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Snail2 controls mesodermal BMP/Wnt induction of neural crest

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SUMMARY

The neural crest is an induced tissue that is unique to vertebrates. In the clawed frog *Xenopus laevis*, neural crest induction depends on signals secreted from the prospective dorsolateral mesodermal zone during gastrulation. The transcription factors Snail2 (Slug), Snail1 and Twist1 are expressed in this region. It is known that Snail2 and Twist1 are required for both mesoderm formation and neural crest induction. Using targeted blastomere injection, morpholino-based loss of function and explant studies, we show that: (1) Snail1 is also required for mesoderm and neural crest formation; (2) loss of *snail1*, *snail2* or *twist1* function in the C2/C3 lineage of 32-cell embryos blocks mesoderm formation, but neural crest is lost only in the case of *snail2* loss of function; (3) *snail2* mutant loss of neural crest involves mesoderm-derived secreted factors and can be rescued synergistically by *bmp4* and *wnt8* RNAs; and (4) loss of *snail2* activity leads to changes in the RNA levels of a number of BMP and Wnt agonists and antagonists. Taken together, these results identify Snail2 as a key regulator of the signals involved in mesodermal induction of neural crest.

KEY WORDS: Snail2, Slug, Snail1, Twist1, Mesoderm, Neural crest induction, BMP, Wnt, *Xenopus*

INTRODUCTION

Neural crest is of interest for both evolutionary and medical reasons. Like the mesoderm, it is an induced tissue, arising at the boundary between the nascent neural plate and the embryonic epidermis (Alfandari et al., 2010; Basch and Bronner-Fraser, 2006; Klymkowsky et al., 2010; Minoux and Rijli, 2010). The neural crest represents an evolutionary innovation (Baker, 2008; Yu, 2010), responsible in part for the diverse cranial morphologies of vertebrates (Hanken and Gross, 2005). It provides a classic example of an epithelial-mesenchymal transition (EMT) and associated apoptotic suppression, and so serves as a model for cancer metastasis (Klymkowsky and Savagner, 2009). Neural crest is also the focus of tetratogenic birth defects in humans, such as those caused by thalidomide (McCredie, 2009) and ethanol (Webster and Ritchie, 1991).

Although there have been reports that mesoderm is not involved in the induction of neural crest in zebrafish (Ragland and Raible, 2004) and chick [(Basch et al., 2006), but see below], there is clear evidence for a role for early mesoderm in neural crest induction in amphibians in general [(Raven and Kloos, 1945), as cited by Baker and Bronner-Fraser (Baker and Bronner-Fraser, 1997)] and, more specifically, in *Xenopus laevis* (Bonstein et al., 1998; Hong et al., 2008; Marchant et al., 1998; Mayor et al., 1995; Monsoro-Burq et al., 2003; Steventon et al., 2009; Steventon et al., 2005). The situation is somewhat less clear in mouse and human, in part because of the challenges associated with experimental studies in the corresponding early embryonic stages (Aggarwal et al., 2010; Carver et al., 2001; Goh et al., 1997; O'Rourke and Tam, 2002; Xu et al., 2000).

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The transcription factors Snail and Twist were first identified in *Drosophila melanogaster*, where they sit at the end of the Toll/Dorsal (NF-κB) signaling pathway (Stathopoulos and Levine, 2002; Valanne et al., 2011). Mutations in *snail* and *twist* lead to defects in mesoderm formation (Leptin, 1991; Thisse et al., 1987). *snail*-type genes encode C_2H_2-type zinc-finger transcription factors. Multiple members of this gene family have been characterized from placozoans through humans (Barrallo-Gimeno and Nieto, 2009). *twist* encodes a basic helix-loop-helix (bHLH)-type transcription factor; these proteins typically act as dimers (Barnes and Firulli, 2009). Both *snail*- and *twist*-like genes are found in the primitive chordate *Ciona intestinalis* (Shi et al., 2005) and in the jellyfish *Podocoryne carnea* (Spring et al., 2002; Spring et al., 2000). A BLAST analysis indicates that both the *Ciona* and *Podocoryne* Snail proteins more closely resemble mammalian Snail2 (Slug) than Snail1 proteins (our unpublished observation). Snail proteins appear to act primarily as transcriptional repressors, binding to DNA E-box (5′-CANNTG-3′) sequences. During *Drosophila* mesoderm specification and patterning, *snail* and *twist* expression are regulated by a molecular cascade involving *dorsal*, which encodes an ortholog of the NF-κB subunit protein RelA (Huguet et al., 1997; Ip et al., 1992). This network involves negative-feedback regulation of *dorsal* through the secreted factor WntD, the expression of which is regulated by Snail and Twist (Ganguly et al., 2005; Gordon et al., 2005). Genomic chromatin immunoprecipitation-microarray studies (Sandmann et al., 2007; Zeitlinger et al., 2007) suggest that Snail and Twist regulate a wide array of target genes: Twist targets almost 25% of all annotated *Drosophila* transcription factors (Sandmann et al., 2007). Interestingly, in the vertebrate *X. laevis*, *snail1*, *snail2* and *twist1* RNAs appear to be 'immediate-early' targets of regulation by the NF-κB subunit protein RelA (Zhang et al., 2006).

In vertebrates, there are two distinct *twist*-like genes: *twist1* and *twist2* (*dermo1*) (Li et al., 1995). *twist1* has been implicated in mesoderm formation, as well as in a number of developmental events. *twist1* haploinsufficiency leads to skeletal dysplasia (Miraoui and Marie, 2010). In the mouse, *Twist1* is required for cranial neural crest migration as well as for the suppression of

apoptosis (Chen and Behringer, 1995; Soo et al., 2002). In humans, mutations in *TWIST* have been implicated in mesenchymal stem cell differentiation and skeletal malformations (craniosynostosis) (Miraoui and Marie, 2010). There are two closely related *snail*-like genes in vertebrates, *snail1* and *snail2* (previously known as *slug*), as well as a number of more distantly related genes (Barrallo-Gimeno and Nieto, 2009; Manzanares et al., 2001; Nieto, 2002). In vertebrates, *snail* gene function was originally studied most intensely in the context of the neural crest (Aybar et al., 2003; Carl et al., 1999; LaBonne and Bronner-Fraser, 2000; Nieto et al., 1994; O'Rourke and Tam, 2002; Tribulo et al., 2004). In the chick, *Snail2* is expressed in both mesoderm and premigratory crest, and appears to be involved in the formation and behavior of both tissues (Nieto et al., 1994). In the mouse, the domains of *Snail2* and *Snail1* expression are switched (Locascio et al., 2002; Sefton et al., 1998) and neither *Snail1* nor *Snail2* appears to be absolutely necessary for either mesodermal or neural crest formation (Carver et al., 2001; Jiang et al., 1998). That said, *Snail1* null mice display a recessive embryonic lethal phenotype with clear gastrulation defects and morphologically abnormal mesoderm (Carver et al., 2001). Whether the roles of *Snail1* and *Snail2* in the early mouse embryo have been subsumed by other genes, such as *Twist1*, *Zeb1* or *Zeb2* (*Sip1*), which encodes an E-box-binding protein implicated in the regulation of EMT and tumor progression (Bracken et al., 2008; Liu et al., 2009; Schmalhofer et al., 2009; Wellner et al., 2009), remains unclear. That complex interactions may be involved is suggested by a report that *Snail2* is required for Twist1-induced EMT in mice (Casas et al., 2011). In the mouse, *Snail2* acts downstream of *Sox9* in trunk neural crest specification (Cheung et al., 2005). *Snail2*$^{-/-}$ mice display white forehead blaze, patchy depigmentation of the ventral body, tail and feet, macrocytic anemia, infertility and a deficiency in white adipose tissue mass (Perez-Mancera et al., 2007). The combination of a conditional *Snail1* null mutation and *Snail2*$^{-/-}$ leads to defects in left-right axis formation but not, apparently, to defects in neural crest formation (Murray and Gridley, 2006).

In *X. laevis*, *snail1*, *snail2* and *twist1* are expressed in the blastula stage embryo (Essex et al., 1993; Mayor et al., 1993; Mayor et al., 2000; Sargent and Bennett, 1990; Zhang and Klymkowsky, 2009). Previously, we presented evidence for a role for *snail2* and *twist1* in mesoderm and neural crest formation (Carl et al., 1999; Zhang et al., 2006; Zhang and Klymkowsky, 2009). The expression of *snail2*, *snail1* and *twist1* in both early mesoderm and neural crest raises a number of issues, illustrated in part by the work of Aybar et al. (Aybar et al., 2003). Based on the behavior of dominant-negative mutant forms of Snail2 and Snail1, they claimed that *snail* (*snail1*) precedes *slug* (*snail2*) in the genetic cascade involved in neural crest specification. Yet morpholino-based studies indicate that inhibition of *snail2* expression disrupts mesoderm formation and leads to a decrease in *snail1* RNA levels in the early embryo (Zhang et al., 2006; Zhang and Klymkowsky, 2009). To resolve these issues, we extended previous work using morpholinos to examine the role of *snail1*, *snail2* and *twist1* in the early *Xenopus* embryo. Blastomere injection and explant studies enabled us to discover distinct roles for *snail2*, as compared with *snail1* and *twist1*, in mesoderm-based induction of neural crest.

MATERIALS AND METHODS

Embryos and their manipulation

X. laevis embryos were staged, and explants and co-explants were generated following standard procedures (Klymkowsky and Hanken, 1991; Nieuwkoop and Faber, 1967; Sive et al., 2000; Zhang et al., 2004). Similar studies were carried out using *X. tropicalis* embryos using animals purchased from Xenopus I following methods analogous to those used in *X. laevis*, and modified as described in Khokha et al. (Khokha et al., 2002). Capped mRNAs were transcribed from linearized plasmid templates using mMessage mMachine kits (Ambion) following the manufacturer's instructions. For 2-cell stage studies, embryos were injected equatorially; for 32-cell stage studies, C2 and C3 blastomeres were co-injected with RNA encoding GFP, and examined at stage 10 to confirm the accuracy of injection. Animal caps were isolated from stage 8-9 blastula embryos in 0.5 MMR (Sive et al., 2000), transferred into wells of a 2% agarose plate (one animal cap per well) and combined with dorsolateral mesodermal zone isolated from stage 10.5 gastrula embryos according to Bonstein et al. (Bonstein et al., 1998). Explant recombinants were harvested when siblings reached stage 18. Images were captured using a Nikon CoolPix 995 camera on a Leica M400 photomicroscope or using a Nikon D5000 camera on a Wild stereomicroscope. Images were manipulated with Fireworks CS4 software (Adobe) using Auto Levels and Curves functions only.

