Development 133, 2757-2770 (2006) doi:10.1242/dev.02391

Maternal control of vertebrate dorsoventral axis formation and epiboly by the POU domain protein Spg/Pou2/Oct4

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Dorsoventral (DV) axis formation of the vertebrate embryo is controlled by the maternal genome and is subsequently refined zygotically. In the zygote, repression of ventralizing Bmp activity on the dorsal side through chordin and noggin is crucial for establishment of a dorsally located organizer. This interplay generates a zygotic Bmp activity gradient that defines distinct positional values along the DV axis. The maternal processes that control expression of the zygotic genes implicated in DV patterning are largely unknown. *spiel-ohne-grenzen* (*spg*/*pou2*) is a maternally and zygotically expressed zebrafish gene that encodes the POU domain transcription factor Pou2, an ortholog of mammalian Oct4/Pou5f1. We show that embryos that are genetically depleted of both maternal and zygotic *pou2* function (MZ*spg*) exhibit extreme DV patterning defects and, independently, a blastoderm-specific arrest of epiboly. Dorsal tissues expand to the ventral side at the expense of ventrolateral tissue in MZ*spg* embryos. Dorsally expressed Bmp-antagonists, such as Chd and Nog1, and Gsc are ectopically activated at ventral levels in MZ*spg*. Lack of ventral specification is apparent very early, suggesting that maternal processes are affected in MZ*spg*. Indeed, maternal *pou2* function is necessary to initiate zygotic expression of ventrally expressed genes such as *bmp2b* and *bmp4*, and for proper activation of *bmp7*, *vox, vent* and *eve1*. A constitutively active Alk8-TGFβ-receptor can ectopically induce *bmp2b* and *bmp4* and rescues the dorsalization of MZ*spg*. This indicates that *pou2* acts upstream of Alk8, a maternally provided receptor implicated in the activation of zygotic *bmp2b* and *bmp4* transcription. Consistent with this possibility, Bmp gene misexpression can rescue MZ*spg* embryos, indicating that TGFβ-mediated signal transduction itself is intact in absence of Pou2. Inhibition of Fgf signaling, another pathway with early dorsalizing activity, can also restore and even ventralize MZ*spg* embryos. The requirement for *pou2* to initiate *bmp2b* expression can therefore be bypassed by releasing the repressive function of Fgf signaling upon *bmp2b* transcription. In transplantation experiments, we find that dorsalized cells from prospective ventrolateral regions of MZ*spg* embryos are non cell-autonomously respecified to a ventral fate within wild-type host embryos. Analysis of *pou2* mRNA injected MZ*spg* embryos shows that *pou2* is required on the ventral side of cleavage stage embryos. Based on the maternal requirement for *pou2* in ventral specification, we propose that ventral specification employs an active, *pou2*-dependent maternal induction step, rather than a default ventralizing program.

KEY WORDS: Zebrafish, *Spg*, Pou2, Oct4, Dorsoventral patterning, Bmp, Epiboly, Maternal effect, Axis formation

INTRODUCTION

During early development, three distinct asymmetries are established in bilaterian embryos, which are precursors to the dorsoventral (DV) and anteroposterior (AP) body axes. Axis formation depends largely on maternal and zygotic genes and involves a series of inductive cell interactions. In the fly, the frog and the zebrafish, an animal-vegetal (AV) polarity emerges upon localization of maternal components, including transcripts, proteins, cytoskeleton or mitochondria, to distinct cytoplasmic parts of the egg (Cheng and Bjerknes, 1989; Gard et al., 1997; Howley and Ho, 2000; Rand and Yisraeli, 2001; Volodina et al., 2003). Cells are endowed with different sets of these determinants and acquire their fates according to their position. AV-asymmetry probably prefigures DV polarity, as removal of vegetal yolk mass leads to lack of dorsal structures in the zebrafish (Mizuno et al., 1999; Ober and Schulte-Merker, 1999). The *Drosophila* AP axis is determined prior to DV patterning, both of which are executed by the activity of various maternal factors (reviewed by Roth, 2003). The chordate DV axis is inverted, and ventral specification involves signaling of Bmps, homologs of invertebrate Dpp (Arendt and Nübler-Jung, 1994; Holley and Ferguson, 1997). Other axis-defining pathways have not been conserved among vertebrates and invertebrates during evolution, and vertebrate DV specification by maternal factors is less well understood.

DV patterning in vertebrates integrates: (1) dorsalizing pathways involving dorsal stabilization of maternal β-catenin (reviewed by Wodarz and Nusse, 1998) (Schier, 2001); (2) ventralizing pathways depending on maternal and zygotic TGFβ signaling (Goutel et al., 2000; Bauer et al., 2001; Mintzer et al., 2001; Sidi et al., 2003) (reviewed by Hammerschmidt and Mullins, 2002) and zygotic Wnt signaling (Ramel and Lekven, 2004); and (3) noncanonical Wnt/Ca^{2+} signaling, which downregulates canonical Wnt signaling (reviewed in Pandur et al., 2002).

The zebrafish DV axis is determined prior to zygotic transcription, at the mid-blastula transition (MBT) (Kane and Kimmel, 1993; Stachel et al., 1993; Strähle and Jesuthasan, 1993). Maternal β-catenin is a key player to initiate dorsal identity (reviewed by Hibi et al., 2002). Specialized cells at the dorsal blastodermal margin, the prospective shield, become induced by the underlying yolk syncytial layer (YSL, the functional equivalent of the amphibian Nieuwkoop center) by maternal factors previously deposited into the egg (reviewed by Sakaguchi et al., 2002). The zebrafish shield is functionally equivalent to the Spemann-Mangold organizer (Shih

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Accepted 4 April 2006

and Fraser, 1996; Saude et al., 2000). A key activity of the shield is the transcriptional repression of ventralizing genes such as Bmp genes, *vox* and *vent*. This repression is mediated by *boz* (*dharma* – Zebrafish Information Network) (Yamanaka et al., 1998; Fekany et al., 1999; Koos and Ho, 1999; Kawahara et al., 2000a; Kawahara et al., 2000b; Leung et al., 2003a). During gastrulation, the coarse DV pattern is refined and involves formation of a Bmp gradient (Wilson et al., 1997; Dosch et al., 1997). This is achieved by dorsally emanating Bmp antagonists such as chordin and Noggin, which binds Bmps and prevents them from activating their receptors (reviewed by Schier, 2001; Hammerschmidt and Mullins, 2002).

The establishment of ventral identity is also controlled by maternal factors and involves the TGFβ factor Radar, which signals through the Alk8/Laf receptor to activate zygotic *bmp2b* and *bmp4* expression (Goutel et al., 2000; Bauer et al., 2001; Mintzer et al., 2001; Payne et al., 2001; Kramer et al., 2002; Sidi et al., 2003). Zygotic *bmp7* expression is probably activated by a different, yet unidentified, maternal pathway (Sidi et al., 2003). The early embryo is predominantly ventrally specified, except for a dorsal region devoid of Bmp gene expression, where the organizer is active. Later, during gastrulation, Bmps autoregulate via the Alk8 receptor (Bauer et al., 2001; Mintzer et al., 2001). Dorsal and ventral factors are crucial to keep the maternally established DV polarity equilibrated, as evidenced by zebrafish mutants lacking activity of Bmp7 (*snh*), Bmp2b (*swr*) or Smad5 (*sbn*), which fail to develop proper ventral tissues because of dorsalization of the embryo, or by ventralized embryos mutated in *chd* (*dino*) or *sizzled* (*ogon/mercedes*) (reviewed by Hammerschmidt and Mullins, 2002).

The zebrafish genome is transcriptionally inactive during the first hours of embryogenesis, and zygotic transcription starts at MBT. Until this stage, development relies on maternal factors deposited in the oocyte (reviewed by Pelegri, 2003). *pou2* encodes the POU domain transcription factor Spg/Pou2 (Belting et al., 2001; Burgess et al., 2002; Reim and Brand, 2002). *pou2* mRNA is uniformly present during initiation of oogenesis and is subsequently confined to the animal oocyte region (Howley and Ho, 2000). After fertilization, *pou2* transcripts are ubiquitously present in the embryo and overlap spatiotemporally with zygotically transcribed *pou2* mRNA from late blastula until midgastrula stages (Takeda et al., 1994; Hauptmann and Gerster, 1995; Reim et al., 2004). *pou2* plays a zygotic role in the establishment of the midbrain-hindbrain organizer (Schier et al., 1996; Belting et al., 2001; Burgess et al., 2002; Reim and Brand, 2002). An earlier *pou2* function for endoderm initiation was detected through analysis of maternal-zygotic (MZ) *spg* mutant embryos (Reim et al., 2004; Lunde et al., 2004).

By analysis of MZ/M*spg* embryos, we describe a novel maternal function of *pou2* in establishing DV patterning and, independently, during epiboly. Our analysis indicates that maternal *pou2* acts upstream of Alk8 signaling, and is required on the prospective ventral side to initiate and maintain zygotic expression of Bmp genes, *vox*, *vent* and *eve1*. Pou2 is therefore among the earliest maternal factors required for the initiation of ventral-specific fate. Based on the maternal requirement for *pou2* in ventral specification, we propose that ventral specification employs an active, *pou2*-dependent maternal induction step, rather than a default ventralizing program.

MATERIALS AND METHODS

Creation of MZ*spg* and M*spg* mutant embryos

To generate embryos that are both maternally and zygotically mutant for *pou2*, mutant adult carriers had to be created. Two strategies have been employed: (1) *pou2* mRNA-mediated rescue of $spg^{hi349-/-}$, as described previously (Reim et al., 2004); and (2) MZ*spg* mutant embryos derived from wild-type females carrying spg^{hi349} mutant germ cells. Primordial germ cells (PGCs) of $spg^{hi349-/-}$ mutant donor embryos were transplanted into wild-type host embryos at midblastula stages. Mutant donor PGCs were labeled by injecting a heterologous *vasa-3′UTR-gfp* mRNA at the one-cell stage (Wolke et al., 2002). Transplanted embryos were screened at 1 dpf for GFP expressing PGCs. To obtain MZ*spg* mutant embryos, transplanted PGC-chimeric females were crossed to $spg^{hi349+/-}$ males. The progeny consists of MZ*spg*, M*spg*, $spg^{+/-}$ and wild-type embryos. The ratio of these genotypes varied, depending on the total number of transplanted PGCs. The most successfully transplanted female carrier produced 80% MZ*spg* embryos per egg lay. Initially, method (1) was not successful, and method (2) was employed instead to produce MZ*spg* embryos for the initial experiments. MZ*spg* embryos showed no difference with respect to their generation strategy.

