Lnx-2b restricts *gsc* expression to the dorsal mesoderm by limiting Nodal and Bozozok activity

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Abstract

Coordinated Nodal-related signals and Bozozok (Boz) activity are critical for the initial specification of dorsal mesoderm and anterior neuroectoderm during zebrafish embryogenesis. Overexpression of Boz expands *gsc* expression into the ventro-lateral marginal blastomeres where Nodal signaling is active, but is insufficient to induce ectopic *gsc* expression in the animal region. We found that overexpression of Boz together with depletion of Lnx-2b (previously named Lnx-like, Lnx-l), but not each manipulation alone, causes robust *gsc* expression in all blastomeres. Furthermore, Nodal-related signals are required for *gsc* expression in embryos with elevated Boz activity. Through targeted injection into single cells at the 128-cell stage we illustrate the role of maternally deposited Lnx-2b to restrict the expansion of *gsc* expression into the presumptive ectodermal region. This report provides a novel mechanism for limiting dorsal organizer specification to a defined region of the early zebrafish embryo.

Keywords

Lnx-2b; Nodal; Bozozok; *goosecoid*; dorsal organizer; dorsal mesoderm

1. Introduction

During early zebrafish development, multiple signaling molecules pattern the embryonic body axis [1,2]. The dorsal organizer, a region that is a source of inductive signals and a major regulator of axis establishment, is formed under the influence of the Wnt and Nodal signaling pathways [1,2]. An early consequence of Wnt signaling is the induction of the homeobox gene *bozozok/dharma/nieuwkoid* (*boz*) in a region that presages formation of the dorsal organizer [1–4]. Among other activities, Boz activates *gsc* expression in the dorsal organizer [5–7] in cooperation with Nodal-related factors Ndr1 (Squint) and Ndr2 (Cyclops). Overexpression of Boz can expand *gsc* expression into the lateral-marginal tissue, where Ndr1 and Ndr2 are present, but fails to induce *gsc* when Nodal signaling is inhibited [8,9]. In contrast, overexpression of Ndr1 induced ectopic *gsc* expression in regions where *boz* is not expressed [5,6,8]. Thus Nodal signaling is absolutely required for *gsc* expression, and enforced Nodal activity appears to be sufficient to induce ectopic *gsc* expression without contribution from Boz, although *gsc* expression in the animal pole region

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was activated only in cells expressing high levels of *Ndr1* [9]. Collectively these observations indicate that *gsc* expression in the marginal blastomeres can be induced by the cooperative activities of Boz and Ndr1. The *gsc* expression domain based on cooperative induction by Boz and Ndr1 appears to be restricted to prospective mesendoderm, even though Ndr1 acts as a long-range morphogen and Boz, even though it is a nuclear protein, is capable of acting in a non-cell autonomous manner [9,10]. Thus a repressing mechanism should exist in the presumptive ectoderm to prevent expansion of the *gsc* expression domain towards the animal pole.

Recently we reported that Lnx-2b (previously named Lnx-like), an E3 ubiquitin ligase containing an amino terminal RING finger and four PDZ domains, restricts the organizer domain by negatively regulating Boz stability [11]. Depletion of Lnx-2b could expand *gsc* expression into the lateral and even ventral margin, but not into the animal region containing future ectoderm. In the present study, we start from the hypothesis that maternally deposited Lnx-2b counteracts Boz and Ndr1-dependent ectodermal expansion of *gsc* expression. We show that overexpression of Boz together with the depletion of Lnx-2b can induce *gsc* expression beyond the marginal blastomeres into the future ectodermal region of the embryo. Further we show that the ectopic *gsc* expression caused by the Boz overexpression in the absence of Lnx-2b is strictly depended on Nodal signaling. Finally, we observed radial expansion of *gsc* expression in cells surrounding a source of Boz and Ndr1 protein under conditions where Lnx-2b translation is inhibited.

2. Material and methods

2.1. Fish embryos

Embryos were obtained from natural spawnings of wild type (AB*). Embryos were raised and harvested according to Kimmel et al. [12].

