Proteolytic Processing as a Regulator of BMP-type Signaling in *Drosophila* Development

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Academic dissertation

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With a solar knife I split the sky
And walk right in between
To search the answers to every “why?”
Where I have seen the unseen

Edlund

CONTENTS

1 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I **Künnapuu, J.**, Björkgren, I. and Shimmi, O. (2009) The *Drosophila* DPP signal is produced by cleavage of its proprotein at evolutionary diversified furin-recognition sites. *Proc. Natl. Acad. Sci. U. S. A.* 106, 8501-8506.

II **Künnapuu, J.**, Tauscher, P., Tiusanen, N., Nguyen, M., Löytynoja, A., Arora, K. and Shimmi. O. Cleavage of the *Drosophila* Screw prodomain is critical for a dynamic BMP morphogen gradient in embryogenesis. Manuscript in preparation.

III **Künnapuu, J.** and Shimmi, O. (2010) Evolutional Imprints on the Sequences of BMP2/4/DPP Type Proteins. *Fly (Austin)* 4, 21-23.

Contributions:

I The author contributed in planning the experiments, conducting almost all the experiments and data analysis, and writing the manuscript.

II The author contributed in planning the experiments, conducting most of the experiments and data analysis, and writing the manuscript.

III The author contributed in writing the manuscript.

2 ABSTRACT

A small set of highly conserved signaling molecules performs a great number of tasks in different animals and developmental contexts. Among them, the bone morphogenetic proteins (BMPs) constitute a group of growth and differentiation factors that are involved in numerous developmental processes affecting cell proliferation, apoptosis and differentiation. In the fruit fly, *Drosophila melanogaster*, three BMP type proteins have been identified, each of which has a homolog in mammals. Decapentaplegic (Dpp) is a BMP2/4 type protein which plays a major role in dorsal-ventral patterning of the early embryo. It participates in midgut development, patterning and growth of imaginal tissues, wing vein formation and maintenance of germline stem cells in the germarium. Dpp is a morphogen which requires a second BMP type protein, Screw (Scw) or Glass bottom boat (Gbb) to be able to form proper concentration gradients in developing tissues. Scw and Gbb belong to the BMP5/6/7/8 subfamily and their expression domains are different; Scw is specifically expressed during the early events of embryogenesis, while Gbb has more functional roles during later stages of fly development, like wing morphogenesis.

BMP type proteins are produced as large proproteins that require proteolytic cleavage prior to secretion and extracellular gradient formation. This study concentrated on the cleavage of Dpp and Scw to reveal the meaning of post-translational modifications in concentration gradient formation and BMP signaling.

Three furin recognition sites were identified in the Dpp proprotein. Mutational analyses indicate that the upstream optimal furin site of the prodomain (furin site (FS) II) is critical for producing ligands and creating a long range concentration gradient in a wing imaginal disc. Cleavage of the other two FSs produce the differently sized Dpp ligands that contribute to BMP gradient formation in the early embryo and wing imaginal disc. It was noted that the cleavage requirements of BMP2/4 type proteins in different species vary to establish species-specific regulation of BMP signaling.

Discovery of the scw^{E1} allele, that causes dominant negative effect in embryos heterozygous for a hypomorphic *dpp* allele, gave more information about how the cleavage patterns of prodomains can contribute to creating diversity in the regulation of signaling. The mutation responsible for the dominant negative function in scw^{E1} was located in the cleavage site that is in the prodomain of Scw. Mutational analyses showed that the mature ligand of Scw^{E1} is produced in lower amounts and in complex with an N-terminal prodomain peptide. Scw^{E1} preferentially binds Dpp and disrupts normal gradient formation possibly through interactions with molecules within the extracellular matrix.

Phylogenetic analyses and functional studies of BMP cleavage mutants propose a mechanism by which post-translational regulation of proproteins modulates BMP signaling.

3 ABBREVIATIONS

after pupariation	AP
Amontillado	Amon
anterior	A
anterior-posterior	A-P
anterior crossvein	ACV
anti-Müllerian hormone	AMH
Bicoid	Bcd
Bone morphogenetic protein	BMP
Brinker	Brk
Crossveinless	Cv
Dally-like	Dly
Decapentaplegic	Dpp
Dorsal	Dl
dorsal	D
dorsal-ventral	D-V
Engrailed	En
extracellular matrix	ECM
Furin	Fur
Furin recognition site	FS
germline stem cell	GSC
Glass bottom boat	Gbb
glycosylphosphatidylinositol	GPI
Hedgehog	Hh
heparan sulfate proteoglycan	HSPG
c-Jun amino-terminal kinase	JNK
large latent complex	LLC
latency associated peptide	LAP
longitudinal vein	L / LV
Mothers against dpp	Mad
Nanos	Nos
Optomotor blind	Omb
Pannier	Pnr
Pentagon	Pent
perivitelline injection	PVI
Phosphorylated Mad	pMad
posterior	P
posterior crossvein	PCV
proprotein convertase	PC
Punt	Put
Saxophone	Sax
Screw	Scw
shibire	*shi*
Short gastrulation	Sog
Shrew	Srw
signal peptide	SP
Spalt	Sal
Thickveins	Tkv
Tolloid	Tld
Tolloid-related	Tlr
Torso	Tor
Transforming growth factor-β	TGF-β
Twisted gastrulation	Tsg
ventral	V
Vestigial	Vg
Viking	Vkg
Zerknüllt	Zen

4 REVIEW OF THE LITERATURE

4.1 Signaling molecules in embryo development

The difference between a cluster of single celled organisms and a cluster of cells in a multicellular organism is in their ability to organize different functions in different cell groups. A single celled organism is responsible for all of the functions that will keep it alive and capable of reproducing. When the amount of cells in a multicellular organism is growing, it is possible for the cell populations to specialize and acquire different tasks in the animal. This cell differentiation is enabled by signaling molecules and organized signaling networks that will help to inform different cell populations about their position and role in the organism. Properties required for this kind of intercellular communication include the ability to secrete proteins and form concentration gradients. These requirements are fulfilled by proteins that are classified as morphogens.

A well-known example of cell fate determination assisted by morphogens is the patterning of the fruit fly, *Drosophila melanogaster*, embryo. The first cues about the spatial orientation and the future head and tail of the embryo are given already before fertilization. Maternal mRNAs are distributed unevenly to give rise to a rough developmental map. First, this map consists of four systems of maternal morphogenetic fields that identify the anterior, posterior and terminal parts, and the dorsal and ventral parts of the embryo (Reviewed in Akam, 1987). Maternal mRNA of a transcription factor Bicoid (Bcd) is provided at the anterior part of the oocyte. After fertilization the translated proteins form a concentration gradient along the anterior-posterior (A-P) axis, and nuclei in the syncytium activate gene expression based on the concentration of Bcd they encounter (Driever and Nusslein-Volhard, 1988). At the posterior pole of the oocyte mRNA of *nanos (nos)* is localized and subjected to a translational control to allow expression of genes required for abdominal development (Andrews *et al*., 2011; Bergsten and Gavis, 1999). Torso (Tor) belongs to a third group of maternal proteins required for A-P patterning. This receptor tyrosine kinase defines the terminal regions of the embryo (Casanova and Struhl, 1989; Klingler *et al*., 1988). Graded nuclear localization of the Dorsal (Dl) morphogen, which is highest at the ventral side of the embryo, specifies the dorsal-ventral (D-V) axis (Reviewed in Reeves and Stathopoulos, 2009). A-P morphogenetic fields give rise to signaling of gap, pair-rule and segment-polarity genes, which comprise the segmentation signaling network. In addition, homeotic genes are activated to specify different segments (Akam, 1987). The D-V system interacts with Bone morphogenetic protein (BMP) -type molecules Decapentaplegic (Dpp) and Screw (Scw) to activate genes that are involved in differentiation of presumptive mesoderm, neuroectoderm and dorsal ectoderm (Arora *et al*., 1994; Reeves and Stathopoulos, 2009).

4.1.1 Morphogens form concentration gradients in tissues

The most representative definition set for a morphogen is the molecule's ability to form a concentration gradient and activate target genes in a concentration dependent manner. Different genes in different cells are turned on and off according to the amount of morphogens they encounter (Reviewed in Rogers and Schier, 2011). Because of this definition, it is understandable that changes in morphogen concentrations cause severe developmental defects. As an example the BMP morphogen gradient, which is required for the D-V patterning in the early development of the *Drosophila* embryo, consists mainly of Dpp molecules, and elevated activity of Dpp leads to development of more dorsal cell fates. The amnioserosa derives from the eight to ten cells that lie adjacent to the

dorsal midline, and is defined by the highest Dpp signaling levels. Injection of *dpp* mRNA makes more laterally situated dorsal ectoderm to acquire amnioserosa cell fate. The absence of Dpp causes ventralization of the embryo (Ferguson and Anderson, 1992; Irish and Gelbart, 1987). The dosage of BMP-4 signaling, a human ortholog for Dpp, has been stated to be affected in some severe disorders. Elevated BMP-4 signaling, caused by mutations in an antagonist *noggin* gene, causes multiple synostoses syndrome, a genetic disease characterized by fusion of the joints (Gong *et al*., 1999). Another severe disease linked to over-activated BMP-4 signaling is fibrodysplasia ossificans progressiva (Kaplan and Shore, 1998; Shafritz *et al*., 1996). This crippling hereditary disorder is characterized by postnatal formation of ectopic bone.

The morphogenetic gradient formation is adjusted on many levels. Transcription and translation are regulated by complicated protein networks. Gradient formation and signaling intensities are regulated by post-translational modifications and binding proteins.

4.2 BMP type signaling in Drosophila

BMPs belong to the Transforming growth factor-β (TGF-β) superfamily of growth and differentiation factor proteins, and they play key roles in developmental processes that are regulated at many different levels. In *Drosophila*, three BMP-type proteins have been found and each of these has a counterpart in vertebrates. Dpp belongs to a subfamily of BMP2/4 type proteins. Scw and its paralog Glass bottom boat (Gbb) belong to the BMP5/6/7/8 subgroup. As is illustrated in Figure 1, these ligand dimers signal through receptor complexes that are formed of two type I, and two type II serine-threonine kinases (Kirsch *et al*., 2000a; Kirsch *et al*., 2000b). After the dimeric ligand binds to the receptor complex of type I receptors Saxophone (Sax) and Thickveins (Tkv), and the type II receptor Punt (Put), the type II receptor phosphorylates the type I receptor (Brummel *et al*., 1994; Letsou *et al*., 1995; Penton *et al*., 1994). This leads to the phosphorylation of the sole intracellular *Drosophila* Smad, Mothers against decapentaplegic (Mad). Phosphorylated Mad (pMad) forms a complex with the co-Smad Medea, which then translocates into the nucleus to regulate expression of target genes (Newfeld *et al*., 1997; Raftery *et al*., 1995).

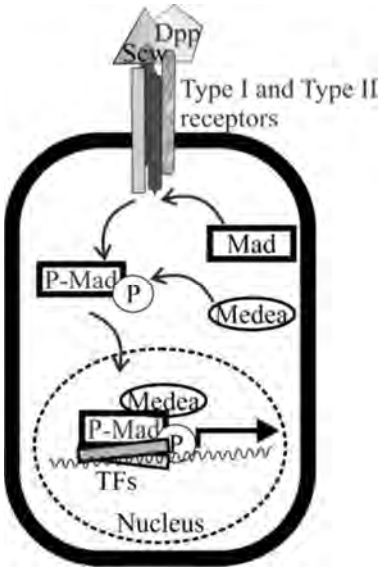

Figure 1. BMP signal is produced through receptor complexes situated on the plasma membrane. Intracellular Mad is phosphorylated by activated receptors. Medea binds pMad to translocate into the nucleus, and in concert with other transcription factors (TFs), controls target gene expression.

4.3 Expression of Dpp

Graded nuclear localization of the protein Dl in *Drosophila* embryo creates the basis for *dpp* expression. As is illustrated in Figure 2 J, Dl activates or represses target gene expression in concentration dependent manner (Reviewed in Reeves and Stathopoulos, 2009). Dl-regulated silencer elements of *dpp* respond to even the lowest levels of nuclear Dl, and that restricts *dpp* expression to dorsal regions (Huang *et al*., 1993). A protease required for Dpp signaling, Tolloid (Tld), is repressed at the same lateral and ventral domains (Kirov *et al*., 1994). Dpp is expressed throughout the dorsal half of the embryo as can be seen from mRNA accumulation in Figure 2 C, but the protein forms a concentration gradient that peaks in dorsal-most cells by the onset of gastrulation in Figure 2 G (Dorfman and Shilo, 2001; Shimmi *et al*., 2005b). Perivitelline injections (PVI), in which an antibody is injected into the space between the cell membrane and the vitelline membrane of live embryos, showed the extracellular accumulation of receptor-bound Dpp on the narrow stripe of the dorsal-most region (Wang and Ferguson, 2005). The pattern of pMad, the output of Dpp signaling follows the dynamic spatial distribution of Dpp during embryo development. pMad staining is broad and shallow (Figure 2 E) during early and mid-stage 5 but sharpens over a 30 min period to form a narrow stripe at the dorsal side (Figure 2 H) by the beginning of stage 6 (Wang and Ferguson, 2005).

