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Ogon/Secreted Frizzled functions as a negative feedback regulator of Bmp signaling

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SUMMARY

The zebrafish mutant *ogon* (also called *mercedes* and *short tail*) displays ventralized phenotypes similar to the *chordino* (*dino*) mutant, in which the gene for the Bmp antagonist Chordin is mutated. We isolated the gene responsible for *ogon* by a positional cloning strategy and found that the *ogon* locus encodes a zebrafish homolog of Secreted Frizzled (Sizzled), which has sequence similarity to a Wnt receptor, Frizzled. Unlike other secreted Frizzled-related proteins (sFrps) and the Wnt inhibitor Dickkopf1, the misexpression of Ogon/Sizzled dorsalized, but did not anteriorize, the embryos, suggesting a role for Ogon/Sizzled in Bmp inhibition. Ogon/Sizzled did not inhibit a Wnt8-dependent transcription in the zebrafish embryo. *ogon/sizzled* was expressed on the ventral side from the late blastula through the gastrula stages. The ventral *ogon/sizzled* expression in the gastrula stage was reduced or absent in the *swirl/bmp2b* mutants but expanded in the *chordino* mutants. Misexpression of *ogon/sizzled* did not dorsalize the *chordino* mutants, suggesting that Ogon/Sizzled required Chordin protein for dorsalization and Bmp inhibition. These data indicate that Ogon/Sizzled functions as a negative regulator of Bmp signaling and reveal a novel role for a sFrp in dorsoventral patterning.

Key words: Zebrafish, Ogon, Sizzled, Bmp, Chordin, Dorsoventral patterning, Feedback inhibitor

INTRODUCTION

One of the most important processes in the generation of vertebrate embryos is the formation of the dorsoventral (DV) and anteroposterior (AP) axes, in which cells at the appropriate positions adopt specific fates. During vertebrate embryogenesis, the dorsal organizer (which is also called Spemann's organizer in amphibian embryos, the node in mice and Hensen's node in chicks), plays a pivotal role in establishing these axes. Molecular identification of the dorsal organizer-derived inductive signals has revealed that Chordin and Noggin, generated from the dorsal organizer, bind to and prevent bone morphogenetic proteins (Bmps) from activating their receptors and thereby inhibit Bmp-dependent ventralization in *Xenopus* embryos (Piccolo et al., 1996; Zimmerman et al., 1996) (reviewed by De Robertis et al., 2000). Interactions between Bmps and the Bmp antagonists at the gastrula stage refine the DV axis in the mesoderm and endoderm, and are also involved in the formation of the neuroectoderm from the dorsal ectoderm. The interaction between Bmps and the Bmp antagonist Chordin is modulated by the metalloproteinase Tolloid (Blader et al., 1997; Piccolo et al., 1997), which cleaves and inactivates Chordin protein and Twisted Gastrulation (TSG), which can make a complex with Bmp and Chordin (Chang et al., 2001; Larrain et al., 2001; Oelgeschlager et al., 2000; Ross et al., 2001; Scott et al., 2001). In addition to Bmp inhibitors, the organizer-derived Wnt inhibitors (such as Dickkopf1, Frzb1 and Cerberus) and Nodal inhibitors (Lefty/Antivin and Cerberus) are thought to prevent the propagation of posteriorizing signals emanating from the mesoderm and endoderm that are involved in formation of the AP axis.

Roles for Bmp signaling in DV axis formation are also supported by analyses of zebrafish mutants that display abnormalities in DV patterning. The dorsalized mutants *swirl* (*swr*) and *snailhouse* (*snh*) have defective *bmp2b* and *bmp7* genes, respectively (Dick et al., 2000; Kishimoto et al., 1997; Schmid et al., 2000). *somitabun*, *captain hook* and *piggy tail* all have defects in the zebrafish gene for Smad5 (*madh5* – Zebrafish Information Network) (Hild et al., 1999; Kramer et al., 2002), which functions as a signal transducer for Bmp signaling. *lost-a-fin* encodes the gene for the type I Bmp receptor Alk8 (Bauer et al., 2001; Mintzer et al., 2001), and *mini fin* encodes Tolloid (Connors et al., 1999). All of these data indicate that Bmp2 and Bmp7 and their signaling play essential roles in the formation of ventral tissues. In contrast to the dorsalized mutants, there are only two mutants, namely *dino* and *ogon*, that have been reported to display clearly

ventralized phenotypes without other abnormalities in the early specification of the dorsal organizer (such as those seen in the *bozozok* mutants) (Hammerschmidt et al., 1996a; Solnica-Krezel et al., 1996). These ventralized mutant embryos display an expansion of ventral tissues, such as the ventral tail fin, posterior somatic mesoderm, blood and pronephron, and a reduction, to various degrees, of the anterior somites and the neuroectoderm. The *dino* (*din*) locus encodes the zebrafish ortholog of Chordin (Chordino; Chd – Zebrafish Information Network), whereas the molecular identity of the *ogon* locus has not yet been elucidated.

Complementation and mapping analyses revealed that *ogon*^{m60}, *mercedes*^{tm305} and *short tail*^{b180} are allelic, and thus commonly referred to as *ogon* (*ogo*) (Miller-Bertoglio et al., 1999). The *ogo*^{m60} and *ogo*^{b180} mutations are deficiencies in the distal part (close to the telomere) of linkage group 25 (LG25), indicating that the *ogo* locus is localized to the deleted region. The N-ethyl-N-nitrosourea (ENU)-induced allele *ogo*^{tm305} displays viable phenotypes, which are less severe than those of the *ogo*^{m60} and *ogo*^{b180} mutants, suggesting that *ogo*^{tm305} is a hypomorphic allele or that *ogo*^{m60} and *ogo*^{b180} harbor the loss of additional gene(s) in the deletion. The *ogo*^{m60} mutant embryo displays neural degeneration in addition to the ventralized phenotypes. The ventralized phenotypes of *ogo* are similar to those of *chordino* mutants, except that the reduction of the anterior neuroectoderm is less severe in the *ogo* than in the *din* mutants. It has also been reported that a maternally derived *ogo* gene contributes to dorsoventral patterning (Miller-Bertoglio et al., 1999). The ventralized phenotypes of *ogo* are fully suppressed by the overexpression of the Bmp antagonists Chordin and Noggin, or the expression of a dominant-negative type II Bmp receptor (Miller-Bertoglio et al., 1999). Epistatic analyses revealed that *swr/bmp2b* and *snh/bmp7* are epistatic to *ogo* in DV patterning (Miller-Bertoglio et al., 1999; Wagner and Mullins, 2002). In ventral tail fin formation, *lost-a-fin/alk8*, is epistatic to *ogo* (Wagner and Mullins, 2002). All of these data consistently indicate that *ogo* encodes a dorsalizing factor that inhibits Bmp signaling either directly or indirectly. In contrast to *chordino*, *ogo* does not show an epistatic relationship with *mini fin/tolloid* (Wagner and Mullins, 2002), suggesting that *ogo* functions differently from *chordin*. It has been reported that elimination of both the zygotic *ogo* and *chordin* genes additively ventralizes the embryo, implying distinct requirements for these genes in DV axis formation (Hammerschmidt et al., 1996a; Miller-Bertoglio et al., 1999). The molecular identification of *ogo* is required to elucidate the precise relationship between *ogo* and *chordin*, and the molecular mechanisms by which *ogo* regulates the formation of the DV axis.

In this study, we isolated the gene responsible for the *ogo* mutants by a positional cloning strategy and found that the *ogo* locus encodes a zebrafish homolog of Secreted Frizzled (Sizzled). The *sizzled* (*szl*) gene was originally identified in *Xenopus* based on its ability to dorsalize the *Xenopus* embryo (Salic et al., 1997). Szl displays sequence similarity with a Wnt receptor Frizzled and is reported to function as an inhibitor of *Xenopus* Wnt8 (XWnt8) (Salic et al., 1997). However, *szl* and a *szl*-related gene *sizzled2* reportedly do not inhibit the activity of XWnt8, suggesting they have a different mode of action in DV axis formation (Bradley et al., 2000; Collavin and Kirschner, 2003). We found that Ogo/Szl functions to inhibit Bmp signaling in a manner that does not involve the inhibition of Wnt8-mediated signaling. Ogo/Szl requires Chordin protein for its dorsalizing activity. In contrast to other dorsalizing factors, *ogo/szl* is expressed on the ventral side and requires Bmp signaling. Our results suggest that Ogo/Szl functions as a negative-feedback regulator of Bmp signaling and provide a novel mechanism by which the DV axis is established during gastrulation.

