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The POU Domain Protein Spg (Pou2/Oct4) Is Essential for Endoderm Formation in Cooperation with the HMG Domain Protein Casanova

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Summary

The gastrulating vertebrate embryo develops three germlayers: ectoderm, mesoderm, and endoderm. Zebrafish endoderm differentiation starts with the activation of *sox17* by *casanova* (*cas*). We report that *spg* (*pou2*/*Oct4*) is essential for endoderm formation. Embryos devoid of maternal and zygotic *spg* function (MZ*spg*) lack endodermal precursors. Cell transplantations show that *spg* acts in early endodermal precursors, and *cas* mRNA-injection into MZ*spg* embryos does not restore endoderm development. *spg* and *cas* together are both necessary and sufficient to activate endoderm development, and stimulate expression of a *sox17* promoter-luciferase reporter. Endoderm and mesoderm derive from a common origin, mesendoderm. We propose that Spg and Cas commit mesendodermal precursors to an endodermal fate. The joint control of endoderm formation by *spg* and *cas* suggests that the endodermal germlayer may be a tissue unit with distinct genetic control, thus adding genetic support to the germlayer concept in metazoan development.

Introduction

In bilateral animals, blastula cells segregate into the three germlayers, ectoderm, mesoderm, and endoderm, during gastrulation (Pander, 1817; von Baer, 1828). Although germlayers provide a useful framework for animal development in view of the organs they generate, their significance as functional units of developing tissue is unclear, and genetic evidence for their importance is scarce. The germlayer concept was also much debated among classical authors for more than 100 years, without apparent resolution (see Oppenheimer, 1940, for a summary). The innermost germlayer in vertebrates, the endoderm, later gives rise to internal organs such as the liver, pancreas, spleen, and intestinal lining. Fate mapping experiments in zebrafish embryos showed that endoderm and mesoderm derive from a common, primordial group of cells, located at the margin of the blastoderm. Because of their common origin, and because mesodermal and endodermal cells are indistinguishable so far by morphology or marker gene expression during their initial development at blastula stages, they are referred to as mesendodermal precursors (Warga and Nüsslein-Volhard, 1999; Maduro et al., 2001; reviewed by Rodaway et al., 2001). Gastrulation starts morphologically with the appearance of the germring, a thickening that forms due to involuting mesendodermal cells at the blastoderm margin. As they involute, cells at the blastoderm margin undergo an epithelium-to-mesenchyme transition within the germring, and form the mesendodermal, so-called hypoblast layer of cells (Figures 1D and 1E; Kimmel et al., 1995).

Endoderm development can be subdivided into two major steps. First, a mesendodermal precursor pool is formed at blastula stages in response to signals from the extraembryonic yolk syncytial layer (YSL) (Rodaway et al., 1999; Chen and Kimelman, 2000; Stainier, 2002), requiring Nodal and other secreted signaling proteins of the TGF-β superfamily (Feldman et al., 1998; Watanabe and Whitman, 1999; Rodaway et al., 1999; Gritsman et al., 1999; Chen and Schier, 2001; David and Rosa, 2001). In response, prospective endodermal transcription factors like *cas, bon, gata5/fau, mezzo*, and the mesodermal transcription factor *ntl* are expressed at the blastoderm margin (Kikuchi et al., 2000, 2001; Dickmeis et al., 2001; Reiter et al., 2001; Aoki et al., 2002; Poulain and Lepage, 2002; reviewed by Warga and Stainier, 2002; Stainier, 2002; Tam et al., 2003). The second phase of early endoderm development starts with gastrulation, when bipotential precursors segregate into endodermal and mesodermal precursor cells. Fgf and Nodal signaling may be involved in distinguishing endoderm from mesoderm (Rodaway et al., 1999). Nodal-responsive genes are initially coexpressed by marginal mesendodermal precursor cells, and expression is subsequently restricted to either mesodermal or endodermal precursors during gastrulation (Warga and Stainier, 2002; reviewed by Rodaway et al., 2001). Interestingly, *cas* expression is confined to a subset of blastomeres within the mesendoderm already at 40% epiboly, and may delineate endodermal precursors before the onset of gastrulation. *cas* encodes the HMG-domain transcription factor Casanova (Cas), which is required to activate the endodermal differentiation markers *sox17* and *foxA2* and to repress mesodermal fate (Alexander and Stainier, 1999; Kikuchi et al., 2001; Aoki et al., 2002; Dickmeis et al., 2001).

The *pou2* gene encodes a POU domain transcription factor and is mutated in *spiel-ohne-grenzen* (*spg*) mutant zebrafish embryos (Schier et al., 1996; Belting et al., 2001; Burgess et al., 2002; Reim and Brand, 2002). Transcripts of *spg(pou2)* are found both in the oocyte

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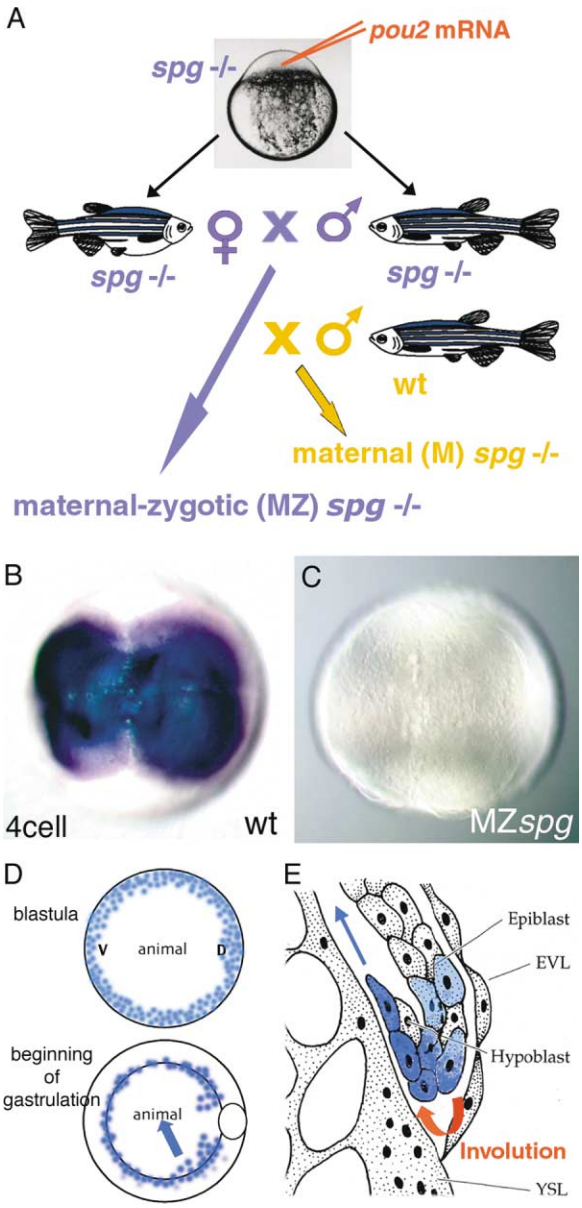

Figure 1. Eliminating Maternal and Zygotic *spg* Function and Endoderm Formation

(A) Homozygous *spg* mutants were rescued by *spg(pou2)* mRNA injection at the 1-cell stage. Viable homozygous adult females were crossed with homozygous males or wild-type males to produce MZ*spg* or M*spg* embryos, respectively.

(B) *spg(pou2)* is maternally supplied to the zygote (C) MZ*spg* embryos never initiate *spg(pou2)* expression.

(D and E) Initial steps in endoderm development. (D) Animal pole view. Mesendodermal precursors (light blue) and endodermal precursors (dark blue) are located at the margin before and after the onset of gastrulation, respectively. (E) Endodermal precursor cells (dark blue) involute and move toward the anterior, animal pole; modified from Kimmel et al. (1995).

and the early zygotic embryo (Takeda et al., 1994; Hauptmann and Gerster, 1995; Howley and Ho, 2000). Zygotic expression is required for early neural development, in particular establishment of the midbrain-hindbrain organizer, but zygotic *spg* mutant embryos also have minor disruptions of mesoderm and endoderm development (Reim and Brand, 2002), suggesting that *spg(pou2)* may have additional functions that are masked by maternally provided mRNA or protein. We therefore removed both the maternal and the zygotic function of *spg(pou2)*. In such maternal-zygotic (MZ) *spg* mutant embryos, we find that *spg(pou2)* is essential for formation of the endoderm. The earliest endoderm-specific expression of *sox17*, *foxA2*, and other endoderm-specific markers is not activated in MZ*spg* mutants, although mesendodermal precursors are present, as judged from normal expression of *cas* and other markers at blastula stages. Transplantation studies show that MZ*spg* mutant cells can develop into mesoderm, but not into endoderm. Importantly, *spg(pou2)* is absolutely required to enable Cas to activate *sox17* in endogenous and ectopic domains. Furthermore, *sox17* promoter-luciferase reporter constructs are activated upon *cas* and *pou2* mRNA coinjection. We suggest that Pou2 and Cas together control the initiation of endodermal germlayer development downstream of Nodal signaling.