Morpholinos and plasmids

Modified morpholino antisense oligonucleotides (MOs) were purchased from Gene Tools. The MO against *snail1* (5′-TTTAGCAGCCGAGCACTGAGTTCCT-3′) was tested for its ability to block the translation of *snail1* RNA using an RNA that contains its target site and encodes a Snail1-GFP chimera. Other MOs used were designed to block the translation of *antipodean/vegT* (5′-ACTTTACATCCGGCAGAGAATGCAT-3′) and *xbra* (5′-GCGCAGCTCTCGGTCGCACTCATTA-3′) RNAs. *snail2* and *twist1* MOs were as described previously (Zhang et al., 2006; Zhang and Klymkowsky, 2009). In addition, a new *snail2* MO was designed (5-TCTTGACCAGAAAAGATCGTGGCAT-3′) that matches the single identified *X. tropicalis snail2* gene perfectly (Vallin et al., 2001).

Plasmids encoding ΔNp63 and $\Delta Np63_{R304W}$ (Barton et al., 2009) were generously supplied by Ethan Lee. Plasmids encoding Sizzled (Salic et al., 1997), Cerberus (Bouwmeester et al., 1996), BMP4, Wnt8, Noggin (Smith and Harland, 1992) and Dickkopf (Dkk) (Glinka et al., 1998) were supplied by Eddy DeRobertis and Richard Harland. Snail2, Snail1 and Twist expression plasmids have been described previously (Zhang et al., 2006; Zhang and Klymkowsky, 2009); a GFP-tagged form of Snail1 was constructed for this project. RNA-injected embryos were selected based on GFP fluorescence.

Immunoblot and in situ hybridization studies

For in situ hybridization studies, digoxigenin-UTP-labeled antisense probes were prepared following standard methods; specific probes for *sox9*, *vegT/antipodean*, *chordin*, *endodermin*, *myoD*, *chd7* and *xbra* RNAs were used. In many cases, embryos were co-injected with RNA (50 pg/embryo) encoding β-galactosidase, the activity of which was visualized in fixed embryos using a brief Red-Gal (Research Organics) reaction in order to identify successfully injected embryos.

RT-PCR and quantitative RT-PCR (qPCR)

RNA isolation, RT-PCR and qPCR analyses were carried out as described previously (Zhang et al., 2003; Zhang et al., 2006). In brief, total RNA was isolated from embryos or dorsal lateral mesoderm regions; cDNA synthesis was performed from 1 μg purified RNA using a Verso cDNA kit (Thermo Scientific) according to the manufacturer's directions. Real-time PCR was carried out using a Mastercycler Epgradient Realplex device (Eppendorf). PCR reactions were set up using DyNAmo SYBR Green qPCR kits (Finnzymes). Each sample was normalized to the expression level of ornithine decarboxylase (*ODC*). The cycling conditions used were: 95°C for 5 minutes; then 40 cycles of 95°C for 15 seconds, 56°C for 15 seconds, 60°C for 30 seconds. The ΔΔCT method was used to calculate real-time PCR results. Primers for RT-PCR analyses were (5′ to 3′, forward and reverse):

snail2, GATGCACATCAGGACACACAC and CTGCGAATGCTCTGTTGCAGT;

snail1, AAGCACAATGGACTCCTT and CCAATAGTGATACACACC;

twist1, AGTCCGATCTCAGTGAAGGGC and TGTGTGTGGCCTGAGCTGTAG;

ODC, CAGCTAGCTGTGGTGTGG and CAACATGGAAACTCACAC;

sox9, TGCAATTTTCAAGGCCCTAC and GCTGCCTACCATTC-TCTTGC;
sizzled, CATGTCCGGAGTCTTCCTGC and GGATGAACGTGTCCA-GGCAG;
cerberus, CCTTGCCCTTCACTCAG and TGGCAGACAGTCCTTT;
wnt8, TGATGCCTTCACTTCTGTGG and TCCTGCAGCTTCTT-CTCTCC;
bmp4, TGGTGGATTAGTCTCGTGTCC and TCAACCTCAGCAG-CATTCC;
dkk, ACGGAAGATGATGACTGTGC and CTCTTGATCTTGCTC-CACAGG;
noggin, AGTTGCAGATGTGGCTCT and AGTCCAAGAGTCTCA-GCA;
frzb1, TGGACTCATTCCTGCTACTGG and AATTGCCAGGATAG-CATTGG;
sfrp2, TCTGTGTGAGCAGGTGAAGG and GTCATTGTCATCCTCG-TTGC;
crescent, GGCTCTCTGCTATGACATTGG and CACACAGACTGCGA-CATGG;
chordin, CCTCCAATCCAAGACTCCAGCAG and GGAGGAGGAG-GAGCTTTGGGACAAG;
fgf8, TGGTGACCGACCAACTAAGC and ACGATTAACTTGGCGT-GTGG;
eomes, TCCTCAGGCTACCAGTACAGC and GCCGGTTACAGAG-GAAGACC;
gata4a, CTGGACTGCTTCTCCATTCG and ACATTGCTCCACAG-TTGACG; and
tbx6, CAGCCAATCAGGAACAAGG and GTTCTGTGCTGCATCT-GTGG.

RESULTS

snail1 is required for both mesoderm and neural crest formation

It is clear that *snail1* RNA is present in *X. laevis* embryos following the onset of zygotic transcription (Mayor et al., 2000; Sargent and Bennett, 1990; Zhang and Klymkowsky, 2009). In past studies, it was reported that injection of *snail1* RNA could partially rescue the effects of *snail2* antisense RNA and *snail2* and *twist1* morphant phenotypes (Carl et al., 1999; Zhang et al., 2006; Zhang and Klymkowsky, 2009), as well as the effects of dominant-negative versions of Snail2 (LaBonne and Bronner-Fraser, 2000). Levels of *snail1* RNA are reduced in *snail2* and *twist1* morphant embryos (Zhang and Klymkowsky, 2009). To determine whether *snail1* expression is involved in early mesoderm formation and/or maintenance, which does not appear to have been examined previously, we designed a morpholino (MO) directed against the 5′ UTR of the single identified *snail1* mRNA in *X. laevis* (see Fig. S1 in the supplementary material). The previously employed *snail2* MO (Zhang et al., 2006) has 12 out of 25 mismatches to the analogous region of the *snail1* mRNA, whereas the *snail1* MO has 17 and 18 out of 25 mismatches to the *snail2a* and *snail2b* mRNAs, respectively, as identified by Vallin et al. (Vallin et al., 2001). As we have yet to identify a functional antibody for *Xenopus* Snail1 (or Snail2 or Twist), we examined the effects of *snail1* MO on the translation of an RNA, UTR-Snail1-GFP, that contains the MO 5′ UTR target sequence. When UTR-Snail1-GFP RNA and *snail1* MO were co-injected into fertilized eggs, there was a reduction in the level of Snail1-GFP protein accumulation (Fig. 1A) and a reduction in the level of Snail1-GFP fluorescence (Fig. 1B,C). RT-PCR (Fig. 1D) and quantitative RT-PCR (qPCR) (Fig. 1E) analyses indicated that injection of *snail1* MO into both blastomeres of a 2-cell embryo led to a reduction in the amounts of both *twist1* and *snail2* RNA.

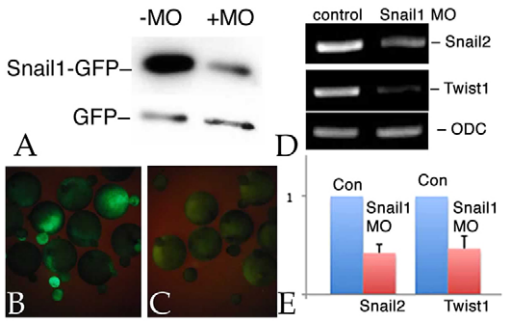

Fig. 1. *snail1* MO effects. *Xenopus* embryos were injected with RNAs encoding myc-tagged GFP (mt-GFP; 50 pg/embryo) and UTR-Snail1-GFP (which latter includes the target of the *snail1* MO) RNAs (600 pg/embryo), either alone or together with the *snail1* MO (7 ng/embryo). (**A**) Immunoblot analysis of stage 11 embryos using an anti-GFP antibody revealed a clear and specific reduction in the accumulation of Snail1-GFP as compared with GFP in the *snail1* MO-injected sample. (**B**,**C**) UTR-Snail1-GFP RNA was injected into one cell of 2-cell embryos either alone (B) or together with the *snail1* MO (C). At stage 11, the *snail1* MO greatly reduced UTR-Snail1-GFP fluorescence. (**D**,**E**) RT-PCR (D) and qPCR (E) analyses indicate that the injection of the *snail1* MO into both cells of 2-cell embryos led to a specific reduction in the levels of *twist1* and *snail2* RNAs at stage 11. Ornithine decarboxylase (*ODC*) RNA was used to control for non-specific effects. Error bars indicate s.d.

Injection of *snail2* antisense RNA, or *snail2* or *twist1* MOs, into one cell of 2-cell embryos leads to the loss of the mesodermal markers *xbra*, *antipodean/vegT* and *myoD*, an increase in the expression domain of the endodermal marker *endodermin* (*edd*), and the loss of expression of the neural crest marker *sox9* (Carl et al., 1999; Zhang et al., 2006; Zhang and Klymkowsky, 2009). Injection of the *snail1* MO produced similar effects: loss of *xbra* (Fig. 2A,A′) and *antipodean/vegT* (data not shown) expression, an increase in *edd* expression (Fig. 2B,B′) and expansion of the *edd* expression domain into the underlying mesoderm of stage 11/12 embryos (Fig. 2C,C′), and the loss of expression of *sox9* (Fig. 2D,E) and *myoD* (Fig. 2F,F′) in later stage embryos. Levels of other neural crest markers, specifically *snail2*, *twist* and *chd7* (Bajpai et al., 2010), were reduced in *snail1* morphant embryos (see Fig. S2 in the supplementary material). A quantitative analysis of the effects of *snail1* MO are presented in Table 1. As noted in past studies, a number of morphant embryos failed to gastrulate, suggesting that later phenotypes are hypomorphic rather than null (amorphic) (see Zhang et al., 2006; Zhang and Klymkowsky, 2009). The effects of the *snail1* MO were rescued by the injection of an RNA encoding Snail1-GFP (Fig. 2G-J), an RNA that does not contain the target sequence of the *snail1* MO. As in the case of *snail2* and *twist1* morphant embryos, the *snail1* MO phenotype was rescued, albeit not as efficiently, by *snail2* and *twist1* RNAs (Fig. 2K). The extent to which *snail2*, *snail1* and *twist1* RNAs rescue the expression of ‘late’ genes, such as *sox9* and *myoD*, was lower than their ability to rescue the expression of the ‘earlier’ genes *xbra* and *edd*, perhaps because later ‘differentiation’ genes are more redundantly regulated.

