Maternally Supplied Smad5 Is Required for Ventral Specification in Zebrafish Embryos Prior to Zygotic Bmp Signaling

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We have previously shown that the maternal effect dorsalization of zebrafish embryos from sbn<sup>dc24</sup> heterozygous mothers is caused by a dominant negative mutation in Smad5, a transducer of ventralizing signaling by the bone morphogenetic proteins Bmp2b and Bmp7. Since sbn<sup>dc24</sup> mutant Smad5 protein not only blocks wild-type Smad5, but also other family members like Smad1, it remained open to what extent Smad5 itself is required for dorsoventral patterning. Here, we report the identification of novel smad5 alleles: three new isolates coming from a dominant enhancer screen, and four former isolates initially assigned to the cpt and pgy complementation groups. Overexpression analyses demonstrate that three of the new alleles, m169, fr5, and tc227, are true nulls (amorphs), whereas the initial dtc24 allele is both antimorphic and hypomorphic. We rescued m169 mutant embryos by smad5 mRNA injection. Although adult mutants are smaller than their siblings, the eggs laid by m169<sup>+/--</sup> females are larger than normal eggs. Embryos lacking maternal Smad5 function (Mm169<sup>+/--</sup> embryos) are even more strongly dorsalized than bmp2b or bmp7 null mutants. They do not respond to injected bmp2b mRNA, indicating that Smad5 is absolutely essential for ventral development and Bmp2/7 signaling. Most importantly, Mm169<sup>+/--</sup> embryos display reduced bmp7 mRNA levels during blastula stages, when bmp2b and bmp7 mutants are still normal. This indicates that maternally supplied Smad5 is already required to mediate ventral specification prior to zygotic Bmp2/7 signaling to establish the initial dorsoventral asymmetry. © 2002 Elsevier Science (USA)

Key Words: Smad5; Bmp2b; Bmp7; dorsoventral patterning; maternal effect; enhancer screen; zebrafish; body size; oogenesis.

INTRODUCTION

Cell tracing experiments have shown that the later fate of a cell in a fish or amphibian embryo depends on its position within the embryo at blastula and gastrula stages (Dale and Slack, 1987; Kimmel et al., 1990). Of particular importance is the position along the future dorsoventral (DV) axis. The system, supplying such positional information and determining differential DV cell fates, is quite well understood (for review, see Hammerschmidt and Mullins, 2002). According to a commonly accepted model, differential cell specification is governed by a morphogenetic gradient of bone morphogenetic proteins (Bmps). These Tgfβ superfamily members (for review, see Hogan, 1996) promote ventral specification in a dose-dependent fashion, while dorsal specification occurs when Bmps are absent or blocked.

The establishment of the Bmp gradient occurs stepwise. In a first phase, most likely under maternal control, a simple initial pattern is set up, with all cells of the early
embryo being ventrally specified, except a rather small dorsal region, where the Spemann organizer is induced. Ventraly specified cells are characterized by the expression of bmp2b and bmp7 (Kishimoto et al., 1997; Nkaido et al., 1997; Dick et al., 2000; Schmid et al., 2000), the Spemann organizer by the expression of Bmp inhibitors like Chordin (Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997). In a second step, driven by long-range signaling of Chordin from the organizer (Hammerschmidt et al., 1996b), this initial pattern becomes transformed from the broad and uniform bmp2b/7 distribution to the aforementioned graded pattern with from ventral to dorsal gradually dropping Bmp levels, eventually leading to differential cell specification.

Genetic evidence for this model of stepwise DV patterning and the essential roles of involved genes comes from dorsalized or ventralized zebrafish mutants (Hammerschmidt et al., 1996a; Mullins et al., 1996; Solnica-Krezel et al., 1996). Two mutants, ichabod and bozozok, interfere with Spemann organizer induction during the first phase of DV patterning, ichabod appears to encode a component of the canonical Wnt signal transduction pathway, which is required to induce Spemann organizer formation in dorsal regions of the blastula (Kelly et al., 2000). One of its activated target genes might be chordin; another target gene is bozozok, which codes for a homeodomain transcription factor required for the repression of bmp2b/7 expression in the organizer region (Fekany et al., 1999; Koos and Ho, 1999; Ryu et al., 2001). How the expression of bmp2b and bmp7 is initiated in the rest of the embryo, however, has been unclear.

Other zebrafish mutants affect the second phase of DV patterning, during which the initial pattern is refined and the Bmp gradient is set up and interpreted by cells along the DV axis of the late blastula and early gastrula embryo. The ventralized phenotype of dino mutants (Hammerschmidt et al., 1996a,b; Fisher et al., 1997) is caused by a null mutation in the Bmp inhibitor Chordin (Schulte-Merker et al., 1997), while mutations in different components of the Bmp signaling system lead to dorsalized phenotypes.

Bmps act as homo- or heterodimers, which bind to a dimeric complex of a type I and a type II transmembrane serine-threonine kinase receptor. Three different type I receptors have been described to mediate Bmp signaling, Alk3, also named BmpR1A (Nkaido et al., 1999a), Alk6, also named BmpR1b (Nkaido et al., 1999b), and Alk8 (Bauer et al., 2001; Mintzer et al., 2001; Payne et al., 2001), which in mammals may correspond to Alk2, also designated as ActR1b, as it was initially found to bind activin, another member of the Tgfβ superfamily (for review, see Massague, 1998). Upon Bmp binding, the type I receptors phosphorylate and activate Smad proteins. Three Smad proteins are specifically involved in the transduction of Bmp signals, Smad1, Smad5 and Smad8, while Smad2 and Smad3 mediate signaling by other members of the Tgfβ superfamily, such as Activins, Nodal, and Tgfβ (Massague, 1998). In their basal state, these Smad proteins are inactive and associated with the cytoplasmic domain of the type I receptors. Upon phosphorylation, Smad proteins dissociate from the type I receptor to form complexes with Smad4 and to translocate to the nucleus, where they participate in transcriptional complexes to activate or repress specific target genes (Massague, 1998; Wotton et al., 1999).

