Mixer/Bon and FoxH1/Sur have overlapping and divergent roles in Nodal signaling and mesendoderm induction

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Summary

Transcription factors belonging to the FoxH1 and Mixer families are required for facets of Nodal signaling during vertebrate mesendoderm induction. Here, we analyze whether zebrafish proteins related to FoxH1 [Schmalspur (Sur)] and Mixer [Bonnie and clyde (Bon)] act within or downstream of the Nodal signaling pathway, test whether these two factors have additive or overlapping activities, and determine whether FoxH1/Sur and Mixer/Bon can account for all Nodal signaling during embryogenesis. We find that *sur* expression is independent of Nodal signaling and that *bon* is expressed in the absence of Nodal signaling but requires Nodal signaling and Sur for enhanced, maintained expression. These results and the association of FoxH1 and Mixer/Bon with phosphorylated Smad2 support a role for these factors as components of the Nodal signaling pathway. In contrast to the relatively mild defects observed in single mutants, loss of both *bon* and *sur* results in a severe phenotype characterized by absence of prechordal plate, cardiac mesoderm, endoderm and ventral neuroectoderm. Analysis of Nodal-regulated proteins reveals that Bon and Sur have both distinct and overlapping regulatory roles. Some genes are regulated by both Bon and Sur, and others by either Bon or Sur. Complete loss of Nodal signaling results in a more severe phenotype than loss of both Bon and Sur, indicating that additional Smad-associated transcription factors remain to be identified that act as components of the Nodal signaling pathway.

Key words: Nodal, Smad, Mesoderm, Endoderm, FoxH1, Mix, Zebrafish

Introduction

Nodal signals induce mesoderm and endoderm and control left-right axis development during vertebrate embryogenesis (Schier, 2003). Nodal signaling is mediated by type I (ALK4, ALK7, TARAM-A) and type II (ActRIIB, ActRIIA) receptor serine/threonine kinases, and requires EGF-CFC proteins as co-receptors. Activation of receptors results in the phosphorylation of regulatory Smad transcription factors such as Smad2, which then associate with Smad4 to translocate into the nucleus. These Smad complexes combine with specific transcription factors to regulate different target genes (Attisano and Wrana, 2000; Hill, 2001; Whitman, 2001; Shi and Massague, 2003). It is generally assumed that the Smad-associated transcription factors determine the specific responses of a cell to a given transforming growth factor β (TGFβ) signal (Hill, 2001; Whitman, 2001; Shi and Massague, 2003). It is unclear, however, how many of these factors are required or sufficient to mediate a particular TGFβ signaling process in vivo. Here, we address this question by analyzing the roles of FoxH1/Sur and Mixer/Bon during Nodal signaling in zebrafish.

Members of the FoxH1 (Fast1, Fast3) and Mix/Bix (Mixer, Milk, Bix3) families are the best characterized partners of phosphorylated Smad2 during embryogenesis (Hill, 2001; Whitman, 2001). FoxH1 proteins are forkhead/winged helix transcription factors that can recruit active Smad complexes to activin responsive elements (AREs) in *Xenopus mix.2*, *xnr1* and other genes (Chen et al., 1996; Watanabe and Whitman, 1999; Osada et al., 2000). Mixer and related Mix/Bix proteins are paired-like homeodomain proteins and can recruit active Smad complexes to the distal element (DE) of the *Xenopus goosecoid* (*gsc*) promoter (Germain et al., 2000; Randall et al., 2002). The interaction of these transcription factors with the activated Smad complex is mediated through a Smad interaction motif (SID in Fast1, SIM in Mixer) (Chen et al., 1997; Randall et al., 2002).

The in vivo roles of FoxH1 have been analyzed genetically in mouse and zebrafish, and through the use of interference approaches in *Xenopus*. *FoxH1* mutant mice have variable but severe phenotypes, including loss of anterior structures, failure to form the node and its midline derivatives, and defects in definitive endoderm formation (Hoodless et al., 2001; Yamamoto et al., 2001). In contrast to *nodal* mutants, however, *foxH1* mutants develop most mesoderm. Blocking antibodies

against *Xenopus* Fast1 led to defects in mesoderm formation, including the inhibition of the mesodermal marker *T/Xbra* and the dorsal marker *gsc* (Watanabe and Whitman, 1999). In addition, Activin-mediated induction of *mix2*, *lim1* and *gsc* is blocked by anti-Fast1 antiserum (Watanabe and Whitman, 1999). Somewhat conflicting results have been reported when *Xenopus* Fast1 and Fast3 activity was knocked down using morpholinos: although gastrulation movements were inhibited in these embryos, most marker genes (including *gsc*, *mix2* and *lim1*) seem to be expressed normally (Howell et al., 2002). Taken together, these results suggest that *Xenopus* FoxH1 might mediate the activation of gastrulation movements and/or mesoderm induction.

Genetic screens in zebrafish have identified mutations in *FoxH1* [*schmalspur* (*sur*)] (Brand et al., 1996; Schier et al., 1996; Solnica-Krezel et al., 1996; Pogoda et al., 2000; Sirotkin et al., 2000). Mutants that lack zygotic *sur* activity (Z*sur*) have variable, relatively mild phenotypes, ranging from randomization of left-right asymmetry but normal early patterning to reduction of prechordal plate and floor plate (Brand et al., 1996; Schier et al., 1996; Solnica-Krezel et al., 1996; Pogoda et al., 2000; Sirotkin et al., 2000). Embryos lacking both maternal and zygotic *sur* function (MZ*sur*) can have more severe but variable phenotypes, including reduction of axial midline structures (Pogoda et al., 2000; Sirotkin et al., 2000) (this report). This phenotype is much milder than the one observed upon complete loss of Nodal signaling, which leads to a lack of all endoderm, head and trunk mesoderm, and ventral neuroectoderm (Feldman et al., 1998; Gritsman et al., 1999; Meno et al., 1999; Thisse and Thisse, 1999).

In the case of *Mix/Bix* genes, misexpression studies in *Xenopus* have shown that members of this family can induce endoderm development but their individual requirements in this process have not been resolved (Ecochard et al., 1998; Henry and Melton, 1998; Lemaire et al., 1998). Supporting a role for *Mix/Bix* genes in endoderm formation, zebrafish mutants for the *mixer*-like gene *bonnie and clyde* (*bon*) (Chen et al., 1996; Stainier et al., 1996; Alexander et al., 1999; Kikuchi et al., 2000) or embryos lacking *bon* activity because of morpholino (MO) injection (Kikuchi et al., 2000) have a dramatic reduction of endoderm. Additional phenotypes include cardia bifida and pericardial edema, but mesoderm induction appears largely normal in these embryos (Stainier et al., 1996; Chen et al., 1996; Kikuchi et al., 2000). In contrast to the *sur* phenotypes, the *bon* phenotype is fully penetrant and largely invariable.

The role of *Mix/Bix* genes in Nodal signaling is complicated by the observation that some of these genes are regulated transcriptionally by Nodal signals. For instance, biochemical studies and sequence analysis indicate that Bon can serve as a binding partner of phosphorylated Smad2 (Randall et al., 2002), suggesting that Bon is a component of the Nodal signaling pathway. Other studies have emphasized that *bon* is a transcriptional target of Nodal signaling (Alexander and Stainier, 1999). In particular, *bon* expression is absent or barely detectable at the onset of gastrulation in the absence of Nodal signaling, suggesting that *bon* is primarily a target of the Nodal signaling pathway rather than a necessary transducer of Nodal signals (Alexander and Stainier, 1999). This raises the question of whether Bon is a Smad-associated component and/or a downstream gene of the Nodal signaling pathway. An

additional level of complexity derives from the observation that some members of the Mix/Bix family, such as mouse Mixl1, appear not to interact with phosphorylated Smad2 (Germain et al., 2000; Randall et al., 2002) but are involved in processes that are regulated by Nodal signaling. For instance, mouse *Mixl1* mutant embryos display complex gastrulation defects and, in chimeras, *Mixl1* mutant cells are largely excluded from endoderm and heart (Hart et al., 2002).

