Title

Schnurri transcription factors from Drosophila and vertebrates can mediate Bmp signaling through a phylogenetically conserved mechanism

Journal

Development, 133(20)

ISSN

0950-1991

Authors

Yao, Li-Chin
Blitz, Ira L
Peiffer, Daniel A
et al.

Publication Date

2006-10-15

DOI

10.1242/dev.02561

Peer reviewed

Development 133, 4025-4034 (2006) doi:10.1242/dev.02561

Schnurri transcription factors from *Drosophila* and vertebrates can mediate Bmp signaling through a phylogenetically conserved mechanism

Li-Chin Yao, Ira L. Blitz*, Daniel A. Peiffer*, Sopheap Phin*, Ying Wang, Souichi Ogata, Ken W. Y. Cho, Kavita Arora† and Rahul Warrior†

Bone Morphogenetic Proteins (Bmps) are secreted growth factors that play crucial roles in animal development across the phylogenetic spectrum. Bmp signaling results in the phosphorylation and nuclear translocation of Smads, downstream signal transducers that bind DNA. In *Drosophila*, the zinc finger protein Schnurri (Shn) plays a key role in signaling by the Bmp2/Bmp4 homolog Decapentaplegic (Dpp), by forming a Shn/Smad repression complex on defined promoter elements in the *brinker* (*brk*) gene. Brk is a transcriptional repressor that downregulates Dpp target genes. Thus, *brk* inhibition by Shn results in the upregulation of Dpp-responsive genes. We present evidence that vertebrate Shn homologs can also mediate Bmp responsiveness through a mechanism similar to *Drosophila* Shn. We find that a Bmp response element (BRE) from the *Xenopus Vent2* promoter drives Dpp-dependent expression in *Drosophila*. However, in sharp contrast to its activating role in vertebrates, the frog BRE mediates repression in *Drosophila*. Remarkably, despite these opposite transcriptional polarities, sequence changes that abolish cis-element activity in *Drosophila* also affect BRE function in *Xenopus*. These similar cis requirements reflect conservation of trans-acting factors, as human Shn1 (hShn1; HIVEP1) can interact with Smad1/Smad4 and assemble an hShn1/Smad complex on the BRE. Furthermore, both Shn and hShn1 activate the BRE in *Xenopus* embryos, and both repress *brk* and rescue embryonic patterning defects in *shn* mutants. Our results suggest that vertebrate Shn proteins function in Bmp signal transduction, and that Shn proteins recruit co-activators and co-repressors in a context-dependent manner, rather than acting as dedicated activators or repressors.

KEY WORDS: Schnurri, Bone Morphogenetic Protein signaling, Bmp, Smad, Transcription, Transcriptional activation, Transcriptional repression, Evolutionary conservation

INTRODUCTION

Bone Morphogenetic Proteins (Bmps) are secreted growth factors of the Tgfβ superfamily that regulate a wide range of biological processes, including specification of embryonic axes, cell fate determination, proliferation and apoptosis. Bmp ligands and receptors have been identified in organisms from sponges and Cnidaria to humans, and elicit their effects in a similar fashion across phyla (Holstein et al., 2003; Massague et al., 2005). Signaling is initiated by ligand binding to a complex of type-I and type-II transmembrane serine/threonine kinase receptors, enabling the type-II receptor to phosphorylate the type-I receptor. The type-I receptor in turn phosphorylates a receptor-regulated member of the Smad (R-Smad) family of intracellular signal transducers. In vertebrates, Bmp signaling is mediated by Smad1, Smad5 and Smad8, whereas in *Drosophila* the sole Bmp-specific Smad is Mothers against dpp (Mad). The activated R-Smad associates with the co-Smad, Smad4 [Medea (Med) in *Drosophila*], forming a heteromeric complex that translocates into the nucleus. R-Smads and co-Smads are structurally similar, possessing amino-terminal MH1 and carboxy-terminal MH2 domains separated by a flexible linker. A conserved β-hairpin motif in the MH1 domain mediates DNA binding, enabling the R-Smad/Smad4 complex to direct transcriptional responses. Co-Smads recognize the sequence GTCT (the 'Smad-binding element' or SBE), whereas Smad1 and Mad preferentially bind GCCGNC (Gao et al., 2005; Johnson et al., 1999; Kim et al., 1997; Xu et al., 1998). Smads bind to these low complexity sites with weak affinity and it is thought that Smad-DNA interactions are enhanced by partnership with other transcription factors that are crucial for promoter selectivity. In support of this idea, reporter constructs containing multimers of SBEs or Smad1 sites alone are incapable of replicating the expression patterns of endogenous Bmp target genes in vivo, although they confer Bmp responsiveness in cell culture (Shi and Massague, 2003; von Bubnoff et al., 2005).

Drosophila Schnurri (Shn) is a large DNA-binding transcription factor with multiple, widely separated zinc finger domains, and was one of the first partners identified for Bmp-specific R-Smads (Dai et al., 2000; Udagawa et al., 2000). Shn localizes to the nucleus and interacts with Mad and Med in response to Dpp signaling. Genetic and phenotypical analysis has established that *shn* is essential for Dpp signaling at many developmental stages and in diverse tissues (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). At the molecular level, Shn uses two modes to regulate Dpp target genes: transcriptional activation and repression. Shn can directly activate a small subset of Dpp-responsive genes, but exerts most of its effect through a relief-of-repression mechanism. Shn inhibits the expression of a transcriptional repressor Brinker (Brk) that, in turn, negatively regulates the expression of most Dpp target genes. Therefore, Dpp signaling leads to the inhibition of *brk* expression and the derepression of batteries of Dpp-responsive genes (Affolter et al., 2001; Marty et al., 2000; Torres-Vazquez et al., 2001; Torres-Vazquez et al., 2000). Shn represses *brk* via the formation of a Shn/Mad/Med complex on defined elements in the *brk* promoter (Muller et al., 2003; Pyrowolakis et al., 2004). Given

Department of Developmental and Cell Biology, and the Developmental Biology Center, University of California Irvine, Irvine, CA 92697, USA.

*These authors contributed equally to this work
†Authors for correspondence (e-mail: karora@uci.edu; rwarrior@uci.edu)

Accepted 3 August 2006

the prominent role of Shn in Dpp signaling, an inevitable question is whether the conservation encountered at other levels of the pathway extends to the cis-regulatory elements and nuclear factors that mediate the transcriptional response to Bmps. Furthermore, *brk*, a primary target of Shn activity, is absent from genomes outside the arthropods, raising the possibility that the involvement of Shn in Bmp signaling may be unique to insects.

Unlike *brk*, Shn-related genes are found in other phyla. In *C. elegans* the lone Shn homolog SMA-9 has been implicated in Bmp signaling, although its mechanism of action is unknown (Liang et al., 2003). Three Shn homologs are found in vertebrates that also contain widely spaced, paired zinc finger domains with high sequence similarity to the fly protein. Shn proteins share additional features, including an unusually large size, overall structural organization and the presence of acidic domains. Human members of this family have been variously referred to as Shn1/HIVEP1/MBP-1/PRDII-BF1/ZAS1, Shn2/HIVEP2/MBP-2/ZAS2 and Shn3/HIVEP3/KRC/ZAS3 (Liang et al., 2003; Wu, 2002). No direct evidence ties vertebrate Shn1 or Shn3 to the Bmp pathway, but Shn2 has recently been shown to promote Bmp-responsive transcription of the PPARγ gene in mouse embryonic fibroblasts. However, Shn2 acts through sites that bear no resemblance to the sequences recognized by the *Drosophila* Shn/Smad complex, and requires co-operativity with the transcription factor C/EBPα (Jin et al., 2006). Thus, whether the mechanism of action of Shn proteins in Bmp signaling is phylogenetically conserved remains an unanswered question.

Here, we demonstrate an unusual and unexpected conservation of the cis- and trans-regulatory elements involved in the response to Bmp signaling. We found that an element from the *Xenopus Vent2* (*Xvent2*) promoter that confers sensitivity to Bmp signaling in vertebrate embryos also directs a Dpp-dependent response in *Drosophila*. However, although the element mediates transcriptional activation in *Xenopus*, it directs transcriptional repression in *Drosophila*. We demonstrate that the closest human Shn homolog, hShn1, can mediate signaling-dependent transcriptional activation through this element in vertebrate cells, implicating it in Bmp signal transduction. Remarkably hShn1 can also mediate signaling-dependent repression in *Drosophila* and rescues patterning defects in *shn* mutant embryos, whereas fly Shn can activate transcription in *Xenopus* assays. This switch in transcriptional output between repression and activation is not a function of differences in the cis-regulatory elements or an inherent property of the human and *Drosophila* proteins, indicating that Shn/Smad complexes are likely to recruit co-activators and co-repressors in a context-dependent manner.

MATERIALS AND METHODS

Drosophila reporter gene analysis

Grh-*lacZ* (provided by Scott Barolo) contains three 8-bp Grh sites upstream of *lacZ* in pH-Pelican (Barolo et al., 2000). *Xvent2*-BRE-*lacZ* contains five 23-bp *Xvent2*-BREs between *Not*I and *Bam*HI sites in Grh-*lacZ*. The 6.0 *brk-lacZ* transgene contains –8.3 to –2.7 kb of the *brk* promoter driving *lacZ* (L.-C.Y., unpublished). All other stocks are described in FlyBase. Embryos and imaginal discs were stained as described (Torres-Vazquez et al., 2000).

