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RESEARCH ARTICLE

Snail2/Slug cooperates with Polycomb repressive complex 2 (PRC2) to regulate neural crest development

Chih-Liang Tien[1], Amanda Jones[2], Hengbin Wang[2], Magda Gerigk[1], Susan Nozell[1] and Chenbei Chang[1,*]

ABSTRACT

Neural crest cells arise from the border of the neural plate and epidermal ectoderm, migrate extensively and differentiate into diverse cell types during vertebrate embryogenesis. Although much has been learnt about growth factor signals and gene regulatory networks that regulate neural crest development, limited information is available on how epigenetic mechanisms control this process. In this study, we show that Polycomb repressive complex 2 (PRC2) cooperates with the transcription factor Snail2/Slug to modulate neural crest development in *Xenopus*. The PRC2 core components Eed, Ezh2 and Suz12 are expressed in the neural crest cells and are required for neural crest marker expression. Knockdown of Ezh2, the catalytic subunit of PRC2 for histone H3K27 methylation, results in defects in neural crest specification, migration and craniofacial cartilage formation. EZH2 interacts directly with Snail2, and Snail2 fails to expand the neural crest domains in the absence of Ezh2. Chromatin immunoprecipitation analysis shows that Snail2 regulates EZH2 occupancy and histone H3K27 trimethylation levels at the promoter region of the Snail2 target E-cadherin. Our results indicate that Snail2 cooperates with EZH2 and PRC2 to control expression of the genes important for neural crest specification and migration during neural crest development.

KEY WORDS: Snail2/Slug, Polycomb repressive complex 2 (PRC2), EZH2, Neural crest, *Xenopus*

INTRODUCTION

The neural crest is a multipotent cell population that arises from the border of the neural plate and the non-neural epidermal ectoderm in vertebrate embryos. Following their induction, neural crest cells migrate along distinct routes throughout the body and differentiate into diverse cell types, such as bone, cartilage, muscle, neuron, glia and melanocytes. The neural crest-derived structures contribute to musculoskeletal elements in the face and the skull, the peripheral nervous system, the cardiovascular septa and valves, the thymus, the pigment cells of the skin and the secretory cells in the adrenal gland. Defects in neural crest development are associated with a variety of human congenital abnormalities, including cleft palate, Waardenburg syndrome, persistent truncus arteriosus and Hirschsprung's disease (Hall, 2009). Detailed knowledge of the molecular control of neural crest development is thus crucial to identify the etiology of neural crest-associated human birth defects and to design proper therapeutic strategies to treat these diseases.

Research over the past two decades has uncovered several signaling pathways and many transcription factors important for neural crest development [reviewed by Rogers et al. (2012); Mayor and Theveneau (2013); Pegoraro and Monsoro-Burq (2013)]. An emerging theme reveals that dynamic signals via bone morphogenetic proteins (BMPs), Wnt and fibroblast growth factors (FGFs) are crucial for neural crest induction and maintenance (Stuhlmiller and Garcia-Castro, 2012). These signals also impact on neural crest cell delamination, whereas ephrin/Eph, Sdf1/Cxcr4 and semaphorin pathways dictate the migration of the neural crest [more extensive reviews can be found in Theveneau and Mayor (2012a,b)]. Multiple transcription factors are activated downstream of the growth factor signals, including members of the Snail family (Snail1 and Snail2/Slug), the SoxE family (Sox8, 9 and 10), the Pax family (Pax3, 7) and the Msx family (Msx1 and 2), among others. These transcription factors act in cascades as well as through feedback regulation to control different aspects of neural crest development, such as specification, epithelial-to-mesenchymal transition (EMT), collective cell migration and differentiation (Meulemans and Bronner-Fraser, 2004; Groves and LaBonne, 2014). Together, they ensure that neural crest cells develop at the right time and location so they can undertake proper movements and differentiate into the correct cell types in suitable tissue environments.

In addition to transcription factors, epigenetic regulators also fundamentally influence gene expression. The epigenetic factors control gene expression via modulation of chromatin structure, often through DNA methylation or histone modification. These modifications regulate chromatin compaction and accessibility of transcriptional machinery to DNA, and thus can affect both local and global gene transcription (Allis et al., 2007). Among the epigenetic factors, the Polycomb group (PcG) proteins are one of the best characterized (Schuettengruber and Cavalli, 2009; Lanzuolo and Orlando, 2012; Di Croce and Helin, 2013; Simon and Kingston, 2013). Originally identified in *Drosophila* as genes that, when mutated, changed the expression of Hox genes and the morphology of body segments, PcG proteins were subsequently found in fungi, plants and metazoan species, including all vertebrate animals (Whitcomb et al., 2007). Targeted gene disruption in mouse shows that null mutants of many PcG genes are embryonically lethal. PcG proteins function in embryonic stem cells to regulate cell self-renewal and differentiation, and altered PcG levels are associated with cancer formation in humans (Sparmann and van Lohuizen, 2006; Bhaumik et al., 2007; Sawarkar and Paro, 2010; Vastenhouw and Schier, 2012; Aloia et al., 2013). PcG proteins form two main multiprotein complexes, Polycomb repressive complex 1 (PRC1) and PRC2. PRC2, which includes the core proteins EED, EZH2 and SUZ12, methylates histone H3 at lysine 27 (K27). The tri-methylated H3K27 (H3K27me3) is frequently associated with closed chromatin conformation; thus, PRC2 is involved mainly in repression of gene

[1]Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, 1720 2nd Avenue S., Birmingham, AL 35294, USA. [2]Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, 1720 2nd Avenue S., Birmingham, AL 35294, USA.

*Author for correspondence (cchang@uab.edu)

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expression. Methylated H3K27 can be recognized by PRC1, which leads to histone H2A lysine 119 ubiquitylation (Schuettengruber and Cavalli, 2009; Lanzuolo and Orlando, 2012; Simon and Kingston, 2013). In *Drosophila*, the PcG-repressive complexes are recruited to chromatin via discrete DNA elements called PREs (Polycomb response elements), which are bound by PRE-associated proteins (Ringrose and Paro, 2004, 2007; Lanzuolo and Orlando, 2012). However, PRE-like sequences have not been identified convincingly in vertebrates, and how PcG proteins recognize their targets remains a focus of intensive research. In addition, owing to early embryonic lethality in mice deficient for many PcG genes, the range of PcG protein functions during embryogenesis is not fully appreciated yet. In particular, it is unknown whether these epigenetic factors play important roles during neural crest development, and if so, whether they regulate cell fate specification or migration of the neural crest.

In this study, we examined the function of PRC2 in neural crest development. We show that EZH2, the catalytic component of PRC2, binds directly to the transcription factor Snail2/Slug (referred to as Snail2 herein). PRC2 and Snail2 collaborate in modulating expression of neural crest genes, and Snail2 fails to expand the neural crest domain in the absence of EZH2 *in vivo*. We further demonstrate that PRC2 regulates proper migration of neural crest cells following their induction. Snail2 and EZH2 co-occupy the promoter region of E-cadherin during neural crest EMT, and the presence of Snail2 is important to maintain H3K27me3 levels at this promoter. Our data suggest that Snail2 recruits PRC2 to control neural crest gene expression during neural crest specification and migration.

