Unexpected functional redundancy between Twist and Slug (Snail2) and their feedback regulation of NF-κB via Nodal and Cerberus

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**A B S T R A C T**

A NF-κB-Twist-Snail network controls axis and mesoderm formation in Drosophila. Using translation-blocking morpholinos and hormone-regulated proteins, we demonstrate the presence of an analogous network in the early Xenopus embryo. Loss of twist (twist1) function leads to a reduction of mesoderm and neural crest markers, an increase in apoptosis, and a decrease in snail1 (snail) and snail2 (slug) mRNA levels. Injection of snail2 mRNA rescues twist’s loss of function phenotypes and visa versa. In the early embryo NF-κB/RelA regulates twist, snail2, and snail1 mRNA levels; similarly Nodal/Smad2 regulate twist, snail2, snail1, and relA RNA levels. Both Twist and Snail2 negatively regulate levels of cerberus RNA, which encodes a Nodal, bone morphogenic protein (BMP), and Wnt inhibitor. Cerberus’s anti-Nodal activity inhibits NF-κB activity and decreases relA RNA levels. These results reveal both conserved and unexpected regulatory interactions at the core of a vertebrate’s mesodermal specification network.

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Introduction

The regulatory network that specifies mesoderm formation appears to be both evolutionarily ancient (Baguñà et al., 2008) and plastic (Hinman and Davidson, 2007). As first described in the dipteran insect Drosophila melanogaster, asymmetric activation of the membrane receptor Toll leads to a gradient of Dorsal/NF-κB transcription factor activity, which in turn leads to regional activation of target genes (Alberga et al., 1991; Jiang et al., 1991; Jiang and Levine, 1993; Leptin, 1991; Papatsenko and Levine, 2005; Ray et al., 1991; Sandmann et al., 2007; Stathopoulos and Levine, 2002a,b; Thisé et al., 1987; Zeitlinger et al., 2007). The products of two of these genes, twist \(^2\) and snail, cooperate with Dorsal to establish the dorsal/ventral axis and mesoderm. The system involves a number of feed-forward and feedback elements. For example, the basic helix-loop-helix transcription factor Twist regulates a substantial fraction of all known transcription factor-encoding genes in Drosophila and acts together with Dorsal/NF-κB to regulate snail expression. snail encodes a zinc-finger transcription factor that negatively regulates wntD, which encodes a secreted Wnt-family protein that negatively regulates Dorsal/NF-κB activity (Ganguly et al., 2005; Gordon et al., 2005).

Many of the components of the Dorsal/Twist/Snail system are evolutionarily conserved. A twist-like gene is expressed in the mesoderm-like cells of diploblastic cnidarians (Spring et al., 2000). twist activity is required for mesoderm formation in the urochordate Ciona intestinalis (Imai et al., 2003; Tokuoka et al., 2005), suggesting that a Twist-dependent circuit was present early in metazoan evolution (Martindale, 2005; Technau and Scholz, 2003). In the mouse, embryos null for twist1 die at embryonic day 11.5 (E11.5) with defects in cranial neural tube formation (Chen and Behringer, 1995; O'Rourke and Tam, 2002). Surprisingly, no direct functional studies of Twist have been carried out in Xenopus laevis since its initial characterization by Hopwood et al. (1989).

