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The type I serine/threonine kinase receptor Alk8/Lost-a-fin is required for Bmp2b/7 signal transduction during dorsoventral patterning of the zebrafish embryo

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SUMMARY

Ventral specification of mesoderm and ectoderm depends on signaling by members of the bone morphogenetic protein (Bmp) family. Bmp signals are transmitted by a complex of type I and type II serine/threonine kinase transmembrane receptors. Here, we show that Alk8, a novel member of the Alk1 subgroup of type I receptors, is disrupted in zebrafish *lost-a-fin (laf)* mutants. Two *alk8*/*laf* null alleles are described. In *laf*^{tm110}, a conserved extracellular cysteine residue is replaced by an arginine, while in *laf*^{m100}, Alk8 is prematurely terminated directly after the transmembrane domain. The zygotic effect of both mutations leads to dorsalization of intermediate strength. A much stronger dorsalization, similar to that of *bmp2b*/*swirl* and *bmp7*/*snailhouse* mutants, however, is obtained by inhibiting both maternally and zygotically supplied *alk8* gene products with morpholino antisense oligonucleotides. The phenotype of *laf* mutants and *alk8* morphants can be rescued by injected mRNA encoding Alk8 or the Bmp-regulated transcription factor Smad5, but not by mRNA encoding Bmp2b or Bmp7. Conversely, injected mRNA encoding a constitutively active version of Alk8 can rescue the strong dorsalization of *bmp2b*/*swirl* and *bmp7*/*snailhouse* mutants, whereas *smad5*/*somitabun* mutant embryos do not respond. Altogether, the data suggest that Alk8 acts as a Bmp2b/7 receptor upstream of Smad5.

Key words: Alk8, Bmp2b, Bmp7, Smad5, Dorsoventral patterning, Lost-a-fin, Zebrafish, Morpholino antisense oligonucleotides

INTRODUCTION

During establishment of the body plan, signaling events regulate cellular behavior and specification. Members of the transforming growth factor β (Tgfβ) superfamily of signaling molecules have been shown to be crucial mediators of a variety of such processes (reviewed by Massagué, 1998). In target cells, Tgfβ signaling is transduced by transmembrane receptors of the serine/threonine kinase family, which themselves regulate the activity of members of the family of Smad transcription factors. Two types of receptors can be distinguished, type I and type II, which form heteromeric complexes upon ligand binding. Ligand binding occurs either in a sequential or a cooperative manner. The sequential mode, as characteristic for Tgfβ and activin receptors, involves direct binding of the ligand to high-affinity type II receptors and subsequent recruitment of type I receptors to the complex, whereas ligands bound in a cooperative mode, such as the bone morphogenetic proteins Bmp2, Bmp4 and Bmp7 or the growth differentiation factor Gdf5, display low affinity to both type I and type II receptors alone, but high affinity to the two receptors together (reviewed in Massagué, 1998). The type II receptors have constitutive kinase activity, leading to phosphorylation and activation of the type I receptor after ligand-induced complex formation. Activated type I receptors themselves phosphorylate and activate Smad proteins, which thereafter enter the nucleus to regulate transcription of target genes.

In line with their distinct functions, type I and type II receptors differ in several structural features. Both have a relatively short (approx. 150 amino acids) extracellular domain that contains 10 or more cysteines to determine the general folding of this region. Three of these cys-residues near the transmembrane domain constitute a specific cluster, the cysteine box, which is conserved in all serine/threonine transmembrane kinase receptors (Wrana et al., 1994). A unique feature of type I receptors is the GS domain, which precedes the kinase domain in the cytoplasmic part of the receptors. The GS domain contains several serine residues that can be phosphorylated by the type II receptors during signal transduction and type I receptor activation. Another special region of type I receptors is the L45 loop, a stretch of 10 amino acids of the kinase domain that determines the specificity of the receptors to the various receptor-regulated Smad proteins (Chen et al., 1998a; Chen et al., 1998b). The sequence of this L45 loop is highly divergent between different subgroups of

type I receptors, such as the Tgfβ receptors (Alk5/TβRI, Alk4/ActRIB, Alk7, etc.), the Bmp receptors (Alk3/BmpRIA, Alk6/BmpRIB and *Drosophila* Thick veins) and the Alk1 group (Alk1, Alk2/ActRIA and *Drosophila* Saxophone), whose endogenous ligands have been a controversial subject (see Discussion). Among the various members of each subgroup, however, the L45 sequences are highly conserved (Chen et al., 1998b).

Members of the Bmp subgroup of Tgfβ proteins are implicated in many different developmental processes, such as early dorsoventral pattern formation of the vertebrate embryo (reviewed in Hogan, 1996). In *Xenopus laevis*, overexpression of *bmp2*, *bmp4* and *bmp7* leads to ventral specification within ectoderm and mesoderm of the embryo (Dale et al., 1992; Jones et al., 1992; Schmidt et al., 1995; Wilson and Hemmati-Brivanlou, 1995). In contrast, disruption of Bmp signaling by cleavage resistant, dominant negative ligands (Hawley et al., 1995) or C-terminally truncated, dominant negative receptors (Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994; Schmidt et al., 1995) causes dorsalization of the embryo.

Genetic evidence that graded Bmp activity is required for a balance between ventral and dorsal development in vertebrates has been provided by studies in zebrafish. The molecular analysis of the dorsalizing mutations *swirl*, *snailhouse* and *somitabun* (Mullins et al., 1996) has shown that zebrafish Bmp2b (Kishimoto et al., 1997; Nguyen, 1998b), Bmp7 (Schmid et al., 2000; Dick et al., 2000) and the Bmp signal transducer Smad5 (Hild et al., 1999) are necessary for ventral development, while dorsal development requires the secreted protein Chordin, an inhibitor of Bmps (Piccolo et al., 1996; Hammerschmidt et al., 1996b) that is disrupted in the ventralized *dino* mutant (Hammerschmidt et al., 1996a; Schulte-Merker et al., 1997). On the ventral side, the activity of Chordin is attenuated by the metalloprotease Bmp1/Tolloid/Minifin (Connors et al., 1999) which cleaves Chordin, thereby promoting Bmp activity (Piccolo et al., 1997; Blader et al., 1997). In contrast to *bmp2b*, *bmp7* and *smad5*, null mutations in *tolloid* cause only a very mild dorsalized phenotype (Connors et al., 1999).

Here, we show that the dorsalized phenotype of *lost-a-fin* zebrafish mutants is caused by null mutations in Alk8 (Yelick et al., 1998), a novel member of the Alk1 subgroup of type I serine/threonine kinase receptors. We provide genetic evidence that Alk8, although clearly distinct from the thus far described 'classical' Bmp type I receptors, is required for the transmission of Bmp2b/7 signaling during dorsoventral pattern formation.

MATERIALS AND METHODS

Cloning of zebrafish *alk8*

RT-PCR with total RNA from early gastrula stage embryos and oligonucleotides corresponding to conserved regions of serine/threonine kinase receptors was used to isolate cDNA fragments encoding various zebrafish type I and type II receptors. With degenerated oligonucleotides encoding YGEVWRG (sense) and KLMKECW (antisense), a 780 bp fragment was amplified, which strongly hybridized with a degenerated oligonucleotide deduced from the internal highly conserved sequence HENILGF. The PCR fragment was cloned using a TA cloning kit (Invitrogen) and used to screen gastrula stage cDNA libraries as described (Bauer et al., 1998), resulting in the isolation of three overlapping clones encoding the type I receptor serine/threonine kinase Alk8 (Accession Number AF292028; Yelick et al., 1998, Accession Number AF038425).

Mapping, linkage analysis, genotyping and RT-PCR analysis

alk8 was mapped on a radiation hybrid panel that was anchored to the genetic zebrafish map (Geisler et al., 1999). Primers used to amplify a genomic *alk8* fragment were AAAACCGACTGCTGAGGAGA (sense) and ATTTGATGGCGTCTCTGTCC (antisense). The *laf* mutation was mapped via its recessive zygotic effect, causing C1-C2 dorsalization in homozygous embryos. A laf^{tm110} carrier fish of the Tü background was crossed with a WIK wild-type fish (Rauch et al., 1997). PCR analysis of genomic DNA from *laf* mutant F_2 embryos for SSLP markers (Knapik et al., 1998) put laf^{tm110} between the markers z17291 (four recombinations in 192 meioses=2.1 cM) and z9234 (2 recombinations in 192 meioses=1.0 cM) on linkage group 2.

