Characterization of Schnurri, an integral component of the Dpp-signaling pathway in *Drosophila melanogaster*

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Müller, B.*, **Hartmann B.***, Pyrowolakis G.*, Affolter M., Basler K. (2003). "Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient." Cell **113**(2): 221-33.

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Summary

Signaling by the transforming growth factor-β (TGF-β) family of ligands plays a crucial role during development and tissue homeostasis in all multicellular organisms. The signal transduction pathway is highly conserved and relatively simple. Ligand mediated receptor activation induces heteromerization and nuclear translocation of the signal mediators, the Smad proteins. Once in the nucleus, the Smad complexes bind to and regulate directly a large number of target genes. Intriguingly, only a few Smad proteins mediate the majority of the complex cellular responses elicited by ligands of the TGF-β family by either transcriptional activation or repression, depending on their associated partners. A number of Smad interacting proteins and modulators have been identified, partly explaining the diversity of transcriptional outcome. Nevertheless, the biological consequences and their role in developmental aspects of TGF-β signaling are poorly understood.

The best characterized member of the TGF-β family in *Drosophila melanogaster* is Decapentaplegic (Dpp). Dpp plays important roles during development and its cellular function has been extensively investigated in genetic experiments. One of the main Dpp-target genes during development encodes a repressor protein, Brinker. Brinker suppresses the transcription of Dpp-target genes and is itself negatively regulated by Dpp-signaling. This down-regulation of *brinker* by Dpp is essential for Dpp target genes to become activated. The molecular basis of the Dpp-mediated repression is elusive; however, genetic studies identified the gene *schnurri* (*shn*) to be required for this activity.

In this thesis, the results of a detailed structure-function analysis of the nuclear zinc finger protein Schnurri are presented. It could be demonstrated that Schnurri protein, together with the *Drosophila* Smads Mad and Medea, forms a signal-dependant DNA-protein complex on short silencer elements within the *brinker* regulatory region. The recruitment of Schnurri to the silencer elements by Mad/Medea is required for the transcriptional regulatory activity of the complex *in vivo*. Two modules within the Schnurri protein were identified that are required and sufficient for Dpp-mediated *brinker* repression *in vivo*. The two entities, a protein-DNA complex formation domain and a domain containing repressor activity, can be separated from each while retaining their function. The Schnurri protein presents the first interaction partner for Mad and Medea in *Drosophila*. Furthermore, Schnurri is the first examples that link TGF-β signaling to tissue-unspecific repression rather than activation.

I: Introduction

1. Introduction

It is still a mystery how a complex organism develops from a single cell, the fertilized egg. Many biologists have been and are mesmerized to explore the great variety of developmental mechanisms involved in the generation of multicellular organisms. A vitally important feature is that cells have to communicate with each other to build and maintain a functional life form. One of the first indications that cells really do communicate with each other, was observed already in 1924 by Spemann and Mangold (*figure1*). In a classical experiment, transplantation of a specific embryonic tissue from one embryo into another could generate a two-headed salamander (Spemann and Mangold 1924). The transplanted tissue was able to induce the fate of the neighboring cells in the host embryo, indicating that cells communicate with each other through secreted signals. In the meantime many of the signaling molecules (mainly protein ligands) and the respective signal receivers (receptors) have been identified and the core components responsible to transduce the signal to its target site have been well characterized. Cell-cell communication via these signaling pathways is involved in many processes during development and tissue homeostasis, and defects within the cascades are the cause of many developmental disorders and diseases. What comes as a surprise is that despite the bewildering number of cell types and patterns found in the animal kingdom, only a few highly conserved signaling pathways are required to generate them. The specificity of these pathways is based on the history of the cell, the intensity and duration of the signal and the cross-regulatory interactions with other signaling cascades. Therefore, it is no surprise that complex signaling networks have evolved to control the diverse processes during animal life.

One of the most thoroughly characterized signaling pathway in developing tissues is that initiated by the transforming growth factor-β (TGF-β) family. The ligands, their receptors and

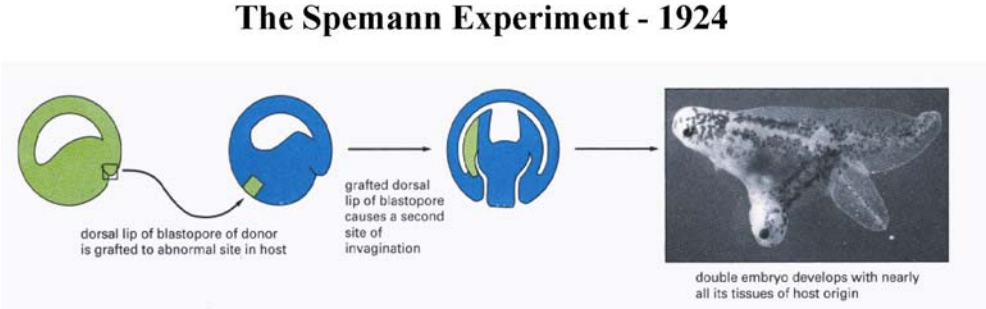


Figure1: Transplantation experiment published by Spemann and Mangold, 1924. Of all tissues in the early gastrula, only the dorsal lip of the blastopore has its fate determined. Transplantation of this tissue into another region initiated gastrulation and embryogenesis in the surrounding tissue, giving rise to two conjoined embryos.

the signal transducers have been intensively investigated in the last years and shown to be highly conserved throughout evolution. Nevertheless, the molecular mechanism regulating the variety of cellular responses through alterations in gene expression patterns is poorly understood. The fruit fly *Drosophila melanogaster* provides an excellent model system to study the mechanism of TGF-β mediated target gene regulation during development by combining *in vivo* and *in vitro* studies. Therefore *Drosophila* has been our choice model organism to study the molecular function of Schnurri, a new factor involved in TGF-β dependant target gene regulation (Müller *et al.* 2003).

2. Overview of the TGF-β signaling field

Signaling by members of the transforming growth factor-β (TGF-β) superfamily of ligands is involved in diverse cellular processes, including cell growth and proliferation, differentiation, apoptosis, morphogenesis, immunity and tissue regeneration in vertebrates and invertebrates (Massagué *et al.* 2000). Mutations in components of the TGF-β signaling pathway are responsible for a number of human diseases, including developmental disorders and numerous components of the signal transduction pathway are tumor suppressors that are functionally mutated in cancer (Massagué *et al.* 2000).

2.1 Morphogen function of TGF-β family members

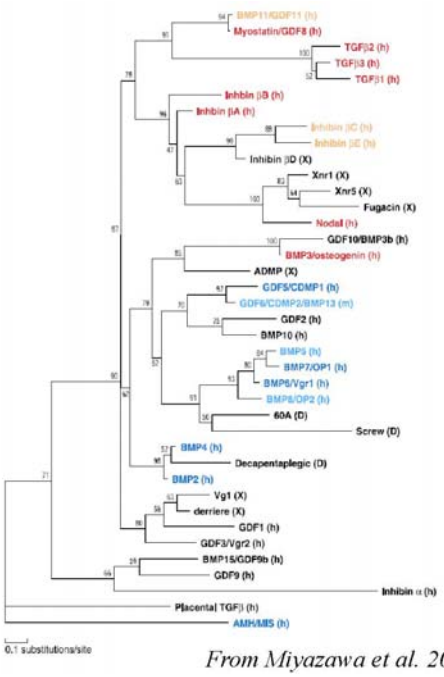
Signaling molecules are able to induce specific cellular responses depending on the developmental state of the cell. In addition to their short range action, a number of signaling molecules, including members of the TGF-β, Wnt and Hedgehog family, have the potential to influence cells at a distant from their source of production in a concentration dependant manner. Molecules with the ability to form such activity gradients are called morphogens ("form giving substances"). Several TGF-β homologues function as morphogens, to set up basic body axes in early development in a broad range of organisms. For example, BMP4 and Activin pattern the mesodermal and ectodermal germlayers in Xenopus (Gurdon *et al.* 1994; Dosch *et al.* 1997). However, the best characterized example of a morphogen is the *Drosophila* TGF-β, Decapentaplegic (Dpp). The Dpp gradient has recently been visualized (Entchev *et al.* 2000; Teleman and Cohen 2000) and its function has been extensively explored in genetic experiments during *Drosophila* development (Tabata 2001). An intriguing question in the morphogen field is how cells respond to different concentrations of ligand. Thus there is also a special interest in the field of TGF-β signal transduction regarding its function as a morphogen.

2.2 The TGF-β signaling pathway

Parallel work in vertebrates, worms and flies has revealed a conserved signaling pathway, which at first glance appears to be surprisingly simple. Considering the bewildering number of target genes it has to regulate, it is no surprise that the core pathway is embedded in complex regulatory networks.

2.2.1 Members of the TGF-β superfamily

The first member of the transforming growth factor-β (TGF-β) superfamily of secreted polypeptide factors, TGF-β1, was discovered approximately 20 years ago. Since then, the family has grown considerably and now comprises over 30 vertebrate members and a dozen or so structurally and functionally related proteins in invertebrates such as worms and flies (*figure2*, Miyazawa *et al.* 2002; Shi and Massagué 2003). They can be grouped into two subfamilies, the TGF-β/Activin/Nodal and the BMP/GDF/MIS (BMP, Bone morphogenic; GDF; growth and differentiation factor proteins; MIS, Müllerian inhibiting substance) subfamily, based on their sequence and the specific signaling pathway that they activate (*figure3B*, Shi and Massagué 2003). Several TGF-β molecules have been identified in *Drosophila* indicating that at least one member of each family is present. Only three of them have been characterized in more detail, all belonging to the BMP family of ligands. Decapentaplegic (Dpp) is more closely related to vertebrate BMP2/4 while Glass bottom boat 60 (Gbb) and Screw are similar to BMP8 (Affolter *et al.* 2001).



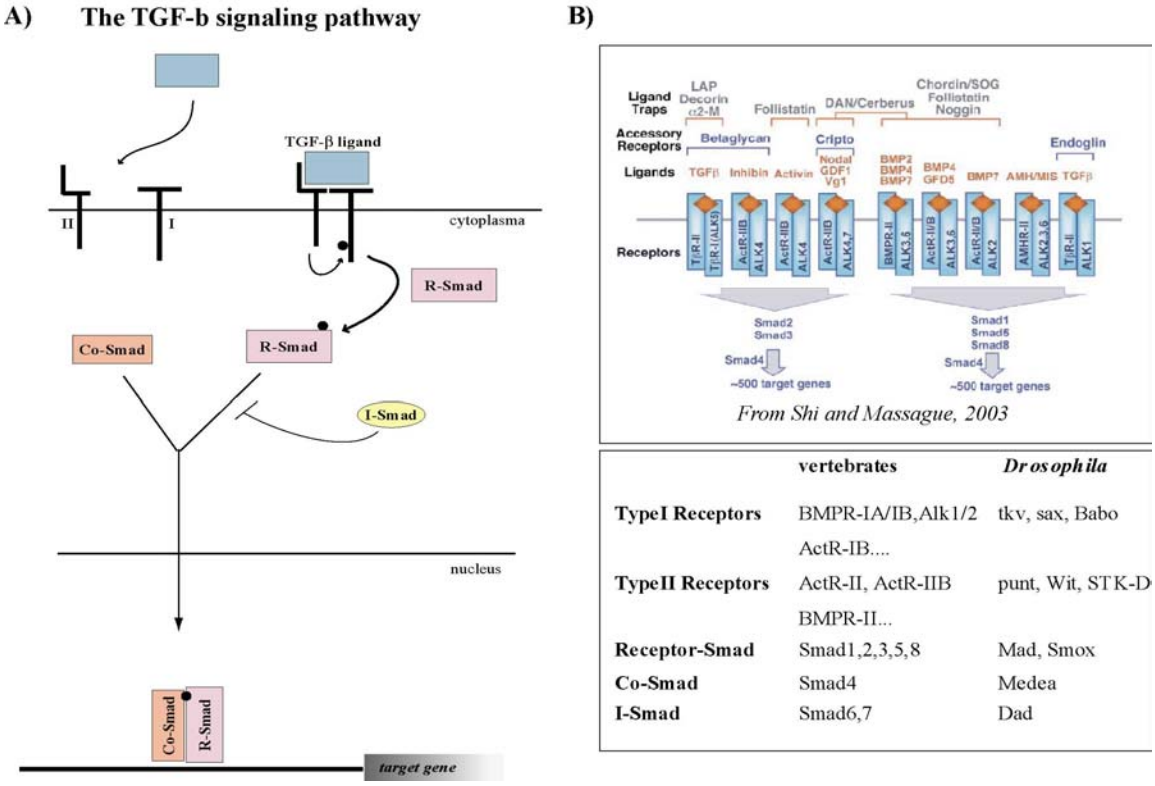
From Miyazawa et al. 2002

Figure2: Phylogenetic tree of the TGF-β superfamily in *human* (h), *mouse* (m), *Xenopus* (X) and *Drosophila* (D). Ligands that activate Activin/TGF-β-Smads or BMP-Smads are shown in red or dark blue, respectively. Ligands that may activate Activin/TGF-β-Smads or BMPSmads, but whose receptors and downstream signaling pathways have not been fully determined, are shown in orange and light blue, respectively. Activins are dimers of inhibin-β chain. OP, osteogenic protein. (*from Miyazawa et al 2002*)

2.2.2 Signal transduction: from the cell surface to the nucleus

The TGF- ligand binds to specific pairs of receptor with serine-threonine kinase activity, known as type I and type II receptors (*figure3A*). Ligand binding to the type II receptor induces the type I and type II receptors to associate. This leads to a unidirectional phosphorylation event in which the type II receptor phosphorylates the type I receptor in its GS-region, thereby activating its kinase domain. The signal is propagated by phosphorylation of the receptor regulated Smad proteins (R-Smads). Smads present a small family of intracellular signal mediators. The phosphorylated R-Smad (pR-Smad) assembles into heteromeric complexes with the common Smad (Co-Smad) in the cytoplasm and subsequently the complex translocates into the nucleus where it participates directly in the modulation of gene expression (Massagué and Wotton 2000; Shi and Massagué 2003).

The ligands can be grouped depending on the R-Smads that they activate (*figure3B*). TGF-



	vertebrates	*Drosophila*
TypeI Receptors	BMPR-IA/IB,Alk1/2 ActR-IB....	tkv, sax, Babo
TypeII Receptors	ActR-II, ActR-IIB BMPR-II...	punt, Wit, STK-D
Receptor-Smad	Smad1,2,3,5,8	Mad, Smox
Co-Smad	Smad4	Medea
I-Smad	Smad6,7	Dad

Figure3: A) Schematic representation of the basic TGF-β signaling pathway. Components of this pathway are highly conserved. Ligand binding leads to the phosphorylation of the type I receptor, which in turn phosphorylates a receptor-Smad (R-Smad). In the phosphorylated state, the R-Smad recruits a common Smad (Co-Smad) and the pR-Smad/Co-Smad complex translocates to the nucleus. Smads posses DNA binding abilities and regulate target genes. **B)** Schematic relationship of the TGF-β ligands, Ligand Binding Traps (LAP), accessory receptors, and the type I and II receptors in vertebrates and the Smad pathway that they activate (*from Shi and Massague 2003*). *Drosophila* homologues of the signaling components which have been characterized are summarized in the table below. Tkv, Thick veins; Sax, Saxophone; Babo, Baboon; Wit, Wishful thinking; Mad, Mother against dpp; Smox, Smad on X; Dad, Daughter against dpp (*for review see Affolter M. et al. 2001*)

β/Activin/Nodal subfamily and their receptors phosphorylate specifically the R-Smads2, 3 whereas the BMP/GDF/MIS ligands trigger Smad1, 5, 8 specific responses (Shi and Massagué 2003).

The first Smad family member was identified in a genetic screen in *Drosophila* and was called "Mother against Dpp" (Mad) (Raftery *et al.* 1995; Sekelsky *et al.* 1995). Instantly, orthologues have been discovered in worms (the *sma* genes) and vertebrates, which were named "Smad" (Sma and Mad) (Derynck and Zhang 1996). The family of Smad proteins can be divided into three distinct classes: receptor regulated Smads (R-Smad; Smad1, 2, 3, 5, 8), the common Smad (Co-Smad; Smad4) and the inhibitory Smads (I-Smad; Smad6, 7). Two major Smad transduction pathways can be activated depending on the ligand and receptors (*figure3A-B*). I-Smads serve as negative regulators of the TGF-β pathway.

Only five R-Smads mediate the majority of complex responses elicited in different tissues by ligands of the TGF-β family. Hence, it is not surprising that an elaborate network of regulators must keep control over the input, activity, and outcome of this system. A multitude of regulatory mechanisms have been recently uncovered that control specificity and diversity of the pathway (for reviews see Massagué and Wotton 2000; Moustakas *et al.* 2001; Shi and Massagué 2003). Since the Smad proteins not only transduce the signal but also regulate target gene responses, they represent the key players within this network being modulated at several levels.

Although the Smad pathway is the main target of TGF-β signaling, there is also evidence for Smad independant TGF-β signal transduction. It has been reported that TGF-β can also activate Rho GTPases (Bhowmick et al 2001), protein phosphatase 2A (Petritsch *et al.* 2000) and MAP kinases (Yu *et al.* 2002; Itoh *et al.* 2003).

2.2.3 Structural features of Smad proteins

The Smad proteins are very similar in structure, consisting of two conserved globular domains, "Mad Homology domain" 1 and 2 (MH1 and MH2), which are separated by a poorly conserved linker region (*figure4,* Massagué and Wotton 2000; Shi and Massagué 2003). The amino terminal MH1 domain exhibits DNA-binding activity and negatively regulates the MH2 domain. Furthermore the MH1 domain has been implicated in nuclear import and interaction with other proteins. A highly conserved β hairpin within the MH1 domain is responsible for DNA contact to the major groove of the DNA (*figure4B*). A minimal Smad Binding Element (SBE: 5`A-G-A-C 3`) has been characterized and the crystal structure of the Smad3-MH1-SBE complex has been solved (Shi *et al.* 1998). Due to the

conserved nature of the β-hairpin as well as the surrounding sequence elements it is believed that all R-Smads can bind to the SBE (Shi *et al.* 1998). An exception is the most common splice form of Smad2 which has a unique 30 residue insertion between the DNA binding β-hairpin and Helix2 and the I-Smads which have a very diverged MH1 domain. Though Smad1, 5 and 8 bind the SBE *in vitro*, binding is inefficient, and they have been demonstrated to bind with higher affinity to G/C rich sequences (Ishida *et al.* 2000). The low specificity of

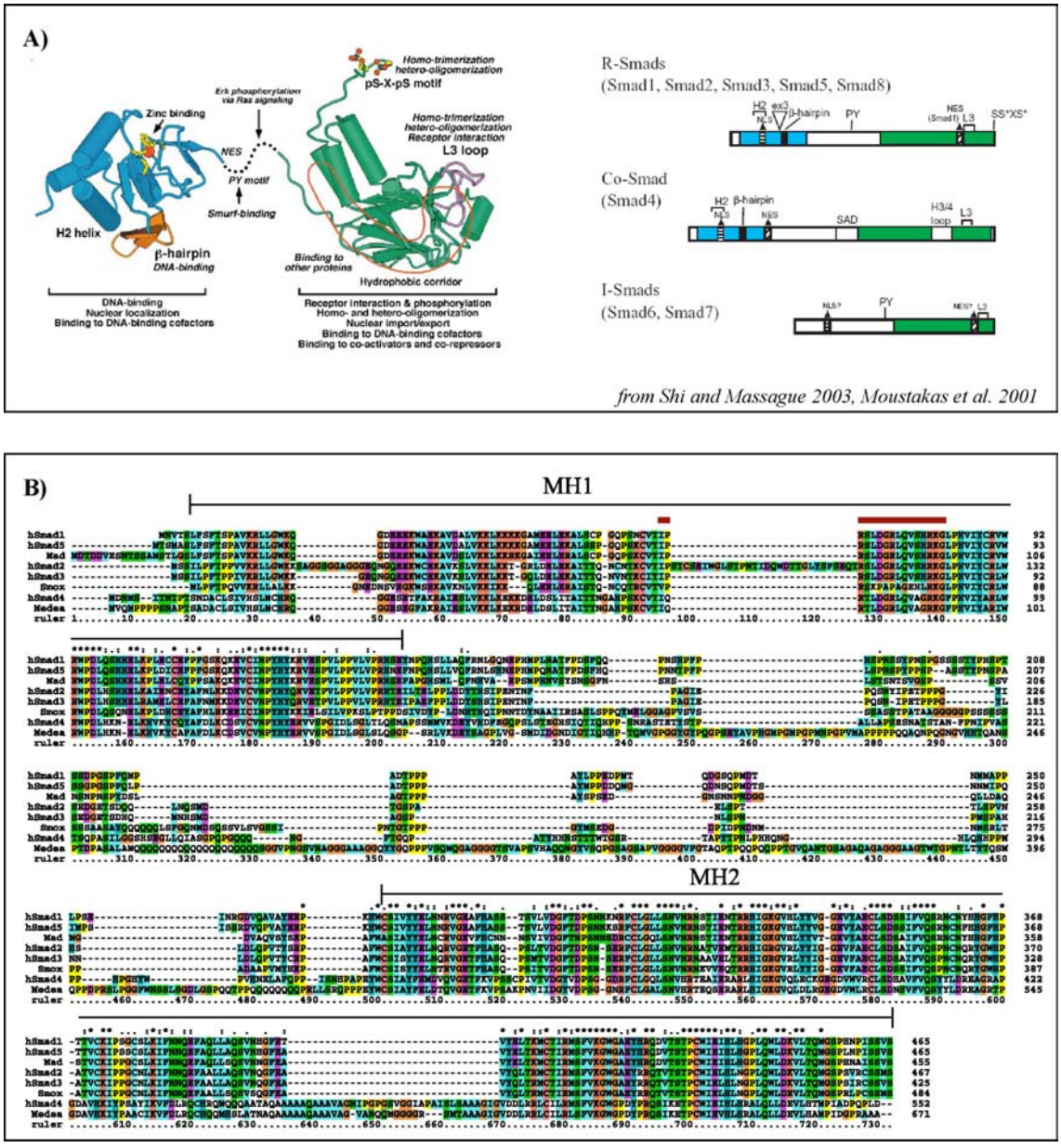


Figure4: A) Structural model of a phosphorylated R-Smad. The MH1 and MH2 domains are colored cyan and green, respectively. The DNA binding hairpinis highlighted in orange. The L3 loop of the MH2 domain is colored purple. The C-terminal pSer-X-pSer motif is shown in ball-and stick representation. On the right, schematic representation of the three subfamilies of Smad proteins (*from Shi and Massaguè, 2003*). **B)** ClustalX alignment of Smad proteins from vertebrates and *Drosophila*. The red bar indicates sequences responsible for DNA binding.

DNA binding by Smads strongly suggests that additional DNA binding factors are necessary to regulate target genes.

The unconserved linker region contains a Smurf interacting motif (PY-motif) which targets Smads for degradation. Interestingly, the linker region is targeted by other signaling pathways for phosphorylation opening possibilities for crosstalk to other cascades (Massague 2003; Shi and Massagué 2003).

The carboxy-terminal MH2 domain is highly conserved among all Smad proteins (*figure4B*). It is responsible for receptor interaction, the formation of homo- and heteromeric complexes with Smad proteins and bears intrinsic transcriptional transactivation activity when fused to Gal4 (Liu *et al.* 1996). The MH2 of R-Smads contains a flexible SSXS motif at the c-terminal end which is phosphorylated by the typeI receptor. Phosphorylation of this motif induces structural changes within the Smad molecule allowing association with the Co-Smad. Similar to the MH1 domain, the MH2 domain has been shown to interact with different proteins. The two domains together offer a wide array of docking surfaces for other cofactors demonstrating that protein-protein interactions are an important feature of the Smad proteins.

Smads can form homo and heteromeric complexes, but the exact stoichometrie of the Smad molecules on DNA is still under debate. Heterodimer and/or heterotrimer models have been suggested and for both models, *in vitro* data exists (Shi and Massagué 2003). It has been proposed, that the stoichometrie depends on the gene promoter context and both a heterotrimer and a heterodimer are possible (Inman and Hill 2002).

2.3 A complex network is responsible for specificity and diversity of TGF-β signaling

TGF-β cytokines are secreted molecules which have no intrinsic directionality when released into the extracellular space. Therefore complex mechanisms have evolved which ensure specificity of the signal. (*figure5,* Massagué and Wotton 2000; Miyazono 2000; Moustakas *et al.* 2001; Shi and Massagué 2003).

Directivity and intensity of the ligand can be achieved at several steps, including the interaction with extracellular matrix proteins, ligand binding proteins or at the level of receptors. For example ligand binding to the receptors is regulated by so-called “ligand-traps”. Ligand traps are soluble proteins that, as shown for Noggin-BMP7 (Groppe *et al.* 2002), bind to the ligand and block the binding to its receptor. An additional class of receptors, typeIII receptors (e.g. endoglin, betaglycan), have been proposed to facilitate or modulate binding of some ligands to the typeI/II receptors. Another class of membrane bound molecules controls ligand access to the receptor. One example is the pseudoreceptor BAMBI (BMP and Activin

receptor membrane bound Inhibitor) which competes with typeI receptor for ligand binding. Also, the differential expression of the TGF-β receptors itself have been reported and represent another possibility to control the specificity of the signal.

The duration and magnitude of the signal in the cell is determined by several mechanisms controlling Smad proteins (*figure5,* Massagué and Wotton 2000; Shi and Massagué 2003). The access to the receptors is controlled by SARA (Smad anchor for receptor activation) a membrane bound protein which specifically recruits Smad2/3 to the receptor for phosphorylation. Both, nuclear localization signals (NLS) and nuclear export signals (NES) were found in several Smad proteins implicating that the cellular localization is regulated as well. The signal can be terminated by dephosphorylation of Smad proteins by yet unidentified phosphatases, or through proteosomal degradation by E3 ubiquination ligases SMURFs (Smad ubiquitination regulatory factor).

The third class of Smad molecules, the inhibitory Smads (I-Smad; Smad6/7) modulate the magnitude and duration of the TGF-β signal by either competing with R-Smads for receptor and Co-Smad interactions or by targeting the receptors for degradation. Their expression can

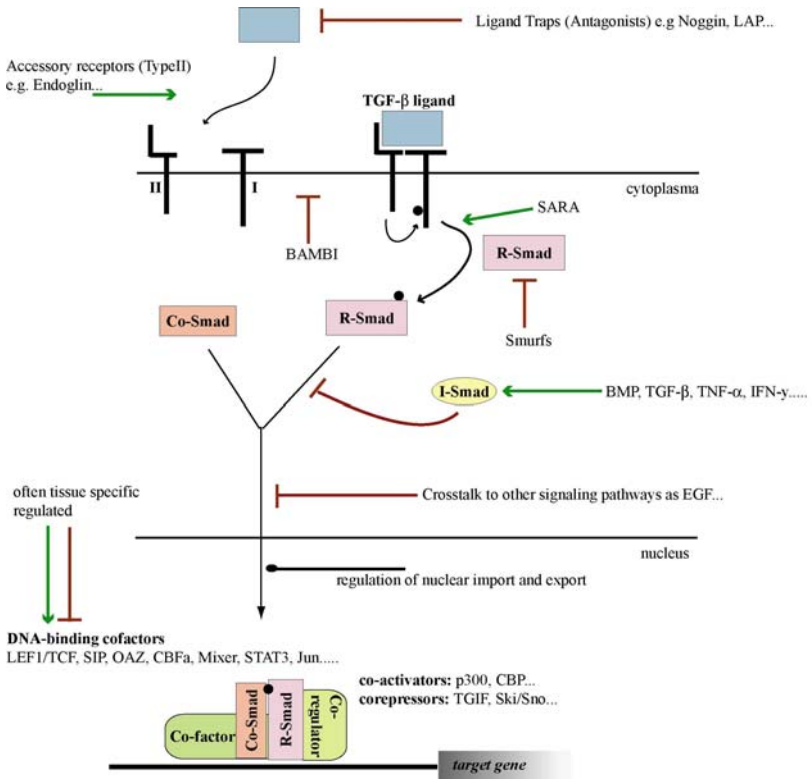


Figure5: A complex network controls the TGF-β pathway. Green arrows indicate an active and red bars an inhibitory function. (*Massague, 2000 and Shi and Massague, 2003)*

be induced by TGF-β signal transduction pathways presenting a negative-feedback mechanism. Furthermore, there is evidence for a possible nuclear function of inhibitory Smads repressing transcription through recruitment the transcriptional corepressor C-terminal binding protein (CtBP) (Lin *et al.* 2003).

2.3.1 Target gene regulation by Smad proteins

The TGF-β signal leads to positive and negative changes in the expression of several hundred genes (Kang 2003) induced by the same set of activated Smad molecules. The binding specificity of the pR-Smad/Co-Smad complex itself is very low (the SBE is predicted to occur once every 256bp in the genome) but target gene regulation is highly specific. Hence, by providing a tissue specific set of nuclear factors, the cell decides which of many potential target genes will respond (*figure5*).

Several factors have been identified, most of them being tissue specific, which target Smad proteins to DNA to elicit specific transcriptional responses. Remarkably, members from many different families of DNA binding proteins, including Fast1, LEF1/TCF, SIP, OAZ, Jun/Fos, E2F and Mixer, have been shown to cooperate with Smad molecules to regulate target genes. (for reviews see, Massagué and Wotton 2000; Moustakas *et al.* 2001; Shi and Massagué 2003). Furthermore, Smad proteins can mediate transcriptional activation or repression depending on their associated partners (usually not DNA-binding proteins). Interaction with co-activators including CBP/p300 and Smif and co-repressors as TGIF, Ski/Sno, CtBP have been characterized. Chromatin remodeling represents one of the mechanisms used to regulate transcription. CBP/p300 has intrinsic histone acetyl transferase activity (HAT) and TGIF and Ski-mediated repression is primarily attributed to histone deacetylase activity.

It becomes clear, that the TGF-β signaling pathway operates as part of a signaling network that collects and integrates diverse environmental cues in the cell. One example is the Ras/MAPK pathway, which phosphorylates four MAPK sites in the linker of Smad,1 antagonizing BMP signaling induced nuclear localization (Massague 2003). Other pathways have also been reported to interfere with the TGF-β pathway such as the TNF-α via NFκB, IFN-γ via STAT1, CamKII, PKC and Wnt/β-catenin (Massagué and Wotton 2000; Miyazono 2000; Moustakas *et al.* 2001; Shi and Massagué 2003).

Signaling by TGF-β stimulates a vast number of cellular responses and the list of Smad interactions which are integrated for an appropriate transcriptional response, is rapidly growing. The number of possible gene regulatory mechanisms, which are mainly characterized in cell culture systems, becomes unlimited and insights in the

endogenous/physiological interplay between components is likely to come from studies involving the whole animal.

3. TGF-β signaling in *Drosophila melanogaster*

Members of the TGF-β family and the components of the pathway have been shown to be highly conserved in animal kingdom. Thus, it is not surprising that homologues have also been identified in *Drosophila* and play a fundamental role during the development of the fly. TGF-β signaling is implicated in a variety of processes ranging from proliferation, cell differentiation, patterning and growth (Affolter *et al.* 2001). *Drosophila* provides an excellent model system to study the role of TGF-β signaling *in vivo*. Mutant fly lines for most of the signal components are available and the power of *Drosophila* genetics provides many tools, from clonal analysis to overexpression experiments, to examine the function of proteins directly in the fly. As a result, a large number of research reports have been published genetically analyzing the components involved in TGF-β signaling. Comparably little has been done concerning the molecular function of these proteins hence only a few Smad interaction partners have been characterized in detail so far.

3.1 *Drosophila* components of the TGF-β pathway

Three of the seven *Drosophila* TGF-β family members have been characterized in more detail: Decapentaplegic (Dpp), Screw and Glas bottom boat 60A (Gbb), all belonging to the BMP family of ligands (Affolter *et al.* 2001). Several typeI and typeII receptors have been described (*see figure3*) including Thick veins (Tkv), Saxophone (Sax) and Baboon (Babo) (typeI receptors) and Punt (Put), Wishful thinking (Wit) and STK-D (typeII receptors). The Drosophila genome contains four genes encoding Smad proteins (*figure3*): two R-Smads, *mother against dpp* (*mad*) most similar to Smad1 and *smad on X* (*smox*) a Smad3 homologue; a single Co-Smad *medea* (*med*) and one I-Smad *daughter against dpp* (*dad*) (Smad6 homologue, (Tsuneizumi *et al.* 1997).

3.2 Dpp signaling during *Drosophila* development

Dpp and BMP4 are functional orthologues (Padgett *et al.* 1993) and signaling by Dpp presents the most eminent TGF-β pathway in *Drosophila* being therefore the best characterized. Decapentaplegic (called decapentaplegic because of its requirement in 15 discs; (Spencer *et al.* 1982) is involved in many aspects of fly development such as maintenance of the germline (Xie and Spradling 1998), mesoderm specification (Staehling-Hampton *et al.* 1994) midgut

induction (Immergluck *et al.* 1990; Tremml and Bienz 1992) tracheal morphogenesis (Vincent *et al.* 1997) establishment of embryonic imaginal disc placodes (Goto and Hayashi 1997), patterning of the adult appendages (Posakony *et al.* 1990; Zecca *et al.* 1995) and growth (Day and Lawrence 2000).

3.2.1 The Dpp signaling pathway

Dpp elicits its effect through activation of the two receptors Tkv (typeI) and Put (typeII). The signal is propagated by phosphorylation of the R-Smad protein Mad which then assembles with the Co-Smad Medea and they translocate into the nucleus to regulate target genes (Affolter *et al.* 2001). Mad and Medea have been shown to bind DNA and their binding sites are essential for target gene regulation (Kim *et al.* 1997; Chen and McKearin 2003b). The Drosophila I-Smad Dad acts in a similar fashion as Smad6, 7 in vertebrates by competing with Mad for binding to the Tkv receptor and antagonizing Mad phosphorylation (Tsuneizumi *et al.* 1997).

3.2.2 Target gene regulation by Drosophila Dpp signaling

A number of Dpp target genes have been described in *Drosophila* in different tissues, predominantly in genetic studies. Enhancers of some of those target genes have been characterized demonstrating that Mad binds to G/C rich sequences, as reported for Smad1 and Smad5, whereas Medea prefers the classical SBE "AGAC" (Kim *et al.* 1997; Chen and McKearin 2003b). Mutations within these sites affect gene expression *in vivo* proving that their binding sites are essential for target gene regulation (Kim *et al.* 1997). However, Mad/Med binding sites are not sufficient to control target gene transcription and additional Mad/Med interaction partners are required. In contrast to the vertebrate field, only a few partners have been characterized in *Drosophila* so far. Among them is the co-activator CBP/p300 which can bind to Mad (Waltzer and Bienz 1998). Characterization of several enhancers revealed a common mechanism for target gene regulation which involves additional factors binding to nearby sites. This factor can be "selector proteins" as it was shown for the *labial*, *tinman* and *even-skipped* enhancers (Grieder *et al.* 1997; Xu *et al.* 1998). Similarly, nearby binding sites for a second, different signaling pathway have been identified which are essential for target gene regulation (Affolter *et al.* 2001). A different mechanism has been reported for *ultrabithorax* (*ubx*), *vestigal* (*vg*) and *optomotor blind* (*omb*). Their Mad binding sites overlap with binding sites for the repressor protein Brinker, where Mad and Brinker compete for binding (Sivasankaran *et al.* 2000; Kirkpatrick *et al.* 2001).

Two relevant examples for the function of Dpp signaling in different developmental processes will be presented in more detail. The secreted ligand Dpp can either induce specific transcriptional responses in neighboring cells (*see 3.2.2*) or can function as a morphogen, signaling over a field of cells inducing target genes in a concentration dependant manner (*see 3.2.3*). The morphogens theory exists for a long time and fascinates biologists until today. Despite the immense interest in the morphogen field, the molecular mechanism are only now starting to be uncovered, primarily performing molecular-genetics in *Drosophila*.

3.2.3 Short range action of Dpp signaling in germ stem cell maintenance

One of the classical examples for the short range action of Dpp is the communication between tissue layers during gut development and has been extensively described by many others (Immergluck *et al.* 1990; Tremml and Bienz 1992). Germ cell maintenance in the female fly is another important process in which Dpp communicates with neighboring cells, inducing a specific fate. The adult ovary contains 14-16 ovarioles, each with a germarium at the tip, within which the germline and somatic cells are located (*figure6*). Two or three germline stem cells, located at the anterior tip of the germarium, divide asymmetrically giving rise to a multipotent stem cell and a cystoblast. The self renewing stem cell stays at the anterior edge of the germarium, whereas the cystoblast undergoes further differentiation into cystocytes the precursor of ovarian follicles. Dpp is expressed in an anterior subset of follicle cells and isimportant for the maintenance and division of the self-renewing stem cell. Mutations of components of the Dpp signaling pathway in the stem cell result in symmetric cell division giving rise to two cystoblasts (Xie and Spradling 1998). A contrary phenotype is observed in flies carrying a mutation in a gene called *bag of marbles* (*bam*) (Ohlstein and McKearin 1997). The germarium of these flies is filled with self-renewing stem cells and cystoblasts do not differentiate. Consistently with the phenotype, *bam* is expressed only in the cystoblasts

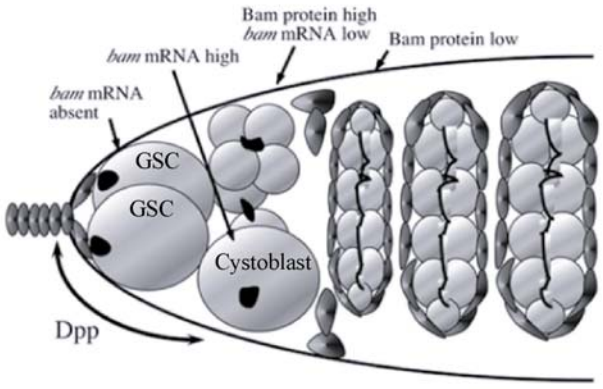


Figure6: Schematic view of the germarium. Dpp is expressed in cells in the anterior membrane. The germ stem cell (GSC) undergoes asymmetric cell division giving rise to a self renewing GSC and a Cystoblast. *bam* transcripts are undetectable in germline stem cells (GSCs) but expression is upregulated in the Cystoblast. (*from Chen and McKearin 2003*)

and it was proposed that *bam* transcription is downregulated in the multipotent stem cell by Dpp. Very recently, the isolation of a "silencer" element within the *bam* regulatory region has been reported and it was demonstrated that Mad and Medea bind to it *in vitro* (Chen and McKearin 2003a; Chen and McKearin 2003b). Although microarray experiments indicate that Dpp signaling not only activates, but also represses target genes, so far, Smads have almost exclusively been demonstrated to promote activation on genes (Shi and Massagué 2003). The *bam* gene in Drosophila presents an example for a repressive function of a Smad complex and it will be interesting to see which other molecule(s) are involved. Another gene which has been reported to be negatively regulated by Dpp signaling in Drosophila is *brinker* (Marty *et al.* 2000; Torres-Vazquez *et al.* 2000). The molecular events regulating *brinker* transcription have not been identified will be the topic of this study. They are especially interesting because *brinker* is also required for the morphogen function of Dpp in the wing imaginal disc.

3.2.4 Dpp acts as a morphogen (in the wing imaginal disc)

Dpp acts as a morphogen during early dorso-ventral patterning of the Drosophila embryo having an analogous function to the vertebrate BMP4 in Xenopus mesoderm specification (Gurdon *et al.* 1994; Dosch *et al.* 1997; Jazwinska *et al.* 1999b). However, the prime example and an excellent model system to study the mechanism underlying morphogen function is the wing imaginal disc. Imaginal discs are flat undifferentiated sheet of cells which give rise to adult organs as the wing, leg or eye. In the wing imaginal disc Dpp is expressed in a narrow stripe of cells along the anterior-posterior boundary, and forms an extracellular protein gradient patterning the whole disc (*figure7A,* Nellen *et al.* 1996; Podos and Ferguson 1999; Strigini and Cohen 1999). The Dpp gradient could recently be visualized for the first time using an Dpp-GFP line (Entchev *et al.* 2000; Teleman and Cohen 2000). Comparable to the Spemann experiment, ectopic expression of Dpp in the wing imaginal disc results in the formation of an additional adult wing (*figure7A,* Zecca *et al.* 1995). Some of the downstream target genes regulated by Dpp have been identified in genetic experiments. Their expression depends on the level of Dpp with *spalt* (*sal*) being expressed only at high levels whereas *optomor blind* (*omb*) and *vestigal* (*vg*) are expressed in broader domains, respectively (f*igure7B,* Nellen *et al.* 1996; Podos and Ferguson 1999). Clonal analysis revealed that their expression depends on the Dpp receptor Tkv, Mad and Medea (Marty *et al.* 2000; Müller *et al.* 2003), and Mad binding sites have been identified in the regulatory element of *vg* (Kim *et al.* 1997) proposing that Dpp directly activates the target genes *vg*, *omb*, and *sal*. An alternative mechanism has emerged with the discovery of Brinker (Brk), a transcription factor

that is required to counteract responses to Dpp (Campbell and Tomlinson 1999; Minami *et al.* 1999; Jazwinska *et al.* 1999a).

