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The ventralized *ogon* mutant phenotype is caused by a mutation in the zebrafish homologue of Sizzled, a secreted Frizzled-related protein

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Abstract

The BMP signaling pathway plays a key role during dorsoventral pattern formation of vertebrate embryos. In zebrafish, all cloned mutants affecting this process are deficient in members of the BMP pathway. In a search for factors differentially expressed in *swirl/bmp2b* mutants compared with wild type, we isolated zebrafish Sizzled, a member of the secreted Frizzled-related protein family and putative Wnt inhibitor. The knockdown of *sizzled* using antisense morpholino phenocopied the ventralized mutant *ogon* (formerly also known as *mercedes* and *short tail*). By sequencing and rescue experiments, we demonstrate that *ogon* encodes *sizzled.* Overexpression of *sizzled*, resulting in strongly dorsalized phenotypes, and the expression domains of *sizzled* in wild type embryos, localized in the ventral side during gastrulation and restricted to the posterior end during segmentation stages, correlate with its role in dorsoventral patterning. The expanded expression domain of *sizzled* in *ogon* and *chordino* together with its downregulation in *swirl* suggests a BMP2b-dependent negative autoregulation of *sizzled.* Indicating a novel role for a secreted Frizzled-related protein, we show that, in addition to the BMP pathway, a component of the Wnt signaling pathway is required for dorsoventral pattern formation in zebrafish.

Keywords: ogon; *sizzled*; Zebrafish; Dorsoventral patterning; Secreted Frizzled-related protein

Introduction

During early embryonic development of vertebrates, the specification of anterior–posterior and dorsoventral cell fates takes place. The bone morphogenetic protein (BMP) signaling pathway plays a central role during patterning of the dorsoventral axis. The opposing gradients of ventralizing BMP ligands and dorsalizing BMP antagonists, such as Chordin, specify the different ventral and dorsal cell fates of the mesoderm (De Robertis et al., 2000). Additionally, the Wnt signaling pathway contributes to dorsoventral patterning through similar antagonistic relationships as the BMP/Chordin system (Moon et al., 1997). In *Xenopus*, Xwnt8 participates in the formation of ventral and lateral mesoderm. Several Wnt inhibitors antagonize the ventralizing XWnt8 activity, such as Frzb-1 or Dkk, which are both secreted by the organizer (reviewed in De Robertis et al., 2000; Moon et al., 1997).

In several zebrafish mutagenesis screens, mutants were identified that display defects in dorsoventral pattern formation (Hammerschmidt et al., 1996; Mullins et al., 1996; Solnica-Krezel et al., 1996). The molecular identification of most of these genes showed that they are components of the BMP pathway, which supports its essential role in dorsoventral pattern formation. The strongly dorsalized mutants *swirl* (*swr*) and *snailhouse* harbor mutations in the *bmp2b* and *bmp7* ligand genes, respectively (Dick et al., 2000; Kishimoto et al., 1997; Nguyen et al., 1998; Schmid et al., 2000). *somitabun* mutants that show a similar dorsalized phenotype are mutant in *smad5*, another member of the BMP signaling pathway (Hild et al., 1999). The weakly dorsalized mutants *lost-a-fin* and *mini fin* harbor mutations in Alk8, a BMP type I receptor (Bauer et al., 2001; Mintzer et al., 2001), and Tolloid, a metalloprotease that can cleave Chordin, thus releasing BMP to bind its receptor (Connors et al., 1999), respectively. The ventralization of the *dino*

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mutant is caused by a mutation in the zebrafish *chordin* gene, therefore designated *chordino (din)* (Schulte-Merker et al., 1997). Lack of its ability to bind and antagonize BMP leads to an expansion of ventral structures.

The zebrafish mutant *ogon* (*ogo*, previously also known as *mercedes* and *short tail*) shows a similar though less severely ventralized phenotype than *din* (Hammerschmidt et al., 1996; Solnica-Krezel et al., 1996). *ogon* mutant embryos are characterized by a duplication of the ventral tail fin and an accumulation of cells in the ventral tail posterior to the yolk extension. However, the molecular identity of *ogon* has not been determined so far. Suppression of the *ogon* mutant phenotype by mRNA injections of BMP antagonists and the observation that *swirl/bmp2b* is epistatic to *ogon* suggested that *ogon* encodes a putative BMP pathway antagonist with dorsalizing activity (Miller-Bertoglio et al., 1999). Double mutant analysis of *ogon;minifin/tolloid* phenotypes resulted in a mutual suppression of each single mutant phenotype, indicating that the antagonistic activity of *ogon* is independent of Tolloid and Chordin (Wagner and Mullins, 2002).

Another pathway involved in dorsoventral patterning is the Wnt signaling pathway. Several studies in *Xenopus* and zebrafish corroborate the idea that Wnt8 is involved in the specification of the ventral and somitic mesoderm (Christian and Moon, 1993; Hoppler et al., 1996; Lekven et al., 2001). In *Xenopus*, ectopic expression of Xwnt8 after mid blastula transition (MBT) in the dorsal marginal zone changes the fate of the dorsal cells to more lateral types of mesoderm (Christian and Moon, 1993). Blocking of Xwnt8 by a dominant-negative construct inhibits the expression of genes normally expressed in the ventrolateral marginal zone (Hoppler et al., 1996). BMP4 cannot substitute for Xwnt8 in somite formation, supporting the idea that zygotic Xwnt8 is required for the specification of the somitic mesoderm (Marom et al., 1999). In zebrafish, Wnt8 is also required in mesoderm patterning. Loss of the *wnt8* locus, which encodes two Wnt8 proteins on a bicistronic transcript, leads to an enlargement of axial mesoderm and a decrease in ventrolateral mesoderm in homozygous mutant embryos (Lekven et al., 2001).

