-F are 28 hour pupal wings showing *cv-2* transcript localization. *cv-2* in situ hybridization in *net*^{1} pupal wings exhibit similar high expression in ectopic veins (F arrowheads), as seen in *nmo* (E).

Figure 18: *nmo* shows no effect on *sog* transcription.



sog in situ hybridization in wildtype (A and D); *nmo*^{DB24}; and *69B-Gal4>UAS-nmo*$^{c5\text{-}1e}$ pupal wings, at 26 hr apf (A-C), and 19 hr apf (D and E). *sog* expression levels appear to be unchanged in all cases.

Chapter Four: Discussion

nmo in vein development

The studies presented above support a regulatory role for Nemo on TGFβ signalling during wing development in *Drosophila*. The phenotypes of *nmo* mutants alone suggest the need for this gene in maintaining correct fates in the wing (Verheyen *et al.*, 2001; Mirkovic *et al.*, 2002). Additionally, *nmo* is expressed in the intervein regions (Verheyen *et al.*, 2001) in pupal wings and may be regulating intervein differentiation. The ectopic vein phenotypes in *nmo* mutant wings coupled with the localization of the transcript in pupal wings gives strong credence to a role in preventing vein formation.

Nemo antagonizes the BMP pathway

As discussed above the specification of vein or intervein fates in the *Drosophila* wing is directed by distinct sets of genes which interact to allow correct distribution of these structures in the wing. Generally intervein-determining genes (such as *bs* and *net*) act to promote intervein fates by suppressing vein-determining genes (such as EGFR) in regions that differentiate into intervein cells. Both *dpp* and *gbb* promote the formation of veins, while *nmo* likely promotes intervein fates. It is possible that *nmo* may interact with the BMP genes to suppress their effects in intervein regions. The interaction between *nmo* and *tkv* (the Type I BMP receptor) (Fig. 9) support the assumption that *nmo* may be an antagonist of TGFβ signalling. Further evidence supporting this line of thought is the similar results (as in the case of *tkv* and *nmo*) obtained using another positive modulator of TGFβ signalling, Mad (Zeng A, unpublished results) which produced a phenotype that

is rescued by *nmo* when both are overexpressed. Also the severity of ectopic veins seen in *nmo* mutants in combination with *dad-lacZ* further argues for a possible inhibitory role of *nmo* on this pathway. Since *dad* was shown to antagonize Dpp signalling (Tsuneizumi *et al.*, 1997), it is possible that both *dad* and *nmo* are needed to effectively inhibit the activities of Dpp in vein development. Perhaps the strongest evidence that implicates *nmo* as a negative regulator is the high levels of ectopic pMad in *nmo* mutant pupal wings. As discussed above, pMad levels represent one of the ways through which BMP signalling activity is measured. Interestingly, pMad levels remain localized mainly within the regular veins but are present in the ectopic veins in *nmo* mutants, clearly demonstrating a role for *nmo* in suppressing BMP signalling, probably at regions where the ectopic veins differentiate in *nmo* mutants.

As explained above, vein formation is well regulated to maintain the species-specific pattern in *Drosophila melanogaster*. Several genes identified as promoting intervein fates have been shown to repress the activities of vein-promoting genes outside the vein-competent domains. A well characterized example is the *bs* gene which is expressed in the intervein regions and inhibits vein formation at the third instar and the pupal stages. Also *net* is shown to inhibit the expression of *rho* in intervein cells (Brentrup *et al.*, 2000). Recall that *rho* is expressed in the vein cells where it mediates EGFR signalling at both larval and pupal stages. Another example is the restriction of EGFR signalling to larval and pupal provein cells by *Notch*. BMPs play prominent roles to promote vein fate in the wing; hence they need to be controlled to allow correct vein patterning. Nemo may be one of several inhibitory genes that may suppress BMP signalling in certain regions of the wing to ensure correct vein specification. The results

presented in this study support such an antagonistic role for Nmo on BMP signalling. However the exact mechanism(s) through which this regulation might be occurring is not known and a number of possibilities are discussed below.

