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The *smad5* mutation *somitabun* blocks Bmp2b signaling during early dorsoventral patterning of the zebrafish embryo

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SUMMARY

Signaling by members of the TGFβ superfamily is thought to be transduced by Smad proteins. Here, we describe a zebrafish mutant in *smad5*, designated *somitabun* (*sbn*). The dominant maternal and zygotic effect of the *sbn*^{tc24} mutation is caused by a change in a single amino acid in the L3 loop of Smad5 protein which transforms Smad5 into an antimorphic version, inhibiting wild-type Smad5 and related Smad proteins. *sbn* mutant embryos are strongly dorsalized, similarly to mutants in Bmp2b, its putative upstream signal. Double mutant analyses and RNA injection experiments show that *sbn* and *bmp2b* interact and that *sbn* acts downstream of Bmp2b signaling to mediate Bmp2b autoregulation during early dorsoventral (D-V) pattern formation. Comparison of early marker gene expression patterns, chimera analyses and rescue experiments involving temporally controlled misexpression of *bmp* or *smad* in mutant embryos reveal three phases of D-V patterning: an early *sbn*- and *bmp2b*-independent phase when a coarse initial D-V pattern is set up, an intermediate *sbn*- and *bmp2b*-dependent phase during which the putative morphogenetic Bmp2/4 gradient is established, and a later *sbn*-independent phase during gastrulation when the Bmp2/4 gradient is interpreted and cell fates are specified.

Key words: Smad5, Bmp2/4 signaling, Dorsoventral patterning, Zebrafish

INTRODUCTION

In amphibia, early D-V patterning of the embryo is a multi-step process governed by maternally and zygotically supplied signaling proteins. Under maternal control, a coarse D-V pattern is set up in the developing mesoderm, consisting of ventral mesoderm spanning most of the marginal region of the blastula, and dorsal mesoderm in a rather small dorsal marginal domain. This initial pattern is subsequently refined by zygotic ventralizing and dorsalizing signals, leading to the specification of intermediate fates. Several signaling proteins have been identified which might be involved in this refinement process: the Bone Morphogenetic Proteins 2 and/or 4 (Bmp2/4), members of the TGFβ superfamily of growth factors, which have ventralizing activities, and their antagonists Chordin, Noggin and Follistatin (for reviews, see Thomsen, 1997; Heasman, 1997). Recent evidence indicates that Bmp2/4 function as instructive morphogens which determine positional identities along the entire D-V axis in a dose-dependent fashion (Dosch et al., 1997), while Chordin, Noggin and Follistatin attenuate this ventralizing activity on the dorsal side of the embryo by physical interaction with Bmp proteins (Piccolo et al., 1996; Zimmerman and Harland, 1996), and thereby establish the putative morphogenetic D-V gradient of Bmp2/4 activity.

Genetic evidence revealing the requirement of Bmp2b and its antagonist Chordin during D-V patterning has recently been obtained in the zebrafish. In large-scale mutant screens at least eight complementation groups have been identified defining eight genes required for dorsoventral development (Mullins et al., 1996; Hammerschmidt et al., 1996a). The phenotype of the strongest of the dorsalized mutants, *swirl*, is caused by null mutations in *bmp2b*, one of the two *bmp2* genes known in zebrafish (Martínez-Barberá et al., 1997; Kishimoto et al., 1997; Nguyen et al., 1998), while the ventralization of *dino* mutants is due to a null mutation in the zebrafish *chordin* gene, therefore designated *chordino* (Schulte-Merker et al., 1997).

In the fruitfly *Drosophila melanogaster*, embryonic D-V patterning is also regulated by structural homologues of Chordin and Bmp2/4, called Short gastrulation (Sog) and Decapentaplegic (Dpp) respectively. Screens for dominant maternal enhancers of *dpp* identified two genes, *mad* (*mothers against decapentaplegic*) and *medea*, which enhance the D-V phenotype of *dpp* mutants (Raftery et al., 1995). *mad* is also

required for later organogenic processes acting downstream of the Dpp receptors to mediate Dpp signaling in target cells (Newfeld et al., 1997; Wiersdorff et al., 1996).

Similar functions as transducers of TGFβ signaling were demonstrated for the Mad-related Sma proteins in the nematode *C. elegans* and Smad proteins in vertebrates (for reviews, see Kretschmar and Massagué, 1998; Attisano and Wrana, 1998). In vertebrates, two Smad proteins, Smad1 and Smad5, show particularly high homology to *Drosophila* Mad. Together with Smad8, they are involved in the mediation of ventralizing signaling by Bmp2/4, the vertebrate homologues of Dpp (Graff et al., 1996; Thomsen, 1996; Liu et al., 1996; Hoodless et al., 1996; Suzuki et al., 1997), while the more distantly related Smad2 and Smad3 proteins mediate signaling by other TGFβ proteins. Smad4 is identical to the tumor suppressor DPC4 (Hahn et al., 1996) and shows high homology to the *Drosophila* Medea protein (Hudson et al., 1998; Wisotzkey et al., 1998; Das et al., 1998). It appears to be a common mediator of all TGFβ superfamily members, interacting with both Smad1- and Smad2-type proteins (Lagna et al., 1996; Zhang et al., 1997). Biochemical evidence indicates that Smad1 and Smad2 proteins form homo-trimers which are recruited and phosphorylated at their C terminus by ligand-activated Bmp- and TGFβ-receptors, respectively. After phosphorylation, these 'receptor-activated' Smad trimers associate with Smad4 trimers, translocate to the nucleus and participate in transcriptional complexes (for reviews, see Kretschmar and Massagué, 1998; Attisano and Wrana, 1998). While the 'receptor-activated' Smads are all positive transducers of TGFβ signaling, two other, more distantly related Smad proteins, Smad6 and Smad7, antagonize TGFβ signaling (reviewed by Whitman, 1997).

The requirement of both Smad2 and Smad4 during early development and mesoderm formation has been recently demonstrated using gene targeting in the mouse (Sirard et al., 1998; Waldrip et al., 1998; Nomura and Li, 1998). Here, we describe a zebrafish mutant in *smad5*, a transducer of Bmp2/4 signaling which is active during early steps of embryonic D-V patterning.

MATERIALS AND METHODS

Cloning of zebrafish *smad5*

Polymerase chain reaction was carried out using a zebrafish gastrula cDNA library (kind gift from David Grunwald) as template and previously reported degenerated primers (Graff et al., 1996) to amplify a 180 bp zebrafish *smad5* cDNA fragment which was used to screen the gastrula cDNA library at high stringency. For further experiments we used a 2.6 kb clone with 365 bp of 5′ UTR, 1395 bp of coding region, giving rise to a predicted protein of 465 aa, and approx. 850 bp of 3′ UTR, including a polyadenylation site and a polyA tail. An in-frame TAA 23 triplets upstream of the start codon indicates that the coding region is complete (sequence not shown, accession number AF127920).

Mapping of *smad5* and *sbn^{tc24}*

smad5 was mapped in F_2 offspring of a Tue × WIK reference cross as described by Rauch et al. (1997), using a *smad5* RFLP (restriction fragment length polymorphism) identified between the P_0 parents of the reference cross.

The *sbn* mutation was mapped via its dominant zygotic effect which was fully penetrant, causing a mild dorsalization. A male *sbn* carrier of the Tue background was mated to a WIK wild-type female and heterozygous F_1 males were crossed to TL wild-type females (Haffter et al., 1996). The SSLP markers linked to *sbn* were indistinguishable in TL and WIK, but different in the Tue (*sbn*) line. Thus, recombinants between the SSLP markers and the *sbn* mutation were scored among the phenotypically wild-type F_2 siblings as heterozygous for the SSLP markers. In addition, a direct linkage analysis of *smad5* and *sbn* was carried out using a tetra-PCR approach as described below.

Amplification and sequencing of the *sbn^{tc24} smad5* mutant allele

RT-PCR with total RNA isolated from the offspring of two *sbn^{tc24}* carriers was performed to amplify three partially overlapping fragments spanning the complete *smad5* open reading frame. The resulting fragments were cloned using a TA cloning kit (Invitrogen); 14 independent clones of each fragment were sequenced, and 7/14 of the clones with the 3′ fragment showed a single missense mutation (ACA→ATA).

