The transcription factor Vox represses endoderm development by interacting with Casanova and Pou2

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SUMMARY
Endoderm and mesoderm are both formed upon activation of Nodal signaling but how endoderm differentiates from mesoderm is still poorly explored. The sox-related gene casanova (sox32) acts downstream of the Nodal signal, is essential for endoderm development and requires the co-factor Pou2 (Pou5f1, Oct3, Oct4) in this process. Conversely, BMP signals have been shown to inhibit endoderm development by an as yet unexplained mechanism. In a search for Casanova regulators in zebrafish, we identified two of its binding partners as the transcription factors Pou2 and Vox, a member of the Vent group of proteins also involved in the patterning of the gastrula. In overexpression studies we show that vox and/or Vent group genes inhibit the capacity of Casanova to induce endoderm, even in the presence of its co-factor Pou2, and that Vox acts as a repressor in this process. We further show that vox, but not other members of the Vent group, is essential for defining the proper endodermal domain size at gastrulation. In this process, vox acts downstream of BMPs. Cell fate analysis further shows that Vox plays a key role downstream of BMP signals in regulating the capacity of Nodal to induce endoderm versus mesoderm by modulating the activity of the Casanova/Pou2 regulatory system.

KEY WORDS: Zebrafish, Endoderm, Fate, Transcription, Nodal

INTRODUCTION
The formation of the three germ layers – ectoderm, mesoderm and endoderm – is crucial for vertebrate development. Whereas our understanding of mesoderm formation has benefited from extensive studies, knowledge about the gene network that regulates endoderm development is still limited. Endoderm and mesoderm can first be distinguished at gastrulation. At the onset of gastrulation, the zebrafish embryo is organized as a cap lying on top of a large yolk sphere. According to fate-mapping studies, the entire zebrafish endoderm, as well as a portion of the mesoderm, arise from the marginal-most four blastomere tiers at this stage, in which endoderm and mesoderm precursors are intermingled, but they soon separate during the involution process that brings both mesoderm and endoderm to a deeper position within the embryo (Kimmel et al., 1990; Warga and Nüsslein-Volhard, 1999; Kimelman and Griffin, 2000).

Work carried out in mice, chicken, frogs and zebrafish has revealed a high degree of conservation in the way that vertebrate endoderm development is controlled. A two-step model of endoderm specification and differentiation was initially proposed in frogs (Yasuo and Lemaire, 1999) and appears to apply at least to zebrafish. During early stages, maternal transcription factors, such as VegT, activate the expression of downstream effectors including Mix.1, Sox17α and several TGFβ/Nodal family members to specify mesendoderm, a common precursor for mesoderm and endoderm. During a second phase, several factors are recruited to induce the endoderm genetic program that leads to endoderm commitment (reviewed by Grapin-Botton and Constand, 2007; Zorn and Wells, 2007). In zebrafish, an equivalent of Xenopus VegT (Xanthos et al., 2001) has not been identified but a maternally deposited signal from the extra-embryonic yolk syncytial layer initiates mesendoderm formation by activating Nodal-type ligands (Feldman et al., 1998; Rebagliati et al., 1998). The activity of Nodal factors appears to be one of the most conserved elements of the pathway leading to endoderm formation (reviewed by Grapin-Botton and Constand, 2007; Zorn and Wells, 2007). Nodal and Nodal-related genes are required for endoderm specification and commitment, and activation of Nodal signaling is sufficient to induce this process (Alexander and Staunier, 1999; Yasuo and Lemaire, 1999; David and Rosa, 2001). Conversely, endoderm formation is inhibited by the extracellular Nodal inhibitor Lefty. Nodal factors bind and act via a type II and a type I serine/threonine kinase receptor as well as an EGF-CFC co-receptor (Renucci et al., 1996; Feldman et al., 1998; Peyriéras et al., 1998; Zhang et al., 1998).

Endoderm formation requires, downstream of the Nodal ligand-receptor interaction, a set of transcription factors, which have been characterized in zebrafish. They include the mix-like homeobox transcription factors Bon (Kikuchi et al., 2000) and Mezzo (also known as Sebox) (Pouain and Lepage, 2002) and the zinc-finger transcription factor Gata5 (also known as Faust) (Reiter et al., 1999; Rodaway et al., 1999; Reiter et al., 2001), which are induced by activation of Nodal signaling. A fourth transcription factor encoded by the sox-related gene casanova (cas; also known as sox32) acts downstream of gata5, bon and mezzo and appears both necessary and sufficient to induce an endodermal fate, at least within mesodendodermal territory (Alexander et al., 1999; Alexander and Staunier, 1999; Dickmeis et al., 2001; Kikuchi et al., 2001; Aoki et al., 2002). cas expression requires, in addition to Nodal signaling activation, the presence of the maternal transcription factor Eomes (Bjornson et al., 2005).

