Development 137, 2671-2681 (2010) doi:10.1242/dev.054130


# SoxB1 transcription factors restrict organizer gene expression by repressing multiple events downstream of Wnt signalling

**Yu-Huan Shih, Cheng-Liang Kuo, Caroline S. Hirst, Chris T. Dee, Yu-Ru Liu, Zulfiqar Ali Laghari and Paul J. Scotting***

## SUMMARY

Formation of the organizer is one of the most central patterning events in vertebrate development. Organizer-derived signals are responsible for establishing the CNS and patterning the dorsal ventral axis. The mechanisms promoting organizer formation are known to involve cooperation between Nodal and Wnt signalling. However, the organizer forms in a very restricted region, suggesting the presence of mechanisms that repress its formation. Here, we show in zebrafish that the transcription factor Sox3 represses multiple steps in the signalling events that lead to organizer formation. Although β-catenin, Bozozok and Squint are known to play major roles in establishing the dorsal organizer in vertebrate embryos, overexpression of any of these is insufficient to induce robust expression of markers of the organizer in ectopic positions in the animal pole, where Sox3 is strongly expressed. We show that a dominant-negative nuclear localisation mutant of Sox3 can cause ectopic expression of organizer genes via a mechanism that activates all of these earlier factors, resulting in later axis duplication including major bifurcations of the CNS. We also find that the related SoxB1 factor, Sox19b, can act redundantly with Sox3 in these effects. It therefore seems that the broad expression of these SoxB1 genes throughout the early epiblast and their subsequent restriction to the ectoderm is a primary regulator of when and where the organizer forms.

**KEY WORDS: Sox3, Organizer, Wnt, *bozozok* (*dharma*), *squint* (*ndr1*), Zebrafish**

## INTRODUCTION

The formation of a single organizer is the central event that initiates the correct patterning of vertebrate embryos. The best-established components of the signalling that promotes the formation of a functional organizer are the Wnt and Tgfβ signalling pathways (Joubin and Stern, 1999; Schier and Talbot, 2005). β-catenin activity, activated by Wnt signalling peptides, becomes restricted to the dorsal side of the embryo. At the same time, Nodal signalling is derived from the marginal region and so is restricted to the more vegetal parts of the embryo. The organizer forms where these two signals overlap. In zebrafish, combined Wnt/Nodal signalling activates expression of the homeodomain factor *bozozok* (*boz*; also known as *dharma*) (*siamois* fulfils this role in *Xenopus*) and the Nodal-related factors *squint* (*sqt*; *ndr1*) and *cyclops* (*cyc*; *ndr2*) (the Xnr genes in *Xenopus*) (Schier and Talbot, 2005). This gene expression marks a region equivalent to the Nieuwkoop centre of *Xenopus* embryos, which is then responsible for the induction of the organizer (Kodjabachian and Lemaire, 1998). Together with the activity of Boz, Nodal factor signalling activates expression of *chordin* (*chd*) and *goosecoid* (*gsc*) (Schier and Talbot, 2005). However, the organizer itself and the various genes that are also expressed in a manner restricted to the vegetal/dorsal region do not occupy identical domains, suggesting that each is restricted by unique control that is not necessarily limited simply by the combination of Wnt/Nodal signals alone. It seems probable that there are also counter signals that restrict the ability of cells to respond to the Wnt/Nodal signals and so help to define the limits of both the Nieuwkoop centre and organizer. In support of this concept, although ectopic activation of the Wnt signalling pathway, or ectopic expression of Boz or Sqt, can induce ectopic expression of organizer markers, none appears able to induce all organizer markers outside of the vegetal region. In general, marker expression is only expanded within the prospective mesoderm/endoderm (Dougan et al., 2003; Leung et al., 2003a; Shimizu et al., 2000). Thus, there might be a repressor that inhibits the ability of these factors to act in the prospective ectoderm. In this study we describe data that support such a role for members of the SoxB1 family of transcription factors.

Sox3 is one of the earliest of the SoxB1 family of transcription factors to be expressed during vertebrate development. In most species analysed, *sox3* expression is first seen throughout the epiblast (Penzel et al., 1997; Rex et al., 1997; Wood and Episkopou, 1999), but is then lost from cells in the marginal region fated to become mesoderm and endoderm (Dee et al., 2007; Okuda et al., 2006; Rex et al., 1997), such that it becomes restricted to the ectoderm. Within the ectoderm, *sox3* expression then becomes restricted to the developing CNS, representing one of the earliest and most generally expressed transcription factors in vertebrate neural development (Penzel et al., 1997; Rex et al., 1997; Wood and Episkopou, 1999; Zhang et al., 2004; Zhang et al., 2003). However, little is known of the role and mechanism of action of Sox3 at early stages prior to the partitioning of ectoderm into neural and non-neural domains.

An indication that Sox3 might be important as early as the period when mesoderm is first specified comes from studies in *Xenopus*, in which Sox3 appeared to directly repress the expression of mesoderm markers, including members of the Nodal family and the homeodomain factor *siamois*, which are central to organizer

Institute of Genetics, Queen's Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK.

*Author for correspondence (paul.scotting@nottingham.ac.uk)

**Accepted 2 June 2010**

formation (Zhang et al., 2004; Zhang et al., 2003). These authors also found that Sox3 was able to repress expression of the Nodal family member *cyc* in zebrafish (Zhang et al., 2004; Zhang and Klymkowsky, 2007).

In this study we set out to analyse the role of Sox3 in the earliest stages of zebrafish development. Loss-of-function strategies, such as the use of mutants or morpholinos, are precluded by the presence of maternal proteins and potential redundancy between related SoxB1 factors (Zhang et al., 2004). We have therefore used both gain-of-function and dominant-negative loss-of-function approaches. We find that overexpression of Sox3 represses genes that are normally expressed in both the mesoderm and organizer, and that this does not appear to be via disruption of β-catenin signalling, but via a less well-described feature of Sox3, that of direct transcriptional repression. In addition, we find that disruption of Sox3 using a dominant-negative construct results in the ectopic induction of markers of the Nieuwkoop centre and organizer in a manner that requires β-catenin activity. We also show that the zebrafish SoxB1 factor Sox19b can function redundantly with Sox3 to repress organizer genes.

## MATERIALS AND METHODS

### Generation of constructs

The coding sequence of zebrafish β-*catenin 2* was amplified using the following primers: forward, 5′-GAATTCCATGGCTAGCCAGGCTGA-3′ and reverse, 5′-TCTAGACAGGTCCGTGTCGAACCA-3′. The PCR product was ligated into the pCR-II vector (Invitrogen) and subcloned into the pCS2-myc-nls vector at the *Eco*RI-*Xba*I sites, creating an in-frame fusion of β-*catenin 2* and *myc* (with the nuclear localisation signal removed). Sox3dNLS (KR36TG, RR50LG, RR107LG) (Li et al., 2007), Sox3N40I (N40I) (Zhang et al., 2003) and βcat2S37A (S37A) (Zorn et al., 1999) were generated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). In order to generate the dominant repressor HMG-EnR and dominant activator HMG-VP16 constructs, the HMG box of *sox3* was cloned in-frame into pBUT-EnR and pBUT-VP16 at the *Xba*I-*Xho*I sites. The Boz-Flash construct for luciferase assays was constructed by inserting the *boz* proximal promoter region (1.2 kb) upstream of the luciferase sequence in the TOP-Flash vector (a gift from Alan Clarke).

### Injection of RNA and morpholinos

Zebrafish embryos were collected and staged according to standard methods (Kimmel, 1995; Westerfield, 2000). Capped mRNAs for microinjection were produced from linearised cDNA template using the mMessage-Machine Kit (Ambion) according to the manufacturer's instructions. Embryos were injected with 50-400 pg of RNA. Antisense morpholino oligonucleotides (MOs) designed to target the 5′ region of β-*catenin 2* (Bellipanni et al., 2006), *boz* (Urtishak et al., 2003) and *sqt* (Maegawa, 2006) were obtained from Gene Tools (Philomath, OR, USA). The MO sequences were: β-*catenin 2* MO, 5′-CCTTTAGCCTG-AGCGACTTCCAAAC-3′; *boz* MO, 5′-TGCCATGTTCAAGTGTAG-GGGTGCC-3′; and *sqt* MO, 5′-ATGTCAAATCAAGGTAATAATCCAC-3′. Embryos were injected with 10-12.5 ng MO in 0.5 nl at the 1- to 2-cell stage.

### Whole-mount in situ hybridisation and immunohistochemistry

Whole-mount in situ hybridisation was carried out as previously described (Jowett and Yan, 1996) using digoxigenin (DIG)-labelled (Roche) or fluorescein-labelled (Roche) riboprobes. Antibodies were detected using BM purple or BCIP (Roche). For the double in situ hybridisation, following the first in situ hybridisation the embryos were refixed before the second probe was detected. Immunostaining was performed according to standard protocols using anti-HA (1/1000; Ab1265, AbCam) and anti-rabbit IgG HRP-labelled secondary antibodies (1:2000; Vector Labs) and visualised with DAB substrate (Vector Labs).

