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Sizzled: a secreted Xwnt8 antagonist expressed in the ventral marginal zone of *Xenopus* embryos

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SUMMARY

An expression cloning screen was used to isolate a novel gene homologous to the extracellular cysteine-rich domain of frizzled receptors. The gene (which we called *sizzled* for secreted frizzled) was shown to encode a soluble secreted protein, containing a functional signal sequence but no transmembrane domains. Sizzled (szl) is capable of inhibiting Xwnt8 as assayed by (1) dose-dependent inhibition of siamois induction by Xwnt8 in animal caps, (2) rescue of embryos ventralized by Xwnt8 DNA and (3) inhibition of XmyoD expression in the marginal zone. Szl can dorsalize *Xenopus* embryos if expressed after the midblastula transition, strengthening the idea that zygotic expression of wnts and in particular of Xwnt8 plays a role in antagonizing dorsal signals. It also suggests that inhibiting ventralizing wnts parallels the opposition of BMPs by noggin and chordin. szl expression is restricted to a narrow domain in the ventral marginal zone of gastrulating embryos. szl thus encodes a secreted antagonist of wnt signaling likely involved in inhibiting Xwnt8 and XmyoD ventrally and whose restricted expression represents a new element in the molecular pattern of the ventral marginal zone.

Key words: *Xenopus*, wnt, frizzled, marginal zone, ventral patterning, dorsalization

INTRODUCTION

Over a dozen related genes are presently known to belong to the *wnt* family (Nusse and Varmus, 1992; Parr and McMahon, 1994). Since the initial identification of *int1* as an oncogene activated by retroviral insertion (Nusse and Varmus, 1982), *wnt* genes have been ascribed roles in a multitude of developmental processes, in a wide range of animals from nematodes to vertebrates. *wnt* genes encode secreted proteins that have so far resisted purification in both soluble and active form, a property that has significantly delayed identification of their receptors. The recent discovery that the *Drosophila frizzled2* gene product can directly bind wingless (wg), and mediate signaling events evoked by it, was made possible by the availability of a soluble wg preparation (Bhanot et al., 1996). *frizzled* genes, of which almost a dozen are already known (Wang et al., 1996) encode seven-spanning transmembrane proteins, with a conserved extracellular cyteine-rich domain (CRD), which is necessary and sufficient for wg binding (Bhanot et al., 1996). The first member of the family, *Drosophila frizzled1* is a tissue polarity gene (Vinson et al., 1989) required for the proper proximodistal orientation of bristles on the body and for the mirror symmetric arrangement of ommatidia in the compound eye (Zheng et al., 1995). Before *frizzled* genes were identified as receptors for wnts, another component of the wg pathway, *dishevelled* was also known as a tissue polarity gene (Krasnow et al., 1995).

The wnt pathway is implicated in early axis induction events and mesoderm patterning in *Xenopus* (Moon, 1993; Kimelman et al., 1992). Although the involvement of specific *wnt* genes in axis specification remains speculative, maternal stores of a downstream component of the wnt pathway, β-catenin, are clearly required for axis formation (Heasman et al., 1994). This is likely due to β-catenin being necessary and sufficient for the formation of a dorsovegetal blastula organizer also known as the Nieuwkoop center (Wylie et al., 1996; Fagotto et al., 1997) which, in turn, induces the gastrula organizer in the overlying dorsal marginal zone (DMZ). The gastrula (or Spemann's) organizer, also known as the dorsal marginal zone, patterns the rest of the equatorial region of the embryo, known as the ventral lateral marginal zone (VLMZ). As a result, the lateral regions of the VLMZ will form muscle and kidney while the most ventral zone will become blood and mesenchyme. The DMZ will develop into prechordal plate and notochord. Ectopic expression of *wnt* genes has dramatically different effects depending on the time and place of expression. Before the mid-blastula transition (MBT) ventral wnt expression (from injected mRNA), induces dorsal structures and results in twinned embryos while post-MBT expression (from injected DNA) results in ventralized embryos (reviewed by Kimelman et al., 1992). *Xenopus wnt8* (*Xwnt8*) is expressed zygotically in VLMZ, being excluded from the organizer (Christian et al., 1991). Xwnt8 inhibition by a dominant-negative construct decreases XmyoD expression in the VLMZ (Hoppler et al., 1996). Xwnt8 therefore normally functions to antagonize dorsal marginal zone (DMZ) signals and to positively regulate lateroventral mesoderm formation, in particular to maintain

XmyoD expression laterally in the region fated to become somitic muscle.

We report here the expression cloning and properties of a secreted protein homologous to the CRD of frizzled genes, but which lacks any obvious transmembrane domain. This gene, which we call *sizzled* (for secreted frizzled) can antagonize Xwnt8 in a number of assays. *sizzled* is expressed in an unusual pattern, in a restricted ventral territory of the marginal zone. Its action in the ventralmost subset of Xwnt8-expressing cells presumably inhibits Xwnt8 signals ventrally. Frzb, a distantly related Xwnt8 inhibitor has recently been described and is expressed in the organizer (Leyns et al., 1997; Wang et al., 1997) in a pattern complementary to that of Xwnt8. Xwnt8 signaling thus appears to be antagonized in both dorsal and ventral marginal zones by the action of frizzled-related secreted factors, subdividing the marginal zone into at least four domains.

MATERIALS AND METHODS

Xenopus embryos

Pigmented and albino *X. laevis* embryos were obtained by in vitro fertilization, dejellied and cultured in 0.1× Marc's Modified Ringer's (MMR) containing 50 μg/ml gentamycin at 16-18°C. Staging of *Xenopus* embryos was done according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Embryos were injected in 1× MMR + 5% Ficoll and then transferred to 0.2× MMR + 5% Ficoll until further manipulations or harvesting. DNA or RNA dissolved in water were injected at a volume of 10 nl per blastomere if injections were done at the 2-4 cell stage or 1 nl per blastomere at the 32-cell stage. Animal cap explants were harvested from stage 8 embryos and cultured in 0.5× MMR + 1mg/ml BSA until the indicated stage reached by control embryos raised at the same temperature.

Transgenic embryos

Transgenic *Xenopus* embryos were generated by restriction enzyme-mediated integration of linearized plasmid DNA into permeabilized sperm nuclei, as developed by Kroll and Amaya (Kroll and Amaya, 1996). Nuclear transplants were done in 0.4× MMR + 5% Ficoll and embryos were raised in 0.2× MMR + 5% Ficoll after approximately stage 7.