MATERIALS AND METHODS

Isolation of *ogon*^{rk1} and mutant fish

ENU-treated AB male fish were mated with wild-type AB female fish to generate F1 female progeny. Haploid embryos were generated by in vitro fertilization using oocytes from the F1 female fish and ultra-violet ray-irradiated sperm. The haploid embryos were fixed at the early segmentation stages and analyzed by whole-mount in situ hybridization with the neural markers *fez-like*, *engrailed3*, *krox20* and a neuronal marker *deltaB*. Two mutants (*rk1* and *rk2*) displaying ventralized phenotypes (reduced neuroectoderm) were obtained by screening 753 F1 females. Complementation analyses using *ogon*^{tm305} and *dino*^{tt250} indicated that *rk1* and *rk2* were allelic to *ogon* and *dino*, respectively. Maternal-zygotic *ogon*^{rk1} embryos were generated by crossing homozygous and fertile male and female *ogon*^{rk1} fish. The genotyping of the *swirl*^{tc300} and *dino*^{tt250} mutants was done as described previously (Dick et al., 2000; Hild et al., 1999). To genotype the *ogon*^{rk1} allele, the genomic fragment containing the splicing donor of the first intron of zebrafish *sizzled* gene was amplified by PCR using rk1-5′ (5′-CCTCGATCTGACGACTTGAGGA-3′) and rk1-3′ (5′-GCCAGTTCTAAATCATGAGCTACAC-3′), and digested with *Rsa*I, which cleaves the PCR product from the wild-type but not from the *ogon*^{rk1} allele.

Positional cloning of *ogon*

ogon^{rk1} heterozygous fish were mated with wild-type India fish to generate F1 families. Homozygous *ogon*^{rk1} mutant embryos were raised from the F1 cross and selected by morphological criteria (expanded ventral tissue). We used samples of their genomic DNA to carry out segregation analyses. We first examined the SSLP markers z1378, z8380, z23415 and z14408, which are located on the distal region of linkage group (LG) 25. This region is reported to be deleted in *ogon*^{m60} and *ogon*^{b180} (Miller-Bertoglio et al., 1999). We found that the SSLP marker z8380 was close to the *ogon* locus (three recombinations out of 2998 meiotic segregations). Using the PCR primers for z8380 as a probe, we obtained BAC and PAC lines: BAC 185L03, BAC 31 and PAC 203B16. We isolated PAC 35K5, PAC 251M11 and BAC 173L18 using the end sequence of PAC 203B16, and we isolated PAC 259J12 using the end sequence of PAC 35K5. We isolated the fragments from the AB and India genomes, which correspond to the end of the PAC and BAC clones, and found polymorphic markers, SSLPs and STSs (sequence-tagged sites, detected by restriction fragment length polymorphisms and PCR). The precise information on the markers is available on request. *ogo/szl* cDNA was isolated by hybridizing a lambda Ziplox zebrafish gastrula cDNA library with the inserts of PAC 203B16, PAC 35K3 and a cosmid generated from PAC 203B16. A genomic fragment and cDNA fragment of *ogon/sizzled* were sequenced by performing shot-gun sequencing and reading the PCR products.

RNA and morpholino oligonucleotide injection

Capped RNAs for Noggin1, Dickkopf1, Chordin, Tlc (a constitutively active type I Bmp receptor), *Xenopus* Frzb-1, *Xenopus* Sizzled and *Xenopus* Crescent were generated as described previously (Furthauer et al., 1999; Hashimoto et al., 2000; Houart et al., 2002; Miller-Bertoglio et al., 1997; Nikaido et al., 1999; Pera and De

Robertis, 2000; Salic et al., 1997). The coding region of zebrafish *sizzled/ogon* cDNA in pZL1 (from the lambda Ziplox clone) was excised and inserted into a modified pCS2+ (pCS2+SN). The *sizzled*^{tm305} cDNA was isolated by PCR from the *ogon*^{tm305} homozygous mutant embryos and inserted into pCS2+SN. The *sizzled* cDNA containing four mispaired nucleotides without amino acid changes was created by PCR and inserted into pCS2+SN. pCS2+SN *sizzled* and *sizzled*^{tm305} were digested with *Asc*I, and the capped RNA was transcribed with SP6 RNA polymerase. The antisense morpholino oligonucleotides used in this study were *szl* MO (5′-ACAGCAGCAGA-CTGAATAGAGACAT-3′) and control MO with four mispaired bases (5′-ACAGgAG-CAcACTGAtTAGAcACAT-3′). The *chordin* MO has previously been published (Nasevicius and Ekker, 2000).

Transcript detection

Whole-mount in situ hybridization was performed using BM purple (Roche) as the alkaline phosphatase substrate. The detection of *six3.2*, *bmp2b*, *chordin*, *goosecoid*, *fused somites/tbx24* and *eve1* was as described previously (Joly et al., 1993; Kobayashi et al., 1998; Miller-Bertoglio et al., 1997; Nikaido et al., 2002; Oxtoby and Jowett, 1993; Stachel et al., 1993). The *Sal*I-*Bam*HI fragment of *sizzled* was subcloned into pZL1. The *sizzled* RNA probe was generated by digestion of pZL1-5′ sizzled and transcription with SP6 RNA polymerase. Photographs were taken using an AxioPlan2 microscope and AxioCam (Zeiss).

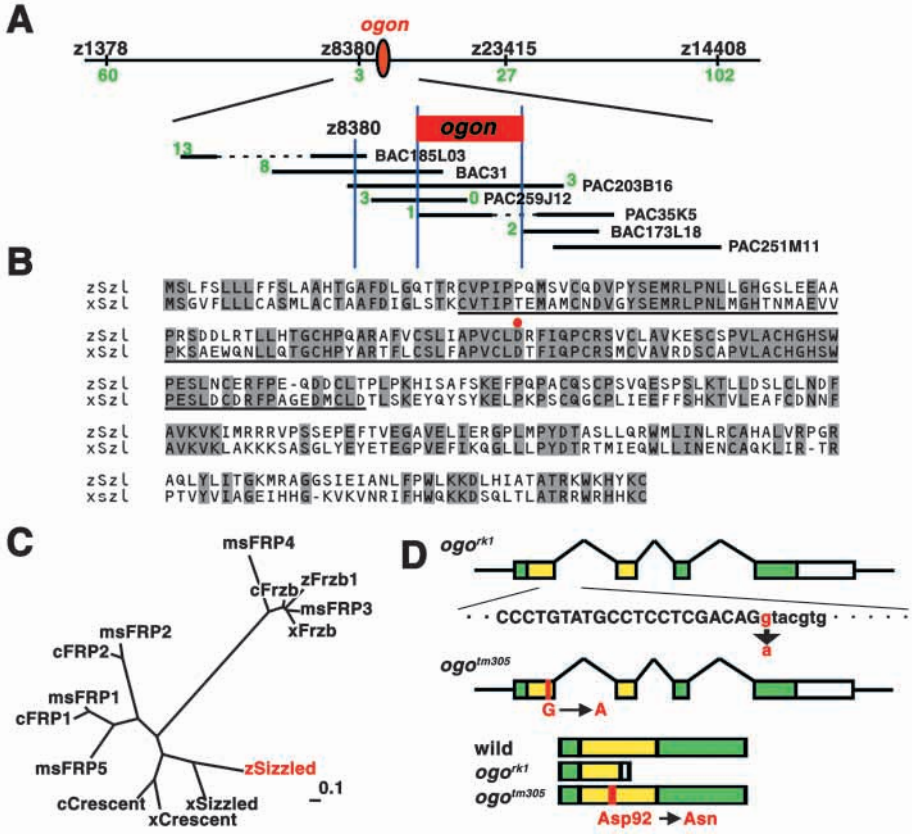

Fig. 1. The *ogon* locus encodes a zebrafish homolog of *secreted frizzled*. (A) The positional cloning of the *ogon* gene. The numbers on the linkage map and the ends of the BAC and PAC clones indicate the number of recombinations in 2998 meiosis. (B) Amino acid sequence of zebrafish Ogon/Sizzled and alignment with *Xenopus* Sizzled. The cysteine-rich domain (CRD) is underlined. The position of Asp92 is indicated (red dot). (C) A phylogenic tree of secreted Frizzled-related proteins (FRPs). z, zebrafish; x, *Xenopus*; c, chick; m, mouse. (D) Mutations in the *ogon/sizzled* genome and the Sizzled proteins of the *ogon*^{rk1} and *ogon*^{tm305} alleles. CRD is indicated by yellow boxes.

