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Dissertation

Die Funktion von Bx42/Skip im TGF- β/Dpp Signal Transduktionsweg

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I Introduction

“As development time and cell proliferation of the anlage proceed, large compartments become split into pairs of smaller ones.”

Garcia-Bellido A., et al.1976

The formation of the *Drosophila* fly from the fertilized egg is a result of different consecutive signals that determine and specify, through gene regulation, a set of cell compartments with their own appropriate genetic address. The genetic address or positional information in a cell compartment describes a state in which defined genes are active or inactive. This specifies cells and also their descendants and becomes fixed in the cell compartments until the formation of a defined spatial pattern (Garcia-Bellido et al., 1973; Garcia-Bellido et al., 1975; Lawrence and Struhl, 1996; Vincent, 1998).

In the developing embryo and larva the genetic address has an important role in determining how cells within a compartment communicate and intermingle with cells in adjacent compartments. It also directs a defined group of cells to form a defined part of the adult body. The imaginal discs of *Drosophila* are a good model to study the establishment of genetic addresses within a group of cells.

Drosophila imaginal discs are simple sac like invaginations consisting of epithelial monolayer structures that give rise to most parts of the adult body. Disc precursors are set aside during embryogenesis. They proliferate extensively during larval development to generate mature imaginal discs (Simcox et al., 1991; Diaz-Benjumea et al., 1993). Imaginal discs are separated into several distinct compartments, for instance the anterior (A) and posterior (P) compartment, with different positional information. This difference is marked by the presence of *engrailed* which is exclusively expressed in posterior cells (Morata et al., 1975; Vincent et al., 1992). The selector gene *engrailed* (*en*) orchestrates the successive steps to pattern the imaginal discs. This occurs in the first step by the activation of *hedgehog* (*hh*) in *engrailed* expressing cells. Hh is a secreted protein that in the anterior cell compartment acts as a short range signal to induce the expression of *decapentaplegic* (*dpp*) in the anterior cells that are adjacent to the A/P boundary (Diaz-Benjumea et al., 1994; Tabata et al., 1994; Felsenfeld et al., 1995). The Dpp molecule, in turn diffuses in both directions to generate a concentration gradient of Dpp, which induces directly and at a distance distinct cellular responses. Consequently Dpp signalling patterns

the discs symmetrically along the A/P axis (Capdevila et al.,1994; Lawrence et al.,1996; Lecuit et al., 1996; Nellen et al., 1996; Dahmann et al.,2000).

1.1 Signals act outside of cells to induce responses inside

Once the activation of the *dpp* gene has occurred at the anterior/posterior boundary by Hedgehog, the secreted Dpp molecule diffuses and forms a gradient in the neighbouring compartment forming a signal transduction pathway. Dpp is the homologue of the human Bone Morphogenic Protein 2/4 BMP2/4 (Massagué, 1998) and both of them are secreted polypeptides that belong to the highly conserved Transforming Growth Factor- β (TGF- β) superfamily (Raftery et al.,1999).

In target cell this extracellular morphogenic molecule activates the intracellular signal transduction machinery by binding to the two transmembrane serine/threonine kinase proteins known as type I receptor and type II receptor. The binding of Dpp to the type II receptor induces the association of type I receptor to the complex. On the cytoplasmic side of the heterodimeric receptor complex the constitutively active kinase of type II receptor phosphorylates the type I receptor at its glycine/serine (GS) rich domain, resulting in the activation of the type I receptor kinase. Once the type I receptor is activated it recognises the R-Smad protein and phosphorylates its C-terminal SSXS domain. Smad proteins are members of an evolutionary conserved signalling transmitter family found in insects, vertebrates and nematodes. Three important subgroups of Smad proteins are identified: the Receptor-regulated Smads (R-Smad), the Common-mediator Smads (Co-Smad) and the Antagonistic or Inhibitory Smads (I-Smad) (Heldin et al., 1997; Massagué,1998; Wrana, 2000) see Table 1.

Tab. 1: Presented are the TGF- β /Dpp and TGF- β /Activin signalling pathways in *Drosophila* and their homologues TGF- β /BMP4 and TGF- β /Activin in vertebrates. Shown are also the three Smad protein subfamilies: the receptor regulating Smads (R-Smad), the common Smads (Co-Smad) and the Inhibitory Smads (I-Smad) that are conserved in *Drosophila* and vertebrates.

	TGF-β subgroups	R-Smad	Co-Smad	I-Smad
<i>Drosophila</i>	Dpp /BMP	Mad	Medea (Med)	Dad
	Activin	dSmad2		
Vertebrate	BMP /Dpp	Smad1 Smad5 Smad8	Smad4 Smad4 β	Smad6 Smad7
	Activin	Smad2 Smad3		

The phosphorylation of R-Smad evokes its dissociation from the receptor complex and its association with Co-Smad (Tsukazaki et al., 1998; Wu et al., 2000). The Co-Smad/R-Smad complex translocates to the nucleus where it regulates the transcription of TGF- β target genes by binding to specific sites on DNA, supported by interaction with other transcription factors (Kim et al., 1997; Zawel et al., 1998; Zhou et al., 1998; Liberati et al., 2001; Moustakas et al., 2001). Dpp signalling can be antagonised by I-Smads, which act in the cytoplasm as TGF- β /Dpp signalling competitive inhibitors at many levels. I-Smads inhibit the binding of R-Smad to the type I receptor, preventing the phosphorylation of R-Smad. Moreover, I-Smads competitively inhibit the binding of activated R-Smads to Co-Smads preventing the transduction of the signal to the nucleus (Nakao et al., 1997; Massagué et al., 2005). Smad6, a mammalian I-Smad protein, binds to activated Smad1 and thus competes with Smad4 for heteromer formation with Smad1. Both Smad6 and Smad7 inhibit TGF- β /BMP signalling, while Smad7 is more potent in inhibiting TGF- β /Activin signals than Smad6 (Hayashi et al., 1997; Itoh et al., 1998) see Figure 1.

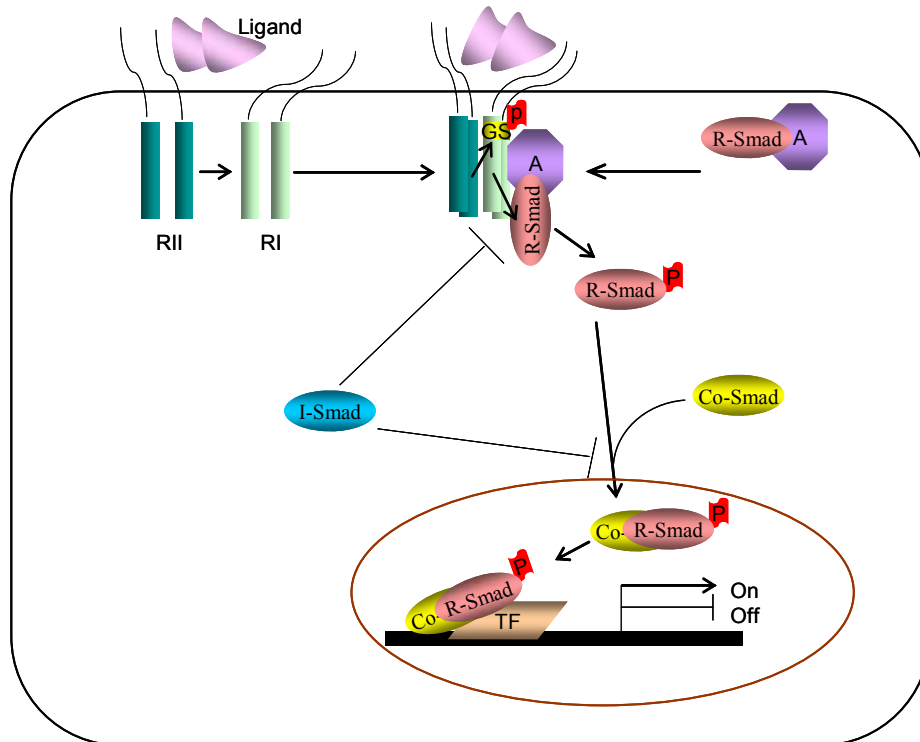


Figure 1: TGF- β /Dpp signalling pathway. The binding of the ligand Dpp to the Type II receptor (RII) results in formation of the heterodimeric type II/type I receptor complex. This leads to the phosphorylation of the type I receptor (RI) by the constitutive active type II receptor kinase at its GS domain. The RI kinase then becomes active and phosphorylates R-Smad, which is recruited to the receptors *via* cytoplasmic Anchor proteins (A). R-Smad changes its conformation and forms a heterodimeric complex with Co-Smad. This complex translocates to the nucleus where it binds to Smad Binding Element (SBE) and regulates in concert with other Transcription Factors (TF) the expression of TGF- β /Dpp target genes. The I-Smad proteins antagonise the TGF- β signalling in two ways. They can inhibit the phosphorylation of R-Smad, or they inhibit the formation of the heteromeric R-Smad/Co-Smad complex.

The sequence analysis of the R-Smad and Co-Smad subgroups led to the identification of two well conserved domains termed Mad Homology 1 (MH1) at the NH₂-terminal region and Mad Homology 2 (MH2) at the COOH-terminal region which are separated with a less conserved linker. Smad MH1 and MH2 display a surface for protein-protein and protein-DNA interaction and exhibit no enzymatic activity (Kim et al., 1997; Feng et al., 2005). The I-Smad proteins however contain only the MH2 domain and have no similarity to the other Smad proteins in their N-terminus. The MH2 domain of the R-Smad family contains a conserved SSXS signature that is a target for the phosphorylation by the RI in the presence of the extracellular signal (Figure 2).

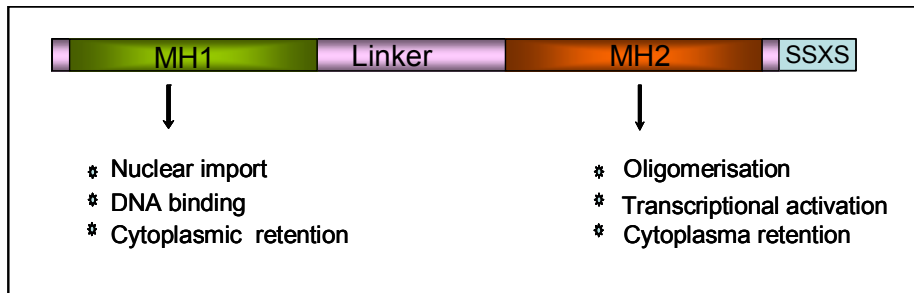


Figure 2: Structure and function of R-Smad and Co-Smad proteins. Both Smads contain the conserved MH1, MH2 domain which are separated with a less conserved linker region. Additionally only the R-Smad proteins have the SSXS signature at their COOH region. This SSXS signature is a target for the type I receptor kinase. The principal functions of each domain are also shown.

1.2 The Positional information conveyed by TGF- β /Dpp signalling

The interpretation of Dpp signalling evokes the activation or repression of target genes in a defined group of cells often in nested domains. This interpretation is specified by the local pre-existing information.

In the wing imaginal discs, Dpp target genes *spalt (sal)*, *optomotor blind (omb)* and *vestigial (vg)* are positively regulated by Dpp signalling. Their expression domains were defined as nested pattern, which are centres on and extends away from the source of *dpp* expression (Lecuit et al., 1996; Nellen et al., 1996). In contrast to these genes *brk* is negatively regulated by the same signal and therefore is expressed in the more lateral parts of the wing where Dpp signalling is low or absent. The expression of *brk* in this region prevents the expression of *spalt* and *omb* in the more lateral region (Jazwinska et al., 1999a; Jazwinska et al., 1999b; Campbell and Tomlinson, 1999; Minami et al., 1999; Marty et al., 2000; Sivasankaran et al., 2000; Affolter et al., 2001) see Figure 3.

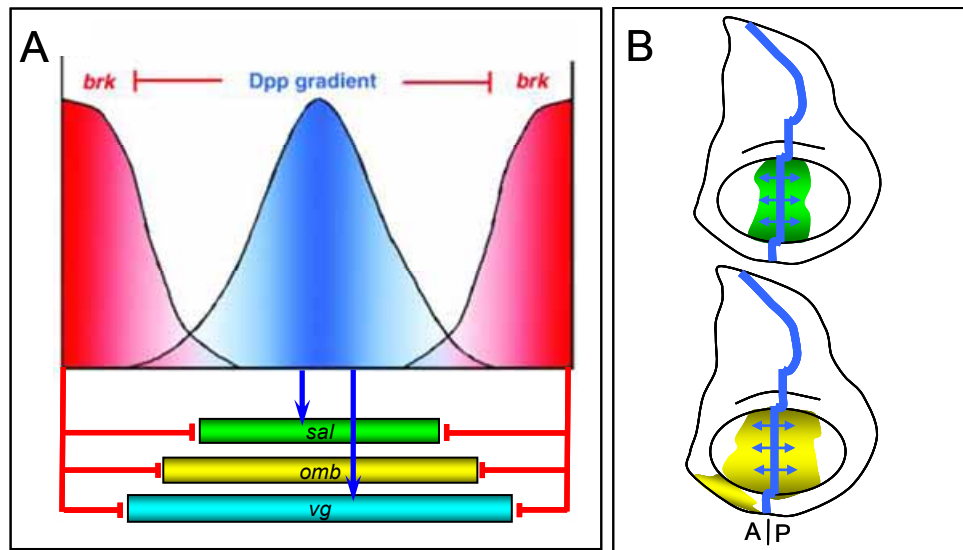


Figure 3: Schematic illustration of the positional information dependent on Dpp signalling in the wing imaginal disc. (A) Dpp is expressed in restricted number of cells anterior to the A/P compartment boundary. It diffuses in both directions from this source to the neighbouring cells in the epithelial sheet forming a long range gradient. In the wing imaginal discs Dpp signalling activates directly the expression of its target genes *optomotor of blind* (*omb*), *vestigial* (*vg*) and *spalt* (*sal*) and represses the expression of the target gene *brinker* (*brk*) in the middle region. *Sal* is expressed in a less broad domain than *omb* and both of this genes are not detectable in the more lateral region. *Vg*, however, is expressed in the entire wing blade primordia. *brk* is expressed in the more lateral region of the wing imaginal disc. *Brk* is responsible for the repression of *sal*, *omb* and *vg* in this region. This results in the formation of nested domains of expression (modified according to Affolter et al., 2001). (B) Schematic view of *Sal* (top) and *Omb* (bottom) expression domains in the wing imaginal disc. Dpp (in blue) is expressed along the anterior/posterior boundary and diffuses in both directions (arrow).

The formation of this kind of gene expression landscapes and cell specific responses is the result of the formation of several different protein complexes containing the Smad proteins as a core and other transcriptional cofactors present in a given cell. The identification of Smad interaction partners at target genes is, therefore, important for the understanding of the Dpp transduction pathway.

Our group previously characterized the *Drosophila* protein Bx42 as a conserved nuclear protein that is essentially required for development. Using RNA-interference we obtained circumstantial evidence for the participation of Bx42 in the TGF- β /Dpp signal transduction pathway (Negeri et al., 2002).

1.3 Bx42: the gene, the protein

The *Drosophila* nuclear protein Bx42 was identified using monoclonal antibodies directed against nuclear proteins. It is localised principally on transcriptionally active sites (Frasch and Saumweber, 1989; Saumweber et al., 1990). Because of an alternative termination, the *Bx42* transcription unit encodes two transcripts of 1.9 and 2.2 kb length. From both transcripts the same putative 547 amino acids protein with a molecular weight of 66 kDa can be expressed. Molecular biological assays such as RNA blots and in situ hybridization on whole mount embryos showed the ubiquitous presence of Bx42 transcripts in all *Drosophila* developmental stages (Wieland et al., 1992). Soon after fertilisation both Bx42 transcripts are detected leading to the assumption that these early transcripts are maternally delivered and stored in the embryo (Wieland et al., 1992). Biochemical assays have shown that Bx42 is a highly hydrophilic protein that contains a high score of charged amino acids.

1.4 Bx42 is evolutionary conserved from yeast to mammals

Bx42 orthologues were identified and characterized in *Saccharomyces cerevisiae* (*Prp45p*; Albers et al., 2003), *Schizosaccharomyces pombe* (*Snw1p*; Ambrozikova et al., 2001), *Dictyostelium discoideum* (*SnwA*, Folk et al., 1996), *Caenorhabditis elegans* (*CeSkip*; Kostrouchova et al., 2002) and in *humans* (*hSkip/NcoA62*; Dahl et al., 1998, Baudino et al., 1998). From the comparison of sequence similarities present in Bx42 orthologues three distinct domains can be distinguished: the N-terminal region that contains an absolutely conserved motive LPXP and a glycine rich box. The central part of the protein was identified as a potential alpha-helical coiled coil. This region contains highly conserved domains consisting of a proline rich box, a SNW signature and a helical repeat. This central region of the protein is also called SNW domain. Finally the C-terminal part is highly charged due to the presence of acidic and basic amino acids and harbours in some species a nuclear localisation signal (NLS).

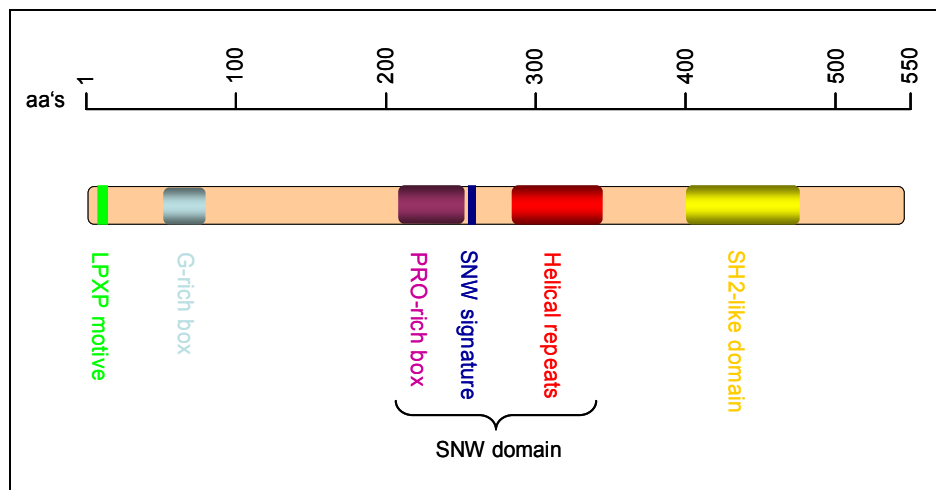


Figure 4: Schematic representation of Bx42 domains. Presented are the highly conserved regions in Bx42. The N-terminus is characterised by the presence of the LPXP motive (aa 7-10) and a glycine-rich box (aa 56-67). The SNW domain consists of a proline-rich box (aa 207-249) which serves as a potential SH3-domain binding site (Folk et al., 1996), the SNW-signature (aa 254-257) and a helical repeat domain (aa 274-333). The C-terminal region is marked by the presence of a SH2-like domain (aa 383-462).

Prp45p/Fun20 of *Saccharomyces cerevisiae* was identified during deletion analysis for chromosome I as an essential gene for growth with unknown function. Hence it was called *Fun20* for Function unknown. This protein lacks part of the N-terminal region that contains the LPXP motive, the glycine rich - and proline rich box but it still contains the SNW sequence, the helical repeat and a highly charged C-terminal region (Harris et al., 1992).

SnwA of *Dictyostelium discoideum* was identified in a screen for genes that contain a SH2 related domain. In contrast to *Fun20/Prp45*, *SnwA*, like Bx42, contains the glycine- and proline-rich box at its N-terminal region (Folk et al., 1996).

Bx42 orthologue was also identified in *Caenorhabditis elegans* that was named *CeSkip*. *CeSkip* is an essential protein which is expressed ubiquitously in all developmental stages of this specie (Kostrouchova et al., 2002).

The human orthologue of Bx42 was identified as an interaction partner of the vitamin D receptor (VDR) in the yeast two hybrid system and was called NcoA62 for Nuclear co Activator with a molecular weight of 62 kDa (Baudino et al., 1998). NcoA62 is present in various human tissues (Baudino et al., 1998). At the same time another group identified the human Bx42 orthologue as a protein that interacts in the two hybrid assay with the oncogene protein Ski and was accordingly termed Skip for Ski Interaction protein (Dahl et

al., 1998). Immunofluorescence analyses of Skip/NcoA62 in expressing cells demonstrated that Skip localises within nuclear speckles (Dahl et al., 1998; Mintz et al., 1999).

Sequence comparison of the various Bx42 homologues showed a high similarity score of more than 95 % in the SNW domain and hence this protein family is called the SNW gene family. This finding suggests that this region could be important to provide an evolutionary conserved biological function.

1.5 The SNW gene family is essential for viability

The essentiality of the SNW gene family for viability and its involvement in various cellular processes was shown in several experiments in different organisms. The deletion of *Prp45p/Fun20* causes lethality of the yeast cells. Interestingly, the N-terminal part of the protein harbouring the SNW domain rescues the lethality caused by deletion of the *Prp45/Fun20* gene in a complementation test. However, a substitution of the three amino acids SNW by three alanines does not affect the N-terminal region to rescue the lethality. Therefore the whole region is necessary for the rescue (Martinkova et al., 2002).

The introduction of CeSkip dsRNA into the gonads of *C. elegans* adults caused a 100 % embryonic arrest in the progeny due to a defect in gastrulation (Kostrouchova et al., 2002). Treatment of *C. elegans* larvae with CeSkip dsRNA generated several postembryonic defects revealing the involvement of *CeSkip* in development of many processes in *C. elegans* (Kostrouchova et al., 2002).

The importance of this gene during development has also been demonstrated in *Drosophila*. The down regulation of Bx42 in early embryos, using RNA interference, causes lethality of the embryo. Furthermore, the tissue specific Bx42-RNAi induction leads to many organ phenotypes (Negeri et al., 2002). These results provide evidence of the early requirement and the important participation of this protein in the morphogenesis in *Drosophila*. In conclusion, these functional analyses delineate not only the essentiality of SNW gene family but also indicate its involvement in several biological processes which will be discussed below.

I.6 The SNW gene family is involved in several biological processes

I.6.1 Involvement in nuclear receptor pathways

Studies in *Drosophila* provided the first evidences of Bx42 acting as a putative transcription factor in the steroid hormone pathway. As mentioned above, Bx42 was detected in a number of transcriptional active puffs on polytene chromosomes of late third instar larvae. One of these sites is the locus of the *Sgs-4* gene (Saumweber et al., 1990; Wieland et al., 1992). This gene is expressed in the salivary gland of third instar larvae and it is important for the anchoring of the pupae to a solid substrate during puparium formation. The activation of this locus depends on the steroid hormone 20-OH ecdysone. Interestingly, the binding of Bx42 to this site depends on the presence of ecdysone and of a 52 bp sequence within the enhancer region of *Sgs-4*, which is also essential for ecdysone hormone binding. The deletion of the 52 bp sequence resulted in a loss of Bx42 binding at the *Sgs-4* locus (Wieland et al., 1992). Whether Bx42 binds directly to the DNA of this locus or is recruited to its target region by binding to transcription factors, for instance Ecdysone receptor (EcR) in response to Ecdysone, still has to be elucidated.

