Daughters against dpp modulates *dpp* organizing activity in *Drosophila* wing development

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The family of TGF-β signalling molecules play inductive roles in various developmental contexts[1]. One member of this family, *Drosophila* Decapentaplegic (Dpp)[2] serves as a morphogen that patterns both the embryo[3,1] and adult[4,5]. We have now isolated a gene, *Daughters against dpp* (*Dad*), whose transcription is induced by Dpp. Dad shares weak homology with *Drosophila* Mad (Mothers against dpp)[6], a protein required for transduction of Dpp signals. In contrast to Mad or the activated Dpp receptor, whose overexpression hyperactivates the Dpp signalling pathway, overexpression of Dad blocks Dpp activity. Expression of Dad together with either Mad or the activated receptor rescues phenotypic defects induced by each protein alone. Dad can also antagonize the activity of a vertebrate homologue of Dpp, bone morphogenetic protein (BMP-4; ref. 7), as evidenced by induction of dorsal or neural fate following overexpression in *Xenopus* embryos. We conclude that the pattern-organizing mechanism governed by Dpp involves a negative-feedback circuit in which Dpp induces expression of its own antagonist, Dad. This feedback loop appears to be conserved in vertebrate development.

The *Drosophila* wing is divided into two compartments along its anteroposterior (A/P) axis. The compartment boundary between these regions serves as the source of an organizing activity that patterns both anterior and posterior compartments[8–10]. This activity is mediated, at least in part, by the long-range action of Dpp[8,11], which is expressed by cells along the A/P compartment boundary (Fig. 1a). Dpp is thought to act as a morphogen to inform target cells of their position along the A/P axis[4,5], but as little is known about how cells interpret the distribution of Dpp protein, we conducted an enhancer trap screen to identify genes whose transcription is controlled by Dpp. We identified two enhancer trap lines in the same locus (89E/F), P1883 and 1(3)1E4, whose expression patterns are similar to those of Dpp during embryonic (data not shown) and imaginal development (Fig. 1b). We designate the gene whose expression is reflected in these enhancer traps *Daughters against dpp* (*Dad*). In these enhancer trap lines, β-galactosidase is expressed in a wide stripe that straddles the A/P compartment boundary of the imaginal discs, in contrast to Dpp, whose expression is confined to the anterior side. This pattern of expression suggests that Dad expression is positively regulated by the secreted Dpp molecule. To test whether Dad responds to Dpp signalling, we examined its expression in P1883 wing discs in which a *UAS-dpp* transgene is transcribed in a ring around a wing pouch under the control of a Gal 4 driver[12] (Fig. 1f). Ectopic Dpp expression resulted in abnormally large discs and in ectopic expression of Dad in a broad ring around a wing pouch (Fig. 1d). Identical results were obtained when another transgene, *UAS-tkv*^{Q253D} (ref. 5), which encodes a constitutively active form of the major type-I Dpp receptor, Thick veins (Tkv), was used (Fig. 1e). In addition, expression of Dad was not detected in cells that lacked a functional Tkv Dpp receptor (Fig. 1g–i). These results indicate that Dpp signalling is necessary and sufficient for Dad expression in the developing wing.

To isolate the *Dad* gene, genomic DNA surrounding the insertion of 1(3)1E4 was cloned by plasmid rescue. An exon-containing genomic fragment was identified by *in situ* (Fig. 1c) and northern hybridization and was used as a probe to isolate complementary DNAs. The longest cDNA is 3.8 kb and contains a putative open reading frame capable of encoding a 63.6K, 568-residue protein. The deduced amino-acid sequence shows limited homology to *Drosophila* Mad[6], a protein that is required for intracellular transduction of the Dpp signal (Fig. 2). A family of structurally related proteins, termed SMAD proteins, has been identified in *Caenorhabditis elegans*[13] and in vertebrates[14]. It has been postulated that these proteins are phosphorylated in response to receptor activation, after which they translocate to the nucleus and function as transcription factors. SMAD proteins share conserved amino- and carboxy-terminal domains separated by a variable proline-rich region. Although Dad shares significant homology with other SMAD family members within the carboxy-terminal domain, the