Injection of mRNAs

mRNAs of *ca-alk8* (30 pg), *bmp2b* (20-30 pg), *bmp4* (20-30 pg) and XFD (400 pg) were injected at the one-cell stage. Embryos depicted in Fig. 6 were injected with 30 pg *pou2* + 100 pg *egfp* mRNA into one or few blastomeres at the 16- to 128-cell stage. At the beginning of shield formation or the beginning of gastrulation, respectively, embryos where photographed individually with the aid of UV light microscopy to visualize EGFP expression, subsequently individually processed in single tubes for *bmp4* in situ hybridization analysis.

Cell transplantations

Wild-type cells were injected with rhodamine-dextrane (M_r 40,000; Molecular Probes), MZ*spg* cells were injected with 100 pg *egfp*-mRNA. Labeled wild-type and MZ*spg* cells were co-transplanted at the shield stage into dorsolateral regions within the germring of MZ*spg*- and wild-type host embryos, respectively. Host embryos were analyzed around tb stage, transplanted cells were monitored by UV light microscopy. Fig. 5: wild-type and MZ*spg* donor cells were rhodamine labeled as described above and transplanted into wild-type embryos between 50% epipoly and shield stage. Chimeric embryos were analyzed 24 hpf for several days and monitored by UV light microscopy.

RESULTS

Loss of *pou2* function disturbs morphogenesis

To investigate an early developmental role for *pou2* we examined the morphology of MZ*spg* and M*spg* mutant embryos devoid of both maternal and zygotic, or maternal *pou2* function only, respectively, and find defects in axis patterning and epiboly (Fig. 1). MZ*spg* embryos were indistinguishable from wild-type embryos during the early cleavage period until sphere stage (Fig. 1A,A′). At dome stage, wild-type embryos undergo their first rearrangements, and during late blastula stages the blastoderm thins and spreads over the yolk cell (Fig. 1B). Doming continues into epiboly, a morphogenetic movement that drives the blastoderm, the enveloping layer (EVL) and the YSL vegetal-wards (Trinkaus, 1951) (reviewed by Kane and Adams, 2002). In MZ*spg* embryos, doming is reduced (not shown), and the blastoderm does not flatten properly (Fig. 1B′). Normal gastrulation commences with the appearance of the circumferential marginal germring and the shield on the dorsal side, reflecting involution of cells around the embryonic margin (Fig. 1C,D). In MZ*spg*, involution and shield formation starts on schedule; however, the blastoderm covers only 40%, rather than 50%, of the yolk (Fig. 1C′,D′). The width of the germring, including the shield, appears thicker when compared with the wild type (Fig. 1D′; arrowheads). During normal epiboly, the vegetal margin of the EVL and the YSL ‘lead the way’ (Fig. 1E,G), trailed by the edge of the blastoderm (Fig. 1E,G). In MZ*spg*, epiboly of the blastoderm is delayed from the start (Fig. 1B′) and ceases at the beginning of gastrulation (Fig. 1E′-H′; red arrows indicate the blastoderm margin). By contrast,

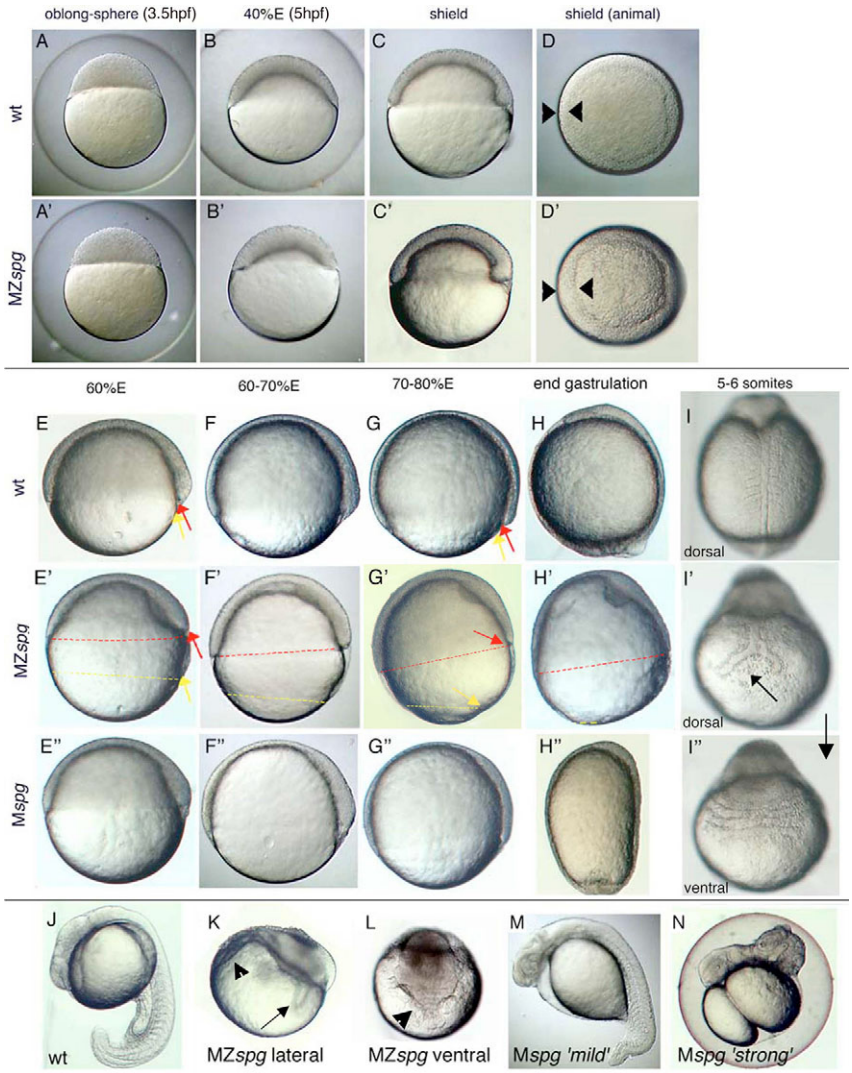

Fig. 1. Live morphology of MZ*spg* and M*spg* mutant embryos. (**A**,**A′**) Until sphere stage, mutants are indistinguishable from wild-type embryos. (**B**,**B′**) Doming and epiboly is inefficient in MZ*spg* embryos and (C,C′) the blastoderm fails to flatten. (**C-D′**) The shield forms on time, but the blastoderm has only reached 40% E in MZ*spg* embryos. Shield and germring are thicker when compared with the wild type. (**E-H′**) Epiboly of the YSL and EVL (yellow arrows) is uncoupled from epiboly of the blastoderm (red arrows) in MZ*spg* embryos, which is stalled when the blastoderm covers around 60% of the yolk. (**I**) The notochord is split in MZ*spg* embryos (**I′**) and the somites fuse on the opposite site (**I″**). (**J-N**) After 1 day of development. (K,L) MZ*spg* embryos display severe morphological abnormalities compared with wild type (J) and exhibit massive cell death (arrow in K indicates the split notochord; arrowhead in K and L indicates ventrally fused somites). (E″-H″) M*spg* embryos recover completely from their initial epiboly defect until the end of gastrulation. Expressivity of the M*spg* phenotype is variable: ‘strong’ M*spg* embryos (M) are dorsalized (H″,N) whereas ‘mild’ M*spg* embryos are hardly dorsalized.

epiboly of the underlying YSL continues normally in MZ*spg* (Fig. 1E′-H′), as does the EVL (Fig. 1E′-H′; yellow arrows indicate the vegetal margin of the YSL and EVL; see Fig. S1L-R in the supplementary material). In the wild type, ventrolateral ectoderm and mesoderm converge to the dorsal side and extend along the AP axis during gastrulation (Fig. 1E-H). This process is disrupted in MZ*spg* (Fig. 1E′-H′), where the prospective head region does not reach the animal pole on schedule but dives vegetally into the yolk (Fig. 1H′). Eventually MZ*spg* mutants develop a dorsalized morphology (Fig. 1I-I″).

Disrupted blastoderm patterning in MZ*spg* embryos

We next investigated mesoderm and ectoderm patterning in MZ*spg* embryos by in situ hybridization analysis. The notochord, the most dorsal mesodermal derivative, forms on schedule in MZ*spg* embryos, though often bifurcated or split (Fig. 1I′), and expresses the notochord marker *ntl* (white arrowhead in Fig. 2D-H′, white arrows in C,E). Split axes are also observed in epiboly mutants (Kane et al., 1996). Round cells line the notochord and the blastoderm margin (arrow in Fig. 1I′), that may have detached from the notochord, as judged by their expression of *ntl* and *foxa1* (Fig. 2D, and not shown). MZ*spg* embryos are severely shortened along their AP axis, as their notochord and *ntl* expression is shortened (Fig. 1I,I′ and Fig. 2C-H′). After gastrulation, paraxial somites form dorsally in the wild type (Fig. 1I). Somites form on schedule in MZ*spg*, but stretch to the ventral side (Fig. 2I″). Accordingly, *myod* expression is observed on the ventral side of MZ*spg* embryos (Fig. 2A,B). *pax2.1*, a marker for the pronephric duct, a ventrolateral derivative, is missing in 19 out of 32 MZ*spg* embryos at tailbud (tb) stage (Fig. 2I,J), or strongly reduced in 9 out of 32 MZ*spg* embryos (not shown). Pronephric *pax2.1* expression is missing at early somite stages in all of the 37 MZ*spg* embryos examined (Fig. 2B; and data not shown). Like *pax2.1*, *gata1* expression in ventral mesoderm is either strongly reduced in 14 out of 30 MZ*spg* embryos, or missing in 16 out of 30 MZ*spg* embryos (Fig. 2K,L; and data not shown). Endodermal differentiation also fails in MZ*spg*, as described elsewhere (Reim et al., 2004; Lunde et al., 2004).