RESULTS

PRC2 transcripts are enriched in the neural and neural crest tissues

To investigate the function of PRC2 in early *Xenopus* embryogenesis, we first examined the expression of PRC2 components at different developmental stages. RT-PCR analysis revealed that all three major components of the PRC2, namely Eed, Ezh2 and Suz12, were expressed constantly throughout early development (data not shown). Whole-mount *in situ* hybridization (WMISH) demonstrated that these genes showed similar patterns of RNA distribution (Fig. 1). At late blastula and early gastrula stages, their transcripts were enriched in the mesoderm and dorsal ectoderm. As development proceeded, their RNAs were downregulated from the mesoderm and maintained in the dorsal ectoderm. During neurulation, their transcripts were detected in neural and neural crest cells. At tailbud and tadpole stages, all three genes were expressed in the neural, migrating neural crest, eye, otic vesicle, olfactory placode and lateral line cells. The expression patterns of Eed, Ezh2 and Suz12 suggested that the three genes probably work together to regulate early mesodermal, neural and neural crest development.

PRC2 regulates neural and neural crest development

Previous studies have shown that PRC2 modulates mesendodermal formation in collaboration with the nuclear factor Geminin (Lim et al., 2011), but whether it also plays roles in neural and/or neural crest development is not clear. To address this issue, we performed targeted injection of PRC2 RNAs or translational blocking antisense morpholino oligos (MOs) specifically into the animal region of early *Xenopus* embryos. This resulted in expression of PRC2 or PRC2-MOs in ectodermal derivatives, so that potential effects of PRC2 on mesendodermal tissue formation could be avoided. The activities of the injected RNAs or MOs were confirmed by histone H3K27 trimethylation assay. It showed increased H3K27me3 levels when PRC2 was ectopically expressed, and decreased H3K27me3 levels when EZH2, the catalytic subunit of PRC2, was knocked down (supplementary material Fig. S1). The RNAs or MOs of each PRC2 core component were then co-injected with the tracer encoding nuclear β-galactosidase into the animal region of one blastomere of two-cell-stage embryos, and their effect on marker expression was examined at the neurula stages by *in situ* hybridization (ISH). The injected side was identified by staining with Red-Gal substrate, and the level of marker expression on two sides of the same embryos was compared. Ectopic expression of individual PRC2 components or all three PRC2 genes together did not drastically alter the expression of the neural markers Sox3 and NRP1, but they did moderately expand the neural plate border and the neural crest markers (supplementary material Fig. S2). The results implied that PRC2 had a low efficiency in regulating expression of the neural and the neural crest genes by itself. Conversely, knockdown of the PRC2 components did not dramatically decrease the expression of the early neural marker Sox3 or the neural plate border genes AP2 and Msx1, but it reduced the expression of the definitive neural marker NRP1 and the neural crest markers Snail2 and Sox9 (Fig. 2). The specificity of the MOs was confirmed by rescuing Sox9 expression in morphant embryos with epitope-tagged PRC2 components that were not targeted by the MOs (supplementary material Fig. S3; and data not shown). Interestingly, knockdown of any single PRC2 component produced similar phenotypes, suggesting that all three proteins need to be present for normal PRC2 activity. This result was consistent with previous reports that all three core proteins are required for functional assembly of PRC2 (Cao and Zhang, 2004; Pasini et al., 2004; Montgomery et al., 2005). Based on this observation, we focused on EZH2 only in the subsequent studies.

EZH2 interacts with the transcription factor Snail2

As PRC2 proteins are not sequence-specific DNA-binding factors, they need to interact with other proteins and/or non-coding RNAs to be recruited to their target genes (Schuettengruber and Cavalli, 2009; Lanzuolo and Orlando, 2012; Simon and Kingston, 2013). To understand better the mechanism of PRC2-regulated ectodermal

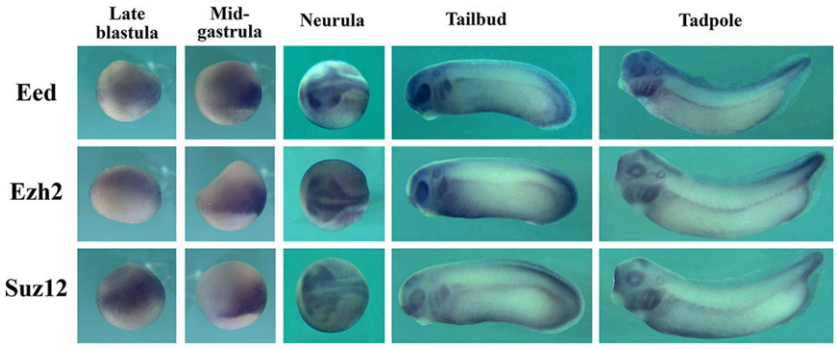

Fig. 1. Expression of the PRC2 core components during early *Xenopus* development. Eed, Ezh2 and Suz12 are expressed similarly in early *Xenopus* embryos. Their transcripts are detected in the pan-mesoderm in late blastulae and early gastrulae embryos, but are shifted to dorsal mesodermal and dorsal ectodermal cells at late gastrula stages. During neurulation, their RNAs are located in neural and neural crest cells. At tailbud and tadpole stages, these genes are expressed in neural, sensory organs and migrating neural crest cells.

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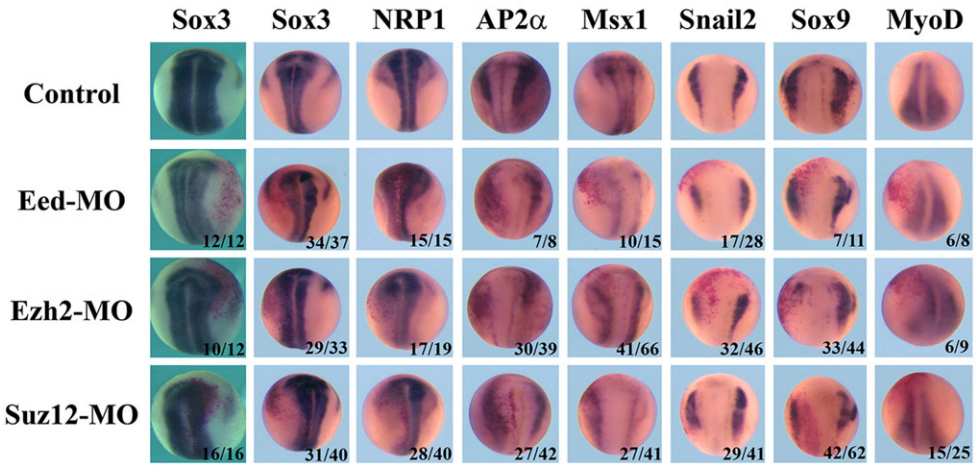

Fig. 2. PRC2 regulates neural and neural crest development. MOs (20 ng) against Eed, Ezh2 or Suz12 were injected unilaterally into two-cell-stage embryos. The embryos were examined at neurula stages by ISH for neural and neural crest markers. Knockdown of either one of the PRC2 proteins reduced the expression of the late neural marker NRP1 and the neural crest genes Snail2 and Sox9, but did not drastically decrease the expression of the early neural marker Sox3 (open neural plate stages, first column from left; late neurula stages, second column from left) or the neural plate border genes AP2α and Msx1. The mesodermal marker MyoD was not inhibited either. Numbers indicate embryos showing the displayed patterns (no reduction for Sox3, AP2α, Msx1 and MyoD, and decreased expression for NRP1, Snail2 and Sox9) over the total number of embryos analyzed.