Loss of mesoderm leads to loss of neural crest

To confirm that loss of mesoderm per se leads to the failure of neural crest induction, as described previously (Bonstein et al., 1998), we disrupted mesoderm formation in two complementary ways. *xbra*

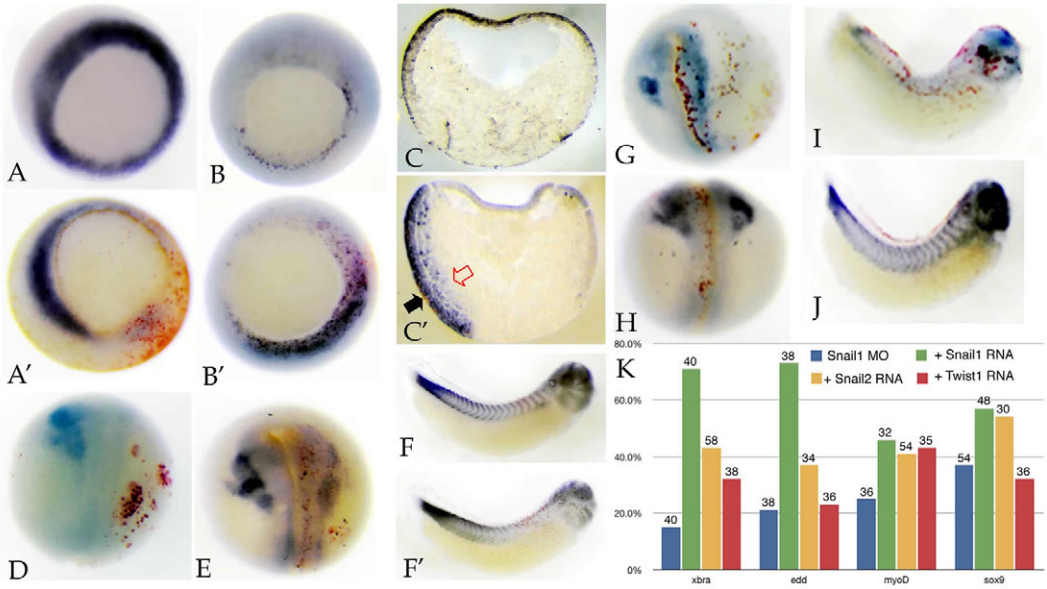

Fig. 2. *snail1* MO effects on germ layer markers. (**A-B′**) *snail1* MO injection (into one cell of a 2-cell embryo) led to the loss of expression (as measured by in situ hybridization) of *xbra* (A′, versus control in A) and to an increase in *endodermin* (*edd*) (B′, versus control in B) RNA levels (at stage 11). (**C**,**C′**) Section analysis revealed that the *edd* expression domain, which is restricted to the superficial region in control embryos (C), extends deeper into the mesodermal region in *snail1* MO-injected embryos (C′, black arrow indicates *lacZ* marker staining, and the red arrow indicates the extent of *edd* staining in deep mesodermal tissue). (**D-F′**) There was a loss of expression of the neural crest/placodal marker *sox9* at stage 17/18 (D, severely affected; E, mildly affected; injected sides to the right), as well as a loss of expression of *myoD* at stage 25, as expressed in myotomal muscle, a mesodermal derivative (F′, versus control in F). (**G-J**) The effects on *sox9* (G,H) and *myoD* (I,J) of *snail1* MO injection (G,I) were rescued by the injection of the Snail1-GFP RNA (H,J), which lacks the *snail1* MO target sequence. Injected sides are shown. (**K**) The percentage of normal embryos plotted with respect to *xbra*, *edd*, *myoD* and *sox9* in situ expression. Blue, *snail1* MO injected; green, *snail1* MO together with *snail1* RNA (600 pg/embryo); yellow, *snail1* MO together with Snail2-GFP RNA (600 pg/embryo); red, *snail1* MO together with Twist1-GFP RNA (600 pg/embryo). The number of embryos examined is indicated above each bar.

(Smith et al., 1991) and *vegT/antipodean* (Stennard et al., 1996; Stennard et al., 1999) encode T-box-type transcription factors involved in mesoderm formation in *X. laevis*. Injection of an *xbra* MO (10 ng/embryo) into one cell of a 2-cell embryo had little effect on the expression of the myotomal marker *myoD*, whereas injection of an *antipodean/vegT* MO led to the loss of *myoD* expression, as described previously (Fukuda et al., 2010) (see Fig. S3 in the supplementary material); neither had any apparent effect on *sox9* expression (data not shown). By contrast, when injected together (5 ng each/embryo), the *xbra* and *antipodean/vegT* MOs caused the loss of *sox9* expression in ~80% of embryos (Fig. 3A-C; Table 2).

That the *antipodean/vegT* MO alone had no effect on *sox9* expression suggested possible compensatory processes. Mesoderm formation involves multiple pathways that act in independent, interdependent and cooperative ways (Koide et al., 2005; Loose and Patient, 2004). A second mesoderm pathway active in *X. laevis* involves the transcription factor p53 and its modulation of TGFβ signaling through interactions with SMAD proteins (Cordenonsi et al., 2003; Cordenonsi et al., 2007; Sasai et al., 2008; Takebayashi-Suzuki et al., 2003). Barton et al. (Barton et al., 2009) described a splice variant of the p53-related protein p63, ΔNp63, that inhibits the p53-SMAD interaction and blocks mesoderm formation in *X. laevis*. Injection of ΔNp63 RNA (~600 pg/embryo) into one cell of a 2-cell embryo led to loss of the neural crest marker *sox9* and of the mesoderm/muscle marker *myoD* in ~50% of embryos (Fig. 3D-G; Table 2). Injection of ΔNp63 RNA into both cells of a 2-cell embryo led to a reduction in *xbra* and *vegT* RNAs, as determined by qPCR, as well as to the reduction of another mesoderm marker, *myf5* (Fig. 3J) (Hopwood et al., 1991). Injection of RNA encoding $\Delta Np63_{R304W}$, a mutated form of ΔNp63 that no longer binds to DNA (Barton et al., 2009), had no effect on *sox9* expression (Fig. 3H,I). When injected together, *xbra* and *antipodean/vegT* MOs and ΔNp63 RNA led to the near complete loss of *sox9* expression (Fig. 3C; Table 2), suggesting that the two pathways (T-box and p53/SMAD) cooperate in terms of mesodermal induction of neural crest (Table 2).

qPCR studies of *xbra* and *antipodean/vegT* morphant (Fig. 3K) and ΔNp63 RNA-injected (Fig. 3L) embryos (both cells of 2-cell embryos injected) revealed decreased levels of *snail1*, *snail2* and *twist1* RNAs. Given that loss of *snail1*, *snail2* or *twist1* expression inhibits mesoderm formation and neural crest induction (see above), we examined whether *xbra* and *antipodean/vegT* MO-induced loss of *sox9* expression could be rescued by the injection

Table 1. Quantitative analysis of the effects of *snail1* MO

		Phenotype (%)			
In situ analysis	Total	Normal	Mild	Moderate	Severe
xbra (stage 11)	44	18	14	32	36
edd (stage 11)	37	20	12	46	22
sox9 (stage 17/18)	62	35	16	21	28
snail2 (stage 17/18)	32	38	0	0	62
twist (stage 17/18)	31	19	10	32	39
chd7 (stage 17/18)	32	25	0	25	50
myoD (stage 25)	32	25	25	28	22

snail1 MO (7 ng/embryo) was injected into one cell of 2-cell embryos and embryos analyzed by in situ hybridization at the indicated stages. The total number of embryos examined is shown for each.