For direct linkage analysis, *alk8* cDNA was amplified from single *laf* mutant embryos and wild-type siblings via RT-PCR with total RNA, using the following conditions and primers: 5 minutes 95°C; 30× (30 seconds at 95°C, 30 seconds at 58°C, 3 minutes at 72°C); 7 minutes at 72°C; sense primer, GCTGAGAGTCACTTGAGGAG; antisense primer GTGAGCGGTCAGTAGTTCTG. For laf^{tm110}, the 1597 bp PCR product was digested with *Fsp*I, resulting in three fragments of 1188, 309 and 100 bp length for the mutant allele and two fragments of 1497 and 100 bp for the wild-type allele. In the case of laf^{m100}, 40 PCR cycles and a *Sal*I digest were performed, yielding fragments of 1126 and 471 bp (mutant) or the uncleaved 1597 bp fragment (wild type). Alternatively, linkage analysis of laf^{tm110} was carried out via PCR with genomic DNA of single embryos. Primers were AGCAAGCGCATGACCTGCTC (sense) and CAGCAGTCGAAGTAGGGTCT (antisense). PCR conditions were 5 minutes at 95°C; 40× (30 seconds at 95°C, 30 seconds at 58°C, 20 seconds at 72°C); 7 minutes at 72°C. *Fsp*I digestion of the 111 bp amplification product resulted in a 44 bp and a 67 bp fragment for the mutant allele, whereas the wild-type allele was not cleaved. The same protocol was used to genotype experimental laf^{tm110} mutants. The *bmp2b* allele swr^{ta72} and the *bmp7* allele snh^{ty68} were genotyped as previously described (Hild et al., 1999; Dick et al., 2000).

Developmental RT-PCR analysis was carried out as previously described (Bauer et al., 1998). To avoid cross reaction with other type I receptors, primers were derived from the highly divergent extracellular domains. *alk8* primers used were ATGGGGCATTGCAGCACCC (sense) and GCAAGTGTGCTGTCTCCAAC (antisense). The resulting 533 bp fragment was blotted and hybridized with the internal oligonucleotide AGCAAGCGCATGACCTGCTC. *BmpR1A*/*alk3* primers used were GCATACGGAGATCTACGGCACG (sense), ATACGTGTGCTTAATGGGAGG (antisense) and CATCAAGAAAAACGGCACCTGC (internal); *BmpR1B*/*alk6* primers were TAAAGGCACGGGCTCGTGG (sense), ATCTCAGTGTGCAGATGGCAG (antisense) and ACTACCTGAAGTCCACCACGC (internal).

Generation of constructs

All constructs used for in vitro transcription were cloned into pCS2+ (Rupp et al., 1994). The coding region of wild-type and mutant forms of *alk8* was amplified by PCR with a proofreading polymerase (Pfu, Stratagene), introducing 5′and 3′ *Eco*RI sites and a Kozak sequence upstream of the start codon. Truncated Alk8ΔC receptors terminating one or 13 amino acid residues after the transmembrane domain were generated via PCR. For the dominant negative version Alk8(K232R), for the constitutively active Alk8CA and for introducing the laf^{tm110} mutation into the truncated *Xenopus* Bmp receptor IA, PCR-based site specific mutagenesis was applied as described (Hild et al., 1999).

RNA synthesis, microinjection and *in situ* hybridization

pCS2-based constructs were linearized with *Not*I, pSP64TS-based constructs with *Bam*HI (*alk3*) or *Xba*I (*bmp2b*). Capped mRNA was

synthesized using the message machine kit (Ambion), and injected into one- to four-cell stage embryos as described (5 nl per embryo; Hammerschmidt et al., 1999). The sequence of the morpholino antisense oligonucleotide *alk8morph1* was CAACTCCTCAA-GTGACTCTCAGCG, complementary to the region from −38 to −17 in the 5′UTR of *alk8* cDNA, *alk8morph2* was GATTCATGT-TTGTGTTCAATTTCCG, complementary to −130 to −105 of the *alk8* cDNA, and the two corresponding four-mismatch control morpholino oligonucleotides *4mm-alk8morph1,2* were CAAgTCC-aCAAGTGACTgTCAcCG and GATaCATcTTTGTGTTCAtTTT-gCG. *bmp2bmorph*, complementary to −4 to +19 of the *bmp2b* cDNA, was CGCGGACCACGGCGACCATGATC. Morpholino oligonucleotides were injected at a concentration of 1-3 mg/ml in 1× Danieau buffer (5 nl per embryo; 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM Hepes pH 7.6). Whole-mount in situ hybridization was carried out as previously described (Hammerschmidt et al., 1996a).

RESULTS

Cloning of *alk8*

Using a degenerate approach to isolate serine/threonine kinase receptors (see Materials and Methods), several cDNA clones were isolated, three of which encode Alk8, a novel type I serine/threonine kinase receptor whose sequence was published during the course of our analysis (Yelick et al., 1998). The extracellular domain of Alk8 is rather divergent from other receptors (30% amino acid identity to the most closely related receptor, human ALK2), whereas the kinase domain is more highly conserved (84% identity with hALK2; Yelick et al., 1998). Within the L45 loop of the kinase domain, the motif that determines the specificity of type I receptors to Smad proteins (Chen et al., 1998a; Chen et al., 1998b), the Alk8 sequence, matches the consensus sequence of the Alk1/2 subgroup of receptors, which is clearly distinct from the subgroup of the classical Bmp receptors (see Introduction; Fig. 1A).

Developmental RT-PCR analysis (Fig. 1B) and whole-mount in situ hybridization (see Fig. 4C and Yelick et al., 1998) revealed that *alk8* is ubiquitously and uniformly expressed from the two-cell stage until day 5, the latest stage examined. Thus, *alk8* displays both maternal and zygotic expression, similar to the putative Bmp type I receptors Alk3/BR1A and Alk6/BR1B (Nikaido et al., 1999a; Nikaido et al., 1999b; Fig. 1B).

Loss of Alk8 function leads to dorsalized, gain of Alk8 function to ventralized phenotypes

As a first step to investigate which signals are mediated by the Alk8 receptor, loss- and gain-of-function experiments were carried out by injecting mRNAs encoding dominant negative and constitutively active versions of Alk8 into wild-type zebrafish embryos. In addition, Alk8 activity was specifically abolished by injecting antisense morpholino oligonucleotides.

For gain-of-function experiments, we first injected synthetic mRNA encoding wild-type Alk8 into wild-type embryos. However, injected embryos developed normally (Table 1), indicating that the concentration of the receptor is not the rate-limiting factor during Alk8-mediated signaling. Therefore, we used a constitutively active version of the receptor, which

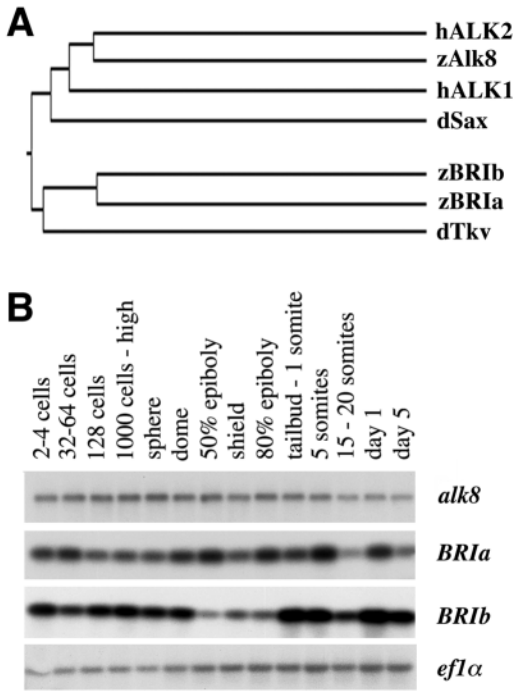

Fig. 1. (A) Phylogenetic tree of zebrafish Alk8 (AF292028), zebrafish Alk3/BmpRIA (AB011826), zebrafish Alk6/BmpRIB (AB020758), human ALK2/ActRIA (Z22534), human ALK1 (Z22533), *Drosophila* Saxophone (U11441) and *Drosophila* Thick veins (U11442), calculated according to the J. Hein method (DNAstar software). (GenBank Accession Numbers are given in brackets.) (B) Temporal expression profile of zebrafish *alk8*, *BR1a* (*alk3*), *BR1b* (*alk6*) and, as control, *ef1α* (Nordness et al., 1994), determined via RT-PCR.

activates signal transduction in the absence of ligand. Such versions can be engineered by exchanging an amino acid residue at a specific position near the GS domain to aspartate (e.g. Wieser et al., 1995). Injection of *alk8CA* mRNA encoding the corresponding (Q204D) mutant receptor into wild-type embryos led to a strong ventralization, morphologically characterized by the absence of head and notochord, and by enlarged blood islands (Fig. 2B). This phenotype was indistinguishable from that obtained upon overexpression of *bmp2*, *bmp4* or *bmp7* (Nikaido et al., 1997; Dick et al., 2000; Schmid et al., 2000).

Conversely, dorsalized phenotypes were obtained upon inhibition of Alk8 using two different approaches. The first was injection of mRNA encoding dominant negative versions of the receptor. C-terminally truncated Alk8 receptors (Alk8ΔC) that lack the cytoplasmic kinase domain only caused weakly dorsalized phenotypes (up to C1) at rather low frequencies (Table 1). In contrast, stronger dorsalized phenotypes up to C5 were obtained with a potential kinase-dead Alk8 version carrying a Lys→Arg exchange in the ATP-binding domain, as originally described for a mutant Tgfβ type I receptor (Bassing et al., 1994; *alk8(K232R)*; Table 1; Fig. 2C).