Apart from Nodal signaling, bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) signaling have also been implicated in the control of endoderm formation and appear to inhibit Nodal signaling action (Pouain et al., 2006). Whereas FGF signaling inhibits Nodal signaling by inducing Cas phosphorylation, the process mediating BMP activity in endoderm formation has
remained unclear. BMPs are also involved in the control of dorsoventral (DV) patterning during gastrulation in frog and fish, a process that is mediated, at least in part, by the Vent (Vox/Vent/Vega/Ved) group of homeobox transcription factors (Kawahara et al., 2000a; Kawahara et al., 2000b; Imai et al., 2001; Shimizu et al., 2002).

Initially, similar to endoderm, mesoderm progenitor formation also relies on the Nodal pathway, a situation found in zebrafish as well as in other vertebrate species (Henry et al., 1996; Sasai et al., 1996; Schier et al., 1997; Hudson et al., 1997; Kofron et al., 1999; Reiter et al., 1999; Agius et al., 2000; Thisbe et al., 2000; Brennan et al., 2001; Dougan et al., 2003; Birsoy et al., 2006), and they express similar molecular markers (Hammerschmidt and Nüsslein-Volhard, 1993; Ecohard et al., 1998; Lemaire et al., 1998; Papaioannou and Silver, 1998; Rodaway et al., 1999; Wardle and Smith, 2004). The differential induction of mesoderm and endoderm seems to rely on different doses/duration of Nodal signaling combined with the activity of other extracellular factors such as BMPs and FGFs, but how these factors act intracellularly in combination to generate the proper ratio between endoderm and mesoderm is not clear.

Cas was identified as a high mobility group (HMG) protein of the Sox family (Dickmeis et al., 2001; Kikuchi et al., 2001) and plays a crucial role in zebrafish endoderm development and differentiation induced by Nodal factors (Aoki et al., 2002). cas mutants completely lack endoderm precursors at the onset of gastrulation, whereas the mesoderm and mesendoderm remain largely normal (Alexander et al., 1999; Dickmeis et al., 2001). In addition, the most marginal cells that are normally fated to endoderm adopt a mesodermal fate in embryos devoid of cas activity (Dickmeis et al., 2001). Thus, cas appears to be a crucial regulator of endodermal versus mesodermal fate and the regulation of its activity is likely to be essential for the proper ratio between endoderm and mesoderm. Sox proteins have been generally considered to function as transcriptional regulators that require physical interaction with their co-factors (Ambrosetti et al., 1997; Wilson and Koopman, 2002) and, in this respect, Cas requires and can synergistically interact with Pou2 (also known as Pou5f1, Oct3 or Oct4) to promote endoderm development by binding to the sox17 promoter (Lunde et al., 2004; Reim et al., 2004; Chan et al., 2009).

To better understand the molecular mechanisms controlling endoderm formation, we carried out a yeast two-hybrid screen for Cas in order to identify the components with which it interacts. Our results demonstrate, for the first time, that Vent group genes, and in particular vox, which is a known effector of DV patterning acting within the BMP signaling pathway, also have an important function in regulating ventral endoderm development and the separation of endoderm from mesoderm and ectoderm.

MATERIALS AND METHODS

Fish maintenance

Zebrafish were kept and staged as described (Westerfield, 1994).

Morpholinos

Morpholinos (MOs) were described previously: vox (Imai et al., 2001), vent (Imai et al., 2001), ved (Shimizu et al., 2002).

Cloning and mutation

The cas cDNA was subcloned into pCS2 (Turner and Weintraub, 1994). D-cas and ΔN-vox were obtained from mutation of cas-pCS2 and vox-pSPE3, respectively, using the Exsite PCR-Based Site-Directed Mutagenesis Kit (Stratagene). The myc tag was subcloned in frame into D-cas-pCS2 or cas-pCS2 at the N-terminus. Enr and VP16 fragments were fused in frame with ΔN-vox-pSPE3. The constructs for making capped mRNA were cloned into different pSPE3 vectors (Roure et al., 2007). Primers used for mutation or cloning were (5′-3′): D-cas-pCS2, CCAGCACAGACTTTGGAGCCACAGGC and ACTGCGATCAAGCTGTCCACAC; ΔN-vox-pSPE3, CACCATGCGAAGAATCTGCTATCTGCAAG and TCAGTGCTAA-TGATGTCGGGGCATCGT.

cDNA library construction and yeast screening

Total RNA was extracted with TRIzol Reagent (Invitrogen) from gastrulation stage wild-type zebrafish embryos. Subsequently, mRNA was isolated using the PolyATtract Kit (Promega). For yeast two-hybrid screening, the cDNA library was constructed using the HybridZIP-2.1 XR cDNA Synthesis Kit and HybridZIP-2.1 XR Library Construction Kit (Stratagene) according to the manufacturer’s instructions except that the cDNAs were inserted into the ACTII phage vector at EcoRI and XhoI sites and in vivo excised into pACTIIa plasmid (Meijer et al., 1998). D-cas cDNA was fused in frame with the Gal4 DNA-binding domain sequence in the pGBK7 vector (Clontech). Yeast strain PJ69-4A (A.H.M.) was used to carry out two-hybrid screening on –LWH +3AT selective medium plates. Yeast transformations were performed by the LiAc method essentially as described (Gietz and Schiestel, 1995). Target plasmids were isolated from positive yeast colonies using the Y-DER Yeast DNA Extraction Reagent Kit (Pierce) and retransformed into E. coli XL-1 blue MRF′ strain for DNA amplification and sequencing.

