SUMOylated SoxE factors recruit Grg4 and function as transcriptional repressors in the neural crest

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A growing number of transcriptional regulatory proteins are known to be modified by the small ubiquitin-like protein, SUMO. Posttranslational modification by SUMO may be one means by which transcriptional regulatory factors that play context-dependent roles in multiple processes can be regulated such that they direct the appropriate cellular and developmental outcomes. In early vertebrate embryos, SUMOylation of SoxE transcription factors profoundly affects their function, inhibiting their neural crest–inducing activity and promoting ear formation. In this paper, we provide mechanistic insight into how SUMO modification modulates SoxE function. We show that SUMOylation dramatically altered recruitment of transcriptional coregulator factors by SoxE proteins, displacing coactivators CREB-binding protein/p300 while promoting the recruitment of a corepressor, Grg4. These data demonstrate that SoxE proteins can function as transcriptional repressors in a SUMO-dependent manner. They further suggest a novel multivalent mechanism for SUMO-mediated recruitment of transcriptional coregulatory factors.

Introduction

The reiterative use of signaling pathways and transcriptional regulatory factors is a hallmark of embryonic development (Raible, 2006; Taylor and LaBonne, 2007). A relatively small number of signaling molecules and transcriptional regulatory proteins must mediate the multiplicity of processes that pattern organs and organisms. Consequently, many developmental regulatory factors are deployed in a context-dependent fashion to direct multiple, diverse, cellular and developmental outcomes.

The neural crest is an excellent system in which to examine the reiterative use of developmental regulatory proteins. Neural crest cells (NCCs) are multipotent progenitors with stem cell properties that give rise to a diversity of cell types essential to the vertebrate body plan (LaBonne and Bronner-Fraser, 1998; Knecht and Bronner-Fraser, 2002; Prasad et al., 2012). After their formation, NCCs undergo an epithelial–mesenchymal transition, acquire migratory and tissue invasive characteristics, and disperse throughout the early embryo where they will contribute to a broad set of derivatives (Duband et al., 1995; Barembaum and Bronner-Fraser, 2005). A variety of transcriptional regulatory proteins have been implicated as key regulators of neural crest development, including the SoxE family transcription factors Sox8, Sox9, and Sox10, high mobility group domain proteins characterized primarily as transcriptional activators (Bowles et al., 2000; Koopman et al., 2004). One or more SoxE factors are required for formation and maintenance of neural crest precursor cells and for directing formation of multiple neural crest derivatives, including craniofacial cartilage, melanocytes, and peripheral glia, in all organisms in which it has been examined (Wegner, 1999; Britsch et al., 2001; Cheung and Briscoe, 2003; Honoré et al., 2003; Haldin and LaBonne, 2010).

An important question about widely deployed factors, such as the SoxE proteins, is how their activities are modulated to ensure that they direct the correct cellular or developmental outcome. Recent work has demonstrated that

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Abbreviations used in this paper: β-gal, β-galactosidase; CBP, CREB-binding protein; Dct, dopachrome tautomerase; EMSA, electrophoresis mobility shift assay; HDAC, histone deacetylase; Mitf, microphthalmia-associated transcription factor; NCC, neural crest cell; RIPA, radioimmunoprecipitation assay; SIM, SUMO-interacting motif; TSA, trichostatin A; VPA, valproic acid.

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SoxE factors can be modulated by the small ubiquitin-like molecule SUMO-1 (Taylor and LaBonne, 2005). SUMO is a small (~10 kD) protein that can be covalently attached to targets in a sequence-directed fashion (Geiss-Friedlander and Melchior, 2007). The effects of SUMOylation depend largely on the function of the targeted protein. SUMOylation of transcription factors can either promote or inhibit DNA binding, alter subcellular localization, and promote or inhibit protein–protein interactions (Girdwood et al., 2004; Lyst and Stancheva, 2007). A small number of SUMO-interacting motifs (SIMs) that can mediate noncovalent interactions with SUMO have been identified (Hecker et al., 2006; Ouyang et al., 2009a). SIMs are found in a diverse set of nuclear and cytoplasmic proteins, with divergent functions making it unclear how specificity is achieved in the response of target proteins to SUMOylation.

SUMOylation of SoxE transcription factors profoundly alters their function in early embryos. SoxE proteins in which the SUMO acceptor site has been mutated to prevent SUMOylation are potent inducers of neural crest precursor cells, whereas SoxE factors with a SUMO moiety constitutively attached inhibit neural crest (Taylor and LaBonne, 2005). Interestingly, SoxE factors also regulate embryonic ear formation, and here, SUMO modification has the opposite effect, promoting expression of ear markers while inhibiting expression of neural crest markers (Taylor and LaBonne, 2005). Despite the dramatic regulatory effects of SUMOylation on SoxE function in the context of NCC formation, the mechanisms via which SUMO modification directs such differences have remained unclear. To begin to elucidate these mechanisms, we focused on formation of a specific neural crest derivative, melanocytes. We have previously demonstrated that both Sox9 and Sox10 can direct the formation of supernumery melanocytes and that SoxE factors that cannot be SUMOylated induce melanocytes more potently (Taylor and LaBonne, 2005). SoxE transcription factors directly regulate the promoters of several genes essential for melanocyte formation, including *microphthalmia-associated transcription factor* (*Mitf*), *tyrosinase*, and *dopachrome tautomerase* (*Dct*; Lee et al., 2000; Elworthy et al., 2003; Jiao et al., 2004; Murisier et al., 2007). The *Dct* promoter contains several binding sites for SoxE transcription factors (Jiao et al., 2004; Ludwig et al., 2004) as well as one for Mitf (Bertolotto et al., 1998), a central melanocyte transcription factor also regulated by SUMOylation (Miller et al., 2005; Murakami and Arnheiter, 2005).

Here, we show that SoxE and Mitf synergistically activate the *Dct* promoter in early *Xenopus laevis* embryos and that SoxE SUMOylation inhibits this activation. We further demonstrate that SUMO dramatically alters recruitment of transcriptional coregulatory factors by SoxE, displacing the binding of coactivators and promoting the recruitment of Grg4. These data provide the first mechanistic evidence that SoxE transcription factors function as context-dependent transcriptional repressors in a SUMO-mediated manner. Our findings further suggest a novel multivalent mechanism for the SUMO-dependent recruitment of coregulatory factors.

Results

SUMOylation-deficient SoxE and Mitf isoforms synergistically induce *Dct* expression

A subset of Sox10-expressing neural crest precursor cells in the posterior neural tube give rise to melanoblasts (Potterf et al., 2001; Aoki et al., 2003). Mitf and Sox10 then function together in these cells to direct the differentiation of melanocytes and the production of melanin (Ludwig et al., 2004; Vance and Goding, 2004; Murisier et al., 2006). The promoter of one enzyme essential for melanin biosynthesis, *Dct*, has been well characterized and contains binding sites for both SoxE factors and Mitf that are essential for synergistic activation by these factors (Ludwig et al., 2004). We took advantage of this to examine the mechanisms through which SUMOylation alters SoxE-dependent transcription.

Because Mitf can also be modified by SUMO (Miller et al., 2005; Murakami and Arnheiter, 2005), we generated Mitf SUMOylation mutants analogous to SoxE SUMOylation mutants we characterized in a previous study (Taylor and LaBonne, 2005). Mitf that cannot be SUMOylated was generated by mutating SUMO acceptors K182/K316 to arginine ($Mitf_{2KR}$), whereas constitutively SUMO-modified Mitf was generated by fusing SUMO-1 in frame to the C terminus ($Mitf_{2KR}$/SUMO; Fig. 1 A). mRNA encoding these mutants was injected into one blastomere of two-cell stage embryos, targeting the neural crest. β-galactosidase (β-gal) was coinjected as a lineage tracer. The effects of mutating the Mitf SUMOylation sites were found to be directly analogous to the effects of mutating SoxE SUMOylation sites with respect to melanocyte formation (Fig. S1); SUMO modification of either SoxE or Mitf inhibits, whereas preventing SUMOylation of these proteins promotes, melanocyte development (Taylor and LaBonne, 2005).

Mitf and SoxE synergistically activate the *Dct* promoter (Ludwig et al., 2004). Because non-SUMOylatable SoxE and Mitf both promote melanocyte formation, we asked whether they retained the ability to synergistically activate *Dct*. mRNA encoding $Mitf_{2KR}$, $Sox9_{2KR}$, or $Sox10_{2KR}$ was injected alone or together into one-cell of two-cell *Xenopus* embryos, and effects on *Dct* expression were examined by in situ hybridization. To better detect synergy, we used concentrations of $Mitf_{2KR}$ and mutant SoxE factors that alone induced little or no ectopic *Dct* ($Sox9_{2KR}$ injected: 0% ectopic expression, $n = 38$ at stage 23, and 2.1%, $n = 48$ at stage 28; $Sox10_{2KR}$ injected: 0%, $n = 28$ at stage 23, and 18.8%, $n = 32$ at stage 28; $Mitf_{2KR}$ injected: 82.5%, $n = 40$ at stage 23, and 95.7%, $n = 47$ at stage 28; Fig. 1 B). When $SoxE_{2KR}$ and $Mitf_{2KR}$ were coinjected, however, ectopic expression of *Dct* was dramatically enhanced ($Sox9_{2KR}$ + $Mitf_{2KR}$ injected: 94.7%, $n = 38$ at stage 23, and 100%, $n = 36$ at stage 28; $Sox10_{2KR}$ + $Mitf_{2KR}$ injected: 85.2%, $n = 27$ at stage 23, and 83.3%, $n = 48$ at stage 28; Fig. 1 B), consistent with SoxE and Mitf mutants synergistically inducing its expression.