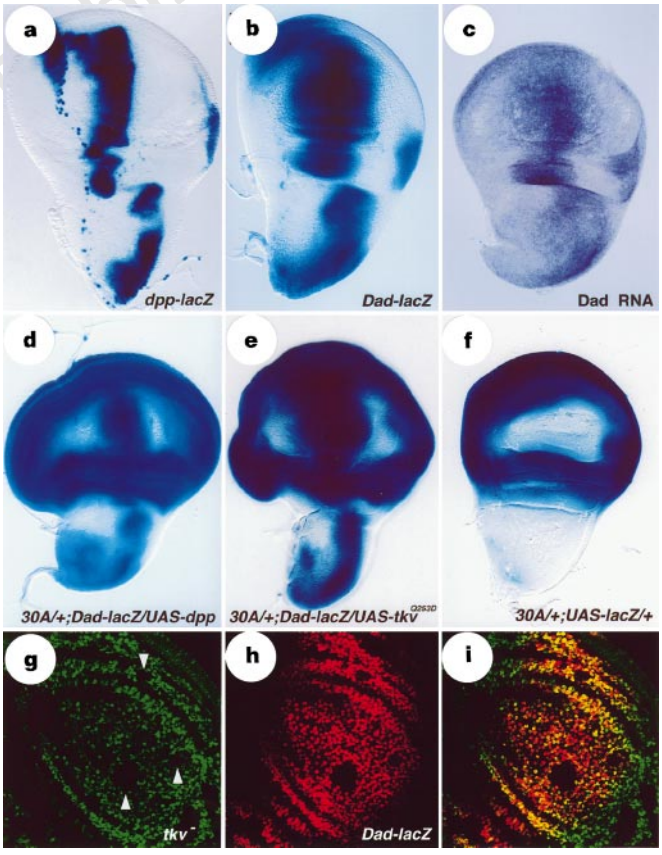
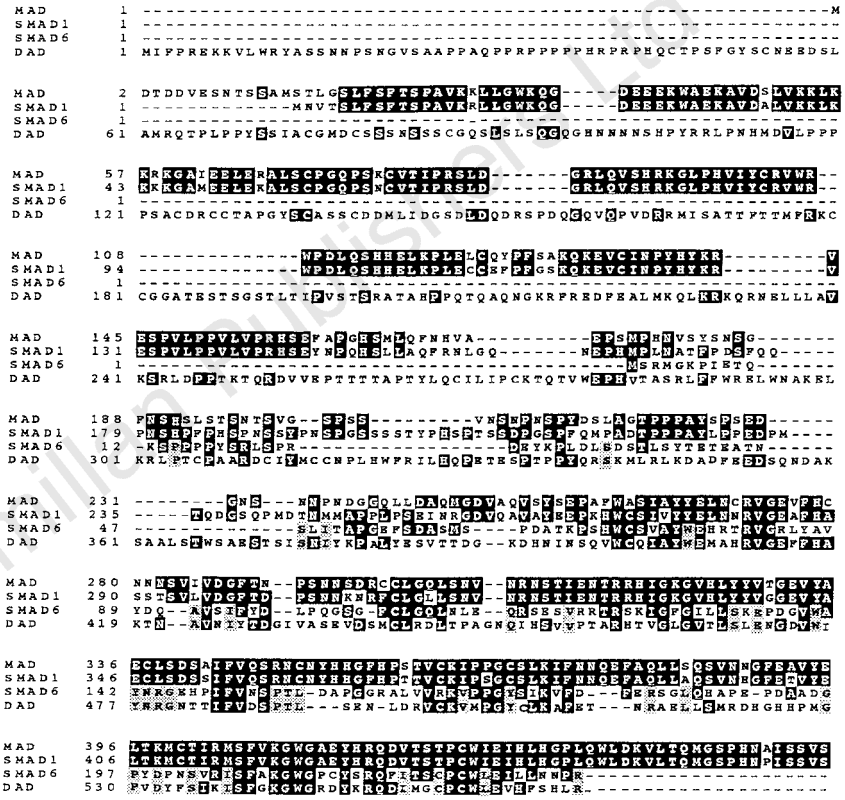


Figure 1 Dad expression is positively controlled by Dpp in the wing imaginal disc. **a**–**c**, Wild-type expression pattern of Dpp revealed by the enhancer trap P1552[31] (**a**) and Dad revealed by the enhancer trap P1883 (**b**) or by *in situ* hybridization (**c**) with a digoxigenine-labelled genomic fragment. The stripe of Dad-expressing cells is broader than that of Dpp. **d**–**f**, Dad expression, as monitored by staining for β-galactodidase activity in P1883, changes correspondingly when Dpp (**d**) or the activated Dpp receptor, Tkv^{Q253D} (**e**) is ectopically expressed under the control of the Gal4 driver 30A. **f**, *UAS-lacZ* expression in strain 30A. **g**–**i**, Dad expression requires Dpp signalling. Clones of cells mutant for *tkv*, identified by the absence of Myc antigen (**g**, green) did not express Dad (**h**) which was monitored by immunostaining against β-galactosidase (red) in P1883. **i**, Superimposed images of **g** and **h**. Mutant clones are marked by arrowheads. All discs shown in this and subsequent figures are wing discs of third instar larvae. Posterior is to the right and ventral is up.

amino-terminal domain is less well conserved. The carboxy-terminal domain of Dad shares the highest homology with human Smad6 (ref. 15), which lacks an amino-terminal homology domain (Fig. 2).