### ChIP-PCR

Embryos at the 1- to 2-cell stage were injected with RNA and collected at 4.5 hours post-fertilisation (hpf). Dechorionated embryos were fixed in 1.85% formaldehyde. After quenching with 2.5 M glycine, embryos were washed and then lysed in 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 0.5% NP40. Nuclei were pelleted in a microcentrifuge at 1000 ***g*** for 5 minutes and resuspended in 50 mM Tris-HCl pH 7.4, 10 mM EDTA, 1% SDS. Two volumes of IP dilution buffer (16.7 mM Tris-HCl pH 7.4, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS) were added and samples sonicated and then centrifuged at 14,000 ***g*** for 10 minutes. Supernatant was incubated with HA beads (HA agarose, A-2095, Sigma) at 4°C overnight. Beads were washed eight times with wash buffer (50 mM Hepes pH 7.6, 1 mM EDTA, 0.7% sodium deoxycholate, 1% NP40, 0.5 M LiCl) and once with 1× TBS [50 mM Tris-HCl (pH 7.4), 150 mM NaCl] and the DNA-protein complex was eluted in 50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS at 65°C overnight. After treatment with proteinase K at 55°C for 2 hours, DNA was precipitated in ethanol. Real-time PCR was carried out using MX3005P MX-PRO (Stratagene) and Brilliant SYBR Green Master Mix Kit (Stratagene) with the following primers: *tubb5*-F, 5′-CCC-AATTTTAAAACACGCCTA-3′; *tubb5*-R, 5′-CGGATGAGGACGATT-TAACC-3′; *boz*-F, 5′-TTGGGACTAGGCTACTGGAAAA-3′; and *boz*-R, 5′-CGCTTTGAAATGAAGACGTG-3′.

### Luciferase assay

Embryos at the 1- to 2-cell stage were injected with luciferase reporter plasmids (Boz-Flash and Fop-Flash) and appropriate RNA and collected at 4 hpf. Dechorionated embryos were lysed in 1× Passive Lysis Buffer (Promega) followed by centrifugation at 14,000 ***g*** for 5 minutes. Supernatant (20 μl) was transferred to a 96-well white microplate (Sterilin) and luciferase activity assayed using the GloMax Luminescence Detection System (Promega).

## RESULTS

### Sox3 can repress markers of the mesoderm and dorsal organizer

We set out to investigate the role of Sox3 in the establishment of the mesoderm and organizer in zebrafish (Fig. 1A). Injection of 50 pg *sox3* mRNA at the 1- to 2-cell stage caused an abnormal thickening on the dorsal side of the embryo by 6.5 hpf (Fig. 1Aa′), a stage at which cells would normally be undergoing involution. A significant number of embryos failed to progress beyond gastrulation (25%), and those that progressed were ventralised, with most lacking substantial head development (Fig. 1Ac′). However, at higher doses few (19% at 100 pg) or no (200 pg) embryos survived past gastrulation.

When analysed at 4 hpf, Sox3 was found to repress markers of both mesoderm and the organizer (Fig. 1B). Expression of the zebrafish Nodal family member *sqt* (Fig. 1Ba′), the homeodomain factor and marker of the organizer *gsc* (Fig. 1Bb′), and the organizer-derived BMP antagonist *chd* (Fig. 1Bc′) were all substantially reduced. We also saw a loss of the later organizer-derived BMP antagonist *noggin 1* (*nog1*) at 8 hpf (Fig. 1Be′) and, by 6 hours of development, we saw large gaps in the expression of the mesoderm marker *no-tail* (*ntl*) (Fig. 1Bd′), coincident with the region where the overexpressed Sox3 was detected (Fig. 1Bd′, inset). It therefore appears that, in addition to its effects on mesoderm, Sox3 also represses genes essential for organizer formation and function.

These data provide a potential explanation for our earlier observation that local injections of wild-type (WT) Sox3 cause bifurcations of the embryonic axis in a small proportion of embryos [generally less than 10% (Dee et al., 2008)], presumably owing to Sox3 repression of organizer gene expression in the centre of their endogenous expression domain and so splitting the organizer.

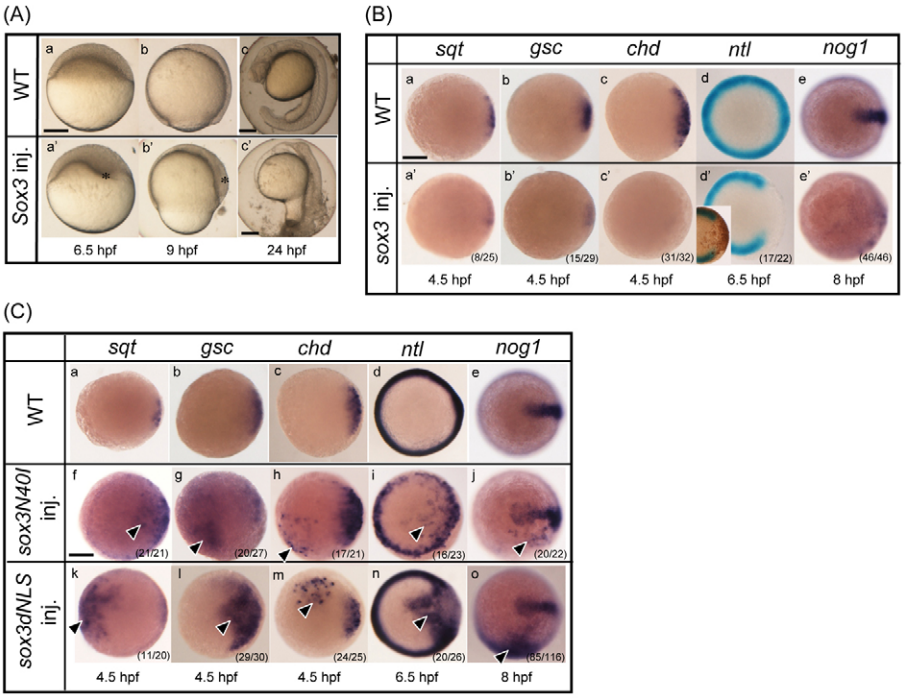


**Fig. 1. Sox3 inhibits gastrulation and represses markers of the zebrafish organizer and mesoderm.** (**Aa-c'**) Injection of *sox3* mRNA at the 1- to 2-cell stage (Aa') caused disruption of the blastoderm/yolk cell border at 6.5 hpf (asterisk). At 9 hpf, *sox3*-injected embryos (Ab') showed delayed gastrulation and a thickening on one side (asterisk) with a pronounced ventralised phenotype in those that survived to 24 hpf (Ac'). (**Ba-e'**) Injected *sox3* caused a dramatic reduction in the expression of *sqt*, *gsc*, *chd* and *nog1* and a substantial gap in the ring of *ntl* expression, which was coincident with the region of injected *sox3*, as shown by staining for HA (brown) (Bd', inset). (**C**) Compared with wild-type (WT) (**a-e**), injection of the dominant-negative forms of Sox3, Sox3N40I (**f-j**) and Sox3dNLS (**k-o**), did not repress the endogenous domains of expression of *sqt*, *gsc*, *chd*, *ntl* or *nog1*, but caused ectopic expression in the majority of embryos (arrowheads). The numbers of embryos showing ectopic expression/number injected are shown bottom right. The period of development is shown below each panel. Gene expression was analysed by whole-mount in situ hybridisation (blue). Animal pole views, except for A which was viewed laterally. Dorsal is to the right, except for Aa, Aa', Bd, Bd', for which orientation cannot be certain. Scale bars: ~200 μm.

## A DNA-binding mutant of Sox3 causes activation, rather than repressesion, of the expression of markers of the organizer

Because some Sox factors, including Sox3, have been shown to have effects via physical interaction with β-catenin (Zorn et al., 1999), we set out to determine whether the effects of Sox3 described above require its transcriptional regulatory activity. To this end, we made a DNA-binding mutant of Sox3 by replacing N40 with I in the HMG domain as described by Zhang et al. (Zhang et al., 2003). This construct (N40I) failed to activate a luciferase reporter construct driven by three consensus Sox binding sites (3×Sox) (Kuhlbroht et al., 1998) (data not shown).

In contrast to WT Sox3 (Fig. 1B), the N40I construct failed to inhibit the expression of *sqt*, *gsc*, *chd*, *ntl* or *nog1* (Fig. 1Cf-j) and gastrulation was unaffected even when injected at substantially higher doses. Western blotting confirmed that the N40I protein was expressed robustly until 12 hpf (data not shown). These data are consistent with the findings of Zhang et al., who showed that the N40I mutant was unable to repress the expression of *siamois* in *Xenopus* embryos (Zhang et al., 2003).

Even more strikingly, embryos injected with the N40I construct exhibited ectopic expression of all of the above markers of the mesoderm and organizer in most injected embryos (Fig. 1Cf-j, arrowheads). This ectopic expression of organizer markers was not simply an expansion of the endogenous domains into more ventral parts of the marginal region, as has been described for *gsc* and *sqt* expression when β-catenin is overexpressed (Dougan et al., 2003), but was seen in both dorsal and ventral aspects of the animal pole.