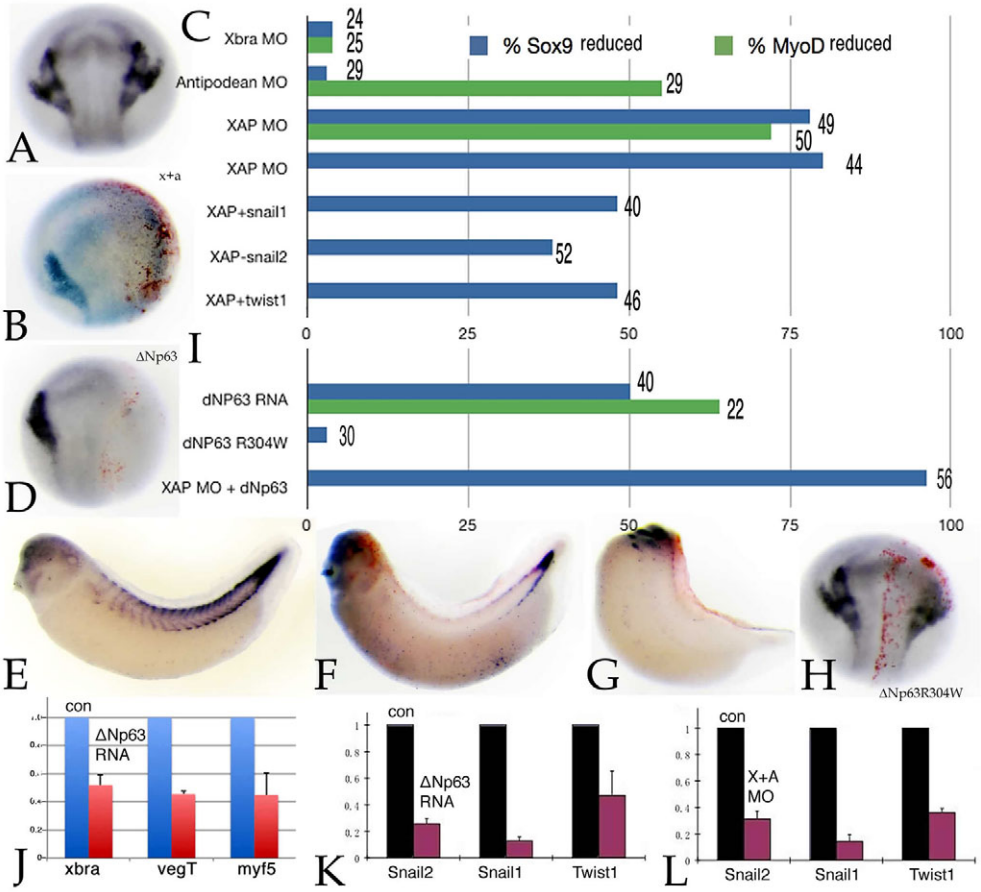

Fig. 3. Loss of mesoderm leads to loss of neural crest. (**A**,**B**) Injection of the *xbra* MO has little effect on *myoD* (stage 25) or *sox9* (stage 17/18) expression. Injection of the *antipodean/vegT* MO causes a decrease in *myoD* expression (Fukuda et al., 2010), but little effect on *sox9* expression (data not shown). Together (XAP), the *xbra* and *antipodean/vegT* MOs block *sox9* (stage 17/18) (B, versus control in A) and *myoD* (data not shown) expression. (**C**) The percentage of embryos with loss of *myoD* or *sox9* staining, with the number of embryos examined shown at the end of each bar. Injection of *snail1*, *snail2* or *twist1* RNAs (600 pg/embryo) produced a modest rescue of *sox9* expression in XAP morphant embryos. (**D-G**) Injection of 600 pg ΔNp63 RNA into one cell of a 2-cell embryo led to the loss of *sox9* (D), *xbra* (not shown) and *myoD* (F, moderate effect; G, severe effect; versus control in E) expression in ~50% of embryos. (**H**) Injection of RNA encoding the mutated and inactive $\Delta Np63_{R304W}$ protein had no effect on *sox9* or *myoD* (data not shown) expression. (**I**) Quantitative presentation of the data shown in D-H. (**J-L**) qPCR analyses of ΔNp63 RNA-injected embryos (both cells of 2-cell embryos injected, analyzed at stage 11) revealed a substantial decrease in the levels of the *xbra*, *vegT/antipodean* and *myf5* mesodermal marker RNAs (J). In both ΔNp63 RNA (K) and XAP MO (L) injected embryos, there were similar decreases in the levels of *snail2*, *snail1* and *twist1* RNAs. Error bars indicate s.d.

of *snail2*, *snail1* or *twist1* RNAs. Such studies produced a modest rescue of *sox9* expression (Fig. 3C). The loss of *sox9* expression in the *xbra* and *antipodean/vegT* morphant and ΔNp63 RNA-injected embryos supports an active role of the mesoderm in neural crest induction in *Xenopus* (Bonstein et al., 1998).

Snail2 is a key regulator of dorsolateral mesoderm-dependent neural crest induction

A limitation of whole- and half-embryo studies is that the injected reagents often influence a range of developing tissues. These studies are also complicated by the fact that the rates of diffusion of different

Table 2. Effects of *xbra* and *antipodean/vegT* (*anti*) MOs and ΔNp63 RNA on mesodermal and neural crest marker expression

	myoD					*sox9*				
		Phenotype (%)					Phenotype (%)			
Treatment*	Total	Normal	Mild	Moderate	Severe	Total	Normal	Mild	Moderate	Severe
xbra MO (10 ng)	24	96	0	4	0	24	96	4	0	0
anti MO (10 ng)	29	45	40	15	0	29	97	0	3	0
xbra MO + *anti* MO (5 ng each)	50	28	24	33	15	49	22	19	38	21
ΔNp63 RNA (600 pg)	25	20	24	24	32	40	50	13	27	10
xbra MO + *anti* MO + ΔNp63 RNA (5 ng each + 600 pg)	36	0	0	50	50	56	4	7	30	59

Phenotypes were analyzed at stage 25 for *myoD* and at stage 17/18 for *sox9*. The total number of embryos examined is indicated for each.
*Values are per embryo.

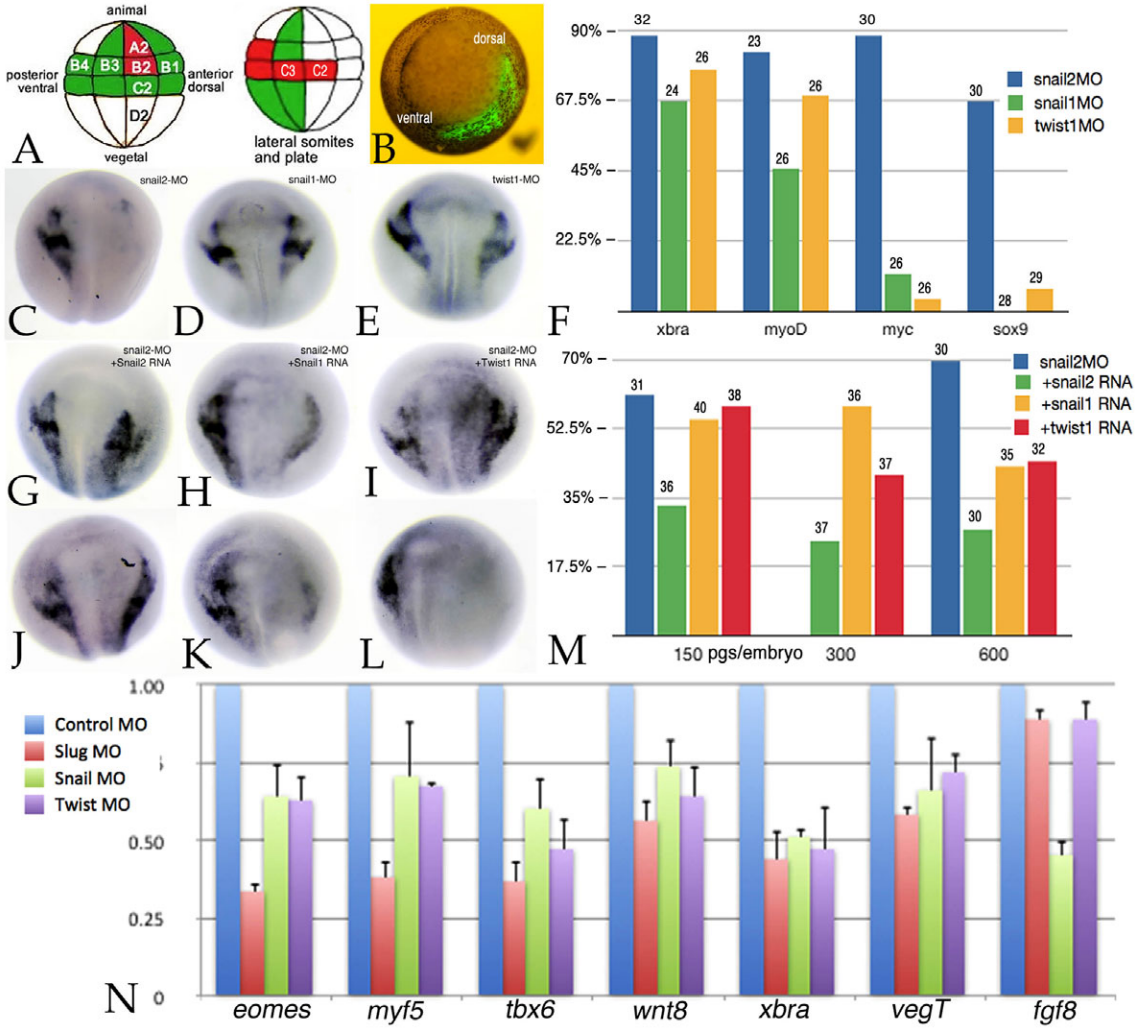

Fig. 4. Target blastomere injection effects. (**A**) Fate map of the 32-cell *X. laevis* embryo, lateral to front, with blastomeres marked with respect to tiers (A-D) and position (1, dorsal; 4, ventral). Blastomeres are marked that make a major (red) or moderate (green) contribution to neural crest (left) or to lateral somites and plate mesoderm (right). (**B**) At the 32-cell stage, this embryo was injected with RNA encoding GFP; the embryo was photographed under bright-field and epifluorescence illumination at stage 11. Ventral pole to front (dorsal and ventral are marked). (**C-E**) C2/C3 blastomeres were injected with *snail2*, *snail1* or *twist1* MOs; at stage 17/18 the embryos were fixed and stained in situ for *sox9* RNA. *sox9* expression was lost in *snail2* morphant embryos (C), but was present in *snail1* (D) and *twist1* (E) morphants. (**F**) The phenotypes of C2/C3 morphant embryos with respect to *xbra*, *myoD*, *c-myc* and *sox9* expression. Bars indicate percentage loss of expression; the number of embryos is indicated above each bar. (**G-I**) The effects of the *snail2* MO could be partially rescued by injection of high levels (600 pg/embryo) of *snail2* (G), *snail1* (H) or *twist1*(I) RNAs. (**J-L**) *snail2* RNA was more effective at rescuing the *snail2* C2/C3 morphant *sox9* phenotype than either *snail1* or *twist1* RNAs. *snail2* C2/C3 morphant embryos were co-injected with 150 pg/embryo of *snail2* (J), *snail1* (K) or *twist1* (L) RNA. (**M**) The rescue of the C2/C3 *snail2* morphant *sox9* phenotype by 150, 300 and 600 pg/embryo *snail2*, *snail1* and *twist1* RNAs. Bars indicate percentage loss of expression; the number of embryos is indicated above each bar. (**N**) The DLMZ of C2/C3 MO-injected embryos was dissected at stage 10.5 and subject to qPCR analysis. The effects on the mesodermal markers *xbra*, *eomes*, *tbx6*, *myf5*, as well as on *wnt8* and *fgf8* RNA levels were analyzed. The results shown are representative of studies carried out in triplicate. Error bars indicate s.d.

reagents (e.g. antibodies, MOs and RNAs) within the embryo differ (Zhang et al., 2004) (our unpublished observations). To circumvent this effect, we used two complementary methods: targeted blastomere injection and combinatorial (sandwich) explant studies. Although it is certainly the case that there is no blastomere within the 32-cell embryo that contributes to a single tissue type or lineage, owing in part to the slow intermixing of cells (Dale and Slack, 1987; Moody, 1987; Nakamura et al., 1978; Wetts and Fraser, 1989), it is possible to favor certain tissues. For example, in the 32-cell embryo, the C2 and C3 blastomeres give rise primarily to paraxial mesoderm and make only moderate contributions to the neural crest. In our studies, we injected the C2/C3 blastomeres (Fig. 4A) with MOs against *snail1*, *snail2* or *twist1* RNAs together with RNA encoding GFP as a lineage tracer. Embryos were sorted at stage 10/10.5 (Fig. 4B) to select those accurately injected. When fixed at stage 18 and stained in situ, we found that *sox9* expression was dramatically reduced in *snail2* morphant embryos, but not in *snail1* or *twist1* morphant embryos (Fig. 4C-F). Subsequent studies indicated that whereas all three MOs led to a reduction in the levels of the mesodermal markers *xbra* (stage 11) and *myoD* (stage 25), only the *snail2* MO produced a decrease in the neural border/early neural crest marker *c-myc* (stage 16) (Bellmeyer et al., 2003) and in the later