Dorsalization is also apparent in the neuroectoderm. *krox20* is normally expressed in rhombomeres 3 and 5; rudimentary expression fuses ventrally in MZ*spg* embryos (Fig. 2E,F, arrows). *gbx2* marks the anterior hindbrain primordium (Fig. 2G, black arrow) and superimposes mesodermal *gbx2* expression (white arrow). Neuroectodermal expression fails in MZ*spg* (Reim and Brand, 2002) (Fig. 2H,H′), while mesodermal *gbx2* expression

fuses opposite of *ntl* expression in MZ*spg* (Fig. 2H,H′, white arrow; inset in H shows a ventroanimal view of an embryo expressing only *gbx2*). Dorsalization is also observed for brain-specific expression of *pax2.1*; however, expression is strongly reduced (arrowhead in Fig. 2J) as its activation requires zygotic *pou2* (Reim and Brand, 2002). The forebrain expression of *otx2* is strongly expanded in MZ*spg* (Fig. 2M,N; *chd* marks the dorsal side), which is already manifested at its onset (inset of Fig. 2M,N). *pax6* expression in the fore- and hindbrain-primordium is as well dorsalized in MZ*spg* (Fig. 2O,P). Non-neural ectodermal expression of *gata2*, *gata3*, *p63* and *dlx3* is lacking in MZ*spg* (Fig. 2Q-V, and data not shown). At late somitogenesis, MZ*spg* embryos are severely malformed, and necrotic cells appear throughout the embryo (Fig. 1J-L), which eventually dies. In summary, MZ*spg* embryos display a striking fusion of somites and expansion of anterior neuroectoderm at the expense of non-neural ectoderm reminiscent of dorsalized phenotypes, as observed (for example) in Bmp-pathway mutant embryos or in embryos with excess Fgf signaling. In contrast to these embryos, MZ*spg* embryos show ventrally fused expression of dorsal markers, which is not completely circumferential, and split notochords, which might be due to the epiboly defect.

To distinguish if MZ*spg* embryos are morphologically abnormal, mostly owing to aberrant patterning, or if they also have a morphogenetic defect as well, we compared the movements of gastrulating wild-type and MZ*spg* cells that were differentially labeled with rhodamine dextrane or EGFP, respectively. A mixture of these cells was transplanted into the lateral germring of host embryos at the early shield stage (Fig. 2W). Transplanted MZ*spg* cells survive and divide in wild-type

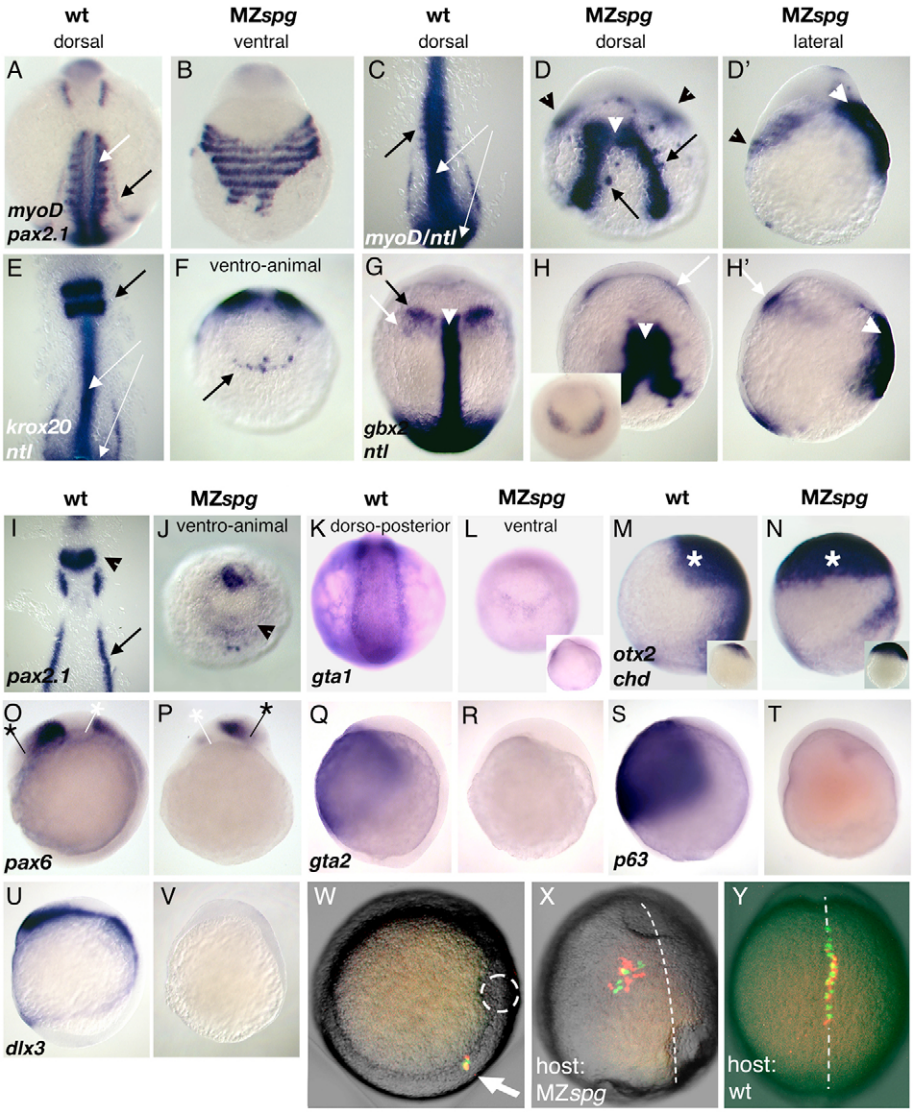

Fig. 2. Meso- and ectodermal gene expression in MZ*spg* embryos. (**A**,**C**) *myod* is expressed in wild-type somites at the eight-somite stage (white arrow). (**B**,**D**,**D′**) Somitic *myod* expression is displaced and fuses ventrally in MZ*spg* (black arrowheads in D,D′). (A,B,I,J) Pronephric *pax2.1* expression (black arrows in A,I) is missing in MZ*spg*. (C,E white arrows; G white arrowhead) *ntl* is expressed in the notochord and tb in wild-type embryos. (D,D′,H,H′, white arrowheads) *ntl* expression is broadened and variably split in MZ*spg* embryos. Single *ntl* expressing cells are found in close proximity to the notochord (black arrows in D). (**E**,**F**) *krox20* expression in rhombomere 3 and 5 (arrow) is severely reduced, and residual expression fuses at the ventral side. (**G**,**H**,**H′**) *gbx2* expression is absent at the MHB in MZ*spg*. Mesodermal *gbx2* expression (white arrows) fuses at the ventral side in MZ*spg*. Inset in H shows ventro-animal view of MZ*spg* solely expressing *gbx2*, which fuses at the ventral side. (**I**,**J**) *pax2.1* expression at the MHB (arrowhead) is strongly reduced and fuses at the ventral side. (**K**,**L**) *gata1* is normally expressed in blood progenitor cells. *gata1* is strongly reduced in MZ*spg*. Inset shows a lateral view of the same embryo. (M-V) Lateral views. (**M**,**N**) At tb stage *otx2* (asterisk) is expressed in the wild-type forebrain and is strongly expanded to the ventral side in MZ*spg*, manifested already at the initial phase of its expression (insets, 40-50% E). (**O**,**P**) *pax6* is expressed within the wild-type forebrain and hindbrain at tb stage. *pax6* domains are radialized in MZ*spg* as indicated by white and black asterisks. (**Q-V**) MZ*spg* embryos lack *gata2*, *p63* and *dlx3* expression at the end of gastrulation. (**W-Y**) Cell movement behavior. Bright-field images of transplanted living embryos were merged with fluorescent images taken at the same focal plane. Transplanted wild-type and MZ*spg* cells are visualized by red or green fluorescence, respectively. (W) Animal view of a chimeric embryo transplanted at the shield stage carrying wild-type and MZ*spg* cells in the dorsolateral germring (white arrow). A broken circle outlines the shield. (X,Y) Dorsal views of tb stage host embryos, which were transplanted as the embryo depicted in W. Broken lines indicate dorsal AP axes, anterior is towards the top. (X,Y) MZ*spg* mutant cells are indistinguishable in their movement behavior from co-transplanted wild-type cells. (X) In MZ*spg* embryos, convergence towards the dorsal midline and extension along the AP axis is affected in both transplanted MZ*spg* and wild-type cells. (Y) Convergence-extension of transplanted MZ*spg* and wild-type cells is normal in wild-type embryos.

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host embryos (Reim et al., 2004). When transplanted into MZ*spg* embryos, wild-type and mutant cells behave the same: they do not converge but stay together in clusters (Fig. 2X), suggesting that cell movement is impaired in the mutant environment. Conversely, MZ*spg* and wild-type cells within a wild-type host undergo normal convergence and extension movements (Fig. 2Y). These results suggest that abnormal morphogenesis of MZ*spg* cells is strictly non cell-autonomous, and not due to a cell-intrinsic movement defect.

pou2 is required to establish DV patterning

Our above analysis suggests that abnormalities of MZ*spg* embryos might stem from early defective Bmp-dependent DV patterning. Indeed, MZ*spg* embryos show a failure of *bmp2b* and *bmp4* initiation and a strong reduction of *bmp7* expression from the beginning (Fig. 3A-C′). *bmp7* is lost in MZ*spg* embryos soon after its initiation. The homeobox genes *vox* and *vent* are ventrolaterally expressed targets of Wnt8 at late blastula stages, and of Bmp activity during gastrulation (Fig. 3D-E) (Ramel and Lekven, 2004;