Thus, DNA binding is necessary for the Sox3-induced repression of these mesoderm and organizer markers, and a DNA-binding mutant might have a dominant-negative function in this context. Indeed, we found that expression of the N40I mutant interfered with the ability of WT Sox3 to activate its target in a luciferase assay (see Fig. S2A in the supplementary material). In order to analyse this phenomenon in further detail, we next examined the effect of an alternative dominant-negative form of Sox3.

## Dominant-negative forms of Sox3 induce ectopic expression of markers of both the organizer and the Nieuwkoop centre

Li et al. (Li et al., 2007) have shown that a version of Sox2 in which the nuclear localisation signals (NLSs) are mutated exhibits a dominant-negative function by interacting with, and so sequestering, the endogenous Sox2 protein (Li et al., 2007). We found that a similar NLS mutant version of zebrafish Sox3, hereafter referred to as Sox3dNLS, was also effective in interfering with the nuclear localisation of WT Sox3 (see Fig. S1 in the supplementary material) and with the ability of WT Sox3 to activate a luciferase reporter construct (see Fig. S2B in the

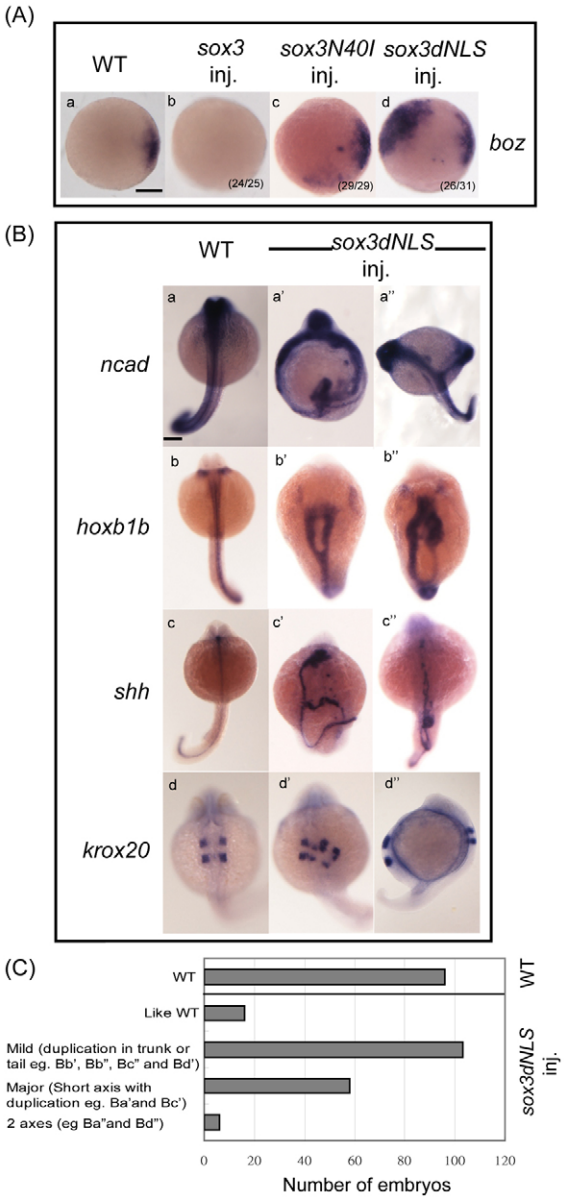


**Fig. 2. Dominant-negative Sox3 constructs induce ectopic expression of *bozozok* and axis duplication.** (**A**) Effect of Sox3 mutants as compared with WT Sox3 (**a**) on *boz* expression by RNA injection at the 1- to 2-cell stage and analysed at 4.5 hpf. Animal pole view, dorsal to the right, except Ab for which orientation cannot be certain. Injection of WT Sox3 caused loss of *boz* expression (**b**). Neither Sox3N40I (**c**) nor Sox3dNLS (**d**) repressed endogenous *boz* expression, but caused ectopic expression of *boz* in the majority of embryos. (**B**) Analysis 24 hours after injection showed that Sox3dNLS caused various extents of axis duplication, as shown by analyzing the expression of *ncad* (*cdh2*) (**a-a″**), *hoxb1b* (**b-b″**), *shh* (**c-c″**) and *krox20* (*egr2*) (**d-d″**). (**C**) The percentage of embryos of each duplication phenotype following injection of Sox3dNLS RNA (*n*=279). Scale bars: ~200 μm.

supplementary material). In addition, the phenotypes generated using the Sox3dNLS mutant (as described below) were rescued by co-expression of the WT protein (see Fig. 4C).

Injection of the Sox3dNLS RNA at the 1- to 2-cell stage failed to repress the endogenous domain of *sqt*, *gsc*, *chd*, *ntl* and *nog1* expression, but resulted in their ectopic expression in a manner very similar to, but stronger than, the N40I DNA-binding mutant described above (Fig. 1Ck-o). Given that the disruption of Sox3 function caused ectopic expression of three independent markers of the organizer, we hypothesised that the effects of overexpressed WT or dominant-negative Sox3 might be due to a single event upstream of all of these genes. The known events that precede the formation of the organizer involve formation of the Nieuwkoop centre. In zebrafish, the homeodomain protein Boz fulfils a similar function to Siamois in *Xenopus*, marking out the cells with Nieuwkoop centre activity and playing a central role in the induction of the organizer. Since there is evidence that Sox3 can repress the expression of *siamois* in *Xenopus* (Zhang et al., 2003), we determined whether *boz* expression was repressed in our experiments. Overexpression of WT Sox3 did indeed cause a dramatic reduction in *boz* expression (Fig. 2Ab), suggesting that this could underlie the loss of later markers of the organizer. In addition, the N40I and Sox3dNLS mutants caused both expansion of the vegetal region and the induction of separate patches of expression of *boz* in the ventral region of the prospective ectoderm (Fig. 2Ac,d). It seems, therefore, that disruption of Sox3 is sufficient to induce cells to adopt a Nieuwkoop centre-like fate, and this could explain why ectopic organizer markers would then be seen. Consistent with this suggestion, embryos injected with the Sox3dNLS RNA (and to a lesser extent with the Sox3 N40I mutant RNA, data not shown) exhibited axis duplication in most injected animals (91%) at 24 hpf (Fig. 2B,C), ranging from localised duplications in the trunk or tail (Fig. 2Ba′-d′,b″,c″) to almost complete independent axes (Fig. 2Ba″,d″). These duplications were remarkably similar to those seen when we injected β-*catenin* RNA (data not shown). It appears, therefore, that Sox3 can repress markers of the mesoderm, Nieuwkoop centre and organizer, whereas interfering with Sox3 function can elicit the ectopic expression of all of these, resulting in axis duplication.

## Repression of Wnt/β-catenin signalling by Sox3 requires transcriptional activity

*boz*, like *siamois* in *Xenopus*, is directly regulated by Wnt signalling via Tcf and β-catenin (Leung et al., 2003b). Likewise, β-catenin regulates *sqt* (Dougan et al., 2003) and it is therefore possible that some of the effects that we see when Sox3 is overexpressed are caused via interaction with the Wnt/β-catenin pathway. However, our initial data using the DNA-binding or dNLS mutants of Sox3 suggest that the repression of the markers of mesoderm and organizer is primarily dependent upon the ability of Sox3 to enter the nucleus and bind DNA. We have also found that WT and mutant forms of Sox3 all repress β-catenin signalling to a similar extent (TOP-Flash assay; see Fig. S3A in the supplementary material). Given that the WT and mutant forms of Sox3 have opposite effects on the expression of endogenous organizer genes and the effects of the Sox3dNLS are rescued by co-injection of WT Sox3 (see Fig. 4C), it is clear that a shared ability to repress β-catenin does not play a central role in the effects of the Sox3 constructs on cell fate. Since the dominant-negative forms of Sox3, which lack the ability to affect transcription directly, do not repress the expression of organizer markers, it seems that the repression of organizer markers by WT Sox3 occurs via transcriptional repression downstream of β-catenin.