Rescue of *shn* mutants

Full-length Shn and hShn1 (Fan and Maniatis, 1990) were subcloned into pUAST (Brand and Perrimon, 1993). *shnP4738* is a null allele (Arora et al., 1995). Progeny from *UAS-Shn/UAS-hShn1*, *shnP4738/CyO* flies crossed to *shnP4738/CyO*; *HS-Gal4* were aged to 8-10 hours and received two 1-hour heat shocks at 37°C, separated by 25°C rest periods. *brk* expression was monitored using the enhancer trap *X-47* (Campbell and Tomlinson, 1999). Whole-mount cuticles were prepared as described (Arora et al., 1995). Homozygous mutants were identified by the absence of *wg-lacZ* or *CyO, Kr>GFP*.

Xenopus reporter gene analyses

p*Xvent2*(–150)LUC and p*Xvent2*-5×BRE(–150)LUC have been described previously (von Bubnoff et al., 2005). The oligonucleotide 5′-CGGCA-GACAGGTTGGAGCCAGCTCGGCAGACAGGTTGGAGCCAGCT-3′ containing a BRE dimer (mutations underlined) was used to generate a PCR product that was subcloned into p*Xvent2*(–150)LUC to create p*Xvent2*-5×BRE(sub2)(–150)LUC. p*Xvent2*-6×BRE(del2)(–150)LUC was similarly created using 5′-CGGCAGAC_GGTGGAGCCAGCTCG-GCAGAC_GGTGGAGCCAGCT-3 (underscores indicate deleted bases). p*Xvent2*-5×BRE(–150)GFP3, p*Xvent2*-5×BRE(sub2)(–150)GFP3 and p*Xvent2*-6×BRE(–150)(del2)GFP3 contain *Bam*HI-*Hin*dIII fragments from the respective reporter plasmids in pCXGFP3 (a gift of Enrique Amaya, University of Manchester). Full-length Shn and hShn1 were subcloned into pBSII-KS, containing the SV40 polyadenylation signal. Capped mRNAs were synthesized using the T3 mMessage Machine Kit (Ambion), including 1 μl GTP/20 μl reaction, diluted into single use 'injection cocktails' containing reporter genes, stored at –80°C and used within 24 hours.

mRNA concentrations/embryo were: luciferase reporters, 200 pg; dominant-negative (Suzuki et al., 1994) and constitutively active (Candia et al., 1997) BmpR, 0.1-2.0 ng; and hShn1 and Shn, 2 ng. Wild-type and mutant BRE reporters were microinjected into the animal poles of two- to four-cell stage embryos and incubated until blastula stages 8-9. Mid-blastula stage animal caps were dissected and homogenized for luciferase assays when sibling embryos reached stages 10.5-11 (Watabe et al., 1995). In situ hybridization and generation of transient transgenic embryos using the sperm nuclear transfer technique have been described previously (von Bubnoff et al., 2005).

Biochemical assays

N-terminal FLAG-Mad and FLAG-Med, TkvA and ShnCT plasmids have been described previously (Gao et al., 2005). PCR fragments 1 (hShn1$^{1-599}$), 2 (hShn1$^{1-702}$), 3 (hShn1$^{496-1121}$), 4 (hShn1$^{1002-1635}$), 5 (hShn1$^{1496-2213}$), 6 (hShn1$^{1756-2544}$) and 7 (hShn1$^{1756-2717}$) were subcloned into the pAWM vector containing an Actin5C promoter and C-terminal 6×Myc epitopes (a gift of T. D. Murphy, Carnegie Institution, Baltimore). Extracts from *Drosophila* S2 cells transfected with individual hShn1 constructs and TkvA/Mad/Med or TkvA/hSmad1/hSmad4 were incubated with anti-FLAG antisera. Immunoprecipitates were run on 4-12% gradient SDS polyacrylamide gels before western blotting and visualization of interacting protein with anti-Myc. Gel shift assays were performed as described (Gao et al., 2005; Kirov et al., 1994; Pyrowolakis et al., 2004). Extracts from *Drosophila* S2 cells transfected with different plasmid combinations were used to shift a *Xvent2* BRE probe (5′-CTAAGAGCT-**GGCTCC**ACCAT**GTCTG**CCGTTAGTTGGCTCA-3′). For supershifts, 60 ng of anti-FLAG (M2, Sigma) or 100 ng of anti-MYC (9E10, Santa Cruz) were added to the binding reactions. GST pull-downs were carried out as described (Dai et al., 2000). Human Smad1 and Smad4 linker+MH2 regions subcloned in pGEX-4T-1 were expressed in *E. coli* BL21. PCR fragments of hShn1 were subcloned into pCITE4A (Novagen) before in vitro translation using the TNT-T7 Coupled Transcription/Translation Kit (Promega).

Molecular phylogeny

Shn proteins used were: *D. melanogaster* DMU31368; *X. tropicalis* Shn1 genome v4.1 gw1.33.114.1, Shn2 gw1.274.19.1, Shn3 ensemble.c_scaffold_478000040; Mouse Shn1 NM_007772, Shn2 NM_010437, Shn3 NM_010657; Human Shn1 NM_002114, Shn2 NM_006734, Shn3, NM_024503. CLUSTALW analysis was performed using the San Diego Supercomputer Center's Biology Workbench.

RESULTS

A vertebrate Bmp response element mediates Dpp signaling in *Drosophila*

To determine whether conservation in the Bmp pathway extends to the cis-regulatory elements and transcription factors involved in the nuclear response, we examined whether a vertebrate Bmp response element (BRE) is sensitive to Bmp signaling in *Drosophila*. The

Xenopus Vent2 gene is one of the best-characterized direct transcriptional targets of Bmp signaling in vertebrates, and is essential for patterning ventral mesoderm and preventing neuralization in the embryo (Onichtchouk et al., 1996). Classical promoter analyses have identified a 53-bp Bmp responsive module located at –191 relative to the initiator ATG (Candia et al., 1997; Hata et al., 2000; Rastegar et al., 1999). Phylogenetic footprinting and in vivo reporter gene analyses have further refined the cis-element to a 23-bp BRE that is both necessary and sufficient for Bmp-dependent activation in animal cap ectodermal explants and transgenic embryos (von Bubnoff et al., 2005). To test whether the 23-bp *Xvent2*-BRE can mediate Bmp responsiveness in *Drosophila*, we generated transgenic lines containing five tandem BREs upstream of *lacZ* in the 3×Grh vector (henceforth referred to as *Xvent2*-BRE-*lacZ*; Fig. 1A). As the Dpp pathway utilizes both direct and indirect relief-of-repression mechanisms to activate gene expression, we chose a single vector that would allow us to assay both outputs. 3×Grh-*lacZ* contains binding sites for Grainyhead (Grh), a transcriptional activator ubiquitously expressed in the ectoderm after embryonic stage 11 (Bray and Kafatos, 1991; Furriols and Bray, 2001). Therefore, 3×Grh-*lacZ* is transcriptionally silent until extended germ band stage, but then drives uniform *lacZ* expression through later embryogenesis and in larval tissues (Fig. 1B-E). Thus, enhancers mediating activation can be assayed prior to stage 11, whereas elements mediating repression can be detected at later stages.

Dpp signaling is crucial in early embryogenesis for the specification of dorsal cell fates and is detected by stage 4 (syncytial blastoderm) in the dorsal embryo (Dorfman and Shilo, 2001). Subsequently, *dpp* is involved in many other aspects of embryonic and larval patterning (Raftery and Sutherland, 1999). We analyzed *Xvent2*-BRE-*lacZ* transgenic lines and found no reporter gene expression prior to stage 11, suggesting that this element cannot direct Dpp-dependent activation in early embryos (data not shown). However, from stage 11 onwards β-galactosidase could be detected in a distinct pattern in both embryos and imaginal discs (Fig. 1F-I). Interestingly, the reporter was downregulated where Dpp signaling is active (Dorfman and Shilo, 2001; Sutherland et al., 2003; Tanimoto et al., 2000). In stage 13 embryos (germband retraction), *dpp* is expressed in the leading edge of the dorsal ectoderm and in

