RESEARCH COMMUNICATION

A crucial component of the endoderm formation pathway, CASANOVA, is encoded by a novel sox-related gene

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casanova (*cas*) mutant zebrafish embryos lack endoderm and develop cardia bifida. In a subtractive screen for Nodal-responsive genes, we isolated an HMG box-containing gene, *10J3*, which is expressed in the endoderm. The *cas* phenotype is rescued by overexpression of *10J3* and can be mimicked by *10J3*-directed morpholinos. Furthermore, we identified a mutation within *10J3* coding sequence that cosegregates with the *cas* phenotype, clearly demonstrating that *cas* is encoded by *10J3*. Epistasis experiments are consistent with an instructive role for *cas* in endoderm formation downstream of Nodal signals and upstream of *sox17*. In the absence of *cas* activity, endoderm progenitors differentiate into mesodermal derivatives. Thus, *cas* is an HMG box-containing gene involved in the fate decision between endoderm and mesoderm that acts downstream of Nodal signals.

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The endoderm germ layer generates the structures of the digestive and respiratory tracts. In addition, endoderm is crucial in the organization and/or induction of neighboring tissues, such as the head and the heart (Grapin-Botton and Melton 2000). In the zebrafish, endoderm derives from cells positioned at the blastoderm margin of the late blastula (Warga and Nusslein-Volhard 1999). Although endoderm and mesoderm progenitors partially overlap, most mesoderm progenitors come from positions relatively far away from the very margin at this stage.

The molecular pathway leading to endoderm formation is only partially understood. Specification of endoderm requires Nodal signaling (Kimelman and Griffin 2000). Zebrafish mutants lacking the Nodal-related fac-

tors Squint (*Sqt*) and Cyclops (*Cyc*) fail to form endoderm (Feldman et al. 1998; Sampath et al. 1998). Similarly, endoderm does not form in embryos defective in both maternal and zygotic components of *one-eyed pinhead* (*MZoepl*), which encodes an EGF-CFC protein required for cells to respond to Nodal signals (Schier et al. 1997; Strähle et al. 1997; Zhang et al. 1998; Alexander and Stainier 1999; Gritsman et al. 1999). In zebrafish, Nodals induce endoderm presumably via activation of the type I TGF β receptor TARAM-A (Tar; Renucci et al. 1996; Peyrieras et al. 1998), the mix-like homeobox transcription factor MIXER (*bonnie and clyde*, *bon*; Kikuchi et al. 2000), and the zinc-finger transcription factor GATA5 (*faust*; Reiter et al. 1999, 2001). Both transcription factors require a third gene, *casanova* (*cas*), to efficiently induce the endoderm-specific *sox17* gene (Alexander and Stainier 1999) and to allow marginal cells to achieve the proper endodermal program. At gastrula stages, *cas* mutant embryos express *sox17* neither in endoderm precursors nor in the forerunner cells, a small group of noninvoluting mesendodermal cells at the dorsal margin (Melby et al. 1996). At later stages, *cas* mutants lack a gut tube and develop a heart condition known as cardia bifida. *cas* activity is required cell-autonomously for endoderm development and endodermal expression of *foxA2* (Alexander and Stainier 1999; Alexander et al. 1999). Thus, *cas* acts within endoderm precursors, downstream of the Nodal signals *Cyc* and *Sqt* and the transcription regulators MIXER and GATA5 but upstream of the transcription factors *FoxA2* (Axial/Hnf3 β) and *SOX17*.

To understand further the events controlling endoderm formation, it is essential to define, in molecular terms, the nature of the *cas* gene and its precise epistatic relationship with the other components of the Nodal pathway. Here, we report the molecular identification of *cas* and show that it encodes an HMG domain-containing SOX factor. Furthermore, we show that, in the absence of *cas* activity, cells normally fated to endoderm develop into mesodermal structures.