development, we screened several candidate transcription factors known to play roles in neural or neural crest development for their ability to interact with the PRC2 components (supplementary material Fig. S4; and data not shown). We found that Snail2, a transcription factor involved in neural crest development, associated with EZH2 in a co-immunoprecipitation (co-IP) assay (supplementary material Fig. S4). This binding was further confirmed via a reciprocal co-IP experiment, using HA-tagged EZH2 and Flag-tagged Snail2 (Fig. 3A). Examination of the other PRC2 proteins revealed that, whereas EED did not interact with Snail2 appreciably when they were co-expressed, EED co-precipitated with Snail2 in the presence of EZH2 and SUZ12 (Fig. 3B). This result indicated that Snail2 probably recruited the entire PRC2 complex via its interaction with EZH2. To assess whether binding of PRC2 to Snail2 is direct in the absence of other co-factors, we constructed a glutathione S-transferase (GST)-Snail2 fusion protein and purified the chimeric protein from cultured *E. coli* with glutathione-agarose beads. When mixed with commercially available purified PRC2 complex, GST-Snail2, but not GST, was able to pull down EZH2 (Fig. 3C). These data suggest that Snail2 interacts directly with EZH2 to recruit the PRC2 complex to their target genes to regulate neural crest development.

Snail2 requires PRC2 to modulate neural crest gene expression

To investigate the functional significance of Snail2 and PRC2 interaction, we first examined the potential synergistic activation of the neural crest genes by combined expression of Snail2 and EZH2. Snail2 expanded several neural plate border and neural crest markers, whereas EZH2 had a weak ability to enhance the expression of these genes. When Snail2 was co-expressed with EZH2, the expression of these markers was similar to that when Snail2 was expressed alone (supplementary material Fig. S5). The result implied that EZH2/PRC2 could not act efficiently outside the Snail2 expression domain to regulate neural crest genes.

As both Snail2 and EZH2 are expressed in neural crest tissues, we next tested whether the activity of PRC2 was required by Snail2 to regulate neural crest markers. We therefore co-expressed Snail2 RNA with EZH2-MO in one blastomere of two-cell-stage embryos, as described above. Marker expression analysis at the neurula stages (stages 17-18) demonstrated that, whereas Snail2 expanded several neural crest genes, it failed to do so in the absence of EZH2 (Fig. 4). The data revealed that EZH2 is required by Snail2 to modulate neural crest gene expression.

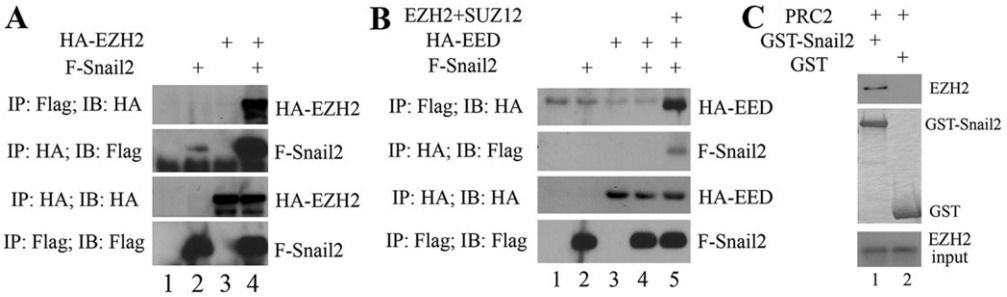

Fig. 3. EZH2 interacts directly with Snail2. RNAs (1-2 ng) encoding tagged and untagged PRC2 proteins and Flag-Snail2 were injected alone or together, and proteins were extracted at gastrula stages 10-11 for IP and western blot analyses. (A) Reciprocal co-IP assay demonstrated that HA-EZH2 interacts with Flag-Snail2 in *Xenopus* embryos. (B) HA-EED and Flag-Snail2 do not co-precipitate. However, in the presence of EZH2 and SUZ12, HA-EED was pulled down by anti-Flag antibody, indicating that Snail2 recruited the entire PRC2 complex. (C) Purified GST-Snail2, but not GST, pulled down commercially available purified EZH2 directly.

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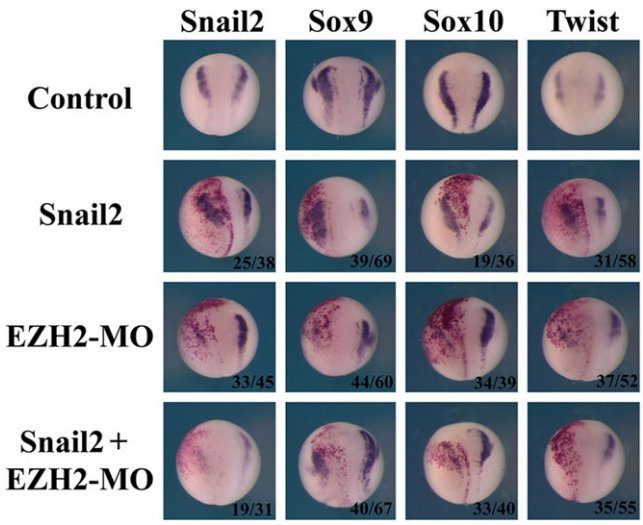

Fig. 4. Snail2 requires EZH2 to regulate neural crest markers. Snail2 RNA (0.15 ng) was injected alone or with 20 ng EZH2-MO unilaterally into two-cell-stage embryos. ISH performed at neurula stages 17-18 revealed that ectopic expression of Snail2 expanded domains of neural crest genes Snail2, Sox9, Sox10 and Twist. However, Snail2 failed to expand the domains of these genes in the presence of EZH2-MO, implying that neural crest expansion by Snail2 required EZH2 function. The numbers of embryos showing the displayed patterns over total embryos analyzed are shown.

