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The b-HLH transcription factor Hes3 participates in neural plate border formation by interfering with Wnt/β-catenin signaling

Chang-Soo Hong[a,b], Jean-Pierre Saint-Jeannet[b,*]

[a] Department of Biological Sciences, Daegu University, Gyeongsan, Republic of Korea
[b] Department of Basic Science & Craniofacial Biology, College of Dentistry, New York University, New York, USA

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ABSTRACT

Hes3 belongs to the Hes basic helix-loop-helix family of transcriptional repressors that play central roles in maintaining progenitor cells and regulating binary cell fate decisions in the embryo. During *Xenopus laevis* development, *hes3* is expressed in the embryonic ectoderm in a horseshoe shape domain at the edge of the developing neural pate. Hes3 mis-expression at early neurula stage blocks neural crest (*snai2*, *sox8*, *sox9* and *sox10*) and cranial placode (*six1* and *dmrta1*) gene expression, and promotes neural plate (*sox2* and *sox3*) fate. At tailbud stage, these embryos exhibited a massive up-regulation of both *sox8* and *sox10* expression, associated with an increase in genes important for melanocytes differentiation (*mitf* and *dct*). Using a hormone inducible construct we show that Hes3 does not induce a pigment cell differentiation program *de novo*, rather it maintains progenitor cells in an undifferentiated state, and as Hes3 expression subsides overtime these cells adopt a pigment cell fate. We demonstrate that mechanistically Hes3 mediates its activity through inhibition of Wnt/β-catenin signaling, a molecular pathway critical for neural crest specification and pigment cell lineage differentiation. We propose that Hes3 at the edge of the neural plate spatially restricts the response to mesoderm-derived Wnt ligands, thereby contributing to the establishment of sharp boundaries of gene expression at the neural plate border.

1. Introduction

At the end of gastrulation the neural plate border (NPB) defines a competence domain, established between the neural plate (NP), prospective central nervous system, and non-neural ectoderm, future epidermis. Within this domain signaling events progressively direct the emergence of two major embryonic structures, the neural crest (NC) and the pre-placodal region (PPR). In the trunk, the NC gives rise to neurons of the peripheral nervous system and pigment cells of the skin, while in the head region the NC forms cartilages and bones of the face and contributes to cranial ganglia (reviewed in Huang and Saint-Jeannet, 2004; Bronner and LeDouarin, 2012). The PPR segregates into cranial placodes that form the paired sensory organs (olfactory epithelium, inner ear and lens), the adenohypophysis, and a subset of cranial ganglia that provide sensory innervation to the orofacial complex (reviewed in Baker and Bronner-Fraser, 2001; Streit, 2007; Park and Saint-Jeannet, 2010; Schlosser, 2010). NPB formation is regulated by several signaling molecules of the Wnt, fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families that need to be precisely modulated in space and time to generate the NC and the PPR (reviewed in Stuhlmiller and García-Castro, 2012; Bae and Saint-Jeannet, 2014; Saint-Jeannet and Moody, 2014; Singh and Groves, 2016). These signaling molecules in turn differentially activate the expression of a subset of transcription factors that uniquely define the molecular identity of these two cell populations and their derivatives (reviewed in Grocott et al., 2012; Simões-Costa and Bronner, 2015).

Hes genes are vertebrate homologs of *Drosophila hairy* and *enhancer of split* genes, forming a family of seven members (*Hes 1–7*). They encode basic helix-loop-helix transcriptional repressors that play essential roles in controlling the maintenance and expansion of stem cell populations, and the timing of their differentiation (reviewed in Kobayashi and Kageyama, 2014). For example, Hes1 and Hes5 expression inhibits neuronal differentiation and promotes proliferation of neural progenitors in the mouse embryonic brain (Ohtsuka et al., 2001), and inactivation of *hes1*, *hes3* and *hes5* genes accelerates neuronal differentiation, depleting prematurely the pool of neural progenitors (Hatakeyama et al., 2004).

In *Xenopus*, Hairy2 also known as Hes4 is expressed at the NPB and has been proposed to mediate Notch signaling during NC induction (Glavic et al., 2004). Hairy2 knockdown blocked NC induction,

* Correspondence to: New York University, College of Dentistry, Department of Basic Science & Craniofacial Biology, 345 East 24th Street, New York, NY 10010, USA.
E-mail address: jsj4@nyu.edu (J.-P. Saint-Jeannet).

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proliferation and differentiation (Nagatomo and Hashimoto, 2007). Hairy2 gain-of-function had a very similar phenotype at early stage, however later in development these cells activated a glial cell differentiation program, suggesting that sustained Hairy2 expression maintained these progenitors in an undifferentiated state (Nichane et al., 2008). During *Xenopus* cranial placode development, another Hes family member, Hes8, has been shown to mediate the inhibition of proneural and neuronal differentiation gene expression in response to Notch signaling (Riddiford and Schlosser, 2017).

In this manuscript we report the expression and function of Hes3 during *Xenopus* development. *hes3* is expressed at the edge of the NP, and Hes3 mis-expression blocks NC gene expression and promotes neural fate. Interestingly later in development these embryos show ectopic activation of a pigment cell differentiation program, suggesting that Hes3 maintains NC progenitors in an undifferentiated state, and overtime these progenitors acquire a pigment cell fate as Hes3 expression subside. Mechanistically we provide evidence that Hes3 mediates its activity by modulating the response to Wnt/β-catenin signaling.

2. Materials and methods

2.1. Plasmid constructs

Xenopus laevis Hes3.L (accession number: XM_018226116.1) full coding region was generated by polymerase chain reaction (PCR) and fused in frame to the human glucocorticoid receptor (GR) ligand binding domain to generate an pCS2+*Hes3GR* construct using the following primers, forward: 5′-ATCGATGCCACCATGGGGACACA TTCCGAACCACGAGAAGA-3′ and reverse: 5′-CTCGAGCCATGGTCT CCACACGTCTTGG-3′. The activity of the fusion protein can regulated by addition of dexamethasone to the culture medium of whole embryos or animal explants (Kolm and Sive, 1995).

