lost-a-fin encodes a type I BMP receptor, Alk8, acting maternally and zygotically in dorsoventral pattern formation

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INTRODUCTION

A bone morphogenetic protein (BMP)/TGFβ signaling pathway is a key component in the generation of diverse cell types along the dorsoventral axis in vertebrates and invertebrates. BMP signaling activity is thought to act as a morphogen, with different activity levels specifying different cell fates along the dorsoventral axis (Dosch et al., 1997; Jones and Smith, 1998; Knecht and Harland, 1997; Mullins, 1998; Neave et al., 1997; Nguyen et al., 1998b; Wilson et al., 1997). The BMP activity gradient is believed to be generated by the diffusion of dorsally expressed, secreted BMP antagonists into ventral regions. A gradient of BMP antagonists thus produces a reciprocal gradient of BMP activity, with highest BMP activity levels in the most ventral regions of the embryo.

A model for BMP signal transduction has been elucidated (reviewed by Massagué, 1998; Whitman, 1998). The BMP ligands bind as dimers to BMP receptors. The receptors are composed of two distinct classes of transmembrane serine-threonine kinases, type I and type II. The receptors are thought to form a tetrameric complex binding two BMP dimers. Upon binding of the BMP ligands to the receptor complex, the type II receptor phosphorylates and activates the type I receptor. The activated type I receptor then directly phosphorylates the downstream receptor-regulated Smad proteins, Smad1, Smad5, and Smad8, which translocate to the nucleus and regulate the transcription of target genes.

SUMMARY

TGFβ signaling pathways of the bone morphogenetic protein (BMP) subclass are essential for dorsoventral pattern formation of both vertebrate and invertebrate embryos. Here we determine by chromosomal mapping, linkage analysis, cDNA sequencing and mRNA rescue that the dorsalized zebrafish mutant lost-a-fin (laf) is defective in the gene activin receptor-like kinase 8 (alk8), which encodes a novel type I TGFβ receptor. The alk8 mRNA is expressed both maternally and zygotically. Embryos that lack zygotic, but retain maternal Laf/Alk8 activity, display a weak dorsalization restricted to the tail and die by 3 days postfertilization. We rescued the laf dorsalized mutant phenotype by alk8 mRNA injection and generated homozygous laf/alk8 mothers to investigate the maternal role of Laf/Alk8 activity. Adult fish lacking Laf/Alk8 activity are fertile, exhibit a growth defect and are significantly smaller than their siblings. Embryos derived from homozygous females, which lack both maternal and zygotic Laf/Alk8 activity, display a strongly dorsalized mutant phenotype, no longer limited to the tail. These mutant embryos lack almost all gastrula ventral cell fates, with a concomitant expansion of dorsal cell types. During later stages, most of the somitic mesoderm and neural tissue circumscribe the dorsoventral axis of the embryo. Zygotic laf/alk8 mutants can be rescued by overexpression of the BMP signal transducer Smad5, but not the Bmp2b or Bmp7 ligands, consistent with the Laf/Alk8 receptor acting within a BMP signaling pathway, downstream of a Bmp2b/Bmp7 signal. Antibodies specific for the phosphorylated, activated form of Smad1/5, show that BMP signaling is nearly absent in gastrula lacking both maternal and zygotic Laf/Alk8 activity, providing further evidence that Laf/Alk8 transduces a BMP signal. In total, our work strongly supports the role of Laf/Alk8 as a type I BMP receptor required for the specification of ventral cell fates.

Key words: Dorsoventral patterning, Alk, Type I receptor, Smad, BMP, TGFβ, Zebrafish
and a reciprocal expansion of dorsal tissue is observed (Schmid et al., 2000). The maternally and zygotically expressed downstream-acting \( smad5 \) gene, when mutated in \( somitabun \) (Hild et al., 1999), causes a similar dorsaled phenotype (Mullins et al., 1996; Nguyen et al., 1998b).

Mutations in these genes in the mouse either do not cause defects in dorsoventral axis formation or the mutants die at a stage prior to establishment of the dorsoventral axis (Chang et al., 1999; Dudley et al., 1995; Luo et al., 1995; Winnier et al., 1995; Yang et al., 1999; Zhang and Bradley, 1996).

In \( Drosophila \), two \( bmp \) family members are required for the specification of dorsal cell fates. Null mutations in the ligand \( decapentaplegic \) (\( dpp \)), the \( Bmp2/4 \) orthologue, eliminate all dorsal structures, whereas null mutations in the ligand \( screw \) eliminate only a subset of dorsal structures. Consistent with their distinct roles in pattern formation, these ligands signal through different type I receptors. \( Dpp \) functions through Thickveins, whereas \( Screw \) signals through Saxophone (Neul and Ferguson, 1998; Nguyen et al., 1998a). In zebrafish, loss-of-function analysis has demonstrated that the ligands encoded by \( swirl/bmp2b \) and \( snh/bmp7 \) are equally required for specifying ventral cell fates (Schmid et al., 2000). The nature of the receptors through which these ligands signal has not yet been defined genetically.

In this study, we determined that the dorsaled zebrafish mutant \( lost-a-fin \) (\( laf \)) is defective in activin receptor-like kinase 8 (\( alk8 \)), a novel TGF\( \beta \) receptor (Yelicik et al., 1998). Our findings from rescue experiments with BMP pathway components and analysis with antibodies specific for the phosphorylated form of the BMP signal transducer, \( Smad1/5 \), show that \( Laf/Alk8 \) acts as a BMP receptor within a \( Bmp2b/Bmp7 \) signaling pathway. To investigate the role of maternally expressed \( laf/alk8 \) mRNA, we generated homozygous mutant \( laf/alk8 \) mothers. Embryos lacking both maternal and zygotic \( Laf/Alk8 \) activity display a strongly dorsaled mutant phenotype similar to the strongly dorsaled mutants \( swirl/bmp2b \) and \( snh/bmp7 \) and \( somitabun/ \( smad5 \), demonstrating that the \( Laf/Alk8 \) receptor is necessary for the specification of nearly all ventral cell fates in the zebrafish embryo.

