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## Molecular integration of *casanova* in the Nodal signalling pathway controlling endoderm formation

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### SUMMARY

Endoderm originates from a large endomesodermal field requiring Nodal signalling. The mechanisms that ensure segregation of endoderm from mesoderm are not fully understood. We first show that the timing and dose of Nodal activation are crucial for endoderm formation and the endoderm versus mesoderm fate choice, because sustained Nodal signalling is required to ensure endoderm formation but transient signalling is sufficient for mesoderm formation. In zebrafish, downstream of Nodal signals, three genes encoding transcription factors (*faust*, *bonnie and clyde* and the recently identified gene *casanova*) are required for endoderm formation and differentiation. However their positions within the pathway are not completely established. In the present work, we show that

*casanova* is the earliest specification marker for endodermal cells and that its expression requires *bonnie and clyde*. Furthermore, we have analysed the molecular activities of *casanova* on endoderm formation and found that it can induce endodermal markers and repress mesodermal markers during gastrulation, as well as change the fate of marginal blastomeres to endoderm. Overexpression of *casanova* also restores endoderm markers in the absence of Nodal signalling. In addition, *casanova* efficiently restores later endodermal differentiation in these mutants, but this process requires, in addition, a partial activation of Nodal signalling.

Key words: *casanova*, Endoderm, Nodal, Cripto, Rescue, Zebrafish

### INTRODUCTION

Endoderm gives rise to the gut, associated organs like the pancreas and the liver, and to the respiratory tract. The molecular mechanisms ensuring proper endoderm specification and differentiation have recently been explored and led to a two-step model, initially proposed in frogs (Yasuo and Lemaire, 1999). In a first step, a combination of maternal signals including the transcription factor VegT allows the transcription of primary specification genes encoding activins, Nodal-related factors and the transcription factor Mix1 (Clements et al., 1999; Zhang et al., 1998a). In a second step, endoderm formation is controlled by cell-cell communication events, regulated in part by the primary specification genes (Yasuo and Lemaire, 1999).

In particular, similar to mesoderm, endoderm formation relies at least on one event of induction, mediated by extracellular signalling molecules, either released from a neighbouring tissue, like the yolk syncytial layer (YSL) in fish, or potentially from presumptive endodermal cells themselves (Rodaway et al., 1999; Yasuo and Lemaire, 1999). Overexpression and dominant interference experiments have

shown roles for TGF $\beta$ -related ligands, Vg1, activins, Nodal-related (Ndr) molecules 1 and 2, and Derrière, as well as their extracellular antagonists, activin/lefty in this induction (Yasuo and Lemaire, 1999). Analyses carried out in mice and fish have highlighted the specific function of a Nodal signalling pathway (Alexander and Stainier, 1999; Schier and Shen, 2000). Mouse embryos mutant for the *nodal* gene and zebrafish mutants in both the *nodal*-related genes *cyclops* (*cyc*) and *squint* (*sqt*) do not develop endoderm or mesoderm (Conlon et al., 1994; Feldman et al., 1998; Zhou et al., 1993). Conversely, a mutation in *lefty* leads to embryos with an excess of mesendodermal tissue (Meno et al., 1999). Endoderm (and mesoderm) formation also requires the function of an EGF-CFC factor encoded by the gene *cripto* in mouse and *one-eyed-pinhead* (*oep*) in zebrafish (Ding et al., 1998; Minchiotti et al., 2000; Schier et al., 1997; Strähle et al., 1997; Xu et al., 1999; Zhang et al., 1998b). Mouse *cripto* mutants and zebrafish embryos lacking maternal and zygotic *oep* expression (MZ*oep* embryos) exhibit phenotypes similar to *nodal* mutants or *cyc*; *sqt* double mutants, indicating that *nodal*-related genes and *cripto/oep* act in the same signalling pathway (Ding et al., 1998; Gritsman et al., 1999; Xu et al., 1999). Further

experiments in zebrafish have demonstrated that *Oep/Cripto* is required as a permissive cofactor downstream of *nodal*-related genes in mesoderm and endoderm formation (Gritsman et al., 1999; Peyri ras et al., 1998).

TGF -related molecules act through the binding to a cell surface type II receptor, followed by the recognition and activation of a type I receptor that conveys the signal intracellularly to activate specific sets of genes (Massague, 2000). Recent biochemical experiments have shown that Nodal signals through the type II receptor ActRIIB and the type I receptor ALK4/ActRIb or the orphan receptor ALK7, but efficient binding of the ligands to the receptors and activation of signalling require the binding of the extracellular EGF-CFC protein Cripto to ALK4/ActRIb and/or ALK7 (Reissmann et al., 2001; Yeo and Whitman, 2001). Consistent with this idea, inactivation of the *alk4/ActRIb* gene led to phenotypes similar to those observed in *nodal* mouse mutants (Gu et al., 1998). Moreover, overproduction of ALK4\* or Tar\*, activated forms of ALK4 or the ALK4-related zebrafish type I receptor Taram-A (Tar), induces mesodermal and endodermal markers (Armes and Smith, 1997; Bally-Cuif et al., 2000; Peyri ras et al., 1998; Renucci et al., 1996; Yasuo and Lemaire, 1999). Last, ectopic expression of Tar\* changes the fate of early zebrafish blastomeres to endoderm and rescues endoderm formation in *oep* embryos, demonstrating that *oep* is required upstream of Tar/ALK4/ActRIb (Gritsman et al., 1999; Peyri ras et al., 1998).

Downstream of Nodal-related signals and their receptors/co-receptors, several types of genes control vertebrate endoderm formation and differentiation. In frogs and fish, overexpression and dominant interference experiments have implicated transcription factors belonging to the Mix/milk/mixer, Bix and Gata families, and the HMG box factor Sox17 in endoderm induction (Hudson et al., 1997; Rosa, 1989; Henry and Melton, 1998; Ecochard et al., 1998; Lemaire et al., 1998; Weber et al., 2000).

Zebrafish mutations affecting endoderm development have opened the way to a definition of the genetic hierarchy by which Nodal-related signals control this process (Alexander and Stainier, 1999). In zebrafish, endoderm progenitors originate from the margin of the embryo and involute soon after the onset of gastrulation (Dickmeis et al., 2001a; Warga and Nusslein-Volhard, 1999). Once involuted, they express *sox17* and *foxa2* (previously known as *axial/HNF3 *) (Alexander and Stainier, 1999; Str hle et al., 1993). Downstream of *cyc*, *sqt* and *oep*, three genes have been defined at the molecular level (*bonnie and clyde* (*bon*), *faust* (*fau*) and *casanova* (*cas*)) that are required for the expression of *sox17* and *foxa2* and for endoderm development. The *fau* and *bon* genes require Nodal signalling for their expression and encode a Gata5-related and a Mixer-related protein, respectively (Kikuchi et al., 2000; Reiter et al., 1999). Mutants in *fau* have fewer endodermal cells and *bon* mutants lose most of them (Reiter et al., 2001). Overexpression of *bon/mixer* can induce a small number of endodermal progenitors in *cyc*;*sqt* double mutants, suggesting that *bon/mixer* acts downstream of Nodal signals (Kikuchi et al., 2000). The *fau/gata5* and *bon/mixer* genes act in parallel on endoderm formation because *fau*;*bon* double mutants have a stronger endodermal phenotype than single mutants and the combination of *bon/mixer* and *fau/gata5* RNA induces more endodermal cells than either RNA alone (Reiter et al., 2001).

The *cas* gene is required for endoderm formation, because

*cas* mutants do not express any known endodermal marker during gastrulation and do not differentiate endodermal derivatives (Alexander et al., 1999; Alexander and Stainier, 1999). Activation of Nodal signalling by ectopic expression of *tar\**, *bon/mixer* or *fau/gata5* fail to induce endoderm in *cas* mutants, indicating that *cas* is required for the action of these Nodal signalling components (Alexander and Stainier, 1999; Reiter et al., 2001). The *cas* gene encodes a novel high mobility group (HMG) protein related to Sox17 (Dickmeis et al., 2001a; Kikuchi et al., 2001) that is expressed in endoderm-like cells during gastrulation. Ectopic expression of *cas* can induce a population of *sox17*-positive cells in MZ*oep* embryos, showing that it can act downstream of Nodal signals, although it is not clear whether it is sufficient to ensure that the fate of early blastomeres is changed to endoderm. In the absence of *cas* activity, endodermal progenitors are respecified to mesoderm (Dickmeis et al., 2001a).