To analyse its function, we ectopically expressed Dad using the Gal4–UAS system and assayed for patterning defects in the wing. When Dad was misexpressed along the wing margin, the wing lost its margin and, in extreme cases, only a tiny winglet formed (Fig. 3b). We obtained similar results with four independent lines and with several different drivers, although the degree of the phenotypic defects varied from line to line (data not shown). Because *dpp* is required not only for pattern formation, but also for cell proliferation, clones of cells that have lost Dpp responsiveness as a result of mutation of genes encoding *tkv* or *punt* (a type II Dpp receptor) do not survive in the wing blade[16]. The similarity in wing phenotype caused by loss of Dpp responsiveness and that caused by ectopic expression of Dad suggest that Dad antagonizes the Dpp signalling pathway. In contrast, conditions that mimicked the effects of hyperactivating the Dpp signalling pathway, such as overexpressing Tkv^{Q253D} or Mad, cause outgrowth of wing tissue (Fig. 3c, d). When Dad was expressed together with either Tkv^{Q253D} or Mad, phenotypic defects caused by overexpression of each protein alone were

Figure 2 Deduced amino-acid sequence of Dad and alignment with *Drosophila* Mad[6], human Smad1 (ref. 32) and Smad6 (ref. 15). Residues identical in more than two sequences are indicated with a black background and residues conserved only between Dad and Smad6 are shaded.



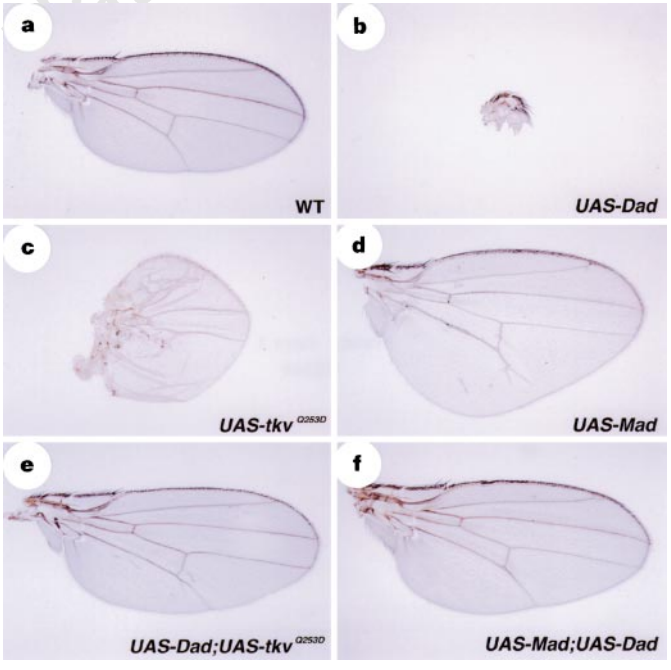


Figure 3 Functional interactions between *Dad*, *tkv*, and *Mad* in adult wings. **a**, Wild type. **b**, Small winglet produced when Dad was expressed ectopically under the control of *vg-Gal4* along the prospective wing margin. **c**, General overproliferation after expression of Tkv^{Q253D} under the control of *vg-Gal4*. **d**, Overproliferation of the P compartment after ectopic expression of Mad under the control of *vg-Gal4*. **e**, Adult wings appeared normal when both Dad and Tkv^{Q253D} were overexpressed under the control of *vg-Gal4*. **f**, Phenotypic rescue after ectopic expression of both Dad and Mad under the control of *vg-Gal4*. Phenotype was variable, from nearly wild type (**f**) to resembling shapes characteristic of the phenotype of overexpression of Mad alone, as in (**d**).

nullified and nearly wild-type wings formed (Fig. 3e, f).

We then examined the effects of ectopic expression of Dad and Mad on a Dpp target gene, *optomotor-blind* (*omb*)[17] which is positively regulated by *dpp* in wing discs. Omb is expressed in a broad stripe straddling Dpp expressing cells, as is Dad, but unlike Dad, it is not expressed along the entire A/P boundary. Clones of cells that expressed Dad or Mad, or both, had effects that were cell autonomous. Omb expression was absent in Dad-overexpressing cells (Fig. 4a–c). When Mad-overexpressing clones fell inside, or near the normal Omb-expression domain, higher level, or ectopic expression of Omb was observed, respectively. In contrast, when Mad-overexpressing cells were situated distal to the endogenous Omb-expression domain, ectopic expression of Omb was not detected (Fig. 4d–f). Thus, overexpressed Mad may be dependent on Dpp signalling for activation whereas elevated levels of Mad may lower the threshold concentration of Dpp required to induce Omb expression. When both Dad and Mad were overexpressed in the same cells, Omb expression was barely affected (Fig. 4g–i). The observation that overexpression of Dad did not reduce expression of endogenous Dpp (data not shown), together with the fact that patterning defects induced by overexpression of Dad were rescued by overexpression of Mad or Tkv^{Q253D}, suggest that antagonism of the Dpp signal by Dad occurs after reception of the Dpp signal at the cell surface and before control of transcription of target genes.