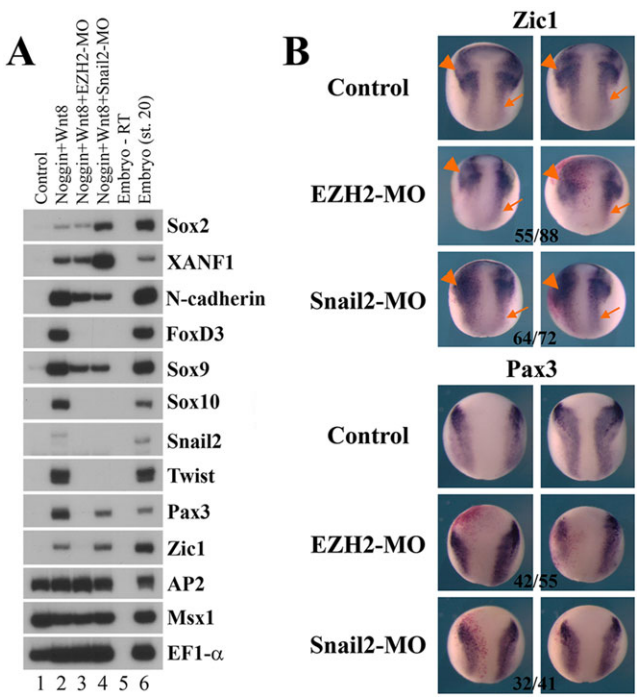

Fig. 5. EZH2 and Snail2 regulate overlapping and distinct markers in *Xenopus*. (A) 10 pg Noggin and 25 pg Wnt8 RNAs, with or without 40 ng EZH2-MO or Snail2-MO, were injected into the animal regions of two-cell-stage embryos. Animal caps were dissected at blastula stage 9 and cultured until neurula stages 19-20. RNA was then extracted for RT-PCR analysis of marker expression. Co-expression of Noggin and Wnt8 induced a panel of neural crest markers in animal caps that were reduced by either EZH2 or Snail2 knockdown. Notably, however, depletion of EZH2 affected more markers (Pax3 and Zic1) than depletion of Snail2, indicating that EZH2 regulates a wider array of gene expression than Snail2. (B) 20 ng EZH2-MO or Snail2-MO was injected unilaterally into two-cell-stage embryos. The embryos were analyzed at neurula stages 16-17 by ISH for expression of Zic1 and Pax3. Knockdown of EZH2 reduced the posterior (arrow), but not the anterior (arrowhead), domain of Zic1 and the expression of Pax3; however, Snail2-MO was ineffective in reducing either Zic1 or Pax3. The numbers of embryos displaying the shown patterns over the total number of embryos analyzed are shown.

Snail2 modulates a subset of genes affected by EZH2

To explore further how Snail2 and EZH2 regulate neural crest markers, we performed an animal cap assay. In this experiment, the neural crest was induced via co-expression of Noggin and Wnt8 in the ectoderm. Ectodermal explants (animal caps) were dissected at blastula stage 9, and expression of the neural plate border and the neural crest genes was analyzed by RT-PCR at neurula stages 19-20. Interestingly, whereas both EZH2 and Snail2 were required for expression of an array of neural crest markers, they displayed differential abilities to modulate the neural plate border genes *Pax3* and *Zic1*. Knockdown of EZH2 abolished expression of these markers in the animal caps, but knockdown of Snail2 either did not change (*Zic1*) or only moderately affected the expression of these genes (Fig. 5A). The results implied that, in addition to collaborating with Snail2 to control neural crest gene expression, PRC2 also interacts with other proteins to regulate neural plate border markers (see Discussion).

To confirm that Snail2 and EZH2 differentially regulate *Zic1* and *Pax3 in vivo*, we next examined their expression in Snail2 or EZH2 morphant embryos. Knockdown of EZH2 reduced Zic1 in the posterior, but not anterior, neural plate border and decreased Pax3 expression. By contrast, knockdown of Snail2 did not appreciably diminish either Zic1 or Pax3 levels (Fig. 5B). Analyses of other neural crest markers, including Snail2, Sox9, Sox10 and Twist, showed that they were similarly reduced in Snail2 and EZH2 morphants (supplementary material Fig. S6). The results indicated that, whereas EZH2 and Snail2 co-modulate many neural crest genes, EZH2 has an additional activity to regulate certain neural plate border genes.

PRC2 regulates migration of the neural crest

Snail2 is shown to influence not only neural crest specification, but also neural crest migration. In our experiments, we noticed that EZH2 morphant embryos that retained Sox10 expression displayed failure in migration of Sox10-expressing cells (data not shown), implying that EZH2 might also regulate neural crest migration. As EZH2 plays an early role in neural crest specification, we sought to block EZH2 activity after neural crest induction to uncover a possible function of PRC2 in neural crest migration. For this purpose, we took advantage of the EZH2-specific inhibitor GSK126, which inhibits EZH2 methyltransferase activity and displays a 1000-fold higher selectivity toward EZH2 than to other methyltransferases (McCabe et al., 2012). We cultured wild-type embryos in DMSO- or GSK126-medium from stages 13-14 onward and examined Sox10 and Twist ISH at tailbud stages 25-26. In DMSO-treated embryos, Sox10- and Twist-expressing cells were seen to migrate in distinct streams in the head. However, treatment with GSK126 led to reduced migration of these cells, especially in the hyoid and the branchial regions (Fig. 6A). Furthermore, Sox10 and Twist were expressed in the dorsal midline in the trunk of DMSO-treated embryos, but this expression was eliminated by treatment with GSK126 (100%, arrow in Fig. 6A). This was consistent with the observation that cranial neural crest development preceded that of the trunk neural crest, and suggested that PRC2 activity is required for induction and/or maintenance of the trunk neural crest during the neurula stages.

To analyze further the requirement of PRC2 in neural crest migration, we also performed neural crest transplant experiments.

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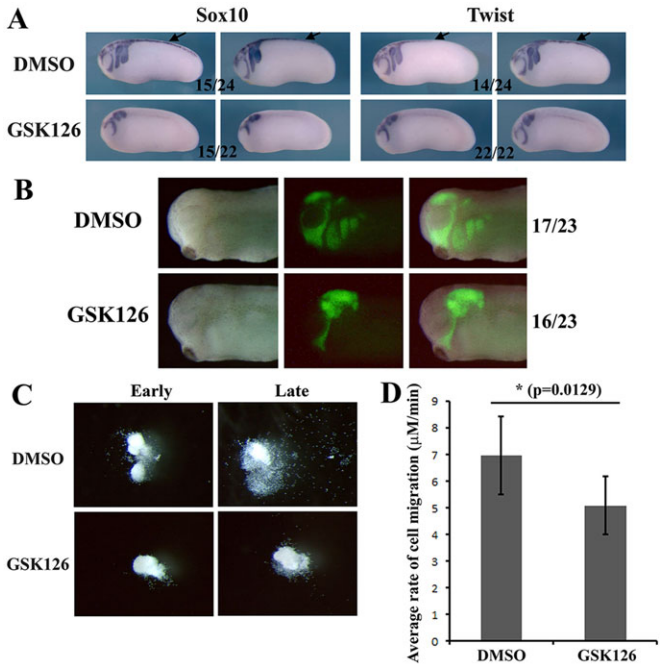

Fig. 6. EZH2 regulates neural crest migration. (A) Wild-type embryos were cultured in medium with DMSO or 50 μM EZH2 inhibitor GSK126 from stages 13-14 onward. The embryos were analyzed by ISH for Sox10 and Twist expression at tailbud stages 25-26. In DMSO-treated embryos, Sox10- and Twist-expressing cells migrated in distinct streams in the head. Treatment with GSK126 resulted in reduced migration of these cells. In addition, Sox10 and Twist expression in dorsal midline of the trunk (arrows) was eliminated by GSK126 treatment. (B) Neural crest tissues from fluorescein dextran-labeled donor embryos were transplanted into unlabeled control embryos at neurula stages 15-16. The embryos were cultured in medium with DMSO or 50 μM GSK126 until tailbud stages 26-28. Migration of the neural crest was examined by fluorescence microscopy. Whereas neural crest from DMSO-treated embryos migrated efficiently in the head, GSK126-treated embryos displayed defects in neural crest migration. (C) Neural crest explants were dissected from stage 15-17 embryos and cultured on fibronectin-coated dishes for about 16 h in the presence of DMSO or 8-10 μM GSK126. Migration of the neural crest was imaged between 2 and 16 h of culture. Whereas neural crest cells dissociated from the core explants and migrated efficiently in DMSO-containing medium, treatment with GSK126 reduced the migration of the neural crest on fibronectin. (D) Tracking of cell migration in time-lapse movies revealed that the velocity of neural crest cell migration was significantly reduced by treatment with GSK126. Four movies each for DMSO- and GSK126-treated samples were analyzed, with 60-170 cells tracked in each movie.