Results and Discussion

To identify new components that act downstream of Nodal/Tar in the zebrafish embryo, a subtractive screen was carried out for Tar-responsive genes (see Materials and Methods). We identified a gene, *10J3*, the expression of which initiates in late blastulae (30% epiboly) at the future dorsal margin of the blastoderm cap (Fig. 1a). Not only the marginal blastomeres but also the underlying yolk syncytial layer (YSL) express *10J3* (Fig. 1b). Expression spreads around the entire margin at subsequent stages (Fig. 1c). At the onset of gastrulation, both the forerunner cells and the endoderm precursor cells, which involute all around the margin and form a population of scattered, flat cells immediately overlying the yolk cell (Warga and Nusslein-Volhard 1999), are positive for the *10J3* transcript. This pattern of expression is maintained until the end of gastrulation (Fig. 1d,e). At subsequent early somitogenesis stages, the lining of Kupfer's vesicle, a tail bud structure derived from the forerunner popula-

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Figure 1. Expression of *10/3*, a Nodal-regulated gene in endoderm and forerunner cells and regulation by Nodal signaling. Expression of *10/3* (a–e) and *sox17* (f) during early embryogenesis. (a,e,f) Dorsal view, animal pole up; (b) lateral view; (c) lateral view, dorsal right; (d) animal pole view, optical section at germ ring level; (f) posterior view, dorsal up. Expression starts at 30% epiboly in a sector of marginal blastomeres (a, arrow) and in the underlying YSL (b, arrow). (c) Until the beginning of gastrulation, both expression domains expand around the margin. (Arrowhead) Start of forerunner cell expression. (d) At the shield stage, expression is detected in hypoblastic endoderm precursor cells (arrows) and in the forerunner cells (not visible). (e) Expression in the endodermal precursors (arrows) and forerunner cells (arrowhead) continues during gastrulation. During the whole gastrulation period, *10/3* and *sox17* expression domains within the blastoderm are very similar. (e,f) 70%–80% epiboly. (g–i) *10/3* expression is regulated by the *nodal* pathway. Embryos are at 60% epiboly. Shown is a dorsal view with the animal pole up. (g) In embryos injected with Fast^{SID} mRNA (400 pg), blastodermal *10/3* expression is strongly reduced. (h) Uninjected control embryo. (Arrow) Endodermal precursors. (i) In embryos, injection of Tar* receptor mRNA (1.2 pg), up-regulates and expands *cas* expression. (j–m) Expression of *10/3* in MZoepe embryos. (j,k) Optical section through the marginal region at 30% epiboly. (l,m) Dorsal view at 70% epiboly. (j) *10/3* is expressed only in the YSL in MZoepe embryos. (Arrowhead) The limit between the blastoderm and the YSL. (k) In wild-type embryos, expression in marginal blastomeres is observed. (l) At 70% epiboly, *cas* expression is lost in MZoepe embryos, but can be restored ectopically by overexpression of Tar* (m).

tion, continues to express the *10/3* gene (data not shown). This pattern of expression is highly reminiscent of that of zebrafish *sox17* (Fig. 1f; Alexander and Stainier 1999) and suggests that the *10/3* gene could play a regulatory role in endoderm development.

The pattern of expression of the *10/3* gene is consistent

with a role downstream of Nodal signals. To verify its dependence with regard to Nodal signals, we expressed the dominant-negative inhibitor of Nodal signaling, FAST-1^{SID} in zebrafish embryos (Muller et al. 2000). As a consequence, *10/3* was significantly down-regulated in injected embryos (Fig. 1g) as compared with controls (Fig. 1h). Also, the *10/3* gene was not expressed during gastrulation in the blastoderm of MZoepe or *sqt/cyc* mutants, which do not form endoderm or forerunner cells (Fig. 1l and data not shown). Conversely, to test the responsiveness of *10/3* to Nodal/Tar, we expressed the constitutively active variant of the receptor Tar* in zebrafish embryos (Renucci et al. 1996; Peyrieras et al. 1998). This led to ectopic up-regulation of the *10/3* gene in wild-type embryos (Fig. 1h,i) and strongly induced *10/3* expression in MZoepe embryos in a large population of deep flat endoderm-like cells and in a small group of superficial cells highly reminiscent of the forerunner cluster (Fig. 1m; Peyrieras et al. 1998). Expression in the YSL, however, was not affected by lack or activation of Nodal signaling (Fig. 1j,k and data not shown). Thus, the expression of the *10/3* gene requires Nodal signals for expression in the endoderm precursors and forerunner cells but not in the YSL.