Here, we analyze the regulation of *bon* and *sur* by Nodal signaling, determine whether *bon* and *sur* have overlapping, additive or antagonistic functions, and test whether Nodal signaling is mediated exclusively by *bon* and *sur*. We find that *sur* expression is independent of Nodal signaling, whereas *bon* is initially expressed in the absence of Nodal signaling but requires Nodal signaling and *sur* for full and maintained expression. We find that MZ*sur*;*bon* double mutants and MZ*sur*;*bon*MO embryos have severe phenotypes not observed upon loss of either *bon* or *sur*. Double mutants lack heart, prechordal plate and ventral neuroectoderm, a subset of the phenotypes seen upon complete loss of Nodal signaling. Analysis of Nodal downstream genes indicates that *bon* and *sur* have both divergent and overlapping functions in gene regulation, and reveals that some Nodal-dependent genes do not require *bon* and *sur* activity. Overall, our study establishes that *sur* and *bon* have both independent and overlapping roles as components of the Nodal signaling pathway but do not account for all effects of Nodal signaling during mesendoderm induction.

Materials and methods

Zebrafish strains
Embryos were staged as described (Kimmel et al., 1995). The following mutant alleles were used: *oep*^{tz57} (Hammerschmidt et al., 1996; Zhang et al., 1998), *bon*^{s9} (Chen et al., 1996; Kikuchi et al., 2000) and *sur*^{m768} (Solnica-Krezel et al., 1996; Schier et al., 1996; Sirotkin et al., 2000; Pogoda et al., 2000). Misexpression studies have indicated that all three alleles are complete loss-of-function mutations (Zhang et al., 1998; Kikuchi et al., 2000; Pogoda et al., 2000; Sirotkin et al., 2000). In addition, a very weak antimorphic phenotype has been described for *bon*^{s9} (Kikuchi et al., 2000). Therefore, we have corroborated results obtained with *bon*^{s9} by using MOs that block *bon*. For simplicity and to distinguish zebrafish genes from mouse and frog genes, we use *bon* and *sur* throughout the text.

### In situ hybridization and phosphorylated-Smad2 detection
In situ hybridization and preparation of RNA probes were performed as described (Schier et al., 1997). Phosphorylated-Smad2 detection was as described (Mintzer et al., 2001).

Microinjection of mRNA and *bon* MO
Synthetic capped *squint* RNA was synthesized and injected as described (Chen and Schier, 2001). Approximately 3 ng of *bon* MO (Kikuchi et al., 2000) dissolved in phenol red buffer was injected into the yolk of one- to two-cell-stage wild-type or MZ*sur* embryos.

Genotyping of *bon* and *sur* fish
Fish were genotyped as described (Chen and Schier, 2001). Primers for *bon* are described in Kikuchi et al. (Kikuchi et al., 2000). Primers for *sur* are 5′-TCACCTTGACTGCAGAATCGG-3′ [fast 330 f2 (Sirotkin et al., 2000)] and 5′-GCCAGGTAAGAGTACGGTGG-TTTGGGATAT-3′ (SurDCWTR2). SurDCWTR2, a dCAP (derived cleaved amplified polymorphic sequence) primer (Neff et al., 1998), introduces an *Eco*RV restriction site into the wild type to give a 205
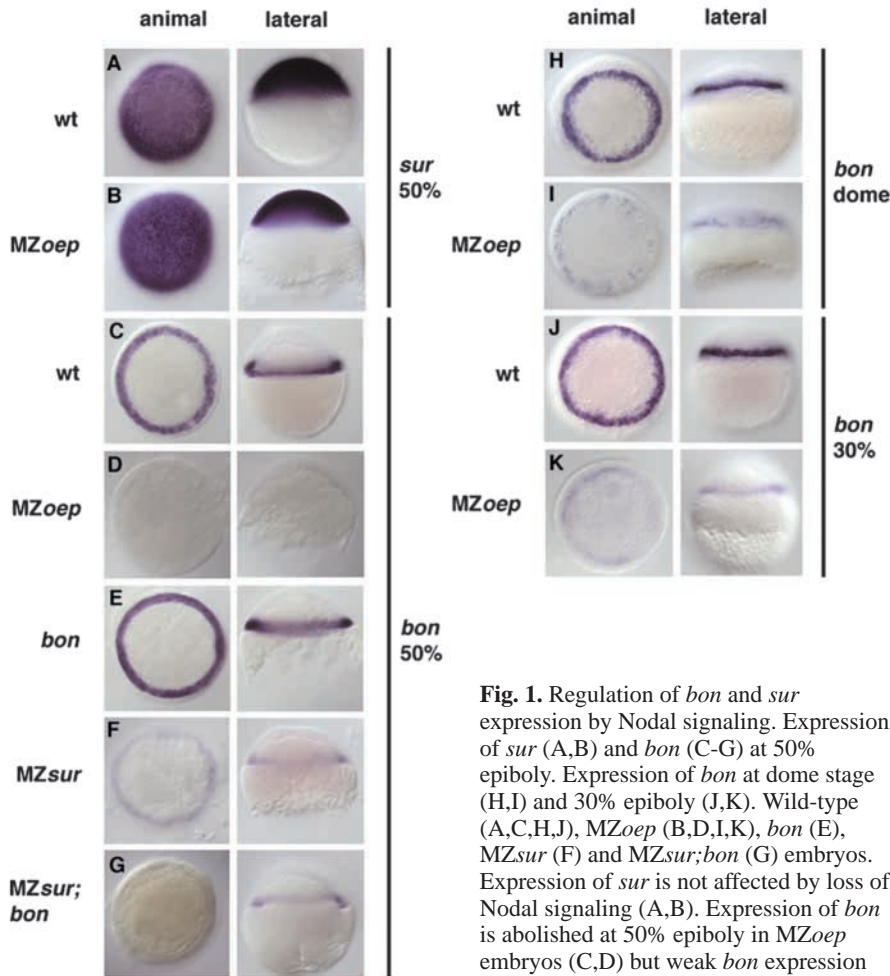
Fig. 1. Regulation of *bon* and *sur* expression by Nodal signaling. Expression of *sur* (A,B) and *bon* (C-G) at 50% epiboly. Expression of *bon* at dome stage (H,I) and 30% epiboly (J,K). Wild-type (A,C,H,J), MZ*oep* (B,D,I,K), *bon* (E), MZ*sur* (F) and MZ*sur;bon* (G) embryos. Expression of *sur* is not affected by loss of Nodal signaling (A,B). Expression of *bon* is abolished at 50% epiboly in MZ*oep* embryos (C,D) but weak *bon* expression can be detected in dome stage (H,I) and 30% epiboly (J,K). Expression of *bon* is not affected in *bon* mutants (E) but reduced in MZ*sur* and MZ*sur;bon* mutant embryos at 50% epiboly (F,G).