Library construction and cloning

A directional cDNA library from stages 14 through 20 *Xenopus* embryos was built in the plasmid vector pCS2+ (Turner and Weintraub, 1994). Capped RNA was synthesized by in vitro transcription from pools of 100-200 independent transformants as described (Krieg and Melton, 1984). 1-2 ng of RNA from each pool were injected into one ventral blastomere of 4-cell stage embryos. Embryos were score visually at stages 11, 14, 18 and 26 for the presence of duplicated axial structures or other observable phenotypes. Pools judged positive were retested and, if still positive, subdivided and sib-selected down to single positive cloned as described by Lustig et al. (1996). Single clones were sequenced on both strands on an automated DNA sequencer.

Constructs

The entire open reading frame of sizzled was amplified by low-cycle number PCR using Pfu polymerase (Stratagene) and cloned as an *Bam*HI-*Xho*I fragment into pCS2+ and as a *Bam*HI-*Cla*I fragment into pCS2+MT (Rupp et al., 1994) thus generating a fusion protein with 6 copies of the myc epitope at the carboxy end.

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was done as described (Harland, 1991) with both digoxigenin- and fluorescein-labeled antisense RNA probes. For double in situ hybridization, the two probes were detected successively. The first probe was usually detected using 5-bromo-4-chloro-3-indolyl phosphate 4-toluidine salt (BCIP) for the color reaction and, after inactivating the alkaline phosphatase, the second probe was detected using Magenta Phos. In single in situ hybridizations, a combination of 4-nitro blue tetrazolium (NBT) + BCIP or BM purple (Boehringer Mannheim) was used for detection. In most injection experiments, 10 pg RNA encoding a nuclear localized GFP was used as a lineage tracer which was subsequently detected in double in situ hybridization using an antisense GFP probe. Embryos were cleared in Murray's clearing reagent; both cleared and uncleared embryos were imaged on either a Zeiss StemI stereoscope or a Zeiss Axiophot equipped with a Sony 3 chip color video-rate CCD camera, controlled by Northern Exposure software (Phase 3 Imaging). Immunofluorescence was performed according to standard methods. The 9E10 monoclonal antibody was used to detect myc-tagged szl in stage 10.5 injected embryos using a cyanine 3-conjugated secondary antibody (Jackson Immunoresearch). Nuclei were stained with DAPI. Confocal images were collected on a Zeiss LSM-410 confocal microscope using a 40x/1.2NA water immersion lens. Coincident Z series were obtained for both the cy3 and the DAPI signal. All images were imported into Adobe Photoshop to make composite figures. Image contrast and brightness were adjusted only to match printer output.

Cell culture

293T cells were cultured using standard tissue culture techniques. pCS2+ szlMT was transfected into 293T cells at 50-60% confluency using Lipofectamine (Gibco BRL). Transfection efficiency was estimated using pCS2+ nuclear GFP(S65T). Secreted szlMT was harvested in low-serum medium (OptiMEM – Gibco BRL). Cells expressing szlMT were judged healthy for the whole duration of the experiment. The possible cellular debris were pelleted from the media after harvesting.

RT-PCR

RT-PCR for siamois was done as described by Brannon and Kimelman (1996). 25 cycles were used to detect the siamois cDNA. Each RT reaction contained RNA extracted from 10-20 animal cap explants or 2 whole embryos. Each PCR reaction corresponded to one animal cap explant or one tenth of a normal embryo. The qualitative nature of the RT-PCR was tested by amplifying serial dilutions of total cDNA and siamois plasmid standards.

RESULTS

Identification of sizzled (szl), a secreted protein homologous to frizzled

Small pools of RNA representing 100-200 independent clones (Lustig et al., 1996) of a *X. laevis* stage 14-20 cDNA library were screened for the ability to generate a duplicated body axis (Lemaire et al., 1995) or any other visible developmental perturbation in *Xenopus* embryos. 1-2 ng of capped synthetic RNA from each pool was injected into one ventral blastomere of 4-cell stage embryos. We screened approximately 300 pools (representing 50,000 independent clones). One of the positive pools reproducibly caused embryos to develop significantly enlarged dorsoanterior structures (most notably large cement glands). Sib selection of this pool resulted in the isolation of a single clone, as little as 10 pg of which was capable of generating the same phenotype in injected embryos. This phenotype

was dramatically enhanced in embryos injected with increasing amounts of RNA (100 pg to 1-2 ng). These embryos (see Fig. 1B; the two left embryos have been injected with 200 pg szl RNA) appeared hyperdorsal, with large heads and cement glands (indicated by arrows in Fig. 1B and also by bottom arrow in Fig. 3B), severely stunted and kinked trunks and tails, wide and misshapen neural plates and folds which often did not close (in Fig. 1B, compare the region between arrowheads in the szl-injected versus the uninjected embryo). Part of the phenotype is reminiscent of the appearance of Xwnt5A-injected embryos (Moon et al., 1993). A histological examination of szl-injected embryos (see Fig. 1C, frontal section through such an embryo) reveals significantly expanded and hypercellular paraxial mesodermal tissue on the injected side, consistent with the presence of an enlarged muscle actin expression domain on that side (see Fig. 3A). The positive clone was sequenced and found to contain a single open reading frame encoding a protein 281 amino acids in length, with a calculated relative molecular mass of 31.8×10^3. In a sequence database search using the BLAST algorithm, the ORF was homologous to an amino-terminal portion of *frizzled* genes which corresponds to the extracellular cysteine-rich domain (CRD); however, although it contained a strong putative signal sequence, it did not contain any obvious transmembrane domain. We named this new gene *sizzled*. While this manuscript was in preparation, a distantly related gene called *frzb* was described in *Xenopus*. The comparison between szl and frzb sequences is shown in Fig. 1A. Although clearly related by sequence and function, the two genes are only 18% identical and have completely different expression patterns (see below).

Szl is a secreted protein

The presence of a putative signal sequence and the absence of an apparent transmembrane domain suggested that szl is secreted. We first determined whether an epitope-tagged szl (generated by attaching 6 copies of the myc epitope to the carboxy terminus) was secreted. The tag did not affect the activity of the protein, as SzlMT had the same effects as wild-type szl in embryo injections (not shown). SzlMT RNA was injected into 2-cell stage embryos and the SzlMT protein was detected by immunofluorescence in stage 10.5 embryos. Confocal micrographs (Fig. 2A and B, showing 2 focal planes of a z-series, located 15 μm apart) demonstrate that szlMT localizes to the cell surface where it tends to aggregate. Little signal can be seen inside the cell except occasional punctate staining suggesting the tagged protein efficiently reaches the cell surface.