Snail-family proteins are expressed in mesoderm throughout the bilateria (Rahimi et al., 2009). A snail-like gene has been implicated in notochord/myotome boundary formation in Ciona (Fujiwara et al., 1998). There appears to be a single snail-like gene in the basal urochordate amphioxus (Langeland et al., 1998). In lamprey, the most basal extant vertebrate, snail RNA is supplied maternally and present throughout early cleavage stages, but was undetected by in situ hybridization until the neural plate stage (Rahimi et al., 2009). In both amphioxus and lamprey, snail is expressed in pre-somatic mesoderm and neural plate. snail loss of function studies have yet to be reported for either amphioxus or lamprey.
Within the vertebrate lineage there appears to have been two gene duplication events, the first giving rise to the snail1/snaI2 and snail3 genes, and the second giving rise to snail1 and snail2 (previously known as slug) genes. Sequence comparison studies indicate that the Snail2 (Slug) proteins are more highly conserved than are Snail1s (Barrallo-Gimeno and Nieto, 2005; Manzanares et al., 2001; Rahimi et al., 2009). In X. laevis snail2 RNA is supplied maternally and is required for mesoderm and neural crest formation (Carl et al., 1999; Mayor et al., 2000; Zhang et al., 2006). Previous studies on snail1 function in X. laevis have involved dominant negative variants of the protein and have focused primarily on neural crest formation (Abyar et al., 2003; LaBonne and Bronner-Fraser, 2000); the interpretation of such studies is complicated by the fact that Snail1 and Snail2 share interaction partners (Langer et al., 2008), bind to similar DNA target sequences (Bolos et al., 2003; Martinez-Estrada et al., 2006), and that the snail2 loss of function phenotype in X. laevis can be rescued by snail1 RNA (Carl et al., 1999; Abyar et al., 2003; Zhang et al., 2006). Mice null for snail1 display mesodermal abnormalities and die between E7.5 and 8.5 (Carver et al., 2002), while mice homozygous for a snail2 null mutation are viable but display a number of adult phenotypes (Jiang et al., 1998; Murray et al., 2007; Savagner et al., 2005). The difference between the role of snail2 in X. laevis and mouse appears to be due, in part, to the shuffling of snail2 and snail1 expression domains (Locascio et al., 2002). Unexpectedly, mice mutant for both snail2 and snail1 functions exhibit left–right axis defects but display apparently normal, at least initially, cranial neural crest formation (Murray and Gridley, 2006). In the mouse, mutations in snail2 and snail1 enhance the phenotypic effects of twist mutations (Oram and Gridley, 2005), arguing for functionally significant interactions between these genes. NF-κB has been implicated in notochord (a mesodermal derivative) formation in Ciona (Kawai et al., 2005; Shimada et al., 2001) and in the teleost Danio rerio (Correa et al., 2004). In X. laevis, inhibition of NF-κB activity disrupts mesoderm and neural crest formation (Beck et al., 1998; Zhang et al., 2006). The Toll system has been implicated in dorsal–ventral axis specification (Armstrong et al., 1998; Prothmann et al., 2000), while the NF-κB subunit protein Rel3 has been found to negatively regulate Nodal signaling (Kennedy et al., 2007). In mouse, and human, there are five NF-κB subunit proteins: RelA, RelB, c-Rel, NF-κB1 and NF-κB2. In the mouse, transgenic reporter studies suggest that NF-κB activity first appears at E12.5 (Schmidt-Ullrich et al., 1996), while nuclear NF-κB has been reported in 1-cell mouse embryos (Nishikimi et al., 1999). Mouse embryonic stem cells express high levels of relA and relB, and lower levels of NF-κB1 and NF-κB2; the regulator of pluripotency Nanog binds to and inhibits NF-κB activity (Torres and Watt, 2008). A number of null mutations in mouse NF-κB genes and accessory factors have been generated. Mice homozygous for a null mutation in relA die at E15, whereas double mutants in nf-κB1 and relA, or relA and c-rel, die at E13, apparently due to defects in their hematopoietic system, a mesodermal derivative (Gerondakis et al., 2006).

An unanswered question is whether a NF-κB/Twist/Snail network is active during early vertebrate embryogenesis. To address this question we used translation-blocking morpholinos and hormone-regulated proteins to study the regulation and functions of twist in early X. laevis embryos. We find that twist function is required for mesodermal and neural crest marker expression, craniofacial morphogenesis, and cell survival. Surprisingly, Snail2 (Slug) rescues twist’s loss of function phenotype and visa versa, and both Twist and Snail2 regulate expression of cerberus, which encodes a secreted Nodal, BMP, and Wnt antagonist. Nodal/transforming growth factor-β (TGFβ) signaling regulates twist, snail2, snail1, and relA RNA levels in a protein synthesis insensitive manner. Through its effects on Nodal signaling, Cerberus inhibits NF-κB activity. Together these data reveal an unexpected functional redundancy between Twist and Snail2 and a double-negative feedback circuit regulating Nodal and NF-κB activities. This network is analogous to, but distinct from, the Dorsal/Twist–Snail/WntD network described in Drosophila.

Materials and methods

Embryos and their manipulation

X. laevis embryos and ectodermal explants (animal caps) were obtained and staged following standard procedures (Klymkowsky and Hanken, 1991; Nieuwkoop and Faber, 1967; Sive et al., 2000; Zhang et al., 2003). Except for the experiment presented in Fig. 2F, all results reported are based on two or more repetitions of the experiments described. Capped mRNAs were transcribed from linearized plasmid templates using mMessage mMachine kits (Ambion) following manufacturer’s instructions. Embryos were typically injected in the animal hemisphere at the one-cell stage for ectodermal explant/animal cap studies, and equatorially at the two-cell stage for other studies. Animal caps were analyzed when control embryos reached stage 11. All images were captured using a Nikon CoolPix 995 Camera on a Leica M400 Photomicroskop. Images were manipulated with Fireworks CS3 software (Adobe) using “auto levels” and “curves” functions only.