Several genes instrumental to the formation of endoderm have been identified but it is now essential to understand how these genes, particularly *cas*, act within the Nodal signalling pathway to ensure proper endoderm development and differentiation. First, the precise position of *cas* in the genetic hierarchy controlling endoderm development and the mechanism by which it influences the cell fate choice at the margin remain to be defined. Second, although Nodal signalling is essential for both endoderm and mesoderm development, it needs to be determined whether these two tissues have the same requirement for the duration and time of activation of the pathway.

By restoring *oep* activity at precise time points in MZ*oep* embryos, we first show that, in contrast to mesoderm development, endoderm development requires sustained Nodal signalling. Then, we show that *cas* is initially expressed in a subdomain of the *bon/mixer* expression domain, that *cas* expression requires a functional *bon/mixer* gene and can be induced in wild-type but not in MZ*oep* embryos upon ectopic *bon/mixer* expression. Last, we have analysed the activities of *cas* when overexpressed in zebrafish embryos and determined its requirements for defined components of the Nodal signalling pathway. We show that *cas* can induce early endodermal markers and repress mesodermal markers in wild-type embryos, and can change the fate of early blastomeres to endoderm. The *cas* gene can also rescue early endodermal markers missing in *oep* and *bon/mixer* embryos, but late differentiation of endoderm requires partial activation of Nodal signalling in addition to *cas* overexpression. Thus, our data allow us to place *cas* within the Nodal signalling pathway downstream of *bon* and demonstrate that *cas* can induce an endodermal fate and differentiation, but requires additional elements of the Nodal signalling pathway to do so.

## MATERIALS AND METHODS

### Zebrafish embryos

Adults were maintained as described by Westerfield (1994). Wild-type and mutant embryos were obtained by natural crosses of wild-type fish and homozygous *oep*<sup>ts57</sup>, heterozygous *bon*<sup>m425</sup> or heterozygous *fau*<sup>s26</sup> mutant fish, respectively (Chen et al., 1996; Hammerschmidt et al., 1996). Embryos were maintained and staged according to Kimmel et al. (Kimmel et al., 1995).

### Cripto production and purification

Recombinant Cripto protein was produced in cell strain 293 as a histidine-tagged fusion protein lacking Cripto amino acid residues +156 to +172 and purified from the conditioned medium by metal chromatography.

### Microinjection

Capped RNAs were synthesized with SP6 polymerase using the mMESSAGE mMACHINE SP6 kit (Ambion) from pCS2cas, pSP64Ttar\*, pCS2gfp, pCS2nls-lacZ, pCN3Xmixer or pSport-zgata5 that carries a full length *fau/gata5* cDNA obtained during a screen for Nodal-inducible genes (Dickmeis et al., 2001b). Purified RNA solutions were injected into wild-type or mutant embryos at the 1/4-cell stage (2 nl) or into one marginal blastomere at the 8/16-cell stage (100 pl) with 0.1% phenol red. In some injections, *gfp* or *nls-lacZ* RNA were added as lineage tracers. We verified that Xmixer RNA was able to rescue endoderm markers in *bon* embryos.

### Whole-mount in situ and immunohistochemical staining

Two-colour whole-mount in situ hybridization and immunohistochemical staining were performed as previously described (Hauptmann and Gerster, 1994).

### Grafting experiments

Donor embryos were injected at the four-cell stage into one blastomere with *gfp* (80 pg) and *nls-lacZ* (120 pg) RNAs as lineage tracers, either alone or combined with *tar\** (1.2 pg) or *cas* (40 pg) RNAs. At sphere stage, 1-20 donor (green) cells were grafted to the margin or to the animal pole of hosts (Ho and Kimmel, 1993). Embryos were then cultured in embryo medium with penicillin 10 U ml<sup>-1</sup> and streptomycin 10 µg ml<sup>-1</sup>.

## RESULTS

### The *casanova* gene is expressed within prospective endoderm progenitor cells

The *cas* gene is first expressed in late blastulae (dome-30% epiboly stage) in a group of superficial dorsal marginal cells (Dickmeis et al., 2001a). At this stage and during the whole gastrulation process, *cas* is also expressed in the YSL (data not shown). About 40 minutes later (40% epiboly), distinct cellular tiers are discernible from vegetal to animal positions, with tier 1 corresponding to the first row of cells at the blastoderm margin and tiers 2 and higher residing further from the margin. At this stage, *cas* expressing cells occupy a superficial position within tiers 1-4 (Fig. 1C,G,U,V). Expression is mosaic and is observed preferentially in cells located close to the margin, with a higher frequency on the dorsal side of the embryo. This spotted pattern is reminiscent of the endoderm fate map established in late blastula (40% epiboly). Indeed, marginal cells located within tiers 1-4 are fated to become endoderm, mesoderm or both, the probability of populating the endoderm increasing with the proximity from the margin and from the dorsal side of the late blastula (Dickmeis et al., 2001a; Warga and Nusslein-Volhard, 1999). Because *cas* is required cell autonomously for endoderm development from blastula stages on, the similarity in the early *cas* expression and the expected position of endodermal precursors strongly suggest that *cas* expression already delineates some of the endodermal precursors.

At the onset of gastrulation (50% epiboly), the mosaic *cas* expression pattern is maintained but *cas*-positive cells are found closer to the margin and a significant proportion of the cells are now found in deep positions, abutting the YSL,

probably as a consequence of involution movements (Fig. 1K,W). In addition, on the dorsal side of the embryo, *cas* is strongly expressed in a group of marginal superficial cells, which do not appear to involute and probably represent the so-called forerunner cell cluster (Cooper and D'Amico, 1996; Melby et al., 1996). During gastrulation, except for the forerunner cells, embryonic expression is found only in cells in a deep position, abutting the YSL, which eventually scatter over its surface (Fig. 1O,S,X,Y). Evidence that these cells are of endodermal nature comes from their flat, star-shaped appearance and from their scattered distribution over the YSL (Alexander and Stainier, 1999; Warga and Nusslein-Volhard, 1999).

To understand the spatial and temporal relationships between *cas* expression and endoderm development, we compared the *cas* expression pattern to those of *bon/mixer*, *fau/gata5* and *sox17* which have important functions in endoderm development. First, *bon/mixer* is expressed before *cas*, at the sphere stage on the dorsal side, both in the YSL and in a ring of marginal cells that expands at 40-50% epiboly to generate a continuous ring encompassing six tiers of marginal cells (Fig. 1A,E,I) (Alexander and Stainier, 1999; Kikuchi et al., 2000). At the shield stage, expression stops (Fig. 1M,Q). Expression of *fau/gata5* initiates slightly later than that of *bon/mixer* (dome stage) in both the YSL and a ring of marginal cells, in a pattern similar to *bon/mixer* (data not shown) (Reiter et al., 2001; Rodaway et al., 1999). At 40% epiboly, *fau/gata5* is expressed in the whole depth of the blastoderm and encompasses four tiers from the margin (Fig. 1B,F,J). At the end of gastrulation, *fau/gata5*-positive cells have adopted a position and a shape typical of endodermal cells (Fig. 1R and data not shown). Comparison of the *cas* early expression domain with those of *bon* and *fau* shows that *bon* initiates expression before *cas* and *fau*, and that *cas* is expressed in a subdomain of *bon*- and *fau*-positive cells.

The *sox17* gene is first expressed at 40% epiboly, in a group of superficial dorsal marginal cells (Fig. 1D,H). This pattern is modified at the shield stage, when two different populations of *sox17*-positive cells can be observed: the forerunner cells and a group of dorsal deep endodermal precursors (Fig. 1P). Later on, *sox17* cells adopt a shape and position very similar to *cas* cells (Fig. 1T). Thus, *cas* is expressed before *sox17* and in a very similar blastodermal domain.

Expression of *cas* represents an interesting basis for understanding the molecular mechanisms leading to the cell fate choice between mesoderm and endoderm. Both endoderm and mesoderm require functional Nodal signalling and are induced by Nodal signals. However, *cas* is expressed in only a small region of the *bon/mixer* and *fau/gata5* expression domains before gastrulation, strongly arguing that specific mechanisms are involved in ensuring proper differential initiation of these markers. In particular, whereas *fau/gata5*, *bon/mixer* and *cas* all require and may be induced by Nodal-related signals, additional mechanisms, yet to be defined, must be postulated to explain the restriction of *cas* initiation to a few marginal cells.