For both dominant negative Alk8 versions, it cannot be ruled out that their effect is caused by interfering with the signaling of other type I receptors, e.g by titrating out shared co-receptors or ligands. For a specific targeting of Alk8, we used a novel antisense approach, injecting antisense

Table 1. Injection studies

Oligo or RNA	Concentration (ng/µl)	Cross	*n*	Strength of dorsalization/ventralization* C5 (%)	C4 (%)	C3 (%)	C2 (%)	C1 (%)	Wild type (%)	V1 (%)	V2 (%)	V3 (%)	V4 (%)	Responsive mutants‡ (%)
alk8	60	+/+×+/+	109	0	0	0	0	0	98	2	0	0	0	–
alk8CA	2	+/+×+/+	105	0	0	0	0	0	3	7	16	29	45	–
alk8ΔC	150	+/+×+/+	166	0	0	0	3	5	92	0	0	0	0	–
alk8(K232R)	45	+/+×+/+	120	14	78	8	0	0	0	0	0	0	0	–
alk8morph1	3000	+/+×+/+	140	0	4	27	15	16	38	0	0	0	0	–
alk8morph1+alk8	3000+60	+/+×+/+	115	0	0	0	7	6	87	0	0	0	0	–
alk8morph1+alk3	3000+60	+/+×+/+	64	0	4	24	6	25	41	0	0	0	0	–
4mm-alk8morph1	3000	+/+×+/+	112	0	0	0	0	0	100	0	0	0	0	–
alk8morph2	3000	+/+×+/+	105	5	89	3	3	0	0	0	0	0	0	–
alk8morph2+alk8	3000+60	+/+×+/+	173	0	0	5	9	19	67	0	0	0	0	–
4mm-alk8morph2	3000	+/+×+/+	104	0	0	0	0	0	100	0	0	0	0	–
–		*laf*+/–×*laf*+/–	435	0	0	0	18	8	74	0	0	0	0	0
alk8	60	*laf*+/–×*laf*+/–	279	0	0	0	2	4	94	0	0	0	0	75
alk3	60	*laf*+/–×*laf*+/–	88	0	0	0	19	6	75	0	0	0	0	0
alk6	60	*laf*+/–×*laf*+/–	86	0	0	16	56	23	5	0	0	0	0	0§
alk8 (tm110)	120	*laf*+/–×*laf*+/–	187	0	0	0	17	10	73	0	0	0	0	0
alk8 (m100)	120	*laf*+/–×*laf*+/–	54	0	0	0	13	13	74	0	0	0	0	0
tXBR1A	150	+/+×+/+	86	0	16	19	38	8	19	0	0	0	0	–
tXBR1A (tm110)	150	+/+×+/+	122	0	0	0	0	0	100	0	0	0	0	–
bmp2b	0.75	*laf*+/–×*laf*+/–	209	0	0	0	15	7	6	12	33	24	3	0
bmp7	6	*laf*+/–×*laf*+/–	110	0	0	0	12	13	42	7	13	13	0	0
smad5	50	*laf*+/–×*laf*+/–	203	0	0	0	2	5	88	5	0	0	0	72
bmp2b	0.75	+/+×+/+	105	0	0	0	0	0	0	19	56	21	4	–
alk8morph2+bmp2b	3000+0.75	+/+×+/+	148	0	25	52	13	10	0	0	0	0	0	7
bmp7	8	+/+×+/+	88	0	0	0	0	0	0	12	40	33	17	–
alk8morph2+bmp7	3000+8	+/+×+/+	110	0	20	47	22	11	0	0	0	0	0	8
smad5	70	+/+×+/+	93	0	0	0	0	0	8	27	43	16	5	–
alk8morph2+smad5	3000+70	+/+×+/+	109	0	0	0	0	0	4	36	40	8	3	100
–		*swr*+/–×*swr*+/–	109	25	0	0	0	2	73	0	0	0	0	–
alk8CA	1	*swr*+/–×*swr*+/–	140	0	0	0	7	2	3	8	21	53	6	100
–		*snh*+/–×*snh*+/–	169	0	22	0	0	0	78	0	0	0	0	–
alk8CA	1	*snh*+/–×*snh*+/–	178	0	0	0	2	0	9	6	27	54	2	100
–		*sbn*+/–×*sbn*+/–	143	0	13	81	6	0	0	0	0	0	0	–
alk8CA	1	*sbn*+/–×*sbn*+/–	153	0	9	87	4	0	0	0	0	0	0	0
bmp2bmorph	3000	+/+×+/+	63	93	7	0	0	0	0	0	0	0	0	–
bmp2bmorph+alk8CA	3000+2	+/+×+/+	111	0	0	0	0	0	0	22	46	26	6	100

*Classification of the strength of dorsalization (from weak (C1) to strong (C5)) and ventralization (from weak (V1) to strong (V4)) was according to Mullins et al., 1996 and Kishimoto et al., 1997. Used alleles were *lost-a-fin laf*^{tm110}, *snailhouse snh*^{ty68}, *swirl swr*^{ta72} and *somitabun sbn*^{dtc24}.

‡Frequency of mutant or morphant embryos showing a response to the injected RNA. Response was defined as a shift of the mutant phenotype to an at least two classes weaker phenotypic strength, e.g. C5 to at least C3 for *swr*, C4 to at least C2 for *snh* or *sbn*, C2 to WT for *laf*. Response frequencies were calculated relative to the expected frequencies of mutant embryos in the various crosses, or relatively to the obtained frequencies in the morpholino oligonucleotide single injections (*alk8morph2* or *bmp2bmorph*).

§Injected *alk6* mRNA leads to a general dorsalization of wild-type embryos.

For injections of *bmp2b*, *bmp7* and *smad5* mRNA into offspring of two *laf* carriers, embryos with the *laf*-characteristic tail phenotype were scored as C2 or C1, independent of ventralized traits in other regions of the body (see text and Fig. 3). In morpholino oligonucleotide/RNA co-injections, injected mRNAs did not contain the 5′ UTR sequences targeted by the morpholinos.

alk8CA, constitutively active Alk8; *alk8ΔC*, C-terminally truncated Alk8; *alk8(K232R)*, Alk8 with Lys-Arg exchange in ATP binding domain; *alk8morph1,2*, *alk8* antisense morpholino oligonucleotides; *4mm-alk8morph1,2*, control morpholino oligonucleotides with four mismatches compared with *alk8morph1,2*; *n*, number of scored embryos; *tXBR1A*, C-terminally truncated *Xenopus* BmpR1A.

morpholino oligonucleotides against the 5′UTR of the *alk8* cDNA. According to recent results obtained in *Xenopus* and zebrafish (Heasman et al., 2000; Nasevicius and Ekker, 2000), such morpholino oligonucleotides lead to a specific inhibition of both maternally and zygotically supplied gene products by interfering with the translation of the corresponding mRNAs. Injection of two distinct *alk8* antisense morpholino 25mer oligonucleotides (*alk8morph1,2*) into wild-type zebrafish embryos resulted in dorsalization up to C5 strength, as judged by morphology and the *krox20* expression pattern (Table 1, Fig. 2D,F-H). Co-injection of wild-type *alk8* mRNA, which by itself had no effect, led to a significant reduction of dorsalization, whereas co-injected *alk3* mRNA had no rescuing effect (Table 1). Furthermore, embryos injected with morpholino oligonucleotides carrying four nucleotide exchanges, compared with *alk8morph1* and *2*, developed normally (*4mm-alk8morph1,2*; Table 1, Fig. 2E). Altogether, these data indicate that the effect of the *alk8* antisense morpholino oligonucleotides is specific, and that Alk8 is an essential component involved in the specification of ventral