Genotyping of *smad5 sbn^{tc24}* and *bmp2b swr^{ta72}*

DNA from single embryos or amputated tail fins of adult fish was prepared according to the method of Westerfield (1994). Genotyping was performed by allele specific tetra-PCR (Ye et al., 1992), identifying wild-type, heterozygous or homozygous mutant genotypes in a single PCR reaction with four primers. Two reverse primers containing the nucleotide of the wild-type or mutant cDNA sequence at their 3′ end were combined with outer primers to give rise to wild-type and mutant-specific DNA fragments of different sizes.

For *sbn^{tc24} smad5*, we used: 94°C (3 minutes), 10 cycles of 94°C (30 seconds), 65°C (45 seconds), 72°C (1 minute), 25 cycles of 94°C (30 seconds), 48°C (45 seconds), 72°C (1 minute), followed by 72°C (10 minutes). Outer sense primer (GCAACTACCATCATGGCTTTC), outer antisense primer (AAAACAAAGGCTTCCTCCCAGG), wild-type sense primer (ACAGACAAGATGTGAC), mutant antisense primer (AGCAGGGGGTGCTTA). For the *bmp2b swr^{ta72}* mutation we used: 94°C (3 minutes), 10 cycles of 94°C (30 seconds), 57.5°C (45 seconds), 72°C (1 minute), 20 cycles of 94°C (30 seconds), 48°C (45 seconds), 72°C (1 minute), followed by 72°C (10 minutes). Outer sense primer (GCTATCATGCTTTCTACTG), outer antisense primer (GTTTTGTTCATTCAACATAAAT), wild-type sense primer (TGT-GGGTGCCGATGA), mutant antisense primer (TTGGGGA-GATTGTTCC).

Northern blotting

Total RNA from staged embryos (Westerfield, 1994) was isolated using Trizol LS reagent (Gibco/BRL). Northern blot analysis was carried out (Bauer et al., 1998) using randomly labeled *smad5* or *smad1* cDNA as probe.

Expression constructs, RNA synthesis and injection into embryos

For the transcription construct p64TS-smad5, the zebrafish wild-type *smad5* coding region containing an upstream Kozak sequence (ACC) was amplified via PCR and cloned into pSP64TS (Krieg and Melton, 1984). To generate p64TS-smad5(sbn), a 325 bp *Bgl*II/*Spe*I fragment of *smad5* cDNA from *sbn^{tc24}* mutant embryos which carries the C→T substitution was used to replace the corresponding fragment of p64TS-WTsmad5. pCS2-smad1(sbn) and pCS2-smad2(sbn) encoding zebrafish Smad1 and zebrafish Smad2 with the *sbn*-specific Thr→Ile mutation were generated via a PCR-based site-directed mutagenesis strategy essentially as described by Ho et al. (1989). To generate pCS2-hsmad4, a *Hin*dIII (blunt-ended)-*Eco*RI fragment of human *SMAD4* (Zhang et al., 1996) was cloned into *Stu*I-*Eco*RI digested pCS2+ (Rupp et al., 1994).

For the expression constructs pXeX-smad5, pCSKA-smad5, pXeX-smad5(sbn) and pCSKA-smad5(sbn), the coding region of wild-type

and mutant *smad5* was amplified and cloned into the *Bam*HI site of pXeX (Johnson and Krieg, 1994) or pCSKA (Harland and Misher, 1988).

For synthesis of capped mRNA, all p64TS constructs were linearized with *Sma*I and all pCS2 constructs with *Not*I, followed by in vitro transcription with SP6 RNA polymerase using the Ambion Message Machine kit. Injections of mRNA and plasmid DNA were carried out as described by Hammerschmidt et al. (1998).

For *Xenopus* experiments, synthetic wild-type *smad5*, wild-type *smad1*, *smad5*(*sbn*) and/or *smad1(sbn)* RNA was injected cell-by-cell into all four animal blastomeres at the 8-cell stage. At stage 9, animal caps were explanted and cultured until sibling embryos reached stage 11.5. Intact caps were harvested and processed for RT-PCR analysis as previously described (Bouwmeester et al., 1996).

Cell transplantation, in situ hybridization, photography

The following zebrafish strains were used: as wild type, TL (Haffter et al., 1996) and Ekkwill (Knapik et al., 1998), and for mutant analyses, the *bmp2b* allele *swirl* (swr^{ta72}) and the *smad5* allele *somitabun* (sbn^{tc24}; Mullins et al., 1996). Cell transplantations were carried out essentially as described by Ho and Kane (1990). Whole-mount in situ hybridization with digoxigenin-labeled RNA probes and photography were according to Hammerschmidt et al. (1996a).

RESULTS

The zebrafish *smad5* cDNA

A full length zebrafish cDNA, designated zebrafish *smad5*, was cloned. Its deduced amino acid sequence shows 91.6 % and 91.4% identity to human and mouse Smad5, 87.9%, 87.4% and 87.0% identity to human, mouse and *Xenopus* Smad1, respectively, and 77.5% identity to rat Smad8, indicating that it is the Smad5 orthologue.

Genetic linkage of *smad5* and the dorsalizing mutation *somitabun* sbn^{tc24}

To investigate whether the phenotype of any of the isolated zebrafish mutants was caused by mutations in the *smad5* gene, the *smad5* cDNA and mutations causing D-V phenotypes were mapped relative to SSLP markers. Mapping with a *Sty*I RFLP in 75 F_2 individuals of the Tue x WIK reference cross positioned zebrafish *smad5* into linkage group 14 (Knapik et al., 1998), 15.9 cM from the SSLP marker z4592 (LOD 10.2), 15.7 cM from z4291 (LOD 10.4) and 2.3 cM from z3290 (LOD 16.0). Of the five investigated dorsalizing mutations (*swirl, somitabun*, *snailhouse*, *lost-a-fin*, *minifin*; Mullins et al., 1996) one, *somitabun* (sbn^{tc24}), mapped to the same region, 5.2 cM from z4592 (LOD 44.6) and 4.4 cM from z4291(LOD 50.3). The genetic distances of *sbn* to the investigated SSLP markers are shorter than for *smad5*. This is expected since the *sbn* mapping strategy only measures male recombination rates, while mixed male and female recombination was used to map *smad5*. The sex-averaged zebrafish map of SSLP markers is reduced in size compared to the zebrafish RAPD map, solely based on female meiosis (Knapik et al., 1998; Johnson et al., 1996).

For a direct linkage analysis between the *sbn* mutation and the *smad5* gene, we designed a tetra-PCR assay that allowed us to distinguish between wild-type and *sbn smad5*, taking advantage of the change of a single nucleotide in the *smad5* coding region of *sbn* mutants (Fig. 1B; see below). Upon genotyping, 50 of 50 embryos showing the zygotic dominant *sbn* phenotype were *smad5* heterozygotes, while 50 of 50 wild-type sibling embryos contained only the *smad5* wild-type allele. In addition, in genotyping experiments of adult males and females, 52 of 52 *sbn* carrier fish were heterozygous for the *smad5* mutation, while 50 of 50 non-carrier silbings were *smad5* wild type. Thus, the *smad5* gene and the mutation causing the zygotic dominant effect of *sbn* are <0.5 cM apart (no recombination in 202 meioses).

somitabun sbn^{tc24} is an antimorph of *smad5*

To investigate the molecular nature of the sbn^{tc24} mutation, *smad5* cDNA was amplified from *sbn* mutant embryos via RT-PCR. Sequencing revealed a single C→T change at nucleotide position 1286 of the *smad5* coding region which leads to a Thr→Ile change at amino acid position 429 in the L3 loop region of the Smad5 MH2 domain. This threonine is conserved in all currently known Smad proteins except the inhibitory *Drosophila* protein Dad (Tsuneizumi et al., 1997) and Smad4, a common transducer of signaling by members of the TGFβ superfamily (Hahn et al., 1996) (Fig. 1A). The L3 loop has been shown to be involved in the interaction of 'receptor-activated' Smad proteins with their respective receptors and with Smad4 (Shi et al., 1997; Lo et al., 1998).