Plasmids and morpholinos

Snail2 (Slug), Snail1 (Snail), VegT, RelA, β-galactosidase, Cerberus, and Cer δ expression plasmids have been described previously (Carl et al., 1999; Zhang et al., 2005a, 2004, 2006). A plasmid containing the X. laevis twist sequence was obtained from Tim Grammer (UC Berkeley) and subcloned via PCR, to form pcS2-Twist-GFP, GR-Twist-GFP, and UTR-Twist-GFP plasmids. Plasmids containing the X. laevis Smad2, Smad4a, and Smad4b sequences as well as a plasmid encoding GR-Smad2 were obtained from Richard Harland (Chang and Harland, 2007); we independently subcloned the Smad2 sequence into the pcS2-GR plasmid to generate pcS2-GR-Smad2. A modified DNA anti-sense oligonucleotide (morpholino) (5′-GGACTCTTCTGCGATCATTTTCC-3′) designed to block translation of the twist mRNA, was synthesized by GeneTools, Inc. and tested for effects on UTR-Twist-GFP RNA, which contains the morpholino’s 5′ UTR translational start region target. The Snail2 (Slug) morpholino has been described previously (Zhang et al., 2006). RNA injected embryos were selected based on GFP-based fluorescence.

Immunoblot and in situ hybridization studies

Immunoblot analysis was carried out as described previously using anti-GFP and antiTCF3c (Zhang et al., 2003) antibodies. For in situ hybridization studies, digoxigenin-UTP labeled anti-sense probes were made following standard methods: specific probes for sox9, vegT/antipodean, snail2, snail1, chordin, endodermim, myoD, and xbra RNAs were used; in the case of Snail1 and Snail2 RNAs, the probes were designed against the 3′ untranslated regions to reduce cross-reaction (Carl et al., 1999; Zhang et al., 2006). In some cases embryos were co-injected with RNA (50 pg/embryo) encoding β-galactosidase; β-galactosidase activity was visualized in fixed embryos using a brief Red-Gal (Research Organics) reaction, in order to identify successfully injected embryos.

Quantitative PCR and RT-PCR

RNA isolation, RT-PCR and quantitative RT-PCR analyses were carried out as described previously (Zhang et al., 2003, 2006). When using hormone-regulated proteins, embryos were treated with dexamethasone (20 μM) in the presence or absence of the protein synthesis inhibitor emetine (100 μg/ml) as described previously (Zhang et al., 2005a,b). Primers for RT-PCR analysis were Twist: U 5′-AGT CCC ATC TCA GTG AAG GCC-3′ D 5′-TGT GTG TCG CCT GAG CTG TAG-3′; primer pairs for xbra, bclxL, and caspase-9 mRNAs are as described in Zhang et al. (2006); we designed new primer pairs for...
snail1 (5'-AAG CAC GTC CTC TTT-3' D 5'-CCA ATA ATG ATA CAC ACC-3' and snail2 (5'-GAT GCA CAT CCG GAC ACA CAC-3' D 5'-CTG CGA ATG CTC TGT TGC AGT-3').

Results

Characterization of early twist, snail1, and snail2 expression

twist, snail1, and snail2 appear to have similar expression domains in gastrula and early neurula stage embryos (Linker et al., 2000). Hopwood et al. (1989) reported that twist RNA appears in the early gastrula (stage 10) embryo and is restricted to mesoderm. It was originally claimed that snail2 (slug) was first expressed in neural crest beginning at stage 17 (Mayor et al., 1995); subsequent studies, however, revealed that snail2 it expressed much earlier (Carl et al., 1999; Mayor et al., 2000; Zhang et al., 2006) and is localized to the dorsal mesodermal region of the early gastrula stage embryo (see below). A similar distribution has been described for snail1 expression in stage 10+ embryos (Carl et al., 1999). In subsequent studies, we have had difficulty in consistently producing clear in situ images of snail1, snail2, and twist RNA distribution in late blastula/early gastrula stage embryos — in each case long development times were required to generate weak signals. This may be due in part to the rather broad expression domains of these genes (see below). We therefore turned to RT-PCR-based analyses to characterize the expression of these genes. twist, snail1, and snail2 RNAs are present in 4 cell (stage 3) and stage 6 embryos, prior to the onset of zygotic transcription at the midblastula transition (stage 8.5); all three are easily detectable (30 cycle PCR) at stage 9 and increase in later stage embryos (Fig. 1A). To define regional expression patterns, early gastrula (stage 10/11) embryos were dissected into dorsal, lateral and ventral marginal equatorial zones (Fig. 1B), twist, like snail2 and snail1, RNA levels are highest in the dorsal and lateral marginal zones of the late blastula/early gastrula stage embryo (Fig. 1C), a result consistent with previous studies (see above). twist, snail1, and snail2 RNAs are sometimes detectable in animal pole/ectodermal explants, but at levels much lower than that present in the equatorial zone (Figs. 1E, F). Both snail1 and snail2 RNA levels are regulated by the NF-κB subunit protein RelA (Zhang et al., 2006); similar studies using a hormone-regulated form of RelA (GR-RelA) indicate that twist RNA levels are regulated in a protein synthesis independent (immediate/early) manner by NF-κB in the late blastula/early gastrula stage embryo (Fig. 1D).