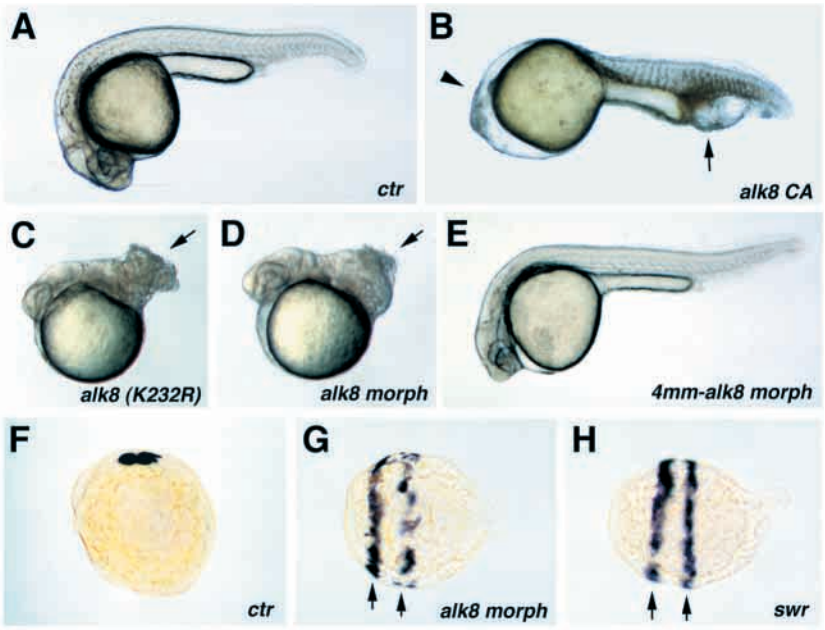

Fig. 2. *alk8* is implicated in ventral specification. All embryos, unless stated otherwise, are genetically wild type. Embryos in A-D are at 36 hours post fertilization, lateral view, head to the left. (A) Uninjected control. (B) Strongly ventralized embryo (V3) after injection of *alk8CA* mRNA. Arrowhead indicates absent head region, arrow indicates enlarged blood islands. (C,D) Strongly dorsalized embryo (C4) after injection of *alk8(K232R)* mRNA (C) or *alk8* antisense morpholino oligonucleotide *alk8morph2* (D). Arrows indicate wound-up trunk. Note that the phenotype is much stronger than that of the *laf* mutant (see Fig. 3B). (E) Wild-type looking embryo after injection of the *alk8* four-mismatch control antisense morpholino oligonucleotide *4mm-alk8morph2*. (F-H) In situ hybridization for *krox20* mRNA (Oxtoby and Jowett, 1993), staining rhombomeres 3 and 5; five-somite stage, lateral view, anterior towards the left, dorsal upwards. (E) Uninjected control; (F) injected with *alk8morph2*; (G) *swirl*/*bmp2b* mutant swr^{ta72}. *alk8morph2*-injected and *bmp2b* mutant embryo display a ventral fusion of both *krox20* stripes (arrows), indicative of C5 dorsalization (compare with Dick et al., 2000).

cell fates during dorsoventral patterning of the zebrafish embryo.

alk8 is mutated in the dorsalized mutant *lost-a-fin (laf)*

We next sought to identify a zebrafish *alk8* mutant. During the large-scale mutant screen, dorsalizing mutations defining six genes required for ventral specification were isolated, *swirl*, *snailhouse*, *somitabun*, *piggy-tail*, *lost-a-fin* and *minifin* (Mullins et al., 1996). For *swirl*, *snailhouse*, *somitabun* and *minifin*, the mutated genes have been identified (see Introduction). As a first step to investigate whether *alk8* might be mutated in one of the two thus far unresolved dorsalized complementation groups *lost-a-fin* (*laf*) or *piggytail* (*pgy*), wild-type *alk8* mRNA was injected into laf^{tm110} and pgy^{ty40} mutants. *laf* mutant embryos display dorsalization of intermediate strength (C1-C2), characterized by a complete loss of the ventral tail fin and an enlarged heart cavity at 36 hours postfertilization (Fig. 3B), while the phenotype of *pgy* mutants can be slightly stronger (up to C3; Mullins et al., 1996). The phenotypes of both mutants are significantly weaker than those caused by *alk8* antisense morpholino oligonucleotides (compare with Fig. 2D) and by mutations in *bmp2b*, *bmp7* or *smad5* (C4-C5; for *smad5*, see Fig. 3M). Injection of *alk8* mRNA led to a striking rescue of the *laf* phenotype (Table 1, Fig. 3C), but not of the *pgy* phenotype (data not shown), while injection of mRNAs encoding the putative Bmp receptors Alk3/BR1A and Alk6/BR1B had no rescuing effect (Table 1).

As a next step, the *alk8* gene and the *laf* mutation were genomically mapped. Using a radiation hybrid panel of the zebrafish genome (Geisler et al., 1999), *alk8* was mapped to LG2 between the markers z3743 and z17291. The *laf* allele laf^{tm110} (see below) was mapped to the same region (Fig. 4A). In addition, direct linkage analysis between the laf^{tm110} mutation and the *alk8* gene was carried out, taking advantage of a restriction fragment length polymorphism (RFLP) generated by the laf^{tm110} mutation. No recombination between laf^{tm110} and *alk8* was found in 280 meioses, indicating that they are located within 0.4 cM. Similarly, no recombination was found between *alk8* and the laf^{m100} allele (see below) in 50 meioses.

Finally, *alk8* cDNA was cloned and sequenced from mutant embryos of the two existing *laf* alleles, laf^{tm110} (isolated during the Tübingen screen; Mullins et al., 1996), and laf^{m100} (isolated during the Boston screen and initially named *grinch*; Solnica-Krezel et al., 1996). The two alleles behave very similarly. They cause phenotypes of identical strengths. In addition, both are zygotic recessive, and no dominant maternal or dominant zygotic effect has been observed.

Sequence analysis of *alk8* cDNA from laf^{tm110} mutant embryos revealed a TGC→CGC mutation causing a Cys→Arg exchange at amino acid position 91 in the extracellular domain of the Alk8 receptor (Fig. 4B). The mutated cysteine is part of a conserved motif, the cysteine-box, which is present in all serine/threonine kinase receptors. In contrast to wild-type *alk8* mRNA (see above), injected laf^{tm110} mutant *alk8* mRNA could not rescue the *laf* phenotype, even when double the amount of mRNA was injected (Table 1). The consequences of the Cys→Arg exchange of the laf^{tm110} allele were also examined in a second assay. A C-terminally truncated *Xenopus* Bmp receptor (XtBRIA; Graff et al., 1994) has been reported to dorsalize zebrafish embryos (Hammerschmidt et al., 1996b), presumably by titrating out Bmp ligand or type II co-receptor. Introduction of the Cys→Arg mutation into this truncated receptor abolished its dorsalizing potential: while injecting wild-type truncated receptor led to severe dorsalization (up to C4), no sign of dorsalization was observed after injection of Cys→Arg mutant truncated receptor (Table 1). This demonstrates that the laf^{tm110} mutation abolishes the ability of the truncated type I receptor to act in a dominant negative fashion, presumably by reducing its ability to bind ligand or type II co-receptor. Together, these results indicate that laf^{tm110} is a strong hypomorph or a null mutation.