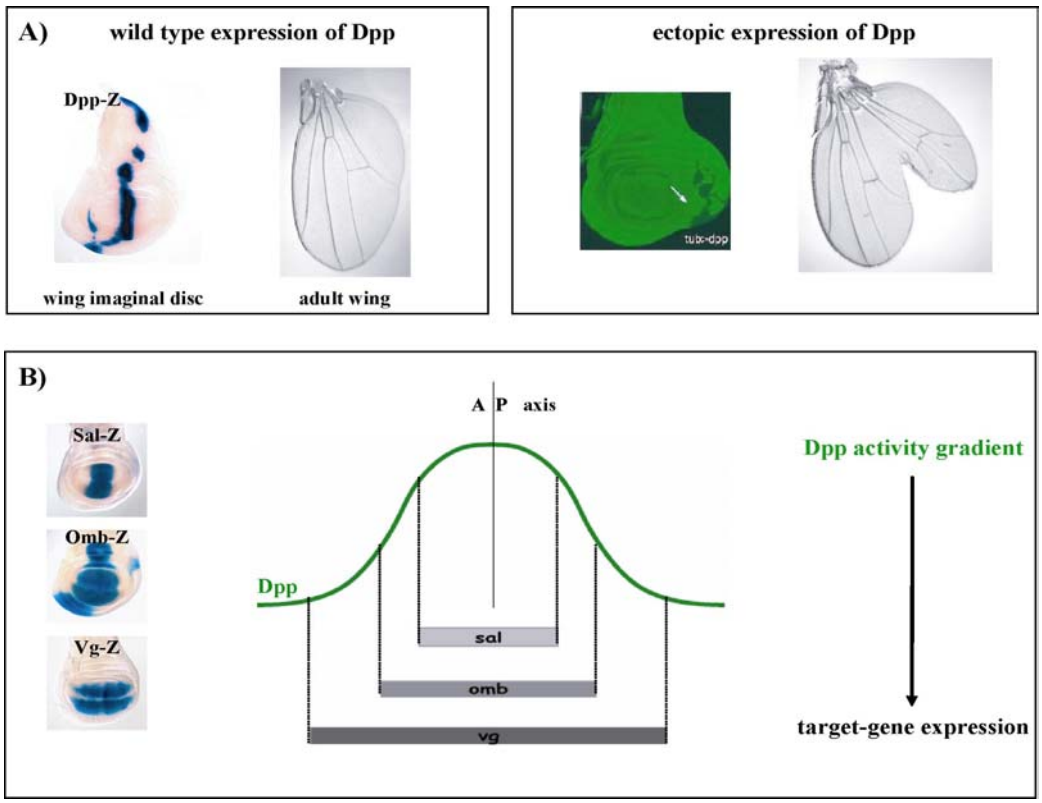


Figure7: A) *left:* wild type wing imaginal disc and adult wing. Expression of Dpp in the wing imaginal disc. *right:* ectopic expression of Dpp in cells (marked by the absence of GFP) driven by the tubulin promotor results in the formation of an additional wing of the adult fly (*Nellen et al. 1996*). **B)** Schematic representation of the Dpp morphogen gradient. Dpp has its highest levels at its source at the anterior posterior boundary with decreasing levels to both nsides. The respective domains of the Dpp target genes *spalt* (*sal*), *omptomotor blind* (*omb*) and *vestigal* (*vg*) are indicated. LacZ staining in the wing imaginal disc is on the left. The Dpp activity gradient results in target gene expression in nested domains.

3.3 The role of the nuclear repressor Brinker in Dpp signaling

Brinker (*Brk*) was identified in a screen for embryonic lethal mutations causing a phenotype similar to ectopic activation of Dpp signaling suggesting a role of Brinker in Dpp signaling (Jazwinska *et al.* 1999a). The same gene was identified through an enhancer trap line that was expressed in a Dpp-responsive manner in the developing wing (Campbell and Tomlinson 1999).

During development, *brinker* expression is absent in regions of high levels of Dpp signaling and genetic experiments confirmed that *brinker* is negatively regulated by Dpp (Marty *et al.* 2000; Torres-Vazquez *et al.* 2000). Brinker is a repressor directly suppressing several Dpp target genes and binding sites have been mapped in various enhancers (Sivasankaran *et al.*

2000; Kirkpatrick *et al.* 2001; Rushlow *et al.* 2001; Saller and Bienz 2001; Zhang *et al.* 2001). Furthermore, genetic data indicates that Dpp target genes are activated by the removal of the repressor Brinker, rather than by Dpp directly (Campbell and Tomlinson 1999; Jazwinska *et al.* 1999a). These findings suggest that it is primarily Brinker that controls Dpp target gene expression and that direct transcriptional activation by the Dpp signal components Mad/Med may only play a subordinate role.

3.3.1 The Brinker protein

Brinker is a nuclear protein of 704 amino acids (*figure8*). In the N-terminal part it contains a N-terminal DNA binding domain of the helix-turn-helix type (HtH). This sequence motif shows weak homology to homeodomains and has been shown to bind the DNA consensus sequence TGGCG C/T C/T (Sivasankaran *et al.* 2000; Kirkpatrick *et al.* 2001; Rushlow *et al.* 2001; Saller and Bienz 2001; Zhang *et al.* 2001), which is present in several Brinker target genes. Brinker acts as a repressor of several Dpp target genes recruiting the corepressors CtBP and Groucho (Hasson *et al.* 2001; Zhang *et al.* 2001). There is no Brinker sequence homolog in vertebrates and no functional homolog is known to date. However given the high conservation between Dpp and TGF-β signaling in other organism it seems highly likely that Brinker relatives will operate elsewhere. An indication of this comes from overexpression experiments in Xenopus indicating that Brinker can also repress BMP targets genes in other organisms (Minami *et al.* 1999).

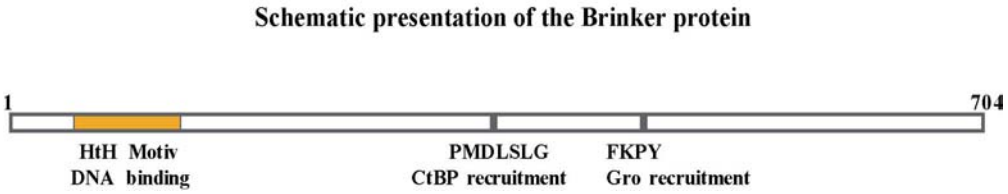


Figure8: The Brinker protein contains a N-terminal DNA binding domain with a helix-turn-helix motif (HtH) and two motifs which have been shown to recruit the corepressors CtBP (C-terminal binding protein) and Groucho (Gro).

3.3.2 Dpp generates an inverse Brinker gradient in the wing imaginal disc

The wing imaginal disc presents an excellent model system to study the function of the morphogen Dpp. Within the last years a considerable amount of genetic data has been collected and a more comprehensive picture emerges about the way morphogens operate. The translation of an extracellular gradient into a specific nuclear response is still poorly

understood. The genetic characterization of *brinker* has been a milestone for this field and offered a new perspective on the underlying mechanism.

The expression of *brinker* in the wing imaginal disc is negatively regulated by Dpp and therefore Brinker is found in lateral regions of the disc (*figure9*). Dpp target genes have been reported to be directly repressed by the Brinker protein and that it is the release of the repressor which allows activation. This lead to the speculation that the Brinker gradient regulates Dpp target genes and the main function of the Dpp activity gradient is to shape a transcriptional *brinker* gradient (*figure9,* Affolter *et al.* 2001).

Very recently the genetic relationship between the Dpp activity gradient, the *brinker* expression profile and the Dpp target genes in the wing imaginal disc were described (Müller *et al.* 2003). Alterations of the Dpp expression levels caused a change in the *brinker* expression pattern with an inverse relationship, demonstrating that Dpp signaling levels control the profile of the *brinker* expression gradient. Furthermore, the expression of low levels of *brinker* in the center of the imaginal disc resulted in the downregulation of the Dpp target gene *spalt*, but not *omb*. In contrast, expression of intermediate levels of *brinker* lead to the repression of both target genes, validating that Brinker levels define the expression

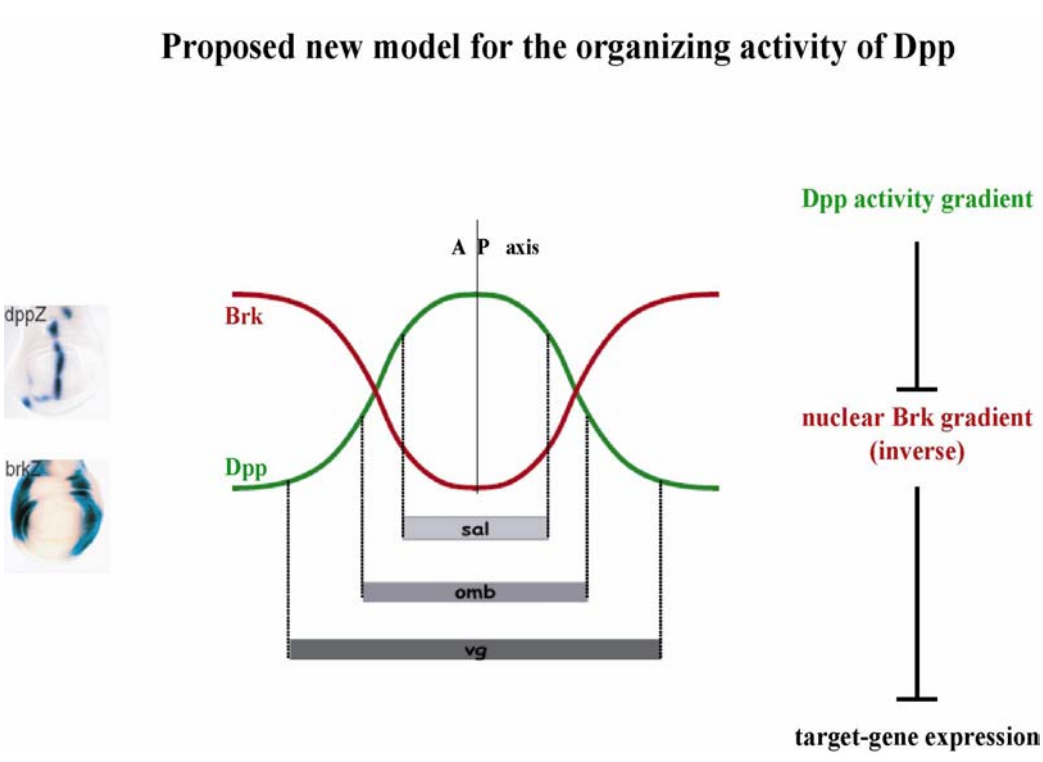


Figure9: Based on the genetic data a new model for the read-out of the Dpp gradient was proposed. The Dpp activity gradient determines an inverse transcriptional *brinker* gradient. Different levels of Brinker control the expression of downstream genes. Brinker adds an additional level to the cascade and the focus is now on the question how the Dpp gradient is translated into an inverse transcriptional *brinker* gradient.

domains of Dpp target genes in the wing.

Taken together, the nuclear protein Brinker presents a crucial player in the regulation of Dpp target genes in several tissues. Consequently, the question of how Dpp regulates target genes has to be rephrased to how Dpp represses *brinker* transcription. The Schnurri protein has been shown to be genetically involved in this process (Marty *et al.* 2000) and the regulatory region of *brinker* has been characterized by B. Müller (Müller *et al.* 2003) revealing a putative target sequence for Dpp mediated downregulation of *brinker* expression.

3.3.3 Dissection of the regulatory region of brinker

In the search for regulatory elements responsible for the Dpp dependant *brinker* expression in the wing imaginal disc the regulatory region of *brinker* has been dissected and analyzed *in vivo* (*figure10,* Müller *et al.* 2003). A 20kb region upstream of the *brinker* transcription locus was scanned and lead to the identification of a fragment B14 which faithfully recapitulated all aspects of late embryonic and larval *brinker* expression. Distal truncations of the B14 fragment lead to the isolation of a ubiquitously active, constitutive enhancer, element E (*brk*E). Three short fragments within B14 (called A, B and S) were identified that possessed

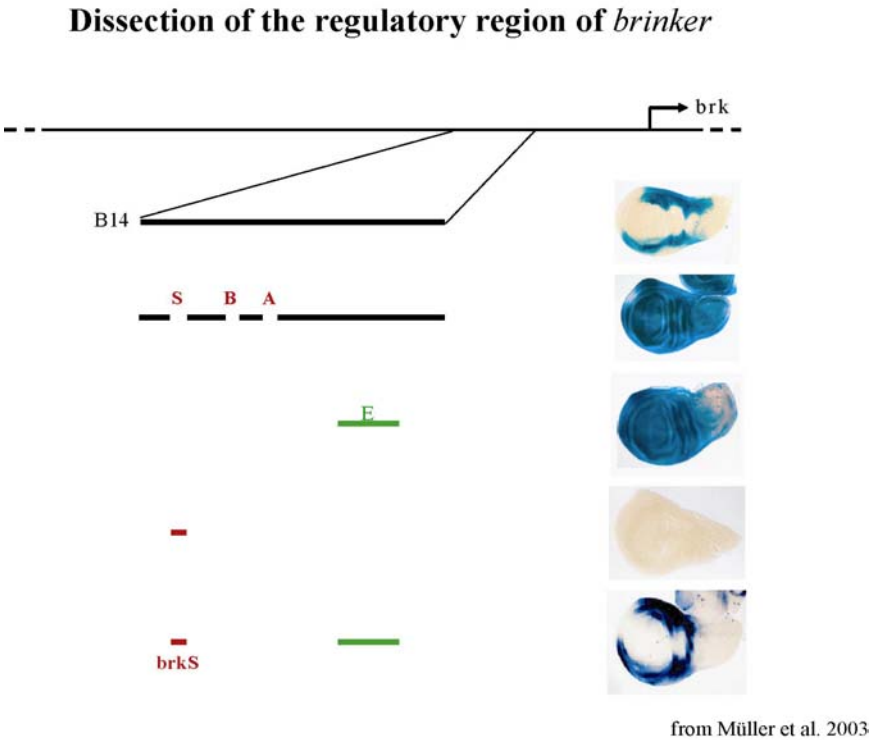


Figure10: Dissection of the brk regulatory region into separable activating and repressing activities. A schematic presentation of the brk upstream region is shown on the top. Fragments were tested for their ability to drive reporter gene expression in transgenic flies. Fragment B14 did faithfully recapitulate all aspects of expression in the wing imaginal disc. Removal of A, B, S from B14 resulted in the ubiquitous expression of the reportergene. A minimal ubiquitous element "E" was isolated. A fusion of Element "E" to the repressive elements A, B or S resulted in the inhibition of reportergene transcription in the Dpp domain (*from Müller et al. 2003*).

Dpp-dependant repressive activities when coupled to the constitutive enhancer *brk*E. The brinker enhancer consists of two separable entities, a ubiquitous active element (*brk*E) and Dpp-regulated repression activities, *brkA, B,* S. Components of the Dpp signaling pathways have been demonstrated to be genetically required for the downregulation of *brk*E via the repressive element *brk*S (Müller *et al.* 2003). In addition to the Dpp core components, the zinc finger protein Schnurri has previously been described to be genetically required for brinker repression (Marty *et al.* 2000).

4. The role of *schnurri* during *Drosophila* development

Schnurri was identified in a screen for embryonic lethal mutations on the second chromosome in Drosophila (Nüsslein-Vollhard *et al.* 1984). In 1995, *schnurri* (*shn*) was cloned and genetically characterized by three groups (Arora *et al.* 1995; Grieder *et al.* 1995; Staehling-Hampton *et al.* 1995). The phenotypes of *schnurri* mutant embryos strongly resemble the phenotypes observed in embryos with defects in the Dpp receptors *tkv* or *punt* suggesting a role of Schnurri in Dpp signaling. Furthermore, Schnurri function was placed downstream in the signaling cascade based on genetic epistasis experiments. Albeit the detailed characterization of the genetic requirements of *schnurri* and its essential role in most Dpp dependant processes its molecular function has not been explored yet. The nuclear localization and the presence of zinc fingers displaying some characteristics of DNA binding zinc fingers indicate a function of Schnurri in target gene regulation.

4.1 *Schnurri* expression pattern and mutant phenotypes

Schnurri mRNA is contributed maternally and the expression profile in the embryo is very dynamic and resembles that of the Dpp receptor *tkv* (Brummel *et al.* 1994; Penton *et al.* 1994). During blastoderm stage, *schnurri* becomes restricted to the dorsal half of the embryo. Later it is detected in the presumptive mesoderm of the invaginating ventral furrow and in the mesoderm throughout germband extension. In germband retracted embryos, *schnurri* is expressed in the endoderm. After midgut fusion it is detected in domains in parasegment (PS) 4 and 7. These domains are transient and disappear before the formation of the midgut constrictions. Dpp was shown not to be required for *schnurri* expression (Grieder *et al.* 1995; Staehling-Hampton *et al.* 1995). Several *schnurri* alleles have been described including two putative null alleles which are the EMS mutant shn^{IB} (see Staehling-Hampton) and the shn^{TD5} allele generated by a P-insertion (Nüsslein-Vollhard *et al.* 1984). These mutants are embryonic lethal and the cuticles show the classical dorsal open phenotype. According to its function in Dpp signaling, most processes which are controlled by Dpp are altered. They

display severe defects in gut morphogenesis consistent with the failure to express critical patterning molecules such as *lab*, *dpp* and *wg*. *Dpp* is transcribed in the visceral mesoderm in PS7, auto regulating its own expression, *wg* expression in PS8 and *lab* in the underlying endoderm. Furthermore ectodermal patterning and wing vein formation are affected. Transcription of Dpp downstream genes is severely altered in *schnurri* mutant embryos and target genes in the wing imaginal disc (*sal*, *omb* and *vg*) are absent in *schnurri* mutant clones. (Arora *et al.* 1995; Grieder *et al.* 1995; Staehling-Hampton *et al.* 1995; Marty *et al.* 2000).
Interestingly, Schnurri is genetically required for stem cell development in the Drosophila ovary, a process which also depends on Dpp signaling as mentioned in 3.1 (Xie and Spradling 1998; Xie and Spradling 2000).

4.2 Schnurri mediates Dpp-dependant repression of *brinker* transcription

Based on genetic experiments, the *schnurri* mutant phenotypes and its nuclear localization it was suggested that Schnurri directly activates Dpp target genes (Torres-Vazquez *et al.* 2001). In 1999 the characterization of the gene *brinker* was published (Campbell and Tomlinson 1999; Jazwinska *et al.* 1999a) uncovering an additional level of complexity in the Dpp signaling cascade. For target genes to be activated, Dpp signaling has to suppress the transcription of the repressor *brinker*. Furthermore, Schnurri was shown to be genetically required for Dpp dependant *brinker* repression, but not for Dpp dependant target gene activation (Marty *et al.* 2000). *Schnurri* mutant embryos displayed highly upregulated levels of *brinker* transcription which could be completely rescued by ectopic co-expression of

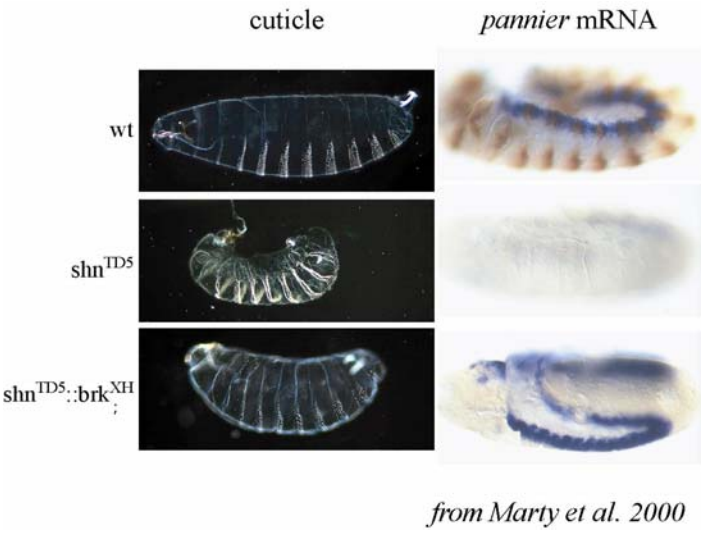


Figure11: Comparison of wild type embryos (upper row) shn^{TD5} homozygoues, (middle row) and $brk^{XH}shn^{TD5}$ homozygous embryos. First columns shows cuticle preparations, the second shows the expression of *pannier* mRNA. (*from Marty et al. 2000*)

schnurri and *dpp* (Marty *et al.* 2000). Moreover, *shn/brk* double mutant embryos almost completely rescued the *schnurri* dorsal open phenotype and expression of several Dpp target genes (*figure11,* Marty *et al.* 2000). Similarly, target genes were normally expressed in double mutant clones in the wing imaginal disc (Marty *et al.* 2000). The fact that *shn/brk* double mutants rescued the *schnurri* phenotype supports the idea that the main function of Schnurri is the repression of *brinker* transcription. Thus, the Dpp signaling pathway bifurcates, downstream of the signal mediating Smad proteins Mad and Medea, into a Schnurri dependant pathway leading to *brinker* repression and a Shn-independant pathway, leading to gene activation (*figure12,* Marty *et al.* 2000).

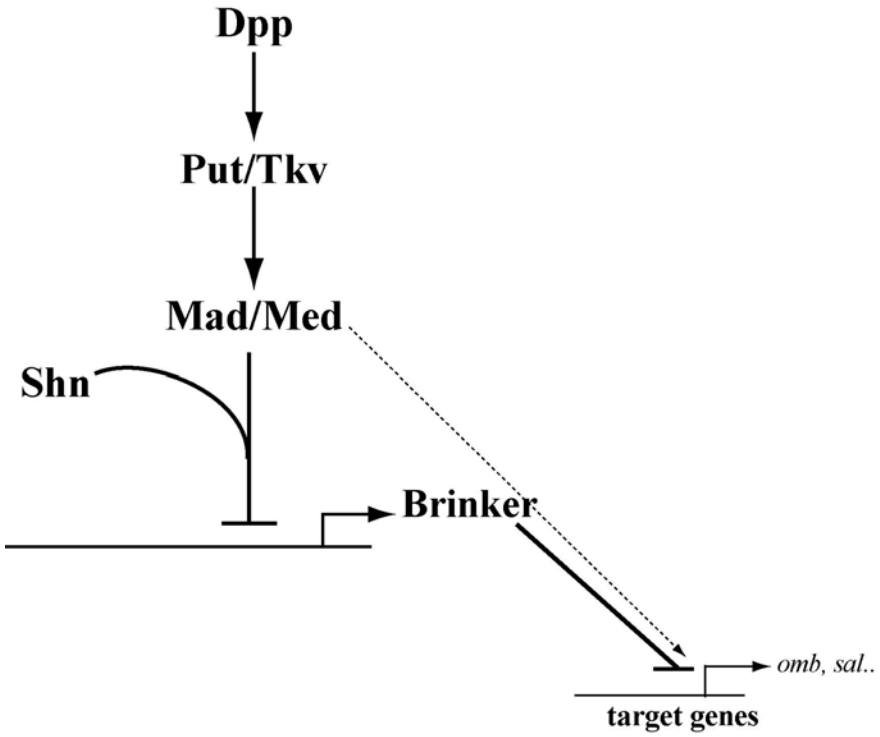


Figure12: Genetic model of the Dpp signaling pathway. Arrows (activation) and bars (repression) denote the genetic relationships. The Mad/Med complex is required for the activation of some target genes and essential for *brinker* repression. Shn is required for Mad/Med-mediated repression of *brk* transcription, but is dispensable for Dpp-mediated target gene activation. (*Marty et al. 2000*)

4.3 Structure of the Schnurri protein

The *schnurri* gene is located on the second chromosome and encodes a large zinc finger protein of 2529 amino acids (*figure13A*). Secondary structure prediction revealed eight zinc finger domains. Seven of those resemble the consensus sequence for Cys_2His_2-type of zinc

fingers (ZF1, 2, 4, 5, 6, 7, 8) and zinc finger3 is of the $Cys_2HisCys$-type. The Cys_2His_2-type of zinc fingers are arranged in three pairs (ZF1/2; ZF4/5; ZF6/7). Zinc finger8 is separated by an unusually long linker region of 22 amino acids from ZF6/7. The zinc fingers are arranged in two widely separated pairs of zinc fingers (ZF1/2 and ZF4/5) and a triplet of fingers (ZF6/7/8) at the C-terminal end. A domain of 17 conserved amino acids, called the region of homology (RH) found in all Schnurri homologues, is located in the N-terminal part of the protein but has no assigned function. A region rich in acidic amino acids is found C-terminal to zinc fingers4/5. Using a yeast-two hybrid approach and *in vitro* pull down assays, a putative Mad-interaction domain was mapped to amino acids 1441-1635 (Dai *et al.* 2000; Udagawa *et al.* 2000).

Zinc finger domains have been shown to contact DNA, but are also capable of protein-protein interaction and RNA binding. A comparison of the zinc finger sequences in Schnurri shows high similarities between the zinc finger pairs ZF1/2, ZF4/5 and ZF6/7 (*figure13B*), whereas zinc finger8 has the highest identity to zinc finger7 and 5. The zinc finger pairs itself are separated by a seven amino acid long linker region which shares similarity to the "TGEKP" type which is found in more then 50% of the Cys_2His_2-type of DNA binding zinc fingers (Wolfe *et al.* 2000; Laity *et al.* 2001). Sequence comparison with the "vertebrate-Schnurri"

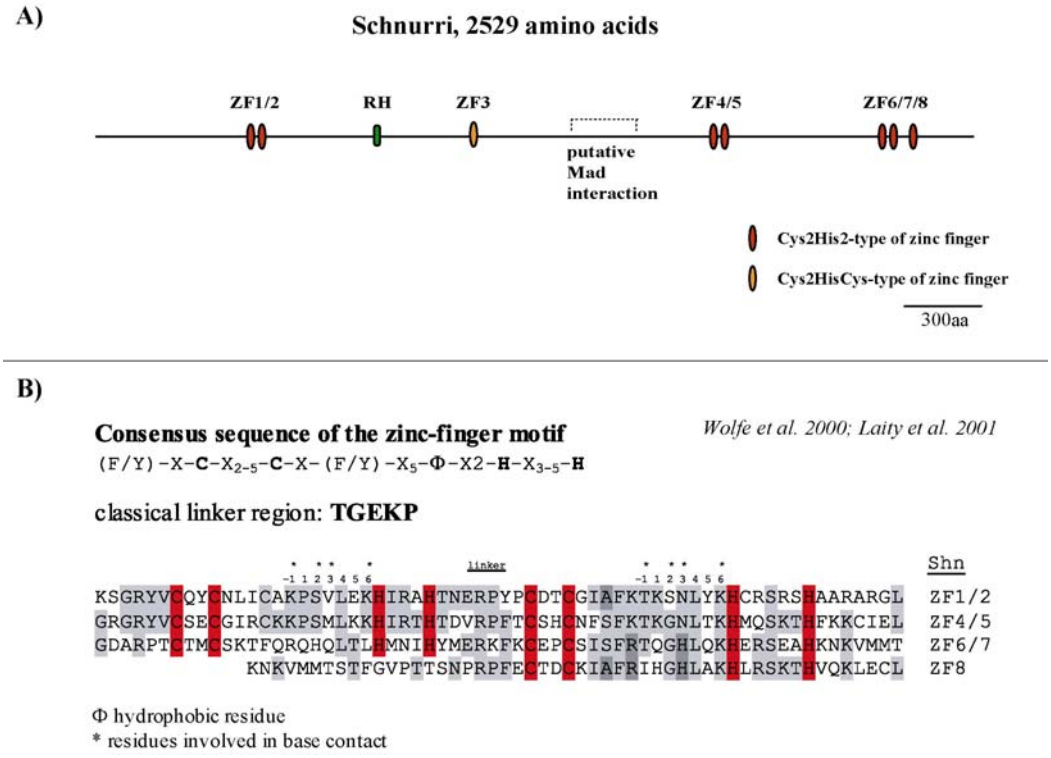


Figure13: A) Schematic presentation of the Shn protein with its respective domains. **B)** *Top*: general structure of a zinc finger domain; *bottom*: sequence comparison between the zinc fingers of Shn. Similar amino acids are highlighted (grey) and cysteine and histidine residues of the zinc finger are marked in red.

zinc fingers (*figure14B*; see chapter 4.4.1), which were originally identified by their ability to bind the consensus site "GGGGATTCCCC" (site for MBP-1; (Baldwin *et al.* 1990) revealed high degree of similarity with the zinc finger pair 1/2 and 4/5.

4.4 Schnurri homologues in other organisms

Sequence homologues of Schnurri are present in vertebrates and C.*elegans* (*figure14A*) including Schnurri1 (MBP-1/PRDII-BF1/αA-CrybB1/HIV-EP1), Schnurri2 (MBP-2/HIV-EP2) and Schnurri3 (HIV-EP3/KRC) in vertebrates and the Sma9 protein in *C.elegans* (Fan and Maniatis 1990; Nakamura *et al.* 1990; Hicar *et al.* 2001; Liang *et al.* 2003). Common to all Schnurri proteins is their large size and the arrangement of two widely separated pairs of zinc fingers which also share high sequence similarity (*figure14*). HIV-EP1 presents the closest vertebrate homologue to Schnurri containing also a conserved zinc finger3 and a region of homology. Interestingly, the C-terminal triplet of zinc fingers6/7/8 is not present in any of the vertebrate homologues. The Schnurri homologue from C.*elegans*, Sma9, has a diverged arrangement of the zinc finger domains, lacking completely the zinc finger pair1/2,

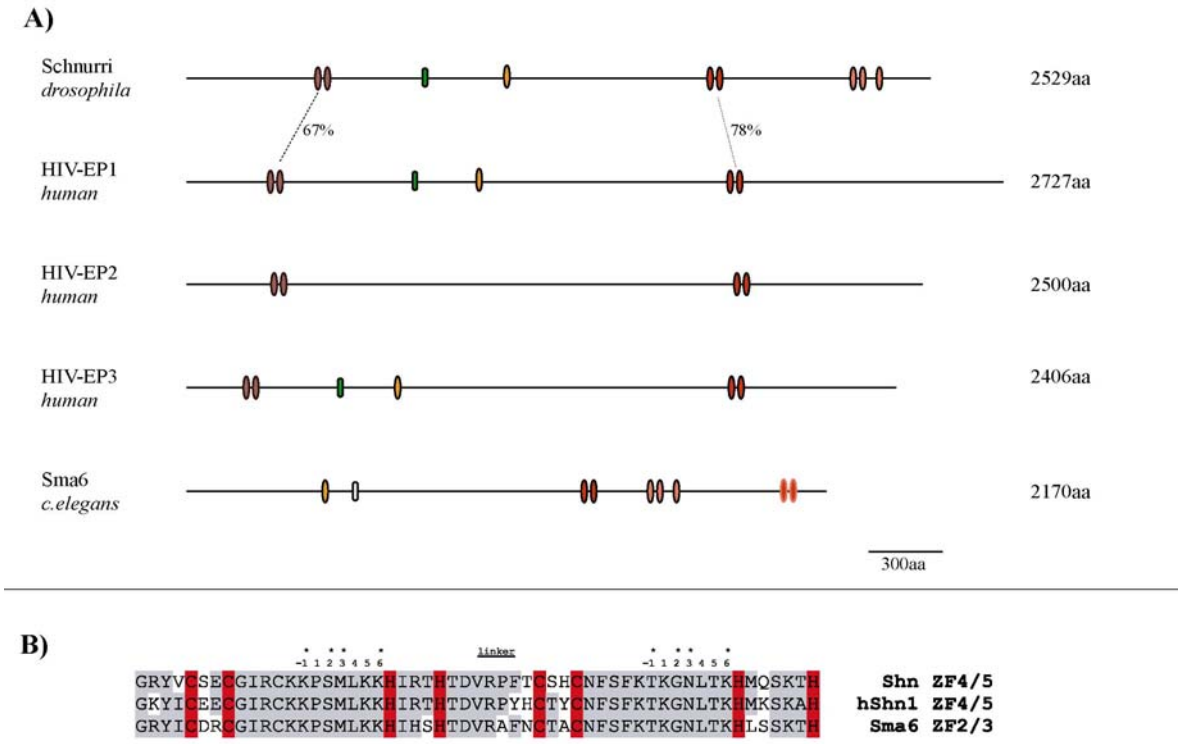


Figure14: A) Schematic presentation of Shn proteins from vertebrates and *C.elegans*. Zinc fingers of the C2H2-type are colored in red-tones. Same color indicates high similarity. The region of homology is depicted in green and the single zinc finger of the C2HC-type in orange. Sequences between the domains are not conserved between the proteins highlighted in grey. **B)** Sequence comparison of zinc finger4/5 of *Drosophila* Shn with their vertebrate counterparts. Cysteine and histidine residues of the respective fingers are labeled in red. Similarities are marked with grey boxes.

but has a triplet of zinc fingers, ZF6/7/8.

The vertebrate Schnurri homologues are DNA binding zinc finger proteins and have been isolated based on their ability to bind to the enhancer of major histocompatibility complex (MHC) classI genes (MBP: MHC-binding protein). However, there is no experimental data suggesting a role in TGF-β signaling. The mouse homologue of Shn3 (KRC) has been implicated in tumor necrosis factor (TNF)-receptor driven responses and interacts with c-jun regulating IL-2 expression in tissue culture (Oukka *et al.* 2002; Oukka *et al.* 2004). A Schnurri2 knock-out mice was generated displaying no developmental phenotypes, but showed defects in the positive selection of thymocytes (Takagi *et al.* 2001). Very recently the C.*elegans sma9* gene was demonstrated to play a role in a TGF-β like pathway in worms (Liang *et al.* 2003).

5. Aim of this study

Most molecular studies on TGF-β signaling were performed in vertebrate cell culture systems often missing the relevance *in vivo*. In contrast, components of the TGF-β signaling pathway in *Drosophila* are mainly characterized *in vivo* exploring their role in the different cellular processes.

The *schnurr*i mutant phenotype and the genetic data obtained manifest the crucial role of the protein in Dpp target gene regulation in several tissues during embryonic development. Genetic analysis also provided a putative target gene which depends both on Dpp signaling and Schnurri activity, namely *brinker*. Nevertheless the molecular mechanisms, which regulate Dpp target genes are unresolved.

The characterization of the molecular role of Schnurri during *Drosophila* development is the subject of this thesis. So far most molecular studies were fruitless mainly because biochemical studies were hampered due to the large size of the protein. Therefore, we decided to perform a deletion analysis of the Schnurri protein to obtain both a smaller, functional protein and to characterize functional domains. To ensure reliability on the deletion approach, different Schnurri constructs were examined *in vivo* for their ability to repress *brinker* in a Dpp dependant manner. Once a shorter version would be identified, classical biochemical experiments will be performed to test for possible protein-DNA interactions and/or protein-protein interactions with Mad/Med.

II: Results

1. Functional analysis of the Schnurri protein

Genetic studies identified *schnurri* (*shn*) as a component of the Dpp-signaling pathway in *Drosophila melanogaster*. Epistatic analysis positioned the function of the gene in the Dpp-signal receiving cell, downstream of the ligand and its receptors. A main function of *schnurri*, and the only one identified so far, is to repress the expression of the transcriptional regulator *brinker* (Marty *et al.* 2000). The elements in the genomic locus of *brinker* responsible for this repression have been well characterized in the wing imaginal disc. Small Dpp-dependant silencer elements (*brk*S) antagonize a constitutive active enhancer (*brk*E) (*figure15,* Müller *et al.* 2003). Although the role of Schnurri in mediating the repression via *brk*S is genetically well established, it is still unknown whether Schnurri acts directly on *brk*S or indirectly through the activation of a repressor. Since the Schnurri protein is mainly localized in the nucleus and contains putative DNA binding zinc fingers (Arora *et al.* 1995; Grieder *et al.* 1995; Staehling-Hampton *et al.* 1995) it is tempting to speculate that Schnurri physically interacts with *brk*S. The Schnurri protein is very large (2529 amino acids) and proved to be refractory for biochemical approaches. In order to investigate the molecular function of Schnurri, a deletion analysis was performed aiming in the identification of smaller, still functional, Schnurri-derivates.

Figure15: Schematic presentation of the regulatory region of *brinker*. (Müller *et al*. 2003). *left*: The constitutive active element (*brk*E) drives *brinker* gene expression in regions were Dpp-signaling is absent. *right:* Silencer element(s) (*brk*S) sense and process the Dpp-signal to confer repression of *brinker* transcription.

1.1 Functional dissection of Schnurri *in vivo*

The strategy was to perform a series of rough deletions of Schnurri including internal deletions of putative domains, to obtain a smaller, molecular useful version of the protein. To assure the significance of the results, the constructs were examined directly *in vivo* for their capability to repress *brinker* transcription.

1.1.1 Brinker repression by ectopic expression of Schnurri in the Drosophila embryo

Genetic experiments in the embryo and in the wing imaginal disc of *Drosophila* revealed, that both Dpp signaling and Schnurri are required for repression of *brinker* transcription (Marty *et al.* 2000). The genetic tools that were established to ectopically repress *brinker* in the embryo presented the basis for the *in vivo* deletion analysis of Schnurri (*figure16*). *Schnurri* mutant embryos displayed highly upregulated levels of *brinker* mRNA compared to wild-type embryos (*figure16 a, b*). A 5kb fragment of the *brinker* genomic region fused to *lacZ* (BM14), exhibited an identical pattern to *brk* mRNA when stained with an anti β-galactosidase antibody in wild-type and s*chnurri* embryos (*figure16 c, d*). Ectopic expression of full length *schnurri* and *dpp* in seven stripes in the embryo, using the Gal4/UAS-system (Brand and

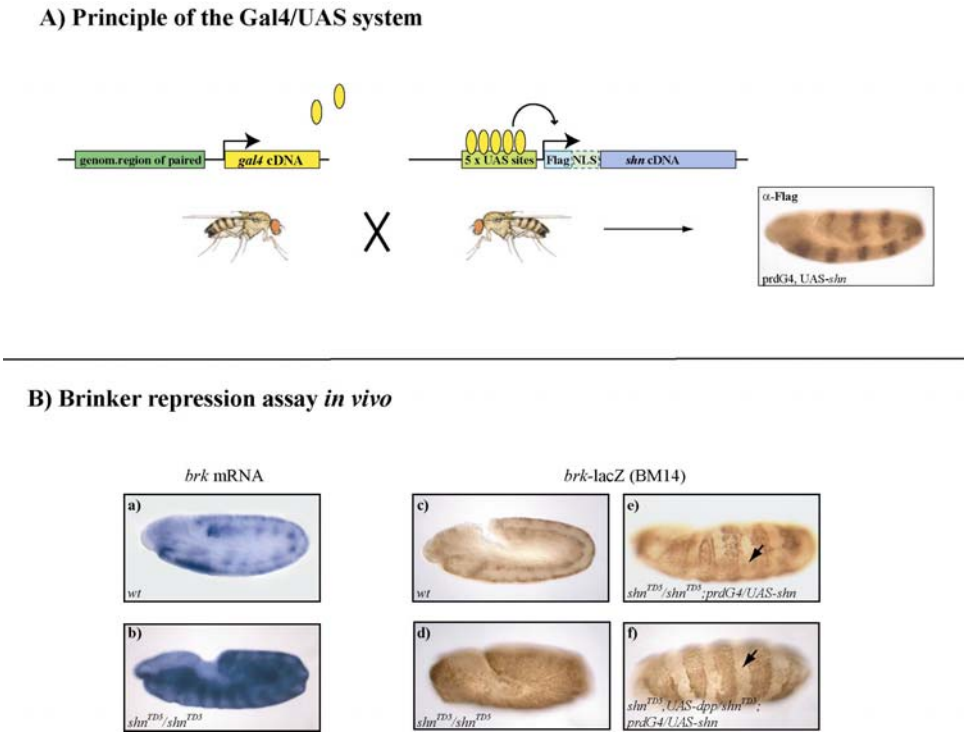


Figure16: A) Flies expressing Gal4 under the control of the genomic *paired* regulatory region (*prd*) were crossed to flies carrying a UAS-*Flag-shn* construct giving rise to embryos which ectopically expressed *shn* in the *paired* domain visualized using an α-Flag antibody. **B)** Embryos were either stained for *brk* mRNA (a,b) or β-Gal using a *brk-lacZ* reporter line (BM14; c-f). Similar results were obtained using either approach. Embryos are viewed from the lateral site (a-d) and from a more ventral position (e-f). *Brk* expression was highly upregulated in mutant embryos compared to wild-type. To assay for Shn function, either *shn* alone or *shn* and *dpp* were overexpressed in a *shn* mutant background (e,f). Ectopic expression of full length *shn* in the *prd* domain in a *shn* mutant background resulted in the repression of lacZ in seven stripes (e). Note that lacZ was not repressed in the most ventral part of the embryo where Dpp was not present endogenously. Ectopic co-expression of *shn* and *dpp* resulted in lacZ repression in seven stripes also in the ventral region (f). Similar results were obtained when embryos were stained for *brk* mRNA (*Marty et al. 2000*).

Perrimon 1993), resulted in the complete downregulation of both endogenous *brinker* (not shown) and *brk-lacZ* in every other segment (*2e, f*). Since Schnurri could also repress the *brk-lacZ* reporter in a Dpp dependant manner it could be excluded that Schnurri acts on a posttranscriptional level.

1.1.2 Deletion analysis of Schnurri in vivo

The Schnurri protein consists of 2529 amino acids, including eight zinc fingers (ZF), a region of homology (RH) and a putative Mad interaction domain (Dai *et al.* 2000). Zinc fingers 4/5 displayed a high similarity to the DNA binding zinc fingers in HIV-EP family proteins and it was speculated that Schnurri might control target genes through direct DNA contact via these pair of zinc fingers (Dai *et al.* 2000). The triplet of zinc fingers at the C-terminal end is present in the fly and worm but not in the vertebrate homologues.

Transgenic fly lines were generated each carrying a defined *UAS-shn-variant* (*figure17*). All constructs were bearing an N-terminal FLAG-epitope. In addition, a nuclear localization signal (NLS) was added to smaller Schnurri constructs to ensure nuclear targeting. A minimum of two individual fly lines for each construct were tested for *brinker* repression in the embryo using the assay described above. Expression and nuclear localization of the *Schnurri* constructs was verified using either in situ hybridization with a *shn*-antisense RNA probe or anti-Flag immunostaining (not shown). Embryos from the cross *shn^{TD5};;prdgal4,BM14 x shn^{TD5},UAS-dpp;;UAS-shn variants* were collected and stained with an anti β-galactosidase antibody for *brk-lacZ* activity. Shn-constructs that repressed the expression of the *brinker* reporter in a similar way as full length Schnurri were scored functional.