downstream target gene of Nodal signaling. By contrast, the ubiquitous maternal and zygotic expression of *sur* has suggested that its expression is not regulated by Nodal signaling (Pogoda et al., 2000; Sirotkin et al., 2000), similarly to *Xenopus FoxH1* (Chen et al., 1996; Watanabe and Whitman, 1999). To explore the regulation of *bon* and *sur* further, we investigated their expression in MZ*oep* mutants, which lack all Nodal signaling (Gritsman et al., 1999). Expression of *sur* is not affected by loss of Nodal signaling (Fig. 1B). By contrast, and in support of previous analyses (Alexander and Stainier, 1999), we found that *bon* expression is abolished or barely detectable at 50% epiboly in MZ*oep* mutants (Fig. 1D). However, we detected weak *bon* expression at earlier stages (dome and 30% epiboly) in MZ*oep* mutants (Fig. 1I,K). These results indicate that *sur* expression is independent of Nodal signaling, whereas maintained and full, but not initial and weak, *bon* expression requires Nodal signaling.
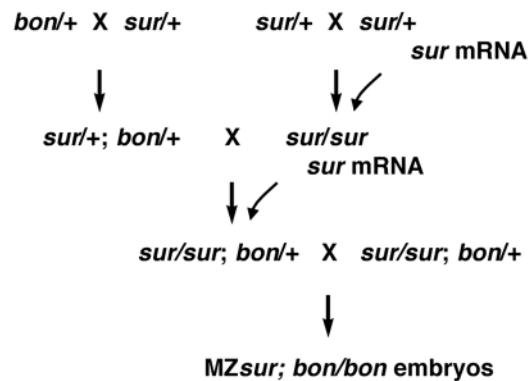
To determine whether *sur* and/or *bon* mediate Nodal signaling to enhance *bon* expression, we analyzed *bon* and MZ*sur* mutants and MZ*sur*;*bon* double mutants (see below). We found that *bon* expression is unaffected in *bon* mutants (Fig. 1E) and strongly, but not completely, reduced in MZ*sur* and MZ*sur*; *bon* mutants (Fig. 1F,G). Expression of *sur* was not affected in any of these mutants (data not shown). Together with previous biochemical studies (Chen et al., 1996; Germain et al., 2000; Randall et al., 2002), these results indicate that Bon and Sur can act as components of the Nodal signaling pathway and that Sur enhances *bon* expression during early embryogenesis.
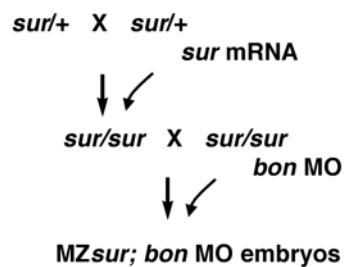
Generation of embryos that lack both *bon* and *sur* activity

The findings that both *bon* and *sur* are expressed in the absence of Nodal signaling, that both Mixer and FoxH1 can interact with phosphorylated Smad2, and that *bon* and *sur* have different phenotypes suggested that *bon* and *sur* might have either additive or overlapping roles during mesendoderm induction and Nodal signaling. To distinguish between these possibilities, we generated embryos that lack both *bon* and *sur* activity using two different approaches (Fig. 2). In a genetic approach (Fig. 2A), we generated embryos that lack both maternal and zygotic *sur* (*sur* is expressed maternally and zygotically) and also lacked zygotic *bon* [*bon* is only expressed zygotically (Kikuchi et al., 2000)]. We crossed *bon*/+ and *sur*/+ heterozygous fish to generate *bon*/+; *sur*/+ double heterozygotes. These were crossed to *sur*/*sur* fish and resulting embryos were injected with wild-type *sur* mRNA to rescue *sur*/*sur* mutants. This allowed us to raise *sur*/*sur*;*bon*/+ fish to adulthood. Intercrosses of these fish result in embryos that lack

base pair band but does not introduce this site into *sur*^*m768*^, resulting in a 235 base pair band. The conditions for genotyping of both *bon* and *sur* were: 94°C for 3 minutes (1 cycle), followed by 45 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, and finally 72°C for 5 minutes. The amplified *sur* product was digested with *Eco*RV and resolved on 2% agarose gels.

Results

Nodal-signaling-dependent and -independent expression of *bon*

Members of the Mixer family have been implicated as both transcriptional downstream genes of Nodal signaling (i.e. transcribed in response to Smad/transcription factor complexes) (Rosa, 1989; Chen et al., 1996; Vize, 1996; Ecochard et al., 1998; Henry and Melton, 1998; Alexander and Stainier, 1999) and components of the Nodal signaling pathway (i.e. as partners of Smads) (Germain et al., 2000; Randall et al., 2002). In the case of *bon*, previous studies have implied that *bon* transcription is almost fully dependent on Nodal signaling (Alexander and Stainier, 1999; Kikuchi et al., 2000). In this scenario, *bon* would initially not be a component of the Nodal signaling pathway but would instead be a

A. Genetic Approach

bon/+ X sur/+   sur/+ X sur/+
                                          sur mRNA

sur/+; bon/+   X   sur/sur
                     sur mRNA

sur/sur; bon/+   X   sur/sur; bon/+

MZ*sur*; *bon/bon* embryos

B. Morpholino Approach

sur/+ X sur/+
              sur mRNA

sur/sur   X   sur/sur
                  bon MO

MZ*sur*; *bon* MO embryos

Fig. 2. Generation of embryos lacking both *bon* and *sur* activity by two different approaches. (A) In the genetic approach, *bon* and *sur* heterozygous fish are crossed to generate *bon* and *sur* double heterozygotes, which are crossed to *sur* homozygous fish. The *sur* homozygous and *bon* heterozygous embryos are injected with *sur* mRNA to rescue *sur* mutants. The intercrossing of these fish results in embryos lacking both *bon* and maternal and zygotic *sur*. (B) In the morpholino approach, *bon*MO is injected into MZ*sur* mutants at the one- to two-cell stage to give rise to embryos lacking both *bon* activity and maternal and zygotic *sur*.

both maternal and zygotic sur activity (MZ*sur*), and one-quarter of embryos are also homozygous for *bon*. These MZ*sur;bon* mutants lack all *sur* and *bon* activity. In a second approach (Fig. 2B), we used a MO antisense oligonucleotide

against *bon* (Summerton and Weller, 1997; Heasman et al., 2000; Nasevicius and Ekker, 2000). Previous studies have shown that this MO efficiently phenocopies the *bon* phenotype (Kikuchi et al., 2000). Injection of *bon*MO into *MZsur* mutants at the one or two cell stage results in MZ*sur;bon*MO embryos.