To investigate the strength of the sbn^{tc24} allele of zebrafish *smad5*, misexpression experiments in *Xenopus* animal cap explants were carried out, comparing the ventral mesoderm-inducing potentials of wild-type and sbn^{tc24} Smad5. Animal caps injected with wild-type *smad5* mRNA contained significant amounts of *Xhox3* mRNA (a ventral marker) and *Xbra* RNA (a pan-mesodermal marker). But no such transcripts could be detected by RT-PCR after injection of sbn^{tc24} *smad5* RNA, so its protein has retained very low or no ventralizing activity (Fig. 1C). Co-expression experiments to ask whether the *smad5* mutation also accounts for the dominant negative effect of *sbn* (see below) revealed that the ventralizing effect of wild-type *smad5* RNA was inhibited by about 80% by equal amounts of co-injected sbn^{tc24} *smad5* RNA, and 90% by twofold amounts (Fig. 1D). To study whether sbn^{tc24} Smad5 might also interfere with other related Smad proteins, sbn^{tc24} *smad5* mRNA was coinjected with zebrafish wild-type *smad1* RNA. In addition, *smad1(sbn)* mRNA, bearing the Thr→Ile mutation found in sbn^{tc24}, was co-injected with wild-type *smad5*. In both cases, the antimorphic version also inhibited the wild-type paralogue, but more weakly than its own wild-type version (Fig. 1E,F).

Expression of *smad1* and *smad5*

The temporal expression patterns of zebrafish *smad1* and *smad5* in wild-type embryos have been investigated by developmental northern blot analysis. The mRNA profile indicates that *smad5* is expressed both maternally and zygotically. Levels of maternally supplied *smad5* mRNA drop to about 50% of their intial value during cleavage and early blastula stages, rise again at the sphere stage after the onset of zygotic gene expression at midblastula stages (Kane and Kimmel, 1993), and decline thereafter, with a strong reduction during early gastrula stages (between shield stage and 80% epiboly, Fig. 2A). *smad1* shows a different temporal expression profile with no mRNA detectable prior to midgastrulation at cleavage, blastula or early gastrula stages (Fig. 2A). The same

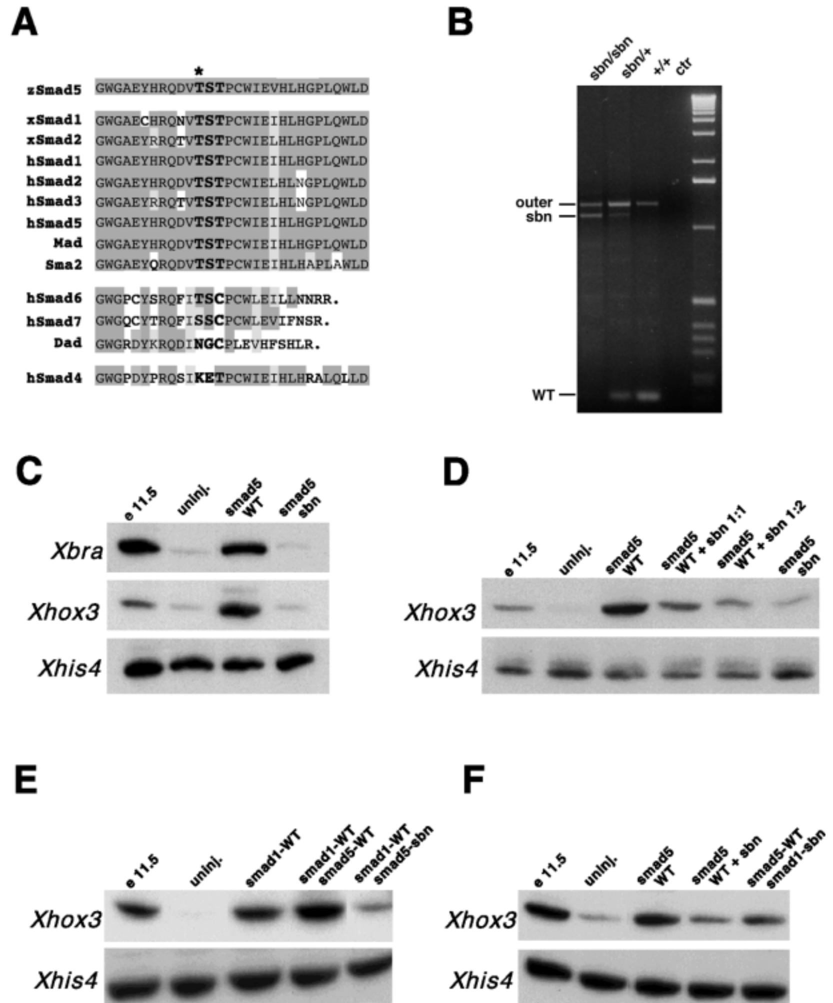

Fig. 1. (A) Sequence of amino acid residues 418-447 (the L3 loop region) of zebrafish Smad5 aligned to the corresponding region of other Smad proteins. Identical and similar amino acids are boxed in dark and light gray, respectively. The motif TST is highlighted in bold. T(429) mutated to I in zebrafish *sbn*^{tc24}Smad5 is indicated with an asterisk. S(430) is mutated in the *Drosophila* Mad allele *mad9* (Sekelsky et al., 1995), and T(431) is not conserved in the inhibitory Smad proteins Smad6, Smad7 and *Drosophila* Dad. (B) Genotyping of rescued adult male fish from a cross of two *sbn* heterozygous parents via tetra-PCR, resulting in an outer *smad5* DNA fragment of 1300 bp, a *sbn*^{tc24} *smad5*-specific fragment of 1150 bp, and a wild-type *smad5*-specific fragment of 158 bp. The 1300 bp and 1150 bp fragments contain a *smad5* intron. Lane 1, *sbn* homozygous mutant; lane 2, *sbn* heterozygote; lane 3, wild type; lane 4, PCR water control; lane 5, DNA size marker (1-kb ladder, Gibco/BRL). Genotypes of the three males were confirmed in crosses to wild-type females. (C) Animal explant assay to monitor the ventralizing activities of wild-type and *sbn* mutant Smad5. *Xbra*, *Xhox3* and *Xhis4* control mRNA levels in *Xenopus* stage 11.5 embryos (e 11.5), uninjected animal caps (uninj.), and animal caps after injection of 600 pg wild-type *smad5* mRNA (smad5-WT) or 600 pg *smad5* RNA bearing the *sbn*^{tc24} point mutation (smad5-sbn), revealed by RT-PCR. (D) *sbn* Smad5 inhibits wild-type Smad5. *Xhox3* and *Xhis4* mRNA levels after injection of 600 pg wild-type *smad5* RNA (smad5-WT), co-injection of 600 pg wild-type *smad5* RNA and 600 pg mutant *smad5(sbn)* RNA (smad5 WT+sbn 1:1), co-injection of 600 pg wild-type *smad5* RNA and 1200 pg mutant *smad5(sbn)* RNA (smad5 WT+sbn 1:2), or injection of 600 pg mutant *smad5(sbn)* RNA (smad5-sbn). (E) *sbn* Smad5 inhibits wild-type Smad1. *Xhox3* and *Xhis4* mRNA levels in animal caps after injection of 600 pg wild-type *smad1* RNA (smad1-WT), co-injection of 600 pg wild-type *smad1* and 1200 pg wild-type *smad5* RNA, or co-injection of 600 pg wild-type *smad1* RNA and 1200 pg mutant *smad5(sbn)* RNA. (F) *sbn* Smad1 inhibits wild-type Smad5. *Xhox3* and *Xhis4* mRNA levels in animal caps after injection of 600 pg wild-type *smad5* RNA (smad5-WT), co-injection of 600 pg wild-type *smad5* RNA and 1200 pg mutant *smad5(sbn)* RNA (smad5 WT+sbn), or co-injection of 600 pg wild-type *smad5* RNA and 1200 mutant *smad1(sbn)* RNA bearing the same point mutation as *sbn*^{tc24} smad5 (smad1-sbn).

temporal *smad1* expression pattern was revealed via developmental RT-PCR analysis (not shown). Studies of the spatial expression pattern of *smad5* by whole-mount in situ hybridizations revealed a uniform and ubiquitous distribution of both maternal and zygotic *smad5* transcripts at cleavage (Fig. 2B), blastula (Fig. 2C) and gastrula stages (Fig. 2D,F).