### Expression of *casanova* requires Nodal signalling and is induced cell autonomously upon Nodal activation

The *cas* gene is not expressed in MZ*oep* embryos and thus

requires Nodal signalling (Dickmeis et al., 2001a). We studied whether the transient activation of the Nodal pathway induced by an early *Oep* function was sufficient to induce a normal level of *cas* expression. Expression of *cas* was analysed in embryos devoid of zygotic *oep* contribution (*Zoep* embryos) (Schier et al., 1997; Strahle et al., 1997). In late blastula (40% epiboly) and during gastrulation, *Zoep*<sup>tz57</sup> homozygous embryos had either a dramatic reduction of the number of *cas* expressing cells or no expressing cells at all within the blastoderm (Fig. 2A-D). Thus, similar to the expression of the endodermal markers *sox17* and *foxa2* and the endodermal differentiation marker *fkf7* (*foxa1*) *cas* expression requires *oep* function and Nodal signalling. However, the early transient Nodal signalling, associated with the maternal *oep* expression is not sufficient to ensure full *cas* expression (Alexander and Stainier, 1999). By contrast, previous work has shown that most mesodermal derivatives form normally in *Zoep* embryos (Schier et al., 1997; Strahle et al., 1997), indicating that

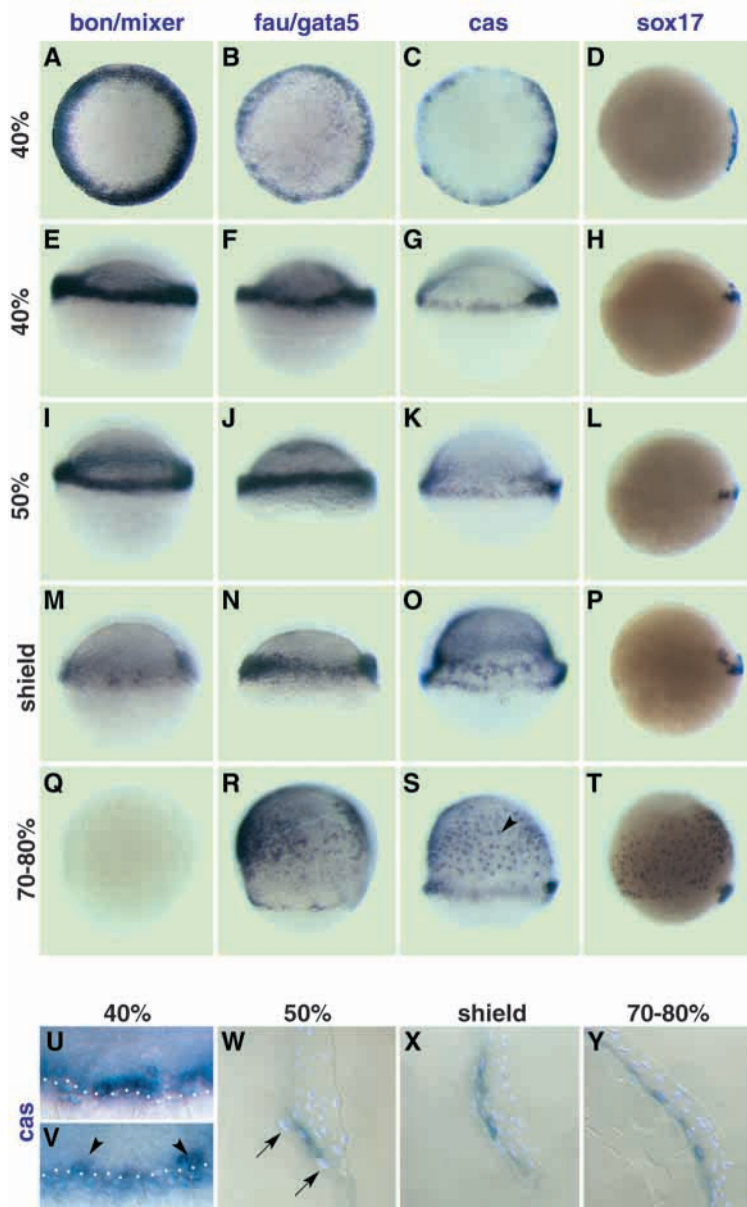
attenuated Nodal signalling is sufficient to allow mesoderm but not endoderm formation.

Expression of *cas* within the blastoderm is induced upon the activation of the Nodal pathway by *Tar\** (Dickmeis et al., 2001a). This induction could be either cell autonomous or non-cell autonomous. To address this issue, we microinjected an RNA encoding the lineage tracer *nls-lacZ* (100 pg) alone as a control or combined with *tar\** RNA (1.2 pg) into early donor embryos. At the late blastula stage (sphere stage), small groups of cells were transferred from donor embryos to the animal pole region of host untreated embryos, which were allowed to develop until mid-gastrulation, fixed and stained for the expression of *cas* and of the lineage tracer. This showed that *tar\** but not *lacZ* induced the expression of *cas* in grafted cells but not in host cells (Fig. 2E,F). Thus, consistent with the endodermal expression of *cas* and the autonomous induction of endodermal progenitors by *Tar\**, *cas* expression is induced in a cell autonomous fashion by activation of the Nodal pathway.

### Endoderm formation and *casanova* expression require sustained Nodal signalling

The analysis of zygotic *oep* mutants indicates that attenuated Nodal signalling is not sufficient to allow endoderm development. *MZoep* embryos do not develop endoderm. We wished to know when activation of Nodal signalling would be required to allow endoderm development in these mutants.

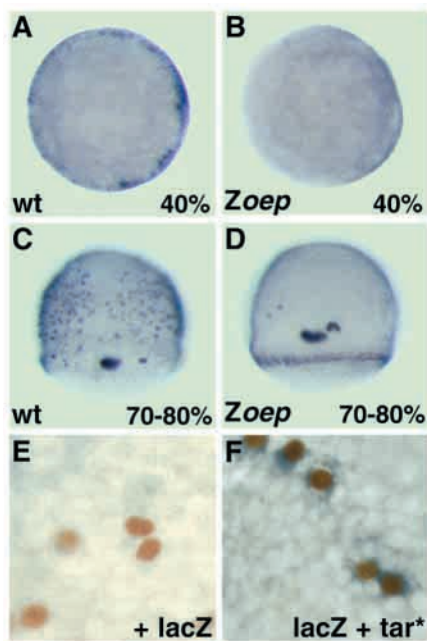
*MZoep* embryos can be fully rescued by microinjection of an RNA encoding a soluble form of



**Fig. 1.** Dynamics of expression of *bon/mixer*, *fau/gata5*, *casanova* and *sox17* genes in wild-type embryos at 40% epiboly (A-H), 50% epiboly (I-L), shield (M-P) and 70-80% epiboly (Q-T) stages. (A-D) Animal pole views; (E-T) lateral views, dorsal to the right. At 40% epiboly, whereas *bon/mixer* (A,E) and *gata5/fau* (B,F) are homogeneously expressed in large marginal domains, *casanova* expression is mosaic and preferentially restricted to the most marginal blastomeres of the dorsal side (C,G). At this stage, *sox17* is expressed only in the superficial and marginal cells of the dorsal side (D,H). At 50% epiboly, expression patterns of *bon/mixer* (I), *gata5/fau* (J) and *sox17* (L) are roughly unchanged. The *casanova* pattern is still mosaic but it is found throughout the margin and in the forerunner cells (K). At the shield stage, *bon/mixer* (M) and *gata5/fau* (N) are expressed in more germ ring blastomeres. Cells expressing *casanova* have begun to involute and abut the YSL, except the forerunner cells, which remain superficial (O). The *sox17* gene is expressed in deep cells abutting the YSL in the dorsal axis (P). After the onset of gastrulation, *bon/mixer* (Q) is no longer expressed, whereas *fau/gata5* (R), *casanova* (S) and *sox17* (T) are expressed in the scattered endodermal cells (arrowhead); *casanova* (S) and *sox17* (T) are still expressed in the forerunner cells. (U,V) Close up of the mosaic pattern of *casanova* at the dorsal margin (U) and lateral margin (V) of embryos at 40% epiboly stage (notice the isolated blastodermal *cas*-positive cells (arrowheads); the dotted lines mark the YSL-blastoderm frontier). (W-Y) Cross sections of embryos following whole-mount in situ hybridization with *casanova* at 50% epiboly (W), shield (X) and 70-80% epiboly stage (Y), animal pole up (arrows point to YSL nuclei). The *cas* positive cells involute at the margin, abut YSL and spread over the whole embryos with a scattered pattern.