Loss of *bon* and *sur* leads to strong and consistent mesendoderm defects

MZ*sur;bon* and MZ*sur;bon*MO embryos have indistinguishable phenotypes at 30 hours postfertilization (hpf; Fig. 3). Although *bon* mutant or *bon*MO embryos have a severe reduction in endoderm (Fig. 3B,C) and MZ*sur* embryos have mild midline defects (Fig. 3D), MZ*sur;bon* (Fig. 3E) and MZ*sur;bon*MO (Fig. 3F) embryos display additional defects not observed in single mutants: the hatching gland and heart are absent, and the ventral forebrain, eyes and floor plate are dramatically reduced. Importantly, this phenotype is highly penetrant and expressive (Fig. 3). Notably, other mesoderm derivatives such as notochord, somites and blood, which are severely affected in the absence of Nodal signaling, appear
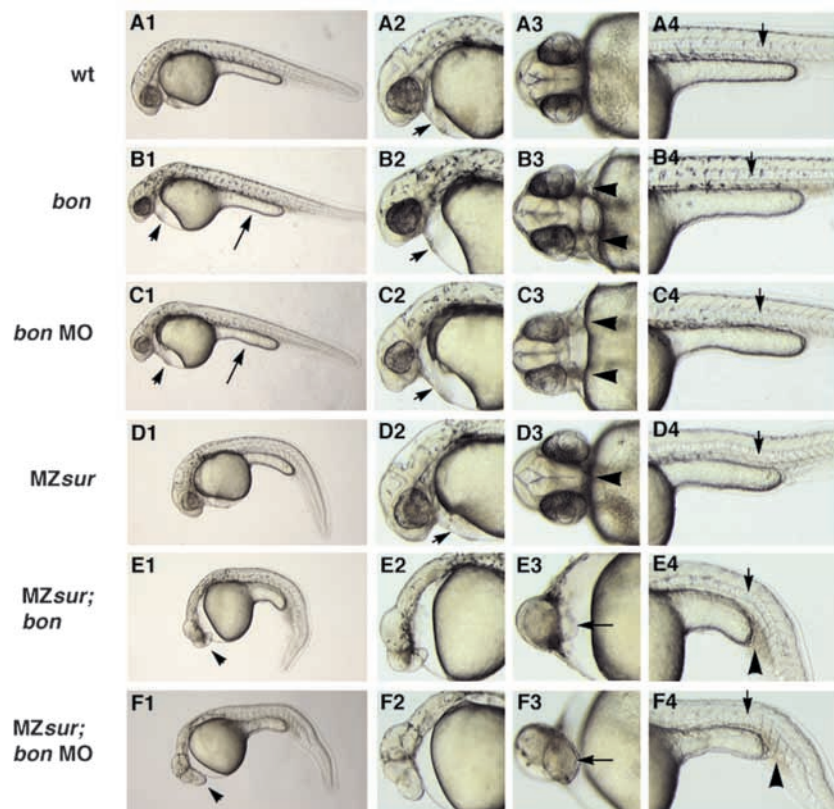

Fig. 3. Phenotypes associated with lack of both *bon* and *sur* activity. Wild-type (A1-4), *bon* (B1-4), *bon*MO (C1-4), MZ*sur* (D1-4), MZ*sur;bon* (E1-4) and MZ*sur;bon*MO (F1-4) embryos at 30 hpf. All images are lateral views except A3-F3, which are ventral views. (A1-F1) Comparison of embryos in a lateral view. The *bon* (B1) and *bon*MO (C1) embryos have pericardial edema (arrowhead) and enlarged yolk extension (arrow). MZ*sur;bon* (E1) and MZ*sur;bon*MO (F1) embryos have an anterior bulb-like structure (arrowhead; also arrow in E3 and F3). (A2-F2) Lateral view of head region, showing the absence of the hatching gland (arrowhead) in MZ*sur;bon* (E2) and MZ*sur;bon*MO (F2) embryos but present in others. (A3-F3) Ventral view of head region, presence of two hearts (cardiac bifida; arrowheads), in *bon* (B3) and *bon*MO (C3), single heart in MZ*sur* (arrowhead in D3) and no heart in MZ*sur;bon* (E3) and MZ*sur;bon*MO (F3) embryos. (A4-F4) Lateral view of trunk and tail region, all embryos display normal notochords (arrow). MZ*sur;bon* and MZ*sur;bon*MO embryos exhibit accumulated blood (arrowhead), owing to defects in circulation. 100% of MZ*sur;bon* embryos lacked hatching gland, heart and ventral CNS (*n*=41). By contrast, 81% of MZsur embryos had a heart, hatching gland and two eyes, 15% had a heart and hatching gland and fused eyes, and 3% had a heart, no hatching gland and fused eyes (*n*=85).
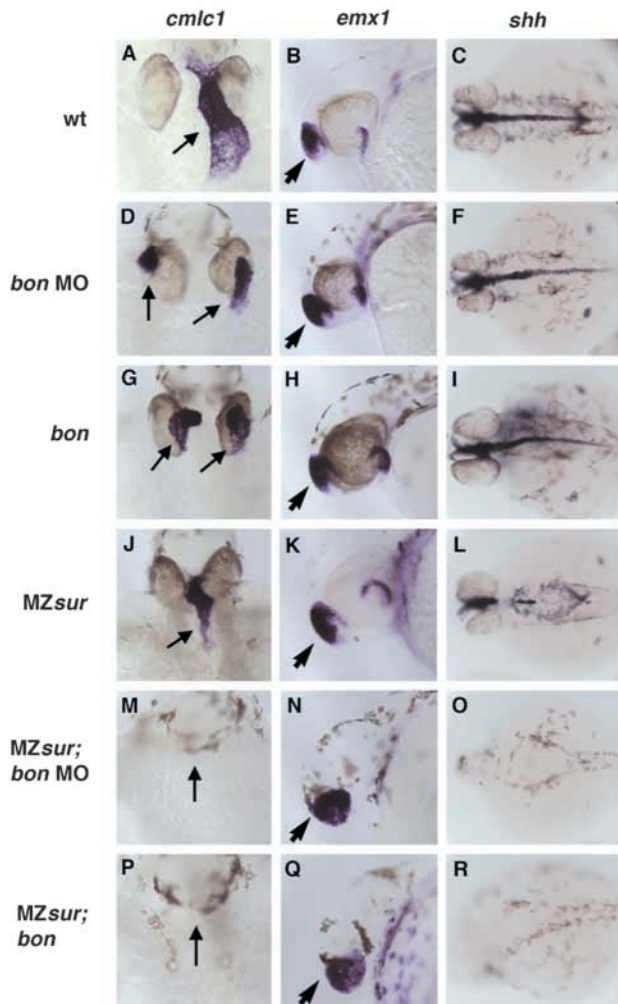
Fig. 4. Roles of *bon* and *sur* in heart and nervous system development. Wild-type (A-C), *bon*MO (D-F), *bon* (G-I), MZ*sur* (J-L), MZ*sur;bon*MO (M-O) and MZ*sur;bon* (P-R) embryos at 30 hpf. Ventral view of *cardiac myosin light chain 1* (*cmlc1*) expression (A,D,G,J,M,P) in a single normal heart in wild-type (A), in two reduced hearts in *bon*MO (D) and *bon* (G), in a single heart in MZsur (J), but not in MZ*sur;bon*MO (M) and MZ*sur;bon* (P) embryos (arrows). Lateral view, anterior to the left, dorsal up, of *emx1* expression in telencephalon (arrowhead in B,E,H,K,N,Q); normal *emx1* expression in wild-type (B), *bon*MO (E), *bon* (H) and MZ*sur* (K), and anterior expression in bulb-like structure in MZ*sur;bon*MO (N) and MZ*sur;bon* (Q). Expression of *shh* in head and trunk region (C,F,I,L,O,R), normal expression in wild-type (C), *bon* (F) and *bon*MO (I) but discontinuous weak expression in MZ*sur* (L) and absence of staining in MZ*sur;bon*MO (O) and MZ*sur;bon* (R), indicating absence of ventral CNS. Also notice the expression in endoderm in wild-type and MZ*sur* embryos.

largely normal and the central nervous system (CNS) is patterned along the anterior-posterior axis. Morphological abnormalities were further confirmed by in situ hybridization using tissue-specific markers (Figs 4, 5). Lack of the prechordal plate-derived hatching gland is evidenced by absence of anterior-most *islet-1* expression domain (Fig. 5D). Lack of myocardial cells is apparent from the absence of the markers *cmlc1* and *cmlc2* (Fig. 4M,P, Fig. 5H). Absence of the
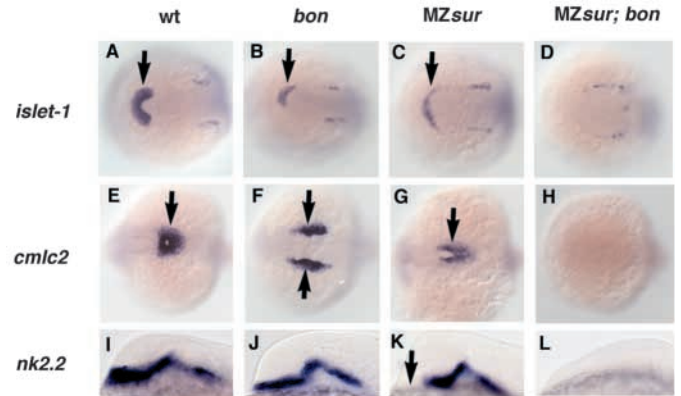