Wnt ligands can be antagonized by several classes of Wnt inhibitors (reviewed in De Robertis et al., 2000). One of those, Sizzled, is a putative Xwnt8 antagonist that belongs to the Wnt inhibitor family of secreted Frizzled-related proteins (sFRP) (Bradley et al., 2000; Salic et al., 1997). Like the other members of this family, such as Frzb-1 (Leyns et al., 1997; Wang et al., 1997) and Crescent (Pera and De Robertis, 2000), Sizzled has dorsalizing activity. However, *sizzled* is expressed in the ventral side of the gastrula (Salic et al., 1997), whereas *frzb-1* and *crescent* transcripts are localized in the Spemann's organizer. Thus, Sizzled has been proposed to antagonize XWnt8 activity in the ventral marginal zone (Salic et al., 1997). This is in contradiction to findings that Sizzled cannot inhibit XWnt8 (Bradley et al., 2000; Collavin and Kirschner, 2003). In *Xenopus*, the expression domains of *sizzled* and *bmp4* overlap in the ventral marginal zone and it was shown that *sizzled* expression is strongly dependent on BMP4 (Marom et al., 1999).

Here, we report the isolation and expression pattern of the *sizzled* gene in zebrafish and we demonstrate that *ogon* encodes *sizzled*. This finding together with the dorsalizing activity of Sizzled provides genetic evidence for an essential role of a member of the Wnt signaling pathway in dorsoventral patterning. We observed a BMP2b-dependent maintenance of *sizzled* expression suggesting a putative mediator role of Sizzled between the BMP and Wnt pathways.

Materials and methods

Fish

Zebrafish stocks were maintained as described previously (Haffter et al., 1996). All experiments were performed by using the following strains: Tübingen wild type, swr^{ta72}, ogo^{tm305}, ogo^{tz209}, and din^{tm84} (Hammerschmidt et al., 1996; Mullins et al., 1996). ogo^{tm305} and ogo^{tz209} embryos are homozygous viable and yield fertile adults. The swr^{ta72} mutation causes the same phenotype as a *swr* null allele (P. Haffter, personal communication). Embryos were staged according to Kimmel et al. (1995). Dorsalized phenotypes were classified as defined by Mullins et al. (1996).

Representational difference analysis

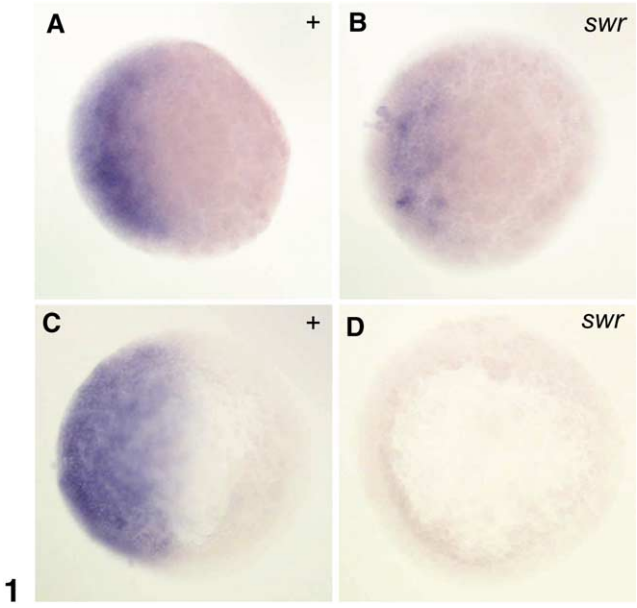
Messenger RNAs of 8-somite-stage sibling and *swirl* mutant embryos were isolated by using Dynabeads mRNA DIRECT Kit (Dynal). Representational difference analysis (RDA) was in principal carried out as described (Hubank and Schatz, 1999), defining sibling and *swirl* mutant cDNA as tester and driver, respectively. The second difference product was generated and cloned into the *Eco*RV site of pBluescript SKII+ (Stratagene). Clones were sequenced and differential expression of the respective genes was tested by in situ hybridization.

In situ hybridization

Whole-mount in situ hybridizations were performed in principle as described (Schulte-Merker et al., 1994). Embryos were stained with BM purple AP Substrate (Roche). After stopping the color reaction, embryos were cleared in 87% glycerol without prior fixation.

Antisense RNA probes of *sizzled* were generated by in vitro transcription using the DIG RNA labeling mix (Roche). As templates for in vitro transcription, we either used the 371-bp fragment isolated from the RDA approach (pBlue-sizzled) or the full-length cDNA clone (pCS2+sizzled).

For sections, whole-mount stained embryos were inbedded in Durcupan (Sigma) and 15-μm sections were performed.



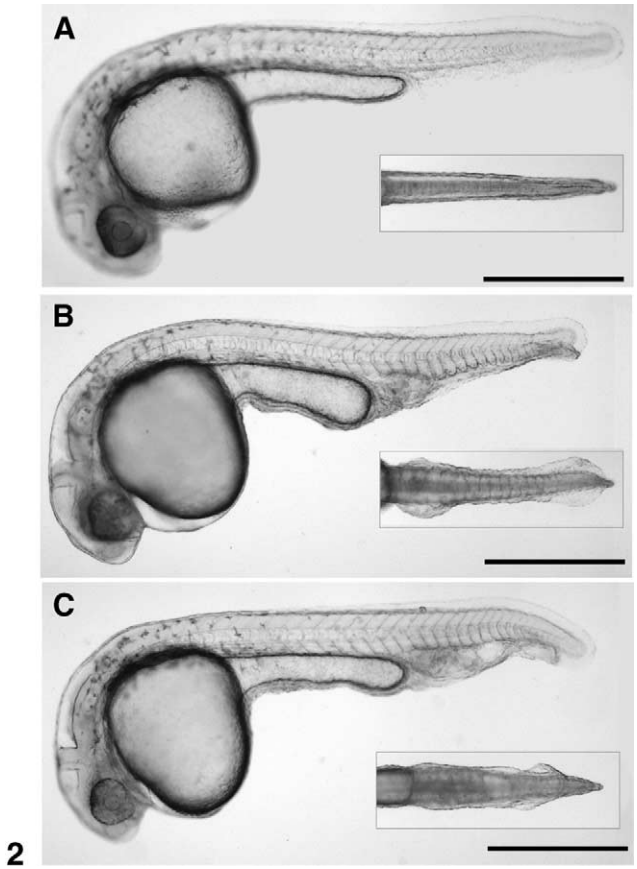


Fig. 1. *Sizzled* expression is downregulated in *swirl* mutant embryos. In situ hybridization with *sizzled* antisense probe on wild type (A, C) and swr^{ta72} homozygous (B, D) embryos. Stages analyzed were 30% epiboly (A, B) and shield stage (C, D). Animal pole view, dorsal is to the right.