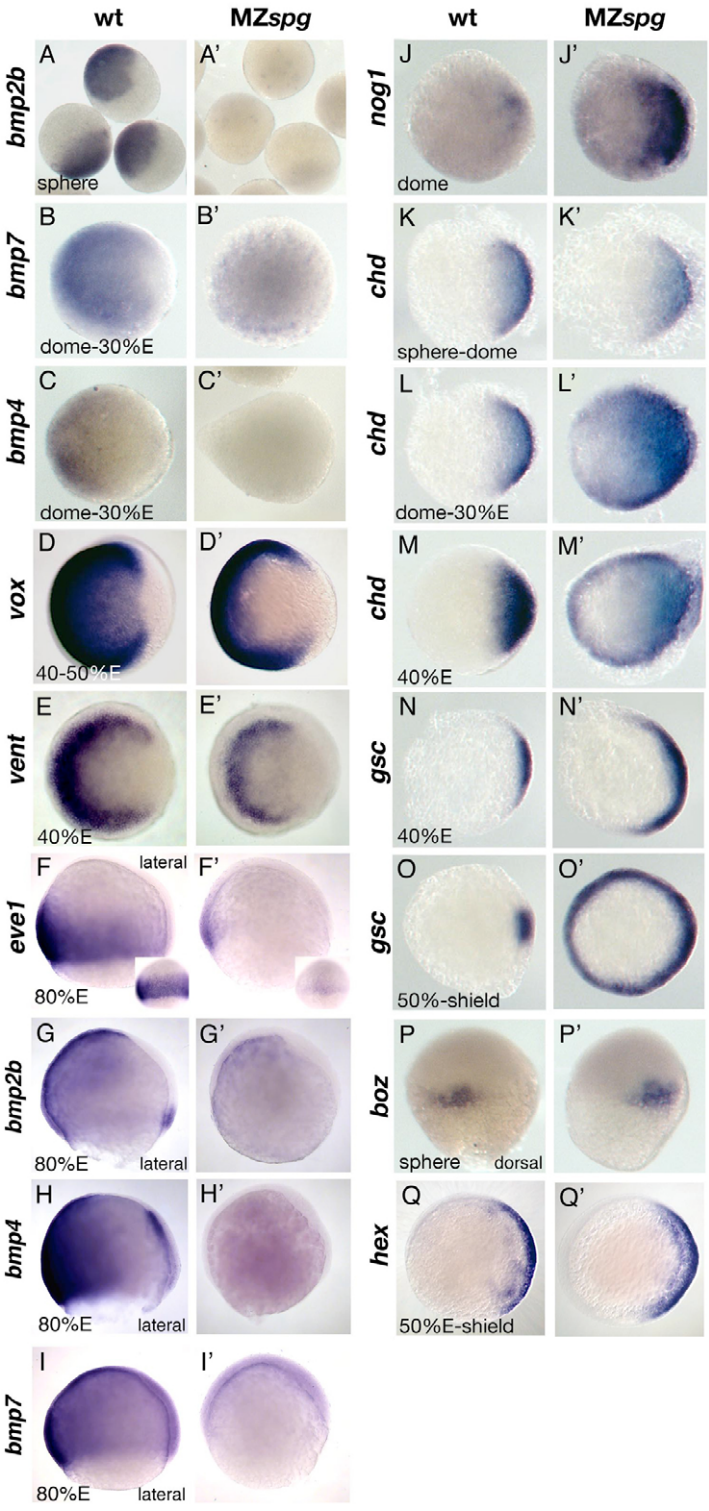

Fig. 3. Establishment of DV patterning is disturbed in MZ*spg*. Embryos are shown in animal views, if not indicated otherwise. (**A**) *bmp2b* initiates at the sphere stage in the wild type. (**A′**) *bmp2b* is absent in MZ*spg* at sphere stage. (**B**) *bmp7* initiates like *bmp2b* at sphere stage. (**B′**) *bmp7* is strongly reduced at its initiation in MZ*spg*. (**C**) *bmp4* is initiated slightly later than *bmp2b* and *bmp7* within the wild type. (**C′**) *bmp4* fails to be initiated in MZ*spg*. (**D**) *vox* is strongly expressed during blastula stages within the wild type and is absent from the dorsal-most region. (**D′**) *vox* is lost in animal regions in MZ*spg*; however, expression remains at the ventrolateral margin. (**E**) *vent* is similarly expressed as *vox* in the wild type during blastula stages. (**E′**) *vent* is strongly reduced in MZ*spg*; however, ventrolateral expression remains. (**F**,**F′**) During gastrulation, *eve1* expression is severely reduced in MZ*spg*. Insets show ventral views. (**G**,**G′**) *bmp2*, (**H**,**H′**) *bmp4* and (**I**,**I′**) *bmp7* fail to be expressed in MZ*spg*. (G′) *bmp2* expression in the YSL is unchanged in MZ*spg*. (**J**) *nog1* is faintly initiated at the dorsal side in the wild type at sphere stage and similarly expressed at dome stage. (**J′**) *nog1* is strongly expressed in MZ*spg* and expands ectopically to ventrolateral regions by dome stage. (**K**,**K′**) *chd* initiates at sphere stage, similar to MZ*spg* embryos. (**L**,**M**) *chd* is similarly expressed slightly later in the wild type. (**L′**) In MZ*spg* *chd* is nearly ubiquitously expressed shortly after initiation. (**M′**) Ectopic expression persists until beginning of gastrulation. (**N**,**O**) *gsc* is expressed at the dorsal margin in the wild-type at blastula stages, and is dorsally confined when the shield forms. (**N′**,**O′**) *gsc* is expanded ventrally within the germ ring by 30-40% E, and is ectopically expressed within the entire germ ring by beginning of shield stage. (**P**,**P′**) *boz* expression in the presumptive dorsal organizer is normal in MZ*spg* embryos. (**Q**,**Q′**) *hhex* expression in the dorso-marginal YSL is normal in MZ*spg*.

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Melby et al., 2000). Both repress transcription of dorsally expressed organizer genes such as *boz*, *chd*, *gsc* or *axial* (*foxa2* – Zebrafish Information Network) in the wild-type blastula (Melby et al., 2000; Melby et al., 1999; Kawahara et al., 2000a; Kawahara et al., 2000b; Imai et al., 2001; Leung et al., 2003a). After normal initiation at sphere or dome stage, respectively, non-marginal expression of *vox* and *vent* are lost and marginal expression is reduced in MZ*spg* from 40% E (epiboly) onwards (Fig. 3D′,E′). Importantly, MZ*spg* embryos are not generally delayed, but initiate expression of unaffected markers on schedule with the wild type at all stages examined. This is substantiated by the proper timing of the initiation of morphological landmarks such as the shield or somites (Fig. 1C,C′,I,I″; Fig. 2A,B).

Genes required for dorsal identity, including the dorsal shield, are *nog1*, *chd* and *gsc*. Nog1 antagonizes Bmp signaling by binding and sequestering Bmp ligands (Zimmerman et al., 1996). *nog1* expression initiates faintly at the dorsal side at oblong-sphere stage in the wild type (Fig. 3J) (Fürthauer et al., 1999). *nog1* expression is stronger and expanded to the ventrolateral side in MZ*spg* embryos shortly after its activation (Fig. 3J′). This might explain the expansion of *otx2* we observe in MZ*spg* (Fig. 2N), as *otx2* is a downstream target of *nog* in *Xenopus* (Gamse and Sive, 2001). Similarly, *chd* expression initiates between sphere and dome stage (Fig. 3K). Initiation of *chd* expression is normal in MZ*spg*, although its ventral limitation is less sharp than in the wild type (Fig. 3K′). Between dome stage and 30% E *chd* strongly expands ventrolaterally in MZ*spg* (Fig. 3L′,M′). *gsc* expands similarly in MZ*spg* after normal initiation at sphere-dome stage; however, ectopic expansion is restricted to the germring (Fig. 3N-O′). Expansion of *nog1*, *chd* and *gsc* lasts during blastula stages in MZ*spg*. A further dorsalizing component is Fgf8, which is expressed from blastula stages onwards. However, *fgf8* expression and activity is normal at blastula and gastrula stages in MZ*spg* embryos, as Fgf target genes such as *spry4*, *erm* and *pea3* are normally expressed (see Fig. S1 in the supplementary material).

During gastrulation, DV patterning remains disrupted in MZ*spg* embryos. After normal initiation, the ventral marker *eve1* is strongly reduced in MZ*spg* from 50% E onwards (Fig. 3F,F'; not shown). *bmp2b*, *bmp4* and *bmp7* expression remain lost within the blastoderm; however, *bmp2b* expression within the YSL is not affected in MZ*spg*, similar to other dorsalized mutants (Fig. 3G-I′). Like *gsc*, *bmp2b* and *bmp4* are also expressed in the wild-type shield during gastrulation, and are maintained in the shield and prechordal plate, respectively. *bmp2b* and *bmp4* initiation within these domains is not affected in MZ*spg* at shield stage, but expression is not maintained during gastrulation (Fig. 3G′,H′; not shown). Other dorsal mesodermal genes expressed in the notochord (*nog1* and *ntl* in the posterior axial mesoderm) or the prechordal plate/hatching gland [*hgg1* (*ctslb* – Zebrafish Information Network) *gsc* or *foxa2* in the anterior axial mesoderm] are ventrolaterally expanded in MZ*spg* (not shown). The prechordal plate did not reach the wild-type level of the animal pole at tb stage (Fig. 1G′,H′, and not shown). Despite the early and strong dorsalization, specific dorsal features remain intact in MZ*spg*. For example, the shield (Fig. 1C-D′), or β-catenin-dependent expression of *boz*, *hhex* and *sqt* was normal in MZ*spg* (Fig. 3P-Q′; not shown).

With regard to DV patterning and epiboly, M*spg* embryos look identical to MZ*spg* embryos until beginning of gastrulation (not shown), but defects in epiboly always recovers until tb stage in M*spg* embryos (Fig. 1E″-H″). Expressivity and penetrance of dorsalization is highly variable in M*spg* (Fig. 1M,N; see Fig. S1 in the supplementary material). 'Strong' M*spg* mutants show severe reduction of non-neural ectoderm (not shown). A variable proportion of M*spg* embryos even restores to the wild-type phenotype (not shown). Z*spg* embryos undergo normal epiboly and display no apparent DV patterning defect (Reim and Brand, 2002), suggesting that zygotic *pou2* is not required to regulate DV specification and epiboly.

Establishment of axial asymmetry

Deposition of maternal mRNAs to the unfertilized egg is a common mechanism to propagate asymmetry to the embryo. In the zebrafish, different mRNA localization patterns reveal asymmetry established along the AV axis during oogenesis, including *pou2* (Howley and Ho, 2000). MZ*spg* embryos lack *pou2* transcripts (Reim et al., 2004), and might therefore derive from improperly polarized oocytes. However, we found that maternally deposited *smad3a/3b/5*, *radar* (*gdf6a* – Zebrafish Information Network) *alk8*, *actRIIb* (*acvr2b* – Zebrafish Information Network), *foxh1*, *zorba* and *brul* (*cugbp1* – Zebrafish Information Network) mRNAs are unaffected in *spg*$^{-/-}$ mutant oocytes and MZ*spg* zygotes (not shown). One possibility is therefore that, rather than controlling asymmetric RNA localization itself, *pou2* might control implementation of asymmetrically deposited information in the oocyte.