Results and Discussion

Removing Maternal and Zygotic *spg* Function

spg(pou2) mRNA is present ubiquitously in the unfertilized oocyte and early zygote (Takeda et al., 1994; Hauptmann and Gerster, 1995; Figure 1B). Zygotic expression is required for early neural development, allowing Fgf8-dependent activation of gene expression during development of the midbrain-hindbrain organizer (Reim and Brand, 2002, and references therein). We generated embryos lacking *spg(pou2)* function maternally and zygotically (MZ*spg*; Figure1A). Injection of *spg(pou2)* mRNA into homozygous mutant embryos for a likely null allele (*spg*^{hi349}, Burgess et al., 2002) at the 1-cell stage rescues the development of these embryos, and allowed us to generate fertile homozygous *spg* mutant adults (Figure 1A). Intercrosses of homozygous mutant females and males generate MZ*spg* mutant embryos which lack maternal and zygotic *spg(pou2)* transcripts (Figure 1C). The phenotype of MZ*spg* mutant embryos is constant regarding expressivity and penetrance in every egg clutch examined. MZ*spg* embryos were phenotypically rescued by *spg(pou2)* mRNA injection at the 1-cell stage and were raised and kept up to 7 dpf (data not shown).

Endoderm Does Not Form in MZ*spg* Embryos

The most prominent defect we observe in MZ*spg* mutants is absence of endoderm (Figure 2). We examined the earliest mesendoderm markers, the transcription factors *cas*, *mez*, *ntl*, *bon*, and *fau(gata5)* by whole-mount in situ hybridization of MZ*spg* embryos at the onset of endoderm formation. These genes are activated in the blastoderm in direct response to Nodal signaling (Reiter et al., 1999, 2001; Alexander and Stainier, 1999; Kikuchi et al., 2000; Poulain and Lepage, 2002; reviewed by Warga and Stainier, 2002). Due to sustained Nodal signaling, mesendodermal marker expression expands circumferentially in the marginal-most tiers of germring cells (Figure 1D). In MZ*spg* mutants, expression of *bon(mix)*, *mez*, *fau(gata5)*, *cas*, and *ntl* is initiated (Figures 2A–2H, and data not shown), indicating that MZ*spg*

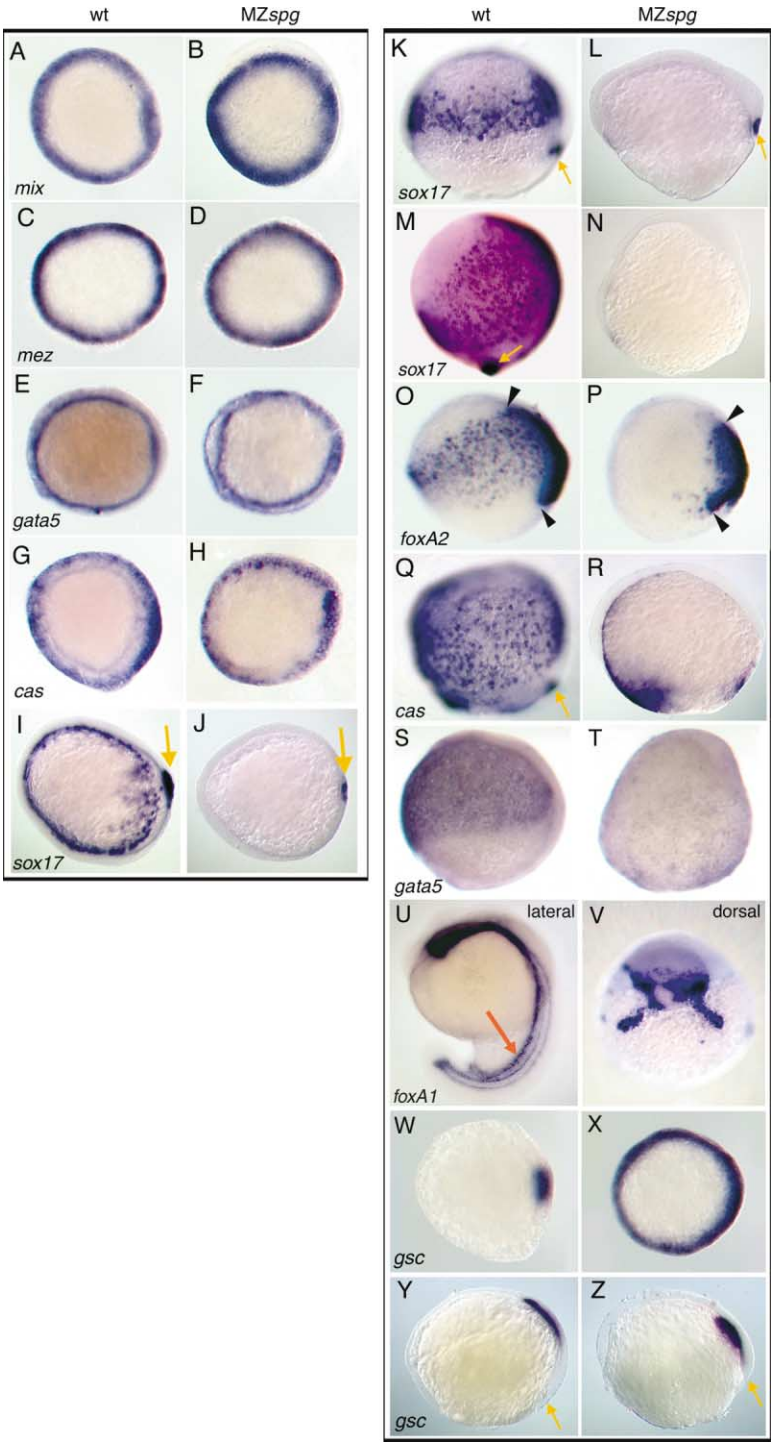

Figure 2. Endoderm Differentiation Is Blocked in MZ*spg* Mutants

(A–H) Expression of *bon(mix)*, *mez*, *fau(gata5)*, and *cas* in endodermal precursor cells at the embryonic margin is normal in late blastula stage MZ*spg* embryos. Animal pole views.

(I–T) Expression of markers for endodermal differentiation is abolished in MZ*spg* mutants. (I and J) Endodermal *sox17* expression is not initiated in MZ*spg* embryos, and is reduced in forerunner cells (arrow). (K–T) Lateral views of gastrulating embryos, genotypes, and markers as indicated. (K and L) Endodermal *sox17* expression is not detected at 60% epiboly. (M and N) *sox17* expression in the endodermal germlayer at the end of gastrulation is missing in MZ*spg* embryos. Similarly, endodermal *foxA2* (O and P), *fau(gata5)* (S and T), and *cas* (Q and R) expression are missing in MZ*spg* embryos. *foxA2* expression in the axial mesoderm (arrowheads) is present in MZ*spg* embryos. *cas* is weakly expressed in the YSL and forerunner cells (arrow) and is enhanced in MZ*spg*. (U and V) *foxA1* is expressed in the prospective gut (arrow), hypochord, and ventral floorplate at late somitogenesis; expression is retained in malformed MZ*spg* embryos probably in hypochord and/or floorplate.

(W and X) *gsc* is normally expressed in the dorsal shield, but expands to ventral levels in MZ*spg*. Animal views, shield stage.

(Y and Z) *gsc* staining in involuted mesoderm at 80% epiboly is similarly expressed in MZ*spg*, though epiboly is delayed (arrows; lateral views).

embryos form a pool of mesendodermal precursor cells in response to Nodal signaling.

In the gastrulating embryo, the earliest endodermal differentiation markers *sox17* and *foxA2* are expressed in a characteristic punctate pattern, which reflects the distribution of endodermal precursor cells (Figures 2K, 2M, and 2O; Strähle et al., 1993; Odenthal and Nüsslein-Volhard, 1998; Alexander and Stainier, 1999). In these cells, expression of *cas*, *bon*, and *fau(gata5)* is maintained (Figures 2Q and 2S; and data not shown). In MZ*spg* embryos, endodermal expression of *sox17* and *foxA2* is abolished (Figures 2L, 2N, and 2P). Moreover, none of the endodermal precursor markers like *cas*, *fau (gata5)*, *bon*, or *mez* is maintained in gastrulating MZ*spg* mutants (Figures 2R and 2T; and data not shown). Forerunner cells are a noninvoluting cell type that may be related to enveloping layer (EVL) and mesoderm, but not endoderm (Cooper and D'Amico, 1996; Warga and Stainier, 2002). In MZ*spg* mutants, forerunner cells were strongly reduced in number and were sometimes lacking

completely (arrow in Figures 2I–2M, 2Q, and 2R). Expression of *foxA2* within dorsal axial mesodermal cells was normal, although the shape of this domain was altered by the disturbed morphology of MZ*spg* mutant embryos (arrowheads in Figures 2O and 2P). MZ*spg* mutants are also abnormally dorsalized and delayed during epiboly, but not generally disrupted in involution, as seen in embryos stained for *gsc*, a marker for dorsal mesoderm (Figures 2W–2Z; G.R. and M.B., unpublished data). Because severely dorsalized mutants nevertheless form endoderm (Warga and Stainier, 2002), these phenotypes probably reflect independent functions of *spg* (*pou2*).