Dorsalized zebrafish mutants in four different Bmp signaling components were identified: Bmp2b/swirl (Kishimoto et al., 1997; Nguyen et al., 1998), Bmp7/scanlhouse (Dick et al., 2000; Schmid et al., 2000), the type I receptor Alk8/lost-a-fin (Bauer et al., 2001; Mintzer et al., 2001), and Smad5/somitabun (Hild et al., 1999). In contrast to bmp2b, bmp7, and alk8, for which null mutations were available, results obtained for the smad5 mutants were ambiguous, as the only known allele was antimorphic, not only blocking Smad5, but also related Smad proteins like Smad1 present in gastrulating zebrafish embryos (Dick et al., 1999; Müller et al., 1999). Thus, the extent to which Smad5 itself is required for DV patterning remained unclear. Also, it remained open in which phases of DV patterning Smad5 is involved. There was some indication that it might act in the second phase downstream of Bmp2b, Bmp7, and Alk8 (for review, see Hammerschmidt and Mullins, 2002); however, other data suggested an earlier role. Thus, only injected smad1 mRNA, but not injected smad5 mRNA, was able to rescue the dorsalization of bmp2b mutants (Dick et al., 1999). Here, we describe novel amorphic smad5 alleles that reveal that maternal Smad5 is required during the first phase to initiate bmp7 expression at midblastula stages, rather than in addition to the second phase of DV patterning to mediate zygotic Bmp2b/7 signaling. Inactivation of maternal Smad5 leads to a stronger dorsalization than caused by the loss of bmp2b and bmp7, suggesting the presence of additional Smad5-dependent factors which act in parallel to Bmp2b and Bmp7 during ventral development.

**MATERIALS AND METHODS**

**Mapping of piggytail pgyl**

The pgy<sup>dy40</sup> mutation, initially isolated in the Tue background, was mapped by scoring the maternal–zygotic dominant dorsalized phenotype (Mullins et al., 1996). A pgy<sup>dy40</sup>/? female was crossed to a WIK wild-type male. Scoring of embryonic phenotypes, isolation of genomic DNA from embryos, and mapping with SSLP markers (Shimoda et al., 1999) were done as described (Gates et al., 1999; Talbot and Schier, 1999). In a genome-wide survey of SSLP markers, we found that pgy was linked to LG 14 marker Z9057 (2 recombinants among 64 meioses), which is located near the smad5 gene (Hild et al., 1999). To directly test linkage between pgy and smad5, a pgy<sup>dy40</sup>/? female was crossed to a TL wild-type male. Embryonic phenotypes were scored and a 438-bp genomic smad5 fragment was amplified from wild-type and mutant embryos, using the primers GCAAGGCGAGTTTTCCTCCCGG (antisense, exon B) and AAAAAAGGCTTCTCTTCCAGG (antisense, exon C). Amplified fragments were digested with XmnI, which cleaves the smad5 allele on the mutant but not the wild-type chromosome, and analyzed by agarose gel electrophoresis. There were no recom-
binants among 109 meioses, indicating that pgy and smad5 are tightly linked.

Screen for Dominant Enhancers of din<sup>1239</sup>/+

Chordin acts as an essential antagonist of Bmp signals during zebrafish gastrulation. din<sup>1239</sup> mutants are strongly ventralized (Hammerschmidt et al., 1996a; Fisher et al., 1997; Miller-Bertolglo et al., 1997; Schulte-Merker et al., 1997). Although the din<sup>1239</sup> mutation has no dominant effect in a wild-type background, din<sup>1239</sup>/+ embryos can develop subtle defects in sensitized backgrounds, e.g., as din/mes double heterozygotes (Hammerschmidt et al., 1996a). To identify similar dominant enhancers, we mutagenized males from the Ekkwill strain (Riley and Grunwald, 1995) with ENU, as described (Mullins et al., 1994). PCR genotyping. PCR of genomic DNA was carried out with i1 or i2 and ACAAAGGCT-TCTTCCAGG (antisense, exon), yielding a 264-bp band, which could be amplified with i1 from fr4/5-mutant, but not wild-type DNA, and with i2 from wild-type, but not fr4/5-mutant DNA. Altogether, no recombination was found in 150 fr5 mutant embryos.

RT-PCR Analyses and Generation of Constructs

Isolation of RNA and RT-PCR to clone full-length smad5 cDNAs from the various mutants was basically carried out as previously described (Hild et al., 1999). After PCR-based introduction of terminal restriction sites, cDNAs were cloned into the expression vector pCS2+ (Rupp et al., 1994).

To investigate whether the gain of splici acceptor site in m169 mutant embryos is preferentially used over the regular acceptor site, RT-PCR was carried out by using the primers described above for the amplification of intron A, yielding a 148-bp fragment in the case of m169 and a 131-bp fragment in the case of wild type. PCR products were separated via electrophoresis on 5% agarose gels and inserted into pCRII (Invitrogen). Insert sizes of individual clones were determined on 5% agarose gels after EcoRI digest.

To determine the relative abundance of m169 mutant transcripts in m169/fr4 transheterozygotes, RT-PCR was carried out to clone the region containing the m169 mutation from mutant and wild-type offspring of a m169/+ mother and a fr4/+ father, as described above (see Fig. 3A, second and third lanes). In addition, the entire RT-PCR products were cloned into pCRII, and inserts of individual clones were PCR-amplified and Hael I restriction-analyzed as described above (see Fig. 3B).