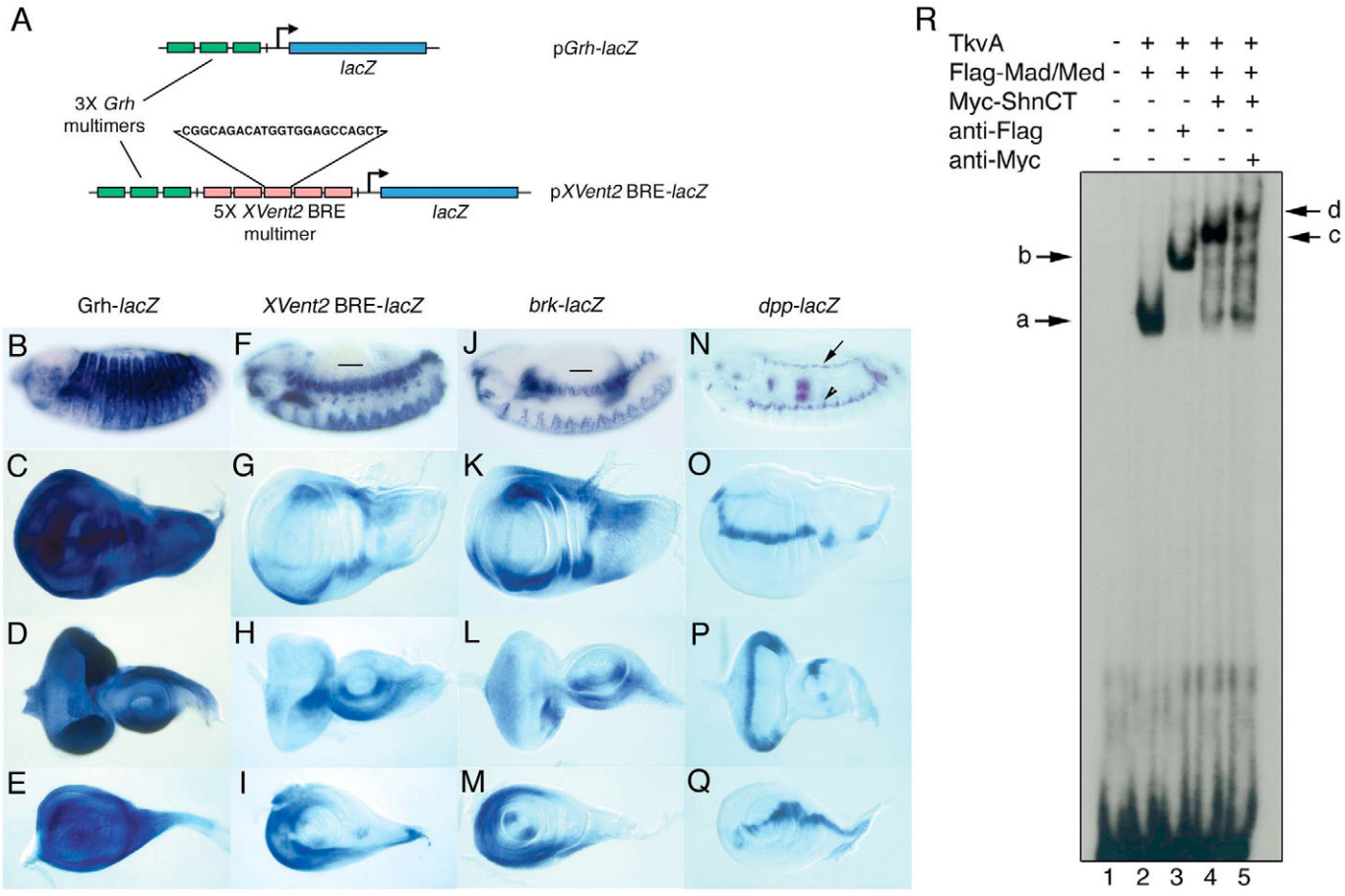

Fig. 1. An *Xvent2*-BRE reporter mimics *brk* expression in *Drosophila* and assembles a Mad/Med/Shn protein-DNA complex. (**A**) Reporter constructs. (**B-Q**) Top row, lateral views of stage 13 embryos; anterior left, dorsal up. Succeeding rows show third-instar wing, eye-antennal and leg imaginal discs stained to visualize *lacZ* expression. *Grh-lacZ* drives nearly ubiquitous expression during (B) late embryogenesis, and (C-E) in imaginal discs. (F-I) The *Xvent2*-BRE-*lacZ* reporter is Bmp sensitive. *lacZ* expression is downregulated at sites of high Dpp signaling. (J-M) Expression of a *brk-lacZ* reporter closely matches expression of *Xvent2*-BRE-*lacZ*. (N) In situ hybridization showing sites of *dpp* expression in the embryo. (O-Q) *dpp-lacZ* expression in imaginal discs. The leading edge of the dorsal ectoderm is marked with a bar in F and J, and an arrow in N; arrowhead in N indicates the boundary between the dorsal and ventral ectoderm. (**R**) Lysates from S2 cells transfected with the indicated plasmids were used to gel shift the BRE probe. The presence of Mad/Med results in a low mobility complex (band a, lane 2) that is further retarded by anti-FLAG (band b, lane 3) or Shn-Myc (band c, lane 4). The latter complex is supershifted by incubation with anti-Myc (band d, lane 5).

the boundary between dorsal and ventral ectoderm, both sites where reporter expression is absent (compare Fig. 1F with 1N). Likewise, *lacZ* is repressed in the medial cells of the wing imaginal disc, and regions of eye-antennal and leg discs subject to Dpp signaling (compare Fig. 1G-I with 1O-Q). At all locations, the spatial and temporal pattern of reporter expression closely replicates that of *brk*, a negatively regulated target of Dpp signaling (Fig. 1J-M). These remarkable parallels strongly suggest that the *Xvent2*-BRE, which activates transcription in vertebrates, mediates transcriptional repression in response to Bmp signaling in *Drosophila*.

A Shn/Mad/Med complex assembles on the BRE

During the course of these experiments, an analysis of the *Drosophila brk* gene revealed that its expression pattern results from two inputs: ubiquitous activation and Dpp-dependent repression (Muller et al., 2003). Repression elements in the *brk* promoter and several other direct Dpp targets contain a GRCGNC(N5)GTCTG consensus, with binding sites for both Mad and Med separated by a spacer of 5 bp (see Fig. 2A). This precise spacing is required for Shn to dock to Mad/Med complexes on the DNA and mediate repression (Gao et al., 2005; Pyrowolakis et al., 2004). A comparison between the *Xenopus* BRE and the *Drosophila* consensus sequence shows a near perfect match (see Fig. 2A). Although this finding suggested a simple explanation for the ability of the *Xenopus* BRE to function in *Drosophila*, there is a single nucleotide difference between the BRE and the Brk consensus (G→T at position 4) that has been reported to prevent Mad/Med binding and formation of the repression complex in flies (Pyrowolakis et al., 2004). This change in a crucial residue raised the possibility that the sequence similarity was purely coincidental and that the BRE-dependent expression pattern arises through a mechanism that is independent of Shn. To test whether BRE function requires Shn, we examined *Xvent2*-BRE-*lacZ* expression in *shn*$^{-}$ embryos. *Xvent2*-BRE-*lacZ* is derepressed throughout the ectoderm from stage 11 onwards, indicating that Shn is crucial for restricted BRE expression (data not shown). Next, we tested whether the *Xenopus* BRE can nucleate assembly of a Shn/Mad/Med complex required for repression. *Drosophila* S2 cells were transfected with constitutively activated Dpp type-I receptor Thickveins (TkvA), epitope-tagged Mad, Med, and a C-terminal fragment of Shn (ShnCT) that has been shown to participate in complex formation (Pyrowolakis et al., 2004). Radiolabelled BRE oligonucleotides were incubated with cell extracts and subjected to

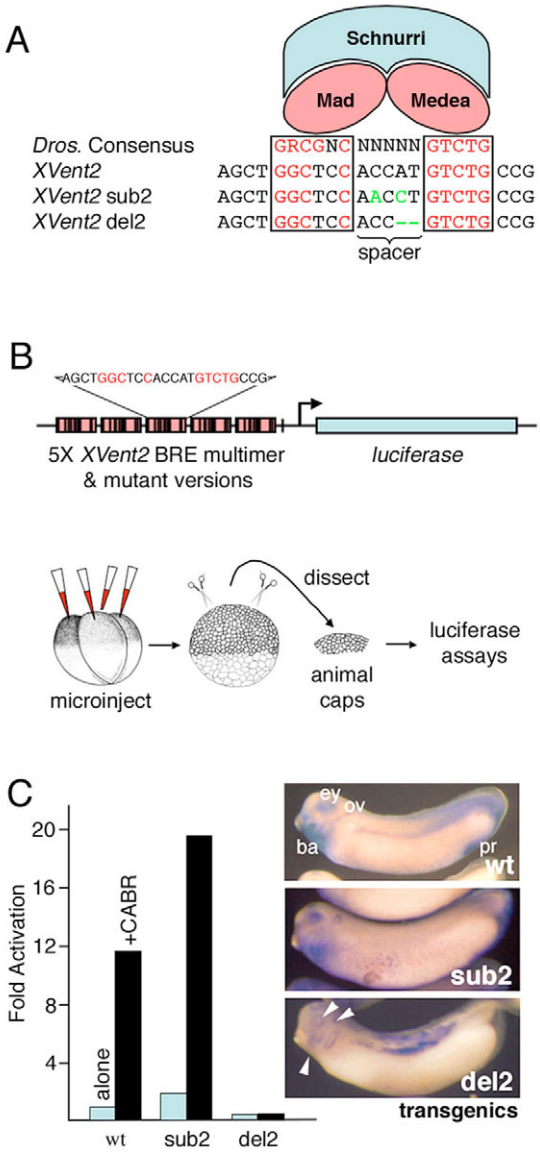

Fig. 2. The *Xvent2*-BRE contains a motif that mediates Bmp responsiveness in *Drosophila*. (**A**) The *Drosophila* element contains Mad and Med sites (boxed) separated by a five-nucleotide spacer that is required for recruitment of Shn. The sequence of Smad sites and their relative spacing are maintained in the *Xvent2*-BRE with one mismatch from the consensus (G→T) at position 4. Point mutations and deletions in the *Xvent2*-BRE variants are marked in green. (**B**) Mutant BREs were tested for their response to Bmp signaling in *Xenopus* animal cap assays. *Xvent2*-BRE-luciferase reporters (top) were microinjected into two- to four-cell stage embryos (bottom), animal cap explants were dissected at stage 8-9 and cultured until siblings reached early gastrula stages, then processed for luciferase assays. (**C**, left) Mutant *Xvent2*-BREs respond identically in *Xenopus* and *Drosophila*. Both the wild-type BRE and *Xvent2*-sub2, which contains two transversions within the five-nucleotide spacer (see A), are stimulated ~10- to 11-fold in response to CABR, compared with reporter alone. By contrast, *Xvent2*-del2 bearing a two-nucleotide spacer deletion (see A) fails to respond. In the experiment shown, 100 pg of CABR mRNA/embryo was used to stimulate expression; however, the *Xvent2*-del2 reporter fails to respond even at 2 ng. All results are presented as fold activation relative to wild type in the absence of CABR. We consistently observed higher luciferase counts for *Xvent2*-sub2 relative to wild type. (C, right) Wild type and *Xvent2*-sub2 respond to endogenous Bmp signaling, whereas the *Xvent2*-del2 mutant does not. Transgenic embryos containing BRE multimers driving GFP are at tailbud stage 31/32. In situ hybridization shows that wild type and *Xvent2*-sub2 mutants direct expression in a pattern similar to the endogenous *Xvent2* mRNA and *Xvent2* transgenes described previously. Transgenic *Xvent2*-del2 embryos had variable expression patterns that are likely to reflect position effects due to random integration sites.

