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SoxE Factors Function Equivalently during Neural Crest and Inner Ear Development and Their Activity Is Regulated by SUMOylation

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Summary

Sox9 and the closely related factor Sox10 are essential for the formation of neural crest precursor cells, and play divergent roles in the process by which these cells are subsequently directed to form specific derivatives. These group E Sox factors have also been implicated in the development of the vertebrate inner ear. Despite their importance, however, the mechanisms that allow SoxE proteins to regulate such a diverse range of cell types have remained poorly understood. Here we demonstrate that during vertebrate development, the activities of individual SoxE factors are well conserved and are regulated by SUMOylation. We show that SoxE mutants that cannot be SUMOylated, or that mimic constitutive SUMOylation, are each able to mediate a subset of the diverse activities characteristic of wild-type SoxE proteins. These findings provide important mechanistic insight into how the activity of widely deployed developmental regulatory proteins can be directed to specific developmental events.

Introduction

Sox proteins are high mobility group (HMG) family transcription factors that regulate diverse developmental processes (Wegner, 1999). The Sox family is divided into subgroups A–J that possess highly homologous HMG-type DNA binding domains but share little overall homology outside of this region (Bowles et al., 2000). All Sox proteins possess transcriptional activation domains; however, they bind DNA with low affinity, and are thought to require DNA binding cofactors to stabilize their interactions with DNA (Kamachi et al., 2000). Several reports have now identified partner proteins that can interact with Sox family members in order to synergistically activate transcription (Bondurand et al., 2000; Lang and Epstein, 2003). Members of Sox group E, comprised of *Sox8*, *Sox9*, and *Sox10*, are further characterized by the presence of two conserved regions (termed E1 and E2) that have been proposed to be protein-protein interaction domains (Bowles et al., 2000).

SoxE factors have emerged as important regulators of the neural crest, a population of migratory, tissue-invasive stem cells that plays a central role in the development of the vertebrate body plan. Neural crest cells migrate extensively, populate diverse regions throughout the embryo, and give rise to a wide range of derivatives that includes most of the neurons and glia of the peripheral nervous system (PNS), melanocytes, and craniofacial cartilage. At neural plate stages, *Sox9* and *Sox10* are expressed by all neural crest precursor cells, although the expression of *Sox9* precedes that of *Sox10* (Aoki et al., 2003; Lee et al., 2004). Later, expression of these two factors in neural crest derivatives becomes nonoverlapping, with *Sox9* expression maintained in cells contributing to facial cartilage and *Sox10* expression restricted to presumptive melanoblasts and glia (Spokony et al., 2002; Aoki et al., 2003; see Figure S1 in the Supplemental Data available with this article online). In vivo, Sox9 and Sox10 likely play divergent roles in the transcriptional control of these different fates (Southard-Smith et al., 1998; Britsch et al., 2001; Stolt et al., 2002); however, the extent to which this is due to any distinct activities possessed by these factors has remained unclear.

In *Xenopus*, morpholino-mediated depletion of either Sox9 or Sox10 leads to a loss of neural crest precursor formation, while overexpression of Sox9 or Sox10 can lead to expansion of the neural crest progenitor domain (Spokony et al., 2002; Aoki et al., 2003; Honore et al., 2003). Thus, by the criteria of their effects on neural crest precursor formation, these two closely related SoxE factors appear to have similar activities. However, it has been reported that overexpression of Sox10 but not Sox9 induces melanocyte formation (Aoki et al., 2003), indicating that each of these factors may also possess some distinct activities. Studies using a number of model organisms have also implicated SoxE factors in inner ear development (Watanabe et al., 2000; Liu et al., 2003; Saint-Germain et al., 2004). In *Xenopus*, *Sox9* is the earliest marker of the newly induced otic placode, which will give rise to the inner ear, and morpholino-mediated depletion of Sox9 leads to a failure of inner ear formation (Saint-Germain et al., 2004). The effects of upregulating SoxE activity on inner ear development have yet to be reported.

Given their overlapping expression patterns and proposed roles in neural crest precursor formation, neural crest lineage diversification, and otic placode formation, it seemed possible that individual SoxE factors might possess some divergent activities, and that the complement of SoxE factors expressed in a cell might play an instructive role in dictating that cell's fate. To test this hypothesis, we expressed Sox9 or Sox10 in early *Xenopus* embryos and compared and contrasted their activities. We found that each factor could direct the formation of neural crest precursors and the development of a range of neural crest derivatives, and we detected no differences in the activities of Sox9 and Sox10 in these assays. Moreover, we found that misexpression of Sox9 or Sox10 frequently resulted in the formation of enlarged or ectopic otocysts, demonstrating that both factors are potent effectors of inner ear formation. A central question in developmental biology is how factors with conserved activity can mediate very different functional outcomes when expressed in dif-

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ferent tissues. To gain insight into the mechanisms by which SoxE proteins might be regulated such that they can direct development of diverse cell types, we carried out a yeast two-hybrid screen that identified SUMO-1 and UBC9 as SoxE-interacting proteins. We report here that the SUMOylation state of SoxE proteins plays a key role in regulating the function of these factors during neural crest and inner ear development. Our findings suggest a novel mechanism by which the activity of these widely deployed developmental regulatory proteins might be directed to specific developmental events.

Results

Sox9 and Sox10 Have Equivalent Effects on Neural Crest Formation

Although Sox9 and Sox10 have recently emerged as key determinants of both neural crest and inner ear development, the extent to which SoxE factors play functionally equivalent roles in these processes has been less clear. We therefore expressed these factors in early *Xenopus* embryos and compared their ability to influence neural crest and inner ear fates. To control for dosage-dependent effects, epitope-tagged forms of both Sox9 and Sox10 were generated. The activities of the tagged and untagged forms of each protein were compared, and the epitope tags were found to have no effect on function (data not shown). Experiments directly comparing Sox9 and Sox10 activity were carried out using the epitope-tagged factors, and protein expression levels were monitored via Western blots. mRNA encoding epitope-tagged Sox9 or Sox10 was injected into one cell of two-cell stage *Xenopus* embryos, and the injected embryos were allowed to develop to neurula stages, when the effects on neural crest precursor cells could be assayed by in situ hybridization. β-galactosidase mRNA was coinjected as a lineage tracer, and the uninjected side of the embryo served as a control for normal development.