2.2. Xenopus embryo injections, explants culture, and dexamethasone treatment

Xenopus laevis embryos were staged according to Nieuwkoop and Faber (1967) and raised in 0.1X NAM (Normal Amphibian Medium; Slack and Forman, 1980). This study was performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The procedures were approved by New York University Institutional Animal Care and Use Committee, under animal protocol # 150201. *hes3GR*, *nog* (Smith and Harland, 1992), *wnt8* (Christian et al., 1991), *ctnnb1* (Funayama et al., 1995) and *β-galactosidase* (*β-gal*) mRNAs were synthesized *in vitro* using the Message Machine kit (Ambion, Austin TX). In whole embryo experiments, synthetic *hes3GR* mRNA (500 pg) was injected in one blastomere at the 2-cell stage (NF stage 2). Injected embryos were cultured in 0.1 X normal amphibian medium (NAM; Slack and Forman, 1980) containing 10 μM dexamethasone (Dex; Sigma-Aldrich, St. Louis, MO) from NF stage 10.5 or 17. Siblings injected with *hes3GR* and cultured in the absence of Dex were used as control. For *wnt8* and *ctnnb1*, plasmid DNA was injected to prevent axis duplication (100 pg and 200 pg, respectively). To identify the injected side, 500 pg of *β-gal* mRNA was coinjected as a lineage tracer and embryos were analyzed by *in situ* hybridization at the appropriate stage. Morpholino antisense oligonucleotides (MO) to knockdown Hes3 function were purchased from GeneTools (Philomath, OR). We used two translation blocking MOs targeting Hes3.L (HES3LMO: AGGTTCGGAATGTGTCCCCATGTTT and HES3MO2: TCCCCATGTTTGAAGGAGTTGGTTT), one splice blocking MO targeting the intron 1-exon 1 junction of Hes3.L and Hes3.S (HES3SMO1: CGCGCAGTACAATATACTGACCTTT), and one translation blocking MO targeting Hes3. S (HES3MO3: CTTGAGGTTCAGAG TGAGTCCCCAT). The MOs were injected in one blastomere at the 2-cell stage either separately or in combination (HES3MO1 +Hes3MO3), to target both Hes3.L and Hes3.S. For the axis duplication assay, embryos were injected with 4 pg of *wnt8* mRNA in the equatorial region in both ventral blastomeres at 4-cell stage (NF stage 3) and analyzed at NF stage 32. For animal explant experiments, both blastomeres at the two-cell stage were injected in the animal pole region, with various combinations of *nog* (400 pg), *wnt8* (50 pg) and *hes3GR* (1 ng). Then explants were dissected at the late blastula stage and immediately cultured *in vitro* for several hours in NAM 0.5X plus 10 μM Dex. For whole embryo injections and animal cap explant assays each experiment was performed on at least three independent batches of embryos.

2.3. Lineage tracing and whole-mount in situ hybridization

Embryos at the appropriate stage were fixed in MEMFA and stained for Red-Gal (Research Organics; Cleveland, OH) to visualize the lineage tracer (*β-gal* mRNA) on the injected side and processed for *in situ* hybridization. Antisense digoxygenin-labeled probes (Genius kit; Roche, Indianapolis IN) were synthesized using template cDNA encoding *hes3*, *snai2* (Mayor et al., 1995), *sox8* (O'Donnell et al., 2006), *sox9* (Spokony et al., 2002), *sox10* (Aoki et al., 2003), *foxd3* (Sasai et al., 2001), *pax3* (Bang et al., 1997), *zic1* (Mizuseki et al., 1998), *dmrta1* (Huang et al., 2005), *sox2* (Mizuseki et al., 1998), *sox3* (Penzel et al., 1997) and *dct* (Aoki et al., 2003). Whole-mount *in situ* hybridization was performed as described (Harland, 1991; Saint-Jeannet, 2017).

2.4. qRT-PCR analysis

For each sample, total RNAs were extracted from 10 animal cap explants using the RNeasy micro RNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. During the extraction procedure the samples were treated with DNase I, to eliminate possible contamination by genomic DNA. The amount of RNA isolated was quantified by measuring the optical density using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). qRT-PCR was performed with 10 ng of total RNAs from animal caps using *Power* SYBR® Green RT-PCR Master Mix (Applied Biosystems, Foster City, CA) on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following primer sets: *snai2*, *sox2, six1,* pax3 (Hong et al., 2007), *sox10* (F: CTGTGAACACAGCATGCAAA; R: TGGCCAACTGACCATGTAAA), *drmta1* (F: TGGAATGTTAC GGGATCCAT; R: AGGCCACTGTGGGACTATTG), *mitf* (F: CA AGAGATGCTGCAAAACCA; R: GCTGTTGGGGAGTCAGACAT) and *dct* (F: AACGGGAAGGAATGAGTGTG; R: GGTCACCAGCCGAT TGTAGT). The PCR conditions were as follows: denaturation 95 °C (15 s), annealing and extension at 60 °C (1 min) for 40 cycles.

3. Results

3.1. Hes3 belongs to the Hes family of transcriptional repressors

Xenopus laevis Hes3 possesses an open reading frame encoding 204 amino acids (Fig. 1A). Outside the b-HLH domain and the N-terminal groucho domain at the amino acid level Hes3 proteins have limited conservation across species (Fig. 1A). Overall *Xenopus laevis* Hes3 (XP_018081605.1) shares 35% identity with human HES3 (NP_001019769.1; Katoh and Katoh, 2004), 32% identity with mouse Hes3 (NP_032263.2; Sasai et al., 1992), 35% identity with zebrafish Hes3 (NP_571155.1; Hans et al., 2004) and 82% identity with *Xenopus tropicalis* Hes3 (XP_004916246.1). Phylogenetically *Xenopus laevis* Hes3 falls into the same clade as mouse and human HES3 (Fig. 1B).