Fig. 2. *Sizzled* knock down phenocopies the *ogon* mutant phenotype. (A–C) One-day-old larvae lateral view, with insets dorsal view. (A) Control injected larvae showing wild type phenotype. (B) Larvae injected with 2 ng *sizzled* antisense morpholino show a duplicated tail and an accumulation of cells in the ventral tail. (C) ogo^{tz209} homozygous larvae display an incomplete duplication of the ventral tail fin and an accumulation of cells in the ventral tail. The scale bars represent 500 μm.

Injection of antisense morpholino oligonucleotides

The 5′ end of the *sizzled* mRNA was identified through virtual sequence extension by using the zebrafish genomic sequences (Sanger Institute), starting from the *Dpn*II fragment isolated from the RDA screen. An antisense morpholino oligonucleotide (AVI Biopharma) designed complementary to the start codon (5′-gcagcagactgaatagagacatc-3′) was dissolved at a concentration of 2 mg/ml in injection buffer (0.4 mM $MgCl_2$, 0.6 mM $CaCl_2$, 0.7 mM KCL, 58 mM NaCl, 25 mM Hepes, pH 7.6). A total of 1 nl was injected at the one cell stage. As a control, a morpholino containing four mismatching bases (5′-gcaacagcctgaagagagatatc-3′) was used.

RACE and full-length cloning of sizzled

The 3′ end of the *sizzled* mRNA was amplified by using primers designed against the sequence of the *Dpn*II-fragment isolated from the RDA experiment (3′1: 5′-caacctgctgggtcacggcagtctggag-3′, 3′2: 5′-tcgtctgctcgctcatcgcccctgtatg-3′), the Marathon cDNA Amplification Kit, and Advantage 2 Polymerase mix (both from Clontech Laboratories). Both strands of four different RACE reactions were sequenced. The full-length sequence has been submitted to GenBank (Accession no. AY189978).

The amplified full-length cDNA of *sizzled* (FLCfor: 5′-tcagtgatgtctctattcagtctgc-3′; FLCrev: 5′-gaatgatgcgcaaaaataagc-3′) from wild type and mutant (ogo^{tm305}, ogo^{tz209}) fish was cloned into the pCS2+ vector, and both strands were sequenced. The modified *sizzled* cDNA was amplified with a forward primer containing five silent mutations (FLCmodfor: 5′-atgtcgctgttaagcctacttctgtttttctctctcg-3′) and FLCrev using pCS2+ sizzled as the template. The resulting PCR product was cloned into the pCS2+ vector, and both strands were sequenced.

RH mapping and mutation analysis

RH mapping of *sizzled* on the T51 panel was performed as described (Geisler et al., 1999). The primers used (RHfor: 5′-gtacccccgttctagcctgt-3′, and RHrev: 5′-atgtccggcgatgtgactat-3′) amplified a 244-bp fragment of genomic DNA covering the exon–intron boundary of exon1. To calculate the map position of the marker's vector, we used "Instant mapping," available at http://zon.tchlab.org/.

To confirm the mutations, RT-PCR using total RNA from individual 1-day-old ogo^{tm305} and ogo^{tz209} mutant embryos was performed (FLCfor and FLCrev primers). Both strands of four independent PCR products for each mutant allele were sequenced.

RNA injection

Capped mRNAs were synthesized from linearized pCS2+-based constructs by using SP6 RNA polymerase of the mMESSAGE mMACHINE Kit (Ambion) and diluted