To determine if szl can be released from cells in a soluble form, we transiently transfected 293T cells with an expression construct in which a simian CMV promoter drives szlMT expression (CS2+szlMT). We then harvested the proteins released by the cells in low-serum media. SzlMT present in the media and the cells, respectively, was analyzed by SDS-PAGE followed by western blotting with the 9E10 monoclonal, which

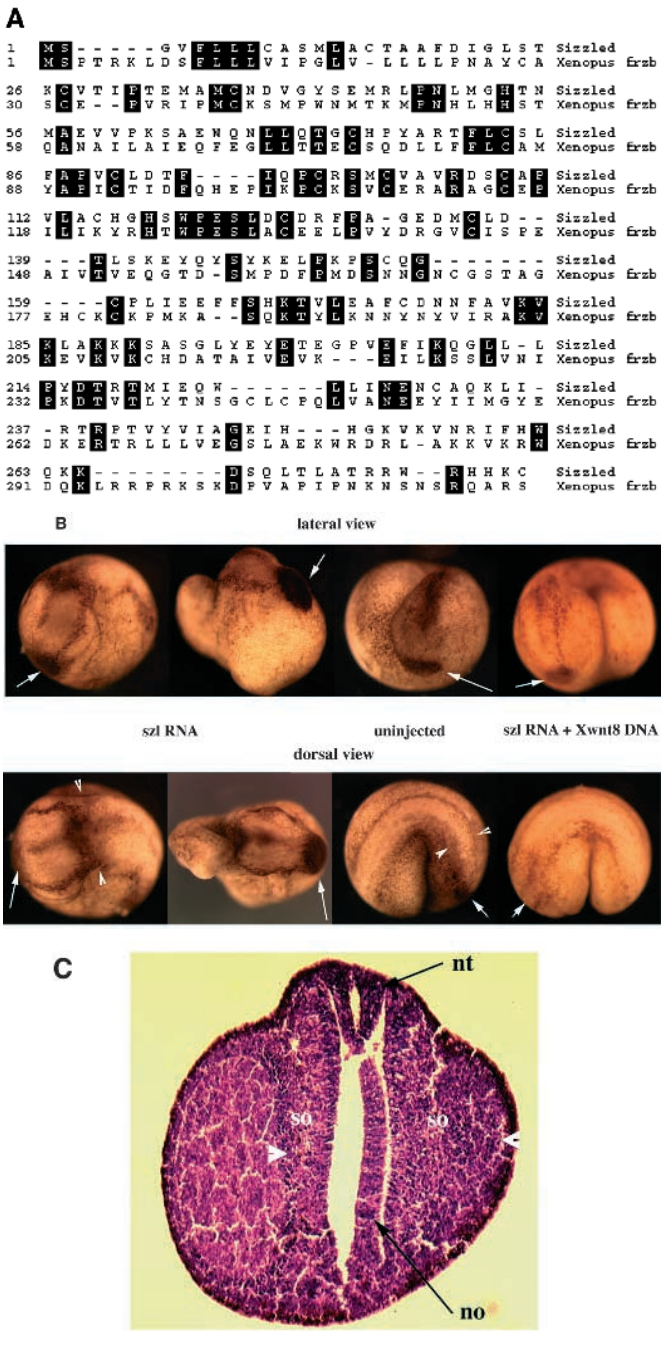

Fig. 1. Expression cloning of *sizzled*, a gene homologous to *frizzled* genes. (A) Sequence comparison between szl and frzb (Leyns et al., 1997; Wang et al., 1997). Conserved amino acid residues are boxed. The two proteins show 18.1% identity at the amino acid sequence level. (B) Phenotype displayed by stage 26 embryos injected with 200 pg of szl RNA into the ventral side at 4-cell stage. Top row of pictures shows lateral views of 4 embryos while the bottom row shows dorsal images of the same embryos. Arrows indicate the position of cement glands. Note the thick and stunted appearance and enlarged cement glands in szl-injected embryos as compared to the uninjected embryo. The neural plate is wide and fails to close properly (compare the area between arrowheads in a szl-injected embryo to that in an uninjected one – bottom row). In the embryo on the right, the phenotype caused by szl RNA injection has been rescued by coinjecting 100 pg of an Xwnt8 expression plasmid (CS2+Xwnt8). (C) Hematoxylin and eosin-stained frontal section through an embryo injected with 100 pg of szl RNA into one dorsal blastomere at 4-cell stage. The injected side is on the right. Note the increased cellularity and larger size of the paraxial mesoderm on the injected side. nt, neural tube; no, notochord; so, somite.

recognizes the myc epitope. As shown in Fig. 2C, szlMT is present in soluble form in the medium (lane 2). An estimated 5-10% of szlMT is secreted; the rest remains associated with the cellular pellet (lane 1).

These findings, which are consistent with szl sequence analysis demonstrate that szl is a secreted protein that localizes to the extracellular space.

Szl dorsalizes embryos when expressed after the midblastula transition

We further characterized the szl overexpression phenotype by injecting szl RNA into the 4 different tiers of blastomeres at 32-cell stage. Although the most pronounced phenotype was observed when szl was injected into tiers A and B, all injected embryos displayed the szl phenotype, consistent with szl being a secreted protein capable of acting at a distance from its source. We next investigated the effects of expressing szl after the mid-blastula transition (MBT) from a plasmid construct in which szl was driven by the simian cytomegalovirus (sCMV) early promoter/enhancer (CS2+szl). DNA-injected embryos (not shown) had the same phenotype as RNA-injected embryos, suggesting that post-MBT expression of szl is sufficient to produce the observed phenotype. Moreover, transgenic *Xenopus* embryos expressing szl under the control of the sCMV promoter/enhancer, generated by the method of Kroll and Amaya (Kroll and Amaya, 1996) showed the same phenotype (not shown).

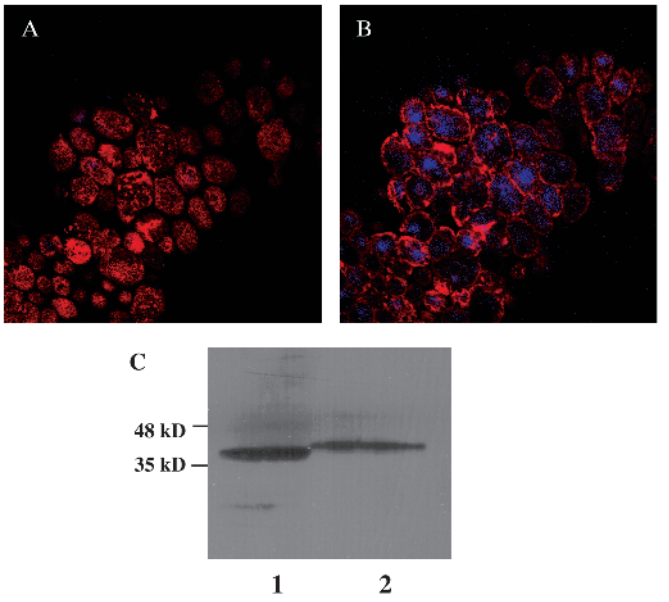

Fig. 2. Sizzled encodes a secreted protein. (A,B) Confocal micrographs of *Xenopus* embryonic (stage 10.5) cells expressing myc-tagged sizzled. Two serial section planes located 15 μm apart are shown. The tagged protein appears red (a secondary antibody conjugated to Cy3 was used for immunofluorescent detection) while the nuclear DNA is blue (stained with DAPI). In A, the cell surfaces are roughly in the optical section plane, which corresponds to the surface of the embryo. Note the cell surface localization of szlMT in B, which shows an optical section plane 15 μm deeper into the embryo. (C) Immunoblot of myc-tagged szl secreted into the medium by 293T cells transfected with CMV-szlMT. Lane 1: cellular pellet; lane 2: tissue culture medium. Note the higher apparent MW of soluble szlMT protein in lane 2.