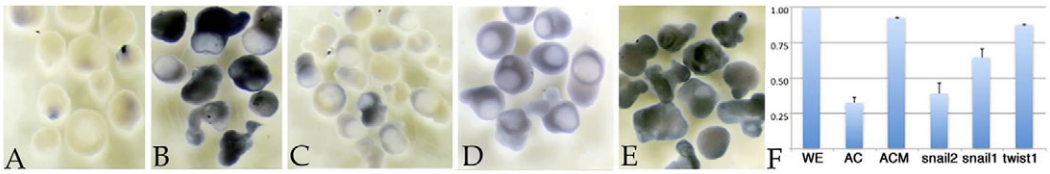

Fig. 5. Dorsal mesoderm-animal cap explant studies. (**A**) When cultured alone, wild-type ectoderm (animal cap) contained little *sox9* RNA, as visualized by in situ hybridization (analysis carried out when control embryos had reached stage 17/18). (**B**) When cultured together with DLMZ from wild-type *Xenopus* embryos, *sox9* RNA accumulated (in the ectodermal region). (**C**) By contrast, DLMZ from *snail2* C2/C3 morphant embryos failed to induce *sox9* RNA. (**D**,**E**) *snail1* morphant DLMZ appears to be intermediate in its ability to induce *sox9* expression (D), whereas *twist1* morphant DLMZ retained the ability of induce *sox9* expression (E). (**F**) qPCR analysis of *sox9* RNA levels supports this general trend. For each condition, 5-15 explants were analyzed when unmanipulated embryos had reached stage 17/18. *ODC* RNA was used as a standard. WE, whole embryo at stage 17/18 (level set to 1); AC, animal cap/ectodermal explant; ACM, animal cap plus DLMZ from uninjected control embryo; snail2, wild-type animal cap plus *snail2* morphant DLMZ; snail1, wild-type animal cap plus *snail1* morphant DLMZ; twist1, wild-type animal cap plus *twist1* morphant DLMZ. The results shown are representative of more than three replications. Error bars indicate s.d.

neural crest marker *sox9* (stage 18) (Lee et al., 2004; Spokony et al., 2002) (Fig. 4F). Preliminary studies in *X. tropicalis* indicate that the same pattern holds true there: C2/C3 *snail2*, but not *snail1* or *twist1*, morphant embryos show a loss of *sox9* expression (see Fig. S4 in the supplementary material).

The effects of C2/C3 *snail2* MO injection on *sox9* expression could be partially rescued by injection of high levels (600 pg/embryo) of *snail2*, *snail1* or *twist1* RNAs (Fig. 4G-I). RNA titration studies indicated that *snail2* RNA was more effective at every dose (150 to 600 pg/embryo) than *snail1* or *twist1* RNAs at rescuing the *snail2* morphant phenotype (Fig. 4J-M).

At stage 11, the dorsolateral mesodermal zone (DLMZ) of C2/C3 injected morphant embryos was dissected and analyzed by quantitative RT-PCR (Fig. 4N). The levels of RNA encoding the mesodermal markers *eomes*, *myf5* and *tbx6* (Hug et al., 1997; Li et al., 2006; Lou et al., 2006; Ryan et al., 1996) were preferentially reduced in *snail2* morphant DLMZ. Interestingly, *fgf8* RNA levels (Fletcher et al., 2006; Fletcher and Harland, 2008; Monsoro-Burq et al., 2003) appeared to be selectively regulated by Snail1. To resolve this and other issues, we have begun RNA SEQ analysis of morphant *X. tropicalis* embryos.

Because C2/C3 blastomeres give rise not only to mesoderm but also to other tissues, including neural crest, we examined inductive interactions between ectoderm (animal cap) and DLMZ explants. On its own, the wild-type animal cap remains as atypical ectoderm, expressing only low levels of the neural crest marker *sox9* compared with whole embryos analyzed at the same stage (Fig. 5A,F). As demonstrated previously, DLMZ induces a number of markers of neural crest, including *snail2*, *foxD3*, *zic5* and *sox9* (Bonstein et al., 1998; Monsoro-Burq et al., 2003). Fig. 5B,F illustrates the effect of wild-type DLMZ on *sox9* expression in explants. *snail2* morphant DLMZ induced a much reduced level of *sox9* expression (Fig. 5C,F), whereas *twist1* morphant DLMZ behaved very much like wild-type DLMZ (Fig. 5E,F). *snail1* morphant DLMZ explants produced a reduced, but readily detectable, level of *sox9* expression (Fig. 5D,F).

Previous studies have implicated FGF, Wnt and BMP signaling in mesodermal induction of the neural crest in *X. laevis* (Monsoro-Burq et al., 2003; Monsoro-Burq et al., 2005; Steventon et al., 2009). We examined this situation in the context of C2/C3 *snail2* morphant embryos. Injection of RNAs (25 pg/embryo) encoding either BMP4 or Wnt8 were able to substantially rescue *sox9* expression (Fig. 6A-D,F). When *wnt8* and *bmp4* RNAs were injected together, there was an improved (although not dramatic) rescue response (Fig. 6E,F). Injection of *fgf8* RNA (25 pg/embryo) alone had little effect on the *sox9* expression phenotype (Fig. 6F), and was not studied further. At lower RNA levels (10 pg/embryo), neither *bmp4* nor *wnt8* RNAs rescued the *snail2* C2/C3 morphant *sox9* phenotype, but together they produced a strong rescue (Fig. 6G). Levels of *chordin* RNA, which encodes a secreted BMP signaling inhibitor (Sasai et al., 1995; Sasai et al., 1994), were upregulated in *snail2* C2/C3 morphant embryos (Fig. 6H,I). qPCR analyses of C2/C3 injected, and subsequently dissected, DLMZ indicate that levels of *wnt8*, *bmp4* and *frzb1* RNAs were decreased, whereas levels of *cerberus*, *sizzled* and *chordin* RNAs were increased in *snail2* morphant tissue; a distinctly different pattern of changes in RNA levels was observed in *snail1* and *twist1* morphant tissues (Fig. 6J).

DISCUSSION

The role of mesoderm in neural crest induction in *X. laevis* is well established (Bonstein et al., 1998; Green et al., 1997; Mayor et al., 2000; Monsoro-Burq et al., 2003; Steventon et al., 2009; Zhang et al., 2006; Zhang and Klymkowsky, 2009). Besides demonstrating that *snail1* is involved in mesoderm formation/maintenance and neural crest induction, here we report that Snail2 plays a distinct role in the DLMZ of the late blastula/early gastrula stage embryo. Loss of Snail2 in this embryonic region led to the loss of expression of the early neural border/neural crest marker *c-myc* and of the late neural crest marker *sox9*. That the effect on neural crest is mediated by inductive signals is supported by 'sandwich'-type explant studies between wild-type ectoderm and morphant DLMZ. Rescue studies suggest that both BMP4 and Wnt8 are essential components of this inductive signal; although this appears to partially contradict the conclusions of Steventon et al. (Steventon et al., 2009), it is likely that the details of the different experimental scenarios influence the behavior of the embryo (for example, differences in the concentrations and potency of the RNAs used, which can be influenced by the efficiency of the in vitro capping reaction.) Our conclusion is bolstered by the observation that the loss of *snail2* activity led to a decrease in the levels of *bmp4* and *wnt8* RNAs, as well as to a reduction in the RNA encoding the selective Wnt inhibitor Frzb1 (Fig. 6J) (Leyns et al., 1997; Wang et al., 1997a; Wang et al., 1997b) and to increases in the levels of RNAs encoding the BMP4 antagonist Chordin, the Wnt and BMP antagonist Sizzled, and the Wnt, BMP and Nodal antagonist Cerberus. *cerberus* is an apparent 'immediate-early' target of Snail2 regulation (Zhang and Klymkowsky, 2009). Together, these

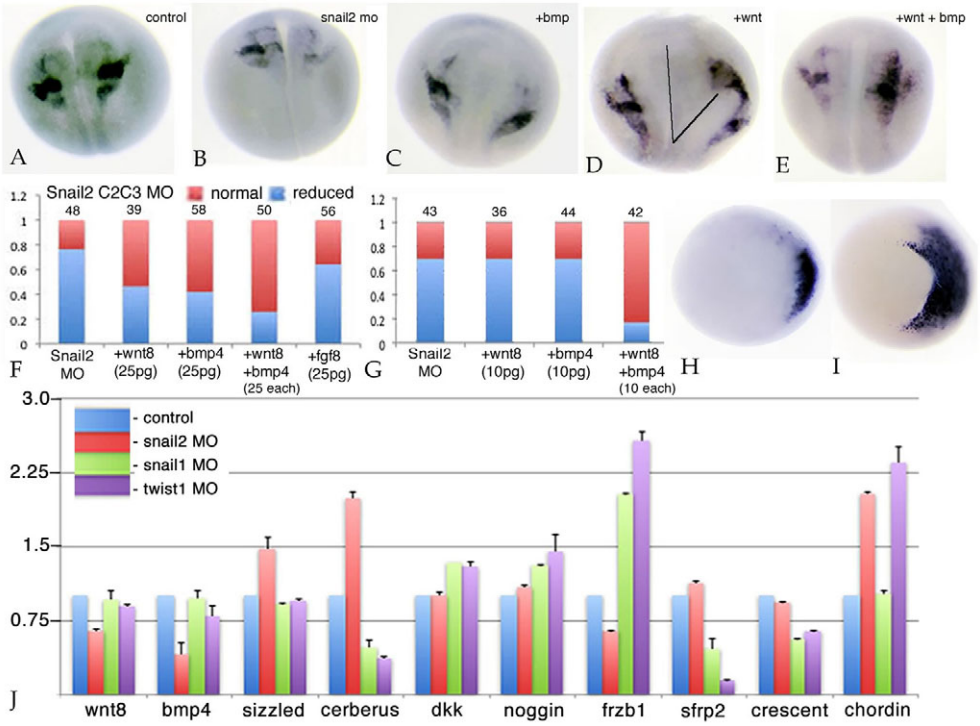

Fig. 6. Further characterization of the *snail2* morphant phenotype. (**A-E**) *Xenopus* C2/C3 blastomeres were injected with *snail2* MO. Compared with uninjected embryos (A), *snail2* morphants displayed a reduction in *sox9* expression at stage 17/18 (B). This reduction was rescued by the injection of 25 pg/embryo *bmp4* RNA (C), *wnt8* RNA (D), or the two RNAs together (E). In the case of *wnt8* RNA injection, there was often evidence of the formation of a secondary axis (D, line to the right). (**F**) The percentage of *snail2* MO C2/C3 injected embryos rescued (red bar) using 25 pg/embryo *fgf8*, *wnt8*, *bmp4* or *wnt8* and *bmp4* (25 pg each) RNAs is shown. (**G**) In a similar study, lower amounts (10 pg/embryo) of *bmp4* and *wnt8* RNAs were used. Rescue was observed only when *bmp4* and *wnt8* RNAs were injected together. (**H**,**I**) In situ analysis indicated that the levels and extent of *chordin* expression increased in stage 11 C2/C3 *snail2* morphant embryos (I, versus control in H). (**J**) At stage 10.5-11, DLMZ from *snail2*, *snail1* or *twist1* morphant C2/C3 dorsolateral zones were dissected, RNA was isolated and subjected to qPCR analysis. This revealed reproducible increases in the levels of *sizzled*, *cerberus* and *chordin* RNAs and decreases in the levels of *wnt8*, *bmp4* and *frzb1* RNAs; distinct patterns of change were observed in *snail1* and *twist1* morphant DLMZ. Error bars indicate s.d.

observations suggest that it is a balance between Wnt and BMP signaling that is critical for mesodermal induction of neural crest.