Cranial neural crest was dissected from fluorescein dextran-labeled embryos and transplanted into unlabeled wild-type embryos at stages 15-16. These embryos were cultured in DMSO- or GSK126-containing medium until the tailbud stages 26-28, and migration of the neural crest was examined by fluorescence microscopy. In DMSO medium, transplanted neural crest migrated in distinct streams toward the ventral region. In GSK126 medium, neural crest did not migrate efficiently and accumulated at the dorsal region (Fig. 6B). These results imply that PRC2 regulates neural crest migration *in vivo*.

To investigate how PRC2 influences the migratory behavior of the neural crest, we next dissected the neural crest explants at neurula stages 15-17 and cultured them in the presence of DMSO or GSK126 on fibronectin-coated dishes. Explants grown in DMSO medium migrated on fibronectin as a collective cell sheet first, before individual cells dissociated from the core tissue and moved about in a random, undirected fashion. Explants treated with GSK126 also migrated as a cohesive cell sheet first. However, as cells dissociated from the explants, they did not spread well on fibronectin and remained largely in round cell shape. These cells tended to have jerky movements and migrated less efficiently (Fig. 6C and supplementary material Movies 1-4). Quantitative analysis of the time-lapse movies revealed that the velocity of cell migration in neural crest treated with GSK126 was significantly reduced; hence, the distance of cell dispersion was also decreased (Fig. 6C,D). The results indicate that EZH2 modulates cell shape, spreading and migratory behavior of the neural crest on fibronectin. Taken together, our *in vivo* and *in vitro* observations indicate that PRC2 regulates neural crest migration subsequently to its initial action in neural crest specification.

PRC2 regulates formation of the craniofacial cartilages

The head neural crest differentiates into craniofacial cartilages among other derivatives (Carl et al., 1999). As EZH2 regulates neural crest specification and migration, we speculated that alteration of EZH2 levels would affect craniofacial cartilages. To confirm this, we analyzed the embryos in which EZH2 was knocked down unilaterally. The head on the MO-injected side of the tadpoles was smaller (Fig. 7A), and Alcian Blue staining revealed that the cartilages were malformed on this side, with shorter and misshaped cartilage elements (Fig. 7B). The phenotype was similar to that seen when Snail2 was knocked down (Fig. 7; see Carl et al., 1999). The results demonstrate that, similarly to Snail2, PRC2 regulates formation of neural crest-derived cartilaginous structures in the head.

Snail2 modulates EZH2 occupancy and histone H3K27 trimethylation at the promoter of its target gene E-cadherin

The biochemical and functional analyses described above suggest that Snail2 recruits PRC2 to its downstream targets to regulate

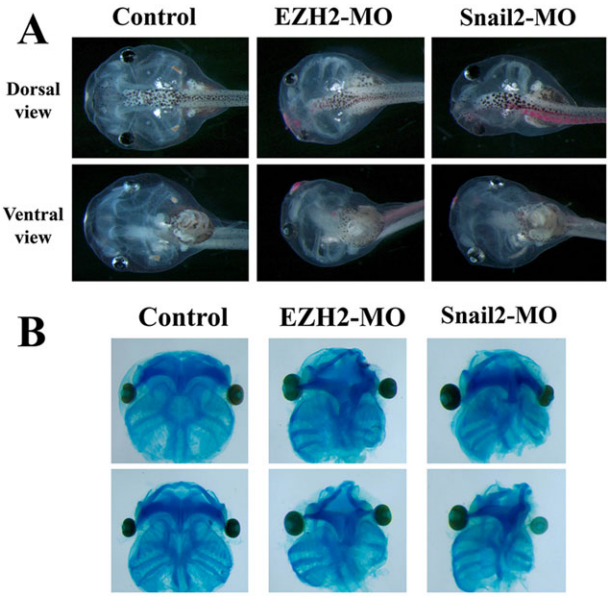

Fig. 7. EZH2 regulates formation of craniofacial cartilages. 20 ng EZH2-MO or Snail2-MO was injected unilaterally into two-cell-stage embryos. The embryos were cultured to the tadpole stages for Alcian Blue staining of the craniofacial cartilages. (A) Knockdown of either EZH2 or Snail2 led to malformation of craniofacial structures on the injected side (labeled by Red-Gal staining). (B) Alcian Blue staining revealed that the neural crest-derived craniofacial cartilages (Meckel's, ceratohyal and ceratobranchial cartilages) were defective on the side injected with EZH2-MO or Snail2-MO (right side of the panels).

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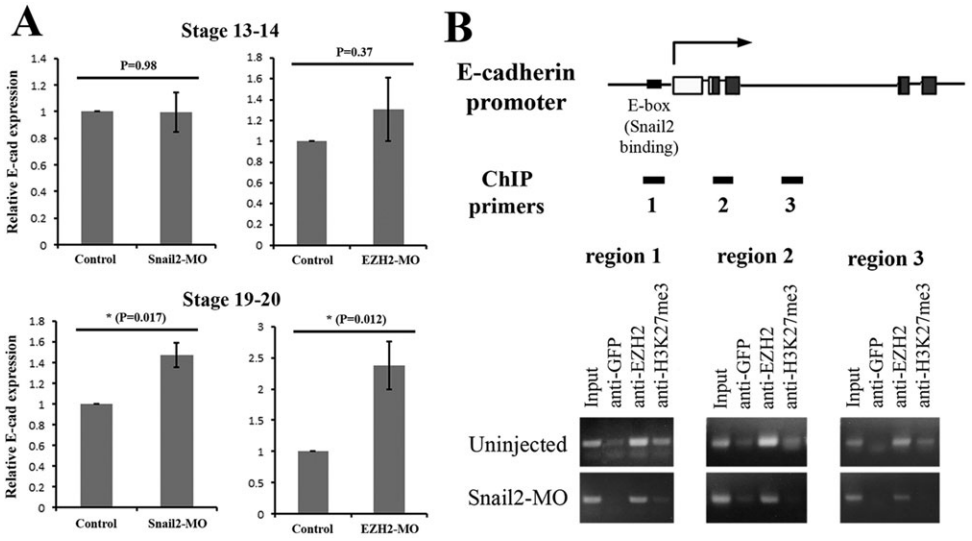