For semiquantitative determination of bmp2b and bmp7 transcript levels in Mm169<sup>+/−</sup> and swr<sup>−/−</sup> embryos (see Fig. 7I), RT-PCR was carried out for 20 cycles, using the primers TCTCGGAGATGCGATCCA-GTACGAG (bmp2b, sense), CACGCTCTCGGATACCTCTCTG (bmp2b, antisense), TTGAACTTGATGTTGTCCT (bmp7, sense), TATACAGTGACATTGACCGG (bmp7, antisense), CCTTATAGACAGCCAAACCC (bmp7, sense), TTGAACTCGGTAGAG (bmp7, antisense), TACCCCTGGAGTGTGAGG (bmp7, sense), and ACTTCGAGCGTGAAG (mp7, antisense). PCR products were separated via 2% agarose gel electrophoresis, blotted, and hybridized with end-labeled internal oligonucleotides (TCTCGGAGATGCGATCCA-GTACGAG, bmp2b; AGGCGAGATAGAGAGG, bmp7-int1; ACCAGTGCTGTA-CATTAGC, bmp7-int2; ACTTCGAGCGTGAAG, mp7, antisense). The following probes were generated as described: smad5 (Hild et al., 1999), bmp7 (Dick et al., 2000), bmp4 and bmp2b (Nikaido et al., 1997), chd (Miller-Bertolglo et al., 1997), gsc (Schulte-Merker et al., 1994), foxb1.2 (Kd3; Odenthal and Nüsslein-Volhard, 1998), otx2 (Li et al., 1994), flh (Talbot et al., 1995), and krox20 (Oxtoby and Jowett, 1993).

In Situ Hybridizations

Whole-mount in situ hybridizations were carried out as previously described (Hammerschmidt et al., 1996a). The following probes were generated as described: smad5 (Hild et al., 1999), bmp7 (Dick et al., 2000), bmp4 and bmp2b (Nikaido et al., 1997), chd (Miller-Bertolglo et al., 1997), gsc (Schulte-Merker et al., 1994), foxb1.2 (Kd3; Odenthal and Nüsslein-Volhard, 1998), otx2 (Li et al., 1994), flh (Talbot et al., 1995), and krox20 (Oxtoby and Jowett, 1993). mRNA synthesis and microinjection into zebrafish embryos was
RESULTS

Identification of Previously Isolated Mutations as smad5 Alleles

We have previously mapped the smad5 gene and the somitabun sbn\textsuperscript{dtc24} mutation to linkage group 14, close to SSLP markers z3290 and z4291 (Hild et al., 1999). The dtc24 mutation has a strong dominant maternal effect, leading to C4 dorsalization of embryos derived from a heterozygous mother (Fig. 1B), independent of the zygotic genotype of the embryos themselves (+/+ , +/− , or −/−). This mutation also exhibits a weak dominant zygotic effect causing an incompletely penetrant C1 to C2 dorsalization of heterozygous embryos (+/−) derived from a wild-type mother and a heterozygous father (Fig. 1C; see legend for definitions of phenotypic classes C1–C4; Hild et al., 1999; Mullins et al., 1996).

A previously isolated dorsalizing mutation maps to the same region as sbn\textsuperscript{dtc24}, the captain hook allele cpt\textsuperscript{m169} (Solnica-Krezel et al., 1996; Neuhauss et al., 1996; Neuhauss, 1996). It leads to a weaker dorsalization than the dtc24 mutation. Homozygous cpt\textsuperscript{m169} embryos display C1–C2 dorsalization (Fig. 1E), while +/− offspring of a +/+− mother are either of wild-type morphology or very weakly dorsalized, characterized by a partial loss of the ventral tail fin ("C0.5"); embryos in (C, J) display slightly stronger dorsalization (C1, characterized by complete absence of ventral tail fin); embryos in (E, L), C2 dorsalization (characterized by absent ventral tail fin and upwards bent tail); embryos in (D, G, I), C3 dorsalization (characterized by absent tail fin and curled tail); and embryo in (B), C4 dorsalization (characterized by curled trunk and tail).
effect of \( \text{sbn}^{\text{fr224}} \), and its interaction with \( \text{swirl} \) and \( \text{pgy} \), it was difficult to assess its allelism, and consequently, the \( \text{dtc24} \) allele was given a tentative independent gene name, somitabun. Our chromosomal mapping (see Materials and Methods) and cloning experiments (see below) now demonstrate that the somitabun and piggytail (pgy) complementation groups correspond to the single gene locus of \( \text{smad5} \).

The strongest of the pgy alleles is \( \text{pgy}^{\text{fr4yo}} \), which causes a C2-to-C3 dorsalization in homozygous mutant embryos (Fig. 1G), pgy\(^{\text{fr2226}} \) and pgy\(^{\text{fr2277}} \) homozygotes are weaker, producing predominantly C2 dorsalized mutants. The pgy alleles also display a variably penetrant, dominant maternal–zygotic effect, leading to a semiviable C0.5/C1 dorsalization in heterozygous embryos derived from heterozygous mothers (Fig. 1H; Mullins et al., 1996). Unlike \( \text{sbn}^{\text{fr224}} \), the pgy mutant alleles do not exhibit a strict dominant zygotic effect. Thus, +/+ embryos from +/+ mothers and –/– fathers are phenotypically wild-type (Mullins et al., 1996).

**Isolation of Novel smad5 Alleles**

In an attempt to identify additional regulators of zebrafish dorsoventral patterning, we carried out a screen for dominant enhancers of the chordin mutation \( \text{din}^{\text{1220}} \) (see Materials and Methods), which—among others—led to the isolation of three new smad5 alleles, \( \text{dfr3} \), \( \text{fr4} \), and \( \text{fr5} \). \( \text{dfr3} \) behaves very similar to \( \text{dti216} \), leading to C3 dorsalized homozygous mutants, and to C1 dorsalization of heterozygous embryos from carrier mothers (Figs. 1I and 1J). The \( \text{fr4} \) and \( \text{fr5} \) alleles behave like \( \text{m169} \), causing C1 dorsalized homozygous mutants (Figs. 1K and 1L), and an incompletely penetrant C0.5 dorsalization of heterozygous embryos from carrier mothers (not shown).

Interestingly, some of the heterozygous \( \text{dfr3} \), \( \text{fr4} \), \( \text{fr5} \), \( \text{tc227} \), and \( \text{m169} \) embryos do not only display ventral tail fin deficiencies, but in addition, subtle ventral tail fin duplications in the posterior tip of the tail (Figs. 1M–1P), a phenotypic trait shared with \( \text{din}^{\text{1220}} \) mutants, and enhanced in double heterozygotes, as generated during the screen (see Materials and Methods and Discussion).