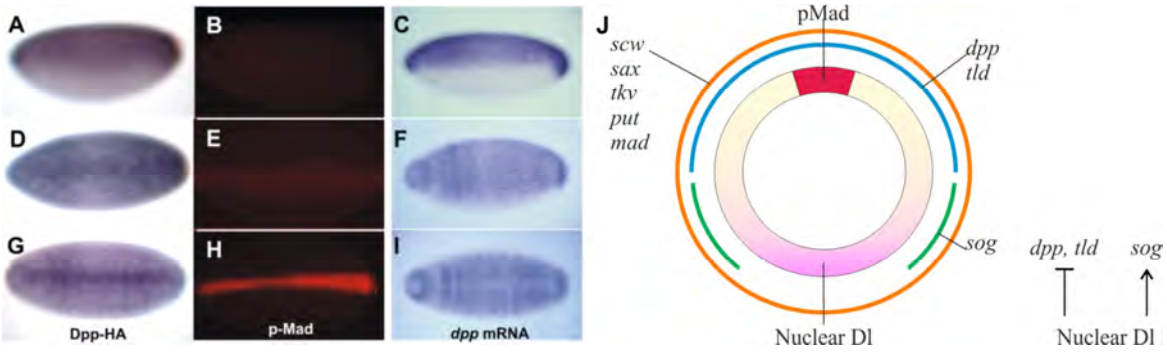

Figure 2. Dpp gradient formation in the blastoderm embryo. (A-I) Dpp-HA staining in a Dpp-HA transgenic embryo (A, D and G), pMad staining (B, E and H), and *in situ* hybridization of *dpp* mRNA (C, F and I) in a wild-type embryo. Early (A-C), middle (D-F), and late (G-I) blastoderm stages, lateral view (A-C) and dorsal view (D-I). Dpp is localized in the dorsal half of the embryo at the early blastoderm stage, as is *dpp* mRNA. The protein is concentrated at the dorsal midline by the onset of gastrulation and sharp pMad staining appears. (J) Cross-section of a *Drosophila* embryo showing the expression of different genes affecting BMP gradient formation. Nuclear localization of Dl (violet) represses the expression of *dpp* and *tld* (blue). Low levels of Dl activate the expression of *sog* (green). The BMP receptors Sax, Tkv and Put, as well as the second BMP type protein Scw are expressed uniformly (orange). The outcome of BMP signaling, staining of pMad can be seen in the dorsal-most cells even though *mad* is expressed uniformly (orange). (Figures A-I are reprinted from (Shimmi *et al*., 2005b), with permission from Elsevier.)

Another tissue that is widely used to study patterning is the *Drosophila* wing imaginal disc. The *Drosophila* wing develops from the larval imaginal disc – a single-layered sac of polarized epithelial cells. The disc is subdivided into anterior (A), posterior (P), dorsal (D), and ventral (V) compartments that are demarcated by different protein expressing cells (Reviewed in Tabata, 2001). The posterior compartment is identified by the expression of *engrailed (en)*. In response to *en* expression, the P cells start to secrete Hedgehog (Hh) which acts as a morphogen and signals to A compartment cells. As is seen

in Figure 3 these two proteins roughly pattern the central domain of the wing blade primordium and induce the expression of *dpp*. In the wing imaginal disc *dpp* is expressed in a stripe of cells adjacent and anterior to the A-P compartment boundary. The chimeric protein of Dpp and green fluorescent protein (GFP) made it possible to visualize the extracellular protein in wing imaginal disc, and it was noted that the protein forms a long range concentration gradient to pattern the wing (Capdevila and Guerrero, 1994; Entchev *et al*., 2000).

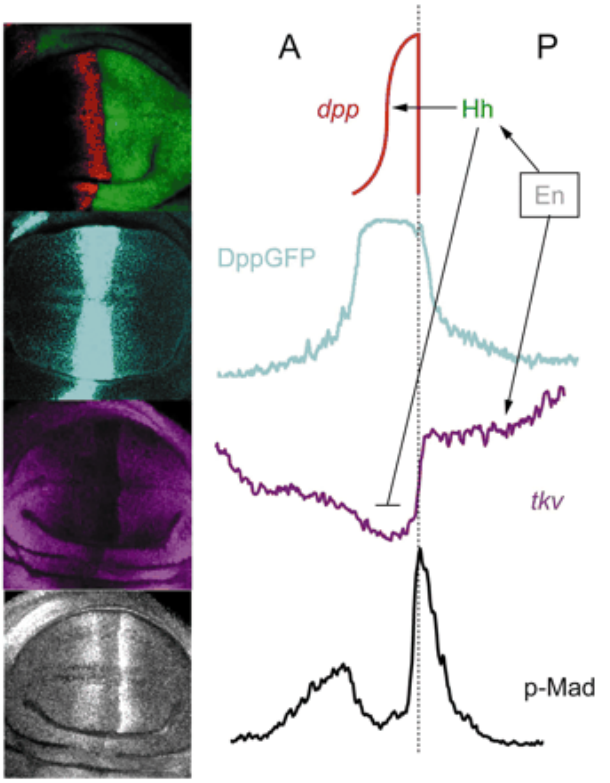

Figure 3. Dpp activity gradient in the wing imaginal disc. Confocal microscopy images (left) and schematic figures (right) showing Dpp gradient formation in the part of the wing imaginal disc that develops into an adult wing. En regulates the posterior (P) expression of *hh* (green) and *tkv* (purple). Hh induces *dpp* expression (red) along the anterior (A)/P border. Dpp diffusion is visualized by GFP-tagged Dpp (blue). The inhibitory effect of Hh on *tkv* expression on the anterior side of the A/P boundary shapes pMad gradient (gray). PMad intensity is highest on the posterior side and in the vicinity of A/P boundary. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics, Tabata, 2001.)

When taking into account the different expression profiles of *dpp* and the different protein distributions in the early embryo and in the wing imaginal disc, it is clear that the expression profile cannot explain why Dpp is found in different concentrations in different places of the tissues. In addition, it was noted that Dpp gradient did not form by simple diffusion. Chimeric GFP-Dpp proteins were created to study diffusion. Secreted GFP and Dpp sequences including Dpp cleavage and secretary transport domains were combined. The chimeric proteins were not able to form a gradient because they lacked the mature Dpp peptide (Entchev *et al*., 2000). This study suggests that there must be other mechanisms that affect Dpp gradient formation, possibly some extracellular molecules interacting with the mature Dpp to enhance/restrict the movement. These mechanisms are presented next.

4.4 BMP gradient formation in the embryo

Since Dpp has been shown to form concentration gradients and regulate target gene expression in a concentration dependent manner, it was classified as a morphogen (Nellen *et al*., 1996; Wharton *et al*., 1993). For a long time it has been known that Dpp can form a sharp concentration gradient in the embryo but the mechanisms behind this were unknown. Even the role of receptors could not shed light on the issue since the Dpp type I receptor Thickveins (Tkv) and the type II receptor Punt (Put) have a uniform maternal distribution (Neul and Ferguson, 1998). The morphogenetic properties of Dpp have intensely been studied in *Drosophila* development and the role of binding proteins has explained many questions concerning the protein's ability to form concentration gradients.

4.4.1 Sog, Tsg, Tld and Srw

Dl protein has dual functions in the regulation of gene expression in the embryo. The same low levels that repress *dpp* expression in ventro-lateral regions, activate the expression of *short gastrulation (sog)* (Markstein *et al*., 2002). This protein binds extracellular Dpp and together with another dorsally expressed protein called Twisted gastrulation (Tsg), inhibits receptor binding (Ross *et al*., 2001). Tld, which is dorsally expressed, is a metalloprotease that cleaves Sog and liberates Dpp for receptor binding and signaling. The embryos which have a genotype of $tld^{-/-}$ fail to develop amnioserosa. The phenotype is due to a loss of Dpp signaling, since no extracellular receptor-bound Dpp was seen in perivitelline space after PVI (Ross *et al*., 2001; Wang and Ferguson, 2005). The expression profiles of *sog* and *tld* explain why there is no Dpp signal in the lateral and ventral domains of the embryo, but not the issue of how the gradient is formed. In fact, the phenotypes of *sog* and *tsg* mutants suggest that these two gene products play an important role during gradient formation. Interestingly, only the peak signaling in the dorsal-most cells is lost in null mutants and low level signaling is spread over the whole dorsal side (Ross *et al*., 2001). In addition, perivitelline injections to examine extracellular Dpp distribution in *sog* and *tsg* mutants showed wider dorsal localization of receptor-bound Dpp (Wang and Ferguson, 2005). It was shown that a small amount of Sog-independent Dpp diffusion occurs but the majority of protein is trapped at the expression site by the receptors. However, the role of Tsg seems to be more complex. Since in *tsg* mutants only a small amount of receptor-bound Dpp was seen in the perivitelline space, it was suggested that Tsg promotes Dpp – receptor interactions (Wang and Ferguson, 2005). Tsg-like protein Shrew (Srw) activity is required for the maximal signaling of Dpp in the dorsal-most cells of the embryo. The expression of *muscle segment homeobox* (*msh*), a homeobox gene specifying the dorsal region of the embryo (Isshiki *et al*., 1997), is broader in *srw* mutants, so that there is more Dpp available for signaling at the lateral domains or Dpp is better able to signal (Bonds *et al*., 2007). Srw's role in signaling can be in localization or activation of the Dpp ligand at the dorsal surface of the embryo. The proposed model for BMP-gradient formation in the early embryo is shown in Figure 4.

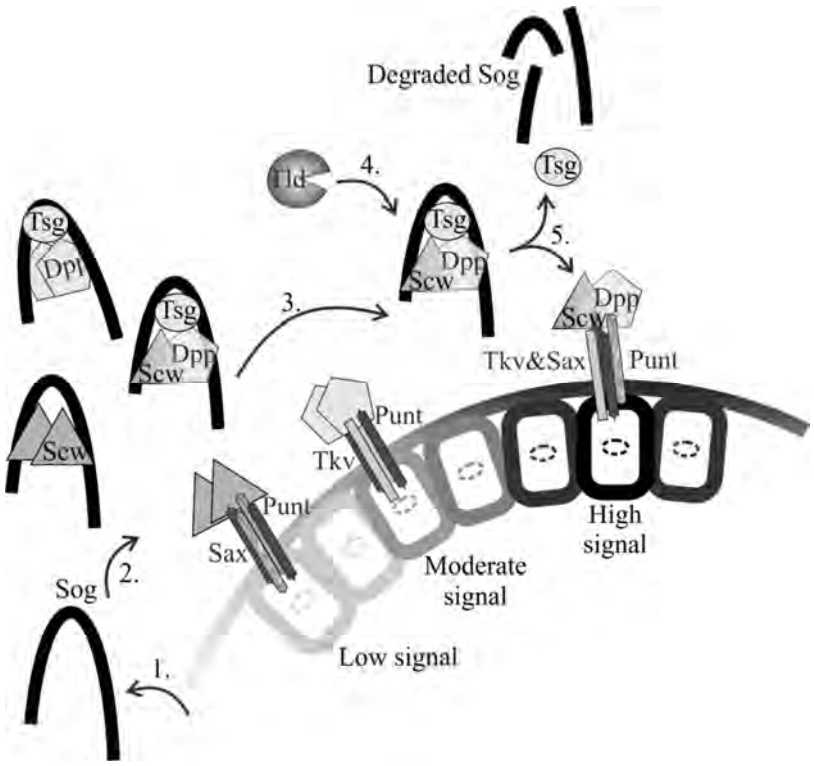

Figure 4. BMP gradient formation and signaling in the early *Drosophila* embryo. Partial cross section of a cellularized embryo is shown. Dpp/Scw dimers form shuttling complexes with Sog and Tsg in the lateral regions of the embryo to inhibit receptor binding (1-3). Tld releases the heterodimers proteolytically (4-5). As Sog concentration is reduced in the dorsal-most regions, more dimers are released by Tld. Dpp homodimers, Dpp/Scw heterodimers, and Scw homodimers participate in BMP gradient formation with differential signaling intensities. BMP signal is produced through receptor complexes situated on the plasma membrane. Dpp signals through Tkv, and Scw signals through Sax. BMP signal increases in dorsal-most cells and activates high-threshold target genes, such as *race* and *zerknüllt (zen)*. Dpp homodimers cause moderate signal when bound to receptors whereas Scw homodimers signal at low intensity. Low level target genes, like *pannier (pnr)*, are transcribed in the dorsolateral regions.