To further examine the context-dependent ability of $Sox9_{2KR}$ and $Mitf_{2KR}$ to induce *Dct* expression, we used *Xenopus* animal

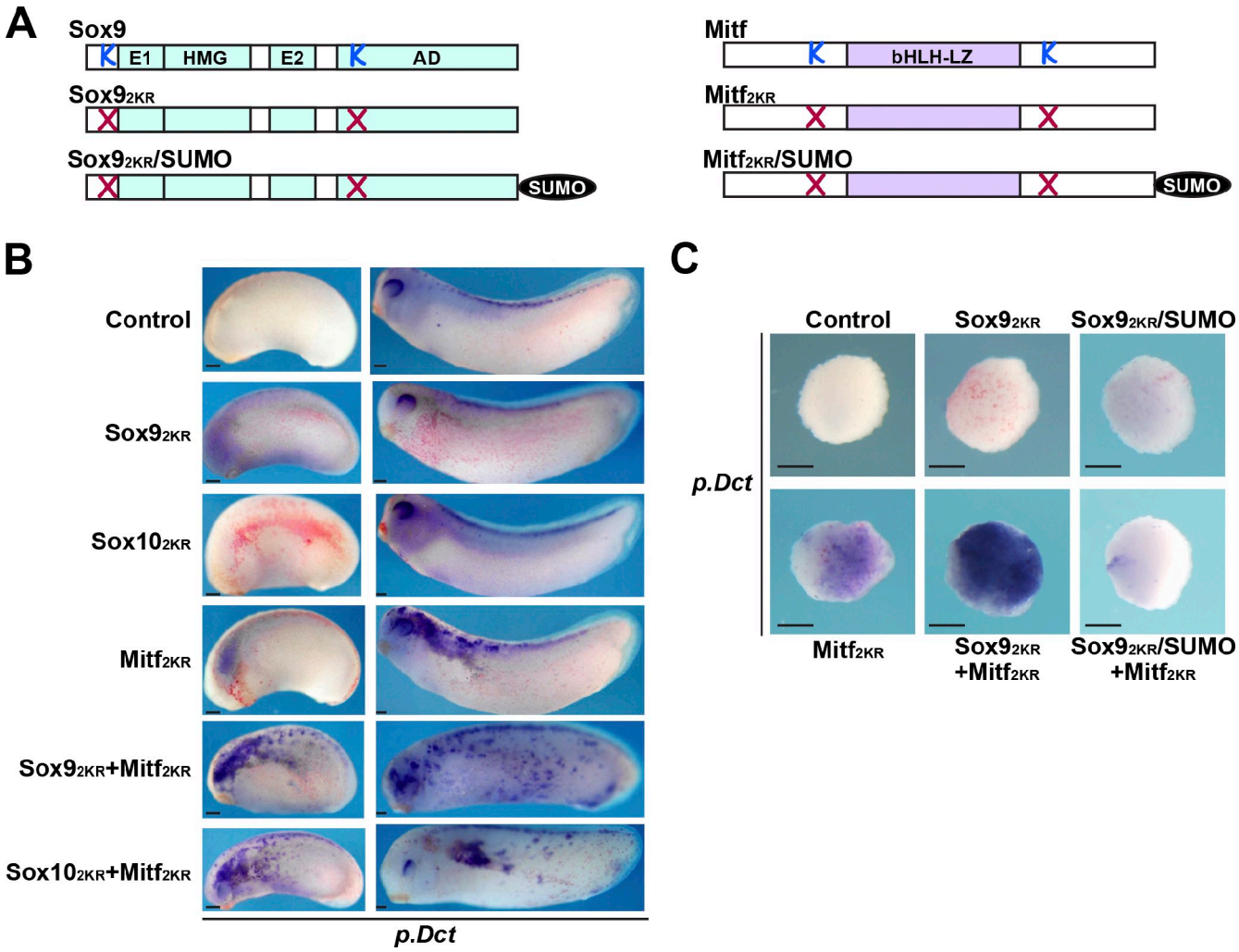

Figure 1. **Effects of SoxE and Mitf isoforms on *Dct* expression in *Xenopus* embryos and animal caps.** (A) Schematic of SoxE and Mitf proteins and SUMOylation mutants. (B) In situ hybridization showing *Dct* expression in embryos injected with SoxE$_{2KR}$, Mitf$_{2KR}$, or both. Both Sox9$_{2KR}$ and Sox10$_{2KR}$ act synergistically with Mitf$_{2KR}$ to induce premature and ectopic *Dct* expression in stage 23 and stage 28 embryos. (C) Animal cap assay showing effects of Sox9$_{2KR}$/SUMO on Mitf$_{2KR}$-mediated induction of *Dct* at stage 28. Sox9$_{2KR}$ enhances, whereas Sox9$_{2KR}$/SUMO blocks, Dct induction. Light red staining represents lineage tracer β-gal. Bars, 200 μm. AD, activation domain; LZ, Leucine zipper.

pole explants (animal caps). Embryos were injected with mRNA encoding Mitf$_{2KR}$, Sox9$_{2KR}$, or Sox9$_{2KR}$/SUMO in both cells at the two-cell stage. Injected embryos were cultured until stage 9, when animal pole ectoderm was explanted and cultured until stage 28, fixed, and processed for in situ hybridization. As expected, animal caps expressing β-gal alone did not induce *Dct* (0%, n = 6). Interestingly, we were unable to find a dose of Sox9$_{2KR}$ (or the analogous Sox10 mutant) sufficient to induce detectable *Dct* expression (0%, n = 8; Fig. 1 C and not depicted). In contrast, Mitf$_{2KR}$ induced weak but consistent *Dct* expression (75%, n = 8), confirming that in at least some cellular contexts, Mitf$_{2KR}$ is sufficient to induce melanocyte formation. When Mitf$_{2KR}$ was coexpressed with even a low dose of Sox9$_{2KR}$, significantly stronger expression of *Dct* was induced (100%, n = 10), indicating that SoxE factors do play a role in melanocyte formation beyond inducing Mitf. Importantly, coexpression of Sox9$_{2KR}$/SUMO blocked induction of *Dct* by Mitf$_{2KR}$ (n = 6), indicating that the inhibitory effects of SoxE SUMOylation extend to other regulatory factors bound to the same promoter.

SUMOylation affects the ability of SoxE and Mitf to synergistically induce *Dct* promoter activity

The aforementioned experiments indicated that melanocyte development would be an excellent context in which to elucidate a more mechanistically detailed understanding of the functional consequences of SoxE SUMOylation. We focused on the *Dct* promoter, which contains six SoxE binding sites, one Mitf binding site, and one Lef-1 binding site (Fig. 2 A), and asked whether a reporter construct driving luciferase expression would be regulated comparably with endogenous *Xenopus Dct* when introduced into embryos. mRNA encoding Mitf$_{2KR}$, Sox9$_{2KR}$, Mitf$_{2KR}$ + Sox9$_{2KR}$, Sox9$_{2KR}$/SUMO + Mitf$_{2KR}$, or Sox9$_{2KR}$ + Mitf$_{2KR}$/SUMO were coinjected with *Dct* promoter DNA, and a control Renilla luciferase construct was coinjected for normalization. Injected embryos were cultured to late neurula stages and processed for luciferase assays. Expression of Sox9$_{2KR}$ led to a modest twofold induction of *Dct* promoter activity (Fig. 2 B), confirming in