**MATERIALS AND METHODS**

**Chromosomal mapping and linkage analysis**

Crosses between a \( T\(\)U strain containing the \( laf^{pm110b} \) mutation and either a polygenic WIK or AB strain were used to generate mapping cross lines. The mapping procedure and the WIK line were described previously (Rauch et al., 1997; Knapp et al., 1996). Pools of about 25 \( F2 \) \( laf^{pm110b} \) and wild-type siblings were collected separately and stored in methanol at \(-20°C\). DNA isolation and PCR were performed as described by Gates et al. (Gates et al., 1999), with similar PCR conditions to those described by Connors et al. (Connors et al., 1999).

Single \( F2 \) mutant and wild-type embryos from \( laf^{pm110b} \) mapping cross lines were tested for segregation of the \( laf^{pm110b} \) mutation from an \( MspI \) RFLP in the \( alk8 \) 3' UTR. The 3' UTR was amplified by PCR with primers: \( alk81944F \): GAGGCAACAAAAAGGACAGACG and \( alk82598b \): AAAAAAGACAGCGTGAGCTTC.

Cloning of \( alk8 \) cDNA and construction of expression vectors

About 50 mutant embryos were collected at 27-30 hours postfertilization (hpf) from a cross between \( laf^{pm110b} \) heterozygotes. Control wild-type embryos from the \( T\(\)U strain (the strain in which the mutation was induced) were also collected at 27-30 hpf. Total RNA was extracted with TRIzol Reagent (Gibco/BRL) according to the manufacturer's instructions. First strand cDNA was synthesized from total RNA using the Superscript Preamplification System for First Strand Synthesis (Life Technologies). At least two independent PCR reactions were conducted on wild-type or mutant embryo cDNA to isolate the full-length coding region of \( alk8 \) (Yelicik et al., 1998). The primers used were: \( alk8147F \): GCCTGAGAGTCCTAGGGGAGTTG and \( alk81755B \): ATATAGGACGCGTGAGCTGCTTAG. PCR conditions were: 94°C, 3 minutes then 40 cycles: 58.5°C, 1 minute; 70°C, 2 minutes; 94°C, 1 minute; followed by 70°C, 20 minutes.

PCR products were subcloned into the pGEM-T Easy Vector System (Promega) according to manufacturer's instructions. Clones from independent PCR reactions were sequenced using \( alk8 \)-specific primers. Sequence analysis was performed using Sequencer software (Gene Codes Corporation). Sequenced \( laf/alk8 \) and \( laf^{pm110b} \) inserts from pGEM-T Easy vectors were subcloned into the EcoRI site of the expression vector pCS2+ (Rupp et al., 1994).

**Genotypic analysis for the \( laf^{pm110b} \) allele**

The missense mutation responsible for the \( laf^{pm110b} \) allele creates a CfoI site, which was used for genotypic analysis. Genomic DNA was isolated from embryos or adult fish according to the method of Gates et al. (Gates et al., 1999 and Connors et al., 1999). DNA was amplified by PCR with primers \( alk8421F \): CTCGCTAGTCAGTTGTGAGGATGCTG and \( alk8498B \): GCAGTCGAAGTAGGGTCTCTTTGG. The PCR product was digested with CfoI and separated on a 3% MetaPhor agarose (BMA, USA) gel.

**mRNA microinjection**

\( p64Tzbmp2 \) (Nikaido et al., 1997), pCS2+\( \cdot \)bmp7 (Schmid et al., 2000), and \( Smad5/pCS2+ \) (G. R. and M. C. M., unpublished) were linearized with I. mRNA was transcribed with the SP6 mMessage mMachne Kit (Ambion). mRNA was injected into the yolk of 1- to 2-cell stage embryos as described by Westerfield (Westerfield, 1995).

**Maternal expression of \( laf/alk8 \)**

50 wild-type embryos from the \( T\(\)U strain were collected at the one cell stage. Total RNA was extracted and used in first strand cDNA synthesis as described above. \( laf/alk8 \) cDNA was amplified by PCR with primers \( alk8844F \): TGTGGAGAGTCATGCTCAATGGCAAGG and \( alk81755B \) (sequence above).

**In situ hybridization**

In situ hybridizations were performed as described previously (Schulte-Merker et al., 1992). The probes used were: \( dix3 \) (Akimenko et al., 1995), \( myoD \) (Weinberg et al., 1996), \( foxb1.2 \) (formerly \( fkd3 \)) (Odenthal and Nüsslein-Volhard, 1998), \( krox20 \) (Otxoby and Jowett, 1999), \( gata2 \) (Detrich et al., 1995), and \( pax2.1 \) (Krauss et al., 1992). Embryos were viewed with a Zeiss Axioplan 2 microscope and scanned with a Progress 3012 digital camera (Kontron Elektroniks). Scanned images were processed with Adobe software.

**Western blot analysis**

Zebrafish embryos were lysed by pipetting in buffer (20 mM Tris pH 8, 50 mM NaCl, 2 mM EDTA, 1 mM NaVO\(_4\), 1% NP-40) with added inhibitors (20 \( \mu \)g/ml aprotinin, 40 \( \mu \)g/ml leupeptin, 4 \( \mu \)g/ml pepstatin, 0.75 \( \mu \)M PMSF, 25 \( \mu \)M \( \beta \)-glycerophosphate). Lysates were centrifuged at 14,000 \( g \) at 4°C for 10 minutes. Supernatants were transferred to new tubes, and equal volumes of 2x Laemmli sample buffer were added. Before analysis, two volumes of sample buffer were added to ensure solubilization of proteins and to facilitate their optimal separation. Samples (1 embryo equivalent per lane) were subjected to 7.5% SDS-polyacrylamide gel electrophoresis and were...
analyzed by western blot analysis as described previously (Faure et al., 2000).