Sequence analysis of the laf^{m100} allele revealed a

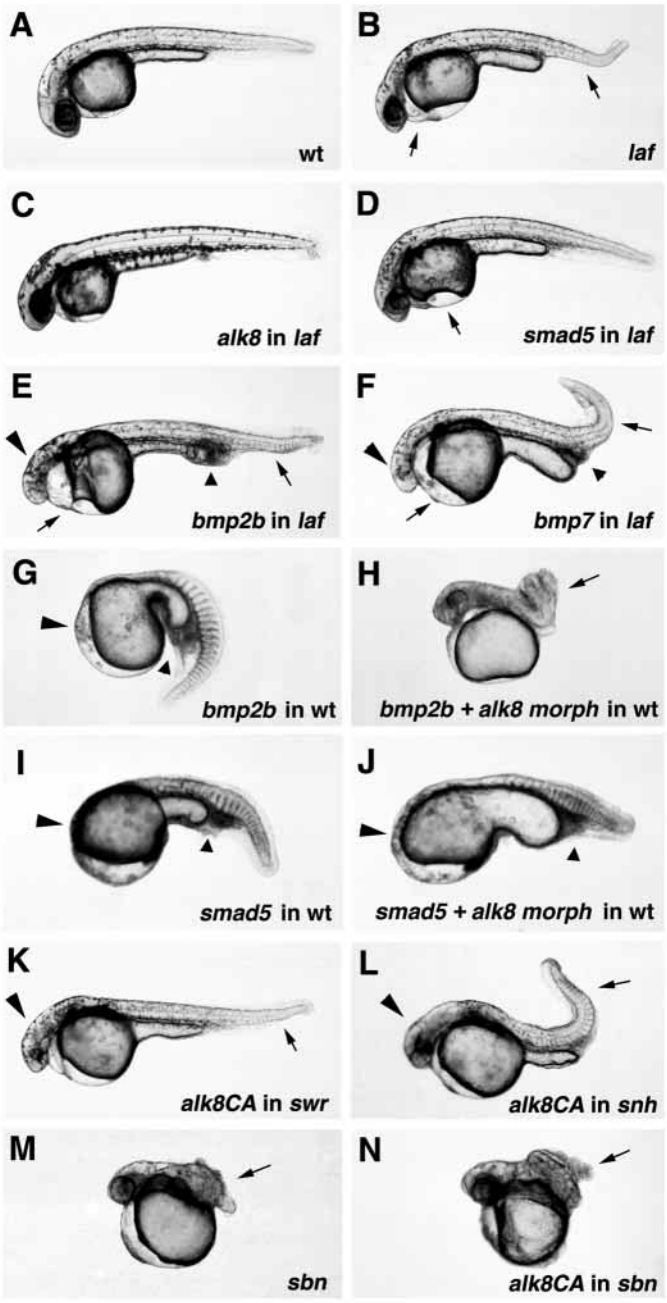

Fig. 3. *alk8* acts downstream of *bmp2b* and *bmp7* and upstream of *smad5*. All embryos are shown at 36 hours post-fertilization, lateral view, head towards the left. The *laf*, *swr* and *snh* embryos shown in panels C-F,K,L were genotyped after photography. (A) Wild-type sibling. (B) *laf*^{tm110} mutant. Arrows indicate absent ventral tail fin and enlarged pericardial cavity. (C) Rescued *laf*^{tm110} mutant, injected with *alk8* mRNA. (D) Rescued *laf*^{tm110} mutant, injected with *smad5* mRNA; arrow indicates starting swelling of the precardial cavity. (E) *laf*^{tm110} mutant, injected with *bmp2b* mRNA. (F) *laf*^{tm110} mutant, injected with *bmp7* mRNA. Arrowheads indicate small head and enlarged blood islands, both signs for ventralization; arrows indicate absent ventral tail fin and enlarged heart cavity, both *laf*-characteristic features (compare with B). (G-J) Genetically wild-type embryos after injection with *bmp2b* mRNA (G), co-injection of *bmp2b* mRNA and the *alk8* antisense morpholino oligonucleotide *alk8morph2* (H), injection of *smad5* mRNA (I), or co-injection of *smad5* mRNA and *alk8morph2* (J). The embryos in G,I,J display strong ventralization (V3; arrowheads indicate absent head and enlarged blood island); the embryo in H shows strong dorsalization (C4; arrow indicates wound-up trunk), similar to the *alk8morph2*-injected embryo shown in Fig. 2D. (K) Rescued *bmp2b* mutant *swr*^{ta72}, injected with *alk8CA* mRNA. (L) Rescued *bmp7* mutant *snh*^{ty68}, injected with *alk8CA* mRNA. Arrows in K,L indicate reduced or absent ventral tail fin, indicating mild dorsalization (C1); arrowheads indicate smaller head, indicating weak ventralization. (M,N) Sibling embryos from *sbn*^{dtc24} heterozygous mother; displaying C4 dorsalization, characterized by wound-up tail and trunk (arrows); (M) uninjected embryo; (N) *alk8CA* mRNA-injected embryo.

CGA→TGA nonsense mutation, introducing a premature stop codon at position 145, two amino acids after the transmembrane domain (Fig. 4B). This leads to a C-terminally truncated protein, similar to the dominant negative *Xenopus* receptor XtBR1A and the engineered truncated Alk8ΔC receptors described above. The resulting protein lacks the entire kinase domain, which is necessary to transmit the signal into the cell. Thus, the *laf*^{m100} allele should have no residual activity and, as expected, injected *laf*^{m100} *alk8* mRNA was not able to rescue the *laf* phenotype (Table 1). Similar to *alk8ΔC* (see above), but unlike *XtBRIA* (see above), injection of *laf*^{m100} *alk8* mRNA into wild-type embryos had no dorsalizing effect, indicating that in contrast to the truncated *Xenopus* type I receptor, truncated Alk8 does not act in a dominant negative manner. This is consistent with the genetic behavior of the mutation, which does not display any dominant effect (Solnica-Krezel et al., 1996). Thus, *laf*^{m100}, like *laf*^{tm110}, appears to be an amorph rather than an antimorph.

One explanation for the missing antimorphic character of the *laf*^{m100} mutation could be instability of mutant *alk8* mRNA (see Discussion for alternative explanation). Both RT-PCR analyses and whole-mount in situ hybridization revealed strongly reduced levels of *alk8* transcripts in *laf*^{m100} mutant embryos (Fig. 4C,D). In contrast, no such reduction was observed in *laf*^{tm110} mutant embryos, ruling out an autoregulatory mechanism (data not shown). Consistently, RT-PCR and restriction analyses of the *alk8* mRNA population in *laf*^{m100}/*laf*^{tm110} trans-heterozygotes revealed an at least 20-fold excess of *laf*^{tm110} transcripts (no *laf*^{m100} allele in 20 investigated RT-PCR clones).

Rescued *laf* mutants develop later defects

While injection of wild-type *alk8* mRNA into *laf/alk8* mutants led to embryos with wild-type appearance at the first day of development (see above; Fig. 3C), these embryos started to develop severe pericardiac edema around 36 hours after fertilization, and died between day 5 and day 10 of development (see Fig. 3D for an example of the starting phase of this phenotype; 10/10 larvae with normal ventral tail fins, but edemas at day 5 of development were genotyped as *laf*^{tm110} homozygotes). This edema phenotype of injected embryos was observed for both *laf* alleles and trans-heterozygotes (24% of offspring of *laf*^{tm110}/+ x *laf*^{tm110}/+ cross, n=350; 28% of offspring of *laf*^{m100}/+ x *laf*^{m100}/+ cross, n=108; 27% of offspring of *laf*^{tm110}/+ x *laf*^{m100} cross, n=218), suggesting that it is not due to closely linked second site mutations. When raising *alk8*-injected offspring of two *laf*^{tm110} carriers and genotyping them as adults, not a single homozygote could be

identified among over 150 tested fish. Therefore, no *laf* homozygous adult females were available to study whether the *laf* mutation has a recessive maternal effect.

alk8 acts downstream of *bmp2b*/*bmp7* and upstream of *smad5*

The dorsalized phenotype of the *laf* mutant suggests that Alk8 acts in a Bmp signal transduction pathway. To further test this notion, injection experiments were carried out in various dorsalized zebrafish mutants. First, we tested the ability of different components of the Bmp signal transduction pathways to rescue the *laf* mutant phenotype. Overexpression of *smad5*, encoding a transcription factor supposed to act downstream of the Bmp receptors, led to a normalization of the *laf* dorsalization to wild-type condition (Table 1; Fig. 3D).

In contrast, *bmp2b* or *bmp7* mRNA, both encoding potential Alk8 ligands, failed to rescue the phenotypic traits of *laf* mutants (Table 1; Fig. 3E,F). Although both mRNAs led to strong ventralization of *laf* mutant embryos characterized by a loss of dorsoanterior fates, the same embryos still displayed severe edema and the absence of the ventral tail fin, the characteristics of *laf* mutants. This demonstrates that, despite their strong ventralizing activity, both Bmp proteins are unable to rescue the defects of *laf* mutant embryos. Consistent results were obtained when mRNA was co-injected with morpholino antisense oligonucleotides in order to interfere with the aforementioned strong dorsalization caused by the maternal-zygotic *alk8* knockdown (see Fig. 2D). *bmp2b* or *bmp7* mRNA, although strongly ventralizing in parallel single injections (Fig. 3G; Table 1), had hardly any effect in co-injections with the dorsalizing *alk8* morpholino antisense oligonucleotides (Fig. 3H; Table 1), indicating that a gain of Bmp2b or Bmp7 function cannot compensate for the effect caused by the loss of Alk8 function. In contrast, co-injection of *smad5* mRNA and *alk8* morpholino antisense oligonucleotides led to ventralized phenotypes (Fig. 3J; Table 1), as after injection of *smad5* mRNA alone (Fig. 3I; Table 1). Altogether, the data suggest that Alk8 is essential to mediate Bmp2/Bmp7 signaling. In contrast, Smad5 action is not blocked by the loss of Alk8 function, suggesting that Smad5 acts downstream of Alk8 or in a parallel pathway.

To distinguish between the two latter possibilities, the constitutively active form of Alk8 described above (Fig. 2B) was applied to *bmp2b*, *bmp7* and *smad5* mutant embryos. Injection of *alk8CA* mRNA into *swirl/bmp2b* (Kishimoto et al., 1997; Nguyen et al., 1998b; Fig. 3K) or *snailhouse/bmp7* (Schmid et al., 2000; Dick et al., 2000) mutant embryos (Fig. 3L) led to an almost complete rescue of the strongly dorsalized phenotype (Table 1). Consistently, *alk8CA* mRNA had a strong ventralizing effect even when co-injected with (normally strongly dorsalizing) *bmp2b* antisense morpholino oligonucleotides (Table 1). In contrast, *somitabun/smad5* mutants (Hild et al., 1999) showed no response to *alk8CA* (Fig. 3M,N; Table 1), indicating that Alk8 requires Smad5 to mediate Bmp signaling.