3.2. Hes3 is expressed at the edge of the developing neural plate

We analyzed the expression of *hes3* by whole mount *in situ* hybridization (ISH) using digoxygenin-labeled RNA probes. At early

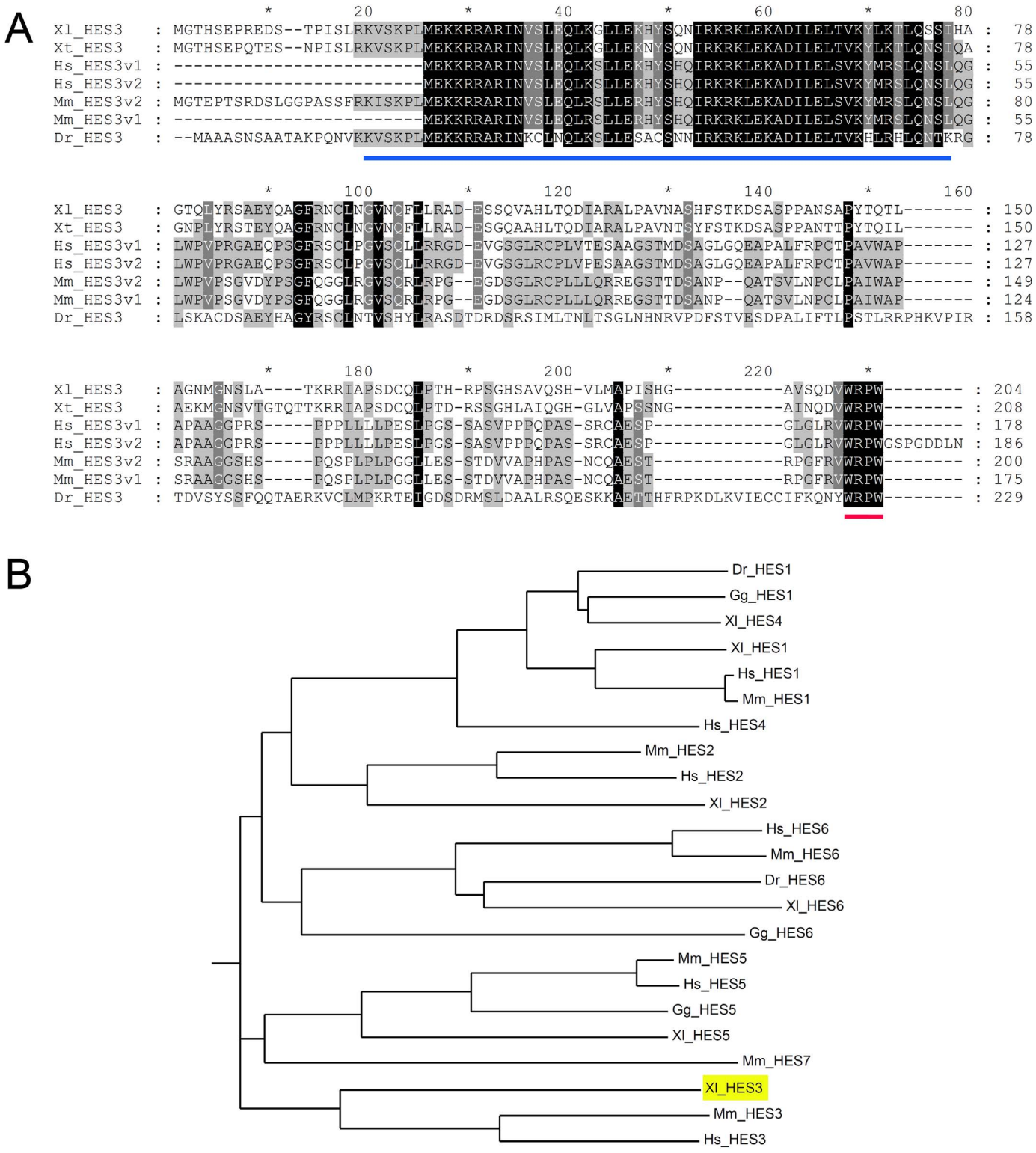


Fig. 1. Sequence comparison of Hes proteins across species. (A) The amino acid sequences of human (Hs), mouse (Mm), zebrafish (Dr), *Xenopus tropicalis* (Xt), *Xenopus laevis* (Xl) *Hes3* genes were aligned using ClustalX2. Hes3 amino acid conservation among species is highlighted in different shades of grey. The b-HLH is underlined in blue and the Groucho-binding domain is underlined in red. (B) Phylogenetic tree of Hes proteins from *Xenopus laevis* (Xl), human (Hs) mouse (Mm), chicken (Gg) and zebrafish (Dr).

gastrula stage (NF stage 10.5), *hes3* is detected in the dorsal anterior ectoderm in a horseshoe shape domain (Fig. 2A). As gastrulation proceeds, *hes3* expression becomes progressively more defined with an anterior domain and two adjacent bilateral domains extending posteriorly, and excluded from the center of the prospective neural plate (Fig. 2B-D). At neurula stage (NF stage 15–20), *hes3* expression levels decrease first in the lateral neural plate and then anteriorly (Fig. 2E-J). Later, at the tailbud stage (NF stage 25–33), *hes3* is confined to the forebrain, hindbrain and spinal cord, and posteriorly to the presomitic mesoderm (Fig. 2K-N). Serial transverse sections of a neurula stage embryo (NF stage 15) highlight the position of *hes3* expression domain in the dorsal ectoderm (Fig. 2O-S). At the same stage, longitudinal sections point to the most rostral *hes3* expression domain, the prospective forebrain (Fig. 2T-U). Double ISH at the neurula stage