Fig. 8. Snail2 regulates EZH2 occupancy and H3K27me3 levels at the promoter of the Snail2 direct target E-cadherin during neural crest EMT. (A) Knockdown of Snail2 or EZH2 did not appreciably affect E-cad expression at early neurula stages 13-14, but resulted in increased E-cad expression at late neurula stages 19-20, when the neural crest underwent EMT to emigrate from the neural tube. Student's *t*-test revealed statistically significant changes in E-cad expression in morphant versus control samples at stages 19-20 ($P<0.05$). (B) Genomic structure around the E-cad promoter and the regions assayed in the ChIP experiments are shown at the top. Snail2-MO (20 ng) was injected into each blastomere of two-cell-stage embryos. Control and injected embryos were harvested at late neurula stages 19-20 for ChIP assay of EZH2 binding and H3K27me3 association with the E-cad promoter. Anti-GFP antibody was used as negative control. Knockdown of Snail2 reduced EZH2 occupancy and decreased H3K27me3 levels at the E-cad promoter, implying that Snail2 recruited EZH2/PRC2 to the E-cad promoter to deposit the repressive chromatin marks during EMT.

neural crest development. One of the best-known Snail2 targets is E-cadherin (E-cad), downregulation of which is required for Snail2-mediated EMT during cell migration, including neural crest migration in *Xenopus* (Peinado et al., 2007; Milet et al., 2013). To examine whether, like Snail2, EZH2 also regulates E-cad expression in neural crest, we dissected neural crest tissues from EZH2 or Snail2 morphant embryos at early (stages 13-14) or late (stages 19-20) neurula stages and analyzed E-cad RNA levels by quantitative RT-PCR (RT-qPCR). Knockdown of either Snail2 or EZH2 did not significantly alter E-cad expression in neural crest at early neurula stages. However, in late neurulae, when neural crest underwent EMT, the level of E-cad transcripts showed a statistically significant increase in both Snail2 and EZH2 morphant embryos (Fig. 8A). Knockdown of EZH2 led to higher levels of E-cad in neural crest (2.4-fold increase over control) than knockdown of Snail2 (1.5-fold increase over control), suggesting that PRC2 might cooperate with additional transcription factors, such as Twist, to further modulate E-cadherin expression (Barriga et al., 2013).

To examine the hypothesis that Snail2 recruits EZH2/PRC2 to the E-cad promoter to influence its expression, we next performed a chromatin immunoprecipitation (ChIP) assay. Analysis of *Xenopus laevis* E-cadherin genomic sequence (www.xenbase.org) revealed nine E-box-like motifs, with two located upstream of the transcription start site (TSS) and the other seven in the intronic regions (supplementary material Fig. S7A). A ChIP assay using Flag-tagged Snail2 showed that, at late neurula stages (stages 19), when neural crest underwent EMT, Snail2 bound preferentially to the E-boxes located around the TSS and at 2.2 kb upstream of TSS, respectively; whereas the other putative E-boxes were devoid of Snail2 (supplementary material Fig. S7B). To examine whether Snail2 binding to the E-cad promoter correlated to EZH2 occupancy and histone H3K27 modification, we next performed a ChIP assay using anti-EZH2 and anti-H3K27me3 antibodies. As genome-wide analyses of PRC2 association with chromatin in mammalian cells revealed peak binding of PRC2 components within 1-2 kb upstream and downstream of the TSS of their target genes (Boyer et al., 2006; Lee et al., 2006; Zhou et al., 2011), we focused on the region surrounding E-cad TSS in the following experiments. In control stage 19-20 embryos, EZH2 was found to occupy the sequences around the TSS of E-cad; and, correspondingly, histone H3K27me3 was found to associate with these sequences (Fig. 8B). Knockdown of Snail2 consistently reduced the binding of EZH2 to E-cad promoter, and this led to a drastic decrease in H3K27me3 levels at this promoter (Fig. 8B). The data thus demonstrate that Snail2 helps to recruit PRC2 to the E-cad promoter to set up repressive histone modification marks that result in inhibition of E-cad expression during neural crest EMT.

DISCUSSION

Epigenetic regulation of gene expression plays crucial roles in many developmental and physiological processes, and its defects are associated with a diverse range of diseases (Sparmann and van Lohuisen, 2006; Bhaumik et al., 2007; Kiefer, 2007; Bogdanovic et al., 2012; Vastenhouw and Schier, 2012; Aloia et al., 2013). Current studies on mammalian epigenetic factors focus on biochemical mechanisms and the functions of these factors in stem cell biology and in pathological conditions such as cancer; however, the roles of epigenetic factors in control of early vertebrate embryogenesis are not fully appreciated. In this study, we take advantage of the *Xenopus* system, in which localized gain- and loss-of-function experiments can be performed by targeted injection, thus avoiding early lethality observed in mouse embryos deficient in PRC2 genes (Faust et al., 1995; O'Carroll et al., 2001; Pasini et al., 2004). Using this model, we show that PRC2 interacts directly with the transcription factor Snail2 and cooperates with it to modulate neural crest specification and migration.

The roles of PcG proteins in vertebrate development have previously been studied in mouse, in which all three PRC2 components regulate

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cell differentiation and gastrulation. Disruption of PRC2 genes results in defects in mesendoderm differentiation in both mouse embryonic stem cells and in mouse embryos and leads to postimplantation lethality [reviewed by Vastenhouw and Schier (2012); Aloia et al. (2013)]. Similarly, PRC2 is shown to regulate mesendodermal formation in *Xenopus* (Lim et al., 2011). Because of theses early phenotypes, the functions of PRC2 in late vertebrate development have not been studied in detail. Expression profiles of both PRC1 and PRC2 components (Fig. 1 and data not shown; see also Reijnen et al., 1995; Aldiri and Vetter, 2009) reveal that PcG transcripts are located primarily in the neural and the neural crest cells during *Xenopus* neurulation, suggesting that they regulate development of these tissues. Consistent with this notion, we show here that PRC2 modulates expression of late, but not early, neural markers and controls neural crest development. As maternally inherited PRC2 proteins might still be present during early neurulation, the lack of any effect on early neural markers in PRC2 morphants might reflect functional compensation by residual PRC2 proteins. It is currently unclear which factors collaborate with the PRC2 complex to control neural specification, but we have identified Snail2 as a direct partner of EZH2 during neural crest development. Snail2 and EZH2 cooperate in modulating neural crest gene expression, and Snail2 fails to expand the neural crest genes in the absence of EZH2. Furthermore, both EZH2 and Snail2 also control migration of the neural crest after the initial cell fate determination event, thus influencing the formation of craniofacial cartilages in tadpoles. Snail2 helps to anchor EZH2 to its target genes to deposit repressive chromatin marks. These data suggest that PRC2 is a central component of the Snail2-dependent transcription program and cooperates with Snail2 to regulate multiple steps during neural crest development.