Although often considered in isolation, there is growing evidence that the BMP and Wnt signaling pathways interact (Itasaki and Hoppler, 2010; Katoh, 2010; Row and Kimelman, 2009). For example, it appears that the secreted Wnt inhibitors Dkk1 and Sost are regulated by BMP signaling (Kamiya et al., 2010) and that BMP signaling requires functional Wnt signaling (Tang et al., 2009). The Wnt signaling modulator Lpr4 interacts with Wise, a secreted and endoplasmic reticulum-localized protein that can either enhance or inhibit Wnt signaling (Guidato and Itasaki, 2007; Guidato et al., 1996; Itasaki et al., 2003). The Lrp4-Wise complex can, in turn, modulate BMP activity (Lintern et al., 2009; Ohazama et al., 2010). It is worth noting that the DLMZ of the late blastula/early gastrula stage *X. laevis* embryo is characterized by the expression of a number of other secreted signaling agonists and antagonists, in addition to *chordin*, *sizzled*, *cerberus*, *frzb1*, *bmp4* and *wnt8*; these include other canonical and non-canonical acting Wnts (Kuhl, 2002), the TGFβ inhibitor Follistatin (Fainsod et al., 1997; Iemura et al., 1998; Tashiro et al., 1991), the BMP inhibitor Noggin (Smith and Harland, 1992) and the Wnt inhibitors Dkk, SFRP2, Sizzled2 and Crescent (Bradley et al., 2000; Glinka et al., 1998; Semenov et al., 2001; Shibata et al., 2000), as well as membrane-bound signaling regulators such as Kremen (Nakamura and Matsumoto, 2008). qPCR analyses support the view that the differences in the effects of *snail1*, *snail2* and *twist1* MOs in the C2/C3 lineage are due to differences in their regulatory targets (Fig. 6J).

It is very likely that these signaling factors interact. For example, Steventon et al. (Steventon et al., 2009) reported that *snail2* RNA levels are reduced in *chordin* morphant embryos. Assuming a simple (and certainly incorrect) linear model for the BMP-Chordin interaction, loss of *chordin* expression would increase BMP signaling activity, thereby mimicking the ventral region of the embryo, where *snail2* (as well as *snail1* and *twist1*) RNA levels are low compared with the dorsal region (Zhang and Klymkowsky, 2009). Dissecting the interactions involved in the mesodermal induction of the neural crest network demands a much more global analysis – an analysis that we have begun by exploiting the recently released genomic data for *X. tropicalis* (Hellsten et al., 2010). Together with RNA and ChIP SEQ data, it should be possible to identify and study, in detail, the differences in the regulatory targets of Snail2, Snail1 and Twist1 in the DLMZ. In this light, it is worth noting that preliminary studies indicate that loss of *snail2* function in the C2/C3 lineage in *X. tropicalis* (see Fig. S3 in the supplementary material) causes a loss of *sox9* expression similar to that observed in *X. laevis*, suggesting that a similar regulatory network is involved in the two species.

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Competing interests statement
The authors declare no competing financial interests.

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References

Aggarwal, V. S., Carpenter, C., Freyer, L., Liao, J., Petti, M. and Morrow, B. E. (2010). Mesodermal Tbx1 is required for patterning the proximal mandible in mice. *Dev. Biol.* **344**, 669-681.
Alfandari, D., Cousin, H. and Marsden, M. (2010). Mechanism of Xenopus cranial neural crest cell migration. *Cell Adh. Migr.* **4**, 553-556.
Aybar, M. J., Nieto, M. A. and Mayor, R. (2003). Snail precedes slug in the genetic cascade required for the specification and migration of the Xenopus neural crest. *Development* **130**, 483-494.
Bajpai, R., Chen, D. A., Rada-Iglesias, A., Zhang, J., Xiong, Y., Helms, J., Chang, C. P., Zhao, Y., Swigut, T. and Wysocka, J. (2010). CHD7 cooperates with PBAF to control multipotent neural crest formation. *Nature* **463**, 958-962.
Baker, C. V. (2008). The evolution and elaboration of vertebrate neural crest cells. *Curr. Opin. Genet. Dev.* **18**, 536-543.
Baker, C. V. and Bronner-Fraser, M. (1997). The origins of the neural crest. Part I: embryonic induction. *Mech. Dev.* **69**, 3-11.
Barnes, R. M. and Firulli, A. B. (2009). A twist of insight-the role of Twist-family bHLH factors in development. *Int. J. Dev. Biol.* **53**, 909-924.
Barrallo-Gimeno, A. and Nieto, M. A. (2009). Evolutionary history of the Snail/Scratch superfamily. *Trends Genet.* **25**, 248-252.
Barton, C. E., Tahinci, E., Barbieri, C. E., Johnson, K. N., Hanson, A. J., Jernigan, K. K., Chen, T. W., Lee, E. and Pietenpol, J. A. (2009). DeltaNp63 antagonizes p53 to regulate mesoderm induction in Xenopus laevis. *Dev. Biol.* **329**, 130-139.
Basch, M. L. and Bronner-Fraser, M. (2006). Neural crest inducing signals. *Adv. Exp. Med. Biol.* **589**, 24-31.
Basch, M. L., Bronner-Fraser, M. and Garcia-Castro, M. I. (2006). Specification of the neural crest occurs during gastrulation and requires Pax7. *Nature* **441**, 218-222.
Bellmeyer, A., Krase, J., Lindgren, J. and LaBonne, C. (2003). The protooncogene c-myc is an essential regulator of neural crest formation in Xenopus. *Dev. Cell* **4**, 827-839.
Bonstein, L., Elias, S. and Frank, D. (1998). Paraxial-fated mesoderm is required for neural crest induction in Xenopus embryos. *Dev. Biol.* **193**, 156-168.
Bouwmeester, T., Kim, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601.
Bracken, C. P., Gregory, P. A., Kolesnikoff, N., Bert, A. G., Wang, J., Shannon, M. F. and Goodall, G. J. (2008). A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. *Cancer Res.* **68**, 7846-7854.
Bradley, L., Sun, B., Collins-Racie, L., LaVallie, E., McCoy, J. and Sive, H. (2000). Different activities of the frizzled-related proteins frzb2 and sizzled2 during Xenopus anteroposterior patterning. *Dev. Biol.* **227**, 118-132.
Carl, T. F., Dufton, C., Hanken, J. and Klymkowsky, M. W. (1999). Inhibition of neural crest migration in Xenopus using antisense slug RNA. *Dev. Biol.* **213**, 101-115.
Carver, E. A., Jiang, R., Lan, Y., Oram, K. F. and Gridley, T. (2001). The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol. Cell. Biol.* **21**, 8184-8188.
Casas, E., Kim, J., Bendesky, A., Ohno-Machado, L., Wolfe, C. J. and Yang, J. (2011). Snail2 is an essential mediator of Twist1-induced epithelial mesenchymal transition and metastasis. *Cancer Res.* **71**, 245-254.
Chen, Z. F. and Behringer, R. R. (1995). twist is required in head mesenchyme for cranial neural tube morphogenesis. *Genes Dev.* **9**, 686-699.
Cheung, M., Chaboissier, M. C., Mynett, A., Hirst, E., Schedl, A. and Briscoe, J. (2005). The transcriptional control of trunk neural crest induction, survival, and delamination. *Dev. Cell* **8**, 179-192.
Cordenonsi, M., Dupont, S., Maretto, S., Insinga, A., Imbriano, C. and Piccolo, S. (2003). Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. *Cell* **113**, 301-314.
Cordenonsi, M., Montagner, M., Adorno, M., Zacchigna, L., Martello, G., Mamidi, A., Soligo, S., Dupont, S. and Piccolo, S. (2007). Integration of TGF-beta and Ras/MAPK signaling through p53 phosphorylation. *Science* **315**, 840-843.
Dale, L. and Slack, J. M. (1987). Fate map for the 32-cell stage of Xenopus laevis. *Development* **99**, 527-551.
Essex, L. J., Mayor, R. and Sargent, M. G. (1993). Expression of Xenopus snail in mesoderm and prospective neural fold ectoderm. *Dev. Dyn.* **198**, 108-122.
Fainsod, A., Deissler, K., Yelin, R., Marom, K., Epstein, M., Pillemer, G., Steinbeisser, H. and Blum, M. (1997). The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. *Mech. Dev.* **63**, 39-50.
Fletcher, R. B. and Harland, R. M. (2008). The role of FGF signaling in the establishment and maintenance of mesodermal gene expression in Xenopus. *Dev. Dyn.* **237**, 1243-1254.
Fletcher, R. B., Baker, J. C. and Harland, R. M. (2006). FGF8 spliceforms mediate early mesoderm and posterior neural tissue formation in Xenopus. *Development* **133**, 1703-1714.
Fukuda, M., Takahashi, S., Haramoto, Y., Onuma, Y., Kim, Y. J., Yeo, C. Y., Ishiura, S. and Asashima, M. (2010). Zygotic VegT is required for Xenopus paraxial mesoderm formation and is regulated by Nodal signaling and Eomesodermin. *Int. J. Dev. Biol.* **54**, 81-92.
Ganguly, A., Jiang, J. and Ip, Y. T. (2005). Drosophila WntD is a target and an inhibitor of the Dorsal/Twist/Snail network in the gastrulating embryo. *Development* **132**, 3419-3429.
Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C. and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357-362.
Goh, K. L., Yang, J. T. and Hynes, R. O. (1997). Mesodermal defects and cranial neural crest apoptosis in alpha5 integrin-null embryos. *Development* **124**, 4309-4319.
Gordon, M. D., Dionne, M. S., Schneider, D. S. and Nusse, R. (2005). WntD is a feedback inhibitor of Dorsal/NF-kappaB in Drosophila development and immunity. *Nature* **437**, 746-749.
Green, J. B., Cook, T. L., Smith, J. C. and Grainger, R. M. (1997). Anteroposterior neural tissue specification by activin-induced mesoderm. *Proc. Natl. Acad. Sci. USA* **94**, 8596-8601.
Guidato, S. and Itasaki, N. (2007). Wise retained in the endoplasmic reticulum inhibits Wnt signaling by reducing cell surface LRP6. *Dev. Biol.* **310**, 250-263.
Guidato, S., Tsai, L. H., Woodgett, J. and Miller, C. C. (1996). Differential cellular phosphorylation of neurofilament heavy side- arms by glycogen synthase kinase-3 and cyclin-dependent kinase-5. *J. Neurochem.* **66**, 1698-1706.
Hanken, J. and Gross, J. B. (2005). Evolution of cranial development and the role of neural crest: insights from amphibians. *J. Anat.* **207**, 437-446.
Hellsten, U., Harland, R. M., Gilchrist, M. J., Hendrix, D., Jurka, J., Kapitonov, V., Ovcharenko, I., Putnam, N. H., Shu, S., Taher, L. et al. (2010). The genome of the Western clawed frog Xenopus tropicalis. *Science* **328**, 633-636.
Hong, C. S., Park, B. Y. and Saint-Jeannet, J. P. (2008). Fgf8a induces neural crest indirectly through the activation of Wnt8 in the paraxial mesoderm. *Development* **135**, 3903-3910.
Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1991). Xenopus Myf-5 marks early muscle cells and can activate muscle genes ectopically in early embryos. *Development* **111**, 551-560.
Hug, B., Walter, V. and Grunwald, D. J. (1997). tbx6, a Brachyury-related gene expressed by ventral mesendodermal precursors in the zebrafish embryo. *Dev. Biol.* **183**, 61-73.
Huguet, C., Crepieux, P. and Laudet, V. (1997). Rel/NF-kappa B transcription factors and I kappa B inhibitors: evolution from a unique common ancestor. *Oncogene* **15**, 2965-2974.
Iemura, S., Yamamoto, T. S., Takagi, C., Uchiyama, H., Natsume, T., Shimasaki, S., Sugino, H. and Ueno, N. (1998). Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *Proc. Natl. Acad. Sci. USA* **95**, 9337-9342.
Ip, Y. T., Park, R. E., Kosman, D., Yazdanbakhsh, K. and Levine, M. (1992). dorsal-twist interactions establish snail expression in the presumptive mesoderm of the Drosophila embryo. *Genes Dev.* **6**, 1518-1530.
Itasaki, N. and Hoppler, S. (2010). Crosstalk between Wnt and bone morphogenic protein signaling: a turbulent relationship. *Dev. Dyn.* **239**, 16-33.
Itasaki, N., Jones, C. M., Mercurio, S., Rowe, A., Domingos, P. M., Smith, J. C. and Krumlauf, R. (2003). Wise, a context-dependent activator and inhibitor of Wnt signalling. *Development* **130**, 4295-4305.
Jiang, R., Lan, Y., Norton, C. R., Sundberg, J. P. and Gridley, T. (1998). The Slug gene is not essential for mesoderm or neural crest development in mice. *Dev. Biol.* **198**, 277-285.
Kamiya, N., Kobayashi, T., Mochida, Y., Yu, P. B., Yamauchi, M., Kronenberg, H. M. and Mishina, Y. (2010). Wnt inhibitors Dkk1 and Sost are downstream targets of BMP signaling through the type IA receptor (BMPRIA) in osteoblasts. *J. Bone Miner. Res.* **25**, 200-210.
Katoh, M. (2010). Network of WNT and other regulatory signaling cascades in pluripotent stem cells and cancer stem cells. *Curr. Pharm. Biotechnol.* **12**, 160-170.
Khokha, M. K., Chung, C., Bustamante, E. L., Gaw, L. W., Trott, K. A., Yeh, J., Lim, N., Lin, J. C., Taverner, N., Amaya, E. et al. (2002). Techniques and