The dorsalization effect seen after szl injections was examined in more detail at the level of both late and early mesodermal markers by double in situ hybridization to detect both the respective marker and the lineage tracer coinjected with szl at 2- or 4-cell stage. When late markers were examined at stages 24-26, the injected side of the embryos showed a strong increase in muscle actin staining (Fig. 3A, compare top embryos to the bottom one). The notochord (as seen by sonic hedgehog and collagen type II staining) was larger but also shorter and thicker than in normal embryos, with rare instances of partial duplications (Fig. 3B, embryo stained for *Xenopus* sonic hedgehog, with the arrow pointing to the secondary notochord). We next investigated how szl injections perturb early marginal zone patterning. Dorsal szl injections markedly expanded the expression domain of *gooscoid* (*gsc*; not shown), a homeobox gene expressed in the first involuting dorsal blastopore cells (Cho et al., 1991) and inhibited the expression of *Xpo* (Fig. 3C; note absence of staining on the left side, where injected szl RNA localizes), a ventroposterior gene (Sato and Sargent, 1991). Abundant ectopic expression of Xnot, a homeobox expressed mainly in the future notochord (von Dassow et al., 1993) was seen in the marginal zone overlapping with injected szl (Fig. 3E, compare injected right side with left side and with the uninjected embryo in D). In normal embryos, the region expressing Xnot elongates dramatically as gastrulation proceeds, paralleling the convergent extension movements in the prospective notochord. In szl-injected embryos, the endogenous Xnot expression fails to extend and is significantly shorter along the anteroposterior axis than in stage-matched uninjected embryos (Fig. 3D; compare to the szl-injected embryo in 3E, which is also delayed in blastopore closure). This suggests szl inhibits convergent extension and provides an explanation for the stunted appearance of szl-injected embryos. The expression of *Xenopus* brachyury in the marginal zone was unaffected by szl injections (Fig. 3F).

Szl defines a new early ventral domain of gene expression in the marginal zone

Though these effects of szl expression on mesodermal markers explain its ectopic effects, they tell us little about its function. We therefore examined the expression pattern of szl by reverse transcriptase PCR (RT-PCR) and whole-mount in situ hybridization (ISH). As determined by RT-PCR, szl starts to be expressed after MBT (not shown) and continues throughout gastrulation and neurulation. We did not detect the presence of maternal szl transcripts. As seen by ISH, just before gastrulation begins (stage 10) szl is diffusely expressed in the animal cap of the embryo. Early during gastrulation the expression levels increase significantly and the szl mRNA becomes restricted to the ventral marginal zone (VMZ) and ventral animal cap. By stage 10.25, szl is found in the ventral blastopore lip where it occupies a sector of approximately 120 degrees (see Fig. 4A), which becomes narrower in stage 10.5 (Fig. 4E) and 11 (Fig. 4J) embryos. Expression is highest near the blastopore lip and decreases on the ventral side toward the animal pole. At later stages, szl shows a wedge of expression in the ventralmost part of the involuting blastopore. At stages in the late teens and early twenties, szl is expressed along the belly, up to and including the heart-forming region (Fig. 4B; arrow points to szl expression in the heart-forming region), an expression pattern that persists in the latest stages examined

(stage 37). In sectioned embryos, szl is expressed in both superficial and deep cells of the VMZ (Fig. 4H; ventral blastopore lip indicated by arrow, with the yolk plug visible above and szl-expressing ventral cells below it).

A number of double ISH were performed in order to relate the expression pattern of szl to that of other genes expressed in the marginal zone. Double ISH for szl and organizer-specific genes such as Xnot (Fig. 4I, magenta staining), pintallavis (not shown) or goosecoid (Fig. 4L, light blue staining) show that at stage 11 szl occupies a sector ventrally about the size of the organizer. Double ISH performed with genes expressed in the entire or just the ventrolateral marginal zone (brachyury – Fig. 4K, magenta staining; XmyoD – Fig. 5A-C, light blue; Xwnt8 – Fig. 4E, light blue; Xvent1 – Fig. 4F,G, light blue) indicated that szl is more ventrally restricted than any of these genes, including Xvent1 (Gawantka et al., 1995) (Fig. 4F,G; note light blue staining representing Xvent1 extends more laterally than szl, their overlap appearing dark blue). The expression domain of szl is contained within that of Xwnt8, which extends more in the dorsolateral direction (Fig. 3E). Szl thus defines a new, ventrally restricted, territory in the marginal zone, adding a new pattern element to the already known molecular regionalization of the marginal zone.

Response of szl to axis perturbation

We next asked how is szl expression affected by treatments that perturb the embryonic axis. Treatment of early blastulae with lithium results in dorsalized embryos. Exposure to lithium ions at the 64-cell stage completely extinguished szl expression in the vicinity of the blastopore (Fig. 4C); expression was seen and persisted throughout gastrulation only in the animal cap of lithium-treated embryos. Ventralized embryos generated by vegetal irradiation early in the first cell cycle showed high levels of szl expression around the entire blastopore (Fig. 4D). These experiments show that szl responds to axis perturbations as expected for a gene involved in ventral patterning events.

Szl cannot rescue UV-ventralized embryos

Two genes expressed in the organizer, *noggin* (Smith and Harland, 1992) and *chordin* (Sasai et al., 1994) have dorsalizing effects on injected embryos. Both noggin and chordin also have potent UV rescuing activities and indeed, noggin was cloned based on this property (Smith and Harland, 1992). In an attempt to define the full-range of phenotypes that szl can elicit, we tested whether szl is capable of rescuing axial structures in UV ventralized embryos. 2-cell stage embryos that received a ventralizing dose of UV during the first cell cycle were injected with up to 2 ng of szl RNA (n=90, not shown). Szl had no detectable effect, all injected UV-irradiated embryos displaying the same 0 to 1 dorsoanterior index (a measure of dorsoanterior development; Kao and Elinson, 1988) as uninjected UV-ventralized embryos. UV irradiation blocks cortical rotation during the first cell cycle and embryos do not form a blastula organizer. Szl thus differs from chd and noggin by not being able to rescue dorsal development in embryos lacking an endogenous blastula organizer.