The new \( \text{dfr3} \), \( \text{fr4} \), and \( \text{fr5} \) mutations fail to complement each other, as well as \( \text{m169} \), \( \text{dti40} \), and \( \text{dtc24} \) (data not shown), suggesting that they are smad5 alleles. We cloned and sequenced smad5 from homozygous mutants of these new alleles, as well as from the pgy and \( \text{m169} \) alleles (see below), carried out linkage analyses (see Materials and Methods), and confirmed that they are all smad5 mutant alleles.

**The smad5 Mutations**

Smad proteins contain two conserved domains, the N-terminal MH1 domain involved in DNA binding, and the C-terminal MH2 domain, implicated in protein–protein interactions, e.g., during oligomerization and binding to the Tgfβ/Bmp type I receptors (Lo et al., 1998). The previously described smad5 allele \( \text{sbn}^{\text{fr224}} \) contains a missense muta-

![FIG. 2.](image.png)
**FIG. 3.** m169 mutant smad5 mRNA is less stable. (A) RT-PCR with RNA from embryos at 24 hpf (lanes 1–4) and in vitro synthesized control mRNA (lanes 5 and 6). Lane 1, 35 cycles (25 cycles did not give a visible band, due to reduced m169 mutant smad5 mRNA abundance; see below); lanes 2–6, 25 cycles. Lane 1, mutant embryos from a cross of two m169+/H11001 carriers. Lanes 2 and 3, mutant and wild-type embryos from a cross of a m169+/H11001 female and a fr4/H11001 male. Lane 4, embryos from a cross of two wild types. From 169/m169 mutants, only the longer version with the 17-bp insert could be amplified, indicating that no wild-type transcripts are made. From m169/fr4 mutants, mainly the wild-type sized fr4-specific transcript was amplified. (B) Random clonal analysis of RT-PCR products from m169/fr4 transheterozygotes, containing a smad5 cDNA fragment with the site mutated in fr4 (see Materials and Methods). Inserts of individual clones were PCR amplified (upper panel) and digested with HaeIII, cutting the m169, but not the fr4 smad5 cDNA fragment, thereby allowing to discriminate between the two different transcript alleles (lower panel). Forty clones contain the fr4-, six clones the m169-specific smad5 cDNA fragment, indicating that the fr4-specific fragment is seven times more abundant. (C–G) smad5 whole-mount in situ hybridization on embryos from a cross of two m169/+ heterozygotes. Shown embryos were genotyped after photography, as described in Materials and Methods; genotype is given in bottom right corner. (C, D) Shield stage, lateral view, dorsal right; (E–G) 70% epiboly stage, lateral view, dorsal right.
FIG. 4. The smad5 alleles m169, tc227, and fr5 are amorphs; dfr3, dti216, dty40, and dtc24 are hypomorphs and antimorphs. (A, B) Effects of different smad5 mRNAs (WT, dtc24, dty40, dfr3, fr5, tc227, m169) injected into wild-type zebrafish embryos to measure possible dominant negative (antimorphic) effects (A), and injected into sbm[H9251 embryos from a cross of a dtc24/+/ female and a +/+ male to measure possible rescues of dorsalization, indicating whether or not the alleles have residual Smad5 activity (hypomorphic or amorphic; B). Embryos were scored at 36 hpf by dorsalized/ventralized morphology (compare with Fig. 1). All bars represent average percentages obtained in at least 3 independent injections and with at least 200 scored embryos. The strength of dorsalization and ventralization is color-coded, as indicated. Ctr, uninjected embryos. (C) RT-PCR analyses of Xenopus animal caps injected with different smad5 mRNAs (WT, dtc24, dty40, dfr3, fr5, m169) for the ventral marker gene XhoX3 and the control marker Xhis3 (compare with Hild et al., 1999). Note the relatively strong XhoX3 band in dtc24-injected caps (left panel), while no band can be detected in m169-injected caps, although in this experiment, more PCR cycles were run (right panel, compare with intensity of wild-type band). ctr, uninjected animal caps; Est22, complete embryos of stage 22.
mutant smad5 transcripts in transheterozygous embryos (Fig. 3B). These results indicate that the reduction of m169 transcripts occurs in cis rather than in trans, suggesting that it results from an intrinsic effect of the mutation on the mutant mRNA (as expected in the case of reduced stability), rather than from an effect of the protein encoded by the mutant transcript (as expected in case of a positive autoregulation of Smad5 on smad5 transcription, which should affect both allelic versions equally).

Activity Tests of the Various smad5 Alleles

Three different assays were performed to determine the activities of the various Smad5 mutations in comparison with wild-type Smad5 and the previously described sbn^{dc24} allele (Hild et al., 1999). To measure possible dominant negative (antimorphic) effects, the different mRNAs were injected into wild-type zebrafish embryos. At the concentrations used, wild-type smad5 had no or a very weak ventralizing effect, while sbn^{dc24} mutant smad5 led to intermediate-strength dorsalization (see Fig. 4A). Of the tested new alleles, dty40 and dfr3 had dominant negative activities; however, they were weaker than that of sbn^{dc24} (Fig. 4A), consistent with the weaker dominant phenotypes of the corresponding mutants. For fr5, m169, and tc227, not even the weakest dominant negative effect could be detected, and all injected embryos displayed wild-type morphology (Fig. 4A). These data suggest that the semi-dominant effects of the m169, fr5, and tc227 mutations (see Fig. 1) are due to haploinsufficiency, rather than to an antimorphic effect of the mutations. To measure residual ventralizing activities of the different smad5 alleles, two assays were performed, both of which are more sensitive than mRNA injections into wild-type zebrafish embryos: (1) Injection into Xenopus embryos and RT-PCR analyses of animal caps for ventral marker gene expression (Fig. 4C); (2) Injection into sbn^{dc24} zebrafish mutant embryos (Fig. 4B). Under the chosen conditions, wild-type Smad5 led to strong expression of the ventral marker gene Xhox3 in Xenopus animal caps (Fig. 4C) and to a rescue of the dorsalization of sbn mutant zebrafish embryos to almost wild-type condition (Fig. 4B). Interestingly, all tested alleles, the strong dominant negative dtc24 also appears to contain most residual ventralizing Smad5 activity. sbn^{dc24} mutant smad5 mRNA could significantly rescue the dorsalization of sbn^{dc24} mutants (Fig. 4B). We think that sbn^{dc24} acts as a