probes for the study of *Xenopus tropicalis* development. *Dev. Dyn.* **225**, 499-510.
Klymkowsky, M. W. and Hanken, J. (1991). Whole-mount staining of Xenopus and other vertebrates. In *Xenopus laevis: Practical Uses in Cell and Molecular Biology*, Vol. 36 (ed. B. K. Kay and H. B. Peng), pp. 419-441. San Diego, California: Academic Press.
Klymkowsky, M. W. and Savagner, P. (2009). Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe. *Am. J. Pathol.* **174**, 1588-1593.
Klymkowsky, M. W., Cortez-Rossi, C. and Artinger, K. B. (2010). Mechanisms driving neural crest induction and migration in the zebrafish and Xenopus laevis. *Cell Adh. Migr.* **4**, 595-608.
Koide, T., Hayata, T. and Cho, K. W. (2005). Xenopus as a model system to study transcriptional regulatory networks. *Proc. Natl. Acad. Sci. USA* **102**, 4943-4948.
Kuhl, M. (2002). Non-canonical Wnt signaling in Xenopus: regulation of axis formation and gastrulation. *Semin. Cell Dev. Biol.* **13**, 243-249.
LaBonne, C. and Bronner-Fraser, M. (2000). Snail-related transcriptional repressors are required in Xenopus for both the induction of the neural crest and its subsequent migration. *Dev. Biol.* **221**, 195-205.
Lee, Y. H., Aoki, Y., Hong, C. S., Saint-Germain, N., Credidio, C. and Saint-Jeannet, J. P. (2004). Early requirement of the transcriptional activator Sox9 for neural crest specification in Xenopus. *Dev. Biol.* **275**, 93-103.
Leptin, M. (1991). twist and snail as positive and negative regulators during Drosophila mesoderm development. *Genes Dev.* **5**, 1568-1576.
Leyns, L., Bouwmeester, T., Kim, S. H., Piccolo, S. and De Robertis, E. M. (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell* **88**, 747-756.
Li, H. Y., Bourdelas, A., Carron, C., Gomez, C., Boucaut, J. C. and Shi, D. L. (2006). FGF8, Wnt8 and Myf5 are target genes of Tbx6 during anteroposterior specification in Xenopus embryo. *Dev. Biol.* **290**, 470-481.
Li, L., Cserjesi, P. and Olson, E. N. (1995). Dermo-1: a novel twist-related bHLH protein expressed in the developing dermis. *Dev. Biol.* **172**, 280-292.
Lintern, K. B., Guidato, S., Rowe, A., Saldanha, J. W. and Itasaki, N. (2009). Characterization of wise protein and its molecular mechanism to interact with both Wnt and BMP signals. *J. Biol. Chem.* **284**, 23159-23168.
Liu, Y., Ye, F., Li, Q., Tamiya, S., Darling, D. S., Kaplan, H. J. and Dean, D. C. (2009). Zeb1 represses Mitf and regulates pigment synthesis, cell proliferation, and epithelial morphology. *Invest. Ophthalmol. Vis. Sci.* **50**, 5080-5088.
Locascio, A., Manzanares, M., Blanco, M. J. and Nieto, M. A. (2002). Modularity and reshuffling of Snail and Slug expression during vertebrate evolution. *Proc. Natl. Acad. Sci. USA* **99**, 16841-16846.
Loose, M. and Patient, R. K. (2004). A genetic regulatory network for Xenopus mesendodermal formation. *Dev. Biol.* **271**, 467-478.
Lou, X., Fang, P., Li, S., Hu, R. Y., Kuerner, K. M., Steinbeisser, H. and Ding, X. (2006). Xenopus Tbx6 mediates posterior patterning via activation of Wnt and FGF signalling. *Cell Res.* **16**, 771-779.
Manzanares, M., Locascio, A. and Nieto, M. A. (2001). The increasing complexity of the Snail gene superfamily in metazoan evolution. *Trends Genet.* **17**, 178-181.
Marchant, L., Linker, C., Ruiz, P., Guerrero, N. and Mayor, R. (1998). The inductive properties of mesoderm suggest that the neural crest cells are specified by a BMP gradient. *Dev. Biol.* **198**, 319-329.
Mayor, R., Essex, L. J., Bennett, M. F. and Sargent, M. G. (1993). Distinct elements of the xsna promoter are required for mesodermal and ectodermal expression. *Development* **119**, 661-671.
Mayor, R., Morgan, R. and Sargent, M. G. (1995). Induction of the prospective neural crest of Xenopus. *Development* **121**, 767-777.
Mayor, R., Guerrero, N., Young, R. M., Gomez-Skarmeta, J. L. and Cuellar, C. (2000). A novel function for the Xslug gene: control of dorsal mesendoderm development by repressing BMP-4. *Mech. Dev.* **97**, 47-56.
McCredie, J. (2009). History, heresy and radiology in scientific discovery. *J. Med. Imaging Radiat. Oncol.* **53**, 433-441.
Minoux, M. and Rijli, F. M. (2010). Molecular mechanisms of cranial neural crest cell migration and patterning in craniofacial development. *Development* **137**, 2605-2621.
Miraoui, H. and Marie, P. J. (2010). Pivotal role of Twist in skeletal biology and pathology. *Gene* **468**, 1-7.
Monsoro-Burq, A. H., Fletcher, R. B. and Harland, R. M. (2003). Neural crest induction by paraxial mesoderm in Xenopus embryos requires FGF signals. *Development* **130**, 3111-3124.
Monsoro-Burq, A. H., Wang, E. and Harland, R. (2005). Msx1 and Pax3 cooperate to mediate FGF8 and WNT signals during Xenopus neural crest induction. *Dev. Cell* **8**, 167-178.
Moody, S. A. (1987). Fates of the blastomeres of the 32-cell-stage Xenopus embryo. *Dev. Biol.* **122**, 300-319.
Murray, S. A. and Gridley, T. (2006). Snail family genes are required for left-right asymmetry determination, but not neural crest formation, in mice. *Proc. Natl. Acad. Sci. USA* **103**, 10300-10304.
Nakamura, O., Takasaki, H. and Nagata, A. (1978). Further studies of the prospective fates of blastomeres at the 32-cell stage of Xenopus laevis embryos. *Med. Biol.* **56**, 355-360.
Nakamura, T. and Matsumoto, K. (2008). The functions and possible significance of Kremen as the gatekeeper of Wnt signalling in development and pathology. *J. Cell. Mol. Med.* **12**, 391-408.
Nieto, M. A. (2002). The snail superfamily of zinc-finger transcription factors. *Nat. Rev. Mol. Cell Biol.* **3**, 155-166.
Nieto, M. A., Sargent, M. G., Wilkinson, D. G. and Cooke, J. (1994). Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science* **264**, 835-839.
Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of Xenopus laevis (Daudin)*. Amsterdam: North-Holland.
O'Rourke, M. P. and Tam, P. P. (2002). Twist functions in mouse development. *Int. J. Dev. Biol.* **46**, 401-413.
Ohazama, A., Porntaveetus, T., Ota, M. S., Herz, J. and Sharpe, P. T. (2010). Lrp4: A novel modulator of extracellular signaling in craniofacial organogenesis. *Am. J. Med. Genet. A* **152A**, 2974-2983.
Perez-Mancera, P. A., Bermejo-Rodriguez, C., Gonzalez-Herrero, I., Herranz, M., Flores, T., Jimenez, R. and Sanchez-Garcia, I. (2007). Adipose tissue mass is modulated by SLUG (SNAI2). *Hum. Mol. Genet.* **16**, 2972-2986.
Ragland, J. W. and Raible, D. W. (2004). Signals derived from the underlying mesoderm are dispensable for zebrafish neural crest induction. *Dev. Biol.* **276**, 16-30.
Row, R. H. and Kimelman, D. (2009). Bmp inhibition is necessary for post-gastrulation patterning and morphogenesis of the zebrafish tailbud. *Dev. Biol.* **329**, 55-63.
Ryan, K., Garrett, N., Mitchell, A. and Gurdon, J. B. (1996). Eomesodermin, a key early gene in Xenopus mesoderm differentiation. *Cell* **87**, 989-1000.
Salic, A. N., Kroll, K. L., Evans, L. M. and Kirschner, M. W. (1997). Sizzled: a secreted Xwnt8 antagonist expressed in the ventral marginal zone of Xenopus embryos. *Development* **124**, 4739-4748.
Sandmann, T., Girardot, C., Brehme, M., Tongprasit, W., Stolc, V. and Furlong, E. E. (2007). A core transcriptional network for early mesoderm development in Drosophila melanogaster. *Genes Dev.* **21**, 436-449.
Sargent, M. G. and Bennett, M. F. (1990). Identification in Xenopus of a structural homologue of the Drosophila gene snail. *Development* **109**, 967-973.
Sasai, N., Yakura, R., Kamiya, D., Nakazawa, Y. and Sasai, Y. (2008). Ectodermal factor restricts mesoderm differentiation by inhibiting p53. *Cell* **133**, 878-890.
Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. M. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.
Sasai, Y., Lu, B., Steinbeisser, H. and De Robertis, E. M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. *Nature* **376**, 333-336.
Schmalhofer, O., Brabletz, S. and Brabletz, T. (2009). E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. *Cancer Metastasis Rev.* **28**, 151-166.
Sefton, M., Sanchez, S. and Nieto, M. A. (1998). Conserved and divergent roles for members of the Snail family of transcription factors in the chick and mouse embryo. *Development* **125**, 3111-3121.
Semenov, M. V., Tamai, K., Brott, B. K., Kuhl, M., Sokol, S. and He, X. (2001). Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. *Curr. Biol.* **11**, 951-961.
Shi, W., Levine, M. and Davidson, B. (2005). Unraveling genomic regulatory networks in the simple chordate, Ciona intestinalis. *Genome Res.* **15**, 1668-1674.
Shibata, M., Ono, H., Hikasa, H., Shinga, J. and Taira, M. (2000). Xenopus crescent encoding a Frizzled-like domain is expressed in the Spemann organizer and pronephros. *Mech. Dev.* **96**, 243-246.
Sive, H. L., Grainger, R. M. and Harland, R. M. (2000). *Early Development of Xenopus laevis: a Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
Smith, J. C., Price, B. M., Green, J. B., Weigel, D. and Herrmann, B. G. (1991). Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.
Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* **70**, 829-840.
Soo, K., O'Rourke, M. P., Khoo, P. L., Steiner, K. A., Wong, N., Behringer, R. R. and Tam, P. P. (2002). Twist function is required for the morphogenesis of the cephalic neural tube and the differentiation of the cranial neural crest cells in the mouse embryo. *Dev. Biol.* **247**, 251-270.
Spokony, R. F., Aoki, Y., Saint-Germain, N., Magner-Fink, E. and Saint-Jeannet, J. P. (2002). The transcription factor Sox9 is required for cranial neural crest development in Xenopus. *Development* **129**, 421-432.
Spring, J., Yanze, N., Middel, A. M., Stierwald, M., Groger, H. and Schmid, V. (2000). The mesoderm specification factor twist in the life cycle of jellyfish. *Dev. Biol.* **228**, 363-375.
Spring, J., Yanze, N., Josch, C., Middel, A. M., Winninger, B. and Schmid, V. (2002). Conservation of Brachyury, Mef2, and Snail in the myogenic lineage of jellyfish: a connection to the mesoderm of bilateria. *Dev. Biol.* **244**, 372-384.