2.2. Microinjection

cDNAs encoding *ndr1* and *boz* open reading frames were subcloned into the modified pcGlobin2 vector [13]. mRNAs were synthesized using the mMESSAGE mMACHINE kit (Ambion Inc.). Indicated RNAs or morpholinos (MO) were injected into the yolk of 1–4 cell stage of embryos. MOs were supplied from Gene Tools, LLC. 1 ng of p53 MO was co-injected with other MOs to inhibit off-target effects [14]. *lnx-2b* MO, 5'-CCTACGCCTCTTTCACAGCTCACAA-3'; *p53* MO, 5'-GCGCCATTGCTTTGCAAGAATTG-3'.

2.3. Single cell injection

A mixture of 2 pg of *ndr1* and 5 pg of *boz* mRNA was injected together with tracers, rhodamine dextran (3%, 10,000 MW) and biotinylated dextran (Molecular Probes), into a single cell of 128 cell wild-type (WT) or *lnx-2b* MO (5 ng) injected embryos. *lacZ* mRNA (7 pg) together with the tracers was injected as a negative control. Injected embryos were raised for 3 hours and fixed with 4% paraformaldehyde at the germ ring stage for whole mount in situ hybridization. Biotin dextran was detected with NovaRed substrate staining (Vector) using standard ABC protocol (Vectastain).

2.4. Whole mount in situ hybridization

Antisense gsc riboprobe was generated using T7 RNA polymerase following the manufacture's instructions (Roche). Proteinase K treatment (10µg/ml) was performed for 1 min at RT. The hybridized probe was detected using pre-absorbed anti-digoxigenin-AP Fab fragments (Roche) diluted (1:2000) in blocking solution (PBS, 0.1% Tween-20, 5% sheep serum, 0.2% Blocking reagent (Roche)).

3. Results and discussion

3.1. Lnx-2b represses ectopic *gsc* expression in the presumptive ectoderm

During early zebrafish development, *boz* expression induced by Wnt/β-catenin signaling mediates dorsal organizer formation by suppressing the expression of ventralizing genes such as *bmp*, *vox* (*vega1*), *vent* (*vega2*) and *ved* [15–20]. Thus overexpression of Boz, through counteracting ventralizing signals, expands *gsc* expression into the ventro-lateral marginal blastomeres in a dose-dependent manner (Fig. 1A–C). However, we have never observed ectopic *gsc* expression beyond the presumptive mesendodermal tissue even after injection of high amounts of *boz* mRNA. Similar to the overexpression of Boz, depletion of Lnx-2b by the injection of translation-blocking MO causes the expansion of *gsc* expression into the mesendoderm at the embryonic shield stage [11] (Fig. 1A, 1D, and 1E). The expanded *gsc* expression elicited by *lnx-2b* MO injection is due to stabilization of endogenously expressed Boz, which leads to gradually expanded expression of *boz* in the marginal blastomeres [11]. Interestingly, we observed strong *gsc* expression in the entire blastoderm including the ectodermal domain when Boz overexpression was coupled with depletion of Lnx-2b (Fig. 1F–H).

Quantitative aspects of the regulation of *gsc* expression by Boz and Lnx-2b are presented in Figure 1I. Classification of *gsc* domains into four categories allowed quantification of the effects. The data show dose dependence for the effect of Boz and of Lnx-2b MO, as well as for the cooperative action of increasing Boz levels while reducing Lnx-2b levels in the embryo. Collectively, our data indicate that Lnx-2b represses ectopic *gsc* expression in the presumptive ectodermal tissue by antagonizing Boz. The environment of the ectoderm is less favorable for *gsc* expression, possibly because it is more distant from the source of Nodal signals, as will be discussed below. In this environment, even the injection of high levels of *boz* RNA cannot induce *gsc* unless the level of Boz protein is increased sufficiently by reduction of Lnx-2b, thereby preventing the ubiquitylation-mediated destruction of Boz that otherwise limits its accumulation [11].

