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Abstract

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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

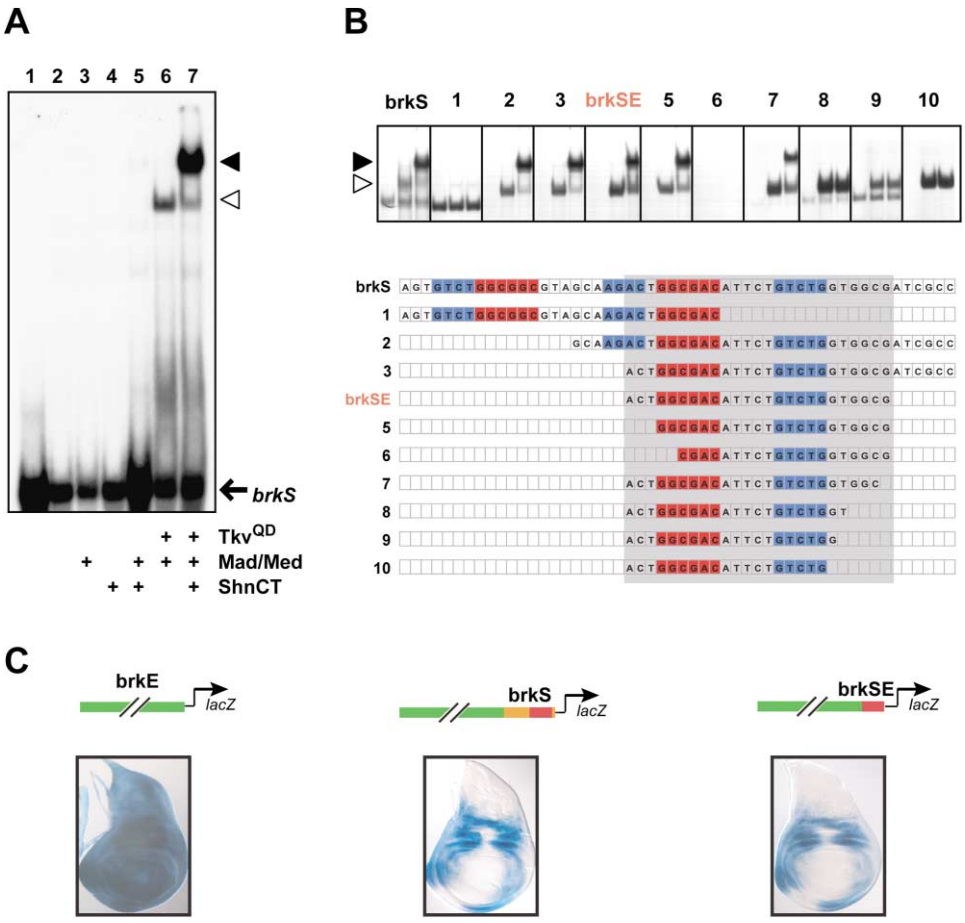

Figure 1. Identification of a Minimal *brk* Silencer Element

(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,