We therefore examined the capacity of the different forms of Sox3 to override the ability of Wnt/β-catenin to increase the endogenous expression of organizer genes in vivo (Fig. 3). Wnt signalling was activated using a constitutively active form of β-catenin 2 (S37A). S37A β-catenin 2 caused robust expansion of

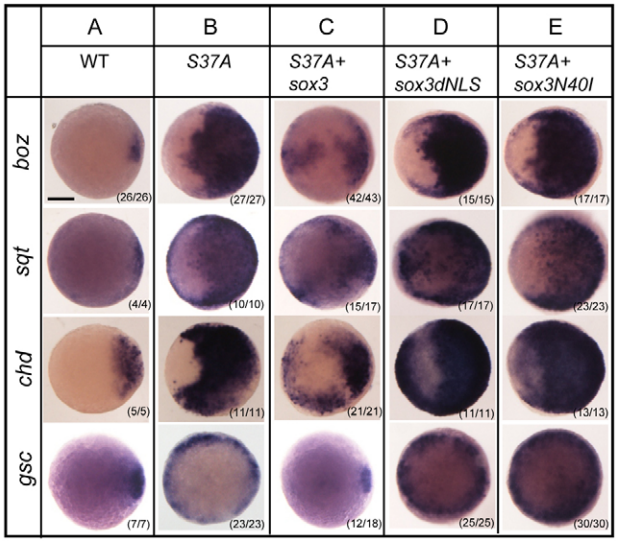


**Fig. 3. Sox3 inhibits Wnt/β-catenin signalling.** WT Sox3 inhibited the ability of S37A β-catenin 2 to activate the expression of *boz*, *sqt*, *gsc* and *chd* in vivo (**A-C**). Neither the dNLS (**D**) nor N40I (**E**) mutants of Sox3 were able to inhibit this response to S37A RNA. Animal pole views, dorsal right. Scale bar: ~200 μm.

*boz*, *sqt*, *gsc* and *chd* expression, but this was substantially inhibited by WT Sox3 (compare Fig. 3B with 3C). However, the dNLS and N40I mutants of Sox3 did not interfere with these effects of S37A β-catenin (Fig. 3D,E). This again indicates that WT Sox3 acts downstream of β-catenin to inhibit the expression of Wnt target genes via its transcriptional regulatory activity, rather than through interfering with β-catenin at the level of protein-protein interaction.

## The effects of dnSox3 require functional Wnt signalling

Although the N40I and Sox3NLS constructs can cause some repression of β-catenin signalling, they also cause the transcriptional activation of organizer/mesoderm markers that are normally dependent on the Wnt pathway. In order to investigate whether the effects of overexpressed mutant Sox3 still require β-catenin signalling, we blocked the β-catenin pathway using two strategies. First, we injected RNA encoding dominant-negative (dn) Tcf3. When dnTcf3 was injected alone at the 1- to 2-cell stage, it caused the dramatic reduction or loss of expression of *boz*, *sqt*, *chd* and *gsc* (compare Fig. 4A with 4D). When co-injected with Sox3dNLS, dnTcf3 was able to entirely inhibit its effects, producing a phenotype almost identical to that of dnTcf3 alone (compare Fig. 4B with 4E). Thus, it seems that activation of gene targets by β-catenin/Tcf signalling is essential for the consequences of Sox3 disruption to be seen. Next, in order to test whether β-catenin itself is necessary for the effects of Sox3dNLS, we injected a morpholino (MO) directed against β-*catenin 2*, which encodes the form of β-catenin that mediates Wnt signalling at these early stages (Bellipanni et al., 2006). This caused the reduction or loss of expression of *boz*, *sqt*, *chd* and *gsc* and dramatically inhibited the effects of Sox3dNLS (Fig. 4F,G). Thus, the ability of Sox3dNLS to induce ectopic organizer gene expression is dependent on β-catenin, despite the fact that this mutant Sox3 can also cause a substantial reduction in β-catenin signalling.

Together, our data suggest a model in which β-catenin signalling is sufficient to activate organizer genes when repression of these genes by Sox3 is relieved by Sox3dNLS overexpression. This is despite the fact that Sox3dNLS also substantially reduces β-catenin signalling (see Fig. S3A in the supplementary material). For this to be true, co-injection of dnTcf3 (which can block the effects of Sox3dNLS) would be expected to reduce the level of β-catenin signalling below that seen when Sox3dNLS is injected alone. This was indeed the case, with dnTCF causing a 4-fold reduction in Wnt signalling (as assessed by the TOP-Flash assay) as compared with injection of Sox3dNLS alone (see Fig. S3B in the supplementary material).

## Induction of the organizer by Sox3dNLS is dependent on Boz and Sqt

Since β-catenin is known to directly regulate *boz* and *sqt* and these factors act upstream of *gsc* and *chd*, it is possible that the effects of dominant-negative Sox3 constructs are entirely due to its activation of *boz* and *sqt*. Alternatively, Sox3 might also act on *gsc* and *chd* independently of Boz and Sqt. We therefore set out to test whether activation of *sqt* and *boz* expression is required for the effects of Sox3dNLS on *gsc* and *chd*.

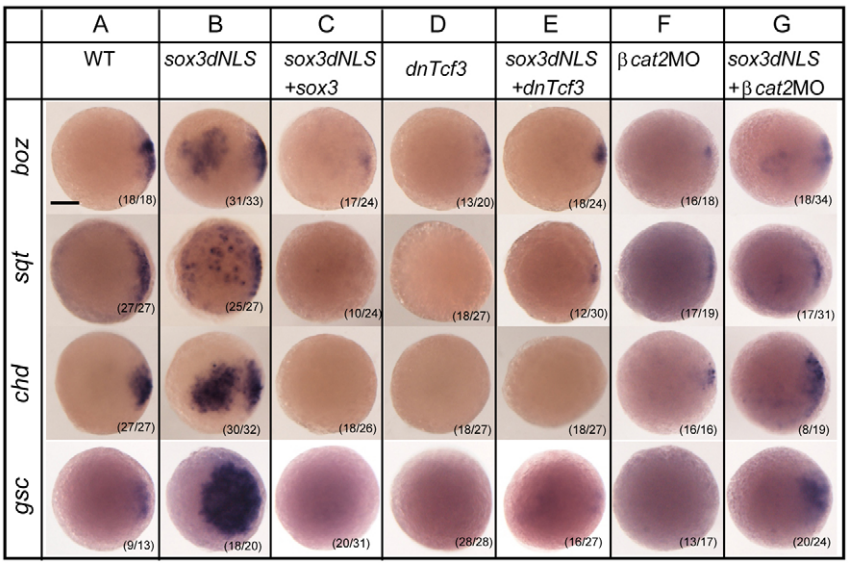


**Fig. 4. The effects of Sox3dNLS require Wnt/β-catenin signalling.** (**A-G**) Ectopic activation of *boz*, *sqt*, *gsc* and *chd* by Sox3dNLS RNA injected at the 1- to 2-cell stage and assayed at 4.5 hpf (B, compare with uninjected WT embryo, A) was blocked by WT Sox3 (C), but also by dnTcf3 (D,E) or a β-catenin 2 MO (F,G). Animal pole views, dorsal right. Scale bar: ~200 μm.

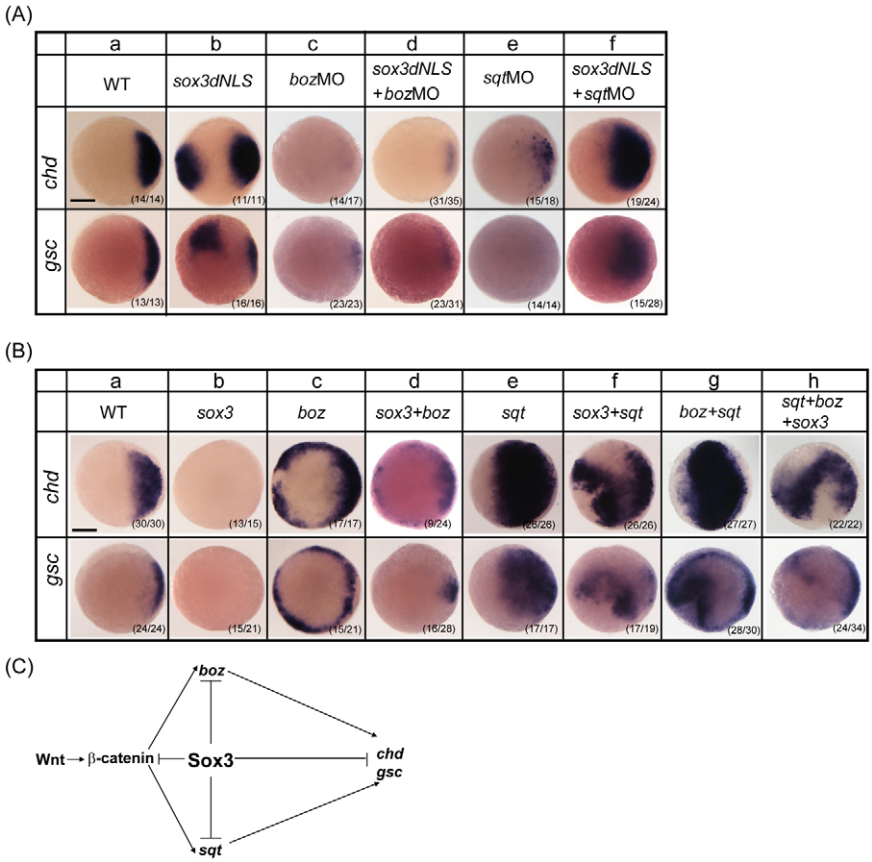


**Fig. 5. Interaction between Sox3, Boz and Sqt.** Animal pole views, dorsal to the right. (**A**) Both endogenous (**a**) and ectopic expression of *gsc* and *chd* induced by Sox3dNLS RNA injected at the 1- to 2-cell stage (**b**) was blocked by both *boz* (**c**,**d**) and *sqt* (**e**,**f**) MOs. (**B**) Compared with uninjected WT embryos (**a**), injection of WT *sox3* RNA at the 1- to 2-cell stage and assayed at 4.5 hpf repressed expression of *gsc* and *chd* (**b**), whereas *boz* and/or *sqt* activated their expression (**c**,**e**,**g**). Co-injection of *sox3* RNA with *boz* (**d**) or *sqt* (**f**) or both (**h**) RNAs resulted in intermediate levels of *gsc* and *chd* expression. (**C**) Model of Sox3 function in pathways that promote organizer formation. T-bars indicate inhibition and arrows indicate requirement for activation. Scale bars: ~200 μm.