3.2. Nodal signaling is a pre-requisite for the expression of gsc

Boz and Nodal-related factors act in parallel to specify dorsal organizer and anterior neuroectoderm formation [6,7,21,22]. Ndr1 and Ndr2, members of TGF-β superfamily, bind to heteromeric type I and type II Activin receptors, which in turn act through the Smad2/Smad3/Smad4 signaling pathway to regulate the transcription of target genes [23]. Nodal signaling is negatively regulated by extracellular inhibitors, for example, Lefty1/Antivin (Lft1) and Lefty2 [24,25]. Because overexpression of Boz failed to induce *gsc* expression in the absence of Nodal signals, Nodal functions are required for the development of dorsal mesoderm [6,7,26]. To determine whether the depletion of Lnx-2b overcomes the suppression of *gsc* expression by the repression of Nodal signaling, we tested *gsc* expression in embryos in which both Nodal signaling and Lnx-2b expression were inhibited. In order to suppress the Nodal signaling we injected *lft1* mRNA into one-two cell stage embryos. Confirming previous reports, Nodal inhibition by Lft1 completely eliminated *gsc* expression in the dorsal mesoderm at the germ ring stage [24,25] (Fig. 2B). Overexpression of Lft1 furthermore inhibited expanded *gsc* expression within the ventro-lateral marginal blastomeres as well as in the entire blastoderm when co-injected with *lnx-2b* MO and *boz* mRNA (Fig. 2C and 2D). Thus inhibition of nodal signaling by Lft1 suppressed all *gsc* expression, irrespective of any manipulation of the levels of Boz and Lnx-2b. These observations strongly support the view that *gsc* expression in the dorsal mesoderm is absolutely dependent on Nodal signaling. Boz and Lnx-2b modulate the induction of *gsc* in opposite directions, but ectopic expression of *gsc* through Boz stabilization via Lnx-2b repression [11] could be achieved only when Nodal signaling is active.

3.3. Lnx-2b restricts the domain of non-cell autonomous action of Nodal and Boz

The cooperative function of Nodal-related factors and Boz seems to be critical for the correct spatio-temporal expression of *gsc* in the dorsal mesoderm [1,4]. The *gsc* gene is expressed only within or close to the domain in which Boz and Nodal factors are expressed. Even though the *ndr1* and *ndr2* genes are expressed, at least at blastula stages, beyond the range of the *gsc* expression domain, the failure of *gsc* induction in lateral marginal blastomeres is likely due to the lack of *boz* expression in this region. In addition, the expression levels of *ndr1* and *ndr2* in the lateral mesoderm might be below the threshold required for activation of *gsc* expression in the absence of Boz activity [6]. However, the *gsc* expression domain does not correspond exactly to the region in which *boz* and Nodal-related factors are co-expressed but extends to a region of neighboring cells in the dorsal mesendoderm. Thus Boz together with Nodal signaling may induce *gsc* expression in a non-cell autonomous manner [10]. As we reported previously, *lnx-2b* MO-dependent expansion of the *gsc* expression domain is due to Boz stabilization resulting in gradual expansion of the *boz* expression domain into the lateral marginal blastomeres [11]. Thus it is plausible that the combined injection of *lnx-2b* MO and *boz* mRNA induced ectopic *gsc* expression beyond the presumptive mesendoderm at the onset of gastrulation stage (Fig. 1F–H and 1I) through the long range effects of Ndr1 [9] and the enhanced level of Boz achieved by depletion of Lnx-2b [11].

We therefore examined whether the capacity of Ndr1 and Boz to transform the presumptive ectoderm into dorsal mesoderm-like tissue is influenced by altered Lnx-2b levels. To provide a local source of Nodal and Boz for inducing *gsc* expression, we injected *ndr1* and *boz* mRNA together with the lineage tracer fluorescein-dextran, into a single animal cell of 128-cell stage normal or *lnx-2b* morphant embryos. As a control, we injected *lacZ* mRNA together with fluorescein-dextran under identical experimental conditions. After 3 hours, the embryos were collected for in situ hybridization with *gsc* (Fig. 3A). *lacZ* injection failed to induce ectopic *gsc* expression in WT embryos (100%, N=11) as well as in *lnx-2b* morphants (100, N=9) (Fig. 3B and 3C). By contrast, ectopic *gsc* expression was detected in cells expressing Ndr1 and Boz (Fig. 3D, 62.5%, N=16). It should be noted that some of the isolated Ndr1 and Boz expressing cells could not drive ectopic *gsc* expression in their neighbors in WT embryos (arrowheads, Fig. 3D). These results suggest that only cell aggregates expressing Ndr1 and Boz may activate *gsc* expression in the presumptive ectoderm, an effect reminiscent of the "community effect" [27]. However, the *gsc* expressing domain became enlarged when *ndr1* and *boz* mRNA were co-injected into a single cell of *lnx-2b* morphant embryos (Fig. 3E, 75%, N=16). In addition, in the morphant embryos we could not detect any isolated cells that express Ndr1 and Boz without inducing *gsc* (Fig. 3E). This observation suggests that every Ndr1/Boz overexpressing cell can induce *gsc* in its neighbors in the environment of Lnx-2b depletion.