Fig. 1. (A) RT-PCR (30 cycles) of embryos at stages 3 (4 cell), 6, 9, 14, and 17 revealed similar levels of twist, snail2, and snail1 RNAs; ornithine decarboxylase (ODC) was used in this and all other such experiments as a control. (B) Equatorial dorsal marginal zone (DMZ), lateral marginal zone (LMZ), and ventral marginal zone (VMZ) regions of early gastrula (stage 10/11) embryos were dissected and analyzed by RT-PCR (28 cycles); (C) twist, snail2, and snail1 RNAs were present at highest levels in the DMZ and LMZ regions, but detectable in the VMZ. (D) Fertilized eggs were injected in the animal hemisphere with RNA encoding GR-Smad2 (500 pg/embryo); at stage 8/9 the protein was activated with dexamethasone (+ Dex). Embryos were analyzed at stage 11 (3 to 4 h after protein activation) and showed an emetine-insensitive increase in twist RNA levels. (E) Fertilized eggs were injected in the animal pole region with RNAs encoding either VegT (100 pg/embryo) or Xnr1 (nodal-related protein 1) (50 pg/embryo) either alone or together with RNA encoding the Nodal-specific signaling inhibitor CerS (300 pg/embryo); ectodermal explants (animal caps) were prepared at stage 8/9 and analyzed at stage 11. Explants from un.injected embryos showed very low levels of twist, snail2, and snail1 RNAs, while both VegT and Xnr1 RNAs induced an accumulation of all three RNAs what was blocked by CerS, suggesting the Nodal dependent regulation of twist, snail2, and snail1 RNA levels. (F) The effects of VegT were not blocked by co-injection of RNAs encoding either the Wnt antagonist Dickkopf (dkk) or the BMP inhibitor Noggin (both injected at 500 pg/embryo). (G, H) Fertilized eggs were injected with GR-Smad2 RNA (600 pg/embryo); animal caps were prepared and treated with dexamethasone and/or emetine and analyzed by standard (G) or quantitative RT-PCR (H) at stage 11. twist, snail2, snail1, and relA RNA levels were increased in an emetine-insensitive manner following dexamethasone activation of Smad2.
VegT/Nodal regulation of twist, snail1, and snail2

Mesendodermal specification in *Xenopus* is dependent upon the maternal RNA/protein VegT, a T-box type transcription factor, acting in part through its effects on Nodal (xnr) gene expression and signaling (Kofron et al., 1999; Rex et al., 2002; Takahashi et al., 2000). Nodals are members of the transforming growth factor-β (TGFβ) family of secreted proteins and act through cytoplasmic Smad proteins to regulate gene expression (Schier, 2003). Using RNAs encoding VegT, the Nodal-related protein Xnr1, and the Nodal inhibitor CerS (Agius et al., 2000), we found that both VegT and Xnr1 RNAs induce the expression of twist, snail2, and snail1 RNAs in ectodermal explants (animal caps) and that CerS blocks these effects (Fig. 1E), indicating that VegT acts through Nodal signaling to induce twist, snail2, and snail1 expression. Neither the wnt antagonist Dickkopf (Glinka et al., 1998) nor the bone morphogenic protein (BMP) antagonist Noggin (Zimmerman et al., 1996) blocked the VegT-induced up-regulation of twist, snail2, or snail1 RNAs in animal caps (Fig. 1F). To examine whether twist, snail2, or snail1 are direct targets of Nodal regulation, we used hormone-regulated forms of the Nodal-regulated Smad, Smad2; one obtained from Richard Harland (Chang and Harland, 2007) and a second that we constructed — both produced similar effects upon activation by dexamethasone. Using both standard and quantitative RT-PCR (Figs. 1G, H), activation of Smad2 led to a protein synthesis independent increase in twist, snail2, snail1, and relA RNA levels. The increase in relA RNA levels may, at least partially, explain why injection of xnr RNAs led to an increase in NfκB activity (see below).

Twist’s role in mesoderm

To examine twist’s role(s) and possible regulatory interactions with snail2 and snail1, we designed and characterized an anti-sense modified oligonucleotide (a morpholino) directed against the translation start region of the twist mRNA. To test this morpholino’s effectiveness, fertilized eggs were injected with 5 to 10 ng/embryo of Twist or control morpholino together with 0.6 ng/embryo UTR-Twist-GFP RNA, which contains the sequence targeted by the Twist morpholino. Embryos were analyzed at the onset of gastrulation (stage 10/11) by either immunoblot analysis using an anti-GFP antibody (Fig. 2A) or by GFP-based fluorescence in living embryos (Fig. 2B); the Twist morpholino produced a dramatic decrease in Twist-GFP protein levels.