gel electrophoresis. We found that Mad and Med decreased the mobility of the BRE fragment (Fig. 1R, lane 2), and addition of Shn further decreased its migration (Fig. 1R, lane 4). Furthermore, both Mad/Med and the Shn/Mad/Med complexes could be supershifted by antisera against FLAG and Myc epitopes on the Smads and Shn, respectively (compare lane 2 with 3, and 4 with 5), confirming the presence of these proteins in the shifted bands. The ability of the *Xvent2*-BRE to assemble a Shn/Mad/Med complex despite the difference in sequence between the *Drosophila* and *Xenopus* elements may be due to compensatory effects from adjacent or flanking residues. This biochemical analysis indicates that the *Xvent2*-BRE can mediate Bmp signaling-dependent transcriptional repression through a mechanism similar to that used by the *brk* element.

The *Xenopus* and *Drosophila* cis-elements share architecture critical for Bmp responsiveness

The similarities between the *Xenopus* BRE and the *Drosophila brk* element, both in sequence and in the ability to assemble a signaling-dependent repression complex, raise an intriguing question. Do these parallels reflect an underlying conservation of the Bmp-responsive transcriptional regulators? To address this issue, we first tested whether mutations that abolish repression by the *brk* element in *Drosophila* correspondingly alter the ability of *Xvent2*-BRE to respond to Bmp signaling in *Xenopus*. Substitutions of individual residues in the Mad and Med sites of the *brk* element interfere with complex formation and affect repression in vivo (Pyrowolakis et al., 2004). In previous studies, we and others have shown that Smad4 binds the BRE and that mutations that compromise the stimulation of *Xvent2* reporters map to regions corresponding to the Smad sites (Hata et al., 2000; Henningfeld et al., 2000; Karaulanov et al., 2004; von Bubnoff et al., 2005). A distinguishing feature of the *Drosophila* element is the requirement for an invariant spacing between the Mad and Med sites that is crucial for the recruitment of Shn to the Smad-DNA complex (Pyrowolakis et al., 2004). If a similar complex binds the *Xenopus* BRE, the spacer sequences could serve as docking sites for a Smad co-factor, such as a vertebrate Shn (see Fig. 1R). We therefore generated BRE-luciferase reporters that alter either the length or the sequence of the *Xvent2* spacer and tested their ability to respond to Bmp signaling in *Xenopus* animal cap assays (Fig. 2B, see Materials and methods). The wild-type *Xvent2*-BRE drives high-level luciferase expression in response to endogenous Bmps that is further increased upon co-injection of a constitutively active Bmp receptor (CABR), demonstrating that the reporter responds to changes in activity of the Bmp pathway (Fig. 2C). Substitution of 2 bp within the spacer without altering its length (*Xvent2*-sub2), does not affect Bmp responsiveness (Fig. 2A,C). By contrast, shortening the spacer by deleting 2 bp (*Xvent2*-del2) rendered the reporter insensitive to stimulation (Fig. 2A,C). These results demonstrate that the *Xvent2*-BRE responds to Bmp stimulation in a manner identical to that observed for the *brk* element in *Drosophila*.

To examine whether the response to endogenous Bmp signaling places similar constraints on BRE architecture, we assessed the activity of these constructs in transgenic *Xenopus* (Kroll and Amaya, 1996; von Bubnoff et al., 2005). Wild-type and mutant BRE multimers were cloned upstream of the *Xvent2* minimal promoter driving GFP, and reporter gene expression was examined by in situ hybridization. The wild-type BRE drives expression at several sites where Bmp4 is transcribed, including the eye, otic vesicle, ventral branchial arches, and the proctodeal region (Fig. 2C) (Karaulanov et al., 2004; von Bubnoff et al., 2005). The *Xvent2*-sub2 mutant drives a similar expression pattern. By contrast, transgenic embryos carrying the *Xvent2*-del2 mutant show random expression patterns, presumably as a result of integration site-dependent position effects. These data reinforce the idea that in vivo (as in animal cap assays) maintenance of an invariant spacer length is crucial for the BRE to respond positively to Bmp signaling in *Xenopus*. Thus, BRE function has the same architectural requirements in *Xenopus* and *Drosophila*, and this constraint on the organization of the element suggests that the recruitment of a Smad-interacting protein analogous to Shn could be essential for BRE activity in vertebrates.

Shn proteins stimulate BRE activity in vertebrate cells

Shn proteins have high homology in the paired C2H2 zinc finger domains but show limited sequence identity outside of these regions (Fig. 3A) (reviewed by Wu, 2002). Generation of a molecular phylogeny of fly, worm, frog, mouse and human proteins indicates that *Drosophila* Shn is most closely related to vertebrate Shn1 (Fig. 3B). Therefore, to investigate whether Shn proteins play mechanistically similar roles in the Bmp pathway, we examined whether human Shn1 (hShn1) could stimulate the BRE-mediated response to Bmp signaling in animal cap assays (Fig. 3C). We found that expression of hShn1 alone resulted in an approximately 4-fold stimulation of the reporter that was further enhanced to 16-fold in the presence of an activated Bmp receptor. This result suggests that vertebrate Shn-related proteins could play an important role in mediating Bmp signaling.

Because the *Xenopus* BRE was Dpp responsive in *Drosophila* embryos, we were interested in determining whether *Drosophila* Shn can also elicit a response in *Xenopus* animal caps. We found that Shn alone did not significantly stimulate the reporter (Fig. 3D). Remarkably, co-injection of Shn and activated Bmp receptor significantly elevated reporter activity (Fig. 3D). Because fly Shn is capable of participating in a Smad complex on the *Xvent2*-BRE in vitro (see Fig. 1R), these data suggest that Shn can function as a transcriptional activator in *Xenopus* embryos. This result is striking as, in *Drosophila*, Shn mediates repression via the same cis-element. This difference in modality suggests that whether Shn drives transcriptional activation or repression is context-dependent rather than an inherent property of the protein.

Vertebrate Shn interacts directly with Smad proteins and assembles a complex on the BRE

The functional similarities between hShn1 and Shn suggest that vertebrate Shn proteins act as Smad co-factors on the *Vent2* BRE. However docking of Shn to the Mad/Med complex requires zinc fingers 6 and 8 (Pyrowolakis et al., 2004), which are not represented in vertebrate Shn proteins (see Fig. 3A). However, we, and others, have shown that *Drosophila* Shn contains at least two additional Smad-interacting domains (Dai et al., 2000; Udagawa et al., 2000), raising the possibility that interactions through other regions contribute to hShn1-Smad complex formation on the *Xvent2*-BRE. We therefore sought to identify and localize potential Smad-interacting regions within hShn1 by co-expressing hShn1 fragments with hSmad1 and an activated Bmp receptor. Co-immunoprecipitation assays identified two separate domains ($hShn1^{1-599}$ and $hShn1^{1756-2544}$) that mediate Smad interactions (Fig. 4A). The ability of hShn1 to bind hSmad1 was independently confirmed in GST pull-down assays. As demonstrated in Fig. 4B, fragments $hShn1^{1-702}$ and $hShn1^{1756-2544}$ bind GST-Smad1 strongly and GST-Smad4 with lower affinity, suggesting that Shn/Smad interactions are conserved in vertebrate proteins. To determine whether hShn1 binding to Smads results in the formation of a DNA-protein complex on the *Xvent2*-BRE, different hShn1

polypeptides were subjected to gel shift analysis using *Drosophila* Smads. We found that hShn1$^{1\text{-}599}$ and hShn1$^{1496\text{-}2213}$ could bind and supershift a BRE oligonucleotide (Fig. 4A,C, lanes 4 and 6). These polypeptides correlate well with fragments that tested positive in the co-immunoprecipitation and GST pull-down experiments, and suggest that the Smad1-interaction domains are located in the region of overlap. Collectively, these data strongly suggest that, despite the difference in modality, vertebrate Shn proteins use a conserved molecular mechanism to mediate the transcriptional response to Bmp signaling.