Fig. 5. Roles of *bon* and *sur* in hatching gland, heart and nervous system development. Wild-type (A,E,I), *bon*MO (B,F,J) MZ*sur* (C,G,K), MZ*sur;bon*MO (D,H,L) embryos at 5S (A-D), 20S (E-H) and 18S (I-L) stages. (A-D) Dorsal view of *islet-1* expression in developing hatching gland (arrow). (E-H) Ventral view of *cardiac myosin light chain 2* (*cmlc2*) expression in a single domain in wild-type (E), in two domains in *bon*MO (F), in a single domain in MZsur (G) but not in MZ*sur;bon*MO (H). Lateral view, anterior to the left, dorsal up, of *nk2.2* expression in ventral neuroectoderm; normal expression in wild-type (I) and *bon*MO (J), reduced anterior expression in MZ*sur* (arrow in K), and lack of expression in MZ*sur;bon*MO (L).

ventral CNS is highlighted by loss of *shh* and *nk2.2* expression (Fig. 4O,R, Fig. 5L). The anterior-most region of the CNS in double mutants expresses *emx1* (Fig. 4N,Q), defining this territory as telencephalon.

To determine whether these phenotypes are already apparent during early embryogenesis, we analyzed the expression of different markers during late gastrulation and early somitogenesis (Fig. 6). At 80-90% epiboly, endodermal markers (*axial*, *casanova* and *sox17*) are absent or severely reduced in double mutant embryos (Fig. 6H,L,P). The average number of *sox17* positive cells was 10 (*n*=4), in contrast to 24 in *bon* mutant embryos (*n*=5). In addition, the prechordal plate marker *gsc* is not expressed in double mutants at the end of gastrulation (Fig. 6D). Similarly, the anterior expression domain of *axial*, corresponding to the prechordal plate, is absent in double mutants (Fig. 6H). Remaining midline expression of *axial* and expression of the notochord marker *ntl* is compressed along the anterior-posterior axis and broader along the dorsal-ventral axis (Fig. 6H,T), suggesting a defect or delay in dorsal convergence. These results reveal essential, overlapping roles for *bon* and *sur* in prechordal plate, heart and ventral CNS formation.
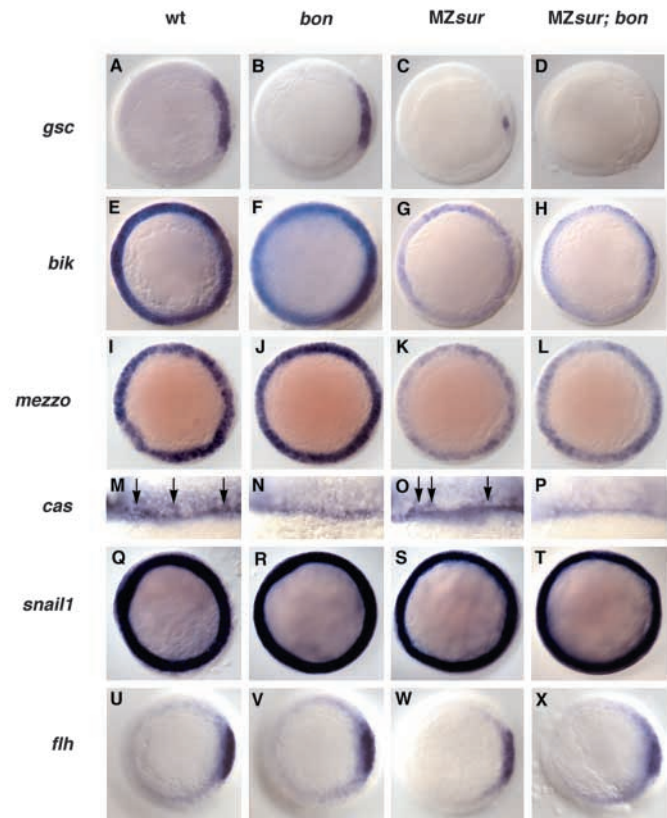
Regulation of Nodal target genes by Bon and Sur

The MZ*sur;bon* phenotype only affects a subset of structures dependent on Nodal signaling. This suggested the hypothesis that Sur and Bon regulate only a specific subset of Nodal-dependent genes (e.g. genes involved in prechordal plate or endoderm formation) but that other genes are not affected by Bon and Sur (e.g. genes involved in mesoderm formation, notochord formation or cell internalization). Alternatively, Bon and Sur might affect the expression of many Nodal-dependent genes but to different extents depending on the level of Nodal signaling required for expression. This would result in the loss
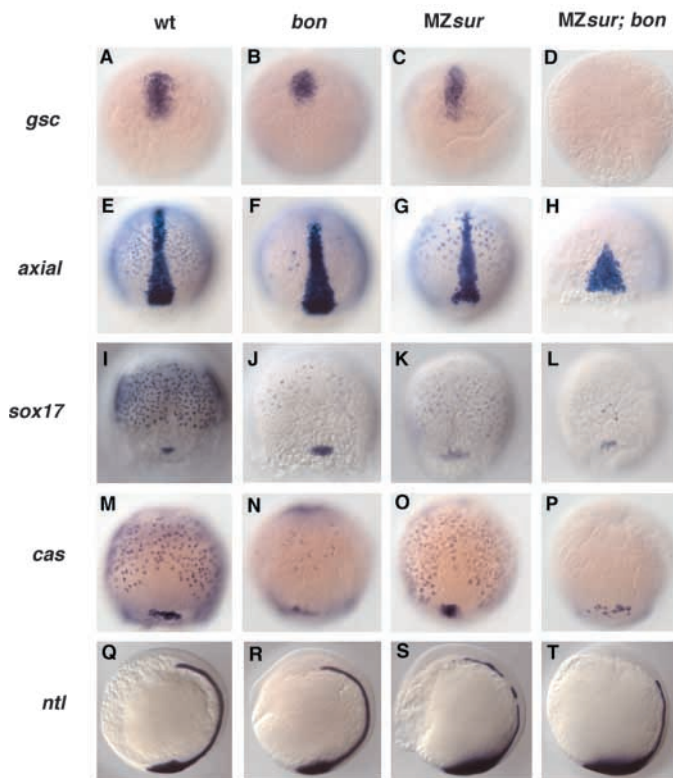
Fig. 6. Roles of *bon* and *sur* in the development of endoderm and prechordal plate. (A-D) Dorsal view of 90% epiboly embryos. (E-L) Dorsal view of 80-90% epiboly embryos. (M-P) Dorsal view of 90% epiboly embryos. (Q-T) Lateral view of three-somite-stage embryos. (A-D) Expression of *goosecoid* in prechordal plate in wild-type (A), *bon*MO (B), MZ*sur* (C) but not in MZ*sur;bon*MO (D). Notice the recovery of *gsc* expression in MZ*sur* at 90% (C) compared with 50% epiboly (Fig. 7C). (E-H) Expression of *axial* in dorsal midline and endoderm progenitors. Endodermal cells are reduced dramatically in *bon* (F) and MZ*sur;bon* mutant (H) embryos but are less reduced in the MZ*sur* mutant embryo (G). In the MZ*sur;bon* mutant embryo (H), anterior expression corresponding to prechordal plate is missing and the remaining midline expression is compressed along the anterior-posterior axis and broadened laterally. (I-L) Expression of the endodermal marker *sox17* is partially reduced in MZ*sur* (K), strongly reduced in *bon* (J) and almost absent in the MZ*sur;bon* mutant embryo (L). Expression of the endodermal marker *casanova* (M-P) is strongly reduced in *bon*MO (N), and almost absent in MZ*sur;bon*MO (P). Notice that the expression of *sox17* and *casanova* in dorsal forerunner cells (vegetal-dorsal expression domain) is not affected. Notochord expression of *ntl* is reduced and discontinuous in MZ*sur* (S) and reduced and compressed in MZ*sur;bon* (T).