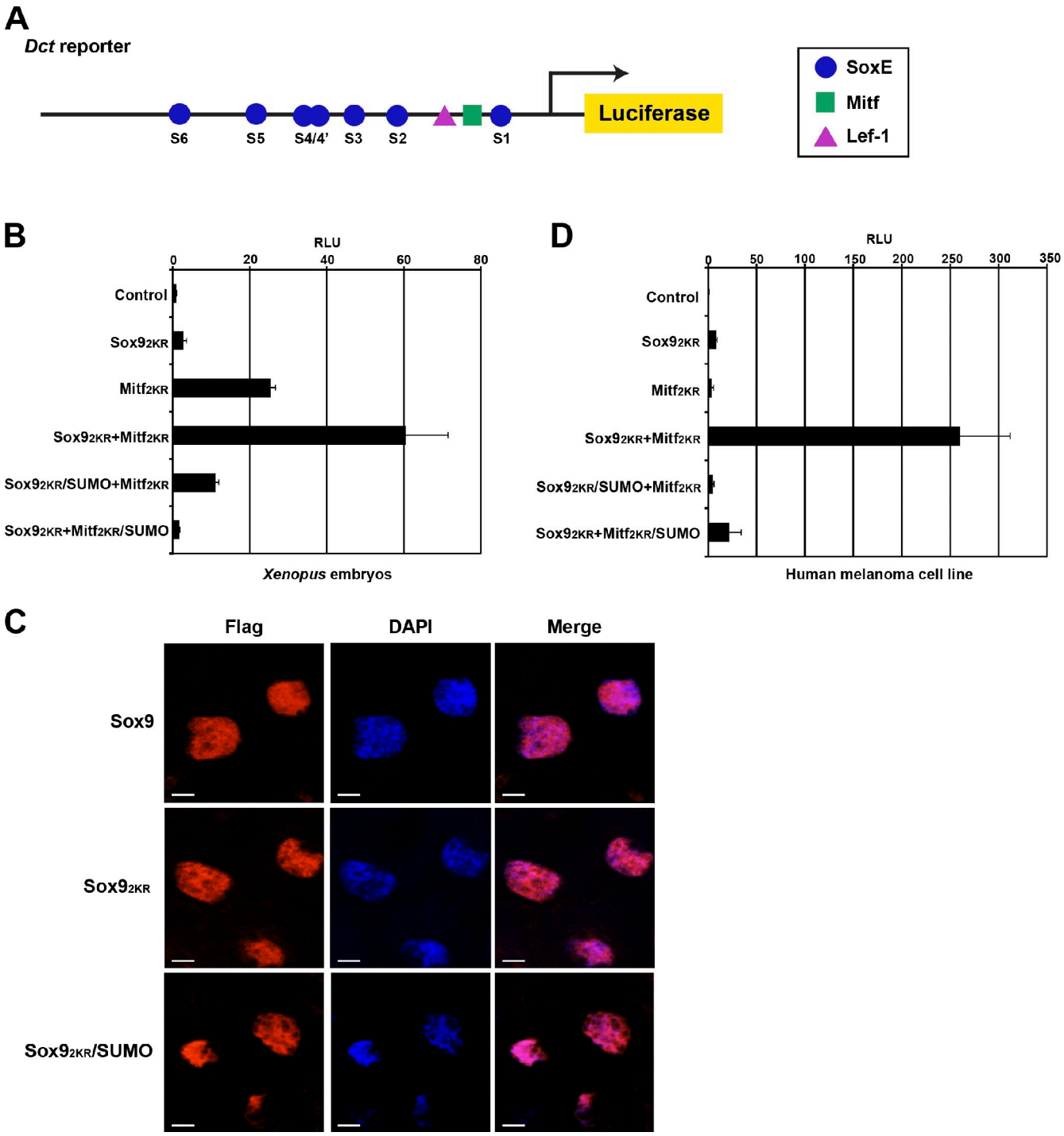

Figure 2. **Effects of Sox9 and Mitf isoforms on *Dct* promoter activity and subcellular localization.** (A) Schematic of the *Dct* promoter/reporter containing a Mitf binding site, a Lef-1 site, and six SoxE binding sites, S1, S2, S3, S4/4′ (dimeric sites), S5, and S6, from right to left. (B and C) Luciferase reporter assay in stage 17 embryo lysates (B) or human melanoma cells (C) using the *Dct* reporter. A Renilla reporter was coinjected for normalization. Relative luciferase activity (RLU) is represented as fold activation relative to the normalized activity of control embryos. Coexpression of non-SUMOylable forms of Mitf and Sox9 activate *Dct* expression synergistically, whereas SUMOylation of either protein significantly reduced reporter activity. (C) Immunofluorescence staining of animal caps dissected from embryos injected with flag-tagged Sox9 isoforms. Nuclei are marked by DAPI. All Sox9 isoforms colocalized with DAPI, suggesting that SUMOylation does not affect cellular localization of Sox9 in *Xenopus* embryos. Error bars represent the standard deviation of the mean of triplicate assays. Bars, 10 µm.

situ hybridization results that SoxE factors alone are not sufficient for significant activation of this promoter. Expression of $Mitf_{2KR}$, in contrast, activated the *Dct* promoter ~27-fold, and combined expression of $Mitf_{2KR}$ + $Sox9_{2KR}$ led to 61-fold activation. When either Sox9 or Mitf was SUMOylated, however, this synergistic activation was abolished. Importantly, and similar to what was observed in animal caps, coexpression of $Sox9_{2KR}$/SUMO significantly reduced promoter activation by

$Mitf_{2KR}$, indicating that the SUMO moiety inhibited Mitf activity even when coupled to a different DNA-bound transcription factor. The subcellular localization of $Sox9_{2KR}$/SUMO was indistinguishable from that of Sox9 or $Sox9_{2KR}$ (Fig. 2 C).

Although having the advantage of developmental context, a caveat of embryo luciferase assays is that the promoter/regulatory factors are in a heterogeneous cellular context, which could theoretically impact experimental outcomes. To ensure that SUMOylation of SoxE factors similarly affected *Dct* promoter activity in a uniform cellular context, we used melanocyte-derived/melanoma cell line, C8161. DNA encoding $Mitf_{2KR}$, $Sox9_{2KR}$, $Mitf_{2KR}$ + $Sox9_{2KR}$, $Sox9_{2KR}$/SUMO + $Mitf_{2KR}$, or $Sox9_{2KR}$ + $Mitf_{2KR}$/SUMO were transiently transfected into C8161 cells together with luciferase reporter plasmids, and cells were cultured for 24 h before lysis (Fig. 2 D). Neither the $Mitf_{2KR}$ nor $Sox9_{2KR}$ alone led to significant activation of *Dct* promoter activity in C8161 cells, indicating that Mitf is insufficient in this cellular context. Cotransfection of $Mitf_{2KR}$ and $Sox9_{2KR}$ synergistically activated the *Dct* promoter ∼260-fold. Importantly, as observed in *Xenopus*, SUMOylated forms of these proteins blocked promoter activity.

SUMO does not regulate SoxE via steric interference

Several mechanisms have been proposed for how SUMO may modulate transcription factor function. One potential mechanism is steric interference, in which addition of a SUMO moiety physically blocks DNA binding or inhibits cooperative assembly of multiple DNA binding factors on cis-regulatory elements. To examine whether SUMOylation blocks DNA binding by Mitf or SoxE, electrophoresis mobility shift assays (EMSAs) were performed using probes containing either both SoxE and Mitf binding sites (S1/M) or the dimeric SoxE binding sites (S4/4′) from the *Dct* promoter and in vitro translated $Mitf_{2KR}$, $Mitf_{2KR}$/SUMO, $Sox9_{2KR}$, and $Sox9_{2KR}$/SUMO proteins. All of the SUMOylation mutant isoforms of Mitf and Sox9 could bind DNA in this assay (Fig. 3, B and C).

We next generated constructs in which the two proteins were covalently linked via a flexible serine/glycine-rich linker (Fig. 3 A). We hypothesized that if the proteins were linked, recruitment of one factor to DNA would necessarily result in recruitment of the second factor. Assuming that the linked factors were as active as their unlinked forms, we could then test whether additionally linking a SUMO moiety could still inhibit promoter activation. When a linked protein in which $Mitf_{2KR}$ was followed by $Sox9_{2KR}$ ($Mitf_{2KR}$/$Sox9_{2KR}$) was expressed in early embryos, ectopic *Dct* expression was induced to a greater or equivalent extent than achieved by the unlinked forms of these proteins expressed at a 50-fold higher concentration (Fig. S2), indicating that that covalently linking these factors enhanced rather than interfered with their activity. To test the hypothesis that SUMO interferes with corecruitment of Mitf and SoxE, we generated linked proteins in which SUMO was inserted at several distinct positions within the fusion construct. $Mitf_{2KR}$/$Sox9_{2KR}$/SUMO, $Mitf_{2KR}$/SUMO/$Sox9_{2KR}$, and SUMO/$Mitf_{2KR}$/$Sox9_{2KR}$ featured SUMO linked at the C terminus, internally, and at the N terminus, respectively. All of these proteins could bind DNA as well as forms lacking the SUMO moiety (Fig. 3, B and C), and none induced ectopic *Dct* expression when expressed in early *Xenopus* embryos (Fig. S2 and not depicted).

mRNA encoding $Mitf_{2KR}$/$Sox9_{2KR}$, $Mitf_{2KR}$/$Sox9_{2KR}$/SUMO, $Mitf_{2KR}$/SUMO/$Sox9_{2KR}$, and SUMO/$Mitf_{2KR}$/$Sox9_{2KR}$ was injected into *Xenopus* embryos together with Dct-luciferase and Renilla luciferase reporters. Consistent with its potency in activating endogenous *Dct* expression, expression of $Mitf_{2KR}$/$Sox9_{2KR}$ induced a 460-fold activation of *Dct*-luciferase (Fig. 3 D). This was significantly greater than the activity of the unlinked factors. Importantly, when SUMO was linked to this fusion protein ($Mitf_{2KR}$/$Sox9_{2KR}$/SUMO, $Mitf_{2KR}$/SUMO/$Sox9_{2KR}$, and SUMO/$Mitf_{2KR}$/$Sox9_{2KR}$), it potently blocked *Dct* promoter activity even though Mitf and SoxE were linked and therefore corecruited. Together, these findings indicate that the effects of SUMO modification are not a consequence of interference with SoxE DNA binding or cooperative recruitment of Mitf, at least in this cellular context.

SUMO-mediated inhibition is not relieved by blocking histone deacetylase (HDAC) activity

Another mechanism via which SUMO has been proposed to promote transcriptional repression is through recruitment of HDAC activity (Hilgarth et al., 2004; Yang and Sharrocks, 2004). HDAC-dependent repression is sensitive to inhibitors such as trichostatin A (TSA) and valproic acid (VPA), and treatment with these compounds at least partially restores promoter activity. We therefore asked whether the loss of *Dct* promoter activity seen with Sox9 or Mitf SUMOylation could be relieved by TSA treatment. Embryos injected with mRNA encoding either $Mitf_{2KR}$ and $Sox9_{2KR}$, $Mitf_{2KR}$ and $Sox9_{2KR}$/SUMO, or $Mitf_{2KR}$/SUMO and $Sox9_{2KR}$ were cultured to stage 11.5, treated with TSA or vehicle, and harvested at late neurula stages to measure *Dct*-luciferase activity. SUMOylation of either Sox9 or Mitf was sufficient to inhibit *Dct* promoter activity; however, no relief of this inhibition was seen after TSA treatment (Fig. 4 A). As a control for effective TSA treatment, sibling embryos were treated with TSA or vehicle in parallel and processed for in situ hybridization. We have previously found that TSA treatment blocks neural crest precursor formation (unpublished data), and consistent with this, TSA-treated embryos displayed a loss of *Slug* expression at stage 17 (Fig. 4 C). To further confirm that SUMO-mediated inhibition of the *Dct* promoter was not completely HDAC dependent, we repeated the TSA experiments in C8161 melanoma cells. As in embryos, treatment of $Mitf_{2KR}$ + $Sox9_{2KR}$/SUMO– or $Mitf_{2KR}$/SUMO + $Sox9_{2KR}$–transfected cells with TSA or VPA failed to restore *Dct* promoter activity (Fig. 4 B and not depicted). Although these results do not exclude involvement of HDAC activity in this process, they indicate that additional mechanisms must also be essential.

SUMO modification prevents SoxE-dependent recruitment of transcriptional coactivators

SoxE factors function as transcriptional activators at least in part by recruitment of CREB-binding protein (CBP)/p300 histone acetyltransferases (Tsuda et al., 2003; Furumatsu et al., 2005).