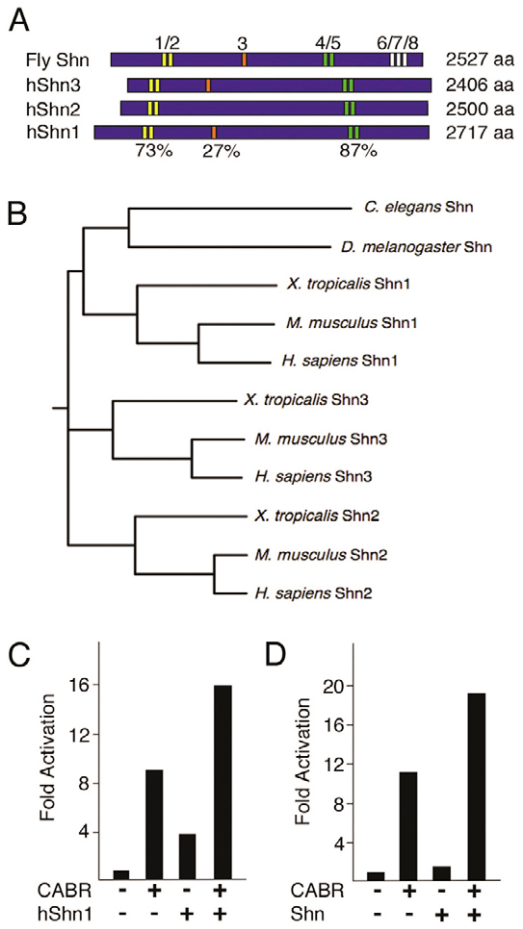

Fig. 3. *Drosophila* Shn and its vertebrate homolog human Shn1 can mediate Bmp-responsive transcriptional activation through the BRE. (**A**) Structural organization of Shn, hShn1, hShn2 and hShn3. Shn contains a total of eight zinc fingers (vertical bars) with characteristic spacing. All zinc finger domains are of the C2H2 type, except for finger 3, which is a C2HC type. All three human proteins contain the first and second set of paired zinc fingers (1/2 and 4/5 in Shn), but lack the triplet set near the carboxy terminus (6/7/8). The C2HC finger is absent from hShn2. Shn and hShn1 share 73% identity in the 1/2, and 87% in the 4/5 paired finger domains, whereas finger 3 shows only 27% identity. The fly and human Shns have minimal identity in the remainder of the protein. By contrast, hShn1, hShn2 and hShn3 share 26-31% sequence identity outside of their finger regions. (**B**) Molecular phylogeny of Shn proteins from *D. melanogaster*, *C. elegans*, *X. tropicalis*, *M. musculus* and *H. sapiens* generated using CLUSTALW. Invertebrate Shn proteins most closely resemble vertebrate Shn1. (**C**) In *Xenopus* animal cap assays, hShn1 stimulates wild-type *Xvent2*-BRE reporter gene expression even in the absence of CABR (i.e. in the presence of endogenous Bmp signaling). However, co-expression of hShn1 together with CABR results in a stronger induction of the reporter. (**D**) *Drosophila* Shn only weakly induces the *Xvent2*-BRE reporter in *Xenopus* animal caps. However, in the presence of CABR, Shn shows a strong induction of the reporter. Results are presented as fold activation relative to basal activity of the wild-type reporter in the absence of Shn proteins or CABR.

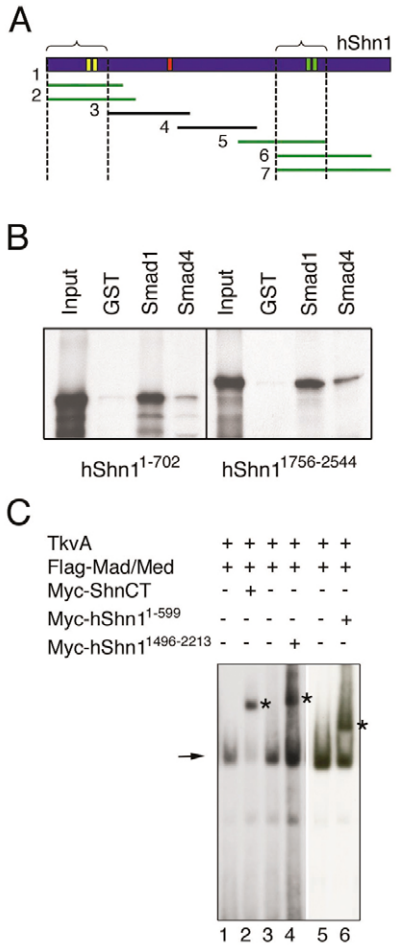

Fig. 4. Human Shn1 contains two Smad interaction domains and can form a complex with Smads on the BRE. (**A**) Summary of hShn1 Smad-interacting regions. hShn1 fragments 1 (hShn1$^{1\text{-}599}$), 2 (hShn1$^{1\text{-}702}$), 5 (hShn1$^{1496\text{-}2213}$), 6 (hShn1$^{1756\text{-}2544}$) and 7 (hShn1$^{1756\text{-}2717}$) (green) co-immunoprecipitate with Mad and Med, whereas polypeptides 3 (hShn1$^{496\text{-}1121}$) and 4 (hShn1$^{1002\text{-}1635}$) (black) do not. Presumptive minimal Smad-interaction domains are bracketed. (**B**) GST pull-down assays using fragments 2 (hShn1$^{1\text{-}702}$) and 6 (hShn1$^{1756\text{-}2544}$) confirm the presence of two Smad-interacting regions in hShn1. Equivalent amounts of GST, GST-Smad1 (MH2 domain+linker) and GST-Smad4 (MH2 domain+linker) were co-incubated with in vitro translated [^{35}S]-methionine-labeled hShn1 polypeptides as indicated. Both hShn1 fragments are specifically retained by Smad1 and Smad4, but not by GST alone. Both hShn1 fragments display a higher affinity for Smad1 than Smad4. (**C**) Gel mobility shifts were performed with radiolabelled *Xenopus* BRE probe. Co-incubation with Mad and Med results in a low mobility complex (arrow) that is further retarded by the addition of a *Drosophila* Shn fragment, ShnCT (asterisk, lane 2). Co-incubation of *Drosophila* Smads with hShn1 polypeptides (hShn1$^{1496\text{-}2213}$ and hShn1$^{1\text{-}599}$) also resulted in retardation of the BRE (asterisks, lanes 4 and 6, respectively), indicating that a Mad/Med/hShn1 complex had formed on the DNA. Both of these fragments contain regions of overlap with the Smad-interacting fragments identified in GST pull-downs (see A). Fragments that lack Smad-interaction domains did not alter probe mobility (data not shown).

hShn1 restores Dpp signaling in *Drosophila shn* mutants

Our data provide strong evidence that parallel cis-regulatory elements and trans-acting factors can mediate Bmp-dependent activation of the *Vent2* gene in *Xenopus* and Dpp-dependent repression in *Drosophila*. Because *Drosophila* Shn activates BRE-reporters in *Xenopus* embryos, but functions primarily as a repressor in flies, we sought to determine whether the converse is also true, by assaying the ability of hShn1 to substitute for *Drosophila* Shn in vivo. We focused on two Dpp-dependent events: down-regulation of *brk* expression in dorsal tissues and differentiation of dorsal epidermis in embryos. In the absence of Shn function, Dpp signaling cannot repress *brk* transcription and *brk-lacZ* is upregulated in all tissues (Fig. 5A,C). In addition, *shn* mutants display a 'dorsal open' phenotype, as the dorsal epidermis fails to differentiate owing to impaired Dpp signaling (Fig. 5B,D). We generated transgenic lines containing hShn1 cDNA downstream of UAS sites and used a heatshock Gal4 (HS>Gal4) driver to express hShn1 ubiquitously in *shn*$^{-}$ mutant embryos. We found that hShn1 effectively represses *brk-lacZ* in regions of the embryo that are subject to high levels of Dpp signaling (Fig. 5G). Thus, although hShn1 is required for transcriptional activation in vertebrate cells, it can direct signaling-dependent repression in a different context. The analysis of whole-mount cuticle preparations reveals that hShn1 expression allows the correct differentiation of the dorsal epidermis and the consequent rescue of the 'dorsal hole' phenotype of *shn* mutants (Fig. 5H). Overall, the severity of the patterning defects is significantly reduced and the morphology of these animals is much closer to that of wild type. Notably, the extent of rescue by hShn1 is comparable to that encountered in control experiments with UAS-driven *Drosophila* Shn (Fig. 5E,F). The ability of hShn1 to compensate for the loss of Shn function in *Drosophila* demonstrates that the human protein can: (1) respond correctly to endogenous Bmp signals to regulate gene expression; (2) do so with enough fidelity to substantially restore embryonic patterning and development; and (3) mediate repression rather than activation depending on the cellular context. These data establish that hShn1 can act in Bmp signaling in the context of an organism, and suggest that it participates in an evolutionarily conserved mechanism for the transcriptional response to Bmp growth factors.

DISCUSSION

The Bmp pathway is an ancient highly conserved signaling module that performs diverse functions in different organismal contexts. Underscoring this conservation, Bmp ligands from humans and the coral *Acropora* can rescue patterning defects in *Drosophila* embryos lacking the Dpp ligand, and fly Dpp can trigger ectopic bone formation in rodents (Hayward et al., 2002; Padgett et al., 1993; Sampath et al., 1993). In this study, we show that both *Drosophila* Shn and its vertebrate homolog Shn1 can use a common mechanism involving interaction with Smads and similar cis-regulatory elements to transduce Bmp signals. Another important finding is that Shn and hShn1 can mediate either transcriptional activation or repression through identical cis-elements, depending on the cellular context. These results demonstrate that Shn proteins are not obligate transcriptional repressors but rather that they recruit co-activators or co-repressors in a cell-type specific fashion.