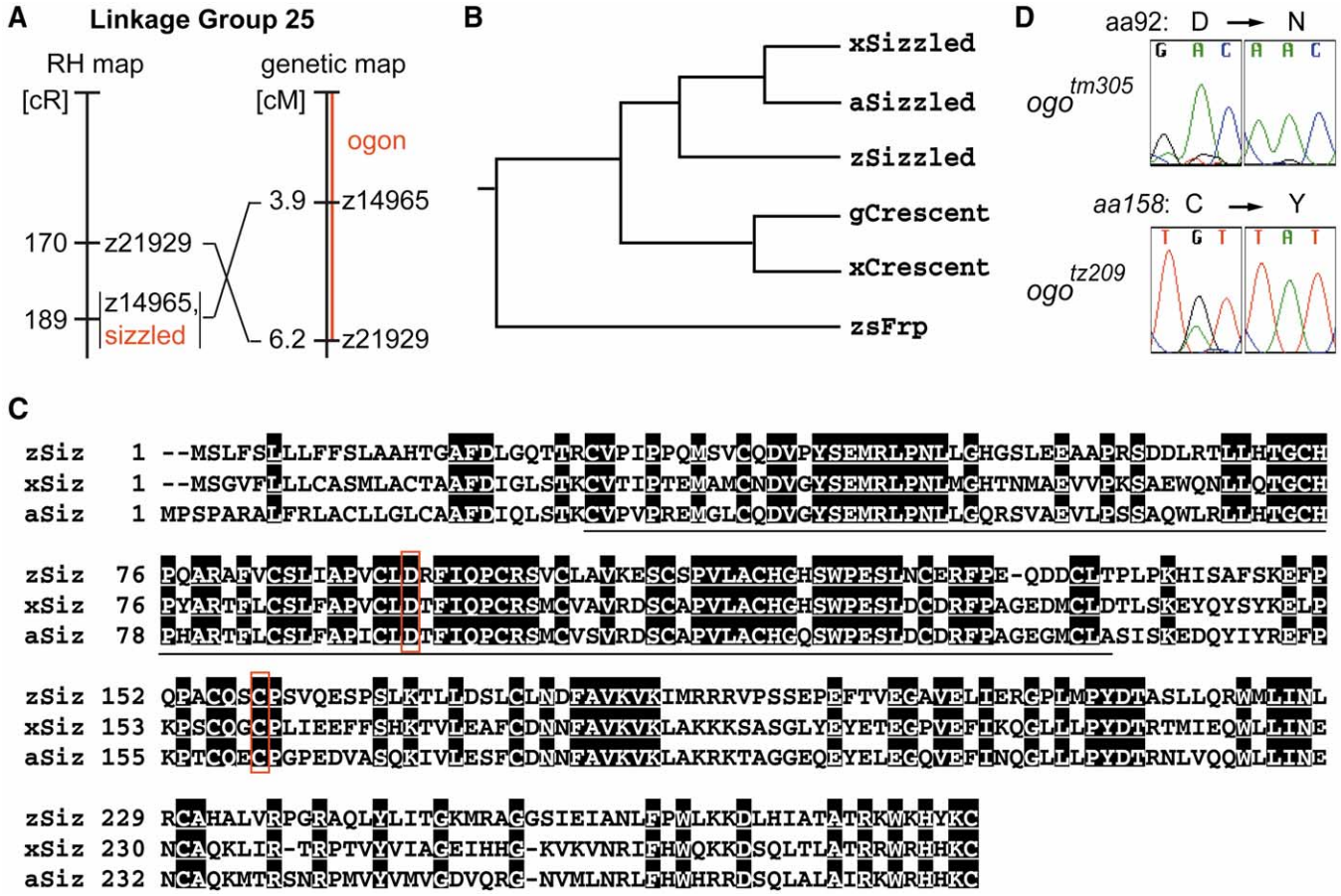


Fig. 3. Linkage of *ogon/sizzled* and mutation detection. (A) Radiation hybrid (T51 panel) and genetic (MGH panel) map of the *ogon* locus on LG25. *Sizzled* and *ogon* map to the distal end of LG25. (B) A phylogenetic tree of Sizzled amino acid sequences reveals an evolutionary conservation between the different members of the sFRP family. a, axolotl; g, gallus; x, *Xenopus;* z, zebrafish. (C) Alignment of the Sizzled protein from zebrafish, *Xenopus*, and axolotl. Dark shaded boxes indicate identical amino acids. CRD domain is underlined. (D) ogo^{tm305} and ogo^{tz209} harbor point mutations that lead to amino acid exchanges at positions 92 and 158, respectively. Amino acid positions 92 and 158 are outlined in red boxes in the alignment in (C).

with water to concentrations of 1, 10, 50, and 100 μg/ml, respectively. For the coinjection of modified *sizzled* mRNA with morpholino, dilutions were done with injection buffer. A total volume of 1 nl was injected into embryos at the one- to two-cell stage.

Results

Identification of sizzled by a RDA approach

In order to identify genes that may be involved in dorsoventral pattern formation, the cDNA RDA approach was chosen. RDA of 8-somite-stage embryos, using wild type as the tester and *swirl* as the driver, led to the isolation of zebrafish *sizzled* (gene identity confirmed by blast search, see below), represented by a 371-bp fragment. At 8-somite-stage, *sizzled* expression is completely absent in embryos mutant for *swirl* (data not shown). In situ hybridization of 30% epiboly and shield stage wild type embryos demonstrates localization of *sizzled* mRNA to the ventral side (Fig. 1A and C). In contrast, in *swirl* mutant embryos, expression of *sizzled* is slightly reduced at 30% epiboly and completely absent at shield stage (Fig. 1B and D). This early down-regulation of mRNA in a *swirl/bmp2b*-deficient situation shows that maintenance of *sizzled* expression is dependent on BMP2b.

Sizzled knock down phenocopies ogon

To investigate the function of *sizzled* in zebrafish, we analyzed the knock down phenotype. The partial sequence of *sizzled* obtained from the RDA could be extended toward the N-terminal end by alignment with genomic sequences. An antisense morpholino oligonucleotide, targeting the start codon of *sizzled*, was injected. While control injections had no effect (Fig. 2A), 100% of larvae ($n = 155$) injected with 2 ng morpholino displayed a weakly ventralized phenotype (Fig. 2B). In 1-day-old fish, the ventral tail fin was duplicated to the tip of the tail and cells had accumulated in the ventral tail. These characteristics of the *sizzled* knock down resembled the phenotype described for the ventralized mutant *ogon* (Fig. 2C) (Hammerschmidt et al., 1996; Solnica-Krezel et al., 1996). This observation suggested *sizzled* as a candidate for the gene mutated in *ogon.*

Table 1
Comparison of the effect of wild type and mutant *sizzled* mRNA on *ogo*^{tm305} embryos

Type of mRNA injected	Amount of mRNA injected (pg)	Phenotypic class (%)					*n*
		Ogo	Weakly ventralized	Wild type	Dorsalized	Abnormal[d]	
Uninjected	–	100	0	0	0	0	35
Control injected[a]	–	100	0	0	0	0	24
ogo^{tz209} *sizzled*	1	100	0	0	0	0	18
	10	95	0	0	5[b]	0	22
	50	95	0	0	0	5	21
ogo^{tm305} *sizzled*	1	100	0	0	0	0	11
	10	100	0	0	0	0	25
	50	85	0	0	0	15	26
Wild type *sizzled*	1	100	0	0	0	0	17
	10	92	0	0	0	8	26
	50	0	34	19	31[c]	16	32

[a] Injection of 1 nl of water.
[b] One embryo with dorsalized C3 phenotype, which we explain as a contamination that occurred during sorting of fertilized versus unfertilized eggs, since injection of higher amounts of *ogo*^{tm305}*sizzled* mRNA showed no dorsalizing activity.
[c] Two C3-, two C4-, and four C5-dorsalized embryos.
[d] Including embryos with gastrulation defects, lysed or necrotic phenotypes. The appearance of this phenotypic class with increasing amount of mRNA suggests a toxic effect of the mRNA.

Ogon and sizzled are linked

To determine whether sizzled mapped to the same chromosomal region as *ogon*, radiation hybrid mapping was performed. "Instant Mapping" calculated z14965 as the closest marker to the *sizzled* gene on the RH map (distance: 6 cR, LOD score: 18.157). The marker z14965 is located at the distal region of linkage group (LG) 25 (Fig. 3A). This corresponded to the interval where the *ogon* mutation was mapped (Miller-Bertoglio et al., 1999). The deletion allele *ogo*^{m60} is lacking the telomeric end of LG25 distal to marker z21929, where z14965 is anchored. Thus, *sizzled* and *ogon* mapped to the same interval.