Lack of endodermal marker gene expression in MZ*spg* embryos could reflect a defect of the prospective mesendoderm to undergo proper endodermal differentiation. Alternatively, migration of endodermal precursor cells within the hypoblast layer could be disrupted, causing failure to differentiate. Endodermal precursor cells become first morphologically visible at the shield stage. In the wild-type, *sox17* is activated in emerging endodermal precursors coincident with their recruitment into the hypoblast (Figure 2I; Alexander and Stainier, 1999). Importantly, expression of *sox17* was not activated in involuting cells of MZ*spg* embryos at the onset of gastrulation (Figure 2J). The same was observed for *foxA2* (data not shown). Because of the abnormal morphology of MZ*spg* embryos at later stages, assaying gut formation in MZ*spg* at pharyngula stages is difficult. *foxA1* is a marker of gut differentiation at somitogenesis stages and is expressed in the wild-type gut primordium (Figure 2U, arrow) and in the overlying, mesodermally derived hypochord and floor plate (Figure 2U; Odenthal and Nüsslein-Volhard, 1998). In MZ*spg* mutants, *foxA1* expression strongly resembled expression of the notochord marker *ntl*, suggesting that the remaining *foxA1* expression we observe is in notochord (Figure 2V). MZ*spg* embryos therefore appear to lack a gut primordium. Together, these findings suggest that MZ*spg* embryos lack differentiating endodermal precursor cells from gastrulation onward. Because gene expression of *cas*, *bon*, *mez*, and *fau(gata5)* is initiated normally at the margin of MZ*spg* mutants at blastula stages, a mesendodermal precursor pool seems to form properly in MZ*spg* embryos. However, the transition from the mesendodermal precursor cell to the endodermal precursor cell appears to fail in MZ*spg* embryos.

Cell-Autonomous Requirement for Endoderm Differentiation

Next, we determined whether the requirement of *spg(pou2)* is spatio-temporally restricted. *spg(pou2)* is strongly and ubiquitously expressed at blastula stages, including endodermal precursor cells. At the onset of gastrulation, *pou2* transcripts quickly recede to the epiblast (Figures 3B and 3C; Takeda et al., 1994; Hauptmann and Gerster, 1995). *spg(pou2)* might then be needed in endodermal precursor cells themselves. Alternatively, *spg(pou2)* might control a hypothetical, epiblast-derived signal that supports endoderm development in the underlying hypoblast. We therefore asked if transplanted wild-type cells are required in the epiblast or in the hypoblast to restore endoderm development in MZ*spg* host embryos. Wild-type donor cells were transplanted from the dorso-lateral, marginal-most region of the embryo into the same position in MZ*spg* host embryos at late blastula stages (Figure 3A). This region contains 51%–80% endodermal precursor cells, which will contribute to endodermal derivatives like the gut (Warga and Nüsslein-Volhard, 1999). Host embryos were then fixed at the end of gastrulation and analyzed for *sox17* expression to assay for endodermal differentiation.

sox17 expression was exclusively observed if transplanted wild-type cells were located within the hypoblast. Because only transplanted cells express *sox17*, *spg(pou2)* acts in a strictly cell-autonomous way (Figures 3D and 3D′; 18 embryos, with $n = 150$ cells in total). *sox17*-positive cells were found in close apposition to the yolk cell of MZ*spg* embryos and displayed the morphology and spacing characteristic of endodermal cells in the wild-type embryo (compare with Figure 2M for wild-type expression pattern of *sox17*). Transplanted wild-type cells located in the mesoderm were characteristically smaller and roundish, and did not express *sox17* (Figure 3D′, arrow). Wild-type cells located in the ectoderm of MZ*spg* embryos never activated *sox17* expression in the underlying hypoblast (Figure 3E; 22 embryos, with $n > 100$ cells in total). *spg(pou2)* is therefore required in a strictly cell-autonomous manner at the transition from mesendodermal precursor cells to endodermal precursors. Moreover, MZ*spg* embryos provide a permissive environment, since wild-type cells can still express *sox17* in MZ*spg* embryos. Conversely, wild-type cells appear unable to stimulate *sox17* expression in MZspg mutant host cells. The cell-autonomous function of *pou2* is in good agreement with the cell-autonomous requirement observed for Cas in endoderm development (Dickmeis et al., 2001; David and Rosa, 2001; Alexander and Stainier, 1999).

Temporal Requirement in Endodermal Development

spg(pou2) mRNA is both maternally and zygotically expressed. Like in MZ*spg* mutants, expression of maternal *spg(pou2)* message is not detected in M*spg* embryos (Figure 4K) until the late blastula stage (Figure 4M). Zygotic *spg(pou2)* expression therefore does not depend on its own maternal gene product. Conversely, in Z*spg*^{hi349} mutant embryos, *spg(pou2)* mRNA levels decrease between 30% epiboly (Figure 4O) and mid-gastrula stage (Figure 4Q). Theoretically, either maternal or zygotic *spg(pou2)* might be most important for endoderm differentiation. We therefore compared endoderm development in wild-type or MZ*spg* embryos with that of embryos lacking zygotic (Z*spg*) or maternal (M*spg*) function alone.

Z*spg* embryos showed a reduced number and intensity of *sox17* expressing cells (Figure 4B). Similarly, M*spg* embryos showed a variably reduced or lost *sox17* expression (Figures 4C and 4D); MZ*spg* embryos completely lacked *sox17* expression (Figure 2N). Therefore, both zygotic and maternal *spg(pou2)* function contribute to *sox17* activation. Z*spg* and "mild" M*spg* embryos at the 20 somite stage express *foxA1* comparable to the

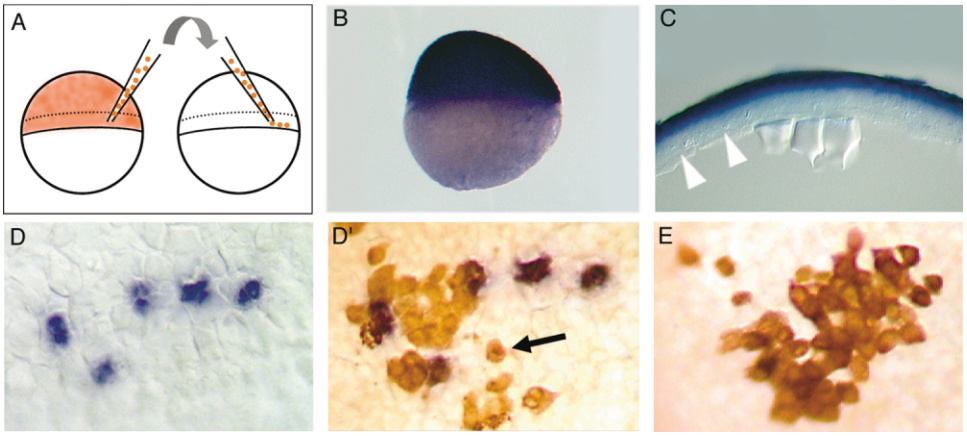

Figure 3. Autonomous Requirement for *spg* in Endoderm Differentiation

(A) Transplantation strategy. (B) Ubiquitous expression of *spg(pou2)* in a wild-type blastula. (C) Restricted expression in dorsal epiblast; arrowheads indicate the border between ectoderm and mesendoderm. Transversal section at 60%–70% epiboly (D and D′). Transplanted wild-type cells located in the mesendodermal primordium of MZ*spg* embryos can express *sox17* (blue staining). (D′) The same embryo as in (D), after detecting transplanted cells by immunochemistry (brown staining). All *sox17*-expressing cells are wild-type (overlay of the brown and blue staining results in purple staining). Cells that are solely brown have a small, mesodermal morphology. (E) Wild-type cells (brown staining) located in the ectoderm of MZ*spg* embryos do not express *sox17*.

wild-type and can form a gut (Figures 4E–4G). The gut is apparently functional since the larvae take up food, transport, and excrete (data not shown). Other endoderm mutants like *fau(gata5)*, *bon*, or Z*oep* also display partially reduced *sox17* expression similarly to Z*spg* and mild M*spg* mutants, express *foxA1* at somitogenesis stages, and form a gut (Reiter et al., 2001; Kikuchi et al., 2001). Moreover, *foxA2* expression also remained in these mutants. Apparently, reduced expression levels of a combination of endodermal markers suffices for endoderm formation. M*spg* embryos displaying a strong mutant phenotype do not form a gut primordium, as seen by loss of *foxA1* expression (Figure 4H). However, in dorsal views, faint and laterally spreading expression of *foxA1* is visible close to the surface of the yolk, which might reflect residual and dorsalized endoderm formation in these embryos (Figure 4I). Expression in nonendodermal *foxA1* domains is present, although morphologically altered in these embryos, probably indirectly due to their strongly dorsalized morphology (Figure 4H; G.R. and M.B., unpublished data).