Functional antagonism between szl and Xwnt8

Since szl resembles the wnt-binding extracellular CRD of frizzled but lacks the transmembrane and intracellular domains of the receptor presumably involved in signal transduction, it follows that szl might act as an inhibitor of wnt signaling. To test this hypothesis, we looked at the interaction between szl and Xwnt8 in three different assays. We chose Xwnt8 because its expression domain contains that of szl as its ventralmost

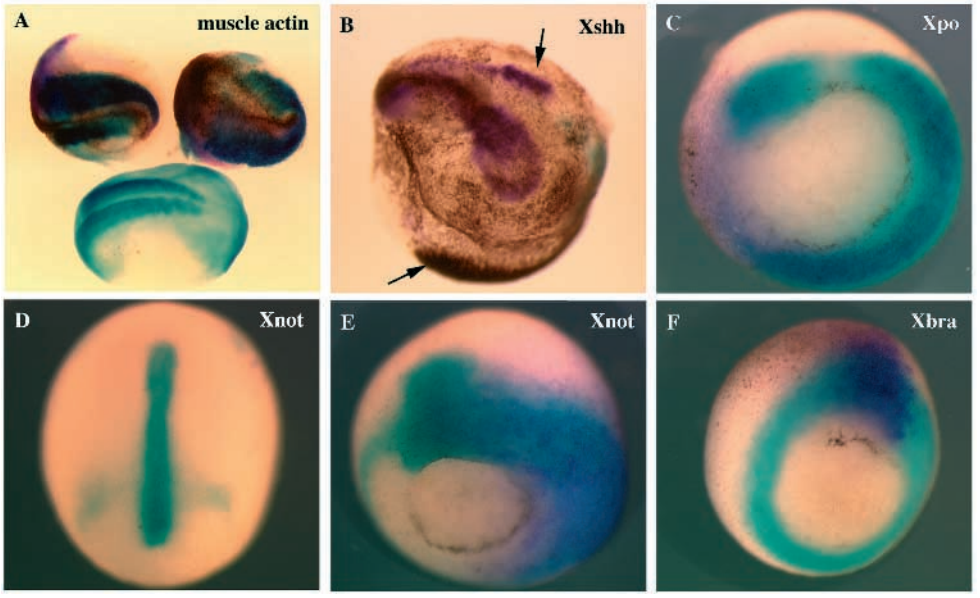

Fig. 3. Szl has dorsalizing activity. (A) Stage 23 szl-injected embryos show increased muscle actin staining on the injected side compared to the uninjected one (top 2 embryos) or to uninjected embryos (bottom). Muscle actin is shown in blue; the localization of injected szl mRNA was followed using a lineage tracer RNA encoding GFP, which was detected using an antisense GFP probe and appears magenta. The top embryos are seen from their dorsal side and have their anterior ends to the left. The left embryo was injected in the right side and the right embryo in the left side (B) Sonic hedgehog (magenta) expression in a szl-injected stage 23 embryo, seen in anterolateral view. The lineage tracer is blue. Note the duplicated but short notochord, indicated by the top arrow. The bottom arrow points to the enlarged cement gland. (C) Inhibition of Xpo (blue), a posteroventral marker, by szl. Xpo expression is extinguished from szl-overexpressing cells (which stain magenta) on the left side of a stage 11.5 embryos. (D) Xnot expression (blue) in a normal stage 12 embryo and (E) in a szl-injected embryo of the same stage. Injected szl is magenta. Note the strong ectopic Xnot expression on the right side of the embryo (which overlaps in part with injected szl and thus appears dark blue) and the blocked elongation of the endogenous Xnot pattern. Also note the delayed blastopore closure in szl-injected embryos compared to the stage-matched uninjected embryo (which makes the embryo in E appear as if it were an earlier stage). (F) Stage 10.25 embryo stained for Xbra (blue) and the lineage tracer coinjected with szl (magenta). Xbra expression is unperturbed by overexpressed szl.