MZ*spg* embryos can respond to Alk8- and Bmp-mediated signaling

In MZ*spg* embryos, Bmp gene expression is either not initiated (*bmp2b*, *bmp4*) or is only partially initiated (*bmp7*), which prompted us to test if signaling pathway(s) leading to Bmp gene initiation are affected in MZ*spg* embryos. Maternally expressed *alk8*/*laf* is the Type I TGFβ-receptor induced by the maternal ligand Radar and, presumably via its nuclear effectors Smad5 or Smad8, activates zygotic expression of *bmp2b* and *bmp4*, but not *bmp7* (Hild et al., 1999; Bauer et al., 2001; Mintzer et al., 2001; Payne et al., 2001; Kramer et al., 2002; Sidi et al., 2003). Transcripts encoding components of this maternal signaling pathway, such as *alk8*, *radar* and *smad5* are normal in MZ*spg* embryos at early cleavage and blastula stages (not shown). To determine at which level *pou2* might act during the establishment of DV patterning, we carried out epistasis experiments by injecting constitutively active (ca-) *alk8* mRNA into MZ*spg* zygotes. Wild-type embryos are ventralized upon ca-*alk8* injection and can be distinguished from non-injected embryos by their spherical shape at the end of gastrulation (Fig. 4A,B) (Bauer et al., 2001). All injected MZ*spg* embryos (n=45) are similarly ventralized to the wild type (Fig. 4B,B′). Expression analysis showed suppression of dorso-axial and neuroectodermal genes, or even ventralization, i.e. loss of somitic marker expression, depending on the amount of injected mRNA (Fig. 4D-F′).

Alk8 is later required for maintenance of Bmp2b/4/7 signal transduction (Bauer et al., 2001). ca-*alk8*-mediated reversion of dorsalization could reflect its function to maintain Bmp signaling during gastrulation, which would not allow a distinction from bypassing its maternal function in the initiation of Bmp gene expression. Therefore, we analyzed early activity of misexpressed ca-*alk8*. We observed an intensified *bmp2b* expression level in injected wild-type embryos at the sphere stage (Fig. 4I,J). MZ*spg* embryos initiate *bmp2b* expression upon ca-*alk8* misexpression at the oblong stage, and intensity of expression is comparable with the wild-type level (Fig. 4I,K). *bmp4* is normally not activated prior to dome stage/30% E (Fig. 4L). ca-*alk8* misexpression therefore elicits premature initiation of *bmp4* in wild-type

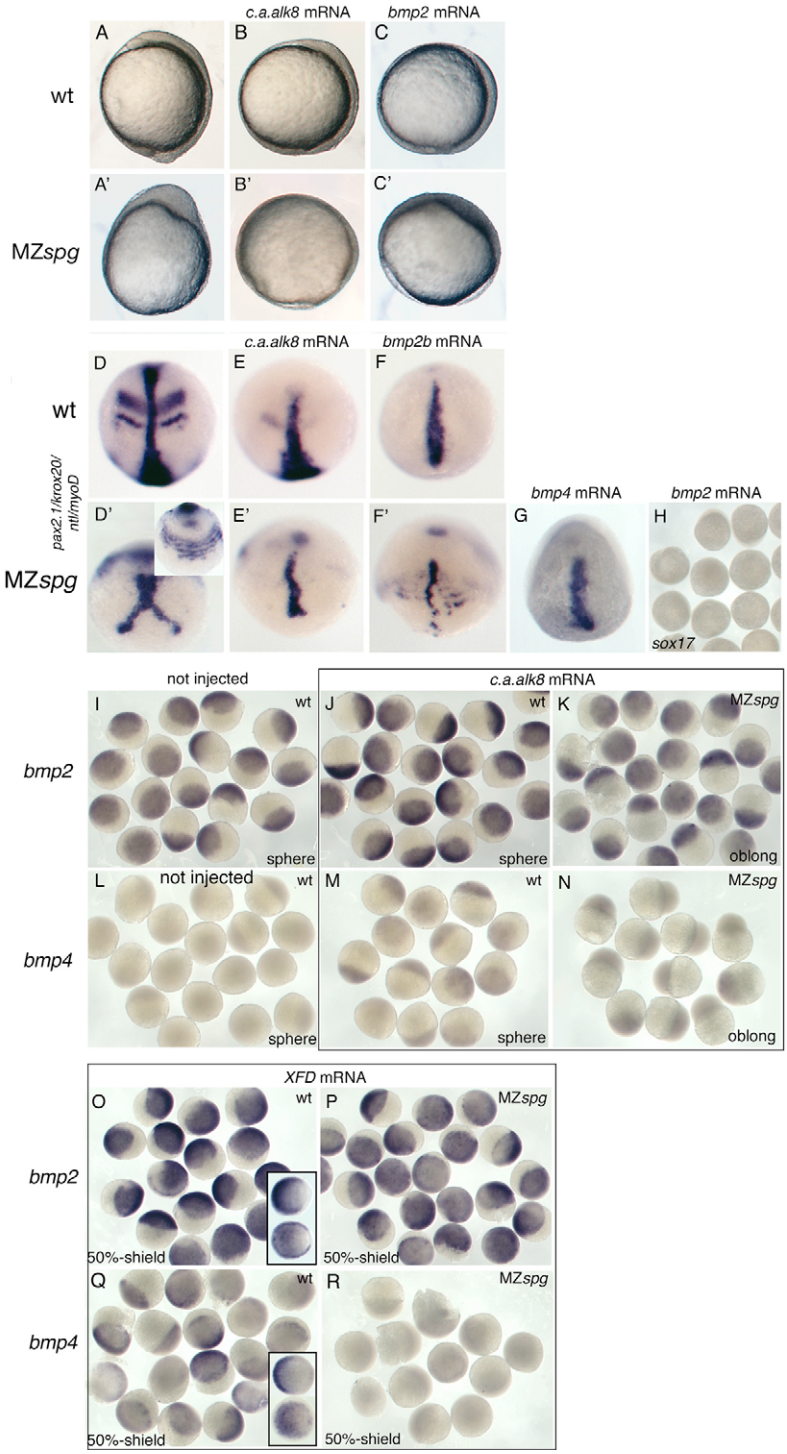

Fig. 4. Epistasis experiments by mRNA misexpression. (**A-B′**) Misexpression of *ca-alk8* leads to a spherical embryonic shape in wild-type and MZ*spg* embryos at the end of gastrulation, indicating ventralization. (**C**,**C′**) A similar ventralization is seen after *bmp2b* misexpression in wild-type and MZ*spg* embryos. (**D-G**) In situ hybridization analysis for *pax2.1, krox20, ntl and myod* expression. (D) Dorsal view of a non injected wild-type embryo. (D′) Non-injected MZ*spg* embryo, dorsal view shows *ntl* expression; inset shows ventral view with somitic *myod* expression in MZ*spg*. (E,E′) Expression of neuroectodermal genes such as *krox20* and *pax2.1*, and of axial or paraxial markers such as *ntl* and *myod* is repressed or lost in MZ*spg* embryos at the end of gastrulation. (F,F′) A similar repression of neuroectodermal and paraxial markers is observed in *bmp2b* misexpressing wild-type and MZ*spg* embryos. (G) *bmp4* misexpression leads to a similar ventralization of MZ*spg* as obtained by *bmp2b* misexpression. (**H**) Injection of *bmp2b* mRNA is not able to rescue endodermal *sox17* expression in MZ*spg* at tb stage. (**I**) Wild-type *bmp2b* expression. (J,K,M,N) Embryos injected with *c.a.alk8*-mRNA. (**J**) ca-*alk8* misexpression slightly enhances *bmp2b* expression in the wild type and restores *bmp2b* initiation in MZ*spg* to the wild-type expression level (**K**). (**L**) *bmp4* is not expressed at sphere stage in the wild type. (**M**) ca-*alk8* misexpression causes slight ectopic *bmp4* expression at oblong-sphere stage in wild-type and MZ*spg* embryos (**N**). (**O-R**) Embryos injected with XFD-mRNA. (O) XFD misexpression in the wild type leads to upregulation of *bmp2b* when compared with *bmp2b* expression of non-injected wild-type embryos (inset; upper embryo, 50% epiboly; lower embryo, shield stage). (P) *bmp2b* expression fully restores in XFD-injected MZ*spg* embryos. (Q) *bmp4* expression is not sensitive to XFD misexpression in wild-type embryos (inset shows non-injected wild-type embryos; upper embryo, 50% epiboly; lower embryo, shield stage). (R) *bmp4* is not restored in XFD-injected MZ*spg* embryos.

embryos at sphere stage and MZ*spg* embryos at oblong stage; however, in both cases the intensity of expression is comparable with the normal *bmp4* initiation level (Fig. 4M,N, compare with Fig. 3C).

swr/bmp2b mutant analysis revealed that Bmp2b is required for self-maintenance of its expression during gastrulation (Kishimoto et al., 1997). Lack of *pou2* prevents *bmp2b* initiation in MZ*spg*. To test effects of exogenous Bmp, *bmp2b* mRNA was injected into MZ*spg* embryos at the one-cell stage. MZ*spg* embryos (50 of 50) responded to Bmp gene injection as observed for ca-*alk8*-injected embryos (Fig. 4C,C′). mRNA injection of *bmp4* into MZ*spg* rescues and ventralizes MZ*spg* embryos in a similar fashion (Fig. 4G; compare with Fig. 4E-F′). Furthermore, *pou2* mRNA injection cannot rescue Bmp gene mutants, and *pou2* is expressed normally in *snh/bmp7*, *sbn/smad5*, *swr/bmp2b* mutant embryos at blastula and gastrula stages (not shown). Together, mutant in situ hybridization and epistasis analysis indicates that Alk8-mediated signal transduction is intact in *pou2*-deficient embryos. Therefore, maternal *pou2* acts upstream of Bmp gene initiation and upstream of maternal Alk8 function.

In contrast to the rescue of DV patterning in MZ*spg* embryos, ca-*alk8*/Bmp gene misexpression could not rescue endodermal *sox17* expression (Fig. 4H; 40 out of 40 injected embryos), which is never expressed in MZ*spg* (Reim et al., 2004). Bmp gene misexpression could not rescue the epiboly defect (Fig. 4C′); however, ca-*alk8* misexpression led to a slight amelioration of epiboly in 50% of injected MZ*spg* embryos (Fig. 4B′).

Fgf-interference suppresses dorsalization of MZ*spg*

pou2 is required in synergy with *fgf8* to activate hindbrain specific genes (Reim and Brand, 2002). With respect to DV patterning the relationship between *pou2* and *fgf8* appears rather antagonistic: *pou2* is required to activate Bmp genes (Fig. 3A-C′), while *fgf8* misexpression represses *bmp2b*, *bmp4* and *bmp7* (Fürthauer et al., 1997; Fürthauer et al., 2004). We therefore asked whether interference with Fgf signaling might cause a reverse effect in *pou2*-deficient embryos, by restoring Bmp gene expression. To analyze the effect of Fgf interference in MZ*spg* embryos, mRNA encoding XFD, a dominant-negative FGFR (Amaya et al., 1991), was injected at the one-cell stage into MZ*spg*. XFD injection caused different effects: *bmp2b* expression is restored and even expanded to the dorsal side at late blastula stages in MZ*spg* embryos, as in injected wild-type embryos (Fig. 4O). By contrast, XFD injection had no effect on *bmp4* expression in the wild type (Fig. 4Q), and *bmp4* was not initiated in XFD-injected MZ*spg* embryos (Fig. 4R). At the same time, ectopic and endogenous expression of *chd*, *gsc* and *nog1* was repressed in MZ*spg* by XFD, as observed in the wild type (not shown) (Amaya et al., 1993). These experiments suggest that in the absence of *pou2* function, *bmp2b* expression, but not *bmp4*, can be restored by releasing Fgf-mediated transcriptional repression of *bmp2b*. This derepression can bypass the requirement for *pou2* to initiate *bmp2b* expression. Therefore, *bmp2b* is probably not directly activated by *pou2*.