In *Drosophila*, Twist collaborates with Dorsal/NF-κB to regulate snail expression. To determine if this is the case in *X. laevis*, fertilized eggs were injected with Twist, Snail2, or control morpholinos and the effects on snail2, twist, and snail1 RNA levels were examined. The accumulation of both snail2 and snail1 RNAs was dependent upon twist function, while twist RNA accumulation was dependent upon snail2 function (Figs. 2C, D). To determine whether these effects are dependent upon ongoing protein synthesis, we generated dexamethasone-regulated forms of Twist (GR-Twist-GFP; this work) and Snail2 (GR-Snail2-GFP); both behave like the wild type polypeptides in the presence of dexamethasone (data not shown for Twist, see Zhang et al., 2006 for data on Snail2). In both cases, that is Twist regulation of snail2 and snail1 RNAs and Snail2 regulation of twist RNA levels, there was a positive interaction that was blocked by the protein synthesis inhibitor emetine (Figs. 2E, F) indicating an indirect regulatory interaction.

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**Fig. 2.** (A, B) Fertilized eggs were injected with RNA encoding UTR-Twist-GFP RNA (500 pg/embryo) either alone (“UN”) or together with the Twist morpholino (“Twist MO”) or a control (“Con MO”), both at 7 ng/embryo; injected embryos were analyzed at stage 11. (A) The Twist morpholino inhibited the accumulation of Twist-GFP, as monitored by immunoblot analysis using an anti-GFP antibody; the control morpholino did not. Blotting with an anti-Tcf3 antibody served as a loading control. (B) Injection of the Twist morpholino inhibited Twist-GFP fluorescence while the control morpholino did not. (C, D) Embryos were injected bilaterally with either Twist or Snail2 morpholinos and analyzed at stage 11; injection of the Twist morpholino (C) inhibited the accumulation of snail2 and snail1 RNAs, while injection of the Snail2 morpholino (D) inhibited the accumulation of twist RNA; injection of a control morpholino had no apparent effect on twist RNA levels. (E, F) Fertilized eggs were injected with either GR-Twist-GFP or GR-Snail2-GFP RNAs (500 pg/embryo); embryos were treated with dexamethasone and/or emetine beginning at stage 8. RNA was isolated at stage 10/11, and levels of snail2, snail1 (E), and twist (F) RNAs were determined by quantitative RT-PCR; the Gr-Snail2 experiment was performed only once. snail2 and snail1 RNA levels were indirectly regulated by Twist, while twist RNA levels were indirectly regulated by Snail2.
To examine the effects of twist loss of function on mesodermal marker expression, one blastomere of a two-cell embryo was injected equatorially with either Twist or control morpholino; early/mid-gastrula (stage 11) embryos were fixed and stained in situ for markers of mesoderm (\textit{xbra} and \textit{antipodean}, a zygotic splice variant of \textit{vegT}) and dorsal mesoderm (\textit{chordin}); in each case, expression was strongly reduced in Twist morphant (that is morpholino-injected) embryos (Figs. 3A–F and Supplemental table). While the expression of mesodermal markers was suppressed, the marker of endoderm, \textit{endodermin}, was expanded (Figs. 3G, H). Injection of a Twist RNA immune to the effects of the Twist morpholino efficiently rescued \textit{xbra} (Figs. 4A, B) and \textit{antipodean/VegT} expression (Supplemental table). Given the similarity in the patterns of expression of \textit{twist} and \textit{snail2} (see above), the fact that both bind to E-box DNA sequences (Castanon and Baylies, 2002; Murre et al., 1989; Takahashi et al., 2004), and the ability of \textit{snail1} RNA to rescue the \textit{snail2} loss of function phenotype (Aybar et al., 2003; Carl et al., 1999; del Barrio and Nieto, 2002; Zhang et al., 2006), we examined the effects of \textit{snail2} RNA on the twist loss of function phenotype and visa versa. In both cases, there was efficient rescue of \textit{xbra} expression in gastrula stage embryos (Figs. 4C–F); a similar pattern of reciprocal rescue was observed with other phenotypes (see below). Preliminary studies indicate that injection of \textit{Snail1} RNA rescues the phenotypic effects of twist loss of function even more efficiently than did \textit{Snail2} RNA (data not shown). Whether this is due to differences in the stability of the two proteins or their activities remains to be determined.