Oep into their YSL (Gritsman et al., 1999), or by injecting a purified recombinant preparation of soluble Cripto protein into the extracellular space (Minchiotti et al., 2001; Minchiotti et al., 2000; Reissmann et al., 2001). We reasoned that Cripto protein injection should readily allow the initiation of Nodal signalling in these embryos because it restored the early marker *bon/mixer* in MZ*oep* embryos within 1 hour from the injection time (data not shown). We then tested when expression of Cripto protein was able to rescue endoderm development in MZ*oep* embryos. Cripto protein or bovine serum albumin (BSA), as a control protein solution, was injected into the extracellular space of early to late MZ*oep* blastula, which were allowed to develop until mid- to late gastrula stages (70-80% epiboly) or 30 hours postfertilization (hpf) and were probed with the early markers *cas*, *sox17* or *foxa2*, or the endoderm differentiation marker *fkf7*. Control injections never led to any rescue (not shown). On the contrary, injections of soluble Cripto protein rescued endoderm development in a dose- and time-dependent fashion. All kinetics experiments were carried out with 2 ng Cripto protein, a dose ten times higher than the minimal dose required to induce full endodermal and embryonic rescue. Four classes of embryos can be recognized according to their degree of endodermal marker restoration. Class I embryos cannot be distinguished from wild-type

embryos (Fig. 3B,F,J). Class II embryos have a reduction in the number of expressing cells whereas class III exhibit a further reduction of expressing cells (less than 50) and class IV exhibit very few, if any, expressing cells (Fig. 3C-E,G-I,K-N). When Cripto was injected before or at the sphere stage, full restoration (class I) was achieved, indicating that Nodal signalling is not essential for endoderm development before this stage (Fig. 4A-C). Injections at dome stage led to a noticeable shift to class II and III embryos (Fig. 4A-C). Injections at 40-50% led to very weak rescue (Fig. 4A-C). Similarly, analysis of *fkf7* expression at 30 hpf showed that Cripto protein injection before or at the sphere stage led to full rescue of *fkf7* endoderm expression, whereas injection at a later stage led to a progressively poorer rescue (Fig. 3O-S, Fig. 4D). In these experiments, posterior mesoderm markers such as the expression of *foxa2* in the axial mesoderm during gastrulation (Fig. 3J-M, asterisk, 98%,  $n=42$ ) or the marker for differentiated somites *myoD* were efficiently rescued (not shown, 100%,  $n=34$ ) up to the onset of gastrulation (40-50% epiboly). Thus, transient Nodal signalling allowed by the maternal *oep* contribution or by injection of Cripto at pregastrula stages is sufficient to ensure mesoderm development but sustained Nodal signalling is required to achieve proper endoderm development.



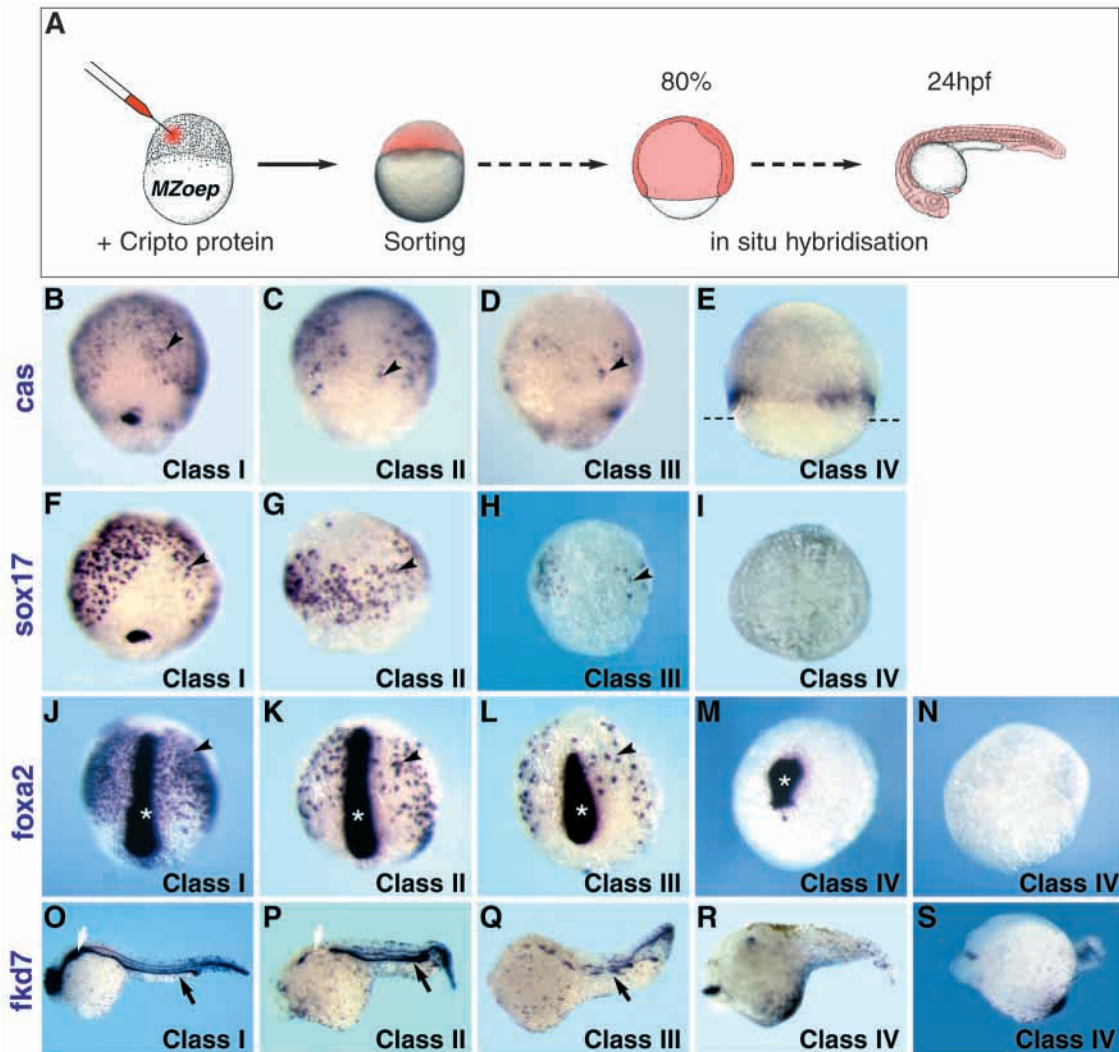
**Fig. 2.** Expression of *casanova* requires Nodal signalling. (A-D) Expression of *casanova* requires zygotic contribution of *oep*. (A,B) Animal pole views, dorsal to the right. (C,D) Dorsal views. Compared with wild-type controls (A,C), *casanova* endodermal expression is not initiated or maintained in blastula (B, 40% epiboly) nor during gastrulation (D, 70-80% epiboly) in *Zsoep* homozygous mutants. Expression in the forerunner cells and YSL is not affected. (E,F) Induction of *cas* upon Nodal signalling activation is cell autonomous. In late blastula, a few wild-type cells expressing *nls-lacZ* alone or combined with *tar\** were transplanted to the animal pole of a host wild-type embryo. During gastrulation (60% epiboly) *tar\** expressing cells transplanted to the animal pole (brown nuclear staining) autonomously express *casanova* (F; 96%,  $n=93$ ) whereas control cells do not (E; 100%,  $n=12$ ).

### Expression of *casanova* requires Bon/Mixer function

To understand in more detail the regulation of *cas* and endoderm development, we analysed whether *cas* expression was dependent on downstream components of the Nodal signalling pathway. The number of cells expressing *cas* was dramatically reduced in the blastoderm of *bon/mixer* embryos both before gastrulation (40% epiboly) and at the end of gastrulation (tail bud) (Fig. 5C-F). Expression of *cas* was also variably and slightly decreased in *fau/gata5* embryos (Fig. 5A,B). Thus, *cas* expression requires Bon/Mixer function and, to a lower extent, *fau/gata5* function. Furthermore, we tested whether *bon* and/or *fau/gata5* could induce *cas* expression in wild-type embryos and whether *bon/mixer* or *fau/gata5* and *cas* could induce each other in MZ*oep* embryos, in which Nodal signalling is inactive. Expression of *cas* induced *fau* (Dickmeis et al., 2001a) but not *bon/mixer* in MZ*oep* embryos (data not shown). On the contrary, overexpression of *Xmixer* in wild-type embryos led to a robust induction of *cas* expression (Fig. 5G,H). Overexpression of *fau/gata5* was also able to induce *cas* expression but the induction appeared weaker and more variable (data not shown). Thus our results confirm that *bon/mixer* and *fau* act upstream of *cas* in the Nodal signalling pathway controlling endoderm formation (Alexander and Stainier, 1999). However, neither *Xmixer* nor *fau/gata5* induced *cas* expression in MZ*oep* embryos (Fig. 5I,J and data not shown). We note, however, that the combination of these two factors induced, in a small number of MZ*oep* embryos a few *cas* expressing cells, which remained in the epiblast (Fig. 5K,L). Altogether, these results suggest that other factors are required in addition to *bon* and *fau*, acting downstream of Nodal signals, to specify and maintain the proper number of *cas* expressing cells (Fig. 5I-L and data not shown).

