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...
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'-GAATTCATGCTAGCGGCTGA-3' and reverse, 5'-CTTAGACAGGGTCCGTAACCA-3'. The PCR product was ligated into the pcRII vector (Invitrogen) and subcloned into the PC82-yc-sls vector at the EcoRI-Xhol sites, creating an in-frame fusion of β-catenin 2 and mcy (with the nuclear localisation signal removed). Sox3NLS (KR36TG, RR50LG, RR107LG) (Li et al., 2007), 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 Kit (Stratagene). In order to generate the dominant repressor HMG-EnR and dominant activator HMG-VP16 constructs, the HMG box of Kit (Stratagene). In order to generate the dominant repressor HMG-EnR and dominant activator HMG-VP16 constructs, the HMG box of Kit (Stratagene). In order to generate the dominant repressor HMG-EnR and dominant activator HMG-VP16 constructs, the HMG box of Kit (Stratagene). In order to generate the dominant repressor HMG-EnR and dominant activator HMG-VP16 constructs, the HMG box of Kit (Stratagene). In order to generate the dominant repressor HMG-EnR and dominant activator HMG-VP16 constructs, the HMG box of Kit (Stratagene). In order to generate the dominant repressor HMG-EnR and dominant activator HMG-VP16 constructs, the HMG box of Kit (Stratagene). In order to generate the dominant repressor HMG-EnR and dominant activator HMG-VP16 constructs, the HMG box of Kit (Stratagene). In order to generate the dominant repressor HMG-EnR and dominant activator HMG-VP16 constructs, the HMG box of Kit (Stratagene). In order to generate the dominant repressor HMG-EnR and dominant activator HMG-VP16 constructs, the HMG box of Kit (Stratagene). In order to generate the dominant repressor HMG-EnR and dominant activator HMG-VP16 constructs, the HMG box of Kit (Stratagene). In order to generate the dominant repressor HMG-EnR and dominant activator HMG-VP16 constructs, the HMG box of Kit (Stratagene).

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 (Utrishak et al., 2007) and sqt (Maegawa, 2006) were obtained from Gene Tools (Philomath, OR, USA). The MO sequences were: β-catenin 2 MO, 5'-CCCTTAGGCT-AGCGACTTCAAAAC-3'; boz MO, 5'-TGCCATGTTCAAGTGTA-3'; and sqt MO, 5'-AGTCAATAAGGATTAAATCACCAAC-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-labellled (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-AATTGGGACGCTTA-3'; tubb5-R, 5'-GGATGAGGACGATT-TAACCC-3'; boz-F, 5'-TGGGACTAGGTCTAGGAAA-3'; and boz-R, 5'-CGCCTTTGAATGAGACGTG-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. 1A'). However, at higher doses few (1% 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. 1Bd') 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.
A DNA-binding mutant of Sox3 causes activation, rather than repression, 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 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 a luciferase reporter construct (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...
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-d') to almost complete independent axes (Fig. 2Ba-d,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 *sgr* (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 expression 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
Sox3 represses organizer formation

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 Sox3NLS, 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 Sox3NLS, 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 Sox3NLS (Fig. 4F,G). Thus, the ability of Sox3NLS 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 Sox3NLS overexpression. This is despite the fact that Sox3NLS 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 Sox3NLS) would be expected to reduce the level of β-catenin signalling below that seen when Sox3NLS 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 Sox3NLS alone (see Fig. S3B in the supplementary material).

Induction of the organizer by Sox3NLS 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 Sox3NLS on gsc and chd.

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.

Fig. 4. The effects of Sox3NLS require Wnt/β-catenin signalling. (A-G) Ectopic activation of boz, sqt, gsc and chd by Sox3NLS 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.
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.

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**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 un.injected 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.

**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. WT HMG-VP16 (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.
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 substantial amount of HA-tagged N40I DNA-binding mutant as additional negative control. 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. 8A-a). In addition, activation of β-catenin signalling, using the S37A mutant, repressed sox3 expression (Fig. 8A-d). 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. 8A-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 SoxB1a/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 soxB1a and soxB1b are expressed at the early stages when they might affect organizer formation (Okuda et al., 2006).
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 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).
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 Spemann’s organizer. These SoxB1 factors appear to 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 mesendoderm markers nil 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 stamois 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?

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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