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 substractive 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 factors 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* (*MZoep*), 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 TGFβ 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/Hnf3β) 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 syncitial 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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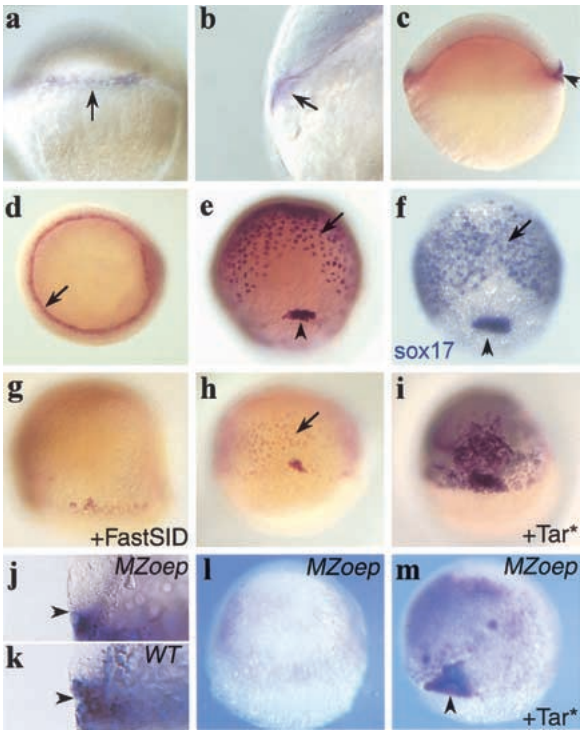

Figure 1. Expression of *10J3*, a Nodal-regulated gene in endoderm and forerunner cells and regulation by Nodal signaling. Expression of *10J3* (*a–e*) and *sox17* (*f*) during early embryogenesis. (*a*,*e*,*f*) Dorsal view, animal pole up; (*b*) lateral view; (*c*) lateral view; dorsal right; (*d*) 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, *10J3* and *sox17* expression domains within the blastoderm are very similar. (*e*,*f*) 70%–80% epiboly. (*g–i*) *10J3* 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 *10J3* 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 10J3 in *MZoep* embryos. (*j*,*k*) Optical section through the marginal region at 30% epiboly. (*l*,*m*) Dorsal view at 70% epiboly. (*j*) *10J3* is expressed only in the YSL in *MZoep* 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 *MZoep* embryos, but can be restored ectopically by overexpression of Tar* (m).

tion, continues to express the *10J3* 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 *10J3* gene could play a regulatory role in endoderm development.

The pattern of expression of the *10J3* 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, *10J3* was significantly down-regulated in injected embryos (Fig. 1g) as compared with controls (Fig. 1h). Also, the *10J3* gene was not expressed during gastrulation in the blastoderm of *MZoep* or *sqt/cyc* mutants, which do not form endoderm or forerunner cells (Fig. 1l and data not shown). Conversely, to test the responsiveness of *10J3* 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 *10J3* gene in wild-type embryos (Fig. 1h,i) and strongly induced *10J3* expression in *MZoep* 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 *10J3* gene requires Nodal signals for expression in the endoderm precursors and forerunner cells but not in the YSL.

Sequencing of the *10J3* 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 *10J3* 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 *10J3* 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 *10J3*-specific primers and sequenced. *cas* mutants were found to harbor a point mutation downstream of the *10J3* HMG-box, leading to premature termination of the *10J3*-encoded protein (Figs. 2b and 3a). This point mutation cosegregates with the *cas* mutant phenotype (Fig. 3b), demonstrating that *10J3* and *cas* are tightly linked. Expression of the wild-type but not the mutant 10J3 protein rescued the expression of the endodermal marker *sox17* in *cas* embryos (Fig. 3c–e). Furthermore, injection of antisense morpholino oligonucleotides specifically directed against *10J3* (*cas*-MO, Fig. 2b) phenocopied the *cas* mutant phenotype: (1) injected embryos lacked cells ex-