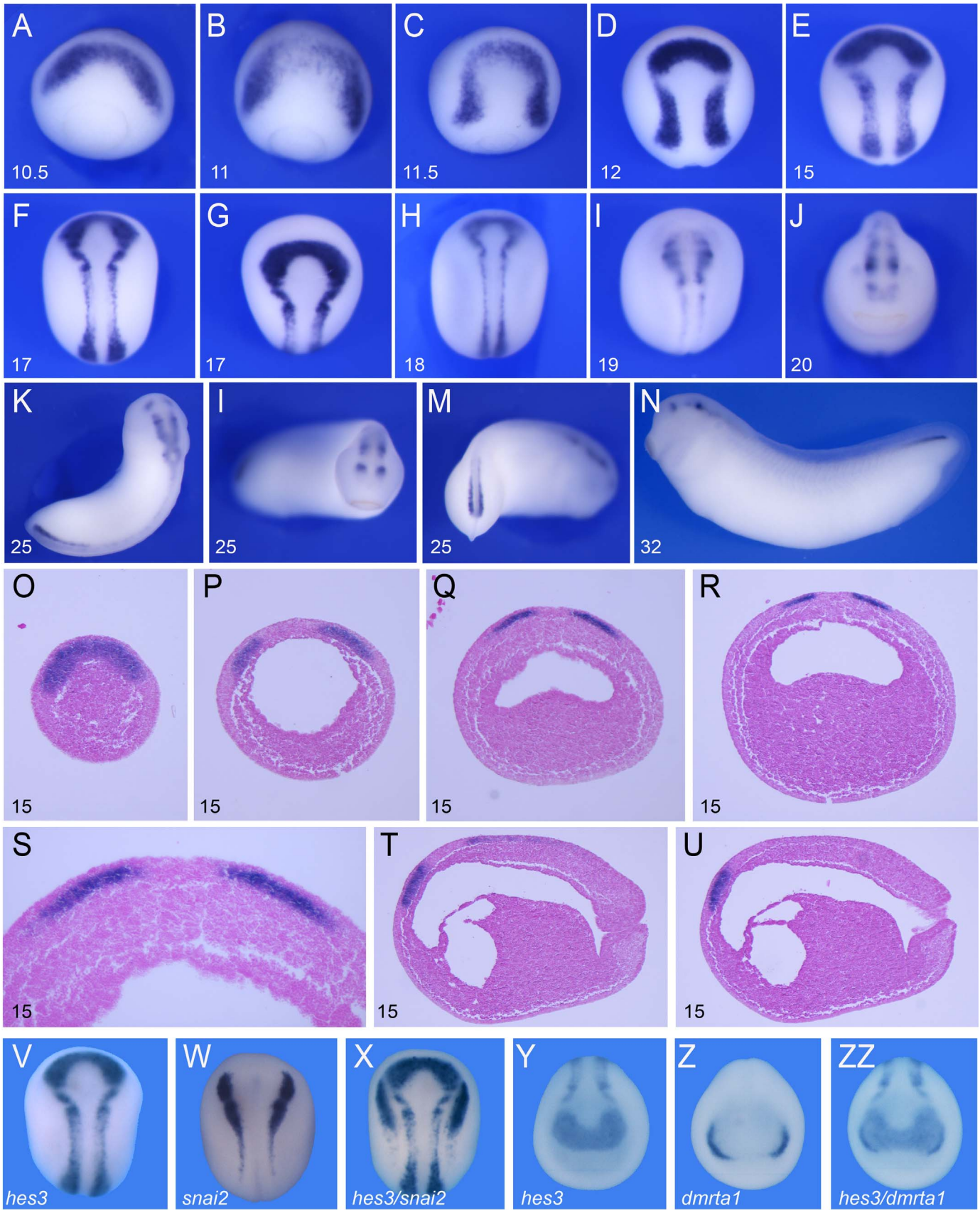


Fig. 2. Developmental expression of *Hes3*. (A-N) Developmental expression of *hes3* visualized by ISH at the gastrula (A-D), neurula (E-J) and tailbud (K-N) stages. (A-I, K) Dorsal views, anterior to top. (J, L) Anterior views, dorsal to top. (M) Posterior view, dorsal to top. (N) Lateral view, dorsal top, anterior to left. (O-R) Serial (anterior to posterior) transverse sections of a neurula stage embryo show the antero-lateral expression domain of *hes3* in the neural plate. Dorsal to top. (S) Higher magnification view of panel (R). (T-U) Longitudinal sections highlight the anterior expression domain of *hes3*. Anterior to left, dorsal to top. (A-U) In each image, the embryonic stage (NF) is indicated in the lower left corner. (V-ZZ) single (V-W, Y-Z) and double (X, ZZ) ISH of neurula stage (NF stage 15) embryos for the genes indicated in the lower left corner of each image. There is no overlap between *hes3* and the NC (*snai2*) or the PPR (*dmrta1*) genes. (V-X) Dorsal views, anterior to top. (X-ZZ) Anterior views, dorsal to top.

(NF stage 17) clearly indicate that *hes3* expression domain does not overlap with *snai2* in the NC (Fig. 2V-X) or *dmrta1* in the PPR (Fig. 2Y-ZZ). In summary, *hes3* is expressed at the anterior and lateral edges of the neural plate in a region that abuts the neural plate border, source of NC and PPR.

3.3. Hes3 mis-expression represses neural plate border fates

To analyze the function of Hes3 we perform gain-of-function in the embryo using a hormone inducible version of *hes3*, in which Hes3 coding region is fused in frame with the human glucocorticoid receptor (GR) ligand-binding domain (*hes3GR*). The activity of the fusion protein is regulated by addition of Dex to the culture medium (Kolm and Sive, 1995). Unilateral injection of 500 pg of *hes3GR* mRNA at the 2-cell stage followed by treatment with Dex at the gastrula stage (NF stage 10.5) resulted in a loss of three NC specifier genes *snai2*, *sox10* and *foxd3*, while the NPB specifiers, *pax3* and *zic1*, were expanded in these embryos. Sibling embryos injected with *hes3GR* and cultured in the absence of Dex had normal *snai2* and *sox10* expression domains (Fig. 3B). We also analyzed the expression of *dmrta1*, a gene expressed in the developing PPR, and found that *dmrta1* was also down-regulated in *hes3GR*-injected embryos (Fig. 3A). The loss of expression of the NC and PPR genes was associated with an anterior and lateral expansion of the NP genes *sox2* and *sox3* (Fig. 3A).

To confirm these observations we used an animal cap explant assay and qRT-PCR. In this preparation activation of the Wnt/β-catenin pathway in conjunction with attenuation of BMP signaling induces NC genes, while attenuation of BMP signaling alone promote PPR and NP fates (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Hong and Saint-Jeannet, 2007). In these explants, the induction of *snai2* and *sox10* by Wnt8 and the BMP antagonist Noggin was significantly repressed by Hes3 expression (Fig. 3C). Similarly, the induction of the placode genes *six1* and *dmrta1* by Noggin was downregulated upon *hes3* injection (Fig. 3D). In both conditions the presence of Hes3 was associated with an increase in *sox2* expression (Fig. 3C-D). Altogether these results indicate that in the embryos and animal cap explants Hes3 promotes neural fate at the expense of NPB fates suggesting that Hes3 may regulate cell fate decisions in the ectoderm.