The finding that *Skip/NcoA62*, in human cells, interacts with the nuclear receptors vitamin D receptor (VDR), retinoid acid receptor (RAR) and retinoid X receptor (RXR) in the two hybrid system (Baudino et al., 1998; Barry et al., 2003) supports the hypothesis that Bx42 binds to the *Sgs-4* locus in association with the Ecdysone receptor and supports the suggestion that the SNW gene family could play an important role in the regulation of nuclear receptor target genes.

In vitro binding assays confirmed the *in vivo* interaction between *Skip/NcoA62* and VDR. These tests also demonstrated that *Skip* binds to VDR in a concentration dependent manner and that the ligand 1,25-Dihydroxyvitamin D3 (1, 25(OH)2D3) is not essential for this interaction, but enhances the formation of the *Skip/VDR* complex *in vitro*. Further studies showed that *Skip* is not only able to form complexes with VDR monomers, but also with VDR homodimers and VDR/RXR heterodimers in a ligand-enhanced manner. Here, *Skip/NcoA62* has a higher affinity to the heterodimer VDR/RXR. Altogether these assays demonstrated an involvement of *Skip/NcoA62* as a coactivator in vitamin D receptor mediated transcription. A C-terminally truncated *Skip* lost its ability to enhance the VDR mediated transcriptional activation, suggesting the presence of a transactivation domain in

the C-terminus of Skip (Baudino et al., 1998; Zhang et al., 2001). These results are in accordance with the finding that Bx42 has a transactivation domain at its C-terminus (Negeri et al., 2002).

I.6.2 Involvement in RNA splicing

Gene expression in cells encompasses several biological processes including the synthesis of the pre-mRNA, the capping or polyadenylation of the 5' and 3' ends respectively and the removal of introns from the pre-mRNA. The mature transcript will then be exported to the cytosol where it will be translated to form the protein. The regulation of gene expression occurs in all of the above mentioned steps. A direct coupling of gene transcription and RNA processing by nuclear receptors has been demonstrated in several recent studies (Monsalve et al., 2000; Auboeuf et al., 2002; Auboeuf et al., 2004a; Auboeuf et al., 2004b; Auboeuf et al., 2005).

Skip/NcoA62 is a transcription coactivator that was detected on the vitamin D receptor responsive promoter of the VDR target gene 24-Hydroxylase by ChiP analysis. Besides in promoter activation Skip also may be required for elongation or RNA processing. Indeed, in several studies Skip was isolated as a component of the spliceosome complex and its interaction with several splicing factors was shown (Mintz et al., 1999; Zhang et al., 2003; Fire et al., 1998). Skip is recruited to the spliceosome before the first catalytic step of splicing, remains bound through the second catalytic step, and still associates with the post-spliceosomal intron complex (Makarov et al., 2002). Evidence for the functional importance of Skip in the RNA splicing processes could also be obtained in mammalian cells (Zhang et al., 2003; Nagai et al., 2004; Bres et al., 2005). Furthermore, the finding that Prp45 is an essential cofactor for pre-mRNA splicing in yeast (Albers et al., 2003) supports the hypothesis that Skip/NcoA62 is a factor linking two different biological processes, transcription and RNA processing.

I.6.3 Interaction with cell cycle components.

Skip was originally isolated as an interaction partner of the oncogene Ski. This interaction was mapped to the highly conserved SNO domain of Ski (Dahl et al., 1998). This region also seems to be necessary for the transforming ability of Ski (Zheng et al., 1997). Ski is an

oncogene protein that was isolated from the avian Sloan Kettering virus. This polypeptide plays a crucial role in cell proliferation and differentiation. It was shown that its expression causes an oncogenic transformation of avian fibroblasts and chicken embryonic cells (Stavnezer et al., 1981; Stavnezer et al., 1986). Furthermore, overexpression of Ski in quail embryos triggers the differentiation of myoblast cells from fibroblasts and thus enhances myogenesis (Colmenares et al., 1989). Additionally, Ski is a nuclear protein that acts in transcription regulation. It binds indirectly through other protein complexes to DNA and enhances or inhibits gene expression depending on the cell type and target promoter (Cohen et al., 1998; Tokitou et al., 1999; Nomura et al., 1999).

In coimmunoprecipitation experiments Ski was detected to interact with the cell cycle protein retinoblastoma (pRb) and inhibits transcriptional repression mediated by pRb (Tokitou et al., 1999). The function of the pRb as a tumour suppressor molecule that regulates the cell cycle by controlling the G1/S transition is well understood. Unphosphorylated pRb captures the E2F protein inhibiting its function. This silences the E2F target genes, which are necessary for the progression of the cell cycle (Nevins, 1992; Weinberg, 1995). Because of this and also the finding that Skip complexes with the muscle specific transcription factor MyoD and Poly A Binding Protein 2 (PABP2), which bind to hypophosphorylated pRb (Kim et al., 2001), it was investigated whether the Ski interacting protein Skip can also bind to pRb to modulate its activity. A strong interaction between pRb and Skip was demonstrated. This interaction could be mapped to the conserved SNW domain of Skip. Additionally, it could be shown that Skip does not act at the pRb expression level but it antagonises, together with Ski, pRb mediated transcriptional activity. Interestingly, Skip interacts with p107 and p130, which belong to the pocket gene family like pRb, and regulates the cell cycle. In cooperation with Ski, Skip can inhibit p130 induced transcriptional repression. This antagonistic effect of Skip and Ski on pRb- and p130- activity resulted in an abrogation of the cell cycle arrest in the G1 phase caused by pRb (Prathapam et al., 2002).

1.6.4 Involvement in signal transduction pathways

Cells receive informations that direct them to proliferate, differentiate, migrate or die. These informations, received from the cells exterior must find their way to the proper location within the cell and trigger the desired action through transcription of a particular

set of genes. Transferring information from the exterior compartment to the cytoplasm and finally to the nucleus involves many components forming signal transduction cascades or pathways.

I.6.4.1 Involvement in the Notch signalling pathway

The Notch signalling pathway forms an important transduction cascade for the proliferation and differentiation of cells in many multicellular organisms. It was first identified in *Drosophila* and after that it was discovered in humans. Notch is a transmembrane protein consisting of an extracellular N-terminal domain (ECN) that interacts with a ligand and an intracellular C-terminal domain (N-IC) that will be cleaved and translocated to the nucleus after binding of a ligand. Ligand binding occurs through direct cell-cell interaction. Once the N-IC is transported to the nucleus it acts on target genes by interacting with the Notch signalling transducer molecule CSL, which stands for CBF1/RBPjk in human, Suppressor of Hairless [Su(H)] in *Drosophila* and Lag-1 in *C. elegans*. CSL proteins are DNA binding proteins that execute two antagonistic functions depending on the presence or absence of an extracellular signal. In the absence of signalling, CSL proteins act as repressors by recruitment of a repressor complex, which consists of SMRT, Sin3A, HDAC and CIR (CBF1 Interacting Repressor) in humans and of Hairless (H), *Drosophila* C-terminal Binding Protein (dCtBP) and Groucho (Gro) in *Drosophila*. In contrast, in the presence of a signal the transmembrane protein Notch is cleaved and the N-IC is translocated to the nucleus, where it binds to CSL and displaces the corepressor complex SMRT/Hairless. In the following a coactivator complex is assembled by binding of coactivators like Histone Acetyl Transferase (HAT) to N-IC (Zhou et al., 2000; Barolo et al., 2002).

A function of human Skip in the Notch pathway was demonstrated using yeast and mammalian two hybrid systems, coimmunoprecipitation and transient expression assay (Zhou et al., 2000). Based on these assays, Skip interacts with CBF1 and with the SMRT repressor, independently of its interaction with CBF1. The significance of these interactions was shown by the fact that Skip represses transcription in a transient expression assay. Furthermore, Skip is able to bind to N-IC in a CBF1 independent manner. The Skip interaction surface on N-IC is distinguishable from the CBF1 binding site which was mapped to the RAM domain of N-IC. The important biological relevance of the interaction

between Skip and N-IC was illustrated by using antisense Skip mRNA and by mutation of the N-IC ankyrin repeat. In such experiments, the myoblast differentiation blockage caused by N-IC overexpression was abrogated in the absence of Skip as well as after the disruption of the Skip interaction site on N-IC. This observation indicates that Skip is necessary to exercise its function as a coactivator for N-IC. From this study it was concluded that Skip could act as an adapter protein for both the N-IC activator complex and the SMRT repressor complex (Zhou et al., 2000).

To address the question if the role of Skip in Notch signalling is conserved throughout evolution, the interaction between Bx42 and CBF1 or N-IC was studied and it could be shown that Bx42 interacts with N-IC and CBF1 as effective as Skip. Moreover, Bx42 mediates transcriptional repression in a transient expression assay (Zhou et al., 2000). These results correspond to genetic studies in *Drosophila* which revealed the involvement of Bx42 in Notch signalling by using Bx42-RNAi (RNA interference) (Negeri et al., 2002). The tissue specific downregulation of Bx42 gave rise to several strong morphological defects, which extended from the embryonic stage to the adult fly depending on the time and tissue of Bx42-RNAi induction. Some of these phenotypes were similar to Notch mutants (Kumar and Moses, 2001; Brennan et al., 1999; De Celis et al., 1994). Additionally, analysis of genetic interactions in the adult wing provided strong evidence for the involvement of Bx42 in the Notch signalling pathway. The simultaneous overexpression of Su(H) and induction of Bx42-RNAi gave rise to an enhancement of the phenotypes that were obtained when only one of this component was induced. Moreover, in the context of Notch signalling in the wing Bx42 seems to act as an activator, since the induction of BX42-RNAi represses the Notch target genes *cut* and *enhancer of split e(spl)m8*.

I.6.4.2 Involvement in the TGF- β signal pathway

TGF- β signalling provides a multifunctional cell-cell signalling pathway, which controls several cell processes through the intracellular signalling effectors Smad proteins (see Table. 1 and Figure. 1). These signal transducer molecules shuttle between the cytoplasm and the nucleus and cooperate with several transcription factors at regulatory sequences to activate or repress transcription.

The oncogene protein Ski is able to form a heterooligomeric complex with TGF- β /Activin comodulators Smad2, Smad3 and the co-Smad, Smad4. Here, Ski antagonises Smad activation of gene expression in response to TGF- β /Activin signalling (Xu et al., 2000; Luo et al., 1999; Luo 2003). Furthermore, an interaction between TGF- β /BMP (Bone Morphogenic Protein), Smad proteins and Ski was demonstrated in both mammalian cells and *Xenopus* embryos. Here, the Ski protein binds to complexes of Smad1/Smad4 or Smad5/Smad4 in a ligand dependent manner and counteracts TGF- β /BMP signalling (Wang et al., 2000). Because of these findings, Leong and co-workers investigated, if the Ski interacting protein (Skip) could interact with Smad proteins and whether it could negatively modulate the TGF- β /BMP signalling pathway accordingly to Ski. Indeed an interaction between Skip with Smad2 or Smad3 as well as with the common Smad protein Smad4 could be demonstrated in *in vivo* and *in vitro* assays (Leong et al., 2001). The SNW domain of Skip seems to be necessary for these interactions. Skip, like Ski, binds the MH2 domain of Smad3. Surprisingly Skip, in contrast to Ski, augments TGF- β transactivation in transient transfection assays of mammalian cells. This enhancement was attenuated by about 80% in a dose-dependent manner by co-transfection with Ski, raising the possibility that the cellular protein ratio of Skip *versus* Ski may play a regulatory role on TGF- β /BMP dependent transcription. The authors suggest a competition between Skip and Ski for a common binding site on Smad (Leong et al., 2001).

Evidence in *Drosophila* for an involvement of Bx42 in TGF- β signalling was obtained from Bx42-RNAi analysis. The tissue-specific induction of Bx42-RNAi caused a deletion or fusion of leg tarsal segments (Negeri et al., 2002). This phenotype is very similar to the hypomorphic mutant phenotype of the Dpp target gene *distal-less* (*dll*) (Cohen et al., 1989; Dong et al., 2000; Panganiban, 2000). In the present thesis I wanted investigated whether Bx42 interacts with components of the TGF- β /Dpp pathway and if these interactions are important in TGF- β /Dpp related developmental processes.

1.7 Aim of my work

Insights into the function of Bx42 in the development of *Drosophila* were obtained from several studies, providing evidence of an early requirement and important participation of this protein in the morphogenesis in *Drosophila* (Negeri et al., 2002). Furthermore, the identification of the human homologue of Bx42, Skip, as an interaction partner of different transcription factors which are components of TGF- β signalling components or Notch signalling as well as RNA splicing components indicates that this gene family may play an important role in the gene regulation of several signal pathways.

The leg phenotypes obtained by Bx42-RNAi showed similarities with the mutant phenotype of the TGF- β /Dpp target gene *distal less (dll)* and also with phenotypes caused by the overexpression of the *Drosophila* Smad protein Medea (Marquez et al., 2001). In all cases, deletion or fusion of leg tarsal segments were observed. This led to the hypothesis, that Bx42 could play a role in TGF- β /Dpp signalling pathway.

The purpose of this work was to elucidate the role of Bx42 in the TGF- β /Dpp signalling pathway. The focus was investigated by different methods.

On one hand, the ectopic expression of Bx42 and the TGF- β /Dpp signalling effectors Mad and Medea were analysed using several driver lines at different temperatures. These results were compared with the obtained Bx42-RNAi phenotypes.

In the other hand the molecular interaction of Bx42 with Mad or Medea was elucidated. These interactions should be proven using the yeast two hybrid system and *in vitro* binding tests. In addition, the genetic interaction between Bx42 and the candidate genes was tested *in vivo* in *Drosophila*. Using the same tools the interaction of Bx42 with the TGF- β /Activin signalling transducer dSmad2 or the oncogene protein dSno was analysed.

The biological significance of this interaction is the third subject of this work. The effect of Bx42-RNAi induction on the expression of the TGF- β /Dpp target genes *distal less (dll)*, *optomotor blind (omb)* and *spalt (sal)* were to be investigated. For this, expression assays were performed on imaginal discs using suitable lacZ reporter genes. Furthermore, *in situ hybridisation* analyses were used to study the effects of Bx42-RNAi on the expression of endogenous target genes at the RNA level.

II Experimental procedure

II.1 General used molecular biological applications

II.1.1 Bacteria- and Yeast strains

The bacteria and yeast strains used for protein-protein interaction are summarised in the Table 2.

Tab. 2: **Bacteria and yeast strains used for protein-protein binding test.** The establishing of the recombinant plasmids used in this work was done in *E. coli* XLI Blue bacteria cells. For the expression of the fusion proteins the strain *E. coli* BL21 was used. The yeast *S. cerevisiae* SFY526 strain was used to test interaction in yeast two hybrid system.

Strains	Genotype	Reportergene	Transformation marker
<i>E. coli</i> XLI Blue	recA1 endA, gyrA96 thi-1 hsdR17 supE44 relA1, lac [F', proAB, lacIqZDM15, Tn10 (Tetr)]c		Amp
<i>E. coli</i> BL21 (DE3) pLysS	<i>E. coli</i> B, F-, dcm, ompT, hsdS(r _B m _B), gall(DE3) [pLysS Camr].		Amp
<i>S. cerevisiae</i> SFY526	MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, can ^r , gal4-542, gal80-538, URA3::GAL1 _{UAS} -GAL1 _{TATA} -LacZ	LacZ	Trp1, leu2

II.1.2 Vectors

All vectors that have been used in this work are listed below (Table 3 and 4). Write down is also a detailed representation of their application. Additionally reference for more details of each vector is also referred. All of these vectors have the ability to be replicated in *E. coli* cells and they allow the selection of cells, which contain them since they give their host bacteria an antibiotic resistance. Moreover, the vectors pGEX-2T, pEt-(Myc)₃-(His)₆ are capable to express GST- and MH- recombinant proteins.

Tab. 3: Vectors, which are used for cloning strategies and for expression of recombinant proteins, are listed in this table. Shown are also the application, the resistance and selection marker and the source of supply of each of these plasmids.

Name	Application	Resistenz-/ Selektionsmarker	Source of supply
pBlue Script	sub cloning	Amp/ LacZ	Stratagene
pET3a -Myc-His	establishing of Myc-His recombinant protein Expression in BL21 E. coli cell	Amp/-	Novagen Rosenberg et al., 1987; Johanna Kaltenhäuser
PCR2.1		Amp, Kan/LacZ	Invitrogen
pGEX-2TK	establishing of GST recombinant protein Expression in BL21 E. coli cells	Amp/-	Pharmacia Biotech Smith and Johnson, 1988
pUAST	Expression in Drosophila (Gal4/Uas System)	Amp/ White Gen	Brand & Perrimon 1993
pII25.7 wc	Helper Plasmid	Amp	

Additionally to the above listed plasmid, two others yeast vectors pGAD424 and pGBT9 were used in the yeast two hybrid system for analysing protein-protein interaction. These plasmids accord the yeast cells to growth on Tryptophan/Leucine (Trp/Leu) missing media. The vectors pGAD424 and pGBT9 were applied to express Activation Domain (AD) fusion proteins and DNA Binding Domain (DBD) fusion proteins respectively (Table 4).

Tab. 4: yeast expression vectors pGAD424 and pGBT9 were the tools for the yeast two hybrid assay. Shown are also the application, the resistance and selection marker and the source of supply of each of these plasmids.

Name	Application	Resistenz-/ Selektionsmarker	Source of supply
pGBT9	establishing of DBD recombinant protein Expression in Yeast cells	Amp/ LEU2	Clontech
pGAD424	establishing of AD recombinant protein Expression in Yeast cells	Amp/LEU2	Clontech

II.1.3 Primer used in this work

Primers for yeast two hybrid system

F1SmaI <i>dSmad2</i>	TTC <u>CCGGGG</u> A ATG TTG CCA TTC ACC CC
R1BamHI <i>dSmad2</i>	GC <u>GGATCC</u> T TAT GAC ATG GAG CTG CAC
F2SalI <i>dSmad2</i> (1-287)	AATC <u>GTCGAC</u> AA ATG TTG CCA TTC ACC CCG
R2PstI <i>dSmad2</i> (1-287)	AATC <u>CTGCAG</u> GTG GTA CAT CAC CGG CG
F3EcoRI <i>dSmad2</i> (129-486)	AT <u>GAATTC</u> CTG TCC ATC CTG GTG CC
R3BamHI <i>dSmad2</i> (129-486)	R1BamHI was used
F4EcoRI <i>dSmad2</i> (287-486)	AT <u>GAATTC</u> CAC GAG CCG GCC TTT TG
R4BamHI <i>dSmad2</i> (287-486)	R1BamHI was used
F1SmaI <i>Mad</i>	TCC <u>CCCGGG</u> AAT GGA CAC CGA CGA TGT G
R1BamHI <i>Mad</i>	CG <u>GGATCC</u> T TAG GAT ACC GAA CTA ATT G
F2SmaI <i>Mad</i> (1-255)	ATT TCC <u>CCC GGG</u> AAT GGA CAC CGA CGA
TGT	
R2BamHI <i>Mad</i> (1-255)	CATA <u>GG ATCC</u> AT AGC TAA CCT GGG CAA CA
F3SalI <i>Mad</i> (145-456)	CATT <u>GTCGAC</u> AT AGT CCG GGT CTC CCG CCA
R3BamHI <i>Mad</i> (145-456)	CATA <u>GGATCC</u> TT AGG ATA CCG AAC TAA TTG
F4SalI <i>Mad</i> (145-255)	F3SalI was used
R4BamHI <i>Mad</i> (145-255)	R2BamHI was used
F1SalI <i>Medea</i>	ATCC <u>GTCGAC</u> AA ATG GGC GGC GGC TCG GGG GC
R1BglII <i>Medea</i>	GA <u>AGATCT</u> A CGG ATT AGG CGG CGG CAC GC
F2SalI <i>Medea</i> (1-466)	
R2BamHI <i>Medea</i> (1-466)	
F3SalI <i>Medea</i> (165-772)	CATT <u>GTCGAC</u> AT TCT CCG GGC ATC GAT CTG
R3BglII <i>Medea</i> (165-772)	CATT <u>AGATCT</u> ACG GAT TAG GCG GCG GCA
F4SalI <i>Medea</i> (466-772)	TATT <u>GTCGAC</u> AT GGA GGC GGTGCG GCT G
R4BglII <i>Medea</i> (466-772)	R3BglII was used
F1EcoRI <i>dSno</i>	TC <u>GAATTC</u> A TGA CCG AAT ACG TGA CG
R1BamHI <i>dSno</i>	GC <u>GGATCC</u> A ACC CAA CGC ACC TTT CT

Primers for Ni-NTA pull down

F5BamHI *Mad* AA GGATCC A TGG ACA CCG ACG ATG TGG AAR5EcoRI *Mad* GC GAAT TC G GAT ACC GAA CTA ATT GCAF5BglII *Medea* TTCATT AGATCT ATG GGC GGC GGC TCG GGR5BglII *Medea* TTC ATT AGATCT CGG ATT AGG CGG CGG CACF5BamHI *Sno* CA GGATCC A TGA CCG AAT ACG TGA CGC CAR5EcoRI *Sno* GC GAAT TC T TAA ACC CAA CGC ACC TTT C**II.1.4 The synthesis of recombinant plasmid**

Part of this work is based on the working with several different recombinant plasmid which are used as the mainly tools for the working with yeast and bacteria. The creation of this recombinant plasmid occurred in the following elementary cloning procedures.

II.1.5 Polymerase chain reaction PCR

Polymerase chain reaction provide a basis to amplify DNA fragments and to create also a new enzyme restriction site in the amplified DNA segment needed for the cloning. It procedures by using specified recombinant primer (see bellow II.1.3)

PCR is performed using the following standard reaction mixture:

Template DNA	50-100 ng
10X amplification puffer	1:10 of the final volume
Mixture of dNTPs	4 μ M
Primer1	10 μ M (\approx 100pmol)
Primer2	10 μ M (\approx 100pmol)
Taq DNA polymerase	5 Units
H2O to a final volume of 50 μ l	

II.1.6 Modification of DNA ends

To clone DNA inserts properly in a specified vector and in order to avoid the recycling of the linear plasmid during ligation it was necessary some time to modify the end of the used DNA vector by attending the plasmid with 0,5-1 U CIP (Calf Intestine Phosphatase) for 30 min at 37°C to removed the phosphate group at its 5' terminal.

Treatment with the DNA polymerase I large klenow fragment for 20 min at 37 °C permit to fill in of 5' overhang and allowed the blend end ligation.

II.1.7 DNA ligation

Joining linear DNA fragments together with covalent bonds is called ligation. More specifically, DNA ligation involves creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another by the ATP- dependent T4 DNA ligase.