A major mesodermal tissue in the larval stage embryo is the somitic mesoderm, which gives rise to dorsal myotome. Studies on myotome formation were complicated, however, by the fact that ~50% of Twist morpholino-injected embryos arrest at gastrulation; this indicates that embryos that successfully progress through gastrulation have a hypomorphic/reduced function rather than a amorphic/null phenotype; the same is true of \textit{Snail2} morphant embryos (Carl et al., 1999; Zhang et al., 2006). That said, both Twist and \textit{Snail2} morphant larvae display a clear anterior loss of the somitic mesodermal marker \textit{myoD} (Figs. 5A–C). As in the case with \textit{xbra} expression, the expression of \textit{myoD} is efficiently rescued in \textit{Snail2} morphant embryos by the injection of either \textit{snail2} (Fig. 5F) or twist (Fig. 5E) RNAs. Similarly, \textit{myoD} expression in Twist morphant embryos are rescued by the injection of either twist (Fig. 5D) or \textit{snail2} RNAs (Fig. 5G). In later stage larvae, unilateral injection of the Twist morpholino led to a consistent bend toward the injected side in surviving embryos (Fig. 5H), presumably due to the absence of mesodermal/myotomal derivatives.

\textbf{Twist loss of function effects on neural crest and its derivatives}

Previous anti-sense/morpholino/dominant negative protein studies established that \textit{snail2} function is required for neural crest formation in both chick (Nieto et al., 1994) and Xenopus (Carl et al., 1999; Mayor et al., 2000; Zhang et al., 2006). Similarly, the unilateral loss of twist function led to the loss of the neural crest markers \textit{sox9} (Fig. 6A), \textit{snail2} (data not shown) and the loss of craniofacial cartilage (Figs. 6B, C) compared to control embryos (Fig. 6D). There was also an apparent decrease in the number of melanocytes in the anterior

\textbf{Fig. 4.} The effect of Twist MO on \textit{xbra} expression (A, C) was rescued by the injection of either Twist-GFP RNA (B) or \textit{Snail2}-GFP RNA (D), both injected at 500 pg/embryo. Similarly, the loss of \textit{xbra} expression in \textit{Snail2} morpholino-injected embryos (E) was rescued by the injection of Twist-GFP RNA (F) (500 pg/embryo). Arrows indicate the β-galactosidase (red) lineage marker, "yp" indicates the yolk plug and "BP" indicates the blastopore — all embryos are oriented similarly, with the dorsal lip oriented to the lower right.

\textbf{Fig. 3.} Compared to uninjected embryos (A, C, E, G), injection of the Twist morpholino (7 ng/embryo) (B, D, F, H) into one-cell of two-cell embryos led to the loss of \textit{xbra} (A, C), \textit{antipodean/VegT} (B, D), and \textit{chordin} (E, F) expression (arrows), and the expansion (arrow) of the expression domain of the endodermal marker \textit{endodermin} (G, H); "yp" marks the yolk plug of gastrula stage embryos, "BP" marks the blastopore — all embryos are oriented similarly, with the dorsal lip oriented to the lower right.
...region, while the trunk melanocytes were clearly present in Twist morphant embryos (see Supplemental Fig. 1). There are two obvious reasons that the observed effect on melanocytes was partial; first the majority of melanocytes arise from the trunk rather than the cephalic neural crest (Aoki et al., 2003; Model and Dalton, 1968) and full inhibition of twist activity blocks gastrulation. This means these embryos display hypomorphic rather than null phenotypes. The effect observed is similar to anterior inhibition of myoD expression (see above). In Twist morphant embryos, expression of sox9 was rescued by either twist (Fig. 6E) or snail2 (Fig. 6I) RNA injection. Similarly, the craniofacial phenotype of Twist morphant embryos can be rescued to various extents by either twist (Figs. 6G–I) or snail2 (Figs. 6J–L) RNAs (supplemental table).

**Apoptotic effects associated with twist loss of function**

Both Snail2 (Slug) (Inukai et al., 1999) and Twist (Maestro et al., 1999) act as negative regulators of apoptosis. There is a dramatic increase in cell death, as visualized by TUNEL staining, in embryos injected with RNA encoding a dominant negative Snail2 protein...
(Tribulo et al., 2004) or the Snail2 morpholino (Zhang et al., 2006) (Fig. 7A). Injection of the Twist morpholino produced a similar increase in apoptosis in neurula stage embryos (Fig. 7C); no change in the number of TUNEL-positive cells was observed in control morpholino-injected embryos (data not shown). The Snail2-dependent increase in apoptosis was suppressed by the injection of Twist-GFP RNA (Fig. 7B) and the Twist-dependent increase in apoptosis was rescued by the injection of Snail2-GFP RNA (Fig. 7D). Snail2 and Twist may regulate apoptosis through similar mechanisms. Like Snail2 (Zhang et al., 2006), Twist positively regulates the levels of xbra and bcxl RNAs (Fig. 7E); bcxl encodes an anti-apoptotic bcl-2 family protein. Studies with GR-Twist-GFP indicate that this effect is dependent upon protein synthesis (data not shown). In contrast GR-Twist-GFP (Fig. 7F), like GR-Snail2-GFP (Zhang et al., 2006), negatively, and in a protein synthesis independent manner, regulates the level of caspase-9 RNA, which encodes a pro-apoptotic protein.