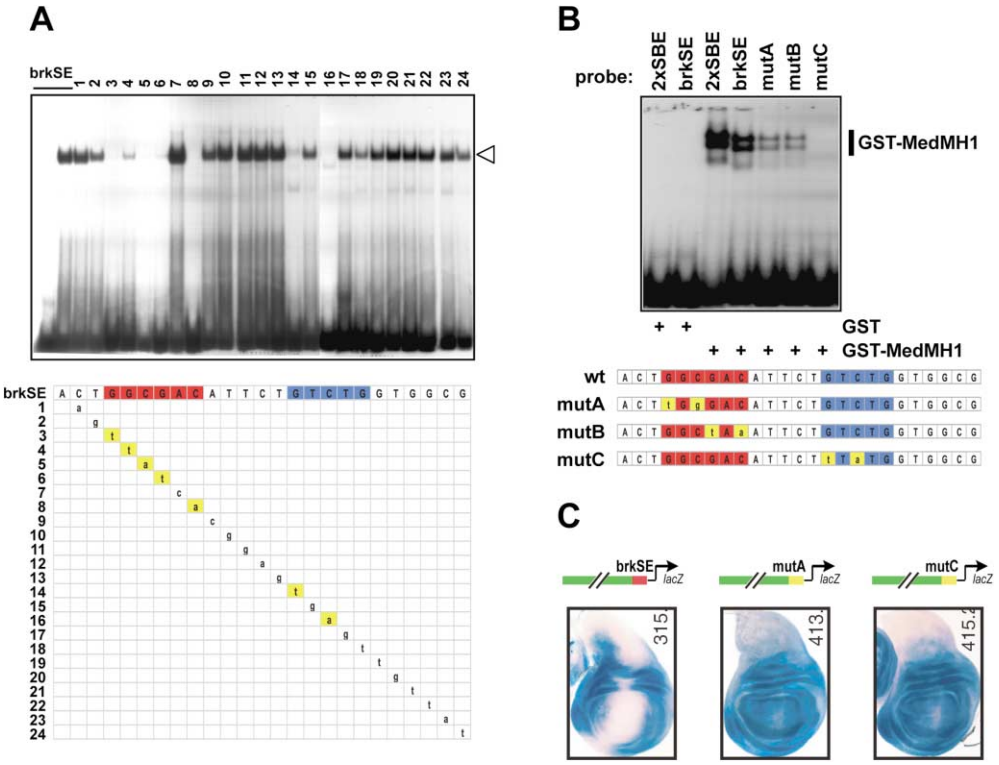

Figure 2. Sequence Requirements for *brkSE*/Mad/Med Complex Formation

(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

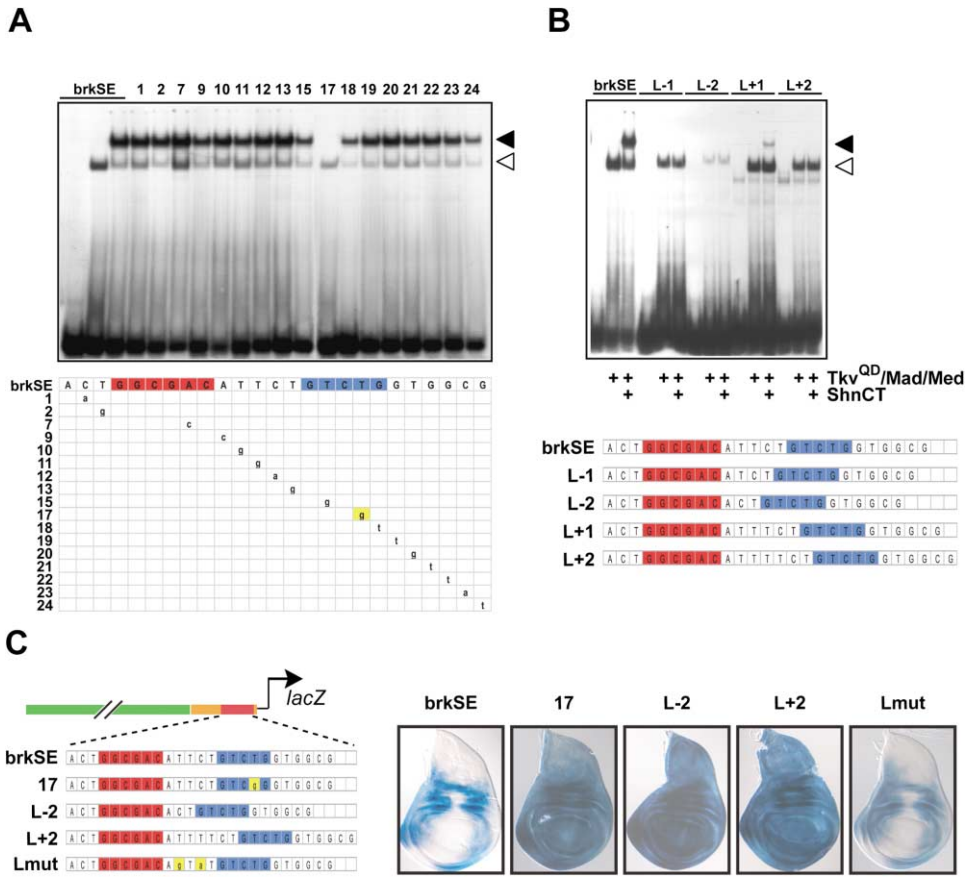

Figure 3. Sequence Requirements for Shn Recruitment to the *brkS*/Mad/Med Complex