This enzyme will ligate DNA fragments having overhanging and will also ligate fragments with blunt ends. The ligation reaction occurs over night at 16°C.

II.1.8 Bacterial Transformation

Bacterial transformation is the process by which competent bacterial cells take up naked DNA molecules. Competent Bacteria are made by treatment with calcium chloride in the early log phase of growth (Hanahan 1983).

II.1.9 Setting up competent cells

The making of competent bacteria occurs according to hanahan protocol (Hanahan 1983) with change:

Streak frozen stock of XL1-Blue bacteria on LB-tet plate and incubated it overnight at 37 °C.

Inoculate a single colony into a 4ml LB medium grow overnight in a shaker at 37 ° C.

The next day set up two flask containing 200 ml LB medium. Into each flask, pipette 2 ml of overnight culture and incubate the cell culture with shaking until it reaches an OD₆₀₀ of

0.4-0.5. Pellet bacteria by spinning at 4000 r.p.m. at 4 °C for 10 minutes then resuspend on ice the pellet gently in 60 ml TFBII. After incubating on ice for 10 min, centrifuge bacteria at 4000 r.p.m., 4 °C for 10 minutes. Resuspend on ice the pellet gently in 8 ml of TFBII and immediately dispense 100 µl of cell suspension into each ice cold microcentrifuge tubes on ice. Once all 8 ml of competent cells are dispensed into microcentrifuge tubes, immediately they will be freeze in liquid nitrogen and stored at -80 °C.

LB medium: Casein hydrolysate (10g/l)
 Yeast extract (5g/l)
 NaCl (10g/l)
 pH 7.0

TBFI solution: 100mM Rubidium Chloride
 50mM Manganese Chloride
 30mM Potassium Acetate
 10mM Calcium Chloride
 15% w/v Glycerol

Adjust pH to 5.8 with acetic acid 2M and Sterilize by filtration.

TFBII solution: 10mM MOPS
 10mM Rubidium Chloride
 75mM Calcium Chloride
 15% w/v Glycerol
 Sterilize by filtration.

II.1.10 Plasmid Transformation and Antibiotic Selection

Immediately to the defrosted competent cells, desired DNA (ligation probe or any plasmid DNA) will be added to the cells and incubated for 30 min on ice. Afterwards, the cells were heat shocked at 42 °C for 90 s. To help the bacterial cells recover from the heat shock, the cells are incubated 30 min with 200 µl non-selective growth LB media. Bacterial cells, which contain the plasmid, were selected by plating them on plate containing the desired antibiotic.

II.1.11 Preparation of plasmid DNA by alkaline lysis

Alkaline lysis is the almost used method for lysing bacterial cells to prepare the transformed DNA plasmid. It occurs in four mainly steps:

Resuspension: transfer a single bacterial colony into 2 ml of LB medium containing the appropriate antibiotic and incubate the culture overnight at 37°C with shaking.

Harvested bacterial cells are then resuspended in 100 µl Tris.HCl/EDTA buffer (solution I) by vigorous vortexing.

Lysis: after the well dispersion of the pellet, the Cells are lysed with 200 µl NaOH/SDS (solution II). Sodium dodecyl sulfate (SDS) solubilizes the phospholipids and protein components of the cell membrane leading to lysis and release of the cell contents. NaOH denatures the chromosomal and plasmid DNA as well as proteins. Do not vortex in this step.

Neutralization: the lysate is neutralized by the addition of 150µl of potassium acetate (solution III). The high salt concentration causes potassium dodecyl sulfate to precipitate. Denatured proteins, chromosomal DNA and cellular debris are coprecipitated in insoluble salt-detergent complexes. Plasmid DNA, being circular and covalently closed, renatures correctly and remains in solution. Precipitated debris are removed by centrifugation at 13,000 rpm for 10 min and the supernatant are transferred to a fresh tube containing 3 M NaAc and absolute ethanol to precipitate DNA plasmid.

Solution I:	50 mM glucose 25 mM Tris.Cl pH 8.0 10 mM EDTA pH8.0
Solution II:	0.2 N NaOH 1% SDS
Solution III:	3M potassium acetate 5 M glacial acetic acid

II.1.12 Genomic DNA extraction (after Ana Dominguez 1996)

About 50-100 flies were homogenized in a 1.5 ml microcentrifuge tube with 0.6 ml of ice cold homogenization buffer. The homogenate was centrifuged for 5 min 13000r.p.m. the pellet was resuspended in 600µl of extraction buffer. Proteinase K and SDS were added to

a final concentration of 100µg/ml and 1% respectively. The mixture was incubated for 1 h at 56°C afterwards the DNA was extracted with chloroform then precipitated with ethanol. The precipitated DNA was dissolved in 300µl of TE buffer treated with pancreatic RNase (20µg/ml) at 37 °C for 30 min. after extraction of the genomic DNA with phenol/chloroform than chloroform alone it was precipitated with absolute ethanol than dissolved in TE buffer.

Homogenization buffer: 0.1 M NaCl
 30mM TRIS-HCl, pH 8.0
 10mM EDTA
 10mM 2-mercaptoethanol
 0.5% Triton X-100

Extraction buffer: 0.1 M NaCl
 0.1 M TRIS-HCl, pH 8.4
 20 mM EDTA

II.2 Protein –protein interaction assays

II.2.1 Yeast two hybrid system

A working stock plate of SFY526 cells will be prepared by streaking a small portion of the frozen glycerol stock onto YPD agar plate this will be incubate 2-3 days at 30 °C. The yeast transformation procedure occurs as follow:

II.2.1.1 Preparation of yeast competent cells

Inoculate 5 ml of YPD medium with a single colony from the fresh prepared working stock platen and incubated the medium at 30°C under shaking at 250 for several hours (5-6).

Transfer the cell culture to 50 ml YPD medium and incubate over night at 30 °C with shacking at 250 rpm until an OD₆₀₀ of 1-2.

Transfer 30 ml of overnight culture to a 300 fresh YPD medium and ensure that the OD₆₀₀ is between 0.2-0.3.

Incubate the culture at 30°C for 3 hr with shaking at 250 rpm until an OD₆₀₀ of 0.4-0.6.

Place cells culture in 50 ml tubes and centrifuge for 10 min at 1000X g (4000 rpm GSA rotor or 2000 rpm in verifuge 3.OR centrifuge) at Room temperature.

After the supernatant was discarded the pellet will be resuspend in 30 ml sterile TE or distilled water.

Centrifuge at 1000 X g for 5 min and resuspend the pellet in 1 ml of freshly prepared, sterile 1X TE/1X LiAc.

YPD medium: 20 g/l Difco peptone
 10 g/l yeast extract
 20 g/l agar (for plate)

TE solution: 200 mM Tris-base
 20 mM EDTA

II.2.1.2 Transformation of the plasmid DNA into Yeast cells

Add to 0.1µl yeast competent cells a mix of 0.1µg plasmid DNA and 0.1 mg herring testes carrier DNA and mix well by vortexing in a fresh tube.

Add 0.6 ml of sterile PEG/LiAc solution to the tube and vortex at a high speed for 10 sec.

Incubate the mixture at 30°C for 30 min

Add 70 µl of DMSO and mix without vortexing.

Heat shock for 15 min in a 42 °C water bath. Chill cells for 2 min on ice and centrifuge them for 5 sec 14, rpm at room temperature.

Remove the supernatant and resuspend cells in 0.5 ml of sterile TE.

Plate the cells on SD agar plate that will select for the desired transformants.

Incubate plate at 30 °C for 2-4 days until colonies appear.

II.2.1.3 β galactosidase assays

II.2.1.3.1 The colony-lift filter assay

This assay used (according to Breeden and Nasmyth, 1985) to detect interaction between two known proteins in a GAL4 two- hybrid system.

For each plate to be assayed, presoak a sterile whatman filter by placing it in 2.5-5 ml of Z buffer/X-gal solution in a clean plate. Using a forceps, place a sterile dry whatman filter over the surface of the plate of colonies to be assayed. Gently rub the filter with the side of the forceps to help the colonies cling to the filter. Orient the filter to the agar by poking holes through the filter into the agar.

Carefully lift the filter off the plate and transfer it, colonies facing up, to a pool of liquid nitrogen and submerge completely the filters for 10 sec then remove it from the liquid nitrogen and allow it to thaw at room temperature this step must be repeated at least three times to lyse the yeast cell walls.

Carefully place the filter, colony side up, on the pre-soaked filter and incubate it at 30°C or at room temperature until the appearance of blue colonies.

II.2.1.3.2 Liquid culture assay using ONPG as substrate

This assay is used to verify and quantify two hybrid interactions.

After determine the positive colonies using the colony lift filter assay a positive colony was picked from the original plate to a 5 ml fresh SD selection medium and incubate at 30°C overnight. On the day of the experiment, dissolve ONPG at 4 mg/ml in Z buffer with shaking for 1-2 hours. Transfer 2ml of the overnight culture to 8 ml of YPD and incubate it for 3-5 hr at 30 °C with shaking 250 rpm until the cells are in mid log phase with OD₆₀₀ of 0.5-0.8.

Place 1.5 ml of culture into each of three microcentrifuge tubes and centrifuge at 14,000 rpm for 30 sec. carefully remove supernatants and add 1.5 ml of Z buffer to each tube and vortex until cells are resuspended. Centrifuge cells and remove supernatants.

Resuspend each pellet in 300 µl Z buffer (the concentration factor is 1.5/0.3= 5 fold) and transfer 0.1 ml of the cell suspension to a fresh tube. Place them in liquid nitrogen until the cell are frozen than place the tube in a 37°C water bath to thaw. Repeat the freeze/thaw cycle two more times to ensure that the cells have broken.

Set up a blank tube with 100 µl of Z buffer.

Add 0.7 ml of Z buffer plus β-mercaptoethanol to the reaction and blank tubes.

Start timer. Immediately add 160 µl of ONPG in Z buffer and incubate them at 30°C.

After the yellow colour develops, add 0.4 ml of 1 M Na₂CO₃ to the reaction and blank tubes.

Centrifuge reaction tubes for 10 min at 14,000 rpm and transfer supernatant to clean cuvettes and measure the OD₄₂₀ of the samples relative to the blank tube. The ODs should be between 0.02-1.0

Calculate β-galactosidase units. 1 unit of β-galactosidase is defined as the amount, which hydrolyzes 1 μmol of ONPG to o-nitrophenol and D-galactose per min per cell.

$$\beta\text{-galactosidase units} = 1,000 \times \text{OD}_{420} / (t \times V \times \text{OD}_{600})$$

Where

t = elapsed time (in min) of incubation

V = 0.1 ml x concentration factor

OD₆₀₀ = A₆₀₀ of 1 ml of culture

Z buffer: 16.1 g/l Na₂HPO₄.7H₂O
 5.50 g/l NaH₂PO₄.H₂O
 0.75 g/l KCl
 0.246 g/l MgSO₄ adjust pH 7.0 and autoclave

X-gal: dissolve X-gal in DMF at a concentration of 20mg/ml.

Z buffer/ X-gal: 100 ml Z buffer
 0.27 ml β-mercaptoethanol
 1.67 ml X-gal stock solution

II.2.2 Ni-NTA pull down assay

II.2.2.1 Expression of recombinant proteins

Genes of interests were cloned in the pGEX2T vector in frame with the GST gene. After analysing of the positive clones the plasmids were transfected in the E-coli BL21 cells and incubated over night at 37°C after to be plated on the appropriate antibiotic plate. Then a single colony from the empty pGEX2T vector and from each recombinant pGEX2T-Mad, pGEX2T-Medea, pGEX2T-SNO, pET-MH-Bx42, and pET-MH-Bx42(SNW to AAA) plasmids was inoculated 2 ml of LB medium and incubated overnight at 37°C with shaking. The culture will be diluted 1:100 into fresh LB medium and grow at 37 °C with shaking until the OD₆₀₀ reaches a value between 0.6- 0.8.

The expression of the recombinant proteins were then induced by adding IPTG (to a final concentration of 0.1 mM) the cultures was incubated at 22 °C for another 90 min with shaking. The cultures were centrifuged at 4000 x g (Heraeus, varifuge 3.OR) for 10 min at

4°C. After where the pellets were suspended in ice cold PBS (50 µl PBS per ml cell cultures) the cells were lysed by adding of 20mg/ml lysozyme to a final concentration of 1mg/ml for 20 min. the bacteria cells were sonicated on ice in short burst 3 times for 16 S at 50 amplitude (Bandelin sonoplus HD70). The fusion proteins were then solubilising by adding of 10% Triton to a final concentration of 1% for 30 min at RT. The probe will be centrifuge at 12,000xg for 10 min at 4°C. After the analysis of the presence of respectively right recombinants proteins on Coomassie staining and SDS-PAGE, the supernatants, cells extracts, were transferred to a fresh tube and stored at -20°C.

PBS: 7 mM Na₂HPO₄
 3 mM NaH₂PO₄
 130 mM NaCl
 pH 7.4

II.2.2.2 In vitro protein interaction test using NI-NTA beads

The prepared cell extract for each GST recombinant protein were incubated with pET-Myc-His recombinant proteins (pET-MH-Bx42 or pET-MH-Bx42(SNW to AAA)) at 4°C overnight under permanent shaking in binding buffer. As a control the empty pGEX2T vector will be incubated in the same work condition with pET-Myc-His recombinant protein. The probes were incubated with shaking at R.T. for additional 2 hours. after that the probes were centrifuged for 5 min at 4 °C 500 g. the beads were washed with washing buffer several times, finally the beads will be incorporate in SDS mix and cooked for 10 min at 95°C than analysed by SDS-PAGE.

Ni-NTA binding buffer: 5 mM Tris pH 8.0
 50 mM NaH₂PO₄
 10 mM imidazol
 NaCl 200 mM (for pGEX2T-Mad, pGEX2T-SNO)
 300 mM (for pGEX2T-Medea)

Ni-NTA washing buffer: 50 mM NaH₂PO₄
 5 mM Tris pH 8.0
 20 mM imidazol
 400 mM NaCl

II.2.2.2.1 SDS- Polyacrylamide Gelelectrophoresis (SDS-PAGE)

The proteins are separated in accordance with their molecular weight in a SDS-PAGE, this occurs with appropriate resolving gel according Laemmli (Laemmli, 1970; Sambrook et al., 1989).

Pour the appropriate resolving gel into the gap between the glass plates. Using a pipette overlay the gel with 0.1 % SDS solution. After polymerization is complete (circa 30 minutes), pour off the 0.1% SDS and add the stacking gel mixture onto the surface of the resolving gel, place the appropriate comb into the stacking gel. Leave it to polymerize (circa 15 minutes). Place the gel into the electrophoresis chamber and fill the chambers with electrophoresis buffer and remove the combs from the stacking gel. Dissolve the protein in SDS loading buffer and heat the sample at 95°C for 10 minutes and load the samples into the bottom of the wells. After gel is running the proteins can be now visualized by coomassie staining or for western blot.

Electrophoresis buffer:	25 mM Tris
	192 mM glycine (electrophoresis grade) pH 8.3
	0.1 % SDS
SDS loading buffer:	1.25 ml 1 M Tris-HCl pH 6.8
	2 ml 20% SDS
	2 ml 87 % glycerol
	1 ml mercaptoethanol
	60 µl bromophenol blue

II.2.2.2.2 Coomassie staining

Fix and stain the gel with Coomassie staining solution for 30 min afterwards destain the gel by successive incubations in Coomassie destaining solution to visualize the band (for a faster destaining, 1-2 hours at 50-60°C)

Coomassie staining solution:	0.25 % coomassie brilliant blue
	50 % methanol
	10 % acetic acid
Coomassie destaining solution:	30 % methanol
	10 % acetic acid

II.2.2.2.3 Western blot

The proteins from the SDS-PAGE are electrophoretic transferred into a nitrocellulose transfer membrane for 90 min at 0.4 mA in blot buffer. Subsequently the membrane is transferred to block buffer and blocked for 30 min under shaking than incubated overnight with the first antibody at 4 °C. The mice anti Myc antibody (9E10) and mice anti GST antibody (Santa Cruz biotechnology) are used in this work as first antibody. After wash step the membrane is incubated for 1-2 h with an alkaline Phosphatase- coupled antibody (1:2000 in block buffer).

The detection of the proteins is occurred by adding the substrate NBT/BCIP (in NBT buffer) to the membrane.

Blot buffer:	25 mM Tris-Base
	192mM glycine
Block buffer:	1 % BSA
	1 x PBS
NBT buffer:	10 mM Tris-HCl pH 9.5
	100 mM NaCl
	10 mM MgCl ₂

II.3 Work with flies

II.3.1 Flies strains

The non transgenic lines used in this work are:

Oregon is the wild type strain and serves as the control
 W1118 in this strain the white gene is mutated. It was used for the germline transformation.

The transgenic lines used to analyze the effect of the ectopic expression of Mad and Medea received from Morata as a gift.

UAS-Medea	2 chromosome
UAS-Mad;Ln2LRGla/Sma ^{CyO}	X chromosome
UAS-Bx42	X chromosome
UAS-Bx42/TM3	3 chromosome
UAS-Bx42/CyO	2 chromosome
UAS-BJ1(265-520)GFP/CyO	2 chromosome

Enhancer trap lines

Omb-lacZ (P{bi-lacZ.pomb ³⁵ })	X chromosome
Dll-lacZ (P{PZ}Dll ⁰¹⁰⁹²)	2 chromosome

Bx42-RNAi transgenic lines used in this work.

Bx42-RNAi/Bx42-RNAi	X chromosome
Bx42-RNAi/CyO	2 chromosome
Bx42-RNAi/TM3	3 chromosome
UAS-Bx42-RNAidll-lacZ/CyO	X; 2 chromosome
Omb-lacZ;Bx42-RNAi/CyO	X; 2 chromosome
UAS-Bx42-RNAiUAS-Medea	X; chromosome
UAS-Mad;Bx42-RNAi/CyO	X; 2 chromosome

The following driver line were used to induced the overexpression as well as the Bx42-RNAi

T80/CyO	2 chromosome
If/CyOwg-lacZ; dpp-Gal4/TM3	3 chromosome
Omb-Gal4/FM7	X chromosome
En-Gal4	2 chromosome
Dll-Gal4 (P{GawB}Dll ^{md23})	2 chromosome
Ptc-Gal4;TM6/MkRs	2 chromosome
Klu-Gal4/TM6	3 chromosome
Brk-Gal4 (P{GAL4}brk3SB)	X chromosome

II.3.2 Acridin orange staining

Imaginal discs from third instar larvae were prepared and collected in ice cold PBS then stained for 5 min in 1µg/µl Acridin orange in PBS. Discs were then rinsed briefly three times with PBS and mounted in PBS and immediately viewed by fluorescent microscope.

II.3.3 Immunostaining of Imaginal discs

Imaginal discs were dissected from third instar larvae in PBS and collected in ice cold PBS. The dissected material will be then placed in fixative solution for 30 min at RT. After fixation the discs will be washed with PBT three times for 20 min each then in block solution for 30 min at RT to prepare the probe for immunostaining. The first antibodies used in this experiment are: rabbit anti β-galactosidase (10mg/ml), which is diluted to 1:10000 in block solution, rabbit anti Spalt (1:20) from Dr. Reinhard Schuh and Z4. The probe will be then overnight at 4 °C with the primary antibody. The day after occur the wash of the probe with 1X PBT three times each 20 min. then occur the incubation for at least 2 hour with the Rhodamin-marked secondary antibody. The imaginal disc were washed three times each 20 min with 1X PBT and prepared in 100% glycerol then viewed by fluorescent microscope.

II.3.4 RNA in situ hybridisation on Imaginal discs

To analyse the effect of Bx42 on the Dpp target genes *in vivo* we used RNA in situ hybridization tool with a non radioactive probe to visualize transcripts of interest in imaginal discs.

II.3.4.1 Preparation of the Dig RNA labelled probe

The DNA to be transcribed was cloned into an appropriate transcription vector that contains SP6 and/or T7 promoters. After linearization of the DNA at a suitable site downstream from the cloned insert. The DNA was purified and concentrated with Zymo DNA purification Kit. 1 µg from the prepared DNA was used for the *in vitro* transcription to generate a large amount of Dig-UTP labelled RNA as described in Dig RNA labelling Kit (Roche, Cat. No, 11 175 025 910). RNA was purified and concentrated with Zymo RNA purification Kit. 40 µl hybridisation buffer was added to the purified RNA and stored at -20°C

II.3.4.2 Preparation of imaginal discs

Imaginal discs will be dissected from third instar larvae in PBS and collected in ice cold PBS. The dissected material will be then placed in fixative solution for 20-30 min at RT. After fixation the discs will be washed with PBT five times for 5 min each. The discs were digested for 3 min at 55 °C with Proteinase K (20µg/ml) in PBT solution and then washed eight times in ice cold PBT for 5 min each at RT. After 20-30 min post fixation with fixative solution the discs were washed in PBT four times for 5 min each at RT.

Fixative solution:	4 % paraformaldehyde in 1 x PBS
PBT:	0.3 % Triton X-100
	1 x PBS

II.3.4.3 Prehybridization and hybridization of the prepared imaginal discs

The prepared Imaginal discs were then Prehybridize by incubating them for 10 min in 1:1 PBT: Hybridization solution at RT then for other 10 min in hybridization solution at RT. The discs were transferred into freshly hybridization puffer and incubated at 55 °C for 1 hr. After prehybridization the discs were hybridized overnight at 55°C with the anti sense DIG (digoxigenin) labelled RNA probe that was diluted 1:10 in Hybridisation buffer and denaturized for 10 min at 80°C (500ng/ml in hybridization buffer). The probe was removed and the discs were washed with hybridization solution for 20 min at 55°C, with a 1:1 mixture of hybridization solution and PBT at 55°C and then with PBT alone five time for 20 min each.

Hybridisation puffer: 50% Formamid
 5X SSC (20X 3M NaCl, 0,3 M Sodium Citrate pH: 7,2)
 100 µg /ml E. coli tRNA
 50µg/ml Heparin
 0,1 % Tween-20
 pH: 4,5

II.3.4.4 Detection of In situ Signal

the discs were incubated with the alkaline phosphatase conjugated goat anti-digoxigenin, (1:2000 in PBT) for at the least 2 hr at RT. Accordingly the disc were washed in PBT four time for 20 min each, then in staining buffer tree times for 5 min each following with the incubation in staining buffer containing 4.5 µl of 4 nitro blue tetrazolium chloride (NBT) and 3.5 µl 5-bromo-4-chloro-3-indoly-phosphat (BICP) per ml of solution. The reaction was stopped by several washes in PBS after the staining was visible. The discs were then transferred to 50% glycerol in PBS for 30 min and then mounted in 100% glycerol.