(12/96) mutant smad5 transcripts in transheterozygous embryos (Fig. 3B). These results indicate that the reduction of m169 transcripts occurs in cis rather than in trans, suggesting that it results from an intrinsic effect of the mutation on the mutant mRNA (as expected in the case of reduced stability), rather than from an effect of the protein encoded by the mutant transcript (as expected in case of a positive autoregulation of Smad5 on smad5 transcription, which should affect both allelic versions equally).

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dominant negative by trapping wild-type Smad5 protein in homomeric complexes with a strongly reduced, but not completely abolished, affinity to Smad4 (Hild et al., 1999).

According to this notion, this reduced Smad4 binding potential can be compensated by increasing the concentration of mutant Smad5 protein, as achieved via the mRNA injections. Some remaining rescuing activity was also found for the weakly dominant negative alleles dty40 and dfr3, whereas the alleles m169, fr5, and tc227 were totally unable to weaken the dorsalization of sbn<sup>−/−</sup> mutants (Fig. 4B). Accordingly, m169 failed to give any detectable Xhox3 expression in Xenopus animal caps, while dfr3, fr4, and fr5 gave rise to a very weak, and dtc24 to a significantly stronger, Xhox3 band (Fig. 4C). Altogether, these results indicate that the m169, fr5, and tc227 alleles are real amorphs, whereas dtc24, dty40, and dfr3 have both antimorphic and hypomorphic potentials.

**m169 Homozygous Adults**

m169 homozygous mutant larvae normally die between day 5 and day 7 of development. However, similar...
to previous reports for swr/bmp2b (Kishimoto et al., 1997; Nguyen et al., 1998), sbn/dtc24/smad5 (Hild et al., 1999), snh/bmp7 (Dick et al., 2000; Schmid et al., 2000), and laf/alk8 mutants (Mintzer et al., 2001), we rescued homozygous m169 mutant embryos by injection of smad5 mRNA at the one- to four-cell stage. When raising injected offspring of two m169/+ parents to adulthood, m169/+/H11001 adult fish were obtained at an average frequency of 3% (20/589), showing that the rescue efficiency was approximately 10%. At an age of 6 months, all obtained m169 homozygous fish were significantly smaller than their heterozygous or wild-type siblings (Figs. 5A and 5B). Nevertheless, both m169−/− males and m169−/− females were fertile and reached sexual maturity at the normal age (4 months).

m169 Mutant Females Produce Abnormally Large Eggs

Despite their reduced body size, m169−/− females lay eggs that are significantly larger than normal eggs (0.9 mm instead of 0.7 mm in diameter, corresponding to 2.1-fold volume). Mm169−/− embryos from homozygous mothers and wild-type fathers remain larger during further development, independent of their own zygotic genotype (Figs. 1C−1L). At the two- or eight-cell stage, it appears that the blastomeres of Mm169−/− embryos are of rather regular size, while it is only the yolk cell which is enlarged (Figs. 5C and 5H). Mm169−/− embryos undergo all morphogenetic movements of gastrulation; however, most likely due to the larger size of the yolk sac, epiboly appears to take longer. Thus, involution starts and the shield is formed when...
the blastoderm has covered 30%, rather than 50%, of the yolk sac (Figs. 5D and 5I). Later, the first somites form before epiboly has finished and the blastopore has been closed (Figs. 5F and 5K). At late gastrula and early segmentation stages, Mm169⁻⁻ embryos display an elongated shape, indicating ectopic extension movements and a strong dorsalization of the embryo (Figs. 5G and 5L).
The Strength of Dorsalization of Mm169ΔΔ Embryos Is Independent of the Zygotic Genotype

Like sbnΔΔΔ mutants, but unlike swr/bmp2b mutants, many Mm169ΔΔ embryos survive beyond 24 hpf. In contrast to sbnΔΔΔ mutants, which are characterized by a curled trunk and tail (Fig. 6B; C4 dorsalization), Mm169ΔΔ embryos fail to form a regularly shaped head. They appear almost perfectly radialized (Figs. 6C and 6D), with the exception of the notochord anlage, which is only present on the dorsal side of the embryo (not shown). We genotyped 46 photographed individual offspring of a cross of a m169ΔΔ female and a m169ΔΔ male (Figs. 6C and 6D; and data not shown), but could not detect any difference in the strength of dorsalization which would have correlated with the genotype of the embryos (+/− or −/−). This indicates the essential role of maternally supplied Smad5, which cannot be complemented by zygotic Smad5.

Mm169ΔΔ Embryos Do Not Respond to bmp2b

We have previously shown that the morphology of sbnΔΔΔ mutants can be rescued to almost wild-type condition by injecting bmp mRNA (Hild et al., 1999; Nguyen et al., 1998). We explained the unexpected ability of an upstream bmp to rescue a downstream smad5 mutant by the presence of other Smads, such as Smad1, acting during later gastrulation stages (Hild et al., 1999). However, smad1 is expressed in a bmp2b/smads5-dependent fashion (Dick et al., 1999), which means that Smad1 could only cause the Bmp susceptibility of sbnΔΔΔ mutants if the early block in Bmp2b/Smad5 signaling was leaky. This notion is further supported by our finding that the sbnΔΔΔ allele can behave like a Smad5 hypomorph in overexpression experiments (see above). In light of these findings, we tested the effect of bmp2b mRNA in the amorphic smad5 allele Mm169. While the strong dorsalization of Mm169ΔΔ embryos could be rescued to a near wild-type condition by injecting smad5 or smad3 mRNA (Figs. 6G and 6H, for gata2), and displayed unaltered dorsalized morphology at 24 hpf (Fig. 6E), though wild-type embryos injected in parallel were strongly ventralized (Figs. 6F, 6I, and 6J). No difference was seen between zygotically homozygous and heterozygous embryos, again pointing to the particular importance of maternally supplied smad5 gene products.