Injection of a *boz* MO alone caused an almost total loss of both *gsc* (100%) and *chd* (82%) expression in most embryos (Fig. 5Ac), consistent with the current model that Boz is an essential upstream factor for the expression of these genes (Kelly et al., 2000; Leung et al., 2003a; Shimizu et al., 2000). When both Sox3dNLS RNA and the *boz* MO were injected together, the outcome was similar to that of *boz* MO injection alone, with generally decreased expression of *gsc* and *chd* (Fig. 5Ad). Thus, it appears that Boz function is absolutely essential for the effect of Sox3dNLS on *gsc* and *chd*.

Injection of a *sqt* MO caused a loss of endogenous *gsc* expression but only reduced *chd* expression (Fig. 5Ae), consistent with published studies (Dougan et al., 2003; Shimizu et al., 2000). Inhibition of Sqt also completely abolished the ectopic *gsc* and *chd* expression that was induced in the ventral part of the animal hemisphere by Sox3dNLS (Fig. 5Af), but did not completely block expansion of the endogenous domain of *gsc* and *chd*.

These data suggest that Boz and/or Sqt could act downstream of Sox3dNLS and therefore explain its effects on *gsc* and *chd*. This would imply that repression of *gsc* and *chd* expression by WT Sox3 is primarily due to its repression of *boz* and/or *sqt*. If Boz/Sqt act downstream of Sox3, then co-injection of *boz/sqt* RNA together with WT *sox3* RNA would be expected to give the same phenotype as *boz/sqt* alone. Although both *sqt* and *boz* RNA (injected either separately or together) were able to rescue the expression of *gsc* and *chd* in the presence of overexpressed WT Sox3 (compare Fig. 5Bb with 5Bd,f,h), the increase in the expression of *gsc* and *chd* was less than that induced by *boz* (Fig. 5Bc) or *sqt* (Fig. 5Be) or a combination of the two (Fig. 5Bg). Sox3 therefore appears to interfere downstream of Boz and Sqt. We propose that Sox3 represses the expression of *boz* and *sqt*, but that it can also repress *chd* and *gsc*, interfering with their activation by Boz and Sqt (see Fig. 5C). This is consistent with our observation that, although Sox3dNLS causes derepression of *gsc* and *chd*, their expression still requires the activity of Boz and Sqt.

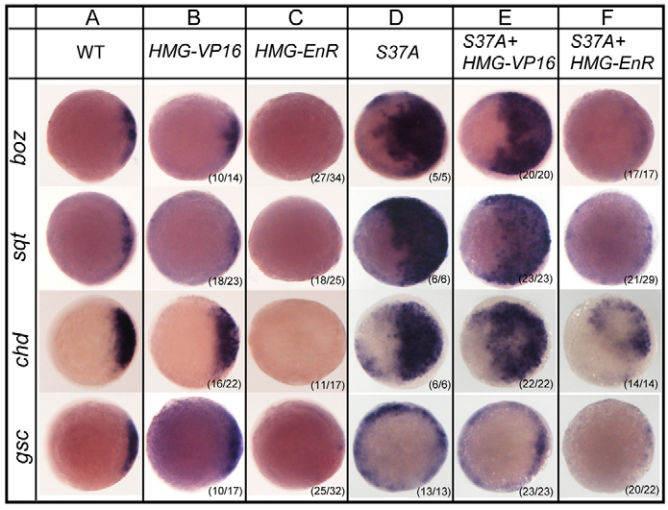


**Fig. 6. HMG-EnR constructs mimic the effects of WT Sox3 on organizer markers.** All embryos are 4.5 hpf, animal pole view, dorsal right. Sox3HMG-EnR (**C**), but not Sox3HMG-VP16 (**B**), repressed all markers (*boz*, *sqt*, *chd*, *gsc*), as compared with controls (**A**). Similarly, Sox3HMG-EnR (**F**), but not Sox3HMG-VP16 (**E**), inhibited the activation of these markers by S37A β-catenin 2 (**D**). Scale bar: ~200 μm.

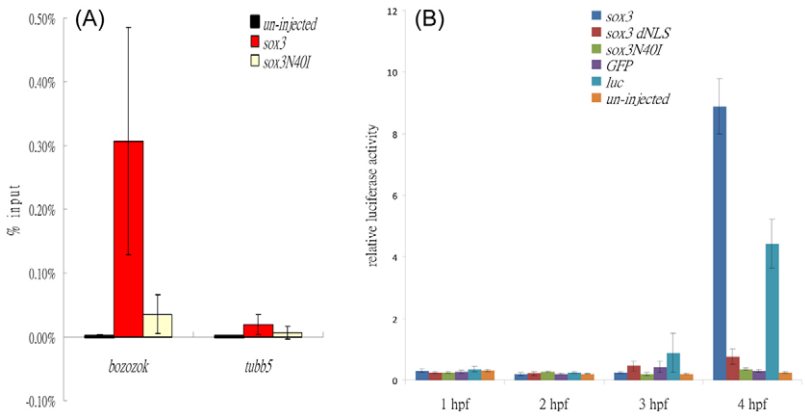


**Fig. 7. Sox3 binds and directly regulates *boz*.** (**A**) ChIP-PCR analysis of the *boz* proximal promoter following immunoprecipitation of HA-tagged Sox3. An HA antibody pulled down the *boz* reporter (but not a *tubb5* target sequence) in embryos injected with HA-tagged Sox3 but not in uninjected embryos. (**B**) Injection of *sox3*, but not the N40I or dNLS mutants of Sox3, robustly activated a luciferase reporter driven by the *boz* 1.3 kb proximal promoter.

## Sox3 acts as a repressor

In order to determine if the repressive effects of Sox3 on the various steps of organizer formation involve direct repression, we examined whether these effects could be mimicked by a dominant repressor construct (Fig. 6). To this end, we generated a Sox3HMG-EnR construct [as described previously (Bylund et al., 2003)]. Injection of this construct mimicked the effect of WT Sox3 on the expression of *boz*, *sqt*, *gsc*, *chd* and *ntl* (compare Fig. 1Ba′-c′ and Fig. 2Ab with Fig. 6C), whereas a Sox3HMG-VP16 constitutive activator construct did not repress these genes (Fig. 6B). Indeed, like WT Sox3 (Fig. 3C), Sox3HMG-EnR, but not Sox3HMG-VP16, was able to completely inhibit the activation of these genes by the constitutively active S37A form of β-catenin 2 (Fig. 6D-F), again showing that Sox3 functions downstream of the Wnt pathway. This suggests that the effect of the WT Sox3 is due to its inherent transcriptional repressor activity.

In order to verify that this repressor activity is direct, we first carried out ChIP-PCR to determine whether Sox3 binds directly to the proximal promoter region of *boz*, the earliest gene affected by Sox3. Tcf, which together with β-catenin activate *boz*, can bind to consensus sites in the 1.3 kb region immediately upstream of the *boz* transcriptional start site. We therefore analysed the same region, which contains several Sox consensus binding sites. Since none of the commercially available antibodies can immune-precipitate endogenous Sox3, we used ectopic expression of an HA-tagged version of Sox3. In order to avoid non-specific Sox3-DNA interactions, we injected an amount of RNA that produced protein at a level below that of the endogenous protein at 70% epiboly. This approach also allowed us to use HA antibody immunoprecipitation (IP) on uninjected embryos and IP of an HA-tagged N40I DNA-binding mutant as additional negative controls. The HA antibody did not precipitate a detectable level of the *boz* promoter in uninjected embryos. IP of the HA-tagged Sox3 gave a robust precipitation of the *boz* promoter fragments, whereas the N40I mutant was over 8-fold less efficient in doing so. IP of either of these Sox3 proteins also failed to pull down any substantial amount of *tubb5*, which was used as a further control (Fig. 7A).

We next showed that Sox3 can activate a luciferase construct driven by the same 1.3 kb *boz* promoter region within 4 hours of injection (Fig. 7B). Similar activation of a reporter construct representing a gene repressed in vivo has been described for other situations, including the regulation of *siamois* by Sox3 in *Xenopus* (Zhang et al., 2003). Together, these data suggest that *boz* is a direct target of repression by Sox3 and, as the expression of *sqt*, *gsc* and *chd* is also repressed within 4 hours of injection of *sox3* RNA and is repressed by the HMG-EnR construct (Fig. 6), these are also likely to be direct effects.