Taken together our data suggest that Lnx-2b maintains the integrity of the ectodermal tissues via suppressing *gsc* expression which can be driven by the coordinated action of Nodal-related factors and Boz. We show that the depletion of Lnx-2b potentiates Boz activity to induce the dorsal mesodermal marker *gsc*. However, Boz alone cannot activate *gsc* expression in the absence of Nodal signaling. In contrast to the consequences of Lnx-2b depletion, *gsc* expression in the organizer was not affected by overexpression of Lnx-2b (data not shown). The failure of suppression of *gsc* expression in the dorsal mesoderm by Lnx-2b overexpression may suggest that co-expression of Nodal and Boz overrides the negative influence of Lnx-2b against *gsc* induction. Alternatively, it is possible that the maternally expressed Lnx-2b is already saturating, so that an additional increase of Lnx-2b does not influence *gsc* expression.

Recently Shih et al. showed that SoxB1 transcription factors, which are expressed in presumptive ectoderm, repress the expression of dorsal organizer markers in the animal pole [28]. This conclusion is supported by the evidence that *soxB1* genes determine dorso-ventral patterning by controlling *bmp2b* and *bmp7* expression [28]. Thus depletion of four *soxB1* genes or overexpression of dominant negative SoxB1 induced similar hyperdorsalized phenotypes [28,29]. Since *lnx-2b* morphants became strongly dorsalized [11], it would be intriguing to test the possible molecular crosstalk between Lnx-2b and SoxB1 factors in their role to delimit the mesendoderm and dorsal organizer formation.

4. Conclusions

In this study we conclude that maternally deposited Lnx-2b is critical to repress the expansion of dorsal organizer genes as exemplified by *gsc*, and thereby delimits the tissues that can respond to Nodal signals and Boz activity.