### Casanova controls endoderm fate

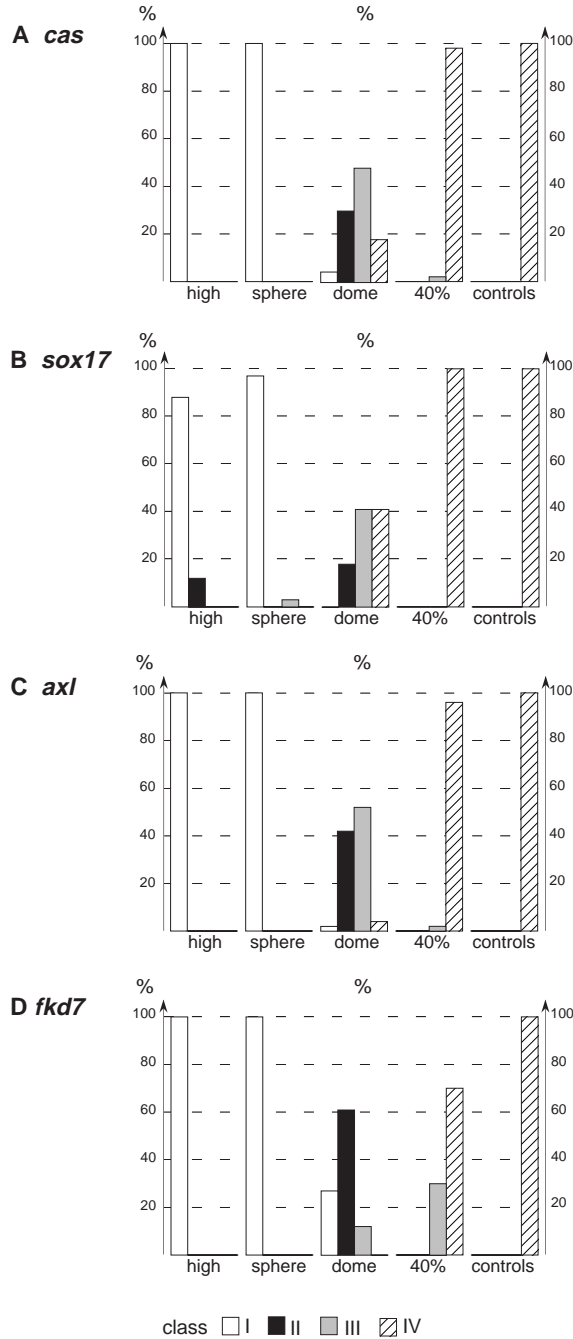
Our understanding of *cas* function for endoderm development



**Fig. 3.** Rescue of endodermal markers in MZoop by Cripto protein injection at different times. (A) Experimental procedure. Synchronized MZoop embryos were injected with Cripto protein together with rhodamine dextran as a tracer at appropriate stages (from high to 40% epiboly). Subsequently, embryos exhibiting homogeneous rhodamine fluorescence were sorted, fixed and analysed by in situ hybridization (B-S). (B-N) Lateral (C-E,G-I,N) or dorsal views. At 80% epiboly, four different classes of expression pattern of *cas* (B-E), *sox17* (F-I) and *foxa2* (J-N) can be identified in Cripto-injected MZoop embryos. When Cripto was injected at 40% epiboly, most embryos do not express *foxa2* in endodermal cells but still express it in axial mesoderm (M). (O-S) Staining of *fkd7* in Cripto-injected MZoop embryos at 30 hpf. Anterior to the left. Four classes were also defined. (O) Class I exhibits almost normal expression of *fkd7* in the pharynx (white arrow) and in the gut (black arrow). (P) Class II exhibits incomplete expression of *fkd7*, often lacking the pharyngeal endoderm (white arrow) but have an almost normal expression in the gut (black arrow). Class III has only a few *fkd7* positive cells (Q, arrow). Class IV shows no endodermal *fkd7* staining (R,S).

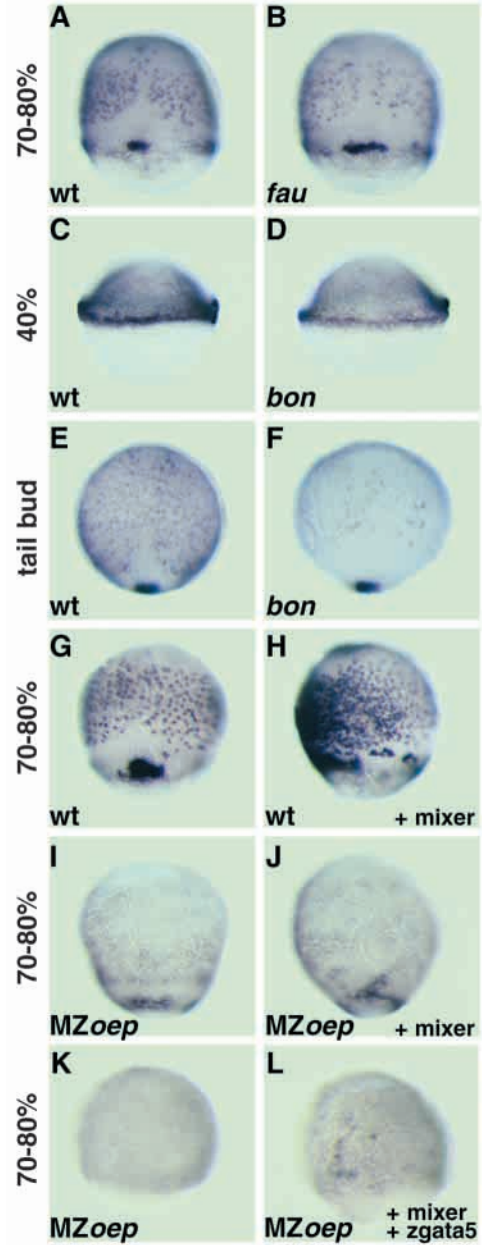
is still partial. Expression of *cas* appears essential to the endoderm versus mesoderm fate choice, but we needed to determine whether *cas* could induce a consistent change of fate of marginal cells to endoderm. To address this issue, we injected the RNA encoding the lineage tracer green fluorescent protein (GFP) alone, as a control, or combined with *cas* RNA and analysed the fate of the expressing cells with two different strategies. In the first strategy, injections were carried out in one marginal cell at the 16-cell stage (Bally-Cuif et al., 2000; Peyri ras et al., 1998). Clones of control marginal cells colonized mostly mesodermal tissues and, to a much smaller extent, endoderm and ectoderm. For instance, dorsal clones mostly populated the notochord, the prechordal plate and the neuroectoderm (Fig. 6A). By contrast, dorsal clones expressing

*cas* RNA appeared to populate the notochord and prechordal plate less frequently, but to populate the pharyngeal endoderm (Fig. 6D, arrowhead) and the forerunner derivatives more frequently (Cooper and D'Amico, 1996; Melby et al., 1996). Colonization of neuroectoderm was not affected by the presence of *cas* RNA. In the second strategy, a small group of cells from injected embryos were grafted to the margin of host untreated blastula (sphere stage) and their fate was analysed at 24 hpf. Grafts from control cells mostly colonized mesodermal tissues (Fig. 6B,C) but a fraction of the embryos (30%,  $n=17$ ) exhibited some grafted cells in the endoderm derivatives such as the pharyngeal endoderm and the gut (not shown). This fraction increased significantly (58%,  $n=20$ ) when grafted cells were derived from *cas* RNA injected embryos and more grafted



**Fig. 4.** Frequency of endodermal markers rescue in MZoep by Cripto-injection at different times, displaying the frequencies corresponding to the four classes described in Fig. 3. Full rescue (Class I) was predominantly observed until sphere stage. Reduction of the number of endodermal cells expressing *cas*, *sox17* or *foxa2* was observed when Cripto protein was injected at dome (Classes II and III). At 40% epiboly, Cripto protein was not able to induce these markers in almost all injected embryos (Class IV). Embryos probed for *foxa2* show that axial mesoderm but not endoderm was rescued when Cripto protein was injected at 40% epiboly (Fig. 3M).

cells appeared to populate endoderm than control grafts (Fig. 6E,F). However, the bulk of grafted cells still populated mostly mesodermal tissues (Fig. 6F). Thus, *cas* expression is sufficient to induce some marginal cells to acquire an endodermal fate.