4.4.1.1 Dynamin fine tuning the Sog distribution

The ventrolateral expression of Sog is the key to inhibiting Dpp signaling in dorsolateral cells and promoting it in the dorsal-most cells. As Figure 4 shows, the net flux of Sog away from its site of synthesis creates the basis for gradient appearance. The notion that Sog is more abundant in ventral side than the dorsal side of the *sog* expression domain suggests that there are also other forces affecting Sog distribution and hence Dpp signaling. The above mentioned dorsally expressed metalloprotease *tld* limits the amount of Sog in Dpp dependent fashion, but there is also a Dpp independent mechanism that limits the active Sog extracellularily. A *shibire (shi)* gene product, Dynamin, was shown to affect the amount of extracellular Sog (Srinivasan *et al*., 2002). This protein is required in the endocytosis related signaling of many growth factors, like Wingless, Epidermal growth factor and even Dpp, as will be discussed later (Bejsovec and Wieschaus, 1995; Entchev *et al*., 2000; Vieira *et al*., 1996). Dynamin-mediated membrane retrieval of Sog is required for fine tuning of the gradient since temperature sensitive shi^{ts} mutants showed elevated levels of Sog protein and greatly reduced pMad levels in the dorsal half of the embryos. This phenotype was partly rescued through a process of injecting the double-stranded RNA of *sog* (Srinivasan *et al*., 2002).

4.4.2 Scw

Another BMP type protein expressed during embryogenesis is Scw and it is required for BMP-signaling in somatic cells before gastrulation (Arora and Nusslein-Volhard, 1992; Arora *et al*., 1994; Dorfman and Shilo, 2001). *Scw* is expressed uniformly as Dpp is expressed only on the dorsal side. The early observation made by Arora *et al.* (1994) in which Scw is only required for dorsal BMP-signaling, suggested that Dpp and Scw must act together, perhaps by forming heterodimers. Indeed, several more recent studies have proven the suggestion right and the researchers managed to piece together the puzzle of different molecules affecting the morphogen gradient formation.

Studies of BMP signaling through the type I receptors have revealed that BMP proteins in *Drosophila* have different preferences for receptor binding (Neul and Ferguson, 1998; Nguyen *et al*., 1998). The mRNA injection assays in embryos performed by Nguyen *et al.* (1998) showed that Dpp signals specifically through Thickveins (Tkv), and Scw is a ligand for Saxophone (Sax). The ventralization of scw^- embryos was rescued by injections of *scw* mRNA, but co-injections with the mRNA of dominant negative form of *sax* reduced the effect of injected *scw*. In contrast, the dominant negative form of *sax* had no effect on Dpp. Alternatively, the dominant negative form of Tkv was able to inhibit the response to both *scw* and *dpp*. These studies are not able to prove the direct interaction between Scw and Tkv since the type I receptors form multimeric complexes for signaling. However, complete loss of *tkv* in the embryo mimics the loss of Dpp function and *sax* mutant embryos mimic scw^- embryos, confirming that the ligands act through different receptor combinations (Nellen *et al*., 1994). In addition, signaling through these receptors seems to have different intensities since injection of *dpp* mRNA can rescue *scw* mutants but *scw* mRNA cannot rescue *dpp* mutants (Nguyen *et al*., 1998).

Although it has been suggested that the heterodimer formation of Scw and Dpp is not required for the biological activity of Scw in the embryo (Nguyen *et al*., 1998), several studies propose the opposite. Nguyen *et al.* showed that it is not necessary to express *dpp* and *scw* in the same dorsal region to achieve the peak signaling at the dorsal-most cells. When *scw* was expressed ventrally under the promoter of *twist* in *scw* null embryos, the embryos were 100 % rescued with four copies of the transgene. As the heterodimer formation is thought to occur inside the cells (Gray and Mason, 1990) the observed rescue was due to Scw-homodimer signaling. The same conclusion was drawn from mRNA injection analysis where *scw* mRNA was injected posteriorly into scw^-/dpp^- embryos expressing Dpp under control of the *even-skipped stripe 2* driver, and pMad staining was recovered (Wang and Ferguson, 2005). On the other hand, the importance of heterodimer formation was verified in a study by Shimmi *et al.* (2005b). They rest their claims on the facts that the heterodimers give stronger signals than Dpp-homodimers, and that heterodimers have a higher affinity to Sog and Tsg to form a shuttling complex. In addition, higher signaling intensities of heterodimers have been observed in mammals. The Dpp ortholog, BMP2 is used as a therapeutic drug to induce bone formation, and it was noted that BMP7, the ortholog for Scw, increases bone formation when the BMP2 and BMP7 ligands are provided as heterodimers (Hazama *et al*., 1995; Wang *et al*., 2012). Heterodimers or not, the importance of Scw for Dpp signaling is indisputable since the absence of Scw reduces receptor binding of Dpp (Wang and Ferguson, 2005) and pMad signal is undetectable in heterozygous null mutants of *scw* (*Df(2L)OD16/*scw^5) (II). In conclusion, Scw is required for BMP morphogen gradient formation. The gradient-forming ligands can be either homodimers or more strongly signaling heterodimers.

4.4.3 Type IV Collagens

More evidence confirming the importance of Dpp-Scw heterodimer formation was found from studies that revealed the role of extracellular matrix (ECM) proteins in Dpp gradient formation. As it had been noted that the movement of Dpp proteins is restricted when the formation of the BMP shuttling complex is inhibited (Eldar *et al*., 2002; Shimmi *et al*., 2005b; Wang and Ferguson, 2005), Wang *et al.* (2008) studied ECM molecules to find out what was stopping the diffusion of Dpp. The two type IV collagens in *Drosophila*, Viking (Vkg) and Dcg1 were shown to bind extracellular Dpp and affect correct signaling during development. Maternally expressed type IV collagens seemed to augment embryonic Dpp signaling. In the germarium of the *Drosophila* ovary, in which Dpp maintains germline stem cells (GSCs) (Xie and Spradling, 1998), Vkg was detected around all the somatic niche cells and GSCs. In hetrozygous Vkg mutants the number of GSCs was increased because of affected Dpp signaling. According to *in vitro* binding assays, Vkg binds Dpp/Scw heterodimers and through this action can limit the amount of free ligands in the tissue. Furthermore, Sog was able to bind Vkg and the addition of Tsg and Sog together was able to release the heterodimers from Vkg. These results suggest that type IV collagens facilitate assembly of the Dpp/Scw-Sog-Tsg complex. A second function of type IV collagens is to promote Dpp/Scw-receptor interactions since increasing amounts of Vkg enhanced ligand-receptor binding (Wang *et al*., 2008).

The revelation of the detailed function of Vkg confirmed the important role of Dpp/Scw heterodimers in BMP gradient formation. Sawala *et al.* (2012) introduced a multistep model for the assembly of the Dpp/Scw-Sog-Tsg shuttling complex on collagen IV. Figure 5 demonstrates how the interplay between collagen IV and the binding sites situated along the four cysteine rich (CR) domains on Sog guide the shuttling complex formation. Dpp but not Scw, can bind collagen IV. This result reveals the primary role for collagen IV in immobilization of the free Dpp. Collagen IV acts as a scaffold to assemble the shuttling complex in three steps; 1) Dpp and Sog bind to collagen IV. 2) Dpp/Scw is transferred onto Sog and the interaction between Scw and Sog disrupts Sog-Vkg interaction through the CR4-domain. This step shows why the Dpp/Scw heterodimer formation is important in BMP gradient formation. While Scw outcompetes collagen IV in binding to Sog, Dpp homodimers are stuck. 3) Tsg releases the shuttling complex by disrupting the bonds between the CR1-domain of Sog and collagen IV. The suggestion by Wang *et al.* (2005) about Tsg's role in promoting Dpp – receptor interactions is overruled by these results. It seems that in *tsg* mutants, the Dpp/Scw heterodimers having higher affinity to Sog (Shimmi *et al*., 2005b) are tightly stuck and thereby, the small amount of receptor-bound Dpp detected by the researchers was possibly caused by Dpp homodimer "escapers".

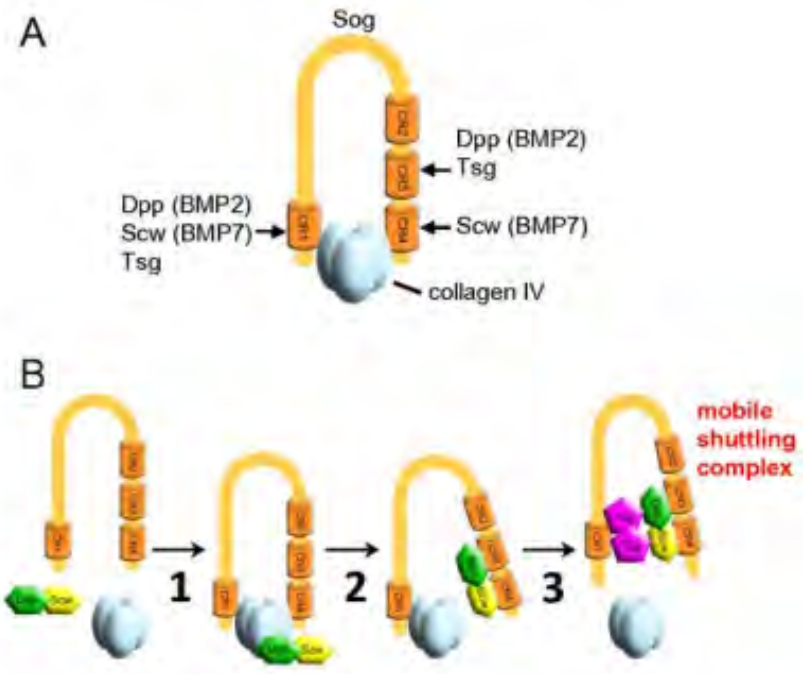

Figure 5. Molecular model for Dpp/Scw-Sog-Tsg shuttling complex formation. (A) Different binding domains on Sog for collagen IV, Dpp, Scw, and Tsg. (B) Model for shuttling complex formation. (Figure reprinted from Sawala *et al*., 2012.)

The molecular model of shuttling complex assembly is in conflict with the conclusion by Neul and Ferguson (1998) where they suggested that Scw activity can be blocked by Sog. This contradiction can be explained by Dpp/Scw heterodimer formation. In their experiments they co-injected mRNA of *sog* with *dpp* or *scw* into *scw*$^-$ embryos and followed Sog's ability to block the rescuing effect of injected ligands. According to their results *sog* mRNA completely blocked the activity of injected *scw* mRNA, but did not have an effect on injected *dpp*. This can be explained if Scw and Dpp form heterodimers before the antagonistic interaction with Sog. Explaining the rescued phenotype of embryos injected with *dpp* and *sog* mRNA is more difficult since the researchers were unable to use *dpp*$^-$ embryos. In addition, the results showing that *scw* mutant embryos can be rescued by overexpression of *dpp* (Arora *et al*., 1994) suggest that signaling of Dpp homodimers requires further studies.

4.4.4 Extracellular matrix

Because Dpp gradient formation varies in different developmental contexts, the different ECM molecules affecting Dpp signaling are interesting targets for studies of morphogen gradient formation and how they contribute to create variation in different tissues. Apart from type IV collagens, other basal lamina components have roles in different developmental stages of *Drosophila*. For example integrins are required for apposition of the amnioserosa and yolk sac to mediate proper germ band retraction and dorsal closure during later embryonic development (Reed *et al*., 2004; Schock and Perrimon, 2003). Then again, heparan sulphate proteoglycans (HSPGs) regulate Dpp movement in the wing imaginal disc during larval development whereas they have no effect on gradient formation during embryogenesis (Akiyama *et al*., 2008; Belenkaya *et al*., 2004; Bornemann *et al*., 2008). Many ECM molecules seem to have very different roles in BMP gradient formation. For example, fibrillins in mouse limb development can control BMP

signaling positively or negatively depending on the cellular context (Arteaga-Solis *et al*., 2001; Nistala *et al*., 2010).

4.4.5 Robustness of the BMP gradient

Formation of a shuttling complex consisting of Dpp, Scw, Sog and Tsg is a prerequisite for the morphogen gradient formation. As the concentration of free Dpp and Scw dimers affects target gene signaling, it is important to have a mechanism that keeps the gradient robust. The events in developmental patterning usually contain feedback loops to buffer against changes in gene expression. Overexpressed gene products can act as inhibitors to silence their own expression or activate their own degradation or storage for later use. The BMP gradient is a good example of this kind of robustness. A mathematical model by Eldar *et al.* (2002) suggests that the coupling of BMP diffusion and Sog degradation leads to a quantitative buffering of perturbations in gene dosage. Indeed, it was shown that the diffusion of free Dpp ligands is restricted to the site of expression and ventrolaterally expressed Sog is the key molecule in the process in which BMP ligands are transported to the dorsal midline (Eldar *et al*., 2002; Shimmi *et al*., 2005b).