Both ogon mutant alleles carry mutations in the sizzled open reading frame

In order to be able to carry out mutation detection, the full-length sequence of *sizzled* was obtained via 3′RACE. A phylogenetic tree from blast search data showed that the cloned gene is more similar to *Xenopus* and axolotl Sizzled than to other sFRP family members, such as Crescent (Fig. 3B). A comparison of the predicted protein sequence with *Xenopus* and axolotl Sizzled revealed an overall amino acid sequence identity of 52 and 49%, respectively (Fig. 3C).

In order to determine whether *ogon* encodes *sizzled*, total RNA from 1-day-old mutant *ogon* embryos was isolated and RT-PCR fragments spanning the translated region of *sizzled* were amplified and sequenced. In both ENU-induced alleles, *ogo*^{tm305} and *ogo*^{tz209}, we found G-to-A transitions that led to exchanges of conserved amino acids in the mature domain of Sizzled (Fig. 3D). In the case of *ogo*^{tm305}, the base substitution alters aspartate at position 92 into an asparagine, while in the case of *ogo*^{tz209}, cysteine at position 158 changes into tyrosine. Thus, point mutations could be detected in *ogo*^{tm305} and *ogo*^{tz209}.

Sizzled mRNA has dorsalizing activity and rescues the ogon mutant phenotype

In order to obtain functional evidence that both *ogon* alleles encode for nonfunctional forms of *sizzled*, we tried to rescue the mutant phenotype by injection of wild type and mutant *sizzled* mRNA. Embryos derived from homozygous

Table 2
Effect of modified *sizzled* mRNA on morpholino injected embryos

Type injected	Amount injected	Phenotypic class (%)					*n*
		Ogo	Weakly ventralized	Wild type	Dorsalized	Abnormal[a]	
Sizzled morpholino	2 ng	100	0	0	0	0	42
mod. *sizzled* RNA	50 pg	0	0	62	28[b]	10	39
Sizzled morpholino/mod. *sizzled* RNA	2 ng/50 pg	28	13	41	15[c]	2	46

[a] Including embryos with gastrulation defects, lysed, or necrotic phenotypes.
[b] One C1-, one C2-, three C3-, one C4-, and four C5-dorsalized embryos.
[c] Three C1-, two C3-, one C4-, and one C4-dorsalized embryos.

ogo^{tm305} mutant parents were injected with 1, 10, and 50 pg each of wild type, *ogo*^{tm305}, and *ogo*^{tz209} *sizzled* mRNA, respectively. Injections of mutant *sizzled* mRNA showed no effect on the *ogon* phenotype (Table 1, and Fig. 4A). Injections of low amounts of wild type *sizzled* mRNA, 1 and 10 pg, also did not result in rescue of the mutant phenotype. However, egglays injected with 50 pg of wild type *sizzled* mRNA displayed a phenotypical shift from ventralized to dorsalized embryos (Table 1). In 32 injected embryos, no embryo with a duplicated ventral tail fin was found, 34% showed a weakly ventralized phenotype displaying only accumulation of cells in the ventral tail, 19% were phenotypically wild type, and 31% displayed C4- and C5-dorsalized phenotypes (Fig. 4B–E).

Injections of wild type *sizzled* mRNA into embryos deriving from parents heterozygous for the *ogo*^{tz209} allele resulted in a similar dorsalization of the larvae. In a total of 78 embryos injected with 10 or 100 pg each of wild type *sizzled* mRNA, no *ogon* mutant phenotype was present, 6% showed a weakly ventralized phenotype, 29% were phenotypically wild type, and 53% displayed dorsalized phenotypes.

In addition, we could rescue the morpholino-induced *ogon*-like phenotype by coinjecting modified *sizzled* mRNA which harbored silent mutations to prevent binding of the morpholino but coded for a functional Sizzled protein (Table 2). In 46 embryos coinjected with 50 pg of modified *sizzled* mRNA and 2 ng of morpholino targeting the start codon, 28% showed the *ogon* phenotype, 13% were weakly ventralized, 41% were phenotypically wild type, and 15% displayed dorsalized phenotypes. Considering the fact that the morpholino has a penetrance of 100% when injected on its own, the coinjection provides convincing evidence that the morpholino specifically inhibits *sizzled.*

These experiments clearly demonstrated that wild type *sizzled* mRNA was able to rescue and dorsalize *ogon* mutant embryos, while mutant forms of *sizzled* failed to do so. This, together with the morpholino-induced phenocopy of *ogon,* and the point mutations identified in the *sizzled* gene in both mutant alleles, leads us to conclude that *ogon* encodes the zebrafish *sizzled* gene.

Misexpression of sizzled in ventralized mutants

To gain more information about the regulation of *sizzled* expression and with the knowledge that *sizzled* expression is downregulated in dorsalized *swirl* mutant embryos (see above Fig. 1), we examined its expression pattern in the ventralized mutants *ogon* and *din* by whole-mount in situ hybridization. Egglays from heterozygous *ogo*^{tz209} and *din*^{tm84} fish were analyzed at late blastula and gastrulation stages. At 30% epiboly, the ventral expression domain of *sizzled* was indistinguishable between wild type and *ogon* mutant embryos (Fig. 5A and B). However, *sizzled* expression was dorsally expanded in embryos mutant for *din* when compared with wild type (Fig. 5C). Similarly at shield stage, the expression domain of *sizzled* was expanded in *ogon* mutant embryos and expanded even further dorsally in *din*