In summary, maternal and zygotic *spg(pou2)* expression overlap in time and are both necessary for proper endoderm formation. The dual requirement might be a safe-guarding mechanism to ensure proper endoderm formation.

spg(pou2) Requirement for Ectopic Nodal Activity

To address the genetic level at which *spg(pou2)* might act, we investigated the relationship between *spg(pou2)* and Nodal signaling, which is essential for endoderm and mesoderm formation. MZ*oep* mutant embryos lacking the Nodal-cofactor EGF-CFC are unable to process Nodal signals, and hence lack all endoderm and most mesoderm (Schier et al., 1997; Gritsman et al., 1999). MZ*oep* mutants can be rescued by injecting *tar** mRNA, encoding a constitutively active variant of the Nodal receptor Taram A (Peyrieras et al., 1998). *tar** misexpression directs the fate of early blastomeres predominantly toward endoderm when misexpressed, as seen by premature and ectopic *sox17* expression already at blastula stages (Figure 5A, upper left inset; Peyrieras et al., 1998).

Nodal signaling is intact in MZ*spg* embryos, as judged from marker expression of mesendodermal precursor cells (Figures 2A–2H) and our transplantation experiments (Figure 3). Accordingly, ligand-independent, constitutive Nodal signaling by Tar* injection caused strong ectopic activation of *mez*, an immediate target of Nodal signaling (Poulain and Lepage, 2002), in MZ*spg* embryos at the sphere stage (Figure 5A, lower left inset). However, the ability to activate *sox17* following *tar** mRNA injection was completely suppressed in MZ*spg* embryos (Figure 5A). Therefore, Nodal signaling cannot bypass the requirement for *spg(pou2)* even when constitutively activated. *spg(pou2)* is expressed normally in Nodal pathway mutants like *oep*, *sqt*, or *cyc* (data not shown), and Nodal pathway components and the Nodal-dependent targets *ntl* and endodermal precursor markers are normally expressed in MZ*spg* embryos (Figure 2, and data not shown). Therefore, *spg(pou2)* acts in parallel to the Nodal signaling pathway.

Joint Requirement for Spg and Cas

cas encodes an HMG domain protein of the Sox-transcription factor superfamily and is necessary for endoderm differentiation (Kikuchi et al., 2001; Dickmeis et al., 2001). *cas* was so far the only mutation that blocks endoderm formation (Alexander and Stainier, 1999). *cas* mutants do not activate any known marker indicative for endodermal precursor development during gastrulation and, consequently, lack all endoderm. As in MZ*spg* embryos, however, gene expression in endodermal precursor cells is not affected in *cas* mutant embryos. Thus, the phenotype of *cas* and MZ*spg* mutants is very similar.

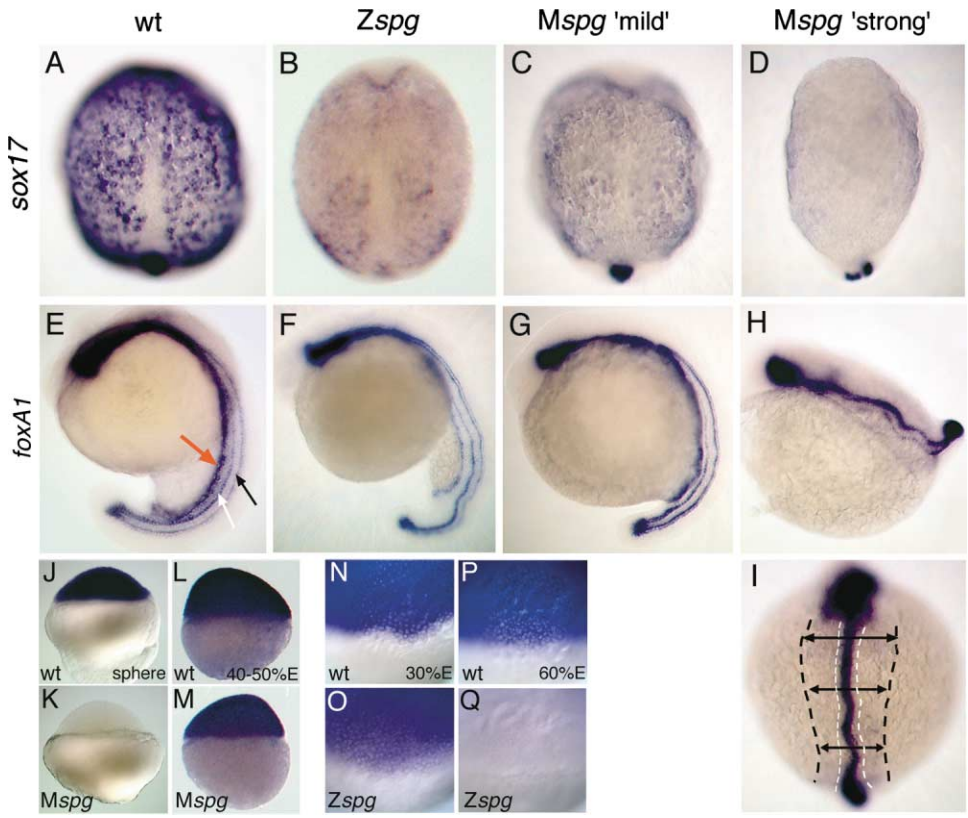

Figure 4. Endoderm Development with Maternal or Zygotic *spg* Function

(A–D) Expression of *sox17* at the tailbud stage, dorsal views. In contrast to the wild-type (A), intensity and number of *sox17* expressing cells are strongly reduced in zygotic (B) and *mild* maternal (D) *spg* embryos, and nearly abolished in *strong* maternal (D) *spg* mutant embryos.

(E–I) Expression of *foxA1* at the 20-somite stage. (E–H) Lateral views. *foxA1* is expressed in the entire prospective gut region (red arrow), the hypochord (white arrow), and floor plate (black arrow) of the wild-type, and expression is reduced in Z*spg* and M*spg* embryos. Endodermal expression cannot be detected in a lateral view. (I) In a dorsal view (anterior to the top), endodermal *foxA1* expression is faintly detected close to the yolk (lateral expansion is marked by arrows and black dotted lines; white dotted lines mark the normal extent).

(J–Q) *spg(pou2)* is ubiquitously expressed in the wild-type blastula, but absent in M*spg* embryos, which commence transcription at late blastula stage (M). Conversely, Z*spg* mutants show normal expression of *spg(pou2)* until late blastula stages (O), which is downregulated from late blastula stages onward (Q).

Unlike other genes necessary for endoderm formation, like *gata5* or *bon*, *cas* expression is sufficient to restore *sox17* expression in embryos lacking Nodal signaling (MZ*oep* embryos; Kikuchi et al., 2001; Aoki et al., 2002), suggesting that Cas is a key activator of *sox17* expression. Moreover, *tar** mRNA misexpression fails to activate *sox17* in *cas* mutants (Alexander and Stainier, 1999), like we observed in MZ*spg* mutant embryos (Figure 5A).

The striking similarity between the phenotype of *cas* and MZ*spg* mutant embryos prompted us to investigate their relationship further. ISH analysis revealed that *cas* expression is initiated normally in MZ*spg* mutants (Figures 2G and 2H), and *spg(pou2)* is expressed normally in *cas* mutants (data not shown). Moreover, *tar** mRNA misexpression could activate *cas* in MZ*spg* mutant embryos at ectopic locations, similar to the ectopic activation of *mez* (data not shown, and Figure 5A, lower left inset). This fits with the notion that *cas* is a direct downstream target of Nodal signaling (Dickmeis et al., 2001; Aoki et al., 2002; Poulain and Lepage, 2002).