In addition to the antagonistic effect of Sog, the gradient is maintained by a positive intracellular feedback circuit. This mechanism can explain the bistability of the BMP gradient, in other words, how the narrow strip of Dpp localization and BMP signaling is achieved. It has been suggested that the extracellular transport system is not enough to create the peak signaling in the dorsal-most cells of the embryo. The steep gradient develops when the received BMP signal is turning on the transcription of some currently unspecified gene that enhances the cell's ability to respond to BMP ligands. This gene product can be a co-receptor that enhances the signaling by increasing the receptor's affinity to bind BMPs. Alternatively, it can be a molecule that down-regulates receptor's activity post-transcriptionally in regions of lower BMP signaling. Few experiments show that positive feedback sharpens Dpp localization; 1) Localized injection of a constitutively active form of *tkv* mRNA but not of wild-type *tkv* mRNA, leads to the accumulation of extracellular Dpp. 2) Blocking signal transduction with *medea* mutants also blocks the sharpening of extracellular Dpp gradient. These experiments suggest that previous activation of BMP signaling enhances future interaction between Dpp and its receptor (Wang and Ferguson, 2005). Another mathematical model describing the robustness of the BMP gradient combines Dpp/Scw heterodimer diffusion and receptor mediated endocytosis of the ligands, and favors the theory of co-receptor related enhancement of signaling (Umulis *et al*., 2006).

The robustness has been challenged in numerous experiments. Changes in Tkv expression have little effect on the shape of BMP gradient (Mizutani *et al*., 2005; Umulis *et al*., 2006; Wang and Ferguson, 2005). Even though it has been shown that the concentration of Dpp is important for the gradient formation and *dpp* is haploinsufficient (Irish and Gelbart, 1987), the concentration of Tkv seems not to cause equally sensitive response. Heterodimer formation between Scw and Dpp can buffer against variations in the receptor concentrations (Shimmi *et al*., 2005b). In addition, heterozygous embryos containing only one functional allele of *scw, sog, tld* or *tsg* are viable (Arora *et al*., 1994; Eldar *et al*., 2002; Mason *et al*., 1997; Nguyen *et al*., 1998). On the other hand, changes in *sog* gene dosage have large effects on the dorsal pMad strip at the final stage of blastoderm (Mizutani *et al*., 2005). When observing the whole picture, e.g. a hatched viable fly, the BMP gradient is robust even though the single components in the gradient seem to affect target gene expression notably.

4.5 BMP gradient formation during wing development

The *Drosophila* wing imaginal disc provides an outstanding environment for studies of morphogen gradients. The primordial wing disc cells are set aside during embryonic development as small clusters of 20-30 cells that invaginate from the embryonic epithelium. As shown in Figure 6 the mature late-third-instar disc consists of some 50,000 cells organized in two distinct surfaces: the thinner peripodial membrane and the thicker folded disc epithelium. The structure of the wing disc and the trajectory of different compartments create an excellent environment for studying the expression of many growth factors. It is possible to visualize proteins in whole mount specimens. Indeed, studies in wing discs made it possible to classify Dpp as a morphogen by comparing the consequences of ectopic expression of the secreted ligand with those of ectopic activation of its constitutively active receptors (Nellen *et al.*, 1996). Genetic manipulations resulting in perturbations of expression patterns can be seen directly as altered phenotypes of wing venation or growth. For example, altering Dpp-mediated BMP signaling changes the size of the intervein region between longitudinal veins L2 and L5, since the positions of L2 and L5 are set according to the Dpp gradient (Reviewed in Blair, 2007).

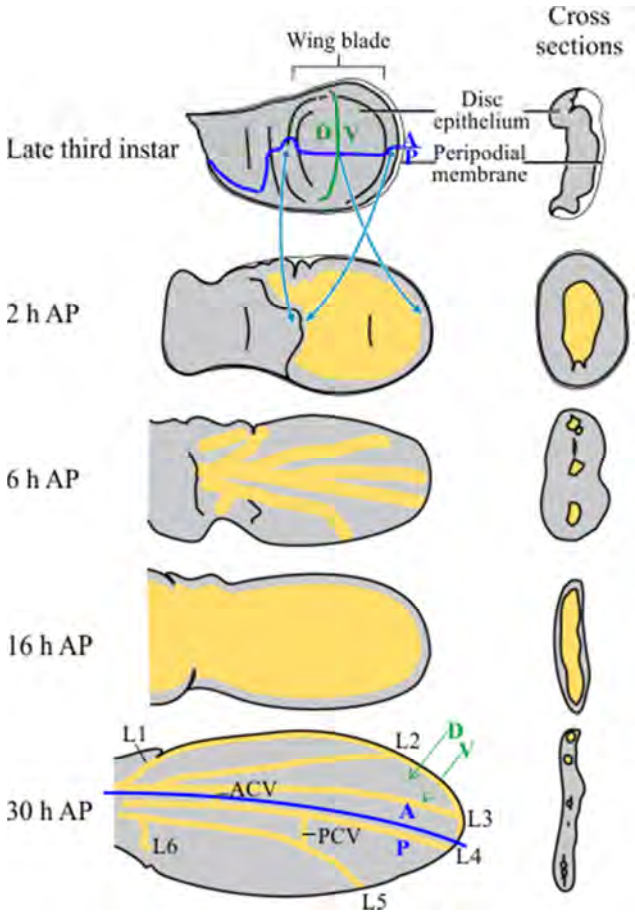

Figure 6. Morphogenesis of the wing. Gaps between the dorsal and ventral wing epithelia are shown in yellow. Blue arrows from late-third-instar wing disc to the wing 2 h after pupariation (AP) show how the basal sides of the dorsal and ventral (marked by green border) wing epithelia come together. A-P border is shown in blue. The positions of future wing veins are marked in the wing 30 h AP; longitudinal veins L1-6, anterior crossvein ACV and posterior crossvein PCV. (Republished with minor modifications with permission of Annual Reviews, Inc, from Blair, 2007, permission conveyed through Copyright Clearance Center, Inc.)

4.5.1 The role of Dpp in the wing imaginal disc

Dpp signaling plays different roles during wing development. In addition to regulating cell fate to specify organ pattern, Dpp also controls organ size during larval stages. During pupal wing development Dpp and the third BMP-family member, Glass bottom boat (Gbb), help to specify vein versus intervein cell fate and this signaling shares many common features with the patterning of early embryo since Dpp/Gbb signaling is tightly regulated by Sog (Matsuda and Shimmi, 2012; Serpe *et al*., 2005). Gbb and its receptor Sax have important roles in shaping the BMP gradient in the wing disc (Bangi and Wharton, 2006a; Bangi and Wharton, 2006b). *gbb* is expressed broadly in the wing pouch but the expression domain along the A-P boundary has a significant role in mediating the Dpp gradient (Khalsa *et al*., 1998; Ray and Wharton, 2001). As Figure 7 shows, cells within the wing pouch respond to different threshold levels of pMad by activating BMP target genes *spalt* (*sal*) and *optomotor blind* (*omb*) at different distances from the A-P boundary (Lecuit *et al*., 1996; Nellen *et al*., 1996). Target gene expression is activated indirectly by repressing *brinker* (*brk*) expression and directly by pMad-Medea (Barrio and de Celis, 2004; Kirkpatrick *et al*., 2001; Minami *et al*., 1999; Muller *et al*., 2003). In the absence of Gbb, Dpp exhibits only short-range signaling. This indicates that Dpp and Gbb interact extracellularily to be able to form wide concentration gradient and activate low level target genes far from the A-P boundary. A reduction in *dpp* expression seems to influence only the high threshold genes in the central domain of the wing pouch (Bangi and Wharton, 2006a).

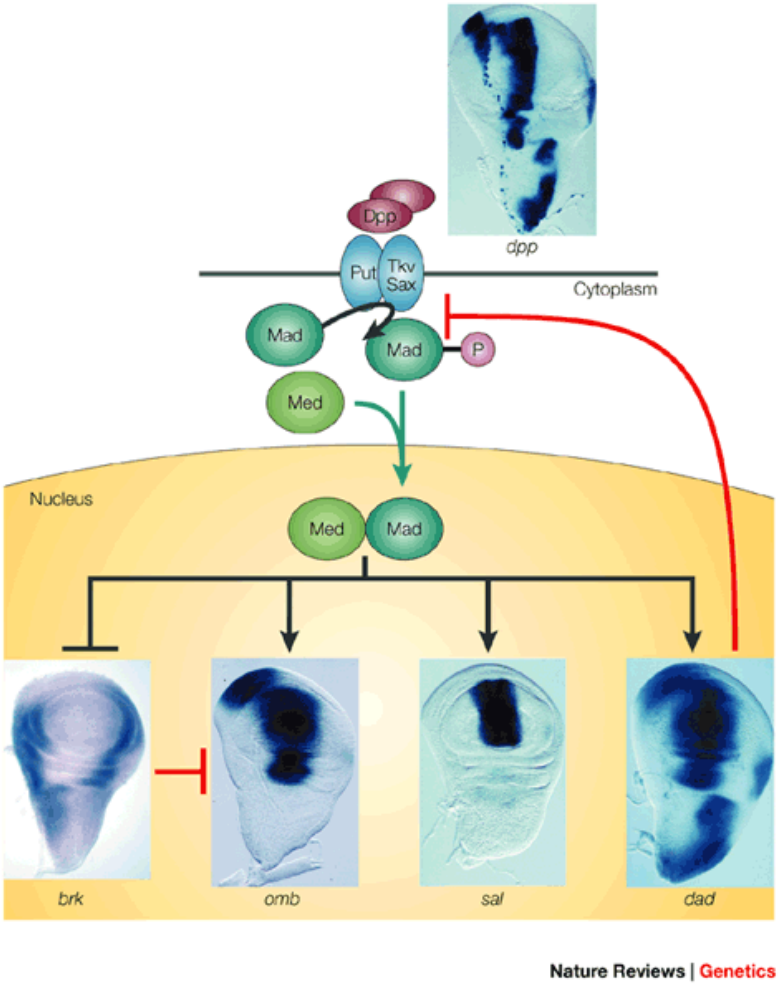

Figure 7. Dpp target gene expression in wing imaginal disc. Expression patterns of *dpp* and its target genes *omb*, *sal* and *daughters against dpp* (*dad*) are shown in imaginal wing discs. Dpp upregulates the expression of *omb*, *sal* and *dad*, and downregulates the expression of *brk*. Dad and Brk function as negative regulators of the pathway: Dad antagonizes receptor mediated phosphorylation of Mad, and Brk represses transcription of *omb*, *sal* and *dad*. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics, Tabata, 2001.)

4.5.2 Receptors and Dpp gradient formation

In the wing imaginal disc, Dpp is expressed in a central strip of cells and diffuses both anteriorly and posteriorly (Entchev *et al*., 2000; Teleman and Cohen, 2000). Since the movement of Dpp cannot be explained by simple diffusion, several mechanisms for Dpp signal transduction have been proposed. The interplay between ligands and receptors seems to play a major role during BMP gradient formation and one of the models suggests that the gradient is formed via intracellular trafficking, planar transcytosis, initiated by receptor mediated endocytosis. Indeed, components affecting endocytosis have an effect on the extent of Dpp gradient: mutations in *clathrin* reduce the active range of Dpp (Gonzalez-Gaitan and Jackle, 1999) and cells lacking Dynamin fail to transduce Dpp signaling (Belenkaya *et al*., 2004; Entchev *et al*., 2000). These results suggest that Dynamin mediated ligand internalization through clathrin coated vesicles is a prerequisite for signaling. Based on the results by Entchev *et al.* (2000) it is tempting to conclude that the main mechanism for Dpp gradient formation would be planar transcytosis since the researchers were unable to detect extracellular Dpp in the wing discs. However, more advanced staining methods revealed that Dpp is diffusing in the extracellular space, even inside the regions of cells mutated for Dynamin. Belenkaya *et al*. (2004) proposed that the extracellular Dpp gradient formation is independent of Dynamin mediated endocytosis since the Dynamin mutant *shibire* does not block Dpp movement but rather inhibits Dpp signal transduction (Belenkaya *et al*., 2004). A mathematical model that takes into account interacting dynamic processes like ligand diffusion, ligand-receptor binding and dissociation, internalization and degradation, favor restricted diffusion as the main morphogen transport mechanism (Lander *et al*., 2002).

The internalization of BMP molecules through receptor mediated endocytosis maintains the gradient by limiting the free movement of ligands. Clonal analysis showed that the shape of the BMP gradient is formed through Dpp's action in repressing the expression of Tkv in the center of the wing disc. High levels of Tkv outside in the wing pouch limit the diffusion of Dpp (Lecuit and Cohen, 1998). In addition to transducing the Dpp signal, the Tkv receptor limits the movement of Dpp by binding to it and acting as a sink.

Conflicting results proposing the role of Sax in BMP gradient formation led to discovery of the dual function of Sax. Several studies suggested that Sax is not needed for Gbb signaling as the wing phenotypes of *sax* mutants did not resemble those of *gbb* mutants, and reducing *sax* gene dose does not enhance the *gbb* partial loss of function wing phenotype, whereas a similar reduction in *tkv* gene dose clearly attenuates Gbb signaling (Khalsa *et al*., 1998; Ray and Wharton, 2001). Bangi and Wharton realized that absence of *sax* resulted in phenotypes that resembled the phenotypes of increased *gbb* activity. In addition to this antagonistic function Sax seemed to enhance Gbb activity. These positive and negative effects were explained by a model where the outcome depends on receptor complex assembly: The Sax/Sax complex binds Gbb without inducing signaling whereas the Sax/Tkv complex leads to activation of the signaling cascade. This way Sax is a modulator of ligand availability (Bangi and Wharton, 2006b).