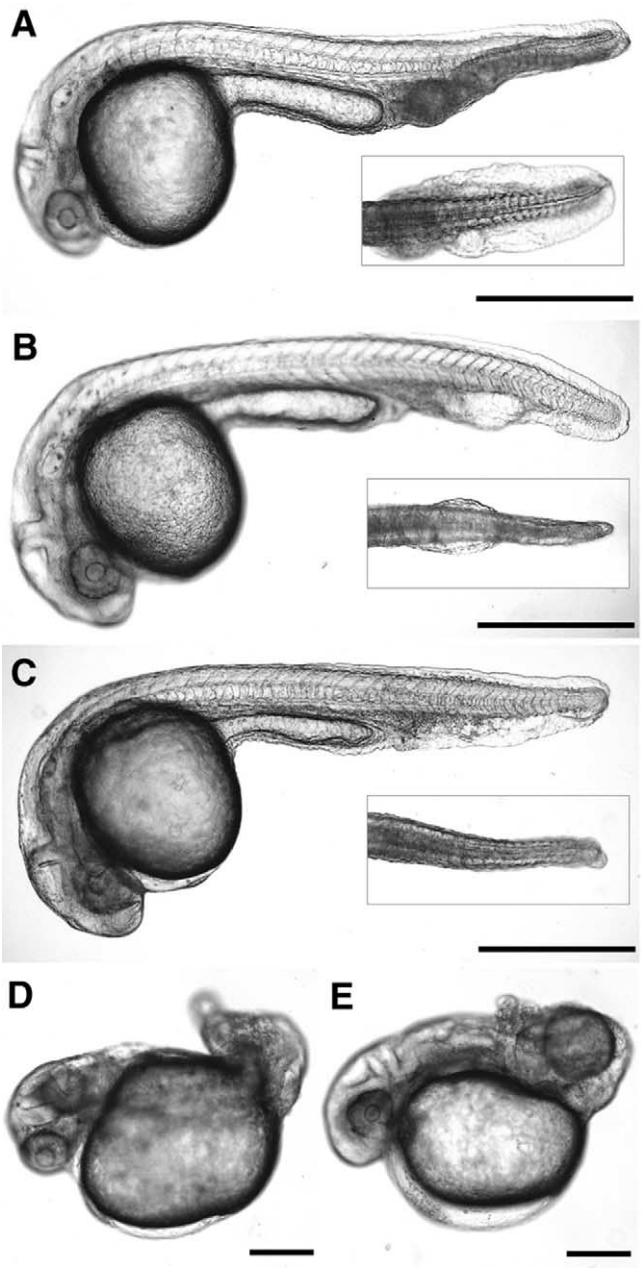
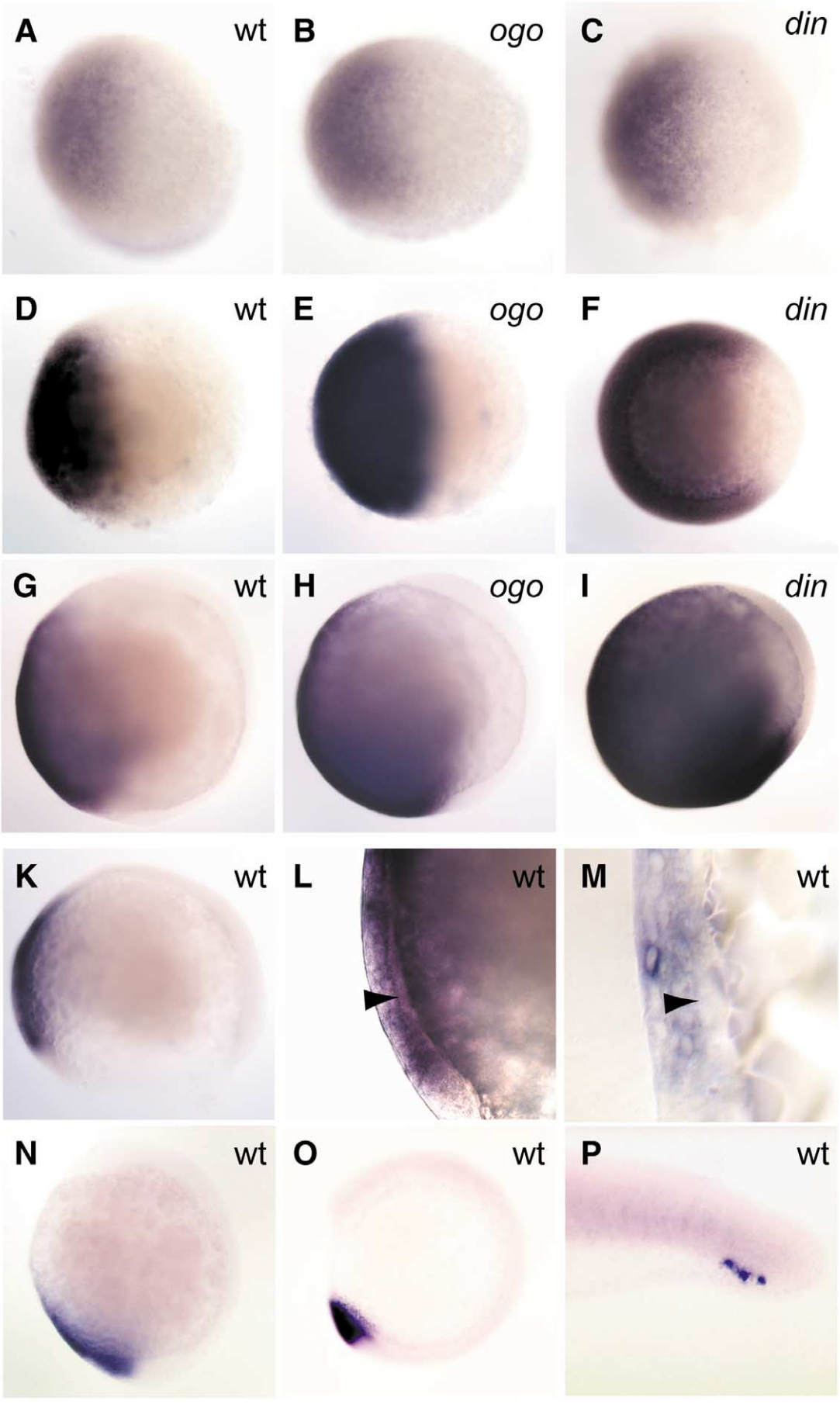