Sequencing of the *10/3* cDNA revealed that it encodes a 307-amino-acid protein belonging to the HMG-domain-containing family of SOX transcription factors (Fig. 2a). Phylogenetic analysis with different tree-building methods shows that it forms a novel member of the F subfamily of *sox* genes, which includes *sox17*, *sox7*, and *sox18* (Fig. 2a; Bowles et al. 2000). The *10/3* gene also shares the exon–intron structure with other members of this subfamily (Wegner 1999; Bowles et al. 2000); a single intron, located at the glycine residue of the consensus sequence K(M/I)LGK in members of the F subfamily (Fig. 2a) separates the HMG domain-encoding exons (Fig. 2b; Bowles et al. 2000). Regions outside the HMG box are not strongly conserved with the exception of a short stretch located near the carboxyl terminus with the consensus sequence EF(D/E)QY. This short domain conserved between SOX7, SOX17, SOX18, and CAS resides within the roughly mapped limits of the activation domain of mouse Sox17 (Kanai et al. 1996).

cas mutants lack endoderm and forerunner cells (Alexander et al. 1999). Expression of *10/3* within these territories prompted us to test whether this gene could be identical to *cas*. Genomic fragments were amplified from wild-type and *cas* embryos with *10/3*-specific primers and sequenced. *cas* mutants were found to harbor a point mutation downstream of the *10/3* HMG-box, leading to premature termination of the *10/3*-encoded protein (Figs. 2b and 3a). This point mutation cosegregates with the *cas* mutant phenotype (Fig. 3b), demonstrating that *10/3* and *cas* are tightly linked. Expression of the wild-type but not the mutant *10/3* protein rescued the expression of the endodermal marker *sox17* in *cas* embryos (Fig. 3c–e). Furthermore, injection of antisense morpholino oligonucleotides specifically directed against *10/3* (*cas*-MO, Fig. 2b) phenocopied the *cas* mutant phenotype: (1) injected embryos lacked cells ex-

Figure 2. *10/3* encodes an HMG box transcription factor. (a) Comparison of the HMG box regions of several vertebrate Sox transcription factors. *10/3* and zebrafish (z) *sox17* encode members of the F subfamily. Similar types of residues are color coded. (*) Residues conserved completely in the alignment (see Materials and Methods). (b) Schematic representation of the *10/3* gene structure. The position of a conserved intron in the HMG box, the *cas*^{ta56} mutation and the morpholino oligonucleotide hybridization region (*cas*-MO) are indicated. GenBank accession no.: *10/3/cas*, AY027650; mouse (m)-*sox18*, AAG48578, human (h)-*sox18*, AB033888; *Xenopus* (x)-*sox7*, D83649; mouse (m)-*sox7*, AB023419; putative human (h)-*sox17*, AK025905; mouse (m)-*sox17*, D49474; *Xenopus* (x)-*sox17α*, AJ001730; x-*sox17β*, AJ001742; and zebrafish (z)-*sox17*, AF168614.

pressing *foxA2* (*axial/HNF3β*; Strähle et al. 1996; Fig. 3f,i) whereas *foxA2* expression was unaffected in the midline of the body axis; (2) *sox17* expression (Alexander and Stainier 1999) was completely abolished by injection of *cas*-MO (Fig. 3g,j); and (3) injected embryos developed cardia bifida at later stages (Fig. 3h,k). In contrast, injection of control morpholinos of distinct sequence did not induce these phenotypes (data not shown). Taken together, these knockdown experiments confirmed that the *10/3* HMG box gene is identical to *cas*. The phenotype of the *cas*^{ta56} allele is likely due to the deletion of the carboxyl terminus of the *10/3* protein, a region that was shown to contain a transcriptional activation domain, including the EF(D/E)QY conserved sequence, and other protein interaction domains in related HMG proteins (van de Wetering et al. 1993; Hosking et al. 1995; Kanai et al. 1996; Sudbeck et al. 1996; Zorn et al. 1999). Below, we will refer to *10/3* as the *cas* gene.