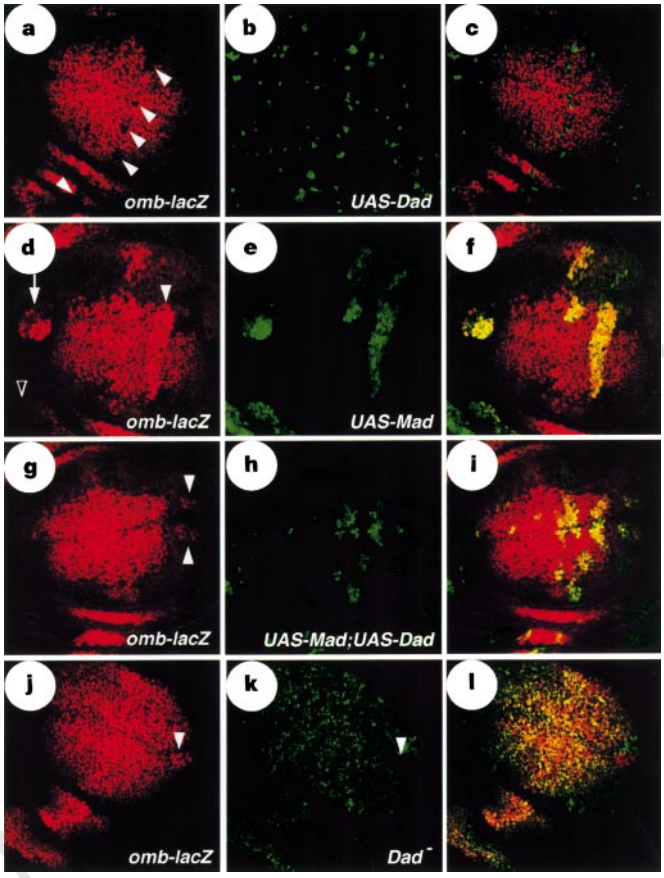


Figure 4 Dad and Mad effects on Omb expression. Clones of cells that overexpressed Dad or Mad or both under the control of *actin-Gal4* were generated by the flp-out technique. Omb expression was revealed by antibody staining against β-galactosidase in wing discs of the omb^{P1} enhancer strain. Omb expression (**a**, red) was lost in clones of cells that ectopically expressed high levels of Dad (revealed by the presence of GFP, green, **b**). **c**, Superimposed images of **a** and **b**. **d**, *Mad*-expressing clones within (arrowhead) or near (arrow) the normal Omb-expressing domain express higher than normal levels of Omb (red). However, Mad-expressing cells distal to the normal Omb-expressing domain, did not express Omb ectopically (**d**, open arrowhead). Mad-expressing cells were marked by the presence of GFP (**e**, green). **f**, Superimposed images of **d** and **e**. **g**, Clones of cells that overexpressed both Dad and Mad did not detectably alter omb expression. Some clones near the normal omb-expressing domain (arrowhead) variably expressed low levels of Omb. Cells expressing both Dad and Mad were marked by the presence of GFP (**h**, green). **i**, Superimposed images of **g** and **h**. **j**–**l**, Clones of cells mutant for *Dad* ectopically express Omb. Omb (**j**, red) was ectopically expressed in clones of cells mutant for *Dad* (arrowhead), identified by the absence of Myc antigen (**k**, green). **l**, Superimposed images of **j** and **k**. Mutant clones are marked by an arrowhead. Clones were generated 48 to 72 h (**d**–**l**) or 72 to 96 h (**a**–**c**) after egg-laying and Omb expression was observed 24 h (**a**–**c**) or 48 h (**d**–**l**) later.

To confirm that Dad represses Omb expression, we analysed somatic *Dad* mutant clones. *Dad* mutants were induced by excising the P element of the 1(3)1E4 enhancer trap line; one of these, $Dad^{271\text{-}68}$, is associated with a deletion of the entire C-terminal domain after amino acid 391. Omb was derepressed autonomously in $Dad^{271\text{-}68}$ clones in wing discs (Fig. 4j–l), indicating that Dad normally represses Omb expression. We infer that Dad negatively modulates the level of Dpp signalling, at least during wing development.