Phenotypes and genetics of *somitabun sbn*^{tc24}

Consistent with the maternal and zygotic expression of *smad5*, the *somitabun* mutation *sbn*^{tc24} has both a dominant maternal and a dominant zygotic effect, which lead to dorsalizations of different degrees.

Heterozygous *sbn* females crossed to either wild-type or heterozygous males give rise to 100% strongly dorsalized embryos (C4 phenotypes; Mullins et al., 1996, see Table 1 for further explanation of the various phenotypes) characterized by a body axis wound up in a snailshell-like fashion at day one of development (Fig. 3A,B). The strength of dorsalization among the different offspring of two *sbn*^{tc24} heterozygous parents was indistinguishable, whether the embryos were homozygous mutant (Fig. 3G), heterozygous (Fig. 3F), or wild-type (Fig. 3E). We were able to rescue *sbn*^{tc24} homozygous mutant embryos and raise them to adulthood (see below), and thus could also generate offspring from homozygous mutant mothers (Fig. 3H). These showed the same degree of dorsalization as embryos from heterozygous mothers, indicating that the dominant maternal effect of the *sbn* mutation is not enhanced by removing the remaining maternal and zygotic contributions of wild-type *smad5* mRNA.

sbn^{tc24} also displays a dominant zygotic effect as seen in 50% of the offspring of a *sbn* heterozygous male and a wild-type female. This dominant zygotic phenotype is viable and is characterized by loss of the ventral tail fin at day 1 of development (C1; Fig. 3C).

Rescue and phenocopy of the *somitabun* phenotype by wild-type and mutant *smad5*

To further confirm that the dorsalization of *sbn*^{tc24} mutant embryos is caused by the mutation found in the *smad5* gene, *smad5* RNA injections were carried out to rescue the mutant

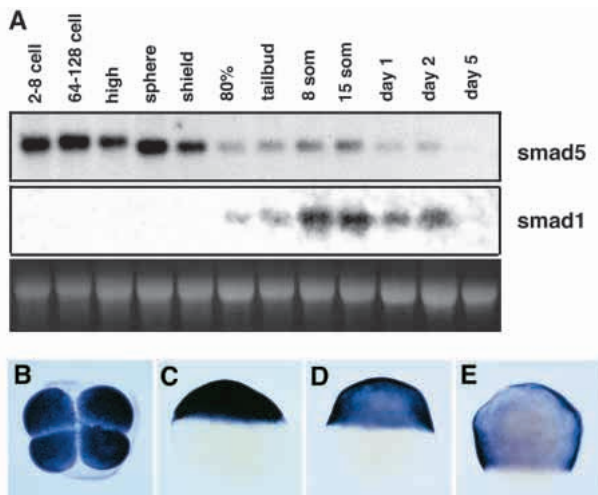

Fig. 2. Expression of *smad5* and *smad1*. (A) Developmental northern analysis with 10 μg of total RNA of the indicated stages, hybridized with a *smad5*- (upper panel) and a *smad1*- (middle panel) specific probe. Lower panel shows ethidium bromide staining of 28S rRNA as loading control. (B-H) Whole-mount in situ hybridization using a *smad5*-specific probe. (B) 4-cell stage, animal view. (C) Sphere stage, lateral view. (D) Shield stage, lateral view, dorsal right. (F) 80% epiboly, lateral view, dorsal right.

phenotype. In addition, mRNA injections of the antimorphic *smad5 (sbn)* allele into wild-type embryos were carried out to phenocopy the *sbn* mutant phenotype.

The dorsalized phenotype of most embryos from a cross of two *sbn* heterozygous parents was rescued in a dose-dependent manner upon injection of wild-type *smad5* mRNA, whereas injection into dorsalized *swr*(*bmp2b*) mutants had no effect (Table 1). A high proportion of the *smad5*-injected *sbn* embryos reached wild-type condition and could be raised to adulthood (Fig. 4A,B; Table 1). For 82 such adult mutants genotyped via tetra-PCR of genomic tail fin DNA, 21/55 males and 13/27 females were wild type (41%), 28/55 males and 8/27 females were *smad5* heterozygous, and 6/55 males and 6/27 females were homozygous mutant (14%) (25% expected wild-type and 25% homozygous mutant fish if rescue efficiency is independent of zygotic genotype). *smad5* homozygous mutant adults of both sexes were fertile, and males gave 100% C1-phenotype embryos when mated to wild-type females.

The *sbn* mutant phenotype could also be rescued, with a weaker frequency, by injecting the closely related zebrafish *smad1* RNA (Table 1); injecting zebrafish *smad2* (A. D. and M. H., unpublished) or *lacZ* mRNA had no effect (Table 1). *sbn* mutants were also rescued by injecting human *smad4* RNA (Fig. 4E,F; Table 1), the putative partner of all 'receptor-activated' Smad proteins.

To phenocopy the *sbn* phenotype, *smad5*(*sbn*) mRNA bearing the mutation found in sbn^{tc24} was injected into wild-type embryos. While 25 pg wild-type *smad5* mRNA had no effect, 25 pg mutant *smad5*(*sbn*) mRNA led to a significant dorsalization up to C4 strength (Fig. 4C,D; Table 1). When the Thr→Ile mutation found in sbn^{tc24} was introduced into other zebrafish *smad* cDNAs, it converted Smad1 into a dorsalizing agent of similar strength to Smad5(*sbn*), whereas mutated *smad2*(*sbn*) RNA injected into wild-type embryos had no effect (Table 1).

The time window of Smad5 action

The weakness of the dominant zygotic effect of sbn^{tc24} compared to its dominant maternal effect suggests that the *smad5* gene product acts at very early stages in development. To appraise the time window of Smad5 action, we carried out both *sbn* rescue and phenocopy experiments, injecting plasmid DNAs that drive *smad5* expression under the control of two promoters with different temporal activation profiles: the *Xenopus* EF1α promoter, which is activated right after midblastula transition when zygotic gene expression starts, and the cytoskeletal actin (CSKA) promoter, which is strongly activated during gastrulation but very weakly expressed at earlier stages (Hammerschmidt et al., 1998). Only when gene expression was under the control of the EF1α promoter, did injection of wild-type *smad5* DNA into *sbn* embryos lead to a rescue, and injection of mutant *smad5* DNA into wild-type

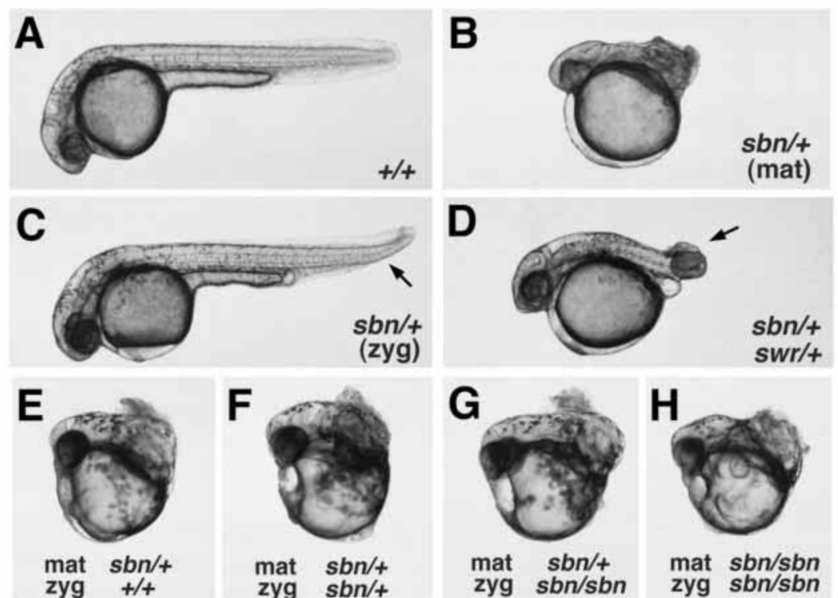

Fig. 3. Later phenotypes of sbn^{tc24} mutant embryos, 36 hpf, lateral view. (A) Wild type; (B) dominant maternal effect of *sbn*, causing strong C4 dorsalization. (C) Dominant zygotic effect of *sbn*, causing weak C1 dorsalization (arrow to ventral tail fin deficiency). (D) *sbn*,*swr* double heterozygote from a cross of a *swr* heterozygous female and a *sbn* heterozygous male, characterized by a loss of the ventral tail fin and a wound-up tail (C3, arrow), indicating a significantly stronger dorsalization than in C. (E-H) The maternal effect of *sbn* in the background of different *sbn* genotypes, 48 hpf, lateral view; (E-G) Embryos from a cross of two *sbn* heterozygous parents; (H) embryo from a cross of a *sbn* homozygous female with a heterozygous male. Both crosses led to offspring with 100% C4 dorsalization. The maternal and zygotic genotypes of individual embryos are indicated. The zygotic genotype was determined by tetra-PCR after photography.