Dorsalized MZ*spg* cells can be corrected by wild-type environment

pou2-dependent control of proper Bmp gene initiation probably underlies the dorsalization of MZ*spg* embryos. Therefore, it should be possible to rescue MZ*spg* cells when transplanted into wild-type embryos. Rhodamine-dextrane labeled MZ*spg* cells where transplanted into the ventral region of wild-type host embryos between 50% E and shield stage. The fate of donor cells was analyzed after 24 hpf. In the wild-type embryo, cells at ventral positions at late blastula stages give rise to ventral derivatives such as blood or the ventral tail fin (Fig. 5A,A′). Similarly, MZ*spg* cells transplanted into wild-type embryos contribute to blood and ventral tail fin, but were not found in dorsal mesodermal tissues (Fig. 5B′-E′). This indicates an ability of strongly dorsalized MZ*spg* cells to differentiate into ventral(-most) mesodermal derivatives in wild-type embryos. This finding illustrates that MZ*spg* cells can show the maximal possible response to ventralizing signals such as Bmps from the wild-type environment.

Spatial requirement for *pou2* to establish ventral gene expression

During early DV patterning stages, *pou2* transcripts are ubiquitously distributed in the blastula embryo, raising the question of where *pou2* is required. Theoretically, dorsalization in MZ*spg* might be explained if Pou2 protein would be required for either: (1) induction of ventral-specifying genes ventrally; (2) repression of dorsal-specifying genes dorsally; or (3) for both. To analyze the spatial requirement for *pou2* function within the early embryo, we injected *pou2* mRNA into single marginal or non-marginal cells of the 16-32-cell stage of MZ*spg* embryos, together with *egfp* mRNA. Co-injected embryos were fixed at shield initiation (Fig. 6A,B) or the beginning of gastrulation (Fig. 6C-N), analyzed for EGFP expression prior to fixation and for *bmp4* expression. *bmp4* is not initiated in MZ*spg* embryos (Fig. 3C′,H′; compare with Fig. 6B′ for wild-type *bmp4* expression) and therefore served as a functional readout for ventral gene expression in these embryos. EGFP expression allowed for spatial allocation of *pou2*-injected cells, in relation to eventual *bmp4* expression domains. We find that *bmp4* expression can be restored only in MZ*spg* embryos when ventral (Fig. 6E-F′,I-J) or ventrolateral blastomeres (Fig. 6A,B,C-D′,G-H) were injected with *pou2* mRNA (19 out of 26 injected MZ*spg* embryos, the remaining embryos did not show *bmp4* expression). Restoration of *bmp4* expression appears cell autonomous in MZ*spg* at the beginning of shield formation (Fig. 6A,B) and at the beginning of gastrulation (Fig. 6C-J). By contrast, *bmp4* expression was never observed in MZ*spg* embryos injected with *pou2* mRNA into dorsal/dorsolateral or non-marginal blastomeres (Fig. 6K-N; 16 out of 16 injected MZ*spg* embryos). Therefore, *pou2* is required from early cleavage stages in the prospective ventral blastomeres to initiate *bmp4*.

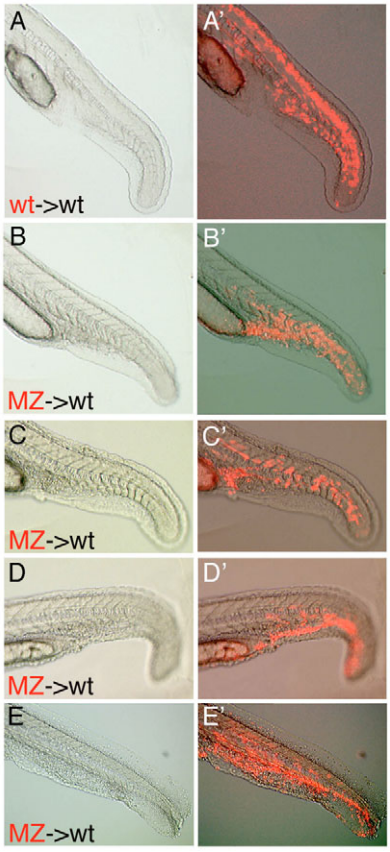

Fig. 5. The fate of dorsalized MZ*spg* cells. (A-E) Bright field images of transplanted living larvae. (A′-E′) UV images merged with bright field images. Wild-type cells (**A**,**A′**) and MZspg cells (**B-E′**) transplanted into the ventral side of wild-type host embryos can be found at pharyngula stages in locations of ventral derivatives, like blood precursors (B′,C′) and ventral tail tissue (D′-E′).

DEVELOPMENT

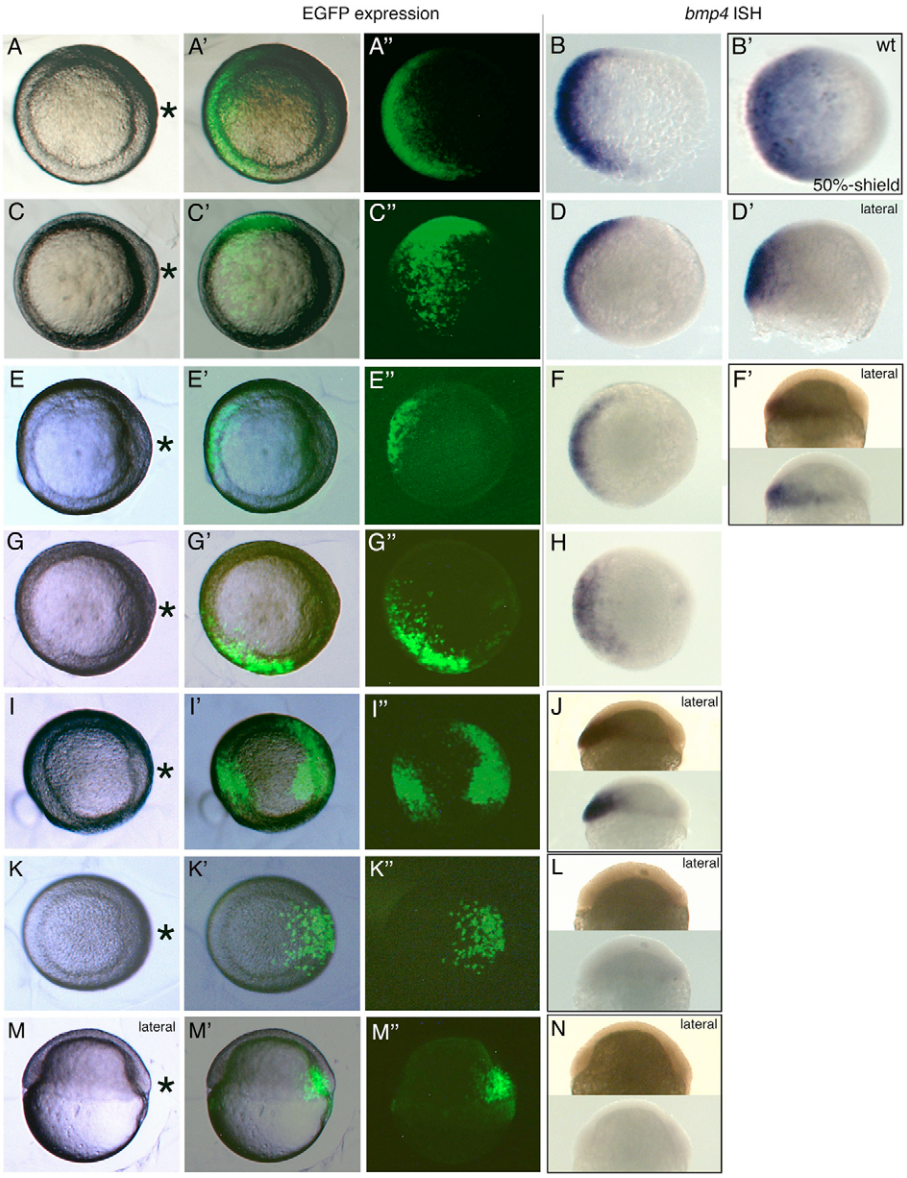

Fig. 6. Spatial requirement for *pou2*. Animal views, if not noted otherwise. (A-M) An asterisk indicates the shield. (F′,H,J,L,N) Upper half with and lower half without phase contrast to show the shield, or expression, respectively. (A′-M′) Bright-field images of injected living embryos were merged with UV images taken with the same adjustment. (A″-M″) UV images show EGFP expression, corresponding to A′-M′. (**A-J**) *pou2* mRNA injection into ventro-marginal blastomeres of MZ*spg* embryos at early cleavage stages can restore *bmp4* expression. (B′) *bmp4* expression at beginning of shield stage in the wild-type embryo. (**K-N**) *pou2* mRNA injection into dorsal or animal blastomeres of MZ*spg* embryos at early cleavage stages cannot restore *bmp4* expression.

DISCUSSION

pou2 is required for the establishment of ventral positional information

Although polarization is morphologically not apparent in the freshly laid zebrafish egg, maternal factors followed by differential zygotic gene expression impose DV asymmetry onto the embryo. Our analysis of MZ*spg* and M*spg* mutant embryos shows that the equilibrium of DV patterning is severely disrupted from the beginning in the absence of maternal *pou2*. As a consequence, cells in ventrolateral regions assume inappropriate dorsal fates soon after MBT (Fig. 3). Loss of *pou2* also interferes with maintenance of Bmp gene expression, as expression of these gene is also lacking at later stages (Fig. 3). This might be due to the failure of Bmp gene autoregulation. Together, this accounts for the later severity of the phenotype, i.e. ventral expansion of paraxial mesoderm and anterior neuroectoderm at the expense of non-neural ectoderm, and loss of ventral specification that is normally required for differentiation of blood and pronephros. The severity of the MZ*spg* phenotype is comparable with the strongest class of dorsalized embryos (Mullins et al., 1996). A proportion of MZ*spg* embryos has residual *gata1* and *pax2.1* expression within the prospective blood and pronephros, respectively. Residual expression of these genes was also observed in embryos devoid of Bmp signaling (Pyati et al., 2005). Unlike other dorsalized mutants, where somites and dorsal neuroectodermal markers extend around the circumference of the embryos, somites in MZ*spg* embryos are displaced from the notochord, which is split. In this aspect, MZ*spg* embryos resemble epiboly mutants (Kane et al., 1996), and altered epiboly may therefore explain the later split notochord and somite defect in MZ*spg* embryos.