Staining buffer: 100 mM NaCl
 50 mM MgCl₂
 0.1 % (v/v) Tween-20
 100 mM Tris pH 9.5

III Results

III.1 Induction of *Bx42*-RNAi causes a penetrant leg phenotype

Since a *Drosophila Bx42* mutant has not been available, *Bx42*-RNA interference was used to investigate the role of *Bx42* during *Drosophila* development. By this tool *Bx42* expression was reduced in different tissues and at different developmental stages using the GAL4/UAS system (Brand and Perrimon, 1993; Negeri et al., 2002).

A cross at 29°C between females, which are homozygous for a UAS-*Bx42*-RNAi transgene on the X chromosome (Negeri et al., 2002) and males containing a third chromosomal *dpp*-Gal4 generated descendants with abnormal legs. The legs of these animals were either missing the tarsal segments T4 and T5, in some cases the third tarsal segments is missing, or exhibited a fusion of the T3 with T4 or T4 with T5 (Figure 5). When the same crosses was performed at 25°C, no effect on leg development was observed.

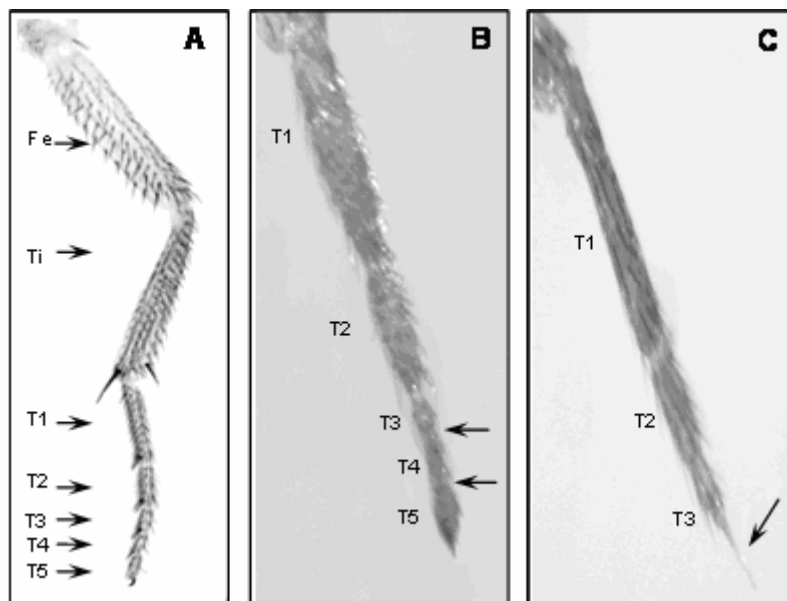


Figure 5: *Bx42* down regulation by *dpp*-Gal4 causes pleiotropic effects in the leg. (A) Wild type leg with the proximal segment Femur (Fe), the Tibia (Ti) and the more distal tarsal segments T1- T5. The reduction of *Bx42* by RNA interference in UAS-*Bx42*-RNAi/+;*dpp*-Gal4/+ animals leads to penetrant leg phenotypes; in (B) a fusion of the tarsal segments T3, T4, T5 is shown. (C) The tarsal segments T4 and T5 are deleted.

The leg phenotype caused by the downregulation of *Bx42* (Figure 5) is similar to the phenotype caused by ectopic expression of the Dpp signal transmitter Mad or Medea (Figure 6). Overexpression of Medea (Co-Smad) using the *dpp*-Gal4 driver line at 29°C

lead either to the deletion of the tarsal segments T3, T4 and T5 (Figure 6B), or to the fusion of the T2 with T3 and T4 with T5 (Figure 6C). Additionally, similar phenotypes were obtained by overexpression the *Drosophila* Dpp signal transducer Mad (R-Smad) with the same driver line at 29°C. Here, the fusion of tarsal segments was also observed in the progeny (Figure 6D).

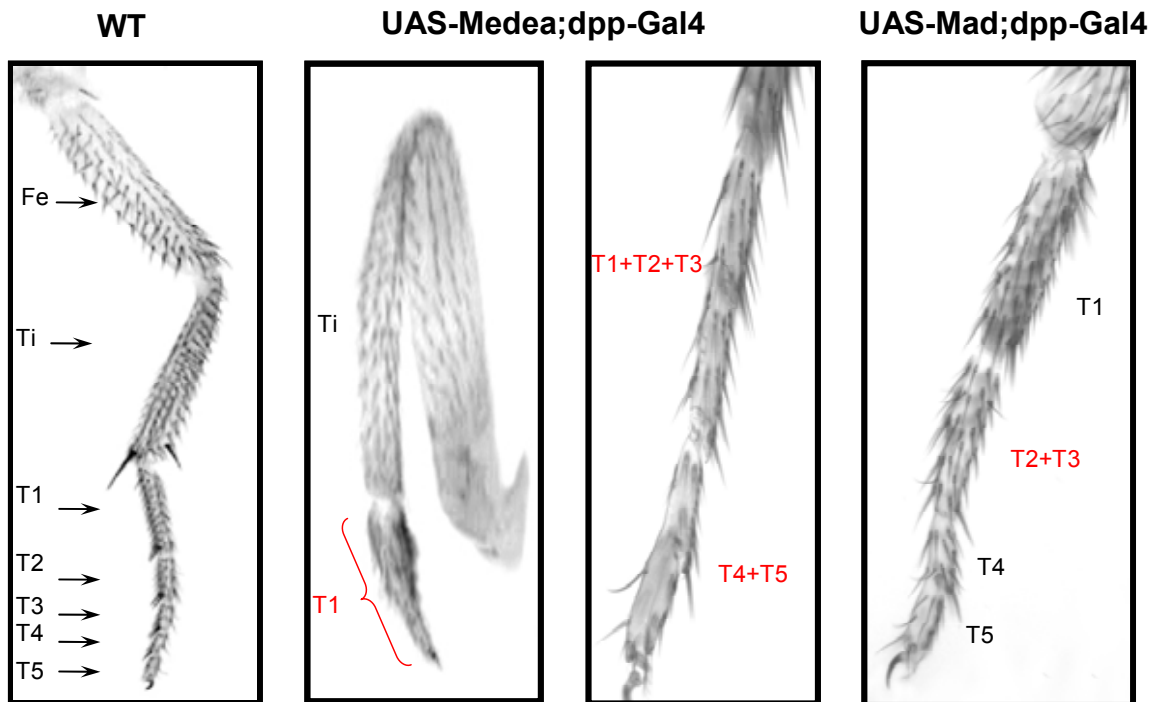


Figure 6: The effects of Mad or Medea overexpression on *Drosophila* leg development. (A) Wild type adult leg with the femur (Fe), Tibia (Ti) and the tarsal segments T1-T5. The overexpression of Medea by dpp-Gal4 (UAS-Medea/+;dpp-Gal4/+) at 29°C causes either (B) deletion of tarsal segments T3, T4 and T5 and deformation of T1 or (C) fusion of the tarsal segments T2 with T3 and T4 with T5. (D) The ectopic expression of Mad with dpp-Gal4 (UAS-Mad/+;dpp-Gal4/+) at 29°C lead also to fusion of tarsal segments T2 with T3 and T4 with T5.

These similarities between the phenotypes caused by Bx42 downregulation and by the overexpression of Mad or Medea lead to the assumption that Bx42 could play a role in the Dpp signalling pathway.

III.2 Bx42 interacts with TGF- β /Dpp signal pathway components

III.2.1 Bx42 interacts with Mad and Medea in yeast

To analyse the hypothesis mentioned above and to elucidate the role of Bx42 in TGF- β /Dpp signalling pathway, the interactions between Bx42 and the TGF- β /Dpp signalling component Mad and Medea were tested using the yeast two hybrid assay.

The yeast two hybrid system is a frequently used tool to study the direct interaction of two proteins in yeast cells. Moreover, the protein domains, which are necessary for the interaction, can be mapped using this assay.

The full length Bx42 protein contains a transactivation domain within its last 55 amino acids, which could lead to false positive results in the yeast two hybrid assay. For this reason the C-terminally deleted Bx42 protein (aa 1-492) Bx42 Δ C was used in this assay. Bx42 Δ C was cloned into the pGBT9 vector that contains a Gal4 DNA Binding Domain (DBD) to generate BD-Bx42 Δ C (Negeri, 2002). The full length Mad and Medea cDNAs were cloned into the pGAD424 vector, which contains the Gal4 Activation Domain (AD). Each of the resultant plasmids, AD-MadFL and AD-MedeaFL, was then cotransformed pairwise with BD-Bx42 Δ C into SFY526 yeast cells. As a positive control SFY526 cells were transformed with the pCL1 vector, which contains the complete Gal4 cDNA. Additionally, SFY526 cells were transformed with the plasmids BD-Bx42 Δ C/AD-Bx42FL as a second positive control, because Bx42 shows homodimerisation in the yeast two hybrid system (Negeri, 2002). For negative controls SFY526 cells were transformed with the empty pGBT9 and pGAD424 yeast vectors. To eliminate the possibility that AD-MedeaFL or AD-MadFL alone could result in a positive signal SFY526 cells were cotransformed with a mixture of each construct and the empty pGBT9 vector. The colonies on the Tryptophan/Leucin plates were tested for their ability to express LacZ in a colony-lift filter assay (see experimental procedure). In the presence of the X-gal substrate the yeast colonies become blue if the proteins in question interact with each other.

Colonies which contained AD-MadFL/BD-Bx42 Δ C displayed a blue colour in the colony-lift assay, indicating that Bx42 Δ C interacts with Mad (Figure 7A). The measured β -Galactosidase activity was 2,6 units for AD-MadFL/BD-Bx42 Δ C (Figure 7B;line 4). Yeast cells that contained AD-MadFL/pGBT9 and the negative controls pGBT9/pGAD424 showed no β -Galactosidase activity (Figure 7B, and data not shown), whereas the positive

controls pCL1 and BD-BX42FL/AD-Bx42 Δ C exhibited a significant β -Galactosidase activity 10,8 units and 7,4 units respectively (Figure 7B; lane 1,2).

These data indicate that MadFL interacts with Bx42 in yeast cells and that the Bx42 C-terminal activation domain is not necessary for the observed interaction.

As mentioned in the introduction the Smad proteins have evolutionary conserved domains, the Mad Homology domains MH1 and MH2 which are separated by a less conserved linker sequence. To identify the domain of the Mad protein interacting with Bx42, the recombinant Mad deletion constructs shown in Figure 7A were cloned in pGAD424 vector and tested for interaction with BD-Bx42 Δ C in SFY526 cells (Figure 7A). The deletions of 200 aa from the C-terminus of Mad (aa 255-455), which includes the MH2 domain, abrogated the interaction of the two proteins. This indicates that the MH2 domain is important for the binding of Bx42 to Mad. Furthermore, deletion constructs lacking either MH1 (AD-Mad Δ MH1) or MH1 and the linker region (AD-Mad Δ (MH1/linker)) still interact with BD-Bx42 Δ C (Figure 7).

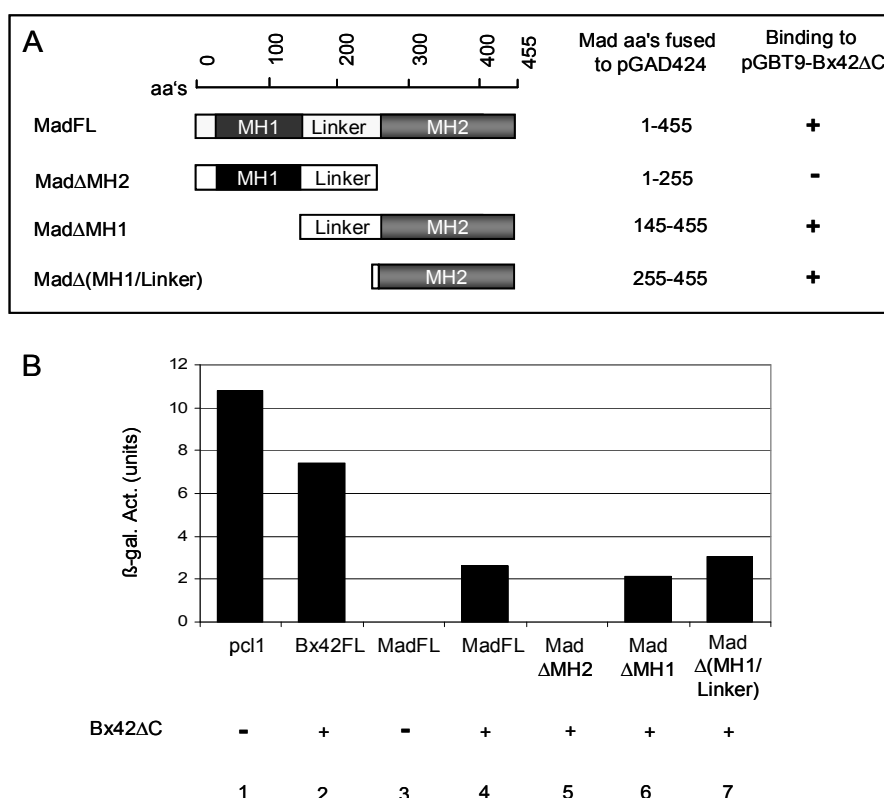


Figure 7: Mad interacts with Bx42 in the yeast two hybrid assay. (A) Schematic representation of MadFL and Mad deletion constructs that were cloned into the pGAD424 vector. The ability of these recombinant constructs to bind to the BD-Bx42 Δ C is indicated. The Mad Δ MH2 deletion fragment that lacks the MH2 domain does not interact with Bx42 Δ C. Mad Δ MH1 and Mad Δ (MH1+linker), which still have the

MH2 domain, interact with Bx42 Δ C, indicating the importance of this domain for the interaction with Bx42. (B) β -galactosidase activity measured in liquid culture of yeast cells transformed with the constructs as indicated. pCL1 and BD-Bx42 Δ C/AD-Bx42FL (column 1 and 2) serve as positive control. AD-Mad FL/pGBT9 show no activity indicating that MadFL has no unspecific effect in this assay (column 3).

These results indicate that neither MH1 nor the linker sequences are required for the tested interaction, while the MH2 domain alone is necessary and sufficient for the direct binding of Bx42 to Mad.

In the same way the interaction between the C-terminal deleted Bx42 protein Bx42 Δ C and the full length Medea protein was demonstrated (Figure 8A). The measured β -galactosidase activity for AD-MedeaFL/BD-Bx42 Δ C was 3,36 units (Figure 8B column 4). The full length Medea cloned in pGAD424 and transformed with pGBT9 in yeast cells exhibited no β -galactosidase activity (Figure 8B column 3). The domain of Medea interacting with Bx42 was mapped using the deletion constructs shown in Figure 8A. These deletion constructs were tested for an interaction with BD-Bx42 Δ C in SFY526 cells. As for Mad it was proven that Medea binds to Bx42 through its MH2 domain and that the MH2 domain is necessary and sufficient to direct the interaction with Bx42 (Figure 8).

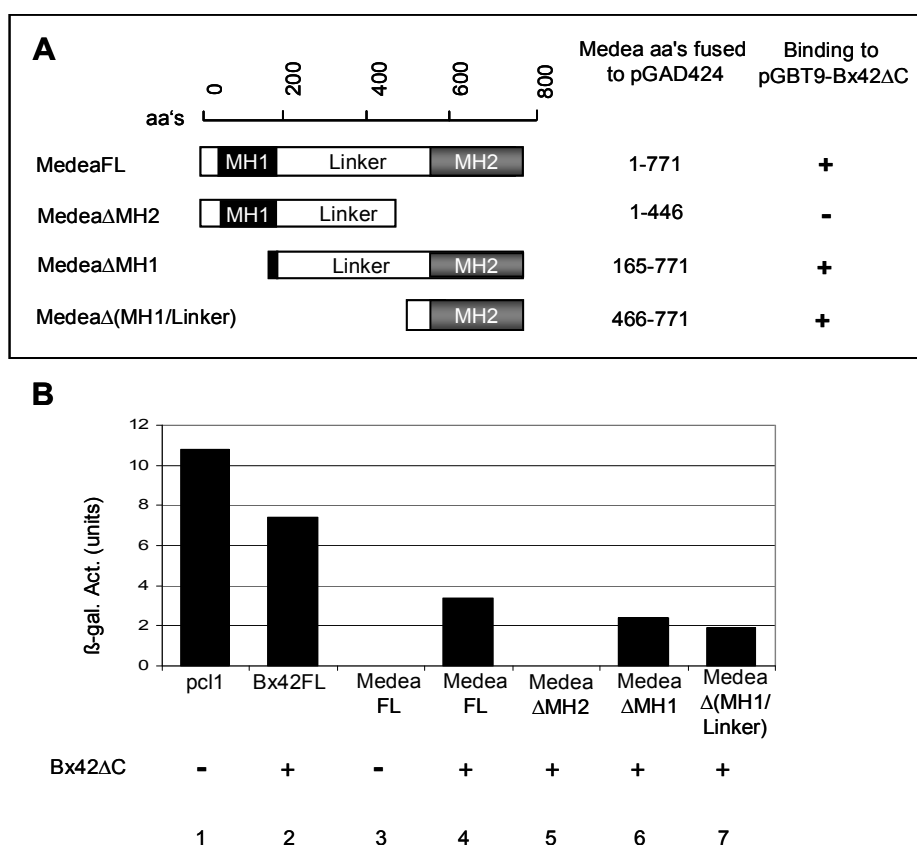


Figure 8: Medea binds to Bx42 in the yeast two hybrid assay. (A) Schematic representation of MedeaFL and Medea deletion fragments, which were cloned into the yeast pGAD424 vector. Shown is also the ability of the recombinant constructs to bind to BD-Bx42ΔC. Only the MedeaΔMH2 fragment that lacks the MH2 domain fails to interact with Bx42ΔC. MedeaΔMH1 and MedeaΔ(MH1/linker) that harbour the MH2+linker and the MH2 domain only, respectively, still interact with Bx42ΔC, indicating the requirement of the MH2 domain for the physical interaction with Bx42. (B) β-galactosidase activity was determined in liquid cultures to measure the relative strength of this interaction. pCL1 and BD-Bx42ΔC/AD-Bx42FL (column 1 and 2) served as positive controls, AD-MedeaFL/pGBT9 (column 3) showed no activity. MedeaΔ(MH1/linker) and MedeaΔ(MH1) have almost the same β-galactosidase activity indicating that the linker fragment has no effect in this interaction.

These results demonstrate that Bx42 interacts with the TGF-β/Dpp signalling transducers Mad and Medea in yeast. The Bx42 interacting domain of Mad as well as of Medea was mapped to their conserved MH2 domains that are necessary and sufficient for the physical interaction.

III.2.2 Bx42 binds Mad and Medea *in vitro*

To confirm the interaction between Bx42 and MadFL as well as Bx42 with MedeaFL obtained in yeast, a Ni-NTA pull down assay was performed. For this aim the Bx42 putative interacting partners MadFL- and MedeaFL- cDNAs were cloned into the pGEX-2T expression vector and expressed as Glutathione S-Transferase (GST) fusion protein in BL21 *E. coli* cells. Each of the fusion proteins GST-MadFL and GST-MedeaFL was tested for an interaction with the fusion protein Myc-His-Bx42FL (MH-Bx42FL) using the Ni-NTA pull-down assay. Equivalent amounts as estimated from western blots of cell lysate of each expressed GST fusion protein were incubated with the same amount of MH-Bx42FL cell lysate and bound to Ni-NTA beads. After extensive washing bound proteins were eluted, separated by SDS-PAGE, blotted to Nitrocellulose and stained with an anti-GST-antibody. As a negative control the GST protein was used in the binding reaction with MH-Bx42FL. The results shown in Figure 9 demonstrate that GST-MedeaFL (lane 1) and GST-MadFL (lane 4) were able to bind MH-Bx42FL. GST protein alone did not interact with MH-Bx42FL (lane 2, 5).

Bx42 protein has an evolutionary conserved domain, the SNW domain that is characterized by the presence of the three well preserved amino acids SNW (serin, asparagin and tryptophan). To address the question if the conservation of these three amino acids are necessary for the interaction a Bx42 mutated protein was generated in which the three amino acids SNW are replaced by three alanines residues this protein is expressed as Myc-His fused protein MH-Bx42FL(AAA). A Ni-NTA Pull-down assay was performed to test if MH-Bx42FL(AAA) is still able to bind GST-MedeaFL and GST-MadFL. The results from this assay are shown in Figure 9 lane 3 and 6. The mutated protein MH-Bx42FL(AAA) was still able to interact with GST-MadFL and GST-MedeaFL. Again, an Interaction with the GST protein was not detectable in this assay (Figure 9, lane 2, 5).

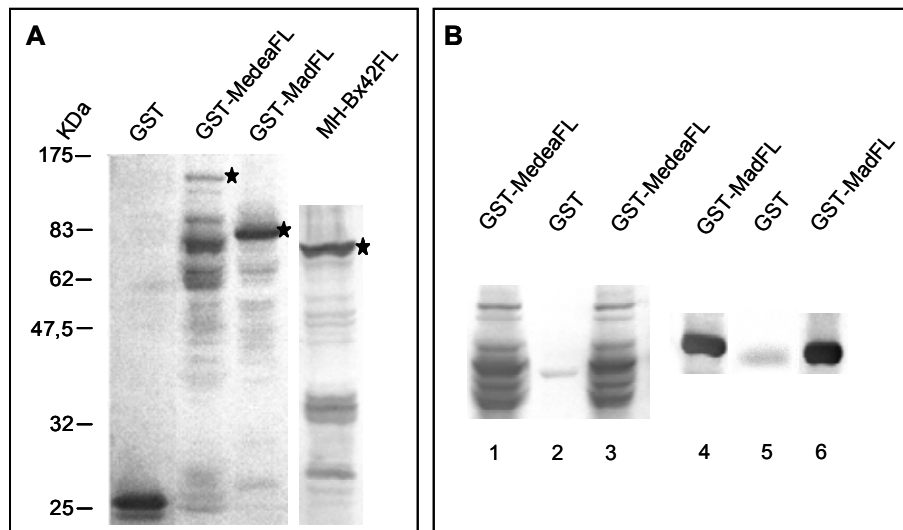


Figure 9: *in vitro* binding of fusion proteins GST-MadFL and GST-MedeaFL with MH-Bx42FL and MH-Bx42FL(AAA). (A) Shown is 10% of input of the Myc-His-Bx42FL recombinant proteins used for the binding reaction and 10% of the input of the GST-MadFL, GST-MedeaFL and GST proteins added to the each binding reaction respectively. Interactions between MH-Bx42FL and GST-MedeaFL (lane 1) or GST-MadFL (lane 4) respectively were visualised by western blots using GST antibody. Lane 3 and 6 show interaction between MH-Bx42FL(AAA) and GST-MadFL or GST-MedeaFL respectively. A negative control was performed using GST protein alone (lanes 2 and 5).

From this data the *in vitro* interaction of the full length Bx42 with both MadFL and MedeaFL was conclusively demonstrated. Additionally, the results established that the conserved SNW sequence is not needed for this interaction.