Initially, Schnurri subfragments bearing large deletions removing at least one of the annotated domains (*figure17b-k*) were tested. The N-terminal part of Schnurri containing zinc fingers 1/2 and the single zinc finger 3 (*figure17 b,* Shn1-909) did not show repression of the reporter *in vivo*. Surprisingly Shn1-1888 which comprises zinc fingers 1/2, zinc finger 3 and the putative DNA binding zinc fingers 4/5, hence exhibiting all the domains conserved in the vertebrate homologues, was unable to repress *brinker in vivo*, suggesting that the C-terminus is required (*c*). A 908 amino acid truncation of the N-terminus, including zinc fingers 1/2 (*figure17 d*; Shn909-2529) did not abolish repression *in vivo*. Shn909-2529 contained zinc finger 3, zinc fingers 4/5, the putative Mad interaction domain and at the very C-terminal end the zinc fingers 6/7/8. Since the highly conserved zinc fingers 4/5 region has been shown to bind DNA and the C-terminal part of Schnurri was essential for repression, the construct Shn909-2529 was believed to be the "minimal" functional Schnurri. Consequently,

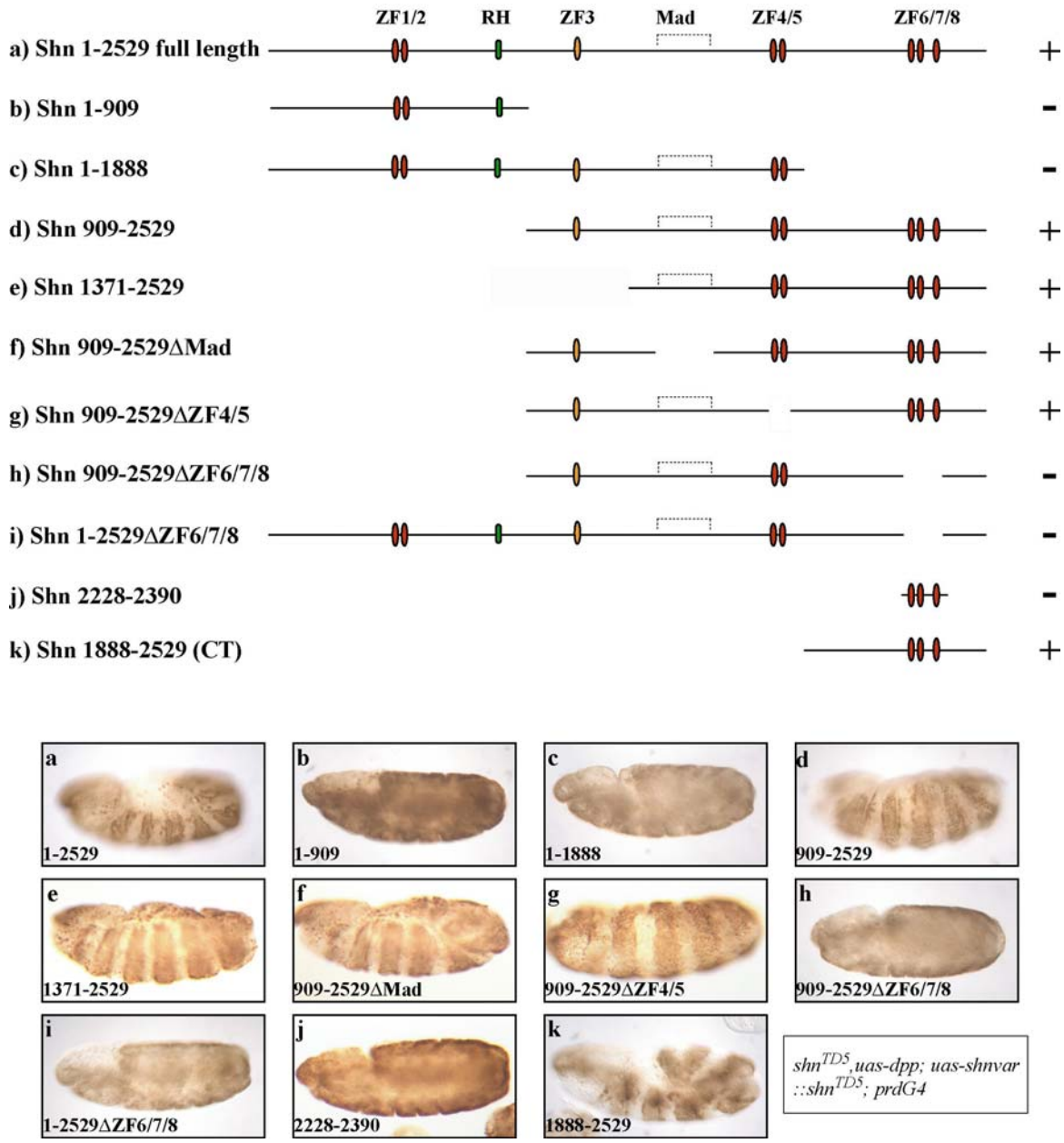


Figure17: Deletion analysis of Shn. Different Shn derivates were analyzed for functionality *in vivo* (see text). The domains are in their relative position in the protein. Embryos were stained for *brk*-lacZ (BM14). *Shn* mutant embryos were recognized by highly upregulated levels of reporter gene expression due to the lack of functional *shn*. The different versions of *shn* were overexpressed together with *dpp* in every other segment using a pairedGal4 driver line to rescue *brk* repression. Repression of *brk*-lacZ was observed in seven stripes for all constructs containing the C-terminal part of the protein including the zinc fingers 6/7/8 (a, d, e, f, g and k). ShnCT (k) presented the smallest functional version of Shn.

truncations and internal deletions of the Shn909-2529 fragment were tested. Deletion of its N-terminal part removing zinc finger 3 and a construct lacking the Mad interaction domain did not interfere with Schnurri repression *in vivo* (*figure17 e, f*). Surprisingly, also an internal deletion removing zinc fingers 4/5 did not affect *brk-lacZ* repression (*figure17 g*). In contrast,

deletion of the unconserved zinc finger triplet 6/7/8 completely abolished repression (*figure17 h*). To further verify this unexpected result, zinc fingers 6/7/8 were also deleted in the context of the full length protein (*figure17,* 1-2529). Indeed this construct was unable to repress the transcription of the *brinker* reporter, confirming that zinc fingers 6/7/8 are essential for repression. Finally and to our surprise a 641 amino acids long C-terminal fragment of Schnurri containing the zinc finger triplet 6/7/8 (*figure17 k,* Shn1888-5229) was able to repress *brinker in vivo*. However, a smaller construct expressing only zinc fingers 6/7/8 (*figure17 j,* Shn 2228-2390) was not capable of *brinker* repression, concluding that zinc fingers 6/7/8 although essential are not sufficient for Schnurri function and that additional flanking sequences are required for proper function.

In summary, the C-terminal 641 amino acids of Schnurri including zinc finger 6/7/8, (from now on referred to as ShnCT) were essential and sufficient for *brinker* repression *in vivo* and presented a solid frame for further molecular studies. Sequence similarity searches with the Shn1888-2529 protein revealed that this domain is present in the Schnurri homologues of the mosquito *Anopheles gambiae* (AgShn) and of *Caenorabditis elegans (sma-9)*. Yet no similar proteins or protein domains were identified in higher eukaryotes so far.

1.3 Mad/Medea/Shn form a complex on the *brinker* silencer element

The discovery of ShnCT, a functional subfragment of Schnurri with a moderate size of 641 amino acids finally permitted biochemical approaches towards the identification of the molecular function of Schnurri in the context of *brinker* repression. Mutant clones in the wing imaginal disc either for the Dpp signal transducers *mad, medea* or the zinc finger protein *schnurri*, resulted in the cell autonomous upregulation of *brinker* transcription (Müller *et al.* 2003). Fortunately, the regulatory region of *brinker* was extensively characterized and an element responsible for Dpp- and Schnurri-dependant repression was isolated and narrowed down to 56bp (*brkS,* Müller *et al.* 2003). The Smad proteins, Mad and Medea have been reported previously to physically interact with DNA and several putative Smad binding sites were present within *brk*S (*figure18*, *top*). So far, DNA binding properties of Schnurri have been assigned to zinc finger 4/5, yet these were not required for *brinker* repression *in vivo*. Nevertheless, a biochemical approach was undertaken to test the possibility of a direct protein-DNA interaction using electrophoretic mobility shift assays (EMSA).

In this assay, a small fragment of radioactive labelled DNA (probe) is incubated with the proteins of interest and the protein/DNA mixture is separated by native polyacrylamide gel electrophoresis. Formation of a DNA-protein complex results in a slower migration of the bound DNA probe compared to the free probe and can be visualized by autoradiography.

For our purposes, radioactive-labelled *brk*S was used as a probe. Epitope labelled versions of Mad, Medea and ShnCT were expressed in Drosophila Schneider cells (^{Flag}Mad, ^{Myc}Medea, and ShnCT ^{V5}). A constitutive active form of the type I Dpp-receptor Tkv (Tkv^{QD}, Nellen *et al.* 1996) was co-expressed to induce Dpp signaling (see below).

In a first set of experiment, lysates from cells expressing the proteins, Mad, Med and ShnCT alone or in combinations were tested for their ability to bind *brkS*. Neither of the proteins was capable of binding to *brk*S by its own (*figure18A, lane3-5*) except for Mad which formed a weak complex with the probe. Intriguingly, lysates of cells overexpressing both Mad and Medea formed a prominent protein complex with *brkS* (*figure18A, lane 7*), suggesting that these proteins bind to the silencer in combination. Use of lysates from cells expressing ShnCT in addition to Mad and Medea resulted in the assembly of a DNA-protein complex of even slower migration properties (*figure18A, lane 8*). Note that this second DNA-protein complex

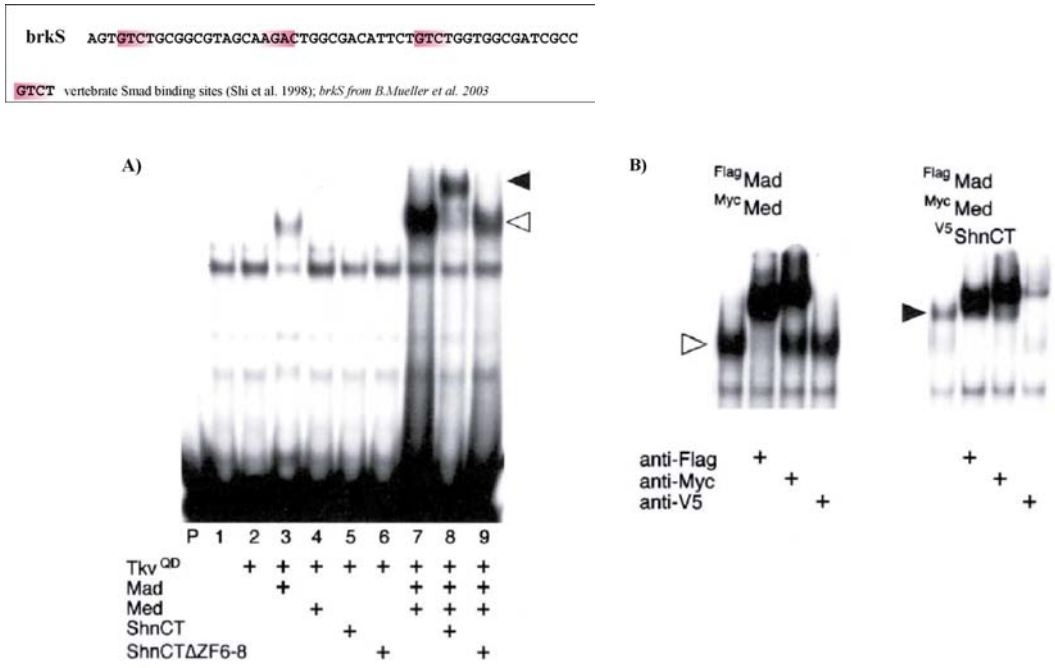


Figure18: The sequence of *brkS* is presented as it was characterized by B. Mueller. The pink boxes indicate putative Smad binding sites within this element. **A)** Lysates of S2 cells transfected with the indicated plasmids were analyzed in EMSA using labeled *brk*S as a probe. Transfection of Mad and Med lead to the formation of a protein/DNA complex of slow mobility (lane 7, indicated by the open arrowhead), which could be further retarded by cotransfecting ShnCT (lane 8, filled arrowhead), but not ShnCTΔZF6-8 (lane 9). Note that in single transfections, only Mad had the ability to form a complex with *brk* S (lanes 3–6). Radiolabeled probe *brk*S was loaded alone (lane P) and after incubation with extract from untransfected cells (lane 1 and 2). **B)** Lysates from cells expressing ^{Flag}Mad, ^{Myc}Med without ^{V5}ShnCT (left), or with ^{V5}ShnCT (right) in combination with Tkv^{QD} were subjected to band shift assays in the presence of the indicated antibodies. Positions of the Mad/Med-complex (open arrowheads) and Mad/Med/ShnCT complex (closed arrowheads) are indicated. In the absence of ShnCT, the complex contained both Mad and Med proteins as evidenced by supershifts with both the anti-Flag and the anti-Myc antibodies. In the presence of ^{V5}ShnCT, the low mobility complex was supershifted by antibodies directed against the V5 epitope.

was formed in the expense of the putative Mad/Med/*brkS* complex.

The only known structural motif within ShnCT is the triplet of zinc fingers 6/7/8 which was already demonstrated to be essential *in vivo*. Indeed a ShnCT construct lacking the zinc finger triplet (ShnCTΔZF) was unable to assemble the complex observed with ShnCT (*figure18A, lane 9*). This data indicated for the first time that Schnurri has the potential to interact physically with *brkS* and that *brinker* may represent a direct target of Schnurri.

To demonstrate the presence of Mad, Medea and ShnCT in the corresponding complexes so-called "supershift" experiments were performed. Specific antibodies against the epitopes fused to each protein were included in the binding reaction and should result in an additional retardation of the DNA-protein complex due to incorporation of the antibody. Lysates from cells expressing ^{Flag}Mad, ^{myc}Medea and ShnCT^{V5} were assayed for the presence of the respective epitopes in supershift analysis. In the absence of ShnCT the complex contained both Mad and Medea proteins (*figure18B, left*). In the presence of ShnCT^{V5} the low mobility complex was additionally supershifted with antibodies against the V5 epitope (*figure18B, right*). From these biochemical experiments it can be concluded that Mad and Medea form a protein complex on the *brinker* silencer. Schnurri was not able to bind to the DNA alone but was recruited to the preassembled Mad/Med/*brk*S complex.

1.3.1 Complex formation is signal-dependant

The signal dependant phosphorylation of the R-Smads in their C-terminal SSXS motif was shown to be required for interaction with the Co-Smad and subsequent nuclear import of the heteromeric complex in the nucleus. Neither Mad nor Medea were capable of binding to *brk*S by themselves suggesting that the signal-mediated heteromerization of the two Smad proteins is required for DNA binding. To test this hypothesis proteins were expressed in Drosophila Schneider cells either in the absence or the presence of Tkv^{QD} and lysates were assayed for their ability to form complexes on *brk*S (*figure19*). Indeed the Mad/Med/*brk*S complex could only assemble when Tkv^{QD} was co-expressed (*compare lanes 2 and 4*). As expected, also the assembly of the

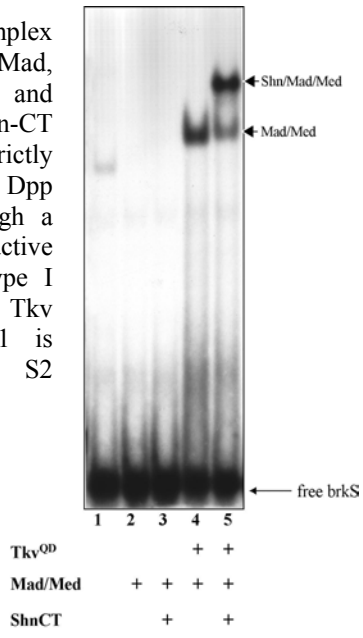


Figure19: Complex formation of Mad, Med on *brk*S and Mad, Med, Shn-CT on *brk*S strictly depends on Dpp signaling through a constitutive active form of the type I receptor Tkv (Tkv^{QD}). Lane1 is non-transfected S2 cell extract

Mad/Med/ShnCT/*brk*S complex was dependant on Tkv^{QD} (*compare lanes 3 and 5*) as formation of the Mad/Medea complex on *brk*S is a prerequisite for the recruitment of ShnCT.

1.3.2 Minimal requirements of Schnurri for complex formation with Mad/Medea on brkS

A number of Smad cofactors have been identified in vertebrates and several of the interaction domains have been analysed in more detail. Schnurri represents the first Mad/Med cofactor described in *Drosophila.* For this, it was particularly interesting to characterize the molecular events on the *brinker* silencer starting with the domain(s) of Schnurri required for complex formation. Although the zinc finger domain of ShnCT was found to be required for recruitment to the Mad/Med/*brk*S complex additional sequences could be equally important. To test this hypothesis, a series of deletion constructs of ShnCT were generated and analyzed in electrophoretic mobility shift assays as described above. Removal of the C-terminal 75 amino acids of ShnCT (ShnCTΔC) did not affect recruitment to the complex. Similarly, a serial deletion from the N-terminus of ShnCT did not abolish the ability of the truncated polypeptides to bind to the Mad/Med/*brk*S complex. Indeed a construct comprising only zinc fingers 6/7/8 was able to form a complex with Mad and Medea on *brk*S. The triplet of zinc fingers, ZF6/7/8, therefore is not only essential but also sufficient for interaction with the Mad/Med/*brk*S complex (*figure20*).

To examine the requirements of the individual zinc fingers mutations of both characteristic cysteine residues were introduced in the context of ShnCT, in order to destroy the three dimensional structure of the domain. Inactivation of zinc finger 6 and zinc finger 8, in the context of ShnCT, abolished complex formation completely, whereas mutations in zinc finger

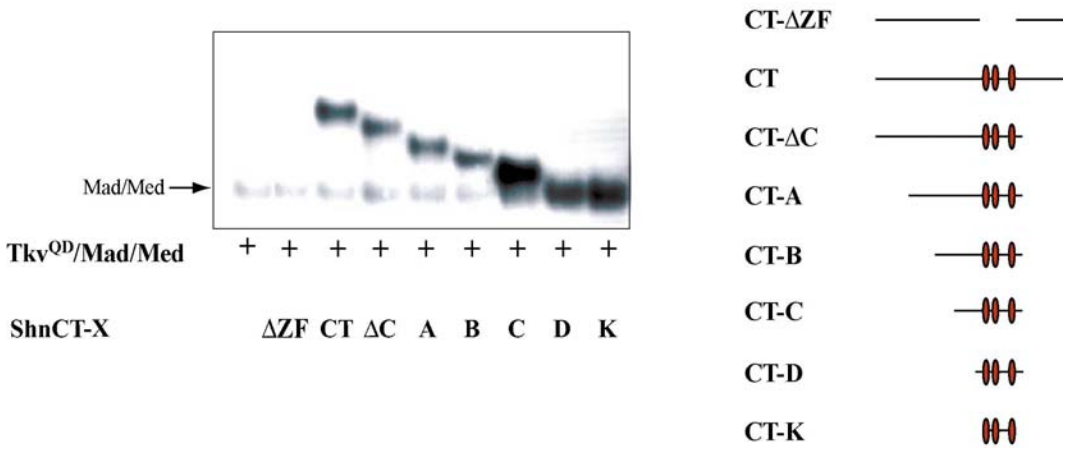


Figure20: Several ShnCT deletions were analyzed in EMSA experiments for their ability to assemble a complex with Mad and Med on *brk*S. ShnCT bearing a deletion of the zinc fingers 6/7/8 was not recruited to Mad/Med/*brk*S, whereas neither N-terminal nor C-terminal truncation did affect this ability. ShnCT-K, which comprises only the zinc finger domain presents the minimal requirement for complex formation.

7 did not show any effect and the triple complex was still formed (*figure21A*).

In addition, the putative DNA contacting amino acids within each zinc finger were mutated to disturb potential DNA contact without affecting the overall structure of the zinc finger domain. Changes in the respective amino acids within zinc finger 6 or zinc finger 8 abolished

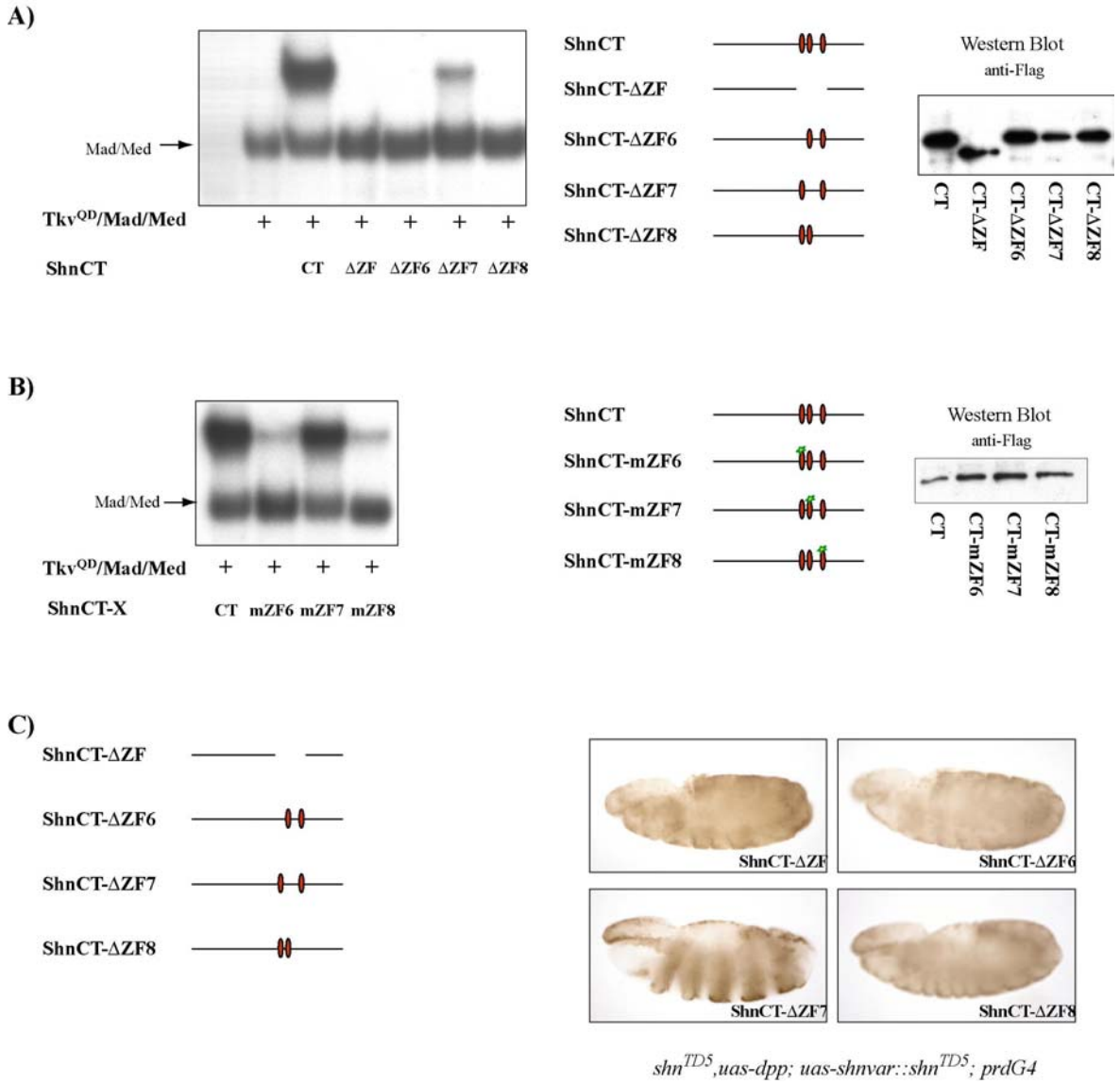


Figure21: A) EMSA: S2 cells were cotransfected with Tkv^{QD}, Mad and Med. Shn derivates were produced using rat reticolocyte lysate for *in vitro* transcription and translation and added separately to each binding reaction to assay for binding to *brk*S. Correct expression of Shn derivates was verified in western blot analysis. Minimal amounts of Mad/Med expressing lysates were used in the reaction to be able to recognize the small complexes which contained smaller Shn version (e.g. ZF6/7/8) compared to ShnCT. **B)** EMSA. ShnCT versions bearing mutations in putative DNA binding amino acids (indicated by a green star) were tested for complex formation with Mad/Med on *brk*S. **C)** *In vivo* assay as described in R1.1.1. Deletion of all three zinc fingers (ZF6/7/8) or only ZF6 or ZF8 resulted in a loss of *brinker* repression activity *in vivo*. However, mutations in ZF7, which did not affect complex formation in EMSA, were still capable of suppressing *brinker in vivo*.

complex formation completely. In line with the observation that zinc finger 7 is dispensable for complex formation, mutations in putative DNA binding residues in this zinc finger did not affect complex formation (*figure21B)*. The results indicate that ZF6 and ZF8 might make contact to DNA, though this would need further proof and will be subject in the discussion. The relevance of the individual zinc fingers in *brinker* repression was also assayed *in vivo*. To this end, transgenic flies bearing UAS versions of ShnCTΔZF6, ShnCTΔZF7 and ShnCTΔZF8 were generated and compared to the *UAS-shnCT* fly for their ability to downregulate the expression of the *brk-lacZ* reporter as described in 1.2 (*figure21C*). The inactivation of the zinc finger 6 or 8 resulted in a complete loss of repressive activity, similar to a ShnCT version were all three zinc finger were deleted (ShnCTΔZF, *figure21C*). In contrast, inactivation of the zinc finger 7 in the context of ShnCT did not affect the activity of the protein. (*figure21C*). The *in vivo* results are in agreement with the biochemical results and assign an essential role for the zinc fingers 6 and 8 in both the assembly of the Mad/Med/Shn/*brk*S complex and the repressive activity of Schnurri.

The biochemical data so far demonstrated that Dpp-signaling induces the assembly of a Mad/Med/Shn complex on the *brinker* silencer element. Since both the protein and the DNA components of this complex were shown to be genetically required for the repression of *brinker* it is very likely that formation of the complex is a prerequisite for transcriptional repression. In support of this correlation, all mutations in Schnurri tested so far that disturb complex formation result in a complete loss of the activity of the protein to repress *brinker*.

1.4 Fine mapping of functional domains within ShnCT using a tissue culture system

The recruitment of Schnurri to a preassembled Mad/Med/*brk*S complex is probably the key event for the complex to achieve repressive activity. Genetic experiments identified a subfragment of Schnurri, ShnCT, which was sufficient to substitute for the full-length protein in the repression of *brinker in vivo*. Within ShnCT a triplet of zinc fingers was found to be required both for the assembly of the complex and for its repressive activity. Interestingly, a ShnCT derivative comprising just the zinc finger triplet although sufficient for complex formation was found to be unable to mediate *brinker* repression *in vivo (see figure17 j)*. This suggests, that the repressive activity of ShnCT maps in a region other than the zinc finger triplet. To identify the domains conferring repressive activity to ShnCT we established a cell culture assay which was established in the lab allowing fast and quantitative analysis of Dpp-induced repression *(Müller et al. 2003)*.

1.4.1. Reconstitution of Dpp signaling in Drosophila cell culture

Schneider S2 cells are originally embryonic Drosophila cells which can be kept in culture and therefore present an "almost" *in vivo* environment. The principle of the system is outlined in figure 22. The activity of the *brinker* silencer element was assayed in the context of an inducible enhancer fused to a minimal promoter and the reporter gene lacZ. This synthetic enhancer contained four tandem Suppressor of Hairless (Su(H)) binding sites. A 30- to 40-fold stimulation of *lacZ* expression was induced upon transfection of S2 cells with plasmids driving the expression of (Su[H]) and a constitutive active form of Notch (N*) (*figure 22B* right panel). *(Kirkpatrick et al. 2001; Müller et al. 2003)*. Simultaneous co-transfection of a plasmid encoding the activated form of the Dpp receptor Tkv (Tkv^{QD}) blocks this activation, in a dosage dependant manner (*figure23B, right panel)*. The effect was strictly dependant of the *brk*S fragment, since TkvQD cotransfection did not affected the Notch/Su[H] response of the control reporter.

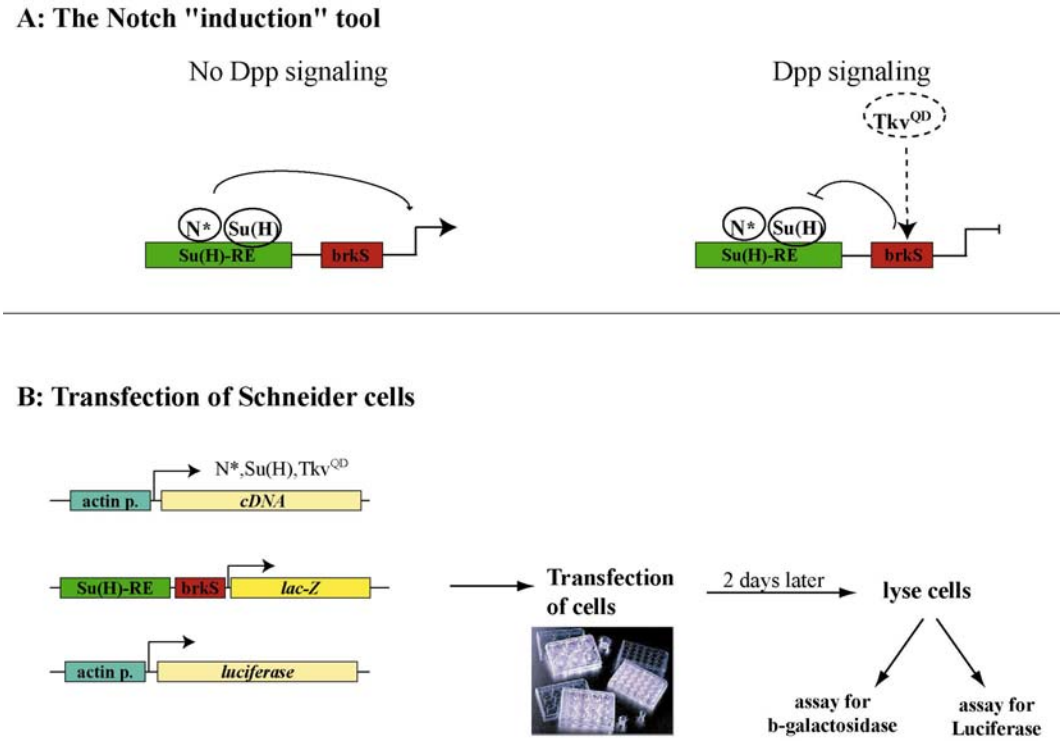


Figure22: A) In cell culture experiments the Su(H)-RE represents the active element and the brkS, which was identified *in vivo* the silencer element. Expression should be high in the presence of N* and Su[H] and the absence of Dpp signaling. Dpp signaling, induced by Tkv^{QD} transfection, should lead to the downregulation of reportergene expression. **B)** Schematic representation of the experimental setup: Proteins are constitutively expressed under the control of the actin promotor (actin p.). The reporter cassette was fused to a reportergene, *lacZ*. *actin-luciferase* was used to standardize the transfection efficiency. Plasmids were transfected and cells were harvested and lysed 2 days after. The activity of *lacZ* (β-galactosidase) and Luciferase activity was messured in an enzymatic reaction. *N*: activated form of Notch; Su[H]: Suppressor of Hairless; RE: response element*

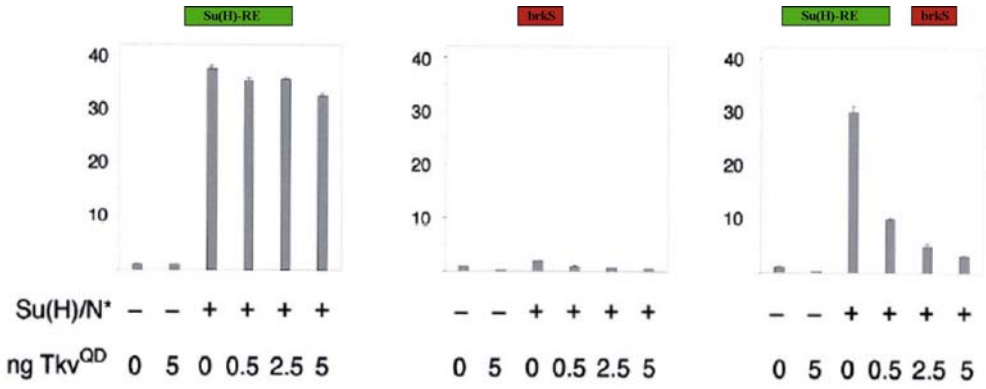


Figure23: β-galactosidase reporter assays in *Drosophila* S2 cells. Reporter plasmids contain the *lacZ* gene under the control of a Suppressor of Hairless response element (Su[H], in green, left), a fragment of the *brk* control region containing the subfragement S (*brk* silencer, in red, middle), or the combination of the two elements (right). These reporters were cotransfected with a combination of plasmids encoding Su(H) and an activated form of Notch (N*). Increasing amounts of a plasmid expressing Tkv^{QD} lead to a stepwise repression of reporter activity (right). Tkv^{QD}-mediated repression is strictly dependant on the presence of the *brk* silencer since it is not observed with the reporter containing only the Su(H) response element (left). Results shown represent the average β-galactosidase activities from transfections done in triplicates (+/- standard deviation) and are expressed as the X-fold activation over the basal activity of each reporter plasmid alone. (*From B. Mueller et al. 2003; G. Pyrowolakis*)

The fact that Tkv^{QD} was sufficient for *brkS* mediated repression in S2 cells implicated that the molecules necessary for signal transduction and repression are endogenously present in this cell line. Indeed depletion of endogenous Mad or Schnurri by RNAi did not interfere with the N*/Su[H] mediated activation of Su(H)-RE but abolished almost completely the repressive effect of Tkv^{QD} (*figure24A*). Treatment of cells with double stranded RNA for S6K as a control, did not interfere with either activation by N*/Su[H] nor with repression by Tkv^{QD} proving the selectivity of this approach (data not shown). Hence Dpp-mediated repression of *brkS* in Schneider S2 cells depended on the signal mediators Mad, Medea and Schnurri, faithfully recapitulating the *in vivo* situation.

The finding that depletion of endogenous Schnurri by RNAi in this system results in a complete inactivation of signal- and *brkS*-mediated repression of the reporter presented the basis for the following "rescue" experiments. To this end Schnurri variants were transfected in S2 cells and tested for their ability to substitute for RNAi-depleted endogenous Schnurri. Indeed, pilot experiments confirmed that ShnCT can restore Tkv^{QD}-mediated repression of the reporter when transfected in Schnurri depleted cells (*figure24B*). Furthermore, and in line with the *in vivo* results, the clustered zinc fingers in ShnCT were critical, since the ShnCTΔZF

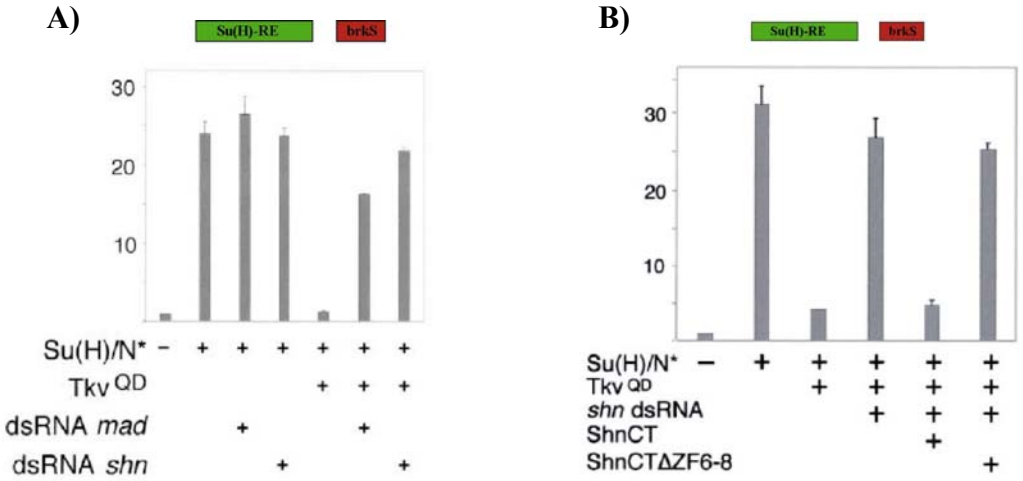


Figure24: A): *Drosophila* S2 cells were cotransfected with a reporter plasmid containing the *brk*S fused to the Su(H)-RE, expression plasmids and dsRNA fragments as indicated below the panel. Tkv^{QD}-mediated repression is blocked when endogenous Mad or Shn are "knocked down" by RNAi. β-galactosidase values are shown as x-fold activation over the basal activity of the reporter plasmid when cotransfected with the empty expression vector. dsRNA fragments are derived from *mad* (nucleotides 658-1230) or the *shn* (nucleotide 5011-5531) coding regions. **B)** S2 cell reporter gene assays. Cells were transfected with the plasmids indicated in combination with *shn* dsRNA to downregulate the expression of the endogenous Shn protein. The loss of Tkv^{QD}-mediated repression caused by *shn* RNAi can by restored by coexpression of ShnCT, but not ShnCTdZF6-8. Note that the dsRNA used does not affect expression of the transfected C-terminal Shn fragments, since it is derived from an upstream part of the *shn* coding region (corresponding to amino acids 1670–1842). Expression of the Shn constructs had no effect on the Notch response as judged by cotransfections with the reporter plasmid containing the Su(H)-response element (data not shown). *(From B. Mueller et al. 2003; G. Pyrowolakis)*

construct lacking this domain could not restore repression (*figure24B*). Similarly the requirements of the single zinc fingers in this assay reflected the *in viv*o requirements. Inactivation of ZF6 and ZF8 but not ZF7 resulted in a complete loss of the ability to restore repression (not shown). Taken together these results prove that the cell culture system presented a reliable and rapid tool for further analysis of Dpp-mediated repression via *brkS*.

1.4.2 Characterization of a "repression domain" (RD) within ShnCT

The Schneider S2 cell culture assay was employed to define regions within ShnCT that are responsible for its repression activity. Truncated versions of ShnCT were tested for their ability to restore Tkv^{QD}-mediated repression of *brkS* in S2 cells depleted for endogenous Schnurri (*figure 25A*). Deletion of the C-terminal part of ShnCT (ShnCTΔC; amino acids 1888-2390) did not affect functionality, suggesting that the region downstream of the zinc finger domain is not essential. Deletion of the N-terminal 113 amino acids (ShnCT-A; amino acids 2001-2390) completely abolished the activity of ShnCT in this assay. Interestingly, this region shows weak but significant similarity to the corresponding region of the *Anopheles gambiae* Schnurri protein (*figure26).* Other regions, with the exception of the sequences of the

zinc finger domains, are clearly less conserved between the two proteins. Within this region of similarity the conservation is highest in a short fragment of 23 amino acids at the N-terminus. Removal of this fragment in ShnCT (ShnCT-H; amino acids 1911-2390) resulted in a reduction but not a complete loss of repressive activity (*figure 25A*).

Direct fusion of the N-terminal 113, or 203 amino acids of ShnCT to zinc fingers 6/7/8 were constructed (ShnCT-G; amino acids 1888-2001+ZFs and ShnCT-F; amino acids 1888-2091+ZFs, respectively). Both fusion constructs were able to completely replace the function of endogenous Schnurri in the S2 cell assay, demonstrating that sequences between the N-terminal 113 amino acids and the zinc finger domain are dispensable (*figure 25A*).

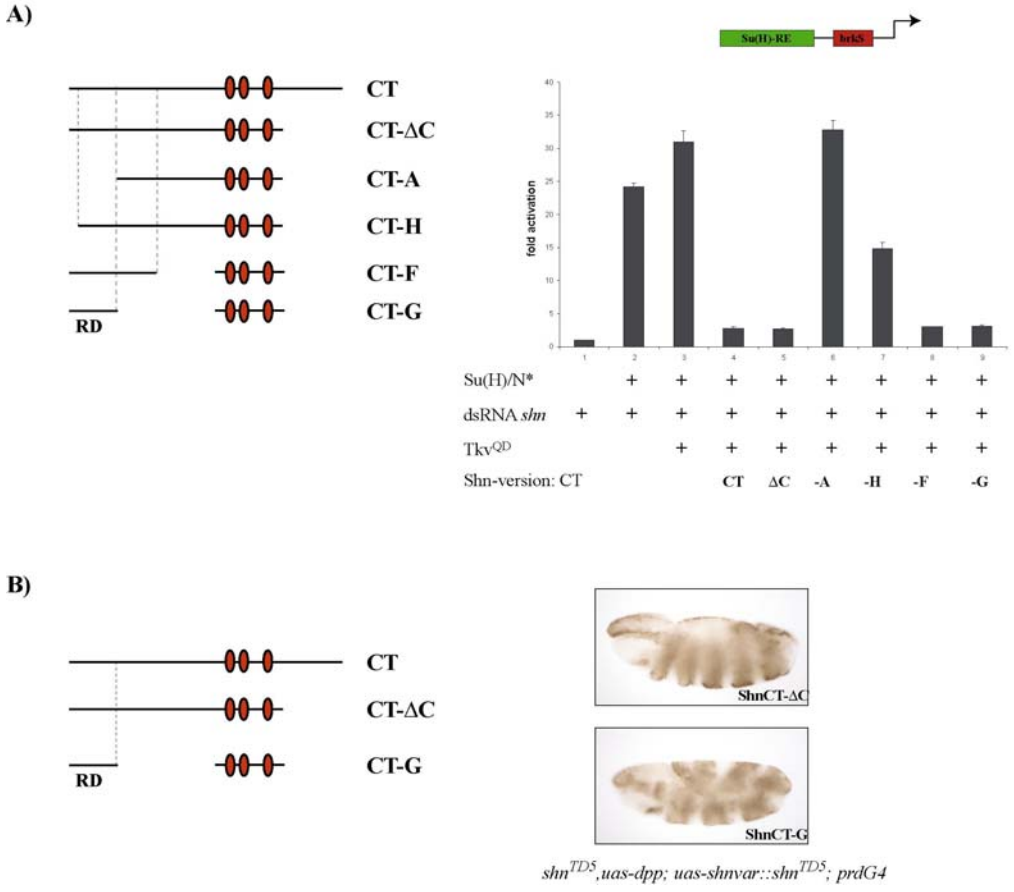


Figure25: A) Truncated versions of ShnCT were assayed for their capacity to rescue Shn function in S2 cell which were depleted of endogenous Shn. Deletion of C-terminal sequences (ShnCTΔC) had no affect on repressive function in this assay. In contrast, truncation of ~100 amino acids N-terminally (ShnCT-A) resulted in the loss of rescue ability. A fusion of this domain to the zinc fingers 6/7/8 again could rescue Shn depletion. **B)** The two constructs, ShnCTΔC and ShnCT-G were also examined for their function *in vivo* in the assay described in the beginning. Both constructs completely repressed *brinker* as it is seen for ShnCT and Shn.