Although previous epistatic analyses have shown that *cas* is a component of the Nodal pathway, essential for endoderm formation, the way it acts within this pathway is not clear. In particular, we wished to understand whether *cas* acts in a permissive or an instructive manner downstream of Nodal signals. *MZoepe* embryos, which lack both maternal and zygotic *oep*, are deficient in Nodal signaling as cells cannot respond to Nodal signals in the absence of *Oep* (Gritsman et al. 1999). As a consequence, these embryos fail to activate endodermal marker genes (Strähle et al. 1997; Schier and Talbot 1998; Alexander and Stainier 1999). To test whether *cas* can by-pass the requirement of Nodal signaling for en-

doderm formation, we injected *cas* mRNA into *MZoepe* embryos. *cas* mRNA led to a strong ectopic activation of *sox17* (Fig. 4a,b) and *gata5* (data not shown) in *MZoepe* embryos. Some *sox17*-positive cells adopted a deep position, suggesting they had involuted (data not shown). Later endoderm differentiation markers were not rescued in these experiments, suggesting that *cas* activity or the activity of other genes might be required during postgastrulation stages to allow proper endoderm differentiation in Nodal-deficient mutants. In contrast to the induction of early endodermal markers, *cas* mRNA did not induce the mesendodermal marker *goosecoid* (data not shown), showing that the effect of *cas* is restricted to the induction of endoderm. Furthermore, *cas* plays a central downstream role in the transduction of Nodal signals, as *Tar*⁺-activated cells are unable to activate the downstream endodermal gene *sox17* in *cas* embryos (Fig. 4c; Alexander and Stainier 1999). As expected, expression of *cas* within *Tar*⁺-activated cells restored the capacity of these cells to express *sox17* (Fig. 4d). Altogether, these results are in agreement with previous epistasis experiments (Alexander and Stainier 1999; Reiter et al. 2001) but also demonstrate that *cas* acts in an instructive manner with regard to endoderm formation as it can induce endodermal markers in the absence of Nodal signals.

To understand how the absence of *cas* activity affects the behavior and fate of marginal cells that express *cas* and that are normally fated to endoderm, we first assessed the expression of *cas* mRNA in *cas* mutants. *cas* mutant embryos express *cas* mRNA in a pattern similar to wild-type embryos at the onset of gastrulation (data not shown). At the end of gastrulation (bud stage), *cas* mRNA was no longer detectable in the blastoderm of mutant embryos (Fig. 4e,f). However expression was still evident in the YSL at the bud stage. Thus, *cas* expression is initiated normally in *cas* mutants. Lack of *cas* blastodermal expression at bud stages could be due to specific loss of the *cas*-expressing cell population in the mutant during gastrulation; however, increased cell death was not apparent. Alternatively, *cas* mutant cells may take up another cell fate. To investigate the fate acquired by endodermal progenitors in *cas* embryos, fate mapping experiments were carried out by injection of the photo-activatable, fluorescent tracer Fluorescein-Dextran (FD) and uncaging of the dye at the 40% epiboly stage in a small group (1 to 5) of blastomeres located immediately at the blastoderm margin (Bally-Cuif et al. 2000). Consistent with published results (Warga and Nusslein-Volhard 1999), we found that most labeled blastomeres in wild-type or *cas* embryos involuted at the onset of gastrulation and migrated within the inner hypoblast germ layer as expected from marginal cells. Around 30 h, clones of labeled cells from wild-type embryos contributed predominantly to endodermal derivatives (45%

Figure 3. CAS is encoded by the *10J3* gene. (a) Sequencing of *cas*^{ta56} and sibling genomic DNA reveals a T-to-G mutation at position 510 that leads to an amber stop codon instead of a tyrosine. (b) Segregation analysis with a CAPS marker reveals cosegregation of the mutation in the homozygous state with *cas* mutant embryos. Cosegregation was observed in all 160 *cas* embryos tested. Representative restriction patterns of five siblings with a wild-type phenotype and five siblings with a *cas* phenotype are shown. (c–e) *10J3* mRNA rescues *sox17* expression in *cas* mutants. Embryos are at 60% epiboly. Shown is a dorsal view with the animal pole up. (e) Uninjected *cas* embryo. (d) *cas* embryo injected with 100 pg of wild-type *10J3* mRNA. (e) *cas* embryo injected with 200 pg of mutant *cas* mRNA. (f–k) Injection of 1 pmole of a morpholino antisense oligonucleotide against *10J3* (*cas*-MO) results in loss of endoderm and forerunner cells and cardia bifida. (f,g,i,j) Dorsal view, 60% epiboly. (h,k) Dorsal view of heart region, anterior down, about 1.5 d of development. (f,i) Expression of *foxA2/axial* in *cas*-MO injected embryos (i) and uninjected wild-type embryos (f). The endodermal expression domain of *foxA2/axial* (arrow) is missing in the *cas*-MO injected embryos, whereas the domain representing the nascent axis remains (arrowhead). (g,j) Expression of *sox17* in *cas*-MO injected embryos (j) and uninjected wild-type embryos (g). Expression in the endoderm (arrow) and forerunner cells (arrowhead) is strongly reduced or absent in *cas*-MO injected embryos. (h,k) *cas*-MO injected embryos exhibit cardia bifida. *nkx2.5* staining reveals two separated heart tubes (arrows) in the *cas*-MO injected embryos (k), whereas the wild-type control shows a single heart tube (h, arrow).