Components of the Dpp signalling pathway are highly conserved between arthropods and chordates[7], so we investigated whether Dad can antagonize signalling by BMP-4, a vertebrate homologue of Dpp. In embryos of *Xenopus laevis*, BMP-4 functions to specify ventral mesodermal and epidermal fates. Blockage of BMP signalling during gastrulation induces the formation of secondary dorsal axes in intact embryos and neuralizes the fate of explanted ectodermal cells[7]. As shown in Fig. 5a–d, similar patterning defects were observed following ectopic expression of Dad in *Xenopus* embryos. Microinjection of Dad mRNA into dorsal blastomeres of 4-cell embryos produced no detectable patterning defects (Fig. 5b), whereas injection into ventral cells induced formation of a secondary axis in 90% of embryos (Fig. 5c). The induced axes contained muscle (Fig. 5c, middle panel) and neural tissue, but not notochord (Fig. 5c, right). In some cases, a cyclopic eye differentiated in the secondary axis (Fig. 5c, right), although the frequency of eye induction varied, ranging from 3–38% in different experiments. Ectodermal explants (animal caps) from Dad-injected embryos elongated and formed darkly pigmented cement glands (Fig. 5d, arrowheads in lower left panel), whereas explants from control embryos retained a rounded epidermal appearance. Dad-injected explants expressed cement gland- (*XAG*) and neural-specific (N-CAM, *OtxA*) genes but not a dorsal mesodermal gene, α-actin or a panmesodermal gene, *Xbra* (Fig. 5d, right), indicating that Dad can directly mediate neural induction in the absence of mesoderm. To test whether Dad can antagonize BMP function, we co-injected BMP-4 and Dad mRNAs into dorsal blastomeres of 4 cell embryos (Fig. 5e). Injection of BMP-4 mRNA alone caused a loss of anterior and dorsal structures, yielding an average dorsoanterior index (DAI; ref. 18) of 1.2 ($n = 30$), whereas co-injection of Dad mRNA almost completely rescued the ventralized phenotype (average DAI = 4.7, $n = 39$). These results suggest that Dad can antagonize BMP signalling in *Xenopus* embryos. Given that components of the Dpp/BMP signalling pathway are highly conserved between insects and vertebrates, it is likely that a homologue of Dad exists that modulates the amplitude or duration of BMP signalling during vertebrate embryogenesis.

Although Dad is a distantly related member of the SMAD family, it is unique among SMADs in antagonizing, rather than transducing, TGF-β-like signals. Oddly enough, Dad appears to participate in a direct negative feedback loop in that it antagonizes the very signalling pathway (that is, Dpp) that is required for induction of its own expression. This relationship between Dpp, which positively regulates the level of expression of Dad, and Dad, which negatively regulates the level of Dpp activity, suggests that the final outcome of Dpp signalling may not be directly proportional to the graded concentration of Dpp protein but may depend on the balance between transduction of Dpp signals by activated Mad, and antag-