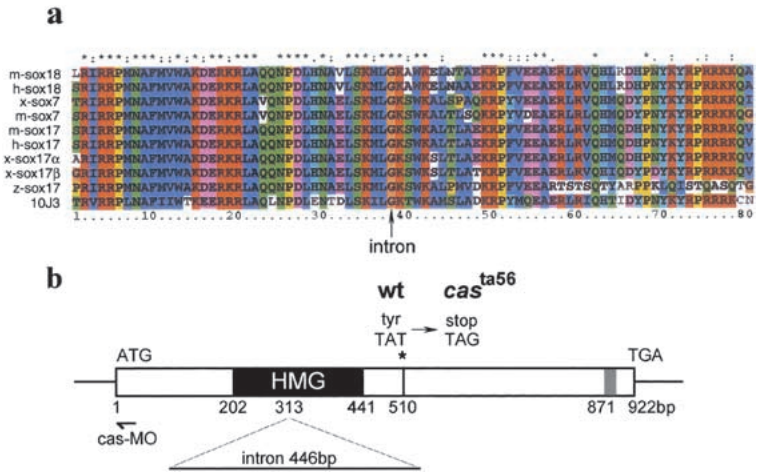

Figure 2. *10J3* encodes an HMG box transcription factor. (*a*) Comparison of the HMG box regions of several vertebrate Sox transcription factors. *10J3* and zebrafish (z) *sox17* encode members of the F subfamily. Similar types of residues are color coded. (*) Residues conserved completely in the alignment (see Materials and Methods). (*b*) Schematic representation of the *10J3* 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.: *10J3*/*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*/*HNF3*β; 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 *10J3* 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 10J3 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 *10J3* 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. *MZoep* 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 endoderm formation, we injected *cas* mRNA into *MZoep* embryos. *cas* mRNA led to a strong ectopic activation of *sox17* (Fig. 4a,b) and *gata5* (data not shown) in *MZoep* 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 photoactivatable, 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%

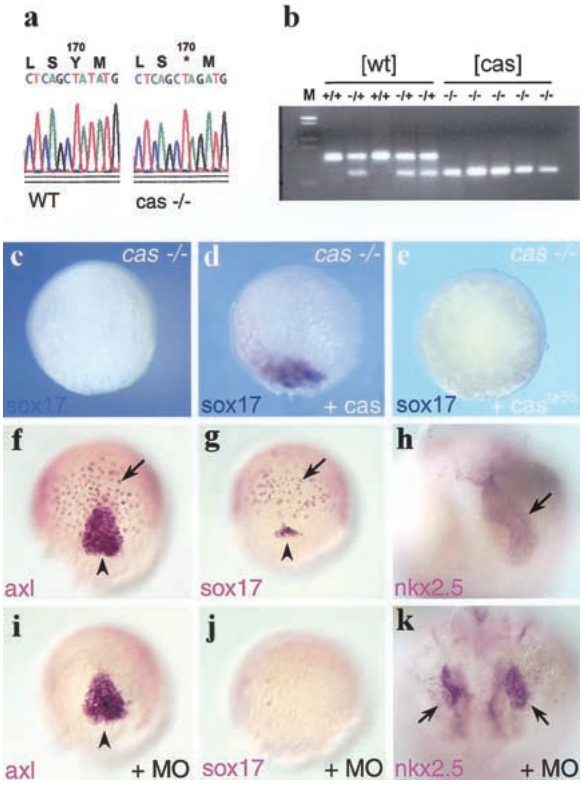

Figure 3. CAS is encoded by the *10J3* gene. (*a*) Sequencing of *cas*^{ta56} and sibling genomic DNA reveals a T-to-G mutation at position 510 that leads to an amber stop codon instead of a tyrosine. (*b*) Segregation analysis with a CAPS marker reveals cosegregation of the mutation in the homozygous 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 mesendodermal derivatives (Fig. 5g), but infrequently 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

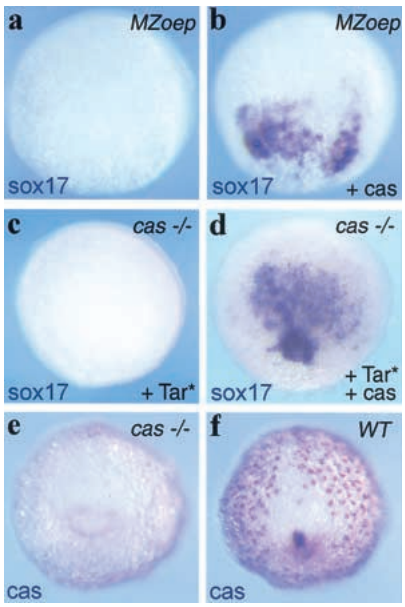

Figure 4. *cas* acts in an instructive manner downstream of nodal signals during gastrulation. (*a–d*) CAS acts in an instructive manner within the Nodal pathway during endoderm induction. *Sox17* expression at 60% epiboly, dorsal view. (*a,b*) Injection of *MZoep* mutant embryos with 100 pg of *cas* mRNA restores *sox17* expression (*b*), which is missing in the uninjected control *MZoep* 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.