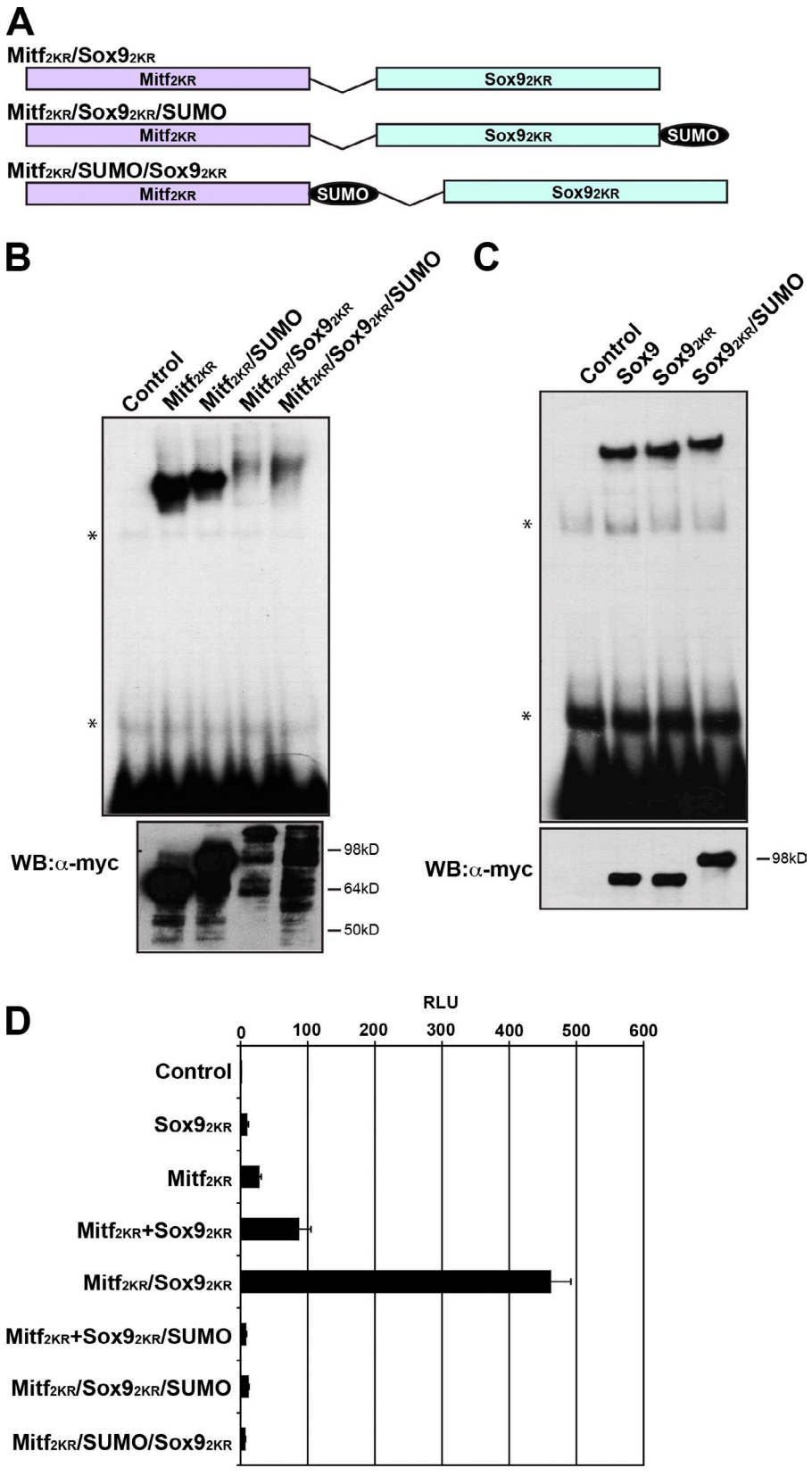

Figure 3. **SUMOylation does not modulate *Dct* promoter activity through steric interference.** (A) Schematic of Mitf/Sox9-tethered constructs used. (B and C) EMSA demonstrating DNA binding by Mitf isoforms, Mitf/Sox9-tethered proteins (B), and Sox9 isoforms (C). In vitro translated proteins were incubated with ^{32}P-labeled probes containing S1/M sites (for Mitf and Mitf/Sox9 isoforms) or S4/4′ sites (for Sox9 isoforms) of the *Dct* promoter. Asterisks denote nonspecific bands. (D) Luciferase reporter assay using stage 17 embryos injected with Mitf/Sox9-tethered constructs or coinjected with Mitf and Sox9 isoforms. SUMO blocks promoter activation even when Mitf and Sox9 are tethered and thus corecruited. Error bars represent the standard deviation of the mean of triplicate assays. RLU, relative luciferase activity; WB, Western blot.

Because SUMOylation is capable of both promoting and inhibiting protein–protein interactions, we hypothesized that one consequence of SUMO modification might be interference with the recruitment of these coactivators. To test this hypothesis, epitope-tagged $Sox9_{2KR}$ or $Sox9_{2KR}$/SUMO was expressed in early embryos; at neurula stages, the endogenous p300 or CBP used was immunoprecipitated from whole-cell lysates, and Western analysis was used to examine interactions with Sox9. Although $Sox9_{2KR}$

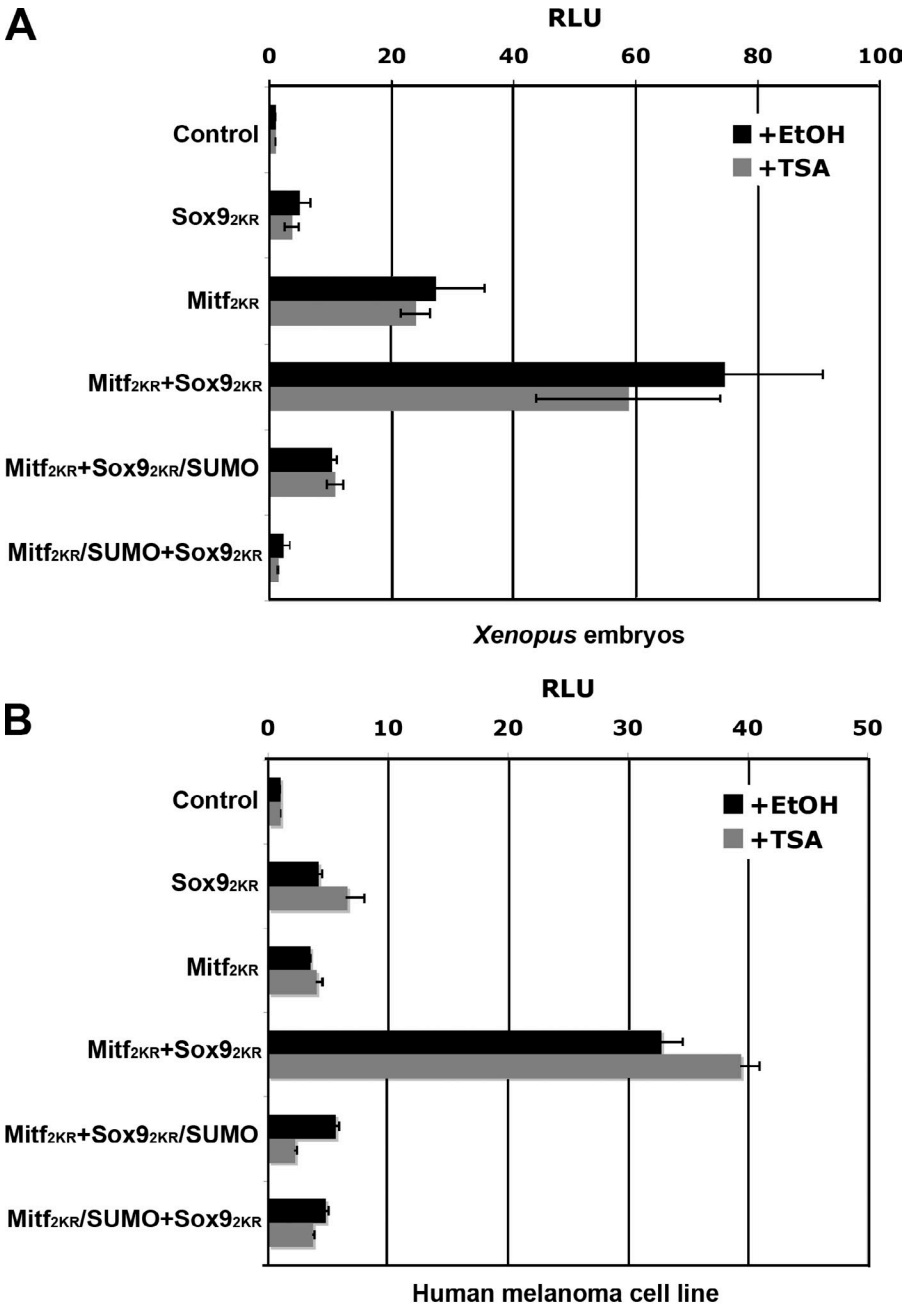

Figure 4. **Blocking HDAC activity does not relieve SUMO-mediated inhibition of the *Dct* promoter.** (A and B) Luciferase reporter assays in stage 17 embryo lysates (A) or human melanoma cells (B) using the *Dct* reporter. Sox9 and Mitf isoforms were coexpressed in the presence or absence of HDAC inhibitor trichostatin A (TSA). Treatment with TSA did not relieve *Dct* inhibition mediated by either Sox9$_{2KR}$/SUMO or Mitf$_{2KR}$/SUMO. Error bars represent the standard deviation of the mean of triplicate assays. (C) In situ hybridization showing TSA treatment was effective, as it caused expected loss of *Slug* expression in stage 17 embryos. Bars, 200 µm. RLU, relative luciferase activity.

was found to interact with both p300 and CBP, no interaction was seen with Sox9$_{2KR}$/SUMO, indicating that SUMOylation interferes with coactivator recruitment (Fig. 5 A). Interestingly, the CBP/p300 binding site localized to the C-terminal activation domain, the predominant site of Sox9 SUMOylation (Tsuda et al., 2003).