4.5.3 Heparan sulphate proteoglycans

As was mentioned above, ECM proteins play crucial roles in BMP gradient formation. Heparan sulphate proteoglycans (HSPGs) of the glypican family are involved in the formation and stabilization of the Dpp gradient. Glypicans are glycosylphosphatidylinositol (GPI)-anchored HSPGs and consist of a protein core to

which heparan sulfate (HS) chains are covalently attached. The HS chains provide binding sites for different growth factors. Especially the role of proteoglycans Dally and Dally-like (Dly) seems to be in the enhancement of Dpp spreading on the cell surface. Clonal analysis showed that extracellular Dpp fails to move across regions that lack both Dally and Dly (Belenkaya *et al*., 2004; Fujise *et al*., 2003). In addition, the proteoglycans seem to have an important role in enhancing the Dpp signal cell-autonomously, possibly by influencing the presentation of Dpp to its receptors (Fujise *et al*., 2003). Additional experiments with a truncated form of Dpp ($Dpp^{\Delta N}$) lacking a short domain at the N-terminus essential for interacting with Dally, suggest that Dally stabilizes Dpp on the cell surface. $Dpp^{\Delta N}$ was more quickly internalized by cells and degraded. It was suggested that Dally may antagonize Tkv in Dpp signaling and inhibit receptor-mediated endocytosis (Akiyama *et al*., 2008).

4.5.4 Dpp and growth regulation

When expressed ectopically, Dpp has impressive effects on organ shape and size. Hence, the studies of Dpp action must include the bipartite aspect that takes into account both Dpp's role in patterning and growth. Usually, patterning by morphogens is linked to the regulation of cell proliferation.

Hypomorphic alleles expressing reduced amounts of Dpp decreased the growth of wing drastically (I; Zecca *et al*., 1995). Alternatively, ubiquitous over-expression of Dpp or its constitutively active Tkv receptor causes massive enlargement of imaginal discs (Capdevila and Guerrero, 1994; Nellen *et al*., 1996; Rogulja and Irvine, 2005). In addition, ectopic *dpp*-expressing clones that are situated along the D-V boundary and include both dorsal and ventral cells can develop into winglets (Zecca *et al*., 1995). Despite this indisputable evidence for supporting Dpp's role in growth promotion, other studies imply that the primary role of Dpp is to ensure the correct architecture of epithelial cells (Gibson and Perrimon, 2005; Shen and Dahmann, 2005). It was noted that decreased Dpp signaling in the distal wing cells or increased Dpp signaling in the proximal wing cells cause apoptosis. The disturbances in Dpp signaling gradient lead to activation of the c-Jun amino-terminal kinase (JNK) apoptotic pathway (Adachi-Yamada *et al*., 1999). Two side-by-side published studies added a new dimension to this apoptotic function. Gibson and Perrimon (2005) used the directed mosaic FLP/FRT system to create *tkv* clones in developing disc epithelia and noticed that clones were consistently presented as cyst-like epithelial extrusions. They favor a more direct role for the Dpp pathway in controlling epithelial morphogenesis by suggesting that the primary phenotype of *tkv* clones is extrusion and that JNK-dependent cell death is a secondary effect, similar to a wound response. Shen and Dahmann (2005) confirmed that Dpp signaling is involved in regulating cytoskeletal organization.

Dpp's role in growth regulation is still unclear. Several models have been presented that include: growth according to the steepness of the Dpp gradient (Day and Lawrence, 2000; Rogulja and Irvine, 2005), growth regulated by an unknown inhibitor expressed by Dpp receiving cells (Serrano and O'Farrell, 1997), mechanical forces and threshold levels required for cell growth in peripheral regions (Hufnagel *et al*., 2007; Shraiman, 2005), and mechanical stretching that stimulates growth in the peripheral regions because of the growth-factor-induced growth in the center (Aegerter-Wilmsen *et al*., 2007). All of the models contain some discrepancy or uncertainty and it is difficult to highlight one among the others. In addition, models containing mechanical forces are purely hypothetical at this moment and require experimental support. However, more convincing results have

previously been gathered using a model that is proposing a circuit motif, termed "expansion-repression". This model explains how the patterning is scaled according to tissue size. It has been shown that the length scale of the Dpp gradient remains proportional to the size of the disc during growth (Wartlick *et al*., 2011). The model by Ben-Zvi *et al.* uses a secreted feedback regulator Pentagon (Pent) to show that two diffusible molecules, a morphogen (Dpp) and an expander (Pent), can scale the morphogen gradient with the tissue size. *pent* is repressed by Dpp and interacts with Dally to control Dpp distribution (Ben-Zvi *et al*., 2011; Hamaratoglu *et al*., 2011; Vuilleumier *et al*., 2010).

4.5.5 Wing vein development

Sog and other extracellular regulators of BMP signaling that were introduced in the context of embryo development have an important role during pupal wing development, particularly in positioning and development of wing veins. Contrary to the larval BMP signaling in wing discs, the venation during pupal stages requires Sog, the protease Tolloid-related (Tlr) and the Tsg-like protein Crossveinless (Cv) (Serpe *et al*., 2005; Shimmi *et al*., 2005a). Especially PCV formation has been studied to find out the transport mechanisms affecting BMP signaling in pupal wings. The PCV is convenient for these studies since *dpp* expression is completely missing from this region and it must be transported from the longitudinal veins (LV) that are maintained by Dpp (Matsuda and Shimmi, 2012; Ralston and Blair, 2005; Yu *et al*., 1996). The BMP5/6/7/8-like protein Gbb likely forms a heterodimer with Dpp to be transported in the developing tissue by the Sog-Cv complex (Shimmi *et al*., 2005a). Heterodimer formation is proposed in a study showing that removal of Gbb expression from adjacent LVs disrupts PCV formation (Ray and Wharton, 2001). Regardless of the ubiquitous expression of *gbb*, only the expression in the LVs where Dpp is expressed is required (Conley *et al*., 2000; Ray and Wharton, 2001). Unlike Scw, Gbb can signal in the absence of Dpp. In addition, it does not cause a synergistic signal with Dpp. Consequently, the major role suggested for Gbb is in the transport of ligands to the prospective PCV region (Shimmi *et al*., 2005a).

4.6 Proteolytic processing

As has been discussed above, morphogen gradient formation is controlled on many levels. After secretion the protein meets different forces that affect its ability to move and transduce signaling. To crown it all, these forces seem to vary according to the tissue or developmental context. At this point of the review we go back a few steps in the course of a morphogen's life and observe an important event in the biosynthesis of many proteins: proteolytic processing.

Numerous proteins are initially synthesized as pro-proteins that require proteolytic processing in the *trans*-Golgi network before they are biologically active (Figure 8). TGF-β-family members are produced as large precursor proteins that need endoproteolytic cleavage. Subtilisin-like proprotein convertases (PCs) are involved in this process by recognizing a short amino acid sequence R-X-K/R-R or R-X-X-R (where R stands for Arginine, K is for Lysine and X means any amino acid) and hydrolyzing the following peptide bond (Creemers *et al*., 1993; Molloy *et al*., 1992; Seidah and Chrétien, 1999; Steiner *et al*., 1992). Different expression profiles of PCs in different tissues or developmental stages may offer an additional dimension for the adjustment of BMP signaling, since protein amount and activity can be controlled by proteolytic cleavage (I;

II; Constam *et al*., 1996). For example, the activity of the *Xenopus laevis* protein Vg1 is controlled by proteolytic processing which is mediated by two distinct PCs. Tightly restricted overlapping expression domains of Vg1 and proteases limit the activity to a specific region. *vg1* mRNA injections have no effect on *X. laevis* patterning since the expression domain of proteases is tightly controlling the maturation of protein (Thomas and Moos, 2007).

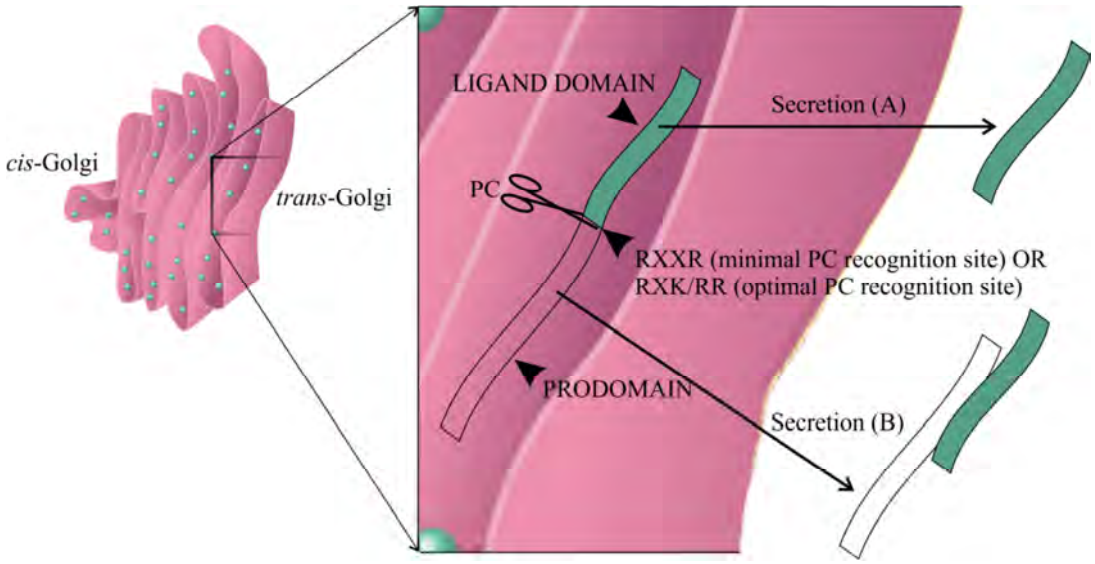

Figure 8. Proteolytic processing. Proteins are synthesized as pro-proteins that require endoproteolytic processing in the *trans*-Golgi network before they are biologically active. Subtilisin-like proprotein convertases (PCs) recognize a short amino acid sequence R-X-K/R-R which is an optimal recognition site, or R-X-X-R which is a minimal recognition site, and catalyze the cleavage of the following peptide bond. After proteolytic processing the ligand domain is transported out of the cell either alone (A) or in complex with the prodomain (B). Ligand domain is shown in green and prodomain is transparent.

In *Drosophila*, three members of PC family have been identified: *Dfurin1* (*Dfur1*), *Dfurin2* (*Dfur2*), and *amontillado* (*amon*) (Roebroek *et al*., 1992; Roebroek *et al*., 1991; Siekhaus and Fuller, 1999). The *Dfur1* gene produces three differently sized proteins with divergent C-terminal sequences. The protein isoforms are called DFurin1, DFurin1-CRR, and DFurin1-X, and no significant differences with regard to the cleavage specificity were found. In contrast, DFur2 showed differential cleavage specificity when compared to the DFur1 isoforms. Also the cleavage efficiency and cellular localization differ between the DFur1 isoforms and DFur2 (DeBie *et al*., 1995; Roebroek *et al*., 1993). *Dfur2* expression is detected in early embryos until the syncytial blastoderm (stage 5), and transient expression is seen in the developing nervous system and tracheal tree, while the expression of *amon* is restricted to the final stages of embryogenesis (stages 15-17) and late pupal and adult stages (Roebroek *et al*., 1995; Siekhaus and Fuller, 1999). Information about the *DFur1* expression profile is incomplete: it is expressed at least during early embryogenesis (Roebroek *et al*., 1991). In addition, *Dfur2* expression has only been studied during embryogenesis but not in larval stages.

4.6.1 The role of the prodomain

It is easy to understand that proteolytic processing is a reasonable way to fine-tune the production of active proteins. Since TGFβ-family members are produced as huge proproteins containing relatively small mature ligand domains, it has led us to consider the role of the huge prodomain.

The role of the large prodomains of TGFβ1 and activin A were studied in cell culture experiments. It was noted that pro-regions have a role in intracellular dimer formation and secretion of the ligands. Thus, the pro-regions aid the folding, disulfide bond formation and secretion of their respective dimers (Gray and Mason, 1990). Later it was shown that the prodomain has an important role in controlling the biological activity of TGFβ. The proteins are secreted as latent complexes consisting of the mature ligand domain and the N-terminal propeptide termed latency associated peptide (LAP). After secretion LAP-TGFβ associates with another binding protein to form a large latent complex (LLC). The formation of LLC enables TGFβ localization within the ECM and subsequent activation. LLCs can be activated for example through integrin binding or protease activity (Annes *et al*., 2003; Yang *et al*., 2007).