These findings suggest that *spg(pou2)* might act in parallel to *cas* to elicit endoderm formation. In order to test this notion, we injected *cas* mRNA into MZ*spg* mutants, and *spg(pou2)* mRNA into *cas* mutants, and examined the embryos for *sox17* expression. Injection of *cas* mRNA elicited strong ectopic *sox17* expression in the wild-type (Figure 5B; Kikuchi et al., 2001; Aoki et al., 2002), but not in MZ*spg* embryos (Figure 5D). Injection of *spg(pou2)* mRNA could not restore *sox17* expression in *cas* mutant embryos (Figure 5E), consistent with the observation that *cas* embryos have normal *spg(pou2)* expression (data not shown). Importantly, when *spg(pou2)* mRNA was coinjected with *cas* mRNA either into *cas*$^{-/-}$ or MZ*spg* mutants, expression of *sox17* was restored and ectopically activated (Figures 5F and 5G; compare with Figure 5B). Due to the high level of activation, the endogenous punctate expression pattern of *sox17* is obscured, comparable to what is observed after *cas* mRNA injection into the wild-type (Figure 5B). Furthermore, the ability of *cas* to restore *sox17* expression after injection into MZ*oep* embryos is totally abolished upon coinjection of a function-blocking morpholino against

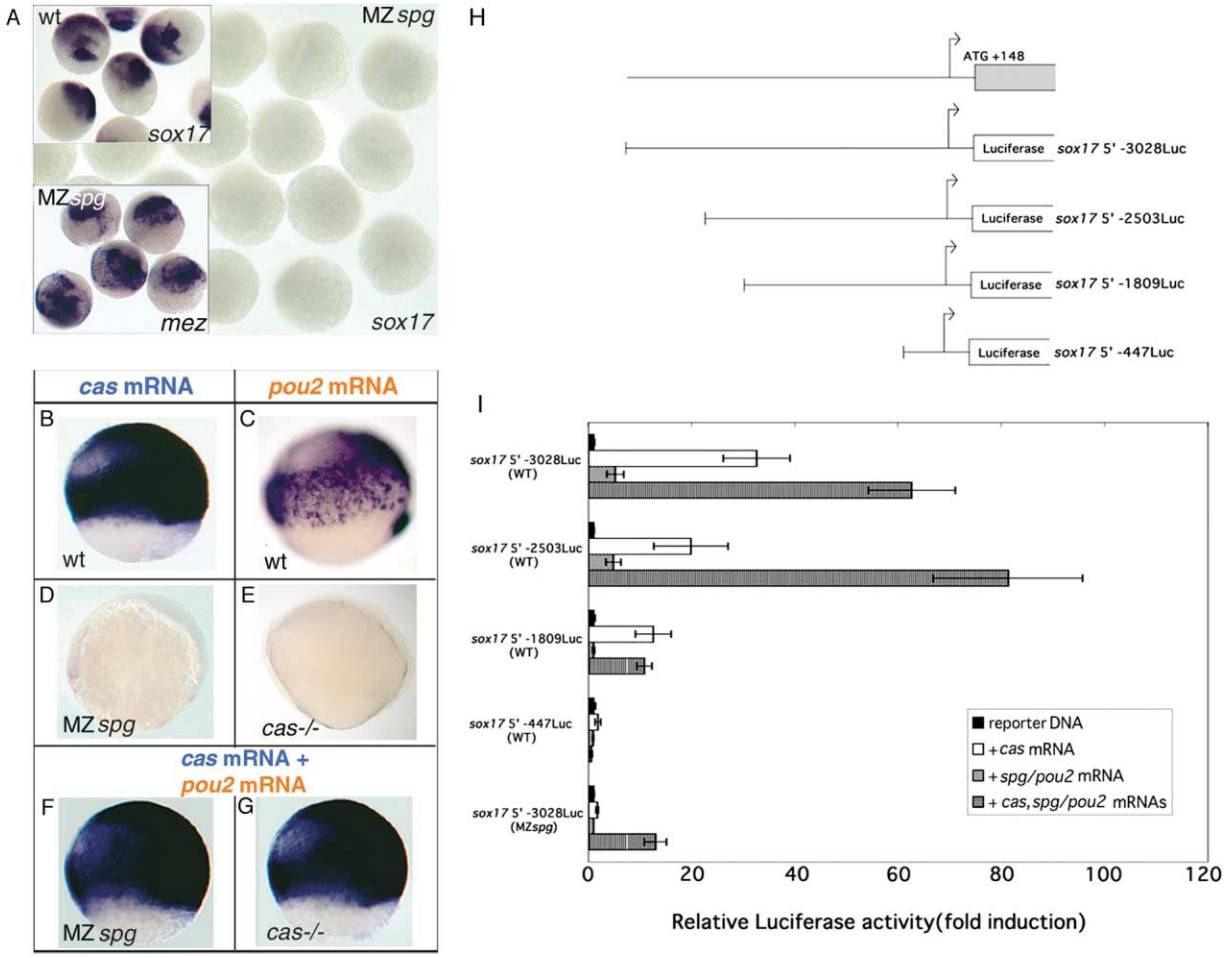

Figure 5. Synergistic Activation of Endoderm Differentiation by *cas* and *spg* Downstream of Ndl Signaling

(A) The constitutively active Ndl receptor TarA* normally elicits ectopic *sox17* expression (upper left inset), but cannot bypass the requirement for *spg* in MZspg embryos. The mesendodermal marker *mez* is still activated in MZ*spg* embryos (lower left inset).

(B–G) After *cas* mRNA injection, *sox17* is activated ectopically in wild-type (B), but not in MZ*spg* embryos at 70%–80% of epiboly (D). *spg* mRNA injection does not activate *sox17* ectopically either in wild-type embryos (C) or in *cas*$^{-/-}$ mutant (E) embryos. (F and G) Importantly, coinjection of *cas* and *spg* mRNA activates *sox17* expression in either *cas*$^{-/-}$ (F) or MZ*spg* mutants (G).

(H) *spg* and *cas* coactivate expression of a *sox17* promoter-luciferase reporter gene. *sox17* promoter region and luciferase reporter constructs. The transcription initiation site was determined by primer extension analysis (data not shown).

(I) Luciferase activity of reporter constructs coinjected with synthetic mRNAs into wild-type (wt) or MZ*spg* embryos at 1-cell stage. Activities are indicated as fold-induction relative to embryos injected with the reporter alone.

spg(pou2) (data not shown). Taken together, these results show that *spg(pou2)* and Cas act together to activate *sox17* expression.

The mRNA injection experiments support the notion that Cas is the main instructive signal for *sox17* activation, but show in addition that this instruction strictly requires *spg(pou2)* as a permissive factor, reminiscent of its permissive role in neuroectodermal development (Reim and Brand, 2002; Burgess et al., 2002). This notion is supported by several findings: (1) Whereas *spg(pou2)* is ubiquitously expressed up to gastrulation, *cas* expression is spatially limited to the blastoderm margin due to its activation by the Nodal pathway. (2) Cas can ectopically elicit endoderm differentiation and transfating of mesoderm into endoderm when misexpressed (Kikuchi et al., 2001; Dickmeis et al., 2001)—which we attribute to the ubiquitous presence of *spg(pou2)*—whereas misexpression of *spg(pou2)* mRNA is not sufficient to trigger endoderm formation (Figure 5C). Finally, because *cas* and *spg(pou2)* transcripts are coexpressed from sphere-stage onward without eliciting endoderm formation, we suggest that an additional, as yet unknown mechanism may normally prevent precocious endoderm formation and activation of *sox17*.

Synergistic Activation of the *sox17* Promoter by Spg and Cas

The *spg(pou2)* or *cas* mRNA injection alone into *cas* or MZ*spg* mutant embryos could not rescue *sox17* expression, suggesting that Pou2 and Cas function synergistically to induce *sox17* expression (Figures 5D and 5E). To test this notion in a different and more quantitative

assay, we isolated the *sox17* promoter region and performed luciferase reporter assays in early gastrula wild-type and MZ*spg* embryos. A fusion construct of the luciferase reporter DNA (*sox17* 5′-3027Luc) was injected with *cas* and/or *spg(pou2)* mRNA(s) into wild-type embryos at the 1-cell stage. The luciferase activity was induced 2- to 16-fold by coinjection with *cas* and *spg(pou2)* mRNAs as compared to injections with either *cas* or *spg(pou2)* mRNA alone (Figure 5I). Although we observed induction of luciferase activity in wild-type embryos, the interpretation is complicated by the presence of endogenous Pou2 protein in these embryos. In a more rigorous test in MZ*spg* embryos, injections of either *spg(pou2)* or *cas* mRNA could not significantly enhance the luciferase activity of the *sox17* 5′-3027Luc reporter DNA (Figure 5I). On the other hand, coinjection of both *spg(pou2)* and *cas* mRNAs with *sox17* 5′-3027Luc reporter DNA strongly enhanced luciferase activity approximately 16-fold, suggesting that Pou2 and Cas cooperate during induction of *sox17* (Figure 5I). Maximal induction of luciferase activity after coinjection into MZ*spg* embryos was lower than after coinjection into wild-type (Figure 5I, *sox17* 5′-3027Luc), which we attribute to the additional presence of endogenous Pou2 and Cas in the wild-type embryos. In summary, our luciferase reporter assay supports the notion that Pou2 and Cas act together to activate *sox17* expression.