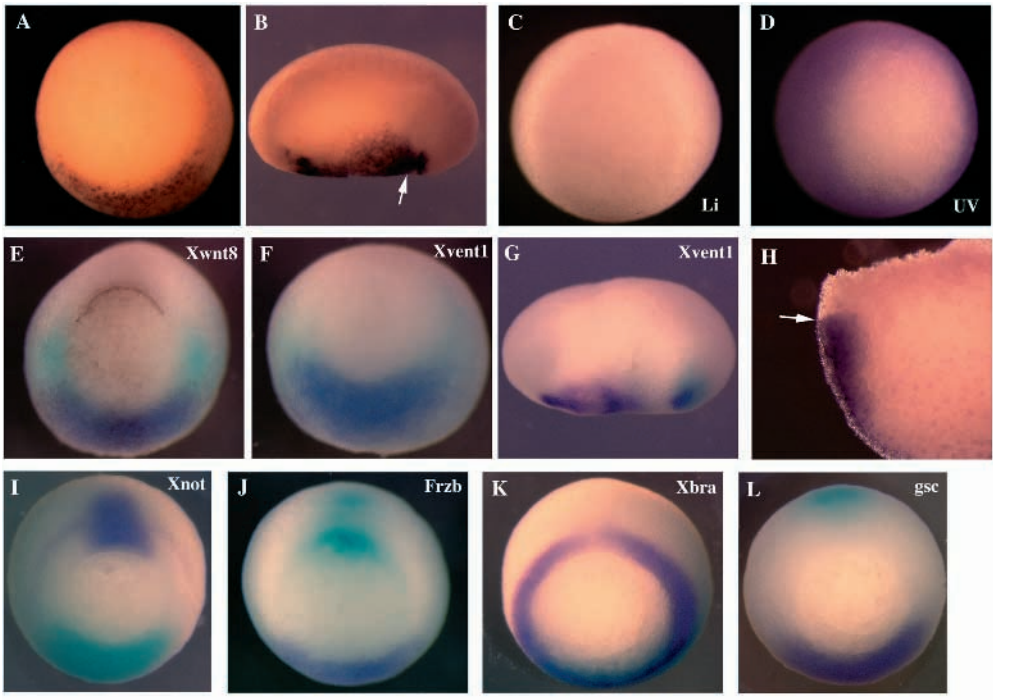

Fig. 4. Expression pattern of sizzled transcripts in *Xenopus* embryos. In all embryos dorsal is up and ventral is down. Szl expression by in situ hybridization (ISH) in (A) a stage 10.25 embryo and (B) a stage 23 embryo. (C) Vegetal view of a stage 11 embryos dorsalized by lithium, a treatment which suppresses szl expression. (D)Vegetal view of a stage 11 UV-ventralized embryo showing szl expression around the entire blastopore. Double in situ hybridizations for szl and (E) Xwnt8 (blue) at stage 10.5, (F) Xvent1 (blue) at stage 10.5, (G) Xvent1 at stage 24, (I) Xnot (blue) at stage 11-11.5, (J) frzb (blue) at stage 11, (K) Xbra (magenta) at stage 10.5, (L) gsc (blue) at stage 11. (H) Section through a stage 11 embryo stained for szl. Note the expression of szl in both superficial and deep layers of the ventral blastopore lip (indicated by arrow). The unstained region above the szl-expressing cells is the yolk plug. Embryos in A, B and H were stained with NBT + BCIP. Embryos in C and D were stained with Magenta Phos. In E-G and I-L staining was done with BCIP (light blue) and Magenta Phos (magenta). The overlap between light blue and magenta appears dark blue.

subdomain so the two proteins can, in principle, interact during development.

To explore this potential antagonism, we first asked whether Xwnt8 can rescue the phenotype caused by szl. Szl RNA (200 pg) was injected into the 2 ventral blastomeres of 4-cell stage embryos either alone or with 100 pg of the CS2+Xwnt8 plasmid DNA which contains the Xwnt8 gene driven by the strong simian CMV promoter/enhancer. Szl RNA-injected embryos were all (n=30) short and hyperdorsal at stage 26 (see the two left embryos in Fig. 1B). When szl RNA (250 pg) was injected together with Xwnt8 DNA (100 pg) ventrally, the embryos appeared normal (n=40), demonstrating that Xwnt8 can rescue the szl overexpression phenotype (see the embryo on the right in Fig. 1B). We next tested whether szl can antagonize Xwnt8 if the two genes are expressed in two adjacent blastomeres rather than in the same one. 100 pg Xwnt8 DNA was injected at the 4 cell-stage into one dorsal blastomere (n=90). Some of the embryos were subsequently injected with 50 pg szl RNA into the ventral blastomere adjacent to the Xwnt8-injected blastomere (n=32) or into the same blastomere (n=30). Embryos injected dorsally with Xwnt8 DNA only were all ventralized (n=28) while szl RNA injections rescued the ventralized phenotype, whether szl was expressed in the same blastomere as Xwnt8 or in the adjacent one (not shown).

As another test of Xwnt8/szl antagonism, we explored the effects of szl overexpression on XmyoD. The expression of XmyoD in the marginal zone of *Xenopus* gastrulae can be inhibited by a dominant-negative Xwnt8 construct (Hoppler et al., 1996). When szl mRNA was injected into one blastomere at the 2- and 4-cell stages and then XmyoD was detected by ISH at stage 10.5-14, szl caused the disappearance or significant reduction of XmyoD on the injected side (Fig. 5D). A role for szl in regulating XmyoD expression is clear from examining the pattern of Xmyo D expression during development. XmyoD is initially expressed throughout the marginal

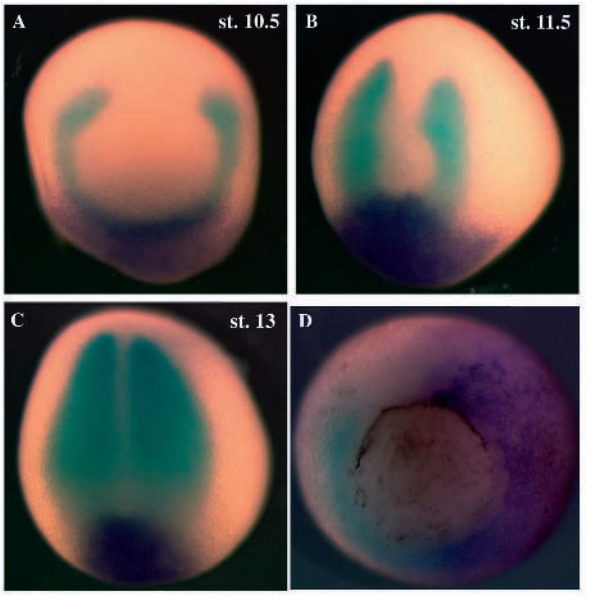

Fig. 5. Szl inhibits XmyoD expression. Double in situ hybridizations showing the relationship between szl and XmyoD expression in (A) stage 10.5 embryo, (B) stage 11.5 and (C) stage 13 normal embryos. sizzled is magenta, XmyoD is light blue. Note how XmyoD becomes gradually extinguished in the ventral sector occupied by sizzled. (D) szl-injected embryo, showing inhibition of XmyoD expression on the injected right side. XmyoD is light blue, the lineage tracer coinjected with szl is magenta.