III.2.3 Interaction of Bx42 with Medea *in vivo*

Both assays shown above exhibit a direct interaction between Bx42 and Medea *in vitro*. In order to prove that these proteins also interact *in vivo*, genetic interactions were tested by simultaneously overexpressing Medea and downregulating Bx42 in *Drosophila*. UAS-Medea;dpp-Gal4 heterozygous flies raised at 29°C showed the formation of a second anterior cross vein. The longitudinal veins (LII, LIII, LIV and LV) were not affected (data not shown). The UAS-Bx42-RNAi;dpp-Gal4 transgenic flies exhibited a penetrant wing phenotype. In these flies the LIII was almost completely deleted and the most distal part of the LIV vein was displaced anteriorly (Figure 10B).

To demonstrate a genetic interaction between Bx42 and Medea, UAS-Bx42-RNAi;UAS-Medea homozygous flies were crossed with the dpp-Gal4 driver line and raised at 29°C.

The wings of adult flies with the genotype *UAS-Bx42-RNAi;UAS-Medea;dpp-Gal4* were analysed. It was observed that the wing phenotype caused by Bx42 downregulation was partially restored. In these flies the wing vein LIII was normally established and the wing vein LIV was formed in its appropriated place (Figure 10C). However, the formation of a secondary posterior cross vein, which is generated by overexpression of Medea, was not suppressed (data not shown).

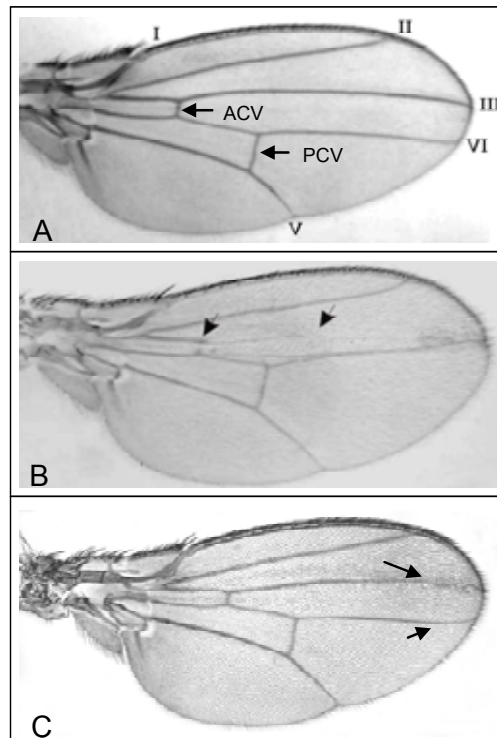


Figure 10: *In vivo* interaction between Medea and Bx42. (A) Adult wild type wing with the longitudinal wing veins (LI, LII, LIII, LIV, LV), the anterior (ACV) and the posterior (PCV) cross veins. (B) Wing phenotype caused by the reduction of Bx42 by induced RNA interference using the *dpp-Gal4* driver at 29°C. Note that the LIII vein and part of the ACV are missing and the LIV vein is displaced anteriorly. (C) Simultaneous overexpression of Medea and induction of Bx42-RNAi with *dpp-Gal4* at 29°C restored the wild type phenotype shown in (A). Both the longitudinal vein LIII and the anterior cross vein are reformed. The longitudinal vein LIV is formed at its original location.

The recovery of the wing phenotype caused by Bx42-RNAi through Medea overexpression indicates that these proteins genetically interact with each other *in vivo* and that the observed *in vitro* interaction is of biological significance in the TGF- β /Dpp signal transduction pathway.

The genetic interaction between Bx42 and Mad was analysed in an analogous way. Heterozygous *UAS-Mad;dpp-Gal4* flies die as pharate adults, exhibiting a strong leg phenotype. However, the simultaneous induction of Bx42-RNAi and the overexpression of

Mad driven by dpp-Gal4 at 29°C had no effect on the leg phenotype and did not rescue the lethality caused by the ectopic expression of Mad alone.

III.3 Bx42 interacts with the TGF- β /Activin pathway component dSmad2

TGF- β /Activin belongs to the TGF- β ligand superfamily. The TGF- β pathway is evolutionary conserved from nematodes to humans. The transmission of the TGF- β /Activin signal from the cell exterior to the nucleus necessitates the signal transducing Smad proteins Smad2 and 3 (see table 1 and paragraph I.1).

The human Bx42 homologue Skip interacts with the human TGF- β /Activin signalling transmitters Smad2 and Smad3 *in vivo* and *in vitro* (Leong et al., 2000). To investigate whether Bx42 also interacts with the *Drosophila* TGF- β /Activin signal transducer dSmad2, yeast two hybrid assay was performed with the C-terminal deleted Bx42 protein Bx42 Δ C, dSmad2 full length and dSmad2 deletion fragments. SFY526 yeast cells were transformed pairwise with the recombinant plasmids BD-Bx42 Δ C/AD-dSmad2FL. For a positive control cells were transformed with pCL1 alone and with BD-Bx42 Δ C/AD-Bx42FL as a second positive control. Cells were also cotransformed with AD-dSmad2FL/pGBT9 as a negative control. A second negative control was also used by transformation of SFY526 yeast cells with the empty vectors pGBT9/pGAD424. Colonies that were grown on the selective media were then tested for the interaction using a colony-lift filter assay.

Results of this assay are summarized in Figure 11. Like its human homologue, Bx42 also exhibits interaction with dSmad2 in yeast. The β -galactosidase activity measured in this assay for BD-Bx42 Δ C/AD-dSmad2FL is 3,84 units (Figure 11B column 4). To map the dSmad2 interacting domain necessary for interaction with Bx42, a yeast two hybrid assay was performed using the dSmad2 deletion constructs that were cloned in pGAD424 (Figure 11A). Yeast cells were transformed with each of the above dSmad2 deletion constructs pairwise with BD-Bx42 Δ C. The dSmad2 Δ MH2 showed no interaction with Bx42 Δ C, indicating that the deletion of the Mad Homology domain 2 (MH2) of dSmad2 abrogated Bx42 binding. In contrast, AD-dSmad2 Δ (MH1+linker) and AD-dSmad2 Δ MH1 deletion constructs that contain a MH2 domain or the MH2+linker domains respectively exhibit an interaction with Bx42 Δ C in this assay. The measured β -galactosidase activity were 2,1 units for AD-dSmad2 Δ MH1/BD-Bx42 Δ C and 2,3 units for AD-

dSmad2 Δ (MH1/linker)/BD-Bx42 Δ C, indicating that the linker has no role in this interaction (Figure 11B).

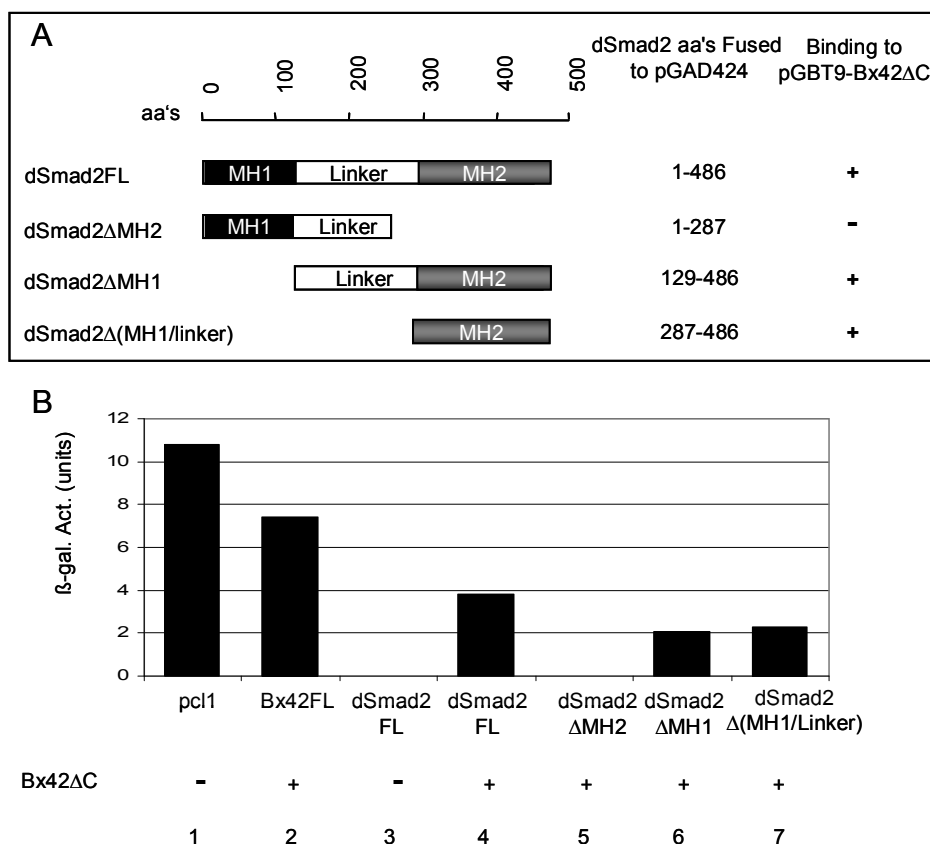


Figure 11: Bx42 interacts with the TGF- β /Activin component dSmad2 in the yeast two hybrid assays. (A) Schematic diagrams of dSmad2FL and dSmad2 deletion constructs used in this assay. All constructs were cloned into the yeast expression vector pGAD424 and expressed as AD-fusion proteins. Each recombinant protein was tested for the interaction with BD-BX42 Δ C in the yeast two hybrid assays. (B) The relative strength of binding of each of these fusion proteins to Bx42 was quantified by measurement of β -galactosidase activity in liquid culture of yeast cells transformed with the constructs as indicated and shown in column 1-7 respectively. Column 1, 2 and 3 were positive controls as mentioned previously.

These results suggest an interaction between TGF- β /Activin signalling pathway effectors dSmad2 and Bx42. As expected from previous experiments with Mad and Medea the well conserved MH2 domain is required for this interaction.

III.4 Bx42 interacts with the oncogene dSno protein

Sno is an oncogenic protein that interacts directly with Smad proteins (Smad2 and Smad3) to repress TGF- β /Activin dependent transcription. Sno is able to interact with human Skip. To address the question if this interaction is also conserved in *Drosophila*, Bx42

interaction with dSno was investigated in the yeast two-hybrid assay. dSnoFL was cloned into an expression vector and transformed together with BD-Bx42 Δ C into yeast cells. As negative controls yeast cells were transformed with the pair of plasmids pGBT9/pGAD424 or AD-dSnoFL/pGBT9. pCL1 and BD-Bx42 Δ C/AD-Bx42FL served as positive controls in this assay. The colonies expressing both of the transformed components were able to grow on the Tryptophan/Leucin plates and they were used for the colony-lift filter assay. The same procedure was done with the dSno deletion constructs shown in Figure 12A. The results from these assays are summarized in Figure 12.

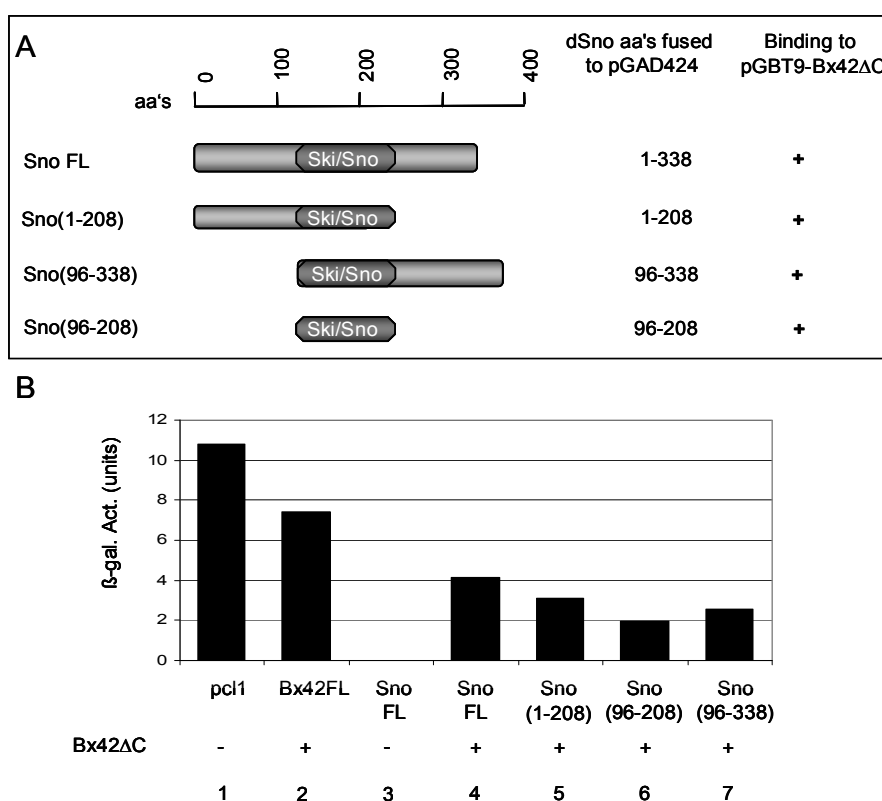


Figure 12: Bx42 interacts with the dSno in the yeast two hybrid assays. (A) Schematic diagram of dSnoFL and dSno deletion constructs. These constructs were cloned and expressed in the yeast vector pGAD424 and each recombinant protein was tested for an interaction with pGBT9-BX42 Δ C in the yeast two hybrid assay. (B) The relative strength of binding of each of these fusion proteins to BX42 Δ C was quantified by measuring the β -galactosidase activity, which is shown in columns 1-7. Columns 1, 2 and 3 were the positive controls used in this experiment as mentioned previously (refer Figures 7,8).

Analogous to the interaction of human Sno with Skip, dSno interacts with Bx42 in the yeast two hybrid assay and for this interaction the amino acids 96-208 of dSno are

required. This region corresponds to the well conserved Ski/Sno domain, which therefore appears to be sufficient for the physical interaction between the dSno and Bx42 proteins.

To further prove the interaction between Bx42 and dSno an *in vitro* pull-down assay was performed using the full length dSno and the full length Bx42 as well as the full length SNW-mutant version of Bx42 (Bx42(AAA)). The full length Bx42 or Bx42FL(AAA) were expressed as (Myc)₃-(His)₆ (MH) tagged fusion proteins and tested for an interaction with GST-dSno. As a negative control binding of MH-Bx42FL to GST alone was tested. GST-dSnoFL and MH-Bx42FL revealed a strong interaction (Figure 13, lane 1). Also Bx42 mutated in its SNW region could still interact with GST-dSnoFL in this assay (Figure 13, lane3). The control showed no specific interaction (Figure 13, lane 2).

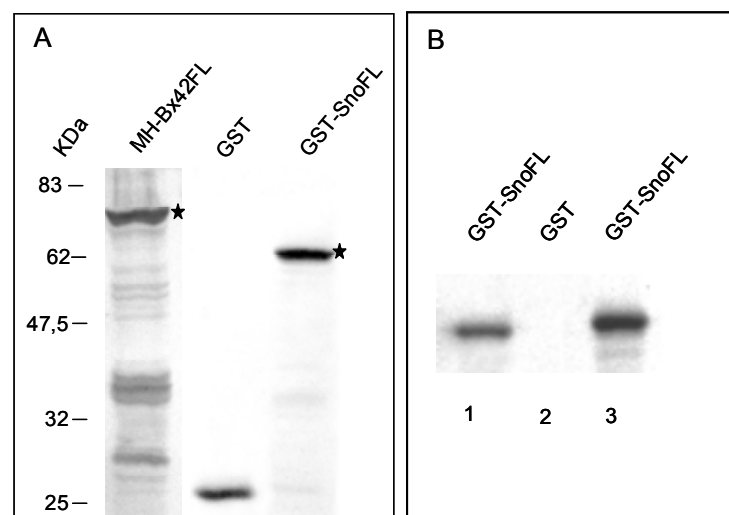


Figure 13: Binding of the fusion proteins MH-Bx42FL or MH-Bx42FL(AAA) to GST-dSnoFL. (A) Shown is 10% of input of the MH-Bx42FL, recombinant proteins used for each Ni-NTA pull down binding reaction and 10% of input of the GST-dSnoFL, and GST alone added to the each binding reaction. Extracts of the analysed proteins were mixed and incubated with Ni-NTA beads. After extensive washing bound proteins were eluted and analysed by western blot using GST antibodies (B). Lane 1 demonstrates interaction between MH-Bx42FL and GST-dSnoFL. Lane 3 shows interaction between MH-Bx42FL(AAA) and GST-dSnoFL. A negative control performed using GST protein alone lane 2.

These data convincingly demonstrate that the interaction between Sno and Skip is evolutionary conserved, since dSno and Bx42 interact in the yeast two hybrid assay as well as in the *in vitro* pull down assay.

III.5 Bx42 downregulation disturbs the expression of TGF- β /Dpp target genes

All *Drosophila* adult appendages develop from the imaginal precursor cells, which are determined during embryogenesis and proliferate during larval stages. This monolayer of epithelial cells form sac like invaginations termed imaginal discs. These discs shape up during metamorphosis in the pupae to form the corresponding adult structures. The leg and wing imaginal discs of *Drosophila* are appropriate tissues to study the expression of Dpp target genes in wild type and Bx42 knockdown flies.

III.5.1 *Omb-lacZ* expression

In order to understand the biological relevance of the observed interaction between Bx42 and the Dpp signalling transducer Mad and Medea, first the effect of Bx42 downregulation on the expression of the Dpp target gene *optomotor blind (omb)* was examined. The *omb-LacZ;Bx42-RNAi/TM6* transgenic line was crossed with *dpp-Gal4* or *omb-Gal4* lines at different temperatures. The imaginal discs from third instar larvae of the progeny *omb-LacZ;UAS-Bx42-RNAi/dpp-Gal4* and *omb-LacZ/omb-Gal4;UAS-Bx42-RNAi* were examined for the expression of the *lacZ* reporter gene by immunostaining with an anti β -galactosidase antibody.

Omb is a T box gene, which has in the wing a large expression domain along the anterior posterior axis where *Dpp* is expressed (Figure 14B). The downregulation of Bx42 clearly changes the expression pattern of *omb* in leg and wing imaginal discs. The activation of Bx42-RNAi by *omb-Gal4* or *dpp-Gal4* results in a complete repression of *omb-LacZ* reporter gene in the leg imaginal discs (Figure 14C and E) and a reduction in its expression in the wing imaginal discs (Figure 14D). The reduction of *omb-lacZ* expression was stronger when Bx42-RNAi was induced by the *dpp-Gal4* driver (Figure 14F). This is an expected result, since *dpp-Gal4* is expressed at earlier stage than *omb-Gal4*. The usage of *omb-Gal4* line, possibly lead to the development of an *omb/Bx42-RNAi* feed back like effect and to a reduction and not a complete elimination of *omb-lacZ* expression in the wing

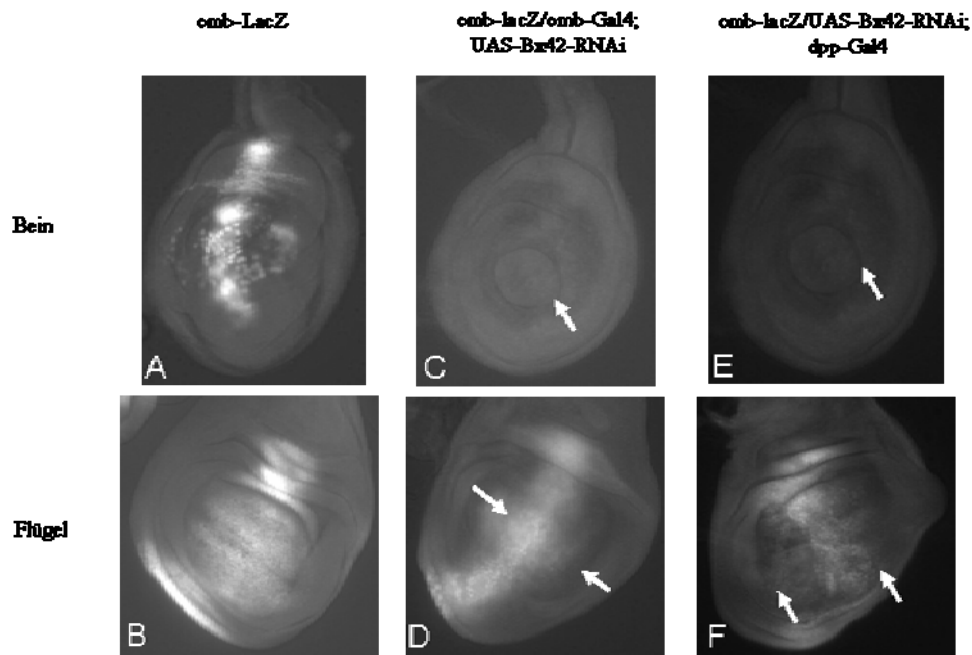


Figure 14: The influence of Bx42-RNAi induction on the expression of the dpp target gene *omb*. *omb* expression is visualised by means of the *omb-lacZ* reporter. The wild type expression of *omb-lacZ* in the leg imaginal disc (A) and in the wing imaginal disc (D). Induction of Bx42-RNAi with the *omb*-Gal4 or *dpp*-Gal4 driver at 29°C attenuates *omb-lacZ* expression in the leg imaginal discs (C and E). (D) *omb-lacZ* expression is restricted to the region immediately flanking the anterior/posterior boundary in the wing imaginal discs of *omb-lacZ/omb-Gal4;UAS-Bx42-RNAi* flies. (F) Reduction of Bx42 by *dpp*-Gal4 driver line at 29°C also causes a strong reduction in *omb-lacZ* expression in the wing imaginal discs.

These results demonstrate that in an *in vivo* reporter gene assay Bx42-RNAi negatively effects the expression of the Dpp target gene *omb*. Therefore in wild type flies Bx42 positively regulates *omb* transcription either directly or indirectly.

III.5.2 *Dll-lacZ* expression

The leg imaginal disc forms in its center a circular epithelial sheet with a distal region that gives rise in the adult leg to more distal segments (tarsal segments). The medial region forms the tibia and the femur and the proximal region generates the more proximal podomere (coxa). The delineation of these regions is regulated by the expression of different regulatory genes. One of these genes is the Dpp target gene *distal-less* (*dll*), which encodes a homeodomain transcription factor. *Dll* is expressed mainly in the distal region of the leg imaginal disc and is required for the formation of the distal leg segment (Cohen et al., 1993; Panganiban, 2000). Genetic studies showed that *Dll* is necessary and sufficient for the formation of distal limb structures in *Drosophila* (Gorfinkiel et al., 1997;

Campbell et al., 1998; Wu and Cohen, 1999). For example, hypomorphic mutants of *dll* cause a loss or fusion of the tarsal segments (Cohen et al., 1989; Dong et al., 2000; reviewed in Panganiban, 2000). As already mentioned, this phenotype is similar to the phenotype caused by Bx42 downregulation (Figure 5B and C).