RESULTS

The *ogon* locus encodes a zebrafish homolog of Secreted Frizzled (Sizzled)

We isolated a novel ENU-induced allele of *ogo*, *ogo*^{rk1}, using a haploid screening procedure. The *ogo* locus was reported to be localized to the distal part of LG25 (Miller-Bertoglio et al., 1999); therefore, we started the positional cloning of *ogo* based on this information. By segregation analysis using the *ogo*^{rk1} allele and simple length polymorphism (SSLP) markers in the region of LG25, we mapped the *ogo* locus close to z8380 (0.1 cM, Fig. 1A). Starting with z8380, we performed chromosomal walking with bacterial artificial chromosomes (BACs) and P1-derived artificial chromosomes (PACs) (Fig. 1A). By further segregation analyses using markers on the BACs and PACs, we found that two PAC clones, 203B16 and 35K5, contained the *ogo* locus. Using inserts from these PAC clones, we screened a gastrula cDNA library and obtained three cDNAs. One of these cDNAs encoded a protein of 282 amino acids, which displayed sequence similarity (52.3% identical at the amino acid level) with *Xenopus* Sizzled (Salic et al., 1997) (Fig. 1B). Owing to similarities with *Xsizzled* in its expression profile and function (described below), the isolated gene is most likely a zebrafish ortholog of *szl*. Like other sFrps, zebrafish Szl has a cysteine-rich domain (CRD) but no transmembrane domain. Sequence comparison revealed that Szl is distantly related to sFrp1-5 and relatively close to Crescent (Fig.1C).

To examine whether mutations in *szl* are responsible for the *ogo* phenotypes, we inhibited the function of Szl by injecting an antisense morpholino oligonucleotide (MO) (Fig. 2; Table 1). The embryos that received injections of the *szl* MO displayed ventralized phenotypes (Fig. 2B), including increases in the blood and ventral tail fin, that were similar to the typical phenotypes of the *ogo* mutant embryos. Injection of the control MO did not have any significant effects (Fig. 2D), and co-injection of a modified *szl* RNA containing four mispaired bases in the MO recognition site or *Xenopus szl* RNA suppressed the ventralized phenotypes caused by the *szl* MO (Fig. 2C; Table 1). Injection of the wild-type *szl* RNA rescued the ventralized phenotypes of the maternal-zygotic (MZ) *ogo*^{rk1} in a dose-dependent manner (Fig. 2F,G; Table 2). We sequenced the genomic DNA and cDNA of *szl* isolated from the *ogo*^{rk1} and *ogo*^{tm305} homozygous mutant embryos. We found a mutation in the splicing donor site of the first intron in the *ogo*^{rk1} allele (Fig. 1D) that disrupted the correct splicing and resulted in truncation at the CRD (data not shown). We found several polymorphisms in the coding region of *szl* in the

Table 1. Rescue of *sizzled* morphant embryos

RNA	*ogon* phenotype (%)	Partially rescued (%)	Normal (%)	Dorsalized (%)	*n*
sizzled MO 5 ng	100	0	0	0	38
sizzled MO 5 ng + 4mis *sizzled* RNA 10 pg	0	25	55	25	20
sizzled MO 5 ng +*Xsizzled* RNA 10 pg	0	28	40	32	25

Five ng of sizzled MO and 10 pg of RNA for zebrafish *sizzled*, which contains four mispaired nucleotide in the MO recognition sequence (4mis *sizzled*), or *Xenopus sizzled* were injected.

Phenotypes of the injected embryos were scored at 24 hpf.

Fig. 2. *sizzled* is responsible for *ogon*. (A) Control 24 hpf embryo. (B) Embryos that received an injection of 5 ng of an antisense morpholino oligonucleotide for *sizzled* (*szl* MO) phenocopied the *ogon* mutant embryos (*n*=38/38). (D) Injection of 5 ng of control MO (*szl 4mis* MO, with four mispaired bases) did not have any significant effect (*n*=45/45). (C) Co-injection of 5 ng of *szl* MO and 10 ng of modified *szl* RNA, which contains four mispaired bases without codon changes in the MO recognition site, rescued the *ogon* phenotype (*n*=11/21). Rescue of maternal-zygotic (MZ) *ogon*^{rk1} by injection of wild-type *szl* RNA (also see Table 1). MZ *ogon*^{rk1} embryos (E) that received an injection of 10 pg of *szl* RNA displayed a dorsalized phenotype, normal phenotype (G) and weakly ventralized phenotype (F) .

ogo^{tm305} allele, one of which introduced an amino acid change at position 92 from aspartate, which is conserved between the zebrafish and *Xenopus* Szl, to asparagine (Fig. 1D). The mutation abrogated the function of Szl (described below). All of these data indicate that *szl* corresponds to *ogo* and that a mutation in the *szl* gene leads to ventralized phenotypes in zebrafish.

Ogon/Sizzled functions as a dorsalizing factor

To examine the function of zebrafish *ogo/szl*, we misexpressed the *ogo*/*szl* RNA in wild-type embryos. The *ogo*/*szl* RNA-injected embryos displayed dorsalized phenotypes (Fig. 3B), similar to those of *swr* and *snh* (mutants with compromised Bmp signaling) (Dick et al., 2000; Kishimoto et al., 1997; Schmid et al., 2000), and of embryos overexpressing the Bmp antagonists Chordin and Noggin1 (Furthauer et al., 1999; Miller-Bertoglio et al., 1997). The *ogo*/*szl* RNA-injected embryos did not show any change in *chordin* and *bmp2b* expression at the early gastrula stage (data not shown), but exhibited ventrally expanded *chordin* expression and reduced *bmp2b* expression at the mid-gastrula stage (Fig. 3D,G). Misexpression of *ogo/szl* did not affect the expression of *goosecoid* in the embryonic shield and the prechordal plate (Fig. 3I,K) or *no tail* expression (data not shown), indicating that Ogo/Szl could dorsalize the embryos without affecting the early specification of the mesoderm and dorsal organizer. Misexpression of *ogo/szl* RNA derived from *ogo*^{tm305} neither dorsalized the embryo (data not shown) nor induced the ventral expansion of *chordin* expression (Fig. 3E), indicating that the mutation in the *szl* gene led to the loss of the dorsalizing activity of Szl.

Ogo/Szl functions differently from the Wnt inhibitor Dkk1 and Crescent

Because most sFrps function as Wnt inhibitors and XSzl is reported to inhibit Wnt8 (Salic et al., 1997), we compared the activity of Ogo/Szl with that of known Wnt inhibitors and a Bmp inhibitor. Overexpression of *ogo/szl* as well as *Xszl* elicited dorsal expansion of the neuroectoderm, similar to the effect of *noggin1* (*nog*) (Fig. 4A). In these embryos, expression

Table 2. Suppression of MZ *ogon* phenotypes by injection of *sizzled* RNA

RNA	*ogon* phenotype (%)	Partially rescued (%)	Normal (%)	Dorsalized (%)	*n*
No injection	100	0	0	0	60
sizzled 10 pg	21	31	24	24	33
sizzled 25 pg	9	21	12	58	33

The indicated amount of *sizzled* RNA was injected into one-cell-stage embryos obtained from crossing homozygous *ogo*^{rk1} fish. The phenotypes were determined at 24 hpf.