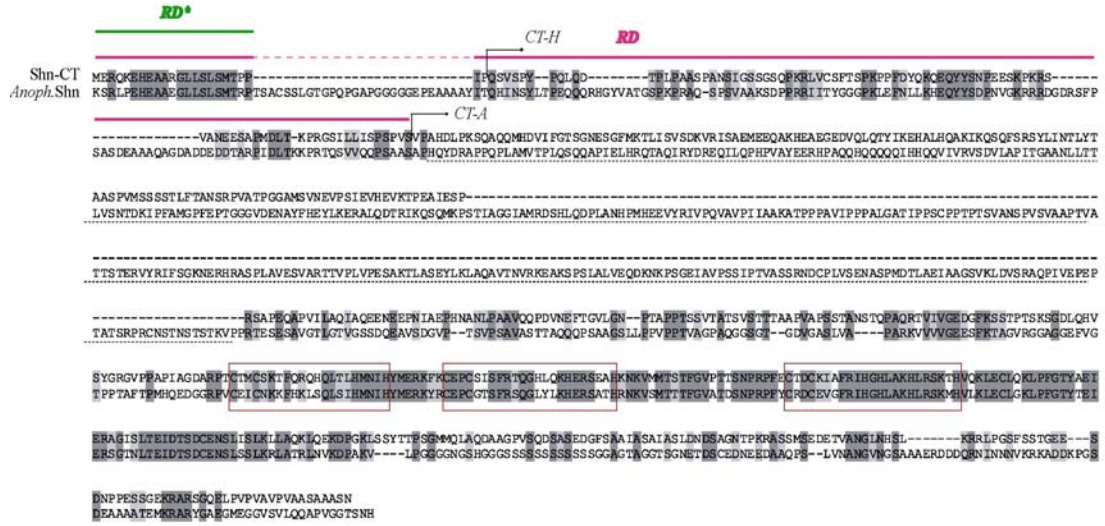


Figure 26: BLAST alignment of ShnCT (1888-2529) with *Anopheles gambae* Shn. Zinc finger 6, 7 and 8 are marked with red boxes. Start of deletion constructs CT-H and CT-A is indicated with arrows. The repression domain (RD) is lined-out in pink. The N-terminal 23 amino acids (R* - green line) show highest similarity compared to *Anoph*. Shn. Dark gray mark identical amino acids, light grey similar amino acids. Sequence of low identity is underlined with dashed line

To verify these results transgenic lines were generated and tested *in vivo* as described in 1.2. In line with the cell culture assay the deletion of the region C-terminal to the zinc finger domain did not abolish the ability of the construct to repress the *brk-lacZ* expression in the *Drosophila* embryo (*figure 25B*). Similarly the direct fusion of the 113 N-terminal region of ShnCT to the zinc finger domain was sufficient for *brk-lacZ* repression.

In summary a region of 113 amino acids (1888-2001) was identified and demonstrated to be essential for the repression activity of Schnurri. This fragment was also shown to be sufficient for repression when combined to the zinc finger triplet of Schnurri. Thus apparently ShnCT exhibits two activities located on two different regions of the polypeptide (*figure27*). The *complex formation* activity maps in the zinc-finger region of Schnurri and is essential for recruitment of Schnurri to the Dpp-inducible Mad/Med/*brk*S complex. The *repression domain* locates in amino acids 1888-2001 of the Schnurri protein and is responsible for the repressive activity of the Mad/Med/Shn/*brk*S complex (*figure27*).

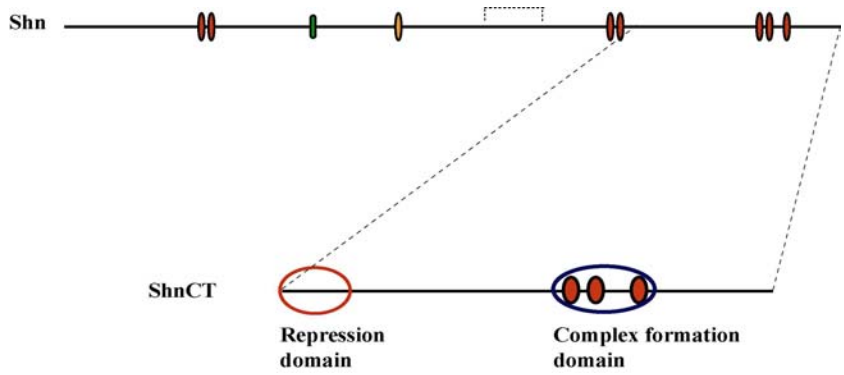


Figure 27: ShnCT is composed of two independant modules: An N-terminal repression domain (red circle) and a complex formation domain (blue circle) which comprises the triplet of zinc fingers.

1.4.2.1. The repression domain of Schnurri (RD) retains activity when targeted to the DNA

A tempting assumption was that the two domains of Schnurri are not dependant on each other to exert their activities. This is indeed the case for the complex formation domain of Schnurri, which was shown to assemble a complex with Mad/Med and *brk*S even when isolated from the rest of the protein. To test if this applies also for the repression domain of Schnurri, the region was fused to well-characterized DNA-binding domains of unrelated proteins and the constructs were tested for repressive activity. A chimeric protein containing the repression domain of Schnurri fused to the DNA-binding domain of Brinker was constructed and expressed in S2 cells. The reporter cassette consisted of four Su(H)-binding sites for Su[H]/N*-inducible activation followed by six binding sites for Brinker fused to the minimal *hsp70* promoter and the *lacZ* gene. Constructs expressing the Brinker DNA-binding domain alone or full-length Brinker were used as negative and positive controls respectively. Indeed expression of increasing amounts of full length Brinker resulted in a stepwise reduction of Su[H]/N*-mediated reporter activity (*figure 28*) while expression of the DNA-binding domain of Brinker alone had no effect (not shown). Expression of the Shn-repression domain (RD) fused to the Brinker DNA-binding domain had an even more profound effect on reporter activity since it repressed *lacZ* expression at even lower concentrations compared to Brinker itself (*figure28*). A fusion of the 23 amino acids (RD*) that are most conserved to *Anopheles* Schnurri, retained some repressive activity when fused to the Brk DNA-binding domain

These results confirm that Schnurri contains a repression domain, which was capable to confer repression when targeted to DNA and confirms our model that it is the recruitment of Schnurri that confers repressive activity to the Mad/Med/Shn/*brk*S complex.

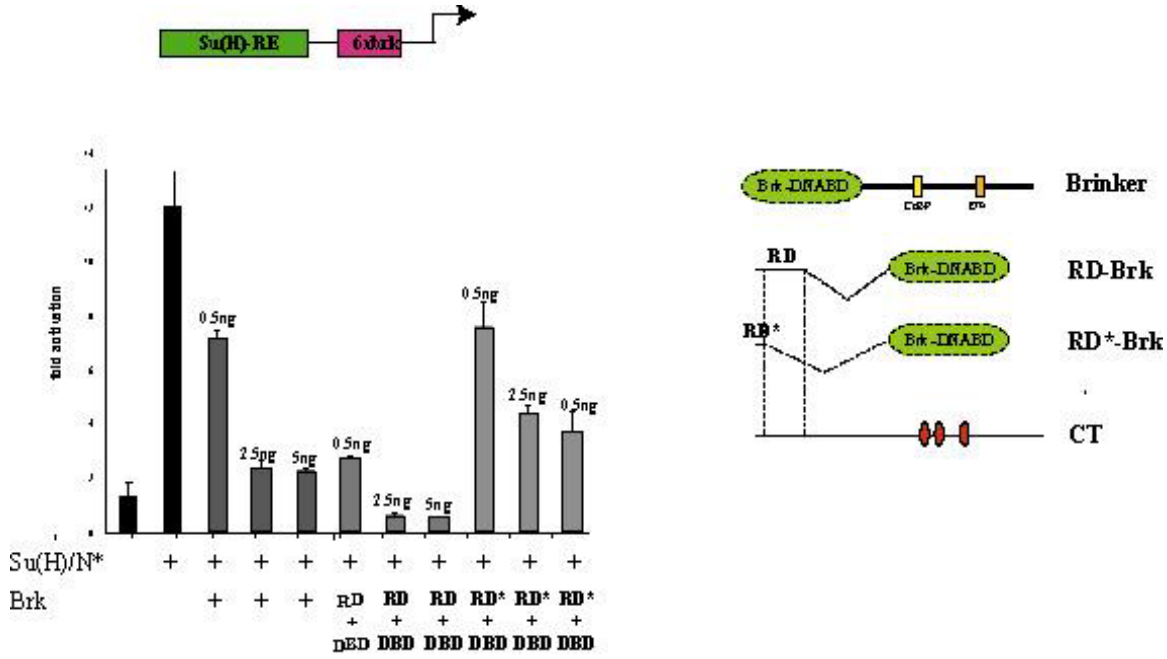


Figure28: A) β-galactosidase assay in Schneider cells. A reporter construct consisting of the Su(H)-RE fused to 6 Brinker binding sites were transfected (Kirckpatrick *et al.* 2001). Reportergene activity was induced upon transfection of Su[H]/N*. Transfection of Brinker resulted in a downregulation of *lacZ* activity. The fusion of the DNA binding domain of Brinker to the repression domain of Shn (RD) or a smaller derivate of RD (RD*) also conferred repression on this element, even more efficiently then Brinker itself. The construct containing the RD compared to RD* was much more effective. A similar relation between those domains was observed when the repressive activity of ShnCT was compared to ShnCT-H.

1.5 Vertebrate Smads assemble a complex with *brk*S and recruit Schnurri

Although no obvious vertebrate homologues of ShnCT exist we asked whether the vertebrate Smads could substitute for the *Drosophila* Smads in complex formation on *brk*S.

To this end the human Smad1 and Smad4, which are the closest homologues to the *Drosophila* proteins Mad and Medea, were tested for their ability to form a complex on the *brinker* silencer and to recruit Schnurri to this complex. Indeed lysate of cells transfected with Tkv^{QD}, Smad1 and Medea were subjected to EMSA with *brk*S as a probe formed a low mobility complex (*figure29, lane 3)*. Furthermore, this complex was able to recruit ShnCT (*figure 29, lane 4*). The same is true for Smad4 which could successfully replace Medea in this assay and assembled a Mad/Smad4/*brk*S and a Mad/Smad4/ShnCT/*brk*S complex (*figure29, lanes 5 and 6*). In addition the two human proteins were able to form a complex in the absence of both *Drosophila* Smads and to recruit ShnCT on this complex (*figure29, lanes 7 and 8*). This result demonstrates that the structural features required for recruitment of Schnurri are conserved also in the Smad proteins of vertebrates. We assume that the

Smad1/Smad4/Shn/*brk*S complex would also possess repressive activity *in vivo*, since the repression domain of Schnurri has been shown to function autonomously.

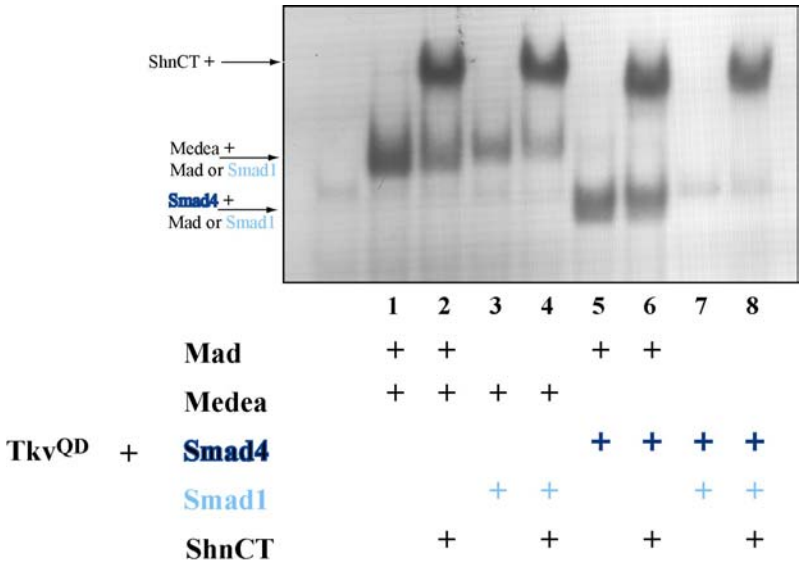


Figure 29: Lysates of cell extracts transfected with the indicated plasmids were analyzed in band shift assays using labeled *brk*S. The Mad/Med and Mad/Med/ShnCT complex is presented in lane1 and 2. Medea forms a complex with Smad1 (lane3) and both assemble ShnCT (lane4). The same result was obtained for the inverse combination of Mad with Smad4 (lane5) and additional ShnCT (lane6). Interestingly Samd1 together with Smad4 (lane7) would complex with *brk*S and also ShnCT was successfully recruited (lane8). note that Smad4 is smaller in size compared to Medea resulting in a smaller complex (e.g. compare lane1 and 5). Correct expression (and size) of the Smad proteins was verified in western blot experiments (not shown).

III: Discussion

Cell-cell interactions through signal-transduction pathways are crucial in the coordination of embryonic development. Signaling by members of the transforming growth factor β (TGF-β) superfamily is involved in a variety of diverse processes during development and at later stages. The core components of the pathway are highly conserved and have been characterized in mouse, worms and flies. The signal is transmitted to the nucleus by Smad proteins that directly regulate target gene expression. Intriguingly, only a small number of Smad molecules control the great diversity of transcriptional responses. The investigation of molecular mechanism responsible for the heterogeneity of transcriptional outputs is crucial to understand the capacity of signaling pathways.

The identification of multiple tissue-specific Smad cofactors in vertebrates offers an attractive concept of target gene regulation. In contrast, molecular studies on the nuclear response to TGF-β signaling by Decapentaplegic (Dpp) in *Drosophila* are rare. This work presents a detailed molecular analysis of the zinc finger protein Schnurri (Shn) which represents the first characterized cofactor for Dpp-mediated target gene regulation in *Drosophila melanogaster*. Moreover the results contribute significantly in understanding the role of signal induced repression which has been only poorly described so far.

1. Schnurri acts as a repressor in Dpp-mediated regulation of *brinker*

Most Dpp-target genes analyzed are repressed by the protein Brinker in the absence of signaling (Campbell and Tomlinson 1999; Minami *et al.* 1999; Jazwinska *et al.* 1999a; Marty *et al.* 2000; Kirkpatrick *et al.* 2001). Brinker itself is negatively regulated by Dpp resulting in the activation of target genes. It appears that, most Dpp target genes are controlled indirectly through the transcriptional downregulation of the repressor *brinker*. Consequently, *brinker* represents an important and tissue-unspecific target of Dpp-mediated gene regulation. The genetic data available substantiate a fundamental role of Schnurri in Dpp-target gene regulation during *Drosophila* development (Arora *et al.* 1995; Grieder *et al.* 1995; Staehling-Hampton *et al.* 1995; Marty *et al.* 2000; Torres-Vazquez *et al.* 2000). Furthermore, Dpp-dependant repression of *brinker* strictly requires *schnurri* function. However, all attempts to define a molecular role of Schnurri have failed so far, mainly because of the large size of the protein which has been refractory to biochemical approaches. To overcome this limitation a deletion analysis of Schnurri was performed to characterize and minimize the sequences required for Dpp-mediated repression of *brinker in vivo*.

1.1 The C-Terminal 640 amino acids of Schnurri are necessary and sufficient for Dpp-dependant *brinker* repression *in vivo*

A series of Shn proteins with terminal truncations and/or internal deletions were tested for their repressive activity in the embryo. Surprisingly, the zinc fingers of Schnurri (ZF1/2, ZF3 and ZF4/5), which are conserved in the vertebrate counterparts and have been demonstrated to possess DNA binding activity *in vitro* (Dai *et al.* 2000), are not required for the transcriptional regulation of *brk in vivo*. Moreover, the unconserved triplet of zinc fingers (ZF6/7/8) is essential for repression *in vivo* since constructs bearing a deletion of ZF6/7/8 in the context of ShnCT or full-length Schnurri protein completely lost of repressive activity. Finally, a 640 amino acid long Shn version (referred to as ShnCT), including only three of the eight zinc fingers (ZF6/7/8) fully recapitulates full length Schnurri function in Dpp-mediated repression of *brinker in vivo* (Müller *et al.* 2003). These findings were additionally confirmed in *Drosophila* cell culture experiments (Müller *et al.* 2003).

1.2 ShnCT is recruited to *brkS* by Mad/Med in a signal dependant manner *in vitro*

The characterization of a 640 amino acid short form of Schnurri (ShnCT) enabled us to examine the molecular role of Schnurri in Dpp-dependant *brinker* repression using biochemical approaches. To our advantage, the regulatory region of *brinker* has been analyzed in detail revealing a constitutive active element (*brk*E) and short silencer elements (*brk*A, B, S). The silencer element *brk*S was further characterized. *Brk*S was sufficient to confer Dpp/Shn-mediated repression *in vivo* and in cell culture (Müller *et al.* 2003) and consequently represented a putative target sequence for Shn/Mad/Med.

EMSA experiments were performed to test this possibility. The proteins Mad, Med and ShnCT were not capable of binding *brk*S on their own. However, the combination of Mad and Med assembled a complex on *brk*S. Furthermore, complex formation was strictly Dpp-dependant indicating that Mad phosphorylation is required to heterodimerize with Medea as it was shown for R-Smad/Co-Smad interactions in vertebrates (Wu *et al.* 2001). Interestingly, ShnCT was able to assemble a complex when Mad and Medea were present, suggesting that Mad/Med recruit ShnCT when positioned on *brk*S. Moreover, ShnCTΔZF, which only lacks the zinc fingers 6/7/8, was not recruited to the Mad/Med complex which is in agreement with the requirements of those zinc fingers *in vivo*.

1.3 Molecular architecture of the Mad/Med/Shn/*brk*S complex

Since complex formation of Mad/Med/*brk*S was strictly dependant on Dpp-signaling, and ShnCT was only recruited to *brk*S in the presence of Mad/Med, it can be inferred that the

complex can only be established in response to Dpp signaling. Moreover, recruitment of Schnurri appears to be specific to Mad/Med/*brk*S, since Mad/Med have been reported to bind to other regulatory sequences, activating the transcription of genes (Kim *et al.* 1997). In the aim to distinguish between Mad/Med-mediated activation and Mad/Med/Shn-mediated repression the molecular architecture of the Mad/Med/Shn/brkS complex was characterized and the requirements were assayed for their relevance *in vivo* (Pyrowolakis *et al.* in prep.).

1.3.1 A 16bp motif within brkS is sufficient and required for function in vivo and in vitro

A detailed mutational analysis of *brk*S was performed by G. Pyrowolakis (Müller *et al.* 2003; Pyrowolakis *et al.* in prep.) uncovering a 16bp consensus sequence in *brk*S (called C6) which is sufficient for Mad/Med/ShnCT complex assembly and still functional *in vivo* (*figure30*). Mad and a Medea binding motifs within C6 were identified and mutations in these sites abolished the formation of a Mad/Med complex. Two signatures could be determined which are essential for Shn recruitment without affecting Mad/Med complex formation. First, mutations of a single nucleotide within the Medea binding site and secondly, the length of the linker region between the Mad and Medea binding sites (*figure30)*. These requirements were further supported by *in vivo* experiments (Pyrowolakis *et al.* in prep.).

Taken together, the *brk*-C6 contains highly specific sequences which determine the assembling of a repressive inactive Mad/Med/Shn versus a repressive active Mad/Med complex.

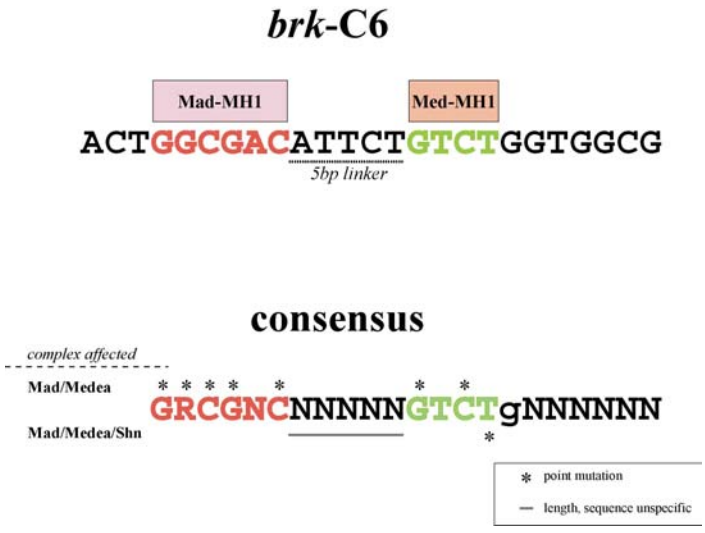


Figure30: *Top:* Sequence of the minimal *brk* silencer element C6. Mad-MH1 and Med-MH1 binding sites are indicate in red and green, respectively. A linker of 5bp separates the two sites. *Bottom:* a detailed mutation analysis of C6 revealed sites which are important for Mad/Med complex formation (upper site of the strand) and others which are required for Shn recruitment to Mad/Med (Note that a double complex is still formed when the latter sites were mutated). The length of the linker was also important for Shn recruitment to Mad/Med.

1.3.2 Requirments for ShnCT to form a complex with Mad/Med on brkS

A first indication of possible motifs in Schnurri required for the formation of a Mad/Med/Shn complex on *brk*S came from the *in vivo* and from cell culture experiments. Shn versions bearing an internal deletion removing zinc fingers 6/7/8 had no detectable repression activity *in vivo* and in cell culture experiments. Furthermore, ShnCTΔZF was no longer able to assemble a complex with Mad/Med on *brk*S in EMSA experiments. Indeed, Schnurri zinc fingers 6/7/8 represented the minimal domain required for complex formation with Mad/Med on *brk*S. The requirements of each zinc finger for triple complex formation were further analyzed. Zinc finger 7 was dispensable for complex formation as well as for *in vivo* function, whereas inactivation of zinc finger 6 and zinc finger 8 abolished both functions of Schnurri. Sequence comparison of the zinc fingers with their homologues in the mosquito *Anopheles* and *C.elegans* revealed highest similarity between zinc finger 8 and less in zinc finger 6 and 7 (*figure31*), which underlined its requirement *in vivo* and *in vitro.*

ShnCT did not exhibit DNA binding properties in the absence of Mad and Medea, but a single nucleotide was identified within *brk*S which is required for Shn recruitment to Mad/Med/*brk*S (Pyrowolakis *et al.* in prep.). Mutations of putative DNA contacting amino acids (Wolfe *et al.* 2000; Laity *et al.* 2001) within the zinc finger motifs did affect complex formation when introduced in zinc finger 6 or zinc finger 8, but had no affect for zinc finger7. Whether these mutations really affected possible DNA interactions has to be confirmed in further experiments. It is to mention that zinc fingers 6/7 represent the more classical pair of zinc finger because of their similarity to the zinc fingers 4/5 and their vertebrate homologues which have been implicated in DNA binding (Omichinski *et al.* 1992). They also contain a TGE/QKP like linker which is typical for DNA-binding zinc finger domains (Wolfe *et al.* 2000; Laity *et al.* 2001). Experiments, which aim to test the possibility of an interaction of Shn with base pairs or the phosphate backbone of brkS, are in progress.

To determine protein-protein interactions between ShnCT and Mad or/and Medea,

Figure31: ClustalX alignment of Shn zinc fingers6/7/8 with the respective zinc fingers in Anopheles and C.elegans. ShnZF8 shows highest conservation among the zinc fingers.

immunoprecipitation experiments were performed. A pilot experiment revealed interactions between Mad and Medea but not with Schnurri, probably due to the lack of *brk*S, which was proposed to induces conformational changes within the Mad/Med complex in order to recruit Schnurri. Further experiments need to be done to explore the relations between Shn and the Mad/Med heteromere.

The requirements for complex formation have been defined on the level off *brk*S (Pyrowolakis *et al.* in prep.) and for Schnurri. Intriguingly, specific sequences within *brk*S dictate the recruitment of Shn to Mad/Med on *brk*S and present the hallmark for Dpp mediated repression. Mad and Medea bound to their respective sites whereas DNA binding was not observed for Schnurri. Probably the most important role for assembling a functional complex involves the direct interaction between Shn and Mad/Med, which seemed to acquire a specific conformation when bound to *brk*S offering binding surfaces for Schnurri. Since the human Smads, Smad1 and Smad4 also assembled a complex with Schnurri on *brk*S, these binding surfaces are highly conserved. The physical contact of Shn with *brk*S might still be required and be induced upon Mad/Med/*brk*S complex formation, possibly to tighten and stabilize the complex.

1.4 Assembly of Mad/Med/Shn on *brk*S is responsible for repression of *brinker in vivo*

Although the physical interactions between Mad/Med/Shn on *brk*S derives from biochemical experiments, the requirement for all these components in vivo was strongly supported by genetic data from the embryo and the wing imaginal disc (Pyrowolakis *et al.* in prep.). First, mutations within *brk*S which affected the Mad/Med/Shn complex but not the Mad/Med complex *in vitro*, were not functional in repressing *brinker* transcription *in vivo*. Secondly, when Dpp input was prevented (hence Mad is neither phosphorylated, nor nuclearly localized, nor associated with Medea), when Schnurri was not present or lacked its C-terminal zinc fingers, *brinker* repression was inactive. The same set of requirements was observed for the formation of the Mad/Med/Shn/*brk*S complex (Marty *et al.* 2000; Müller *et al.* 2003). Moreover, it is the concurrence of all of these conditions that appeared to provide the exquisite specificity of the Dpp regulated repression of gene transcription.

1.5 Schnurri acts as a repressor in Mad/Med-mediated target gene regulation

Most regulatory events ascribed to Smad proteins to date concern signaling induced activation of target gene transcription. Though Smad proteins have the ability to recruit co-activators or corepressors (Massagué and Wotton 2000) it is rather unlikely that they provide bias toward

activation or repression per se. In the case of *brinker* the formation of a Mad/Med complex was demonstrated to be essential but not sufficient for transcriptional repression and we propose that Schnurri acts as the "switch factor" for Mad/Med towards repression.

The triplet of zinc fingers 6/7/8 in Schnurri has been shown to be sufficient for complex formation, but not for repression *in vivo*, suggesting the existence of an additional domain required for the downregulation of *brinker* transcription. A series of N-terminal deletions of ShnCT revealed a 100 amino acid sequence that was essential for repression and retained its activity when fused to zinc fingers 6/7/8 directly *in vivo*. Moreover, this domain confered repression when fused to other DNA binding domains proving that a 100 amino acid domain within ShnCT works independant of the *brinker* context. Taken together, the data of this work demonstrated that the repressive activity of the Mad/Med/Shn complex is provided by the Schnurri protein. Furthermore, the Schnurri protein has a modular structure consisting of two domains, a complex formation domain and a repression domain, which can function independant of each other (Pyrowolakis *et al.* in prep.).

1.5.1 Read-out of the Dpp morphogen via Mad/Med/ShnCT on silencer elements

In the wing imaginal disc of *Drosophila*, Dpp acts as a morphogen with its highest levels at the anterior posterior boundary, forming a gradient to both sides. It has been shown that the Dpp gradient determines, with an inverse relationship, different levels of *brinker* expression (Müller *et al.* 2003). The levels of brinker expression are dependant on the levels of Dpp and on the net balance of silencer (*brk*S) and enhancer activities (*brk*E). For example two silencer elements (*brk*S or C6) fused in tandem to the ubiquitous element *brk*E result in higher sensitivity to Dpp signaling and consequently in higher repressive activity (Müller *et al.* 2003, figure5). The *brinker* genomic region contains a total of ten such C6-like silencer elements which contain the necessary sequences to assemble a Mad/Med/Shn complex and it is possible that they possess different affinities for the repressive complex (Pyrowolakis *et al.* in prep). Although biochemical experiments only considered the ON/OFF situation for complex formation, it is tempting to assume that it is the number of silencer element occupied by the repressive complex, which in turn depends on the levels of phosphorylated Mad, which determines the expression levels of *brinker*.

1.5.2 Schnurri dependant repression is tissue independant

Mad/Med/Shn mediated repression of *brinker* has been observed in diverse tissues including the embryonic ectoderm, the wing imaginal disc and also *Drosophila* Schneider cells (Müller *et al.* 2003; Pyrowolakis *et al.* in prep.). Moreover, the *brinker* silencer element did not only

specifically affect the constitutive *brk* enhancer but diminishes transcriptional activation when fused to heterologous enhancers (Müller *et al.* 2003). We could demonstrate that the repressive activity of the Mad/Med/Shn/*brk*S complex is provided by a 100 amino acid domain within Shn. This domain functions independantly when fused to other DNA binding domains (Brinker DNA-binding domain and Gal4). Taken together, the cofactor(s) associated with this domain, if any, seem to be tissue unspecific (Müller *et al.* 2003) and independant of associated enhancers or sequences (Pyrowolakis *et al.* in prep.). Therefore, it is likely that the Mad/Med/Shn/brkS complex interferes directly with events at the promoter or contains enhancer independant chromatin remodeling activity. A variety of molecular mechanisms have been described which are used by different transcription factors to bring about transcriptional repression. One way of classification is to distinguish between short-range and long-range repression, whereas short range repression means in a distance not more than 100bp to the promoter. The CtBP (C-terminal binding protein) presents a prominent example of such corepressors. A long range repressor (e.g. Groucho/Tup1) makes a promoter resistant to the influence of all enhancers, even if those enhancers are located thousands of base pairs from the repressor binding site (Courey and Jia 2001). Repression can be achieved by several mechanisms, including interference with the basal transcriptional machinery, counteracting the activity of positively acting transcription factors or by re-organizing chromatin structures. Often more than one mechanism can be used by the same (co)-repressor. For example both, long-range and short-range corepressors, have been associated with chromatin modification by histone deacetylases (HDAC) (Courey and Jia 2001; Gaston and Jayaraman 2003).
A putative dCtBP binding site (consensus: PxDLS, Nibu *et al.* 1998) is present in the repression domain of Schnurri (PMDLT), though dCtBP has been described to be a short-range co-repressor. Nevertheless, as it was shown for other repressors (Gaston and Jayaraman 2003), Schnurri might well exploit more then one repressive mechanism and the identification of interacting proteins with the repression domain is therefore essential.

Very little is known about TGF-β induced repression in vertebrates. Intriguingly, most of the immediate gene responses to TGF-β signaling have been linked to transcriptional activation rather then to TGF-β dependant repression. Equally, Ski, Sno and TGIF act by the inhibition of TGF-β dependant gene activation rather then by direct repression (Massagué and Wotton 2000; Shi and Massagué 2003). The only example so far presents the E2F4/5 protein which associates with Smad4 and Smad2/3 to target c-myc for repression (Chen *et al.* 2002). In

Drosophila, the repressor Schnurri links the Dpp-signaling to target gene repression. Disruption of Schnurri-mediated repression in *Drosophila* results in severe defects.

2. Dpp/Shn-mediated repression via small silencer element(s) is not restricted to *brinker*

First indication of *brinker* independant function of Schnurri came from studies in the Drosophila ovary (Xie and Spradling 1998). This year, two studies were published presenting genetic and biochemical evidence for the Dpp dependant repression of the gene *bag of marble*s (*bam*) (Chen and McKearin 2003a). *Bam* transcription is downregulated by Dpp in the multipotent stem cell through a small *bam* silencer element on which Mad and Medea binding sites were characterized (Chen and McKearin 2003b). Sequence comparison of the bam silencer and the brkS revealed a high degree of conservation containing all essential signatures. Indeed, a complex of Mad/Med and ShnCT did assemble on this element (Pyrowolakis *et al.* in prep.). Furthermore, the *bam* silencer element conferred repression to heterologous enhancers in the wing imaginal disc and in Schneider cells similar to *brk*S (Pyrowolakis *et al.* in prep.). These findings together with the genetic requirements for Schnurri and Dpp in germcell development demonstrate that bam is a target gene of Mad/Med/Shn.

The possibility that Dpp mediated repression by Mad/Med/Shn via small silencer elements represents a general mechanism to repress the transcription of genes motivated us to conduct a genome wide search for such silencer sites (Pyrowolakis *et al.* in prep.). The search revealed numerous candidates and some of them are currently investigated. Preliminary results support the activities of silencer elements in other genes, suggesting that those genes are also suppressed by the Dpp activity during Drosophila development. It becomes evident that Dpp-mediated repression executed by the ubiquitously expressed repressor Schnurri presents a common mechanism to suppress genes within Dpp signaling domains throughout development (*figure32*). Hence the importance of the repressive activity of Mad/Med/Shn becomes apparent. Target genes are suppressed in the Dpp activity domain as shown for *bam* and *brinker* (Sivasankaran *et al.* 2000; Kirkpatrick *et al.* 2001; Rushlow *et al.* 2001; Pyrowolakis *et al.* in prep). Brinker additionally regulates Dpp target genes ("indirect" target genes) some of which require additional positive Dpp-input (*vestigal* and *spalt* in the wing imaginal disc). Besides that, Mad/Med might also regulate target genes in a Schnurri and Brinker independant manner, for example recruiting other (tissue specific) cofactors, similar

to TGF-β target gene regulation in the vertebrate field. Therefore, what seemed in the beginning to represent the main target of Mad/Med/Shn mediated repression, *brinker*, appears to be one among many. Nevertheless, the *brinker* genomic region seems to be exceptional since it contains several of those silencer elements, which might well be a feature especially acquired for the Dpp morphogen function during imaginal disc development in *Drosophila*.
It will be interesting to see whether such a signal-induced repression mechanism exists also in other signaling pathways and whether Schnurri orthologues are present in TGF-β signaling in vertebrates.

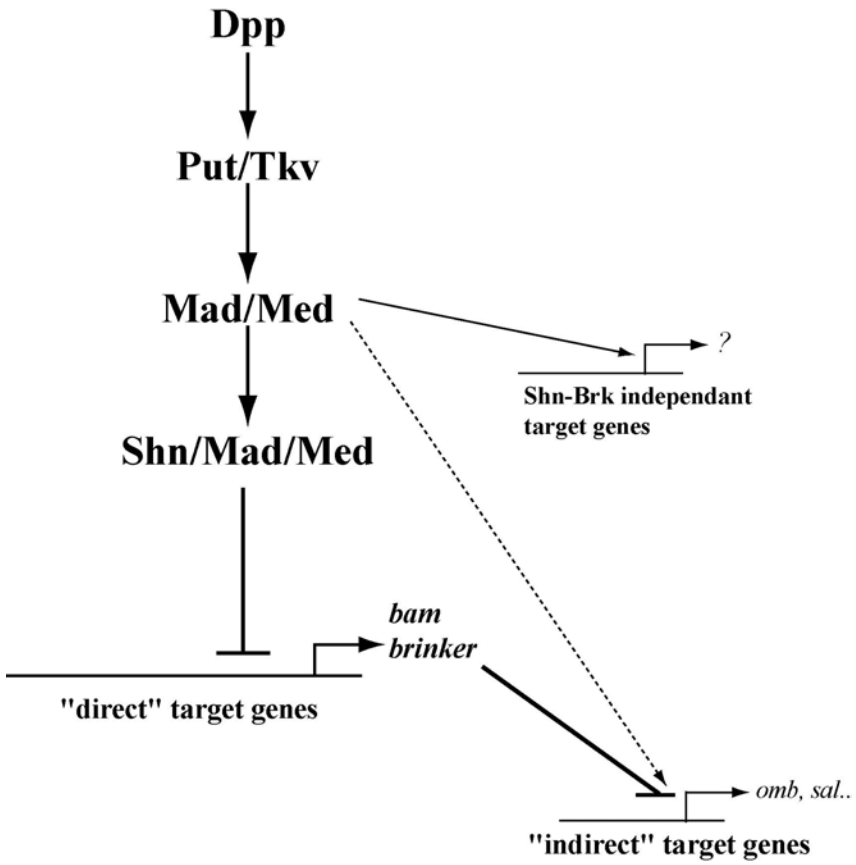


Figure32: Novel model of target gene regulation by the Dpp signaling pathway in Drosophila melanogaster. The repressive Shn/Mad/Med complex has a dual role during development. Genes, which should not be expressed in the Dpp activity domain, are repressed by Shn/Mad/Med. In addition, Dpp target genes are directly repressed by Dpp such as bam and brinker. The downregulation of brinker results in the activation of "indirect" target genes. Some of those target genes have been demonstrated to require additional, positive Dpp input. Target genes might also be directly activated by Mad/Med probably by association with cofactors as it is the case for many TGF-β target genes in vertebrates.

2.1 Schnurri: a fly specific cofactor for TGF-β signaling?

ShnCT, which has been demonstrated to be sufficient for Dpp-dependant *brinker* repression *in vivo* and in tissue culture, displays no sequence similarity with any of its vertebrate homologues. Moreover, the triplet of zinc finger responsible for complex formation with Mad/Med on the *brinker* silencer element is not present in other Schnurri proteins. Also, general sequence comparison revealed no striking similarity to any vertebrate genes neither for the Shn complex formation domain nor the repression domain. Consistent with this, a Brinker homologue has not been identified in vertebrates, suggesting that the branch of target gene regulation by Dpp, involving Shn and Brk, is insect specific. However, a triplet of zinc fingers is present in *C. elegans* Sma-9 which has recently been demonstrated to function in a TGF-β like pathway (DBL-1 pathway) in worms in the regulation of body size and male tail development (Liang *et al.* 2003).

Notwithstanding, there is some, though weak, evidence for a Schnurri/Brinker-like mechanism in vertebrates. Drosophila Brinker has been reported to suppress BMP-4 target genes in Xenopus. Injection of *brinker* mRNA in Xenopus embryos at the two-cell stage, resulted in a typical dorsalized phenotype of the injected embryos (Minami *et al.* 1999), postulating that a *brinker*-like activity is also present in higher eukaryotes. In addition ShnCT assembled in a complex with Smad1 and Smad4 on the *brinker* silencer element, indicating that the surfaces in Smads for Schnurri recruitment have been conserved during evolution. The characterization of the complex formation domain of Schnurri was a first attempt to define structural requirements for further searches for vertebrate homologues. It will also be interesting to see whether *brk*S-like motifs are present in TGF-β regulated genes in other organisms and if these genes are targeted for repression by TGF-β.

3. Schnurri, a multi-functional protein?

The *Drosophila* Schnurri protein consists of 2529 amino acids and eight zinc finger motifs. The C-terminal 640 amino acids containing an unconserved triplet of zinc finger are essential and sufficient to function as a repressor in Dpp-mediated *brinker* repression *in vivo*. Though genetic data demonstrate that the main function of Schnurri during *Drosophila* development is to suppress the transcription of *brinker*, it is unlikely that this is the only function. While there is no experimental data to date, it is hard to imagine that a large protein containing several zinc finger domains accomplishes its function with only one fourth of its structure (ShnCT). Interestingly a single exon encodes for ShnCT in *Drosophila* as well as in *Anopheles* suggesting that ShnCT might have been joined to the rest of Schnurri during evolution. In the

same line, C. *elegans* Sma-9 has a diverged arrangement of the zinc fingers, each of them encoded by a single exon. In addition *sma-9* possess multiple alternatively spliced variants suggesting different protein isoforms with potentially different subcellular localizations and molecular functions (Liang *et al.* 2003).

Multi-zinc finger proteins have been demonstrated before to acquire more than one independant function. One example is OAZ (Hata *et al.* 2000), a 30-zinc finger protein, which functions as a transcriptional partner of Olf-1/EBF in olfactory epithelium and lymphocyte development through a cluster of zinc fingers different from those which have been demonstrated to interact with Smad2 and *Xvent-2* in the BMP signaling pathway. Thus, the mutually exclusive use of OAZ in two different signal transduction cascades illustrates the dual role of multi zinc finger proteins during development. Accordingly other function(s) of Schnurri in *Drosophila* can be anticipated which involve the C-terminal 1900 amino acids (ShnNT) including five zinc finger domains (ZF1-5) and are probably Dpp-independant. Indeed, ShnNT, and not ShnCT, is similar to the vertebrate Schnurri proteins which have been implicated to participate in IL-2 transcriptional activation (KRC, Oukka *et al.* 2004), TNF signaling (KRC, Oukka *et al.* 2002) and the process of positive selection during T-cell development (mShn2, Takagi *et al.* 2001). So far, the only molecular data comes from studies on KRC (Shn3) demonstrating that KRC is induced upon T-cell receptor signaling and interacts with c-jun to augment AP-1-dependant (activator protein 1) IL-2 transcription (Oukka *et al.* 2004). An attractive model would be that ShnCT acts as a repressor in Dpp-signaling during *Drosophila* development, independant of ShnNT, whereas ShnNT would be involved in homeostasis processes such as immunity or hemocyte development and maintenance. Following this idea, phenotypes caused by the loss of ShnNT would only become evident during adult life of the fly and were therefore not observed in the developmental stages examined so far. A genomic rescue, comparing the phenotypes of a Schnurri rescue with the full length protein to ShnCT, will illustrate whether *brinker* repression is the only function of Schnurri during *Drosophila* development.

As for the future, the focus will be on the identification of possible other functions of Schnurri and the molecular analysis of the mechanism by which Shn-mediates repression.

IV: Materials and Methods

1. Molecular techniques

Standard molecular biology techniques were applied following the procedures described in Maniatis (Sambrook *et al.* 1989). *E. coli XL1 blue* bacteria were used for plasmid amplification. Commercial kits were used for extraction and purification of DNA as well as for small scale and large scale isolation of plasmid DNA from *E.coli* (Qiagen or Sigma). PCR based constructs were sequenced using automated sequencing (ABI PRIM 310 Genetic Analyzer).

1.1 Cloning of Schnurri derivates

Schnurri derivates were generated by polymerase chain reaction, PCR (*see 1.2*) following the strategies presented in Figur1. For all constructs the EcoRI restriction site was used at the 5`prime end (followed directly by the start codon ATG) and the XhoI site at the 3`prime end. The sequence of all PCR-based constructs was verified by sequencing. Depending on the experimental context, Shn derivativess were cloned into the pUAST vector to generate transformant fly lines, the pcDNA3-vector (Invitrogen) for *in vitro* expression of proteins and into pAc5.1BV5/His (Invitrogen) for constitutive expression in Schneider cells. The following Shn-derivates (in pcDNA3) were a kind gift of Kawabata (Udagawa *et al.* 2000): Shn1-909; Shn1-1888; Shn909-1888; Shn909-2529, Shn1888-2529, Shn1-2529 (all internal EcoRI and XhoI sites are mutated in these versions).