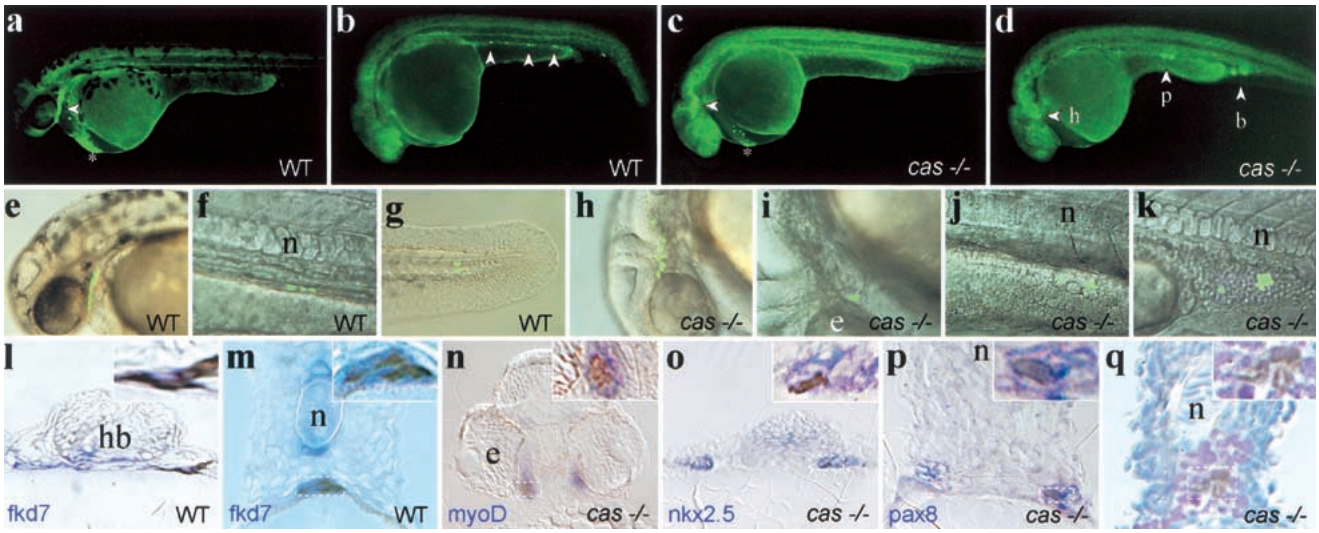

Figure 5. Marginalmost blastomeres are transfated to mesoderm in *cas* embryos. One (*b*,*d*) or five (*a*,*c*) blastomeres of the most marginal row were labeled at 40% epiboly by uncaging the photoactivatable FD. At a later stage (30–36 h), embryos were photographed under low magnification epifluorescence (*a–d*, whole-mount side views, anterior to the left) or high magnification (*e–k*) to visualize cells harboring uncaged FD. Higher magnification views were superimposed on Nomarski pictures (*e–k*). (Open arrowheads) Fluorescent cells or groups of cells. (*a*,*b*,*e–g*) Fate acquired by marginalmost precursors in wild-type embryos. Progenitors contributed cells to the pharyngeal endoderm in the head (*a*,*e*) and the gut in the trunk (*b*,*f*), in addition to the hatching gland (*a*, ⋆) and tail mesendoderm (*g*; see Bally-Cuif et al. 2000) as well as occasional mesoderm cells (blood in *b*). (*c*,*d*,*h–k*) Principal fates acquired by marginalmost precursors in *cas* embryos. Progenitors contributed mostly to head mesenchymal cells (*c*,*h*), myocardial cells (*d*,*i*), trunk pronephric duct cells (*d*,*j*), and blood (*d*,*k*) as well as hatching gland cells (*c*, ⋆). (*l–q*) Then, embryos were fixed, stained with antifluorescein antibodies to reveal uncaged FD (brown/black) and by in situ hybridization with tissue specific markers (blue/purple) and analyzed by cross-section. A close-up view is provided in the upper right corner of each section and corresponds to the region included in the dotted rectangle. Wild-type cells are located in the pharyngeal endoderm (*l*) and the gut (*m*) and express*fkd7* (*l*,*m*). *fkd7* is also expressed in the hypochord and the ventral brain. *cas* cells are located in mesoderm-derived tissues including head muscles (*n*), the heart primordia (*o*), the pronephric duct (*p*), and blood (*q*). These tissues are revealed by the markers indicated in the lower left corner, except for in *q*, in which blood is revealed by counterstaining with hematoxylin and eosin. (b) Blood; (e) eye; (h) heart; (hb) hindbrain; (n) notochord; (p) pronephros.