The Pregastrulation Phenotype of Mm169ΔΔ Embryos

In light of the importance of smad5 gene products of maternal origin, we analyzed the pregastrulation phenotype of Mm169ΔΔ embryos in more detail. smad5 displays strong maternal expression in embryos from wild-type mothers (Figs. 7A and 7E; Dick et al., 1999; Hild et al., 1999). In contrast, Mm169ΔΔ eggs appear to be devoid of smad5 mRNA (Fig. 7F), consistent with the aforementioned instability of m169 mutant smad5 transcripts (Fig. 3).

For the sbnΔΔΔ allele, no requirement for Smad5 prior to zygotic Bmp signaling was revealed (Hild et al., 1999). Here, we carried out whole-mount in situ hybridizations and RT-PCR analyses for bmp7 and bmp2b mRNA, two early markers for ventrally specified zebrafish cells (Kishimoto et al., 1997; Nguyen et al., 1998; Schmid et al., 2000; Dick et al., 2000). In situ hybridizations revealed significantly reduced bmp7 mRNA levels in Mm169ΔΔ blastulas, whereas no reduction was detectable in sbnΔΔΔ, bmp2b/swr, or bmp2b/snh mutants (Figs. 7G and 7H; and data not shown). Consistent results were obtained in RT-PCR analyses, in which offspring of m169ΔΔ females were compared with offspring of homozygous swr/bmp2 mutant parents (Kishimoto et al., 1997; Nguyen et al., 1998) and with wild-type embryos at late blastula stages (sphere; Fig. 7I). These analyses revealed significantly reduced bmp7 mRNA levels in Mm169ΔΔ embryos, while bmp2b mRNA levels were normal. In contrast, swr/bmp2b mutant embryos of the same stage displayed normal bmp7 levels (Fig. 7I). At the onset of gastrulation (shield stage), both bmp2b and bmp7 mRNA levels in Mm169ΔΔ mutants are more strongly reduced than in swr/bmp2b mutants (Figs. 7J–7O), consistent with previous results obtained in a comparison of bmp2b and bmp7 expression in sbnΔΔΔ and swr/bmp2b mutants (Hild et al., 1999; Schmid et al., 2000). Together, these results suggest that maternally supplied Smad5 functions in early ventral specification of zebrafish blastomeres prior to zygotic Bmp2b/Bmp7 signaling. The nature of the signals mediated by such maternal Smad5 remains unclear. Like Smad5 itself, they are likely to be of maternal origin. A prime candidate is the Gdf6-related Radar, which has been previously reported to be maternally expressed and to have ventralizing activity (Goutel et al., 2000). Other candidates are Bmp4 and Bmp7, whose mRNAs are also present in zebrafish oocytes, whereas bmp2b mRNA appears to be absent (Figs. 7B–7D; see also Discussion).

Marker Gene Expression in Gastrulating Mm169ΔΔ Embryos

Consistent with the situation at pregastrula stages, Mm169ΔΔ embryos are more strongly dorsalized than bmp2b/swr and bmp7/snh embryos during gastrulation and later stages of development. At shield stage, the dorsal organizer region, marked by the expression of goosecoid (Stachel et al., 1993) and chordin (Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997), is slightly enlarged in Mm169ΔΔ embryos, but normal in bmp2b and bmp7 mutants (Figs. 8A–8D; and data not shown); Miller-Bertoglio et al., 1997; Mullins et al., 1996). Similarly, at midgastrula stages, the expression domain of the neural marker foxb1.2 is ventrally fused both in anterior (animal) and in posterior (marginal) regions of Mm169ΔΔ embryos, but only in posterior regions of bmp2b mutants (Figs. 8E–8G). Furthermore, at the one- to two-somite stage, expression of flh...
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(Talbot et al., 1995), a marker of dorsal diencephalon (Figs. 8I and 8J), is absent in Mm169⁻/⁻ embryos (Fig. 8K). A similar phenotype has previously been reported for wildtype embryos injected with maximum amounts of noggin mRNA (Barth et al., 1999), whereas in null bmp2b, bmp7 (shn<sub>212</sub>, Schmid et al., 2000), and bmp2b; bmp7 double mutants, flh is present, but shifted ventrally (Fig. 8L; and data not shown; Barth et al., 1999). In addition, Mm169⁻/⁻ embryos display ectopic flh expression in ventral regions of the mesoderm, indicating ectopic ventral notochord specification (Fig. 8M), whereas bmp2b, bmp7, and bmp2b; bmp7 double mutants show regular mesodermal flh expression restricted to the dorsal midline (Fig. 8N; and data not shown). Altogether, the data indicate that Mm169⁻/⁻ embryos are more strongly dorsalized than any of the thus far described zebrafish bmp mutants. Still, this strong dorsalization appears to solely result from blocked Bmp signaling, as a similar phenotype can be achieved by Bmp inhibition via introduction of the Bmp antagonist Noggin (Barth et al., 1999).

In addition, and unlike bmp2b mutants, late gastrula and early segmentation Mm169⁻/⁻ embryos display a general compression of the head region, as for instance indicated by the krox20 expression pattern in the hindbrain (Figs. 8O and 8P), although all anterior marker genes are expressed, such as otx2 (Fig. 8H), six3.1, and anf (data not shown). This head compression does not seem to be a secondary consequence of the delayed epiboly movements of Mm169⁻/⁻ embryos, as it is also observed in embryos injected with smad5 antisense morpholino oligonucleotides, which are of normal size and display normal epiboly (Lele et al., 2001).

DISCUSSION

We have previously shown that the strongly dorsalized phenotype of embryos from somitabun sbn<sub>9524</sub> mothers is caused by dominant negative mutation in smad5, leading to a block in Bmp signaling (Hild et al., 1999). Since dtc24 mutant mRNA could also block the ventralizing activity of other Smad family members, it remained open to which extent Smad5 itself is essential for ventral development. Therefore, it was important to identify and examine amorphic smad5 alleles.