Abbreviations

Boz   Bozozok

Gsc   Goosecoid

Lft1   Lefty1

WT   wild type

Acknowledgments

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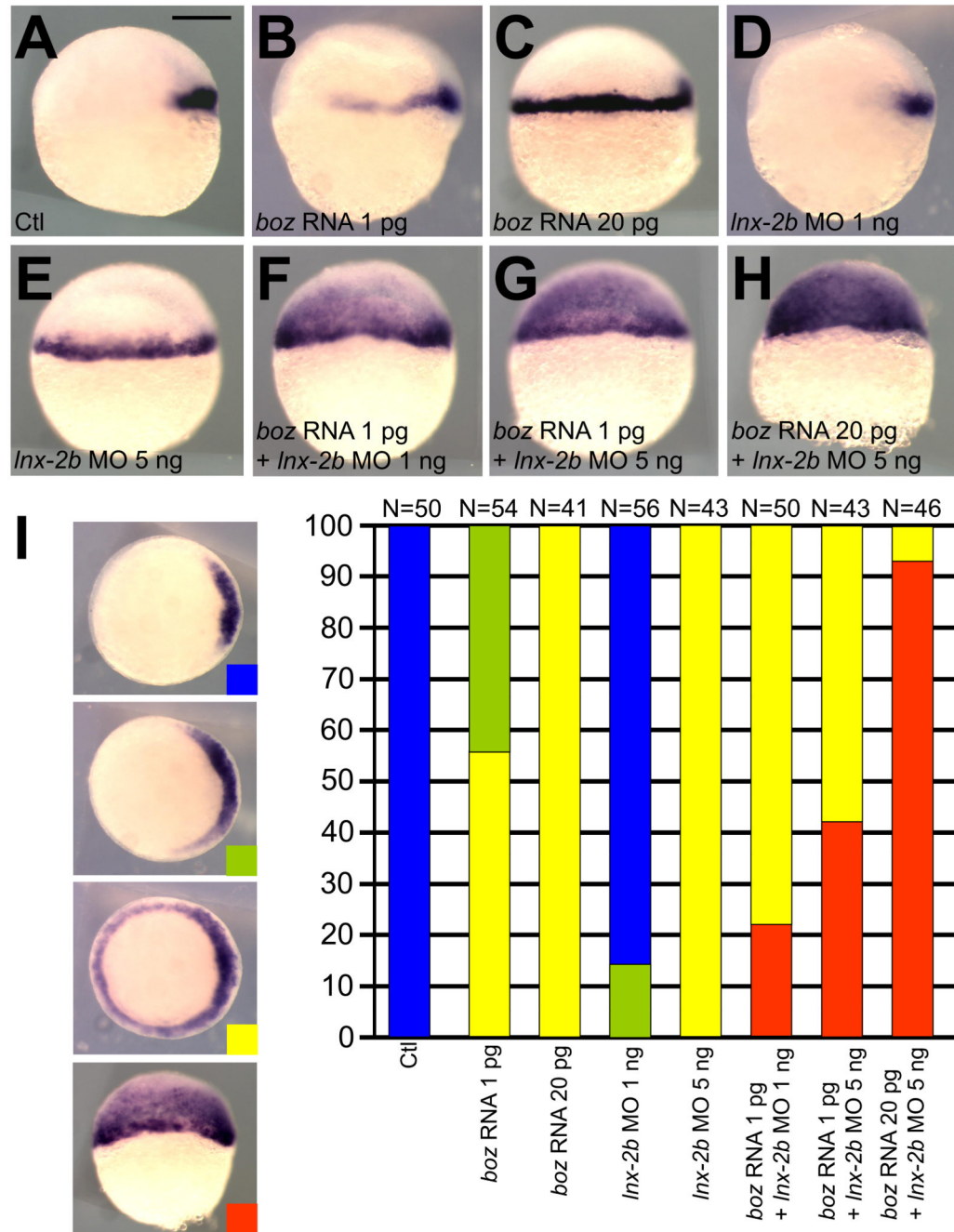
Fig. 1.
Overexpression of Boz and concomitant depletion of Lnx-2b cause ectopic *gsc* expression in the presumptive ectoderm. (A) Control uninjected embryo; *gsc* is expressed in the dorsal organizer. (B) Embryo injected with 1 pg of *boz* mRNA; the *gsc* expression domain is laterally expanded. (C) Embryo injected with 20 pg of boz mRNA; the *gsc* expression domain includes the entire margin. (D) Embryo injected with 1 ng of *lnx-2b* MO; slight lateral expansion of *gsc*. (E) Embryo injected with 5 ng *lnx-2b* MO showing similar *gsc* expansion to (C). (F) One pg of *boz* mRNA was co-injected with 1 ng of *lnx-2b* MO; 22% of embryos (N=50) showed ectopic expanded expression of *gsc* in animal (presumptive ectodermal) cells. (G) One pg of *boz* mRNA co-injected with 5 ng of *lnx-2b* MO; 42% of