In DV patterning *pou2* might: (1) act as a repressor of dorsal gene expression; (2) activate ventral-specific genes, such as Bmp genes, required to restrict dorsalizing factors at blastula stages; or (iii) act both as repressor of dorsal and as an activator of ventral genes. At the time when dorsal markers start to expand to the ventral side (*nog1* at sphere-dome stage, followed by *chd* and *gsc* at dome stage-30% E), the transcriptional initiation of ventralizing genes such as *bmp2b/7/4* fails in MZ*spg* embryos. Concomitant initiation of *eve1*, *vent* and *vox* expression is unaffected in MZ*spg* embryos. From late blastula stages, however, maintenance expression of *vox* and *vent*,

as well as of *eve1*, fails in MZ*spg* mutants, indicating that *pou2* is required for activation and maintenance of ventral-specific genes. Normal expression of *eve1*, *vox* and *vent* is not sufficient to prevent *nog1* expansion at blastula stages. *nog1* overexpression is sufficient to cause ectopic germring expression of *gsc* and *eve1* reduction (Fürthauer et al., 1999). In accordance with this, uncontrolled *nog1* expression in MZ*spg* might cause ectopic *chd* and *gsc* expression. *nog1* is unlikely to cause repression of *bmps* from their onset, as *bmp2b/7* expression is already affected before *nog1* becomes expanded in MZ*spg* embryos. *nog1* gain-of-function experiments would clarify whether *nog1* would be sufficient to repress Bmp gene initiation.

In contrast to Bmp genes, induction of *vox and vent* is independent of zygotic gene expression but becomes partially *wnt8* dependent during late blastula and Bmp gene-dependent during gastrula stages, respectively (Kawahara et al., 2000a; Kawahara et al., 2000b; Ramel and Lekven, 2004). The partial loss of *vox/vent* expression might cause ectopic expansion of *chd* and *gsc* in MZ*spg* embryos at late blastula stages. This is similar to loss of both *vox* and *vent* function, which causes ectopic expansion of *chd* and *gsc* (Imai et al., 2001), and probably explains why embryos mutated in Bmp ligands, such as *swr/bmp2b* or *snh/bmp7* do not ectopically express *chd* or *gsc* until gastrulation. Taken together, *pou2* functions in parallel pathway(s) to activate ventral-specific genes, and is required to maintain *vox*, *vent* and *eve1*. *pou2* might repress *nog1* independently of any relay by bmp genes, as *nog1* expression is normal in Bmp gene mutant embryos.

pou2 acts upstream to Alk8/Bmp-mediated signaling

Type I TGFβ receptors, including Alk8, mediate TGFβ signaling (reviewed by Hammerschmidt and Mullins, 2002). Alk8 transduces a maternal signal via the nuclear effector Smad5/8 that is required for initiation of *bmp2b* and *bmp4*, but is dispensable for *bmp7* activation. The maternal signal for Alk8 might be Radar, a GDF-subgroup member of Bmps (Rissi et al., 1995; Sidi et al., 2003). *alk8/laf*, *radar* and *smad5* are maternally and ubiquitously provided throughout early wild-type development (Mintzer et al., 2001; Goutel et al., 2000; Sidi et al., 2003; Dick et al., 1999; Hild et al., 1999). Smad5 is required to relay a maternal signal also to initiate *bmp7* expression at blastula stages, and amorphic *smad5* mutants exhibit strong maternal-zygotic effects, where embryos are earlier and more strongly dorsalized when compared with all thus far described DV patterning mutants, including compound *bmp2b/7*-mutant embryos (Hild et al., 1999; Schmid et al., 2000; Kramer et al., 2002). Later, during gastrulation, Smad5 also mediates auto-/crossregulation of *bmp2b/4/7* (Kishimoto et al., 1997; Schmid et al., 2000; Hild et al., 1999; Kramer et al., 2002). In case of *bmp7* initiation, Alk3 and Alk6 are putative maternal TGFβ receptors; however, the ligand(s) of this signaling system are so far unknown (reviewed by Wilm and Solnica-Krezel, 2003).

Similar to the loss of *smad5* and *radar* function, lack of ventral induction and dorsalization is very early manifested in MZ*spg* embryos. Furthermore, *radar*-morphant embryos display ectopic expression of *chd* after normal initiation, which is probably due to reduced Bmp gene expression in these embryos (Sidi et al., 2003). As *chd* and *gsc* expression is normal in *bmp2b* mutants, Radar probably acts either via its zygotic target *bmp4* or by other factor(s) to repress dorsal genes. Although maternal transcripts of *alk8*, *smad5* and *radar* are present at normal levels in MZ*spg* embryos, they are not sufficient to mediate proper Bmp gene activation. However, we did not analyze their translational or post-translational expression, which might be affected in MZ*spg*. Injection of an activated Alk8 receptor or *bmp2b/4* is able to rescue and ventralize MZ*spg* embryos. Therefore, TGFβ signaling can function in absence of Pou2, arguing that *pou2* does not act downstream of Alk8 signaling to activate *bmp2b/4*. Instead, Pou2 probably acts upstream of the Alk8, Smad5 and, eventually, Bmp signaling, which would be in accordance with the strong dorsalization in MZ*spg* embryos.

Ventral requirement for *pou2* to initiate Bmp gene expression

pou2 transcripts are ubiquitously present in the early embryo; however, their uniform distribution does not reveal a distinct spatial requirement for *pou2* in DV patterning. We analyzed the spatial requirement of *pou2* by *pou2* mRNA injection into MZ*spg* embryos at early cleavage stages (Fig. 6). In the injection assay, we found that *pou2* is needed in ventral or ventrolateral blastomeres, respectively, from early cleavage stages onwards to restore *bmp4* expression.

Maternal *pou2* antagonizes Fgf-mediated Bmp gene repression

Fgfs play an important role on the dorsal side, as dorsal XFD injections elicit more severe dorsalization than do ventral injections (Isaacs et al., 1994). In addition to organizer-derived secreted factors, Fgf signaling also modulates Bmp activity by transcriptional suppression (Fürthauer et al., 1997; Fürthauer et al., 2004). Antagonism between Egf/Fgf and Bmp signaling is thought to converge on Smad1, which is phosphorylated by MAPK, further preventing nuclear accumulation of Smad1 (Kretzschmar et al., 1997; Koshida et al., 2002; Pera et al., 2003). In turn, repression of Bmps at the protein level causes breakdown of Bmp gene-autoregulation in *Xenopus* and mouse (Ghosh-Choudhury et al., 2001; von Bubnoff and Cho, 2001). However, we did not observe overactivated or ectopic expression of Fgf genes or their downstream targets *spry2/4* and *pea3* in MZ*spg* embryos at any stages, suggesting that the failure of Bmp gene activation does not involve ectopic Fgf signaling. In spite of normal dorsal Wnt signaling and elevated expression of organizer molecules such as Nog1 and Chd, blocking Fgf signaling was sufficient to repress dorsal development in MZ*spg* embryos, which is reminiscent of interference with dorsal Wnt signaling in *Xenopus* (Gerhart et al., 1989). In particular, expression of *bmp2b*, but not *bmp4*, could be rescued in MZ*spg* embryos. At the same time, *chd*, *nog1* and *gsc* could be repressed. This suggests that dorsalizing pathways crossregulate each other, and removal of one dorsalizing component causes breakdown of the maintenance of other dorsalizing pathways. Moreover, in the absence of Fgf signaling, *pou2* is dispensable for *bmp2b* expression at late blastula stages. By derepression, i.e. repression of an Fgf-dependent repressor, *pou2* might therefore indirectly activate *bmp2b* expression at late blastula stages, which might already apply for *bmp2b* initiation. Fig. 7 summarizes the function of *pou2* during establishment of DV patterning.

Dorsal organizer function is normal in MZ*spg*

Cognate dorsalized Bmp gene mutants show normal gene expression within the dorsal organizer, suggesting that early maternal events in DV patterning and organizer establishment are normal in these mutants. By contrast, dorsal expression of *gsc*, *nog1*, *chd*, including the organizer, is aberrant in the absence of *pou2*. For example, *gsc* expansion in MZ*spg* is similar as in LiCl-treated embryos and embryos with overactivated canonical Wnt

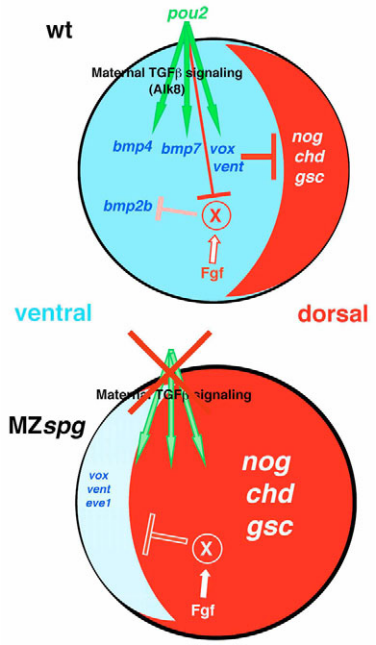

Fig. 7. Spg/Pou2 is a maternally provided regulator of DV patterning. Schematic top view of blastula embryos. Arrows indicate a regulatory relationship that need not be direct. *pou2* is required upstream of maternal TGFβ/Bmp signaling to activate ventral-specifying genes such as *bmp2b*, *bmp4* and, partially, *bmp7*. *pou2* is also required to maintain *vox*, *vent* and *eve1* from late blastula stages onwards. In addition, *pou2*-dependent *bmp2b* expression involves repression of an Fgf-dependent repressor, either at the onset of *bmp2b* expression or at later blastula stages. In MZ*spg* embryos, ventral-specifying genes completely or partially fail to be initiated, causing the embryo to be dorsalized. Specifically, ventral Bmp activity fails to establish, causing ectopic expression of dorsal organizer-derived genes *nog1*, *chd* and *gsc*.