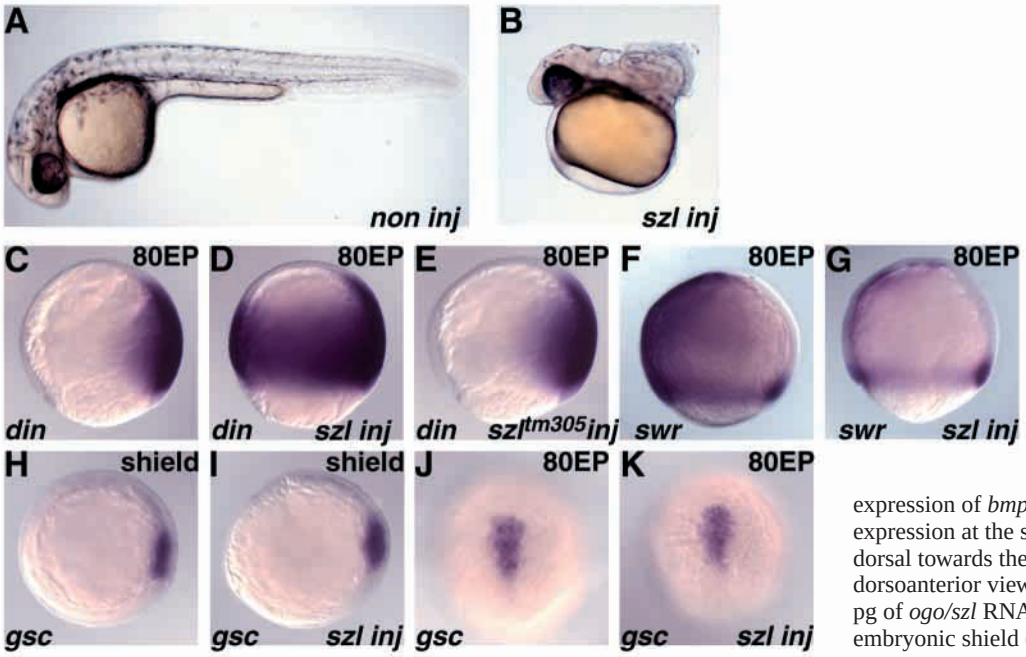

Fig. 3. Ogon/Sizzled is a dorsalizing factor. (A) Control 30 hpf embryo. (B) Injection of 100 pg of *ogo/szl* RNA dorsalized wild-type embryos. (C-E) *chordin* expression at the 80% epiboly stage. Lateral views with dorsal towards the right. Control (C). Injection of 100 pg of *ogo/szl* RNA (D) but not *ogo/szl*^{tm305} RNA (E) elicited the ventral expansion of *chordin* (*din*) expression. (F,G) *bmp2b/swirl* (*swr*) expression at 80% epiboly stage. Lateral views with dorsal towards the right. Control (F). (G) Injection of 100 pg of *ogo/szl* RNA decreased the expression of *bmp2b/swirl*. (H-K) *goosecoid* (*gsc*) expression at the shield (H,I; animal pole views with dorsal towards the right) and 80% epiboly stages (J,K, dorsoanterior views). (H,J) Control. Injection of 100 pg of *ogo/szl* RNA did not affect *gsc* expression in the embryonic shield (I) or prechordal plate (K).

of the forebrain-specific gene *six3.2* and the mid-hindbrain marker *pax2.1/no isthmus* was expanded ventrally and formed a circular expression domain. By contrast, the overexpression of the Wnt inhibitor Dkk1 and the sFrp XCrescent reduced or abolished the *pax2.1* expression and induced posterior but not ventral expansion of the *six3.2* expression (Fig. 4A), indicating that these Wnt inhibitors had an anteriorizing activity on the neuroectoderm (Erter et al., 2001; Hashimoto et al., 2000; Lekven et al., 2001). Similarly, misexpression of XFrzb-1 and Tlc did not appear to dorsalize the zebrafish embryos (data not shown) (Houart et al., 2002). This is consistent with the involvement of Wnt8 and Wnt8 inhibitors in anterior-posterior (AP) patterning rather

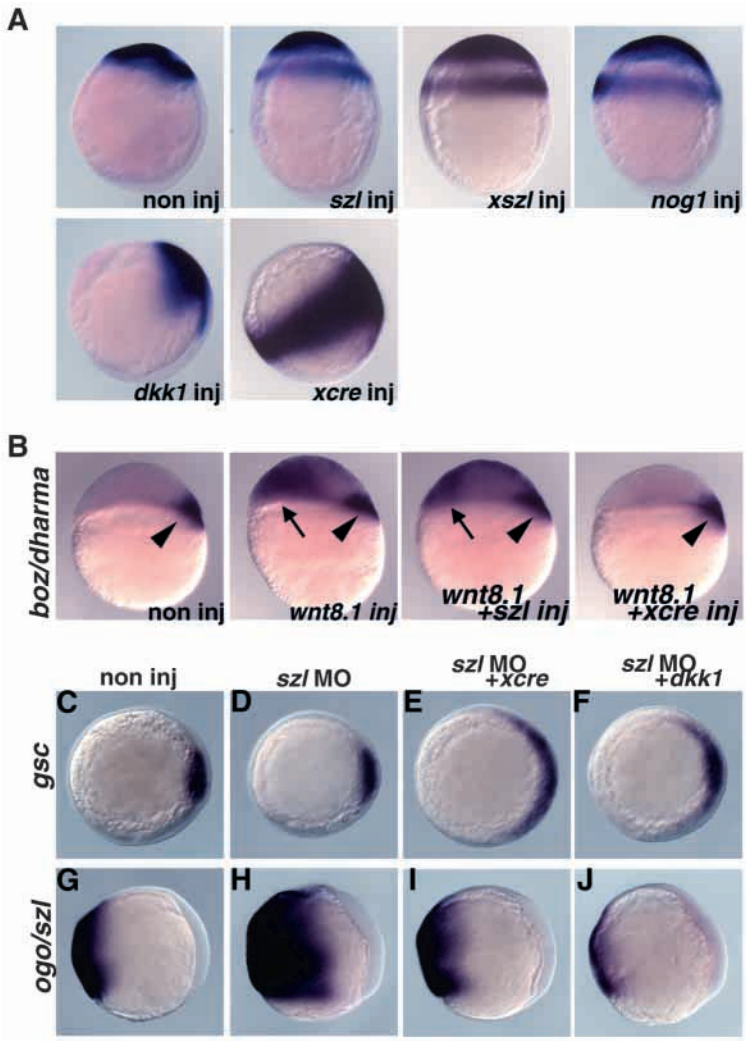

Fig. 4. Ogon/Sizzled functions differently from the Wnt inhibitor Dkk1 and other Frps. (A) *ogo/szl* induced ventral expansion, but not anteriorization, of the neuroectoderm. Embryos that received injections of *ogo/szl* (100 pg), *noggin1* (*nog*, 50 pg), *dickkopf1* (*dkk1*, 50 pg) or *Xcrescent* (*xcre*, 600 pg) were fixed and stained with the forebrain-specific marker *six3.2* and the mid-hindbrain boundary marker *pax2.1/no isthmus*. Lateral views with dorsal to the right. (B) Ogo/Szl does not inhibit Wnt8. One nanogram of *ogo/szl* RNA or 600 pg of *Xenopus crescent* RNA was co-injected with 40 pg of *wnt8.1* RNA. Wnt8.1-dependent ectopic expression of *bozozok (boz)/dharma* (indicated by arrows) was inhibited by *Xcrescent* (n=53/58) but not by *ogo/szl* (n=9/67). Endogenous *boz/dharma* expression is indicated by arrowheads. (C-J) Effects of *Xcre* and *dkk1* overexpression on *szl* morphant embryos. Embryos that received injections of *szl* MO (5 ng) or *szl* MO (5 ng) together with *Xcre* RNA (25 pg) or *dkk1* RNA (12.5 pg) were fixed at shield (C-F) and 80% epiboly stages (G-J), and stained with *gsc* or *ogo/szl*, respectively. *Xcre* and *dkk1* suppressed expansion of *ogo/szl* expression, which was observed in *szl* morphant embryos, but concomitantly elicited expansion of *gsc* at shield stage.

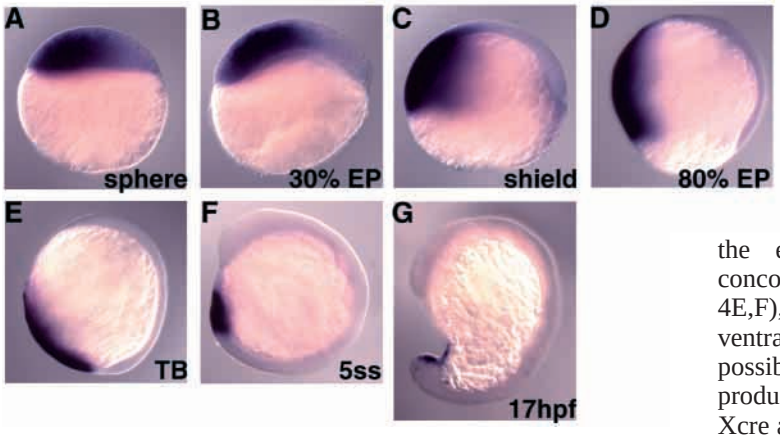

Fig. 5. *ogon/sizzled* expression. Expression of *ogo/szl* during zebrafish development: sphere (A), 30% epiboly (EP; B), shield (C), 80% epiboly (D), tailbud (TB; E), five-somite (5 ss; F) and mid-segmentation (17 hpf; G) stages. Lateral views with dorsal towards the right.