Regulation of signalling

Tkv

As reviewed earlier, the Type I receptor is subject to regulation to prevent ligand-independent signalling. This regulation could be used as a means of suppressing ligand-induced signalling by regulators. Nemo may therefore be acting on the cytoplasmic region of Tkv to lock it in a non-functional state which could explain the rescue of the *UAS-tkv* phenotype by *UAS-nmo*. On the other hand, *nmo* may interact with, and inhibit other components of the pathway to produce the same effect on Tkv as observed.

dad

Dad has been shown to specifically antagonize Dpp signalling (Tsuneizumi *et al.*, 1997); if *nmo* also acts to inhibit BMP signalling, then the effect of losing both *nmo* and *dad* might be enough to permit uncontrolled TGFβ signalling resulting in the more severe ectopic wing vein phenotype (Fig. 9D). However, it is possible that Dad interacts with Nemo physically, implying they both need each other's activity to fully exert their effects on TGFβ signalling. Such a co-operation between I-Smads and other proteins has been seen by others. For example, Yasuda *et al* demonstrated that Tob protein is able to repress BMP signalling in *Xenopus* through its interaction with I-Smads (Yasuda *et al.*, 2003). Smurfs also induce nuclear export of I-Smads and have been shown to mediate their inhibitory activity on TGFβ signalling in vertebrates (Ebisawa *et al.*, 2001). There is

no evidence so far which identified an activator for Dad, and Nemo could be a potential candidate. This interaction between Dad and Nemo if true, may be necessary to activate Dad's binding to the receptor or Mad. It may also allow Dad to be recognized by Dsmurf, which will induce its nuclear export. We are currently doing biochemical studies to determine whether nmo and dad might be interacting physically and the relevance of this possible interaction on Dad's inhibitory role in TGFβ signalling.

Mad

Mad presents yet another potential source of crosstalk between the BMP pathway and Nemo. R-Smads are targeted by several proteins to regulate TGFβ signalling. These Smads contain MAPK phosphorylation sites (PXSP sequence) in their linker region (Itoh *et al.*, 2000; Massague, 1998) (Fig. 2). MAPK phosphorylation of Smads at these sites prevents the nuclear accumulation of Smad complexes (Kretzschmar *et al.*, 1997a, 1999) and is believed to be a means of regulating TGFβ signalling (Massague, 1998). In vertebrate cells, the MAPK Erk under the influence of Ras phosphorylates the R-Smads on their linker domain, and prevents their ligand-induced nuclear import, resulting in impaired TGFβ signalling. It is also believed that other kinases can equally target these Erk phosphorylation sites in Smads (reviewed in Derynck and Zhang, 2003). In view of this, it may be true that Nemo phosphorylates Mad in these regions to impair BMP signalling. This is quite interesting, as analysis between mouse *Nlk* and Erk reveal some similarity between these proteins (Brott, 1998). Additionally, A. Zeng (Verheyen lab) recently found that Nemo binds Mad in cell culture, raising the possibility of Nemo acting through Mad to affect TGFβ signalling. It is possible that Nemo phosphorylates Mad at MAPK sites in its linker region; preventing it from translocating to the nucleus

and thus inhibiting BMP target genes. As discussed earlier, Smurfs target Smads for proteosome mediated-degradation; and recognize Smads through a PY motif in the linker region (Fig. 2). It is possible that Nemo may be required to phosphorylate Mad to allow DSmurf to recognize and degrade it. One may also argue that the binding of Nemo to Mad is likely to prevent the phosphorylation of Mad by the receptors. Any of these, if true might explain the elevated pMad levels in the absence of functional Nemo.

Transcriptional regulation of mediators

In theory, it is possible that Nemo inhibits transcriptional activity of the Smads on target genes as a means of suppressing signalling; especially since Nlk plays a similar role in regulating Wnt signalling in vertebrates (Ishitani *et al.*, 1999). As mentioned earlier, Nlk is found to inhibit the transcriptional activity of Smads indirectly by repressing the Smad transcriptional coactivator, CBP/p300 (Yasuda *et al.*, 2003). The level of ectopic pMad staining seen in the absence of Nemo activity however suggests that Nemo might be functioning upstream, or at the level of Mad; or may influence the transcription of mediators or regulators of TGFβ signalling.