Stathopoulos, A. and Levine, M. (2002). Dorsal gradient networks in the Drosophila embryo. *Dev. Biol.* **246**, 57-67.
Stennard, F., Carnac, G. and Gurdon, J. B. (1996). The Xenopus T-box gene, Antipodean, encodes a vegetally localised maternal mRNA and can trigger mesoderm formation. *Development* **122**, 4179-4188.
Stennard, F., Zorn, A. M., Ryan, K., Garrett, N. and Gurdon, J. B. (1999). Differential expression of VegT and Antipodean protein isoforms in Xenopus. *Mech. Dev.* **86**, 87-98.
Steventon, B., Carmona-Fontaine, C. and Mayor, R. (2005). Genetic network during neural crest induction: from cell specification to cell survival. *Semin. Cell Dev. Biol.* **16**, 647-654.
Steventon, B., Araya, C., Linker, C., Kuriyama, S. and Mayor, R. (2009). Differential requirements of BMP and Wnt signalling during gastrulation and neurulation define two steps in neural crest induction. *Development* **136**, 771-779.
Takebayashi-Suzuki, K., Funami, J., Tokumori, D., Saito, A., Watabe, T., Miyazono, K., Kanda, A. and Suzuki, A. (2003). Interplay between the tumor suppressor p53 and TGF beta signaling shapes embryonic body axes in Xenopus. *Development* **130**, 3929-3939.
Tang, N., Song, W. X., Luo, J., Luo, X., Chen, J., Sharff, K. A., Bi, Y., He, B. C., Huang, J. Y., Zhu, G. H. et al. (2009). BMP-9-induced osteogenic differentiation of mesenchymal progenitors requires functional canonical Wnt/beta-catenin signalling. *J. Cell. Mol. Med.* **13**, 2448-2464.
Tashiro, K., Yamada, R., Asano, M., Hashimoto, M., Muramatsu, M. and Shiokawa, K. (1991). Expression of mRNA for activin-binding protein (follistatin) during early embryonic development of Xenopus laevis. *Biochem. Biophys. Res. Commun.* **174**, 1022-1027.
Thisse, B., el Messal, M. and Perrin-Schmitt, F. (1987). The twist gene: isolation of a Drosophila zygotic gene necessary for the establishment of dorsoventral pattern. *Nucleic Acids Res.* **15**, 3439-3453.
Tribulo, C., Aybar, M. J., Sanchez, S. S. and Mayor, R. (2004). A balance between the anti-apoptotic activity of Slug and the apoptotic activity of msx1 is required for the proper development of the neural crest. *Dev. Biol.* **275**, 325-342.
Valanne, S., Wang, J. H. and Ramet, M. (2011). The Drosophila Toll signaling pathway. *J. Immunol.* **186**, 649-656.
Vallin, J., Thuret, R., Giacomello, E., Faraldo, M. M., Thiery, J. P. and Broders, F. (2001). Cloning and characterization of three Xenopus Slug promoters reveal direct regulation by Lef/beta-catenin signaling. *J. Biol. Chem.* **287**, 30350-30358.
Wang, S., Krinks, M., Lin, K., Luyten, F. P. and Moos, M., Jr (1997a). Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. *Cell* **88**, 757-766.
Wang, S., Krinks, M. and Moos, M. J. (1997b). Frzb-1, an antagonist of Wnt-1 and Wnt-8, does not block signaling by Wnts -3A, -5A, or -11. *Biochem. Biophys. Res. Commun.* **236**, 502-504.
Webster, W. S. and Ritchie, H. E. (1991). Teratogenic effects of alcohol and isotretinoin on craniofacial development: an analysis of animal models. *J. Craniofac. Genet. Dev. Biol.* **11**, 296-302.
Wellner, U., Schubert, J., Burk, U. C., Schmalhofer, O., Zhu, F., Sonntag, A., Waldvogel, B., Vannier, C., Darling, D., zur Hausen, A. et al. (2009). The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat. Cell Biol.* **11**, 1487-1495.
Wetts, R. and Fraser, S. E. (1989). Slow intermixing of cells during Xenopus embryogenesis contributes to the consistency of the blastomere fate map. *Development* **105**, 9-15.
Xu, W., Edmondson, D. G., Evrard, Y. A., Wakamiya, M., Behringer, R. R. and Roth, S. Y. (2000). Loss of Gcn5l2 leads to increased apoptosis and mesodermal defects during mouse development. *Nat. Genet.* **26**, 229-232.
Yu, J. K. (2010). The evolutionary origin of the vertebrate neural crest and its developmental gene regulatory network-insights from amphioxus. *Zoology (Jena)* **113**, 1-9.
Zeitlinger, J., Zinzen, R. P., Stark, A., Kellis, M., Zhang, H., Young, R. A. and Levine, M. (2007). Whole-genome ChIP-chip analysis of Dorsal, Twist, and Snail suggests integration of diverse patterning processes in the Drosophila embryo. *Genes Dev.* **21**, 385-390.
Zhang, C. and Klymkowsky, M. W. (2009). Unexpected functional redundancy between Twist and Slug (Snail2) and their feedback regulation of NF-kappaB via Nodal and Cerberus. *Dev. Biol.* **331**, 340-349.
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.
Zhang, C., Carl, T. F., Trudeau, E. D., Simmet, T. and Klymkowsky, M. W. (2006). An NF-kappaB and slug regulatory loop active in early vertebrate mesoderm. *PLoS ONE* **1**, e106.