**Immunohistochemistry**

Zebrafish embryos were fixed in 4% paraformaldehyde in PBS overnight. After transfer to PBT (PBS plus 0.1% Triton X-100 and 2 mg/ml BSA), they were manually dechorionated with forceps and were dehydrated stepwise into 100% methanol for storage. After rehydration into PBT and PBTD (PBT plus 1% DMSO), embryos were blocked in PBT and PBTD plus 10% normal goat serum (NGS, Jackson ImmunoResearch Laboratories, Inc.) that had been dialyzed against PBS to remove sodium azide. Embryos were then incubated with primary antibody (1:10) in PBT and PBTD plus 10% NGS at 4°C overnight. After washing with four changes over 2 hours in PBTD plus 1% NGS, embryos were incubated with secondary goat anti-rabbit IgG-HRP antibody (1:250, Boehringer Mannheim) in PBTD plus 10% NGS for 2 hours at room temperature and was washed again. Staining was developed with ImmunoPure Metal-enhanced DAB Substrate Kit (Pierce), as per manufacturer’s instructions, for 1-2 minutes. For microscopy and photography, embryos were cleared in benzy alcohol:benzylbenzoate (1:2) and then mounted in Canada Balsam:methyl salicylate (9:1). Images were photographed as described above.

**RESULTS**

*lost-a-fin is a mutation in the TGFβ type I receptor alk8*

We have previously described mutants of several genes that are essential for specifying ventral regions of the zebrafish embryo. One of these dorsalized mutants not yet characterized is *lost-a-fin* (*laf*) ( Mullins et al., 1996; Solnica-Krezel et al., 1996), which we examine here. We can first detect the *laf* zygotic mutant phenotype morphologically at bud stage (end of gastrulation), as a slight protrusion of the tail bud (data not shown). The phenotype becomes more pronounced during somitogenesis, as the tail bud protrudes further and does not extend as far around the yolk as in wild-type embryos, maintaining a more vegetal position than wild type (Fig. 1A,B). At 1 dpf (day postfertilization) *laf* mutant embryos lack the ventral tail fin and vein (Fig. 1C,D), but display a tail of normal length. Affected embryos fail to develop blood circulation, probably due to loss of the tail vein, and die by 3 dpf.

As a first step to determine the molecular nature of the *laf* mutation, we mapped the mutation to a chromosomal position using simple sequence length polymorphic (SSLP) markers. We found that the *laf* mutation is located on linkage group 2 in the 1.3 cM interval between SSLP markers Z21490 and Z11023 (Fig. 2A). The TGFβ type I receptor, *alk8* (Yelick et al., 1998), had also been linked to Z11023 using a radiation hybrid panel (WashU-Zebrafish Genome Resources Project; Geisler et al., 1999; Fig. 2A). Since other dorsalized mutants had been found to be components of a TGFβ signaling pathway, the linkage of *alk8* and *laf* to the same marker, made *alk8* a good candidate for the *laf* gene.

To test directly if *laf* corresponds to the *alk8* gene, we first examined linkage of *alk8* to the *laf* mutation. We followed the segregation of an RFLP in the 3’ UTR of *alk8* in mutant *laf* and sibling F2 embryos from a mapping cross. In the 96 meioses tested, the polymorphism always segregated with the mutation, placing the *laf* mutation within 1 cM of the *alk8* gene (data not shown).

To determine if *laf* is a mutation in the *alk8* gene, we cloned and sequenced the *alk8* cDNA from *laf* mutant embryos. The *alk8* cDNA from mutant embryos contains a single missense mutation, which changes a highly conserved cysteine in the extracellular domain in an arginine residue (Fig. 2B). Type I and type II TGFβ receptors possess 10 characteristic cysteine residues in their extracellular domains (reviewed by Massagué, 1998). The 3 most cysteine residues are highly conserved in their positions relative to the transmembrane domain. The third of these cysteines is altered in the *laf* mutant. This cysteine has been shown to form an intramolecular disulfide bond in the human type I BMP receptor IA and the murine type II activin receptor (Greenwald et al., 1999; Kirsch et al., 2000). The highly conserved nature of this residue and its presumptive disulfide bond formation in all type I and type II receptors indicates a critical role for it in receptor function. Therefore, substitution of this amino acid is likely to greatly impair receptor activity.

To demonstrate that the *laf* and *alk8* mutation is responsible for the *laf* mutant phenotype, we examined the rescuing activity of both wild-type and *laf* mutant *alk8* mRNA following injection into 1- to 2-cell stage *laf* mutant embryos. We measured rescue as a lessening of the *laf* mutant phenotype from its class 2 dorsalized phenotype (Fig. 1D), to a weaker class 1 dorsalization or a wild-type phenotype. Wild-type *alk8* mRNA rescued the ventral tail mutant phenotypes in greater than 95% of the *laf* mutant embryos (Fig. 2C). As little as 30 pg of wild-type *alk8* mRNA per embryo was sufficient for rescue. In contrast, an almost fivefold larger amount (140 pg/embryo) of mutant *laf* mRNA failed to rescue *laf* mutant embryos, thus demonstrating the critical nature of the mutated cysteine residue in *laf*. The linkage, cDNA sequence analysis, and rescue assays together demonstrate that *laf* is a mutation in the *alk8* gene.

**The Laf/Alk8 type I receptor is a component of a BMP signaling pathway**

The *alk8* gene encodes a novel TGFβ type I receptor, which was first identified by Yelick et al. (Yelick et al., 1998), in the zebrafish. It is most related to Alk2, with 65% overall identity. TGFβ receptors are considerably more divergent in their extracellular domains compared to their intracellular kinase domains. This is also true for Alk8 and human ALK2, which are only 30% identical in their extracellular domains (see also Yelick et al., 1998). In examining the phylogenetic relationship of Alk8 to other TGFβ type I receptors, we find that Alk8 is most closely related to a group of receptors, which include the vertebrate Alk1 and Alk2, and fly Saxophone type I receptors (Newfeld et al., 1999, data not shown). Depending on the nature of the type II receptor, Alk1 can bind activin and TGFβ (Lux et al., 1999; Oh et al., 2000), and Alk2 can bind activin and Bmp7 ligands (Attisano et al., 1993; ten Dijke et al., 1994; Yamashita et al., 1995) and can activate BMP signaling pathways (Armes and Smith, 1997; Faure et al., 2000). Molecular-genetic studies in *Drosophila* indicate that Saxophone transduces the activity of the Screw and Gbb-60A BMP-related ligands (Haerry et al., 1998; Neul and Ferguson, 1998; Nguyen et al., 1998a). The ability of various TGFβ
family ligands to bind or activate these type I receptors brings into question the nature of the signal(s) that Alk8/Laf transduces.