In vitro binding assay

S35-labeled proteins were translated in vitro with the Flexi Rabbit Reticulocyte Lysate Kit (Promega) from capped mRNA that was synthesized by the mMESSAGE mMACHINE Kit (Ambion) and purified using the RNasy Kit (Qiagen). Immunoprecipitation (IP) was performed with Protein G-agarose (Roche) and the 9E10 anti-c-myc or 3F10 anti-HA antibody (Roche) essentially following the recommendations for the co-IP procedure of these products (washing strength of NaCl 0.5-1 M).

Microinjection and in situ hybridization

Capped mRNA was injected into zebrafish early embryos as described (Peiryéras et al., 1998). In situ hybridization was performed as described (Hauptmann and Gerster, 1994). In Figs 3-7, representative embryos are shown.

qRT-PCR

Primers were designed using Primer Express 3.0 (Applied Biosystems). The amplified region included an intron to avoid amplifying genomic DNA. In the reverse transcription, 15 bp oligo(dT) (Promega) was used under the conditions suggested by the M-MLV Reverse Transcription Kit (Invitrogen). qRT-PCR was performed with Platinum SYBR Green qPCR SuperMix-UDG with the ROX Kit (Invitrogen) in an Applied Biosystems 7500 real-time PCR system. β-actin was used as an internal control to normalize different samples. qRT-PCR results were averaged from at least triplicate reactions (up to 11) carried out on RNA extracted from at least two independent batches of embryos. Primers were as follows (5′-3′, forward and reverse): αgata5, CCAGCACAGGGAA-CTGTCGACCTGCAAGCATGG and GGGACGCCAGGGAA-CTCCTCAG and ACTGCGATCAAGCTGTCCACAC; ΔN-vox-pSPE3. The constructs for making capped mRNA were cloned into different pSPE3 vectors (Roure et al., 2007). Primers used for mutation or cloning were (5′-3′): D-cas-pCS2, CCAGCACAGACTTTGGAGCCACAGGC and ACTGCGATCAAGCTGTCCACAC; ΔN-vox-pSPE3, CACCATGCGAAGAATCTGCTATCTGCAAG and TCAGTGCTAA-TGATGTCGGGGCATCGT.

Transplantations and fate analyses

Transplantations were carried out essentially as described (David and Rosa, 2001). GFP-positive cells from donor embryos injected at the 8-cell stage in one of the eight cells with Tar* + GFP or Tar* + GFP + vox RNA were grafted into the margin of untreated embryos at the sphere stage. Embryos were then analyzed by combined brightfield and epifluorescence microscopy at 30 hours (hpf) or at 3 days (dpf) post-fertilization.

Statistical analyses

Results from qRT-PCR and cell number counting were analyzed by the t-test using the relevant control conditions (see figure legends). Results from embryo counting (Figs 3 and 4) were analyzed by the χ2 test.
RESULTS
Deletion of the conserved EFDQYLN amino acid sequence abolishes Cas endoderm-inducing activity without altering its expression or subcellular localization

In order to identify new partners for Cas we carried out a yeast two-hybrid screen. Since Cas is thought to bind DNA and act as a transcription factor, it might have generated false positives by activating, in the absence of binding partners, transcription of the target gene in yeast. We thus first generated and tested a modified version of cas, D-cas, from which we removed the conserved EFDQYLN peptide located in the C-terminal region of the protein that is likely to act as an activation domain (Fig. 1A). Consistent with this idea, wild-type cas overexpression induced strong sox17 ectopic expression (Fig. 1B,C), whereas D-cas was unable to do so (Fig. 1D).

To ensure that D-cas retained the capacity to be expressed, we overexpressed in zebrafish embryos an N-terminal myc-tagged D-Cas variant. Immunohistochemistry showed that D-Cas was predominantly located in the nucleus, as expected (Fig. 1F). To confirm that myc-D-cas can be efficiently expressed, myc-D-cas was in vitro translated, resolved on an SDS-PAGE gel and probed by western blot with anti-myc antibodies. Immunoprecipitation (IP) results demonstrated that myc-D-Cas is expressed as fully as wild-type Cas in vitro (Fig. 1G).

Altogether, these results show that the deletion of a short conserved amino acid sequence can abolish the endoderm-inducing activity of Cas without altering its expression or subcellular localization.