## Mutual repression between Sox3 and Boz/Sqt

In vertebrate development, the establishment of mutually exclusive domains of expression often involves mutual repression between genes expressed in those domains. It has also been shown previously that Nodal signalling is required in zebrafish for the exclusion of *sox3* expression from the margin (Bennett et al., 2007). We therefore carried out a careful comparison of the earliest expression of *sox3*, *boz* and *sqt* (Fig. 8A). *Sox3* expression was relatively weak and patchy at 3.5 hpf, becoming much stronger and uniform by 4 hpf, with the notable exception of a gap in expression on the dorsal side coincident with the region where *boz* expression appeared (compare Fig. 8Aa with 8Ac and 8Ba). Expression of *sqt* was also first seen at very low levels on the dorsal side of the margin at 3.5 hpf and then more strongly by 4 hpf, with weak expression around the margin (Fig. 8Ab). Between 4.5 and 5.5 hpf, *sqt* expression was strong in the margin where *sox3* became excluded, as shown by double in situ hybridisation (Fig. 8Bb).

We therefore analysed whether, in addition to Sox3 repressing *sqt* and *boz* expression, Sqt and Boz could reciprocally repress the expression of *sox3*. Injection of either *sqt* or *boz* RNA caused robust inhibition of *sox3* expression (Fig. 8Ca-c). In addition, activation of β-catenin signalling, using the S37A mutant, repressed *sox3* expression (Fig. 8Cd). Inhibition of *sox3* expression by S37A β-catenin 2 did not appear to be indirect, i.e. by activating the expression of *boz* and/or *sqt*, as the repression of *sox3* by overexpression of S37A was unaffected by co-injection of MOs directed against both *boz* and *sqt* (Fig. 8Ce-g). Hence, it seems that there is reciprocal repression at all levels, with Sox3 repressing β-catenin 2 activity and negatively regulating transcription of both *boz* and *sqt*, and β-catenin, Boz and Sqt all independently repressing expression of *sox3* (see Fig. 8D).

## Redundancy between Sox3 and Sox19a/b

The experiments above showed that Sox3 could repress the expression of genes associated with the organizer and that a dominant-negative form of Sox3 could cause ectopic activation of these genes and axis duplications. However, we were concerned that this activity might not be restricted to Sox3 and that the dominant-negative construct could also affect other members of the SoxB1 subfamily. Of the other SoxB1 factors, only *sox19a* and *sox19b* are expressed at the early stages when they might affect organizer formation (Okuda et al., 2006). We

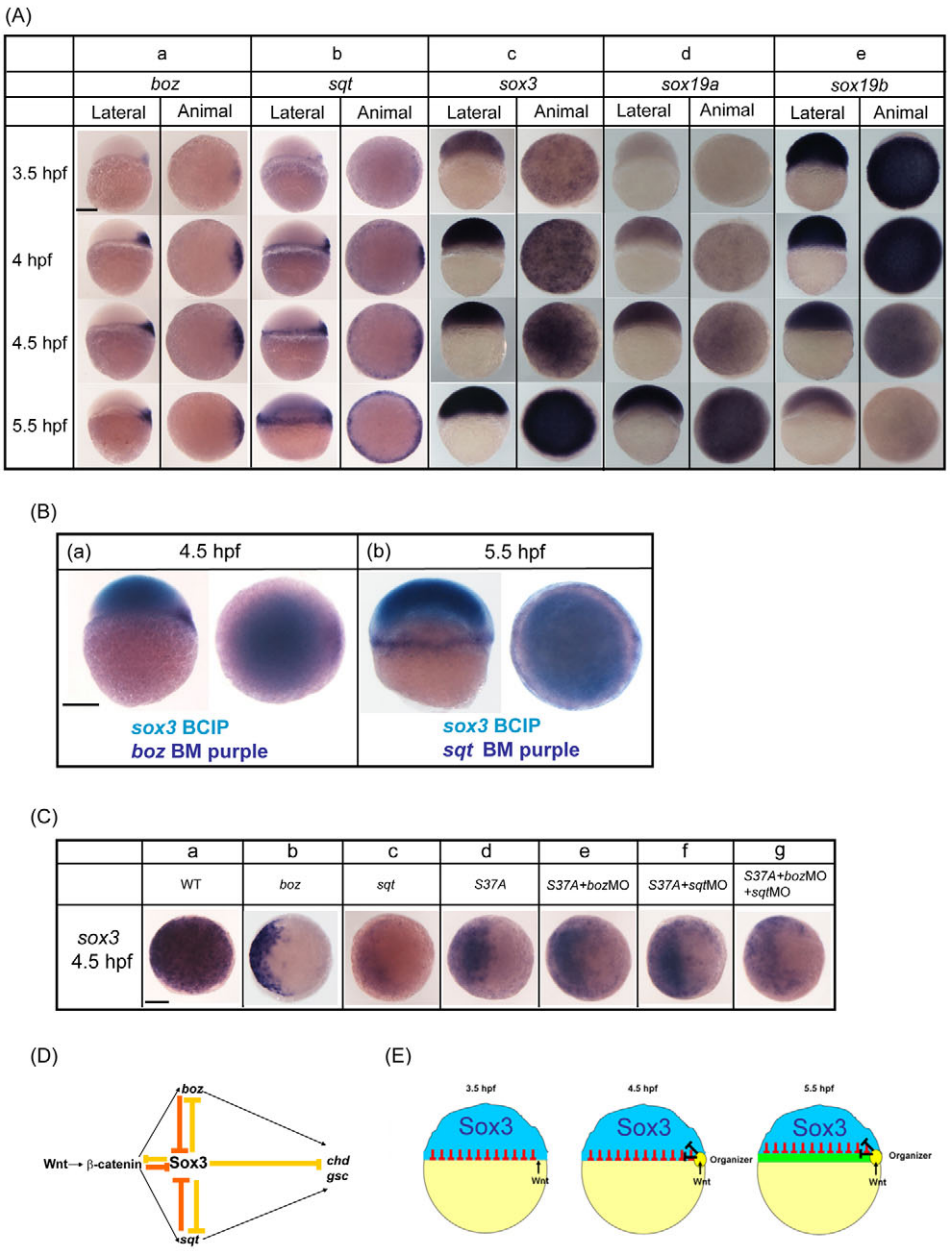


**Fig. 8. Reciprocal expression and transcriptional repression between Sox3, β-catenin 2, Boz and Sqt.** (**A**) Lateral and animal views, dorsal right, showing endogenous expression of *boz* (**a**), *sqt* (**b**), *sox3* (**c**), *sox19a* (**d**) and *sox19b* (**e**). (**B**) Double in situ hybridisation for *boz*/*sox3* (**a**) or *sqt*/*sox3* (**b**). *sox3* is detected with BCIP (pale blue), *boz*/*sqt* with BM purple. (**C**) Effect of S37A, Boz and Sqt on *sox3* expression. Animal view of *sox3* expression at 4.5 hpf, dorsoventral orientation cannot be certain. Endogenous *sox3* expression (**a**) was repressed by injection of *boz* (**b**), *sqt* (**c**) or S37A β-*catenin 2* (**d**) RNA. MOs directed against *boz* and/or *sqt* did not affect the ability of S37A β-*catenin 2* RNA to inhibit the expression of *sox3* (**e-g**). (**D**) Model for the role of Sox3 in restricting organizer formation. T-bars indicate mutual repression between Sox3 and β-catenin 2, *boz* and *sqt*. (**E**) Taking our experimental data together with the endogenous domains of expression illustrated above, this leads us to a model in which at 4-4.5 hpf, high levels of Wnt signalling on the dorsal side (yellow) together with vegetal Nodal signalling inhibit *sox3* expression and induce expression of *boz*, which can also repress the expression of *sox3* in the dorsal margin. By 5.5 hpf, *sox3* is further depleted throughout the whole margin by Nodal signalling, allowing mesendoderm to form (green). Away from the margin, in the prospective ectoderm, Nodal signalling is too weak to repress *sox3* expression and so Sox3 remains present (blue), inhibiting the expansion of mesoderm and organizer into this region (red bars). Scale bars: ~200 μm.

therefore analysed their expression in more detail at early stages using in situ hybridisation (Fig. 8A). Consistent with published RT-PCR data (Okuda et al., 2006), *sox19b* was present at high levels as maternal RNA from the 1-cell stage (data not shown). However, unlike *sox3*, *sox19b* expression remained in the prospective mesendoderm at 4-4.5 hpf, after which expression declined dramatically by 5.5 hpf (Fig. 8Ae). *sox19a* was not present as maternal RNA but appeared weakly by 4 hpf, when it was expressed throughout the epiblast and, like *sox3*, its expression was lost from the prospective mesendoderm between 4 and 4.5 hpf, with this pattern maintained at 5.5 hpf (Fig. 8Ad). Next, we showed that, like Sox3 (Fig. 9B), both Sox19a and Sox19b could repress expression of the organizer genes (Fig. 9C,D), but to a lesser extent than Sox3. However, only Sox19b could also effectively rescue the effects of the Sox3dNLS construct. Whereas WT Sox3 and Sox19b inhibited the ectopic expression of *boz*, *sqt*, *chd* and *gsc* induced by Sox3dNLS (Fig. 9F,H), Sox19a was only able to rescue the effect on *boz* (Fig. 9G). Since these data suggested that Sox19b might act redundantly with Sox3 to repress organizer genes, we next examined whether Sox3dNLS could also interfere with the transcriptional regulatory function of Sox19b. We found that the Sox3dNLS construct inhibited the ability of Sox19b to activate a luciferase reporter construct in COS7 cells in much the same way as it affected Sox3 (see Fig. S2C in the supplementary material). This suggested the possibility that the ectopic organizer gene expression induced by Sox3dNLS might be due not only to interference with the ability of Sox3 to repress its target genes, but might also involve interaction and interference with Sox19b.