To test whether Laf/Alk8 functions as a component of a BMP signaling pathway, we assayed for the ability of the Bmp2b and Bmp7 ligands and the Smad5 intracellular signal transducer to rescue the laf/alk8 dorsalized mutant phenotype. We reasoned that if Laf/Alk8 is a BMP receptor in this pathway, then the downstream effector, Smad5, is predicted to rescue the laf/alk8 mutant phenotype, whereas the upstream-acting Bmp2b and Bmp7 ligands should not.

We overexpressed bmp2b and bmp7 mRNAs (Nikaido et al., 1997; Schmid et al., 2000) and observed no rescue of laf/alk8 mutant embryos. Approximately the same fraction of mutants was found in the injected as the uninjected embryos (Table 1). One concern in performing these experiments is that the injected mRNA may be degraded by the time tail tissue specification occurs and consequently no rescue would be possible. To control for this possibility, we injected amounts of bmp2b or bmp7 mRNA sufficient to cause duplications of the ventral tail fin in the wild-type sibling embryos (40% and 23% for bmp2b and bmp7, respectively), indicating that the mRNA is present at the time of tail patterning. Moreover, to determine if a small number of mutants was rescued, but not detectable by a significant change in the number of mutant embryos, we genotyped all the non-mutant embryos for the laf/alk8 mutation in one experiment. We never found a homozygous laf/alk8 embryo that had a wild-type or ventralized phenotype (0/97 for bmp2b and 0/64 for bmp7; Table 1), consistent with the Laf/Alk8 receptor functioning downstream of the Bmp2b and Bmp7 ligands.

In contrast, injection of smad5 mRNA (G. R. and M. C. M., unpublished) into laf/alk8 mutant embryos reduced the percentage of class 2 dorsalized embryos from an expected 25% to 17.7% (Table 1). This reduction is consistent with smad5 rescuing a fraction of the laf/alk8 mutant embryos. As unambiguous proof that smad5 can rescue laf/alk8 mutant embryos, we genotyped all the class 1 and wild-type embryos from a smad5 injection experiment. All the class 1 embryos and 5% of the phenotypically normal or ventralized embryos (n=101) were laf/alk8 homozygotes, demonstrating that smad5 can rescue the laf/alk8 dorsalized mutant phenotype. The low fraction of rescued laf/alk8 mutant embryos may be due to the lower activity of the non-phosphorylated, overexpressed smad5. A type 1 BMP receptor normally phosphorylates and activates Smad5. Therefore, the rescue we observed probably represents the ability of Smad5 to function without phosphorylation when highly overexpressed. Our findings are consistent with the Laf/Alk8 receptor functioning within a BMP signaling pathway and acting downstream of the Bmp2b and Bmp7 ligands and upstream of the receptor-associated Smad, Smad5.

**Maternal expression of laf/alk8**

The ability of Smad5 to rescue laf/alk8 mutants, while Bmp2b and Bmp7 cannot, raises the possibility that the Laf/Alk8 receptor transduces the Bmp2b and/or Bmp7 signal. However, the bmp2b/swirl and bmp7/shn dorsalized mutant phenotypes are much more severe than the laf/alk8 mutant phenotype, which is restricted to the tail. The different strengths of mutant

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**Table 1. Ability of bmp2b, bmp7 and smad5 mRNA to rescue the laf/alk8 homozygous mutant phenotype**

<table>
<thead>
<tr>
<th>RNA</th>
<th>n*</th>
<th>% Normal or ventralized</th>
<th>% Phenotype</th>
<th>% phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>bmp2b</td>
<td>367</td>
<td>77.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>bmp7</td>
<td>187</td>
<td>77.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>smad5</td>
<td>226</td>
<td>80.1</td>
<td>5.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Uninjected</td>
<td>149</td>
<td>76.3</td>
<td>ND</td>
<td>0</td>
</tr>
</tbody>
</table>

*Data represents the compilation of multiple experiments. mRNA was injected into 1-2 cell stage embryos from a cross between laf/alk8 homozygotes.

†Mutant phenotypes were scored according to Mullins et al., 1996.

‡All phenotypically normal or ventralized embryos from one experiment were genotyped for laf/alk8: 0/97 bmp2b-injected, 0/64 bmp7-injected, and 5/99 smad5-injected embryos were laf/alk8 homozygotes.

§Class 1 embryos from one experiment were genotyped for laf/alk8: 2/2 embryos were laf/alk8 homozygotes.
phenotypes suggest that, (1) Laf/Alk8 may transduce only a subset of the Bmp2b/Bmp7 signal, (2) another receptor functions redundantly to Laf/Alk8 early in development and/or (3) that maternal laf/alk8 expression may be present and functioning early in development. Since other type I TGFβ receptors are maternally expressed (Nikaido et al., 1999a; Nikaido et al., 1999b), we investigated whether laf/alk8 is as well. We examined laf/alk8 mRNA by RT-PCR at the 1-cell stage, well before zygotic transcription commences during the tenth cell cycle (Kane and Kimmel, 1993). Consistent with a previous report (Yelick et al., 1998), we found that laf/alk8 is a maternally expressed message (data not shown).

**Rescue of laf/alk8 mutant embryos to homozygous adults**

To address the in vivo role of maternal laf/alk8 expression, we sought to generate homozygous laf<sup>tm110b</sup> adult females and analyze their progeny for a maternal-effect mutant phenotype. Such females would produce eggs that lack maternally derived Laf/Alk8 activity. Since laf homozygotes die by 3 dpf, we generated homozygous adults by injecting alk8 mRNA and rescuing mutant embryos from a cross between laf<sup>tm110b</sup> heterozygotes. Phenotypically wild-type embryos at 1 dpf (including >95% of the laf/alk8 mutants) were raised to adults and then genotyped for the laf<sup>tm110b</sup> allele. Of the 149 fish genotyped, 147 were either laf<sup>tm110b</sup> heterozygotes or wild type and only two fish were laf<sup>tm110b</sup> homozygotes. This number is significantly below the expected 25% or 37 homozygotes. In addition, the laf<sup>tm110b</sup> homozygotes identified were runts, about two-thirds the size of their siblings (Fig. 3A,B). We then selectively genotyped the small fish from approximately 1000 embryos injected and raised to adults and found that eight of 15 small fish were laf<sup>tm110b</sup> homozygotes.