Cas binds Vox and/or Pou2

HMG domain-containing Sox proteins play a key role in the regulation of embryonic development and in cell fate control, and frequently regulate their target genes by cooperating with partners in a cell-specific manner (Wegner, 1999; Bowles et al., 2000; Kamachi et al., 2000). We thus searched for putative Cas partners by carrying out a yeast two-hybrid screen using D-Cas as bait. Among candidate partners, two clones were of particular interest. One of the clones encoded pou2, a gene already known to genetically interact with cas (Reim et al., 2004; Lunde et al., 2004), thus confirming the specificity of our screening approach. Among other potential partners, Vox was identified as one of the strongest, but neither Vent (also known as Vega2) nor Ved could be identified as strong Cas interactors in this assay, even when their cDNA was specifically transformed into yeast to perform the two-hybrid test.

The capacity of the Vox and Pou2 proteins to bind Cas was confirmed using an in vitro binding assay coupled to IP, following in vitro translation of the candidate RNAs (Fig. 2A, lanes 2-4). Interestingly, Ved was also able to bind Cas in this assay, although because Ved could bind GFP this prevented us from identifying it as a bona fide interactor (Fig. 2C). Vent did not bind Cas consistently or reproducibly. Using the same assay, we also found that Pou2 binds Vox (Fig. 2A, lane 5).

Homo- or heterodimerization has been considered a means by which transcription factors can regulate target genes with limited DNA binding sites and some of the homeodomain proteins work in this way (Chan and Mann, 1996; Ryan and Rosenfeld, 1997; Smit et al., 2000). We thus tested whether Vox can homodimerize. Consistent with this notion, Vox co-precipitated with Vox-HA (Fig. 2B). This result was confirmed by yeast two-hybrid analysis (data not shown). Altogether, these results demonstrate that Vox can form heterodimers with Cas and/or Pou2, and that Vox can form homodimers as well, raising the possibility that Vox, Cas and Pou2 can form a multicentric complex.

We then attempted to define the region of the Vox protein that is required for interaction with its different partners. Vox is composed of a central homeodomain flanked by N- and C-terminal domains. In vitro binding assays were carried out with tagged forms of either Cas, Pou2 or Vox and radiolabeled variants of Vox lacking either its N-terminus (ΔN-Vox) or C-terminus (ΔC-Vox) (Fig. 2D), which are efficiently translated in vitro (data not shown). Whereas ΔN-Vox retained the capacity to bind either Cas, Pou2 or Vox, ΔC-Vox appeared unable to do so (Fig. 2E-G). Thus, the Vox C-terminal domain is essential for binding to these three proteins. However, similar experiments involving a fusion between GFP and the Vox C-terminus showed that this latter domain itself is not sufficient to allow binding to Cas, Pou2 or Vox (data not shown). Thus, the integrity of Vox is important for the interaction with its co-factors. The Vox C-terminus is thus necessary, but not sufficient, to allow physical interaction between Vox partners.

Fig. 1. Structure, endoderm-inducing activity and subcellular localization of wild-type cas and a deletion variant. (A) D-Cas contains a deletion of the conserved EFDQYLN peptide located in the C-terminal region (red in the wild type) that is likely to act as an activation domain. cas and D-cas RNAs were used for injection. (B-F) Zebrafish embryos were left uninjected (B) or injected with the RNA indicated bottom right and probed by in situ hybridization for sox17 (B-D) or by immunohistochemistry with anti-myc antibody (E,F). The inset in F indicates the position of myc-positive nuclei (arrows). (G) Immunoprecipitation (IP) for myc-D-Cas and myc-Cas. The myc-Cas and myc-D-Cas proteins are detected at 45 kDa.
Vox inhibits Cas activity and acts as a repressor

We then probed the potential effect of vox, ved and vent on cas function. Cas is a key regulator of endoderm development, and in particular can strongly upregulate the endoderm marker sox17 when overexpressed in zebrafish embryos (Alexander et al., 1999; Dickmeis et al., 2001; Kikuchi et al., 2001; Aoki et al., 2002). We ectopically expressed cas alone or together with vox or ved or vent by introducing RNAs into zebrafish embryos and probed for sox17 expression by in situ hybridization. qRT-PCR was performed in parallel to allow independent quantification of the effects of overexpression. Injection of cas RNA alone led to large ectopic domains of sox17 expression during gastrulation (in 46% of embryos, n=127; Fig. 3B,F), whereas co-expression of vox (16.7%, n=191; P<0.005) or ved (28%, n=64; 0.05<P<0.1) but not vent (37.5%, n=56; P>0.2) reduced both the occurrence and the size of the ectopic domains (Fig. 3C-F). qRT-PCR analyses gave similar results, although, in contrast to the in situ analyses, vent also appeared to have some effect, the discrepancy being potentially explained by the fact that it is not possible to precisely quantitate the size of the ectopic domain by in situ analysis. Thus, vox, ved and vent inhibit the sox17- and endoderm-inducing activity of cas.

vox is required for cas activity and has been reported to synergistically enhance the capacity of cas to induce endoderm (Lunde et al., 2004; Reim et al., 2004; Chan et al., 2009). We tested the capacity of vox to inhibit cas sox17-inducing activity in the presence of an excess of vox. As previously reported, overexpression of cas and vox led to embryos that exhibit more frequent ectopic sox17 domains (63%, n=51) than with overexpression of cas alone (46%, n=127) (Fig. 3H,I). By contrast, overexpression of cas, vox and vent inhibited this effect (16.6%, n=48; P<0.005), as seen by in situ hybridization, indicating that vox can also inhibit the combination of cas and vox (Fig. 3J). This conclusion is supported by the qRT-PCR results (Fig. 3K).