From these data it remained unclear whether both Sox3 and Sox19b normally repress organizer formation and so whether both must be blocked to elicit the ectopic expression of organizer markers. However, we have found that MOs against *sox3* alone do not elicit an early phenotype (Dee et al., 2008). It

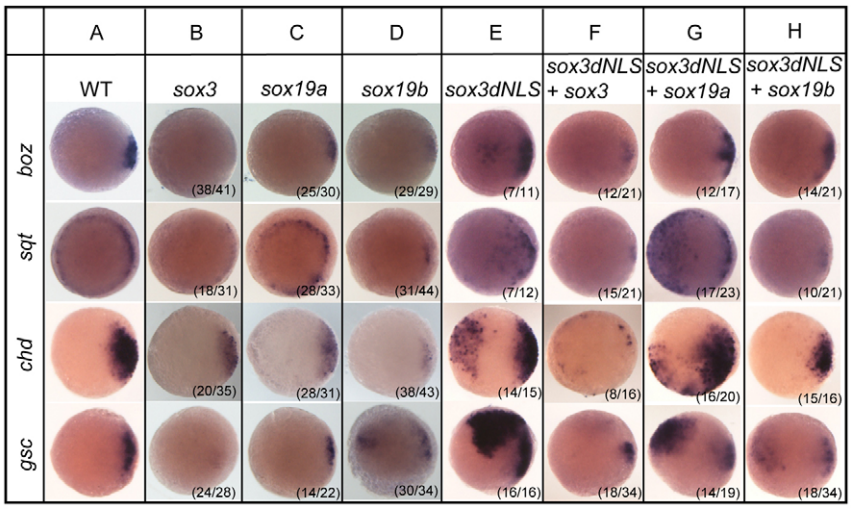


**Fig. 9. Comparison of the effects of Sox3, Sox19a and Sox19b on the expression of organizer genes.** All embryos are at 4.5 hpf; animal pole view, dorsal to the right. Compared with uninjected WT embryos (**A**), overexpression of Sox3 (**B**), Sox19a (**C**) or Sox19b (**D**) resulted in the loss or reduction of expression of *boz*, *sqt*, *chd* and *gsc*. Like Sox3 (**F**), Sox19b (**H**) was largely able to rescue the effect of Sox3dNLS (**E**), whereas Sox19a was not (**G**).

therefore seems likely that a maternal protein or a second factor, most probably Sox19b, must also be inhibited in order to elicit the expression of ectopic organizer markers. Given the presence of *sox19b* RNA from the 1-cell stage, which declines dramatically between 4.5 and 5.5 hpf, it seems likely that Sox19b protein is also present during this early period (there is no antibody currently available with which to assess this), precluding the effective use of MOs to study its function. It is not perhaps surprising then that injection of *sox3* and *sox19b* MOs in combination also failed to mimic the effects of Sox3dNLS or to cause any early morphological phenotype (data not shown).

## DISCUSSION

We have shown that Sox3 inhibits multiple steps in the pathways that induce a dorsal organizer in zebrafish. Dominant-negative Sox3 constructs were able to induce the ectopic expression of organizer markers and the formation of a duplicated axis. Hence, the early expression of Sox3 throughout the epiblast and early ectoderm provides a mechanism to restrict organizer formation to the correct time and place. This was shown to be through its transcriptional repression of target genes, rather than through interference with β-catenin. The observation that neither a DNA-binding mutant nor an NLS mutant repressed those targets that are inhibited by the WT Sox3 protein, and our data showing that Sox3 binds directly to the *boz* gene, indicate that Sox3 directly represses at least some of its target genes. The simplest interpretation of the effects seen when the dominant-negative forms are overexpressed is that these proteins interfere with the ability of WT Sox3 to reach its transcriptional targets. We have also shown that *sox19b*, which is expressed even earlier in development than *sox3*, can also repress organizer gene expression. The possible presence of maternal Sox19b protein might explain why MOs against the SoxB1 genes do not cause significant disruption of early developmental events.

### The intersection between Sox3 and β-catenin

The Wnt effector proteins Tcf and Lef, with which β-catenin binds to DNA, are HMG family factors closely related to the Sox proteins. Hence, it was not surprising when the first studies in *Xenopus* indicated that some Sox factors, including Sox3, could compete with Tcf and Lef for binding to β-catenin and so interfere with Wnt/β-catenin signalling (Zorn et al., 1999). Since this first observation, several Sox factors have been shown to interact with β-catenin, sometimes repressing its function and other times activating targets in cooperation with it (Sinner et al., 2004; Zorn et al., 1999). Where a Sox protein repressed Wnt signalling, this was shown to involve degradation of the β-catenin protein (Sinner et al., 2004).

In our study, we have verified that Sox3 can interfere with Wnt signalling independently of its transcriptional activity, but this does not seem to play a role in the effects of Sox3 upon the downstream targets studied in this report. In fact, disruption of Sox3 through the dominant-negative Sox3 constructs can induce the ectopic expression of genes that require β-catenin activity. This occurs with constructs that we know also inhibit β-catenin signalling and in a region (the ventral part of the animal hemisphere) where β-catenin is believed to be already low. It therefore seems that the level of β-catenin that is required for these genes to be activated when Sox3 function is disrupted is very low. This should lead to a reassessment of the role of β-catenin/Wnt signalling in regions where its activity was previously thought to be insignificant.

### Induction of the organizer

The data described in this study indicate a central role for Sox3/Sox19b in establishing the earliest organizing centres of vertebrates, which are the functional equivalents of the Nieuwkoop centre and Spemann's organizer. These SoxB1 factors appear to repress the expression of genes of the mesoderm, Nieuwkoop centre and organizer, suggesting that they act as master repressors of non-ectodermal fate. In the normal embryo, both *sox3* and *sox19b* are initially expressed throughout the epiblast and they are then excluded from the dorsal marginal region where cells become specified to form the equivalent of the Nieuwkoop centre and later organizer. Our data, together with that of Bennett et al. (Bennett et al., 2007), suggest a model in which Nodal-related signals in the marginal cells repress transcription of SoxB1 genes such that, by 5.5 hpf, SoxB1 gene expression is strong throughout the prospective ectoderm, but is largely excluded from the prospective mesendoderm including the organizer (see Fig. 8D,E). The presence of SoxB1 factors throughout the prospective ectoderm then restricts formation of the mesoderm, Nieuwkoop centre and organizer to their normal marginal position. SoxB1 factors achieve this by directly repressing three sequential steps in the signalling that promotes organizer formation: by inhibiting β-catenin, by repressing the expression of *boz*/*sqt*, and by

repressing the expression of organizer markers such as *gsc* and *chd*. Thus, Sox3/Sox19b appear to represent comprehensive inhibitors of organizer formation.

The importance of this inhibition by SoxB1 factors is supported by our observation that disrupting their function in the prospective ectoderm results in the ectopic expression of the mesoderm markers *ntl* and *sqt*, the Nieuwkoop marker *boz*, and the organizer markers *chd* and *gsc*. This also results in these embryos developing a second axis, including duplication of the CNS. Overall, our data show that the effects of dominant-negative Sox3 on the induction of ectopic markers of Nieuwkoop centre-like activity and the organizer are as strong, or stronger, than the effects of ectopic β-catenin signalling or Boz and Sqt combined. Although overexpression of Boz expands the expression of *gsc* and *chd* ventrally, unlike the ectopic expression induced by dominant-negative Sox3, they remain restricted to the vegetal region (Kelly et al., 2000; Leung et al., 2003a; Shimizu et al., 2000); we suggest that this is because of repression of these markers by Sox3/Sox19b in the ectoderm. One reason why the organizer itself is normally restricted vegetally is because it requires mesendoderm determinants and dorsal modifiers. This would explain why Boz simply expands the marginal expression of genes such as *chd* and *gsc*. The fact that dominant-negative Sox3 can induce the expression of these markers in the animal hemisphere might be because it can also induce some aspects of mesendoderm fate, presumably those that are necessary for subsequent organizer formation.