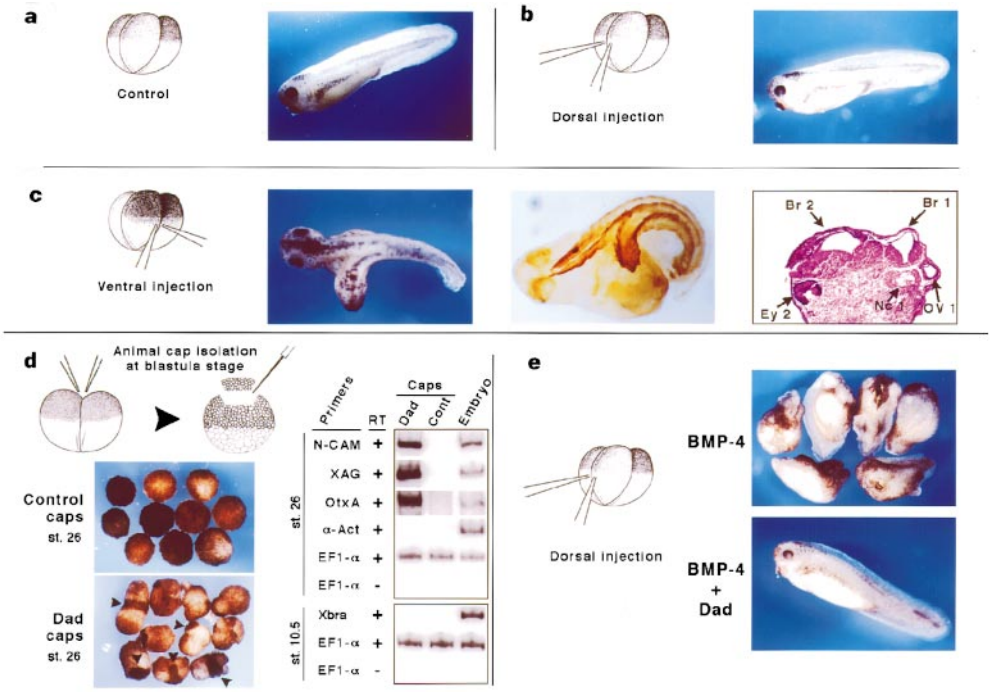


Figure 5 Dad antagonizes BMP-4 activity in *Xenopus* embryos. **a**–**c**, Dad induces a partial secondary axis in *Xenopus* embryos. 200 pg synthetic RNA encoding Dad was injected into two blastomeres near the dorsal or ventral marginal zone of 4 cell embryos as shown schematically. Embryos injected dorsally (**b**) ($n = 69$; 7% showed nonspecific abnormality) are indistinguishable from uninjected sibling embryos (**a**) ($n = 216$; 4% showed nonspecific abnormality) whereas 90% of embryos injected ventrally developed with a partial secondary axis (**c**) ($n = 86$ from 4 independent experiments; 10% showed nonspecific abnormality) in which muscle was detected by immunostaining with 12/101 antibody (middle panel). A transverse section (**c**, right panel) through the anterior end of a twinned embryo shows the presence of brain and eye (Br 2 and Ey 2, respectively) and absence of notochord in the secondary axis, and the presence of brain, otic vesicle and notochord (Br 1, OV 1, Nc 1, respectively) in the primary axis. **d**, Dad directly induces neural tissue in the absence of mesoderm in ectodermal explants (animal caps). Dad mRNA (200 pg) was injected near the animal pole of 2-cell embryos as shown, and animal caps were explanted at the blastula stage and cultured in isolation until sibling embryos reached stage 10.5 or stage 26. Dad-injected caps (Dad caps, lower left panel) form cement glands (arrowheads), whereas control caps (upper left) remain epidermal. Explants were analysed by reverse transcription with the polymerase chain reaction (RT-PCR) for expression of neural- (OtxA, N-CAM), cement gland- (XAG) or mesodermal- (α-actin, Xbra)-specific gene expression. Dad-injected caps expressed neural, but not mesodermal marker genes. **e**, Dad antagonizes BMP-mediated ventralization. Dorsal injection of BMP-4 mRNA (500 pg) leads to a loss of all dorsal and anterior structures; co-injection of Dad mRNA (250 pg) completely rescues the ventralized phenotype.

onism of Dpp signals by Dad. Mechanistically, Dad may interact directly with Mad to modulate Dpp signalling because co-expression of Dad and Mad rescue phenotypic defects induced by either protein alone. Precedence for direct interaction between different members of the SMAD family exists in that the SMADs form multimeric complexes[19]. Alternatively, Dad may antagonize signalling by interacting directly with the receptors. A vertebrate SMAD protein, Smad2, stably associates with receptors and blocks TGFβ-dependent transcriptional responses when its conserved three C-terminal serine residues are substituted with alanine residues[20]. In contrast, wild-type Smad2 transiently associates with receptors and transduces the signal. Dad does not have these C-terminal serines and may stably associate with receptors. A new member of SMADs, Smad7, which does not have these C-terminal serines, was reported to inhibit TGF-β signalling by interacting stably with the receptor[21]. Our data suggest the existence of a Dad negative feedback circuit that might stabilize the gradient of positional information emanating from Dpp expressing cells. □

Methods

Cloning of the *Dad* gene. Genomic DNA adjacent to the P-element transposon in strain 1(3)1E4 was isolated by plasmid rescue. A 3.7-kb fragment of the rescued plasmid was used to isolate genomic clones from a λ phage library (gift from Y.-N. Jan). Genomic fragments containing exons were identified by northern blotting and fragments that hybridized to RNA species with a distribution identical to the expression pattern of β-galactosidase in 1(3)1E4 and P1883 were used to screen a 4–8-h embryonic cDNA library from N. Brown. Three independent cDNA clones were sequenced on both strands using an automated sequencer.

Ectopic expression. Transgenes and the enhancer trap line used were *UAS-dpp* and *vestigal-Gal4* (gift from S. Morimura), *UAS-tkv*^{Q253D} (ref. 5), *UAS-Mad*[22], and *omb*^{P1} (ref. 17). *UAS-Dad* was constructed with a 3.5 kb fragment containing the entire coding region inserted into pUAST[12]. Discs from the larvae of the genotype *30A/+*; *P1883/UAS-dpp* (Fig. 1d), *30A/+*; *P1883/UAS-tkv*^{Q253D} (Fig. 1e) and *30A/+*; *UAS-lacZ/+* (Fig. 1f) were stained for β-galactosidase activity. Wings from the adults of the genotype *vg-Gal4/UAS-Dad* (Fig. 3b), *vg-Gal4/+*; *UAS-tkv*^{Q253D}*/+* (Fig. 3c), *UAS-Mad/+*; *vg-Gal4/+* (Fig. 3d), *vg-Gal4/UAS-Dad*; *UAS-tkv*^{Q253D}*/+* (Fig. 3e) and *UAS-Mad/+*; *vg-Gal4/UAS-Dad* (Fig. 3f) were examined. Dad- or Mad-expressing clones (Fig. 4) were made by combination of a Flp-out technique and Gal4/UAS system[23]. Briefly, excision of Flp-out cassette by Flp from AyGal4 places the Gal4 coding sequence just downstream to the actin 5C promoter which had been separated by the Flp-out cassette from the Gal4 coding sequence. Discs from larvae of the genotype *y w hsFlp omb*^{P1}*/y w*; *AyGal4 UAS-GFP/UAS-Dad* (Fig. 4a–c), *y w hsFlp omb*^{P1}*/y wUAS-Mad*; *AyGal4 UAS-GFP/+* (Fig. 4d–f), and *y w hsFlp omb*^{P1}*/ y wUAS-Mad*; *AyGal4 UAS-GFP/UAS-Dad* (Fig. 4g–i) were immunostained for lacZ expression. The Gal4-expressing clones also expressed UAS-GFP.