In various systems, Sox factors have been reported to bind to adjacent or overlapping elements with Pou domain transcription factors to specifically activate or repress transcription of target genes, respectively (reviewed in Ryan and Rosenfeld, 1997; Wilson and Koopman, 2002). As an example, DNA binding studies and crystal structure determination could show functional cooperativity between murine Oct4 and Sox2 on the *Fgf4* enhancer (Ambrosetti et al., 2000; Remenyi et al., 2003). Therefore, one explanation for the observed cooperativity between Cas and Pou2 could be that both factors bind to the *sox17* promoter directly. In an attempt to gain more evidence in favor of or against this hypothesis, we investigated the sequence of our *sox17* promoter more closely. Deletion analysis showed that luciferase induction was abolished in deletion mutant construct *sox17* 5′-1809Luc, suggesting that a Pou2/Cas-synergy-dependent 700 bp promoter region from –2502 to –1809 is critical for synergy between Pou2 and Cas (Figure 5I). Within this 700 bp element, Pou2/Cas synergy-dependent activation could be furthermore confined to about 100 bp by luciferase assay, which harbored two putative binding sites for HMG domain containing factors adjacent to each other. When we mutated these two putative binding sites, respectively, we found that Pou2/Cas-dependent luciferase activation mediated by the 100 bp promoter element was abolished (unpublished data). This suggests an indirect synergistic regulation of the 100 bp *sox17* promoter element by Pou2/Cas. The exact molecular mechanism of *sox17* activation remains to be determined. Our findings are compatible with the possibility of a direct activation of *sox17* by Pou2 and Cas serving a remote, "early" *sox17* enhancer element, which has not been subject to our examination. As soon as Sox17 protein is expressed, Sox17 itself could take over to maintain its own expression, involving a "late" promoter. This model would be in accordance with the observation that *pou2* expression is shut down at the beginning of gastrulation, and Pou2/Cas might be used initially to prime *sox17* expression.

MZ*spg* Mutant Cells Develop into Mesoderm

Our previous results demonstrate that both in *cas* and MZ*spg* mutant embryos, mesendodermal precursors cannot undergo endodermal differentiation. What happens to endodermal precursor cells of MZ*spg* embryos at late blastula stages in which endodermal differentiation is intercepted? To address this question, we performed transplantation experiments to investigate the fate of these precursor cells. Cells located in the dorso-lateral margin of wild-type embryos later predominately colonize endoderm-, and only rarely mesoderm-derived tissues (Warga and Nüsslein-Volhard, 1999). Labeling of transplanted cells into this area with rhodamine-dextrane allowed us to track them in the living embryo, which we examined at pharyngula stages. Wild-type cells transplanted into the dorso-lateral mesendodermal primordium predominantly colonized the gut, and with lower frequency mesodermal derivatives (Figures 6A and 6A′, and data not shown; n = 15 embryos). In contrast, MZ*spg* mutant cells were unable to populate endodermal derivatives. Instead, MZ*spg* cells contributed to mesodermal tissue like the hypochord or the notochord (Figures 6B and 6C; n = 30 embryos). We have not observed cell death of MZ*spg* cells transplanted into wild-type embryos, supporting the notion that bipotential mesendodermal precursor cells exist also in MZ*spg* mutant embryos. MZ*spg* cells of unknown type did populate the branchial arches (data not shown), a tissue containing both mesodermal and endodermal cell types. Indeed, anterior, i.e., respiratory tract endoderm, and posterior, digestive tract endoderm, may be differently regulated and may not initially depend on *cas* (Piotrowski and Nüsslein-Volhard, 2000; Kanai-Azuma et al., 2002; Warga and Stainier, 2002).

We propose a model in which *spg(pou2)*, together with Cas, is required for triggering the decision of mesendodermal precursors to develop into endoderm (Figure 6). Wild-type mesendodermal precursor cells have both Cas and Pou2. We suggest that the lack of *spg(pou2)* biases development of mesendodermal precursors toward a mesodermal fate. This model can account for the depletion of the endodermal precursor population in MZ*spg* mutants early in development, when wild-type precursor cells normally change their state of differentiation. The model also predicts that cells normally destined to form endoderm instead form mesoderm in MZ*spg* mutants. Since the number of endodermal precursors is very small relative to that of mesodermal precursors (Warga and Nüsslein-Volhard, 1999), it would be difficult to observe an appreciable increase of mesoderm at the expense of endoderm in MZ*spg* embryos; however, this notion is supported by our observation that transplanted MZ*spg* cells form mesoderm instead of endoderm even when placed into the endodermal primordium of wild-type embryos (Figures 6B and 6C).

Cas not only stimulates endoderm formation, but also represses mesodermal markers like *ntl* when overexpressed (Aoki et al., 2002). Accordingly, overexpression

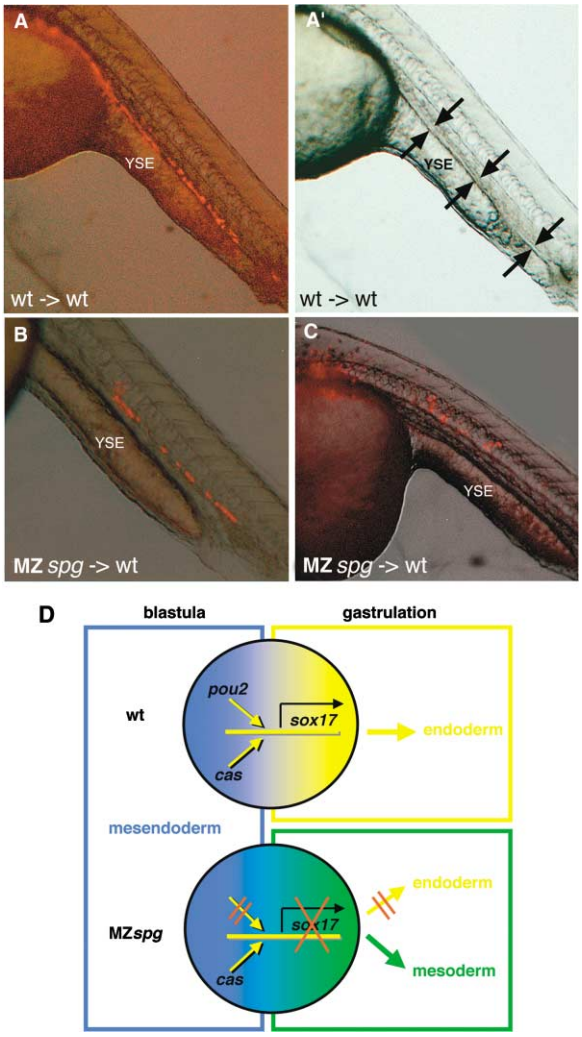

Figure 6. Transplanted MZspg Cells Are Unable to Form Endoderm and Form Mesoderm Instead

Bright-field images of living embryos at pharyngula stages, superimposed with flourescent images of the same focal plane; transplanted cells show red fluorescence. (A) Control: wild-type cells transplanted into the prospective endoderm of wild-type embryos colonize predominately endodermal derivatives like the gut, and with lower frequency mesodermal structures (not shown). (A′) Bright field image of (A). Arrows indicate the gut tube next to the yolk (YSE).

(B and C) MZ*spg* cells transplanted into the prospective endodermal region of wild-type embryos populate mesodermal derivatives: hypochord (B) or notochord (C), but not gut.

(D) *spg(pou2)* is essential for commitment to the endodermal precursor lineage and acts cooperatively with *cas* to activate *sox17* expression. In the absence of functional *spg(pou2)*, MZ*spg* mutant cells instead become mesoderm.

of *mez* mRNA leads to repression of *ntl* via overactivation of *cas* (Poulain and Lepage, 2002). Interestingly, in the absence of Cas, *mez* overexpression elicits ectopic induction of *ntl*, suggesting a role for *mez* in the choice between mesoderm and endoderm (Poulain and Lepage, 2002). In the wild-type, Cas may therefore be required to downregulate *ntl* in endodermal precursors, suggesting that these cells require both the activation of *sox17* and the repression of *ntl* at the transition from the blastula to the gastrula to become endodermal precursor cells. This raises the possibility that *cas* and *spg(pou2)* might also cooperate to repress mesoderm differentiation. To test this notion, we injected MZ*spg* embryos with *cas* mRNA and assayed for *ntl* expression. Interestingly, *ntl* expression was repressed by *cas* in absence of functional *spg(pou2)* (data not shown). Therefore, repression of the pan-mesodermal marker *ntl* was not sufficient to allow endoderm differentiation. We speculate that Cas cooperates in this case with another, as yet unknown POU family member.

A Conserved Role for Spg (Oct4) in Endoderm Formation?