## Sox3 and the organizer in other vertebrates

The expression of *sox3* appears similar across a range of vertebrate species, whereas Sox19a/b are only found in zebrafish. As in zebrafish, *sox3* is expressed throughout the epiblast of *Xenopus* and chick and is then lost in cells that undergo gastrulation. Indeed, even prior to gastrulation, *Sox3* expression is weak in the region of the blastopore lip in early gastrula stage *Xenopus* embryos (Penzel et al., 1997) and is also lost from the region equivalent to the organizer, prior to the start of gastrulation, in chick embryos (Rex et al., 1997). It is therefore feasible that Sox3 plays a similar role in all these species.

In *Xenopus*, it is clear that Sox3 can repress both *nodal* and *siamois* expression (Zhang et al., 2003) and so does seem to be involved in these events. Although the mechanism by which Sox3 interferes in these processes has not been analysed in detail, it seems to require DNA binding (Zhang et al., 2003). It therefore seems probable that *Xenopus* Sox3 does indeed play a similar role to that described here. Less is known of the earliest events that set up the organizer in chick. However, in their review comparing the events that lead to organizer formation in various vertebrates, Joubin and Stern commented that in chick "an antagonism between the node and the periphery of the embryo imposes spatial restrictions on the organizer, thereby confining it to just the centre of the embryo" (Joubin and Stern, 1999). They also observed that although certain signals, including BMPs, may repress regeneration of the organizer at later stages, an alternative repressive influence must exist at earlier stages. The expression of *Sox3* in chick is consistent with just such a role, repressing the organizer except at the midline.

Given the overarching and dominant role that Sox3 appears to play in regulating when and where the organizer forms, many new questions arise for future investigation. How does Sox3 function as a repressor? How many more genes does Sox3 repress in this context? Finally, as Sox3 also activates genes associated with neural development, how does Sox3 determine which targets to repress and which to activate?

**Acknowledgements**
We thank Elaine Thorpe and Dave Reffin for their technical support and Alan Clarke, Martin Gering and the DSHB at the University of Iowa for plasmids and antibodies. This work was supported by a BBSRC Stem Cell Initiative grant (BBS/B/14744). Z.A.L. was supported by a Pakistan HEC scholarship (University of Sindh).

**Competing interests statement**
The authors declare no competing financial interests.

**Supplementary material**
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.054130/-/DC1

**References**

**Bellipanni, G., Varga, M., Maegawa, S., Imai, Y., Kelly, C., Myers, A. P., Chu, F., Talbot, W. S. and Weinberg, E. S.** (2006). Essential and opposing roles of zebrafish {beta}-catenins in the formation of dorsal axial structures and neurectoderm. *Development* **133**, 1299-1309.
**Bennett, J. T., Joubin, K., Cheng, S., Aanstad, P., Herwig, R., Clark, M., Lehrach, H. and Schier, A. F.** (2007). Nodal signaling activates differentiation genes during zebrafish gastrulation. *Dev. Biol.* **304**, 525-540.
**Bylund, M., Andersson, E., Novitch, B. G. and Muhr, J.** (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nat. Neurosci.* **6**, 1162-1168.
**Dee, C. T., Gibson, A., Rengifo, A., Sun, S. K., Patient, R. K. and Scotting, P. J.** (2007). A change in response to Bmp signalling precedes ectodermal fate choice. *Int. J. Dev. Biol.* **51**, 79-84.
**Dee, C. T., Hirst, C. S., Shih, Y.-H., Tripathi, V. B., Patient, R. K. and Scotting, P. J.** (2008). Sox3 regulates both neural fate and differentiation in the zebrafish ectoderm. *Dev. Biol.* **320**, 289-301.
**Dougan, S. T., Warga, R. M., Kane, D. A., Schier, A. F. and Talbot, W. S.** (2003). The role of the zebrafish nodal-related genes squint and cyclops in patterning of mesendoderm. *Development* **130**, 1837-1851.
**Joubin, K. and Stern, C. D.** (2001). Formation and maintenance of the organizer among the vertebrates. *Int. J. Dev. Biol.* **45**,165-175.
**Jowett, T. and Yan, Y. L.** (1996). Double fluorescent in situ hybridization to zebrafish embryos. *Trends Genet.* **12**, 387-389.
**Kelly, C., Chin, A. J., Leatherman, J. L., Kozlowski, D. J. and Weinberg, E. S.** (2000). Maternally controlled beta-catenin-mediated signaling is required for organizer formation in the zebrafish. *Development* **127**, 3899-3911.
**Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F.** (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.
**Kodjabachian, L. and Lemaire, P.** (1998). Embryonic induction: is the Nieuwkoop centre a useful concept? *Curr. Biol.* **8**, R918-R921.
**Kuhlbroht, K., Herbarth, B., Sock, E., Hermans-Borgmeyer, I. and Wegner, M.** (1998). Sox10, a novel transcriptional modulator in glial cells. *J. Neurosci.* **18**, 237-250.
**Leung, T., Bischof, J., Soll, I., Niessing, D., Zhang, D., Ma, J., Jackle, H. and Driever, W.** (2003a). bozozok directly represses bmp2b transcription and mediates the earliest dorsoventral asymmetry of bmp2b expression in zebrafish. *Development* **130**, 3639-3649.
**Leung, T. C., Soll, I., Arnold, S. J., Kemler, R. and Driever, W.** (2003b). Direct binding of Lef1 to sites in the boz promoter may mediate pre-midblastula-transition activation of boz expression. *Dev. Dyn.* **228**, 424-432.
**Li, J., Pan, G., Cui, K., Liu, Y., Xu, S. and Pei, D.** (2007). A dominant-negative form of mouse SOX2 induces trophectoderm differentiation and progressive polyploidy in mouse embryonic stem cells. *J. Biol. Chem.* **282**, 19481-19492.
**Maegawa, S., Varga, M. and Weinberg, E. S.** (2006). FGF signaling is required for β-catenin-mediated induction of the zebrafish organizer. *Development* **133**, 3265-3276.
**Okuda, Y., Yoda, H., Uchikawa, M., Furutani-Seiki, M., Takeda, H., Kondoh, H. and Kamachi, Y.** (2006). Comparative genomic and expression analysis of group B1 sox genes in zebrafish indicates their diversification during vertebrate evolution. *Dev. Dyn.* **235**, 811-825.
**Penzel, R., Oschwald, R., Chen, Y., Tacke, L. and Grunz, H.** (1997). Characterization and early embryonic expression of a neural specific transcription factor xSOX3 in Xenopus laevis. *Int. J. Dev. Biol.* **41**, 667-677.
**Rex, M., Orme, A., Uwanogho, D., Tointon, K., Wigmore, P., Sharpe, P. and Scotting, P.** (1997). Dynamic expression of the chicken Sox2 and Sox3 genes in ectoderm induced to form neural tissue. *Dev. Dyn.* **209**, 323-332.
**Schier, A. F. and Talbot, W. S.** (2005). Molecular genetics of axis formation in zebrafish. *Annu. Rev. Genet.* **39**, 561-613.
**Shimizu, T., Yamanaka, Y., Ryu, S.-L., Hashimoto, H., Yabe, T., Hirata, T., Bae, Y.-k., Hibi, M. and Hirano, T.** (2000). Cooperative roles of Bozozok/Dharma and Nodal-related proteins in the formation of the dorsal organizer in zebrafish. *Mech. Dev.* **91**, 293-303.

**Sinner, D., Rankin, S., Lee, M. and Zorn, A. M.** (2004). Sox17 and beta-catenin cooperate to regulate the transcription of endodermal genes. *Development* **131**, 3069-3080.
**Urtishak, K. A., Choob, M., Tian, X. B., Sternheim, N., Talbot, W. S., Wickstrom, E. and Farber, S. A.** (2003). Targeted gene knockdown in zebrafish using negatively charged peptide nucleic acid mimics. *Dev. Dyn.* **228**, 405-413.
**Westerfield, M.** (2000). *The Zebrafish Book. A Guide For The Laboratory Use Of Zebrafish (Danio rerio).* Eugene, OR: University of Oregon Press.
**Wood, H. B. and Episkopou, V.** (1999). Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. *Mech. Dev.* **86**, 197-201.
**Zhang, C. and Klymkowsky, M. W.** (2007). The Sox axis, Nodal signaling, and germ layer specification. *Differentiation* **75**, 536-545.
**Zhang, C., Basta, T., Jensen, E. D. and Klymkowsky, M. W.** (2003). The beta-catenin/VegT-regulated early zygotic gene Xnr5 is a direct target of SOX3 regulation. *Development* **130**, 5609-5624.
**Zhang, C., Basta, T., Hernandez-Lagunas, L., Simpson, P., Stemple, D. L., Artinger, K. B. and Klymkowsky, M. W.** (2004). Repression of nodal expression by maternal B1-type SOXs regulates germ layer formation in Xenopus and zebrafish. *Dev. Biol.* **273**, 23-37.
**Zorn, A. M., Barish, G. D., Williams, B. O., Lavender, P., Klymkowsky, M. W. and Varmus, H. E.** (1999). Regulation of Wnt signaling by Sox proteins: XSox17 alpha/beta and XSox3 physically interact with beta-catenin. *Mol. Cell* **4**, 487-498.