endodermal cells, 43 % mesendodermal cells, 12% mesodermal cells, $n = 361$ cells in 53 wild-type embryos), such as the pharyngeal endoderm (Fig. 5a,e,l), the gut (Fig. 5b,f,m) as well as head (hatching gland; Fig. 5a, *) and tail mesodermal derivatives (Fig. 5g), but infre-

quently to mesodermal structures. In contrast, labeled clones developed mostly into mesodermal derivatives in *cas* mutant embryos (0% endodermal cells, 38% mesendodermal cells, 62% mesodermal cells, $n = 269$ cells in 31 *cas* mutant embryos), such as head mesenchymal cells (Fig. 5c,h,n), the heart primordia (Fig. 5d,i,o), the pronephros (Fig. 5d,j,p), blood (Fig. 5d,k,q), and somites (data not shown). *cas* mutant cells also contributed to the hatching gland, which is present in *cas* embryos. We conclude that, in the absence of a functional *cas* gene, the marginalmost endodermal progenitors are respecified to a mesodermal/mesendodermal fate and that the function of *cas* in this process is to ensure the proper allocation of endodermal progenitors to endoderm-derived tissues.

Endodermal progenitors appear to be initially specified in the *cas* mutant. Indeed, *cas* embryos normally express the early endodermal markers *gata5* (Reiter et al. 1999, 2001), *mixer (bon)* (Kikuchi et al. 2000), *her5* (Bally-Cuif et al. 2000), and *cas* itself before the onset of gastrulation, indicating that the field from which endodermal progenitors originate has been properly defined in the mutant. However, *cas* embryos do not express the late

Figure 4. *cas* acts in an instructive manner downstream of nodal signals during gastrulation. (a–d) CAS acts in an instructive manner within the Nodal pathway during endoderm induction. *Sox17* expression at 60% epiboly, dorsal view. (a,b) Injection of *MZoepl* mutant embryos with 100 pg of *cas* mRNA restores *sox17* expression (b), which is missing in the uninjected control *MZoepl* mutant embryo (a). (c,d) Coinjection of 1.2 pg of *Tar** and 40 pg of *cas* mRNA restores the endoderm inducing activities of *TAR** in the *cas* mutant background (d). No *sox17* expression can be detected in control *cas* mutant embryos injected with 1.2 pg of *Tar** RNA only. (e,f) *cas* regulates its own expression from the beginning of gastrulation onward. Embryos are at the bud stage. Shown is a posterior view with the dorsal side up. (e) Blastodermal expression is lacking in *cas* mutants. (f) Sibling control. The genotype of each embryo is indicated in the upper right corner of each panel.

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Morpholinos (Nasevicius and Ekker 2000) purchased from GeneTools (Corvallis, USA) were as follows: *cas*-MO, 5'-CAGGGAGCATCCGGTC GAGATACAT-3' and control-MO, 5'-CCTCTTACCTCAGTTACAAT TTATA-3'.

These oligonucleotides were diluted in H₂O to a concentration of 0.5 mM and mixed 2 : 1 with phenol red prior to injection.

To map the fate of cells at the blastoderm margin, embryos were injected with caged-Fluorescein Dextran (FD, 20 ng, Molecular Probes). At 40% epiboly, FD was uncaged with a microbeam laser (photonics instruments) in one cell at the blastoderm margin. Embryos were doubly stained by immunohistochemistry with antifluorescein antibody coupled to peroxidase (Feldman 2000) and by in situ hybridization with the relevant probes (Hauptmann 1994). References for the probes can be obtained at http://www.zfish.uoregon.edu/cgi-bin/ZFIN_jump?record=JUMPTOGENE.

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