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

Tazu O. Aoki1,*, Nicolas B. David1,*, Gabriella Minchiotti2, Laure Saint-Etienne1, Thomas Dickmeis3, Graziella M. Persico2, Uwe Strähle3, Philippe Mourrain1 and Frédéric M. Rosa1,†

1U 368 INSERM, Ecole Normale Supérieure, 46, rue d’Ulm, F-75230 Paris Cedex 05, France
2International Institute of Genetics and Biophysics, CNR, Via G. Marconi, 12-80125 Naples, Italy
3Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, BP 163, F-67404 Illkirch Cedex, CU de Strasbourg, France

*These two authors contributed equally to this work
†Author for correspondence (e-mail: rosa@wotan.ens.fr)

**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 TGFβ-related ligands, Vg1, activins, Nodal-related (Ndr) molecules 1 and 2, and Derrière, as well as their extracellular antagonists, antivin/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 zebralif 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., 1998). *Mouse cripto* mutants and zebrafish embryos lacking maternal and zygotic *oep* expression (M*Zoe*p embryos) exhibit phenotypes similar to *nodal* mutants or cyc;*sqt* double mutants, indicating that *nodal*-related genes and *criptoloep* act in the same signalling pathway (Ding et al., 1998; Gritsmann 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; Peyriéras et al., 1998).

TGFβ-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 ActRIB 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; Peyriéras 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; Peyriéras 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 Stainier, 1999). In zebrafish, endoderm progenitors which Nodal-related signals control this process (Alexander and Stainier, 1998; Ecochard et al., 1998; Lemaire et al., 1998; Weber et al., 2000). The cas gene leads to phenotypes similar to those observed in nodal mouse mutants (Gu et al., 1998).

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 MZoep 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 MZoep 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>57</sup>, heterozygous bon<sup>tt21</sup> or heterozygous fau<sup>ts</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, pSP64Tar*, pCS2gfp, pCS2nlslacZ, 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 sar+ (1.2 pg) or cas (40 pg) RNAs. At sphere stage, 1-20 donor (green) cells were grafted to the tracers, either alone or combined with tar*.

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) (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 MZoep 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 (Zoop embryos) (Schier et al., 1997; Strahle et al., 1997). In late blastula (40% epiboly) and during gastrulation, Zoep<sup>−/−</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 fkd (fox1) 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 oep expression is induced in a cell autonomous fashion by activation of the Nodal pathway. Whether the transient activation of the Nodal pathway induced by Tar* (Dickmeis et al., 2001a). We studied whether the transient activation of the Nodal pathway induced 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 sufficient to allow endoderm development. MZoop embryos do not develop endoderm. We wished to know when activation of Nodal signalling would be required to allow endoderm development in these mutants.

MZoop embryos can be fully rescued by microinjection of an RNA encoding a soluble form of Nodal.

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**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 (Grisman 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 MZoep 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 MZoep embryos. Cripto protein or bovine serum albumin (BSA), as a control protein solution, was injected into the extracellular space of early to late MZoep 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 fkd7. 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 fkd7 expression at 30 hpf showed that Cripto protein injection before or at the sphere stage led to full rescue of fkd7 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.

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 MZoep embryos, in which Nodal signalling is inactive. Expression of cas induced fau (Dickmeis et al., 2001a) but not bon/mixer in MZoep 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 MZoep embryos (Fig. 5I,J and data not shown). We note, however, that the combination of these two factors induced, in a small number of MZoep 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

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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 Zoep 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 nlslacZ 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).
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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; Peyriéras 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
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.

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
central or one marginal cell at the 8/16-cell stage in wild-type embryos and analysed the expression of early endodermal markers \textit{sox17}, \textit{fau/gata5}, \textit{foxa2} and mesodermal markers \textit{tbx6} and the Brachyury orthologue \textit{ntl} (Hug et al., 1997; Schulte-Merker et al., 1993). Overexpression of \textit{nls-lacZ} did not alter the expression of any of these markers (Fig. 6G,I,K,O,Q). However, \textit{cas} expression in marginal cells led to an increased number of endodermal cells expressing \textit{sox17} and \textit{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 β-galactosidase, used as a lineage tracer). By contrast, mesodermal markers \textit{ntl} (O,P) and \textit{tbx6} (Q,R) are repressed by overexpression of \textit{cas} (arrowheads in P and R: 86%, \(n=21\) and 78%, \(n=18\), respectively). Conversely, overexpression of \textit{ntl} represses \textit{cas} expression at the margin (arrowhead in T, compare with S).

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

We then wished to know whether \textit{cas} alone was sufficient to allow an endodermal fate choice downstream of Nodal signalling. To this aim, \textit{cas} RNA was injected at the 8/16-cell stage into one marginal cell of \textit{MZoep}\textsuperscript{1257} embryos, \textit{Zoep}\textsuperscript{1257} or \textit{bon}\textsuperscript{m425} homozygous embryos. Embryos were probed during gastrulation with the endodermal markers \textit{sox17} and \textit{foxa2} or at 24 hpf with the endoderm differentiation marker \textit{fkd7}. Expression of \textit{cas} was able to restore the early endodermal markers \textit{sox17} and \textit{foxa2} in \textit{MZoep}, \textit{bon/mixer} (Fig. 7H-O) and

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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 fkd7. Endoderm fkd7-positive structures were efficiently restored upon cas overexpression in Zoep embryos (Fig. 7B,E,F; 88%, n=42), but were not in MZoep 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 MZoep embryos, this downregulation of bon/mixer could be responsible for the failure of cas to restore late fkd7 endodermal expression. However, fkd7-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 MZoep 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 MZoep 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 MZoep 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 MZoep 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. MZoep or wild-type embryos were injected with a lineage tracer (gfp for MZoep embryos and rhodamine for wild-type embryos) alone or combined with cas RNA. By the blastula (spherodome 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, MZoep 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.

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 fkd7 (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 MZoep embryos. Consistent with this idea, cay 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 cay expressions are downregulated during this crucial period of development in MZoep 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/lefly. Antivin/lefly inhibited endoderm formation at low doses and mesoderm formation at higher doses (Thise and Thise, 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 bonm425 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 MZoep 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 MZoep 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 markersntl 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 MZoep mutants, whereas it rescued the same derivatives very efficiently in a Zoep mutant. Similarly, activation of Nodal signalling in MZoep 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 MZoep 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.

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REFERENCES
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