DISCUSSION

The dorsalization of *laf* mutants is caused by null mutations in Alk8

In the past two years, two Bmp ligands (Bmp2b, Bmp7) and one downstream transcription factor (Smad5) have been shown to be essential for ventral development during dorsoventral pattern formation of the zebrafish embryo. Here, we have identified a receptor, the serine/threonine kinase Alk8, as an additional essential component. Two mutant alleles are described: *lost-a-fin laf*^{tm110} from the Tübingen screen and *laf*^{m100} from the Boston screen, both of which appear to be null mutations.

laf^{m100} encodes a C-terminally truncated receptor that lacks its entire cytoplasmic kinase domain. In contrast to similar versions of other type I receptors such as *Xenopus* and zebrafish Alk3/BR1A (Graff et al., 1994; Nikaido et al., 1999a), however, truncated *laf*^{m100} Alk8 does not act in a dominant fashion. This could be due to a specific degradation of mutant *laf*^{m100} *alk8* RNA caused by the premature stop codon, in line with the strongly reduced *laf*^{m100} *alk8* mRNA levels found in mutant embryos. Recent findings indicate that such nonsense-mediated decay is a widely used mechanism to eliminate mRNAs with nonsense mutations (reviewed in Czaplinski et al., 1999). Alternatively, the absent dominant effect of the *laf*^{m100} mutation could be explained by differences in the mechanisms of signal transduction used by Alk8 and other type I receptors, such that only Alk8 requires its intracellular domain to bind ligand and/or co-receptor. Such a role of the cytoplasmic domain would explain why even the engineered C-terminally truncated versions of Alk8, which lack all possible 3′ elements associated with nonsense-mediated decay (Czaplinski et al., 1999), had hardly any dorsalizing effects, whereas the kinase-dead version Alk8(K232R), which carries just a single amino acid exchange in the ATP-binding domain, was strongly dorsalizing.

Alk8 acts as a Bmp2b/7 receptor upstream of Smad5

Previously, the function of Alk8 and potential ligands was unknown. No *alk8* orthologs have been described from other vertebrate species (Yelick et al., 1998). Sequence comparison with other type I receptors revealed that Alk8 is rather divergent from the hitherto described Bmp receptors Alk3/BmpRIA and Alk6/BmpRIB. Alk8 shares highest similarity with Alk2, previously known as ActRIA, and the other members of the Alk1 subgroup of Tgfβ receptors (Massagué, 1998; Chen and Massagué, 1999). For Alk2, a role during signaling by Bmps as well as by other Tgfβ proteins has been suggested. It has been shown to bind activin and Tgfβ1, and to mediate activin-like responses in cell culture studies (Attisano et al., 1993; Ebner et al., 1993; ten Dijke et al., 1994; Yamashita et al., 1995). In addition, Alk2 is capable of binding Bmp7 (ten Dijke et al., 1994; Liu et al., 1995) and possibly Bmp2/4, although with lower affinity (Liu et al., 1995). Functional studies in *Xenopus* indicate that Alk2 is involved in the induction of ventral cell types, similar to Bmp2 and Bmp4 (Armes and Smith, 1997). In line with these findings, it was shown that Alk2, despite its different L45 loop (see Introduction), can phosphorylate and activate Smad1, a transducer of Bmp2/4 signals, but not Smad2, a transducer of Tgfβ and activin signals (Macias-Silva et al., 1998; Chen and Massagué, 1999), similar to the Smad specificities of the classical Bmp receptors Alk3/BmpRIA and Alk6/BmpRIB (Kretschmar et al., 1997).

Another prominent member of the Alk1 subgroup of Tgfβ receptors is *Drosophila* Saxophone (Massagué, 1998).

Previous reports have described Saxophone as a receptor of the *Drosophila* Bmp2/4 homolog Decapentaplegic (Dpp, Brummel et al., 1994; Nellen et al., 1994). However, more recent findings indicate that Saxophone mediates signaling by the more distantly related Tgfβ protein Screw (Neul and Ferguson, 1998; Nguyen et al., 1998a) and the Bmp7-related protein Gbb-60A (Chen et al., 1998a; Khalsa et al., 1998; Haerry et al., 1998), while Dpp signaling is transduced by the serine/threonine kinase receptor Thick veins (Neul and Ferguson, 1998; Nguyen et al., 1998). This suggests that Saxophone might be a receptor of Bmp7-like ligands. Interestingly, Dpp and Screw have synergistic roles during embryonic dorsoventral patterning (Neul and Ferguson, 1998; Nguyen et al., 1998a), and the same is true for Dpp and Gbb-60A during wing formation of *Drosophila* (Khalsa et al., 1998; Haerry et al., 1998), similar to the equivalent roles of the related vertebrate proteins Bmp2b and Bmp7 during dorsoventral patterning of the zebrafish embryo (Dick et al., 2000; Schmid et al., 2000). In summary, there are hints pointing to Alk8-related proteins as a distinct class of Bmp receptors, while other data suggest other functions.

Here, we provide genetic data pointing to a role of zebrafish Alk8 as a receptor for Bmp2b and Bmp7. The *alk8* loss-of-function phenotype indicates that Alk8 is required for the specification of ventral fates during dorsoventral pattern formation, similar to Bmp2b, Bmp7 and the Bmp signal transducer Smad5 (Kishimoto et al., 1997; Nguyen et al., 1998b; Schmid et al., 2000; Dick et al., 2000; Hild et al., 1999). In addition we found that Smad5, but not Bmp2b and Bmp7 can rescue the *alk8* loss-of-function phenotype, while Alk8 can rescue the *bmp2b* and *bmp7*, but not the *smad5*, mutant phenotypes, demonstrating that Alk8 acts downstream of Bmp2b and Bmp7 and upstream of Smad5. These data, however, do not exclude the possibility that Alk8 serves as a receptor for other ligands such as Bmp4. Biochemical studies are in progress to clarify this point.

The requirement of maternally supplied *alk8* mRNA

The phenotypes caused by the zygotic effect of null mutations in *alk8* are relatively weak compared with those caused by the loss of the two putative Alk8 ligands Bmp2b and Bmp7 (Kishimoto et al., 1997; Nguyen et al., 1998b; Dick et al., 2000; Schmid et al., 2000) and the putative downstream transcription factor Smad5 (Hild et al., 1999). This indicates that some, but not all Bmp2b/7 signaling depends on zygotic Alk8, a notion supported by our results obtained in injection studies and double mutant analyses. Injected *bmp2b* and *bmp7* mRNA, although unable to rescue the Alk8-dependent development of the ventral tail fin, can ventralize all other body regions of *laf* mutant embryos. Similarly, loss of the Bmp2/7 inhibitor Chordin (Hammerschmidt et al., 1996b; Schulte-Merker et al., 1997; Dick et al., 2000) in *din*/*laf* double mutants leads to reduced head sizes and enlarged blood islands as in *din* single mutants and after *bmp2/7* overexpression, while the *laf*-specific ventral tail fin deficiency is maintained (H. B. and M. H., unpublished observation). Altogether, these data indicate that – except during ventral tail fin specification – Bmp2b/7 signals can use receptors that act in parallel to those encoded by zygotically generated *alk8* mRNA.

It appears that these partially redundant receptors are encoded by maternally supplied *alk8* mRNA (see Fig. 1B), similar to the situation found for the putative downstream component Smad5: loss of zygotic *smad5* gene products leads to a very weak dorsalization (C1); loss of maternal gene products to a very strong dorsalization (C4-5; Hild et al., 1999; T. Mayr and M. H., unpublished observations). To study a possible recessive maternal effect of the *laf* mutations, homozygous *laf* mutant females need to be generated, which unfortunately has failed so far, due to later lethality of *alk8* mRNA-injected *laf* embryos (see below). As an alternative approach to target both maternally and zygotically supplied *alk8* mRNA, we injected antisense morpholino oligonucleotides. *alk8morph* injections led to dorsalization up to C5, which is as strong as that caused by the loss of *bmp2b*, *bmp7* or *smad5* function, and significantly stronger than the C1/C2