A gene with a *cas*-like function has not yet been described in chick or mammals, raising the question whether a Cas/Spg-type of mechanism could operate in endoderm formation also in these species. We have previously suggested that *spg(pou2)* is the likely ortholog of the murine *Oct4* gene on the basis of chromosomal synteny and phylogenetic sequence comparisons (Burgess et al., 2002), and have shown that murine *Oct4* can replace the missing function during neural patterning of *spg(pou2)* mutant embryos (Reim and Brand, 2002). Mammalian Oct4 is well known for its function in stem- and germ-cell development (reviewed in Pesce and Schöler, 2000, 2001). Zebrafish use a different mode of germ cell determination than mice (Johnson et al., 2003), suggesting that the germ cell function of the ancestral Oct4 gene may have been lost in the teleost lineage, and indeed we found that expression of the germ cell marker *vasa* occurs normally in MZ*spg* embryos (data not shown). Oct4 requirement in preimplantation development has so far precluded an analysis of a possible function during definitive endoderm development. Evidence in mice, however, suggests that Oct4 may also function in endoderm formation, similar to what we report here. Oct4 physically interacts in vitro with the Forkhead domain protein FoxD3 on promoter elements of endoderm-specific genes like *FoxA1* and *FoxA2*, where it acts as a corepressor of endodermal differentiation of ES cells in culture (Guo et al., 2002). Interestingly, in addition to its role in ES cell maintenance, Oct4 has recently been implicated in initial differentiation events in ES cell development (Pan et al., 2002). Reminiscent of *spg(pou2)*'s function in zebrafish, elevated levels of murine Oct4 can initiate endodermal differentiation in an inducible cell culture system (Niwa et al., 2000). Moreover, analysis of *Oct4* knockout mice revealed that Oct4 is involved in early differentiation of primitive endoderm from the hypoblast. In this context, a transient increase of Oct4 within the primitive, premigratory hypoblast activates *Osteopontin*, which is required for migratory endoderm formation at peri-implantation stages (Botquin et al., 1998). Intriguingly, Oct4 elicits transcriptional activation, whereas recruitment of the HMG transcription factor Sox2 to a DNA binding site not adjacent, but overlapping with the Oct4-POU domain binding site counteracts activation by Oct4. Therefore, Oct4 seems essential for early cell fate decisions. Subsequently, downregulation of Oct4 after fate decision is necessary for terminal differentiation. This is reminiscent of the expression of *spg(pou2)* in zebrafish

endoderm development: high levels of *spg(pou2)* expression seem to endow precursor cells at late blastula stages with the competence to enter a precursor phase and to adopt a more differentiated state upon gastrulation. Later, during gastrulation, *spg(pou2)* expression is shut down in the differentiating endoderm. In other species like the frog or chick, *cas* orthologs or true orthologs of *spg(pou2)* have not been described. The gene historically named *pou2* in *Xenopus* belongs to another subclass (Hudson et al., 1997). However, as in the zebrafish, *Xenopus* and mouse *sox17* genes are important components of the endodermal pathway (Hudson et al., 1997; Kanai-Azuma et al., 2002).

The germlayer theory proposed by von Baer (1828) suggests that triploblastic metazoans developed from diploblastic, ectoderm, and endoderm containing ancestors. In the ontogenetic remnants of this transition, the vertebrate embryo primarily develops two germlayers, the ectoderm and a bipotential mesendoderm, and then mesoderm and endoderm emanate from this common layer. In zebrafish, the bipotential mesendoderm requires both *pou2* and *cas* to undergo differentiation into the endodermal lineage. The Cas-Pou2 system seems to act in a truly germlayer-specific fashion, at the time the endodermal germlayer is first formed. We suggest that these findings provide genetic support for the significance of the endodermal and mesodermal germlayers as important transient units of tissue formation. A diploblastic, gastrula-like animal may be the developmental archetype of metazoan animals (von Baer, 1828; see Hall, 1992); it will be interesting to further determine to which extent the Cas-Pou2 system is also conserved in other animals.

Experimental Procedures

Fish Maintenance and Mutants

Zebrafish were kept and staged as described (Westerfield, 1994; Kimmel et al., 1995). MZ*oep* fish were obtained as described (Gritsman et al., 1999). MZ*spg* carriers for the spg^{hi349} null allele (Burgess et al., 2002) were obtained by rescuing homozygous embryos injected at the 1-cell stage with 30 pg *spg(pou2)* mRNA to adulthood; genotype was confirmed by PCR. To generate MZ*spg* mutant embryos, homozogous carriers were intercrossed; for M*spg* mutant embryos, homozygous females were crossed with wild-type males. mRNA injections were done at the 1-cell stage as described (Reim and Brand, 2002).

Sox17 Promoter

Genomic clones were isolated and subcloned from a BAC library (Genome Systems, Inc.) using *sox17* cDNA probe. Luciferase fusions were to a PCR-generated NcoI site at the AUG in *sox17*, and subcloned into pGL3 (Promega). Deletion mutants were constructed by restriction digestion and PCR. The transcription initiation site was determined by primer extension analysis with *sox17* primer GGC GCGTATCCTTATTGATCACCGATGAAACC. For luciferase assays, 50 pg of reporter DNA and synthetic mRNAs (*cas*, 3 pg; *spg*, 30 pg) was coinjected into wild-type or MZ*spg* embryos. *Renilla* luciferase DNA (2 pg) under control of CMV promoter was coinjected as an internal control. 30 injected embryos were harvested at the shield stage (6 hpf) and luciferase activity was determined using the Pica-Gene luciferase assay system (Toyo Inki) by five measurements each.

Transplantations

Cells from donors fluorescently labeled with Rhodamine-dextrane (Molecular Probes D-1817) were transplanted into unlabeled hosts as described (Reim and Brand, 2002), fixed after gastrulation, and processed for ISH as described (Reifers et al., 1998). After ISH, embryos where first photographed and transplanted cells were then visualized using Vectastain (VectorLabs).

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References

Alexander, J., and Stainier, D.Y. (1999). A molecular pathway leading to endoderm formation in zebrafish. Curr. Biol. *9*, 1147–1157.

Ambrosetti, D.C., Schöler, H.R., Dailey, L., and Basilico, C. (2000). Modulation of the activity of multiple transcriptional activation domains by the DNA binding domains mediates the synergistic action of Sox2 and Oct-3 on the fibroblast growth factor-4 enhancer. J. Biol. Chem. *275*, 23387–23397.

Aoki, T.O., David, N.B., Minchiotti, G., Saint-Etienne, L., Dickmeis, T., Persico, G.M., Strähle, U., Mourrain, P., and Rosa, F.M. (2002). Molecular integration of *casanova* in the Nodal signalling pathway controlling endoderm formation. Development *129*, 275–286.

Belting, H.G., Hauptmann, G., Meyer, D., Abdelilah-Seyfried, S., Chitnis, A., Eschbach, C., Soll, I., Thisse, C., Thisse, B., Artinger, K.B., et al. (2001). *spiel ohne grenzen/pou2* is required during establishment of the zebrafish midbrain-hindbrain boundary organizer. Development *128*, 4165–4176.

Botquin, V., Hess, H., Fuhrmann, G., Anastassiadis, C., Gross, M.K., Vriend, G., and Schöler, H.R. (1998). New POU dimer configuration mediates antagonistic control of an osteopontin preimplantation enhancer by Oct-4 and Sox-2. Genes Dev. *12*, 2073–2090.

Burgess, S., Reim, G., Chen, W., Hopkins, N., and Brand, M. (2002). The zebrafish *spiel-ohne-grenzen (spg)* gene encodes the POU domain protein Pou2 related to mammalian Oct4 and is essential for formation of the midbrain and hindbrain, and for pre-gastrula morphogenesis. Development *129*, 905–916.

Chen, S., and Kimelman, D. (2000). The role of the yolk syncytial layer in germ layer patterning in zebrafish. Development *127*, 4681–4689.

Chen, Y., and Schier, A.F. (2001). The zebrafish Nodal signal Squint functions as a morphogen. Nature *411*, 607–610.

Cooper, M.S., and D'Amico, L.A. (1996). A cluster of noninvoluting endocytic cells at the margin of the zebrafish blastoderm marks the site of embryonic shield formation. Dev. Biol. *180*, 184–198.

David, N.B., and Rosa, F.M. (2001). Cell autonomous commitment to an endodermal fate and behaviour by activation of Nodal signalling. Development *128*, 3937–3947.

Dickmeis, T., Mourrain, P., Saint-Etienne, L., Fischer, N., Aanstad, P., Clark, M., Strähle, U., and Rosa, F. (2001). A crucial component of the endoderm formation pathway, CASANOVA, is encoded by a novel *sox*-related gene. Genes Dev. *15*, 1487–1492.