Since BMPs are produced as large proproteins containing comparatively small ligand domains and, unlike TGFβ are secreted as active ligands right after processing, the role of huge prodomain has raised interest. Domain swap experiments by Constam and Robertson (1999) revealed that the structure of a prodomain can influence the stability of the ligand. For example chimeric BMP4 or Dorsalin proteins containing the prodomain of Nodal were degraded much faster than their natural counterparts. On the contrary, prodomain of Dorsalin enhanced Nodal stability and this was due to the association of prodomain with its mature protein. According to these results prodomains may influence the half-life of the mature protein and limit the range of signaling.

BMP7 is secreted as a stable complex consisting of a growth factor dimer noncovalently associated with two propeptides. Here, the propeptides do not cause latency as was shown to be the case for TGFβ. On the contrary, the prodomains target the growth factor to fibrillin-1 and the peptides are displaced upon ligand binding to the type II receptor (Gregory *et al*., 2005; Sengle *et al*., 2008b). The same kind of targeting role for prodomains is seen in many other BMP family members. For example BMP9, BMP10, growth and differentiation factor (GDF)-5 and GDF8 were shown to form complexes with their prodomains (Brown *et al*., 2005; Sengle *et al*., 2008a). Binding studies revealed that fibrillin-1 serves as a universal high affinity docking site for the propeptides (Sengle *et al*., 2008a). These results provide again a new function for a prodomain; targeting to the extracellular matrix. In addition, Fritsch *et al.* (2012) showed that the BMP7 prodomain carries a species specific function since the full length BMP7 is unable to rescue *gbb* mutants in *Drosophila* even though the chimeric construct carrying the pro domain of *gbb* and the ligand domain of BMP7 is fully functional and rescues *gbb* mutants in flies.

4.6.2 Cleavage of BMP4

The first indication of the PCs' role in the activation of BMP-proteins was already obtained in 1996 by Constam *et al*. They studied the expression patterns of different PCs and BMPs in mouse embryos. It was noted that the expression profiles of PC4 and PC6 overlapped with many BMPs during limb development. Later, Cui *et al.* (1998) showed that injected PC inhibitor blocked the proteolytic processing of BMP4 and led to

dorsalization of mesoderm and direct neural induction of *Xenopus laevis* embryos. Furin and PC6 were shown to be the responsible processing enzymes.

Jan L. Christian's lab discovered a new dimension of proteolytic processing. They studied cleavage of pro-BMP4, an ortholog of Dpp, and found that the proprotein is cleaved sequentially at two cleavage sites that are recognized by Furin and other PCs (Constam and Robertson, 1999; Cui *et al*., 1998; Cui *et al.*, 2001). As can be seen in Figure 9, an initial cleavage at an optimal furin consensus motif (R-S-K-R) cuts the bonds between the mature ligand domain and the prodomain, but does not release the ligand for signaling. The first cleavage results in formation of a protein complex that contains the prodomain noncovalently attached to the mature ligand domain. This complex is less active and signals at shorter range. The mature ligand is released if the second cleavage takes place at an upstream minimal furin motif (R-I-S-R). If the second cleavage is inhibited, the protein complex is targeted to the lysosome for degradation either within the biosynthetic pathway or within the endocytic pathway following receptor activation and internalization. Analysis of mice carrying a point mutation that prevents processing of the upstream site showed severe loss of BMP4 activity in some tissues. This phenotype was not caused by reduced ligand levels, since tissues that are sensitive to BMP4 dosage, like limb, dorsal vertebrae and kidney, developed normally, whereas testes and germ cells were affected. These studies demonstrate that cleavage at the upstream site is essential for normal development and may selectively occur in a tissue-specific manner (Cui *et al*., 2001; Degnin *et al*., 2004; Goldman *et al*., 2006).

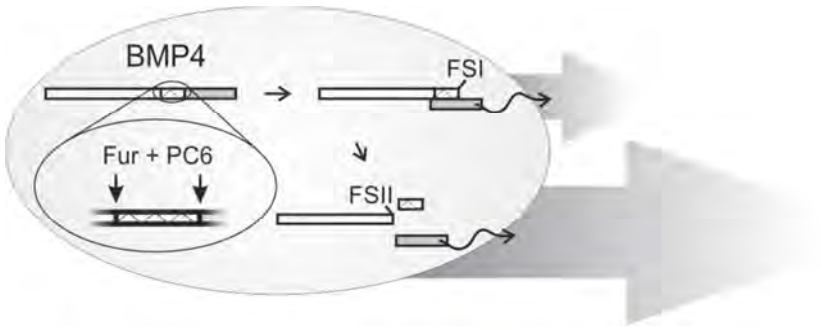

Figure 9. Maturation process of BMP4. BMP4 proprotein is cleaved by Furin and/or PC6 at the optimal furin site FSI. The prodomain remains in contact with the ligand and the complex is degraded quickly after secretion. Thus the molecule can signal only in short range. In case the complex is processed at the FSII site, the mature ligand domain is released and it is involved in long range signaling. The ligand domain of BMP4 is marked in gray. (Reprinted from III.)

4.6.3 Gbb and Scw

The *Drosophila* Gbb and Scw proteins belong to the BMP5/6/7/8 subfamily and have three and four PC cleavage sites, respectively, which are shown in Figure 10. Two of the cleavage sites are situated at the junction between the prodomain and ligand domain, and are called Main and Shadow sites. The third site within the prodomain is called Pro site. The Pro2 site of Scw is situated upstream of Pro site (Fritsch *et al*., 2012).

In both proproteins cleavage at the Main site is required for cleavage at the Shadow site and consequently for efficient ligand production. Dissimilarities in processing

requirements were observed when Main or Pro cleavage mutants were tested in *Drosophila* development. It was noted that Gbb must be processed at either the Pro or Main site to be functional *in vivo*. On the contrary, processing at both Pro and Main sites is essential for Scw function. In addition, mutation of the Pro site reduces the amount of secreted mature Scw, and the protein is secreted in complex with its N-terminal prodomain fragment. The Pro2 site of Scw was suggested not to be processed according to Fritsch *et al.* (2012) even though mutations in this site seemed to have some effect on ligand production in cell culture. Mutation at Pro2 reduced the amount of Pro-cleaved intermediate forms. Our studies show that Pro2 is cleaved, and the intermediate form produced through cleavage of Main and Pro2 sites (Pro-mutant) is detected in secreted fractions (II).

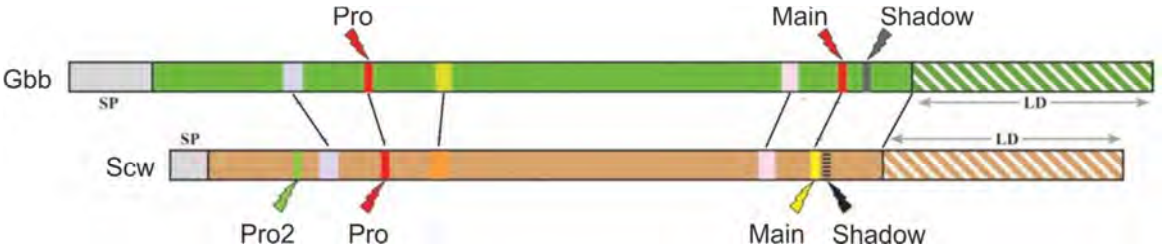

Figure 10. Cleavage sites of Scw and Gbb proproteins. Gbb (green) contains three furin cleavage sites. Scw (tan) has four potential cleavage sites. Hatched boxes show ligand domains (LD) and gray boxes indicate the signal peptides (SP). Conserved sequence motifs are shown in blue, ochre and pink. (Modified from Fritsch *et al*., 2012.)

The mature ligands of Scw and Gbb are mainly produced through cleavage at the Shadow site, since constructs carrying mutation at this site produce ligands that are slightly bigger than their wild type counterparts. In addition, hypomorphic gbb^4 mutants were rescued by genomic *gbb* that produces ligands processed from the Shadow site only and mutation of the Shadow site shows reduced Scw function in rescue experiments (Fritsch *et al*., 2012). Akiyama *et al.* (2012) published opposing results related to the functionality of the ligand produced by the Shadow site cleavage. According to their results the smaller 14 kD product of Gbb was undetectable in Western blot analysis by their antibody. However, they may have misinterpreted their results since they did not include a Shadow mutant in their analysis.

The Pro site has drawn some attention recently. It was noticed that this site is conserved among many family members. Mutations at the Pro site of hBMP4, hBMP15, and anti-Müllerian hormone (AMH) have been linked to cleft lip with or without palate, premature ovarian failure, and persistent Müllerian duct syndrome, respectively (Dixit *et al*., 2006; Imbeaud *et al*., 1994; Suzuki *et al*., 2009). Akiyama *et al.* (2012) discovered that Gbb is present in tissues in two molecular forms; a 328-amino acid form produced by the cleavage at the Pro site, and a 130-amino acid mature ligand. Even though Pro and Main site mutations in Gbb caused no apparent phenotypes on flies (Fritsch *et al*., 2012), it seems that the resulting protein products have different signaling activities and signaling ranges in tissues. Signaling activity was reduced by 50 % when the Pro site was mutated. In addition, the 328-amino acid protein could influence cells distant from where it was produced (in a posterior part of the wing disc), as the smaller 130-amino acid mature ligand could not. The abundance of the different forms of Gbb varied among different tissues, implying that differential processing could account for tissue-specific behaviors of BMP gradient (Akiyama *et al*., 2012).

4.7 Conservation of BMP type proteins

The number of proteins belonging to the BMP family, and the shared similarities in structure and function are indicators of evolution through duplication and divergence. A vertebrate genome may contain approximately 20 BMP-type proteins that can be divided into distinct subgroups according to their function. The main subgroups, BMP2/4/Dpp and BMP5/6/7/8/Gbb/Scw, are represented in bilateria and the closest outgroup to the Bilateria, the phylum Cnidaria (Fritsch *et al*., 2010; Hayward *et al*., 2002; Van der Zee *et al*., 2008). Classification and prediction of the hypothetical ancestors for evolutionary trees has been difficult, and thus revealing the components of the major signaling pathways has given a tool to study the developmental relationships of different phyla.

The mature BMP4 ligand forms a dimer through one of the cysteine residues found in the ligand domain. The other six cysteines are involved in intramolecular disulfide linkages (McDonald and Hendrickson, 1993). This structure is probably maintained in all BMP2/4/Dpp like proteins since the seven cysteine residues are conserved among different species. In addition, conservation of the ligand domain is evident, since the BMP ligands are functional when ectopically expressed in other organisms. *Drosophila* Dpp can induce bone formation in mammalian cells and the human BMP4 rescues patterning defects in *Drosophila dpp* mutant embryos (Padgett *et al*., 1993; Sampath *et al*., 1993). The reef building coral *Acropora millepora* belongs to phylum Cnidaria, and identification of the orthologous BMP2/4/Dpp gene in this organism revealed compelling similarity of ligand domains in such distant animals like the African clawed frog *Xenopus laevis* (80%). Sequence similarity between *D. melanogaster* and *A. millepora* was 67%. This is enough for maintaining the three-dimensional structure of the *A. millepora* ligand and being functional in developing *Drosophila* embryos (Hayward *et al*., 2002).

Functional conservation of BMP5/6/7/8/Gbb/Scw ligands has also been studied. The conserved function of Gbb between arthropods and vertebrates was seen in experiments with chimeric constructs fusing *D. melanogaster* Gbb with the ligand domains of human BMP5, BMP6, or BMP7. The constructs were able to rescue *gbb* mutant flies. On the other hand, Scw function is not even conserved within the higher Diptera (Fritsch *et al*., 2010).

Phylogenetic analyses suggest that *Drosophila* Scw arose from a unique duplication of an ancestral *gbb* after the separation of the mosquitoes and the higher Diptera and continued to evolve rapidly (Fritsch *et al*., 2010; Van der Zee *et al*., 2008). The distinct expression patterns of *scw* and *gbb* and their roles in different developmental contexts suggest that the appearance of *scw* is important for *Drosophila* embryogenesis. When combining *scw* cis-regulatory sequences with *gbb*, Gbb is not able to replace Scw in the early dorsal-ventral patterning. Vice-versa, expression of Scw under the control of *gbb* regulatory sequences showed at least partially rescued phenotypes. Fritsch *et al.* (2010) suggested that the differences in the functions of Scw and Gbb must lie downstream of secretion of the ligand and upstream of receptor binding. Indeed, the extracellular binding proteins responsible for the sharp Dpp/Scw gradient formation in the early embryo emerged at the same time with *scw* in the linage leading to the higher Diptera, and evolved rapidly to maintain Scw function in the embryo (Fritsch *et al*., 2010).

Despite the vast changes in BMP repertoire, the basic signaling mechanisms have remained unchanged. The signaling cascade has co-opted new functions to meet the evolutionary pressure. In addition, the conserved system defining the polarity of the

dorsal-ventral axis suggests that the main features in the BMP signaling pathway are maintained unchanged. In vertebrates the dorsal and ventral poles have inverted during evolution so that the ventral region of *Drosophila* is homologous to the dorsal side of the vertebrate. Despite this inversion, the signaling molecules and their antagonists have remained unchanged. In *X. laevis* BMP4 is expressed ventrally and the Sog ortholog, Chordin, acts dorsally to set the BMP signaling gradient (Arendt and Nubler-Jung, 1997; De Robertis and Sasai, 1996).