than in DV patterning (Erter et al., 2001; Lekven et al., 2001). These data indicate that Ogo/Szl functions similarly to the Bmp inhibitor but not the Wnt inhibitors, at least when these molecules are misexpressed. We next examined whether Szl inhibits the functions of *wnt8*, *wnt5/pipetail* or *wnt11/silberblick*, which are known to be expressed at the blastula and gastrula stages (Heisenberg et al., 2000; Kelly et al., 1995; Makita et al., 1998; Rauch et al., 1997). Overexpression of *ogo/szl* did not inhibit the *wnt8*-dependent ectopic expression of *bozozok/dharma* (Fig. 4B), a target of the Wnt canonical pathway (Ryu et al., 2001), but the overexpression of *Xcrescent* did. Furthermore, the overexpression of *ogo/szl* did not inhibit the *wnt5*- and *wnt11*-dependent inhibition of convergent extension (data not shown). This is consistent with the idea that Wnt5 and Wnt11-mediated signaling is not involved in DV patterning (Heisenberg et al., 2000; Rauch et al., 1997). All of these data indicate that Ogo/Szl does not inhibit the function of Wnt8 in zebrafish and that it functions differently from other sFrps and from the Wnt inhibitor Dkk1. We further examined whether Wnt inhibitors rescue the ventralized phenotypes of the *szl* MO-injected embryos. The *szl* MO-injected embryos displayed expansion of ventral *ogo/szl* expression at the late gastrula stage (Fig. 4H), as *ogo/szl* is expressed on the ventral side, in a Bmp signal-dependent manner (described below). Overexpression of Xcre or Dkk1 in the *szl* MO-injected embryos suppressed the expansion of *ogo/szl* expression (Fig. 4I,J), but concomitantly elicited the expansion of *gsc* expression (Fig. 4E,F), indicating that Xcre and Dkk1 can suppress the ventralized phenotypes caused by the loss of Ogo/Szl, but possibly through the expansion of the dorsal organizer, which produces Bmp inhibitors. However, it remains possible that Xcre and Dkk1 have a similar dorsalizing activity, which does not depend on Wnt8 inhibition, as Ogo/Szl does.

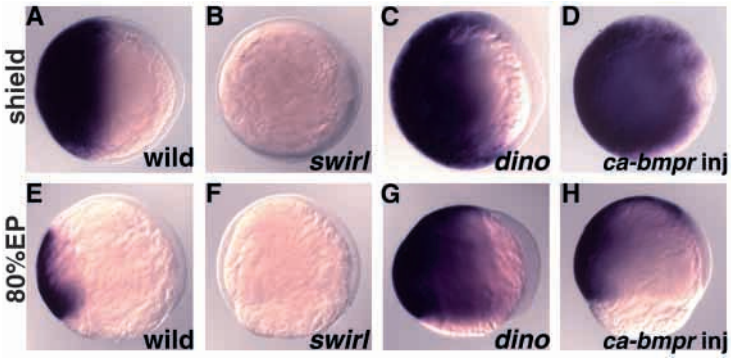

Fig. 6. *ogon/sizzled* is regulated by Bmp signaling. *ogo/szl* expression in wild-type embryos (A,E), *swirl*^{tc300} (B,F) and *dino*^{tt250} mutant embryos (C,G), and embryos that received injections of 200 pg of constitutively active Bmp receptor IA RNA (D,H) at the shield (A-D) and 80% epiboly (E-H) stages. Animal pole views with dorsal towards the right (A-D), and lateral views with dorsal towards the right (E-H).

Bmp-dependent expression of *ogo/szl* in the ventral blastoderm

Maternally deposited *ogo/szl* transcripts were detected weakly by RT-PC) but not by in situ hybridization (data not shown). This is consistent with a hypothesized role of the maternal *ogo* gene (Miller-Bertoglio et al., 1999). Zygotic expression of *ogo/szl* was detected on the ventral side from the late blastula stage (Fig. 5). The ventral expression continued through gastrulation. At the segmentation stages, *ogo/szl* expression was specifically detected in the ventral tail fin, which is strongly affected in the *ogo* mutant embryos. The ventral expression of *ogo/szl* during the gastrula stages was severely diminished or absent in the *swr/bmp2b* mutant embryos (Fig. 6B,F). By contrast, *ogo/szl* expression was dorsally expanded in the *chordino* (*din*) mutant embryos (Fig. 6C,G) and in embryos that received an injection of RNA for a constitutively active Bmp receptor (Fig. 6D,H). These data indicate that *ogo/szl* expression is positively regulated by the Bmp signal during gastrulation.

Ogo/Szl requires Chordin for dorsalization and inhibition of Bmp signaling

Because Chordin is a dorsalizing factor that is indispensable for dorsalization (Oelgeschlager et al., 2003; Schulte-Merker et al., 1997), we examined whether Chordin is involved in the Ogo/Szl-dependent dorsalization and Bmp inhibition. We injected 200 pg of *ogo/szl* RNA into embryos obtained from crossing *din* heterozygous fish; this treatment induced strong dorsalization in the wild-type and *din* heterozygous embryos (*din*/+; Fig. 7A). However, overexpression of *ogo/szl* in the *din* homozygous embryos (*din/din*) did not cause dorsalization, and the *ogo/szl*-overexpressing *din/din* embryos displayed strongly ventralized phenotypes, similar to the *din* mutant embryos, indicating that Chordin is required for the *ogo/szl*-dependent dorsalization. We next examined whether Ogo/Szl regulates the expression of *chordin* (Fig. 7B). We overexpressed Ogo/Szl in the *din* mutant and in *chordin* MO-injected embryos to exclude the involvement of autoregulated *chordin* expression (Fig. 7B). The loss of Chordin protein, either after injecting the *chordin* (*din*) MO or in the *din* mutant, led to the reduction or absence of the lateral expression of *chordin*, as reported previously (Miller-Bertoglio et al., 1997; Schulte-Merker et al.,