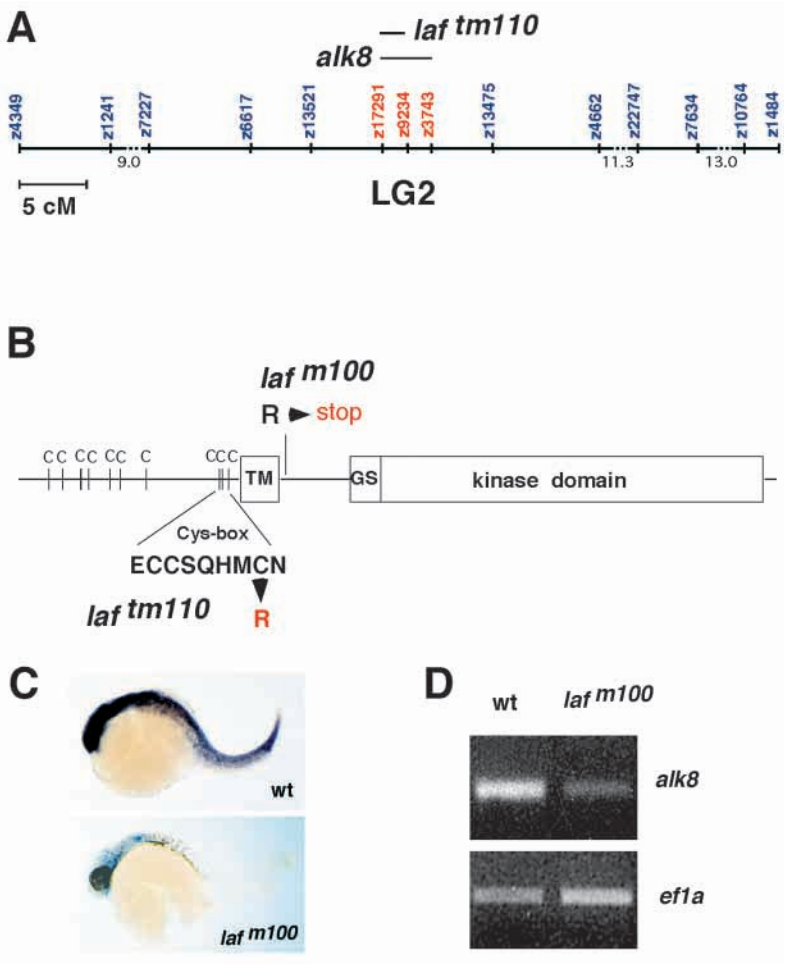

Fig. 4. *lost-a-fin* is *alk8*. (A) Genomic position of the zebrafish *alk8* gene and the *lost-a-fin* mutation *laf*^{tm110}. (B) Alk8 protein; the mutations found in *laf*^{tm110} and *laf*^{m100} mutant Alk8 are indicated with arrowheads. TM, transmembrane domain; GS, type I receptor-specific GS domain. (C,D) *alk8* expression in *laf*^{m100} mutant and wild-type sibling embryos at 36 hours post-fertilization, detected via whole-mount in situ hybridization (C), and via RT-PCR analysis, with *ef1a* as control (D).

dorsalization caused by the zygotic effect of the *laf* mutations. In contrast to the C1/C2 *laf* mutants (see above), the C5 *alk8* morphants do not respond to co-injected *bmp2b* or *bmp7* mRNA. This indicates that dorsoventral patterning and Bmp2b signal transduction does indeed depend on maternally supplied *alk8* gene products that can partly compensate for the loss of zygotic *alk8* in mutant offspring from heterozygous mothers. According to this notion, the missing zygotic supply of wild-type *alk8* mRNA leads to a progressively increasing reduction in the overall concentration of Alk8 receptors that affects only those cell fates specified the latest and/or requiring strongest Bmp signaling (as is indeed the case in *laf* mutants).

A second reason for the observed weak phenotype of *laf* mutants could be partial redundancy of Alk8 with other receptors such as the 'classical' Bmp type I receptors Alk3/BmpR1A and Alk6/BmpR1B, whose mRNAs are present during all relevant stages. However, this possibility appears quite unlikely in the light of preliminary results, according to which targeting of either receptor with morpholino antisense oligonucleotides has no dorsalizing or *alk8*-enhancing effect (H. B. and M. H., unpublished observations).

Possible later functions of *laf/alk8*

In addition to dorsoventral pattern formation, *alk8* might be required for later developmental processes. This is indicated by results obtained in our *laf* rescuing experiments. Upon injection of *alk8* mRNA, the *laf*-specific dorsoventral defects were normalized in most of the injected *laf* mutants. However, in contrast to all other studied dorsalized mutants, none of these embryos survived to adulthood. Rather, they developed pericardiac edema and died at larval stages. More detailed analyses of such *alk8* mRNA-injected *laf* mutants are under way to address possible later functions of the Alk8 receptor.

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NOTE ADDED IN PROOF

In the accompanying paper (Mintzer et al., 2001), similar results on the essential role of Alk8/Laf during dorsoventral pattern formation of the zebrafish are described. Mintzer et al. succeeded in raising and breeding homozygous *laf* mutant females. The offspring of such homozygous mothers are strongly dorsalized, which correlates well with our results obtained using morpholino antisense oligonucleotide injections.

REFERENCES

Armes, N. A. and Smith, J. C. (1997). The ALK-2 and ALK-4 activin receptors transduce distinct mesoderm-inducing signals during early *Xenopus* development but do not co-operate to establish thresholds. *Development* **124**, 3797-3084.

Attisano, L., Carcamo, J., Ventura, F., Weis, F. M., Massagué, J. and Wrana, J. L. (1993). Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* **75**, 671-680.

Bassing, C.H., Yingling, J.M., Howe, D.J., Wang, T., He, W.W., Gustafson, M.L., Shah, P., Donahoe, P.K. and Wang, X.-F. (1994). A transforming growth factor β type I receptor that signals to activate gene expression. *Science* **263**, 87-89

Bauer, H., Meier, A., Hild, M., Stachel, S., Economides, A., Hazelett, D., Harland, R. M. and Hammerschmidt, M. (1998). Follistatin and Noggin are excluded from the zebrafish organizer. *Dev. Biol.* **204**, 488-507.

Blader, P., Rastegar, S., Fischer, N. and Strähle, U. (1997). Cleavage of the BMP-4 antagonist Chordin by zebrafish Tolloid. *Science* **278**, 1937-1940.

Brummel, T. J., Twombly, V., Marques, G., Wrana, J. L., Newfeld, S. J., Attisano, L., Massagué, J., O'Connor, M. B. and Gelbart, W. M. (1994). Characterization and relationship of Dpp receptors encoded by the *saxophone* and *thick veins* genes in *Drosophila*. *Cell* **78**, 251-261.

Chen, Y., Riese, M. J., Killinger, M. A. and Hoffmann, F. M. (1998a). A genetic screen for modifiers of *Drosophila decapentaplegic* signaling identifies mutations in *punt*, *mothers against dpp* and the BMP-7 homologue, *60A*. *Development* **125**, 1759-1768.

Chen, Y.-G., Hata, A., Lo, R. S., Wotton, D., Shi, Y., Pavletich, N. and Massagué, J. (1998b). Determinants of specificity in TGF-β signal transduction. *Genes Dev.* **12**, 2144-2152.

Chen, Y. G. and Massagué, J. (1999). Smad1 recognition and activation by the ALK1 group of transforming growth factor-β family receptors. *J. Biol. Chem.* **274**, 3672-3677.

Connors, S. A., Trout, J., Ekker, M. and Mullins, M. C. (1999). The role of *tolloid*/*minifin* in dorsoventral pattern formation of the zebrafish embryo. *Development* **126**, 3119-3130.

Czaplinski, K., Ruiz-Echevarria, M. J., González, C. I. and Peltz, S. W. (1999). Should we kill the messenger? The role of the surveillance complex in translation termination and mRNA turnover. *BioEssays* **21**, 685-696.

Dale, L., Howes, G., Price, B. M. J. and Smith, J. C. (1992). Bone morphogenetic protein 4: a ventralizing factor in early *Xenopus* development. *Development* **115**, 573-585.

Dick, A., Hild, M., Bauer, H., Imai, Y., Maifeld, H., Schier, A., Talbot, W., Bouwmeester, T. and Hammerschmidt, M. (2000). Essential role of Bmp7 (*snailhouse*) and its prodomain in dorsoventral patterning of the zebrafish embryo. *Development* **127**, 343-354.

Ebner, R., Chen, R. H., Lawler, S., Zioncheck, T. and Derynck, R. (1993). Determination of type I receptor specificity by the type II receptors for TGF-beta or activin. *Science* **262**, 900-902.

Geisler, R., Rauch, G.-J., Baier, H., van Bebber, F., Broß, L., Davis, R. W., Dekens, M., Finger, K., Fricke, C., Gates, M. A. et al. (1999). A radiation hybrid map of the zebrafish genome. *Nat. Genet.* **23**, 86-89.

Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J. and Melton, D. A. (1994). Studies with a *Xenopus* BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals in vivo. *Cell* **79**, 169-179.

Haerry, T. E., Khalsa, O., O'Connor, M. B. and Wharton, K. A. (1998). Synergistic signaling by two BMP ligands through the SAX and TKV receptors controls wing growth and patterning in *Drosophila*. *Development* **125**, 3977-3987.

Hammerschmidt, M., Pelegri, F., Mullins, M. C., Kane, D. A., van Eeden, F. J. M., Granato, M., Brand, M., Furutani-Seiki, M., Haffter, P., Heisenberg, C.-P. et al. (1996a). *dino* and *mercedes*, two genes regulating dorsal development in the zebrafish embryo. *Development* **123**, 95-102.

Hammerschmidt, M., Serbedzija, G. N. and McMahon, A. P. (1996b). Genetic analysis of dorsoventral pattern formation in the zebrafish: Requirement of a BMP-like ventralizing activity and its dorsal repressor. *Genes Dev.* **10**, 2452-2461.

Hammerschmidt, M., Blader, P. and Strähle, U. (1999). Strategies to perturb zebrafish development. *Meth. Cell Biol.* **59**, 87-115.

Hawley, S. H. B., Wünnenberg-Stapleton, K., Hashimoto, C., Laurent, M. N., Watabe, T., Blumberg, B. W. and Cho, K. W. Y. (1995). Disruption of BMP signals in embryonic *Xenopus* ectoderm leads to direct neural induction. *Genes Dev.* **9**, 2923-2935.