The cpt and pgy Complementation Groups

In this paper, we describe seven additional smad5 alleles: three new isolates, and four previously described mutations, m169, tc227, dti216, and dti40. Furthermore, we have determined that the tentative assignment of somitabun and piggytail as independent genes can now be resolved into a single complementation group. The m169 allele, isolated in an independent screen, was previously assigned to the captain hook (cpt) complementation group, consisting of two alleles, m169 and m52 (Solnica-Krezel et al., 1996). m52 has been identified as an allele of the Bmp inhibitor dino/chordin (Miller-Bertoglio et al., 1999), which is in line with our isolation of novel smad5 alleles in a dino enhancer screen (see also below). Together, it appears that cpt and pgy are not independent complementation groups.

The Novel Amorphic smad5 Alleles and the Kinetics of smad5 mRNA Generation and Decay

Of the seven newly described smad5 alleles, three proved amorphic in different overexpression assays, lacking any residual ventralizing (hypomorphic) and any dorsalizing (dominant negative, antimorphic) activity.

For the m169 allele, we could further show that the mutant smad5 mRNA is extremely unstable. This allowed us to investigate the kinetics of the generation and decay of maternal and zygotic wild-type smad5 transcripts. In the offspring of two m169 heterozygotes, mutant embryos display strongly reduced transcript levels at the onset of gastrulation, whereas the wild type siblings do not, although they were both supplied with the same amount of maternal transcripts. Thus, the differences are due to the decay of zygotic m169 transcripts, indicating that zygotic smad5 transcription must start during blastula stages. It also shows that, by the onset of gastrulation, smad5 transcripts are largely of zygotic origin. Since the total levels of smad5 transcripts at early gastrula stages are not higher than in freshly laid eggs (Hild et al., 1999), it can be further concluded that maternally supplied wild-type smad5 transcripts are degraded during the first 5 h of development.

Smad5 Is Essential for Ventral Development and Bmp Signal Transduction

In contrast to the strongly dorsalized offspring of dtc24 heterozygous mothers (C4), homozygous embryos deriving from mothers heterozygous for the amorphic smad5 mutations are only mildly dorsalized (C1–C2; Fig. 1). This is due to maternal wild-type smad5 gene products, which largely compensate for the loss of zygotic smad5. To obtain the smad5 null phenotype, we generated m169⁻/⁻ adult females. m169 homozygous females gave offspring which were more strongly dorsalized than all thus far described mutants (C6; Fig. 6; for review, see Hammerschmidt and Mullins, 2002). Since m169 is a null mutation, we can conclude that Smad5 is absolutely essential for ventral specification, a statement which had not been possible after the analysis of the dtc24 allele, an antimorph that could have caused the dorsalization indirectly by inhibiting Smad1, Smad8, or other related family members present in gastrulating zebrafish embryos (Dick et al., 1999; Müller et al., 1999; M.N., Z. Lelé and M.H., unpublished data). Also, we can conclude that Smad5 is essential for Bmp signaling, since Mm169 mutants, in contrast to Mdtc24 mutants, do not respond to injected bmp2b mRNAs. Interestingly, this Bmp2b unresponsiveness is independent of the zygotic genotype of the Mm169 mutants. Thus, heterozygous embryos do not respond to injected bmp2b mRNA, although

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they should acquire zygotic smad5 mRNA from blastula stages onwards. This indicates that Smad5 must act during very early stages of zebrafish development, most likely before zygotic Bmp2b is present. Therefore, we would like to propose a model, according to which the dependence of Bmp2b signaling on Smad5 might be twofold. First, Smad5 might act as a direct transducer of Bmp2b signaling downstream of the Bmp2b signal, as previously proposed (Dick et al., 1999; Hild et al., 1999). Alternatively or in addition, Smad5 could act before Bmp2b comes into the play, downstream of a thus far unknown ligand to induce the expression of a component of the Bmp2b signaling pathway, such as the Bmp2b partner Bmp7 (Schmid et al., 2000; see below).

A Ventralizing Role of Smad5 Prior to Zygotic Bmp2b/7 Signaling

Further support for such an earlier function of Smad5 comes from results obtained in a comparison of the dorsalized phenotypes of maternal m169 and zygotic bmp2/7 mutants (neither bmp2b nor bmp7 mutations do not have a maternal effect; Kishimoto et al., 1997; Dick et al., 2000; Schmid et al., 2000). Mm169 mutants are not only more strongly dorsalized than bmp2b, bmp7, and bmp2b; bmp7 double mutants during gastrula stages, their dorsalization is also manifested much earlier than in mutants lacking zygotic Bmp2b signals. Thus, Mm169−/− embryos display significantly reduced bmp7 mRNA levels during blastula stages, when bmp2b mutants are still normal. Interestingly, bmp2b mRNA levels appear to be normal in Mm169 mutant embryos of the blastula stage, suggesting that Smad5 is particularly involved in mediating signals promoting early zygotic expression of bmp7. This appears to happen before the onset of zygotic smad5 expression at blastula stages, since the phenotype is solely caused as a maternal effect and cannot be compensated by zygotic smad5 gene products. Together, the data indicate the presence of a Smad5-dependent signaling process required to set up the initial broad bmp7 expression pattern during blastula stages, thereby shedding first light on the thus far open question how ventral specification is initiated during the first phase of DV patterning (see Introduction).

The signals initiating the expression of bmp7 (through Smad5) and bmp2b are currently not known. Like smad5, they are most likely of maternal origin. Bmp4 and Bmp7 are good candidates, as their mRNAs are present in developing oocytes. However, maternal Bmp7 mutants are of wild-type morphology, and maternal–zygotic Bmp7 mutants are not more strongly dorsalized than zygotic mutants (Dick et al., 2000; Schmid et al., 2000), indicating that Bmp7 is not essential for this early ventralizing process. Another candidate is Radar, a maternally expressed Bmp found in freshly laid eggs, which has early ventralizing activity (Goutel et al., 2000).