3.4. Hes3 mis-expression induces ectopic pigment cells differentiation

We next analyzed the long-term consequences of Hes3 mis-expression on embryonic development. While at the neurula stage *sox10* expression was completely abolished in *hes3GR*-injected embryos (Fig. 3A), surprisingly a few hours later (NF stage 25) these embryos exhibited a massive upregulation of *sox10* expression that persisted at least up to stage 31 (Fig. 4A). Because sox10 is critical for pigment cell lineage specification (Aoki et al., 2003; Harris et al., 2010; Kelsh, 2006), we analyzed the expression of *dct*, a gene required for melanocyte development. We found that the ectopic population of *sox10*-expressing cells was also *dct*-positive, suggesting that Hes3 regulates a pigment cell differentiation program. At stage 41, sibling tadpoles had supernumerary pigment cells (Fig. 4A).

To determine whether this expanded cell population resulted from an increase in cell proliferation, we analyzed the number of cells immunopositive for phospho-histone H3 (pHH3) in *hes3GR*-injected embryos. We found no difference in the number of mitotic cells in the injected *versus* control sides of these embryos (not shown). We next investigated whether this cell population arose at the expense of other cell types. We observed a marked reduction in neural (*sox2*, *sox3* and *pax6*) and placode (*dmrta1* and r*unx1*) gene expression in *hes3GR*-injected embryos at the tailbud stage (Fig. 4B), suggesting that the expanded progenitor population formed at the expense of neural and placode cells.

To test whether Hes3 was sufficient to promote pigment cell fate, 2-cell stage embryos were injected in the animal pole region with *hes3GR* mRNA, animal explants dissected at the blastula stage (NF stage 9), treated with Dex, cultured *in vitro* for 10, 15 or 22 h and analyzed for gene expression by qRT-PCR. In these explants *sox10* and *mitf* expression was significantly upregulated after 22 h in culture (Fig. 4C), while *dct* was largely unchanged at this time point. Because *dct* is downstream of sox10 and mitf it may require longer culture time for its activation. Interestingly, in this explant preparation Hes3 was also sufficient to induce *pax3* (NPB specifier), *snai2* (NC specifier) and sox2 (neural) expression after 22 h in culture (Fig. 4D).

3.5. Hes3 maintains neural crest progenitors in an undifferentiated state

We expanded our analysis to include other *soxE* genes expressed in the developing NC, namely *sox8* and *sox9,* which direct development of trunk NC-derivatives and NC-derived craniofacial skeleton, respectively (Hong and Saint-Jeannet, 2005; Weider and Wegner, 2017). We observed that the expression of both genes was also repressed at the neurula stage in hes3GR-injected embryos (Fig. 5A-B). At the tailbud stage *sox8* expression was strongly up-regulated in a pattern similar to *sox10* (Fig. 5A). The expansion of *sox8* and *sox10* expression appeared between stage 17–20 and stage 20–23, respectively. Sox8 is an upstream regulator of sox10 (O'Donnell et al., 2006), and therefore as expected sox8 expansion precedes that of sox10 in Hes3-injected embryos. In contrast *sox9* expression was not expanded in these embryos (Fig. 5B).

To determine whether Hes3 activates *de novo* a pigment cell differentiation program in the embryo, we injected *hes3GR* mRNA at the 2-cell stage, treated the embryos with Dex either pre- (stage 10.5) or post- (stage 17) NC specification and analyzed *sox9* and *sox10* expression at the tailbud stage. While Dex treatment before NC specification expanded *sox10* expression as described above, the addition of Dex after NC specification had no impact on the expression of *sox9* or *sox10* (Fig. 6), indicating that at this stage the mere expression of Hes3 is not sufficient to activate a pigment cell differentiation program as these cells are no longer competent to respond to Hes3 mis-expression.

Based on these observations, we propose that Hes3 mis-expression in NC progenitors maintains these cells in an undifferentiated state. Overtime, as Hes3 expression levels decrease, NC progenitors start to differentiate and adopt a pigment cell fate (*sox10*- and *dct*-positive cells). Interestingly, these cells failed to express *sox9* a gene typically associated with skeletogenic cranial NC cells (Spokony et al., 2002).

3.6. Hes3 regulates Wnt/β-catenin signaling

Our gain-of-function experiments suggest that Hes3 activity is not compatible with NC progenitor formation. Consistent with this view, Hes3 is not expressed in the NC at the end of gastrulation, rather it is confined to a domain of the NP immediately medial to the NC territory (Fig. 2), and raising the possibility that Hes3 may participate in the establishment of the NP/NC boundary. Because Wnt/β-catenin signaling is essential to specify the NC we posited that Hes3 might interfere with Wnt/β-catenin signaling to prevent expansion of the NC territory towards the neural plate. To test this possibility we analyzed the ability of Wnt8 and Ctnnb1 to expand the expression of *snai2* and *sox10* when co-expressed with Hes3. As previously shown (Hong et al., 2008), unilateral injection of Wnt8 or Ctnnb1 plasmid DNA resulted in an anterior expansion of *snai2* and *sox10* expression domains (Fig. 7A). Co-expression of Hes3 in these embryos completely abrogated the NC-inducing activity of Wnt8 or Ctnnb1 (Fig. 7A). Consistent with this function, Hes3 expression also strongly repressed Wnt8-mediated activation of a Wnt-responsive TOP-FLASH reporter in animal cap explants (Fig. 7B). Furthermore, in another assay, axis duplication by Wnt8, we were able to show that the ability of Wnt8 to induce