The *brk* element is a phylogenetically conserved cis-regulatory motif

Our data indicate that a common set of simple parameters – the presence of Smad1 and Smad4 binding sites with a 5-bp separation – govern the ability of the *Xvent2*-BRE to respond to Bmp signaling in both vertebrate cells and in *Drosophila*. We establish that the BRE can assemble a Smad/Shn complex despite a G→T substitution at position 4 (see Fig. 2A). This was unexpected, as the change affects one of the two GNCN sites in the *brk* consensus site that are thought to bind Mad (Gao and Laughon, 2006; Gao et al., 2005). One possibility is that protein-protein interactions render the presence of both GNCN sites unnecessary. Additionally, it has recently been shown that an A→C substitution at position 5 in the *brk* element enhances Mad binding (Gao and Laughon, 2006). Interestingly the *Xvent2*-BRE also contains a C at this position that could play a compensatory role and aid complex

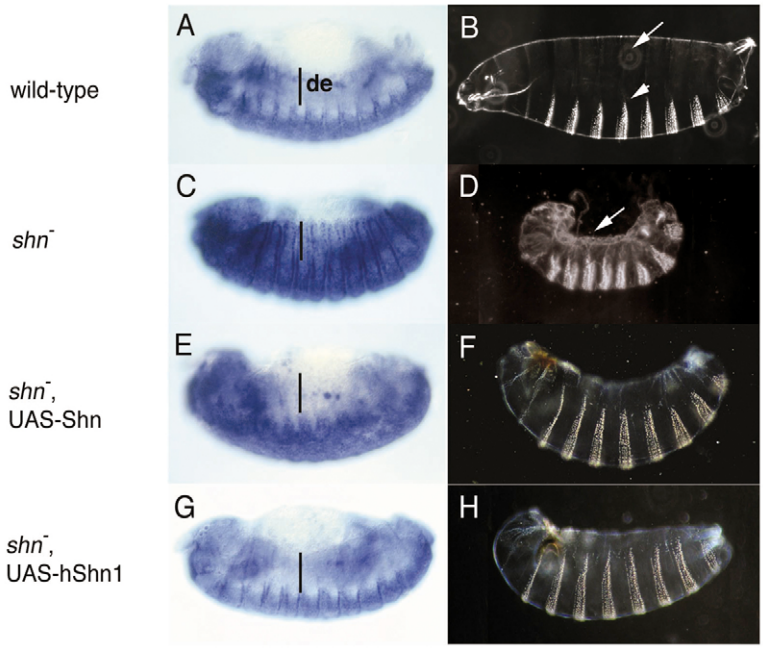

Fig. 5. Human Shn1 can compensate for loss of Shn function in *Drosophila* embryogenesis. Lateral views of *Drosophila* embryos showing *brk-lacZ* expression at stage 13 (left), and darkfield images of differentiated cuticle (right). Anterior left, dorsal up. (**A**) In wild-type embryos, the *brk-lacZ* reporter is expressed ventrally but is downregulated in the dorsal ectoderm (de, vertical bar) in response to Dpp signaling. (**B**) In wild-type animals, the thoracic and abdominal segments differentiate denticle belts (arrowhead) characteristic of the ventral epidermis, whereas the dorsal epidermis contains fine dorsal hairs (arrow). (**C**,**D**) In *shn* mutants, *brk-lacZ* expression is derepressed (C), and the cuticle displays a characteristic 'dorsal open' phenotype (arrow) owing to the failure of dorsal epidermal differentiation (D). (**E-H**) Rescue of *shn*^{P4738} null embryos by UAS-Shn and UAS-hShn1. In control experiments, a UAS-Shn transgene driven by the heat-shock Gal4 driver can respond to endogenous Dpp signaling and repress *brk-lacZ* expression in the dorsal ectoderm (E). It can also rescue the morphological defects in *shn*^{P4738} mutants (F). Rescued embryos differentiate a dorsal ectoderm and therefore show a closed and contiguous dorsal cuticle. Remarkably, UAS-hShn1 is as effective as *Drosophila* Shn in compensating for the loss of endogenous Shn function (compare G,H with E,F).

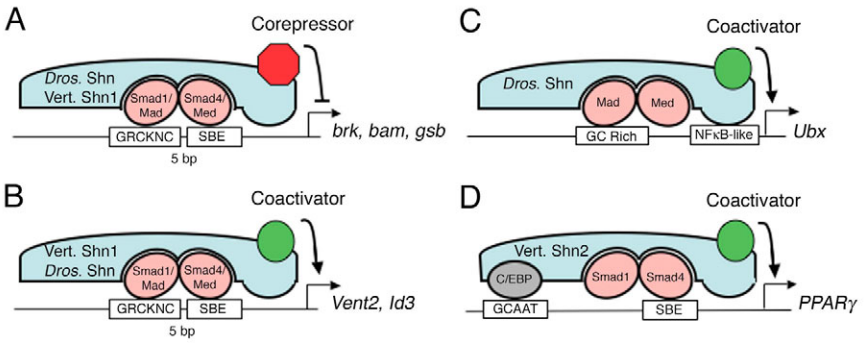

Fig. 6. Shn proteins contribute to Bmp signaling by functioning as scaffolding factors. Shn proteins provide a framework that integrates Smads, co-activators/co-repressors and other transcription factors. The large size of Shn proteins may provide the flexibility to recognize different partners and to act through a variety of cis-elements. On genes such as *Xvent2*, *Id3*, *brk*, *bam* and *gsb*, that contain the GRCKNC(N5)GTCTG consensus, Smad1/Mad and Smad4/Med bind to GRCKNC and GTCT sites, probably as a heterotrimeric complex (Gao et al., 2005) (not represented in this figure). Shn/Shn1 interaction with the MH2 domains of Smads could stabilize the complex and provide docking sites for cell/tissue-specific co-repressors, as in **A**, or co-activators, as in **B**. Shn binding in A and B is highly sensitive to the spacing between the Smad sites indicating steric constraints (Gao et al., 2005; Pyrowolakis et al., 2004). (**C**) Shn promotes activation of *Ubx* in the *Drosophila* midgut through a promoter element that contains sites for Mad and an NFκB-like site directly bound by Shn. In this context, there is no apparent requirement for Med binding to DNA. (**D**) In contrast, the mouse PPARγ enhancer that is activated by Shn2 requires sites for Smad4 and C/EBPα, but does not contain Smad1 motifs (Jin et al., 2006). The sensitivity of these enhancers to alterations in spacing between binding sites has not been tested.

formation. Thus, our study suggests a modified consensus recognition site GRCKNC(N5)GTCTG for the Shn/Smad complex. The conservation of both sequence and function in the BRE is unexpected. For example, *even skipped* (*eve*) stripe 2 enhancers from the related species *D. yakuba* and *D. melanogaster*, which drive expression in similar patterns and are functionally equivalent, show considerable sequence divergence, suggesting that 'turnover' in transcription factor binding sites and their relative spacing is likely to be common, with co-evolutionary changes being necessary to retain essential functions (Ludwig, 2002; Tautz, 2000). The stability of the Shn/Smad site differs from the *eve* element and is reminiscent of the cross-phyla conservation of enhancers in the Dorsal/NFκB and wound healing pathways in *Drosophila* and vertebrates (Gonzalez-Crespo and Levine, 1994; Mace et al., 2005).

Incorporation of the Shn/Mad/Med sequence into target gene promoters appears to be a widely used strategy in *Drosophila*. Genome-wide searches reveal that in addition to *brk*, the motif is present flanking a number of genes that are downregulated by Dpp. In two instances, *bag of marbles* and *gooseberry*, functional studies have validated that the *brk* consensus is crucial for repression (Pyrowolakis et al., 2004). The extent to which the BRE confers Bmp responsiveness in vertebrates remains to be established. In addition to *Xvent2*, this element is precisely retained in the promoters of the related *Xvent1B* and *Xvent2B* genes, as well as in the human and zebrafish homologs *VENTX2*, *Vox* and *Vent*. The BRE consensus is also present in the Bmp-responsive enhancers of the *id1*, *id2*, *id3* and *id4* genes (Karaulanov et al., 2004; von Bubnoff et al., 2005). These elements in the *id* genes are also conserved between humans, rats, mice, fish and frogs, which strongly suggests that the BRE represents an ancient and functionally important motif. Limited searches have identified BRE-like sequences in several other Bmp-responsive genes (Q. Zeng and K.W.Y.C., unpublished), although more extensive bioinformatics combined with in vivo verification will be required to gauge the full spectrum of genes regulated by Shn proteins. It is likely that Shn proteins regulate only a subset of Bmp targets through BRE-like motifs, as close matches to the consensus are absent from several characterized Bmp-responsive promoters. This is not unexpected, as an important function of Smad-interacting cofactors would be the provision of cell, tissue and promoter specificity.

A previous study has implicated another Smad-interacting factor, OAZ, in activation of *Xvent2* transcription (Hata et al., 2000). Although it remains possible that OAZ may contribute to *Xvent2* expression in some tissues, the OAZ expression pattern is insufficient to explain all aspects of *Xvent2* synexpression with Bmps. In addition, the OAZ site GCTCCA (that overlaps with the GRCKNC Smad1 motif) was retained in the *Xvent2*-del2 mutation that abolished Bmp responsiveness in animal caps and embryos (see Fig. 2), which suggests that OAZ may not have a crucial role in *Xvent2* regulation.