SUMOylation promotes interaction of SoxE with Grg4

The observation that Sox9 SUMOylation leads to loss of p300/CBP binding provides one mechanism for SUMO-dependent inhibition of target promoters such as Dct. However, our data suggested that effects of Sox9$_{2KR}$/SUMO extend beyond simple

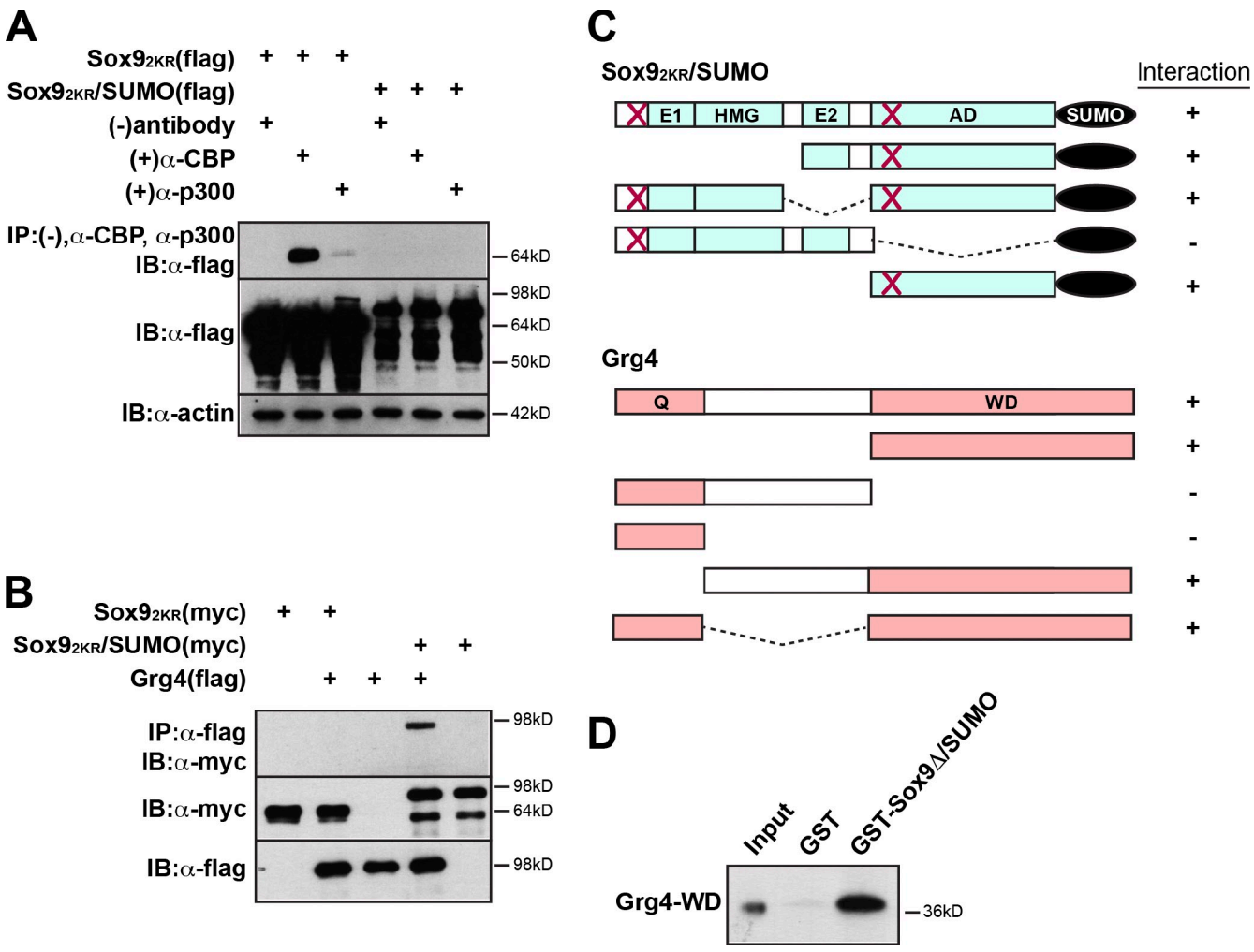

Figure 5. **SUMOylation of Sox9 alters recruitment of transcriptional cofactors.** (A) Coimmunoprecipitation from embryo lysates expressing flag-tagged Sox9 isoforms. Immunoprecipitation used antibodies against endogenous CBP or p300 followed by Western analysis with α-flag. A mock antibody treatment (−) was used as a negative control. SUMOylation blocks interaction with CBP or p300. (B) Coimmunoprecipitation from embryos injected with Myc-tagged Sox9 isoforms and flag-tagged Grg4. Immunoprecipitation used a flag antibody followed by Western analysis with α-Myc. Sox9$_{2KR}$/SUMO, but not Sox9$_{2KR}$, interacts with corepressor Grg4. (C) Schematic summarizing results of interaction experiments using Grg4 and SoxE/SUMO deletion constructs. The Grg4-WD40 domain and the activation domain plus SUMO moiety of SoxE/SUMO are sufficient for interaction. (D) GST pull-down experiment demonstrating direct interaction between the Grg4-WD40 domain and the Sox9 activation domain (AD)–SUMO fusion. IB, immunoblot; IP, immunoprecipitation.

loss of transactivation activity. For example, coexpressed Sox9$_{2KR}$/SUMO reduced the level of promoter activity below what was achieved by Mitf$_{2KR}$ alone, indicating that the SUMO moiety affected Mitf function even when coupled to a different DNA-bound transcription factor (Fig. 2 B), and similar effects were observed in animal caps (Fig. 1 C). These results suggested that loss of "activator" function was insufficient to explain the effects of Sox9$_{2KR}$/SUMO and that acquisition of repressor function was also involved.

Groucho/TLE proteins function as corepressors in multiple developmental processes and have been implicated as a node of cross talk between multiple signaling pathways (Hasson and Paroush, 2006; Cinnamon and Paroush, 2008). These proteins often interact with bimodal transcription factors and can serve as switches from activator to repressor states. Two Groucho family proteins, Grg4 and Grg5, are expressed ubiquitously in early *Xenopus* embryos (Molenaar et al., 2000) and thus present at a time/place consistent with modulating SoxE function. To determine whether Grg4 could be recruited by SoxE proteins, embryos were injected with mRNA encoding epitope-tagged Grg4 together with Sox9$_{2KR}$ or Sox9$_{2KR}$/SUMO and subjected to coimmunoprecipitation. Interestingly, Sox9$_{2KR}$ showed little or no association with Grg4, whereas Sox9$_{2KR}$/SUMO strongly interacted with Grg4 (Fig. 5 B). Together with the CBP/p300 results, these findings indicate that SUMOylation of SoxE promotes loss of interaction with transcriptional coactivators and gain of interaction with corepressor Grg4.

Interaction of Sox9$_{2KR}$/SUMO and Grg4 requires the activation domain of Sox9 and WD40 domain of Grg4

SoxE factors have been mainly categorized as transcriptional activators and have not previously been shown to interact with

corepressors. To better characterize this interaction, we mapped the domains required for interaction between $Sox9_{2KR}$/SUMO and Grg4. A series of $Sox9_{2KR}$/SUMO isoforms were generated in which one or more key functional domains were deleted (Fig. 5 C). Deletions were coexpressed with Grg4 in early *Xenopus* embryos, and interactions were assessed via coimmunoprecipitation. Deletion of the activation domain in $Sox9_{2KR}$/SUMO led to a loss of its ability to interact with Grg4. Conversely, a construct consisting only of the activation domain fused in frame to SUMO retained the ability to interact with Grg4 (Fig. 5 C and Fig. S3). Together, these findings demonstrate that the SUMOylated activation domain is both necessary and sufficient for Grg4 recruitment. The activation domain includes the previously mapped p300/CBP-interacting region (Tsuda et al., 2003), suggesting that Grg4 may displace p300/CBP upon Sox9 SUMOylation.

To similarly determine the domains of Grg4 required for $Sox9_{2KR}$/SUMO binding, a Grg4 deletion series was generated. Grg4 contains a highly conserved N-terminal poly-Q domain critical for oligomerization and other protein–protein interactions and a C-terminal WD40/β-propeller domain also involved in multiple protein–protein interactions. The region between these domains is not highly conserved but has been proposed to contain sequences important for corepressor activity (Chen and Courey, 2000; Jennings and Ish-Horowicz, 2008). Interaction between Grg4 was found to require the WD40 domain, and this domain is sufficient to mediate interaction (Fig. 5 C and Fig. S3). Similar results were also obtained using $Sox10_{2KR}$/SUMO or $Sox8_{2KR}$/SUMO (Fig. S4), and wild-type SoxE proteins behaved like $SoxE_{2KR}$ in this assay (Fig. S5). To determine whether the interaction between Grg4 and $Sox9_{2KR}$/SUMO is direct, a Sox9 deletion construct containing only the E2 and activation domains was expressed in *Escherichia Coli* as a GST fusion (GST-Sox9Δ/SUMO). GST pull-down assays demonstrated that GST-Sox9Δ/SUMO directly interacts with the in vitro translated Grg4-WD domain (Fig. 5 D).

Grg4 repression of neural crest formation and *Dct* promoter activity is dependent on the ability of SoxE factors to be SUMOylated

If Grg4 recruitment plays an important role in the loss of *Dct* promoter activation seen with $Sox9_{2KR}$/SUMO, we would expect that forms of Sox9 that cannot be SUMOylated would show less Grg4 sensitivity than would forms of Sox9 that retain the ability to be modified. To test this hypothesis, we coexpressed $Mitf_{2KR}$ with either wild-type Sox9 or $Sox9_{2KR}$ in the presence or absence of Grg4 and assayed Dct-luciferase activity. Promoter activation by wild-type Sox9, which can be SUMOylated endogenously, was significantly inhibited by Grg4 coexpression. In contrast, the ability of Grg4 to inhibit *Dct* promoter activity mediated by a form of Sox9 that cannot be SUMO modified ($Sox9_{2KR}$) is significantly diminished (Fig. 6 A). SUMOylation is a highly dynamic and reversible process. De-SUMOylation is mediated by SUMO-specific isopeptidases, such as Senp proteins. Overexpression of Senp1b effectively prevented Sox9 SUMOylation (Fig. S5). When Sox9 and Senp1b were coexpressed, Grg4 could not inhibit *Dct* promoter activity (Fig. 6 B), further demonstrating that Grg4-mediated repression of *Dct* depends on Sox9 SUMOylation.