**Fig. 5.** Expression of *bon/mixer* is required for *casanova* expression. (A,B) Dorsal views, 70-80% epiboly. Compared with the control (A), *casanova* is normally expressed in most *faust* mutant embryos, but is sometimes slightly reduced (B). (C,D) Lateral views, 40% epiboly. (E,F) Dorsal views, tail bud. Compared with the control (C,E), there are many fewer *cas* positive cells in *bon* mutant embryos from late blastula (D) to late gastrula (F). (G-J) Dorsal views, 70-80% epiboly. Compared with the control (G,I), *Xmixer* overexpression increases the number of cells expressing *casanova* in wild-type embryos (H) but not in MZoep mutants (J). (K,L) Combination of *Xmixer* and *fau/gata5* (L, compare with control K) induces, in a few embryos (5%,  $n=20$ ), a very small number of *cas* positive cells, which never reach the YSL.

**Expression of *casanova* induces endodermal markers and represses mesodermal markers**

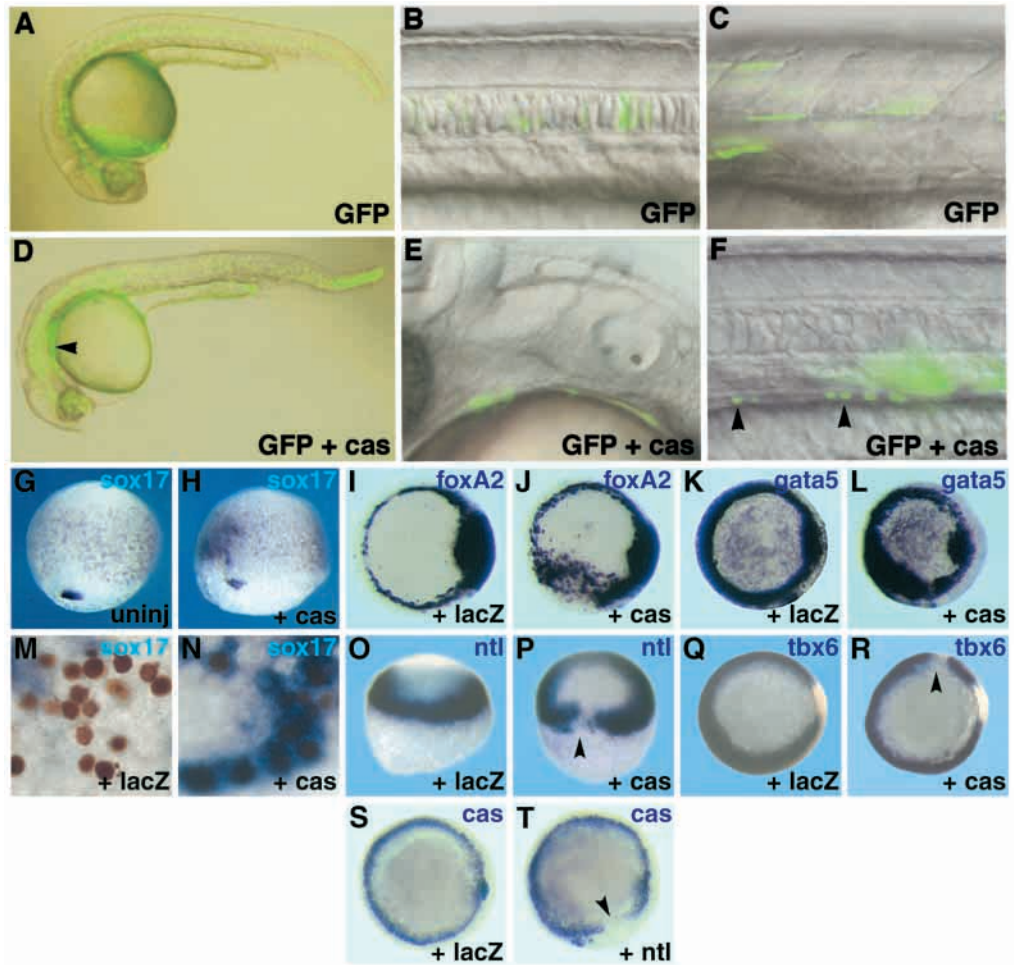
To understand how *cas* causes the observed change of cell fate, we injected *cas* RNA or *nls-lacZ* RNA as a control into one

**Fig. 6.** Expression of *cas* represses mesodermal markers, induces endodermal markers and can change the fate of cells to an endodermal identity.

(A-F) Anterior to the left, dorsal to the top. (A,D) Clonal progeny of one marginal blastomere injected with *gfp* alone (A) or combined with *cas* (D) RNA at the 16-cell stage. At 24 hpf, expression of *cas* leads to a more frequent colonization of endodermal territories, like pharynx (arrowhead in D). (B-C, E-F) This ability to change the cell fate was confirmed by transplant experiments. By blastula stage, a few cells expressing only the *gfp* gene (B-C) or both *gfp* and *cas* (E-F) were transplanted to the margin of host embryos. At 24 hpf, cells expressing *cas* more frequently became involved in endodermal derivatives such as pharynx (E) and gut (arrowhead in F). Dorsal (G-H, O-P) and animal pole (I-L, Q-R) views of shield and 75% epiboly embryos.

(G-L) Compared with the *lacZ* injected siblings (G,I,K), injection of *cas* RNA at the 8/16-cell stage induces expression of endodermal markers *sox17* (H; 100%,  $n=23$ ), *foxa2* (J; 92%,  $n=12$ ) and *gata5* (L; 93%,  $n=15$ ). (M,N) This induction is cell autonomous, as

revealed by transplant experiments (the nuclei of grafted cells has been stained in brown by immunodetection of nuclear  $\beta$ -galactosidase, used as a lineage tracer). By contrast, mesodermal markers *ntl* (O,P) and *tbx6* (Q,R) are repressed by overexpression of *cas* (arrowheads in P and R; 86%,  $n=21$  and 78%,  $n=18$ , respectively). Conversely, overexpression of *ntl* represses *cas* expression at the margin (arrowhead in T, compare with S).