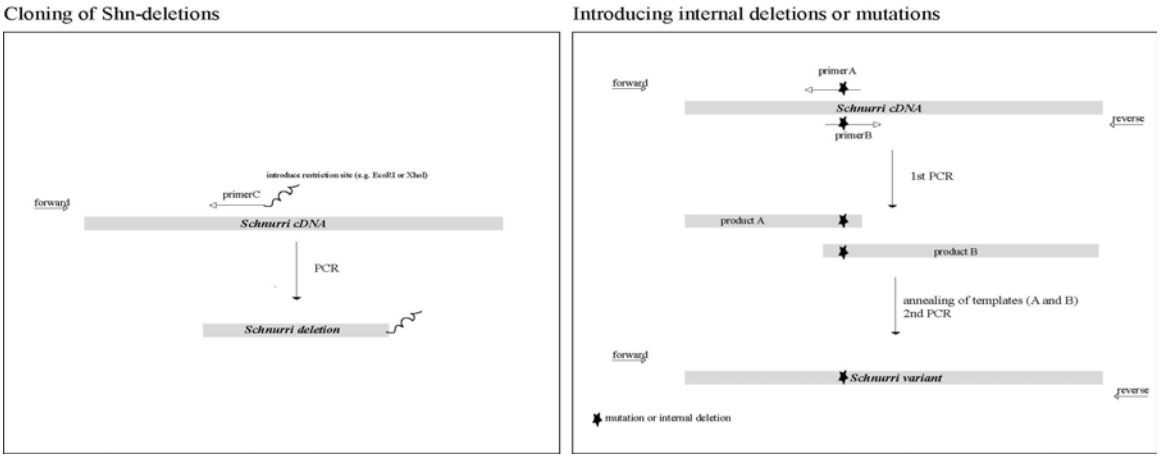


Figure1:

1.1.1 Introducing internal deletions and single zinc finger mutations into the Schnurri protein

Internal deletions were generated by PCR (Figure1) removing exactly those amino acids which have been annotated to be part of the structure to be removed. For example, zinc fingers were deleted from their first cysteine to the last histidine residue. Single zinc fingers were inactivated by introducing mutations in the codons for the first two characteristic cysteine residues to change the coding sequence to another amino acid (ZF6: C to G, C to G; ZF7: C to S, C to G; ZF8: C to S, C to G). For constructs bearing mutations in the putative DNA contacting amino acids *(Wolfe et al. 2000),* mutations were introduced at the following positions 6, 3, 2,-1. Amino acids were mutated to alanines (ZF6: LQHR; ZF7: KHGT; ZF8: KHGI - amino acids in positions 6, 3, 2, -1, respectively).

1.1.2 Polymerase chain reaction reactions (PCR) to generate Schnurri derivates

PCR reactions were typically carried out in a thermocycler (Techne Thermal Cycler) using thin wall 500µl PCR tubes. A 50µl reaction typically contained 1µl of each primer (10µM), 10µl polymerase buffer (Roche), 200µM dNTP mix, 1µl Pfu Turbo DNA polymerase (Stratagene) or Dynazyme (Bioconcepts) and variable amounts of template. The following PCR cycles were generally applied: 1cycle 94°C for 2min; 2 cycles 94°C-30sec, 35°C-30sec, 72°C-xsec (~60sec per 1kb); 25 cycles 94°C-30sec, 54°C-30sec, 72°C-xsec (~60sec per 1kb) and a final 10min at 72°C. PCR reactions were purified (Qiaex gel extraction kit or Qiaquick PCR purification kit - Qiagen) for further use.

1.2.1 Restriction digests of PCR products and vectors:

1µg of vectors was digested for 1.5h at 37°C in a volume of 30µl. PCR products were digested in a volume of 50µl for ~4h at 37°C. Subsequently, the DNA was purified through agarose gel electrophoresis.

1.1.3 DNA ligations and bacterial transformations

Ligation (T4 DNA Ligase, Biolabs) and bacterial transformation were carried out using standard protocols. All constructs carried ampicillin resistance for later bacterial selection. Bacterial colonies were amplified in an overnight culture and plasmids were isolated using a Miniprep kit (Sigma). The presence of the insert was verified by restriction digest before isolating large amounts of plasmids following the protocol of the Midiprep kit (Qiagen).

1.2. Electro-mobility shift assays (EMSA):

All reactions were carried out at room temperature if not indicated differently. Big gels (glass plates ordered for our purpose directly at the manufactory) had a matrix volume of approximately 90ml and small gels 70ml.

1.2.1 Annealing of Oligonucleotides:

The double stranded oligonucleotide probe was generated by annealing 40µmol of each single stranded oligonucleotide (*brkS*: 5` AGTGTCTGCGGCGTAGCAAGACTGGCGACATTC TGTCTGGTGGCGATCGCC 3`) in a 100µl 1xTE. The mixture was incubated at 80°C for 10 min in a water bath and then cooled down slowly to room temperature (2-5hours).

1.2.2 Labeling of Oligos:

The ^{32}P-labeled oligonucleotide probe was generated by filling in overlapping oligonucleotides (a minimum of one T overhang was required) in the presence of [α-^{32}P]ATP and 200µM dCTP/dGTP/dTTP, 2µl of 10xKlenow buffer (see for recipe, Sambrook *et al.* 1989) and 1µl Klenow enzyme (final volume of 20µl). The reaction was incubated at room temperature for 30 min. Labeled oligonucleotides were separated from not-incorporated nucleotides using Quickspin columns G25 (Roche)

1.2.3 Binding reaction and gel electrophoresis:

10,000-20,000 cpm of probe were mixed with ~30µg of proteins (4µl of cell extract - *see 3.3* or 4µl of *in vitro* translated proteins - *see 3.5*), in 1x Binding Buffer (5x: 25mM Tris-HCl [pH7.8], 30% glycerol, 400mM KCl, 50mM MgCl2, 50µM ZnCl2, 0.25% NP-40) in a total volume of 25µl and kept for 30min on ice. Protein-DNA complexes were separated from free probe on 4% nondenaturing polyacrylamide gels (Acrylamide Biorad 40%, 29:1, 0.5x TBE, 1.2% APS, 0.6µL/ml TEMED) at room temperature for 130min at 160V in 0.5x TBE. For supershift experiments the following antibodies were added to the binding reaction: 20ng of monoclonal anti-Flag (M2, Sigma), 8ng of monoclonal anti-Myc (9B11, Cell signaling), 0.5 µg monoclonal Anti-V5 (Invitrogen). Dried polyacrylamide gels were visualized by autoradiography (Fuji, SuperRX7543).

1.2.4 In vitro transcription of proteins:

Proteins were *in vitro* translated using the TNT®T7 Coupled Reticulocyte Lysate System (Promega) following the instruction of the manual. For that purpose cDNAs were usually

cloned in the pcDNA3 vector (Invitrogen) which contains a T7 promotor 3`prime to the multiple cloning site and the Flag-Epitope (Figure 2).

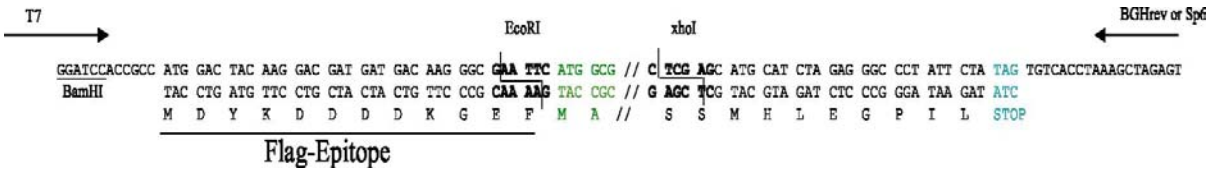


Figure2: Multiple cloning site of the pcDN3 containing the Flag-epitope

1.2.5 Western Blot Experiments:

Western Blot analysis was performed to verify the presence of the different proteins in S2 cell extracts or the *in vitro* translated proteins. Samples were separated via 10% denaturing polyacrylamide gelectrophoresis (SDS-PAGE) following standard protocols (Sambrook *et al.* 1989). Proteins were electro-transferred from the gel onto a nitrocellulose membrane (Schleicher&Schnell) usually overnight at 4°C at 30mA. Antibodies were used in the following dilutions: α-Flag: 1:1000; α-Myc: 1:4000; α-V5: 1:5000. The presence of the antibodies was visualized using the ECL-detection kit (Amersham Pharmacia Biotech).

1.3. Drosophila Schneider cell culture

S2 cells were propagated in S2 Cell Growth Medium (Schneiders Drosophila Medium, Gibco) supplemented with 10% FCS (Fetal calf serum, Gibco), 1xGlutamine and 1xPen/Strep (Gibco); note that cells were very sensitive to the quality of FCS. Cells were usually kept in 75cm^2 tissue culture flasks at 25°C. S2 cells grow both in suspension and attached to the flask. Cells were passaged at a 1:2 dilution into a new flask once they achieved a cell density of 6 x10^6 cells/ml (usually every other day). The cells that are attached to the flask were easily dislodged through mild pipetting. The cells grew well as long as they were not subcultured to a very low density.

S2 cell reporter plasmids containing the *brk* silencer were generated by inserting 100 bp of the 3`end of fragment C (comprising subfragment S) between the EcoRI and Asp718 sites in hsplacCasper (G. Pyrowolakis) and 4xSuh-lacZ (Kirkpatrick *et al.* 2001). Epitope-tagged versions of Tkv^{QD}, Mad, Med, and Shn-versions were cloned in the vector pAc5.1B/V5His (Invitrogen) for constitutive expression under the control of the actin5c promoter. Plasmids for constitutive expression of Su[H] and a constitutive active form of Notch (N*) were a gift from A. Laughon.

1.3.1 Plasmid transfection of S2 cells:

1.5 x10^6 cells/ml were transfected with a total of 200ng plasmid in 12-well microtiter plates and incubated for 48h at 25°C before assayed for β-galactosidase and luciferase activity (Effectene Transfection Reagent, Qiagen). For the reportergene assays the following amounts of plasmids were transfected: 30ng of reporter, 30ng of pAc5.1-Su[H], 30ng pAc5.1-N*, 2.5ng (or the indicated amounts) pAc5.1Tkv^{QD}, 3ng pAc5.1Shn, 25ng dsRNA (for RNAi treatment) and 5ng of a plasmid constitutively expressing firefly luciferase (pAc-Luc) for normalization of transfection efficiency. Empty parental expression vector (pAc5.1B/V5His) was used to adjust the total DNA amount to 200 ng per transfection.

1.3.2 RNAi treatment of S2 cells

(protocol from Worby et al. 2001; Science`s stke: www.stke.org).

A) Preparation of dsRNA: dsRNA fragments were generated corresponding to nucleotides 658–1230 and 5011–5531 of the *mad* and *shn* open reading frames, respectively. The MEGAscript T7 kit (Ambion) was used to synthesize dsRNA from a PCR template. A reaction mix (8µl purified PCR template, 2µl 10x reaction buffer, 2µl each ATP, CTP, GTP, UTP mixes, 2µl enzyme mix) was incubated at 37°C for 4-6h (see manufactors protocol). dsRNA was precipitate at -20°C with 3M sodium acetate solution and 2.5 volumes of ethanol for at least 30min. Samples were centrifuged for 30min in a microcentrifuge at maximum speed (4°C). RNA pellets were air dried and resuspended in DEPC-treated water. The amount of dsRNA was estimated at 260nm.

B) pretreatment of cells with RNAi: For the "Shn-rescue assay" S2 cells were pretreated with dsRNA 24h before transfection. 1x 10^7 cells (1 x10^6 cells/ml) were transferred to 10ml (1/2V) DMEM-medium including ~15µg of dsRNA and incubate at 25°C for 30-60min to allow uptake of dsRNA. Then, 10ml (1vol) of S2 Growth Medium containing 20%FCS was added and incubated for another 24h before transfection.

1.3.3 Preparation of S2 cell extracts:

Cells were harvested 48h after transfection by thoroughly pipetting and transferred directly into a 15ml polypropylene tube (wells were additionally washed with 2ml of PBS to collect remaining cells). Cells were pelleted at 700g for 3min and the supernatant was discarded. After an additional washing step with 2ml PBS cells were lysed for 10min on ice in 50µl of the appropriate lysis buffer (for EMSA: 100mM Tris-Cl [pH7.5], 1mM DTT, 1%TritonX100;

for S2-cell culture assay: 25mM Gly-Gly pH7.8, 15mM MgSO4, 4mM EGTA, 1mM DTT, 1% TritonX100). Cell debris were removed by centrifugation for 10min at 4°C.

1.3.4 Reporter gene assays:

The Luciferase assay was used to normalize the transfection efficiency. 10µl of extracts (luciferase counts should be within 0.5-5x10^6) were added to a 350µl reaction mix (25mM Gly-Gly[pH7.8], 10mM $MgSO_4$, 2mM ATP) and immediately measured in a luminometer (Microlite TLX1, Dynatech laboratories; count time 10sec; 50µl injections). The luminometer was filled with injection mix (25mM Gly-Gly[pH7.8], 0.2mM Luciferin) directly before use.
β-Galactosidase assay: β-galactosidase assays were performed in 96 well multititer plates in a microplate reader (Thermomax). 10µl of extracts were mixed with 200µl of Z-buffer (100mM Na_3PO_4[pH7.4], 1mM MgCl2, 0.314%β-mercaptoethanol, 0.5mM CPRG) and the OD change was measured over 1h at 37°C with a peak absorbance of 574-578nm.

1.3.4.1 Statistics

The β-galactosidase values were normalized by dividing to the luciferase counts (lac/luc). Experiments were usually performed as duplicates or triplicates and the mean values and the standard deviation were calculated using Excel (Microsoft corporation). Results present the average β-galactosidase activity expressed in x-fold activation. Individual experiments were repeated at least once to confirm the results obtained.

2. Fly work

2.1 Fly lines

y w, //, prdG4, BM14/TM3 (recombinant line generated by T. Marty)
w, shn^{TD5}/CyO
w, shn^{TD5}, *UAS-dpp*/Cyo (recombinant line generated by T. Marty)
y w, //, *UAS-shnvariants*/TM3

2.2 Ectopic expression of Shn derivatives in flies:

All *Shn* derivatives were cloned in a pUAST vector using EcoRI and XhoI restriction sites (Figure 3). All UAS-*shn* derivates carried the Flag epitope at their N-terminus. The nuclear localization signal (NLS) was fused to all *shnCT* variants to assure proper cellular localization. Transformant fly lines were balanced and at least two independant fly lines were tested for each Shn-version for their ability to repress *brinker in vivo*. The following crossing scheme was setup for *brinker* repression *in vivo* (Marty *et al.* 2000).

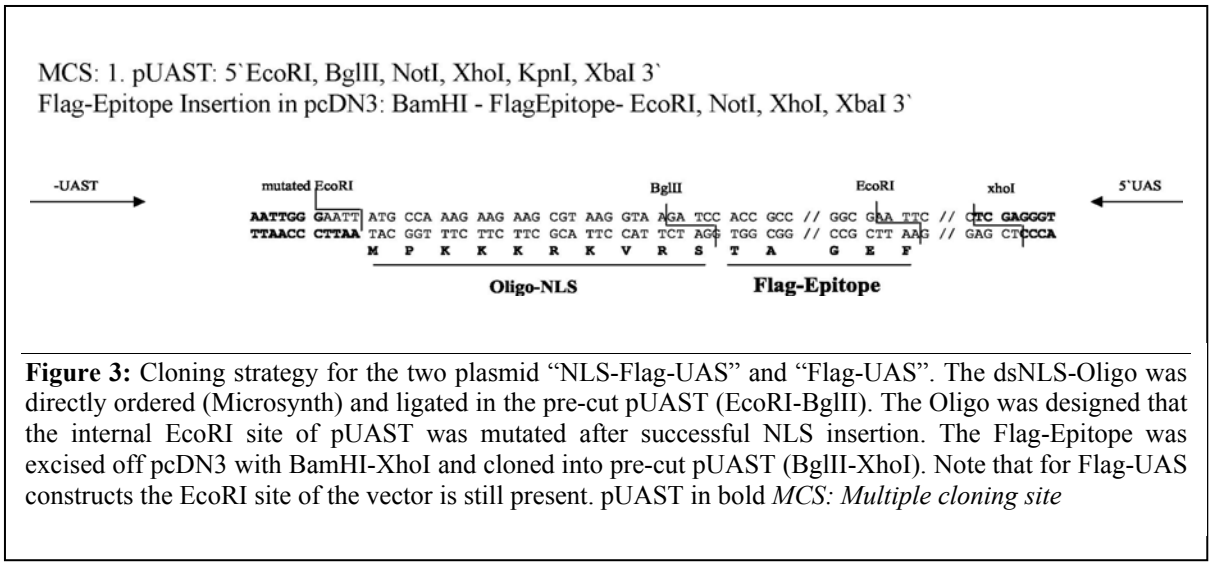
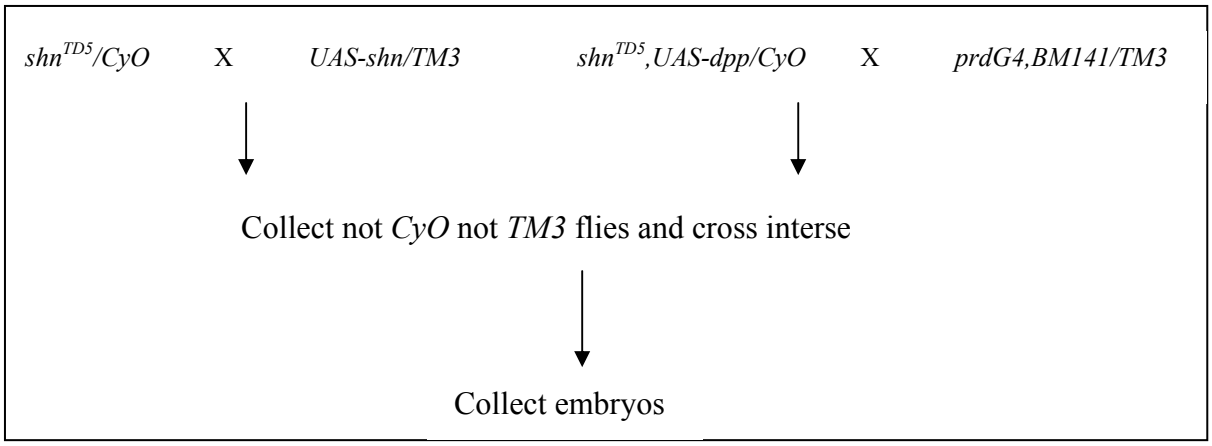


Figure 3: Cloning strategy for the two plasmid "NLS-Flag-UAS" and "Flag-UAS". The dsNLS-Oligo was directly ordered (Microsynth) and ligated in the pre-cut pUAST (EcoRI-BglII). The Oligo was designed that the internal EcoRI site of pUAST was mutated after successful NLS insertion. The Flag-Epitope was excised off pcDN3 with BamHI-XhoI and cloned into pre-cut pUAST (BglII-XhoI). Note that for Flag-UAS constructs the EcoRI site of the vector is still present. pUAST in bold *MCS: Multiple cloning site*



2.3 Embryo collection and fixation

Embryos were collected overnight (12-14h) at 25°C on grape juice plates. They were dechorionized in 4% chlorax solution for 3min, washed with 120mM NaCl, 0.03% TritonX-100 and fixed in 500µl heptane and 500µl fixative for 25min. Subsequently the fixative was discarded and replaced with 700µl methanol to remove the viteline membrane of the embryos. Embryos were transferred to methanol and stored at -20°C until further use.

2.4 Immunohistochemistry and whole mount in situ hybridisation

The following primary antibodies were used: monoclonal anti-Flag antibody (diluted 1:3000; M2, Sigma) and anti-β-Galactosidase (diluted 1:500; Promega). Embryos were fixed and immunostained by incubation with the first antibody followed by second biotin-conjugated antibody (diluted 1:500, Vector) (Patel 1994). The distribution of the secondary antibody was revealed by using the horseradish peroxidase ABC kit (Vectastain). mRNA was detected by in situ hybridization to whole-mount embryos as described by Tautz and Pfeifle, 1989 (Tautz and Pfeifle 1989) with minor modifications.

Aknowledgements:

- G. Pyrowolakis for sharing the S2cell culture plasmids and first establishing and the introducing the system to me (Müller *et al.* 2003; Pyrowolakis *et al.* in prep)
- Thomas Marty for diverse fly lines and cloning for some Shn constructs into pUAST (Marty *et al.* 2000)
- M. Kawabata for Shn plasmids (Udagawa *et al.* 2000)
- A. Laughton for diverse S2cell culture plasmids (Kirkpatrick *et al.* 2001)
- Ten Dijke, P. for kindly providing the human Smad cDNA`s (Itoh *et al.* 2000)
- Nicole Grieder for the original UAS-shn fly line (Grieder *et al.* 1995)

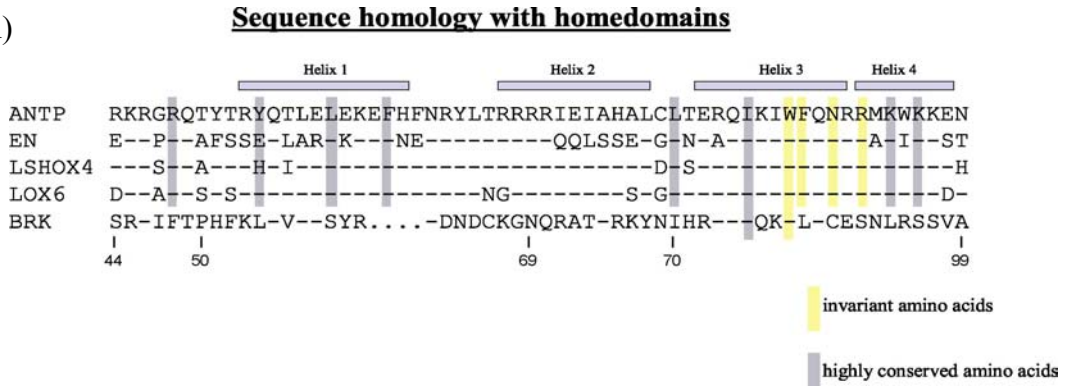
NMR structure of the DNA binding domain of Brinker

In collaboration with Florence Cordier, Marco Rogowski, Stefan Griszek

The nuclear protein Brinker protein plays an important role in Dpp signaling in *Drosophila*. It has been demonstrated that all Dpp target genes analyzed so far, are repressed by Brinker in the absence of Dpp signaling (Jazwinska *et al.* 1999a; Jazwinska *et al.* 1999b; Affolter *et al.* 2001; Kirkpatrick *et al.* 2001). Binding sites for the repressor have been identified and characterized in several of those target genes revealing a G/C rich consensus binding site (GGCGT/CT/C) (Sivasankaran *et al.* 2000). The DNA binding domain of Brinker has been identified and localized at the N-terminus of the protein (amino acids 44-99). Sequence comparison revealed weak similarity to known homeodomains though most of them are within helix 3 which makes the main DNA contact in homeodomains *(figure1, Jazwinska et al. 1999a, F. Cordier)*.

In a collaboration with the lab of S. Griszek (**F. Cordier** and M. Rogowski) the structure of the DNA binding domain of Brinker was solved by NMR and the DNA contacting amino acids were determined. Biochemical approaches (e.g. EMSA), *Drosophila* cell culture and probably *in vivo* studies were considered to confirm the structural analysis. A 100 amino acid long peptide (Brk1-101) was used in NMR experiments.

A)



B)

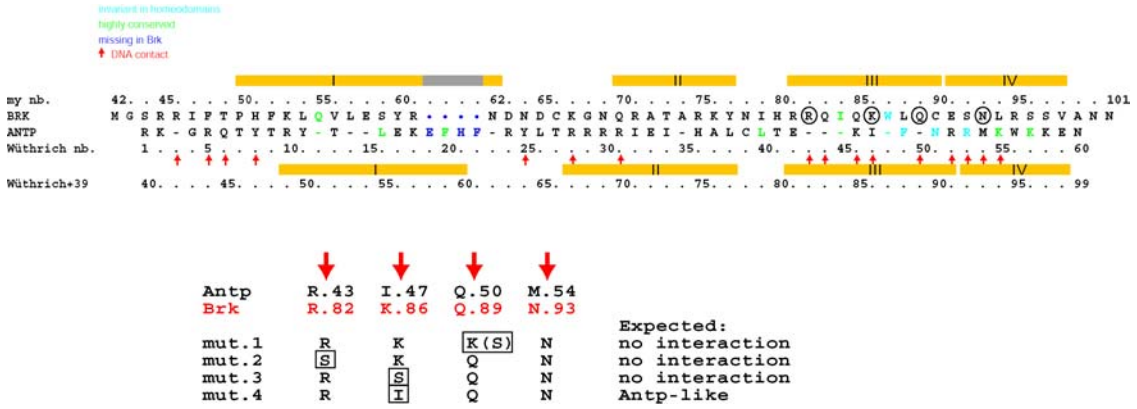


Figure1: A) Sequence alignment of known homeodomains with the Brinker DNA binding domain. The secondary structure is indicated on the top. Lines indicate similar amino acids and points lack of an amino acid. **B)** Comparison of Antennapedia homeodomains with the DNA binding domain of Brinker. Red arrowheads indicate amino acids which were demonstrated to make Antp-DNA contact. The yellow bars indicate the helices. Amino acids 82, 86 and 89 of Brinker were mutated and tested for their influence on the repressive activity of Brinker in Schneider cell assays (see figure3)

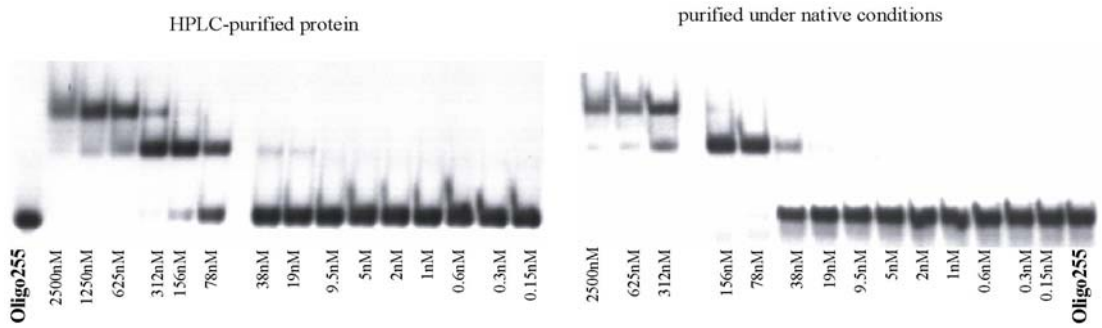


Figure 2: The Brk1-101 protein was purified under two different conditions (M. Rogowski) and tested for binding in EMSA experiments using the „omb-Oligo" (255, Sivasankaran et al. 2000) and different concentration of the purified Brk DNA binding domain. The K_D was estimated to 30 ~60x10^{-9}M. The KD of Antennapedia was experimentally determined to approximately 1.5 x10^{-9}M (Affolter *et al.* 1990). The method of purification did not influence the binding affinities of the Brk protein.

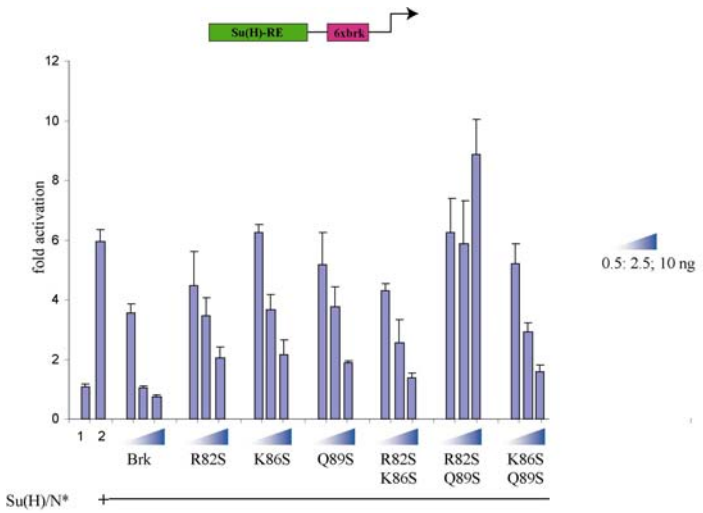


Figure3: S2 cell culture assay using the "Notch-induction tool" as described before. The Su(H) response element was fused to six Brinker consensus binding sites (Kirkpatrick *et al.* 2001), coupled to a minimal promoter hsp70 fused to the reportergene lacZ. Transfection of Su[H]/N* result in the activation of reportergene activity (lane 1 & 2). Increasing amounts of Brinker, or Brinker carrying mutations in putative DNA binding amino acids, were cotransfected (0.5ng; 2.5ng; 10ng).

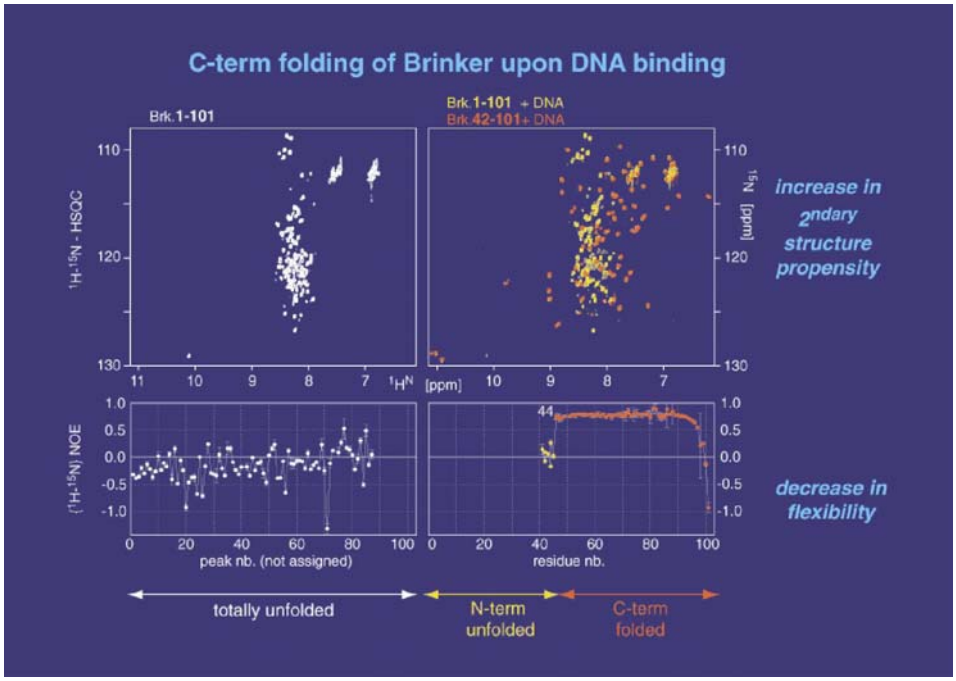


Figure4: *contributed by Florence Cordier*; *Top, left:* 1H-15N HSQC spectrum of the free Brinker (construct 1-101) typical for an unfolded protein (weak dispersion of the peaks). *Top, right:* 1H-15N HSQC spectra of the complexes Brinker/DNA (construct 1-101 in blue and 42-101 in red). Folding of the protein upon DNA binding is evidenced by a larger dispersion of the peaks. In Brk[42-101], the whole protein is folded. In Brk[1-101], only the C-terminal part is folded, and the peaks are exactly superimposed to the one of Brk[42-101]. *Bottom:* 1H-15N NOE are indicators of the dynamics in proteins (more flexible --> small NOE ; and more rigid --> NOE about 0.8). *Left:* the small NOE values are typical for unfolded (usually very flexible) proteins. *Right:* folding upon DNA binding induces more rigidity of the C-terminal part of Brk[1-101] and of the full protein Brk[1-42].

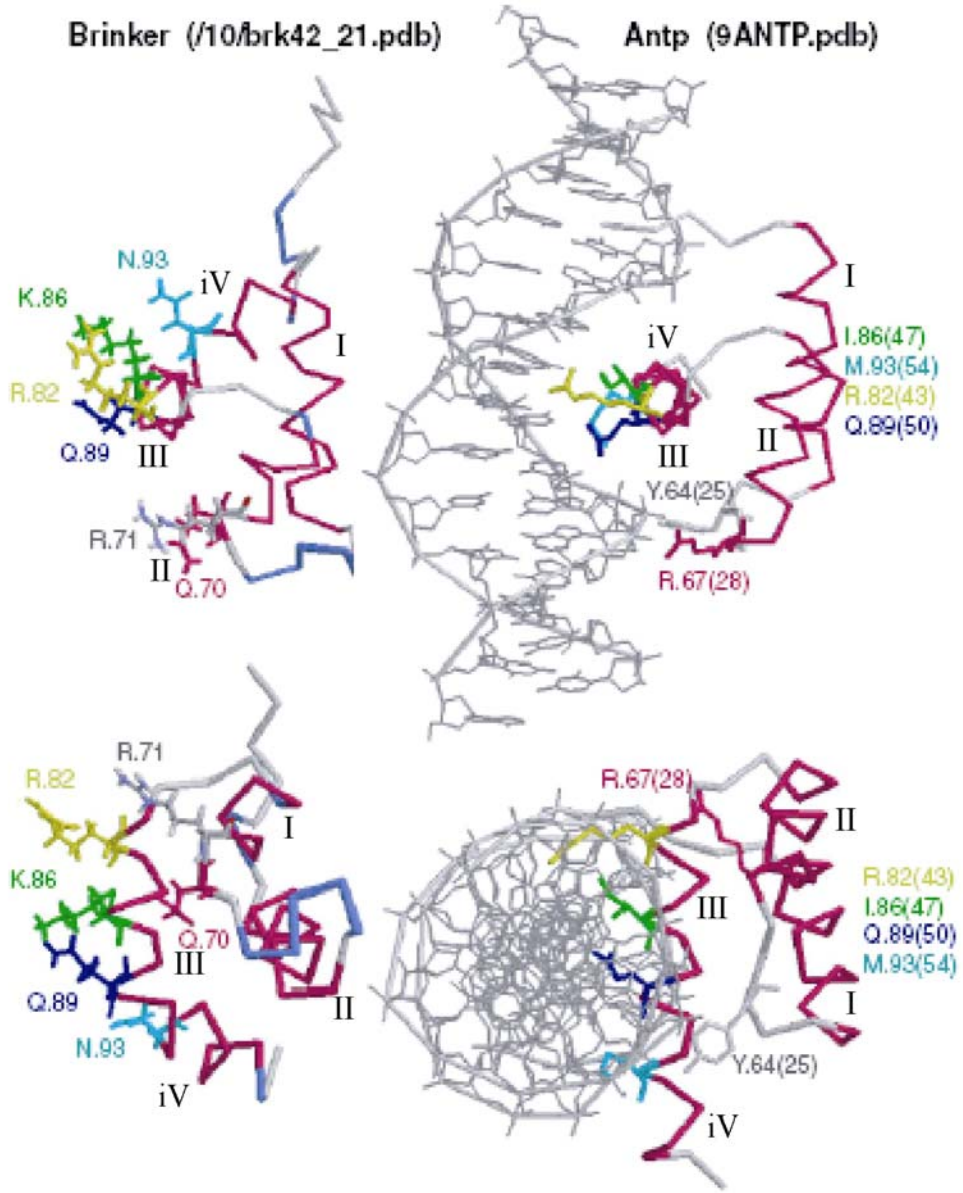


Figure5: Structure of the Antennapedia homedomain in respect to the DNA (right). The side chain of amino acids making DNA contact are colored and most of them lay within helix 3. The respective amino acids in the Brk-DNA binding domain are labeled with identical colors (left). Helices are labeled in red. Roman letter indicate the number of the helix. Note that the Brk-DNA contacts present putative contacts based on the Antp-DNA complex. The DNA contacts of Brk were not assigned at this time point.

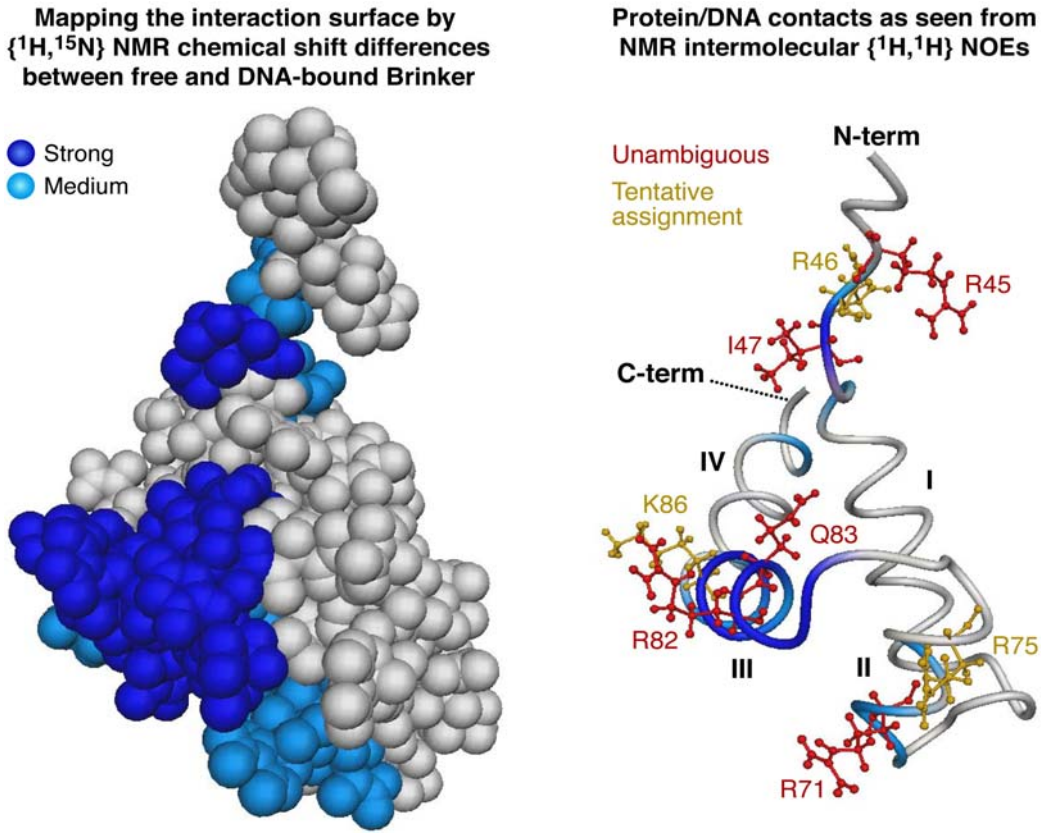


Figure6: *by F. Cordier* To determine the Brinker-DNA contacts a 2D 13C-edited/12C,14N-filtered NOESY experiments were done. *On the left:* 2D 15N-HSQC spectrum in the free form (at 5C) and in the complex showing differences of the (amide proton and nitrogen) chemical shifts. *On the right:* intermolecular NOEs contacts. The side chains of the DNA-contacting amino acids are colored. Red, unambiguous assignments and in gold, tentative assignments.

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List of Publications

Callaerts P, Lee PN, **Hartmann B**, Farfan C, Choy DW, Ikeo K, Fischbach KF, Gehring WJ, de Couet HG.HOX genes in the sepiolid squid Euprymna scolopes: implications for the evolution of complex body plans. *Proc Natl Acad Sci U S A.* **2002,** Feb 19;99(4):2088-93.

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Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient. *Cell* **2003**,113(2): 221-33
***equal contribution**

Pyrowolakis, G., **Hartmann B.**, Müller, B., Basler K. and Affolter M.
A simple molecular complex mediates widespread BMP-induced repression during *Drosophila* development. *Dev.Cell* **2004**, 7: 229-240

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Erklärung

Ich erkläre hiermit, dass die Dissertation „Characterization of Schnurri, an integral component of the Dpp-signaling pathway in *Drosophila melanogaster*“ nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Fakultät eingereicht wurde

Britta Hartmann

Basel, den 24.01.2004

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Conversion of an Extracellular Dpp/BMP Morphogen Gradient into an Inverse Transcriptional Gradient

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Summary

Morphogen gradients control body pattern by differentially regulating cellular behavior. Here, we analyze the molecular events underlying the primary response to the Dpp/BMP morphogen in *Drosophila*. Throughout development, Dpp transduction causes the graded transcriptional downregulation of the *brinker* (*brk*) gene. We first provide significance for the *brk* expression gradient by showing that different Brk levels repress distinct combinations of wing genes expressed at different distances from Dpp-secreting cells. We then dissect the *brk* regulatory region and identify two separable elements with opposite properties, a constitutive enhancer and a Dpp morphogen-regulated silencer. Furthermore, we present genetic and biochemical evidence that the *brk* silencer serves as a direct target for a protein complex consisting of the Smad homologs Mad/Medea and the zinc finger protein Schnurri. Together, our results provide the molecular framework for a mechanism by which the extracellular Dpp/BMP morphogen establishes a finely tuned, graded read-out of transcriptional repression.

Introduction

It was proposed more than a century ago that the organization of cell and body patterns might be controlled by concentration gradients of "form-producing" substances or morphogens (Morgan, 1897; Turing, 1952; Wolpert, 1989). Only recently has it been possible to demonstrate that secreted proteins of the Wnt, Hedgehog, and transforming growth factor-β (TGFβ) families specify positional information by this mechanism (reviewed by Gurdon and Bourillot, 2001).

Particularly compelling evidence for the existence of an extracellular morphogen gradient comes from studies on the developing wing imaginal disc of *Drosophila*, where a localized source of the BMP2/4 homolog Decapentaplegic (Dpp) is expressed in a stripe of cells along the anteroposterior compartment boundary and exerts a direct and long-range organizing influence on both the anterior and posterior halves (reviewed by Strigini and Cohen, 1999; Podos and Ferguson, 1999). In addition to controlling growth, Dpp induces the expression of different target genes above distinct threshold concentrations. These targets include *vestigial* (*vg*), *optomotor-blind* (*omb*), and *spalt* (*sal*), are expressed in progressively narrower domains, define the primordium of the wing blade, and control important aspects of pattern, differentiation, and survival (Kim et al., 1996; Grimm and Pflugfelder, 1996; Sturtevant et al., 1997; reviewed by Podos and Ferguson, 1999).

An understanding of how morphogen gradients operate requires answers to two different questions. How do concentration gradients arise, and how do cells interpret different morphogen concentrations? While recent efforts in the field focused on the problem of how Dpp protein spreads through tissue (Ramírez-Weber and Kornberg, 1999; Entchev et al., 2000; Teleman and Cohen, 2000), we are here concerned with the question of how a Dpp gradient is converted into transcriptional outputs.