To date all ten of the identified laf/alk8 homozygotes are runts. We have assayed growth capacity of the small homozygotes by growing them and similarly sized small, non-mutant siblings in parallel under ideal growth conditions. Homozygotes remained small, while their similarly sized siblings achieved normal size within 2 months, suggesting that the homozygotes have an intrinsic growth defect that persists into adulthood. It is likely that this defect is caused by loss of Alk8/Laf activity, rather than a second linked mutation. Based on the number of fish we genotyped, a second mutation would have to lie within approx. 1.3 cM of laf<sup>tm110b</sup>, which is highly improbable based on the mutation frequency used in the original ENU mutagenesis procedure that induced this mutation (Mullins et al., 1994). In addition to their small size, some homozygotes exhibit an enlargement in the region of the heart (3/10) and swirl when they swim.

In order to gain insight into the reduction in the number of homozygotes at 3 months of age, we followed the development of rescued homozygous embryos and their siblings. Embryos from an intercross of laf<sup>tm110b</sup> heterozygotes were injected with alk8 mRNA at the 1- to 2-cell stage. Embryos that were phenotypically wild type at 1 dpf were followed for

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**Fig. 2.** laf<sup>tm110b</sup> maps to linkage group 2 (LG 2) and is a mutation in the alk8 gene. (A) Position of laf on LG2 between markers Z21490 and Z11023. Radiation hybrid mapping analysis linked alk8 to Z11023 on LG2.

(B) Schematic of Laf/Alk8 protein and laf<sup>tm110b</sup> mutation. Black lines in the extracellular domain represent cysteine residues.

(C) Injection of synthetic wild-type laf/alk8 and laf<sup>tm110b</sup> mRNA into embryos from a cross between two laf<sup>tm110b</sup> heterozygotes. The embryos were grouped into phenotypic classes: wild-type, class 1 (C1) dorsalization, and class 2 (C2) dorsalization (Mullins et al., 1996).
developed abnormalities until 9 dpf, then sacrificed and genotyped. No consistent, specific abnormalities were observed morphologically in the homozygotes compared to their siblings (heterozygotes and wild types), except that only 29% (8/28) of the homozygotes displayed inflated swim bladders by 9 dpf, compared to 82% (118/144) of the non-mutant siblings. Lack of swim bladder inflation is a frequent secondary effect of a mutation. These results suggest that there is a defect in some homozygotes occurring prior to 9 dpf that we did not detect morphologically.

**Maternal-zygotic laf/alk8 mutants are strongly dorsalized**

To determine the role of maternally and zygotically derived Laf/Alk8 (MZ-Laf/Alk8) activity, we examined the progeny from crosses between homozygous females and males. Such MZ-laf/alk8 mutant embryos display a phenotype morphologically similar to the class 5 swirl/bmp2b mutant phenotype (Mullins et al., 1996). The phenotype is apparent by 95% epiboly and is quite striking by the 1-somite stage; mutant embryos are ovoid rather than round and the notochord appears broadened (Fig. 4A-D). The phenotype becomes more dramatic during somitogenesis, when the somites are expanded medially and laterally and the second and more posterior somites extend from the notochord to the ventral side where they fuse (Fig. 4E,F). The embryos die between the approx. 15-somite stage, we investigated the expression of pax2.1/myoD/munoD around the axis (Nguyen et al., 1998b; Schmid et al., 2000).

Crosses between homozygous laf/m110b females and wild-type males demonstrate that there is no strict maternal function for Laf/Alk8. All embryos from such crosses display a wild-type phenotype. Thus, zygotic expression of laf/alk8 is sufficient to compensate for the loss of maternal laf/alk8 activity during early development. Similarly, maternal laf/alk8 expression can substitute for some, but not all zygotic laf/alk8 expression, since weak dorsalized mutants are observed in 25% of the progeny of crosses between heterozygotes. Only loss of both maternal and zygotic activity reveals the full extent of Laf/Alk8 function in early development.

We examined the expression of a variety of marker genes by whole-mount in situ hybridization to determine the strength of the dorsализation in MZ-laf/alk8 mutant embryos. At the approx. 5-somite stage, we investigated the expression of myoD in the somitic mesoderm (Weinberg et al., 1996) and pax2.1 in the pronephric system (Krauss et al., 1992) as markers of dorsal and ventral mesodermal derivatives, respectively. At this stage, myoD is expressed in 2 stripes of adaxial cells and in medial-lateral stripes of somitic mesoderm (Fig. 5A; Weinberg et al., 1996). In MZ-laf/alk8 mutant embryos, the myoD adaxial stripes are present and relatively normal, while the first medial-lateral somite stripe is dramatically expanded laterally and all subsequent stripes circumscribe the embryo (Fig. 5C), consistent with the morphology of live mutant embryos (Fig. 4F). In contrast to the expanded dorsal mesoderm, we found pax2.1 expression absent from the pronephric system, a ventral mesodermal derivative (Fig. 5A,C,D).

We investigated the expression of krox20 in rhombomeres 3 and 5 of the hindbrain (Oxtoby and Jowett, 1993), and pax2.1 in the midbrain/hindbrain boundary, both of which are dorsal ectodermal derivatives. We found that prospective rhombomeres 3 and 5 are expanded, encircling the dorsoventral axis of MZ-laf/alk8 mutant embryos (Fig. 5B,D). The mid/hindbrain boundary expression of pax2.1 is also expanded laterally, but only circumscribes a minority of MZ-laf/alk8 mutant embryos (Fig. 5B,D, data not shown). In contrast, in null mutant swirl/bmp2b and snh/bmp7 embryos, both the midbrain and hindbrain tissues extend circumferentially around the axis (Nguyen et al., 1998b; Schmid et al., 2000). Thus, MZ-laf/alk8 mutants are less strongly dorsIALIZED than null swirl/bmp2b and snh/bmp7 mutants.