We next examined the consequences of Grg4 misexpression for neural crest development. We hypothesized that if Grg4 was the major mediator of $Sox9_{2KR}$/SUMO function, it might be expected to at least partially phenocopy the effects of $Sox9_{2KR}$/SUMO misexpression. In support of this hypothesis, Grg4 misexpression was found to inhibit expression of early neural crest markers, such as *Sox10* (100%, n = 39; Fig. 6 C) similar to what is seen with $Sox9_{2KR}$/SUMO (Taylor and Labonne, 2005). Grg4 could also at low frequency lead to the formation of structures resembling ectopic ears (31.4%, n = 86). Grg4 serves as a corepressor for many transcription factors, thus its effects on neural crest development might be unrelated to recruitment by $Sox9_{2KR}$/SUMO. If Grg4-mediated repression of neural crest is caused by recruitment by SUMO-modified SoxE proteins, Grg4 should be unable to inhibit neural crest induction by a Sox9 that cannot be SUMO modified ($Sox9_{2KR}$). To test this, we expressed $Sox9_{2KR}$ under conditions in which it causes a modest expansion of *Sox10* expression (98.7%, n = 77; Fig. 6 D). *Sox10* expression is greatly inhibited after Grg4 overexpression (100%, n = 55). Importantly, however, Grg4 does not inhibit *Sox10* expression in $Sox9_{2KR}$-injected embryos (85.9%, n = 99). Moreover, a fusion protein in which Grg4 has been linked in frame to $Sox9_{2KR}$ is also capable of inhibiting neural crest formation (78.8%, n = 52; Fig. 6 E), demonstrating that targeting of Grg4 to SoxE sites in target promoters is sufficient to account for its effects. Together, these experiments show that association with SoxE factors is both necessary and sufficient for the ability of Grg4-mediated repression of neural crest formation.

Grg4 is recruited by $Sox9_{2KR}$/SUMO via a multivalent interaction mechanism

The consensus SIM is characterized by a hydrophobic core flanked by acidic amino acids and in some cases serine residues and is found in a diverse set of nuclear and cytoplasmic proteins with divergent functions (Hecker et al., 2006; Kerscher, 2007; Perry et al., 2008). Despite its recruitment by $Sox9_{2KR}$/SUMO, Grg4 lacks an identifiable SIM. A specific subset of amino acids on SUMO have been identified as essential for interaction with the consensus SIM, and mutations were characterized that disrupt these interactions (Baba et al., 2005; Hecker et al., 2006). To further challenge the apparent lack of SIM-like sequence in Grg4, we introduced these mutations into the SUMO moiety of $Sox9_{2KR}$/SUMO ($Sox9_{2KR}$/$SUMO_{mut}$). Although these mutations abolished SIM-dependent interactions with Ubc9, no significant difference was noted in the ability of $Sox9_{2KR}$/SUMO and $Sox9_{2KR}$/$SUMO_{mut}$ to interact with Grg4 (Fig. 7 A).

Most DNA binding factors that recruit Groucho-related proteins possess small peptide motifs, such as a WRPW or eh1 (engrailed homology 1), that mediate this interaction (Jennings et al., 2006; Jennings and Ish-Horowicz, 2008). As neither SUMO nor SoxE factors possess previously characterized Groucho-recruiting motifs, we examined their

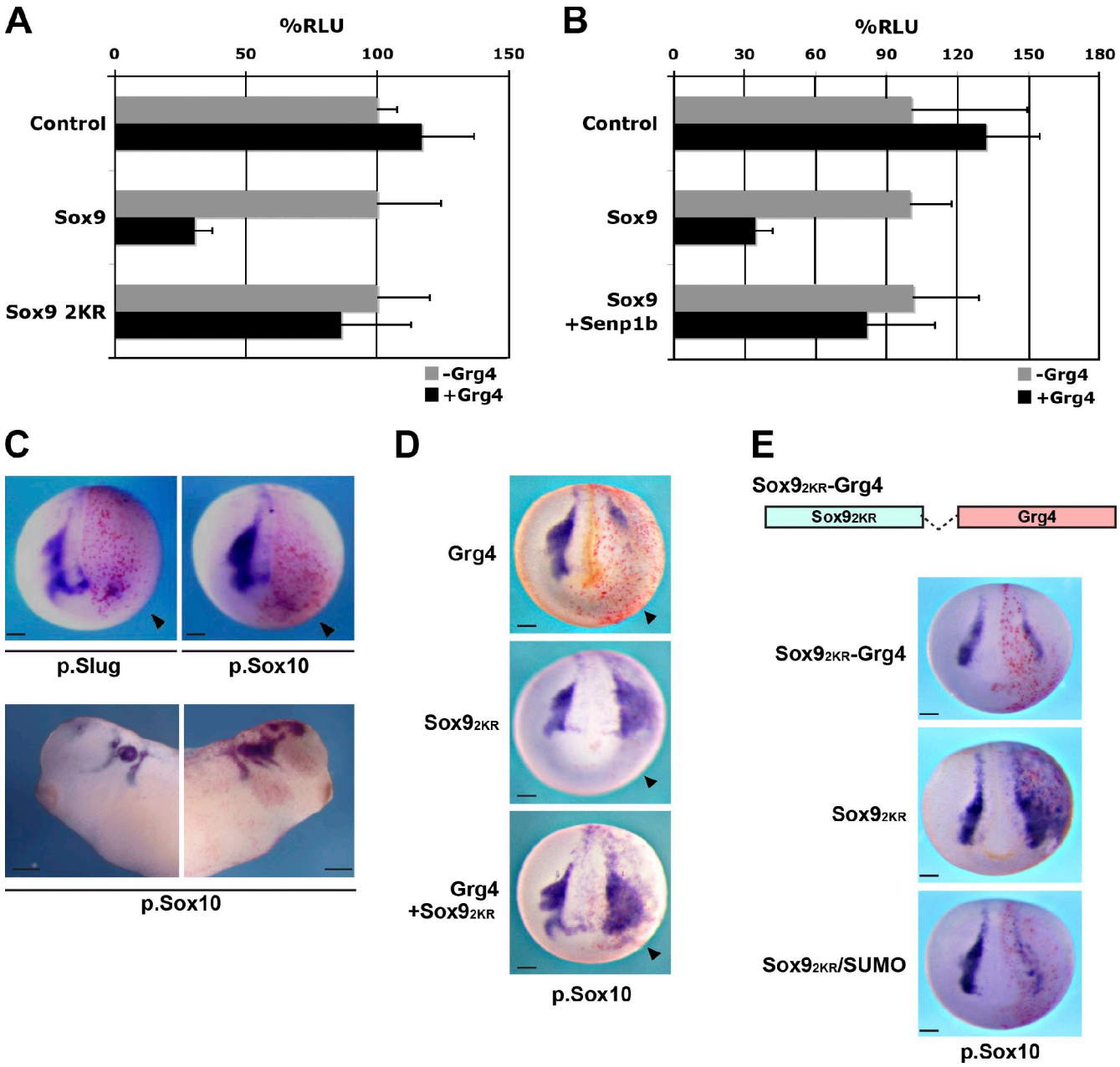

Figure 6. **Grg4-mediated repression of the *Dct* promoter and neural crest formation are dependent on SoxE SUMOylation.** (A) Luciferase assay measuring *Dct* reporter activity in embryos expressing either wild-type Sox9 or Sox9$_{2KR}$. Grg4 represses *Dct* promoter activity mediated by wild-type Sox9 but not the form that cannot be SUMOylated. (B) Luciferase assay measuring *Dct* reporter activity in embryos expressing wild-type Sox9 either in the presence or absence of SUMOylase Senp1b. Grg4 can repress *Dct* promoter activity mediated by wild-type Sox9 only in the absence of Senp1b. (C, top) In situ hybridization of showing Grg4-mediated inhibition of neural crest markers *Slug* and *Sox10* at stage 17. (bottom) Ectopic ear formation was observed in Grg4-injected embryos at stage 28. (D) In situ hybridization showing effects of Grg4, Sox9$_{2KR}$, or both on *Sox10* expression. Grg4 cannot repress Sox9$_{2KR}$-mediated neural crest formation. (E, top) Schematic of Sox9$_{2KR}$-Grg4–tethered construct. In situ hybridization showing effects of Sox9$_{2KR}$-Grg4, Sox9$_{2KR}$, or Sox9$_{2KR}$/SUMO on *Sox10* expression. Tethered Sox9$_{2KR}$-Grg4 phenocopies the effects of Sox9$_{2KR}$/SUMO. Arrowheads denote the injected side of embryos. Error bars represent the standard deviation of the mean of triplicate assays. Bars, 200 µm. RLU, relative luciferase activity.

relative ability to bind Grg4. Significantly, we found that neither SUMO nor SoxE$_{2KR}$ alone efficiently interacted with Grg4, whereas interaction with SoxE$_{2KR}$/SUMO was robust (Fig. 7 B and Fig. S4). Thus, physiologically significant interactions with Grg4 require surfaces on both SoxE and SUMO. These data suggest that SUMO provides one component of a bipartite interaction domain and only promotes stable association with Grg4 in the context of a second interaction surface on SoxE. Interactions mediated by a multivalent mechanism allow SUMO to be deployed as a highly versatile posttranslational modification, allowing for interactions with a greater diversity of cellular targets in a context-dependent manner with the context provided by the SUMOylated substrate.