Considering this finding the role of Bx42 in the regulation of *dll* was investigated. For this aim the expression of *dll-lacZ* reporter gene was compared in leg imaginal discs prepared from wild type controls and UAS-Bx42-RNAi,*dll-lacZ*;dpp-Gal4 transgenic lines. Control leg imaginal discs exhibit the wild type *dll-lacZ* expression in the central region of the discs (Figure 15A). After the induction of Bx42-RNAi with the dpp-Gal4 driver a complete repression of *dll-lacZ* was observed in the distal region of the leg imaginal disc (Figure 15B). These results indicate that in addition to *omb*, Bx42 positively regulates the expression of the TGF- β /Dpp target gene *dll*.

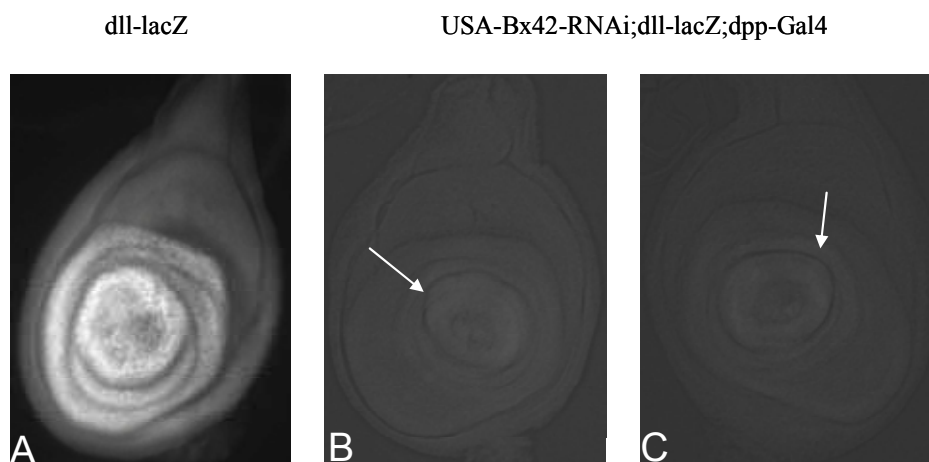


Figure 15: Influence of Bx42-RNAi induction on the expression *dll-lacZ*. (A) Wild type *dll* expression in third instar leg imaginal discs visualised by means of the *dll-lacZ* reporter. Dll is expressed in the distal region of leg imaginal discs that leads to the formation of the tarsal segments. The reduction of Bx42 with the dpp-Gal4 driver at 29°C leads to a complete elimination (B) or a strong reduction (C) of *dll-lacZ* expression in the leg imaginal discs.

To investigate at which step of gene regulation Bx42 affects *dll* expression, a RNA in situ hybridisation approach was carried out to examine if the *dll* transcript is diminished by Bx42 downregulation. For this purpose, a 994 bp Dig-labelled Dll-RNA probe was hybridized to third instar larval wing imaginal discs. The downregulation of Bx42 with the en-Gal4 driver, which is expressed in the posterior compartment of the wing imaginal disc, causes a size reduction of the posterior region of the wing and an elimination of *dll* transcripts in this region (Figure 16C). Moreover, wing imaginal discs from Bx42-

RNAi/omb-Gal4 transgenic larvae exhibit the absence of *dll* transcripts in the central region of the wing, where *omb* normally is expressed. *dll* transcripts were detectable in this line only in the lateral regions where Bx42-RNAi was not induced (Figure 16B). Finally, the effect of Bx42 knockdown on the expression of the endogenous *dll* gene in leg discs was studied. In the wild type leg imaginal discs *dll* transcripts were detectable in the more distal region.

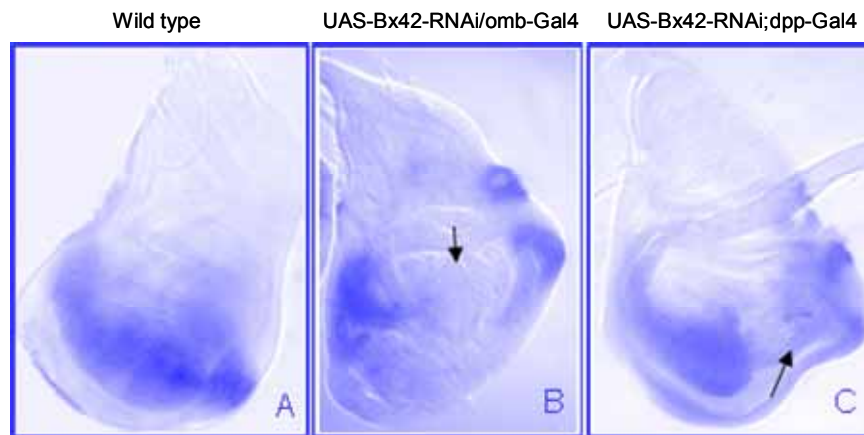


Figure 16: In situ hybridisation with a Dig-labelled *dll*-RNA probe to third instar larvae wing imaginal discs. (A) The wild type expression of the *dll* transcript along the dorsal/ventral axis within a third instar larval wing imaginal disc. (B) The induction of Bx42-RNAi with omb-Gal4 results in loss of the *dll* transcript in cells expressing *omb*. (C) A similar result was obtained by using en-Gal4 line to down regulate BX42. *Dll* transcription was attenuated in a significant part of the posterior region where normally *en* is expressed.

In comparison leg imaginal discs of Bx42 knockdown larvae driven by en-Gal4 and omb-Gal4 had reduced signal intensity for *dll* under the same hybridisation conditions (data not shown).

These data indicate a positive role of Bx42 in the regulation of *dll* transcription at the promoter level.

III.5.3 *Spalt* expression

The direct involvement of Bx42 in the TGF- β /Dpp signalling pathway was also investigated by the analysis of the expression of another Dpp target gene *spalt* (*sal*). In order to determine whether there is an obligatory requirement for Bx42 in mediating *sal* expression, Bx42-RNAi transgenic flies were crossed with omb-Gal4 or dpp-Gal4 driver at

28°C and the expression of *spalt* was visualized in the wing imaginal discs of UAS-Bx42-RNAi/omb-Gal4 and UAS-Bx42-RNAi;dpp-Gal4 larvae by indirect immunofluorescence. Spalt is a zinc finger transcription factor that normally is expressed within the wing pouch in a broad region centered over the Dpp expression domain (Figure 17A). After the induction of Bx42-RNAi with omb-Gal4 the expression of *sal* was attenuated in Bx42-RNAi/omb-Gal4 wing imaginal discs (Figure 17B). When Bx42-RNAi was induced by dpp-Gal4, *sal* expression was also abolished in a stripe of cells corresponding to the *dpp* expression domain (Figure 17c).

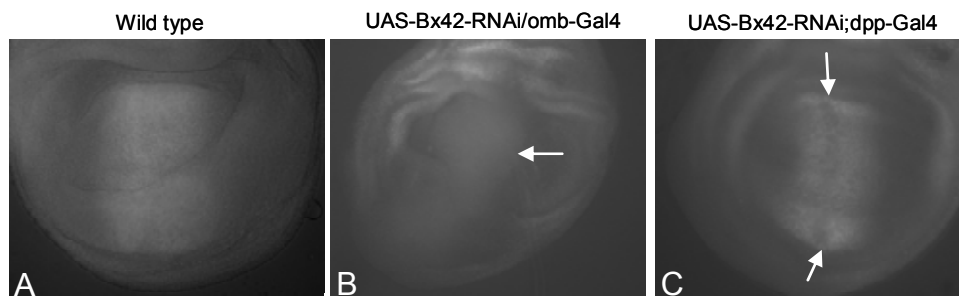


Figure 17: *Bx42* is required for the activation of the Dpp target gene *spalt* (*sal*). (A) Immunostaining showing Sal expression in a wild-type third-instar wing imaginal disc. (B) Expression of *sal* is altered in the wing imaginal disc pouch (arrow) by downregulation of Bx42 using the omb-Gal4 driver. (C) Expression of *sal* is affected in a narrow region corresponding to the domain where Bx42-RNAi is induced by dpp-Gal4.

These results suggest an important requirement of Bx42 for the expression of the Dpp target gene *spalt*.

IV Discussion

IV.1 Bx42 –RNAi causes a penetrant leg phenotype

Using Bx42-RNAi it was possible to understand more about the function of the *Drosophila* Bx42, since the phenotypes that were obtained could be interpreted in favour of an involvement of Bx42 in several biological processes and different signalling pathways (Negeri et al., Negeri et al., 2002). Based on some of these phenotypes it was argued that Bx42 is an essential component in Notch signalling pathway, that it interacts *in vivo* with the Notch signal pathway components Su(H) and N-IC and positively regulates the expression of Notch target genes such *e(Spl)m8* and *cut* (Negeri et al., 2002).

One of the Bx42 knockdown phenotypes exhibited a deletion or fusion of leg tarsal segments (see Figure 1B and C) without affecting the medial and the more proximal structures. These phenotypes are similar to phenotypes caused by viable *dll* alleles of different severity. Weak *dll* mutant alleles lead to the fusion of tarsal segments, whereas intermediate *dll* mutant alleles result in loss of the tarsal segments (Cohen et al., 1998; Dong et al., 2000; Panganiban et al., 2002). Additionally, the induction of ectopic *medea* expression using *ptc-Gal4* similarly generates tarsal segment deletions (Marquez et al., 2001). Moreover, the formation of the more distal parts of the leg is affected if the Dpp signal transducers Mad or Medea are overexpressed by using *dpp-Gal4*. Here, deletion or fusion of tarsal segments occurred (see Figure 2B, C and D).

In the leg, Dpp and Wingless signalling pathways, which are expressed dorsally along the anterior/posterior boundary and ventrally in the anterior compartment, respectively (Campbell et al., 1993) organize the formation of the proximal/distal axis by acting synergistically to promote the regulation of leg specific genes *homothorax (hth)*, *dachshund (dac)* and *distal-less (dll)* in a concentration-dependent manner (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997; Abu-Shaar and Mann, 1998; Milan and Cohen, 2000). In leg imaginal discs, *hth* is expressed in the more proximal region that corresponds to the proximal leg segment; *dac* is expressed in the intermediate ring corresponding to the presumptive femur and tibia whereas *dll* is present in the distal domain that gives rise to the tarsal segments and distal tibia. Mutations in any of these genes disturb leg patterning, leading to the absence of those parts in which the genes are

normally expressed (Gorfinkiel et al.,1997; Campbell et al.,1998; Wu and Cohen, 1999; Dong et al., 2000).

dll is the earliest marker known that is specific for leg formation. *dll* is essential for the establishment of the leg proximal/distal coordinates. It is expressed in the distal region of overlapping high level expression of *Wg* and *Dpp* (Goto and Hayashi 1997; Gorfinkiel et al.,1997; Williams 1998; Dong et al., 2000). The input from both pathways is essential and necessary for the wild type expression of *dll*. The absence of one of these signalling components causes an up-regulation of *dll* expression (Diaz-Benjumea et al.,1994; Galindo et al., 2002).

The reduction of *Bx42* under the control of *dpp-Gal4* affects the development of the more distal part of the leg corresponding to the tarsal segments whose development is controlled by *distal-less*. Therefore it was investigated whether this phenotype correlated with an altered *dll* expression. Indeed, *Dll* was completely missing or very weak in the leg imaginal discs of *UAS-Bx42-RNAi;dll-lacZ;dpp-Gal4* larvae compared to wild type (see Figure 11). The variability in the reduction of *dll* expression might be the results of a varying strength of *Bx42* knockdown. Moreover, this variability in *dll* expression is mirrored in the adult leg phenotype. The failure to form tarsal segments may be explained by an alteration of *dll* expression. On the other hand, the fusion of tarsal segments may be explained by the presence of a minimum amount of *Dll* protein that is necessary for the formation of the tarsal segments but which may be not sufficient for the segmentation.

The *Drosophila* leg is composed of nine segments; the coxa, trochanter, femur, tibia and the five tarsal segments. Each segment is separated from the next by a joint or boundaries. The formation of each boundary needs an input from Notch signalling. Notch is activated in the most distal cells of each segment, which are distally adjacent to the expression domains of the Notch ligands *Serrate* (*Ser*) and *Delta* (*Dl*), and specifies them as joint forming cells. Loss of function of Notch and Notch ligands leads to deletion of joint structures and fusion of leg segments. It has been shown that Notch null mutant clones or clones of cells double mutant for *ser* and *dl* fail to form joint structures in adult leg (De Celis et al., 1998; Rauskolb and Irvin, 1999; Rauskolb, 2001; Kojima et al., 2004). The restriction of the *ser* and *dl* expression domains to a narrow repeated ring is a result of the combined activities of the genes *homothorax*, *dachshund* and *distal-less* (Rauskolb, 2001). In the tarsal segments *Dll* represses the expression of the Notch ligand *Serrate*.

Spineless-aristapedia (*ss*) and *bric-a-brac* (*bab*) are positively regulated by *Dll*. *Ss* encodes a bHLH-PAS family transcription factor and is required for patterning of the distal part of

the first tarsal (T1) and second through fourth tarsal segments (T2-T4). Flies mutant for *ss* shown an unsegmented tarsus (Duncan et al., 1998). *Bric-a-brac (bab)*, which is required specifically for distal limb segmentation, is expressed in the tarsal segments T1 to T4. *bab* null animals also cause the fusion of tarsal segments (Godt et al., 1993; Chu et al., 2002). Since *bab* and *ss* mutants promote the fusion of tarsal segments, it was suggested that they are key regulators by which the expression of *serrate* in the tarsal segments is regulated. Together *Ss* and *Bab* may either antagonise the repression of *serrate* by *Distal-less* or they may convert the repressor *Dll* to an activator (Rauskolb, 2001).

Strong *bab* mutants show a fusion of the tarsal segments T2 with T3 and T4 with T5 similar to the phenotype generated by *Bx42*-RNAi induction. The reduction of *Bx42* results in a reduction of *dll* expression. This change of *dll* expression may abrogate the activation of *bab* and/or *ss*. At the same time the amount of *Dll* may still be sufficient to maintain the repression of *ser* resulting in the fusion of tarsal segments (Figure 15C and 18).

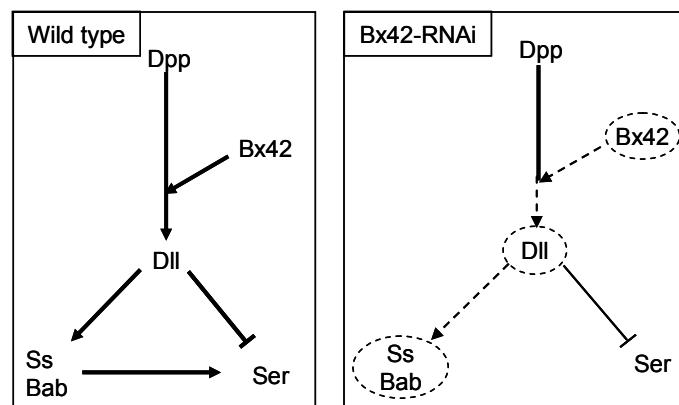


Figure 18: Bx42 may act indirectly through Dll to regulate the expression of *ss*, *bab* and *ser*. (Left) In the wild type situation Bx42 may act together with the Dpp signalling transducer Mad and Medea to regulate positively *dll* expression. In tarsal segments Dll represses *ser* and activates *ss* and *bab*. These proteins may act together to activate *ser* expression in joints. This leads to a proper segmentation of the more distal part of the leg. (Right) The knockdown of Bx42 by RNAi appears to lead to the destruction of Dpp signalling. A result of this is the strong reduction of *dll* expression in leg imaginal discs. The remaining Dll is then unable to activate *ss* and *bab* but it may be still enough to represses *ser*, leading to the formation of unsegmented tarsi.

However, this hypothesis must be proven by studying the expression of *ser*, *bab* and *ss* in leg imaginal discs of *Bx42* knockdown animals. *Bx42* could also act directly to activate *ser*, *bab* and/or *ss* genes.

IV.2 Bx42 interacts with Mad and Medea

The data discussed above lead to the question how Bx42 regulates the expression of *Dll* and whether Bx42 plays a role in Dpp and/or Wg signalling pathways.

Decapentaplegic (Dpp), a homolog of the vertebrate Bone Morphogenic protein (BMP2/4), is essential for patterning and cell fate specification in *Drosophila*. Dpp signalling triggers the nuclear accumulation of Mad and Medea, which regulate the expression of target genes by the binding of the Mad/Medea complex to the regulatory regions of *vestigial (vg)* and *spalt (sal)*, which are activated, or to the regulatory region of *brinker (brk)* which is repressed. Additionally, the Dpp signal transducers Mad and Medea act indirectly in the wing imaginal discs, through negative regulation of *Brinker*, to repress Dpp target genes *optomotor of blind (omb)*, *vestigial (vg)* and *spalt (sal)*.

Homozygote Mad mutants cause a loss of distal leg segments (Sekelsky et al., 1995; Galindo et al., 2002). This phenotype mimics the effects caused by Bx42-RNAi induction. Interestingly, the overexpression of Mad and Medea under the control of *dpp-Gal4* results in a similar phenotype that differs only in its severity (Figures 6B, C and D). Taken together, these data suggest the possibility of an involvement of Bx42 in Dpp signalling. To examine this hypothesis the contribution of Bx42 in this pathway was investigated by studying the interaction of Bx42 with Mad and Medea using the yeast two hybrid assay. It was shown by this assay that Bx42 associates with Mad and Medea. Moreover, the importance of the MH2 domains of Mad and Medea for the interaction with Bx42 was demonstrated (see Figures 7, 8).

The physical interaction between Bx42 and Mad or Medea was also confirmed *in vitro*. Mad and Medea are able to bind to the C-terminally truncated Bx42 (Bx42(Δ C)) (see Figure 9). These results are consistent with previous data demonstrating an interaction of fox-tapeworm *Echinococcus multilocularis* EmSkip, a novel member of the SNW gene family, with the MH2 domain of EmSmadB that is the orthologue of Smad1 and Smad5 in human and of Mad in *Drosophila* (Gelmedin et al., 2005).

The region of Bx42 that is necessary for the interaction with the Smad proteins is as yet unidentified. The highly conserved three amino acids SNW could be excluded to be essential for the interaction with Mad and Medea, as Bx42FL(AAA), a Bx42 mutated protein in which the three amino acids SNW are replaced with three alanines, is still able to interact with Mad or Medea (Figures 5, lane 3 and 6). These results are consistent with an analysis of the Prp45p/Fun20 SNW signature in *Saccharomyces cerevisiae*. Deletion of

Prp45p/*Fun20* is lethal and this lethality can be rescued by expression of the Prp45p/*Fun20* N-terminal part harbouring the SNW domain. However, a substitution of the three amino acids SNW by three alanines does not affect the N-terminal region in its ability to rescue the lethality. Therefore, the whole region is essential for the function of Prp45p/*Fun20* and the role of the SNW motive remains elusive (Martinkova et al., 2002).

IV.2.1 The genetic interaction between Bx42 and Medea

The tissue specific knockdown of Bx42 uncovers the essential functions of Bx42 in various *Drosophila* developmental stages. Tissues specific phenotypical defects and abnormal organ development suggest that Bx42 may play a role in the regulation of a number of different cellular signalling (Negeri et al., 2002).

The downregulation of Bx42 by dpp-Gal4 in anterior cells along the anterior/posterior boundary, the region where *Dpp* is usually expressed in the wing imaginal disc, results in an adult wing phenotype that is characterized by the deletion of the longitudinal vein III (LIII), a part of the anterior cross vein (ACV) and a displacement of the more distal part of the longitudinal vein IV (LIV) to anterior region (see Figure 10A and B). This phenotype is completely rescued by the simultaneous overexpression of the Dpp signalling component Medea, suggesting an *in vivo* interaction between Bx42 and Medea. This result supports previous data concerning the *in vitro* interaction of Bx42 and Medea (see Figures 8 and 9). The fact that the overexpression of Medea compensates the reduction of Bx42 indicates that both proteins probably act synergistically in the Dpp pathway for a proper formation of wing veins. One explanation of this observation is that increased levels of Medea may enhance the ability of the Mad/Medea activator complex to signal even in the absence of the cofactor Bx42. In this model, Bx42 might stabilize the Mad/Medea complex. The reduction of this stabilizer might cause the disruption of the Mad/Medea complex and eventually a loss of the Dpp signalling. An increased amount of Medea might substitute the stabilizing effect of Bx42 by favouring oligomerization between Mad and Medea.

Taken together, these data demonstrate that the Bx42/Mad and Bx42/Medea interactions were apparent in *vivo* and *in vitro*, as shown in this work by yeast two hybrid protein-protein studies and Ni-NTA pull-down assays. It was proven that the evolutionary well conserved SNW signature within Bx42 is not a motive that is used for the interaction of

Bx42 with the Dpp signal pathway components Mad and Medea, but the whole SNW domain may be necessary for these interactions.

IV.3 Bx42 interacts with TGF- β /Activin pathway component dSmad2

The interaction of human Skip with the TGF- β /BMP signalling components Smad1 and Smad5 has so far not been demonstrated. However, it was shown that Skip interacts with the TGF- β /Activin pathway components Smad2 and Smad3 and positively modulates TGF- β /Activin dependent transcription. The Skip/Smad interaction is mediated by the MH2 domain within Smad3 (Leong et al., 2001).

The Ski and Sno oncoproteins have been shown to negatively modulate TGF- β /Activin signalling through an interaction with the N-CoR repressor complex (Luo et al., 1999) and Smad3 (Leong et al., Leong et al., 2001). Like Skip, Ski-Sno interacted with the MH2 domain of Smad3, suggesting that the opposing transcriptional effects of Skip and Ski/Sno may involve competition for Smad3 binding between Skip and Ski-Sno (Leong et al., 2001).

The analysis of the interaction between Bx42 and the *Drosophila* TGF- β /Activin component dSmad2 in the yeast two hybrid assay indicates that Bx42 binds dSmad2 and that the MH2 domain within dSmad2 is essential and sufficient for this interaction (Figure 11). This interaction seems to be evolutionally conserved since it has been shown also in *Echinococcus multilocularis*. Here EmSkip interacts with EmSmadA, the orthologue of dSmad2 (Gelmedin et al., 2005). The interaction of the SNW gene family (Bx42, Skip and EmSkip) with the TGF- β /Activin pathway components Smad is conserved in humans, *Drosophila* and *Echinococcus multilocularis*. In humans the biological meaning of the Smad/Skip interaction was elucidated, since Skip augments TGF- β /Activin dependent transactivation in mammalian cells (Leong et al., 2001).

Thus, as Bx42 interacts with dSmad2 in yeast cells and with the oncogene protein dSno *in vivo* and *in vitro* (see Figures 11, 12 and 13) it is also possible that Bx42 may be able to modulate TGF- β /Activin signalling in *Drosophila* through Smad2 by the same mechanisms as its human homologue Skip.