Fig. 4. Injection of wild type *sizzled* mRNA results in dorsalization and therefore rescue of *ogo*^{tm305} homozygous embryos. One-day-old *ogo*^{tm305} homozygous larvae injected with 50 pg RNA at one- to two-cell stage. Lateral view, with insets dorsal view. (A) Control injection with *ogo*^{tm305} *sizzled* mRNA showing no effect on embryos mutant for *ogo*^{tm305}. (B–E) Wild type *sizzled* mRNA injection resulting in rescue and dorsalization of *ogo*^{tm305} mutants. (B) Weakly ventralized embryo with accumulation of cells in the ventral tail. (C) Rescued wild type phenotype. (D, E) Examples of dorsalized C3 (D) and C4 (E) phenotypes. The scale bars represent 500 μm (A–C) and 200 μm (D, E), respectively.

mutants (Fig. 5D–F). The dorsal expansion of *sizzled* expression persisted in mutant versus wild type embryos at bud stage (Fig. 5G–I). In wild type embryos, *sizzled* expression was restricted to the ventral side. In contrast, the expression domain of *sizzled* covered the ventral half in *ogon* and almost the whole embryo in *din* mutant embryos. Equivalent results were obtained by using *siz-*


A wt
B ogo
C din
D wt
E ogo
F din
G wt
H ogo
I din
K wt
L wt
M wt
N wt
O wt
P wt

zled knockdown embryos (data not shown). Taken together, *sizzled* expression was expanded in *ogon* and *din* mutants, with a stronger effect in the more strongly ventralized mutant *din.*

We additionally analyzed *sizzled* expression in wild type embryos in more detail and at later stages. The ventral expression in 75% epiboly stage embryos is predominantly localized in the epiblast (Fig. 5K–M). Sections through the ventral side of an embryo of this stage revealed that *sizzled* is also weakly expressed in the hypoblast layer (Fig. 5M). During the segmentation period, expression of *sizzled* becomes further restricted to the posterior part of the embryo (Fig. 5N–P). At 24 h postfertilization (hpf), *sizzled* is only expressed in few cells in the ventral part of the tail tip (Fig. 5P). We could not detect *sizzled* expression after 24 hpf.

Discussion

Identification of sizzled in zebrafish

We have performed RDA, a PCR-based subtractive hybridization method (Hubank and Schatz, 1999), to identify downstream target genes of *swirl/bmp2b* in zebrafish. Using this screen, we have cloned a gene that belongs to the sFRP-family and shows highest sequence similarity to *Xenopus sizzled*, a putative Wnt inhibitor (Salic et al., 1997). Like other sFRPs, it contains a putative signal sequence and a cysteine-rich domain (CRD), but lacks any transmembrane domains. It is expressed on the ventral side during late blastula and early gastrulation stages like *Xenopus sizzled,* but unlike other sFRPs, for example *frzb-1*, which is expressed in the Spemann's organizer (Leyns et al., 1997; Wang et al., 1997). Based on the close sequence similarity and the expression pattern, we conclude that the cloned zebrafish gene is orthologous to tetrapod *sizzled.*

Ogon is a mutant in the sizzled gene

We demonstrate here that the *ogon* mutant phenotype is caused by a mutation in the zebrafish *sizzled* gene. First, *ogon* and *sizzled* map to the same interval, the distal end of LG25. Second, the morpholino knock down of *sizzled* phenocopies the *ogon* mutant phenotype. Third, in both ENU-induced alleles analyzed, we found point mutations in the open reading frame, which led to amino acid exchanges in positions highly conserved in the sFRP-family (Bradley et al., 2000). Fourth, *ogon* mutant embryos were rescued by injection of wild type *sizzled* mRNA, while mutant mRNA was ineffective. Taken together, the available evidence strongly supports the notion that *sizzled* is encoded by the *ogon* locus.

Allelic strength of ogo^{tm305} and ogo^{tz209}

Our results indicate that ogo^{tm305} is likely to be a strong hypomorphic or possibly a null allele. On the one hand, the ogo^{tm305} mutant and *sizzled* knock down phenotypes are identical (compare Fig. 4A with Fig. 2B: both with ventral fin duplication to the tip of the larvae; injection of higher amounts of morpholino does not result in stronger phenotypes). On the other hand, the identified amino acid exchange affects the CRD domain, which is likely to be involved in ligand binding (Bhanot et al., 1996). In accordance with the observation that the phenotype of ogo^{tz209} is weaker than that of ogo^{tm305} mutant embryos (compare Fig. 2C with Fig. 4A; Hammerschmidt et al., 1996), the mutation in this allele affects a cysteine-residue in the coding region C-terminal to the CRD domain, suggesting that the ogo^{tz209} allele is weak hypomorphic. The ogo^{tm305} allele was reported to be hypomorphic due to its weaker phenotype compared with the deletion alleles (Miller-Bertoglio et al., 1999). It is likely that the deletions in the ogo^{m60} and ogo^{b180} mutants affect additional genes that result in stronger phenotypes (Wagner and Mullins, 2002). We therefore suggest that ogo^{tm305} is a strong hypomorphic allele, though we cannot exclude the possibility that ogo^{tm305} is a null allele.

The expression domain of sizzled correlates with its role in dorso-ventral patterning and the ogon phenotype

Sizzled transcripts were detected between late blastula stage and 24 hpf zebrafish embryos. Throughout gastrulation, *sizzled* is expressed in a broad domain on the ventral side of the embryo, namely in the epiblast and to a lesser extent in the hypoblast. During this period of development, cellular identities along the dorsoventral axis are determined. The temporal expression pattern of *sizzled* supports the idea of Sizzled playing a role in dorsoventral pattern formation. During the segmentation period, *sizzled* expression becomes restricted to the posterior part of the tail. This spatial restriction of *sizzled* transcripts corresponds to the phenotypic effects in *ogon* mutant embryos.