To determine if the dorsализation observed during somitogenesis stages in MZ-laf/alk8 mutant embryos is caused...
Fig. 5. Gene expression alterations in whole-mount in situ hybridizations of MZ-laf mutant embryos reveal a strongly dorsalized phenotype. Expression of *myoD*, *pax2.1* and *krox20* in 5- to 6-somite stage wild-type (A,B) and MZ-laf mutant (C,D) embryos. (A) Dorsal view, with anterior to the top; (B-F,I-L) lateral views, with dorsal to the right. (A) Somitic (sm) and adaxial expression of *myoD* and pronephric (pn) expression of *pax2.1*. (B) *pax2.1* expression in the midbrain/hindbrain boundary (mhbb) and *krox20* expression in rhombomeres 3 (r3) and 5 (r5) of the hindbrain. Arrow in C denotes the failure of the first somite to extend to the most ventral region of the embryo. In contrast, all subsequent somites circumscribe the embryo. In MZ-laf embryos expression of *krox20* in rhombomeres 3 and 5 is expanded, encircling the embryo (C,D). Arrowhead in D indicates that the midbrain/hindbrain boundary *pax2.1* expression domain in MZ-laf mutants is expanded compared to wild type, but does not extend to the most ventral region of the embryo. Expression of *foxb1.2* (formerly *fkd3*) in wild-type (E,G) and MZ-laf (F,H) embryos at 70% epiboly (mid-gastrulation). (G,H) Vegetal views with dorsal to the right. In F and H *foxb1.2* expression is expanded ventrally, the expansion is more pronounced in more vegetal regions (F). In F-L, the asterisks marks the expression domain of *goosecoid*, which appears normal in MZ-laf mutants. Expression of *gata2* in wild-type (I) and MZ-laf (J) embryos. Expression of *dlx3* in wild-type (K) and MZ-laf mutant (L) embryos.

Reduced signaling through Smad1/5 in MZ-laf/alk8 mutants

Our overexpression results with BMP pathway components, together with genetic and phenotypic analyses, indicate that *laf/alk8* encodes a receptor that participates in BMP signaling during dorsoventral patterning. To examine directly how activation of the BMP signaling pathway is affected in MZ-laf/alk8 mutants, we investigated the amount and spatial distribution of phosphorylated Smad1 and Smad5, the intracellular transducers of BMP signaling, during gastrulation. Antibodies that specifically detect the carboxy-terminal, activating phosphorylations of Smad1 and Smad2 proteins at conserved SSXS motifs have been characterized and shown to be specific (Persson et al., 1998). In *Xenopus* embryos, endogenous Smad1 phosphorylation is enhanced by overexpression of BMP ligands or constitutively active Alk2* or Alk3* receptors, and is inhibited by overexpression of the truncated BMP receptor ΔBRK or the extracellular BMP antagonist Noggin (Faure et al., 2000). Smad1 and Smad5 share identical C-terminal epitopes, and the anti-phosphoSmad1 antibody also recognizes phosphoSmad5 (M. W., unpublished). Phospho-specific Smad1/5 antibodies can therefore be used to visualize the level of activity, temporal regulation, and spatial distribution of BMP signals in early embryos.

Levels of phosphoSmad1/5 and phosphoSmad2 were examined by western blot analysis in gastrula stage wild-type and MZ-laf/alk8 mutant embryos collected at shield stage, 70% epiboly, and 90% epiboly. In western blot analysis, two bands are detected by anti-2Smad1/5 antibodies in lysates of zebrafish embryos. By changes in pattern formation during gastrulation, we investigated the expression of dorsal and ventral gastrula markers. We found that during mid-gastrulation stages (70% epiboly) the expression of the dorsal marker *foxb1.2* (formerly *fkd3*; Odenthal and Nüsslein-Volhard, 1998) is expanded laterally and encircles the embryo in marginal regions (Fig. 3E-H). Similar to *swirl/bmp2* and *snh/bmp7* mutants (Mullins et al., 1996), the expression of *goosecoid* in the most dorsal-anterior mesoderm appears normal, indicating that the most dorsal mesoderm is not affected (Fig. 5I-L). We examined the expression of *dlx3* and *gata2* at 70% epiboly, which are expressed in the ventral non-neural ectoderm (Akimenko et al., 1994; Detrich et al., 1995). In MZ-laf/alk8 mutant embryos *gata2* expression is absent (Fig. 5I,J). In most MZ-laf/alk8 mutants *dlx3* expression is absent, while in the remaining embryos it is severely reduced (Fig. 5K-L; data not shown). The expansion of gastrula dorsal tissue accompanied by a...
Fig. 6. Maternal-zygotic laf/alk8 mutants exhibit markedly reduced signaling through Smad1/5, but not Smad2. (A) laf/alk8 is required for normal levels of phosphorylated Smad1/5. Wild-type (WT) and MZ-laf mutant (laf) embryos were examined by western analysis at three gastrula stages: shield, 70% epiboly, and 90% epiboly. PhosphoSmad1/5 levels are significantly reduced in mutant embryos (top). Total Smad1/5 levels are not altered in mutants (upper middle). PhosphoSmad2 levels are not reduced in MZ-laf mutant embryos (lower middle). Cytoskeletal actin serves as a loading control (bottom). (B-E) laf/alk8 is necessary for asymmetric distribution of phosphorylated Smad1/5 across the dorsoventral axis. Wild-type and MZ-laf embryos were examined at 70% epiboly by immunohistochemical analysis. Brown staining indicates detection of phosphorylated Smad1/5. Both animal (B,C) and lateral (D,E) views are shown; dorsal is to the right. In wild-type embryos, phosphoSmad1/5 signal is greatly enriched on the ventral side of the embryo, opposite the dorsally localized shield (B,D). In MZ-laf mutants, which are dorsalized, this signal is greatly reduced (C,E).