Like all members of the TGFβ superfamily, Dpp assembles at the cell surface a receptor serine/threonine kinase complex comprising subunits known as the type I and type II receptors, encoded by the genes *thick-veins* (*tkv*) and *punt*, respectively (reviewed by Massagué, 1998; Podos and Ferguson, 1999; Tabata, 2001). The binding of Dpp to its receptors triggers the phosphorylation of Tkv by Punt and in turn enables Tkv to recognize and phosphorylate the Smad protein Mad (Raftery and Sutherland, 1999; Tanimoto et al., 2000). Phosphorylation releases Mad from cytoplasmic retention, allowing its association with the related factor Medea (Med) (Hudson et al., 1998; Inoue et al., 1998; Wisotzkey et al., 1998) and subsequent translocation into the nucleus, where the two proteins are involved in the transcriptional regulation of target genes (reviewed by Massagué and Wotton, 2000; Affolter et al., 2001).

Mad and Med possess DNA binding activities that have been implicated in the recognition of a regulatory element in the *vg* gene (Kim et al., 1997). Hence, *Drosophila* Smad proteins have been proposed, in analogy to their vertebrate counterparts, to directly activate the Dpp targets *vg*, *omb*, and *sal*. An alternative mechanism has recently emerged with the unexpected discovery of Brinker (Brk), a transcription factor that is required to counteract responses to Dpp (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a; Minami et al., 1999). Loss of Brk function causes overproliferation and ligand-independent, ectopic expression of the Dpp targets *vg*, *omb*, and *sal*. *brk* expression itself is negatively regulated by Dpp, such that peripheral cells in the wing disc express high and central cells undetectable levels of Brk. These findings raise the possibility that it is primarily the repressive function of Brk that controls growth and Dpp target gene expression and that direct transcriptional activation by Mad may only play a subordinate role.

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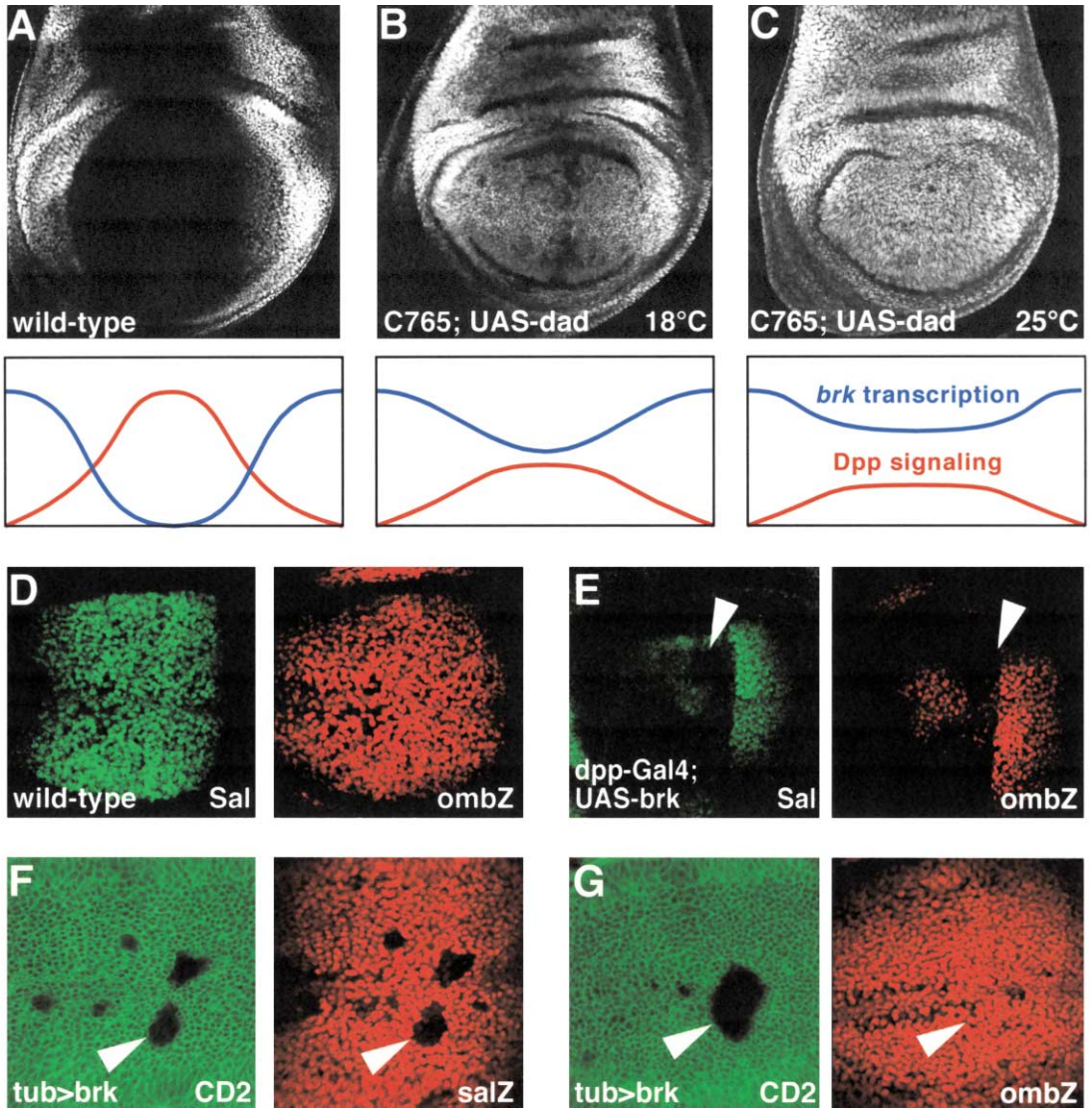

Figure 1. The Dpp Signaling Gradient Determines the Profile of *brk* Expression, Which in Turn Defines the Activity States of Dpp Target Genes

(A–C) Confocal sections of wing discs are shown (dorsal up, anterior to the left). *brk* expression is visualized by means of the *brk-lacZ* reporter *X47* in wild-type (A) and when Dpp signaling is downregulated by expressing the inhibitory Smad *Daughters against dpp* (*Dad*, [B and C]). The low-level ubiquitous *C765-Gal4* line was used to induce a *UAS-dad* transgene, which results in a shallower, and hence better detectable, *brk* gradient. Below each panel our interpretation is shown in the form of diagrams, which indicate the inverse relation between Dpp signaling levels (red) and *brk* expression levels (blue).

(D–G) Different Brk levels define distinct combinations of target gene expression. (D) Wild-type expression patterns of the Dpp target genes *sal* (in green) and *omb* (in red) are shown in wing primordia. (E) High levels of ectopic Brk expression were obtained with *dpp-Gal4 UAS-brk*. These levels of Brk repress the expression of both *sal* (E, left) and *omb* (E, right). The domain of *dpp-Gal4* activity is broader than the domain of endogenous *dpp* expression, hence the widespread effect in the anterior compartment. *dpp-Gal4*, rather than *actin5c>Gal4*-expressing clones, was used in this experiment to drive *UAS-brk* expression, because clones ectopically expressing substantial levels of *brk* rapidly undergo apoptosis in the wing pouch epithelium. (F and G) Low levels of ectopic Brk were obtained with a *tubulinα1>CD2>brk* construct. Clones expressing *brk* under the *tubulinα1* promoter (*tub>brk*) are marked by the absence of CD2 staining (in green). In such clones, *sal* expression is repressed (F), but *omb* expression is unaffected (G).

Here, we provide strong support for this view by studying the role and establishment of the Brk gradient. We find that the output of Dpp signaling and the action of the zinc finger protein Schnurri (Shn), both of which have been implicated by genetic means in the regulation of *brk* (Marty et al., 2000; Torres-Vazquez et al., 2000), converge on defined silencer elements of *brk*. The resulting inverse expression gradient of nuclear Brk protein has the capacity to differentially regulate *omb* and *sal*. Our results provide the molecular framework for a mechanism in which the extracellular Dpp gradient is converted into primary nuclear outputs via the generation of an inverse transcriptional gradient of *brk* by means of Shn-dependent silencer elements.

Results

Dpp Signaling Levels Control the Profile of the Brk Expression Gradient

High levels of Dpp signaling prevent the expression of the *brk* gene (Campbell and Tomlinson, 1999; Minami et al., 1999). In contrast, *brk* is readily transcribed in cells situated far away from a Dpp source or in cells with an experimental block in the Dpp transduction pathway (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a; Minami et al., 1999). In leg and wing imaginal discs, lateral cells, expressing maximal levels of *brk*, and central cells, in which *brk* expression cannot be detected, are separated by a seemingly narrow stripe of cells with graded *brk* expression. To explore whether position and spatial extent of this population are sensitive to Dpp signaling levels, we altered the presumptive Dpp signaling gradient by ubiquitously expressing the inhibitory Smad6 homolog Dad (Tsuneizumi et al., 1997). Low Dad levels cause a significant expansion of the *brk*-expressing domains toward the center of the disc (Figure 1B, *C765-Gal4 UAS-dad* at 18°C) with an extended, shallow gradient of *brk* levels. Higher levels of Dad (Figure 1C, same genotype at 25°C) produce an even more pronounced effect with cells along the entire anteroposterior (AP) axis expressing *brk*. We interpret these observations as indication that different levels of Dpp signaling determine, with an inverse relationship, different levels of *brk* expression. These experiments taken together with the genetic requirement of *brk* for regulating target genes (data not shown) suggest that the functional Brk gradient extends beyond the domain in which graded *brk* expression can be detected with reporter genes in wild-type.

Brk Expression Levels Control the Activity States of Dpp Target Genes

The Dpp target genes *vg*, *omb*, and *sal* are expressed in nested domains with progressively narrower widths of activity along the AP axis. The expression of all three of these genes is subject to repression by Brk in lateral regions of the wing disc (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a; Minami et al., 1999), raising the possibility that different levels of *brk* alone are able to specify distinct combinations of activity states of these genes. To address this possibility, we asked whether low levels of ectopic Brk expression can repress *sal*, but not *omb*, transcription, whereas high levels of Brk levels would repress both genes. High levels of ectopic Brk expression in the center of the disc were obtained by using a *UAS-brk* transgene in conjunction with a *dpp-Gal4* driver. Low levels of Brk were expressed by the weak constitutive promoter from the *tubulinα1* gene in marked clones of cells. As shown in Figure 1E, the *dpp-Gal4 UAS-brk* transgenes cause repression of both *sal* and *omb* transcription. In contrast, the lower levels of *brk* produced by *tubulinα1>brk* repress only *sal*, while *omb* transcription is not affected. Hence, different levels of Brk expression can elicit distinct outputs.

Together, the experiments described so far imply that the transcriptional control of *brk* is a key event in the interpretation of the Dpp morphogen gradient. In order to understand how this morphogen gradient becomes translated into different cell fates, we focused on the question of how Dpp generates an inverse transcriptional gradient of *brk* expression.

Dissection of the *brk* Regulatory Regions into Separable Enhancer and Repression Activities

Our first efforts were directed toward isolating the regulatory elements of the *brk* gene that ensure proper expression levels along the AP axis in response to Dpp signaling. We scanned the 20 kb region between the *brk* transcription unit and its upstream neighboring locus for such elements (Figure 2A). Restriction fragments from genomic *lambda* phages were cloned into a *lacZ* reporter P element and assayed for regulatory activity in vivo. This led to the identification of fragment B14, which faithfully recapitulates all aspects of late embryonic and larval *brk* expression (Figure 2A).

Interestingly, we found that distal truncations of B14 caused a progressive widening of the lateral expression domains toward the center of wing imaginal discs, while the levels of expression remained constant (Figure 2A). This observation suggested to us that the *brk* enhancer consists of two separable entities, a ubiquitously active, constitutive enhancer element located in the proximal half and a regulated repression activity encoded by the distal half.

Both activities were narrowed down by an extensive series of reporter constructs, a small subset of which is shown in Figures 2B–2D (for details, see legend to Figure 2). Three short fragments (called A, B, and C) were identified that possess repression activities when coupled to the constitutive enhancer represented by construct B38 (Figures 2B and 2C). The most potent of these short elements, fragment C, was further dissected into a 53 bp element (Figure 2D), referred to as S (S for *silencer*, see below). Its repression function is encoded in a nonredundant manner, as point mutations abolish its activity (Figure 2D and Experimental Procedures).

The dissection of 20 kb of potential regulatory sequences into two discrete minimal elements with opposite activities, which together reconstitute the hallmarks of *brk* expression, establishes the basis for our molecular studies. As described below, fragment C and its shorter derivative S serve as a paradigm to study the regulation of *brk* repression.

A Signaling-Regulated Silencer: The *brk* Repression Element Can Operate Independently of the *brk* Enhancer, but Its Activity Depends Strictly on Dpp Input

The activities of the *brk* regulatory elements were analyzed in diverse imaginal and embryonic tissues (Figure 3). Invariably, repression activity was maximal in vicinity of well-characterized sources of Dpp, suggesting that this activity is dependent on Dpp signaling. To confirm this apparent requirement for Dpp input, the repression activity was monitored in wing disc cells lacking the Dpp type I receptor Thick-veins (Tkv). *tkv* mutant cells autonomously lost repression activity (data not shown, but see below), indicating that this repression is strictly regulated by Dpp signaling.

Reporter constructs exhibiting spatially decreased domains of repression can therefore be regarded as less

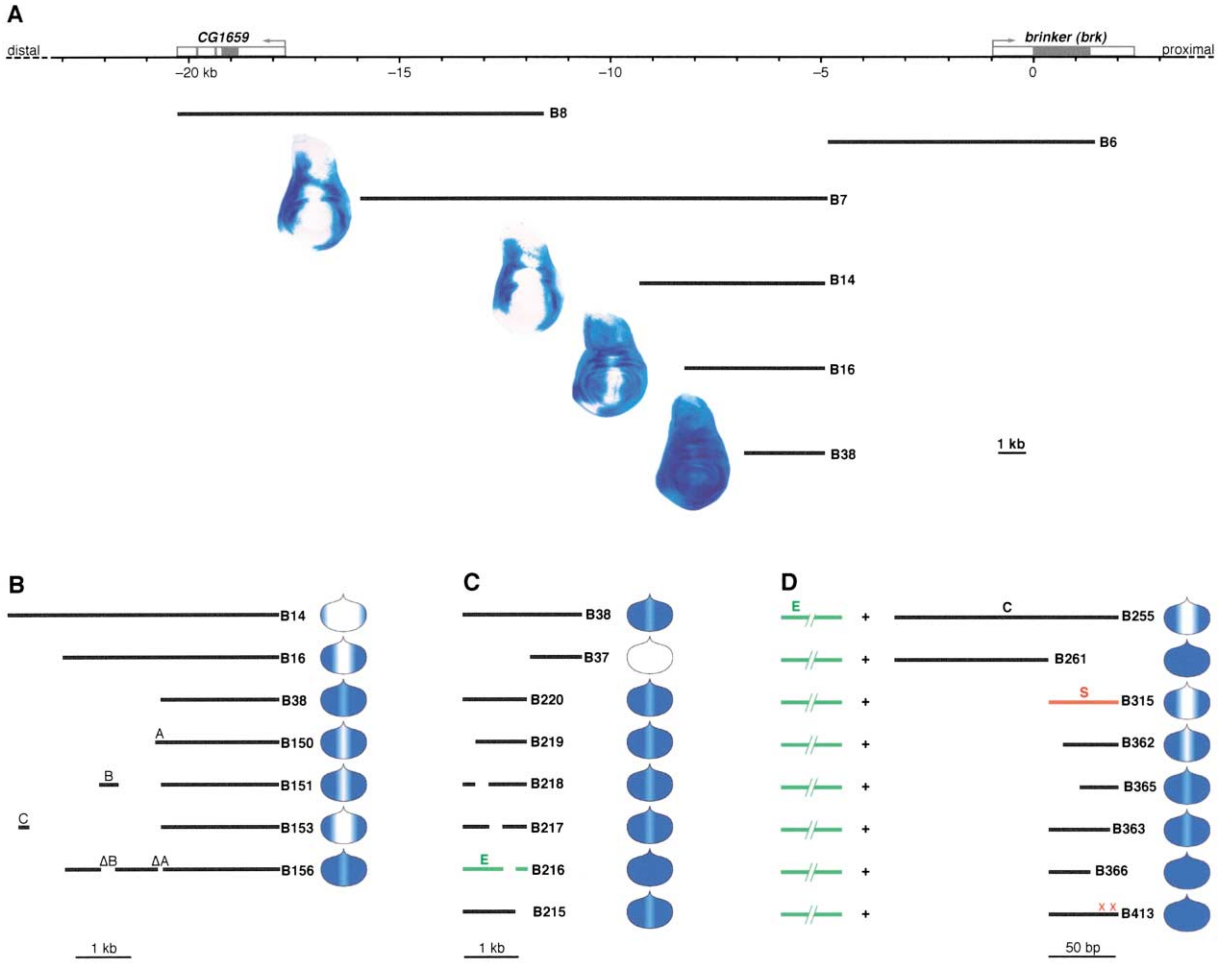

Figure 2. Dissection of the *brk* Regulatory Region into Separable Activating and Repressing Activities

(A) A map of the upstream region of the *brk* locus is shown on top. Restriction fragments B6, B7, and B8 were tested for their ability to drive reporter gene expression in transgenic animals. Fragment B8 did not cause any detectable expression, and no transgenic animals were obtained from fragment B6, likely due to toxicity. Fragment B7 faithfully recapitulated all aspects of *brk* expression and was further reduced in size, leading to the 5 kb fragment B14 that still drives *brk*-like expression. Distal truncations of fragment B14 resulted in a progressive widening of the lateral expression domains in imaginal discs (see B14, B16, and B38), suggesting that B14 contains repression elements in its distal part and a constitutively active enhancer in its proximal part (represented by fragment B38). In all panels, only a small subset of the constructs tested are shown.

(B) Three fragments A, B and C were identified in the distal part of B14 to cause repression in central regions of the wing disc (in combination with the constitutively active enhancer B38), as shown in constructs B150, B151, and B153. Among the three fragments, C showed strongest activity. If sequences A and B are removed from B16 (i.e., construct B156) repression activity is almost completely lost and expression is like that of B38.

(C) Fragment B38 still shows slightly reduced expression in the center of the disc where Dpp signaling is highest. In an attempt to obtain an enhancer fragment that is uniformly expressed, B38 was further dissected. This lead to the identification of B216 (shown in green) that is evenly and ubiquitously expressed in the wing pouch and therefore provides a sensitive tool to test other fragments for their ability to mediate regulated repression. We call B216 "E" for *enhancer*.

(D) Fragment C (see Figure 2B) was chosen for further analysis. The repressive activity of C and of its derived subfragments was assayed in combination with E (Figure 2C), and was localized to a 53 bp subfragment, referred to as "S" (for *silencer*, shown in red). The activity of S was strongly reduced and became unstable by further terminal deletions (B362, B365, B363, and B366). In addition, systematic point mutations throughout S identified base pairs that are essential for repression activity (exemplified by B413), leading us to conclude that S represents a minimal fragment. For nucleotide sequences of S and B413, see Experimental Procedures.

sensitive toward Dpp input. In all tissues examined, the decrease in sensitivity of such reporters is similar to that observed in wing discs (Figure 3), indicating that the *brk* repression element operates throughout embryonic and imaginal stages to perceive the activity state of the Dpp signal transduction pathway.

So far, the *brk* repression element has only been assayed in the context of the constitutive *brk* enhancer, which is part of the same regulatory region in the *Drosophila* genome. We sought to test whether this negative regulatory element can impose Dpp-dependent repression on heterologous enhancers. Below, we use three diverse enhancers in three different systems to provide evidence that this is indeed the case.

First, we used a previously characterized regulatory element of the *dpp* locus, which directs uniform expression within the pouch region of wing imaginal discs (Müller and Basler, 2000). When the *brk* repression ele-

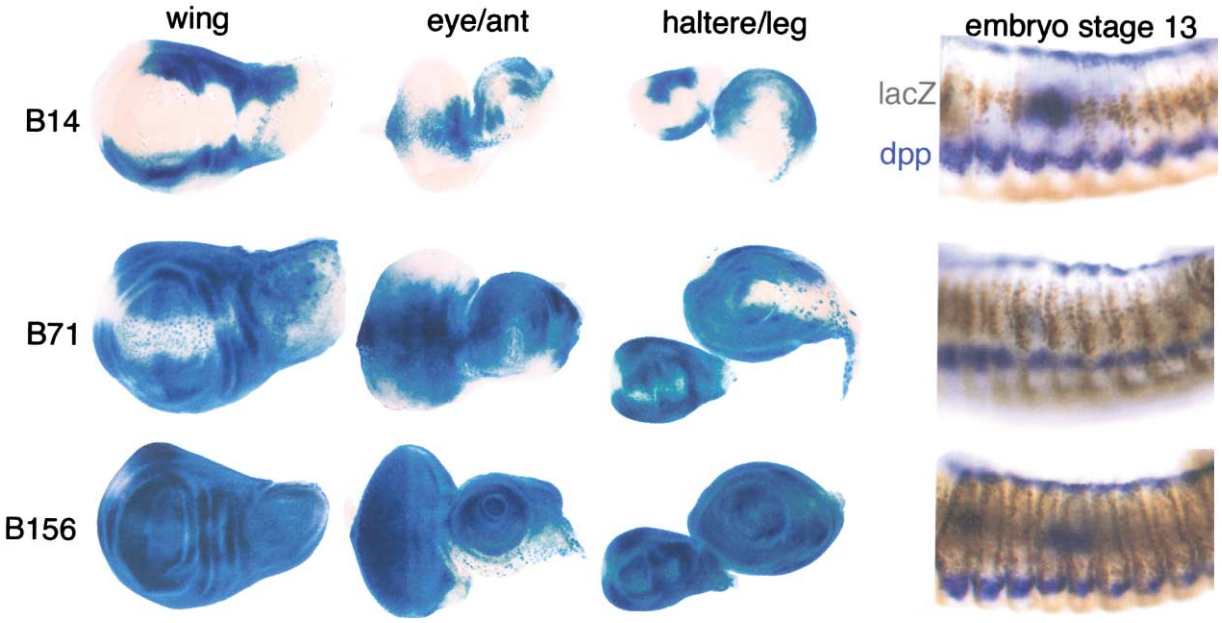

Figure 3. Throughout Embryonic and Larval Tissues, the Activity State of the Dpp Signaling System Is Integrated by the *brk* Regulatory Region

Expression of *brk* reporter constructs with different sensitivities to Dpp signaling are shown: wild-type sensitivity (B14, top row), decreased sensitivity (B71, middle row), and almost absent sensitivity (B156, lowest row). In all larval tissues analyzed (i.e., eye, antennal (ant), haltere, and leg imaginal discs), as well as in mid- and late-embryonic tissues, the sensitivity of the reporter constructs to Dpp is similar to that observed in wing discs, judged by the gap between the expression domains of *dpp* (data not shown for discs) and the reporters. In the embryo (the rightmost panels) *dpp* mRNA expression is shown in blue, and the antibody staining in brown detects the *lacZ* expression of the reporter constructs. Wing discs are oriented with their anterior side up and dorsal to the right.

ment is linked to this enhancer, transcriptional activity is confined to the lateral edges of the wing pouch (Figure 4A). Second, we assayed the embryonic *even-skipped-stripe-2* enhancer (Small et al., 1992) in isolation of, and combination with, the *brk* repression element. This enhancer is normally active in a circumferential band of cells in the early blastoderm stage. However, when linked to *brk* repression elements, the *even-skipped-stripe-2* enhancer is repressed in dorsal regions of the embryo (Figure 4B), where cells are exposed to high levels of Dpp (Ferguson and Anderson, 1992). Finally, we assayed the activity of the *brk* repression element in the context of a Notch-responsive enhancer in *Drosophila* tissue-culture cells. This synthetic enhancer shows a 30- to 40-fold stimulation of reporter gene expression upon transfection of S2 cells with plasmids driving the expression of Suppressor of Hairless (Su[H]) and a constitutively active form of Notch (Kirkpatrick et al., 2001). Simultaneous cotransfection of a plasmid encoding the activated form of the Dpp receptor Tkv (Tkv^{QD}, see Nellen et al., 1996) blocks this activation, in a manner strictly dependent on the presence of the *brk* repression fragment (Figure 4C). Thus, S2 cells are capable of transducing Tkv input and converting it into transcriptional regulation. The repression mediated by the *brk* element in this system occurs in the context of a heterologous enhancer located on transfected plasmid DNA.

Together with our finding that the *brk* repression element can mediate Dpp-dependent repression independently of its orientation or position (data not shown), the above-described results allow us to call this element a "signaling-regulated silencer."

The Net Balance of Silencer and Enhancer Activities Determines the *brk* Expression Levels

The results presented so far indicate that the levels of *brk* expression determine the fate of wing cells along the AP axis and that these levels are defined by three parameters: (1) the degree of activation of the Dpp transduction pathway, (2) the "strength" of the constitutive *brk* enhancer, and (3) the repressive activity of the *brk* silencer at any given degree of Dpp signaling. This model raises the prediction that altering any of the three parameters, while leaving the other two fixed, should have a direct impact on the spatial profile of *brk* expression. In the first result section, we have tested the effects of altering the Dpp signaling levels. We next set out to alter the activity of the *brk* silencer (S). As shown in Figure 5, an increase of its copy number results in a progressive lateral shift of those cells that express high detectable levels of reporter gene activity in the wing disc. Conversely, the duplication of the constitutive *brk* enhancer (E) has the opposite effect and leads to an expansion of reporter gene expression toward the disc center at a given number of *brk* silencer elements. Hence, it is the net balance of the two opposing regulatory forces that determines the level of *brk* expression at any given level of Dpp signaling.

brk Silencer Activity Depends on Mad, Med, and Schnurri Function

Over the past few years, a fairly detailed picture has emerged of how target genes are activated in response to ligands of the TGFβ, BMP, and Activin families in a stage- and tissue-specific manner (reviewed by Mas-

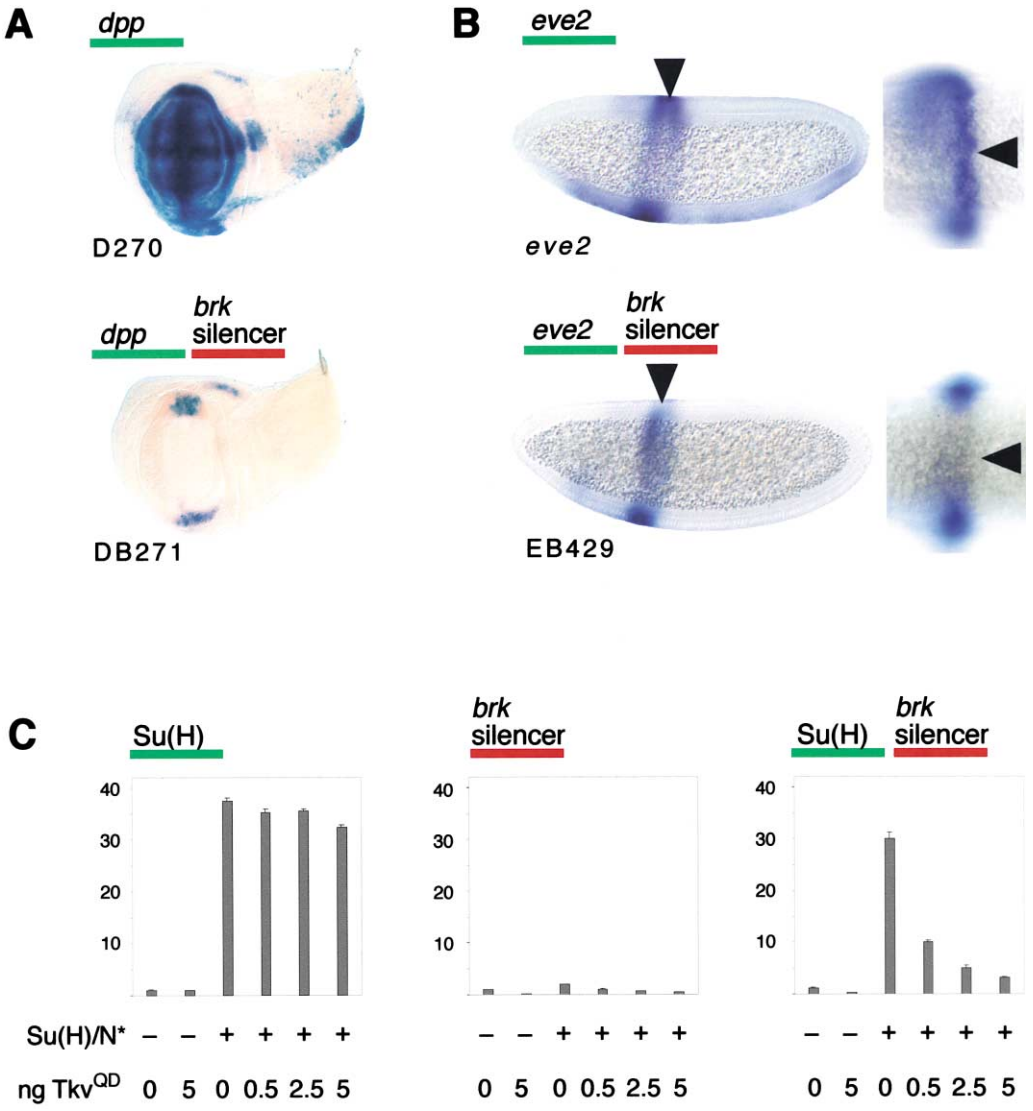

Figure 4. The *brk* Silencer Imposes Repression on Heterologous Enhancers Active in Discs, in the Early Embryo, and in S2 Cells

(A) Construct D270 is derived from a minimal *dpp* enhancer that lacks repressive inputs of both Ci and Engrailed (see Figure 2 of Müller and Basler, 2000) and drives reporter expression in the entire wing pouch. The addition of *brk* silencer fragments in construct DB271 leads to a repression in central domains where Dpp signaling occurs.

(B) A blastoderm-stage embryo in which reporter gene expression is driven by the *even-skipped-stripe-2* enhancer (*eve2*, Small et al., 1992) is shown on top, either in a lateral view (to the left) or dorsal view (to the right, higher magnification). The *eve2* enhancer is fully active on the dorsal side of the embryo (arrowheads). The addition of *brk* silencer elements (construct EB429) causes a repression in dorsal domains where Dpp signaling is highest (arrowheads). The anterior sides of embryos are oriented to the left.

(C) β-galactosidase reporter assays in *Drosophila* S2 cells. Reporter plasmids contain the *lacZ* gene under the control of a Suppressor of Hairless response element (Su[H], in green, left), a fragment of the *brk* control region containing the subfragement S (*brk* silencer, in red, middle), or the combination of the two elements (right). These reporters were cotransfected with a combination of plasmids encoding Su(H) and an activated form of Notch (N*). Increasing amounts of a plasmid expressing Tkv^{QD} lead to a stepwise repression of reporter activity (right). Tkv^{QD}-mediated repression is strictly dependent on the presence of the *brk* silencer since it is not observed with the reporter containing only the Su(H) response element (left). β-galactosidase values were normalized by cotransfecting 5 ng of a plasmid expressing luciferase as an internal standard. Results shown represent the average β-galactosidase activities from transfections done in triplicates (± standard deviation) and are expressed as the X-fold activation over the basal activity of each reporter plasmid alone.

sagué and Wotton, 2000; Attisano and Wrana, 2002). To explore how input by the BMP homolog Dpp causes repression rather than activation of *brk* transcription, and how it can do so in virtually all cells of an organism, we set out to analyze this process by genetic and biochemical means. We first assayed the requirements for the known Dpp signal transduction components in the above-described context in which the *brk* silencer represses transcription driven by the heterologous wing blade enhancer from the *dpp* locus. As shown in Figures 6A–6C, wing cells require the activities of the *tkv*, *Mad*, and *Med* genes to repress reporter gene expression. In addition, the *brk* silencer also depends on Shn function, as *shn* mutant cells ectopically express high levels of

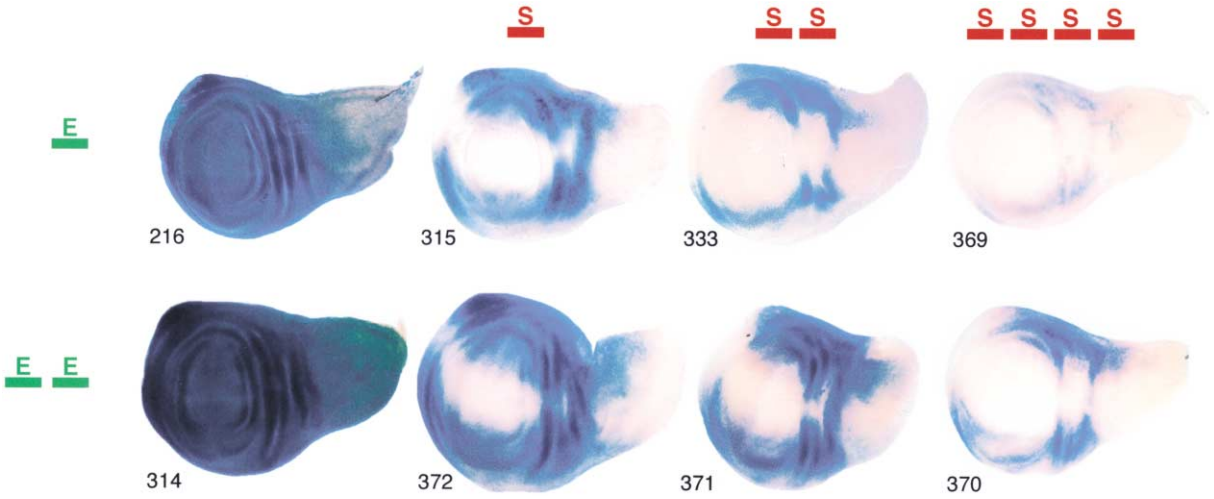

Figure 5. The Net Balance of *brk* Enhancer and Silencer Activities Determines the Transcriptional Output Levels in a Given Cell

None, one, two, or four copies of *brk* silencer elements (S, shown in red) were combined with one (top row) or two (bottom row) *brk* enhancer elements (E, shown in green). An increase in the copy number of S results in a progressive lateral shift of those cells that express high detectable levels of reporter gene activity (both rows, from left to right). Conversely, the duplication of enhancer element E has the opposite effect and leads to an expansion of reporter gene expression at a given number of *brk* silencer elements (compare top row with bottom row). Discs are oriented with their anterior side up.

the reporter gene (Figure 6D). The same requirements were observed in cultured cells, where Tkv^{QD} activity was no longer able to abolish Notch-induced activation of reporter gene expression when either endogenous *Mad* or endogenous *shn* functions were knocked-down by RNAi (Figure 6F). Addition of either double-stranded RNA had no effect, however, on the Notch-stimulated induction in the absence of Dpp signaling.

The C-Terminal 640 Amino Acids of Shn Are Necessary and Sufficient for Dpp-Dependent *brk* Repression In Vivo and In Vitro

Our observations that repression by the minimal *brk* silencer shows the same requirements in S2 cells as in vivo (i.e., an activated Dpp receptor, Mad, Med, and Shn) prompted us to analyze this process biochemically with epitope-tagged proteins. However, all our attempts to detect significant amounts of full-length Shn protein in extracts from embryos or from transfected S2 cells failed. The Shn protein is very large (2529 amino acids, Arora et al., 1995; Grieder et al., 1995) and proved to be refractory to biochemical manipulation in our hands. To overcome this limitation, we searched for shorter derivatives of Shn that retained the ability to mediate Dpp-dependent repression of *brk*. In a series of Shn proteins with terminal truncations and/or internal deletions, we identified one short form, referred to as ShnCT, which retained the key properties of full-length Shn. Like transgene-derived full-length Shn, ShnCT is able to repress transcription of the endogenous *brk* gene (data not shown), as well as that of the B14 reporter gene (Figure 7A), in *shn* null mutant embryos in a Dpp-dependent manner. ShnCT comprises the C-terminal 640 amino acids and thus three of the eight Shn zinc finger motifs. In contrast, ShnNT, which comprises all but the 640 residues of ShnCT, or ShnΔZF6-8, which only lacks the three C-terminal zinc fingers, has no detectable rescuing activity (Figure 7A), indicating that these structural motifs play a crucial role in repression via *brk* silencer elements.

To confirm that the same C-terminal Shn sequences are able to mediate *brk* repression in our S2 cell assay, we expressed ShnCT in cells treated with double-stranded RNA against the central portion of endogenous *shn* mRNA and, hence, substituted endogenous Shn protein with ShnCT. Dpp-dependent repression was fully recovered under these conditions (Figure 7B). Furthermore, and as observed in vivo, the three clustered zinc fingers in ShnCT are critical for this rescue of repression, validating our Shn reagent as well as our cell-based transcription assay.

The *brk* Silencer Element Assembles a Shn/Mad/Med Complex

Since all three proteins that are required for Dpp-dependent repression by genetic criteria contain putative DNA binding domains (i.e., Mad, Med, and Shn), we set out to test their ability to molecularly interact with the *brk* silencer element in extracts of Tkv^{QD}-expressing S2 cells. Electrophoretic mobility shift assays indicated that neither ShnCT nor Med was able to form a stable protein/DNA complex (Figure 7C), although both proteins were readily expressed (data not shown). Transfection with a Mad-encoding plasmid resulted in the formation of a detectable protein/DNA complex (Figure 7C, lane 3), but a more prominent complex of similar mobility was obtained upon expression of Mad in combination with Med (Figure 7C, lane 7). Coexpression of ShnCT with Mad and Med led to the formation of a complex of even slower mobility (lane 8), suggesting that ShnCT is recruited to the *brk* silencer element with the help of Mad and Med. In contrast, the complex that formed in the presence

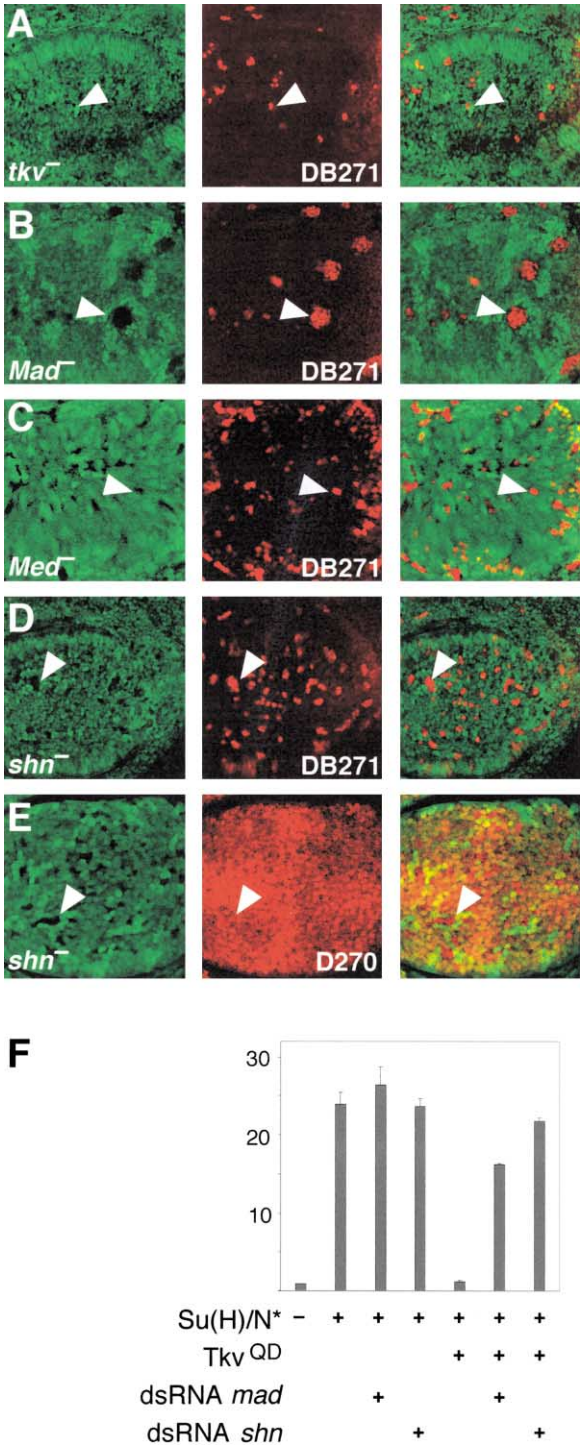

Figure 6. *brk* Silencer Activity Depends on Tkv, Mad, Med, and Shn Function

(A–D) Expression of reporter DB271 (described in Figure 4A) in wing discs with *tkv*, *Mad*, *Med*, and *shn* mutant clones. The left shows the expression of the marker gene (green), the loss of which indicates mutant genotypes. In the middle, the β-galactosidase expression of DB271 is visualized (red). A merge of both images is shown to the right. Expression of DB271 is strongly upregulated in medial *tkv*, *Mad*, and *Med*, as well as in *shn* mutant clones.

(E) In contrast, expression of D270 is not affected by these genotypes. D270 lacks *brk* silencer elements and is expressed ubiquitously in the wing pouch. Representative for the *tkv*, *Mad*, *Med*, and *shn*, only the results for *shn* mutant clones are shown. Arrowheads point to exemplary clones.

(F) *Drosophila* S2 cells were cotransfected with a reporter plasmid containing the *brk* S element fused to the Su(H)-response element, expression plasmids and dsRNA fragments as indicated below the panel. Tkv^{QD}-mediated repression is blocked when endogenous Mad or Shn are "knocked down" by RNAi. β-galactosidase levels are shown as X-fold activation over the basal activity of the reporter plasmid when cotransfected with the empty expression vector. dsRNA fragments are derived from the *Mad* (nucleotides 658–1230) or the *shn* (nucleotides 5011–5531) coding regions.

of Mad and Med was not retarded in its mobility by concomitant expression of the ShnCT variant lacking the three clustered zinc fingers (lane 9).

To investigate the molecular composition of the low mobility protein/DNA complexes, we cotransfected Tkv^{QD}, ^{Flag}Mad, ^{Myc}Med, and ^{V5}ShnCT and assayed for the presence of the Flag, Myc, or V5 epitope tags by supershift analysis upon addition of the appropriate antibodies. In the absence of ShnCT, the complex contained both Mad and Med proteins as evidenced by supershifts with both the anti-Flag and the anti-Myc antibodies (Figure 7D). In the presence of ^{V5}ShnCT, the low mobility complex was additionally supershifted by antibodies directed against the V5 epitope. However, when the same low mobility complex was produced with an untagged version of ShnCT, no increase in mobility was observed upon addition of the anti-V5 antibody, confirming the specificity of the assay (data not shown).