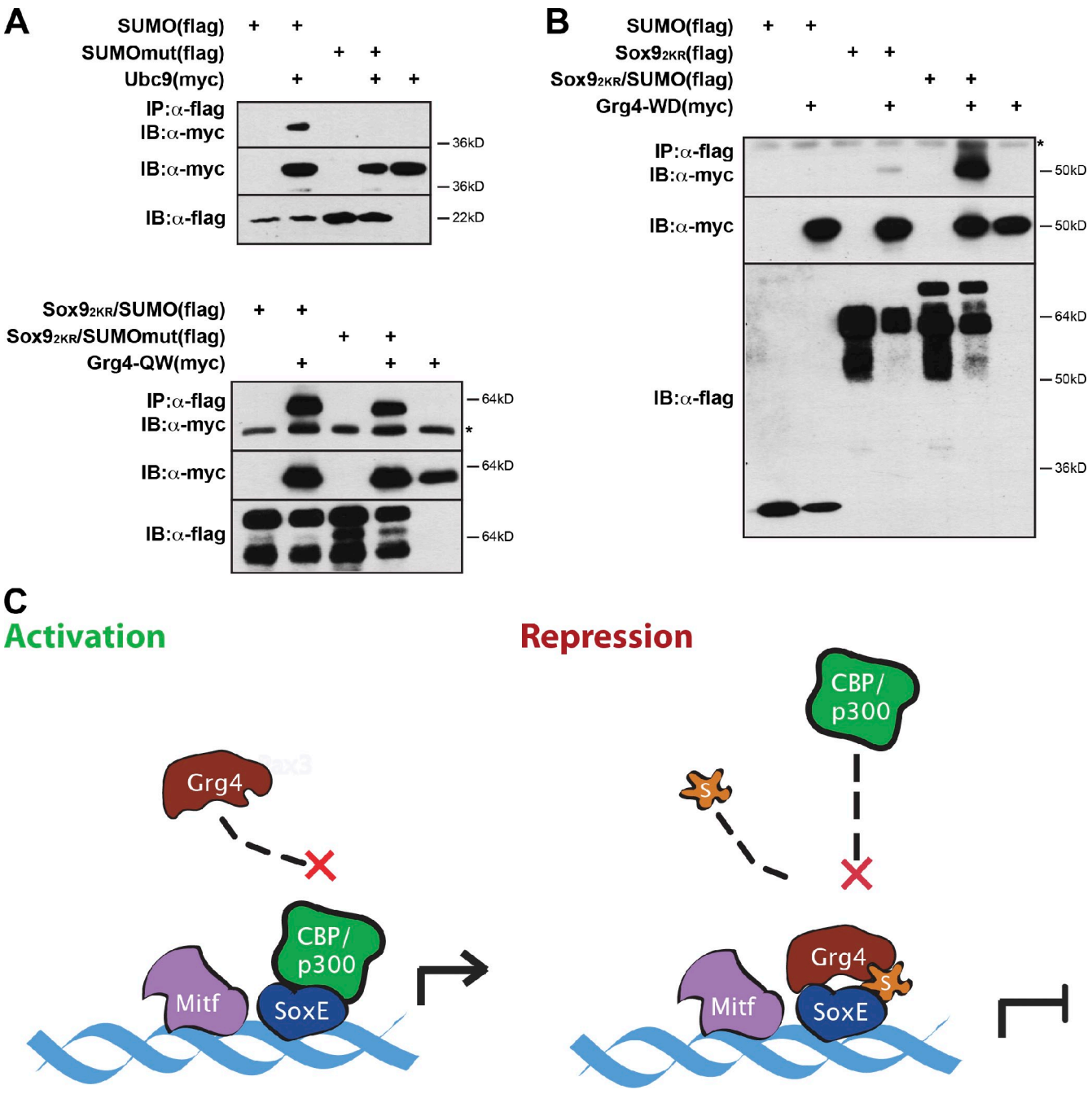

Figure 7. **SoxE-SUMO recruits Grg4 by a novel bivalent mechanism.** (A) Mutation of an essential SIM-interacting residue on the SUMO moiety ($SUMO_{mut}$ and $Sox9_{2KR}/SUMO_{mut}$) abolishes interaction with UBC9 but not with Grg4QW, which includes the poly-Q and WD domains. (B) Coimmunoprecipitation from *Xenopus* lysates showing that neither SUMO nor Sox9 is sufficient for robust interaction with Grg4. Strong interaction requires surfaces on both SUMO and Grg4. (A and B) Asterisks denote IgG bands. (C) Model describing the mechanism underlying SUMO-mediated modulation of SoxE function. SUMOylation of SoxE converts it from a transcriptional activator to a repressor by displacing coactivator CBP/p300 and recruiting corepressor Grg4 via a multivalent interaction.

Discussion

During development, many essential proteins are used reiteratively and play distinct roles in different cellular contexts. How such proteins are regulated to perform diverse functions in a context-dependent manner remains poorly understood. Although combinatorial control with other regulatory factors is clearly one mechanism for generating such functional versatility, it is increasingly clear that posttranslational modifications play an important role as well (Prasad et al., 2012).

In this study, we use the reiteratively deployed SoxE factors to further investigate this phenomenon. SoxE proteins are essential regulators of both neural crest precursor and otic placode formation. Later, SoxE proteins are involved in instructing multipotent NCCs to adopt a subset of derivative fates, including cartilage, glia, and melanocytes (Haldin and LaBonne, 2010).

We have previously shown that posttranslational modification by SUMO interferes with the neural crest–inducing abilities of SoxE proteins and promotes ear formation (Taylor and LaBonne, 2005).

Studies in cell culture have shown that the melanocyte promoting basic helix–loop–helix transcription factor Mitf is also a target for SUMOylation (Murakami and Arnheiter, 2005; Taylor and LaBonne, 2005). Here, we show that SUMOylation of either SoxE or Mitf interferes with melanocyte development. SoxE and Mitf synergistically activate expression of the melanocyte factor *Dct*, and SUMOylation of either protein inhibits this activation. In the case of SoxE, we show that SUMOylation leads to the remodeling of transcriptional coregulatory complexes, with a loss of association with coactivators such as p300/CBP and the recruitment of corepressor Grg4 (Fig. 7 C).

SoxE factors play key roles in the development of several cell types, including melanocytes, chondrocytes, and oligodendrocytes. Until recently, these proteins have been predominantly characterized as constitutive transcriptional activators. The C-terminal region of SoxE factors has been identified as the activation domain, and in vitro studies show that deletion of this domain completely abolishes the activation of target promoters (Potterf et al., 2000; Aoki et al., 2003). SoxE-dependent activation has been linked to the recruitment of coactivators p300/CBP (Tsuda et al., 2003; Imamura et al., 2005). We find here that it is non-SUMOylated SoxE factors that associate with p300/CBP and that SUMOylation leads to a loss of coactivator recruitment.

More recently, a handful of studies have suggested that SoxE factors may also act as negative regulators of gene expression. One recent study proposed a DNA binding–independent mechanism in which SoxE factors inhibit gene expression via interactions with other transcription factors (Dupasquier et al., 2009). Cruz-Solis et al. (2009) observed that overexpression of Sox10 leads to transcriptional repression of at least one target gene in a glutamate-dependent manner. It has also been shown that Sox9 expression can suppress *Vegfa* and *Spp1* during bone development (Hattori et al., 2010; Peacock et al., 2011); however, the mechanism behind the observed inhibitory effect was not determined. Here, we demonstrate that SUMOylated SoxE proteins can actively repress the *Dct* promoter and prevent Mitf-mediated activation in trans, both by preventing p300/CBP binding and promoting recruitment of the corepressor Grg4. This is the first mechanistic evidence demonstrating that SoxE proteins function as bimodal/context-dependent transcriptional regulators. The ability of SoxE factors to act as both positive and negative regulators of target promoters likely contributes to their functional versatility. It will be important to determine how the switch between these two modes is regulated, and such studies are currently underway.

Groucho family proteins are widely used transcriptional corepressors. In vertebrate embryos, these proteins play roles in neural development, ear development, somitogenesis, osteogenesis, and hematopoiesis among other processes (Cinnamon and Paroush, 2008; Jennings and Ish-Horowicz, 2008). Interestingly, other neural crest regulatory factors, including lymphoid enhancer factor/T cell factor, FoxD3, and Pax2, have also been shown to interact with Grg4 (Brantjes et al., 2001; Wagner et al., 2006; Yaklichkin et al., 2007). Given our findings with SoxE, it is possible that Groucho may act as a common node for regulating many transcriptional repression events during neural crest development. Indeed, it has been suggested that Grg4 may act as an integration point for information from several cellular signaling pathways, many of which also regulate neural crest development (Hasson and Paroush, 2006; Cinnamon and Paroush, 2008).

A growing number of studies have shown that posttranslational modification by SUMO plays an important role in development and disease processes (Baek, 2006; Meulmeester and Melchior, 2008). A variety of mechanisms have been proposed for how SUMOylation modulates substrate function, including changes in protein localization, stability, and partner interactions. When appended to transcription factors, SUMOylation most frequently leads to a decreased transactivation of target genes, although there are exceptions to this (Gill, 2005; Ouyang et al., 2009b). In the current study, we show that SUMOylation of SoxE (or Mitf) repressed *Dct* expression during melanocyte development. Our findings demonstrate that SUMOylation of SoxE factors abolishes their interaction with transcriptional coactivators p300/CBP and leads to recruitment of corepressor Grg4. This is the first evidence that Groucho can be recruited by SUMOylated proteins and that SoxE proteins can assemble corepressor complexes. In future studies, it will be important to demonstrate using chromatin immunoprecipitation assays the assembly of a SoxE-SUMO–dependent regulatory complex on target promoters and to determine when, where, and how SUMOylation of SoxE factors occurs.

Importantly, SUMOylation of target proteins does not always lead to Grg4 recruitment, as previous studies on HIPK2 have shown that SUMOylation disrupts its interaction with Groucho (Sung et al., 2005). Thus, interactions between Groucho and SUMOylated proteins are context dependent. SUMOylation is also associated with recruitment of corepressor complexes distinct from Grg4. For example, Ouyang and Gill (2009) and Ouyang et al. (2009a) demonstrated binding of corepressor complex LSD1–CoREST1–HDAC to SUMO2/3. The mechanism by which specificity is conferred on SUMO-mediated protein–protein interactions is a question of profound importance in a wide array of fields. Importantly, SUMO has not previously been shown to recruit Grg4, and Grg4 does not possess the characterized SIM possessed by most factors shown to interact noncovalently with SUMO (Hecker et al., 2006). Similarly, neither SoxE nor SUMO possesses previously defined Groucho-binding motifs (Jennings et al., 2006). Mutating characterized SIM-interacting residues on SUMO did not interfere with binding of Grg4, suggesting a SIM-independent interaction. Indeed, we find that neither SUMO nor SoxE alone interact with Grg4 with high affinity and that high affinity Grg4 interaction depends on surfaces provided by both SUMO and its substrate, in this case SoxE. Use of a multivalent interaction mechanism permits SUMO to be deployed as a more versatile posttranslational modification, allowing interaction with a greater diversity of cellular targets in a context-dependent manner.