DISCUSSION

_**lost-a-fin encodes the type I BMP receptor alk8**_

Several lines of evidence lead us to conclude that _laf_ encodes the type I TGFβ receptor _alk8_. _laf_ and _alk8_ are closely linked in the zebrafish genome. The _laf<sup>tm110b</sup>_ allele contains a missense mutation that is predicted to disrupt a disulfide bridge in the extracellular domain of Alk8. This predicted disulfide bond is highly conserved among all type I and type II TGFβ receptors. Furthermore, mRNA overexpression of _laf_ rescues the _laf_ dorsalized mutant phenotype, whereas overexpression of the mutant _laf<sup>tm110b</sup>_ mRNA does not.

We conclude that Laf/Alk8 functions as a BMP receptor for the following three reasons. First, the zygotic and maternal-zygotic _laf_ mutant phenotypes are similar to those of known components in a BMP signaling pathway. The loss of the ventral tail fin in zygotic _laf_ mutants is similar to the dominant _swirl/bmp2b_, the dominant zygotic _somitabun/smad5_, and the recessive _mini fin/tolloid_ tail fin phenotypes (Connors et al., 1999; Mullins et al., 1996). Furthermore, the strongly dorsalized _MZ-laf_ mutant phenotype displays dorsoventral patterning defects similar to homozygous _swirl/bmp2b_, _somitabun/smad5_ and _snailhouse/bmp7_ mutant embryos.

represent the superimposition of postranscriptionally modified Smad5 and Smad1. The nature of the putative modification resulting in distinctly migrating forms of Smad5 is not known, but these isoforms appear to be identically regulated by BMP signaling. We find that Smad1/5 phosphorylation is maximal in wild-type embryos at 70% epiboly, whereas Smad2 phosphorylation is maximal earlier at 50% epiboly.

In MZ- _laf/alk8_ mutant embryos, phosphoSmad1/5 levels are markedly decreased compared to wild-type controls at all stages examined (Fig. 6A). However, total Smad1/5 protein levels are not altered in MZ- _laf/alk8_ mutants (Fig. 6A), indicating a defect in the phosphorylation of Smad1/5 proteins. In contrast, Smad2 phosphorylation, which is mediated by an activin/Nodal signaling pathway, is not reduced in these mutant embryos (Fig. 6A), consistent with a specific decrease in BMP signaling in MZ- _laf/alk8_ mutant embryos. Thus, the Laf/Alk8 receptor is specifically required for normal levels of Smad1/5 phosphorylation, providing strong evidence for Laf/Alk8 acting as a BMP receptor.

We examined the spatial distribution of phosphorylated Smad1/5 at mid-gastrulation (70% epiboly) by immunohistochemical analysis of wild-type and MZ- _laf/alk8_ mutant embryos (Fig. 6B-E). We observed punctate staining, consistent with the predicted nuclear localization of phosphorylated Smad1/5 (Massagué, 1998) (data not shown). In wild-type embryos, phosphorylated Smad1/5 is greatly enriched on the ventral side (Fig. 6B,D). In embryos lacking both maternal and zygotic Laf/Alk8 activity, phosphorylated Smad1/5 is barely detectable (Fig. 6C,E). Smad1/5 phosphorylation, and therefore presumably BMP signaling, are graded across the dorsoventral axis of the zebrafish gastrula, and the maternal–zygotic _laf/alk8_ mutation disrupts this pattern by substantially decreasing BMP signaling. The Laf/Alk8 receptor is therefore required for the asymmetric distribution of phosphorylated Smad1/5 across the dorsoventral axis of the early zebrafish embryo.
null mutant phenotypes. First, it is possible that the laf tm110b domain (M. Hammerschmidt, personal communication) that predicted to generate a truncated protein lacking the kinase mutants, similar to the hypomorphic Although the alk8, laf genes are expressed ubiquitously throughout gastrula stages (Dick et al., 1999; Müller et al., 1999; Yelick et al., 1998), we show that phospho-Smad1/5 is localized to ventral regions during gastrulation (Fig. 6B,D). This demonstrates that activation of the BMP signaling pathway is ventrally localized, as it is in Xenopus (Faure et al., 2000). Moreover, we show that this asymmetric distribution of phosphoSmad1/5 depends on Alk8/Laf activity (Fig. 6C,E). All of this evidence strongly supports a role for Laf/Alk8 as a type I receptor transducing BMP-specific signals within a gastrula Bmp2b/Bmp7/Smad5 signaling pathway.

The lost-a-fin maternal-zygotic mutant phenotype

Embryos lacking both maternal and zygotic Laf/Alk8 activity display a dramatic expansion of dorsal-laterally derived structures around the circumference of the embryo and a concomitant loss of ventral tissues. This reduction of ventral and expansion of dorsal cell types is reflected in alterations of gene expression during gastrulation and indicates a defect in pattern formation. The strength of the dorsalization in MZ-laf mutants is intermediate between swirl/bmp2b or snh/bmp7 null mutants and a hypomorphic snh/bmp7 mutant, snh568a. Notable differences between MZ-laf and null swirl/bmp2b and snh/bmp7 mutants are the less severe expansion of the mid/hindbrain boundary and first somite in most MZ-laf mutants, similar to the hypomorphic snh568a mutant (Mullins et al., 1996; Schmid et al., 2000). During gastrulation in MZ-laf mutants, expression of the dorsal, neuroectodermal marker foxb1.2 circumscribes the dorsoventral axis marginally, but does not do so in the most animal regions (Fig. 5E-H), in contrast to swirl/bmp2b mutants (Nguyen et al., 1998b). Similar to the hypomorphic snh568a mutant, MZ-laf mutants lack expression of the ventral marker gata2 (Fig. 5LJ), but retain dlx3 expression in a reduced ventral domain in some mutants (Nguyen et al., 1998b). However, the phenotype is stronger than snh568a mutants in that Krox20 expression in rhombomeres 3 and 5 circumscribes the dorsoventral axis. The MZ-laf mutant phenotype likely represents an intermediate dorsalization between null swirl/bmp2b and snh/bmp7 mutants and the hypomorphic snh/bmp7 mutant, snh568a.