We found that both Sox9 and Sox10 were able to increase the formation of neural crest precursor cells, as evidenced by expanded expression of markers such as *Slug* and *Sox9* (Figures 1A–1D), consistent with previous reports (Aoki et al., 2003; Honore et al., 2003). Significantly, however, we also noted that neural crest markers were sometimes inhibited in Sox9- and Sox10-injected embryos. For example, in an experiment in which most embryos showed expanded expression of neural crest markers, we also noted embryos in which expression of the same markers had been inhibited (Figures 1E and 1F) (Sox9-injected: 75% increased, 10% decreased, n = 47; Sox10-injected: 68% increased, 8% decreased, n = 38). This suggested that in the embryos showing a decrease, Sox9 or Sox10 activity might have led to the formation of some other cell type at the expense of neural crest precursors. When in the same experiment 5-fold higher levels of Sox9 or Sox10 were expressed, this inhibition of neural crest precursor formation was noted more frequently than at lower doses (Sox9-injected: 45% increased, 34% decreased, n = 29; Sox10-injected: 40% increased, 45% decreased, n = 40). Importantly, we observed no differences in the abilities of Sox9 and Sox10 to expand or inhibit the formation of neural crest precursor cells. Moreover, the observation that the differences in these phenotypes were not fully dose dependent suggested the possibility that the activity of SoxE proteins might be dynamically regulated, perhaps by posttranslational modification.

Sox9 and Sox10 Promote Melanocyte and Glial Formation and Inhibit Neuronal Differentiation

We next compared the ability of Sox9 and Sox10 to direct the formation of specific neural crest derivatives. In particular, we were interested in comparing the effects of these two proteins on development of melanoblasts and glia, as previous studies have suggested that Sox10 is an important determinant of these lineages (Southard-Smith et al., 1998; Kelsh et al., 2000; Britsch et al., 2001; Stolt et al., 2002). Sox9 and Sox10 were expressed at levels that predominantly expand the neural crest progenitor pool, and injected embryos were harvested at tailbud stages for in situ hybridization with markers of developing melanoblasts or allowed to develop to stages when formation of differentiated melanocytes could be directly evaluated. In these experiments, we found that both Sox9 and Sox10 potently induced the formation of melanoblasts as evidenced by scattered ectopic cells on the embryonic flank that expressed melanocyte markers such as *Sox10*, *Mitf*, and *Trp2* (Figures 1G and 1H and data not shown; Sox9-injected: 51% of embryos, n = 129; Sox10-injected: 53% of embryos, n = 103), as well as by the formation of supernumerary melanocytes at swimming tadpole stages (see below, and data not shown).

At the stages examined in these experiments, *Sox10* expression marks three cell populations: melanoblasts, glia, and the developing ear. In addition to numerous ectopic melanoblasts in caudal regions, we noted dramatically increased *Sox10* expression in cranial regions. Such staining is consistent with an increased number of cells adopting glial and/or otic fates. To distinguish between these possibilities, we examined expression of *FoxD3* at stage 28, when it serves as a glial marker (Kelsh et al., 2000; Gilmour et al., 2002). We found that both Sox9- and Sox10-injected embryos showed significantly enhanced *FoxD3* staining in the cranial ganglia (Figures 1I, 1J, 1M, and 1N) (Sox9-injected: 69% increased, n = 55; Sox10-injected: 74% increased, n = 46). Together, these findings indicate Sox9 and Sox10 can both direct the formation of at least two cell types commonly associated with Sox10 function, melanocytes and glia.

Recent work has implicated Sox10 in the maintenance of multipotency in neural crest stem cells and in the inhibition of neuronal differentiation (Paratore et al., 2002; Kim et al., 2003). While these studies did not address whether other SoxE factors had similar activities, more recent work has demonstrated that forced expression of Sox9 blocks neuronal differentiation of neural crest cells in avian embryos (Cheung and Briscoe, 2003). We therefore asked whether Sox9 or Sox10 could influence the adoption of neuronal fates in *Xenopus* neural crest cells. We found that both factors inhibited the differentiation of *N-tubulin*-expressing cells in

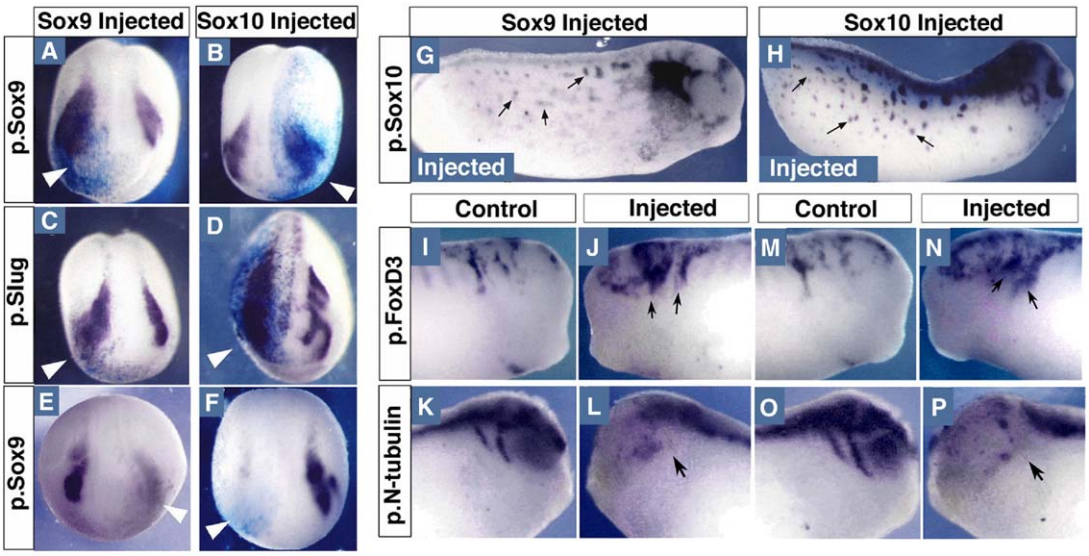

Figure 1. Effects of Sox9 and Sox10 on Neural Crest Development

(A–F) In situ hybridization examining the expression of neural crest markers *Sox9* (A, B, E, and F) and *Slug* (C and D) in Sox9- (A, C, and E) or Sox10- (B, D, and F) injected embryos. Injection of either Sox9 or Sox10 frequently leads to an increase in neural crest precursor formation (A–D, white arrowhead) but could also result in loss of neural crest precursor formation (E and F, white arrowhead).