IV.4 The function of Bx42 in vein formation

“The individual identity of each vein as revealed by specific genes expressed in their presumptive territories implies that one particular vein can be affected independently of the others.”

De Celis and Diaz-Benjumea, 2003

Veins are important structures that provide rigidity to the adult wing. Vein cells differ from the neighbouring cells by being small and by secreting a dark pigmented cuticle. There are two vein categories, the longitudinal veins (LI, LII, LIII, LIV and LV) that run, in constant position, along the anterior-posterior wing axis and two cross veins. The anterior cross vein (ACV) connects the LIII with LIV and the posterior cross vein (PCV) bridges LIV with LV (Figure 10A). The development of veins in their correct position occurs in three main developmental steps that are regulated by several signalling pathways (Bier, 2000; De Celis and Diaz-Benjumea, 2003a; De Celis, 2003b; Molnar et al., 2006).

The first step of vein patterning is the subdivision of the presumptive wing blade into defined domains and the establishment of the provein and intervein territories in a process regulated by Dpp and Hh signalling (Mullor et al., 1997; Nestoras et al., 1997; Mohler et al., 2000). These two pathways trigger the expression of several provein specific transcription factors such as the products of the *iroquois* complex (*iro-c*) in the LI, LIII and LV provein region and *knirps* (*kn*) in the LII provein region (Gómez-Skarmeta et al., 1996; Lunde et al., 1998; De Celis et al., 2000). Additionally, Dpp and Hh signalling determine the intervein domains by regulating and limiting other factors, for instance *blistered* (*bs*) that is expressed only in interveins (Montagne et al., 1996).

Following the establishment of the initial pattern of gene expression in proveins and interveins a second developmental step takes place within the proveins. This step is characterised by the expression of several members of the Epidermal Growth Factor Receptor (EGFR) and the activation of the Notch signalling pathway, leading to the subdivision of each provein in a central region that will differentiate as vein, where EGFR signalling is active, and boundary provein cells adjacent to the veins where Notch signalling prevents vein differentiation (Sturtevant et al., 1993; De Celis et al., 1997; Bier, 2000; De Celis and Diaz-Benjumea, 2003a; De Celis, 2003b). The third and final step

in vein formation occurs during the pupal stage and is controlled by Dpp signalling that is sufficient for veins differentiation in this stage (De Celis et al., 1997; De Celis,2003b).

The reduction of Bx42 in the wing altered the normal vein formation including the disappearance of the longitudinal vein LIII. This phenotype could be the consequence of an alteration of one or more of the above discussed stages in vein development. However, the *dpp-Gal4* driver line used in this assay is expressed exclusively in larvae; therefore vein formation has only been affected in larval stages and not in pupae where vein differentiation occurs. The effect of Bx42 downregulation on vein development possibly takes place during the first step where Hh signalling acts either alone or together with Dpp signalling to establish the right place for each vein or in the second step where Notch and EGFR signalling are involved to define vein domains. The fact that Bx42-RNAi induction by *dpp-Gal4* leads to the loss of the LIII vein and not LIV indicates that Bx42 might regulate the expression of LIII-specific genes. *Iroquois* products are detectable in LIII and not in LIV and a change in their expression would explain this phenotype. This assumption is supported by the finding that the LIII vein is missing in *iro* mutant (De Celis et al., 2000).

Additionally to Hh signalling, Dpp signalling is required for the activation of the *Iro*-complex genes, *Araucan (ara)* and *Caupolican (caup)* in the medial wing. These genes are needed for the formation of vein LIII (Gomez-Skarmeta et al., 1996a; Gomez-Skarmeta et al., 1996b).

The phenotype caused by Bx42 knockdown in the wing could be explained by an alteration of Dpp signalling leading to the loss of expression of the LIII vein specific transcription factors *Iro-C*. In this context, rescue of this phenotype by overexpression of *Medea* could be due to a bypass in the requirement of Bx42 in the activation of *iro-c* in the presence of high levels of *Medea* through enhanced Dpp signalling. However, this hypothesis must be examined by analysing *iro-c* expression in Bx42 knockdown animals.

IV.5 Bx42 controls the expression of Dpp target gene optomotor blind

Once the expression of Dpp is activated in response to Hh signalling in the anterior cells adjacent to the anterior-posterior boundary, a long range Dpp signalling takes place to pattern wing and leg imaginal discs along its anterior-posterior axis. Dpp is a secreted

molecule that diffuses from its expression source to the neighbouring cells anteriorly and posteriorly where it contributes by the regulation of target genes to the establishment of different genetically specified domains. Dpp is the ligand of the membrane receptors Type I and II, which transmit the signal from the exterior to the interior cells by the phosphorylation of Mad. Phosphorylated Mad transfers Dpp signalling forward to the nucleus where the responsiveness to this signalling occurs by activation of the target genes such *omb*, *sal* or repression of *brk* (Lawrence and Struhl, 1996; Grimm et al., 1996; Lecuit et al., 1996; Kim et al., 1997; Singer et al., 1997).

Optomotor blind (omb) belongs to the T box family genes. In the leg *omb* is expressed in the dorsal domain and is required for dorsal leg development (Maves et al., 1998), whereas in the wing *omb* is expressed in a relatively broad domain and is detected in the wing pouch and in the presumptive hinge region (see Figure 3B and 14D). As *omb* is downstream of Dpp signalling, loss of this signalling leads to a change of *omb* expression. Indeed, clones homozygous for Mad fail to express *omb*. In a similar manner, *omb* expression is lost in Dpp type I receptor Thick vein (Tkv) mutant clones (Penton et al., 1994; Wisotzkey et al., 1998). Moreover, elimination of Dpp by the temperature sensitive mutant Hh^{ts} leads to a reduction of *omb* expression (Grimm et al., 1996). *Omb* is required for the development of the central and more distal wing regions and its absence leads to the destruction of the wing proximodistal axis (Grimm et al., 1996; Lecuit et al., 1996; Del Alamo Rodriguez et al., 2004).

In this work the interaction of Bx42 with the Dpp signalling components Mad and Medea was demonstrated (Figures 7, 8, 9 and 10). Additionally, the involvement of Bx42 in the regulation of the Dpp target gene *dll* was shown (Figure 15). These data support the assumption that Bx42 is an essential Dpp signalling component that acts together with Mad and Medea to modulate Dpp target genes.

To assess the role of Bx42 in the regulation of other genes downstream of Dpp, the effect of Bx42 downregulation on the expression of *optomotor blind (omb)* in the wing and leg imaginal discs was examined using *omb-lacZ* reporter gene. The expression of the *lacZ* reporter is reduced in wing discs and is totally eliminated in the leg imaginal discs (Figure 14) indicating that also in this case Bx42 is an essential factor for the maintenance of *omb* expression. The change in *omb* expression is reflected in the adult wing phenotype. Bx42-RNAi induction in wing imaginal discs by *omb-Gal4* at 18 °C results in destruction of the proximodistal axis and at 25 °C in a loss of the central region of the wing (Negeri, 2002).

These phenotypes are similar to those generated in *omb* mutants (Del Alamo Rodriguez et al., 2004).

Previous data reported that the wing phenotype of *omb* mutants is caused by JNK-mediated apoptosis (Adachi-Yamada, et al., 1999; Del Alamo Rodriguez et al., 2004). To investigate whether the Bx42 phenotype is a result of apoptosis, wing imaginal discs were stained with Acridin orange which revealed a massive cell death in the expression domain of *omb*, mainly in the distal-most region of the wing discs (data not shown). Cell death is likely an indirect effect of *Bx42* downregulation and it is probably a consequence of the elimination of *omb* expression as a JNK-mediated cell death occurred in *omb* mutant discs (Del Alamo Rodriguez et al., 2004). This result suggests that Bx42 is involved in the regulation of the Dpp target gene *omb* in the wing and in the leg by interacting with Mad and Medea.

Interestingly the analysis of the lacZ reporter expression in wing imaginal discs from *omb-lacZ/UAS-Bx42-RNAi;dpp-Gal4* animals showed an unexpected strong reduction of expression of the reporter in the whole wing blade, that means in regions where Dpp is not expressed. An explanation for this is a cell-non-autonomous effect of Bx42. Probably Bx42 acts positively in the regulation of *dpp*. However this assumption should be demonstrated by analysing Dpp expression in Bx42 mutant animals.

Dissection studies of the cis regulatory sequence of *omb* revealed the presence of a minimal wing regulatory region, the WF12 enhancer. WF12-lacZ still responds to Dpp signalling and mimics *omb* expression in the wing pouch (Sivasankaran et al., 2000). This enhancer harbours a consensus sequence that is a target for the repressor Brinker as well as for an as yet unknown activator. This enhancer would be an ideal tool to investigate whether Bx42 regulates *omb* via binding to the WF12 enhancer.

IV.6 Bx42 is necessary for the normal expression of spalt in the wing blade

Drosophila spalt (sal) is an important developmental regulator that encodes a zinc finger protein. The expression of *sal* in the presumptive wing blade is first detected in discs of early third instar larvae. In late third instars, *sal* is expressed in a set of cells centered on the Dpp expression region (De Celis et al., 1996). Studies showed that *tkv/brk* and *mad/brk* double mutant clones, which at the same time lack the repressor (Brk) and the activators of the Dpp pathway (Tkv or Mad), still express *sal*, though at lower levels than in wild type.

This indicates that Dpp signalling is necessary for the formation of the normal expression pattern of *sal* gene and that an additional input is needed for wild type expression of *sal* in the wing pouch. It was suggested that this activation is provided by the wing marker Vestigial (Vg) (Kim et al.,1997; Guss et al., 2001; Baena-Lopez et al., 2003; Barrio et al., 2004).

The induction of Bx42-RNAi by the dpp-Gal4 resulted in the total elimination of *sal* expression in the region where Dpp is expressed, suggesting that the signals that regulate positively the expression of this gene, namely Vestigial and Dpp signalling are disturbed. Indeed, reduction of Bx42 by RNA interference using dpp-Gal4 caused the elimination of Vg in the Dpp expression domain (Negeri, 2002). That means that the Vg input for *sal* activation in this region was abolished. These data indicate the involvement of Bx42 in Dpp signalling. Downregulation of Bx42 results in the disruption of this signalling and so of an activator of *sal*, probably resulting in the complete elimination of *sal* expression in the cells where the induction of Bx42-RNAi occurs.

Taken together, these data demonstrate that Bx42 regulates positively the expression of Dpp target genes *omb*, *dll* and *sal*, probably by interacting with Mad and Medea. However, the question if Bx42 is able to interact with the Mad/Medea heterooligomer to form a ternary complex and if this interaction occurs in the cytoplasm or in the nucleus are yet unknown (Figure19).

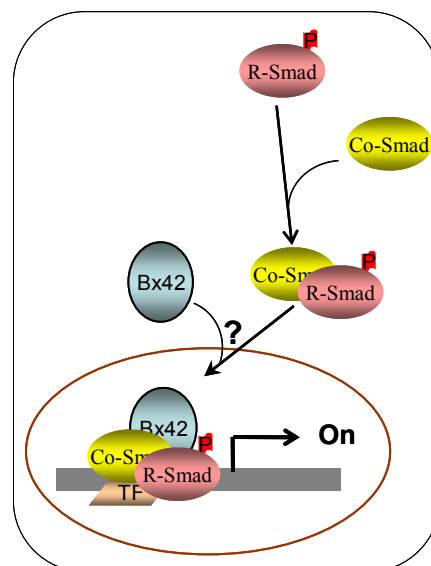


Figure 19: Schematic representation of the Dpp signalling pathway and the role of Bx42 in the regulation of Dpp target genes. The phosphorylation of R-Smad induces the formation of a heterooligomer complex with Co-Smad in the cytoplasm. The interaction of Bx42 with the

complex R-Smad/Co-Smad could occur either in the nucleus or in the cytoplasm. The formed complex binds then to the DNA and activates Dpp target genes.

IV.7 Bx42 is not a general coactivator in *Drosophila*

Studies of the effect of Bx42 downregulation by RNA interference on the Notch target genes *cut* and *enhancer of split m8* [*e(spl)m8*] demonstrated the importance of Bx42 in the activation of Notch target genes (Negeri 2002). Additionally, it was shown that Bx42 is involved in the regulation of the TGF- β /Dpp target genes *omb*, *spalt* and *dll* by acting as an activator at the promoter level.

To address the question if Bx42 is a general coactivator in *Drosophila*, the effect of Bx42-RNAi induction on the expression of *z4* was studied. Z4 is a zinc finger protein that binds to the interband chromatin of polytene chromosomes and is ubiquitously expressed in imaginal discs (Eggert et al., 2004). Immunostaining of wing imaginal discs of UAS-Bx42-RNAi/*omb*-Gal4 larvae with an anti-Z4 antibody showed no change in the expression of *z4* in comparison to wild type imaginal discs (data not shown). This data indicate that Bx42 does not act as a general coactivator of genes.

In this work, evidence is presented implicating Bx42 in the positive regulation of the expression of a set of genes. Moreover, the induction of Bx42-RNAi caused a strong cell death in imaginal discs. Therefore, an alternative explanation could be that the massive cell death observed in Bx42-RNAi imaginal discs is the cause but not the consequence of the observed alterations in *dll*, *omb* and *sal* expression. However, the fact that Z4 staining in wing imaginal discs is not affected by Bx42-RNAi induction is evidence that an altered expression of *dll*, *omb* and *sal* is not a general effect due to cell death, but a consequence of the specific regulatory capacity of Bx42.

V Literature

- Z1 Abu-Shaar and Mann, Generation of multiple antagonistic domains along the proximodistal axis during *Drosophila* leg development *Development* 125 1998 (19) 3821-3830
- Z2 Adachi-Yamada, et al., Distortion of proximodistal information causes JNK-dependent apoptosis in *Drosophila* wing *Nature* 400 1999 6740)
- Z3 Affolter et al., Nuclear interpretation of Dpp signalling in *Drosophila* *EMBO J.* 20 2001 (13) 3298-3305
- Z4 Albers et al., Identification and characterization of Prp45p and Prp46p, essential pre-mRNA splicing factors *JD. RNA* 9 2003 (1) 138-150
- Z5 Ambrozkova et al., the fission yeast ortholog of the coregulator SKIP interacts with the small subunit of U2AF *Biochem. Biophys. Res. Commun.* 284 2001 1148-1154
- Z6 Angelini D. R., Kaufman TC., Insect appendages and comparative ontogenetics *Dev. Biol.* 286 2005 (1) 57-77
- Z7 Auboeuf et al., A subset of nuclear receptor coregulators act as coupling proteins during synthesis and maturation of RNA transcripts *Mol. Cell Biol.* 25 2005 (13) 5307-5316
- Z8 Auboeuf et al., Differential recruitment of nuclear receptor coactivators may determine alternative RNA splice site choice in target genes *Proc. Natl. Acad. Sci. USA* 101 2004a (8) 2270-2274
- Z9 Auboeuf et al., CoAA, a nuclear receptor coactivator protein at the interface of transcriptional coactivation and RNA splicing *MOL. Cell Biol.* 24 2004b (1) 442-453
- Z10 Auboeuf et al., Coordinate regulation of transcription and splicing by steroid receptor coregulators *Science* 5592 2002
- Z12 Baena-Lopez et al., Genetic requirement of vestigial in the regulation of *Drosophila* wing development *Development* 130 2003 (1) 197-208
- Z13 Barolo et al., Default repression and Notch signalling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of hairless. *Genes Dev.* 16 2002 (15) 1964-1976

- Z14 Barrio et al., Regulation of spalt expression in the *Drosophila* wing blade in response to the Decapentaplegic signalling pathway. *Proc. Natl. Acad. Sci. USA* 101 2004 (16)
- Z15 Barry et al., Interactions of SKIP/NCoA-62, TFIIB and retinoid X receptor with vitamin D receptor helix H10 residues. *J. Biol. Chem.* 278 2003 (10) 8224-8228
- Z16 Baudino et al., Isolation and characterization of a novel coactivator protein, NcoA-62, involved in vitamin D-mediated transcription. *J. Biol. Chem.* 273 1998 16434-16441
- Z17 Bier, Drawing lines in the *Drosophila* wing: initiation of wing vein development. *Curr. Opin. Genet. Dev.* 10 2000 (4) 393-398
- Z18 Brand and Perrimon, Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118 1993 (2) 401-415
- Z19 Brennan et al., Wingless modulates the effects of dominant negative notch molecules in the developing wing of *Drosophila*. *Dev. Biol.* 216 1999 (1) 210-229
- Z20 Bres et al., A human splicing factor, SKIP, associates with P-TEFb and enhances transcription elongation by HIV-1 Tat. *Genes Dev.* 19 2005 (10) 1211-1226
- Z21 Campbell et al., The role of the homeobox genes *aristaless* and *Distal-less* in patterning the legs and wings of *Drosophila*. *Development* 125 1998 (22) 4483-4493
- Z22 Campbell and Tomlinson, Transducing the Dpp morphogen gradient in the wing of *Drosophila*: regulation of Dpp targets by *brinker*. *Cell* 96 1999 553-562
- Z23 Campbell et al., Axis specification in the developing *Drosophila* appendage: the role of *wingless*, *decapentaplegic*, and the homeobox gene *aristaless*. *Cell* 74 1993 (6) 1113-1123
- Z24 Capdevila et al., Targeted expression of the signalling molecule *decapentaplegic* induces pattern duplications and growth alterations in *Drosophila* wings. *EMBO* 13 1994 (19) 4459-4468
- Z25 Chu et al., Limb type-specific regulation of *bric a brac* contributes to morphological diversity. *Development* 129 2002 (3) 695-704
- Z26 Cohen et al., Allocation of the thoracic imaginal primordia in the *Drosophila* embryo. *Development* 117 1993 597-608

- Z27 Cohen et al., A domain necessary for the transforming activity of SnoN is required for specific DNA binding, transcriptional repression and interaction with TAF(II)110
Oncogene 17 1998 (19) 2505-2513
- Z28 Cohen et al., Distal-less encodes a homeodomain protein required for limb development in *Drosophila* *Nature* 338 1989 (6214)
- Z29 Colmenares et al., the ski oncogene induces muscle differentiation in quail embryo cells *cell* 59 1989 (2) 293-303
- Z30 Gomez-Skarmeta et al., Araucan and caupolican provide a link between compartment subdivisions and patterning of sensory organs and veins in the *Drosophila* wing. *Genes Dev.* 10 1996 (20) 2935-2945
- Z31 Crozatier M., Glise B., Vincent A. Patterns in evolution: veins of the *Drosophila* wing. *Trends Genet.* 20 2004 (10) 498-505
- Z32 Dahl et al., The Ski oncoprotein interacts with Skip, the human homolog of *Drosophila* Bx42. *Oncogene* 16 1998 1579-1586
- Z33 Dahmann et al., Opposing transcriptional outputs of Hedgehog signalling and engrailed control compartmental cell sorting at the *Drosophila* A/P boundary. *Cell* 100 2000 (4) 411-1-422
- Z34 De Celis, Pattern formation in the *Drosophila* wing: the development of the veins. *Bioessays* 25 2003b (5) 443-451
- Z35 De Celis et al., Funktion of the spalt/spalt-related gene complex in positioning the veins in the *Drosophila* wing. *Mech. Dev.* 91 2000 (1-2) 31-41
- Z36 De Celis et al., Notch signalling regulates veinlet expression and establishes boundaries between veins and interveins in the *Drosophila* wing. *development* 124 1997 (10) 1919-1928
- Z37 De Celis et al., Developmental basis for vein pattern variations in insect wings *Int. J. Dev. Biol.* 47 2003a (7-8) 653-663
- Z38 De Celis et al., Modification of the notch function by *Abruptex* mutations in *Drosophila melanogaster* *Genetics* 136 1994 (1) 183-194
- Z39 De Celis et al., Notch signalling mediates segmentation of the *Drosophila* Leg. *Development* 125 1998 (23) 4617-4626

- Z40 De Celis et al., A gene complex acting downstream of dpp in *Drosophila* wing morphogenesis. *Nature* 30;381 1996 (6581)
- Z41 Del Alamo Rodriguez et al., the role of the T-box gene *optomotor-blind* in patterning the *Drosophila* wing *Dev. Biol.* 268 2004 (2) 481-492
- Z42 Diaz-Benjumea et al., Cell interaction between compartments establishes the proximal-distal axis of *Drosophila* legs. *Nature* 372 1994 (6502) 175-179
- Z43 Diaz-Benjumea et al. Interaction between dorsal and ventral cells in the imaginal disc directs wing development in *Drosophila* *Cell* 75 1993 (4) 741-752
- Z44 Dong et al., Coexpression of the homeobox genes *Distal-less* and *homothorax* determines *Drosophila* antennal identity *Development* 127 2000 (2) 209-216
- Z45 Duncan et al., Control of distal antennal identity and tarsal development in *Drosophila* by *spinless-aristapedia*, a homolog of the mammalian dioxin receptor. *Genes Dev.* 12 1998 (9) 1290-1303
- Z46 Eggert et al., Identification of the *Drosophila* interband-specific protein Z4 as a DNA-binding zinc-finger protein determining chromosomal structure. *J. Cell Sci* 117 2004 (18) 4253-4264
- Z47 Felsenfeld et al., Positional signalling by *hedgehog* in *Drosophila* imaginal disc development *Development* 121 1995 (1) 1-10
- Z48 Feng et al., Specificity and versatility in *tgf-beta* signalling through *Smads*. *Annu. Rev. Cell. Dev. Biol.* 21 2005 659-693
- Z49 Fire et al., Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans* *Nature* 391 1998 (6669) 806-811
- Z50 Folk et al., The homolog of chromatin binding protein *Bx42* identified in *Dictyostelium*. *Gene* 181 1996 229-231
- Z51 Frasch and Saumweber, Two proteins from *Drosophila* nuclei are bound to chromatin and are detected in a series of puffs on polytene chromosomes. *Chromosoma* 97 1989 (4) 272-281
- Z52 Galindo et al., Leg patterning driven by proximal-distal interactions and *EGFR* signalling. *Science* 297 2002 (5579) 256-259
- Z53 Garcia-Bellido et al., Genetic control of wing disc development in *Drosophila* *Symp.* 29 1975 161.182