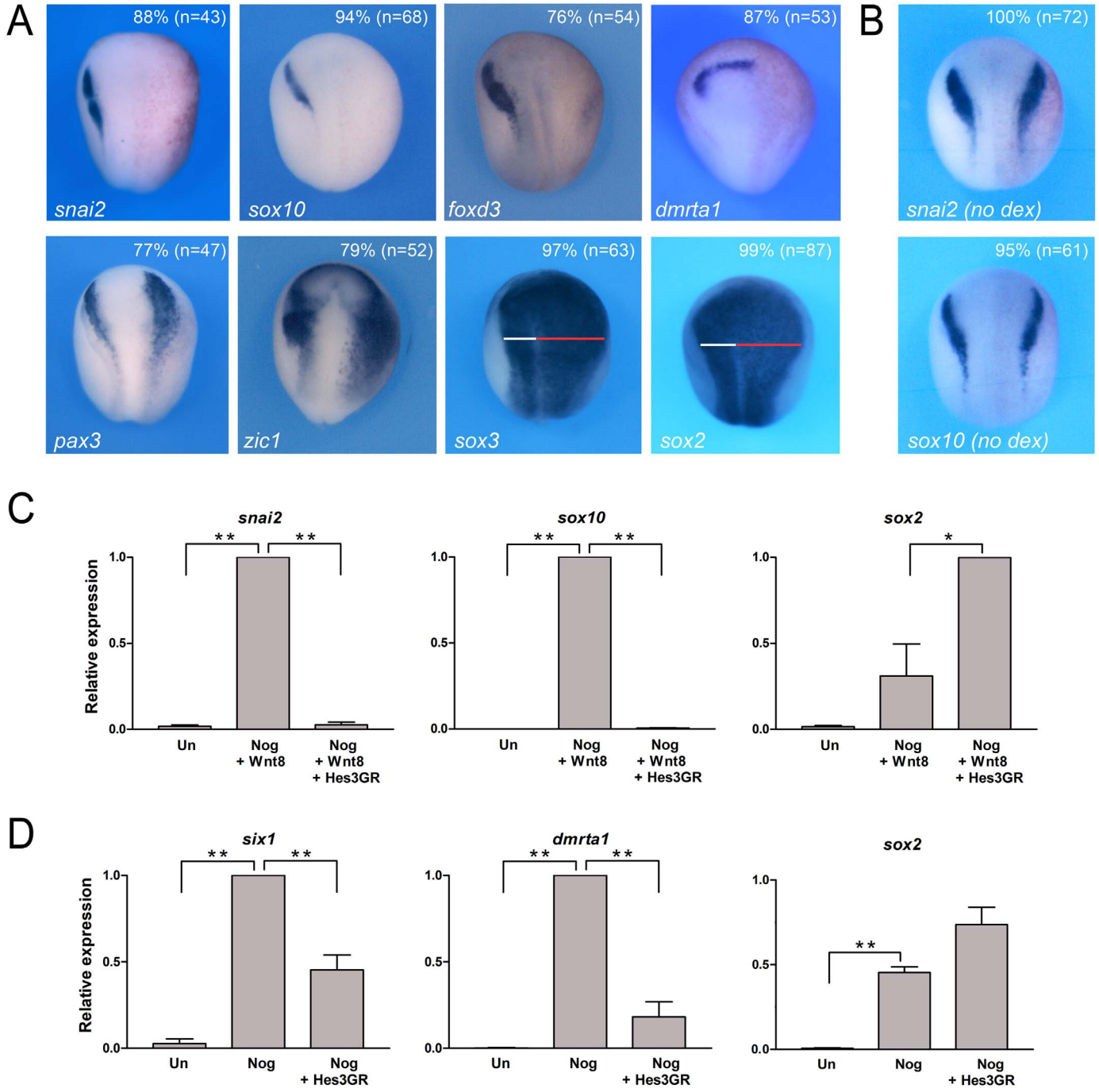
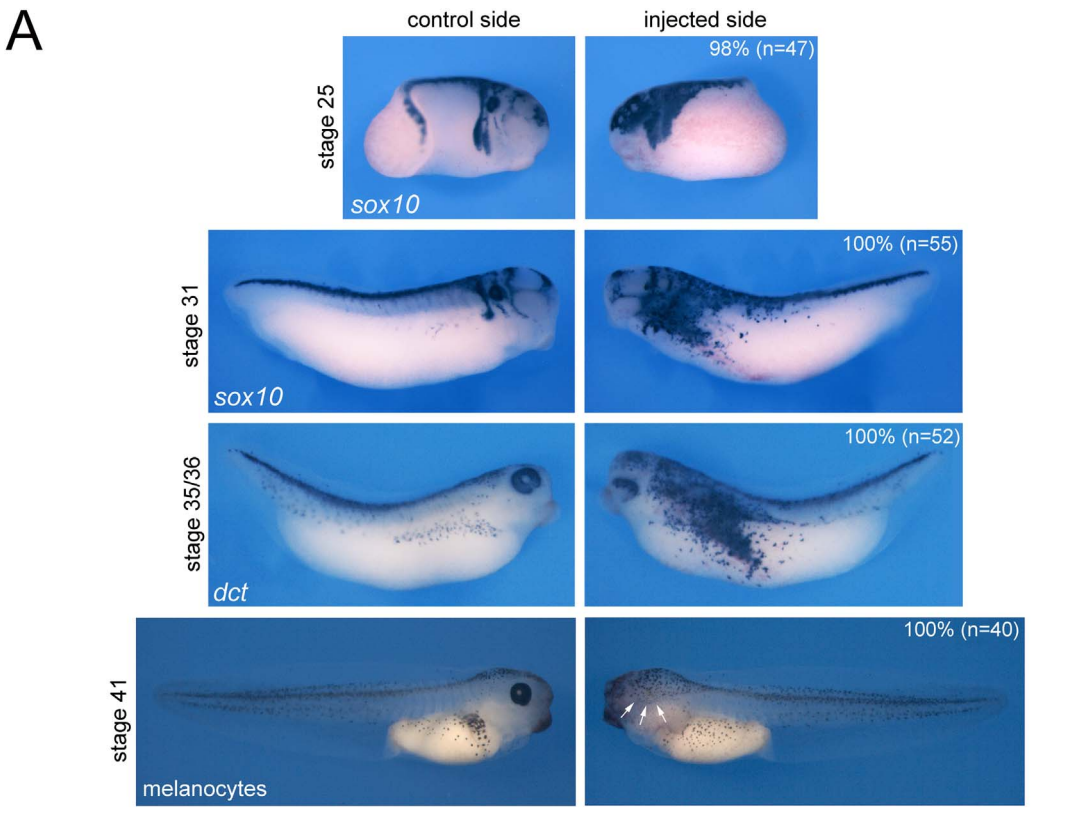