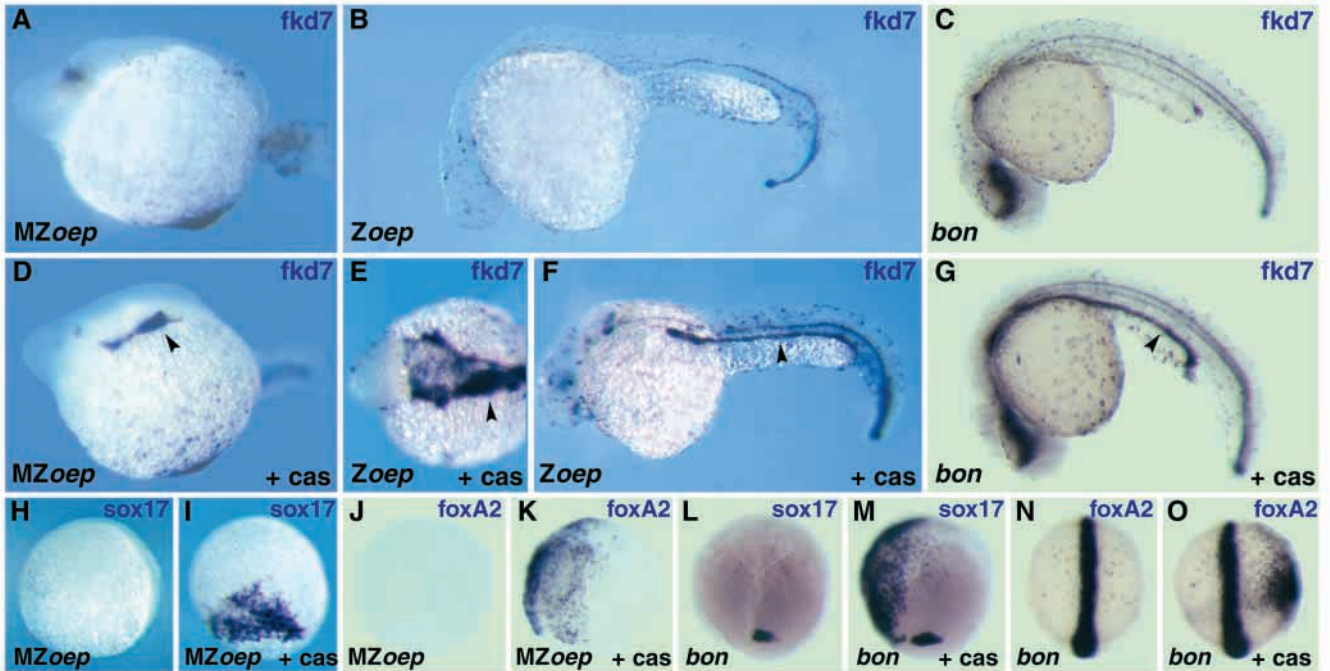


central or one marginal cell at the 8/16-cell stage in wild-type embryos and analysed the expression of early endodermal markers *sox17*, *fau/gata5*, *foxa2* and mesodermal markers *tbx6* and the Brachyury orthologue *ntl* (Hug et al., 1997; Schulte-Merker et al., 1993). Overexpression of *nls-lacZ* did not alter the expression of any of these markers (Fig. 6G,I,K,O,Q). However, *cas* expression in marginal cells led to an increased number of endodermal cells expressing *sox17*, *gata5* and/or *foxa2* (Fig. 6H,J,L). By contrast, ectopic expression of endodermal markers in embryos injected in central cells was only transient, probably reflecting the requirement for additional marginal signals in endoderm specification (data not shown). Induction was cell autonomous because, when *cas* expressing cells were grafted to the margin of host embryos, grafted cells but not neighbouring cells expressed the endodermal markers (Fig. 6M,N). In addition, consistent with the fact that, in *Xenopus*, endoderm specification genes can repress mesodermal genes (Lemaire et al., 1998), *cas* expression led to the downregulation of *tbx6* and *ntl* expression in the blastoderm (Fig. 6P,R). Because the overexpression of the *ntl* homolog *Xbra* leads, in *Xenopus*, to the downregulation of the endodermal marker *mix.1* (Lemaire et al., 1998), we

also tested whether overexpression of *ntl* would affect *cas* expression. Consistent with the above, *ntl* inhibited the blastodermal expression of *cas* (Fig. 6S,T), suggesting that *ntl* and *cas* are involved in cross-regulatory interactions, probably leading to the generation of mutually exclusive domains during gastrulation. Altogether, our results on *cas* overexpression show that it acts at gastrulation in marginal cells to activate endodermal and forerunner specific genes autonomously and to repress mesodermal specific genes.

#### Expression of *casanova* restores late differentiation of endoderm but requires functional Nodal signalling

We then wished to know whether *cas* alone was sufficient to allow an endodermal fate choice downstream of Nodal signalling. To this aim, *cas* RNA was injected at the 8/16-cell stage into one marginal cell of MZoep<sup>tz57</sup> embryos, Zoep<sup>tz57</sup> or bon<sup>m425</sup> homozygous embryos. Embryos were probed during gastrulation with the endodermal markers *sox17* and *foxa2* or at 24 hpf with the endoderm differentiation marker *fkf7*. Expression of *cas* was able to restore the early endodermal markers *sox17* and *foxa2* in MZoep, bon/mixer (Fig. 7H-O) and



**Fig. 7.** Expression of *casanova* rescues late differentiation of endoderm and early endodermal marker expression in *oep* and *bon* mutants. Compared with 24 hpf MZ*oep* (A), *Zoep* (B) and *bon* (C) controls, which are deficient in endodermal structures and in the endodermal differentiation marker *fkf7*, *casanova* overexpression can sometimes restore the differentiation of pharynx in MZ*oep* mutants (D) (11%,  $n=97$ ) and efficiently restores differentiation of pharynx and gut in *Zoep* (E,F) (88%,  $n=42$ ) and *bon* mutants (G) (100%,  $n=17$ ), as evidenced by *fkf7* expression (arrowheads). Overexpression of *casanova* also increases the number of cells expressing the early endodermal markers *sox17* and *foxa2* in MZ*oep* (H-K) and *bon* (L-O) embryos.

*Zoep* embryos (not shown). Thus, *cas* does not require active Nodal signalling or Bon/Mixer function to induce these early endoderm markers. Because *cas* is induced by *bon/mixer* and requires Bon/Mixer function to be expressed, this places *cas* activity downstream of *bon/mixer* in endoderm formation.

We obtained different results when analysing the marker *fkf7*. Endoderm *fkf7*-positive structures were efficiently restored upon *cas* overexpression in *Zoep* embryos (Fig. 7B,E,F; 88%,  $n=42$ ), but were not in MZ*oep* embryos (Fig. 7A,D; 11%,  $n=97$ ). Similar results were obtained with late pharyngeal endoderm markers *foxa2* and *shh* (data not shown). Because *bon/mixer* embryos do not differentiate endoderm and *bon/mixer* is strongly downregulated in MZ*oep* embryos, this downregulation of *bon/mixer* could be responsible for the failure of *cas* to restore late *fkf7* endodermal expression. However, *fkf7*-positive endodermal structures were also restored in *bon/mixer* embryos overexpressing *cas* (Fig. 7C,G). Thus, *cas* can restore endoderm differentiation in endoderm deficient mutants but this activity requires a component activated by Nodal signalling independently of Bon/Mixer function.

We then determined whether Nodal signalling was required within *cas* expressing cells to allow endoderm differentiation. The lineage tracer *gfp* RNA was injected alone or together with *cas* RNA into MZ*oep* or wild-type embryos and few or single cells were grafted at mid-blastula stages from injected embryos at the margin of host wild-type embryos (Fig. 8A,B). During gastrulation and independent of the genotype or the RNA injected, grafted cells involuted and a portion of them remained in contact with the YSL (Fig. 8C,D), thereby adopting the expected position of endodermal progenitors during

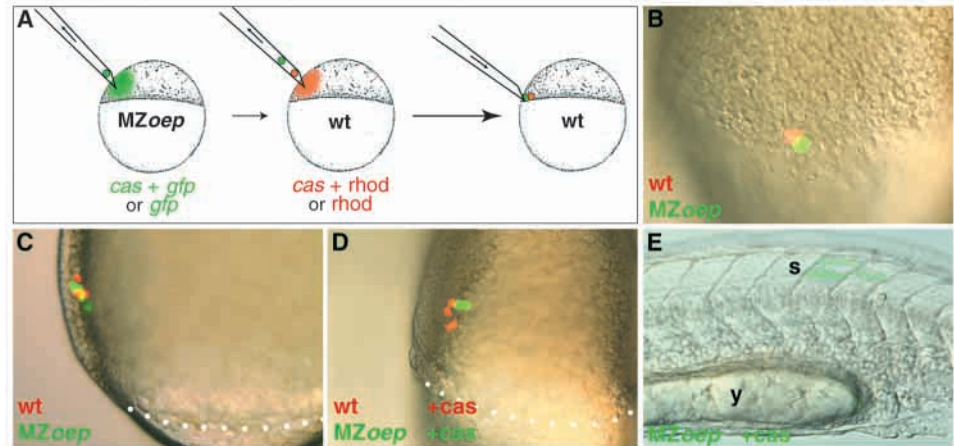
gastrulation. In older embryos, whereas *cas* expressing wild-type cells could significantly populate endodermal derivatives such as the gut and pharyngeal endoderm (Fig. 6), *cas* expressing MZ*oep* cells were unable to do so except very infrequently (Fig. 8E). Thus Nodal signalling is required at least within *cas* expressing cells to allow them to populate endodermal derivatives. Altogether, our results show that *cas* acts on the cell fate decision between endoderm and mesoderm by activating early endoderm markers independently of Nodal signalling. It also restores endoderm differentiation in endoderm deficient mutants but this restoration requires, in an autonomous fashion, some active Nodal signalling independently of Bon/Mixer function.