Fig. 7. Different regulation of Nodal target genes by *bon* and *sur*. All embryos are at 50% epiboly; animal pole view except M-P, which are lateral views. (A-D) Expression of *gsc* in prechordal plate progenitors is weakly reduced in *bon* (B), strongly reduced in MZ*sur* (C) and absent in MZ*sur;bon* mutant embryos (D). (E-H) Expression of *bik* is not affected in *bon* (F) but downregulated in MZ*sur* (G) and MZ*sur;bon* (H) mutant embryos. (I-L) Expression of *mezzo* is not affected in *bon*MO (J) but downregulated in MZ*sur* (K) and MZ*sur;bon*MO (L) embryos. (M-P) Expression of *cas* in endodermal progenitors (arrows) is strongly reduced in *bon* (N) and MZ*sur;bon* (P) mutant embryos. Notice that *cas* expression in the yolk syncytial layer is not regulated by Nodal signaling. Expression of *snail* (Q-T) and *flh* (U-X) are not affected in any of the mutants.

of structures that require high levels of Nodal signaling. To test these possibilities, we investigated the regulation of different Nodal target genes in *bon*, MZ*sur* and MZ*sur;bon* embryos at 50% epiboly, the onset of gastrulation (Fig. 7). We analyzed the expression of *goosecoid* (*gsc*) (a marker of prechordal plate progenitors; Fig. 7A-D), *no tail* (*ntl*) (a pan-mesodermal marker; data not shown), *bhikhari* (*bik*) (a retroelement containing FoxH1 binding sites; Fig. 7E-H), *mezzo* (a mix-like gene implicated in mesendoderm formation; Fig. 7I-L) (Poulain and Lepage, 2002), *bon* (Fig. 1), *casanova* (*cas*; a marker of endoderm progenitors) (Dickmeis et al., 2001;

Kikuchi et al., 2001; Sakaguchi et al., 2001) (Fig. 7M-P), *snail1* (a marker of internalizing mesendoderm; Fig. 7Q-T) and *floating head* (*flh*; a marker of notochord progenitors; Fig. 7U-X).

These genes show four distinct classes of response: (1) *ntl*, *snail1* and *flh* are expressed normally in *bon*, MZ*sur* and MZ*sur;bon* embryos; (2) *bik*, *mezzo* and *bon* are expressed in *bon* but strongly downregulated in MZ*sur* (Poulain and Lepage, 2002) and MZ*sur;bon* embryos; (3) *cas* expression in endoderm precursors is slightly reduced in MZ*sur* and strongly impaired in *bon* (Kikuchi et al., 2001) and MZ*sur;bon* embryos; (4) *gsc* is weakly reduced in *bon* mutants, strongly reduced in MZ*sur* mutants (Pogoda et al., 2000; Sirotkin et al., 2000) and undetectable in MZ*sur;bon* mutants. As described above, *gsc* expression in MZ*sur* mutants recovers at later stages in a *bon*-dependent manner (Fig. 6C). These results establish a differential dependence of different Nodal target genes on Bon and/or Sur and reveal a correlation between the lack of a
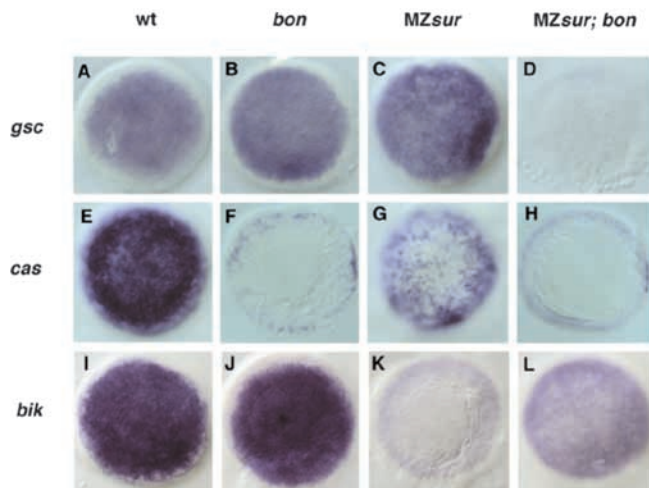
Fig. 8. Regulation of ectopic activation of Nodal target genes by *bon* and *sur*. (A-L) *squint* RNA was injected in different genetic backgrounds and assayed for gene activation at 50% epiboly. (A-D) *gsc* is induced in *bon* (B) and MZ*sur* (C) but not in MZ*sur;bon* mutants (D). (E-H) *cas* is induced in MZ*sur* (G) but not in *bon* (F) and MZ*sur;bon* (H) embryos. (I-L) *bik* is induced in *bon* (J) and MZ*sur;bon* and MZ*sur;bon*/+ (J and not shown) but not in MZ*sur* (K) mutant embryos.

Fig. 9. Regulation of Smad2 phosphorylation by *bon* and *sur*. Western-blot analysis of wild-type, MZ*sur*, *bon*MO and MZ*sur;bon*MO embryos at dome and shield stage. At dome stage, MZ*sur* and MZ*sur;bon*MO embryos have lower phosphorylated Smad2 levels but *bon*MO embryos are not affected. Phosphorylated-Smad2 levels recover by shield stage. Detection of actin serves as loading control.
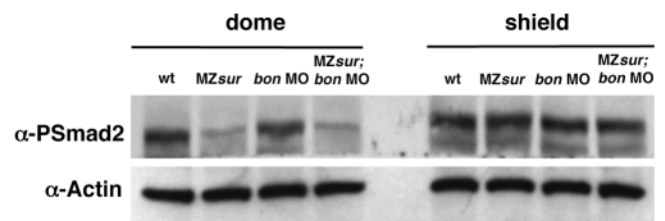
given cell type and the regulation of genes marking specific progenitors at the onset of gastrulation.

Ectopic activation of Nodal target genes is regulated by Bon and Sur

As an additional test of the requirement for Bon and Sur in mediating Nodal signaling, we determined the response of *ntl*, *cas*, *gsc* and *bik* to the ectopic activation of the Nodal signaling pathway (Fig. 8). RNA for the Nodal signal Squint was injected at the one- to two-cell stage and gene response was assayed at 50% epiboly. The expression of *ntl* was induced in *bon*, MZ*sur* and MZ*sur;bon* embryos (data not shown). The expression of *cas* was induced in MZ*sur* but not in *bon* or MZ*sur;bon* embryos (Fig. 8E-H). The expression of *bik* was induced in *bon* but not in MZ*sur* embryos (Fig. 8I-L). Surprisingly, the expression of *bik* was weakly induced in MZ*sur;bon* and MZ*sur;bon*/+ (data not shown) embryos, suggesting that *bon* might act as a repressor of *bik* at high levels of Nodal ligand and in the absence of *sur*. Finally, the expression of *gsc* was not induced in MZ*sur;bon* mutants but was activated in *bon* and MZ*sur* mutant embryos (Fig. 8A-D). These results provide further evidence for independent and overlapping functions of Bon and Sur in the regulation of Nodal downstream genes.