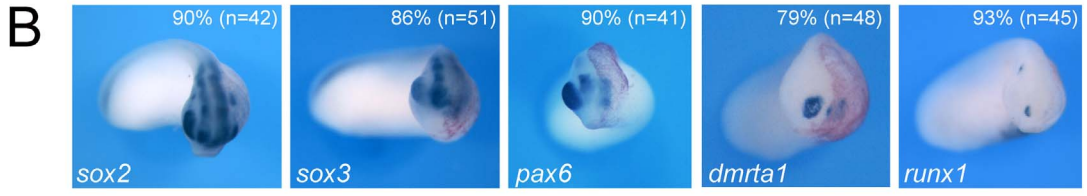
Fig. 3. Hes3 represses NPB fates. (A) Unilateral injection of *hes3GR* mRNA (500 pg) at the 2-cell stage followed by Dex treatment at the gastrula stage (NF stage 10.5) blocked *snai2*, *sox10*, *foxd3* and *dmtra1 expression*, while the expression domain of *pax3* and *zic1* was expanded. In these embryos, the neural plate genes *sox2* and *sox3* were also expanded (red line). (B) Injection of *hes3GR* mRNA in the absence of Dex had not effect on *snai2* and *sox10* expression. (A-B) Dorsal views, anterior to top. Injected side is to the right. The frequency of the phenotypes shown (%) and the number of embryos analyzed (n) are indicated in the upper right corner. (C) qRT-PCR analyses indicate that Hes3GR blocks NC induction (*snai2* and *sox10*) by Wnt8 and Noggin in animal cap explants, and promote neural plate fate (*sox2*). (D) Similarly, Hes3GR blocks PPR gene induction (*six1* and *dmtra1*) by Noggin in animal cap explants. Values are normalized to *ef1α* and presented as mean ± s.e.m. Statistically significant changes are indicated; (*) $p < 0.05$ and (**) $p < 0.01$ (Student's *t*-test), from three independent samples.

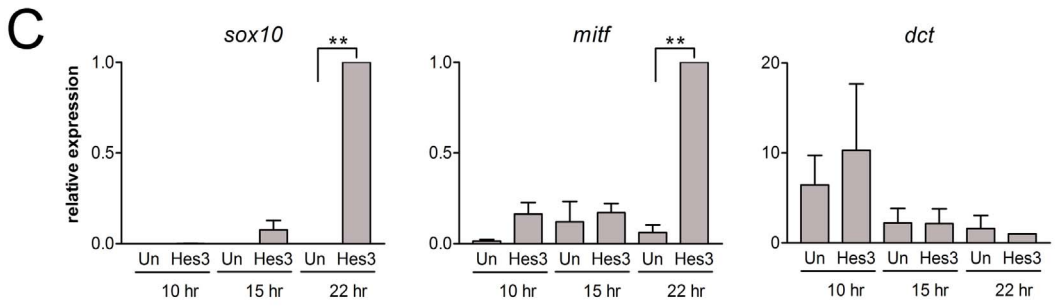
secondary axis was strongly repressed by co-expression of Hes3 (Fig. 7C). Therefore, in three different assays we demonstrate that Hes3 interferes with Wnt/β-catenin signaling, presumably by rending Hes3-expressing cells refractory to Wnt ligands.

4. Discussion

Here we report the expression and function of Hes3, a member of the Hes family of basic helix-loop-helix transcriptional repressors, during *Xenopus* development. *hes3* is expressed at the edge of the developing NP, adjacent to the prospective NC and PPR territories. Hes3 mis-expression blocks NC gene expression and promotes neural fate. Later in development these embryos show ectopic activation of a pigment cell differentiation program. These results suggest that Hes3 maintains NC progenitors in an undifferentiated state, and as Hes3 protein levels subside overtime these progenitors adopt a pigment cell fate. Mechanistically we provide evidence that Hes3 mediates its activity by interfering with the response to Wnt/β-catenin signaling


A
control side
injected side
stage 25
sox10
98% (n=47)
stage 31
sox10
100% (n=55)
stage 35/36
dct
100% (n=52)
stage 41
melanocytes
100% (n=40)


B
sox2
90% (n=42)
sox3
86% (n=51)
pax6
90% (n=41)
dmrta1
79% (n=48)
runx1
93% (n=45)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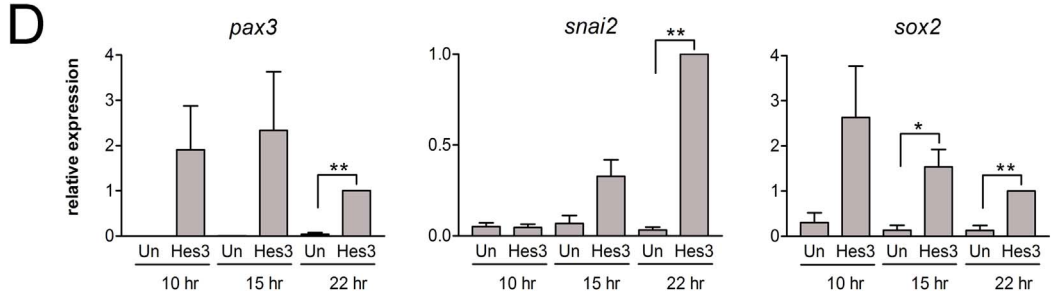
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pax3
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Un Hes3 Un Hes3 Un Hes3
10 hr 15 hr 22 hr