(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

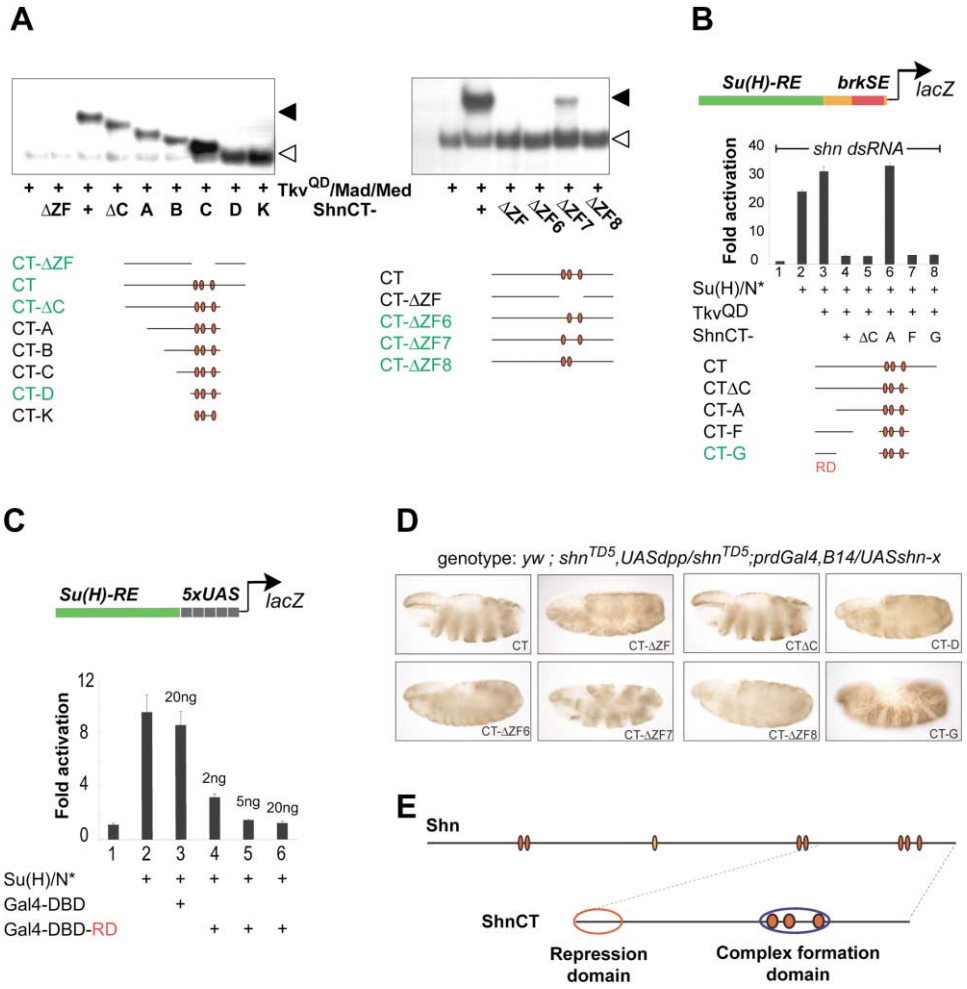

Figure 4. Modular Architecture of the Schnurri Protein

(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

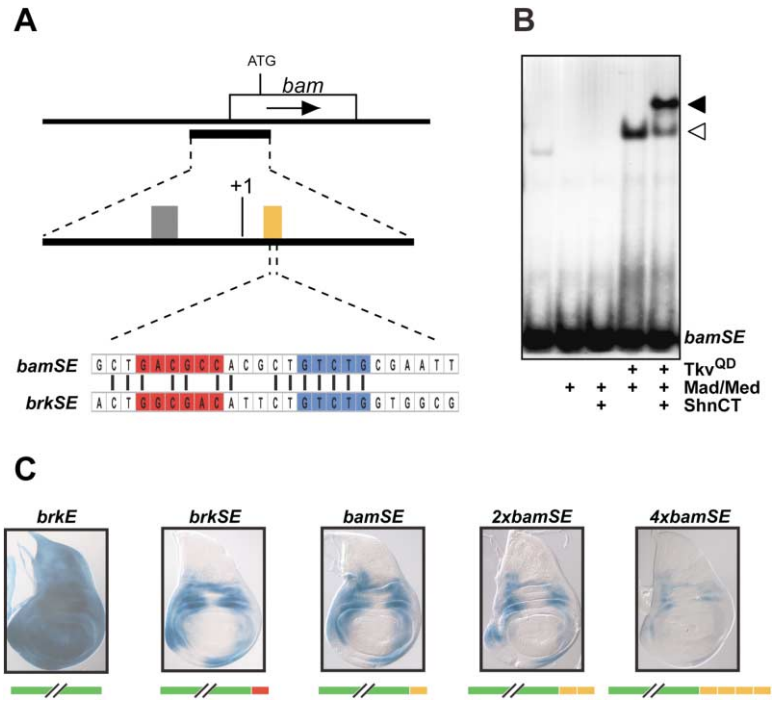

Figure 5. *bam* Contains a Functional Dpp- and Shn-Dependent SE

(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

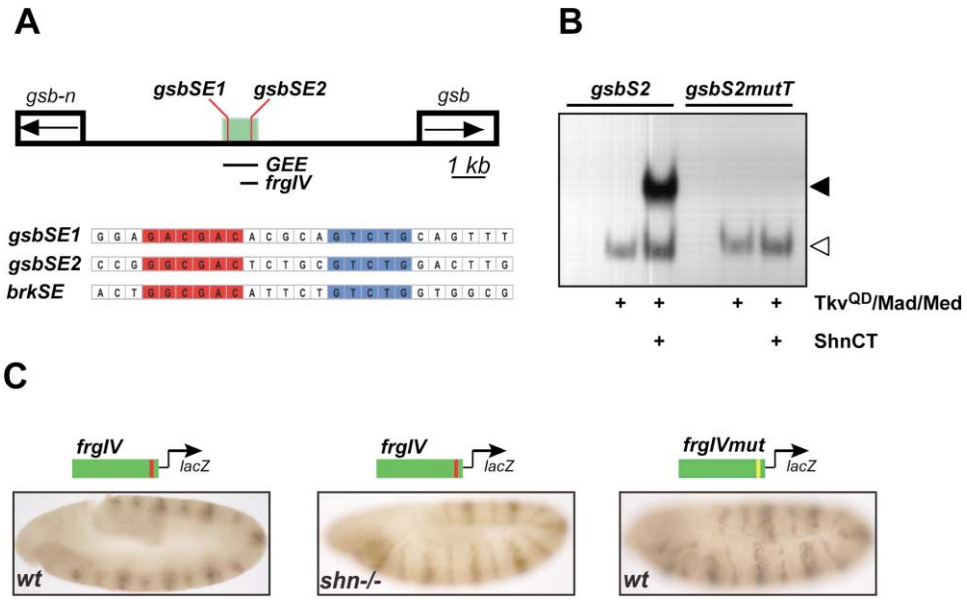