Table 1. RNA and DNA injection studies

injected RNA/DNA	cross	pg/emb	*n* exp	*n*	%C5	%C4	%C3	%C2	%C1	%WT	%V1	%V2	%V3	%V4/5
smad5RNA	*sbn*/+f×*sbn*/+m	25	2	234	0	40	19	18	15	8	0	0	0	0
smad5RNA	*sbn*/+f×*sbn*/+m	50	20	1071	0	19	10	15	13	43	0	0	0	0
lacZRNA	*sbn*/+f×*sbn*/+m	50	2	115	0	100	0	0	0	0	0	0	0	0
smad5RNA	+/+f×+/+m	25	5	469	0	0	0	0	0	100	0	0	0	0
smad5RNA	*swr*/+f×*swr*/+m	50	5	404	25	0	0	0	0	75	0	0	0	0
smad5RNA	*sbn*/*sbn*f×*sbn*/*sbn*m	50	2	83	0	12	8	15	20	45	0	0	0	0
smad1RNA	*sbn*/+f×*sbn*/+m	50	2	97	0	52	20	13	11	4	0	0	0	0
smad2RNA	*sbn*/+f×*sbn*/+m	50	2	88	0	100	0	0	0	0	0	0	0	0
hsmad4RNA	*sbn*/+f×+/+m	25	1	124	0	43	19	23	5	10	0	0	0	0
smad5(*sbn*)RNA	+/+f×+/+m	25	6	318	0	26	21	21	17	15	0	0	0	0
smad5(*sbn*)RNA	+/+f×+/+m	50	2	44	0	71	29	0	0	0	0	0	0	0
smad1(*sbn*)RNA	+/+f×+/+m	50	2	134	0	42	18	11	10	19	0	0	0	0
smad2(*sbn*)RNA	+/+f×+/+m	50	2	165	0	0	0	0	0	100	0	0	0	0
pXeX-smad5	*sbn*/+f×+/+m	20	3	128	0	89	5	4	2	0	0	0	0	0
pXeX-smad5	+/+f×+/+m	20	1	88	0	0	0	0	0	100	0	0	0	0
pCSKA-smad5	*sbn*/+f×+/+m	20	2	112	0	100	0	0	0	0	0	0	0	0
pCSKA-smad5	+/+f×+/+m	20	1	87	0	0	0	0	0	100	0	0	0	0
pXeX-smad5(*sbn*)	+/+f×+/+m	40	2	111	0	3	8	5	15	68	0	0	0	0
pCSKA-smad5(*sbn*)	+/+f×+/+m	40	1	56	0	0	0	0	0	100	0	0	0	0
bmp2bRNA	*sbn*/+f×*sbn*/+m	20	2	116	0	0	0	0	0	0	0	89	11	0
bmp2bRNA	*sbn*/+f×*sbn*/+m	1	1	62	0	84	8	6	2	0	0	0	0	0
bmp4RNA	*sbn*/+f×*sbn*/+m	20	1	21	0	0	0	0	0	0	0	0	5	95
bmp4RNA	*sbn*/+f×*sbn*/+m	1	1	125	0	84	14	2	0	0	0	0	0	0
Xbmp4RNA	*sbn*/+f×*sbn*/+m	20	1	58	0	0	0	0	0	0	0	90	10	0
Xbmp4RNA	*sbn*/+f×*sbn*/+m	4	2	71	0	37	24	28	11	0	0	0	0	0
Xbmp4RNA	*sbn*/+f×*sbn*/+m	1	4	266	0	75	14	6	5	0	0	0	0	0
Xbmp4RNA	*sbn*/*sbn*f×*sbn*/*sbn*m	2.5	3	120	0	10	26	21	43	0	0	0	0	0
pCSKA-hBmp4	*sbn*/+f×*sbn*/+m	50	2	78	0	6	0	0	1	0	7	5	37	44

n exp, number of experiments; *n*, number of scored embryos. C1-C5 represent dorsalized phenotypes off increasing strength, as described in Mullins et al. (1996) and Kishimoto et al. (1997), V1-V5 ventralized embryos of increasing strength, as described in Kishimoto et al. (1997). C5 is the strongest dorsalized phenotype, characterized by football-shaped embryos at the end of gastrulation and rupture of the yolk sac around the 15-somite stage. C4 to C1 were defined based on the phenotype at 36 hours after fertilization. C4 embryos are characterized by a body axis that is wound up in a snail shell-like fashion. In C3 embryos, the tail is wound up, while the trunk is normal. C2 is characterized by a complete loss of the ventral tail fin and a bent-up tip of the tail. C1 embryos display a partial loss of the ventral tail fin,while the shape of the entire embryo is normal. In all experiments, at least 50 sibling embryos were kept as uninjected controls. All control embryos from crosses with *sbn*/+females were C4. Mutant alleles used were: sbn^{tc24}, swr^{ta72}.

Abbreviations: emb, embryo; f, female; m, male; + and WT, wildtype; pXeX, plasmid driving expression under control of the *Xenopus* EF1 alpha promoter; pCSKA, plasmid driving expression under control of cytoskeletal actin promoter; hsmad4, human smad4; hbmp4, human bmp4; Xbmp4, *Xenopus* bmp4. (*sbn*) indicates mutant *smad* RNA or DNA encoding protein with the T(429)I exchange found in sbn^{tc24} Smad5.

embryos to a phenocopy of the *sbn* phenotype. No effect was observed upon injection of the corresponding CSKA constructs (Table 1). These data suggest that *smad5* acts after midblastula transition and before gastrulation.

sbn(smad5) interaction with *swr(bmp2b)*

Smad5 has been previously described as a putative mediator of ventralizing signaling by Bmps. In the zebrafish, the *bmp2b* null mutations swr^{ta72} and swr^{tc300} lead to dorsalized embryos (Kishimoto et al., 1997; Nguyen et al., 1998) very similar to *sbn* mutant embryos, indicating that *bmp2b* and *smad5* are both involved in ventral development. In contrast to *sbn*, the effect of swr^{ta72} is purely zygotic, suggesting that Bmp2b has no maternal function (Kishimoto et al., 1997). To investigate whether *smad5* and *bmp2b* interact genetically, *sbn*,*swr* double mutant embryos were generated. While crosses between *sbn* heterozygous males and wild-type females led to 50% weakly