Clonal analysis. Clones of cells mutant for *tkv* were made with the Flp-FRT technique. Discs from *y w hsFlp/+*; *tkv*^{a12} *FRT40/πMyc FRT40*; *P1883/+* larvae were immunostained for β-galactosidase and Myc protein. *tkv*^{a12} *FRT40* was a gift from K. Basler. Clones of cells mutant for *Dad* were made by γ-ray irradiation (1,350 rads from a ^{137}Cs source). Discs from *yw omb*^{P1}*/+*; *FRT82πMyc/Dad*$^{271\text{-}68}$ larvae were immunostained for β-galactosidase and Myc proteins.

***Xenopus* embryo culture and manipulation.** *Xenopus* eggs were obtained and embryos microinjected and cultured as described[24]. Histological analysis,

immunostaining of whole embryos (12/101 antibody was obtained from the Developmental Studies Hybridoma Bank, NICHD), and RT-PCR analysis of RNA extracted from cultured animal caps was done as described[25] except that 14-μm sections were cut and PCR cycles were as follows: 95 °C for 5 min followed by a variable number of cycles (determined empirically for each primer pair) at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. Control reactions in which reverse transcriptase was omitted were run in parallel for all samples. The sequences of *EF1-α*, *N-CAM*[26]; α-actin[27]; *OtxA*[28]; *XAG1* (ref. 29); *Xbra*[30] primers have been reported previously.

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1. Hogan, B. L. M. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10,** 1580–1594 (1996).
2. Padgett, R. W., St Johnston, R. D. & Gelbart, W. M. A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-β family. *Nature* **325,** 81–84 (1987).
3. Ferguson, L. F. & Anderson, K. V. Decapentaplegic acts as a morphogen to organize dorsal–ventral pattern in the *Drosophila* embryo. *Cell* **71,** 451–461 (1992).
4. Lecuit, T. *et al.* Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing. *Nature* **381,** 387–393 (1996).
5. Nellen, D., Burke, R., Struhl, G. & Basler, K. Direct and long-range action of a DPP morphogen gradient. *Cell* **85,** 357–368 (1996).
6. Sekelsky, J., Newfeld, S., Raftery, L., Chartoff, E. & Gelbart, W. Genetic characterization and cloning of *Mothers against dpp*, a gene required for *decapentaplegic* function in *Drosophila melanogaster*. *Genetics* **139,** 1347–1358 (1995).
7. Ferguson, E. L. Conservation of dorsal–ventral patterning in arthropods and chordates. *Curr. Opin. Genet. Dev.* **6,** 424–431 (1996).
8. Zecca, M., Basler, K. & Struhl, G. Sequential organizing activities of *engrailed*, *hedgehog* and *decapentaplegic* in the *Drosophila* wing. *Development* **121,** 2265–2278 (1995).
9. Tabata, T., Schwartz, C., Gustavson, E., Ali, Z. & Kornberg, T. B. Creating a *Drosophila* wing *de novo*, the role of *engrailed*, and the compartment border hypothesis. *Development* **121,** 3359–3369 (1995).
10. Lawrence, P. A. & Struhl, G. Morphogens, compartments, and pattern: lessons from *Drosophila*? *Cell* **85,** 951–961 (1996).
11. Capdevila, J. & Guerrero, I. Targeted expression of the signaling molecule *decepentaplegic* induces pattern duplications and growth alterations in *Drosophila* wings. *EMBO J.* **13,** 4459–4468 (1994).
12. Brand, A. H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118,** 401–415 (1993).
13. Savage, C. *et al. Caenorhabditis elegans* genes *sma-2*, *sma-3* and *sma-4* define a conserved family of transforming growth factor-β pathway components. *Proc. Natl Acad. Sci. USA* **93,** 790–794 (1996).
14. Massague, J., Hata, A. & Fang, L. TGF-β signalling through the Smad pathway. *Trends Cell Biol.* **7,** 187–192 (1997).
15. Riggins, G. J. *et al.* Mad-related genes in the human. *Nature Genet.* **13,** 347–349 (1996).
16. Burke, R. & Basler, K. Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing. *Development* **122,** 2261–2269 (1996).
17. Grimm, S. & Pflugfelder, G. O. Control of the gene optomotor-blind in *Drosophila* wing development by *decapentaplegic* and *wingless*. *Science* **271,** 1601–1604 (1996).
18. Kao, K. R. & Elinson, R. P. Dorsalization of mesoderm induction by lithium. *Dev. Biol.* **132,** 81–90 (1989).
19. Lagna, G., Hata, A., Hemmati-Brivanlou, A. & Massagé, J. Partnership between DPC4 and SMAD in TGF-β-signalling pathways. *Nature* **383,** 832–836 (1996).
20. Macias-Silva, M. *et al.* MADR2 is a substrate of the TGFβ receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* **87,** 1215–1224 (1997).
21. Hayashi, H. *et al.* The Mad-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. *Cell* **89,** 1165–1173 (1997).
22. Newfeld, S. J., Chartoff, E. H., Graff, J. M., Melton, D. A. & Gelbart, W. M. *Mothers against dpp* encodes a conserved cytoplasmic protein required in DPP/TGF-β responsive cells. *Development* **122,** 2099–2108 (1996).
23. Ito, K., Awano, W., Suzuki, K., Hiromi, Y. & Yamamoto, D. The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development* **124,** 761–771 (1997).
24. Moon, R. T. & Christian, J. L. Microinjection and expression of synthetic mRNAs in *Xenopus* embryos. *Technique* **1,** 76–89 (1989).
25. Cui, Y., Tian, Q. & Christian, J. L. Synergistic effects of Vg1 and Wnt signals in the specification of dorsal mesoderm and endoderm. *Dev. Biol.* **180,** 22–34 (1996).
26. Kengaku, M. & Okamoto, H. bFGF as a possible morphogen for the anteroposterior axis of the central nervous system in *Xenopus*. *Development* **121,** 3121–3130 (1995).
27. Hemmati-Brivanlou, A. & Melton, D. A. Inhibition of activin receptor signaling promotes neuralization in *Xenopus*. *Cell* **77,** 273–281 (1994).
28. Lai, C.-J., Ekker, S. C., Beachy, P. A. & Moon, R. T. Patterning of the neural ectoderm of *Xenopus laevis* by the amino-terminal product of hedgehog autoproteolytic cleavage. *Development* **121,** 2349–2360 (1995).
29. Blitz, I. L. & Cho, K. W. Y. Anterior neurectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene *orthodenticle*. *Development* **121,** 993–1004 (1995).
30. Hemmati-Brivanlou, A., Kelly, O. G. & Melton, D. A. Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77,** 283–295 (1994).
31. Tabata, T. & Kornberg, T. B. Hedgehog is a signalling protein with a key role in patterning in *Drosophila*. *Cell* **76,** 89–102 (1994).
32. Liu, F. *et al.* A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature* **381,** 620–623 (1996).