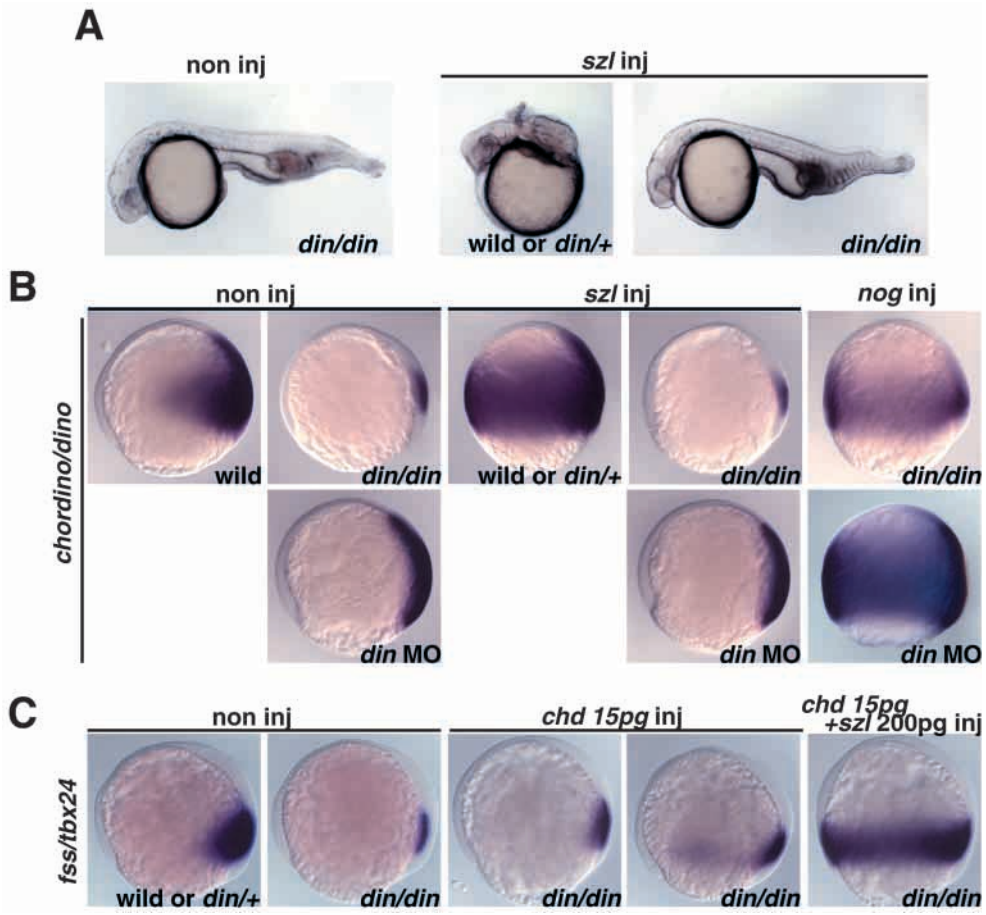

Fig. 7. Requirement for Chordin in Ogon/Sizzled-mediated Bmp inhibition. (A) Injection of *ogo/szl* RNA did not dorsalize the *dino/dino* homozygous embryos. Embryos obtained by crossing heterozygous *dino*^{tt250} fish received injections of 200 pg of *ogo/szl* RNA. The wild-type and heterozygous *din* embryos (*din*/+) displayed dorsalized phenotypes, but the homozygous *din* embryos (*din/din*) displayed ventralized phenotypes at 24 hpf (*din/din* was confirmed by genotyping), which are typical phenotypes for the *din/din* embryos (right). (B) *chordin* expression was not regulated by *ogo/szl* in the absence of Chordin protein. Two hundred picograms of *ogo/szl* RNA or 50 pg of *noggin1* RNA was injected into embryos from the *din*/+ cross, or embryos received an injection of 2 ng of *chordin* MO (*din* morphant). The embryos were fixed at the 80% epiboly stage and stained with the *chordin* probe. Lateral views, dorsal towards the right. The *din* mutant and *din* morphant embryos displayed reduced or an absence of lateral *chordin* expression. Overexpression of *nog* but not *ogo/szl* in these embryos rescued and expanded the lateral expression of *chordin*. (C) Ogo/Szl acts cooperatively with Chordin to dorsalize the embryos. Embryos from the *din*/+ cross received injections of 15 pg *chordin* RNA alone or 15 pg *chordin* RNA and 200 pg *ogo/szl* RNA. The embryos were fixed at the 80% epiboly stage and stained with a probe for *fused somites* (*fss)/tbx24*, which marks the paraxial mesoderm, followed by genotyping. *fss* expression in the *chordin* RNA-injected embryos was slightly rescued or ectopically expanded, compared with that in the uninjected *din/din* embryos, but was weaker than in the wild-type or *din*/+ embryos. *fss* expression was strongly expanded in the *chordin* and *ogo/szl* RNA-co-injected embryos.

1997). The misexpression of Noggin1, which binds directly to and inhibits the function of Bmp2/4, rescued and expanded the lateral expression in *din* mutant and *din* morphant embryos (Fig. 7B). By contrast, the misexpression of *ogo/szl* neither rescued the lateral expression nor affected the *chordin* expression at any developmental stage in the absence of the Chordin protein, indicating that *ogo/szl* does not regulate the expression of *chordin* in the absence of Chordin.

These data suggest that Ogo/Szl functions to modulate the activity of Chordin. To address this issue, we examined whether Ogo/Szl functions cooperatively with Chordin in DV patterning. We misexpressed *ogo/szl* and *chordin* in *din* mutant embryos and examined the morphology and expression of *fused somites (fss)/tbx24*, which marks the paraxial mesoderm (Nikaido et al., 2002) (Fig. 7C; Table 3). *fss* expression was reduced in the *din* mutant embryos. Injection of a sub-optimal amount (15 pg) of *chordin* RNA slightly rescued or induced the ectopic expression of *fss* in the *din* mutant embryos, but the total expression of *fss* in these embryos was less than in the wild-type and *din* heterozygous embryos. Co-injection of 15 pg *chordin* RNA with 200 pg *ogo/szl* RNA elicited a strong ventral expansion of *fss* in the *din* mutant embryos. Injection

Table 3. Cooperation of Ogon/Sizzled and Chordin in dorsalization

RNA	Ventralized (%)	Normal (%)	C1 (%)	C2 (%)	C3 (%)	C4-5 (%)	*n*
sizzled 200 pg	24	0	0	0	0	76	181
chordin 15 pg	16	60	14	5	4	1	160
chordin 15 pg+*sizzled* 200 pg	1	0	1	0	3	95	161
noggin1 50 pg	0	0	0	0	0	100	73

Embryos obtained by crossing heterozygous *dino*^{tt250} fish received injections of *ogo/szl* and/or *chordin* RNA. The injected embryos were classified by morphology into categories C1-C5 (where C1 was weak and C5 strong dorsalization) (Mullins et al., 1996). Despite the genotype, the co-injection of both RNAs induced strong dorsalization compared with the single RNA injection of either, indicating that the co-expression of *ogo/szl* and *chordin* causes dorsalization in *din/din* mutant embryos.

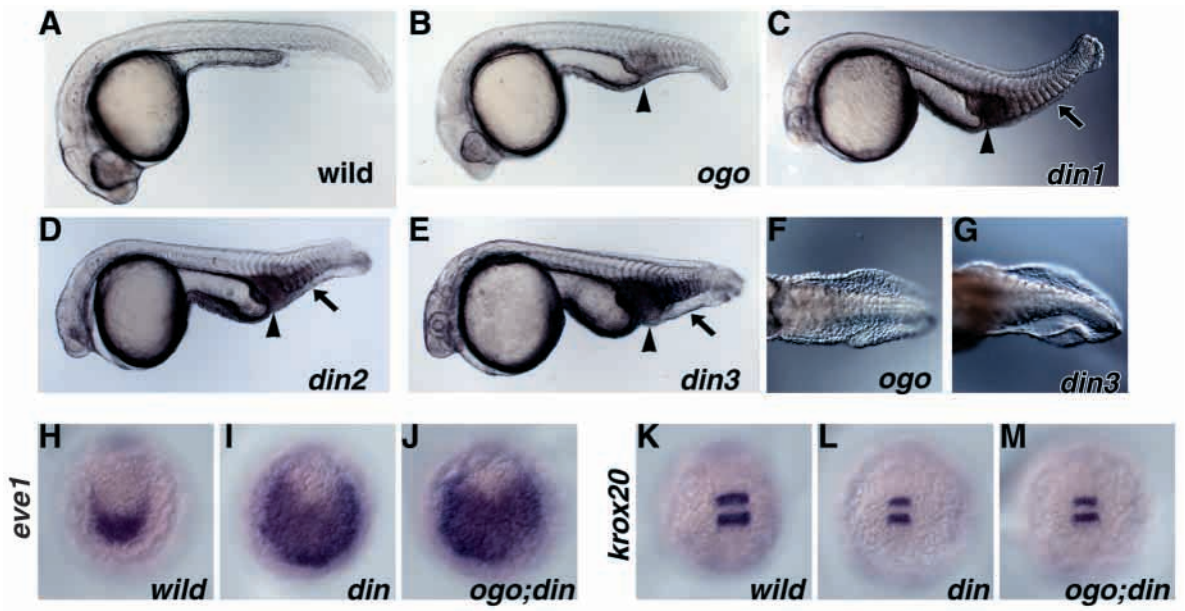

Fig. 8. *ogon* does not enhance the *chordino* phenotypes. (A-G) Embryos obtained from crossing *ogo*^{rk1}/+;*din*^{tt250}/+ parents (crosses of five different parent pairs) were assigned to five groups by morphological inspection at 24 hpf. (A-E) Lateral views with anterior towards the left and (F,G) ventral views of tail region. The numbers of embryos in each morphological category and their genotypes are shown in Table 4. (B,F) *ogo*-like embryo, displaying typical *ogo* phenotypes: caudal cell accumulation (containing blood cells, arrowhead) and expansion of the ventral tail fin (the ventral tail fin was expanded laterally), but no reduction in anterior neuroectoderm. (C-E,G) *din*-like embryos, displaying a reduction in the anterior neuroectoderm and caudal cell accumulation (arrowhead). These embryos displayed variable phenotypes in the tail fin (arrows): loss (*din1*, C), reduction (*din2*, D) or expansion (*din3*, E,G) of the ventral tail fin. (H-M) Expression of *eve1* and *krox20* in the eight-somite stage wild-type, *din*, and *ogo;din* embryos. (H-J) Vegetal pole views with dorsal towards the top and (K-M) anterior-dorsal views. *din* mutant embryos show variable expansion of *eve1* expression (I) and the additional *ogo* mutation did not enhance this expansion (J). The *ogo* mutation did not increase the reduction of *krox20* expression, which marks rhombomeres 3 and 5, in the *din* mutant embryos (L,M).

of 15 pg *chordin* RNA weakly rescued the ventralized phenotype of *din* at a low penetrance, but 200 pg of *ogo/szl* did not (Table 3). Co-injection of 15 pg *chordin* RNA with 200 pg *ogo/szl* RNA led to strong dorsalization regardless of the genotypes of the injected embryos. Because the misexpression of *ogo/szl* could not induce the expression of endogenous Chordin in the *din* mutant embryos, the data simply indicate that Ogo/Szl functions cooperatively with Chordin in DV patterning.