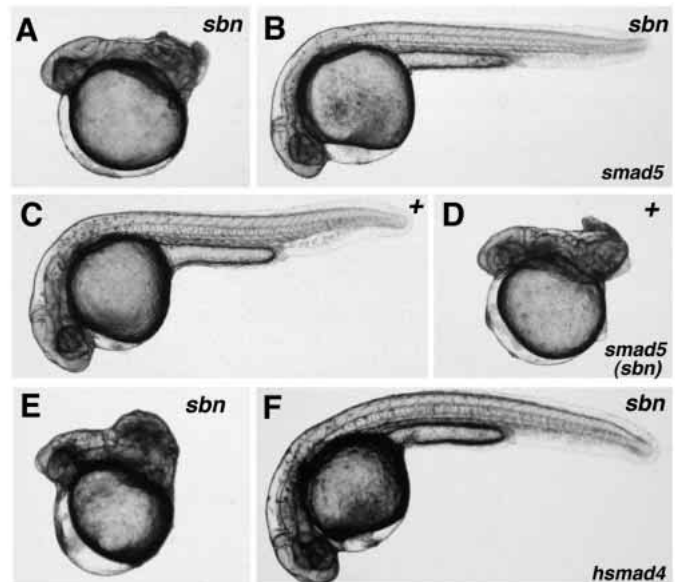

Fig. 4. Rescue and phenocopy of sbn^{tc24} mutant phenotype. The genotype of embryos is indicated in the upper right corner, the injected RNA in the lower right corner; 36 hpf, lateral view. (A,C,E) Uninjected controls; (B,D,F) injected embryos. (A,B,E,F) Rescue of *sbn* phenotype upon injection of wild-type *smad5* mRNA (A,B) or human *SMAD4* mRNA (E,F) into embryos from a cross of two *sbn* heterozygous parents. (C,D) Phenocopy of *sbn* phenotype upon injection of *smad5* mRNA, bearing the *sbn* mutation, into wild-type embryos.

dorsalized (C1) and 50% wild-type embryos, and crosses between wild-type males and *swr* heterozygous females to 100% wild-type embryos, a significantly stronger dorsalization was found in a quarter of the offspring of *sbn* heterozygous males and *swr* heterozygous females (25% C3, 23% C1, 52% wild type; *n*=588; 7 crosses; compare Mullins et al., 1996). Genotyping of 20 individuals of each class identified the C3 embryos as *sbn*,*swr* double heterozygotes (Fig. 3D), while the C1 embryos were *sbn* heterozygous and wild type for *swr*. This indicates that the dominant zygotic effect of the *smad5* mutation sbn^{tc24} is substantially enhanced by the loss of one functional *bmp2b* allele. The same appears to be true for the dominant maternal effect of *sbn*. Crosses between *sbn* heterozygous females and *swr* heterozygous males yielded 50.5% more strongly dorsalized embryos (C5; see Table 1), and 49.5% C4 embryos (*n*=327; 2 crosses), compared to 100% C4 embryos in crosses between *sbn* heterozygous females and wild-type males. Genotyping revealed that 10 of 10 tested C5 embryos were *swr* heterozygotes, while 10 of 10 C4 embryos were *swr* wild type.

The effect of *sbn* on early *bmp2b* expression

Analysis of *swr* mutant embryos has revealed that *bmp2b* is required for the maintenance of its own expression (Kishimoto et al., 1997). To investigate the role of *smad5* on *bmp2b* expression, we compared the *bmp2b* expression pattern in *sbn* mutant, *swr*(*bmp2b*) mutant, and wild-type embryos.

At the sphere stage, approx. 1 hour after the onset of zygotic transcription, the *bmp2b* expression patterns of both *sbn* and *swr* mutants were indistinguishable from that of wild-type embryos (Fig. 5A-C). At the onset of gastrulation (shield stage), however, both mutants display a dramatic reduction in *bmp2b* transcripts (Fig. 5D-F). Thus, the initial induction of *bmp2b* expression is not affected by the maternal effect of the *sbn* mutation, but the later, Bmp2b-dependent maintenance of *bmp2b* expression appears to be impaired.

Differential effects of exogenous Bmp2/4 in *somitabun* mutant embryos

The comparison of the *bmp2b* expression patterns in *sbn* and *swr* (*bmp2b*) mutant embryos suggests that the *sbn* mutation blocks the aforementioned positive Bmp2b autoregulation. To test this idea more directly, the effects of exogenous Smad5 and Bmp2/4 on *bmp2b* expression in *sbn* mutant embryos were analyzed. *smad5* (Fig. 5H; compare with 5D), but not *Xbmp4* mRNA (Fig. 5I), injected into *sbn* mutant embryos led to a rescue of *bmp2b* mRNA levels at early gastrula stages, while increased *bmp2b* mRNA levels were observed after *Xbmp4* RNA injection in wild-type embryos. This suggests that the *sbn* mutation acts downstream of Bmp2b, blocking the mediation of Bmp2/4 signaling during late blastula and early gastrula stages.

In contrast to this unresponsiveness in early *bmp2b* expression, *bmp2/4*-injected *sbn* mutant embryos displayed a striking response in their morphology, first apparent at the end of gastrulation. Depending on the amounts of injected zebrafish *bmp2b* or *bmp4* or *Xenopus bmp4* mRNA, the strong dorsalization of *sbn* mutants could be significantly normalized (C4 to C1), or even converted to a ventralization (C4 to V3, Table 1). Since this effect was also obtained with embryos deriving from two *sbn* homozygous mutant parents, it can be ruled out that the rescue by Bmp2/4 was mediated by residual maternal or zygotic wild-type Smad5 (Fig. 6A,B; Table 1).

To determine when during development exogenous Bmp2/4 can override the effect of the *sbn* mutation, we expressed human *bmp4* under the control of the cytoskeletal actin promoter in *sbn* mutant embryos. In contrast to the corresponding *smad5* transgene, expression of the human *bmp4* gene under this promoter led to a striking rescue and even ventralization of *sbn* mutant embryos (Fig. 6C,D; Table 1), suggesting that the *sbn*-independent response of the zebrafish embryo to exogenous Bmp2/4 occurs during gastrula stages.

somitabun mutant cells behave normally in a wild-type environment

In the *bmp2/4* RNA injection experiments described above, the actual in vivo concentrations of Bmp2/4 protein are unknown. To investigate whether *sbn* cells can respond to Bmp2/4 under physiological conditions, cell transplantation experiments were carried out. Labeled cells from either wild-type embryos or embryos deriving from a cross of two *sbn* heterozygous or two *sbn* homozygous mutant parents were transplanted into wild-type embryos. Homochronic transplantations were carried out at the sphere stage, when *sbn* mutant embryos still show normal marker gene expression, or at the shield stage, shortly after the onset of gastrulation when *sbn* mutants display a dramatic loss in *bmp2b* mRNA levels. In all cases, donor cells gave rise to blood (Fig. 6E,F) and ventral tail fin (Fig. 6G,H), tissues completely absent in *sbn* mutant embryos. The frequencies of ventral tissue contribution were similar, independently of whether donor cells were wild-type or *sbn* mutant (see Table 2), and whether they were transplanted at late blastula or early gastrula stages. Furthermore, individual host embryos with blood cells deriving from both donors were obtained after simultaneous transplantation of differently labeled wild-type and *sbn* mutant cells (not shown). This indicates that in a wild-type environment, *sbn* mutant cells can form the ventral-most derivatives, thereby showing the maximal possible response to endogenous ventralizing signals. Thus, in contrast to the early mediation of Bmp2/4 signaling, the final specification of ventral cell fates can occur in the absence of functional Smad5.

DISCUSSION

Many recent publications deal with the function of Smad proteins as mediators of TGFβ signaling. Direct genetic evidence for this function was provided for *Drosophila* Mad,

Table 2. Cell transplantation studies

Cross for donors	*n*	% vtf	% blood
sbn/+×*sbn*/+	51	26	12
sbn/*sbn*×*sbn*/*sbn*	33	30	15
+/+×+/+	18	22	11

20-100 cells from embryos of the indicated donor crosses were transplanted into wild-type embryos of the same stage. Approximately 70% of the transplantations were carried out at the sphere stage and 30% at the shield stage. *n*, number of evaluated chimeric embryos; % vtf, % blood, portion of chimeric embryos with transplanted cells in the ventral tail fin or the blood.