Autoregulation of Nodal signaling involves Sur but not Bon

In the analysis of regulatory networks, it is often difficult to distinguish between direct and indirect regulatory effects. For instance, in the case of FoxH1, many genes have been suggested to be directly regulated, based on overexpression assays in the presence of cycloheximide (Watanabe and Whitman, 1999). However, it has also been proposed that the major role of FoxH1 in zebrafish might be in the autoregulation of Nodal signaling and not necessarily in the regulation of

downstream genes (Pogoda et al., 2000). This latter scenario postulates that Bon and Sur regulate Nodal signals or other components of the Nodal signaling pathway to allow full Nodal signaling. The activation of other downstream genes would then be mediated by other components in the Nodal signaling pathway. Therefore, we directly tested the extent of Nodal signaling activity by assaying Smad2 phosphorylation (Fig. 9). In the absence of Nodal signaling (MZ*oep* mutants or *lefty* overexpression), no phosphorylated Smad2 is detectable (data not shown). Although MZ*sur* mutants initially have less phosphorylated Smad2 than wild-type embryos, phosphorylated Smad2 levels have recovered by the shield stage (Fig. 9). Loss of *bon* does not influence phosphorylated Smad2 levels. Importantly, despite the much more severe phenotype of MZ*sur;bon*MO embryos, no difference in phosphorylated Smad2 levels is observed compared with MZ*sur*. These results indicate that the stronger phenotype of MZ*sur;bon*MO embryos compared with MZ*sur* mutants is not caused by a decrease in overall Nodal signaling activity.

Discussion

Regulatory relationships between Nodal signaling, Bon and Sur

Previous studies have shown that members of both the Mixer and the FoxH1 families can associate with phosphorylated Smad2 to confer recognition of specific cis elements (Chen et al., 1996; Germain et al., 2000; Hill, 2001; Whitman, 2001; Randall et al., 2002). Additional studies have indicated that some *Mixer*-like genes are transcriptionally regulated by Activin/Nodal signaling (Rosa, 1989; Vize, 1996; Chen et al., 1996; Ecochard et al., 1998; Henry and Melton, 1998; Alexander and Stainier, 1999). In particular, genetic studies in zebrafish have led to the view that *bon* is predominantly a transcriptional target of Nodal signaling, not a component of the pathway (Alexander and Stainier, 1999; Kikuchi et al., 2000; Poulain and Lepage, 2002). Our studies clarify the regulatory interactions between Nodal signaling, Sur and Bon. Our results, together with biochemical studies (Chen et al., 1996; Watanabe and Whitman, 1999; Osada et al., 2000; Germain et al., 2000; Randall et al., 2002), suggest that Sur is a component of the Nodal signaling pathway, whereas Bon is both a component and a transcriptional target of Nodal signaling (Fig. 1). In particular, we find that there is an early

Nodal-signaling-independent, albeit reduced, expression of *bon* at the blastula margin. It is not clear how this activation is achieved. Studies in *Xenopus* have demonstrated that the transcription factor VegT can act as a maternal vegetal determinant and activate *mix*-like genes (Yasuo and Lemaire, 1999). However, functional homologs of VegT have not been identified in zebrafish. Embryological experiments by Chen and Kimelman (Chen and Kimelman, 2000) have led to the suggestion that a secreted factor derived from the extraembryonic yolk syncytial layer induces gene expression at the margin, independently of Nodal signaling. It is thus conceivable that this unknown signal also induces *bon* expression in the absence of Nodal signaling.

Although not required for the initiation of *bon* expression, Nodal signaling is essential for normal *bon* expression (Alexander and Stainier, 1999) (Fig. 1). At the onset of gastrulation, *bon* expression is lost or barely detectable in the absence of Nodal signaling. The enhancement and maintenance of *bon* expression is in part mediated by *sur*, because MZ*sur* mutants display reduced *bon* expression. The downregulation of *bon* might explain the reduced expression of the endodermal markers *axial* and *sox17* in MZ*sur* mutants (Pogoda et al., 2000; Sirotkin et al., 2000) (Fig. 6), because *bon* is required for *axial* and *sox17* expression (Alexander and Stainier, 1999; Kikuchi et al., 2000). The low levels of *bon* in MZ*sur* embryos are apparently sufficient for many processes that are disrupted upon complete loss of both *sur* and *bon* in MZ*sur;bon* embryos. For instance, cardiac mesoderm, endoderm, prechordal plate and ventral neuroectoderm form in MZ*sur* embryos despite the lower levels of *bon*. It is conceivable that the phenotypic variability observed in MZ*sur* mutants (Pogoda et al., 2000; Sirotkin et al., 2000) (Fig. 3) is in part caused by slightly varying levels of *bon* in these mutants. Reduction of *bon* expression is not as severe in MZ*sur* or MZ*sur;bon* mutants as in MZ*oep* mutants (Fig. 1), indicating that factors other than Sur and Bon are also involved in Nodal signaling to enhance *bon* expression (see below). In contrast to *bon*, *sur* expression is not affected by loss of Nodal signaling. Taken together, these results indicate that both Sur and Bon are initially expressed in responsive cells independently of Nodal signaling and can thus serve as components of the Nodal signaling pathway. Nodal signaling, in part mediated by Sur but not Bon, then further enhances and maintains *bon* expression, allowing efficient activation of Bon target genes.

The finding that Bon can associate with phosphorylated Smad2 (Randall et al., 2002) and is initially expressed independently of Nodal signaling also offers an explanation for the finding that Bon is not able to activate Nodal target genes such as *cas* in the animal region of the blastula (Kikuchi et al., 2000). We suggest that Bon is only active upon association with phosphorylated Smad2, and this association is Nodal dependent. In turn, Bon might restrict the expression domain of some targets of Nodal signaling, because *bon* is expressed only in cells at the margin. For example, *cas* and *sox17* are only expressed in the domain where high levels of Nodal signaling overlap with and induce *bon* expression. Ectopic expression of *bon* extends the territory of *cas* and *sox17* expression, but this domain is still within the normal range of Nodal signals (Kikuchi et al., 2000; Chen and Schier, 2001). Interestingly, these observations are reminiscent of the Dorsal-dependent

regulation of a subset of target genes in the *Drosophila* embryo. High levels of Dorsal induce expression of the transcription factor Twist in the ventral-most region of the embryo. Dorsal and Twist then act together to activate a group of ventrally expressed target genes such as *snail* (Ip et al., 1992; Stathopoulos and Levine, 2002). Analogously, phosphorylated Smad2 might activate *bon* in the margin region of the zebrafish embryo. Phosphorylated Smad2 and Bon would then associate in marginal-most cells where phosphorylated Smad2 levels are high and specifically regulate vegetally expressed target genes. Hence, both Dorsal and phosphorylated Smad2 appear to induce transcriptional activators to regulate a specific set of target genes. It is tempting to speculate that this strategy is a general mechanism to translate the graded activity of a transcription factor into discrete downstream responses.