Materials and methods

DNA constructs, embryological methods, and cell lines

XSox9 was isolated from stage 17 cDNA using a low copy number PCR and a high fidelity polymerase (Tgo; Roche). Murine Mitf (gift of D. Lang, University of Chicago, Chicago, IL) and XSox9 were cloned into pCS2 vectors that add either five N- or C-terminal Myc tags or three N-terminal flag tags. All constructs were confirmed by sequencing. The Sox9 K61R and K365R mutations and Mitf K182R and K316R mutations were generated via mutagenesis kit (QuikChange; Agilent Technologies). $Sox9_{2KR}$/SUMO and $Mitf_{2KR}$/SUMO were created by ligating SUMO-1 in frame C terminal to the full-length $Sox9_{2KR}$ or $Mitf_{2KR}$ mutants using PCR methods. Fusions proteins were inserted into pCS2-Myc. The linked versions of these constructs were created by similar PCR methods and inserted into the pCS2 N-terminal Myc-tagged vector. The linker region was inserted in frame between constructs and is a glycine-rich repeat. The portion of the linker construct that has SUMO-1 fused upstream of $Mitf_{2KR}$ was created in an analogous manner to the $Mitf_{2KR}$/SUMO construct but with SUMO-1 ligated to the N terminus of $Mitf_{2KR}$. *Xenopus* Grg4 was a gift from H. Clevers (Hubrecht Institute, Utrecht, Netherlands). Grg4 was subcloned into the pCS2 variant with either five N-terminal Myc tags or three N-terminal flag tags using PCR methods. $Sox9_{2KR}$/SUMO and Grg4 deletion constructs were generated by PCR and subsequently subcloned to the pCS2 vector. $Sox9_{K365R}\Delta E1$,high mobility group/SUMO was also fused to the pGEX-6P-1 vector (GE Healthcare) to create the GST-Sox9Δ/SUMO construct. All constructs were confirmed by sequencing and express as full-length constructs in embryo lysates (Fig. S2 and not depicted). All results shown are representative of at least three independent experiments. RNA for injection was produced in vitro from linearized plasmid templates using the synthesis kit (mMESSAGE mMACHINE; Ambion). mRNA concentrations injected were in the range of 5–50 pg. Collection, injection, and in situ hybridization of *Xenopus* embryos were as previously described (Bellmeyer et al., 2003). Lineage tracer β-gal was detected by Red-Gal substrate (Research Organics) and demarks the cells that received injected messages. Location of modulated cells, as marked by Red-Gal staining, at neurula and tadpole stages is dependent on the region of the embryo targeted by injection. Animal cap explants (of ∼0.5 × 0.5 mm) were manually isolated using forceps and a hair knife from the animal pole of blastula (stage 8) devitellinated embryos that had previously been injected at the two-cell stage with the indicated mRNA. Animal caps were cultured at room temperature in Marc's modified ringers in agar-coated dishes until the stage indicated and then fixed in formaldehyde for 30 min before being processed for in situ hybridization (LaBonne and Bronner-Fraser, 1998). Embryo and explant images were collected on a dissecting microscope (SZX12; Olympus) fitted with a dark-field Plan Fluorite 0.5× objective (Olympus) and a microscope digital camera system (DP12; Olympus) at room temperature. Composite images were assembled using Photoshop (Adobe). Human melanoma cell line C8161 was provided by M. Hendrix (Northwestern University, Evanston, IL). C8161 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Corning).

Luciferase (and TSA and VPA) assays

The luciferase constructs, *Dct*-luciferase, were a gift from D. Lang, and the Renilla construct was a gift of C. Horvath (Northwestern University, Evanston, IL). The *Dct*-luciferase reporter contains the ∼3.2-kb mouse *Dct* promoter. In brief, the luciferase and Renilla constructs (DNA) were injected alone or in combination with RNA into both cells of a two-cell *Xenopus* embryo. Embryos were cultured until stage 17, collected in 10-embryo sets, and lysed in 500 µl of passive lysis buffer using the reporter assay system kit (Dual-Luciferase; Promega). Cell transfection experiments were performed with 800 ng DNA total per well transfected, 100 ng of each reporter, and 300 ng of each construct. Wells lacking experimental constructs were transfected with the appropriate amount of empty vector. Cells were transfected using Lipofectamine Plus (Invitrogen). Assays were performed using a TD-20/20 luminometer (Turner Biosystems) or the Glomax luminometer (Turner Biosystems). TSA experiments were performed in *Xenopus* embryos by adding TSA (Sigma-Aldrich) at a final dilution of 1:25,000 of a 2-mg/ml stock solution at stage 11 and were cultured to stage 17. The control embryos were treated with ethanol. For cell culture experiments, TSA was added at a final dilution of 1:15,000 of a 2-mg/ml stock solution. Treated cells were cultured overnight and stopped at the 24 h mark (TSA treatment for a total of 16 h). Control wells were treated with ethanol. VPA treatments were performed in *Xenopus* embryos by adding VPA at a final concentration of 50 mM at stage 11 followed by culture to stage 17. Control embryos were treated with water.

Western blot analysis and coimmunoprecipitation assays

For Western blots, one cell of a two-celled embryo was injected, harvested at stage 8, and lysed in lysis buffer (PBS + 1% NP-40) supplemented with phenylmethylsulfonyl fluoride, aprotinin, and leupeptin. For SUMOylation assays, flag-tagged SUMO-1 (Taylor and LaBonne, 2005) was coexpressed with Myc-tagged SoxE proteins, and injected embryos were cultured to gastrula stages for Western blot analysis. Shifted species were detected by blotting for the Myc epitope. For coimmunoprecipitation assays, mRNAs were injected into both cells of a two-celled embryo, collected at stage 8, and lysed as previously stated in this paragraph. The immunoprecipitation or "pull" antibody was added to the lysate at a dilution of 1:250 (CBP and p300 immunoprecipitations) or 1:500 (all other immunoprecipitations). Also added to the lysate was radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) supplemented with phenylmethylsulfonyl fluoride, aprotinin, and leupeptin. The antibody/lysate mixture was incubated by rocking at 4°C for 2 h (CBP and p300 immunoprecipitations) or on ice for 2 h (all other immunoprecipitations), upon which protein A–Sepharose beads were added (Sigma-Aldrich) and then rocked for an additional 2 h at 4°C (p300 [N-15]: sc-584X; and CBP [A-22]: sc-369X antibodies; Santa Cruz Biotechnology, Inc.). Beads were washed three times with RIPA buffer, resuspended in lysis buffer, and resolved by SDS-PAGE. Proteins were detected using antibodies against the epitope tags (Myc [9E10; Santa Cruz Biotechnology, Inc.]; flag [affinity purified M2; Sigma-Aldrich]; or Actin [Sigma-Aldrich]).

Immunofluorescence staining

Animal pole ectoderms (animal caps) were dissected at stage 9 from blastula stage embryos expressing flag-tagged Sox9 isoforms, fixed, and stored in 100% EtOH. For antibody staining, animal caps were slowly rehydrated into PBS, blocked with 10% sheep serum/PBS, and incubated with flag antibody at 1:1,000 dilution in 10% sheep serum/PBS at 4°C. After extensive PBST washes, animal caps were incubated with Alexa Fluor 594 secondary antibody (Invitrogen) at 1:250 dilution along with DAPI at 1:1,000 in 10% sheep serum/PBS for 3 h at room temperature. After washing in PBST (PBS and Tween), caps were mounted in gelvatol for image collection with an microscope (Axiovert 200M ApoTome; Carl Zeiss) with Plan-Neofluar 40× objective lenses (Carl Zeiss). Images were captured with a camera (AxioCam MRm; Carl Zeiss) with the AxioVision operation system (Carl Zeiss) at room temperature. Composite images were assembled using Photoshop and Illustrator (Adobe).

EMSA

The probe for Mitf and linked construct EMSAs contains the S1 and Mitf site from the mouse *Dct* promoter (up, 5′-CTTAGGGTCATGTGCTAACAAAGAGGATTTCTC-3′; down, 5′-GAGAAATCCTCTTTGTTAGCACATGACCCTAAG-3′). Probes were labeled with γ-[^{32}P]ATP and purified using microcolumns (ProbeQuant G-50; GE Healthcare). Proteins were in vitro translated with the rabbit reticulocyte lysate system (Promega). Proteins were incubated with poly dI/dCs (Sigma-Aldrich) and EMSA buffer (50% glycerol, 5 mM DTT, 0.5 mg/ml BSA, 10 mM $MgCl_2$, 375 mM NaCl, 100 mM Hepes, and 50 µg/ml single-strand DNA) for 5 min at room temperature. The labeled probe was then added to the mixture and incubated for 30 min at room temperature. Samples were resolved on 5% TBE (Tris base, boric acid, and EDTA)/acrylamide gels and imaged using autoradiography. For SoxE, the probe used contains the dimeric binding site S4/4′ from the Dct promoter (Stolt et al., 2008). Sox9 and its SUMOylation mutant were transcribed and translated in vitro using the quick coupled transcription/translation system (TNT; Promega). Proteins were incubated with 0.05 µg/µl poly-dG/dCs (Sigma-Aldrich), 5 mM DTT, 0.15 µg/µl BSA, and mobility shift buffer (5% glycerin, 2 mM DTT, 5 mM $MgCl_2$, 25 mM NaCl, and 10 mM Hepes), and the labeled probe was incubated on ice for 20 min. Samples were resolved on 5% TBE/acrylamide gels and imaged using autoradiography.

Purification of GST proteins and GST pull-down assay

GST proteins were expressed in BL21 stain of *E. coli*, sonicated, and purified with glutathione-agarose (Sigma-Aldrich). Protein induction and bead attachment were verified by SDS-PAGE and Coomassie staining. Grg4-WD (amino acids 479–766) was transcribed and translated in vitro using the quick coupled transcription/translation system (TNT) in the presence of [^{35}S]methionine. 8% of the reaction mixture was kept as the input. The remainder was incubated with glutathione bead–bound GST fusion proteins for 2 h at 4°C in lysis buffer in a 500-µl volume. Glutathione-agarose was washed four times with RIPA buffer, and bound proteins were released by

boiling in SDS sample buffer, analyzed by SDS-PAGE, and imaged using autoradiography.

Online supplemental material

Fig. S1 and Fig. S2 show the effects of Mitf isoforms and Mitf/Sox9-tethered constructs on *Dct* expression, respectively. Fig. S3 shows coimmunoprecipitations mapping the domains required for interaction between Sox9/SUMO and Grg4. Fig. S4 demonstrates interactions between Sox8/Sox9/Sox10 and Grg4 and demonstrates Sox8 SUMOylation. Fig. S5 shows the effect of Senp1b on Sox9 SUMOylation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201204161/DC1.

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