(G and H) Both Sox9 and Sox10 induce the formation of supernumerary and ectopic melanocytes (black arrows).

(I–P) An increase in *FoxD3*-expressing glia (I versus J; M versus N) and a decrease in *N-tubulin*-expressing neurons in the cranial ganglia (K versus L; O versus P) are found in Sox9- (I–L) and Sox10- (M–P) injected embryos. Light blue or red stain is the lineage tracer β-gal.

the cranial ganglia, even at doses that significantly increased formation of *FoxD3*-expressing glia in sibling embryos (Figures 1K, 1L, 1O, and 1P) (Sox9-injected: 69% decreased, n = 49; Sox10-injected: 72% decreased, n = 42).

Sox9 Can Rescue Neural Crest Formation in Sox10-Depleted Embryos

The above data demonstrate that in overexpression assays, Sox9 and Sox10 are functionally equivalent with respect to their ability to mediate neural crest formation. However, these experiments do not exclude the possibility that these factors perform equivalently in these assays secondary to an ability to positively cross-regulate each other's expression. For example, the ability of Sox9 to induce pigment cells might be due to its ability to induce expression of endogenous Sox10, which would then initiate a program of melanocyte differentiation. To explore this possibility, we compared the ability of Sox9 and Sox10 to rescue neural crest development in embryos depleted for Sox10. Embryos injected at the eight-cell stage with morpholinos targeting Sox10 show reduced or absent expression of early neural crest markers (Figures 2A and 2B; 90% inhibited, n = 59). Subsequent injection of mRNA encoding either Sox9 or Sox10 significantly rescued formation of neural crest progenitors in these embryos (Figures 2C–2F; Sox9: 18% inhibited, n = 40; Sox10: 21% inhibited, n = 52). These findings confirm that Sox10 does not have functions during neural crest precursor formation that cannot be compensated for by Sox9. Importantly, when rescued embryos were allowed to develop to tailbud stages, we found that Sox9 was still able to induce formation of ectopic melanocytes despite Sox10 depletion (Figures 2I and 2J). This finding demonstrates that the ability of Sox9 to direct melanocyte formation is not secondary to an ability to activate *Sox10*.

Sox9 and Sox10 Direct the Formation of Enlarged and Ectopic Otic Vesicles

Because SoxE factors have also been implicated in otic placode formation, we compared the effects of Sox9 and Sox10 activity on inner ear development. The consequences of SoxE misexpression on the development of this cell type have not been previously reported. We found that injection of mRNA encoding either factor frequently led to expanded expression of otocyst markers, or "enlarged ears" (Figures 3A and 3B) (Sox9-injected: 55% enlarged, n = 94; Sox10-injected: 61% enlarged, n = 154). Moreover, in 3%–5% of cases, Sox9- and Sox10-injected embryos developed supernumerary otocysts, such that between two and four distinct otic vesicles formed on a single side of the injected embryo (Figures 3C, 3D, and 3F). To ask whether the appearance of enlarged or ectopic otic vesicles correlated with an increase in size of the otic placode at neural plate stages, we examined the expression of *Pax8*, which is among the earliest markers of this structure (Heller and Brandli, 1999). We found that both Sox9 and Sox10 could expand the domain of *Pax8* expression corresponding to this placode (Figures 3G and 3H) (Sox9-injected: 52% increased, n = 50; Sox10-injected: 48% increased, n = 62). Similarly, both factors could mediate increased *Dlx3* expression in the developing ear at stage 25 (data not shown). Importantly, however, Sox9 and Sox10 did not differ in their ability to induce

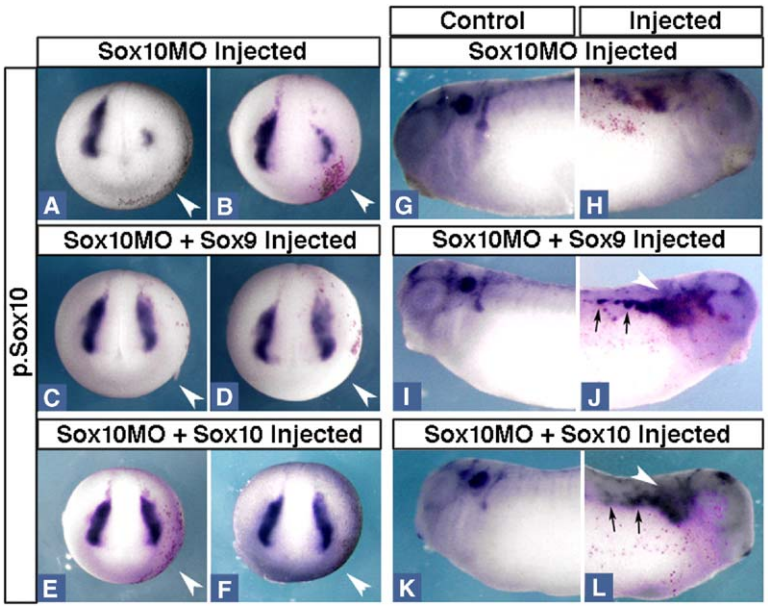

Figure 2. Sox9 and Sox10 Rescue Neural Crest Development in Sox10-Depleted Embryos

Embryos injected with Sox10 morpholinos (MO) show reduced or absent expression of early neural crest markers such as *Sox10* (A and B) and reduced otic vesicles and cranial neural crest (G and H). These defects could be rescued equally well by Sox9 or Sox10 (C–F and I–L). Sox9 retains its ability to induce ectopic melanocytes in Sox10-depleted embryos (J, black arrows). Light red stain is the lineage tracer β-gal.

the formation of enlarged or ectopic ears, providing further evidence that these factors function equivalently.

UBC9 and SUMO-1 Are SoxE-Interacting Factors

The above findings further highlight the question of how the activity of SoxE family proteins is regulated during the formation of diverse cell types such as the neural crest or otic placode. To address this, we sought to identify interacting proteins that might modify the function of these proteins during development. We carried out a yeast two-hybrid screen, using a Sox10 cDNA truncated before the activation domain as bait and a *Xenopus* gastrula stage cDNA library as prey. This screen identified a number of SoxE-interacting proteins, among which were 7 isolates of the small ubiquitin-like modifier SUMO-1 and 27 isolates of the E2 SUMO-conjugating enzyme UBC9, and these factors were also found to interact when the Sox9 N-terminus was used as bait (Figure 4A and data not shown). Together, these findings suggested the intriguing possibility that SoxE activity might be regulated posttranslationally via SUMOylation.