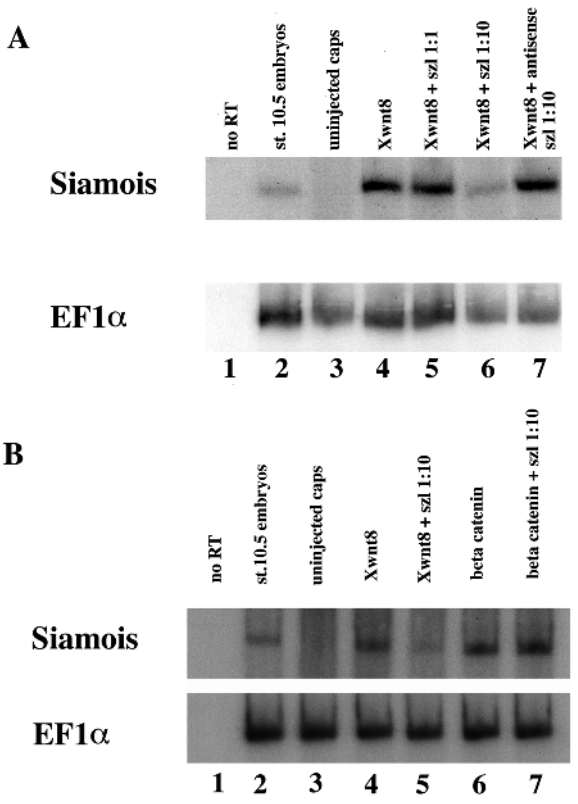

Fig. 6. (A) Szl blocks siamois induction by Xwnt8 in animal cap explants in a dose-dependent manner. RT-PCR analysis of siamois expression in stage 10.5 animal pole explants (caps) and embryos. Lane 1: no reverse transcriptase; lane 2: whole uninjected embryo; lane 3: uninjected caps; lane 4: caps injected with 250 pg Xwnt8 RNA; lane 5: caps injected with 250 pg Xwnt8 RNA + 250 pg szl RNA; lane 6: caps injected with 250 pg Xwnt8 + 2.5 ng szl RNA; lane 7: caps injected with 250 pg Xwnt8 and 2.5 ng antisense szl RNA. (B) Szl can block siamois induction in animal caps by Xwnt8 but not by β-catenin. Stage 10.5 animal pole explants were analyzed for siamois expression by RT-PCR. Lane 1: no reverse transcriptase; lane 2: stage 10.5 embryos; lane 3: uninjected caps; lane 4: caps injected with 250 pg Xwnt8 RNA; lane 5: caps injected with 250 pg Xwnt8 RNA + 2.5 ng szl RNA; lane 6: caps injected with 250 pg β-catenin RNA; lane 7: caps injected with 250 pg β-catenin RNA + 2.5 ng szl RNA.

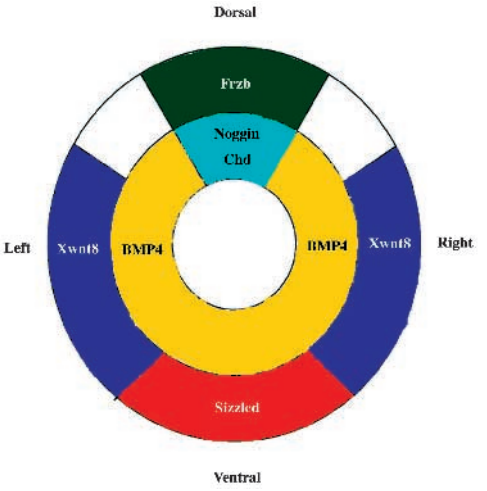

Fig. 7. Diagramme representing the expression patterns of Xwnt8, BMP4 and their secreted antagonists in the marginal zone of *Xenopus* gastrulae, seen from the vegetal pole. For clarity, the secreted components of the Xwnt8 and BMP4 pathways are shown as two non-overlapping concentric rings. In reality, BMP4 overlaps with Xwnt8, frzb overlaps with chordin and noggin while sizzled overlaps with both Xwnt8 and BMP4.

zone and is later turned off in the dorsal lip and ventrally, persisting in the lateral marginal zone (Frank and Harland, 1991), ultimately limiting the extent of muscle formation. As seen in double ISH for XmyoD and szl, the two expression domains initially overlap but they start segregating until they are completely separated around stage 13, with szl contained in the ventral wedge from which XmyoD became excluded (Fig. 5A-C). This dynamic behavior of the two genes together with the inhibition of XmyoD by szl suggest that szl could be involved in limiting the ventral expansion of the XmyoD expression domain. Although we found XmyoD to be inhibited by ectopically expressed szl, the amount of muscle actin staining on the injected side was increased (Fig. 3A). This apparent discrepancy could be due to the fact that, although an early patterning event involving XmyoD is inhibited by szl, the later muscle differentiation process is augmented in hyperdorsal szl-injected embryos by recruitment of more ventral mesodermal tissues to become muscle. In addition, there is no simple correlation between the amounts of myoD RNA and muscle tissue: XmyoD overexpression results only in very modest muscle increase (Rupp et al., 1994) while myoD gene disruption does not reduce muscle formation (Rudnicki et al., 1993).

The homeobox gene *siamois* (Lemaire et al., 1995) is induced by Xwnt8 in animal cap explants (Brannon and Kimelman, 1996; Carnac et al., 1996; Fagotto et al., 1997) thus offering a more quantitative assay for wnt signaling, in a simple explant system. We coinjected Xwnt8 RNA with increasing amounts of szl RNA, cut animal cap explants at stage 8 and harvested them at stage 10.5 after culturing them in isolation, in a neutral medium. Fig. 6A shows by RT-PCR analysis that, szl was capable of inhibiting siamois expression seen in response to Xwnt8 at high but not low levels of injected szl RNA (compare lanes 6 to 5 and 7). If szl inhibits Xwnt8 by direct interaction, a component of the wnt pathway downstream of Xwnt8 such as β-catenin should not be inhibited by szl. We tested this prediction in our animal cap assay, by coinjecting equal amounts of Xwnt8 and β-catenin RNA, respectively with szl RNA and assaying siamois expression. As shown in Fig. 6B, a dose of szl RNA that was capable of inhibiting siamois expression induced by Xwnt8 did not appreciably decrease the amount of siamois transcript induced by β-catenin in animal cap explants.

DISCUSSION

We have used a small-pool cDNA expression cloning strategy (Lustig et al., 1996) to identify genes capable of producing visible developmental defects in *Xenopus* embryos. Although axis duplication is one obvious and dramatic phenotype, other subtler perturbations can be reproducibly obtained. Using this screen we have cloned *sizzled*, a novel gene encoding a

frizzled-like protein, based on its ability to generate embryos with enlarged dorsoanterior structures (head and cement gland). However, szl lacks any apparent membrane-spanning sequence, while members of the *frizzled* family are seven membrane-spanning receptors. Szl contains an obvious N-terminal signal sequence. Combined with the subcellular localization of tagged szl protein in the embryo and its presence in the media of cultured cells expressing it, this strongly argues that szl is a secreted protein.

Szl can antagonize Xwnt8

Frizzled proteins are strong candidates for receptors of the wnt family of secreted glycoproteins. The extracellular cysteine-rich domain (CRD) of Dfz2 alone can bind wingless (wg) but does not transduce the wg signal (Bhanot et al., 1996) so szl, which shows homology to fz CRD was expected to inhibit wnt signaling by titrating the amount of wnt protein that can bind to the receptor(s). Indeed, by 3 different assays we demonstrated an antagonism between szl and Xwnt8: (1) Xwnt8 DNA can rescue embryos dorsalized by szl and conversely – szl rescues embryos ventralized by Xwnt8 DNA injections if the two genes are either expressed in the same blastomere or in adjacent ones; (2) szl inhibits the expression of XmyoD which is known to require Xwnt8 (Hoppler et al., 1996) and (3) szl can block the induction of siamois by Xwnt8 but not by β-catenin in animal cap explants. The above suggest szl can inhibit Xwnt8, perhaps by direct binding to it, in a similar manner to Xwnt8 binding to *Xenopus* frzb (Leyns et al., 1997; Wang et al., 1997). Xwnt8 is not yet available in soluble form to test binding to szl. We have been unable to detect szl binding to *Drosophila* wg.