injected embryos (N=43) displayed graded expansion of *gsc* into the animal region. (H) Co-injection of 20 pg *boz* mRNA and 5 ng *lnx-2b* MO; 93% of embryos (N=46) showed strong ectopic *gsc* expression. (I) Dorsalization was determined by *gsc* expression at the germ ring stage, showing synergism between *boz* mRNA and *lnx-2b* MO. The classification is depicted by different colors (Blue, unaffected; Green, moderate expansion; Yellow, circumferential *gsc* expression; Red, ectopic *gsc* expression into animal region). (A–H) Shield stage. Lateral view, dorsal is right. Scale bar, 200 µm.
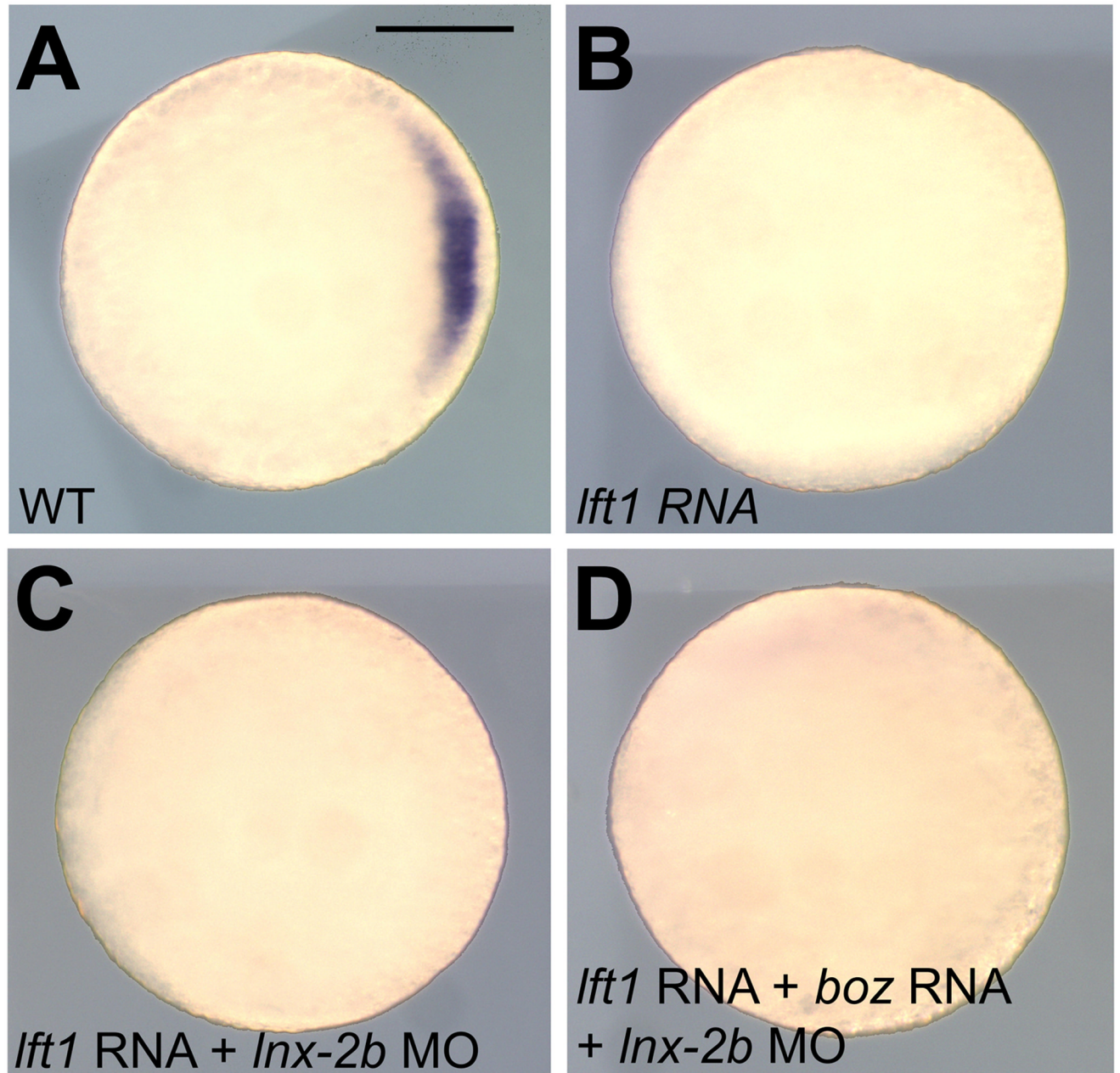
Fig. 2.
Nodal-related signals are a prerequisite for *gsc* induction. (A) Control uninjected embryo; *gsc* was detected in dorsal mesodermal cells. (B) Embryo injected with 25 pg *lft1* mRNA; *gsc* expression was eliminated. (C) Embryo injected with 25 pg of *lft1* mRNA and 5 ng *lnx-2b* MO. Depletion of Lnx-2b did not recover *gsc* expression. (D) Embryo injected with 25 pg of *lft1* mRNA, 20 pg *boz* mRNA and 5 ng *lnx-2b* MO showed no *gsc* expression. (A–D) Germ ring stage. Animal pole view, dorsal is right. Scale bar, 200 µm.