Examination of the *Xenopus* Sox9 and Sox10 sequences showed that they each possess two conserved SUMOylation consensus sites, ΨKXE (Gill, 2004). The first of these sites is located just N-terminal to the E1 domain, while the second site is located within the C-terminal activation domain (Figure 4B). Because not all proteins containing this motif are SUMOylated in vivo (Hay, 2005), we asked whether *Xenopus* Sox9 and Sox10 are SUMOylated in *Xenopus* embryos. When Sox9 or Sox10 were expressed in the presence or absence of epitope-tagged SUMO-1, Western blot analysis indicated that Sox9 and Sox10 could each be SUMOylated on two sites, and that each modification leads to a distinct and distinguishable shift in mobility on SDS-PAGE gels (Figures 4C and 5A). Blotting against the myc tag on Sox9 or Sox10 following immunoprecipitation of SUMO-1 (flag) confirmed that the slower mobility SoxE isoforms represent SUMOylated products (Figure 3D), and SUMO modification of Sox9 has also recently been observed in human embryonic kidney cells (Komatsu et al., 2004). We found that at gastrula stages, Sox10 is SUMOylated at lysine 44 in the N terminus as well as at lysine 333 in the activation domain. In Sox9, the predominant site of SUMOylation is lysine 365 in the C terminus, although the protein can also be SUMOylated on lysine 61. The identities of these modified residues were confirmed by mutating one or both reactive lysines in the hypothesized SUMOylation sites to arginine in order to block their SUMOylation. Experiments in which Sox9 and Sox10 isoforms carrying these lysine mutations were coexpressed with SUMO-1 in early embryos confirmed that these were the only SUMOylation sites in these proteins (Figures 5B and 5C). Consistent with this, Sox9 and Sox10 proteins lacking these lysine residues no longer interact with SUMO-1 or UBC9 in yeast (Figure 4A). Interestingly, when only a single modified lysine in Sox10 was mutated, only one of the two slower migrating Sox10 species was lost (Figure 5C). These findings indicate that the mobility differences apparent by SDS-PAGE reflect which site in the protein has been SUMOylated, rather than the number of SUMO moieties appended.

SUMOylation Modulates SoxE Function during Neural Crest Development

More than half of all identified SUMO substrates are transcriptional regulatory proteins, and SUMOylation can either up- or downregulate the activity of these factors (Girdwood et al., 2004). Having confirmed biochemically that SoxE proteins are SUMOylated in *Xenopus* embryos, we sought to better understand how SUMOylation modulates SoxE function during neural crest development. We initially focused these studies on Sox9, as this protein displayed only one predomi-

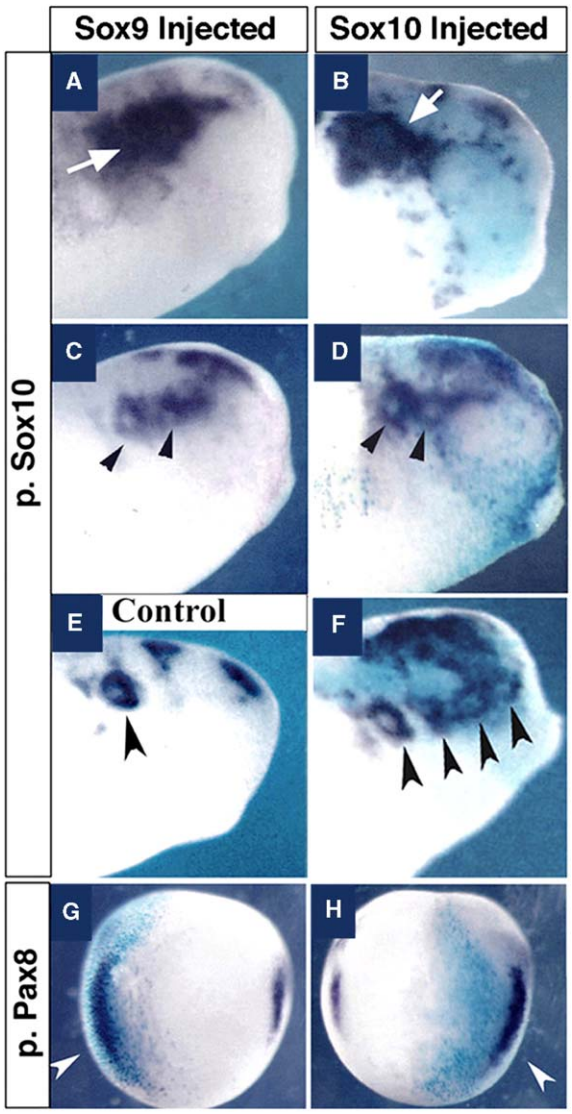

Figure 3. Sox9 and Sox10 Induce Enlarged and Ectopic Ear Structures

(A–F) In situ hybridization showing *Sox10* expression in the ears of stage 28 embryos injected with Sox9 (A and C) or Sox10 (B, D, and F). Expression of either of these factors consistently leads to the formation of enlarged ears (A and B, white arrows) and/or to the formation of one or more ectopic ears (C, D, and F, black arrowheads).

(G and H) Increased expression of *Pax8*, which marks the otic placode (white arrowhead) in Sox9- (G) or Sox10- (H) injected embryos at stage 13. Light blue or red stain is the lineage tracer β-gal.

nant site of SUMO incorporation. In addition to our loss-of-SUMOylation mutants, we generated a form of Sox9 which had SUMO-1 fused in-frame to its C terminus (Figure 6A). Such fusions have been found to mimic the constitutively SUMOylated state of a protein and have proven particularly useful when the native SUMOylation site occurs near the terminus of a protein of interest, as it is in Sox9 (Ross et al., 2002; Holmstrom et al., 2003; Long et al., 2004). In order to facilitate interpretation of these experiments, we ensured that the fused SUMO moiety would represent the only SUMOylation of the protein by appending it to the double-lysine mutant to create Sox9^{K61,365R}/SUMO-1.