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Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling

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TGF-β signals from the membrane to the nucleus through serine/threonine kinase receptors and their downstream effectors, termed SMAD proteins[1]. The activated TGF-β receptor induces phosphorylation of two such proteins, Smad2 and Smad3 (refs 2–6), which form hetero-oligomeric complex(es) with Smad4/DPC4 (refs 5–10) that translocate to the nucleus[2,4,5,7], where they then regulate transcriptional responses[11,12]. However, the mechanisms by which the intracellular signals of TGF-β are switched off are unclear. Here we report the identification of Smad7, which is related to Smad6 (ref. 13). Transfection of Smad7 blocks responses mediated by TGF-β in mammalian cells, and injection of Smad7 RNA into *Xenopus* embryos blocks activin/TGF-β signalling. Smad7 associates stably with the TGF-β receptor complex, but is not phosphorylated upon TGF-β stimulation. TGFβ-mediated phosphorylation of Smad2 and Smad3 is inhibited by Smad7, indicating that the antagonistic effect of Smad7 is exerted at this important regulatory step. TGF-β rapidly induces expression of Smad7 mRNA, suggesting that Smad7 may participate in a negative feedback loop to control TGF-β responses.

Smad7 was identified as an expressed sequence tag (EST) related to known SMAD proteins. Sequence analysis of isolated mouse and human cDNAs predict that mouse Smad7 and human Smad7 have 426 amino-acid residues with 98% identity (Fig. 1a). Smad7 is most related to Smad6 (ref. 13), with 36% and 56% sequence identities in the amino-terminal domain and the carboxy-terminal Mad homology (MH)2 domain, respectively. The Smad7 amino-terminal domain shows only weak similarity to the MH1 domains found in Smad1 to Smad5. RNA blot analysis with a Smad7 probe revealed one main transcript of approximately 4.4 kilobases (Fig. 1b). Among the tissues analysed, the highest expression of *Smad7* was found in the lung.

In order to investigate whether Smad7 modulates the responsiveness to TGF-β, we transfected the TGFβ-inducible p3TPLux luciferase reporter construct, which contains the PAI-1 promoter, into Mv1Lu mink epithelial cells in the absence or presence of Smad7 cDNA. Smad7 was found to inhibit TGFβ1-induced luciferase activity (Fig. 2a). Moreover, the induction of p3TPLux response by constitutively active variants of the TGF-β receptor TβR-I and a structurally related type IB receptor for activin (ActR-IB), when transfected in R-mutant cells, was also inhibited by co-transfection with Smad7 (Fig. 2b, and data not shown). Thus our results indicate that Smad7 is a potent negative regulator of p3TPLux response induced by both TβR-I and ActR-IB. In addition, Smad7, but not