From these biochemical experiments, we conclude that Mad, Med, and Shn form a protein complex on the *brk* silencer element. Nuclear translocation of Mad and Med requires the activation of the Dpp signaling pathway (Raftery and Sutherland, 1999). Since Shn is only recruited to the *brk* silencer element in the presence of Mad and Med, it can be inferred that the complex can only be established in response to Dpp signaling. Moreover, our finding that both transcriptional repression as well as complex formation critically depend on the presence of the C-terminal three zinc finger motifs supports the notion that the *brk* silencer element controls *brk* expression by assembling a Mad/Med/Shn multiprotein complex.

Discussion

Dpp's ability to organize cellular patterns serves as a paradigm for the existence and mode of action of extracellular morphogen gradients. Most notably, Dpp gradients control cell fates along the dorsoventral axis of the early embryo and along the anteroposterior axis of imaginal discs (reviewed by Podos and Ferguson, 1999). In addition to its capacity to act at long range, Dpp elicits distinct outputs at different concentrations (Nellen et al., 1996; Lecuit et al., 1996; Ferguson and Anderson, 1992). BMP activity gradients have also been implicated in the control of vertebrate body pattern, particularly in the establishement of the dorsoventral axes of the early mesoderm, neural tube, and retina (Holley and Ferguson, 1997; Lee and Jessell, 1999; Sakuta et al., 2001). Major interest is devoted, therefore, to the mechanisms

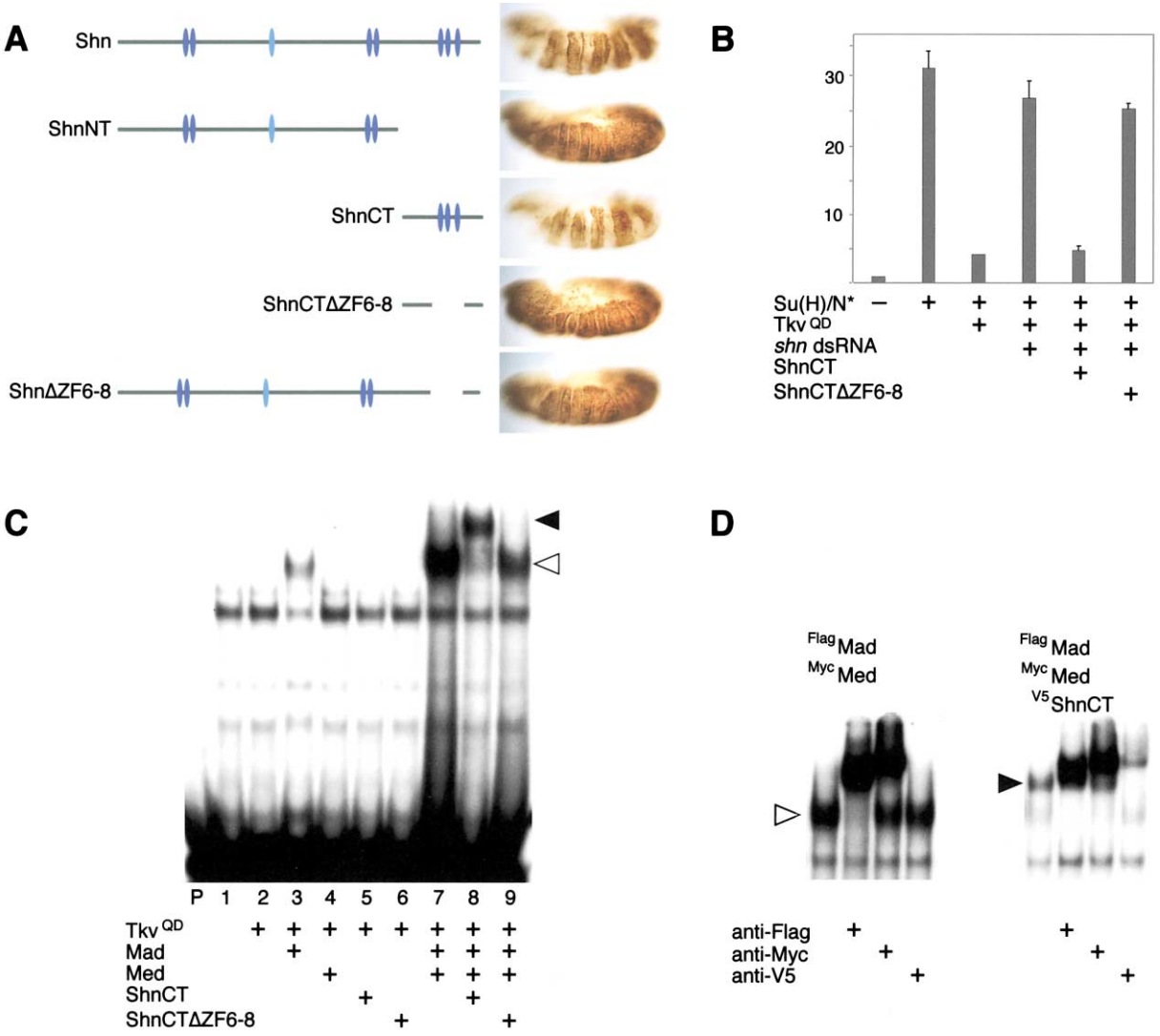

Figure 7. The Carboxy-Terminal Part of Shn Is Both Essential and Sufficient for Dpp-Dependent Repression In Vivo and in Cultured Cells and Forms a Complex with the *brk* Silencer, Mad, and Med

(A) Schematic representations of the Shn derivatives tested are shown to the left (blue ovals indicate zinc finger of the C2H2-type, light blue of the C2HC-type). Embryos transgenic for the illustrated *UAS-shn* constructs were tested for their ability to repress the expression of B14 or *brk* in vivo. The *UAS-shn* constructs were expressed in a shn^{TD5} mutant background together with *UAS-dpp* using a *paired-Gal4* driver. β-galactosidase expression of the B14 reporter is shown in stage 14/15 embryos to the right (assay described in Marty et al., 2000). The same results were obtained when these genotypes were assayed for *brk* transcript levels by in situ hybridization (data not shown). Shn, full-length (1–2529); ShnNT, N-terminal portion (1–1888); ShnCT, C-terminal portion (1888–2529); ShnCTΔZF6-8, C-terminal portion lacking zinc fingers 6 to 8 (i.e., residues 2263–2352); ShnΔZF6-8, full-length protein lacking zinc fingers 6 to 8 (i.e., residues 2263–2352). All Shn-fragments contained a Flag-epitope and expression of the tagged proteins was verified by antibody stainings of embryos (data not shown).

(B) S2 cell reporter gene assays. Cells were transfected with the plasmids indicated in combination with *shn* dsRNA to downregulate the expression of the endogenous Shn protein. The loss of Tkv^{QD}-mediated repression caused by *shn* RNAi can by restored by coexpression of ShnCT, but not ShnCTΔZF6-8. Note that the dsRNA used does not affect expression of the transfected carboxy-terminal Shn fragments, since it is derived from an upstream part of the *shn* coding region (corresponding to amino acids 1670–1842). Expression of the Shn construct has no effect on the Notch response as judged by cotransfections with the reporter plasmid containing the Su(H)-response element (data not shown).

(C) Lysates of S2 cells transfected with the indicated plasmids were analyzed in band shift assays using labeled *brk* fragment C as a probe. Transfection of Mad and Med leads to the formation of a protein/DNA complex of slow mobility (lane 7, indicated by the open arrowhead), which can be further retarded by cotransfecting ShnCT (lane 8, filled arrowhead), but not ShnCTΔZF6-8 (lane 9). Note that in single transfections, only Mad has the ability to form a complex with *brk* S (lanes 3–6). Radiolabeled probe S was loaded alone (lane P) and after incubation with extract from untransfected cells (lane 1). The Shn/Mad/Med complex can also be observed in transfection experiments in which no TkvQD is expressed (data not shown), suggesting that phosphorylation of Mad is not a prerequisite for complex formation in vitro.

(D) Lysates from cells expressing ^{Flag}Mad, ^{Myc}Med without ^{V5}ShnCT (left), or with ^{V5}ShnCT (right) in combination with Tkv^{QD} were subjected to band shift assays in the presence of the indicated antibodies. Positions of the Mad/Med-complex (open arrowheads) and Mad/Med/ShnCT-complex (closed arrowheads) are indicated. In the absence of ShnCT, the complex contained both Mad and Med proteins as evidenced by supershifts with both the anti-Flag and the anti-Myc antibodies (Figure 7D, left). In the presence of ^{V5}ShnCT, the low mobility complex was supershifted by antibodies directed against the V5 epitope.

by which Dpp/BMP signaling controls gene expression. Important advances have recently been made by the discovery that a significant aspect of Dpp target gene control involves the repressive action of Brk, whose expression itself is regulated by Dpp (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a; Minami et al., 1999; Marty et al., 2000). Here, we first confirm and extend these findings by showing that the Dpp signaling system shapes an inverse profile of Brk expression, which serves as a mold for casting the spatial domains of Dpp target genes. Thus, the question of how the Dpp morphogen gradient is converted into transcriptional outputs can be largely reduced to the question of how Dpp generates an inverse transcriptional gradient of *brk* expression. We applied an unbiased approach to this problem by isolating the regulatory elements of *brk*. We then identify and characterize a protein complex that binds to and regulates the activity of these elements in a Dpp dose-dependent manner.

The Two Key Elements of *brk* Regulation

Dissection of the *brk* locus revealed two separable elements with opposite properties: a constitutive enhancer and a morphogen-regulated silencer. Both elements have a direct effect on the level of *brk* expression, and it is the net sum of their opposing forces that dictates the transcriptional activity of *brk* in any given cell. In this sense, expression of the *brk* gene behaves like a spring that is compressed by Dpp signaling. Its silencer and enhancer embody the variable compressing and constant restoring forces, respectively. As stated by Hooke's law, an increased elastic constant (e.g., two copies of the constitutive enhancer) either shifts the *brk* levels toward those normally present at more lateral positions or necessitates a correspondingly higher compressing force (e.g., more silencer elements or higher levels of Dpp signaling). Given the central role Brk plays in controlling growth and pattern together with the direct impact of the two regulatory elements on *brk* levels, it appears inevitable that their quantitative properties must exhibit a fine-tuned evolutionary relationship with each other and with those of the Dpp transduction system. It appears, furthermore, that both the *brk* enhancer as well as the *brk* silencer elements represent ideal substrates for evolutionary changes in morphology.

The Molecular Events at the *brk* Silencer

Based on our combined genetic and biochemical analysis, we propose that upon Dpp signaling the following key players meet at the *brk* silencer elements to execute repression: the Smad proteins Mad and Med and the zinc finger protein Shn. The role of Shn must be to direct the signaling input provided by Mad and Med into transcriptional silencing. In principle, two scenarios can be envisaged by which Shn fulfills this task. Shn could possess repressor activity (presumably via recruitment of corepressors) but lack the ability to bind the *brk* silencer and, hence, depend on Mad/Med for being targeted to its site of action. Alternatively, Shn could be prebound to the silencer, but only be capable of recruiting corepressors upon interaction with Mad/Med. Based on our observation that a Shn/DNA complex cannot be detected in the absence of Mad/Med, we favor the first of these two possibilities. The molecular architecture of the protein complex binding to the *brk* silencer as well as the DNA sequences providing the specificity for the local setup of this complex remain to be determined in detail.

An additional protein, which appears to influence the events at the *brk* silencer, is Brk itself. Genetic experiments indicate that Brk negatively modulates its own expression, forming a short regulatory loop that contributes to the final shape of the Brk gradient (Hasson et al., 2001). This autoregulatory action occurs also via the *brk* silencer element (B.M., unpublished data), suggesting that Brk directly participates in the protein-protein or protein-DNA interactions at this site.

Most regulatory events ascribed to Smad proteins to date concern signaling-induced activation of target gene transcription. In the case of the *brk* silencer Shn could be regarded as a "switch factor" that converts an inherently activating property of Smad proteins into transcriptional repression activity. Indeed, it has been shown that Smad proteins have the ability to recruit general coactivators with histone acetyl transferase activity (reviewed by Massague and Wotton, 2000). However, in an alternative and more general view, Smad proteins per se may provide no bias toward activation or repression. Their main function may be to assemble transcriptional regulatory complexes involving other DNA binding proteins and endow these complexes with additional DNA binding capacity. Such associated DNA binding factors would not only determine target site specificity, but, by their recruitment of either coactivator or corepressor proteins, also define the kind of regulatory influence exerted on nearby promoters (Chen et al., 2002). Since Shn directs Mad/Med activity toward repression, we hypothesize the existence of at least one other such Mad/Med partner in *Drosophila* to account for Mad/Med-mediated activation of gene expression. Such Mad/Med-mediated activation appears to be required for peak levels of *sal* and *vg* transcription (Marty et al., 2000; Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a), as well as for defining gene expression patterns in domains where *brk* expression is completely repressed, e.g., close to the Dpp source of the dorsal embryonic ectoderm (Ashe et al., 2000; Jazwinska et al., 1999b).

The Specificity of Signaling-Regulated Repression

At the heart of our model is the direct causal relationship between the formation of a Shn/Mad/Med/*brk*-silencer complex and the silencing of *brk* gene transcription. Although the two observations have been derived from different experimental data sets (biochemical versus genetic, respectively), there is a firm correlation between the requirements for either event to occur. *brk* is not repressed when either (1) the *brk* silencer elements are lacking or mutated, when (2) Dpp input is prevented (and hence Mad is neither phosphorylated, nor nuclearly localized, nor associated with Med), or when (3) Shn is not present or is deprived of its C-terminal zinc fingers. The same set of requirements was observed for the formation of the Shn/Mad/Med/*brk* complex. Moreover, it is the concurrence of all three of these conditions that appears to provide the exquisite specificity to the Dpp-

regulated silencing of gene transcription. (1) It only occurs in conjunction with a functional *brk* silencer, or an equivalent element. (2) There is an absolute requirement for Dpp input in Shn-mediated silencing. Not even a partial repressor activity of Shn was observed in cells that do not receive Dpp signal (e.g., loss of *shn* function in cells situated in lateral-most positions of the wing disc does not cause a further upregulation of *brk* transcription). (3) Shn represents only one of several zinc finger proteins expressed in Dpp receiving cells, yet none of the other proteins is able to substitute for Dpp-mediated repression. A major determinant for the specificity with which Shn engages in the signaling-dependent protein/DNA complex appears to be the triple zinc-finger motif. Although it is likely that this structural feature is required for contacting specific nucleotides on the *brk* silencer, we can currently not exclude the possibility that some of the zinc fingers mediate protein-protein interactions between Shn and Mad, Med or other cofactors.

While all of the above-discussed elements contribute to the specificity of signaling-regulated repression, it is important to emphasize that one possibility for specificity has not been exploited. The *brk* repression element does not specifically impinge upon the constitutive *brk* enhancer but promiscuously diminishes transcriptional activation by heterologous enhancers. It is likely, therefore, that the *brk* repression element interferes directly with events at the promoter, a property that may permit it to function as a bona fide silencer.

From an Extracellular Gradient to a Nuclear Gradient to Growth and Thresholds

A fundamental characteristic of any morphogen system is that cells at different positions in the concentration gradient respond in qualitatively different ways. Cells must be able to activate different sets of genes at different threshold concentrations. The simplest way by which cells could produce two distinct responses at different threshold concentrations would be the employment of two kinds of receptors of different affinity for the morphogen. This mechanism does not appear to apply for the Dpp morphogen gradient, where Tkv and Punt appear to mediate both low- and high-threshold responses (see Gurdon et al., 1998). Thresholds could also be imposed at any downstream event in the signal transduction cascade. To our surprise, it appears that in the case of the Dpp morphogen, no such gates are in place, and the transcription of the *brk* gene is a negative image of the Dpp gradient. Thus, while our findings provide mechanistic insights into how an extracellular protein gradient is converted into a nuclear gradient of gene activity, they pass the burden of generating threshold effects on to downstream events. Several morphogen gradients operating in the early syncytial embryo, however, have been sufficiently well studied to explain the mechanistic principles of how a gradient of transcriptional activity can specify thresholds of gene activity and tissue differentiation (Struhl et al., 1989; Driever et al., 1989; Struhl et al., 1992; Jiang and Levine, 1993; Hoch and Jäckle, 1993).

A key difference between such embryonic transcriptional gradients and that of *brk* concerns the nature of their outputs: while all of them affect cellular patterns, Brk also controls growth. Flattening the *brk* gradient during development has catastrophic effects: reducing its high end causes overgrowth (Campbell and Tomlinson, 1999), and increasing its low end causes growth arrest (Jaźwińska et al., 1999a; B.M., unpublished data). It may be this fundamental role in growth control that prohibits a discontinuous conversion of the Dpp morphogen gradient into its first transcriptional output. The identification of the elusive growth target(s) controlled by the Brk gradient represents one of the major challenges in the field.

Experimental Procedures

Reporter Transgenes

Inserts of reporter constructs B6 to B38 are derived from genomic lambda phages (G5 and G17, gifts from G. Campbell) and were subcloned into the P element reporter plasmid pX27 (Ségalat et al., 1994). Inserts of B71 to B220 were obtained by PCR, using B14 as a template. Constructs B255 and B261 consist of B216 plus a PCR fragment representing C (B255) or part of C (B261). Constructs B261 to B413 consist of B216 plus a double-stranded oligonucleotide derived from C. The sequence of the C subfragment S is as follows (from distal to proximal): AGTGTCTGGCGGCGTAGCAAGACTGGC GACATTCTGTCTGGTGGCGATCGCC. B413 contains a mutated form of S (mutated bases in lower case): AGTGTCTGGCGGCGTAG CAAGACTGGCGACATTCTtTaTGGTGGCGATCGCC. The insert of D270 is a chimera of constructs 10ΔG and 10-En-mut as shown in Figure 2 of Müller and Basler (2000). For construct DB271, fragment C was inserted at the 5′ position of the *hsp70* promoter of D270. The *eve-stripe-2* enhancer is represented by the MSE construct as published in Small et al. (1992). To obtain EB429, four copies of S were cloned into the EcoRI site of the MSE construct.

Marked Clones of Mutant Cells

Clones of mutant cells were generated by Flp-mediated mitotic recombination, subjecting late second or early third instar larvae to a 35°C heat-shock for 30 min. All mutant alleles used are molecular nulls. Genotypes of dissected larvae were as follows. *Mad* mutant clones: y w hsp70-flp; Mad[12] FRT40/ubi-nlsGFP FRT40; DB271. *Med* mutant clones: y w hsp70-flp; FRT82 e Med[1]/FRT82 2xhsp70-πmyc; DB271. *tkv* mutant clones: y w hsp70-flp; tkv[a12] FRT40/ubi-GFP FRT40; DB271. *shn* mutant clones: y w hsp70-flp; FRT42 shn[TD5]/FRT42 hsp70-GFP; D270 or DB271. *tub>brk* clones: y w omb-lacZ hsp70-flp; tub>CD2,y+>brk and y w hsp70-flp; CyO[sal-lacZ], tub>CD2,y+>brk.

Immunohistochemistry

Imaginal discs from third instar larvae were fixed and stained by standard techniques. Antibodies were rabbit polyclonal anti-β-Gal (Cappel), mouse anti-cMyc (1-9E10.2, Santa Cruz Biotechnology), anti-rabbit 594 Alexa and anti-mouse 488 Alexa fluorescent secondary antibodies (Molecular Probes). To detect β-galactosidase activity, third instar larval discs were fixed and subjected to a standard X-gal color reaction for 2 hr at 37°C. For all X-gal stainings shown in this study, at least four independent transgenic lines were analyzed at standardized reaction conditions (2 hr at 37°C), and a representative disc was chosen for presentation.

S2 Cell Plasmids

S2 cell reporter plasmids containing the *brk* silencer were generated by inserting 100 bp of the 3′ end of fragment C (comprising subfragment S) between the EcoRI and Asp718 sites in hsplacCasper and 4xSuh-lacZ (Kirkpatrick et al., 2001). Epitope-tagged versions of Tkv^{QD}, Mad, Med, ShnCT, and ShnCTΔZF were cloned in the vector pAc5.1B/V5His (Invitrogen) for constitutive expression under the control of the *actin5c* promoter. Plasmids for constitutive expression of Su(H) and activated Notch were a gift from A. Laughon. dsRNA fragments were generated corresponding to nucleotides 658–1230

and 5011–5531 of the *Mad* and *shn* open reading frames, respectively.

Transfections and Reporter Gene Assays

For reporter gene assays 1.5×10^6 S2 cells were transfected with a total of 200 ng of DNA using the Effectene Transfection Reagent (Qiagen) (20 ng reporter plasmid, 5 ng of a plasmid constitutively expressing firefly luciferase, the indicated amount of expression plasmids and pAc5.1B/V5His to bring total DNA to 200 ng). For RNAi experiments 50 ng of the appropriate dsRNA fragment were cotransfected. Cells were lysed 48 hr after transfection for β-galactosidase and luciferase assays.

Band Shift Assays

The ^{32}P-labeled oligonucleotide probe (corresponding to silencer fragment S, see above) was generated by annealing and filling in overlapping oligonucleotides in the presence of [α-^{32}P]ATP. Epitope-tagged proteins were expressed in *Drosophila* S2 cells transfected with 100 ng of each expression plasmid. After 48 hr, cells were lysed in 100 μl of 100 mM TrisHCl (pH 7.8), 0.5% Triton X-100, 1 mM DTT, and protease inhibitors. For mobility shifts, 30 μg of protein was mixed with 10,000–20,000 cpm of probe in Binding Buffer (5×: 25 mM Tris-HCl [pH 7.6], 30% glycerol, 400 mM KCl, 50 mM $MgCl_2$, 50 μM $ZnCl_2$, 0.25% NP-40). Binding was allowed to proceed for 30 min on ice. Protein-DNA complexes were separated from free probe on 4% nondenaturing polyacrylamide gels (at room temperature for 130 min at 160V in 0.5 × TBE). For supershifts, the following antibodies were added to the binding reaction: 20 ng of monoclonal anti-Flag (M2, Sigma), 8 ng of monoclonal anti-Myc (9B11, Cell Signaling), 0.5 μg monoclonal anti-V5 (Invitrogen).

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A Simple Molecular Complex Mediates Widespread BMP-Induced Repression during *Drosophila* Development

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Summary

The spatial and temporal control of gene expression during the development of multicellular organisms is regulated to a large degree by cell-cell signaling. We have uncovered a simple mechanism through which Dpp, a TGFβ/BMP superfamily member in *Drosophila*, represses many key developmental genes in different tissues. A short DNA sequence, a Dpp-dependent silencer element, is sufficient to confer repression of gene transcription upon Dpp receptor activation and nuclear translocation of Mad and Medea. Transcriptional repression does not require the cooperative action of cell type-specific transcription factors but relies solely on the capacity of the silencer element to interact with Mad and Medea and to subsequently recruit the zinc finger-containing repressor protein Schnurri. Our findings demonstrate how the Dpp pathway can repress key targets in a simple and tissue-unrestricted manner in vivo and hence provide a paradigm for the inherent capacity of a signaling system to repress transcription upon pathway activation.

Introduction

A small number of signaling pathways (Wnt, TGFβ, Hedgehog [Hh], receptor tyrosine kinases [RTKs], Notch [N], Jak/STAT, and nuclear hormone receptors) control the majority of cell fate decisions during development of multicellular organisms (Barolo and Posakony, 2002; Gerhart, 1999). Each pathway is used repetitively during development and regulates distinct target genes in different developmental contexts. Although these signaling pathways are extremely diverse in their complexity and biochemical mechanisms of signal transduction, recent studies have revealed several fundamental similarities in the logic of how these pathways control gene expression (Barolo and Posakony, 2002). Three functionally conserved properties of these signaling cascades, "default repression," "activator insufficiency," and "cooperative activation," appear to allow signals to activate genes selectively and in a tissue-specific manner. In the absence of the ligand, default repression limits the ability of weak local activators bound to signal-dependent enhancers to activate target genes before signal transduction occurs. Transcriptional activation requires the cooperation of nuclear signal mediators with tissue-restricted factors, providing both for specificity and for selectivity of gene induction during development and homeostasis.

Although considerable progress has been made in the molecular analysis of how signaling pathways activate target genes, less is known about how extracellular signals actively repress gene transcription and how DNA binding site context discriminates between activation and repression. The best-studied case for signal-induced repression comes from the Toll signaling pathway in *Drosophila*, where the effector of the pathway, Dorsal, can repress a number of genes in a context-dependent manner (Courey and Jia, 2001; Stathopoulos and Levine, 2002). In the TGFβ-signaling pathway, molecular scenarios for ligand-induced repression have also been described. A complex containing Smad3, E2F4/5, DP1, and p107 exists in the cytoplasm, moves into the nucleus in response to TGFβ, associates with Smad4, and recognizes a composite Smad-E2F binding site in c-*myc* for repression (Chen et al., 2002). Similarly, Smad3 can physically cooperate with ATF3 and repress the transcription of the gene *Id*, an inhibitor of differentiation (Kang et al., 2003). In these two cases, the Smad proteins bind to or repress target genes cooperatively with the help of two different transcriptional regulators and two distinct *cis*-regulatory elements. A somewhat different scenario has been reported for a particular case of BMP-induced repression, in which Smad-dependent recruitment of a histone deacetylase/Sin3A complex accounts for the repressor activity of the Nkx3.2 protein (Kim and Lassar, 2003). Also in this case, repression relies on a tissue-restricted factor, Nkx3.2.

Transcriptional repression has also been analyzed in the context of the Dpp/BMP morphogen readout in *Drosophila*. Dpp signaling target genes are repressed in the absence of the ligand by the default repressor Brinker (Brk), which is not part of the signal transduction pathway proper (Affolter et al., 2001; Jazwinska et al., 1999; Minami et al., 1999; Raftery and Sutherland, 1999). To overcome this repression, a silencing mechanism is employed through which the activated Dpp signaling pathway represses *brk* transcription in many different tissues throughout development (Marty et al., 2000; Müller et al., 2003). Here we molecularly define this silencing mechanism and its minimal DNA sequence element. We show that transcriptional repression does not require cell-specific input, but depends on the capacity of a short *cis*-acting silencer element (SE) to bind the *Drosophila* Smad proteins Mad and Medea with high affinity. The precise sequence and spacing of the Mad and Medea binding sites allow the SE to recruit the zinc finger protein Schnurri, which brings along repressive activity. A combination of in vitro and in vivo assays with mutated minimal *brk* SE allowed us to derive a

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consensus sequence for a functional SE element. Genome-wide searches using this consensus sequence identified SEs both in genes known to be repressed by Dpp and in many other genes. We show in two cases that these SEs indeed repress transcription in a signaling-dependent manner via the same molecular complex we defined for the *brk* SE. Our findings reveal the existence of a repression system that relies on the organization of Smad binding motifs into a Smad/Shn complex-recruiting element. This system not only overcomes Brk-mediated default repression but also directly downregulates key developmental targets in many tissues in a strictly signal-dependent manner without apparent reliance on cooperation with cell type-specific transcription factors. The identification of a *cis*-regulatory signature for Dpp-dependent repression now allows for a genome-wide analysis of potential target genes and the study of their contribution to the biological effects of this important signaling pathway in *Drosophila*.

Results

Mad and Medea Directly Bind to a Dpp Morphogen-Dependent Silencer Element of the *brk* Gene

We have previously identified a 52 bp *cis*-regulatory sequence upstream of the *brk* gene that mediates Dpp-dependent transcriptional repression in vivo and in cultured S2 cells (Müller et al., 2003). We named the element the *brk* silencer (*brkS*) and showed that it forms a protein-DNA complex with the two Dpp signal mediators Mad and Medea (Med) and the zinc finger protein Shn. Mad and Med only bind to *brkS* upon activation of the Dpp signaling cascade (Figure 1A), and the formation of this signal-induced complex is a prerequisite for Shn recruitment; Shn does not bind the *brkS* on its own (in our transfection assays we used a short version of Shn, ShnCT, which contains only the C-terminal 600 amino acids of the 2500 amino acid full-length Shn protein; see below and Müller et al., 2003).

In order to identify binding sites for individual proteins on *brkS* and to gain insight into the transcription regulatory capacity of the element, we first aimed at the isolation of the smallest version of the silencer that is still capable of establishing the protein-DNA complex in vitro and to provide Dpp-dependent repression in vivo. We deleted sequences from the 5′ or the 3′ end of the 52 bp silencer and tested the shortened elements for complex formation (Figure 1B). A 25 bp sequence was capable of efficiently assembling a signal-induced multiprotein-DNA complex in the presence of all three proteins (Figure 1B, *brkSE*). Using differently tagged versions of the Mad and Med proteins and supershift analysis, we determined that the stoichiometry of the complex was 1:1, i.e., the multiprotein-DNA complex consists of a single molecule of each protein per double-stranded DNA element (data not shown). When tested in vivo, this short DNA sequence was able to repress transcription of a *lacZ* reporter construct driven by the strong, ubiquitous *brk* enhancer (see Müller et al., 2003) in the center of the wing disc, where high levels of Dpp signaling occur (Figure 1C).

To identify functionally relevant base pairs in this short element, which we refer to as *brkSE* in the following (for *brk Silencer Element*), we generated a systematic series of point mutations and tested the effect of these nucleotide substitutions on protein-DNA complex formation (Figure 2A). Since the assembly of a Mad/Med complex is a prerequisite for the recruitment of ShnCT, we first tested mutations for alterations in the formation of a Mad/Med complex. This analysis identified two regions of importance, highlighted in red and blue in Figure 2A. The blue region consists of a GTCTG motif, a sequence previously identified as a binding site for vertebrate Smad3 and Smad4 and called the minimal Smad binding element (SBE; Shi and Massague, 2003; Shi et al., 1998; Zawel et al., 1998). The red region contains a GC-rich element with similarity to the Mad binding sites identified by Laughon and colleagues (Kim et al., 1997). When tested in vivo, mutations in the red and the blue elements abolished Dpp-dependent repression (Figure 2C), linking complex formation in vitro to gene repression in vivo.

To determine whether the red and blue boxes represented Mad and/or Med binding sites, we made use of Mad MH1 and Med MH1 DNA binding domains produced in bacteria; full-length Mad or Med produced in S2 cells do not bind *brkSE* alone, presumably because the MH2 domain inhibits the MH1 domain (Kim et al., 1997). While the Mad MH1 domain recognized both sites with equal affinity (data not shown), binding of the Med MH1 domain was selectively lost upon mutations in the GTCTG sequence (Figure 2B). Based on this result and on the 1 to 1 stoichiometry of Mad and Med in the protein-DNA complex, we infer that the GTCTG site is bound by Med, while the GC-rich site is bound by Mad.

The Spacing but Not the Sequence between the Mad and the Med Binding Site Is Important for Shn Recruitment

Shn is recruited to the *brkSE* by the Mad/Med complex. Therefore, each mutation in the silencer that abolished the formation of a Mad/Med complex also abolished the formation of a triple complex with ShnCT (data not shown). In order to determine whether Shn binding imposed additional sequence constraints on the *brkSE*, we tested all mutant oligonucleotides that still allowed formation of the Mad/Med complex for Shn recruitment. Surprisingly, none of the mutations that mapped outside or between the Mad and Med binding sites (the red and blue boxes, respectively) interfered with the formation of the ShnCT-containing protein-DNA complex (Figure 3A). Only a single point mutation in the Med binding site (GTC**T**G to GTC**G**G) abolished the formation of the triple Mad/Med/ShnCT complex, despite its ability to recruit Mad and Med (Figure 3A, probe 17). When tested in vivo, introduction of this single point mutation in the *brkSE* destroyed the capacity to repress transcription upon Dpp signaling, indicating that Shn recruitment is essential for repression to occur in vivo (Figure 3C, probe 17).

We also noticed that Shn recruitment in vitro was abolished when the sequences 3′ to the GTCTG motif were deleted (see Figure 1B, probes 8–10). This suggests that Shn interacts with the 3′ region in a sequence-nonspecific manner, possibly involving the phosphate backbone (see below).

As shown above, mutations in the linker segment between the Mad and Med binding sites did not affect the

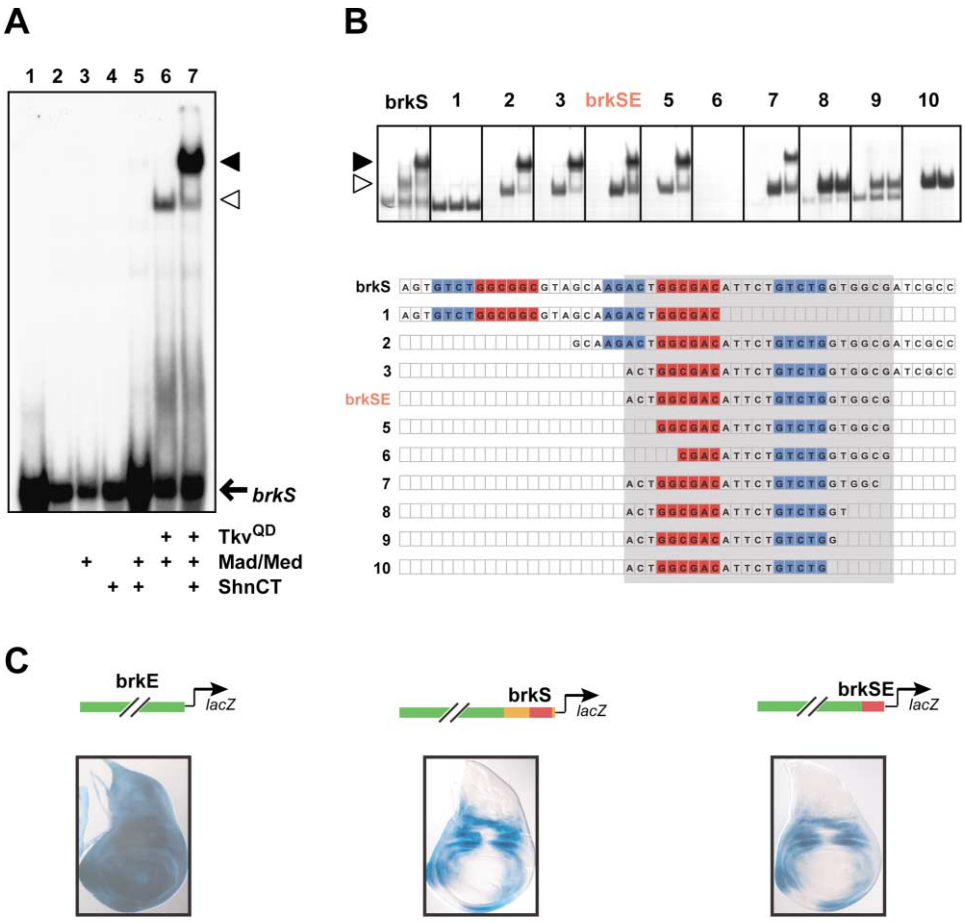

Figure 1. Identification of a Minimal *brk* Silencer Element

(A) Electrophoretic mobility shift assays (EMSA) with lysates of S2 cells transfected with the indicated expressions plasmids and radiolabeled *brk Silencer* (*brkS*) as a probe. The Mad/Med and the Mad/Med/ShnCT complexes on *brkS* are indicated by open and closed arrowheads, respectively. Note that the assembly of both complexes required Dpp signaling, brought about by cotransfection of a constitutive active version of the Dpp receptor Tkv (Tkv^{QD}; compare lanes 3 and 5 to lanes 6 and 7). ShnCT was not able to bind to *brkS* alone (lane 4) but was recruited by the Mad/Med/*brkS* complex (lane 7). Radiolabeled *brkS* probe loaded alone or after incubation with extracts of nontransfected cells is shown in lanes 1 and 2, respectively.

(B) EMSA with subfragments of *brkS*. Each radiolabeled probe was incubated with extracts of nontransfected cells (left lane) or extracts of cells transfected with Tkv^{QD}/Mad/Med without (middle lane) or with (right lane) extracts containing ShnCT. The identification numbers above the radiographies represent the probes used; the exact sequences are shown below the radiographies. Potential Smad binding sites are highlighted in red (GC-rich element) or in blue (Smad binding element [SBE] of the sequence GTCT or GTCTG). The region boxed in gray represents a minimal element for complex formation (*brk Silencer Element* [*brkSE*]) chosen for further characterization. Note that 3′ deletions of this element resulted in a loss of ShnCT binding to the complex (closed arrowhead) while Mad/Med complex formation (open arrowhead) was unaffected, suggesting that this region is involved in Shn recruitment.

(C) Repressive activity of *brkSE* in vivo. Wing imaginal discs from third instar larvae of transgenic flies carrying the illustrated reporter constructs were stained for β-galactosidase activity. The minimal *brkSE* (deep red) was comparable to *brkS* (red) in its ability to repress the *brk* enhancer (*brkE*; green) in the Dpp domain at the anterior/posterior boundary of the disc. The *brkE* alone drives expression of *lacZ* uniformly throughout the wing imaginal disc (left). Wing imaginal discs are oriented with their anterior side to the left and their dorsal side up.

establishment of the multiprotein complex on the *brkSE*. However, it has been shown in several cases, in which the formation of protein-DNA complexes depends on different DNA binding components, that the spacing between the sites to which individual partner proteins bind is critical for cooperative binding (Smith and Johnson, 1992). To investigate the relevance of the spacing of the Mad and Med sites for efficient double and/or triple complex formation, mutant *brkSEs*, in which one or two nucleotides were inserted or deleted between the Mad and Med sites, were tested for Mad and Med binding as well as for Shn recruitment. Strikingly, all insertion or deletion mutants were still able to form a Mad/Med complex but failed to recruit ShnCT (Figure 3B). When tested in transgenic embryos, a perfect correlation between Shn recruitment in vitro and repression in vivo was observed (Figure 3C); only the element maintaining the natural 5 bp spacing between the Mad and Med sites was functional, and *brkSE* versions with linker deletions or insertions were inert. We also tested an element carrying two point mutations in the linker sequence between the Mad and Med sites, and, in line with the results obtained in the mobility shift assays, we found that these mutations do not affect the function of the element,

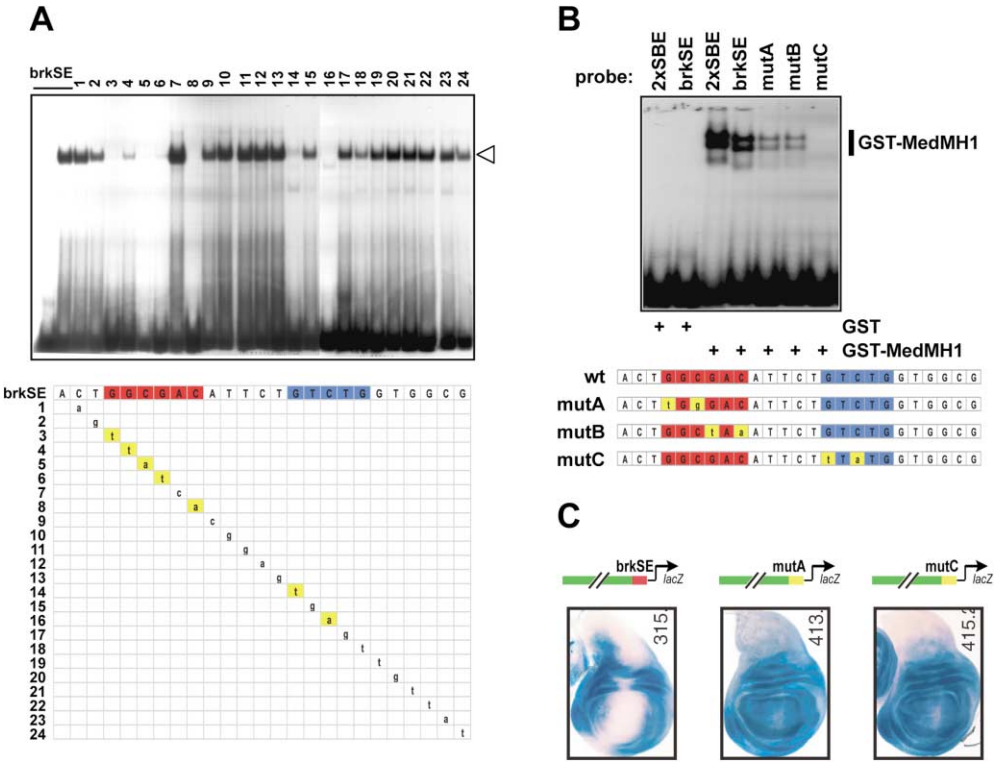

Figure 2. Sequence Requirements for *brkSE*/Mad/Med Complex Formation

(A) Double-stranded probes each bearing a single point mutation (1–24) were compared to *brkSE* for their ability to assemble a Mad/Med complex (open arrowhead) when challenged with lysates from cells transfected with Tkv^{QD}, Mad, and Med. Point mutations that affect complex formation are highlighted in yellow and map exclusively either in the GC-rich motif (red, mutations 3, 4, 5, 6, and 8) or the SBE motif (blue, mutations 14 and 16). As a control, *brkSE* was incubated with an extract from nontransfected S2 cells (first lane).

(B) Med binds to the SBE of the *brkSE*. The MH1 domain of Med was purified as a GST fusion from bacteria and assayed for binding to *brkSE* or to *brkSE* versions, in which either the GC-rich region (red) or the SBE (blue) were inactivated by point mutations. While binding was observed both with the intact *brkSE* and its derivatives bearing mutations in the GC-rich motifs (MutA and MutB), the GST-MedMH1 polypeptide failed completely to interact with the *brkSE* carrying two point mutations in the SBE (MutC). In a control reaction, GST-MedMH1 bound strongly to a probe bearing two copies of the SBE motif (2×SBE). In all cases where binding was detected, two differently migrating complexes containing the GST-MedMH1 protein were observed. This has also been reported in similar experiments using a GST construct of the MH1 domain of the vertebrate homolog of Med, Smad4 (Zawel et al., 1998) and could be due to homodimer formation of the fusion proteins via the GST-moiety.