endoderm specification markers *sox17* and *foxA2* in deep, nonaxial, cells (Alexander and Stainier 1999; Alexander et al. 1999), suggesting that cells from the endodermal field are unable to acquire a proper endodermal fate in subsequent stages. *cas* might control the capacity of cells to involute during gastrulation and reach the blastoderm, as is observed in Nodal mutants (Feldman et al. 2000). This hypothesis is not consistent with our observation that marginal cells do involute in *cas* mutant embryos. On the contrary, the fact that marginal *cas* cells normally fated to endoderm adopt a mesodermal fate, combined with the fact that *cas* induces endodermal markers in Nodal-deficient mutants strongly supports the idea that the function of *cas* is to specify the endodermal identity of marginal cells downstream of Nodal signals.

Materials and methods

Fish stocks and embryo production

Mutant alleles used were: *cas*^{z321}, a mutation identified among the progeny of *swirl* heterozygous fish obtained from the Tuebingen stock center. This allele proved identical to the *cas*^{ta56} allele and was renamed *cas*^{ta56} (Chen et al. 1996) and *oep*^{m134} (Schier et al. 1996). *MZoep* was generated as described previously (Gritsman et al. 1999).

In situ analysis

In situ hybridization was performed as described (Hauptmann and Gerster 1994).

Cloning of cas *and mapping of the mutation*

The *10f3* cDNA was identified in a subtractive screen for TARAM-A inducible genes in zebrafish gastrula. A partial cDNA (MPMGP637J0310) was identified on a macroarray of a shield stage library (RZPD library no. 637; Clark et al. 1999). Details of the screen will be published elsewhere. A gastrula-stage cDNA library (provided by T. Lepage) was screened with the *10f3* probe to obtain full-length cDNAs, which were confirmed by RACE on mRNA and analysis of genomic DNA. A stop codon resides 42 bp upstream of the first ATG, indicating that the isolated cDNA clone most likely contains the entire open reading frame.

Alignments and phylogenetic tree building were carried out by use of the CLUSTAL X program (Thompson et al. 1997).

To identify the mutation in the *cas*^{ta56} allele, genomic DNA from mutants was amplified by PCR and sequenced. Cosegregation of the point mutation with the *cas* mutant phenotype was monitored by use of a CAPS marker (codominant cleavable amplified polymorphic sequences): A reverse oligonucleotide carries a point mutation generating a *Bgl*II site in conjunction with the point mutation in *cas*^{ta56} but not with the wild-type *cas* allele. By use of a forward oligonucleotide, a 112-bp fragment is amplified irrespective of the genotype, which is not cleaved in the wild-type allele but is cleaved into 82- and 30-bp fragments in the mutant allele.

Oligonucleotide sequences were as follows: reverse (mutated) primer, 5′-GGGCCGCTGAGGGGCTTG ACCTTGAAAGAT-3′ and forward primer, 5′-ACGAAAGTGCAACAAGCGGTGCAGCAAGATG-3′.

Microinjection and cell fate determination

The *cas* cDNA was subcloned into pCS2 (Turner and Weintraub 1994). The *cas*^{ta56} mutation was introduced into this plasmid. Synthesis of capped mRNA and microinjection into zebrafish early embryos were as described (Peyrieras et al. 1998).

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

These oligonucleotides were diluted in H_2O 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/cgibin/ZFIN jump?record=JUMPTOGENE.

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