We first wished to ascertain whether the SUMOylation mutants affected Sox9 protein stability, as SUMOylation of targeted lysines in some proteins regulated by ubiquitin-dependent proteolysis results in stabilization of those proteins (Hay, 2005). We did not expect this to be the case for Sox9 and Sox10, however, as we have found these proteins to be very stable when expressed in *Xenopus*, and have been unable to detect their ubiquitination (data not shown). Nevertheless, to directly ask whether blocking SUMOylation alters Sox9 stability, we expressed wild-type Sox9 and Sox9^{K61,365R} such that equivalent protein levels were detected at blastula stages, and then compared the expression levels of these proteins over time. We detected no difference in protein stability between wild-type Sox9 and the double-lysine mutant (Figure 5D and data not shown), making it unlikely that SUMOylation regulates SoxE stability in this system.

To examine the effects of Sox9 SUMOylation on neural crest development, mRNA encoding wild-type Sox9, Sox9^{K61,365R}, or Sox9^{K61,365R}/SUMO-1 was injected into one cell of two-cell stage embryos and effects on neural crest precursor cells were assayed by in situ hybridization at neurula stages. These proteins were epitope-tagged and were expressed at equivalent levels as determined by Western blot. When the expression of markers such as *Slug* and *Sox10* were examined in these embryos, we found that Sox9^{K61,365R} and Sox9^{K61,365R}/SUMO-1 had dramatically different effects on neural crest precursor formation. For example, when expressed at levels at which the wild-type protein mediates a modest increase in *Sox10* expression, Sox9^{K61,365R} consistently induced a dramatic increase in *Sox10* expression (97% increased, n = 81). Conversely, Sox9^{K61,365R}/SUMO-1 strongly inhibited *Sox10* expression (97% inhibited, n = 82) (Figure 6B). Given that wild-type SoxE proteins can both positively and negatively influence the formation of neural crest progenitors (Figure 1), these results suggest the SUMOylation state of the expressed protein may be an important determinant of these different outcomes.

Sox9^{K61,365R} and Sox9^{K61,365R}/SUMO-1 were also found to differentially affect the formation of neural crest derivatives. For example, as with wild-type Sox9, embryos injected with Sox9^{K61,365R} developed numerous scattered *Sox10*-expressing melanoblasts on the injected side of the embryo (92%, n = 121). By contrast, Sox9^{K61,365R}/SUMO-1 was unable to induce ectopic melanoblasts (0%, n = 162) (Figure 6C, black arrows). Similarly, both wild-type Sox9 and Sox9^{K61,365R} mediate the formation of supernumerary differentiated melanocytes on the injected side of the embryo, while Sox9^{K61,365R}/SUMO-1 does not (Figure 6D). Also like wild-type Sox9, we found that Sox9^{K61,365R} could rescue neural crest development in Sox10-depleted embryos (Figure 6E; Sox10MO: 93% inhibited, n = 28; Sox10MO+Sox9^{K61,365R}: 29% inhibited, n = 34). Importantly, however, Sox9^{K61,365R}/SUMO-1 is not inactive in these assays, as it strongly induced ectopic Sox10 expression in cranial regions (Figure 6C, white arrowheads). Some of this staining was consistent with en-

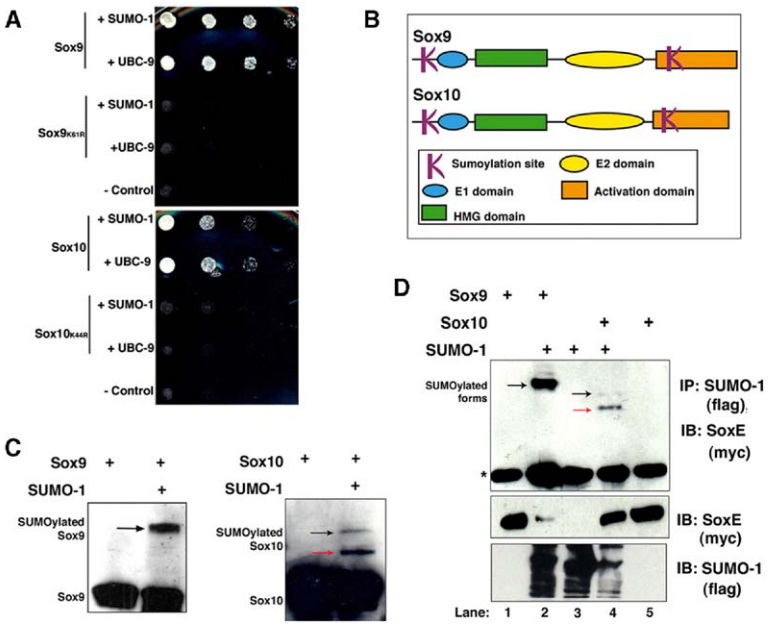

Figure 4. Sox9 and Sox10 Are SUMOylated

(A) Growth of serially diluted cultures on selective media showing that both Sox9 and Sox10 (activation domain deleted) interact with UBC9 and SUMO-1 in a yeast two-hybrid assay. Mutation of the SUMOylation sites prevents interaction.

(B) Schematic of SoxE protein domains.

(C) Western blot of lysates prepared from embryos injected as noted showing modification of Sox9 or Sox10 with SUMO-1. Arrows mark reduced mobility forms resulting from conjugation to one or more SUMO moieties.

(D) Lysates from embryos injected with Sox9 or Sox10 alone, or together with SUMO-1, were immunoprecipitated (IP) with antibodies against the epitope tag in SUMO-1 (flag) and then immunoblotted (IB) using antibodies against the epitope tag on the SoxE factors (myc), confirming that more slowly migrating SoxE species are SUMOylated. Direct immunoblotting of the crude lysate with either myc or flag antibodies served as input control (bottom panels).

larged otocysts, although staining corresponding to an increase in cranial glia was also apparent (Figure S3). To confirm that our findings with Sox9 SUMOylation mutants were generally applicable to SoxE factors, we made an analogous set of mutations in Sox10 to create Sox10^{K44,333R} and Sox10^{K44,333R}/SUMO-1. We found that the effects of overexpressing these Sox10 SUMOylation mutants closely mimicked the effects of their Sox9 counterparts (Figure S2).