Feedback regulation of NF-κB Nodal by twist and snail2 regulation via Cerberus

Activation of GR-Slug-GFP leads to an indirect up-regulation of relA, rel2, and rel3 RNAs, while relA RNA levels and 3xκB RNAs (Fig. 8F) increased in response to a decrease in Snail2 RNA levels in an emetine-insensitive manner. In animal caps, both Cerberus and CerS RNAs (Fig. 8F) increased 3xκB-regulated 3xκB luciferase reporter activity; co-injection of either Cerberus or CerS RNAs blocked this effect. Injection of both Cerberus and CerS RNAs (Fig. 8F) increased 3xκB reporter activity, an effect that was blocked by both injection of both Cerberus and CerS RNAs (Fig. 8F). Nodal signaling acts through activation of

Fig. 7. At stage 16 there were few TUNEL-positive cells in control (uninjected or control morpholino-injected) embryos (data not shown). Unilateral injection of Snail2 (A) or Twist (C) morpholinos led to a dramatic increase in TUNEL staining. Co-injection of Twist-GFP RNA suppressed the increase in TUNEL-positive cells in Snail2 morphant embryos (B), while Snail2-GFP RNA suppressed TUNEL staining in Twist morphant embryos (D). Arrows indicate injected sides of the embryos, which are oriented anterior up. (E) Bilateral injection of the Twist morpholino leads to an increase in caspase-9 and a decrease in bcxl and xbra RNA levels. (F) In animal caps prepared from fertilized eggs injected with RNA encoding GR-Twist-GFP; when activated, there was an emetine-insensitive decrease in the levels of caspase-9 RNA. This effect is similar to that produced by Snail2 (Zhang et al., 2006).

Fig. 8. (A) Animal caps were prepared from fertilized eggs injected with RNA encoding GR-Twist; when activated, there was an emetine-insensitive decrease in the levels of cerberus RNAs. Quantitative RT-PCR analysis using GR-Twist-GFP (B) or GR-Snail2-GFP (C) indicates that both Twist and Snail2 inhibited the accumulation of cerberus RNA in a protein synthesis independent manner. (D) Injection of cerberus RNA reduced the level of the relA RNA as determined by RT-PCR. (E) In animal caps, both Cerberus and CerS (300 pg RNA/embryo) inhibited the activity of the 3xκB luciferase reporter, normalized to TK-Renilla luciferase activity; injection of a control GFP RNA (500 pg/embryo) had no effect on reporter activity. (F) Injection of xnr1 RNA activated the NF-κB-regulated 3xκB luciferase reporter, while co-injection of either cerberus or CerS RNAs blocked this effect. Injection of Sma2, Sma4b (G) or Sma4a (H) RNAs (500 pg/embryo) led to an increase in 3xκB luciferase reporter activity; co-injection of CerS RNA did not block Sma4b activation of the 3xκB reporter. The effect of Sma4a was reproducibly lower than that of either Sma2 or Sma4b.
Smads; injection of RNAs encoding Smad2, Smad4b (Fig. 8G), and to a lesser extent Smad4a (Fig. 8H) led to an increase in 3xκ-B reporter activity. As expected, since CerS acts extracellularly while Smads act intracellularly, co-injection of CerS RNA failed to inhibit Smad4b RNA induced activation of 3xκ-B (Fig. 8G). As described above, the increase in the level of relA RNA in response to the activation of GR-Smad2 was not inhibited by emetine (Fig. 1H).

**Discussion**

Based on the Twist morphant phenotype, we conclude that twist function is required for mesoderm formation in *Xenopus* as it is in *Drosophila*. Rather surprising, however, was the ability of Twist and Snail2 to regulate the accumulation of each other’s RNAs and to rescue each other’s loss of function phenotypes. This observation builds on the previously reported ability of Snail1 to rescue the Snail2 loss of function phenotype in *Xenopus* (Aybar et al., 2003; Carl et al., 1999; Zhang et al., 2006). Even partial functional redundancy between Twist, Snail2 and Snail1 may be developmentally and physiologically significant given their roles in the induction and progression of various forms of epithelial–mesenchymal transition and the metastasis of cancer cells (Gupta et al., 2005), the regulation of apoptosis, and their ability to generate stem cell like behaviors in immortalized human epithelial cells (Mani et al., 2008). For example, it is possible that the apparent absence of an early neural crest phenotype observed in snail and slug (snail2) double null mouse embryos (Murray and Gridley, 2006) may be due in part to the rescuing effects of Twist, or other proteins with similar functions, such as the Smad-interacting proteins (SIPs) which, like Twist, Snail2, and Snail1, bind to E-box sequences, display effects on epithelial–mesenchymal transitions similar to that of Twist/Snail2/Snail1 and regulate Smad activity (Papin et al., 2002; van Groenven et al., 2006; Verschueren et al., 1999). This conclusion is supported by the observation that the twist null phenotype in *Drosophila* is ameliorated by an early “burst” of snail expression and that over-expression of snail suppresses some aspects of the twist loss of function phenotype (Seher et al., 2007; Simpson, 1983).