5 AIMS OF THE STUDY

Cells in a multicellular organism must be able to differentiate and respond to the needs of changing environments by expressing a relatively small set of proteins. The different responses caused by the same secreted proteins are achieved by complicated regulatory systems. The experimental fact of BMP ligand domain conservation raises the question of how evolutionarily conserved molecules can be involved in a wide array of different developmental events. Transcriptional regulation and extracellular events affecting ligand availability have been extensively studied, but the post-translational modifications, like proteolytic cleavage, have received less attention.

To understand better the impact of proteolytic cleavage on the regulation of protein activity we specifically studied:

I, III The cleavage patterns of BMP2/4/Dpp type proteins, and how the three cleavage sites of *Drosophila* Dpp coordinate maturation of ligands and contribute to signaling *in vivo*.

II Diversification of cleavage motifs in BMP5/6/7/8/Gbb/Scw subfamily prodomains, and the functional purpose for the appearance of the prodomain cleavage site in Scw protein.

6 MATERIALS AND METHODS

The materials and methods used in these studies are summarized here. For a more detailed description see the appropriate article. The original publications are referred to by Roman numerals.

Cell culture and recombinant protein expression (I, II)

The *Drosophila* cell line Schneider 2 (S2) was used in recombinant protein expression experiments. The cells were transfected in M3 medium (Sigma) supplemented with Insect Supplement Medium (Sigma), and dimethyldioctadecylammonium bromide (DDAB, Fluka, (Han, 1996)) or Fugene HD (Roche) were used for transfections. Plasmids used for recombinant protein production are listed in Table 1.

Table 1. Plasmids used in cell culture.

Plasmid	Description	Source or reference	Used in
dpp-HA	Contains 3xHA-tag in the ligand domain	Shimmi *et al.*, 2005b	I, II
dpp^{MFSI}-HA	FSI site is mutated, RNKR to GNKG	I	I
dpp^{MFSII}-HA	FSII site is mutated, RLRR to GLRG	I	I
dpp^{MFSIII}-HA	FSIII site is mutated, RSIR to GSIG	I	I
$dpp^{MFSI/III}$-HA	FSI and FSIII sites are mutated	I	I
$dpp^{MFSII/III}$-HA	FSII and FSIII sites are mutated	I	I
$dpp^{MFSI/II}$-HA	FSI and FSII sites are mutated	I	I
dpp^{hr4}-HA	$G^{1205} > A$ ($G^{402} > E$)	II	II
mad-Flag	Was used in BMP-signaling assay. Contains Flag-tag for quantification.	Shimmi *et al.*, 2005b	I, II
scw-HA	Contains HA-tag in the ligand domain	Shimmi *et al.*, 2005b	II
HA-scw-Flag	Contains HA-tag in the prodomain and Flag-tag in the ligand domain	Shimmi *et al.*, 2005b, II	II
HA-scw^{MFSI}-Flag	FSI site is mutated, $RFKR^{271}$ to GFKG	II	II
HA-scw^{MFSII}-Flag	FSII site is mutated, $RPRR^{54}$ to GPRG	II	II
HA-scw^{E1}-Flag	E1 site is mutated, $C^{271} > T$ ($R^{91} > C$)	II	II
HA-$scw^{MFSI/E1}$-Flag	FSI and E1 sites are mutated	II	II
HA-$scw^{E1/MFSII}$-Flag	E1 and FSII sites are mutated	II	II
tsg-His	Was used in BMP-signaling assay	Shimmi *et al.*, 2005b	II
sog-Myc	Was used in BMP-signaling assay	Shimmi *et al.*, 2005b	II
gfp	Secreted form of GFP used as a loading control	Shimmi *et al.*, 2005b	II

Conditioned media were collected after 3-5 days after transfection and used as supernatants in further analyses. The cells were lysed in lysis buffer (I).

Western blotting (I, II)

Protein samples were heated at 95°C for 5 minutes, separated in SDS-PAGE gels, and transferred to a PVDF membrane (Millipore). Blots were pre-incubated with 5% milk and incubated with the antibodies presented in Table 2.

Table 2. Antibodies used in Western blot analyses.

Antigen	Antibody	Suorce or reference	Used in
HA	Mouse monoclonal (12CA5)	Roche	I, II
Flag	Mouse monoclonal (M2)	Sigma	I, II
β-Tubulin	Mouse monoclonal	Sigma	I
pMad	Rabbit PS1 polyclonal antiserum	Dr. P. ten Dijke (The Netherlands Cancer Institute, Amsterdam, The Netherlands)	I, II
DFurin1	Rabbit polyclonal antiserum	Roebroek *et al.*, 1993	I
DFurin2	Rabbit polyclonal antiserum	Roebroek *et al.*, 1993	I
GFP	Rabbit polyclonal	Santa Cruz Biotechnology	II
Myc	Rabbit polyclonal (C14)	Santa Cruz Biotechnology	II
RGS-His	Mouse monoclonal	Qiagen	II

HRP-conjugated goat anti-rabbit IgG (Jackson Immuno Research Laboratories), HRP-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories), anti-rabbit IRDye 800 (LI-COR) and anti-mouse IRDye 680 (LI-COR) were used as secondary antibodies. The blots were developed using Super Signal (Pierce) for Film and LAS-3000 (Fujifilm). The intensities of the bands were quantified by an Odyssey Infrared Imaging System (LI-COR) or Aida Image Analyzer.

Immunoprecipitation (I, II)

Supernatants from the cells transfected with plasmids expressing recombinant proteins were incubated with Heparin Sepharose 6 Fast Flow (GE Healthcare) (I) for 2 h at RT or anti-Flag M2 Affinity Gel (Sigma) for O/N at 4°C (II). The Heparin Sepharose beads were washed four times with 20 mM Hepes-Na (pH 7.2), and incubated with 20 mM Hepes-Na and 0.5 M NaCl (pH 7.2) for 10 min at RT for elution. The anti-Flag M2 gel was washed and the bound ligands were eluted with Flag peptide (Sigma) according to manufacturer's instructions.

BMP signaling assay (I, II)

Supernatants of transfected cells (I) or eluted fractions containing IP-purified heterodimers (II) were mixed with S2 cells that had been transfected with mad-Flag expressing plasmid. Supernatant of the sog-Myc and tsg-His expressing cells was included to see the antagonistic effect (II). After 3 h incubation at RT the cells were collected and lysed in SDS-sample buffer.

RNA interference and quantitative RT-PCR (I)

The PCR primers for making dsRNA are listed in Table 3. dsRNA was synthesized with the MEGAScript High Yield Transcription Kit (Ambion) and purified with the RNeasy Mini Kit (Qiagen). 4 μg dsRNA / 1 ml cell suspension were used to knock down target gene expression.

Table 3. PCR-primers for making dsRNA. T7 is 5'-TAATACGACTCACTATAGGGAC.

Target gene	Forward primer	Reverse primer
Dfurin1	T7-GCAAAGATCCTCTGTGGCA-3'	T7-ATTGCTCCCGGAACTGC-3'
Dfurin2	T7-GCTAGAGGCCAATCCGGAA-3'	T7-CCTTCTCGCCCCAAAAGTG-3'
amontillado	T7-CCACATGGAGCTGGCTGTT-3'	T7-CCTGACTTTGCCGCCAT-3'

Quantitative RT-PCR was used to measure RNAi efficiency. The RNA of transfected cells was isolated with TRIzol (Invitrogen), treated with RQ1 RNase-free DNase (Promega), and used for cDNA synthesis by M-MLV reverse transcriptase (Promega). A LightCycler 480 SYBR Green I Master (Roche) was used for RT-PCR. The PCR-primers for RT-PCR are presented in Table 4.

Table 4. RT-PCR primers.

Target gene	Forward primer	Reverse primer
Dfurin1	5'-CGATGCGGTTGCCAAGGATC	5'-TGGCGTCCACCATCGACATG
Dfurin2	5'-TGCGTTACCTAGAGCATGTCCAATG	5'-TTGAGGTAACTGGCAAAGCTATCCG
amontillado	5'-GGGTCAGAATGGCGGCAAAG	5'-TTCGCCGGCACAACGAGTTC

Transgenic animals (I, II)

Drosophila lines carrying *dpp* transgenes under the control of the yeast Gal4 UAS (upstream activation sequence) were generated. The UAS sequence results in cDNA transcription when yeast Gal4 is expressed in *Drosophila* cells (Brand and Perrimon, 1993). UAS-*dpp* was constructed by M. Hoffmann (Haerry *et al*., 1998). The QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used to generate mutations in UAS-*dpp*-HA. Several independent lines of each pUAS construct were obtained by P-element mediated germline transformation (I).

A 4882-bp genomic DNA fragment containing the *scw* locus was amplified by PCR, and mutations were generated by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Wild-type or cleavage mutants of genomic *scw* lines were obtained by the injection of pattB-genomic *scw*, using the PhiC31 site-specific integration system at chromosomal position 86Fb (Bischof *et al*., 2007). The scw^{E1} and dpp^{hr4} flies were obtained from L. Raftery (II).

Other mutant strains, and strains carrying genetic markers or chromosome balancers (dpp^{d6}/CyO;*dpp*-Gal4/Tm3*Sb*, dpp^{d14}/CyO, A9-Gal4, Df(2L)OD16, scw^{5}/CyO, dpp^{H46}/CyO, CyO *ftz-lacZ*) were obtained from Bloomington Drosophila stock center or Drosophila Genetic Resource Center (I; II).

Immunostaining and *in situ* hybridization (I, II)

Staining of late 3rd instar larval wing discs (I) and 0-4 h embryos (II) were carried out following standard procedures. *In situ* hybridization of wing imaginal discs (*Dfur1, Dfur2* and *amon*, I) and whole-mount embryos (*zen*, *pnr*, *Msh*, *race*, and *lacZ*, II) was performed with digoxigenin-labeled RNA probes and visualized as blue alkaline phosphatase precipitates (Shimmi *et al*., 2005b). Mutant embryos were identified by lack of hybridization of *lacZ* transcripts produced from the Cyo, *ftz-lacZ* balancer chromosome (II).

Phylogenetic analyses (II)

BMP5/6/7/8/Gbb/Scw type protein sequences were downloaded from the Ensembl database and aligned with the program PRANK (Loytynoja and Goldman, 2005). Phylogenetic analyses were first performed for the paralog subgroups. The full phylogenetic tree was inferred from a reduced alignment containing only the sequences of ligand domains and the most conserved parts of the prodomain and, for comparison, from an alignment containing only the sequences of the ligand domains. The tree with the best arrangement for the paralog subgroups and a small number of individual sequences were searched with the program RAxML (Stamatakis, 2006) using the PROTGAMMAJTT model. 100 bootstrap replicates were performed and support values were projected to the maximum likelihood tree.

7 RESULTS AND DISCUSSION

The *Drosophila* Dpp signal is produced by cleavage of its proprotein at three furin recognition sites (I)

Drosophila Dpp is synthesized as an inactive 588-amino acid precursor protein. After dimerization and proteolytic cleavages, the active C-terminal mature forms are secreted from the cells. In contrast to the single mature form of the vertebrate ortholog BMP4, Dpp is secreted in two molecular forms, named Dpp23 and Dpp26, according to their size. Since only one cleavage had been localized to occur after the $RSIR^{456}$-motif (Shimmi *et al*., 2005b), we created HA-tagged *dpp* constructs to clarify the maturation process of Dpp. Potential cleavage sites were identified by searching for consensus furin recognition sites (-RXXR- or -RXR/KR-) in protein sequences. Three furin cleavage sites were found to be conserved in Dpp proteins of Diptera and they were mutated for more precise analyses. Cell culture experiments showed that the 90 kD precursor form of Dpp is cleaved at Furin recognition site II (FSII) situated in the prodomain to create an intermediate form which is rapidly processed at FSIII or FSI to produce the Dpp23 or Dpp26. Figure 11 illustrates the proposed model for Dpp cleavage. Surprisingly, all the cleavage products including the intermediate form processed only at FSII, were able to bind receptors and activate the BMP signaling pathway.

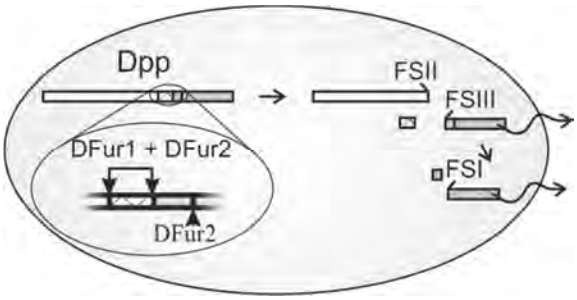

Figure 11. Maturation of *Drosophila* Dpp. The precursor is simultaneously cleaved by DFurin1 and DFurin2 at the optimal furin recognition site FSII and the minimal furin recognition site FSIII. This produces a biologically active Dpp26 that can be further cleaved by DFurin2 at the optimal furin recognition site FSI, to produce Dpp23. The ligand domain of Dpp is marked in gray. (Reprinted from III.)