SUMOylation of SoxE Proteins Is Important for Inner Ear Development

Given our findings that SoxE proteins could mediate otic placode formation, we compared the ability of Sox9^{K61,365R} or Sox9^{K61,365R}/SUMO-1 to modulate development of this tissue. In contrast to its effects on neural crest precursor formation, we found that Sox9^{K61,365R}/SUMO-1 increased the size of the otic placode, as evidenced by an expanded domain of *Pax8* expression (47% increased, n = 44). Conversely, Sox9^{K61,365R} inhibited *Pax8* expression in placodal regions (61% decreased, n = 55) (Figure 6F). Consistent with this, when we examined expression of *Dlx3* in the otic vesicle at tailbud stages, we found that Sox9^{K61,365R} inhibited its expression (50% decreased, n = 51), whereas Sox9^{K61,365R}/SUMO-1-injected embryos frequently showed expanded regions of *Dlx3* expression (55% increased, n = 47) (Figure 6G). These

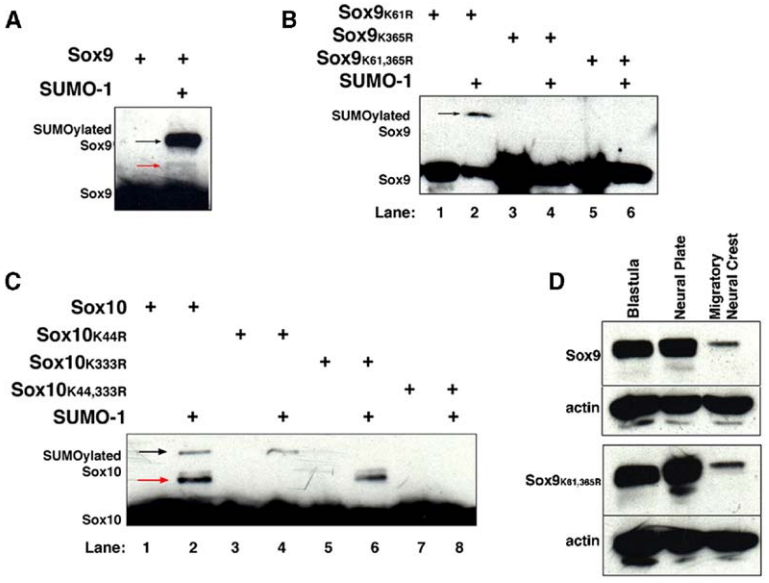

Figure 5. Identification of SoxE SUMOylation Sites

(A) Western blot of lysates prepared from stage 11 embryos expressing wild-type or lysine mutant Sox9 proteins. One major and one minor SUMO-modified form of Sox9 are noted at this stage (black and red arrows).

(B) Mutation of the reactive lysine in the C-terminal SUMOylation site eliminates the major modified species.

(C) Western blot of lysates prepared from embryos expressing wild-type or lysine mutant Sox10 proteins. Two SUMO-modified forms of Sox10 of different mobilities are noted (black and red arrows). Each single lysine mutation eliminates only a single one of these species.

(D) Western blot showing that mutation of SUMOylation sites does not affect Sox9 protein accumulation.

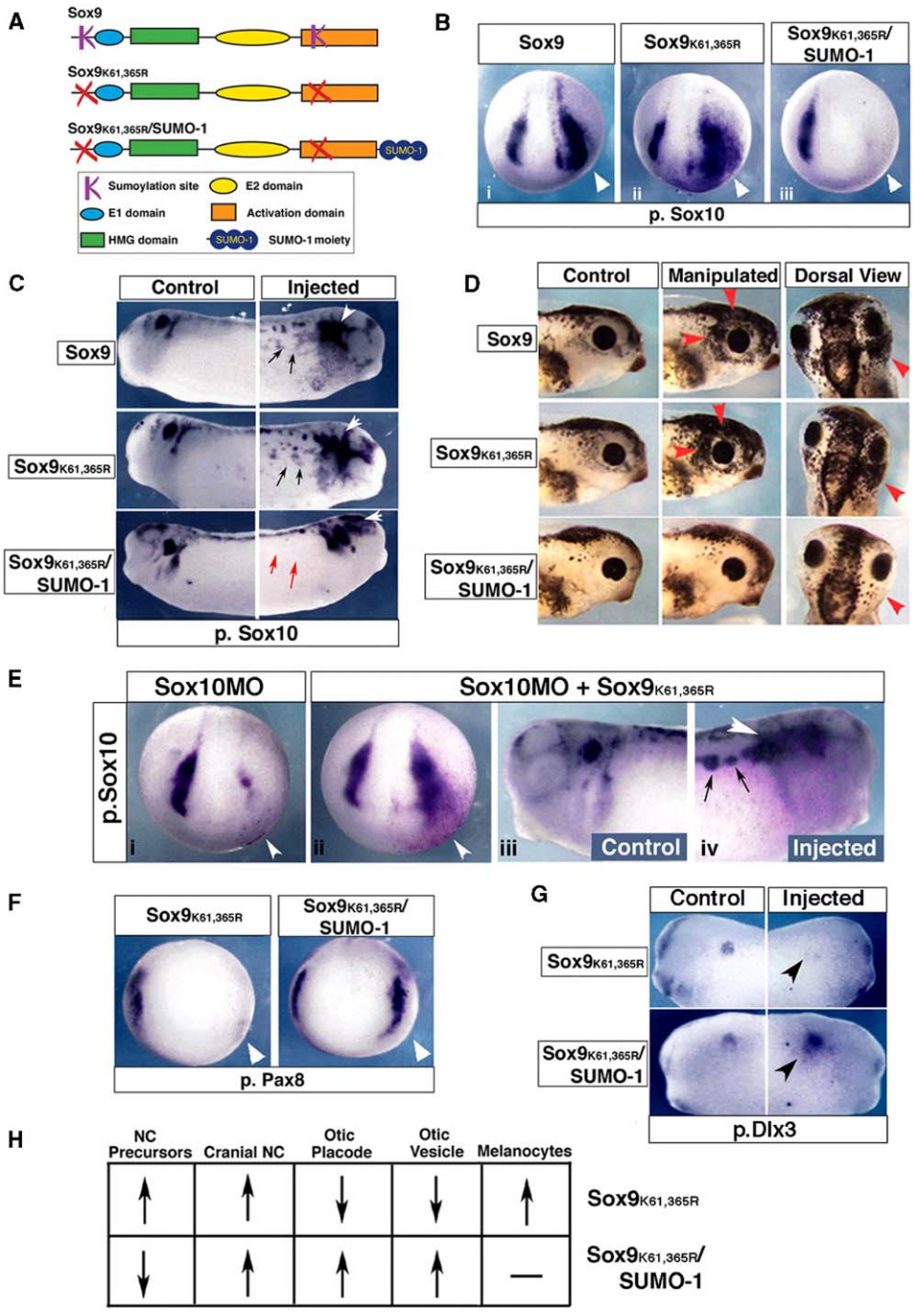

Figure 6. Regulation of Sox9 Activity by SUMOylation

(A) Schematic showing expressed Sox9 isoforms.