Szl is a potent dorsalizer

Szl was cloned based on its dorsalizing activity. Both RNA and DNA injections of szl, as well as transgenic expression in *Xenopus* embryos, led to the same dorsalized phenotype, suggesting that post-MBT expression of szl is sufficient for dorsalization. Wnts and in particular Xwnt8 ventralize embryos if expressed after MBT (Christian et al., 1991). With Xwnt8 being expressed zygotically in the ventrolateral marginal zone (VLMZ), this implies Xwnt8 might normally be involved in inhibiting dorsal signals. Our findings that szl can inhibit Xwnt8 and dorsalize embryos post-MBT strengthens this idea of wnts playing a role in counteracting signals from the dorsal side of the early embryo.

Two classes of secreted proteins have been shown thus far to be capable of ventralizing *Xenopus* embryos: Bone Morphogenetic Proteins (BMPs) and wnts. BMP4 can be inhibited through direct binding by noggin (Zimmerman et al., 1996) and chordin (Piccolo et al., 1996). Our studies of szl show that Xwnt8, belonging to the second class of ventralizing secreted factors can be inhibited by szl, presumably by direct binding, paralleling the antagonism seen between BMP4 and noggin/chordin. The recent description of frzb, a gene distantly related to szl but expressed in the gastrula organizer (Leyns et al., 1997; Wang et al., 1997) argues that dorsalization by szl might be mimicking a process normally controlled by frzb. It also points to a caveat of misexpression experiments where the observed phenotype is in conflict with the expression pattern of a gene. Szl is, however, the first known example of a ventrally localized gene that can doralize embryos after the MBT.

Szl and morphogenesis

Superimposed on their dorsalized phenotype, szl-injected embryos display morphogenetic defects similar to those produced by Xwnt5A (Moon et al., 1993), a dominant-negative Xwnt8 (Hoppler et al., 1996) or a dominant-negative *Xenopus* dishevelled construct (Sokol, 1996). Most prominently, embryos are short and the axis is kinked. Examination of the early Xnot expression, which marks cells fated to become notochord and thus displaying the most pronounced convergent extension movements revealed a marked decrease in the anteroposterior dimension of the Xnot expressing territory. This hinted at the inhibition of convergent extension as a possible explanation for the stunted phenotype. Beta catenin, the vertebrate homologue of *Drosophila* armadillo and downstream of wnt and dishevelled in the wnt pathway, interacts with the cytoplasmic domain of cadherins. Cadherins are involved in calcium-mediated adhesion between cells. Wnt signaling, acting through β catenin can modulate cell-cell adhesion (Hinck et al., 1994; Moon et al., 1993b; Peifer et al., 1993) and we speculate that inhibiting wnts could result in changes in cellular adhesion which, in turn, could perturb normal cellular intercalation and convergent extension. Whereas we do not know if szl actually operates to dampen convergent extension movements in *Xenopus* embryos, it should be noted that expression of szl occurs in a region where these movements are the least pronounced in the whole marginal zone. It will be of interest to explore the effect of szl and that of other inhibitors of the wnt pathway on cellular behavior in dorsal marginal zone explants undergoing convergent extension.

What is szl doing on the ventral side?

In apparent contrast to the dorsalizing activity of szl, the gene is mainly expressed in the ventral blastopore lip where it occupies a sector that becomes narrower as the blastopore closes and involutes. Additionally, szl responds to lithium and UV treatments in a manner consistent with its ventral expression. A similar situation is encountered in the case of the Anti-Dorsalizing Morphogenetic Protein (ADMP, a TGF-β family member; Moos et al., 1995), a molecule with ventralizing activity expressed in the organizer. These two examples of genes with expression patterns contrasting with their ectopic activity point to the existence of both positive and negative regulators of dorsal and ventral development, respectively. To our knowledge, szl is more restricted ventrally than any other transcript presently known, revealing a more detailed molecular pattern in the VLMZ. It will be of interest to elucidate what upstream signals control the localized expression of szl, perhaps by analyzing the promoter region of the gene in transgenic frog embryos. Xwnt8 has been strongly implicated in maintaining the expression of XmyoD in *Xenopus* embryos (Hoppler et al., 1996). Following the expression patterns of szl and XmyoD during and after gastrulation showed that szl and XmyoD initially overlap in the VMZ but XmyoD expression becomes gradually extinguished in the territory expressing szl and in two narrow stripes of tissue flanking this region. This dynamic expression pattern and our finding that szl can, by blocking Xwnt8, inhibit XmyoD expression, place szl as a candidate for restricting the expression of XmyoD to the lateral parts of the marginal zone which are fated to become muscle, thus refining marginal zone

pattern. A similar role is perhaps played by frzb in the dorsal marginal zone (Leyns et al., 1997; Wang et al., 1997). Taken together, these two novel secreted proteins point to a regionalization of the marginal zone based on the activity level of the wnt pathway so that wnts would be fully active laterally and their activity inhibited dorsally and ventrally (see Fig. 7). This early regionalization prefigures later differentiation events, with muscle being limited to the dorsolateral domains, notochord to the extreme dorsal domain and blood and mesenchyme to the extreme ventral sector.

Until recently, the predominant view on the VLMZ, based on early organizer extirpation and grafting experiments, was that of a gastrula region undergoing default ventral development in the absence of dorsalizing signals. That ventral development is an active process was suggested by functional studies on BMP4 and Xwnt8, both broadly expressed in the VLMZ. More recently, two ventralizing homeobox genes expressed in the VLMZ, *Xvent1* (Gawantka et al., 1995) and *Vox1* (Schmidt et al., 1996) were proposed to act downstream of *BMP4*. Little is known about how the two ventralizing pathways initiated by BMP4 and Xwnt8 interact in the VLMZ of *Xenopus* embryos. Overexpressing BMP4 can, in fact, inhibit Xwnt8 expression (Schmidt et al., 1995, and our unpublished observations by in situ hybridization). The localization and activities of szl unveil a more complex picture of ventral patterning. Szl could define a region where Xwnt8 signaling is inhibited but the BMP4 pathway would be functional whereas laterally to the szl-expressing zone both pathways would operate. BMP4 could thus act either alone or in conjunction with Xwnt8 to pattern the VLMZ. Additional levels of refinement of this pattern could be provided by the localization and diffusibility of szl protein (both unknown at this point). We speculate that the increasingly complex pattern in the VLMZ might be indicative of a ventral organizing center (VOC), located 180 degrees from the organizer. The marginal zone could in principle be viewed as consisting, in part, of a mosaic of territories (Fig. 7) characterized by different levels of activity of the wnt and BMP pathways, generated by different local ratios of BMP4, Xwnt8 and their secreted antagonists.

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