It was noticed that the cleavage at FSII is a prerequisite for producing mature ligands since mutations inhibiting the processing of FSII led to significantly lower amounts of mature ligands in cell culture and in wing imaginal discs. In addition, transgenic flies carrying mutations at FSII (Dpp^{MFSII}-HA) were unable to develop full sized wings when their wing development was perturbed by a hypomorphic combination dpp^{d6}/dpp^{d14}. At the same time flies overexpressing wild type Dpp-HA, Dpp^{MFSI}-HA or $Dpp^{MFSI/III}$-HA were able to develop normal sized wings. Wing imaginal discs were stained with HA antibody to see how the ectopically expressed Dpp mutant proteins were produced and distributed. Conventional staining showed that both the wild type and mutated Dpp were stably produced. Extracellular staining, however, revealed that Dpp^{MFSII}-HA proteins failed to form a protein gradient.

RNA interference (RNAi) of PCs was used to understand how the cleavages at FSI-III are regulated. dsRNAs against the three known PCs in *Drosophila* were co-transfected with *dpp*-HA and the resulting cleavage products were analyzed by Western blotting. It was noted that Amon did not participate in Dpp cleavage. This was reasonable because *in situ*

hybridization of wing imaginal discs showed that *amon* is not expressed in the tissue during the time when Dpp cleavage is required. *Dfur1* and *Dfur2* expression in wing imaginal discs was clearly visible after *in situ* hybridization, and their roles in Dpp cleavage turned out to be partly overlapping. Based on the results of the RNAi analysis in S2 cells we propose a cleavage model that is presented in Figure 11. DFur1 prefers the cleavage of FSII and FSIII and their cleavage is closely linked. DFur2 can cleave FSI and is also capable of cleavage at FSII and FSIII.

We considered the role of the two Dpp ligands in *Drosophila* development. Akiyama *et al.* (2008) used a truncated form of Dpp ($Dpp^{\Delta N}$) lacking a short domain at the N-terminus essential for interacting with Dally, to prove that Dally stabilizes Dpp on the cell surface. $Dpp^{\Delta N}$ was more quickly internalized by cells and degraded. It was suggested that Dally may antagonize Tkv in Dpp signaling and inhibit receptor-mediated endocytosis. Akiyama *et al.* (2008) were able to show different binding affinities of the two Dpp ligands in a Heparin binding assay. We were unable to reproduce this result and concluded that there are no differences in the binding affinities of Dpp23 and Dpp26 towards HSPGs. Nevertheless, the two forms of Dpp may have other yet unknown differential functions in different tissues with different combinations of ECM molecules. For instance, it would be potentially beneficial to study the different Dpp ligands in embryo development since Sawala *et al.* (2012) suggested that the bigger form of Dpp (Dpp26) would bind better to collagen IV than the smaller Dpp23. Apparently the two Dpp ligands have similar binding affinities to Scw (II) and would therefore participate in gradient formation differently; circumventing the shuttling complex formation and signaling at short range.

Sopory *et al.* (2010) were able to show that in contrast to wing imaginal discs, midgut development does not require cleavage at the FSII. This shows that sequential cleavage of BMP4/Dpp precursors is a mechanism for regulating BMP signaling levels in different tissues.

Cleavage of the *Drosophila* Scw prodomain is critical for a dynamic BMP morphogen gradient in embryogenesis (II)

Raftery *et al.* (1995) performed genetic screens to identify genes required to maximize *dpp* signaling during dorsal-ventral patterning of *Drosophila* embryo. Screens for dominant enhancers of partial loss-of-function mutations in *dpp* revealed among others a new allele of *scw*, named scw^{E1}. Loss-of-function mutations in *scw* or deficiency strains lacking *scw*, are viable in combination with hypomorphic *dpp* alleles, thus the failure of scw^{E1} to complement a range of *dpp* alleles has been interpreted as interfering with some aspect of Dpp signaling in a dominant negative manner (Arora *et al*., 1994). To find out what is the affected link between scw^{E1} and BMP signaling we sequenced the allele and identified a mutation in the prodomain of Scw (nucleotide C^{271} > T (amino acid R^{91} > C)) that inhibited cleavage of the proprotein. These findings led us to study the relationship between the Scw prodomain cleavage and BMP signaling.

We wanted to understand how cleavage of the Scw proprotein contributes to its role in embryonic patterning *in vivo*. Genomic *scw* constructs carrying point mutations that affect the cleavage of the proprotein were generated. We examined the ability of different cleavage mutants of Scw to restore function in a *scw* mutant background and noticed that all the cleavage sites are necessary to create normal BMP signal in the developing embryo. Only the wild type genomic *scw* construct was able to rescue a loss-of-function *scw* mutant, scw^{5}*/Df(2L)OD16*. Closer examination of BMP target gene expression and staining of pMad in embryos showed that the cleavage sites of Scw appear to be

differentially utilized for Scw signaling *in vivo*. The absence of pMad and high threshold targets, *zen* and *race*, in the dorsalmost cells argues that loss of the FSI (Main site in Fritsch *et al*., 2012) impacts the ability of the ligand to contribute to peak levels of BMP signaling, however, this form of Scw is able to provide minimal BMP signaling, probably through partial activity of the longer form of Scw. By contrast, the E1-mutant (Pro site in Fritsch *et al*., 2012) is unable to substitute for any aspect of Scw function.

On top of that, scw^{E1}, but not scw^{MFSI}, showed genetic interaction with a hypomorphic dpp^{hr4} allele. The scw^{E1} allele and a hypomorphic *dpp* allele (dpp^{hr4} scw^{+}/dpp^{+} scw^{E1}) result in synthetic lethality despite the presence of a functional copy of each gene (Raftery *et al*., 1995). So we analyzed BMP signaling in animals carrying one copy of mutated genomic *scw* in hypomorphic *dpp* background. Target gene expressions revealed that mutations affecting the cleavage of the mature ligand (MFSI) have no definite effect on signaling *in vivo* whereas scw^{E1} seems to have a dominant negative effect on BMP signaling in a genetic background that is compromised for Dpp.

To be able to understand the dominant negative interactions between Scw^{E1} and Dpp we analyzed protein production and biochemical properties of the cleavage mutants in S2 cell culture. Mutated E1 produced mature ligands in notably smaller quantities. In addition, the mature ligand was secreted with prodomain peptides that result from the cleavage at the FSII or signal peptide (SP) processing site. We noticed that cleavage mutants of Scw are equally functional *in vitro* since they are able to form heterodimers with Dpp and bind to receptors to activate the signaling cascade. In addition, signaling was blocked by Sog and Tsg. The notions concerning the amount of mature ligand produced by scw^{E1} led us to believe that the dominant negative effect in embryogenesis may arise from reduced amounts of Dpp/Scw heterodimers. We hypothesized that Scw^{E1} could proportionately impact secretion of Dpp/Scw^{E1} heterodimers. This assumption was soon overruled since we noticed that Scw^{E1} preferentially forms heterodimers with Dpp and they are efficiently secreted. Quantifications showed that very limited amounts of mature ligands produced by scw^{E1} are able to pull down significant amounts of Dpp in co-immunoprecipitation analyses.

The dominant negative behavior of mutants has been shown before in the TGFβ superfamily of proteins. This effect was explained by heterodimer formation; the mutated protein dimerizes with the wild type protein and promotes their degradation within the cell (Lopez *et al*., 1992). In case of scw^{E1} the dominant negative effect seems to be based on the same mechanism. Our results strongly support that cleavage-resistant Scw binds Dpp more efficiently than its wild type counterpart. Still, the properties or mechanisms that inhibit BMP signaling in the embryo remain to be solved. We propose that the extra peptides that are associated to the Scw ligand after the processing at FSI and FSII/SP may interfere with interactions with the ECM. This kind of functional role for the prodomain has already been described for BMP7 where the attached prodomain peptide targets the ligand to the ECM. As fibrillin-1 has been shown to serve as a universal high affinity docking site for propeptides of many BMPs (Sengle *et al*., 2008a), it would be worthwhile to study the interactions between fibrillin-1 and Scw or Scw^{E1}.

BMP2/4 and BMP5/6/7/8 subfamily proteins have evolutionarily diversified cleavage sites (I, II, III)

Phylogenetic analyses have placed BMPs into several subgroups amongst which the BMP2/4/Dpp and BMP5/6/7/8/Gbb/Scw groups are the most uniform. The ligand domains of BMPs in the subgroups are well conserved, but the prodomains have largely been

considered unconserved. The conservation of the ligand domains suggests that they are not tolerant to mutations through evolution. The stringent requirements for having the right three-dimensional structure to be able to bind receptors have set limitations to the evolutionary development of proteins. Recent studies have started to pay more attention to the structure of prodomains and researchers have discovered the functional and evolutionary meaning of the variation in the sequences of prodomains.

We have found that the cleavage patterns of proproteins in BMP2/4/Dpp subfamily can be categorized in four different types (I; III). Cnidaria BMP2/4/Dpp is considered to be a prototype (type I) containing only one optimal furin site. All the bilaterian ligands belonging to the BMP2/4/Dpp subgroup have two or three furin recognition sites. These were further categorized according to the nature of their cleavage; Type II ligands contain an upstream minimal (-RXXR-) furin site and an optimal (-RXK/RR-) site adjacent to the ligand domain, whereas type III proteins have optimal furin sites both upstream and adjacent to the ligand. Type IV has evolved a third cleavage site (minimal) between the two optimal sites. Our experimental studies combined with the results by Sopory *et al.* (2010) and Goldman *et al.* (2006) clearly prove that the Dpp maturation process has acquired new features through the evolutionarily diversified cleavage sites and belongs to the group of Type IV ligands. The FSII cleavage is essential for Dpp function in wing and leg development, but not in the gut, whereas analysis of BMP4 function in mice demonstrated an opposite requirement for FSII cleavage: FSII mutations did not affect limb development, whereas the development of testis and germ cells was affected (Goldman *et al*., 2006; Sopory *et al*., 2010). In addition, the evolutionarily conserved FSI cleavage in Dpp may no longer be crucial, although the precursor is cleaved at the FSI *in vivo*, indicating that function of the FSI has been lost. However, the relevance of FSI cleavage requires additional studies, since the role of the two Dpp ligands has not been clarified yet.

Our studies concentrating in Scw cleavage reveal a unique mechanism by which post-translational modification of Scw modulates Dpp signaling. Sequence alignment and phylogenetic analyses of the BMP5/6/7/8 subfamily indicate that the furin cleavage motifs in the proprotein are highly diversified, through gain and loss of sequence motifs. The differential signaling capacity of wild type Scw and Scw^{E1} provides further insights into how the diversification of cleavage motifs in the BMP5/6/7/8 subfamily prodomains could be exploited as modules for post-translational regulation of BMP signaling.

The evolutional path of *scw* is interesting since it has evolved rapidly after arising from a duplication of an ancestral *gbb*, following the separation of the mosquitoes and the higher Diptera (Fritsch *et al*., 2010; Van der Zee *et al*., 2008). Closer comparison of the Gbb and Scw functions in different developmental contexts reveals the meaning of evolutional diversification of the proproteins. The different requirements for proteolytic cleavage in these two proproteins can explain their differential function to a certain degree, but additional studies are required to understand the biophysical and chemical properties of the cleavage products. For example, the role of ECM may provide the missing link in the context. The content of ECM molecules varies in different tissues and thus could require different binding affinities between ECM and BMPs, and differential cleavage patterns could provide a means to meet the requirements.

8 CONCLUSIONS

Our studies reveal that evolutionary changes in prodomain cleavage patterns of BMP type proteins can be a means of exploiting conserved ligands in differential processes. Furin cleavage sites in BMP precursors are tolerant to mutations acquired through evolution and have adapted to different systems in divergent species. Functionally conserved ligand domains indicate that the signaling pathway and especially the ligand-receptor interactions are maintained unaltered during evolution. Since the development of different tissues in variable animals requires some fine-tuning of the BMP-signaling to achieve different outcomes, the variable cleavage patterns in the prodomains of BMPs are a biologically sensible way to adjust signaling suitable for different contexts. Further studies specifying the significance of the cleavage sites of BMP proteins in variable species would increase our knowledge of how nature modifies proteins to create biological diversity.

In addition, it is reasonable to pay more attention on the cleavage patterns of other growth factors as well. When developing antibodies for research or for clinical use, it is extremely important to study the whole maturation process of the target protein. Antibodies have become an ever more important group of therapeutic substances for treating cancer (Reviewed in Carter, 2001). The efficacy of the drug is often improved by reducing the size of the antibody and by allocating the substance to specific region of the target protein. Differential processing in variable tissues may provide a tool for tissue specific targeting of the antibodies in clinical approaches, but it must be taken into account that differential cleavage of the target protein may lead to inefficient allocation of the antibody.

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