(B) In situ hybridization showing neural crest expression of *Sox10* in Sox9- (i), Sox9^{K61,365R}- (ii), and Sox9^{K61,365R}/SUMO-1- (iii) injected embryos.

(C) In situ hybridization showing ectopic *Sox10*-expressing melanoblasts (black arrows) in stage 28 embryos injected with wild-type Sox9 or Sox9^{K61,365R}. Sox9^{K61,365R}/SUMO-1-injected embryos never develop ectopic melanoblasts (note absence of these cells in region of red arrows) but do show increased *Sox10* expression in cranial regions (white arrowheads; compare injected and control sides of same embryos).

(D) Close ups of the heads of the swimming tadpoles injected with wild-type Sox9, Sox9^{K61,365R}, or Sox9^{K61,365R}/SUMO-1. Both wild-type Sox9 and Sox9^{K61,365R} mediate formation of supernumerary differentiated melanocytes on the injected sides of the embryos (red arrowheads), while Sox9^{K61,365R}/SUMO-1 is unable to do so.

(E) Sox9^{K61,365R} can rescue early (ii) and late (iv) aspects of neural crest formation in Sox10MO-injected embryos (white arrowheads), and retains its ability to induce ectopic melanocytes (iv, black arrows).

(F) *Pax8* expression in the otic placode of stage 13 embryos previously injected with Sox9^{K61,365R} or Sox9^{K61,365R}/SUMO-1.

(G) *Dlx3* expression, which marks the otocyst, in stage 28 embryos previously injected with Sox9^{K61,365R} or Sox9^{K61,365R}/SUMO-1.

(H) Table summarizing the effects of Sox9^{K61,365R} and Sox9^{K61,365R}/SUMO-1 in these assays. In panels showing in situ hybridization, light red or blue stain is the lineage tracer β-gal.

findings underscored that the constitutively SUMOylated form of Sox9 has activities that are distinct from those possessed by the unmodified protein. Moreover, our results strongly suggest that the SUMOylation state of SoxE factors plays a central role in determining whether these factors will mediate neural crest or otic placode formation, most likely by regulating their choice of partner interactions.

Discussion

Work in a number of model organisms has detailed the expression domains of individual SoxE factors and the embryonic consequences resulting from the loss of this expression (reviewed in Hong and Saint-Jeannet, 2005). By contrast, very little is known about the comparative activities of these factors. Sox9 and Sox10, together with Sox8, most likely arose via duplication of a single ancestral SoxE factor, and the most commonly employed explanations for the retention of such duplicate genes during vertebrate evolution are neofunctionalization and subfunctionalization. Neofunctionalization assumes that the duplication event frees one copy of the gene from selective pressure to maintain essential functions, allowing this copy to evolve new functions. While examples of neofunctionalization have been reported (McClintock et al., 2001), subfunctionalization may be a more common explanation for the high retention rate of duplicate genes. Under the duplication-degeneration-complementation (DDC) model of subfunctionalization, the reciprocal loss of aspects of the ancestral expression pattern in each of the duplicates could account for the selective pressure to maintain both copies (Lynch and Force, 2000).

Although *Sox9* and *Sox10* are initially expressed in all neural crest precursors in *Xenopus*, their later expression is restricted to distinct subsets of neural crest derivatives in all model organisms examined, consistent with a role for subfunctionalization in retaining these paralogs subsequent to their duplication. While neofunctionalization suggests some degree of functional divergence, duplicate genes maintained as a result of subfunctionalization are likely to retain similar activities. To ask whether this is the case for Sox9 and Sox10, we compared their activities by expressing them in early *Xenopus* embryos and assaying their effects on neural crest and inner ear development. We also compared their abilities to rescue morpholino-mediated depletion of Sox10. Significantly, our experiments detected no differences in the activity of these factors. However, in the course of this work, we uncovered several previously unreported activities possessed by these factors. First, we found that in addition to its previously described ability to induce the formation of neural crest progenitor cells, Sox9 can also induce the formation of both melanocytes and cranial glia (Figure 1), cell types typically associated with Sox10 function. Most strikingly, we found that both Sox9 and Sox10 were capable of inducing the formation of enlarged or ectopic ears (Figure 3).

Because our findings strongly suggested that the functional activity of SoxE paralogs has been largely conserved, we sought to determine how these proteins might be regulated such that they can direct the formation of cell types as distinct as the neural crest and otic placode. The identification of UBC9 and SUMO-1 as SoxE-interacting proteins suggested one mechanism by which the activity of these factors might be regulated posttranslationally, and we confirmed biochemically that both of these factors undergo SUMOylation in *Xenopus* embryos (Figures 4 and 5). While Sox10 can be modified at two sites, Sox9 is predominantly modified on a single site in the C terminus (K365), and our experiments to date have failed to identify functional consequences of mutating the N-terminal SUMO acceptor site of either factor (our unpublished data). While we cannot rule out that SUMOylation at this site modifies SoxE function in a manner not detected by our assays, in lamprey, the prototype model for the basal vertebrate, all three identified SoxE paralogs have only the C-terminal SUMOylation consensus site (D. McCauley, personal communication), emphasizing the primary importance of this site.

Although SUMOylation was first described as a posttranslational modification almost 10 years ago, the cellular consequences of SUMO modification remain poorly understood at the molecular level, and appear to differ significantly from substrate to substrate. Moreover, SUMO modification of different sites within the same protein can have different consequences for that protein's activity (Poukka et al., 2000; Gill, 2004; Hay, 2005). Among the reported effects of SUMOylation on transcription factors are the modulation of protein-protein interactions, protein-DNA interactions, and protein localization, as well as the regulation of protein stability via antagonization of ubiquitination (Gill, 2004; Hay, 2005). The consequences of SUMOylation for transcriptional activity are also diverse. In a number of cases, SUMO modification of transcriptional activators inhibits their potency as activators (Girdwood et al., 2003; Gill, 2004); however, SUMO modification of other proteins leads to an increased ability to activate transcription (Gostissa et al., 1999; Rodriguez et al., 1999; Goodson et al., 2001; Hong et al., 2001).