signaling, which provoke ectopic axes (Stachel et al., 1993). However, dorsalization in MZ*spg* does not include ectopic organizer activity. *boz* is among the earliest genes initated dorsally around MBT by maternal Wnt/nuclear β-catenin signaling and is normally required to initiate organizer-specific gene expression (Koos and Ho, 1999; Yamanaka et al., 1998; Fekany et al., 1999; Ryu et al., 2001; Leung et al., 2003a; Leung et al., 2003b). *boz* represses *bmp2b* and mutually represses *vox/vent*, thereby defining the extent of the initial dorsal organizer (Melby et al., 2000; Kawahara et al., 2000a; Kawahara et al., 2000b; Leung et al., 2003a). However, *boz* and another β-catenin-dependent gene, *hhex* (Ho et al., 1999), are not expanded, but are normally expressed in MZ*spg* (Fig. 3). The reduction of *vox* and *vent* in MZ*spg* embryos seems therefore not responsible for lateral expansion of *boz*, as previously indicated (Imai et al., 2001). Rather, *ved* is upregulated upon reduction of *vox/vent*, and able to repress *boz* (Gilardelli et al., 2004). Downstream of β-catenin, Nodal signaling is required for the function of the dorsal organizer, and the Nodal-related factor Sqt induces *chd* and *nog* (reviewed by Schier, 2001). However, expression of *sqt* and *cyc*, as well as of Nodal signaling is normal in MZ*spg* embryos (Reim et al., 2004). Therefore, lack of *pou2* does not cause ectopic function of the dorsal organizer. The broadened shield and various aberrant forms of axial tissue in MZ*spg* embryos, including split and incomplete duplications, might be a consequence of reduced convergence combined with affected epiboly. Altogether, ventral expansion of *nog*, *chd* and *gsc* at late blastula stages are not sufficient to induce an ectopic organizer activity in MZ*spg* embryos. Beside the organizer, *gsc* and *chd* are also expressed in non-axial mesendoderm during blastula stages, which might be predominantly affected in MZ*spg* embryos. In addition, ectopic organizer activity might require deregulation of further organizer-specific genes like *boz*, *sqt* or *cyc*.

Aberrant migration of MZ*spg* cells can be explained by disrupted DV patterning

Positional information controls both cell fates and movements. The Bmp gradient controls morphogenetic gastrulation movements of convergence-extension (CE) (reviewed by Myers et al., 2002). Highest Bmp activity at the ventral side specifies ventral regions and inhibits CE movements. Lowest Bmp activity at the dorsal side enforces extension, but reduces convergence movements, and intermediate Bmp activity at lateral regions of the embryo favor both convergence and extension. Therefore, Bmp activity tightly links DV patterning to CE movements. Wild-type and MZ*spg* cells migrate in the same manner, which strictly depends on the genotype of the host embryo, but not of donor cells (Fig. 3). The cell non-autonomous convergence defect of MZ*spg* cells is probably attributable to loss of Bmp gene function, which also leads to pronounced extension movements, most obvious at the dorsal axis of wild-type and dorsalized embryos (Fig. 3) (Myers et al., 2002). However, normal extension along the AP axis is probably obscured by stalled blastodermal epiboly in MZ*spg* embryos.

pou2 is cell-autonomously dispensable at later stages of ventral differentiation

At the beginning of gastrulation, the position of a cell correlates to its prospective fate (Kimmel et al., 1990; Ho and Kimmel, 1993). We analyzed the fate of dorsalized MZ*spg* cells from prospective ventral positions within wild-type embryos (Fig. 5). The MZ*spg*/wild-type chimera analysis shows a dispensability of cell autonomous *pou2* for late DV patterning. Therefore, a wild-type environment is able to bypass non-autonomously, by providing secreted Bmps, the early requirement of *pou2* for Bmp gene activation. This result is reminiscent to *smad5/sbn* function: *sbn* mutant cells transplanted into wild-type embryos can give rise to ventral tissues, indicating that in a wild-type environment these cells can differentiate properly (Hild et al., 1999). Alternatively, *pou2* expression in the wild-type environment could exert a cell non-autonomous function on transplanted MZ*spg* cells, as *pou2* is expressed in the tail region of wild-type embryos during somitogenesis (Hauptmann and Gerster, 1995). Similar to Smad5 function, ultimate specification of ventral fates is independent of cell autonomous *pou2*, in contrast to initiation of *bmp2b/4* at blastula stages, which is *pou2* and *smad5*-dependent.

Epiboly is differentially affected in MZ*spg* embryos

Epiboly is a morphogenetic movement of the zebrafish embryo in which the blastoderm engulfs the spherical yolk (reviewed by Kane and Adams, 2002). Movement of the YSL occurs independently of blastoderm epiboly, and the EVL is linked to the YSL, whereby the yolk may act as a towing motor of epiboly (Trinkaus, 1951). This is in accordance with our findings, as EVL and YSL undergo normal epiboly in MZ*spg* in spite of arrested blastoderm epiboly. It is therefore unlikely that the blastoderm is merely passively 'stretched out' between these two layers. The epiboly phenotype of MZ*spg* is

reminiscent of epiboly mutants such as *hab*, *ava*, *law* and *weg*, which eventually arrest epiboly of deep blastodermal cells (Kane et al., 1996). These mutants are allelic series of *E-cadherin/hab* (Kane et al., 2005). The gap between the YSL/EVL and the blastoderm begins to widen at 80% E in *hab*, whereas in MZ*spg* a much stronger delay between the leading EVL/YSL and the lagging deep blastodermal cells is obvious from shield stage onwards. In *ava* mutants, deep cells escape into the space between the YSL and the EVL. We similarly observe scattering of mesodermal MZ*spg* cells (Fig. 1I′, Fig. 2D,H) that might therefore be linked to the epiboly phenotype. This indicates that cell adhesion is impaired in MZ*spg* and other epiboly deficient embryos; however, *E-cadherin* transcription is normal in MZ*spg* during blastula and gastrula stages (not shown). The molecular basis of the affected epiboly in MZ*spg* is currently unknown.

Maternal *pou2* and MHB development

Previous analysis of Z*spg* embryos showed that zygotic *pou2* is required to establish the MHB organizer (Schier et al., 1996; Belting et al., 2001; Burgess et al., 2002; Reim and Brand, 2002). The MHB appears slightly more impaired in MZ*spg* when compared with strong M*spg* or Z*spg* embryos (Fig. 2F,J, and not shown), although altered DV patterning and epiboly certainly contribute indirectly to the phenotype in MHB development, we cannot rule out that residual maternal Pou2 protein might also have a subtle function in MHB development.

Three independent maternal functions of *pou2*

MZ*spg* embryos display failure of initial endoderm differentiation (Reim et al., 2004; Lunde et al., 2004), of initial DV patterning and epiboly. These components of the MZ*spg* mutant phenotype probably reflect independent *pou2* functions. (1) In MZ*spg* embryos DV patterning is disturbed shortly after MBT, whereas endoderm development is only disrupted later, from the beginning of gastrulation onwards. (2) Embryos affected in Bmp signaling can differentiate into endoderm, and endoderm mutants do not fail in DV patterning. (3) DV patterning mutants, including strongly dorsalized M*spg* embryos, undergo normal epiboly, and epiboly-defective embryos have normal DV and endoderm differentiation (Kane et al., 1996; Bruce et al., 2005). (4) Mutants affected in endoderm formation, including strongly dorsalized M*spg* embryos, have no arrest in epiboly, and vice versa. (5) Rescue of dorsalization in MZ*spg* restored DV patterning without any rescue of defective endoderm or epiboly. However, we cannot rule out a slight improvement of epiboly in *ca-alk8* injected MZ*spg* embryos, which might be Bmp gene-independent. (6) MZ*spg* cells transplanted into wild-type embryos show similar morphogenetic movements as wild-type cells and adopt positions according to DV positional values of the wild-type host, suggesting that MZ*spg* cells can overcome their epiboly and DV patterning defects. By contrast, in a similar transplantation experiment, endodermal differentiation remains defective (Reim et al., 2004). In summary, we suggest that the phenotype of MZ*spg* mutant embryos reflects a mosaic of independent functions of *pou2* in different processes, including patterning as well as morphogenesis during early development.

pou2 and *Oct4* in early development

Oct4/Pouf51 is a functional ortholog of *pou2* in mammals (Burgess et al., 2002; Reim and Brand, 2002), raising the issue of whether mammalian Oct4 has a similar function as its zebrafish ortholog. Murine *Oct4* is thought to be required for totipotency of stem cells, and in *Oct4* mutant mice, extra-embryonic tissue forms at the expense of an embryo proper (Pesce and Schöler, 2000). This seems not to be the case in zebrafish, at least with respect to embryonic somatic and germ stem cells: embryonic tissue develops; primordial germ cells develop normally in MZ*spg* embryos (G.R. and M.B., unpublished). Oct4 is also required for the transition of the primitive, premigratory into migratory hypoblast (Botquin et al., 1998) and functions in early embryonic differentiation of the primitive endoderm and the germ line (Niwa et al., 2000; Pesce and Schöler, 2000), reminiscent of the function of *pou2* in controlling endodermal versus mesodermal fate choice (Lunde et al., 2004; Reim et al., 2004). A putative zygotic role in DV patterning in mammals might be obscured by the requirement for Oct4 before implantation. Recent observations in mammals suggest the presence of maternally derived asymmetries determining cleavage patterns (Zernicka-Goetz, 2004). Germline inactivation of Oct4 revealed its requirement for germ cell survival (Kehler et al., 2004), which so far precluded further analysis of Oct4 in early asymmetry of the mammalian egg. However, *Xenopus* XPou-25, which belongs to the same protein subclass as Pou2 and Oct4, can stimulate transcription of *Xvent-2B* and prevents differentiation of neuroectodermal and mesodermal tissues when misexpressed (Cao et al., 2004). Although the role of *pou2* has been mainly studied by loss-of-function experiments, the *Xenopus* data are reminiscent of the role of *pou2* in activation of ventral-specifying genes, neural patterning and differentiation into endoderm.

We thank members of the Brand laboratory for discussions; Günter Junghanns, Evelyn Lehmann and Marika Fischer for excellent fish care; Max Fürthauer, Matthias Hammerschmidt and our anonymous referees for comments on the manuscript; Erez Raz for providing *vasa-3′UTR-gfp* plasmid; and the zebrafish community for sharing many reagents. This work was supported by funds from the EU (Zf models), DFG SFB 655 and the Human Frontiers Science Program to M.B.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/14/2757/DC1

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