Shn proteins display context-dependent modalities

A striking feature of the Shn complex assembled at the BRE is that it leads to opposite outputs in *Drosophila* and *Xenopus*. In *Drosophila*, the *brk* element appears to exclusively mediate repression both in cell culture and in vivo (Gao et al., 2005; Muller et al., 2003). Repression is dependent upon recruitment of Shn to a Mad/Med complex, and this suggested that Shn might function as an obligate repressor (Pyrowolakis et al., 2004). Consistent with this view, a 1-bp increase or decrease in the *brk* spacer prevents Shn recruitment and changes the response from repression to activation in cell culture assays (Gao et al., 2005). The results presented in this work do not fit such a model. Although Shn/Mad/Med bound to the *Xenopus* BRE mediates repression in *Drosophila* (see Fig. 1), in *Xenopus* Shn elicits activation through the same response element (see Fig. 3D). Thus the crucial determinant of whether the complex mediates activation or repression appears to be the cellular milieu rather than the presence of Shn. Human Shn1 similarly behaves as a transcriptional repressor in flies and as an activator in *Xenopus* (see Fig. 3B, Fig. 4) further emphasizing the fact that the transcriptional polarity of Shn proteins may depend on differential interaction with co-repressors and co-activators (Fig. 6). Furthermore, there is evidence that *Drosophila* Shn need not be an obligate repressor in flies. We have previously shown that Shn can directly stimulate the expression of *Ubx*, a Dpp target gene, both in vivo and in cell culture (Dai et al., 2000) (see Fig. 6). Likewise, Shn proteins may not be obligate activators in vertebrate cells. Mouse Shn1 represses expression of collagen2α1 through the recruitment of SPEN, a protein that interacts with co-repressors SMRT/NCoR and HDAC (Yang et al., 2005). Thus, the outcome of Shn interactions with the

BRE may not be a species-specific phenomenon. Instead, our observations suggest that the modality of Shn is regulated in a cell-type specific manner. This has several ramifications. First, it identifies Shn co-activators and co-repressors as additional, and as yet uncharacterized, players in the Bmp pathway. Second, the ability of human Shn proteins to repress genes in *Drosophila* leads us to speculate that at least some of the co-repressors are evolutionarily conserved, and that vertebrate Shn proteins may also be involved in the downregulation of Bmp targets.

In Shn, zinc fingers 6 and 8 that mediate complex formation with Mad/Med and an adjacent 113 amino acid domain are essential for repression (Pyrowolakis et al., 2004). Surprisingly, hShn1 that does not contain either the Smad interaction domain or the 'repression domain' can nevertheless effectively repress *brk-lacZ* expression in flies (see Fig. 5). As we have demonstrated, hShn1 can assemble a complex on the BRE through two independent Smad-interacting regions (see Fig. 4C), thus it appears that co-repressor recruitment may depend on structural motifs/charge interactions that are not apparent from the primary sequence.

Shn function in vertebrate Bmp signaling

Our results show that Shn1 can transduce a Bmp signal in *Xenopus* and *Drosophila* embryos, as well as in cell culture (see Figs 3, 5; S.P. and R.W., unpublished). The transcript distribution of *Shn1* in *Xenopus* has significant overlap with, and could explain, the Bmp-dependent synexpression pattern of the *Xvent2* gene from which the BRE was derived (Durr et al., 2004; Niehrs and Pollet, 1999; Onichtchouk et al., 1996). However, a complete understanding of the role of Shn proteins in vertebrates will require loss-of-function analyses. Recent studies of knockouts for the closely related *Shn2* gene has shown that it contributes to Bmp-responsive transcriptional activation of PPARγ2, a key regulator of adipocyte differentiation. Consistent with this, although *Shn2* null mice are viable and fertile, they have reduced adipose tissue. Interestingly, although Shn2 also forms a complex with Smad1/Smad4 in response to Bmp signaling, it mediates transcriptional activation via a distinct mechanism (Jin et al., 2006). The sequence recognized by the complex does not resemble the BRE and in fact lacks a Smad1 site. Instead, activation of PPARγ2 expression by Shn2 requires sites for Smad4 and the transcription factor C/EBP-α (see Fig. 6). Mice lacking *Shn3* are also viable and fertile, but they show defects in immune function. Shn3 has been shown to act downstream of the T cell and Tnf receptors (Oukka et al., 2002; Oukka et al., 2004), but whether these events are linked to Bmp signaling remains to be explored.

All three vertebrate Shn proteins contain the zinc finger domains implicated in Smad interaction (Pyrowolakis et al., 2004; Jin et al., 2006) (this work), raising questions about how related Shn proteins contribute to Bmp signaling. One possibility is that all Shn proteins can interact with Smads but that each paralog recruits different sets of cofactors. Thus, each Shn protein would regulate a distinct subset of targets, allowing Bmp signaling to control a wide range of target genes in a selective manner. Alternatively, vertebrate Shn proteins may share the ability to interact with common co-factors and have redundant or partially overlapping functions. In light of this, it is interesting that *Shn2* and *Shn3* mutant mice are viable, in contrast to the embryonic lethality of mutations in Bmp ligands, receptors and Smads (reviewed by Zhao, 2003). Mouse *Shn1* and *Shn2* are widely expressed in overlapping tissues during embryogenesis and in adults, whereas *Shn3* shows restricted expression, suggesting that it may have a more limited role (reviewed by Wu, 2002). A more complete understanding of how Shn proteins contribute to Bmp signaling in mice will require the analysis of mouse *Shn1* knockouts, as well as the generation of double and triple null animals. Our attempts to knockdown Shn1 function in *X. tropicalis* using splice-site directed morpholino oligonucleotides have been unsuccessful (I.L.B. and K.W.Y.C., unpublished). Given that all three genes are ubiquitously distributed in gastrula stage embryos and continue to be expressed throughout early development (Durr et al., 2004), one possibility is that vertebrate Shn proteins are functionally redundant. Alternatively, because the morpholinos do not target mature mRNA, the absence of a phenotype may be due to perdurance of the maternal message. The conservation of the size and organization of Shn-like proteins suggests that their structural properties may contribute to their function. One possibility is that they act as molecular scaffolds for the assembly of the transcriptional machinery that mediates Bmp responsiveness (see Fig. 6). Shn proteins could also serve an architectural function by promoting bending or looping of the DNA, by binding at more than one location.

We gratefully acknowledge Rene Bernards, Tom Maniatis, Scott Barolo and Alan Laughon for generously sharing reagents, and Douglas Bornemann for insightful discussion and comments on the manuscript. Support was provided by grants from the NIH (GM-55442) and March of Dimes (FY2-06-135) to K.A., the NICHD (HD-29507) to K.W.Y.C., and the UC Cancer Research Co-ordination Committee (CRCC 34384) to R.W.

References

Affolter, M., Marty, T., Vigano, M. A. and Jazwinska, A. (2001). Nuclear interpretation of Dpp signaling in Drosophila. *EMBO J.* **20**, 3298-3305.

Arora, K., Dai, H., Kazuko, S. G., Jamal, J., O'Connor, M. B., Letsou, A. and Warrior, R. (1995). The Drosophila *schnurri* gene acts in the Dpp/TGFβ signaling pathway and encodes a transcription factor homologous to the human MBP family. *Cell* **81**, 781-790.

Barolo, S., Carver, L. A. and Posakony, J. W. (2000). GFP and beta-galactosidase transformation vectors for promoter/enhancer analysis in Drosophila. *Biotechniques* **29**, 726-732.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Bray, S. J. and Kafatos, F. C. (1991). Developmental function of Elf-1: an essential transcription factor during embryogenesis in Drosophila. *Genes Dev.* **5**, 1672-1683.

Campbell, G. and Tomlinson, A. (1999). Transducing the Dpp morphogen gradient in the wing of Drosophila: regulation of Dpp targets by *brinker*. *Cell* **96**, 553-562.

Candia, A. F., Watabe, T., Hawley, S. H., Onichtchouk, D., Zhang, Y., Derynck, R., Niehrs, C. and Cho, K. W. (1997). Cellular interpretation of multiple TGF-beta signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads. *Development* **124**, 4467-4480.

Dai, H., Hogan, C., Gopalakrishnan, B., Torres-Vazquez, J., Nguyen, M., Park, S., Raftery, L. A., Warrior, R. and Arora, K. (2000). The zinc finger protein schnurri acts as a Smad partner in mediating the transcriptional response to decapentaplegic. *Dev. Biol.* **227**, 373-387.

Dorfman, R. and Shilo, B. Z. (2001). Biphasic activation of the BMP pathway patterns the Drosophila embryonic dorsal region. *Development* **128**, 965-972.

Durr, U., Henningfeld, K. A., Hollemann, T., Knochel, W. and Pieler, T. (2004). Isolation and characterization of the Xenopus HIVEP gene family. *Eur. J. Biochem.* **271**, 1135-1144.

Fan, C. M. and Maniatis, T. (1990). A DNA-binding protein containing two widely separated zinc finger motifs that recognize the same DNA sequence. *Genes Dev.* **4**, 29-42.

Furriols, M. and Bray, S. (2001). A model Notch response element detects Suppressor of Hairless-dependent molecular switch. *Curr. Biol.* **11**, 60-64.