The consequences of SUMO modification can also be highly context dependent. For example, on some promoters, SUMOylation of Smad4 results in transcriptional repression, while on other promoters this modification has been found to enhance Smad4-dependent transcriptional activation (Long et al., 2004). Once the effects of SUMOylation are better understood, it may prove to be the case that, like other aspects of transcriptional regulation, such promoter context-dependent effects are the rule rather than the exception. Indeed, given our findings that SoxE isoforms that cannot be SUMOylated or that mimic a constitutively SUMOylated state have distinct effects on neural crest and otic placode formation, cellular context appears likely to play an important role in determining SoxE function in these two cell types.

No direct targets of SoxE transcriptional regulation have been identified at early stages of neural crest development; however, a number of promoters are known to be directly regulated by one or more of these factors during neural crest differentiation. During melanocyte development, Sox10 binds the Dct/Trp2 promoter in cooperation with MITF (Potterf et al., 2001; Ludwig et al.,

2004; Murakami and Arnheiter, 2005), whereas during the development of glia this factor regulates both the Po and MBP promoters (Peirano et al., 2000; Stolt et al., 2002, 2004). Similarly, Sox9 binds to and regulates the Col2a1 promoter during chondrogenesis (Lefebvre et al., 1997; Tsuda et al., 2003). In all cases where they have been examined molecularly, SoxE factors have been shown to function as transcriptional activators on target promoters. It will therefore be important to determine how overexpression of constitutively SUMOylated forms of Sox9 or Sox10 leads to such a dramatic downregulation of early neural crest markers such as Sox10 and Slug (Figure 6; Figure S2). An important first step in this direction will be identification of direct targets of SoxE regulation during these stages.

Insight into potential mechanisms via which SUMO modification might regulate SoxE activity may also derive from recent work on MITF. In that study, SUMO modification of MITF was found to have no effect on the regulation of promoters containing a single MITF binding site but suppressed synergistic activation of promoters with multiple binding sites (Murakami and Arnheiter, 2005). If SUMOylation regulates SoxE-mediated transcription by selectively modulating cooperative interaction among factors constituting transcriptional complexes, then our findings suggest that distinct types of complexes may be deployed in tissues such as the neural crest and the inner ear, and future work will explore this possibility. Elucidating the precise mechanisms through which SUMOylation modifies SoxE activity may have broad implications for understanding how developmental regulatory factors with conserved activity can mediate very different functional outcomes when expressed in different tissues.

Experimental Procedures

DNA Constructs and Embryo Methods

A partial *XSox10* cDNA was isolated from an arrayed cDNA library, and a full-length clone was generated by 5′RACE (BD Clontech, Mountain View, CA). *XSox9* was isolated from stage 17 cDNA using low copy number PCR and a high-fidelity polymerase (Tgo; Roche, Indianapolis, IN). cDNAs were cloned into a pCS2 variant that adds five C-terminal myc tags (gift of R. Davis) and confirmed by sequencing. The Sox9 K61R and K365R mutations and Sox10 K44R and K333R mutations were generated using the Quick Change method (Stratagene, La Jolla, CA; primer sequences available upon request). hSUMO-1 was inserted in the vector pCS2-FlagN and used for in vivo assays. Sox9^{K61,365R}/SUMO-1 and Sox10^{K44,333R}/SUMO-1 were created by ligating SUMO-1 in-frame C-terminal to the full-length Sox9^{K61,365R} or Sox10^{K44,333R} mutants using PCR methods, inserting a proline and a glycine between the two sequences. The fusion proteins were N-terminally epitope tagged by insertion into vector pCS2-Myc (provided by D. Turner). All constructs were confirmed by sequencing. All results shown are representative of at least two independent experiments. RNA for injection was produced in vitro from linearized plasmid templates using the Message Machine kit (Ambion, Austin, TX). mRNA concentrations injected were in the range of 5–50 pg. Collection, injection, and in situ hybridization of *Xenopus* embryos were as described (Bellmeyer et al., 2003). *Sox10* probe is directed against the 3′UTR of the message and does not recognize the coding region construct used for misexpression experiments. Constructs for making Pax8 and Dlx3 probes were provided by A. Brandli and T. Moreno, respectively.

Yeast Two-Hybrid Assays

Sox10 amino acids 1–333 inserted in the vector pEG202 was used as bait to screen a gastrula stage yeast two-hybrid library in the pJG4-5 vector (gift of S. Sokol) essentially as described (Itoh et al., 2000). Positive interactors were recovered from yeast, shuttled through bacteria, and retested in yeast for stringency of interaction by growth on selective medium and by assaying β-gal activity. Clones that retested were sequenced, and a number of these were identified as either *Xenopus* UBC-9 or *Xenopus* SUMO-1 (GenBank accession numbers BC046273 and Z97073, respectively). Baits consisting of Sox9 amino acids 2–305, Sox9^{K61R} amino acids 2–305, and Sox10^{K44R} amino acids 1–333 were also constructed in pEG202, and interactions with both SUMO-1 and UBC9 were compared by plating serial dilutions of the transformants on selective medium.

Western Blots and SUMOylation Assays

Wild-type or mutant Sox9 or Sox10 proteins were expressed in the presence or absence of SUMO-1, and embryos were collected at gastrula stages unless otherwise noted. For Western blots, embryos were lysed in RIPA buffer (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with phenylmethylsulfonyl fluoride, aprotinin, leupeptin, N-ethylmaleimide, cytochalasin B, and 1,10-phenanthroline. Samples were resolved on SDS-PAGE and proteins were detected using antibodies against the epitope tags (Myc: 9E10; Santa Cruz Biotechnology, Santa Cruz, CA; Flag: M2; Sigma-Aldrich, St. Louis, MO). For loading controls, blots were stripped and reprobed for actin (α-actin; Sigma-Aldrich). Secondary antibodies were horseradish peroxidase coupled and detected by chemiluminescence (Pierce, Rockford, IL).

Morpholino Oligonucleotide Rescue Experiments

A Sox10 morpholino antisense oligonucleotide designed against the 5′UTR and coding region of *Xenopus* Sox10 (5′-AGCTTTGGT CATCACTCATGGTGCC-3′, Sox10MO) was obtained from Gene Tools, LLC (Philomath, OR). To deplete Sox10, 10 ng of Sox10MO was injected into a single blastomere at the eight-cell stage. For rescues, mRNA encoding epitope-tagged forms of either Sox9, Sox10, or Sox9^{K61,365R} was subsequently injected, together with mRNA encoding lineage tracer β-gal. Injected embryos were cultured to the indicated stage, and harvested for in situ hybridization.

Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/9/5/593/DC1/.

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