It has previously been reported that disruption of both *ogo* and *chordin* leads to more severely ventralized phenotypes than is seen in the single mutants (Hammerschmidt et al., 1996a; Miller-Bertoglio et al., 1999). This is inconsistent with the Chordin-dependency of the dorsalizing activity of Ogo. We generated *ogo;din* double mutant embryos by crossing fish bearing the *ogo*^{rk1} and *din*^{tt250} alleles, and examined their phenotypes and the expression of the ventral marker *eve1* and the neuroectoderm marker *krox20* (markers for rhombomeres 3 and 5). We also examined the genotypes of some of these embryos. The *din*^{tt250} mutant embryos obtained from crossing *ogo*^{rk1}/+;*din*^{tt250}/+ parents displayed reduced anterior neuroectoderm (narrow stripes of *krox20* expression), the accumulation of caudal cells, including blood cells, and showed variable phenotypes in the ventral tail fin, from reduction to expansion (Fig. 8C-E,G,L; Table 4), as reported previously (Miller-Bertoglio et al., 1999). We found that neither heterozygous nor homozygous mutations of *ogo*^{rk1} in the *din*^{tt250} mutant enhanced the morphologically ventralized phenotypes, nor did the mutation enhanced the expansion of *eve1* expression or the reduction of *krox20* expression in the

Table 4. *ogo* does not enhance *din* phenotypes

		ogo genotype (%)			*din* genotype (%)	
Phenotype	*n*	+/+	*ogo*/+	*ogo/ogo*	*din/din*	+/+, din/+, +/+
Wild-type	1047	ND	ND	ND	ND	ND
ogo-like	355	ND	ND	ND	ND	ND
*din*1	225	22	49	29	100	0
*din*2	106	20	54	26	100	0
*din*3	129	20	56	24	100	0

Embryos obtained from crossing *ogo*^{rk1}/+;*din*^{tt250}/+ parents (crosses of five different parent pairs) were categorized into five groups: wild-type (Fig. 8A), *ogo* (Fig. 8B, F), *din*1 (Fig. 8C), *din*2 (Fig. 8D) and *din*3 (Fig. 8E,G), by morphological inspection at 24 hpf in a similar way to the previous publication (Miller-Bertoglio et al., 1999). After recording the phenotypes, the genotypes for *ogo* and *din* were determined.

ND, not determined.

din^{tt250} mutant embryos. Taken together with the results of our misexpression studies in the *din* mutant embryos, we conclude that Chordin is required for the dorsalizing activity of Ogo/Szl.

DISCUSSION

The zebrafish *sizzled* gene corresponds to *ogon*

The interaction between Bmps and Bmp antagonists plays an important role in DV patterning during early vertebrate embryogenesis. Many organizer-specific genes and/or Bmp antagonists have been molecularly identified; however, only *chordin* (*chordino*) and *ogon* have been shown genetically to be required for the early DV patterning in zebrafish (Hammerschmidt et al., 1996a; Schulte-Merker et al., 1997; Solnica-Krezel et al., 1996). This is likely to be due to redundant functions among Bmp inhibitors at the gastrula stages. By contrast, Chordin and Ogon would be expected to have non-redundant functions in the DV patterning. Genetic and phenotypic analyses of *ogo* mutants predict that the *ogo* locus encodes a dorsalizing factor that inhibits Bmp signaling. In this report, we demonstrated that a zebrafish ortholog of *sizzled* corresponds to *ogo*. First, the *szl* gene was located in the *ogo* locus (Fig. 1A). We found mutations in the *szl* gene for two alleles, *ogo*^{rk1} and *ogo*^{tm305}, that disrupted the dorsalizing activity of Szl (Figs 1, 3). The loss of function of Szl caused by injecting the *szl* MO phenocopied the *ogo* mutant embryos, and the injection of *szl* RNA suppressed the ventralized phenotype of the *ogo* mutants (Fig. 2). All these data indicate that the loss of function of *szl* leads to the ventralized phenotype observed for the *ogo* mutant alleles.

The phenotypes of the *ogo*^{m60} and *ogo*^{b180} mutant embryos are more severe than the phenotype of the *ogo*^{tm305} mutant embryos (Miller-Bertoglio et al., 1999). *ogo*^{m60} and *ogo*^{b180} are deficiencies in the chromosome, and *ogo*^{tm305} is suggested to be a hypomorphic allele. However, misexpression of large amounts of the *szl* gene from *ogo*^{tm305} did not dorsalize the embryo (Fig. 3E), suggesting that *ogo*^{tm305} is a functionally null allele. The loss of Szl protein following the injection of the *szl* MO led to ventralized phenotypes similar to those of the *ogo*^{m60} and *ogo*^{b180} mutants (Fig. 2), indicating that the loss of function of the single gene *szl* is solely responsible for the ventralized phenotypes of *ogo*. The embryonic lethality of *ogo*^{m60} and *ogo*^{b180} is probably due to the deletion of additional gene(s) in LG25.

A contribution from maternally derived *ogo* has been reported (Miller-Bertoglio et al., 1999). Consistent with this, we detected maternally deposited *ogo/szl* transcripts by RT-PCR (data not shown), and embryos receiving injections of large amounts of *szl* MO display phenotypes similar to those of maternal-zygotic (MZ) *ogo* mutant embryos (Miller-Bertoglio et al., 1999) (Fig. 2). However, as discussed below, the dorsalizing activity of *ogo/szl* requires the presence of *chordin*, which is expressed after the mid-blastula transition (Miller-Bertoglio et al., 1997). Maternally provided Ogo/Szl should support the function of the zygotic Ogo/Szl in dorsalization.

ogon/sizzled expression depends on Bmp signaling

Expression of *ogo/szl* was detected in the ventral blastoderm from the late blastula through the gastrula stages (Fig. 5). Ventral expression of *ogo/szl* at the gastrula stages strongly depended on Bmp signaling (Fig. 6). Zygotic Bmp signal-dependent expression has been reported for *bmp2b/swr* and *bmp7/snh* (Dick et al., 2000; Kishimoto et al., 1997; Schmid et al., 2000). The ventral expression of *bmp2b* and *bmp7* decreases after the mid-gastrula stage in the *swr* and *snh* mutant embryos, suggesting that the ventral expression of these genes depends only on Bmp signaling after the mid-gastrula stage. Similarly, the ventral *ogo/szl* expression was not affected at the late blastula stage in the *swr* and *din* mutant embryos (data not shown). These data indicate that ventral *ogo/szl* expression after the mid-gastrula stage depends on zygotic Bmp signaling. However, the *ogo/szl* expression was restricted to the ventral side from the time of its initiation at the late blastula stage. It has recently been shown that maternally provided *smad5* is involved in the early specification of ventral tissue at the late blastula stage (Kramer et al., 2002). Ventral *ogo/szl* at the late blastula and early gastrula stages might be regulated by the maternally derived Bmp signal. Alternatively, the early ventral expression of *ogo/szl* might be regulated by the interaction between the dorsal-specific homeobox gene *bozozok* and ventrally expressed homeobox genes *vox* (previously *vega1*), *vent* (previously *vega2*) and *ved*, which play a role in early DV specification before the zygotic Bmp signaling occurs (Imai et al., 2001; Kawahara et al., 2000a; Kawahara et al., 2000b; Shimizu et al., 2002).

Compared with the expression of *bmp2b*, *bmp4* and *bmp7*, *ogo/szl* expression was restricted to the more ventral blastoderm (Fig. 5). Similarly, in *Xenopus* embryos, the expression of *szl* and *szl2* is confined to the ventral-most part of the ventral blastoderm (Bradley et al., 2000; Salic et al., 1997). The injection of increasing amounts of *bmp4* RNA and the injection of *chordin* MOs in *Xenopus* embryos revealed that a high level of Bmp signaling is required for the expression of *szl* (Marom et al., 1999; Oelgeschlager et al., 2003). Thus, the ventral expression of *sizzled* is regulated by a mechanism that is conserved between zebrafish and *Xenopus*, and a high level of Bmp signaling activity, which exists in the ventralmost part of the blastoderm, is required for the expression of *sizzled* on the ventral side. Promoter analyses of *ogo/szl* will clarify this issue.