Bon and Sur have overlapping roles in prechordal plate, heart and endoderm formation

Although a plethora of factors has been identified that interact with regulatory Smads, an in vivo requirement for these factors during vertebrate development has been established in only a few cases (Brand et al., 1996; Chen et al., 1996; Schier et al., 1996; Solnica-Krezel et al., 1996; Stainier et al., 1996; Kikuchi et al., 2000; Pogoda et al., 2000; Sirotkin et al., 2000; Hoodless et al., 2001; Yamamoto et al., 2001). Our double mutant analysis now provides evidence that partners of regulatory Smads have overlapping roles in vivo (Figs 3-8). Formation of heart, prechordal plate and ventral neuroectoderm are only mildly affected in *bon* or MZ*sur* embryos but are severely disrupted in embryos lacking both *sur* and *bon* activity. Moreover, although the penetrance and expressivity of MZ*sur* mutants are variable, loss of *sur* and *bon* leads to fully penetrant and expressive phenotypes.

The wider roles for *bon* revealed in MZ*sur;bon* mutants are also supported by the phenotypes of embryos lacking both *bon* and *mezzo* (Poulain and Lepage, 2002) or *bon* and *spadetail* (Griffin and Kimelman, 2002) activity. Loss of the T-box transcription factor *spadetail* and *bon* results in loss of myocardium, indicating overlapping roles of these genes during cardiac development (Griffin and Kimelman, 2002). Mezzo is another member of the Mix family but, in contrast to Bon, does not contain phosphorylated Smad interaction motifs and thus appears to act exclusively downstream of Nodal signaling (Poulain and Lepage, 2002). Loss of *mezzo* and *bon* results in heart and prechordal plate defects, suggesting overlapping roles of these two genes in the formation of these structures (Poulain and Lepage, 2002). Although removal of Mezzo enhances the *bon* phenotype, we have found no enhancement of MZ*sur;bon* embryos upon depletion of Mezzo (S.Z. and A.F.S., unpublished).

Bon and Sur regulate separate and common target genes

The requirements for *sur* and *bon* are already reflected before gastrulation in the regulation of downstream genes (Figs 7, 8). We found Nodal-regulated genes whose expression requires *bon* but not *sur* (*cas*), *sur* but not *bon* (*bhikhari, bon, mezzo*), *bon* or *sur* (*gsc*), or neither *bon* nor *sur* (*flh, ntl, snail1*). It is as yet unclear whether all these genes are directly regulated by Nodal signaling, but studies in *Xenopus* indicate that at least some of these genes might be direct targets. Experiments

involving cycloheximide and/or VP16 fusion constructs suggest that Mixer-like proteins can directly regulate *gsc* (Germain et al., 2000) and FoxH1 can directly activate *mix.2*, *Xbra*, *lim-1* and *gsc* (Chen et al., 1996; Watanabe and Whitman, 1999; Osada et al., 2000). Similarly, zebrafish *cas*, *mezzo* and *ntl* appear to be directly regulated by Nodal signaling (Poulain and Lepage, 2002). Moreover, *bik* elements contain binding sites for FoxH1 (Vogel and Gerster, 1999) and the zebrafish *gsc* promoter contains sequences resembling Mixer binding sites (McKendry et al., 1998). These observations suggest that Nodal signaling leads to the activation of genes regulated by Bon or Sur, Bon and Sur, or neither Bon nor Sur.

The use of different transcription factors, such as Sur and Bon, associating with phosphorylated Smad2 allows Nodal signaling to diverge downstream of receptor activation. For instance, and as outlined above, the restricted expression of *bon* might contribute to the restricted expression of Nodal-regulated genes implicated in endoderm formation. Indeed, we might speculate that, during evolution, specific genes have come under the control of Nodal signaling by the phosphorylated-Smad2-mediated recruitment of different transcription factors. In this scenario, subsets of genes were initially regulated by transcription factors independently of phosphorylated Smad2. Interaction with and eventual dependence on phosphorylated Smad2 would then usurp these factors into the Nodal signaling pathway. Intriguingly, some members of the *mix* family are independent of phosphorylated Smad2, whereas others interact with phosphorylated Smad2 (Rosa, 1989; Vize, 1996; Chen et al., 1996; Ecochard et al., 1998; Henry and Melton, 1998; Alexander and Stainier, 1999; Germain et al., 2000; Hill, 2001; Whitman, 2001; Randall et al., 2002). Moreover, FoxH1-VP16 fusion proteins can regulate Nodal targets in the absence of Nodal signaling (Watanabe and Whitman, 1999; Pogoda et al., 2000). It is thus conceivable that ancestral Mixer- and FoxH1-like proteins were active independently of phosphorylated Smad2 and have only recently been recruited into the Nodal signaling pathway. Support for this model is also provided by the observation that Forkhead transcription factors, but not Activin/Nodal signals, are involved in endoderm formation in *Caenorhabditis elegans* and *Drosophila* (Gaudet and Mango, 2002; Stainier, 2002).

Limited roles for Sur and Bon in Nodal autoregulation

It has been speculated that Sur might exclusively enhance the expression of Nodal signals (Pogoda et al., 2000). This suggestion was based on the observation that the expression of the Nodal genes *cyclops* and *squint* appears to be downregulated in MZ*sur* mutants. In apparent contradiction to this, however, a Sur-VP16 fusion can rescue aspects of the MZ*oep* mutant phenotype (Pogoda et al., 2000). These mutants are unable to transmit Nodal signals (Gritsman et al., 1999) and so Sur-VP16-mediated activation of *cyclops* and *squint* would not have any effect, indicating that, in this context, Sur must regulate other genes to rescue MZ*oep* mutants. Our results also indicate that a purely autoregulatory role of Sur is unlikely. In particular, we find that phosphorylated-Smad2 levels are reduced at dome but not shield stage in MZ*sur* embryos (Fig. 9). These observations are consistent with results in *Xenopus*, in which a FoxH1-Engrailed fusion construct has no effect on

overall phosphorylated Smad2 levels during gastrulation (Lee et al., 2001). It is possible that the effects on phosphorylated Smad2 levels are relatively minor, because FoxH1 does not only regulate the expression of Nodal ligands but also feedback inhibitors of Nodal signaling, such as Lefty and Cerberus (Whitman, 2001; Hamada et al., 2002; Schier, 2003). Therefore, the net effect of elimination of FoxH1 on phosphorylated Smad2 levels might be quite limited (Fig. 9). In addition, no change in phosphorylated Smad2 levels is seen upon blocking *bon* in wild-type or MZ*sur* mutants. This indicates that the much more severe phenotype of MZ*sur;bon* mutants is unlikely to be due to the reduced activity of *cyclops*, *squint* or other components of the Nodal signaling pathway upstream of Smad2 phosphorylation.

Multiple aspects of Nodal signaling are independent of Bon and Sur

Previous studies have identified several transcription factors that interact with Smad proteins to regulate the expression of specific genes (Massague and Wotton, 2000; Whitman, 2001; Hill, 2001). It is unclear how many of these factors are required or sufficient to mediate a particular TGFβ signaling process in vivo. We find that the defects in morphology and gene regulation observed in MZ*sur;bon* double mutants represent only a subset of the phenotypes observed upon complete block of Nodal signaling. In particular, Nodal mutants lack all trunk mesoderm, including blood, pronephros, somites and notochord, and display disrupted expression of genes such as *snail1*, *flh* and *ntl* (Feldman et al., 1998; Gritsman et al., 1999). These defects are not observed in MZ*sur;bon* double mutants, establishing that Bon and Sur cannot account for all Nodal signaling during mesendoderm induction. The p53 tumor suppressor has recently been implicated in the regulation of a subset of Nodal target genes (Cordenonsi et al., 2003). However, blocking *p53* in wild type does not lead to mesendoderm defects in zebrafish (Langheinrich et al., 2002) and depletion of p53 in MZ*sur;bon* embryos does not enhance the phenotype (J.T.B. and A.F.S., unpublished). Our results thus indicate that at least one additional Smad-associated transcription factor remains to be identified as a component of the Nodal signaling pathway.

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