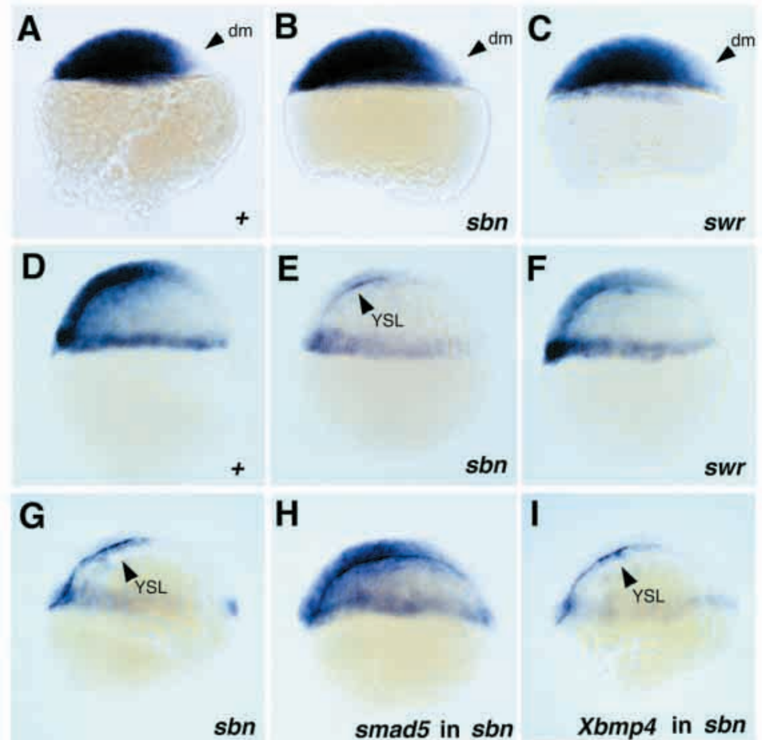

Fig. 5. Expression of *bmp2b* in *sbn* and *swr* mutant embryos (A-F) and after injection of *smad5* or *Xbmp4* mRNA in *sbn* mutants (G-I). All embryos are shown in a lateral view, dorsal side right. (A,D) Wild-type siblings of *swr* mutants; (B,E) *sbn* mutants from cross of *sbn* heterozygous female with wild-type male. Embryos were fixed and stained in parallel to the *swr* and wild-type embryos shown; (C,F) *swr* mutants. (A-C) Sphere stage; presumptive dorsal mesoderm (dm) devoid of *bmp2b* staining is marked with arrowheads. (D-F) Shield stage; the *sbn* mutant (E) displays severely reduced *bmp2b* staining, and the *swr* mutant (F) slightly reduced *bmp2b* staining in the blastoderm; the staining in the yolk syncytial layer (YSL, arrowhead in E) appears normal. (G-I) *sbn* mutant embryos, shield stage, after injection of *smad5* (H; 50 pg/embryo) or *Xbmp4* (I; 4 pg/embryo) mRNA. Similar results as in I were obtained for approx. 100 embryos in each of three independent injection experiments. Of the *Xbmp4*-injected *sbn* sibling embryos that were raised to day 2, 53% (*n*=45) showed a strong morphological response, appearing wild-type or ventralized (see Fig. 6).

which mediates Decapentaplegic (Dpp) signaling during midgut and eye development (Newfeld et al., 1997; Wiersdorff et al., 1996). In addition to these later zygotic functions, *mad* is also required for early D-V patterning of the fly embryo, and mutants lacking both maternally and zygotically contributed *mad* gene products exhibit the same ventralized phenotype as *dpp* mutant embryos (Das et al., 1998).

Here, we show that in the zebrafish one of the homologues of *mad*, *smad5*, is involved in the mediation of Bmp2/4 signaling during early phases of embryonic D-V pattern formation. Like *Drosophila mad*, zebrafish *smad5* mRNA is both maternally and zygotically supplied. The antimorphic *smad5* allele *somitabun* (sbn^{tc24}) leads, when maternally supplied, to a strong dorsalization, similar to the phenotype caused by loss of the ventralizing signal Bmp2b (Kishimoto et al., 1997; Nguyen et al., 1998), indicating a role of *smad5* in early ventral development. Smad5 may also be involved in later developmental processes, but its later function is not completely indispensable, as suggested by rescue experiments of *smad5* mutant embryos with exogenous wild-type *smad5* RNA, which is usually degraded by early somitogenesis stages (Hammerschmidt et al., 1998). While uninjected embryos from

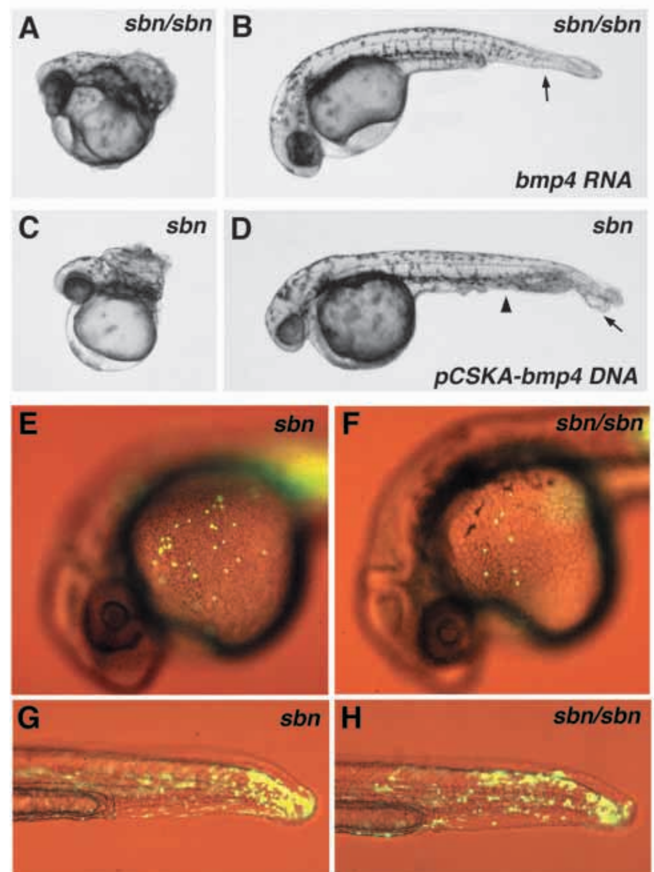

Fig. 6. Rescue of ventral cell fates in *sbn* mutant cells by exogenous *bmp4* mRNA or DNA (A-D) and wild-type environment (E-H); 36 hpf, lateral view. (A,B) Injection of 1 pg *Xbmp4* mRNA into a *sbn* homozygous mutant embryo from a cross of two homozygous mutant parents. The injected embryo displays a normalization of the *sbn* mutant phenotype from C4 (A) to C1 (B, arrow indicates absence of ventral tail fin). (C,D) Injection of 50 pg pCSKA-bmp4 DNA into an embryo from a cross of two *sbn* heterozygous parents. The *sbn* mutant phenotype is converted from a strong dorsalization (C4, panel C) to a weak ventralization (V1, panel D; see slight duplication of the ventral tail fin (arrow) and the enlarged blood island (arrowhead). (E-H) Chimeric embryos with fluorescein-labeled cells from embryos of a *sbn*/+ × *sbn*/+ cross (E,G) or a *sbn*/*sbn* × *sbn*/*sbn* cross (F,H) transplanted into wild-type recipients. Donor cells give rise to blood (E,F) and ventral tail fin (G,H) (see also Table 2).

two *sbn* heterozygous parents display dorsalizations of equal strengths, irrespective of their zygotic genotype, rescued homozygous mutant adults were obtained with a lower than expected frequency, indicating a lower survival rate compared to their *sbn* heterozygous and wild-type siblings.

The antimorphic *smad5* allele *somitabun* sbn^{tc24}

The alterations in Smad5 activity caused by the *somitabun* mutation have been investigated in *Xenopus* animal cap explants, measuring the ventral mesoderm-inducing properties of different Smad5 and Smad1 alleles – although such assays may not reflect the in vivo function of Smad1/5 proteins, as the initial induction of ventral mesoderm is unaffected in *sbn* mutant zebrafish embryos. In contrast to the wild-type version, sbn^{tc24} Smad5 showed no or a very weak ventralizing activity. In addition, co-expression experiments in *Xenopus* animal caps and comparison of *sbn* homozygous and heterozygous mutant zebrafish embryos suggest that sbn^{tc24} Smad5 acts like an antimorph that inactivates most of wild-type Smad5 protein when present in equimolar ratios. Together, these results suggest that embryos from *sbn* mutants lack all or at least most of Smad5 activity.