Gao, S. and Laughon, A. (2006). DPP-responsive silencers contain overlapping mad binding sites. *J. Biol. Chem*. Doi:10.1074/jbc.M603371200.

Gao, S., Steffen, J. and Laughon, A. (2005). Dpp-responsive silencers are bound by a trimeric Mad-Medea complex. *J. Biol. Chem.* **280**, 36158-36164.

Gonzalez-Crespo, S. and Levine, M. (1994). Related target enhancers for Dorsal and NF-κB signaling pathways. *Science* **264**, 255-258.

Grieder, N. C., Nellen, D., Burke, R., Basler, K. and Affolter, M. (1995). *schnurri* is required for Drosophila Dpp signaling and encodes a zinc finger protein similar to the mammalian transcription factor PRDII-BF1. *Cell* **81**, 791-800.

Hata, A., Seoane, J., Lagna, G., Montalvo, E., Hemmati-Brivanlou, A. and Massague, J. (2000). OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. *Cell* **100**, 229-240.

Hayward, D. C., Samuel, G., Pontynen, P. C., Catmull, J., Saint, R., Miller, D. J. and Ball, E. E. (2002). Localized expression of a dpp/BMP2/4 ortholog in a coral embryo. *Proc. Natl. Acad. Sci. USA* **99**, 8106-8111.
Henningfeld, K. A., Rastegar, S., Adler, G. and Knochel, W. (2000). Smad1 and Smad4 are components of the bone morphogenetic protein-4 (BMP-4)-induced transcription complex of the Xvent-2B promoter. *J. Biol. Chem.* **275**, 21827-21835.
Holstein, T. W., Hobmayer, E. and Technau, U. (2003). Cnidarians: an evolutionarily conserved model system for regeneration? *Dev. Dyn.* **226**, 257-267.
Jin, W., Takagi, T., Kanesashi, S. N., Kurahashi, T., Nomura, T., Harada, J. and Ishii, S. (2006). Schnurri-2 controls BMP-dependent adipogenesis via interaction with Smad proteins. *Dev. Cell* **10**, 461-471.
Johnson, K., Kirkpatrick, H., Comer, A., Hoffmann, F. M. and Laughon, A. (1999). Interaction of Smad complexes with tripartite DNA-binding sites. *J. Biol. Chem.* **274**, 20709-20716.
Karaulanov, E., Knochel, W. and Niehrs, C. (2004). Transcriptional regulation of BMP4 synexpression in transgenic Xenopus. *EMBO J.* **23**, 844-856.
Kim, J., Johnson, K., Chen, H. J., Carroll, S. and Laughon, A. (1997). Drosophila Mad binds to DNA and directly mediates activation of *vestigial* by Decapentaplegic. *Nature* **388**, 304-308.
Kirov, N., Childs, S., O'Connor, M. and Rushlow, C. (1994). The Drosophila dorsal morphogen represses the tolloid gene by interacting with a silencer element. *Mol. Cell. Biol.* **14**, 713-722.
Kroll, K. L. and Amaya, E. (1996). Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* **122**, 3173-3183.
Liang, J., Lints, R., Foehr, M. L., Tokarz, R., Yu, L., Emmons, S. W., Liu, J. and Savage-Dunn, C. (2003). The Caenorhabditis elegans schnurri homolog sma-9 mediates stage- and cell type-specific responses to DBL-1 BMP-related signaling. *Development* **130**, 6453-6464.
Ludwig, M. Z. (2002). Functional evolution of noncoding DNA. *Curr. Opin. Genet. Dev.* **12**, 634-639.
Mace, K. A., Pearson, J. C. and McGinnis, W. (2005). An epidermal barrier wound repair pathway in Drosophila is mediated by grainy head. *Science* **308**, 381-385.
Marty, T., Muller, B., Basler, K. and Affolter, M. (2000). Schnurri mediates Dpp-dependent repression of brinker transcription. *Nat. Cell Biol.* **2**, 745-749.
Massague, J., Seoane, J. and Wotton, D. (2005). Smad transcription factors. *Genes Dev.* **19**, 2783-2810.
Muller, B., Hartmann, B., Pyrowolakis, G., Affolter, M. and Basler, K. (2003). Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient. *Cell* **113**, 221-233.
Niehrs, C. and Pollet, N. (1999). Synexpression groups in eukaryotes. *Nature* **402**, 483-487.
Onichtchouk, D., Gawantka, V., Dosch, R., Delius, H., Hirschfeld, K., Blumenstock, C. and Niehrs, C. (1996). The Xvent-2 homeobox gene is part of the BMP-4 signalling pathway controlling [correction of controling] dorsoventral patterning of Xenopus mesoderm. *Development* **122**, 3045-3053.
Oukka, M., Kim, S. T., Lugo, G., Sun, J., Wu, L. C. and Glimcher, L. H. (2002). A mammalian homolog of Drosophila schnurri, KRC, regulates TNF receptor-driven responses and interacts with TRAF2. *Mol. Cell* **9**, 121-131.
Oukka, M., Wein, M. N. and Glimcher, L. H. (2004). Schnurri-3 (KRC) interacts with c-Jun to regulate the IL-2 gene in T cells. *J. Exp. Med.* **199**, 15-24.
Padgett, R. W., Wozney, J. M. and Gelbart, W. M. (1993). Human BMP sequences can confer normal dorsal-ventral patterning in the Drosophila embryo. *Proc. Natl. Acad. Sci. USA* **90**, 2905-2909.
Pyrowolakis, G., Hartmann, B., Muller, B., Basler, K. and Affolter, M. (2004). A simple molecular complex mediates widespread BMP-induced repression during Drosophila development. *Dev. Cell* **7**, 229-240.
Raftery, L. A. and Sutherland, D. J. (1999). TGF-beta family signal transduction in Drosophila development: from Mad to Smads. *Dev. Biol.* **210**, 251-268.
Rastegar, S., Friedle, H., Frommer, G. and Knochel, W. (1999). Transcriptional regulation of Xvent homeobox genes. *Mech. Dev.* **81**, 139-149.
Sampath, T. K., Rashka, K. E., Doctor, J. S., Tucker, R. F. and Hoffmann, F. M. (1993). Drosophila transforming growth factor beta superfamily proteins induce endochondral bone formation in mammals. *Proc. Natl. Acad. Sci. USA* **90**, 6004-6008.
Shi, Y. and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.
Staehling-Hampton, K., Laughon, A. S. and Hoffmann, F. M. (1995). *Drosophila* protein related to the human zinc finger transcription factor PRDII/MBPI/HIV-EP1 is required for *dpp* signaling. *Development* **121**, 3393-3403.
Sutherland, D. J., Li, M., Liu, X. Q., Stefancsik, R. and Raftery, L. A. (2003). Stepwise formation of a SMAD activity gradient during dorsal-ventral patterning of the Drosophila embryo. *Development* **130**, 5705-5716.
Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K. and Ueno, N. (1994). A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early Xenopus embryo. *Proc. Natl. Acad. Sci. USA* **91**, 10255-10259.
Tanimoto, H., Itoh, S., ten Dijke, P. and Tabata, T. (2000). Hedgehog creates a gradient of DPP activity in Drosophila wing imaginal discs. *Mol. Cell* **5**, 59-71.
Tautz, D. (2000). Evolution of transcriptional regulation. *Curr. Opin. Genet. Dev.* **10**, 575-579.
Torres-Vazquez, J., Warrior, R. and Arora, K. (2000). schnurri is required for dpp-dependent patterning of the Drosophila wing. *Dev. Biol.* **227**, 388-402.
Torres-Vazquez, J., Park, S., Warrior, R. and Arora, K. (2001). The transcription factor Schnurri plays a dual role in mediating Dpp signaling during embryogenesis. *Development* **128**, 1657-1670.
Udagawa, Y., Hanai, J., Tada, K., Grieder, N. C., Momoeda, M., Taketani, Y., Affolter, M., Kawabata, M. and Miyazono, K. (2000). Schnurri interacts with Mad in a Dpp-dependent manner. *Genes Cells* **5**, 359-369.
von Bubnoff, A., Peiffer, D. A., Blitz, I. L., Hayata, T., Ogata, S., Zeng, Q., Trunnell, M. and Cho, K. W. (2005). Phylogenetic footprinting and genome scanning identify vertebrate BMP response elements and new target genes. *Dev. Biol.* **281**, 210-226.
Watabe, T., Kim, S., Candia, A., Rothbacher, U., Hashimoto, C., Inoue, K. and Cho, K. W. (1995). Molecular mechanisms of Spemann's organizer formation: conserved growth factor synergy between Xenopus and mouse. *Genes Dev.* **9**, 3038-3050.
Wu, L. C. (2002). ZAS: C2H2 zinc finger proteins involved in growth and development. *Gene Expr.* **10**, 137-152.
Xu, X., Yin, Z., Hudson, J. B., Ferguson, E. L. and Frasch, M. (1998). Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the Drosophila mesoderm. *Genes Dev.* **12**, 2354-2370.
Yang, X., Li, J., Qin, H., Yang, H., Li, J., Zhou, P., Liang, Y. and Han, H. (2005). Mint represses transactivation of the type II collagen gene enhancer through interaction with alpha A-crystallin-binding protein 1. *J. Biol. Chem.* **280**, 18710-18716.
Zhao, G. Q. (2003). Consequences of knocking out BMP signaling in the mouse. *Genesis* **35**, 43-56.