To determine which domain of Vox is required to inhibit cas function and if, in particular, the C-terminal domain that is required for binding to Cas is important, cas RNA was injected alone or co-injected with either vox, ΔN-vox or ΔC-vox RNA into wild-type embryos, which were then probed for sox17 expression during gastrulation or analyzed by qRT-PCR. Compared with cas RNA injections, both deleted forms led to a similar percentage of embryos exhibiting ectopic domains (cas + ΔN-vox: 43.4%, n=53, P>0.5; cas + ΔC-vox: 45.4%, n=44, P>0.9), a result consistent with the qRT-PCR analysis, demonstrating that these deletions lead to a decrease in Vox inhibitory activity (Fig. 4A-F). Thus, both the N-terminus and the C-terminus appear to be essential for Vox to inhibit cas endoderm-inducing activity. Furthermore, the reduction in the inhibitory activity of ΔC-vox is consistent with the fact that Vox binding is required to inhibit cas activity.

Vox (which is also known as Vegai3) has been reported to act as a repressor of dharma (also known as box or dha) activity in zebrafish DV patterning and its N-terminal domain is required for this repressive activity (Kawahara et al., 2000a; Kawahara et al., 2000b). To determine whether Vox acts as an activator or repressor of cas activity, we fused ΔN-vox with either the repressor domain of Engrailed (EnR-ΔN-vox) or the activation domain of VP16 (VP16-ΔN-vox). Similar to, but more efficiently than the ectopic expression of full-length Vox (18.5%, n=243), expression of EnR-ΔN-vox mRNA repressed sox17 (56%, n=46; P<0.005; Fig. 4H,I), whereas VP16-ΔN-vox barely attenuated sox17 expression during gastrulation, both in terms of the number of embryos affected and the reduction of the sox17-positive domain (15.8%, n=38; P=0.2; Fig. 4J). A similar result was obtained by qRT-PCR (Fig. 4K). Thus, our results show that Vox is a strong repressor of cas endoderm-inducing activity.

Vox is an endogenous regulator of endoderm development

We then tested whether vox, vent or ved could regulate endogenous endoderm formation. First, we checked the expression pattern of vox during gastrulation. vox is expressed in a large domain centered on the ventral side. Similarly, vent and ved are progressively excluded from dorsal territories but encompass prospective endoderm territories on the ventral side (Kawahara et al., 2000a; Melby et al., 2000). Analysis of sections of zebrafish gastrula showed that vox is expressed throughout the depth of the blastoderm in this domain, and particularly in the deepest blastodermal cells that encompass endoderm (data not shown). Thus, cas and vox, vent and ved are expressed in overlapping domains, consistent with a cell-autonomous interaction, potentially by physical interaction in the case of vox and cas.

Gain- and loss-of-function strategies were carried out by vox, vent and ved RNA or MO injection into zebrafish embryos. Injection of either vox, vent or ved RNA induced a decrease in sox17 expression (Fig. 5C, arrowhead; SD) and in two other endoderm markers: axial (also known as foxa2) (Fig. 5G,H) and gata5 (Fig. 5L). By contrast, vox but not vent or ved MO injection (Imai et al., 2001; Shimizu et
al., 2002) led to a percentage of the population (17%, \( n = 67 \)) exhibiting a significantly higher number of sox17-positive or axial-positive cells than the uninjected control (Fig. 5B,D,F,H; Table 1).

We then determined whether vox inhibits mesoderm as well as endoderm development in early stage zebrafish embryos by analyzing the expression of the pan mesodermal marker ntl (Schulte-Merker et al., 1994) at shield stage, at which time endoderm becomes committed (Ho and Kimmel, 1993; David and Rosa, 2001). Neither vox MO (Fig. 5J) nor vox mRNA (Fig. 5K) could alter the expression domain of ntl at this stage.

Taken together, our results show that Vent group genes can downregulate the endoderm population but that only vox appears to be required for regulating the size of the endodermal, but not the mesodermal, domain during early gastrulation.

The inhibition of ventral endoderm progenitor formation by BMPs is mediated by vox

BMPs are required for zebrafish endoderm formation. BMP signals have been shown to control endoderm DV patterning and to inhibit endoderm formation on the ventral side of the zebrafish gastrula (Tiso et al., 2002; Poulain et al., 2006). However, the molecular mechanism responsible for the inhibitory action of BMPs on ventral endoderm is unknown. Vent group genes act upstream and/or downstream of BMP signals to control DV patterning of the gastrula, suggesting that they could also act upstream or downstream of BMP signals to control the generation of ventral endoderm in the early gastrula (Imai et al., 2001; Shimizu et al., 2002). To address this issue, we tested the potential epistatic relationship between vox, ved and vent and BMP signals in the control of endoderm formation.