However, this loss of wild-type Smad5 activity is not necessarily the basis for the *sbn* mutant phenotype, since *sbn* mutant Smad5 protein could also inhibit other Smad proteins, as shown in the animal cap assay for *smad5* and *smad1*. The observed dorsalization of *sbn* mutant embryos could equally well result from a general inhibition of Smad1, Smad5 and other as yet unidentified additional members of the Smad1/5 family. It is thus currently impossible to state to what extent *smad5* itself is actually required for early dorsoventral patterning, although preliminary data suggest that it might be essential: another mutation, *captain hook* (cpt^{m169}; Solnica-Krezel et al., 1996), appears to interfere specifically with zygotic *smad5* expression, and leads to a mild dorsalization similar to that caused by the zygotic effect of *sbn* (A. D. and M. H., unpublished results).

Possible mechanisms of sbn^{tc24} Smad5 function

There are several hints as to how the point mutation found in sbn^{tc24} Smad5 may cause its dominant negative effect molecularly. According to current models, Smad5 proteins normally exist as homo-trimers which upon activation by Bmp receptors bind to Smad4 trimers to form transcriptionally active regulators. The mutated threonine residue T(429) of Smad5 is located at a solvent-exposed position within a region of the MH2 domain, the L3 loop. This loop is involved in the interaction of receptor-regulated Smad proteins with the kinase domain of the TGFβ receptors and with Smad4 (Shi et al., 1997; Lo et al., 1998). According to in vitro studies carried out with Smad2, replacement of the threonine corresponding to T(429) in Smad5 by lysine or alanine causes a dramatic reduction in the affinity to Smad4 and TGFβ receptors, while the homo-trimer formation is unaffected (Lo et al., 1998). Similar properties have been proposed for the naturally occurring inhibitory Smad6 protein, which can bind to Smad1, but not to Smad2 or Smad4 (Hata et al., 1998). In its L3 loop, Smad6 protein differs only in a few amino acid residues from the sequence conserved in the receptor-regulated Smad protein, including a T(431)C change of two amino acids downstream of T(429). Apparently, the T(429)I change in sbn^{tc24} Smad5 yields an inhibitory version of Smad5 protein, trapping wild-type Smad5 protein in signaling-incompetent trimers whose interaction with either the Bmp2/4 receptors or the Smad4 trimers is strongly impaired. Our observation that the maternal effect of the *sbn* mutation could be rescued by injecting high amounts of human *smad4* mRNA indicates that interaction with Smad4, rather than activation by the Bmp receptors is affected by the *sbn* mutation.

The epistasis between *smad5* and *bmp2/4*

Co-expression experiments in *Xenopus* animal cap explants with a dominant negative version of the Bmp receptor have indicated that Smad1 and Smad5 act downstream of Bmp signaling. In this study, we analyzed the relationship between *bmp2b* and *smad5* by genetic tests. The enhancement of the dominant effect of the *sbn* mutation by the loss of one *bmp2b* wild-type allele in *sbn,swr* double heterozygotes indicates that *smad5* and *bmp2b* interact during ventral specification of the early zebrafish embryo. Their epistatic relationship cannot be addressed in such double mutant analyses because mutations in both genes lead to a similar phenotype. Epistasis analyses are further complicated by the existence of an autoregulatory positive feedback loop of Bmp2b on its own expression (Kishimoto et al., 1997) which, as shown in this work, depends on Smad5. Thus, during the maintenance phase of *bmp2b* expression, Bmp2b appears to act both upstream and downstream of Smad5. Accordingly, exogenous Bmp2/4 failed to rescue the early defects of *smad5* mutants on *bmp2b* expression, and exogenous Smad5 failed to rescue the *bmp2b* mutant phenotype.

In contrast to its maintenance, the initiation of *bmp2b* expression in *smad5* mutant blastula embryos occurs normally, indicating that *smad5* plays no role in its induction, and possibly not prior to Bmp2b action at all. Accordingly, exogenous *smad5* expressed after midblastula transition can compensate for the loss of endogenous Smad5 activity. Together, these data support a model according to which *smad5*, despite its maternal expression and the maternal effect of the *sbn* mutation, has no maternal function per se. Rather, *smad5* gene products appear to be present so early to ensure that cells are competent to process Bmp2b signaling when zygotic *bmp2b* expression starts.

The three phases of D-V pattern formation

These findings define three distinct phases of D-V patterning: in the first phase, an initial coarse D-V pattern is set up, when the future dorsal mesoderm, the equivalent of the amphibian Spemann organizer, is induced in a small dorsal marginal domain characterized by the expression of *chordino* and opposed by the expression of *bmp2b* in the rest of the embryo. This pattern is most likely set up under maternal control, and is independent of Bmp2b and Smad5, although in *Xenopus*, Bmp2/4 and Smad1/5 are dicussed as maternal components involved in the induction of ventral mesoderm (Graff et al., 1996).

In the second phase, which is under zygotic control and dependent on Bmp2b and Smad5, this initial D-V pattern is refined, leading to the transformation of the broad and uniform expression of *bmp2b* to a graded pattern with ventral-to-dorsal progressively dropping *bmp2b* mRNA levels. This

transformation is governed by the antagonizing action of Bmp2b and its mediator Smad5 on one side and the Bmp2b-antagonist, Chordino, on the other side. In *swirl*(*bmp2b*) and *somitabun*(*smad5*) mutant embryos, *bmp2b* mRNA levels drop throughout the entire embryo, indicating that *bmp2b* and *smad5* are involved in the maintenance of *bmp2b* expression, while in *chordino* mutant embryos, the initial broad *bmp2b* expression pattern is maintained (Hammerschmidt et al., 1996b), indicating that Chordino signaling from the dorsal side is required for the clearing of *bmp2b* mRNA in dorsolateral regions of the late blastula embryo. Judging from in situ hybridization patterns in wild-type and mutant embryos, the establishment of this Bmp2b gradient, which is thought to have morphogenetic character defining positional values and cell fates along the D-V axis, appears completed by the onset of gastrulation.

Several observations point to a function of Bmp2/4 beyond this time point, defining a third phase of D-V patterning. In *Xenopus*, the morphogenetic action of Bmp4 is thought to occur at early gastrula stages (Jones et al., 1996). There are indications that the same might be true in zebrafish. Here, embryos lacking functional Bmp2b (Hammerschmidt et al., 1996b) or Smad5 (this work) can be rescued by injecting a DNA construct with the expression of Bmp4 under the control of the cytoskeletal actin promoter, which is active during gastrulation (Hammerschmidt et al., 1998). Furthermore, cells from shield stage *smad5*-deficient embryos transplanted into wild-type embryos can be rescued to form the ventral-most derivatives like blood or ventral tail fin, tissues normally missing in mutant embryos (this work).

This suggests that Bmp2/4 function during D-V pattern formation is twofold. First, during blastula stages Bmp2b serves to ensure the maintenance of its own expression, a prerequisite for establishing the morphogenetic Bmp2/4 gradient by the antagonistic action of Bmp2b and Chordino. The morphogenetic Bmp2/4 action itself, to determine cell fates along the D-V axis in a dose-dependent fashion, seems to take place later, during early gastrula stages, after the gradient has been set up.

The *smad5* mutation *sbn*^{tc24} described here nicely dissects these two phases of zygotic Bmp2/4 action, as it affects just the early phase, when the Bmp2/4 gradient is set up, whereas the later mediation of Bmp2/4 signaling to induce final cell fate specifications can occur normally.

This dispensability of Smad5 function at later stages might be caused by the presence of other mediators of Bmp2/4 signaling with redundant functions, such as e.g. Smad1, which starts to be made during early gastrula stages. However, we find that Smad1 can also be inhibited by Smad5(sbn), so this redundant transducer of Bmp2/4 signaling could be another Smad protein, or even a completely unrelated transcription factor. In any case our results indicate that there must be an additional, Smad5(sbn)-independent transcription factor downstream of Bmp2/4 specifically involved in the third phase of D-V patterning, the interpretation of the putative morphogenetic Bmp2/4 gradient.

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