Sog has been shown to inhibit BMP signalling, especially in wing development (Yu *et al.*, 1996). The complexity of the genetic interaction between *nmo* and *sog* as well as *nmo*'s lack of influence on *sog* transcription probably rules out *sog* as a possible mediator of *nmo*'s effect on the BMP pathway. Further genetic studies using loss of function alleles of *sog* may be important in making this assumption.

The Cv-2 protein has been linked with the BMP pathway as a positive modulator (Conley et al., 2000); and the results presented here (Fig. 14E) support the view that *cv-2*

is acting upstream of BMPs and may be essential to their function. This leaves Cv-2 in an important position in the BMP pathway, and its levels may be essential in regulating the pathway. Nemo's effect on *cv-2* transcription raises the possibility that it may be interacting with the BMP pathway through its regulation of *cv-2* transcription. It is likely that *nmo* downregulates *cv-2* transcription in the interveins; and its absence is what causes elevated transcription of *cv-2* as seen in the ectopic veins (refer to Fig. 16C). Double mutants of *nmo* and *cv-2* show more of the *cv-2* phenotype (Fig. 15H) implying that *cv-2* may be downstream of Nemo function; probably at the level of transcriptional regulation. It is striking that both *cv-2* expression and pMad levels are elevated in the *nmo* ectopic veins. One may conclude then that lack of the inhibitory effect of Nemo on *cv-2* message may lead to hyperactivation of the BMP pathway (accounting for the high pMad levels) in regions that correspond to the extra veins. It may also be true that Nemo is affecting both events simultaneously to effectively control signalling.

A drawback to the assumption above is that *cv-2* transcription is equally affected by another intervein-determining gene; *net* (Fig. 17F). However, BMP-induced activity may be controlled through the suppression of *cv-2* transcription in the intervein region by certain genes that promote intervein fate, including *net* and *nmo*. This perception could be true, given that the *cv-2* transcript is localized specifically in the crossveins, and appear only in the ectopic veins in both *net* and *nmo* mutants. This specific localization will imply that the ectopic veins seen in these mutants are of the crossvein fate. Insect wings are thought to have arisen from ancestors with many veins that have undergone progressive loss of veins through evolution (de Celis and Diaz-Benjumea, 2003). It is suggested that crossveins might have arisen as a result of fusion between some

longitudinal veins during evolution (Garcia-Bellido and de Celis, 1992; de Celis and Diaz-Benjumea, 2003). Probably Drosophila have evolved certain genes (including *cv-2*) to maintain the crossveins; and others (including *net* and *nmo*) to prevent reversal to the ancestral state (i.e. appearance of ectopic veins) through the repression of *cv-2* transcription. Cv-2 may in turn function through the BMP pathway (among others) to promote the crossvein fate as demonstrated already (Conley *et al.*, 2000) and in this study. Therefore *net* and *nmo* may both inhibit BMP signalling by virtue of repressing *cv-2* transcription. There is no evidence that *net* functions to inhibit BMP signalling; probably pMad staining of *net* mutants could be useful in testing this possibility.

The TGFβ pathway is controlled by various regulators to ensure correct signal levels in the cell. Nmo and its homologues are emerging as regulators of signalling pathways to mediate correct development in various organisms. Results from this study suggest potential involvement of *nmo* in regulating TGFβ signalling in the *Drosophila* wing. The initial evidence that connected *nmo* to the TGFβ pathway is through Tak1, but studies so far indicate that dTak1 might not be playing any role in wing development (Mihaly et al 2001; Vidal et al., 2001). *nmo*'s effect on wing development may therefore be independent of Tak1. Recently two additional *tak*-like genes have been identified in *Drosophila*, so their characterization will be useful to determine whether the *tak/nmo* pathway contributes to wing vein patterning.

In this study however *nmo* appears to function as an inhibitor of the TGFβ pathway in wing development. This activity is presumably essential to prevent BMP-induced ectopic vein formation in the wing. Further studies will be important to address the question of how *nmo* specifically interacts with the pathway. This regulatory role of

nmo may be of high significance in addressing developmental conditions that may be due to excessive TGFβ signalling. It will be interesting to see if this possible interaction occurs in other organisms, including vertebrates.

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