Feldman, B., Gates, M.A., Egan, E.S., Dougan, S.T., Rennebeck, G., Sirotkin, H.I., Schier, A.F., and Talbot, W.S. (1998). Zebrafish organizer development and germ-layer formation require Nodal-related signals. Nature *395*, 181–185.

Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W.S., and Schier, A.F. (1999). The EGF-CFC protein One-eyed pinhead is essential for Nodal signaling. Cell *97*, 121–132.

Guo, Y., Costa, R., Ramsey, H., Starnes, T., Vance, G., Robertson, K., Kelley, M., Reinbold, R., Schöler, H., and Hromas, R. (2002). The embryonic stem cell transcription factors Oct-4 and FoxD3 interact

to regulate endodermal-specific promoter expression. Proc. Natl. Acad. Sci. USA *99*, 3663–3667.

Hall, Z.W. (1992). An Introduction to Molecular Neurobiology (Sinauer).

Hauptmann, G., and Gerster, T. (1995). *pou-2*-a zebrafish gene active during cleavage stages and in the early hindbrain. Mech. Dev. *51*, 127–138.

Howley, C., and Ho, R.K. (2000). mRNA localization patterns in zebrafish oocytes. Mech. Dev. *92*, 305–309.

Hudson, C., Clements, D., Friday, R.V., Stott, D., Woodland, H.R., Gad, J.M., Tajima, Y., Taya, C., Kurohmaru, M., Sanai, Y., et al. (1997). Xsox17α and -β mediate endoderm formation in Xenopus. Cell *91*, 397–405.

Johnson, A.D., Crother, B., White, M.E., Patient, R., Bachvarova, R.F., Drum, M., and Masi, T. (2003). Regulative germ cell specification in axolotl embryos: a primitive trait conserved in the mammalian lineage. Philos. Trans. R. Soc. Lond. B Biol. Sci. *358*, 1371–1379.

Kanai-Azuma, M., Kanai, Y., Gad, J.M., Tajima, Y., Taya, C., Kurohmaru, M., Sanai, Y., Yonekawa, H., Yazaki, K., Tam, P.P., and Hayashi, Y. (2002). Depletion of definitive gut endoderm in *Sox17*-null mutant mice. Development *129*, 2367–2379.

Kikuchi, Y., Trinh, L.A., Reiter, J.F., Alexander, J., Yelon, D., and Stainier, D.Y. (2000). The zebrafish *bonnie and clyde* gene encodes a Mix family homeodomain protein that regulates the generation of endodermal precursors. Genes Dev. *14*, 1279–1289.

Kikuchi, Y., Agathon, A., Alexander, J., Thisse, C., Waldron, S., Yelon, D., Thisse, B., and Stainier, D.Y. (2001). *casanova* encodes a novel Sox-related protein necessary and sufficient for early endoderm formation in zebrafish. Genes Dev. *15*, 1493–1505.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. (1995). Stages of embryonic development of the zebrafish. Dev. Dyn. *203*, 253–310.

Maduro, M.F., Meneghini, M.D., Bowerman, B., Broitman-Maduro, G., and Rothman, J.H. (2001). Restriction of mesendoderm to a single blastomere by the combined action of SKN-1 and a GSK-3β homolog is mediated by MED-1 and -2 in *C. elegans*. Mol. Cell *7*, 475–485.

Niwa, H., Miyazaki, J., and Smith, A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet. *24*, 372–376.

Odenthal, J., and Nüsslein-Volhard, C. (1998). fork head domain genes in zebrafish. Dev. Genes Evol. *208*, 245–258.

Oppenheimer, J.M. (1940). The non-specificity of the germ-layers. Q. Rev. Biol. *15*, 1–27.

Pan, G.J., Chang, Z.Y., Schöler, H.R., and Pei, D. (2002). Stem cell pluripotency and transcription factor Oct4. Cell Res. *12*, 321–329.

Pander, C. (1817). Beyträge zur Entwicklungsgeschichte des Hühnchens (Würzburg).

Pesce, M., and Schöler, H.R. (2000). Oct-4: control of totipotency and germline determination. Mol. Reprod. Dev. *55*, 452–457.

Pesce, M., and Schöler, H.R. (2001). Oct-4: gatekeeper in the beginnings of mammalian development. Stem Cells *19*, 271–278.

Peyrieras, N., Strähle, U., and Rosa, F. (1998). Conversion of zebrafish blastomeres to an endodermal fate by TGF-β-related signaling. Curr. Biol. *8*, 783–786.

Piotrowski, T., and Nüsslein-Volhard, C. (2000). The endoderm plays an important role in patterning the segmented pharyngeal region in zebrafish (Danio rerio). Dev. Biol. *225*, 339–356.

Poulain, M., and Lepage, T. (2002). Mezzo, a paired-like homeobox protein is an immediate target of Nodal signalling and regulates endoderm specification in zebrafish. Development *129*, 4901–4914.

Reifers, F., Böhli, H., Walsh, E.C., Crossley, P.H., Stainier, D.Y.R., and Brand, M. (1998). Fgf8 is mutated in zebrafish *acerebellar* mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Development *125*, 2381–2395.

Reim, G., and Brand, M. (2002). *spiel-ohne-grenzen/pou2* mediates regional competence to respond to Fgf8 during zebrafish early neural development. Development *129*, 917–933.

Reiter, J.F., Alexander, J., Rodaway, A., Yelon, D., Patient, R., Holder, N., and Stainier, D.Y. (1999). Gata5 is required for the development of the heart and endoderm in zebrafish. Genes Dev. *13*, 2983–2995.

Reiter, J.F., Kikuchi, Y., and Stainier, D.Y. (2001). Multiple roles for Gata5 in zebrafish endoderm formation. Development *128*, 125–135.

Remenyi, A., Lins, K., Nissen, L.J., Reinbold, R., Schöler, H.R., and Wilmanns, M. (2003). Crystal structure of a POU/HMG/DNA ternary complex suggests differential assembly of Oct4 and Sox2 on two enhancers. Genes Dev. *17*, 2048–2059.

Rodaway, A., Takeda, H., Koshida, S., Broadbent, J., Price, B., Smith, J.C., Patient, R., and Holder, N. (1999). Induction of the mesendoderm in the zebrafish germ ring by yolk cell-derived TGF-beta family signals and discrimination of mesoderm and endoderm by FGF. Development *126*, 3067–3078.

Rodaway, A., Patient, R., David, N.B., and Rosa, F.M. (2001). Mesendoderm. an ancient germ layer? Cell *105*, 169–172.

Ryan, A.K., and Rosenfeld, M.G. (1997). POU domain family values: flexibility, partnerships, and developmental codes. Genes Dev. *11*, 1207–1225.

Schier, A.F., Neuhauss, S.C.F., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D.Y.R., Zwartkruis, F., Abdelilah, S., Stemple, D.L., Rangini, Z., et al. (1996). Mutations affecting development of the embryonic zebrafish brain. Development *123*, 165–178.

Schier, A.F., Neuhauss, S.C., Helde, K.A., Talbot, W.S., and Driever, W. (1997). The *one-eyed pinhead* gene functions in mesoderm and endoderm formation in zebrafish and interacts with *no tail*. Development *124*, 327–342.

Stainier, D.Y. (2002). A glimpse into the molecular entrails of endoderm formation. Genes Dev. *16*, 893–907.

Strähle, U., Blader, P., Henrique, D., and Ingham, P.W. (1993). *axial*, a zebrafish gene expressed along the developing body axis, shows altered expression in cyclops mutant embryos. Genes Dev. *7*, 1436–1446.

Takeda, H., Matsuzaki, T., Oki, T., Miyagawa, T., and Amanuma, H. (1994). A novel POU domain gene, zebrafish *pou2*: expression and roles of two alternatively spliced twin products in early development. Genes Dev. *8*, 45–59.

Tam, P.P., Kanai-Azuma, M., and Kanai, Y. (2003). Early endoderm development in vertebrates: lineage differentiation and morphogenetic function. Curr. Opin. Genet. Dev. *13*, 393–400.

von Baer, V.E. (1828). Entwicklungsgeschichte der Tiere. Beobachtung und Reflexion (Königsberg: Bornträger).

Warga, R.M., and Nüsslein-Volhard, C. (1999). Origin and development of the zebrafish endoderm. Development *126*, 827–838.

Warga, R.M., and Stainier, D.Y. (2002). The guts of endoderm formation. Results Probl. Cell Differ. *40*, 28–47.

Watanabe, M., and Whitman, M. (1999). The role of transcription factors involved in TGFbeta superfamily signaling during development. Cell Mol. Biol. (Noisy-le-grand) *45*, 537–543.

Westerfield, M. (1994). The Zebrafish Book, 2.1 edn. (Oregon: University of Oregon Press).

Wilson, M., and Koopman, P. (2002). Matching SOX: partner proteins and co-factors of the SOX family of transcriptional regulators. Curr. Opin. Genet. Dev. *12*, 441–446.