In addition to Snail2, PRC2 probably interacts with other transcription factors to control neural crest development. This is indicated by our results showing that PRC2 modulates additional genes that are not affected by Snail2 (Fig. 5). Indeed, it has been reported that EED, one of the core components of PRC2, binds directly to YY1, a transcription factor shown to regulate neural crest development (Satijn et al., 2001; Morgan et al., 2004). EZH2 also interacts in mammalian cells with *Msx1* (Wang et al., 2011), a neural plate border gene known to modulate neural crest markers (Tribulo et al., 2003; Monsoro-Burq et al., 2005). Snail1 can also interact with PRC2 (Herranz et al., 2008). Furthermore, Aebp2, a Gli-type zinc-finger protein that associates with PRC2, is shown to influence tissues derived from the neural crest in mouse (Kim et al., 2011). These studies imply that PRC2 can cooperate with multiple nuclear factors to control the neural crest transcription program. The results also point to the intriguing prospect that PcG proteins are targeted to chromatin in vertebrates mainly via their diversified binding partners. In *Drosophila*, defined Polycomb response elements (PREs) are shown to bind distinct transcription factors, such as PHO, GAGA factor, Pipsqueak, Zeste, Grainyhead, Dsp1 and Sp1-KLF, which help to recruit the PcG proteins to their target genes (Ringrose and Paro, 2004, 2007). In vertebrates, no definitive PREs have been identified, and it has remained an enigma how PcG proteins recognize their targets. Different mechanisms have been proposed, including non-coding RNAs, unmethylated CpG islands around the promoters, other chromatin modification and remodeling proteins, and existing histone marks [Peng et al., 2009; Shen et al., 2009; da Rocha et al., 2014; Kaneko et al., 2014; Mozzetta et al., 2014; also reviewed by Lanzuolo and Orlando (2012); Simon and Kingston (2013)]. In our experiments, overexpression of PRC2 components in the ectoderm did not appreciably alter neural gene expression and only moderately increased neural crest markers, and this did not lead to obvious morphological defects in the resulting embryos (supplementary material Fig. S2; and data not shown). The results imply that generic mechanisms, such as CpG islands, are insufficient to anchor PcG proteins stably to chromatin for persistent effect on gene expression and cell phenotype. Other recruiting factors might be required. It is possible that no common principle can account for PcG targeting in vertebrates. Instead, diversification in both PRC complexes, including duplication of PcG components and inclusion of other facilitating factors, such as JARID2 and PCL, and PcG-interacting molecules, including non-coding RNAs and unmodified CpG DNA elements in addition to proteins, helps to determine recruitment of PcG-repressive complexes to their target chromatin in a cell type-specific manner. This would require the presence of cell-specific recruiting factors for proper PcG function in a particular cell (e.g. the neural crest), and allow cell type-specific regulation of PcG function by intrinsic and extrinsic signals (Badeaux and Shi, 2013).

Although the traditionally ascribed activity of PRC2 is to methylate histone H3K27, it has been reported recently that PRC2 can also methylate non-histone proteins to affect their function. PRC2 methylates GATA4 in cardiac cells and attenuates the transcriptional activity of GATA4 by reducing its interaction with the p300 acetyltransferase (He et al., 2012). It is possible that PRC2 might also methylate Snail2 to influence its activity. Interestingly, Snail2 contains an RKS (arginine-lysine-serine) motif that is similar to that surrounding histone H3K27. To examine whether PRC2 methylates Snail2, we performed an *in vitro* protein methylation assay. Using commercially available reconstituted PRC2, we showed that PRC2 efficiently methylated histone in purified nucleosomes, but it did not modify purified GST-Snail2 protein (supplementary material Fig. S8A). When Flag-tagged Snail2 was expressed in *Xenopus* embryos, we detected with a pan-methylated lysine antibody that a minority of the tagged Snail2 could be methylated *in vivo*. However, the level of modified Snail2 was not affected when EZH2 was knocked down with the antisense MO (supplementary material Fig. S8B). Thus, both our *in vitro* and *in vivo* results imply that PRC2 does not methylate Snail2 directly. Instead, Snail2 appears to recruit PRC2 to its downstream targets to modify the chromatin structure at the promoter region. ChIP assays reveal that Snail2 and EZH2 co-occupy the promoter region of the Snail2 target E-cadherin during EMT, and knockdown of Snail2 reduces both EZH2 binding and H3K27me3 levels at the E-cadherin promoter (Fig. 8). The data suggest that, during neural crest development, PRC2 acts in a conventional way to control histone modification and chromatin conformation to regulate expression of genes crucial for neural crest specification and migration, and Snail2 acts as an important recruiting factor to target PRC2 to the promoter regions of these genes.

Besides the Polycomb group proteins, other epigenetic factors can also modulate neural crest development. Several distinct epigenetic programs, including histone modification, chromatin remodeling and non-coding RNA (e.g. miRNAs), have been implicated in different steps of neural crest development in various species (Liu and Xiao, 2011; Prasad et al., 2012; Strobl-Mazzulla et al., 2012; Mayanil, 2013). For example, it has been shown that the histone H3K9me3/H3K36me3 demethylase JmjD2A regulates neural crest specification in the chick (Strobl-Mazzulla et al., 2010), and the PRC1 component Ring1b specifically modulates craniofacial chondrocyte differentiation in zebrafish without affecting early induction and migration of the neural crest (van der Velden et al., 2013). Several components of chromatin remodeling complexes, such as Brg1 and CHD7, have

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also been reported to influence neural crest induction and/or differentiation (Eroglu et al., 2006; Bajpai et al., 2010; Weider et al., 2012; Li et al., 2013). Furthermore, both microRNA-processing enzymes and several specific microRNAs have been shown to regulate neural crest migration or affect neural crest-derived tissues, such as cranial and cardiovascular structures (Prasad et al., 2012; Strobl-Mazzulla et al., 2012; Mayanil, 2013). A more recent report also indicates that Ezh2 is required for craniofacial skeleton development in mouse (Schwarz et al., 2014). How these diverse epigenetic programs are integrated, to what extent these various epigenetic factors crosstalk to each other, and how these epigenetic mechanisms work with transcriptional machinery to control gene expression, cell identity and behavior during neural crest development are some of the key remaining questions that await further in-depth investigation.

MATERIALS AND METHODS

Embryo manipulations, ISH and animal cap assay

Embryos were obtained, maintained and microinjected with capped RNAs or antisense MOs as described (Nie and Chang, 2007). EED, EZH2 and SUZ12 coding sequences were amplified from gastrula-stage cDNA and inserted into the pCS105 vector. RNAs were synthesized using a mMessage mMachine transcription kit (Ambion). Snail2-MO and SUZ12-MO were used as described (Zhang et al., 2006; Lim et al., 2011). EED-MO and EZH2-MO sequences were: 5′-AGCTTCCGACATGTTCCACCGCC-3′ and 5′-TTCCCCGTCTGGCCCATGATTATCC-3′, respectively. For ISH, 20 ng of MO was injected with 0.2 ng nβGal RNA into one blastomere of two-cell-stage embryos. Injected embryos were collected at neurula stages for marker expression analysis by ISH as described (Harland, 1991). For MO rescue, 20 ng MO was co-injected with 1 ng RNA encoding tagged PRC2 core component, and embryos were processed for ISH as described. For animal cap assay, 10 pg noggin and 25 pg Wnt8 RNAs were injected alone or with 40 ng Snail2-MO or EZH2-MO at the two-cell stage. Animal caps were dissected from blastula stage 9 embryos and cultured until neurula stages 19-20 for RNA extraction and RT-PCR.