Sizzled has dorsalizing activity in zebrafish

Xsizzled can dorsalize *Xenopus* embryos if expressed after MBT and is capable of inhibiting XWnt8. This most likely occurs in the ventral mesoderm, where *Xsizzled* is

Fig. 5. *Sizzled* expression is localized to the ventral side of wild type embryos and expands dorsally in *ogon* and *dino* mutant embryos. In situ hybridization with *sizzled* antisense probe on wild type (A, D, G, K–P), ogo^{tz209} (B, E, H), and din^{tm84} (C, F, I) embryos. Stages analyzed were 30% epiboly (A–C) and shield stage (D–F), animal pole view, dorsal is to the right, and tailbud stage (G–I), lateral view, dorsal is to the right. (K–M) 75% epiboly stage embryos, lateral view, dorsal is to the right. (L) Optical section of the ventral side of the embryo shown in (K). (M) Section through the ventral side of a whole-mount stained embryo such as shown in (K). Note boundary between epiblast and hypoblast layers (arrowheads in L and M). In wild type embryos, *sizzled* expression gets restricted to the posterior end of the tail at 1-somite and 8-somite stage (N and O, both lateral view, dorsal is to the right) and 24 hpf (P, lateral view of tail).

expressed during gastrulation (Salic et al., 1997). However, Bradley et al. (2000) were unable to observe suppression of *Xwnt8* mRNA effects by *sizzled*. In a recent study, Collavin and Kirschner (2003) confirmed that Sizzled cannot block activation of the canonical Wnt pathway by XWnt8. It was also reported that secreted Wnt binding proteins can activate the Wnt pathway, such as FZD4S, a soluble-type splicing variant of Frizzled-4 (Sagara et al., 2001). We show that zebrafish *sizzled* also has potent dorsalizing activity resulting in C5 dorsalized phenotypes. Together with the ventral localization of its transcripts and the ventralized phenotype of the *ogon/sizzled* mutant, it seems likely that zebrafish Sizzled has a Wnt antagonizing function in the ventral mesoderm.

It will be interesting to find out which ventralizing Wnt ligand is anatagonized by the binding of Sizzled in zebrafish. Obviously, zebrafish Wnt8 is a candidate to investigate due to the spatially and temporally overlapping expression patterns of *wnt8* and *sizzled* (Kelly et al., 1995) and its role in patterning of the mesoderm shown by Wnt8 mutant analysis (Lekven et al., 2001). However, recent findings in *Xenopus* make this interaction unlikely (Collavin and Kirschner, 2003). Also yet to be identified ventralizing Wnt molecules, which have been suggested by Itoh and Sokol (1999), need to be considered as ligands for Sizzled. So far, only the molecular similarity of Sizzled to the extracellular domains of Frizzleds support the idea that Sizzled acts as a Wnt antagonist. The possibility that Sizzled does not interact with Wnt ligands at all cannot be ruled out, but seems unlikely to us.

Sizzled negatively autoregulates its own expression

Zebrafish *sizzled* expression is downregulated early in embryos deficient in *swirl/bmp2b*. Conversely, the expression domain of *sizzled* is dorsally expanded in *ogon* mutant embryos. These data indicate that the maintenance of *sizzled* expression requires a defined threshold level of BMP2b activity. In *swirl/bmp2b* mutants, the transcript level of *bmp2b* is downregulated (Kishimoto et al., 1997) and obviously insufficient for the maintenance of *sizzled* expression, while upregulation of *bmp2b* transcript in *ogon* (Miller-Bertoglio et al., 1999) accompanies expanded expression of *sizzled*. The increased expansion of *sizzled* expression in *din* mutants compared with *ogon* is consistent with their stronger ventralized phenotype. These data suggest that Sizzled negatively autoregulates its own expression in a BMP2b-dependent way.

In *Xenopus*, *sizzled* expression is also strongly dependent on BMP activity (Marom et al., 1999). *Xsizzled* transcript was normally localized to the ventral-most marginal zone, where the highest levels of BMP4 activity are present. Injections of high levels of *bmp*4 mRNA led to a dorsal expansion of the *sizzled* expression domain, whereas inhibition of BMP signaling eliminated *sizzled* expression. Our findings in zebrafish are consistent with these results.

Sizzled: a mediator for the interaction of the Wnt and BMP pathways?

So far, all cloned zebrafish mutants known to be essential for the patterning of the dorsoventral axis are deficient in members of the BMP signaling pathway. Interestingly, we identified *sizzled*, a member of the sFRP-family, as the gene mutated in *ogon*. This finding is consistent with the prediction that *ogon* encodes a dorsalizing factor that antagonizes BMP in a Tolloid- and Chordin-independent way (Miller-Bertoglio et al., 1999; Wagner and Mullins, 2002). Obviously, the genetic evidence that a putative Wnt inhibitor is required for patterning of the ventral mesoderm raises the question how the Wnt and BMP pathways interact during this process.

Interaction of the Wnt and BMP pathways during mesoderm patterning has already been discussed in *Xenopus* (Hoppler and Moon, 1998), where it was shown that *Xwnt8* expression is regulated by BMP2/4 signaling. However, the interaction of XWnt8 and BMP2/4 does not function in a simple linear pathway as overexpression of *Xwnt8* cannot compensate for an inhibition of BMP2/4 function, but *bmp2/4* overexpression in embryos with blocked Wnt signaling rescues the expression of ventral markers (Hoppler and Moon, 1998). The mechanism of the interaction between the BMP and Wnt pathways needs to be elucidated.

Based on the available data, a mediator role for Sizzled during this process is possible. First, BMP maintains Sizzled, as evident by analysis of *sizzled* transcript in *swirl* mutant embryos. Second, Sizzled potentially inhibits Wnt signaling, as predicted from its molecular nature, through binding to a yet to be identified Wnt ligand. This inhibition probably occurs in the ventral most marginal zone to separate the ventral mesoderm from the somitogenic region, a process requiring Wnt8 activity. Third, Sizzled antagonizes BMP signaling, possibly by negatively regulating *bmp* transcription, as *bmp2b* expression is expanded in an *ogon*-deficient situation. Fourth, the latter in turn negatively autoregulates the transcription of *sizzled*, as discussed above.

In summary, *ogon/sizzled* is on the one hand funneling into the Wnt signaling pathway, while it is on the other hand regulated by the BMP signaling pathway. Therefore, *ogon/sizzled* is at a key position to connect both the BMP and Wnt signaling pathways.

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