- Z54 Garcia-Bellido et al., Developmental compartmentalisation of the wing disk of *Drosophila* Nat. New Biol. 245 1973 251-253
- Z55 Garcia-Bellido A., et al., Developmental and compartmentalization in the dorsal mesothoracic disc of *Drosophila*. Dev. Biol. 48 1976 132-147
- Z56 Gelmedin et al., *Echinococcus multicularis*: cloning and characterization of a member of the SNW/SKIP family of transcriptional coregulators. Exp. parasitol. 111 2005 (2) 115-120
- Z57 Godt et al., Pattern formation in the limbs of *Drosophila*: bric a brac is expressed in both gradient and wave-like pattern and is required for specification and proper segmentation of the tarsus. Development 119 1993 (3) 799-812
- Z58 Gomez-Skarmeta et al., Araucan and caupolican provide a link between compartment subdivisions and patterning of sensory organs and veins in the *Drosophila* wing. Genes Dev. 10 1996 (20)
- Z59 Gorfinkiel et al., The homeobox gene Distal-less induces ventral appendage development in *Drosophila* Genes Dev. 11 1997 (17) 2259-2271
- Z60 Goto and Hayashi, Specification of the embryonic limb primordium by graded activity of Decapentaplegic. Dev. 124 1997 (1) 125-132
- Z61 Grimm et al., Control of the gene optomotor-blind in *Drosophila* wing development by decapentaplegic and wingless Science 271 1996 (5255) 1601-1604
- Z62 Guss et al., Control of a genetic regulatory network by a selector gene. Science 292 2001 (5519) 1164-1167
- Z63 Hanahan Studies on transformation of *Escherichia coli* with plasmids J. Mol. Biol. 166 1983 (4) 557-580
- Z64 Harris et al., Molecular analysis of *Saccharomyces cerevisiae* chromosome I. on the number of genes and the identification of essential genes using temperature-sensitive-lethal mutations. J. Mol. Biol. 225 1992 (1) 53-65
- Z65 Heldin et al., TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 390 1997 (6659) 465-471
- Z66 Hayashi et al., The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signalling. Cell 89 1997 1165-1173

- Z67 Itoh et al., Transforming growth factor- β 1 induces nuclear export of inhibitory Smad7. *J. Biol. Chem.* 273 1998 29195-21201
- Z68 Jazwinska et al., The *Drosophila* gene *brinker* reveals a novel mechanism of Dpp target gene regulation *Cell* 96 1999a (4) 563-573
- Z69 Jazwinska et al., The role of *brinker* in mediating the graded response to Dpp in early *Drosophila* embryos. *Development* 126 1999b (15) 3323-3334
- Z70 Jenster et al., Steroid receptor induction of gene transcription: a two-step model. *Proc. Natl. Acad. Sci. USA* 94 1997 (15) 7879-7884
- Z71 Kokura et al., The Ski protein family is required for MeCP2-mediated transcriptional repression *J. Biol. Chem.* 276 2001 (36) 34115-34121
- Z72 Kim et al., *Drosophila* Mad binds to DNA and directly mediates activation of *Vestigial* by *Decapentaplegic* *Nature* 388 1997 (6639) 304-308
- Z73 Kim et al., The product of an oculopharyngeal muscular dystrophy gene, poly(A)-binding protein 2, interacts with SKIP and stimulates muscle-specific gene expression. *Hum. Mol. Genet.* 10 2001 (11) 1129-1139
- Z74 Kojima et al., the mechanism of *Drosophila* leg development along the proximodistal axis *Dev. Growth Differ.* 46 2004 (2) 115-129
- Z75 Kostrouchova et al., SKIP is an indispensable factor for *Caenorhabditis elegans* development. *Proc. Natl. Acad. Sci. USA* 99 2002 (14) 9254-9259
- Z76 Kumar and Moses, EGF receptor and Notch signalling act upstream of *Eyeless/Pax6* to control eye specification *Cell* 104 2001 (5) 687-697
- Z77 Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4 *Nature* 227 1970 (5259) 680-685
- Z78 Lawrence and Struhl, Morphogens, compartments, and pattern: lessons from *Drosophila*? *Cell* 85 1996 951-961
- Z79 Lecuit et al., Two distinct mechanisms for long-range patterning by *Decapentaplegic* in the *Drosophila* wing *Nature* 381 1996 387-393
- Z80 Lecuit and Cohen, Proximal-distal axis formation in the *Drosophila* leg *Nature* 388 1997 (6638) 139-145

- Z81 Leong et al., Ski-interacting protein interacts with Smad proteins to augment transforming growth factor-beta-dependent transcription. *J. Biol. Chem.* 276 2001 (21) 18243-18248
- Z82 Liberati et al., An essential role for Mad homology domain 1 in the association of Smad3 with histone deacetylase activity. *J. Biol. Chem.* 276 2001 (25) 22595-22603
- Z83 Lunde et al., The Knirps and knirps-related genes organize development of the second wing vein in *Drosophila*. *Development* 125 1998 (21) 4145-4154
- Z84 Luo, Negative regulation of BMP signalling by the ski oncoprotein. *J. Bone Joint Surg. Am.* 2003 39-43
- Z85 Luo et al., The Ski oncoprotein interacts with the Smad Proteins to repress TGF-beta signalling. *Genes Dev.* 13 1999 (17) 2196-2206
- Z86 Makarov et al., Small nuclear ribonucleoprotein remodeling during catalytic activation of the spliceosome. *Science* 298 2002 (5601) 2205-2208
- Z87 Marquez et al., Transgenic analysis of the Smad family of TGF-beta signal transducers in *Drosophila melanogaster* suggests new roles and new roles and new interactions between family members. *Genetics* 157 2001 (4) 1639-1648
- Z88 Martinkova et al., Funktional mapping of *Saccharomyces cerevisiae* Prp45 identifies the SNW domain as essential for viability. *J. Biochem.* 132 2002 (4) 557-563
- Z89 Marty et al., Schnurri mediates Dpp-dependent repression. *Nature Cell Biol.* 2 2000 745-749
- Z90 Massagué, TGF-beta signal transduction. *Annu. Rev. Biochem.* 67 1998 753-791
- Z91 Massagué et al., Smad transcription factors. *Genes Dev.* 19 2005 (23) 2783-2810
- Z92 Maves et al., A molecular basis for transdetermination in *Drosophila* imaginal discs: interactions between wingless and decapentaplegic signalling. *Development* 125 1998 (1) 115-124
- Z93 Milan and Cohen, Subdividing cell populations in the developing limbs of *Drosophila*: do wing veins and leg segments define units of growth control? *Dev. Biol.* 217 2000 (1) 1-9
- Z94 Minami et al., brinker is a target of *Drosophila* that negatively regulates Dpp-dependent genes. *Nature* 398 1999 242-246

- Z95 Mintz et al., Purification and biochemical characterization of interchromatin granule clusters EMBO 18 1999 (15) 4308-4320
- Z96 Mohler et al., Activation of knot (kn) specifies the 3-4 intervein region in the Drosophila wing Development 127 2000 (1) 55-63
- Z97 Molnar et al., A gain-of-function screen identifying genes required for vein formation in the Drosophila melanogaster wing Genetics 174 2006 (3) 1635-1659
- Z98 Monsalve et al., Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1. Mol. Cell 6 2000 (2) 307-316
- Z99 Montagne et al., The Drosophila serum response factor gene is required for the formation of intervein tissue of the wing and is allelic to blistred Development 122 1996 (9) 2589-2597
- Z100 Morata et al., Control of compartment development by the engrailed gene in Drosophila Nature 255 1975 (5510) 614-617
- Z101 Moustakas et al., Smad regulation in TGF-beta signal transduction J. Cell Sci. 114 2001 (24) 4359-4369
- Z102 Mullor et al., Hedgehog activity, independent of decapentaplegic, participates in wing disc patterning. Development 124 1997 (6) 1227-1237
- Z103 Nagai et al. SKIP modifies gene expression by affecting both transcription and splicing. Biochem. Biophys. Res. Commun 316 2004 (2) 512-517
- Z104 Nakao et al., Identification of Smad7, a TGF-beta-inducible antagonist of TGF-beta signalling Nature 389 1997 (6651) 631-635
- Z105 Negeri D., et al. Inducible RNA interference uncovers the Drosophila protein Bx42 as an essential nuclear cofactor involved in Notch signal transduction Mech. Dev. 117 2002 (1-2) 151-162
- Z106 Nellen et al., Direct and long-range action of a Dpp morphogen gradient Cell 85 1996 (3) 357-368
- Z107 Nestoras et al., Role of knot (kn) in wing patterning in Drosophila Genetics 147 1997 (3) 1203-1212
- Z108 Nevins, E2F: a link between the Rb tumor suppressor protein and viral oncoprotein Science 258 1992 (5081) 424-429

- Z109 Nomura et al., Ski is a component of the histone deacetylase complex required for transcriptional repression by Mad and thyroid hormone receptor. *Genes Dev.* 13 1999 (4) 412-423
- Z110 Panganiban, Distal-less function during drosophila appendage and sense organ development. *Dev. Dyn.* 218 2000 (4) 554-562
- Z111 Panganiban et al. Development function of the Distal-less/Dlx homeobox genes. *Development* 129 2002 (19) 4371-4386
- Z112 Penton et al., Identification of two bone morphogenetic protein type I receptors in Drosophila and evidence that Brk25D is a decapentaplegic receptor *Cell* 78 1994 (2) 239-250
- Z113 Prathapam et al., Skip interacts with the retinoblastoma tumor suppressor and inhibits its transcriptional repression activity. *Nucleic Acids Res.* 30 2002 (23) 5261-5268
- Z114 Raftery et al., TGF-beta family signal transduction in Drosophila development: from Mad to Smads. *Dev. Biol.* 210 1999 (2) 251-268
- Z115 Rauskolb, The establishment of segmentation in the Drosophila leg *Development* 128 2001 (22) 4511-4521
- Z116 Rauskolb and Irvin, Notch-mediated segmentation and growth control of the Drosophila leg. *Dev. Biol.* 210 1999 (2) 339-350
- Z117 Sambrook et al., Cold spring harbor laboratory press *Molecular cloning: a laboratory manual* 1989 2 NY
- Z118 Saumweber et al., Two puff-specific proteins bind within the 2,5 kb upstream region of the Drosophila melanogaster Sgs-4 gene *Chromosoma* 99 1990 (1) 52-60
- Z119 Sekelsky et al., Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. *Genetics* 139 1995 (3) 1347-1358
- Z120 Seth S. Blair, Lineage compartments in Drosophila *Current Biology* 13 2003 (14) 548-551
- Z121 Simcox et al., Establishment of imaginal discs and histoblast nests in Drosophila *Mech. Dev.* 35 1991 (1)

- Z122 Singer et al., Signalling through both type I Dpp receptors is required for anterior-posterior patterning of the entire *Drosophila* wing. *Development* 124 1997 (1) 79-89
- Z123 Sivasankaran et al., Direct transcriptional control of the Dpp target omb by the DNA binding protein Brinker *EMBO* 19 2000 (22) 6162-6172
- 124 Stavnezer et al., Generation of transforming viruses in cultures of chicken fibroblasts infected with an avian leukosis virus *J. Virol.* 39 1981 920-934
- Z125 Stavnezer et al., Transforming Sloan-Kettering viruses generated from the cloned v-ski oncogene by in vitro and in vivo recombinations. *J. Virol.* 57 1986 (3)
- Z126 Sturtevant et al., the *Drosophila* rhomboid gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signalling pathway *Genes Dev.* 7 1993 (6) 961-973
- Z127 Tabata et al., Hedgehog is a signalling protein with a key role in patterning *Drosophila* imaginal discs. *Cell* 76 1994 (1) 89-102
- Z128 Inoue et al., Interplay of signal mediators of decapentaplegic (Dpp): molecular characterization of mothers against dpp, Medea, and daughters against dpp. *Mol. Biol. Cell.* 9 1998 (8) 2145-2156
- Z129 Tokitou et al., Viral ski inhibits retinoblastoma protein (Rb)-mediated transcriptional repression in a dominant negative fashion. *J. Biol. Chem.* 274 1999 (8) 4485-4488
- Z130 Tsukazaki et al., SARA, a FYVE domain protein that recruits Smad2 to the TGF-beta receptor. *Cell* 95 1998 (6) 779-791
- Z131 Tulasiram P., et al., Skip interacts with the retinoblastoma tumor suppressor and inhibits its transcriptional repression activity *Nucleic Acids Research* 23 2002 5261-5266
- Z132 Van den Heuvel M., et al., Cell patterning in the *Drosophila* segment: engrailed and wingless antigen distributions in segment polarity mutant embryos. *Dev. Suppl.* 1993 105-114
- Z133 Vincent et al., The State of engrailed expression is not clonally transmitted during early *Drosophila* development. *Cell* 68 1992 (5) 923-931
- Z134 Vincent, Compartment boundaries: Where, why and how? *Int. J. Dev. Biol.* 42 1998 (3) 311-315

- Z135 Wang et al., Ski represses bone morphogenetic protein signalling in *Xenopus* and mammalian cells. *Proc. Natl. Acad. Sci.* 97 2000 (26) 14394-14399
- Z136 Weinberg, The retinoblastoma protein and cell cycle control. *Cell* 81 1995 (3) 323-330
- Z137 Wieland et al., The *Drosophila* nuclear protein Bx42, which is found in many puffs on polytene chromosomes, is highly charged *Chromosoma* 101 1992 (8) 517-525
- Z138 Williams, Distalless expression in crustaceans and the patterning of branched limbs *Dev. Genes Evol.* 207 1998 (7) 427-434
- Z139 Wisotzkey et al., Medea is a *Drosophila* Smad4 homolog that is differentially required to potentiate Dpp responses *Development* 125 1998 (8) 1433-1445
- Z140 Wrana, Crossing Smads *Sci. STKE* 2000
- Z141 Wu et al., Structural basis of Smad2 recognition by the Smad anchor for receptor activation *Science* 287 2000 (5450) 92-97
- Z142 Wu J., et al., Proximal distal axis formation in the *Drosophila* leg: distinct functions of teashirt and homothorax in the proximal leg *Mech. Dev.* 94 2000 (1-2) 47-56
- Z143 Wu and Cohen, Proximodistal axis formation in the *Drosophila* leg: subdivision into proximal and distal domains by Homeothorax and Distal-less *Development* 126 1999 (1) 109-117
- Z144 Xu et al., Ski acts as a co-repressor with Smad2 and Smad3 to regulate the response to type beta transforming growth factor *Proc. Natl. Acad. Sci.* 97 2000 (11) 5924-5929
- Z145 Yeon-jeong K., et al., The product of an oculopharyngeal muscular dystrophy gene, poly(A)-binding protein2, interacts with SKIP and stimulates muscle-specific gene expression. *Human Molecular Genetics* 10 2001 (11) 1129-1139
- Z146 Zawel et al., Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol. Cell* 1 1998 (4) 611-617
- Z147 Zhang et al., Nuclear coactivator-62 kDa/Ski-interacting protein is a nuclear matrix-associated coactivator that may couple vitamin D receptor-mediated transcription and RNA splicing *J. Biol. Chem.* 278 2003 (37) 35325-35336

- Z148 Zhang et al., Ternary complexes and cooperative interplay between NCoA-62/Ski-interacting protein and steroid receptor coactivators in vitamin D receptor-mediated transcription *J. Biol. Chem.* 276 2001 (44) 40614-40620
- Z149 Zheng et al., Identification of a core functional and structural domain of the v-Ski oncoprotein responsible for both transformation and myogenesis *Oncogene* 15 1997 (4) 459-471
- Z150 Zhou et al., Characterization of human FAST-1, a TGF-beta and activin signal transducer. *Mol. Cell* 2 1998 (1) 121-127
- Z151 Zhou et al., Skip, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC to facilitate NotchIC function *Mol. Cell. Biol.* 20 2000 (7) 2400-2410

VI Abbreviation

A	<u>A</u> nterior
AA	<u>A</u> mino <u>A</u> cid
LiAc	<u>L</u> ithium <u>a</u> ccetat
ATP	<u>A</u> denosin <u>t</u> riphosphat
Bp	<u>B</u> asen <u>p</u> aar
OD	<u>O</u> ptische <u>D</u> ichte
DEPC	<u>D</u> iethylpyro <u>c</u> arbonat
ONPG	<u>O</u> - <u>N</u> itrophenyl- β - <u>D</u> Galactopyranosid
DNA	<u>D</u> esoxyribo <u>n</u> ukleins <u>ä</u> ure
PBS	<u>P</u> hosphate- <u>B</u> uffered <u>S</u> aline
dNTP	<u>D</u> esoxynukleotid <u>t</u> riphosphat
PCR	<u>P</u> olymerase <u>C</u> hain <u>R</u> eaction
DTT	<u>D</u> ithiothreitol
PEG	<u>P</u> olyethylenglykol
EDTA	<u>E</u> thylendiamin <u>t</u> etra <u>a</u> ccetat
PMSF	<u>P</u> henylmethy <u>s</u> ulfony <u>l</u> fluorid
ELISA	<u>E</u> nzyme <u>L</u> inked <u>I</u> mmuno <u>S</u> orbent <u>A</u> ssay
FCS	<u>F</u> etal <u>C</u> alf <u>S</u> erum
RT	<u>R</u> everse <u>T</u> ranskription
LB	<u>L</u> uria <u>B</u> ertani
P	<u>P</u> osterior
En	<u>E</u> ngrailed
Hh	<u>H</u> edghog
Dpp	<u>D</u> ecapentaplegic
BMP4	<u>B</u> one <u>M</u> orphogenic <u>P</u> roteins 4
TGF- β	<u>T</u> ransforming <u>G</u> rowth <u>F</u> actor- β
RI	<u>T</u> ype I receptor
RII	<u>T</u> ype I receptorI
GS	<u>G</u> lycine/ <u>S</u> erine
R-Smad	<u>R</u> eceptor-regulated <u>S</u> mad
Co-Smad	<u>C</u> ommon-mediator <u>S</u> mad

I-Smad	<u>I</u> nhibitory <u>S</u> mad
TF	<u>T</u> ranscription <u>F</u> actors
SBE	<u>S</u> mad <u>B</u> inding <u>E</u> lement
MH1	<u>M</u> ad <u>H</u> omology 1
MH2	<u>M</u> ad <u>H</u> omology 2
Mad	<u>M</u> other <u>A</u> gain <u>D</u> pp
Sal	<u>S</u> palt
Omb	<u>O</u> ptomotor <u>B</u> lind
Vg	<u>V</u> estigial
Brk	<u>B</u> rinker
RNA	<u>R</u> ibonucleinsäure
RNAi	<u>R</u> NA <u>I</u> nterferenz
kDa	<u>K</u> ilodalton
Skip	<u>S</u> ki <u>I</u> nteracting <u>P</u> rotein
NLS	<u>N</u> uclear <u>L</u> ocalisation <u>S</u> ignal
Fun20	<u>F</u> unction <u>U</u> nknown
CeSkip	<u>C</u> aenorhabditis <u>e</u> legans Skip
VDR	<u>V</u> itamin <u>D</u> <u>R</u> eceptor
NcoA62	<u>N</u> uclear <u>c</u> o <u>A</u> ctivator 62
dsRNA	<u>D</u> ouble <u>S</u> trand RNA
<i>C. elegans</i>	<u>C</u> aenorhabditis <u>e</u> legans
Sgs4	<u>S</u> alivary <u>G</u> land <u>S</u> ecretion 4
EcR	<u>E</u> cdysone <u>R</u> eceptor
RAR	<u>R</u> etinoid <u>A</u> cid <u>R</u> eceptor
RXR	<u>R</u> etinoid <u>X</u> <u>R</u> eceptor
1, 25(OH)2D3	1,25-Dihydroxyvitamin D3
mRNA	<u>M</u> essenger <u>R</u> NA
ChiP	<u>C</u> hromatin <u>I</u> mmunoprecipitation
pRb	<u>P</u> rotein <u>R</u> etinoblastoma
PABP2	<u>P</u> oly <u>A</u> <u>B</u> inding <u>P</u> rotein 2
ECN	<u>E</u> xtra <u>C</u> ellular <u>N</u> -terminal
N-IC	<u>N</u> otch <u>I</u> ntracellular <u>C</u> -terminal
Su(H)	<u>S</u> uppressor of <u>H</u> airless
CIR	<u>C</u> BF1 <u>I</u> nteracting <u>R</u> epressor

H	<u>H</u> airless
dCtBP	<u>D</u> rosophila <u>C</u> -terminal <u>B</u> inding <u>P</u> rotein
Gro	<u>G</u> roucho
HAT	<u>H</u> istone <u>A</u> cetyl <u>T</u> ransferase
E(spl)m8	<u>E</u> nhancer of <u>spl</u> m8
Dll	<u>D</u> istal <u>l</u> ess
E. coli	<u>E</u> scherichia <u>c</u> oli
S. cerevisiae	<u>S</u> accharomyces cerevisiae
GST	<u>G</u> lutathion <u>S</u> - <u>T</u> ransferase
MH	Myc-His
AD	<u>A</u> ctivation <u>D</u> omain
DBD	<u>D</u> NA <u>B</u> inding <u>D</u> omain
Trp	Tryptophan
Leu	Leucin
dNTP	2'- <u>d</u> eoxy ribonucleosid-5'- <u>t</u> riphosphat
CIP	<u>C</u> alf <u>I</u> ntestine <u>P</u> hosphatase
RT	<u>R</u> oom <u>T</u> emperature
ATP	<u>A</u> dinosinetriphosphat
Rpm	<u>R</u> otation pre <u>m</u> inute
PAGE	<u>P</u> olyacrylamidgelelectoohorese
PEG	<u>P</u> olyethylene <u>G</u> lycol
Lac	<u>L</u> actose
NBT	<u>N</u> itrobluetetrazoliumchlorid
X-gal	5-Brom-4-chlor-3-indolyl- β -D-galactopyranosid
SDS	<u>S</u> odium <u>D</u> odocyl <u>S</u> ulfate
BICP	5- <u>b</u> romo-4- <u>c</u> hloro-3- <u>i</u> ndoly- <u>p</u> hosphat
T(1-5)	Tarsal segments 1-5
Ti	<u>T</u> ibia
Fe	<u>F</u> emur
FL	<u>F</u> ull <u>L</u> ength
L(I-V)	<u>L</u> ongitudinal vein
ACV	<u>A</u> nterior <u>C</u> ross <u>V</u> ein
PCV	<u>P</u> osterior <u>C</u> ross <u>V</u> ein
Dac	<u>D</u> achshund

Hth	<u>H</u> omothorax
Ss	<u>S</u> pineless-aristapidia
Bab	<u>B</u> ric- <u>a</u> - <u>b</u> rac
Ser	<u>S</u> errate
Iro-c	<u>I</u> roquois <u>c</u> omplex
Kn	<u>K</u> nirps
Bs	<u>B</u> listered
EGFR	<u>E</u> pidermal <u>G</u> rowth <u>F</u> actor <u>R</u> eceptor

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Statement

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und ohne Verwendung anderer Hilfsmittel und Hilfern als der in der Arbeit angegebenen verfaßt habe.

Berlin, den 15/12/2006

.....
El Hachoumi Mounia

Scientific congresses

- 1) Annual Meeting of the German Genetics Society, Martin Luther University Halle, October 4-6, 2001
- 2) 9th Regional Drosophila Meeting, Ruhr University Bochum, Molecular cell Biochemistry, June 21-22, 2002
- 3) 10th Regional Drosophila Meeting, University of Regensburg, June 4-5, 2004
- 4) 5. GFE School, for Gesellschaft für Entwicklungsbiologie, Schloß Reisenburg bei Ulm, September 23-25, 2004