In the context of cancer, our results suggest an unexpected connection to the report by Postovit et al. (2008) who reported that the inhibition of Nodal activity by human embryonic stem cells suppressed the “tumorigenic phenotype of aggressive cancer cells”. In *X. laevis* both Twist and Snail2 negatively regulate cerberus, which acts through its anti-Nodal activity to inhibit NF-κB activity. A similar regulatory loop, if it exists in tumor cells, would enhance both Nodal and NF-κB activity. Pushing the analogy with Xenopus further, increased Nodal and NF-κB activity would act to induce twist, snail2, and snail1 expression, leading to both migratory and anti-apoptotic phenotypes. While it is clearly important to remember that our understanding of the dynamics of the Nodal-NF-κB-Twist-Snail2-Snail1-Cerberus network in *Xenopus* is incomplete (Fig. 9), it does suggest that it could be a key driver of metastatic behavior in other systems.

The ability of both Snail2 and Twist to negatively regulate cerberus (a BMP inhibitor) was somewhat unexpected, given that Mayor et al. (2000) had reported that the injection of Snail2 RNA activated rather than repressed cerberus expression (although the effect of Snail2 on cerberus expression was weak, at least as judged by their Fig. 5G). In contrast, their observation that Snail2 RNA induced an increase in chordin expression is consistent with our observation of the loss of chordin expression in Snail2 morphant embryos. The system, however, is known to be complex. Based on microarray studies of Snail2 morphant embryos, we find that Snail2 negatively regulates the levels of sizzled RNA (data not shown). Sizzled, a secreted factor originally identified as a Wnt inhibitor (Salic et al., 1997) has more recently been found to negatively regulate Xolloid, which acts as an inhibitor of the BMP inhibitor Chordin (Lee et al., 2006). Snail2 could therefore act through sizzled, cerberus, and other regulatory targets to inhibit BMP activity in the most dorsal region of the late blastula/early gastrula stage embryo, and perhaps later in development as well.

How do we reconcile our results with previous studies, using dominant negative proteins, that suggested that slug and twist act downstream of snail during neural crest formation (Aybar et al., 2003)? First, all three genes are clearly expressed in the embryo prior to gastrulation with similar kinetics and a similar spatial distribution (Linker et al., 2000); all three are up-regulated in a Nodal/Snail2 dependent manner by VegT in animal caps. Whether they are regulated directly at the transcriptional level, or indirectly at the post-transcriptional level or via microRNAs requires further analysis. Second, as we reported previously, Snail2 is able to induce, but not sustain, the expression of a number of neural crest markers in animal caps (Zhang et al., 2006). Finally, it is clear that there is a mutual dependence and functional redundancy between the three proteins. Inhibition of snail2 and twist function blocks mesoderm and neural crest formation; preliminary studies, using anti-sense snail1 RNA injection, suggest that snail1 function is also required for mesoderm formation, and can rescue twist and snail2 loss of function phenotypes (data not shown).

The presence of an NF-κB-Nodal-Snail2/Twist-Cerberus feedback loop in *Xenopus* (Fig. 9) is reminiscent of the situation in *Drosophila*, where Snail inhibits expression of wntD, which encodes an inhibitor of NF-κB activity/dorsal expression (Ganguly et al., 2005; Gordon et al., 2005). While the mechanism of WntD action remains unclear, the effects are similar. In *Xenopus* the pathway appears to involve an intermediate, the secreted Nodal-associated proteins, which in turn regulate NF-κB at least in part through effects on relA expression. Given the regional differences in the distribution of the components of this feedback system, as well as the impact of other regionally localized factors and targets, it is difficult to predict the detailed behavior of the system without data that can be obtained only through an extensive regional and time-resolved analysis. That said, it is clear that in the absence of Twist or Snail2, the formation of mesoderm is greatly reduced, endodermal marker expression is expanded, neural
crest formation does not occur, myotome formation is disrupted, and apoptosis increases dramatically.

In sum, the discovery and initial characterization of the Nodal/NF-κB-Snail2/Twist-Cerberus network in Xenopus, and its similarity to the Nodal/Twist-Snail/WntD network in Drosophila, suggests that versions of these networks are involved in a wide range of cellular systems, ranging from cancer cells (Gupta et al., 2005; Postovit et al., 2008; Yang et al., 2004) to somatic stem cells (Mani et al., 2008), and that regulatory and therapeutic manipulations of this network are likely to produce complex outcomes that can only be predicted based on a more detailed spatial and temporal analysis and modeling of the underlying network.

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Appendix A. Supplementary data


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