(C) Mutations in the Mad or Med binding sites affect the activity of *brkSE* in vivo. Wing imaginal discs from transgenic animals carrying the illustrated reporter constructs were stained for β-galactosidase activity. Mutations that inactivate Mad (MutA) or Med (MutC) binding in vitro result in a complete abolishment of repressive activity in vivo.

suggesting that this linker sequence might not be used to recruit further proteins to the silencer in vivo. These experiments demonstrate that the spacing but not the sequence between the Mad and Med sites is important for Dpp-dependent repression.

From the experiments presented thus far, we conclude that a short sequence element, *brkSE*, containing a Mad and a Med binding site of defined sequence and spacing is sufficient to recruit ShnCT protein and to provide Dpp-dependent repression to the *brk* enhancer in vivo.

Shn Is a Modular Repressor Protein

Shn codes for a large protein containing eight zinc fingers (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). ShnCT, the C-terminal 600 amino acids of Shn including zinc fingers six to eight, is sufficient to repress *brk* transcription in vivo upon Dpp signaling (Müller et al., 2003). To delineate the sequences of ShnCT that are required for this activity, we generated a series of deletion mutants producing shorter versions of ShnCT and tested their capabilities to form protein-DNA complexes with Mad and Med in vitro and to repress transcription in vivo. Since we have previously shown that the C-terminal zinc finger cluster of ShnCT is essential for complex formation (Figure 4A; Müller et al., 2003), we asked whether sequences N- or C-terminal to the zinc fingers were also important. When tested in vitro, efficient complex formation was observed with a minimal ShnCT protein containing only the zinc finger cluster, demonstrating that the flanking sequences are not essential for the recruitment of Shn to the *brkSE* via the Mad/Med complex (Figure 4A). Inactivation of individual zinc fingers showed that a major role in complex formation was attributed to zinc fingers 6 and 8 (Figure 4A), while zinc finger 7 was dispensable.

To test altered ShnCT proteins for their repression potential in cultured cells, we depleted S2 cells of endogenous Shn using double-stranded RNA targeted against the 5′ end of the *shn* transcript and then assayed

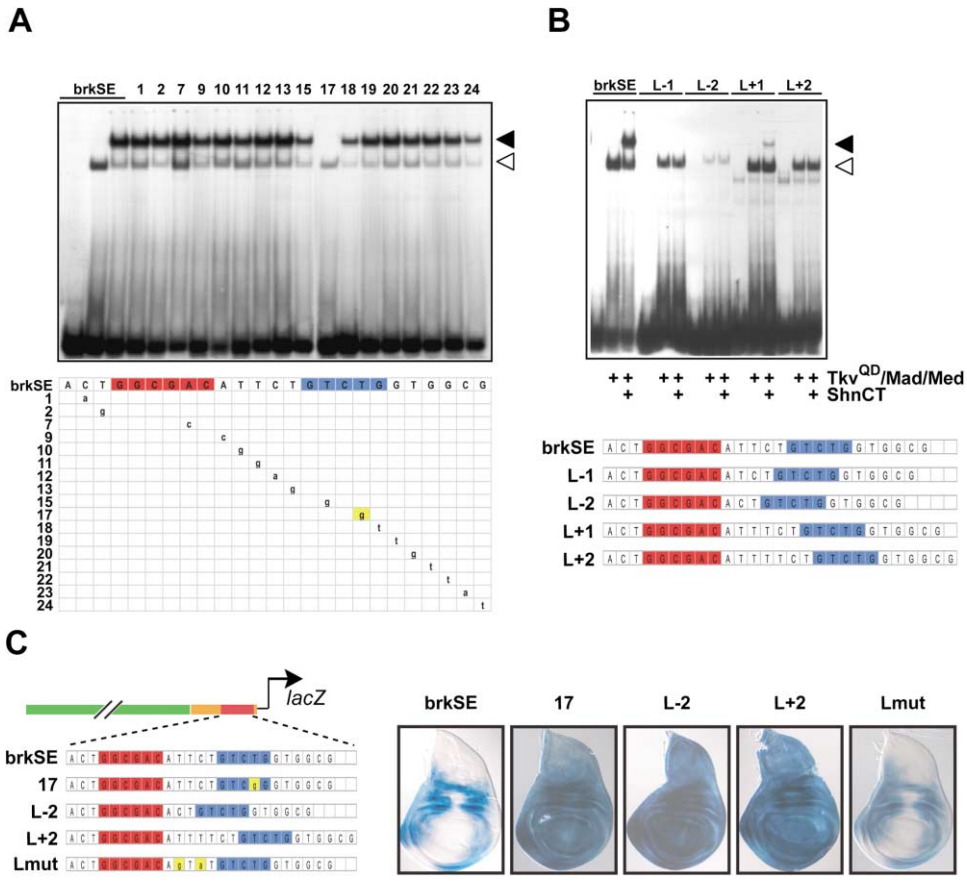

Figure 3. Sequence Requirements for Shn Recruitment to the *brkS*/Mad/Med Complex

(A) Point mutants of *brkSE* that still allow the assembly of a Mad/Med complex were tested for their ability to recruit ShnCT in band shift assays. Each probe (same numbering as for Figure 2A) was incubated with extracts from cells expressing Tkv^{QD}, Mad, and Med in combination with an extract from cells expressing ShnCT in order to induce the formation of Mad/Med (open arrowhead) and Mad/Med/ShnCT complexes (closed arrowheads). A single point mutation (GTCTG in GTCGG, highlighted in yellow in probe 17) abolished ShnCT recruitment to the *brkSE*/Mad/Med complex completely. The two first lanes are control reactions in which the *brkSE* probe was incubated with lysates from nontransfected cells or cells transfected with Tkv^{QD}, Mad, and Med.

(B) The spacing of the Mad and Med binding sites affects ShnCT recruitment. Band shift assays with lysates from cells expressing the indicated proteins and *brkSE* derivatives, in which the DNA linker between the Mad (red) and the Med (blue) binding sites was shortened (L−1, L−2) or lengthened (L+1, L+2) by one or two nucleotides.

(C) Expression of a *lacZ* gene under the control of *brkE* fused to the indicated versions of the *brkSE* was visualized by β-galactosidase staining of wing imaginal discs. Mutations of the *brkSE* affecting ShnCT recruitment (Figure 3A, mutation 17; or Figure 3B, L−2 and L+2) resulted in the loss of Dpp-induced repression as compared to wild-type *brkSE*. In contrast, mutations that affect the sequence but not the length of the linker did not influence the repressive activity of *brkSE* (Lmut).

the capacity of variant proteins to reinstall Dpp-dependent repression. We found that the most N-terminal sequences in ShnCT (amino acids 1–114, corresponding to position 1888–2001 in full-length Shn) were critically involved in repression (Figure 4B); not surprisingly, the zinc fingers 6 and 8 were also required for repression (not shown), since in their absence ShnCT can not be recruited to the silencer element by Mad and Med.

The N-terminal sequences of ShnCT might be required to induce a conformational change in the Mad and/or Med proteins, allowing them to interact with transcriptional corepressors; alternatively, these Shn residues might interact with such proteins themselves and confer repression to the silencer. To address this issue, we asked whether the N-terminal repression domain of ShnCT was transferable to an unrelated DNA binding domain. Indeed, the Shn repression domain was functional when fused to the DNA binding domain of GAL4 in cultured cells (Figure 4C), demonstrating that this protein region has an inherent capacity to repress transcription.

To confirm that the same sequence requirements we defined in mobility shift assays (complex formation) and in S2 cells (repression) also defined the functional requirements for repression via ShnCT in vivo, we tested a selection of critical ShnCT versions for Dpp-dependent repression of *brk* in the *Drosophila* embryo. Transgenes encoding modified ShnCT proteins were expressed together with a Dpp transgene in stripes perpendicular to the anterior-posterior axis in *shn* mutant embryos; the capability of these transgenes to repress *brk* transcription was then tested by revealing the expression of a *lacZ* reporter driven by *brk* regulatory sequences (see Experimental Procedures). Indeed, we found that zinc fingers 6 and 8 were crucial for Dpp-dependent repression, while zinc finger 7 as well as the sequences

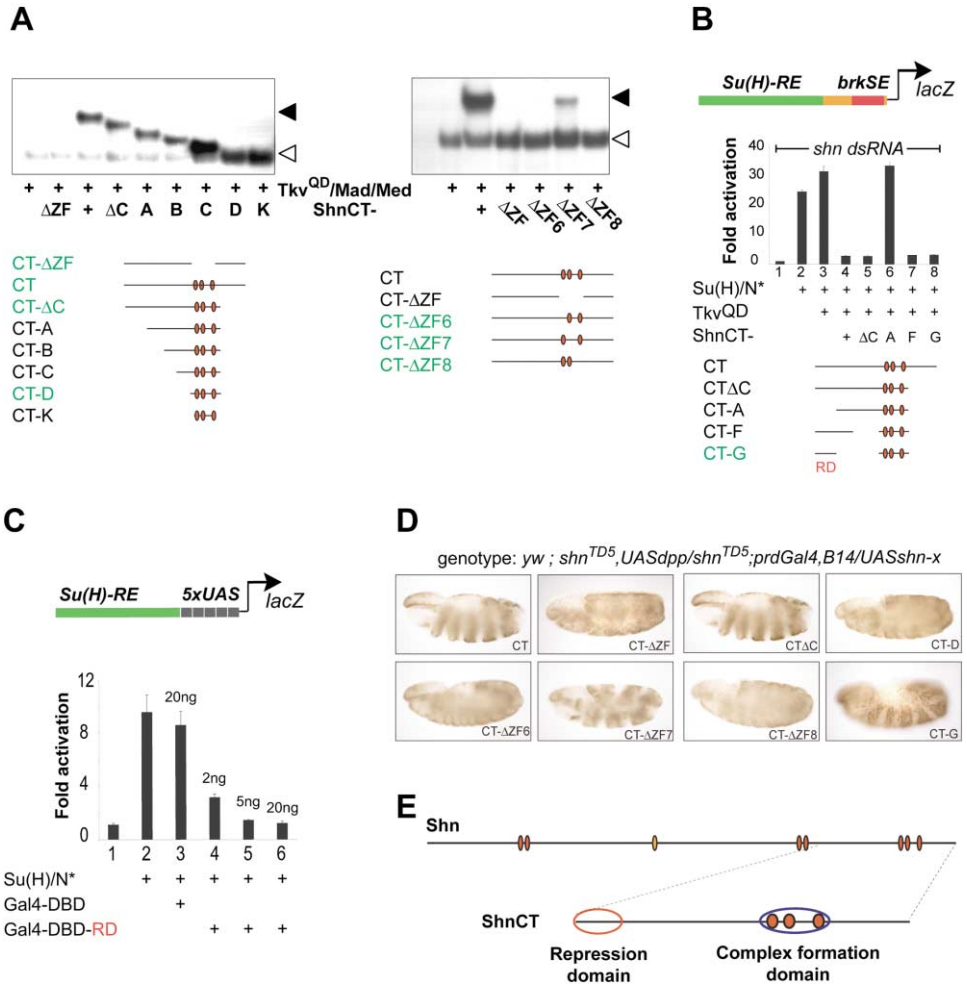

Figure 4. Modular Architecture of the Schnurri Protein

(A) EMSA with lysates of Tkv^{QD}/Mad/Med-transfected S2 cells and in vitro translated subfragments or mutant versions of ShnCT (ShnCT corresponds to amino acid 1888–2529 of full-length Shn, red circles represent the C2H2-type zinc fingers of the protein). The deletion analysis of ShnCT revealed that the triplet of zinc fingers is sufficient for complex formation (ShnCT-K). Zinc fingers 6 and 8 are essential for complex formation. The *brkSE*/Mad/Med and *brkSE*/Mad/Med/ShnCT complexes are indicated with open and closed arrowheads, respectively. Expression of the polypeptides was verified by Western blots (not shown).

(B) Reporter gene assays in S2 cells using a reporter plasmid containing the *brkSE* element fused to a *suppressor of Hairless* response element (Su(H)-RE). Cells were treated with *shn* dsRNA to downregulate endogenous *shn* prior to transfection of plasmids encoding Su(H) and activated Notch (N*). In the absence of functional versions of Shn (i.e., ShnCT-A, bar 6), Tkv^{QD} cotransfection failed to counteract Su(H)/N*-induced activation of the reporter. Repression was restored in the presence of functional versions of Shn (bar 4, 5, 7, and 8). The fusion of the N-terminal 114 amino acids of ShnCT (referred to as repression domain, RD) to zinc finger 6/7/8 generated a minimal ShnCT version (ShnCT-G) that retained similar repressive capacity as ShnCT (compare bar 4 to bar 8).

(C) Reporter assays with S2 cells transfected with the indicated reporter and expression plasmids. N*/Su(H)-mediated activation (bar 2) was not affected by cotransfection of the DNA binding domain of Gal4 (Gal4-DBD, bar 3) but was gradually inhibited by cotransfecting increasing amounts of a Gal4 DBD-ShnRD construct (bars 4–6).

(D) *shn* constructs highlighted in green in (A) and (B) were co-expressed with *dpp* in seven stripes in the embryo using a *prdGal*/*UAS* system and tested for repression of the *brk* reporter B14 (Marty et al., 2000; Müller et al., 2003). β-galactosidase expression is shown in stage 11–13 embryos (lateral views, anterior to the left, dorsal up).

(E) Schematic presentation of the Shn protein. Domains essential for recruitment of the protein to the *brkSE*/Mad/Med complex (complex formation domain) and for repressive activity (repression domain) are highlighted.

C-terminal to the zinc finger cluster were dispensable (Figure 4D). A mini ShnCT protein containing only the N-terminal repression domain and the zinc finger cluster was able to repress *brk* transcription in the presence of Dpp in vivo (Figure 4D), confirming that the important protein determinants on ShnCT included the repression domain as well as the zinc finger cluster (Figure 4E).

Functional Mad/Med/Shn-Dependent Silencers Are Found in Other *Drosophila* Genes

The results presented so far demonstrate that the minimal *brkSE* contains a GC-rich Mad binding site and a GTCTG site bound by Med. When these two sites are appropriately spaced (5 bp between the Smad sites) and conform to a sequence of the following consensus

within an endogenous gene (*gsb*-enhancer *lacZ* fusions). We find that the minimal functional silencer contains a distinct, single binding site for each of the two signal mediators, Mad and Med. Med binds to a GTCTG site, previously recognized as a high-affinity site for Smad binding (Shi and Massague, 2003). Mad binds to a different, GC-rich sequence. Upon binding of Mad and Med, the zinc finger protein Shn is recruited to the protein-DNA complex, bringing along a highly effective repression domain. Although ShnCT contains three essential zinc fingers, it does not bind the silencer element in the absence of Mad and Med. Our data suggest that even in the triple protein complex, Shn might bind DNA with moderate sequence specificity, since we identified only a single nucleotide position, which is essential for Shn recruitment. However, a number of other *cis*-regulatory elements that bind Mad and Med (derived from the *vestigial*, *labial tinman*, and *ubx* genes [Kim et al., 1997; Marty et al., 2001; Thuringer et al., 1993; Xu et al., 1998]) failed to recruit Shn (data not shown), demonstrating the exquisite selectivity of the element defined here.

Part of this selectivity is accounted for by the specific spacing and orientation of the Mad and Med binding sites in the silencer. Deletion and insertion of single base pairs between the two sites abolish Shn recruitment in vitro and Dpp-dependent repression in vivo, although such alterations still allow the efficient formation of a Mad/Med complex. These findings suggest that Shn recruitment requires a specific steric positioning of amino acid residues in the Smad signal mediators. Strikingly, GTCTG- and GC-rich elements were also found to be crucial for the activation of the Id gene by BMP signaling, but in this case the spacing between the GTCTG- and the GC-rich sites is much larger, and additional factors might be involved in the signal-dependent activation of the Id gene (Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002). A more recent study also links these two elements to transcriptional activation of the BMP4 synexpression group in *Xenopus* (Karaulanov et al., 2004). It is tempting to speculate that simple sequence elements similar to the one we identified here in several *Drosophila* genes might be involved in the repression of genes by BMP signaling. Interestingly, human Smad1/5 and Smad4 do form a complex with ShnCT on the *Drosophila* silencer element from *brk* (data not shown); however, a mammalian protein sharing clear homology with Shn in the C-terminal three zinc fingers has not been identified so far.

Repression by the Dpp-Dependent Silencer: Simplicity at the Functional Level

The Dpp-dependent SE allows cells in the developing organism to read out the state of the Dpp signaling pathway. This readout is relatively straightforward because the SE participates in a single switch decision, that is, either to repress (bind Mad/Med and recruit Shn along with its repression domain) or not to repress (not bind Mad/Med, thus failing to recruit Shn). This decision is critically dependent upon one major parameter: the amount of available nuclear Smad complex. For the SE to be functional in vivo, it only needs to interact with a Mad/Med heteromer in those regions of the genome that are actively transcribed; genes that are not active in a given tissue do not need to be repressed by Dpp signaling. This might be one of the main characteristics explaining why such a simple sequence element can have operator-like function in vivo; the element only needs to be recognized by the relevant *trans*-acting factors in open and active chromatin regions.

Occurrence of the Dpp-Dependent Silencer Element: Conservation at the Functional Level

We have identified a minimal Dpp-dependent silencer element derived from the *brk* gene, demonstrated that it functions in vivo in a single copy, and defined its interaction with relevant *trans*-acting factors. Based on the results of this analysis, we were able to derive a consensus sequence, GRCGNCN(5)GTCTG, which allowed us to scan the entire *Drosophila* genome for potential additional elements. We identified approximately 350 sites, which, when assayed using transgenic approaches in vivo or in cell culture, should function in a manner analogous to the SEs isolated from the *brk* regulatory region. Strikingly, and likely significantly, our in silico search revealed that the *brk* gene contains a total of ten SEs, three of them in regions that have been shown to respond to Dpp-dependent repression (regions B and C and the enhancer; see Figure 6A; Müller et al., 2003). Since *brk* transcription responds to (or can respond to) Dpp signaling in all tissues examined so far (Affolter et al., 2001), *brk* might require a SE in the vicinity of each of the different enhancers driving expression in distinct tissues. Alternatively, the readout of the Dpp morphogen gradient might require several SEs, each contributing to the graded repression by Dpp signaling.

Interestingly, our subsequent analysis of two genes containing such Dpp-dependent SEs demonstrated that these elements function in these transcription units the same way as they do in the *brk* regulatory region. Therefore, the same molecular principle underlies morphogen readout (*brk* repression), germline stem cell maintenance (*bam* repression), and restriction of gene expression to the ventral side of the developing embryo (*gsb* repression). When the SEs from these three genes are aligned, all the parameters we determined to be important for complex formation and for repression are conserved; at all other positions, different base pairs were found in different SEs (Figure 7A). In addition, several genes harboring silencer elements are expressed in the wing imaginal disc in a pattern similar to *brk* (data not shown) or are known to be repressed by Dpp signaling (Dobens et al., 2000; Dobens and Raftery, 2000). In contrast, SEs were not found in the vicinity of enhancers known to be activated by Dpp signaling.

Clearly, our findings implicate that Dpp-induced, Shn-dependent repression via SE elements is a key aspect of development (Figure 7B). The readout of the *brk* gradient contributes to growth and patterning of appendages, and the repression of *bam* in the germline is essential for the maintenance of germline stem cells. To what extent the repression of *gsb* contributes to proper cell fate determination along the dorsoventral axis will have to be determined by rescuing the *gsb* phenotype with a transgene lacking the *gsbSE*. However, we have previously observed that *wingless* (*wg*) expression expands from ventral positions to the dorsal side in *shn* mutant

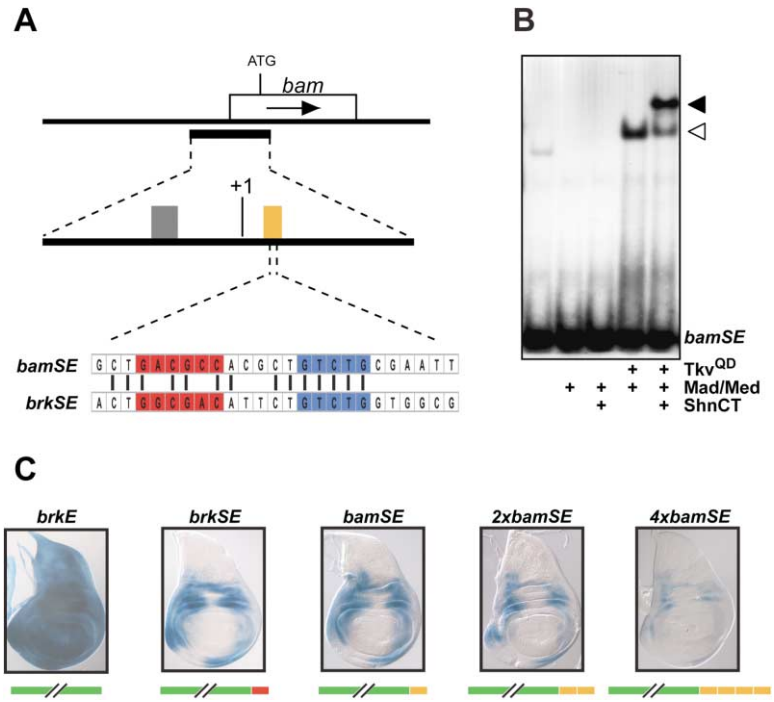

Figure 5. *bam* Contains a Functional Dpp- and Shn-Dependent SE

(A) Schematic illustration of the genomic locus of *bam*. Expression of *bam* is controlled by a positive acting element (gray), active both in cystoblasts and germline stem cells, and a negative acting element, which inhibits expression specifically in the stem cells (orange). Sequence comparison of the latter and the *brkSE* predicts the existence of a Shn-dependent SE (*bamSE*).

(B) EMSA using radiolabeled *bamSE* as a probe and lysates of S2 cells expressing the indicated proteins. The *bamSE* triggered complex formation with the proteins Mad, Med, and ShnCT. Similarly to the *brkSE*, complex formation was only seen upon cotransfection of the Tkv^{QD}-expressing plasmid.

(C) The *bamSE* is capable of repressing the *brkE* in a Dpp-dependent manner in the wing imaginal disc. β-galactosidase staining of wing imaginal discs from flies transgenic for the illustrated reporter constructs. The SE from the *bam* gene could functionally replace the *brkSE* in the Dpp-mediated repression of the *brkE* in the wing imaginal disc. Note that increasing the copy number of the *bamSE* (*2×bamSE* and *4×bamSE*) resulted in a progressive increase in the sensitivity of the reporter for Dpp.

(GRCGNCN(5)GTCTG), Mad and Med recruit ShnCT to the silencer, and the N-terminal sequences of ShnCT confer a strong repression potential to the silencer element.

When the consensus sequence GRCGNCN(5)GTCTG was used to scan the *Drosophila* genome (see Experimental Procedures), approximately 350 putative silencer elements (SEs) were identified. Remarkably, the *brk* gene, from which the SE was initially isolated, turns out to be the only gene that contains more than two such elements (10 in total) in its vicinity; it is possible that all these elements contribute to shape the transcription profile of *brk*, which displays an inverse gradient with regard to the Dpp morphogen gradient (see Discussion). Several other genes in the vicinity of silencer consensus sequences attracted our attention. Below, we will describe two such genes and show that, unexpectedly, the molecular principle underlying Dpp-induced transcriptional repression of *brk* is also used for the direct downregulation of other key developmental genes.

Germline Stem Cells Are Maintained by Shn Recruitment to an SE in the *bam* Gene

The first SE that caught our attention was located in the 5′ untranslated region of the *bag of marbles* (*bam*) gene, which encodes the key regulator determining asymmetric division of the *Drosophila* germline stem cell (Figure 5A). The protein Bam is both necessary and sufficient for cystoblast differentiation, and *bam* transcription is specifically repressed in germline stem cells by Dpp signaling via a discrete transcriptional silencer element in the *bam* transcription unit (Chen and McKearin, 2003a, 2003b; Song et al., 2004). It has been shown that Mad and Medea bind to this element, but why this binding would result in transcriptional repression rather activation remains unanswered (Chen and McKearin, 2003a; Song et al., 2004). The sequence similarity between the *brkSE* and the *bamSE* suggested that they share functional properties, i.e., the capability to recruit ShnCT via Mad and Med and provide Dpp-dependent repression to heterologous transcription units. Indeed, we found that the *bamSE* formed a ShnCT-containing protein-DNA complex with high affinity when Dpp signaling is activated (Figure 5B). When inserted between the *brk* ubiquitous enhancer and the *lacZ* gene, the *bamSE* repressed transcription just like the *brkSE* element (Figure 5C). We conclude that the molecular paradigm identified for the *brkSE* also applies to the *bamSE* and that this mechanism underlies the maintenance of germline stem cells by Dpp (Xie and Spradling, 1998). In line with these results, it has been shown that *shn* is genetically required for germline stem cell maintenance (Xie and Spradling, 2000). Hence, we conclude that Dpp represses the transcription of genes other than *brk* in a direct manner with the help of Shn.

Dpp Directly Represses *gsb* Transcription in the Dorsal Ectoderm

One of the most prominent functions of Dpp and its vertebrate homologs in the development of multicellular animals is the organization of the dorsoventral axis and the repression of neurogenesis (Bier, 1997; Lee and Jessell, 1999; Munoz-Sanjuan and Brivanlou, 2002; Raftery and Sutherland, 2003). Despite this conserved role of Dpp in the fly and in higher vertebrates, little is known about the molecular basis of dorsoventral axis formation and neural suppression by Dpp in *Drosophila*, and few

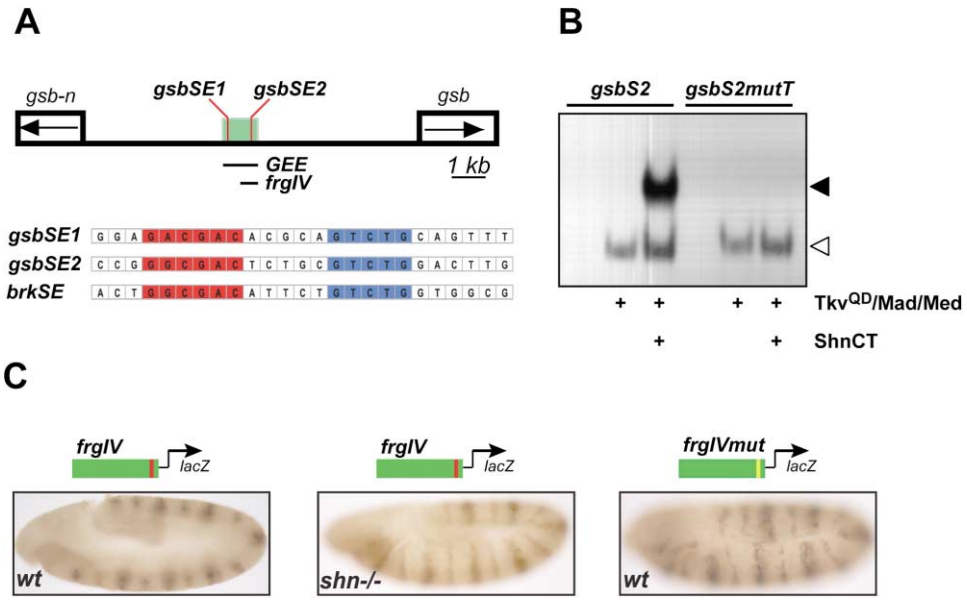

Figure 6. The Early Expression of *gsb* Is Controlled by Dpp- and Shn-Dependent SEs

(A) Schematic drawing of the gsb genomic locus. Early embryonic expression of gsb is driven by a ~1 kb long enhancer (GEE; green) containing two potential SEs (gsbSE1 and gsbSE2; red). A 500 bp subfragment of the *gsb* early enhancer (frg IV, black line) has also been shown to faithfully recapitulate early expression of *gsb* and contains a single SE (*gsbSE2*).

(B) The *gsbSEs* assembles a Mad/Med/ShnCT complex. Both the *gsbSE1* (not shown) and the *gsbSE2* probe (left panel) served as templates for the formation of Mad/Med (open arrowheads) and a Mad/Med/ShnCT complexes (closed arrowheads) when incubated with extracts of cells expressing the corresponding proteins in combination with Tkv^{QD}. As shown for the *brkSE*, recruitment of ShnCT was abolished when the conserved T residue of the SBE motif (GTCTG) was replaced by a G (right panel, *gsbSE2mutT*).

(C) Ventral restriction of *gsb* expression is mediated by the silencer element. Early embryonic expression of *gsb* visualized by β-gal staining of wt or *shn* embryos carrying the *lacZ* reporter under the control of either frgIV or a frgIV version bearing an inactivating mutation in the Med binding site of *gsbSE2* (frgIVmut). The segmental stripes of *gsb* expression were restricted to the ventral ectoderm in wild-type embryos (left) but were significantly expanded in *shn* mutant embryos and invaded the dorsal ectoderm (middle). The same dorsal expansion in *shn* mutants was observed with a *lacZ*-reporter under the control of the 1 kb long GEE (not shown). Inactivation of the *gsbSE2* in the reporter frgIVmut (right) resulted in the same dorsal expansion of *lacZ* expression. Embryos are orientated with the anterior end to the left and the dorsal side up.

direct target genes have been isolated that would provide detailed insight into these important functions of Dpp. Therefore, we were intrigued by our finding that one of the genes, in which we identified two SEs using bioinformatics, corresponds to the segment polarity gene *gooseberry (gsb)*. Segmental *gsb* expression is limited to the ventral side of the early *Drosophila* embryo where it is critical for proper CNS formation and specifies a number of well-defined neuroblasts in the neuroectoderm (Li and Noll, 1993). Enhancer elements driving early, ventral expression of *gsb* were identified and characterized; interestingly, both *gsbSE*s map within the enhancer driving ventral expression (Figure 6A; Li and Noll, 1994). Subsequent analysis led to the identification of a smaller enhancer driving ventral expression (frgIV), and this enhancer still contains one of the SEs (Bouchard et al., 2000). *gsbSE*-derived oligonucleotide probes promoted the assembly of a Mad/Med/ShnCT triple complex (Figure 6B), and the recruitment of ShnCT by Mad/Med depended on the same nucleotide in the GTCTG sequence as it did in the *brkSE*.

To provide in vivo functional evidence for the *gsbSE*, we first analyzed the expression of the short *gsb* enhancer (frgIV) in *shn* mutant embryos. While expression of this enhancer was limited to the ventral side in wild-type embryos, the activity was expanded to cells in the dorsal half in *shn* mutant embryos (Figure 6C). The same phenomenon was observed in wild-type embryos when *lacZ* expression was driven by a frgIV version, in which we mutated the single SE (Figure 6C). These findings strongly support the notion that the expression pattern of *gsb* is limited to the ventral side by Dpp-dependent transcriptional repression provided by the *gsbSE*. Therefore, the same, simple molecular paradigm controls repression of *brk*, *bam*, and *gsb*.

Discussion

Architecture of the Dpp-Dependent Silencer: Simplicity at DNA and Protein Level

One of the primary events controlled by the Dpp morphogen gradient during growth and patterning of imaginal discs is the establishment of an inverse gradient of *brk* expression. We have previously shown that *brk* expression is controlled by two opposing activities, a ubiquitous enhancer and a Dpp-dependent silencer (Müller et al., 2003). Here, we identified the minimal requirements for a functional silencer complex, both at the DNA and at the protein level. Importantly, we have demonstrated that the minimal element functions in vivo when assayed in the vicinity of a strong enhancer (the *brk* enhancer) or when present in a single copy in chimeric transgenes (*brk* enhancer-*bamSE* fusions) or from

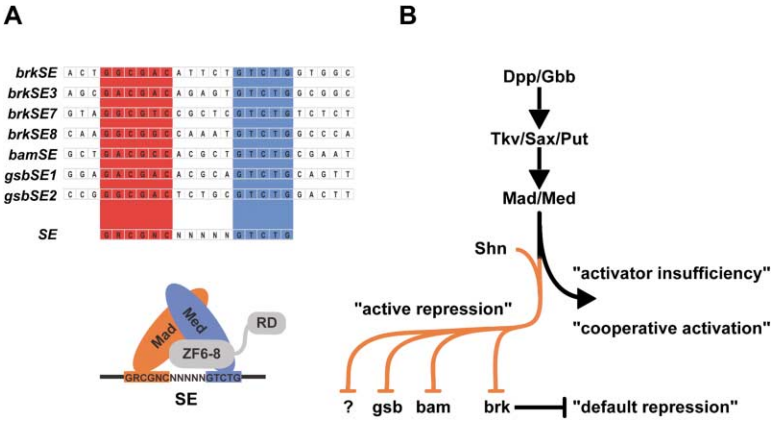

Figure 7. Dpp Represses Several Key Developmental Genes via a Simple DNA Sequence Element and a Mad/Med/Shn Complex

(A) Alignment of functional silencer elements derived from the *brk*, *bam*, and *gsb* genes. Nucleotides conserved in the consensus shown below (SE) correspond to the positions that we found to be essential for the formation of a Mad/Med/Shn complex.

(B) The Dpp pathway actively represses key developmental genes with the help of the zinc finger protein Shn. Repressed target genes contain *cis*-acting SEs that bind Mad and Med and recruit Shn. Gene repression is tissue nonspecific, in contrast to gene activation, which relies on "cooperative activation." The Dpp signaling pathway is equipped with an inherent capacity to repress gene transcription upon pathway activation via a simple, well-defined sequence element, the SE.

embryos (Grieder et al., 1995). Since *gsb* activates *wg* transcription (Li and Noll, 1993), the expansion of *gsb* (in the absence of the *gsbSE*) possibly leads to the expansion of *wg* and subsequently to the alteration of dorsoventral cell fate assignments.

It is important to note that genes repressed by a signaling pathway will not easily be identified in genetic screens because the loss-of-signaling phenotype does not correspond to the loss-of-function phenotype of a repressed gene; in the absence of the signal, such genes are ectopically expressed, leading to a locally restricted gain-of-function phenotype of the corresponding gene. Moreover, since these specific, local patterns of missexpression are likely to result in different phenotypes than widespread overexpression would, simple gain-of-function screens for candidate targets of signal-mediated repression are unlikely to offer straightforward results. Since we have identified the target sequence of Dpp/Shn-mediated repression, we can now scan the genome and identify potential target genes by expression studies and enhancer dissection. It is likely that we will identify additional Dpp-repressed genes using this approach, and this will allow the painting of a much clearer picture of the gene network controlled by Dpp signaling.

Differences between Dpp-Induced, Shn-Dependent Repression and Other Signal-Induced Repression Mechanisms

As outlined in the Introduction, only a few cases of signal-induced repression have been studied at the molecular level. In most of these cases, repression relies on cooperative action of cell type-specific transcription factors with nuclear signal mediators (Chen et al., 2002; Kang et al., 2003; Kim and Lassar, 2003). The DNA elements that have been demonstrated to mediate repression of particular genes have not been demonstrated to be important for the regulation of other genes, and genome-wide identification of potential target genes using a bioinformatic approach might therefore be difficult, if not impossible.

The Dpp-dependent repression system we identified in this study relies on the organization of Smad binding motifs into Smad/Shn complex-recruiting SEs. The simplicity of these SEs and their capacity to repress transcription in different tissues argues that they function in the absence of tissue-restricted factors. The simple consensus sequence of the SE provides a signature for Dpp-dependent repression, allowing for a genome-wide analysis of potential target genes. Confirmed Dpp-repressed target genes can then be expressed ectopically under the control of the appropriate SE-mutated enhancers to assess the biological importance of repression in a given tissue.

Experimental Procedures

Plasmid Constructs

Reporter constructs containing derivatives of the *brinker* or the *bam* silencer elements were generated by inserting double-stranded oligonucleotides between the wing-specific *brk* enhancer (*brkE*) and the *hsp70* minimal promoter using the SpeI and Asp718 sites in the vector B216 (Müller et al., 2003). The subfragment IV of the *gsb* early embryonic enhancer was amplified by PCR using plasmid 9E9P4Z as a template (Li and Noll, 1994) and inserted between the XbaI and Asp718 sites of plasmid pX27 (Segalat et al., 1994) upstream of the *hsp70* minimal promoter and the *lacZ* gene. Mutations converting the Med binding site from GTCTG into **AATT**G or GTC**GG** were generated by PCR. For the generation of transgenic flies, fragments of the s*hn* cDNA were cloned into the pUAST vector in-frame with a nuclear localization signal (NLS) followed by an N-terminal FLAG epitope. For in vitro transcription and translation, *shn* fragments were cloned into pcDNA3 (Invitrogen) in-frame with an N-terminal FLAG epitope. For constitutive expression in *Drosophila* S2 cells, Shn versions with a C-terminal V5 epitope were cloned in the vector pAc5.1B/V5His (Invitrogen). All *shn* constructs were generated by inserting PCR fragments into the EcoRI and XhoI sites of the vectors described above (ShnCT, amino acids 1888–2529 of Shn; ShnCTΔZF, 1888–2257 fused to 2358–2529; ShnCT-ΔC, 1888–2387; ShnCT-A, 2001–2387; ShnCT-B, 2091–2387; ShnCT-C, 2226–2387; ShnCT-D, 2254–2387; ShnCT-K, 2254–2355; ShnCT-F, 1888–2095 fused to 2254–2387; ShnCT-G, 1888–2004 fused to 2254–2387). To inactivate the individual zinc fingers of ShnCT in the constructs ShnCTΔZF6, ShnCTΔZF7, and ShnCTΔZF8, the two first characteristic cysteine residues of each zinc finger were converted to alanine. Plasmids for constitutive expression of luciferase, Su(H), activated Notch (N*), ^{FLAG}Mad, ^{myc}Med, and activated Tkv (Tkv^{QD}) as well as the reporter plasmids containing the Su(H) response elements with or without the *brkS* have been described (Kirkpatrick et al., 2001; Müller et al., 2003). The reporter *Su(H)-5xUAS* was constructed by inserting a PCR fragment containing five tandem binding sites for Gal4 between the Su(H) response element and the minimal *hsp70* promoter in the reporter *Su(H)-lacZ*. To generate the plasmid Gal4DBD, a fragment coding for the DNA binding domain of Gal4 (amino acids 1–147) was amplified by PCR using the yeast two-hybrid vector pAS2.1 (Clontech) as a template and inserted into the Asp718 and EcoRI

sites of pAc5.1B/V5His. Subsequently, a fragment corresponding to amino acids 1888–2095 of Shn was fused to the Gal4 DNA binding domain by insertion between the EcoRI and XbaI of the Gal4DBD plasmid to generate the Gal4DBDShnRD expression plasmid. The MH1 domains of Mad and Med (amino acids 1–147 and 16–355, respectively) were fused to the GST moiety in the plasmid pGEX4T.1 (Pharmacia). The integrity of all constructs was verified by sequencing analysis.

Fly Stocks and Transgenes

shn^{TD5} mutant allele was used in this study. pUAST-*shn* constructs and *lacZ* reporter plasmids were introduced into w^{1118} by standard P element transformation. Three to six independent transgenic lines were established for each construct. Missexpression of *shn* versions together with *dpp* in stripes in the embryo was achieved by employing the *paired*-Gal4 driver line as described (Marty et al., 2000).

Transfections and Reporter Gene Assays

Drosophila S2 cells were maintained in Schneider's insect medium (Invitrogen) supplemented with 10% Fetal Calf Serum and were transfected with the Effectene Transfection Reagent (Qiagen). For reporter gene assays, 2×10^6 cells were transfected with a total of 200 ng plasmid DNA (20 ng reporter plasmid, 5 ng of a plasmid constitutively expressing firefly luciferase, the indicated amounts of expression plasmids, and the parental vector pAc5.1B/V5His to bring the total amount of DNA to 200 ng). For RNAi experiments, cells were treated with dsRNA corresponding to nucleotides 5011–5531 of the *shn* cDNA 24 hr prior to transfection as described elsewhere (Clemens et al., 2000). Cells were lysed 48 hr after transfection and lysates were assayed for β-galactosidase and luciferase activity as described previously (Müller et al., 2003).

Production of Proteins for Electrophoretic Mobility Shift Assays

For electrophoretic mobility shift assays (EMSA), 4×10^6 cells were cotransfected with 50 ng Tkv^{QD}- and each 175 ng Mad- and Med-expression plasmids or with 400 ng of a ShnCT-expression plasmid. Cells were harvested 48 hr after transfection, lysed in 100 mM Tris (pH 7.8), 1 mM DTT, and 0.5% TritonX100 supplemented with a protease inhibitor cocktail (Complete, Roche) for 10 min at 4°C, and cleared by centrifugation. ShnCT and its subfragments used in the EMSA shown in Figures 4A and 4B were produced in vitro using the TNT T7 Quick Coupled Transcription/Translation System (Promega) and pcDNA3-*shn* plasmids as template. Proper expression was assayed in immunoblots using antibodies against the epitope tags of the constructs. Recombinant GST-MadMH1 and GST-MedMH1 were expressed in *E. coli BL21* cells and purified using Glutathione-Sepharose beads (Pharmacia) according to the manufacturer's protocols.

Electrophoretic Mobility Shift Assays

Radioactive-labeled probes were generated by annealing and filling in partially overlapping oligonucleotides in the presence of [α-^{32}P] ATP. Binding reactions were carried out in 20 μl of 100 mM KCl, 20 mM HEPES (pH 7.9), 20% glycerol, 1 mM DTT, 0.3% BSA, 0.01% NP40 containing 10,000 cpm probe, and 1 μg dIdC. As a protein source, 50 μg or 30 μg total protein of cleared lysates from S2 cells transfected with Tkv^{QD}/Mad/Med and ShnCT, respectively, were used. Purified GST-fusion proteins were added at a final concentration of 50 ng/μl. In the case of in vitro translated proteins, 1 μl of the reticulocyte extract of a standard 50 μl reaction was used. After incubation for 30 min at 4°C, the reactions were analyzed by nondenaturing 4% polyacrylamide gel electrophoresis followed by autoradiography.

Antibody and β-Galactosidase Staining

Embryos were collected at 25°C, fixed, and stained with anti-FLAG (M2, Sigma) or anti-β-galactosidase (Promega) antibodies using standard protocols. Wing imaginal discs were dissected from third instar larvae and stained for β-galactosidase activity with standard X-gal color reactions for 2 hr at 37°C. For all stainings shown in this study, at least three independent transgenic lines were analyzed.

Computer-Assisted Search for Silencer Elements

Putative target genes for Dpp- and Shn-mediated repression were identified by screening the entire *Drosophila* genome sequence with the consensus GRCGNCNNNNNGTCTG using the program FLY ENHANCER (freely available at http://flyenhancer.org [Markstein et al., 2002]).

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