Figure 6. The Early Expression of *gsb* Is Controlled by Dpp- and Shn-Dependent SEs

(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

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

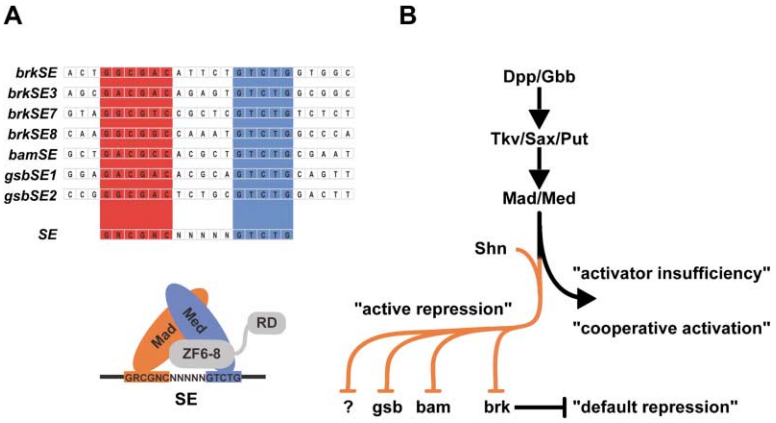

Figure 7. Dpp Represses Several Key Developmental Genes via a Simple DNA Sequence Element and a Mad/Med/Shn Complex

(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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