First, bmp2, bmp4 and bmp7 RNAs (Poulain et al., 2006) were injected into zebrafish embryos together with, or without, MOs directed against ved, vent or vox (Fig. 6B-E). In ~20% (\( n = 113 \)) of the embryos, BMP overexpression led to a gap in the ventral endoderm and to a marked decrease in endoderm precursor numbers (Fig. 6B; Table 2A). Co-injection of vox, but not vent or ved, MO inhibited this effect (Fig. 6C-E; Table 2A), both by reducing the number of embryos presenting gaps and by restoring the proper number of cells in the remaining embryos, suggesting that vox is required downstream of BMP to control ventral endoderm formation. Co-injection of vent, ved and vox MO did not lead to a stronger effect than vox MO alone (data not shown).

Conversely, BMP signaling inhibition by expression of a dominant-negative mutant of BMP receptor, \( \Delta \mathrm{mBMPR} \) (Suzuki et al., 1994), induced an expansion in the sox17-positive progenitor domain in a fraction (27%, \( n = 86 \)) of the embryos (Fig. 6F; Table 2A).
Vox inhibits Cas

Endoderm is induced by the activation of Nodal signaling, the activity of which is mediated by cas. Activation of Nodal signaling or overexpression of Cas is sufficient to induce an endoderm fate in early zebrafish blastomeres. Conversely, reduction in Nodal signaling or inhibition of cas activity leads to the reduction/absence of the endoderm fate. In addition, loss of cas function leads to a change in the fate of the most marginal cells of the gastrula from endoderm to mesoderm. Since vox regulates the activity of cas in endoderm formation, we wondered whether it is also involved in controlling the fate of endodermal cells.

Overexpression of the constitutively active form of the type I transforming growth factor (TGFβ) or the ALK4-related zebrafish type I receptor taram-a (also known as acvr1b) (Tar*) can promote endoderm formation and transfect naïve cells in wild-type embryos (Peyriéras et al., 1998; Alexander and Stainier, 1999; David and Rosa, 2001; Nair et al., 2007). To determine whether Vox controls the endoderm fate, we injected a mixture of Tar* mRNA and GFP mRNA as a tracer, with or without vox RNA, into donor wild-type embryos and transplanted GFP-positive cells into the margin of host unlabeled embryos at the sphere stage (David and Rosa, 2001). As previously described, GFP-positive cells, in the absence of vox RNA, almost systematically adopt an endodermal fate and are located in classical endodermal locations in 30-hour embryos, including the pharyngeal endoderm, the gut and the mesendodermal hatching gland (Fig. 7A-D; Table 3). Co-expression of vox dramatically altered this fate and prevented cells from participating in endoderm territories. By contrast, vox co-expressing cells appeared as mesodermal (predominantly muscle and mesenchyme) as well as ectodermal (hindbrain, posterior neural tube, otic vesicle and epidermis) derivatives (Fig. 7E-J; Table 3).

We conclude from this experiment that vox controls the endoderm fate that is generated by the activation of Nodal signaling.

DISCUSSION

Cas, Pou2 and Vox physically interact

To better understand how the activity of the sox-related protein Cas is regulated, we searched for binding partners for Cas. Several potential partners were identified by yeast two-hybrid screens, two of which are described here, both of which were confirmed by co-IP. The first partner is Pou2, a result fully consistent with the potential partners were identified by yeast two-hybrid screens, two of which are described here, both of which were confirmed by co-IP. The first partner is Pou2, a result fully consistent with the

Table 1. Inhibition of vox activity causes the formation of an excess of endodermal precursors at 75% epiboly

<table>
<thead>
<tr>
<th>MO injected at the 1-cell stage</th>
<th>Control</th>
<th>vox</th>
<th>vent</th>
<th>ved</th>
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<tbody>
<tr>
<td>Average number of sox17-positive cells per embryo</td>
<td>340±22</td>
<td>430±33***</td>
<td>320±35*</td>
<td>378±52**</td>
</tr>
<tr>
<td>Number of embryos analyzed</td>
<td>14</td>
<td>10</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Number of embryos with apparent cell number phenotype</td>
<td>1</td>
<td>11</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Total number of embryos</td>
<td>70</td>
<td>67</td>
<td>69</td>
<td>67</td>
</tr>
<tr>
<td>Average number of axial-positive cells per embryo</td>
<td>278±30</td>
<td>370±21***</td>
<td>260±42</td>
<td>251±34*</td>
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<tr>
<td>Total number of embryos</td>
<td>84</td>
<td>83</td>
<td>74</td>
<td>71</td>
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</table>

Embryos injected with MOs as indicated were sorted into those exhibiting a wild-type phenotype and those with an apparent modification in cell number, of which a portion were used for counting cells positive for sox17 or axial expression. ***P<0.005, **P<0.05, *P<0.1, versus uninjected controls.