## DISCUSSION

### Expression of *cas* and endoderm formation require sustained Nodal signalling

We wished to determine when Nodal signalling should be activated to ensure endoderm development. *Oep* function was activated in MZ*oep* embryos at precise time points by microinjection of Cripto protein into the extracellular space. Several conclusions can be drawn from these experiments. First, consistent with the fact that embryos with only zygotic *oep* contribution develop normally, reintroduction in MZ*oep* mutants of *Oep* function and Nodal signalling, after the onset of zygotic transcription, allowed the restoration of endoderm development (Gritsman et al., 1999). Second, the degree of endoderm restoration was time dependent, leading to poorer

**Fig. 8.** Functional Nodal signalling is required cell autonomously to allow *Cas* to change the cell fate. (A) Experimental procedure. MZ*oep* or wild-type embryos were injected with a lineage tracer (*gfp* for MZ*oep* embryos and rhodamine for wild-type embryos) alone or combined with *cas* RNA. By the blastula (sphere-dome stage), one mutant cell and one wild-type cell were transplanted to the margin of a wild-type host (B). During gastrulation, both mutant and wild-type cells, expressing *cas* or not, involute and remain close to or in contact with the YSL (C,D). The dotted line indicates the position of the margin. (E) Anterior to the left, dorsal to the top. At 24 hpf, MZ*oep* cells hardly ever took part in endodermal derivatives (only six intestinal cells in one embryo, out of 24 embryos), whereas wild-type cells expressing *cas* frequently participated in the endoderm (58%,  $n=31$ ; Fig. 6). Abbreviations: s, somites; y, yolk.



rescue when *Cripto* was injected at progressively later stages. In this process, the expression of *cas* and of other endodermal markers exhibited a strong correlation, showing that they obeyed similar regulatory mechanisms. These results are in good agreement with the fact that *cas* acts upstream of *sox17* and is required for the endodermal expressions of *foxa2* and *fkf7* (Alexander et al., 1999; Alexander and Stainier, 1999). Expression of *cas* and endoderm development were fully restored when *Cripto* was injected before or at the sphere stage, demonstrating that *Oep* function and Nodal signalling are not required for endoderm development before this stage in MZ*oep* embryos. Consistent with this idea, *cyc* and *sqt* begin to be expressed throughout the margin at this stage in wild-type embryos, suggesting that endoderm formation also requires Nodal signals from the sphere stage in wild-type embryos (Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998). Moreover, both *sqt* and *cyc* expressions are downregulated during this crucial period of development in MZ*oep* embryos, providing an explanation for the absence of endoderm (Meno et al., 1999).

In these experiments, mesoderm and endoderm exhibited different requirements: past the 40% epiboly stage, mesoderm could still be restored whereas endoderm could not. Thus, whereas transient *Oep* function and Nodal signalling are sufficient for mesoderm formation, sustained *Oep* function is required to ensure endoderm development. This differential requirement for Nodal signalling in the development of endoderm versus mesoderm was also observed upon attenuation of Nodal signalling by overexpression of the Nodal antagonist *antivin/lefty*. *Antivin/lefty* inhibited endoderm formation at low doses and mesoderm formation at higher doses (Thisse and Thisse, 1999). This different requirement for Nodal signalling can be explained in terms of dose or time of exposure, or both (Dyson and Gurdon, 1998; Gritsman et al., 2000). Whatever the mechanism involved, our results clearly show that activation of the Nodal signalling cascade at the proper time is a crucial factor for proper endoderm development.

#### The *bon/mixer* gene acts upstream of *cas*

We have examined the requirements for specific components

acting downstream of Nodal signalling. During gastrulation, *fau/gata5* embryos exhibit a variable reduction in the number of *cas* endodermal cells. Thus, similar to *sox17*, *fau/gata5* appears required for the proper expression of *cas*. However, the reduction in the number of cells observed is modest, suggesting that, similar to what has been proposed for the regulation of *sox17*, *fau/gata5* may act in parallel with other genes, downstream of Nodal-related genes, to control *cas* expression (Reiter et al., 2001).

The *bon/mixer* gene is also required for *cas* expression. Consistent with the requirement for *bon/mixer*, overexpression of *Xmixer* increased the number of *cas*-expressing cells in wild-type embryos. However, two sets of evidence lead us to postulate the implication of an additional Nodal signalling component in the induction of *cas*. First, *bon/mixer* embryos harbour, during gastrulation, a few *cas* expressing cells. Although this could mean that *bon<sup>m425</sup>* is not a null allele, it could also suggest the existence of another gene acting in parallel to *bon/mixer* to induce *cas*. Second, and consistent with the latter hypothesis, overexpression of *Xmixer* cannot induce *cas* expression in MZ*oep* embryos. Thus, although *bon/mixer* clearly acts upstream of *cas* and appears to be capable of inducing its expression, it requires additional factors activated by Nodal-related signals to do so. Such factors probably include *fau/gata5* but probably also other partners, because combined expression of *fau* and *Xmixer* is unable to rescue a normal number of *cas* expressing cells, in deep position, in embryos deficient in Nodal signalling.

#### Expression of *casanova* is sufficient to induce early endoderm and to repress early mesoderm markers

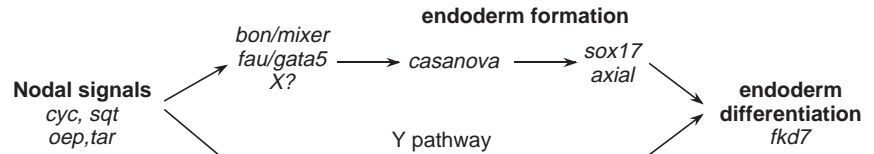
The overexpression of *cas* is sufficient, in the absence of Nodal signalling, to induce the endodermal marker *sox17* (Dickmeis et al., 2001a). This may mean either that *cas* is a regulator of *sox17* or has a wider function in the induction and/or differentiation of endoderm. Here, we provide the evidence that *cas* has a more general role in endoderm formation, downstream of Nodal signals. First, we show that overexpression of *cas* is sufficient to induce several endodermal markers. This induction is cell autonomous, consistent with the fact that both *cas* and *sox17* are induced

autonomously by Tar\* (this work) (David and Rosa, 2001). Second, the induction of early endodermal markers is also observed in MZ*oep* embryos, so *cas* can function in the absence of Nodal signalling, extending our previous results (Dickmeis et al., 2001a). Third, the induction of early endodermal markers by *cas* does not require Bon/Mixer function. Altogether, our results show that *cas* acts downstream of *bon* in the induction of endodermal markers. We also show that *cas* overexpression leads to the inhibition of the expression of the mesodermal markers *ntl* and *tbx6*. Whether the inhibition of mesoderm formation is mediated by *cas* itself or by a gene acting with or activated by *cas* remains unclear because none of *sox17*, *fau/gata5* and *foxa2* have been reported to inhibit mesoderm formation. Thus, consistent with the fact that marginal cells normally fated to endoderm have their fate changed to mesoderm in the absence of *cas* activity, *cas* appears to achieve a dual function, i.e. to inhibit mesoderm formation and to induce endoderm formation.

### Expression of *casanova* changes the fate of marginal blastomeres and restores endoderm differentiation in Nodal mutants

Expression of *cas* can increase the contribution of marginal cells to endoderm, probably at the expense of mesoderm. However, this effect is modest because most *cas* expressing cells remain in the mesoderm. It is possible that higher doses of *cas* RNA induce a better change of fate of marginal cells to endoderm but, owing to the incompatibility of the lineage tracing and *cas* overexpression, we have not been able to address this specific point.

Because *cas* was able to induce a spectrum of endoderm markers in the absence of or reduced Nodal signalling, we tested whether it could also restore the formation of late endoderm derivatives. We found that *cas* rescued, at a very low frequency, small domains of late endodermal derivatives in MZ*oep* mutants, whereas it rescued the same derivatives very efficiently in a *Zoep* mutant. Similarly, activation of Nodal signalling in MZ*oep* embryos at doses that do not allow endoderm formation also enabled *cas*-mediated restoration of late endoderm derivatives (data not shown). This indicates that *cas* is sufficient to induce early endoderm progenitors in the absence of Nodal signalling but that their later differentiation requires some other component of Nodal signalling, Y, which is not itself sufficient to allow endoderm formation (Fig. 9 shows the adaptation of the model proposed by Alexander). Late activation of Nodal signalling mediated by Cripto injections is not sufficient to allow *cas*-mediated endoderm differentiation, suggesting that the additional component must either be provided at an early stage or induced by sustained Nodal signalling (data not shown). In addition, this component appears required within *cas* expressing cells because *cas*-expressing MZ*oep* cells grafted into wild-type embryos do not efficiently populate endoderm derivatives. Altogether our results combined with previous work show that endoderm formation and differentiation requires sustained Nodal signalling and is mediated by the Nodal inducible *sox*-related gene *casanova* in conjunction with other components activated by the Nodal signalling pathway.



**Fig. 9.** A model for endoderm formation and differentiation, adapted from Alexander and Stainier (Alexander and Stainier, 1999). Our work suggests the involvement of an unknown factor (X) acting with *bon/mixer* and *fau/gata5* for *cas* induction, and it also shows the requirement for an independent Y pathway induced by Nodal signalling to allow proper endoderm differentiation.

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