There are at least two possible reasons why the MZ-laf mutant phenotype is less severe than the swirl/bmp2b and snh/bmp7 null mutant phenotypes. First, it is possible that the laf<sup>fm110b</sup> mutant allele is not a null allele. A second allele of laf, laf<sup>pm100</sup>, exhibits an identical, recessive zygotic mutant phenotype to laf<sup>fm110b</sup> (Solnica-Krezel et al., 1996; unpublished). The laf<sup>pm100</sup> allele contains a nonsense mutation, predicted to generate a truncated protein lacking the kinase domain (M. Hammerschmidt, personal communication) that we presume represents a null allele. The identical zygotic mutant phenotypes suggest that laf<sup>fm110b</sup> is a null or strong hypomorphic mutation. It is possible that laf<sup>pm110b</sup> is a strong hypomorph with some residual activity that can establish a very small ventral domain in MZ-laf mutant embryos.

A second possible reason for the less severe dorsalization in MZ-laf mutants compared to swirl/bmp2b and snh/bmp7 null mutants is that other type I BMP receptors may function in dorsoventral patterning in the embryo. Two additional BMP type I receptors, BMPR-IA (Alk3) and BMPR-IB (Alk6), are expressed ubiquitously during gastrulation in the zebrafish (Nikaido et al., 1999a; Nikaido et al., 1999b). Either one or both of these receptors may have overlapping functions to Alk8/Laf and/or act independently of Alk8/Laf in dorsoventral patterning. Overexpression of truncated, dominant-negative forms of the BMPR-IA receptor or its homologues cause dorsalized phenotypes in both zebrafish and Xenopus embryos (Graff et al., 1994; Nikaido et al., 1999a; Suzuki et al., 1994). However, the truncated BMPR-IA receptor may also inhibit other type I BMP receptors, including the Alk8 receptor, as found for other dominant-negative proteins. Thus, these experiments do not distinguish between the functions of individual type I BMP receptors. In contrast, our loss of function analysis demonstrates a key role for the Alk8/Laf BMP type I receptor in vertebrate dorsoventral patterning. The near absence of phosphoSmad1/5 throughout gastrulation indicates that nearly all, if not all, gastrula BMP signaling requires Alk8/Laf receptor activity.

The roles of BMP type I receptors in the mouse

In the mouse, mutants of two type I BMP receptors, Alk2 (ActRIA) and Alk3, and one type II BMP receptor (BMPR-II) have been generated (Beppu et al., 2000; Gu et al., 1999; Mishina et al., 1999; Mishina et al., 1995). For all of these mutants a primary defect occurring prior to or very early in gastrulation has made it difficult to determine the role of these BMP receptors in dorsoventral pattern formation. An interesting case, however, is Alk2 mutant embryos, which arrest during gastrulation with a severe deficit in mesoderm formation. When the mesoderm defect in Alk2 receptor mutants is rescued by wild-type extraembryonic tissues, the mutants display a truncation of posterior regions (Gu et al., 1999; Mishina et al., 1999). Some mouse Bmp4 mutants that survive beyond the egg cylinder stage display a similar posterior truncation (Winnier et al., 1995), which could reflect a dorsalization of the embryo. Further studies are required to discern the nature of the posterior truncation. Although it is possible that the Alk2 receptor plays a similar role to zebrafish Alk8/Laf in dorsoventral patterning, it would be interesting to determine the function of an Alk8 orthologue in the mouse.

Evolutionary conservation in dorsoventral patterning

In Drosophila, two BMP type I receptors, Thickveins and Saxophone, act in the transduction of the Dpp and Screw signals, respectively (Neul and Ferguson, 1998; Nguyen et al., 1998a). However, Dpp and Screw together with their respective receptors play non-equivalent roles in dorsoventral pattern formation, unlike the Bmp2b and Bmp7 ligands in the zebrafish. Thickveins and Dpp act in the establishment of all dorsal cell fates, while Saxophone and Screw specify only a
subset. Here we show that the Laf/Alk8 receptor, which is more similar to Saxophone phylogenetically than Thickveins, plays an extensive role in dorsoventral pattern formation, more similar to Thickveins in the fly. It is possible that all Bmp2b and/or Bmp7 signaling acts through the Laf/Alk8 type I receptor in dorsoventral patterning; however, this does not exclude the role of additional type I receptors in this process. In the fly, Thickveins, Saxophone, and two Punt type II receptor subunits are proposed to form a tetrameric complex together with one Dpp and one Screw homodimer (Neul and Ferguson, 1998; Nguyen et al., 1998a). Hence, additional type I receptors may function together with the Alk8/Laf receptor. Further studies are required to determine the roles of other BMP type I receptors in dorsoventral patterning in vertebrates.

Additional roles of lost-a-fin in development

Our rescue of the zygotic laf dorsalized mutant phenotype by alkl mRNA injection points to at least one additional role for laf/alkl beyond dorsoventral pattern formation. Non-viable laf mutants that have been rescued for their dorsaled phenotype, exhibit a low rate of swim bladder inflation at 9 dpf compared to sibling controls (29% vs. 82%), without other gross discernable abnormalities. Lack of swim bladder inflation is a common secondary effect of a mutation. In addition, all identified homozygous laf adults are small, developing to about two-thirds the size of their wild-type siblings. Although the rescued, non-viable laf mutants at 9 dpf are of normal size, it is possible that the non-viability defect is related to the later size defect of homozygous laf adults or, alternatively, these defects reflect two independent functional processes.

It is important to note that rescued homozygous swirl/bmp2b and snh/bmp7 mutant embryos survive at rates more comparable to wild type and are of normal size as adults. This difference suggests that Laf/Alk8 is a receptor for a ligand other than Bmp7 or Bmp2b after dorsoventral patterning, or that in the absence of one ligand the other ligand can compensate. None of the mouse BMP ligand or BMP receptor mutants have been reported to exhibit a general growth defect as adults; however, such a function may only be revealed following rescue of an early lethal defect, as we have done here. Interestingly, several C. elegans mutants exhibit a small adult body size and the mutated genes are known components of a BMP signaling pathway (sma-2, sma-3, sma-4, sma-6, daf-4, and dbI-1; reviewed in Patterson and Padgett, 2000). It is possible that the function of the Laf/Alk8 receptor in regulating body size is related to the function of this C. elegans BMP pathway. In summary, our analysis suggests a new developmental function for BMP signaling in general growth or size regulation in vertebrates, a role we are currently investigating.

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**lat/alk8 receptor in dorsoventral patterning**