Bmp antagonist versus Wnt antagonist

Ogo/Szl has sequence similarity with the Wnt receptor Frizzled, suggesting a role for Ogo/Szl in Wnt inhibition. However, the overexpression of Ogo/Szl did not inhibit the Wnt8-dependent ectopic expression of *bozozok* (Fig. 4). Misexpression of the Wnt8 inhibitor Dkk1 and the sFrp Crescent anteriorized the neuroectoderm, but did not dorsalize the embryo efficiently, whereas misexpression of Ogo/Szl dorsalized the embryo but did not anteriorize the neuroectoderm, unlike the Bmp inhibitor Noggin 1 (Fig. 4). These data suggest that Ogo/Szl functions as a Bmp inhibitor rather than as a Wnt inhibitor.

We found that overexpression of Crescent and Dkk1 suppressed the ventralized phenotypes of *szl* MO-injected embryos (Fig. 4). However, Crescent and Dkk1 also elicited expansion of the dorsal organizer (Fig. 4) (Hashimoto et al., 2000), which produces the Bmp inhibitors Chordin and Noggin 1. Thus, Crescent and Dkk1 might substitute for the function

of Ogo/Szl indirectly by expanding the expression domain of the organizer-derived Bmp inhibitors. It remains possible that Crescent and Dkk1 (less likely) might have a dorsalizing activity other than expansion of the dorsal organizer, just as Ogo/Szl does. However, Ogo/Szl did not inhibit the Wnt8 activity; therefore, the dorsalizing activity of Ogo/Szl should not depend on Wnt8 inhibition.

In zebrafish, the loss of *wnt8* or of *tcf3/headless*, which functions to inhibit Wnt8 signaling, strikingly affects the AP patterning in the neuroectoderm in addition to causing abnormalities in the DV patterning (Erter et al., 2001; Kim et al., 2000; Lekven et al., 2001). The phenotypes of the *ogo* mutant and the *ogo/szl*-overexpressing embryos were different from those of embryos with high (e.g. *headless* mutant embryos) and low (*wnt8* morphant embryos) Wnt8 activities, respectively, further supporting the idea that Ogon does not function to inhibit Wnt8 signaling. In addition to *wnt8*, there are several Wnt genes reported to be expressed at the blastula and gastrula stages in zebrafish. Among them, *wnt5/pipetail* and *wnt11/silberblick*, which activate a non-canonical Wnt signal, are known to be involved in convergent-extension movements during gastrulation (Heisenberg et al., 2000; Rauch et al., 1997), and thus it is unlikely that the dorsalizing activity of Ogo/Szl is due to the inhibition of Wnt5 and Wnt11. Consistent with this, misexpression of *ogo/szl* did not affect the *wnt5*- or *wnt11*-mediated inhibition of convergent extension (data not shown). Similarly, it was reported that *Xenopus szl2* does not inhibit the activities of *Xenopus* Wnt3a, Wnt5a and Wnt8 (Bradley et al., 2000). All of these data indicate that Ogon/Szl and *Xenopus* Szls promote dorsalization through interactions with factors other than the Wnts.

How does Ogon/Sizzled inhibit Bmp signaling?

Ogo/Sizzled requires the Chordin protein to dorsalize embryos (Fig. 7), indicating that Ogo/Szl displays a mode of action that is completely different from that of other Bmp antagonists. This finding is consistent with a previous report that misexpression of *Xenopus sizzled* cannot rescue UV-treated ventralized embryos, which should not express *chordin* (Salic et al., 1997).

How does Ogo/Szl inhibit Bmp signaling in a Chordin-dependent manner? Our data and the data previously published imply the mode of function of Ogo/Szl: (1) *din* and *ogo* mutant embryos have similar ventralized phenotypes (Hammerschmidt et al., 1996b; Miller-Bertoglio et al., 1999; Solnica-Krezel et al., 1996); (2) the ventralized phenotypes of *ogo* can be suppressed by the expression of Chordin, Noggin and a dominant-negative Bmp receptor (Miller-Bertoglio et al., 1999), but the *din* phenotypes cannot be suppressed by misexpression of *ogo/szl* (Fig. 7); (3) overexpression of *ogo/szl* did not inhibit Wnt8 activity (Fig. 4); (4) overexpression of *ogo/szl* induced a similar phenotype to that of *noggin1* but not that of *dkk1* or *crescent* (Fig. 4); (5) low levels of *chordin* could act synergistically with *ogo/szl* in dorsalization (Fig. 7); (6) a mutation in *ogo* did not enhance the ventralized phenotypes of *din* embryos (Fig. 8); and (7) loss of *tolloid/mini fin* can suppress the *ogon* tail phenotype (Wagner and Mullins, 2002). All of these results indicate that Ogo/Szl can augment the activity of Chordin, by inhibiting an inhibitor of Chordin, by directly making Chordin more active, or by modulating the Bmp signal so that it becomes more susceptible to the Chordin-mediated inhibition. The dorsalizing activity of the Chordin protein is regulated by different mechanisms: the chordin protein level is regulated through processing by Tolloid-related metalloproteinases, and Chordin interacts physically and functionally with Bmp and Twisted Gastrulation (Tsg) to modulate Bmp activity (De Robertis et al., 2000). Tolloid-related proteins and Tsg might be involved in the function of Ogo/Szl. Alternatively, Ogo/Szl may function in parallel with Chordin. Both Ogo/Szl and Chordin are required for the formation of posterior dorsal tissues, and the loss of either Ogo/Szl or Chordin might lead to ventralization. In this scenario, the lowering of the Bmp signal by Chordin might work cooperatively with Ogo/Szl to dorsalize the embryo.

A mutation in the cysteine-rich domain (CRD) of the Ogo/Szl in ogo^{tm305} implies an essential role for the CRD in the activity of Ogo/Szl. As the CRD of Frizzled is known to interact with Wingless and Wnts (Bhanot et al., 1996), it is still possible that Ogo/Szl functions by inhibiting unidentified Wnt(s). The identification of proteins that interact with Ogo/Szl will shed light on the mechanisms by which Ogo/Szl inhibits Bmp signaling and regulates the specification of the DV axis.

Ogon/Sizzled functions as a negative-feedback regulator of Bmp signaling

Many feedback inhibitors play roles in the early patterning of vertebrate embryogenesis (Freeman, 2000). The Antivin/Lefties (Lefy1 and Lefy2) function as feedback inhibitors for the Nodal-related molecules in zebrafish, *Xenopus* and mice (Bisgrove et al., 1999; Cheng et al., 2000; Meno et al., 1999; Thisse and Thisse, 1999). Sprouty4 and Sef function in FGF signaling (Furthauer et al., 2002; Furthauer et al., 2001; Tsang et al., 2002). An inhibitory Smad, Smad7 and Bambi/Nma function as feedback inhibitors of Bmp signaling (Grotewold et al., 2001; Nakayama et al., 1998; Onichtchouk et al., 1999; Souchelnytskyi et al., 1998). *ogo/szl* is regulated positively by Bmp signaling and in turn Ogo/Szl inhibits Bmp signaling, indicating that Ogon/Szl functions as a negative-feedback regulator of Bmp signaling. In contrast to the other feedback inhibitors described above, Ogo/Szl function requires the Chordin protein. *ogo/szl* is expressed on the ventral side of the embryo in a Bmp-signal-dependent manner, whereas *chordin* is expressed on the dorsal side and is negatively regulated by Bmp signaling (Hammerschmidt et al., 1996b). Thus, *ogo/szl* and *chordin* are regulated in completely opposite manners, but cooperate in inhibiting the Bmp signal. The Ogo/Szl and Chordin proteins that are diffused from the ventral and dorsal sides might be colocalized at a specific position along the DV axis and function there cooperatively to inhibit the Bmp signal. In support of this idea, Chordin and Szl2 appear to diffuse a long distance from their source (Bradley et al., 2000; Jones and Smith, 1998). Alternatively, molecules that function downstream of the Ogo/Szl-mediated signaling might interact with Chordin or unknown regulator(s) of Chordin to inhibit Bmp signaling. In any case, the functional interaction between Ogo/Szl and Chordin provides a precise positional cue to cells along the DV axis during gastrulation.

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