Heasman, J., Kofron, M. and Wylie, C. (2000). β-catenin signaling activity dissected in early *Xenopus* embryos: a novel antisense approach. *Dev. Biol.* **222**, 124-134.

Hild, M., Dick, A., Rauch, J. G., Meier, A., Bouwmeester, T., Haffter, P.

and Hammerschmidt, M. (1999). The *smad5* mutation *somitabun* blocks Bmp2b signaling during early dorsoventral patterning of the zebrafish embryo. *Development* **126**, 2149-2159.

Hogan, B. L. M. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10**, 1580-1594.

Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, W. V. E. and Hogan, B. L. M. (1992). DVR-4 (bone morphogenetic protein-4) as a posterior ventralizing factor in *Xenopus* mesoderm induction. *Development* **115**, 639-647.

Khalsa, O., Yoon, J. W., Torres-Schumann, S. and Wharton, K. A. (1998). TGFβ/BMP superfamily members, Gbb-60A and Dpp, cooperate to provide pattern information and establish cell identity in the *Drosophila* wing. *Development* **125**, 2723-2734.

Kishimoto, Y., Lee, K.-H., Zon, L., Hammerschmidt, M. and Schulte-Merker, S. (1997). The molecular nature of *swirl*: BMP2 function is essential during early dorsoventral patterning. *Development* **124**, 4457-4466.

Knapik, E. W., Goodman, A., Ekker, M., Chevrette, M., Delgado, J., Neuhauss, S., Shimoda, N., Driever, W., Fishman, M. C. and Jacob, H. J. (1998). A microsatellite genetic linkage map for zebrafish (*Danio rerio*). *Nature Genet.* **18**, 338-343.

Kretschmar, M., Liu, F., Hata, A., Doody, J. and Massagué, J. (1997). The TGF-β mediator Smad1 is directly phosphorylated and functionally activated by the BMP receptor kinase. *Genes Dev.* **11**, 984-995.

Liu, F., Ventura, F., Doody, J. and Massagué, J. (1995). Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. *Mol. Cell. Biol.* **15**, 3479-3486.

Macias-Silva, M., Hoodless, P. A., Tang, S. J., Buchwald, M. and Wrana, J. L. (1998). Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. *J. Biol. Chem* **273**, 25628-25636.

Maeno, M., Ong, R. C., Suzuki, A., Ueno, N. and Kung, H. (1994). A truncated bone morphogenetic protein receptor alters the fate of ventral mesoderm to dorsal mesoderm: roles of animal pole tissue in the development of the ventral mesoderm. *Proc. Natl. Acad. Sci. USA* **91**, 10260-10264.

Massagué, J. (1998). TGF-β signal transduction. *Annu. Rev. Biochem*. **67**, 753-791.

Mintzer, K., Lee, M. A., Runke, G., Trout, J., Whitman, M. and Mullins, M. C. (2001). *lost-a-fin* encodes a type I BMP receptor, Alk8, acting maternally and zygotically in dorsoventral pattern formation. *Development* **128**, 859-869.

Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Brand, M., van Eeden, F. J. M., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C.-P. et al. (1996). Genes establishing dorsoventral pattern formation in the zebrafish embryo: the ventral specifying genes. *Development* **123**, 81-93.

Nasevicius, A. and Ekker. S.C. (2000). Effective targeted gene knockdown in zebrafish. *Nat. Genet.* **26**, 216-220.

Nellen, D., Affolter, M. and Basler, K. (1994). Receptor serine/threonine kinases implicated in the control of *Drosophila* body pattern by *decapentaplegic*. *Cell* **78**, 225-237.

Neul, J. L. and Ferguson, E. L. (1998). Spatially restricted activation of the SAX receptor by SCW modulates DPP/TKV signaling in *Drosophila* dorsal-ventral patterning. *Cell* **95**, 483-494.

Nguyen, M., Park, S., Marqués, G. and Arora, K. (1998a). Interpretation of a BMP activity gradient in *Drosophila* embryos depends on synergistic singnaling by two type I receptors, SAX and TKV. *Cell* **95**, 495-506.

Nguyen, V. H., Schmid, B., Trout, J., Connors, S.A., Ekker, M., Mullins, M.C. (1998b). Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a *bmp2b/swirl* pathway of genes. *Dev. Biol.* **199**, 93-110.

Nikaido, M., Tada, M., Saij, T. and Ueno, N. (1997). Conservation of BMP signaling in zebrafish mesoderm patterning. *Mech. Dev.* **61**, 75-88.

Nikaido, M., Tada, M., Takeda, H., Kuroiwa, A. and Ueno, N. (1999a). In vivo analysis using variants of zebrafish BMPR-1A: range of action and involvement of BMP in ectoderm patterning. *Development* **126**, 181-190.

Nikaido, M., Tada, M. and Ueno, N. (1999b). Restricted expression of the receptor serine/threonine kinase BMPR-IB in zebrafish. *Mech. Dev.* **82**, 219-222.

Nordness, S., Krauss, S. and Johansen, T. (1994). cDNA sequence of zebrafish (*Brachydanio rerio*) translation elongation factor-1 alpha: molecular phylogeny of eukaryotes based on elongation factor-1 alpha protein sequences. *Biochim. Biophys. Acta* **1219**, 529-532.

Oxtoby, E. and Jowett, T. (1993). Cloning of the zebrafish *krox-20* (*krx-20*) and its expression during hindbrain development. *Nucleic Acids Res.* **21**, 1087-1095.

Piccolo, S., Y., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). A possible molecular mechanism for Spemann organizer function: inhibition of ventral signals by direct binding of Chordin to BMP-4. *Cell* **85**, 589-598.

Piccolo, S., Agius, E., Lu, B., Goodman, S., Dale, L. and DeRobertis, E. M. (1997). Cleavage of Chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. *Cell* **91**, 407-416.

Rauch, G.-J., Granato, M. and Haffter, P. (1997). A polymorphic zebrafish line for genetic mapping using SSLPs on high-percentage agarose gels. *Tech. Tips Online* T01208,

Rupp, R. A. W., Snider, L. and Weintraub, H. (1994). *Xenopus* embryos regulate the nuclear localization of XMyoD. *Genes Dev.* **8**, 1311-1323.

Schmid, B., Fürthauer, M., Conners, S. A., Trout, J., Thisse, B., Thisse, C. and Mullins, M. C. (2000). Equivalent genetic roles of *bmp7*/*snailhouse* and *bmp2b*/*swirl* in dorsoventral pattern formation. *Development* **127**, 957-967.

Schmidt, J. E., Suzuki, A., Ueno, N. and Kimelman, D. (1995). Localized BMP-4 mediates dorsal/ventral patterning in the early *Xenopus* embryo. *Dev. Biol.* **169**, 37-50.

Schulte-Merker, S., Lee, L. J., McMahon, A. P. and Hammerschmidt, M. (1997). The zebrafish organizer requires *chordino*. *Nature* **387**, 862-863.

Solnica-Krezel, L., Stemple, D. L., Mountcastle-Shah, E., Rangini, Z., Neuhauss, S. C. F., Malicki, J., Schier, A., Stanier, D. Y. R., Zwartkruis, F., Abdelilah, S. and Driever, W. (1996). Mutations affecting cell fates and cellular rearrangements during gastrulation in the zebrafish. *Development* **123**, 67-80.

Suzuki, A., Thies, R. S., Yamayi, N., Song, J. J., Wozney, J. M., Murakami, K. and Ueno, N. (1994). A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early *Xenopus* embryo. *Proc. Natl. Acad. Sci. USA* **91**, 10255-10259.

ten Dijke, P., Yamashita, H., Sampath, T. K., Reddi, H., Estevez, M., Riddle, D. L., Ichijo, H., Heldin, C.-H. and Miyazono, K. (1994). Identification of type I receptors for Osteogenic Protein-1 and Bone Morphogenetic Protein-4. *J. Biol. Chem.* **269**, 16985-16988.

Wieser, R., Wrana, J. and Massagué, J. (1995). GS domain mutations that constitutively activate TβR-1, a downstream signaling component in the TGFβ receptor complex. *EMBO J.* **14**, 2199-2208.

Wilson, P. A. and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* **376**, 331-333.

Wrana, J. L., Tran, H., Attisano, L., Arora, K., Childs, S. R., Massagué, J. and O'Connor, M. B. (1994). Two distinct transmembrane serine/threonine kinases from *Drosophila melanogaster* form an activin receptor complex. *Mol. Cell. Biol.* **14**, 944-950.

Yamashita, H., ten Dijke, P., Huylebroeck, D., Sampath, T. K., Andries, M., Smith, J. C., Heldin, C. H. and Miyazono, K. (1995). Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *J. Cell. Biol.* **130**, 217-226.

Yelick, P. C., Abduljabbar, T.S. and Stashenko, P. (1998). zALK-8, a novel type I serine/threonine kinase receptor, is expressed throughout early zebrafish development. *Dev. Dyn.* **211**, 352-361.