Excluding cells belonging to the nascent axis.
the presence of Pou2 binding sites within the sox17 promoter region and neighboring the Cas binding sites. However, the requirement for pou2 for cas activity could be direct or indirect. Our binding results favor a direct physical interaction of Cas with Pou2 to induce endoderm. This is also consistent with the known requirement of sox-related transcription factors for Pou domain-containing partners. For instance, the Pou domain factor Oct3 (also known as Pou5f1) interacts with the HMG domain protein Sox2 and collaborates with Sox2 to bind the Fgf4 enhancer (Dailey et al., 1994; Ambrosetti et al., 1997; Ambrosetti et al., 2000). In vivo, Sox2-Oct3 complex formation is essential for transcriptional regulation of Fgf4. The identification of Cas as one of the major Cas binding partners validates the relevance of our screening approach.

Using the same approach, we also identified another homeobox-containing gene, vox, as a cas binding partner. By contrast, neither Ved nor Vent appears to efficiently and specifically bind Cas. Thus, within the three Vent group genes, only vox appears to be a cas binding partner. This is in contrast to the fact that Vox, Vent and Ved efficiently bind the Gsc transcription factor, suggesting that binding to Gsc and to Cas might involve different regions of the Vent group proteins. Consistent with this idea, comparison of the Vox C-terminal region, a domain required for the interaction with Cas, with the Vent/Ved C-terminal regions failed to reveal any strong homology (Kawahara et al., 2000b) (our results). Structure-function analyses and, in particular, domain swapping experiments are required to further define the regions of the Vent group proteins that are crucial for interaction with Cas.

We further show that, in addition to binding to Cas, Vox also binds to Pou2 and itself. It is thus possible that Vox, Cas and/or Pou2 are assembled into a multimeric complex or that there is an equilibrium between binary complexes involving either of these components, with different activities. Attempts to detect in vivo binding between Cas and Vox or Pou2 or both have been unsuccessful, raising the possibility that the functional interactions between these proteins are indirect. However, several reports have shown that co-activator interactions with Pou or Sox proteins can often be weak and transient and thus a direct functional interaction remains a likely scenario at this stage, particularly because of our in vitro interaction results (Wang et al., 2006; Van den Berg et al., 2010; Engelen et al., 2011). Further biochemical experiments are needed to resolve this point.

**Vox controls endoderm formation**

Overexpression of vox (and, more generally, the Vent group genes) and cas shows that vox inhibits the capacity of cas to induce sox17 expression. In this process, Vox behaves as a repressor and its N-terminus is required, at least in part, in the repressive activity. This situation is strikingly similar to the genetic interaction between Vent group genes and gsc, in which the Vent proteins also physically interact with Gsc and behave as repressors of gsc transcription. However, the biological significance of this latter physical

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**Table 2. vox acts downstream of BMP to repress endoderm precursor formation at 75% epiboly**

<table>
<thead>
<tr>
<th>RNA injected</th>
<th>No</th>
<th>Bmp2,4,7</th>
<th>Bmp2,4,7</th>
<th>Bmp2,4,7</th>
<th>Bmp2,4,7</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO injected</td>
<td>No</td>
<td>No</td>
<td>vox</td>
<td>vent</td>
<td>ved</td>
</tr>
<tr>
<td>Average number of sox17-positive cells per ventral half of embryo</td>
<td>98±10</td>
<td>12±11</td>
<td>82±15***</td>
<td>16±4</td>
<td>19±12</td>
</tr>
<tr>
<td>Total number of sox17-positive cells per embryo</td>
<td>294±35</td>
<td>192±14</td>
<td>264±29***</td>
<td>202±33</td>
<td>214±25**</td>
</tr>
<tr>
<td>Number of embryos analyzed</td>
<td>10</td>
<td>12</td>
<td>10</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Number of embryos exhibiting an apparent variation in cell number</td>
<td>2</td>
<td>23</td>
<td>10</td>
<td>36</td>
<td>50</td>
</tr>
<tr>
<td>Total number of embryos</td>
<td>45</td>
<td>113</td>
<td>94</td>
<td>101</td>
<td>124</td>
</tr>
</tbody>
</table>

**B. Vent group gene overexpression represses endoderm precursor formation when BMP signaling is inhibited**

<table>
<thead>
<tr>
<th>RNA injected</th>
<th>ΔmBMPR</th>
<th>ΔmBMPR + vox</th>
<th>ΔmBMPR + vent</th>
<th>ΔmBMPR + ved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average number of sox17-positive cells per ventral half of embryo</td>
<td>163±23</td>
<td>24±9***</td>
<td>86±10***</td>
<td>65±13***</td>
</tr>
<tr>
<td>Total number of sox17-positive cells per embryo</td>
<td>417±46</td>
<td>234±33***</td>
<td>343±24***</td>
<td>319±31***</td>
</tr>
<tr>
<td>Number of embryos analyzed</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Number of embryos exhibiting an apparent variation in cell number</td>
<td>23</td>
<td>74</td>
<td>38</td>
<td>23</td>
</tr>
<tr>
<td>Total number of embryos</td>
<td>86</td>
<td>87</td>
<td>84</td>
<td>92</td>
</tr>
</tbody>
</table>

Embryos injected with MOs or RNAs or both, as indicated, were sorted into those exhibiting a wild-type phenotype and those with an apparent modification in cell number, of which a portion were used for counting sox17-positive cells. Both ventral and total cell numbers per embryo were counted to ensure that variations did not result from improper localization of cells. ***P<0.005, **P<0.05, samples are compared to Bmp2,4,7-injected (A) or ΔmBMPR-injected (B) embryos.
interaction has not been explored, probably owing to the absence of an assay for gsc transcriptional activity at the time.