Various Bmp receptors might be involved in this early process. One of them is Alk8 (Payne et al., 2001; Yelick et al., 1998). Like smad5, alk8 is both maternally and zygotically expressed and absolutely essential for ventral specification, as indicated by the strongly dorsalized phenotype of MZ1alf/alk8 mutants (Bauer et al., 2001; Mintzer et al., 2001). However, there are crucial differences between smad5/m169 and alk8/laf mutants. While in the case of smad5, loss of maternal gene products (Mm169−/−) leads to strong dorsalization independently of the zygotic genotype of the embryos, both maternal and zygotic alk8 gene products have to be affected to yield strongly dorsalized laf mutants (Mintzer et al., 2001). In other words, zygotic alk8 gene products can compensate for the loss of maternal contribution, whereas zygotic smad5 products cannot. Also, in contrast to Mm169−/− embryos, the dorsalization of MZ1alf−/− embryos is not stronger than that caused by the loss of zygotic Bmp2b and/or Bmp7 signaling. Thus, it appears likely that Alk8 is required later than Smad5, only being essential for mediating zygotic Bmp2b/7 signaling. In this case, the early Smad5-dependent signals would be mediated by other maternally expressed type I receptors which act instead of, or in addition to Alk8 (thereby making Alk8 dispensable), such as BmpR1b/Alk6 or BmpR1a/Alk3 (Nikaido et al., 1999a,b; Goutel et al., 2000; Bauer et al., 2001).

Is Smad5 Also Involved in Antagonizing Ventral Specification?

Although zygotic smad5 gene products do not compensate for the loss of maternal smad5 function, they are essential for normal development, as indicated by the zygotic m169 phenotype (Zm169), affecting both DV patterning and other processes. Concerning DV patterning, it might appear surprising that the zygotic effect of m169 and other smad5 alleles does not only cause partial loss of ventral tail fin tissue, indicative for mild dorsalization, but also subtle duplications in adjacent posterior regions of the ventral tail fin (Figs. 1M–1O). Ventral tail fin duplications are normally interpreted as signs for a weak ventralization, pointing to a role of the mutated gene as a Bmp antagonist. However, this does not necessarily have to be true for smad5. In all smad5 alleles, including the three amorphs, the duplications are only found in heterozygous embryos, suggesting that they result from a reduction in the dose of smad5 gene products (haploinsufficiency). Normally, the concentration of the Bmp ligands is the limiting factor of Bmp signaling, while components of the signal transduction pathway, including Smad5, are present in excess. In Zm169−/− embryos, however, the reduced Smad5 levels appear to become limiting in those cells requiring highest Bmp signals (highest phospho-Smad5 levels), while all other cell types can specify normally. Along these lines, we propose that the ventral tail fin cells missing in Zm169−/− mutants require maximum phospho-Smad5 levels, followed by the adjacent posterior-most cells of the ventral tail fin, which require slightly lower levels. In the mutant, maximum phospho-Smad5 levels cannot be reached, due to the overall reduction in Smad5 protein. As a result, cells
normally forming anterior ventral tail fin tissue would adopt the fate of their posterior neighbors, which require Bmp signaling levels that can still be achieved under the reduced Smad5 conditions. In sum, this leads to the combined gain and loss of cells in adjacent regions of the ventral tail fin. Thus, we need not hypothesize that the ventralized traits of weak smad5 mutants are due to a Bmp-antagonistic role of Smad5.

This same line of reasoning can be explained why smad5 and chordin mutations enhance each other in double heterozygotes (the novel smad5 alleles were isolated in a dino enhancer screen; see Materials and Methods). In addition to its early role as a Bmp antagonist, Chordin can promote Bmp signaling when acting in concert with the Chordin-protease Tolloid to carry Bmps into ventral-most regions of the tailbud-stage embryo. As a consequence, loss of chordin in dino mutants causes subtle ventral tail fin deficiencies adjacent to ventral tail fin duplications more posteriorly (Hammerschmidt and Mullins, 2002; Wagner and Mullins, 2002), very similar to the combined phenotype of smad5 mutants. Thus, the reduced capability to generate highest Bmp concentrations in ventral-most regions of the tailbud due to reduced Chordin activity, in combination with reduced levels of Smad5, should synergistically promote “second class” ventral fates at the expense of ventral-most fates in double heterozygous embryos, as observed.

Later Roles of Zygotic Smad5 during Zebrafish Growth and Oogenesis

In mouse, Smad5 is not required for DV patterning, but for multiple later processes (Chang et al., 1999, 2000; Yang et al., 1999; Chang and Matzuk, 2001). Similarly, zebrafish Smad5 appears to fulfill various later roles. It remains ubiquitously expressed throughout segmentation stages (C.K., T.M., and M.H., unpublished data). In addition, we found strong expression in the ovaries of mature females, in oocytes of all developmental stages (Fig. 5A).

Consistent with the oogonal smad5 expression, we found defects during oogenesis of m169 mutant females, which laid larger eggs than their siblings. In particular, the volume of the yolk appears increased. This suggests that Smad5 might be a negative regulator of oocyte growth during the vitellinogenic phase of oogenesis.

In addition, Smad5 appears to positively regulate the general growth of juvenile fish. Rescued m169 mutant zebrafish grow more slowly and are significantly smaller than their wild-type siblings, similar to the phenotype described for rescued alk1/alk2 mutants (Mintzer et al., 2001). Together, these data suggest that Alk8/Smad5-dependent Bmp signaling is an essential component regulating zebrafish growth. Interestingly, a similar effect of Bmp signaling has been found in the nematode Caenorhabditis elegans. Here, loss-of-function mutations in Bmp homologues, a type I receptor, and the Smad proteins SMA-2, -3, and -4 lead to reduced body length (for review, see Patterson and Padgett, 2000). Further analyses of Zm169 embryos will help to shed more light on the diverse roles of zygotic Smad5 during zebrafish development.

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