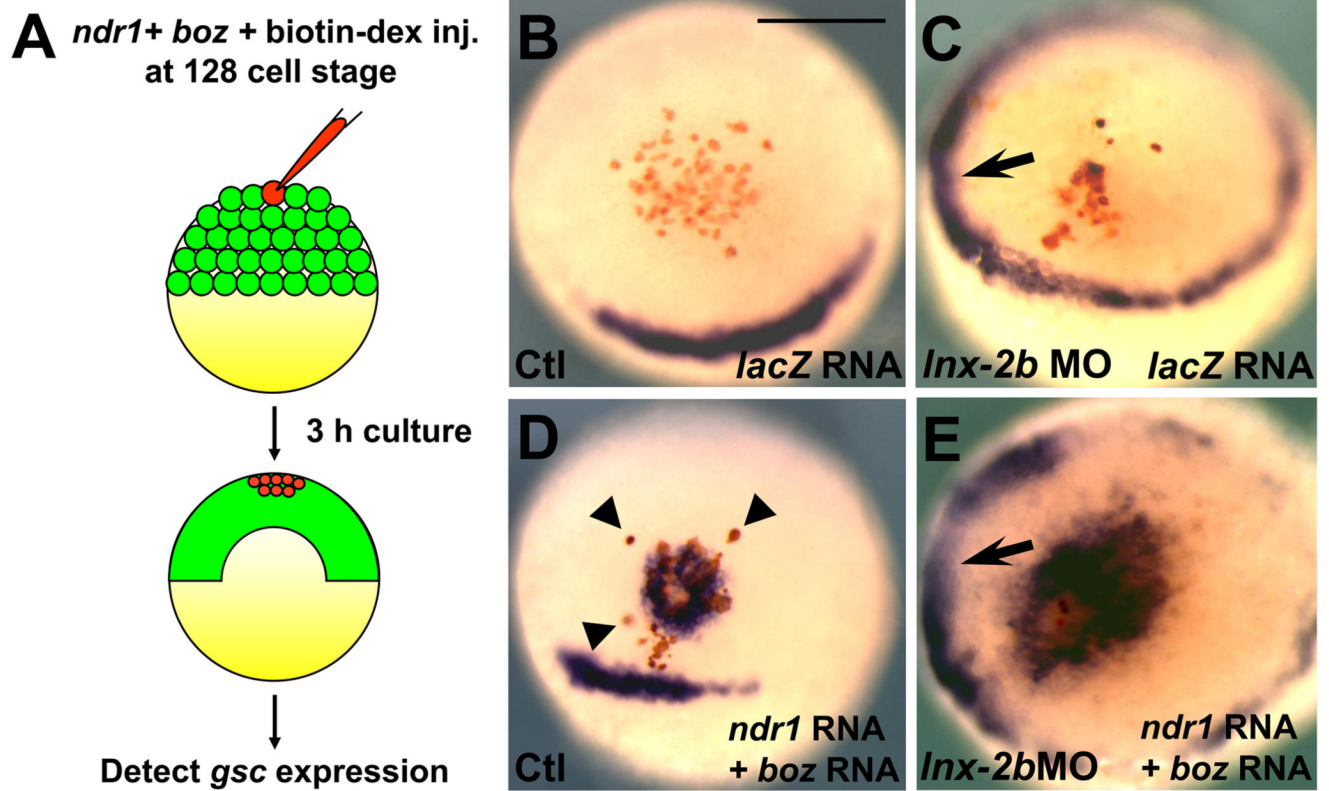
Fig. 3.
Lnx-2b antagonizes Nodal/Boz-dependent *gsc* expansion. (A) Schematic representation of the experimental procedures. The indicated RNA(s) was co-injected with fluorescein-dextran (red) into a single cell at the 128-cell stage of control or *lnx-2b* morphants (embryos unjected with 5 ng *lnx-2b* MO at the 1–2 cell stage). Embryos were fixed 3h later for *in situ* hybridization. (B) *lacZ* mRNA injected control embryo. (C) *lacZ* mRNA injected *lnx-2b* morphant. Note that *gsc* was expressed in the ventro-lateral margin (arrow) but not in animal cells expressing LacZ. (D) Two pg of *Ndr1* and 5 pg of *boz* mRNA was injected into a single cell, leading to a tight region of *gsc* expression and a few separate *gsc*-positive cells (arrowhead). (E) The same mix of *Ndr1* and *boz* mRNA as in (D) was injected into one cell of *lnx-2b* morphants. A large *gsc*-positive region is seen in the animal hemisphere. Arrow indicates expanded *gsc* expression at the margin. (B–E) Germ ring stage. Animal pole view. Scale bar, 200 µm.