Since \textit{sox17} is a direct target of \textit{cas} and its transcriptional regulatory region has been partly defined, we were able to approach this problem in more detail. Although we cannot exclude an indirect action of \textit{vox} on \textit{cas}, two arguments are in favor of a simple model in which Vox binds Cas and/or Pou2 and prevents transcription of \textit{sox17}. First, analysis of the \textit{sox17} regulatory region shows that it contains several potential binding sites for Vox within 100 bp of the Pou2 or the Cas binding sites. Removal of one or two of these binding sites leads to increased expression of \textit{sox17}, suggesting that this region indeed contains target elements for a repressor (data not shown). Second, Vox binds both Cas and Pou2 and removal of the Vox C-terminus prevents binding and leads to a reduction in Vox inhibitory activity, consistent with the idea that binding is required for this latter process. However, whereas binding is no longer inhibitory activity, consistent with the idea that binding is required to mesoderm in the absence of functional \textit{cas}. However, the fact that ectoderm derivatives are also generated in the presence of \textit{vox} could suggest that \textit{vox} is also involved in repressing the endodermal fate. Taken together, our results show that \textit{vox} together with \textit{cas} and \textit{pou2} control the endoderm versus mesoderm/ectoderm fate by regulating \textit{cas} transcriptional activity, presumably by direct physical interaction.

\textbf{Vox mediates the activity of BMPs on endoderm}

Interference with \textit{vox} activity leads to an enlarged endodermal domain, consistent with its inhibitory effect on \textit{cas} function. This phenotype is highly reminiscent of the action of BMPs on endoderm development. Since \textit{vox}, \textit{vent} and \textit{ved} are regulators and effectors of BMP signals (Kawahara et al., 2000a; Kawahara et al., 2000b; Shimizu et al., 2002; Gilardelli et al., 2004), \textit{vox} could act upstream and/or downstream of BMPs in endoderm regulation. By epistatic analysis, we find that \textit{vox} acts downstream of, or in parallel to, BMPs, a situation that is consistent with the fact that \textit{vox} is induced by BMPs during gastrulation (Ladher et al., 1996; Melby et al., 1999). Interestingly, \textit{vox} involvement in endoderm repression downstream of BMPs does not seem to strictly parallel its role in

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|c|c|c|c|c|}
\hline
Injection & \multicolumn{2}{c|}{Pharyngeal} & \multicolumn{2}{c|}{Intestine} & \multicolumn{2}{c|}{Hatching} & \multicolumn{2}{c|}{Muscle} & \multicolumn{2}{c|}{Mesenchyme} & \multicolumn{2}{c|}{Mesenchyme} & \multicolumn{2}{c|}{Posterior} & \multicolumn{2}{c|}{Otic} & \multicolumn{2}{c|}{Otic} & \multicolumn{2}{c|}{Epidermis} \\
\hline
Tar* + GFP & 12 & 11 & 10 & 6 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & & & & & & & & \\
Tar* + GFP + vox & 27 & 1 & 0 & 1 & 13 & 7 & 9 & 5 & 2 & 2 & & & & & & & & & \\
\hline
\end{tabular}
\caption{\textit{vox} inhibits endodermal fates}
\end{table}

The phenotype of grafted cells was scored when host embryos had reached 30 hpf and was confirmed at 3 dpf. Indicated is the number of embryos with at least one cell participating in a given tissue. In each embryo, the number of cells incorporated into each type of derivative could not be precisely determined.

ICM, intermediate cell mass.
DV gastrula patterning. First, vox does not appear to be essential for DV gastrula patterning but has been shown to act non-redundantly with vent and ved in this process. Second, in contrast to their common activity in DV gastrula patterning, although ved and vent can inhibit endoderm formation when BMP signaling is reduced, only vox appears required for BMP inhibition of ventral endoderm development. Thus, vox appears to be a specific repressor of endoderm development within the Vent group, while this group has a more general role in gastrula DV patterning as a whole.

In conclusion, our results together with those of others show that proper endoderm development relies on the integration of three distinct signaling pathways: the Nodal pathway, which is responsible for the induction of cas, and the FGF and BMP pathways, which further control cas transcriptional activity. Whereas FGF signaling negatively regulates Cas by phosphorylation, BMPs act via induction of the vox repressor, which is a binding partner for both cas and pou2.

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Competing interests statement
The authors declare no competing financial interests.

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Vox inhibits Cas

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