Co-IP, western blot and *in vitro* pull-down assays

RNAs (1-2 ng) encoding Flag-Snail2, EZH2/HA-EZH2, HA-EED and Suz12 were injected into one-cell-stage embryos. The embryos were collected at gastrula stages 10-11 and lysed with buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10% glycerol and 0.5% Triton X-100, pH 7.5. Embryonic lysate was divided equally and reciprocal immunoprecipitation was performed with mouse 1:1000 anti-Flag (Sigma, F3165) or mouse 1:250 anti-HA antibodies (Covance, MMS-101P). For *in vivo* histone methylation assay, proteins from the head of injected embryos were extracted at neurula stages 20-21 using 1 ml cold lysis buffer (10 mM Tris-HCl, 50 mM sodium bisulfite, 1% Triton X-100, 10 mM $MgCl_2$ and 8.6% sucrose, pH 6.5). Protein pellets were washed three times with the lysis buffer and once with buffer containing 10 mM Tris-HCl and 13 mM EDTA (pH 7.4). Total histones were extracted with 0.1 ml 0.2 N HCl at 4°C overnight and precipitated with 1 ml acetone before being re-dissolved in water. Western blot was performed using rabbit anti-Flag (1:1000; Sigma, F7425), rabbit anti-HA (1:1000; Covance, PRB-101P), rabbit anti-histone H3 (1:3000; Abcam, ab1791), rabbit anti-H3K27me3 (1:1000; Cell Signaling Technology, 9756), mouse anti-EZH2 (1:1000; BD Biosciences, 612667) and rabbit anti-methylated-lysine (1:500; Abcam, ab23366) antibodies. For the *in vitro* pull-down assay, a GST-Snail2 fusion protein was constructed by inserting the coding region of Snail2 into the pGEX-KG vector and expressed in BL21 Star *Escherichia coli*. GST and GST-Snail2 were extracted from bacteria after 3 h IPTG induction in exponentially growing bacteria culture, purified on glutathione-agarose beads (Sigma, G4510) and incubated with the PRC2 complex (BPS Bioscience, 51003). After extensive washing, proteins bound by GST or GST-Snail2 were eluted in SDS-PAGE loading buffer, separated by gel electrophoresis and analyzed by EZH2 western blot. For details on *in vitro* methylation assay see supplementary material methods.

Neural crest migration assays

For *in vivo* neural crest migration analysis, wild-type embryos were incubated in DMSO or 50 μM GSK126 (Xcess Biosciences, M60071-2) medium from early neurula stages 13-14 onward. The embryos were collected at the tailbud stages 25-26 for ISH of Sox10 and Twist, which reflected the pattern of neural crest migration. Alternatively, cranial neural crest was dissected from stage 15-16 donor embryos labeled with the fluorescent tracer fluorescein dextran and transplanted into wild-type recipient embryos, followed by incubation in medium containing DMSO or 50 μM GSK126. The pattern of neural crest migration was examined by fluorescence microscopy at the tailbud stages 26-28, using a Zeiss M2BIO stereomicroscope with an attached epifluorescent light source. For the *in vitro* neural crest migration assay, cranial neural crest explants were dissected from stage 15-17 embryos and cultured on fibronectin-coated dishes in DFA medium with DMSO or 8-10 μM GSK126. Migration of the neural crest was observed and imaged between 2 and 16 h post explantation, using a Zeiss M2BIO microscope equipped with a SPOT INSIGHT camera. Time-lapse microscopy was performed at 2 min intervals for 1-3 h. Quantitative analysis of cell movements was performed using NIH Image J MultiTracker Plugin software, which tracked cell coordinates, cell migration trajectories and the length of cell migration simultaneously for multiple cells. Four time-lapse movies each for DMSO- and GSK126-treated samples were analyzed, with ~60-170 cells tracked in each movie. Student's *t*-test was performed to assess the statistical significance of migration velocity difference between DMSO- and GSK126-treated samples.

Cranial cartilage staining with Alcian Blue

Embryos unilaterally injected with 20 ng Snail2-MO or EZH2-MO at the two-cell stage were cultured to tadpoles before fixation in paraformaldehyde and dehydration in ethanol. They were then stained in Alcian Blue solution (0.04% Alcian Blue in 10 mM $MgCl_2$ and 80% EtOH) for five days. After washing in ethanol and clearing in glycerol/KOH, the cranial cartilages were exposed by manual dissection of the head and imaged with Zeiss M2BIO microscope.

RT-qPCR

Neural crest tissues were isolated from stage 13-14 or stage 19-20 wild-type, EZH2 morphant or Snail2 morphant embryos. RNA extraction and reverse transcription were performed as described (Nie and Chang, 2007). RT-PCR, using a radioactive nucleotide, was first performed to confirm the expression of the neural crest markers Sox9 and Twist in all samples. The process also revealed the presence of a very low level of epidermal keratin (EK) in our samples. As epidermal cells also express E-cad, we corrected our analysis by taking into account the contribution of E-cad from the epidermal cells. We thus assessed the levels of E-cad, EF-1α and EK by qPCR, and calculated relative E-cad expression in the neural crest by deducting the E-cad contribution from the epidermis: qPCR was carried out using ABI Prism 7500 system (Applied Biosystems) and RT^2 SYBR Green ROX qPCR Mastermix (Qiagen). Melting curve analysis revealed a single peak in each reaction. The data were analyzed using threshold cycle (C_t) differences. The relative expression (RQ) of EF-1α ($RQ_{EF\text{-}1\alpha}$) was considered as $2^{-[Ct(EF\text{-}1\alpha,\ samples)-Ct(EF\text{-}1\alpha,\ control)]}$ for each sample, and the relative expression of E-cad in the neural crest was calculated as $2^{-[Ct(E\text{-}cad)-Ct(EK)]}/RQ_{EF\text{-}1\alpha}$ for each sample. Results from at least three independent experiments were analyzed by Student's *t*-test and the statistical significance of the changes was determined by the *P*-values ($P<0.05$ was considered significant).

ChIP assay

ChIP assay was performed using the method described by Blythe et al. (2009). Embryos injected with 1 ng Flag-Snail2 RNA or 40 ng Snail2-MO were collected at late neurula stages 19-20. A total of 100-120 embryos were used, and the lysates were split into two or three parts for IP with rabbit anti-EZH2 (1:100; Cell Signaling Technology, 5246), rabbit anti-H3K27me3 (1:50; Cell Signaling Technology, 9733), mouse anti-Flag (1:100; Sigma, F3165) or rabbit anti-GFP (1 μg; Fisher Scientific, OSE00003G) antibodies. PCR was performed using the following protocol: 95°C for 30 s, 56°C for 40 s and 72°C for 40 s for 32-36 cycles. The following PCR primers were used: region 1,

forward: ATGCCTCCTCGCATTTCTGA, reverse: GCAAACATTCCCAC-CACCCA; region 2, forward: TATTGGTGGGCTCTT-TGGGC, reverse: GCC-TGAAGGGTCTAAGCCAG; region 3, forward: TGTTGCTCACG-AGCTACTGG, reverse: TCTCTGTTTGCGGCCATGAT.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
C.-L.T. and C.C. performed most of the experiments; A.J. and H.W. helped with the *in vitro* protein methylation experiment and advised on GST-fusion protein purification and the ChIP assay; M.G. and S.N. helped with quantitative PCR analysis. C.T. and C.C. wrote, and all the other authors commented on, the manuscript.

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Supplementary material
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