Fig. 4. Hes3 mis-expression induces ectopic pigment cells differentiation. (A) Unilateral injection of *hes3GR* mRNA (500 pg) at the 2-cell stage followed by Dex treatment at the gastrula stage (NF stage 10.5) induced ectopic *sox10* (NF stages 25 and 31) and *dct* (NF stage 35/36) expression. Sibling embryos at the tadpole stage (NF stage 41) showed supernumerary pigment cells in the head region (arrows). In all panels, lateral views are shown, dorsal to top. (B) At the tailbud stage, *hes3GR*-injected embryos show a decrease in neural (*sox2*, *sox3* and *pax6*) and placode (*dmrta1* and *runx1*) gene expression. Anterior views, dorsal to top. Injected side is to the right. (A-B) The frequency of the phenotypes shown (%) and the number of embryos analyzed (n) are indicated in the upper right corner. (C) In animal cap explants, Hes3 is sufficient to activate *sox10* and *mitf* expression (22 h in culture), while *dct* expression was unchanged over the same period of time. (D) Hes3 is also sufficient to induce *pax3* (NPB specifier), *snai2* (NC specifier) and sox2 (neural) genes after 22 h in culture. Values are normalized to *ef1α* and presented as mean ± s.e.m. Statistically significant changes are indicated; (*) $p < 0.05$ and (**) $p < 0.01$ (Student's *t*-test), from three independent samples.

in these progenitors. We propose that Hes3 at the lateral edge of the NP participates in the establishment of the NP/NC boundary by restricting the response to mesoderm-derived Wnt ligand, a major NC-inducing signal in vertebrates (Fig. 8).

Our attempts to knockdown Hes3 function in the embryo to confirm these observations were unsuccessful. We designed several morpholino antisense oligonucleotides, and their microinjections in the embryo alone or in combination did not significantly affect the expression of genes expressed in the ectoderm at the neurula stage (not shown). It is likely that a compensatory mechanism by other Hes family members may account for the lack of an early phenotype in these embryos. This is not uncommon for this class of molecules as

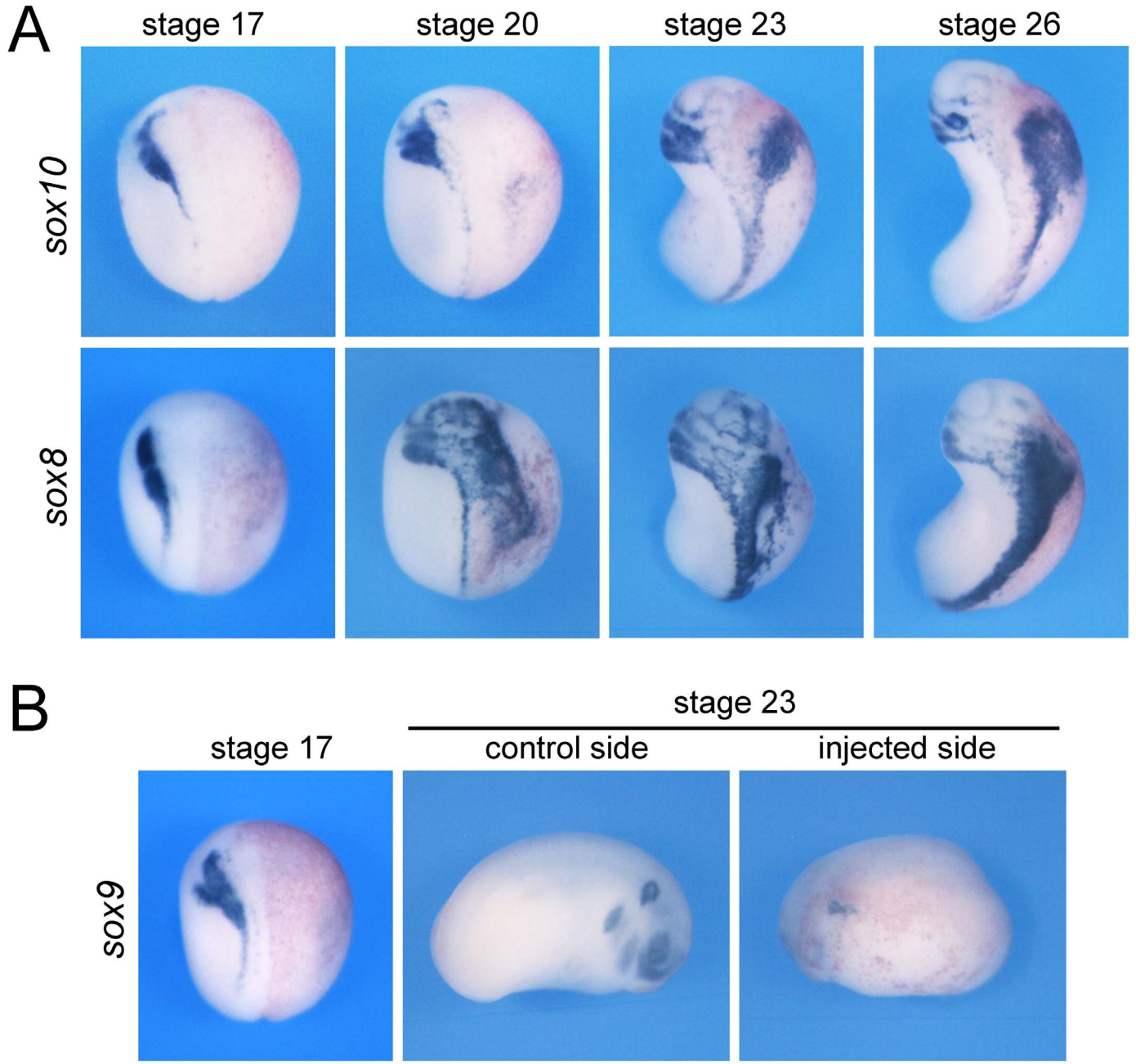

Fig. 5. Hes3 mis-expression differentially affects *soxE* gene expression. (A) *sox10* and *sox8* follow a similar expression pattern upon Hes3 mis-expression in the embryo. Their expression is lost at the neurula stage (NF stage 17), but progressively reactivated as development proceeds, NF stage 17–20 for *sox8* and NF stage 20–23 for *sox10*. (B) Under the same conditions, *sox9* expression is also lost at the neurula stage (NF stage 17), however its expression is not reactivated at the tailbud stage (NF stage 23). (A-B) Dorsal views, anterior to top. Injected side is to the right. (B) Stage 23 lateral views, dorsal to top, anterior to right (control side), anterior to left (injected side).

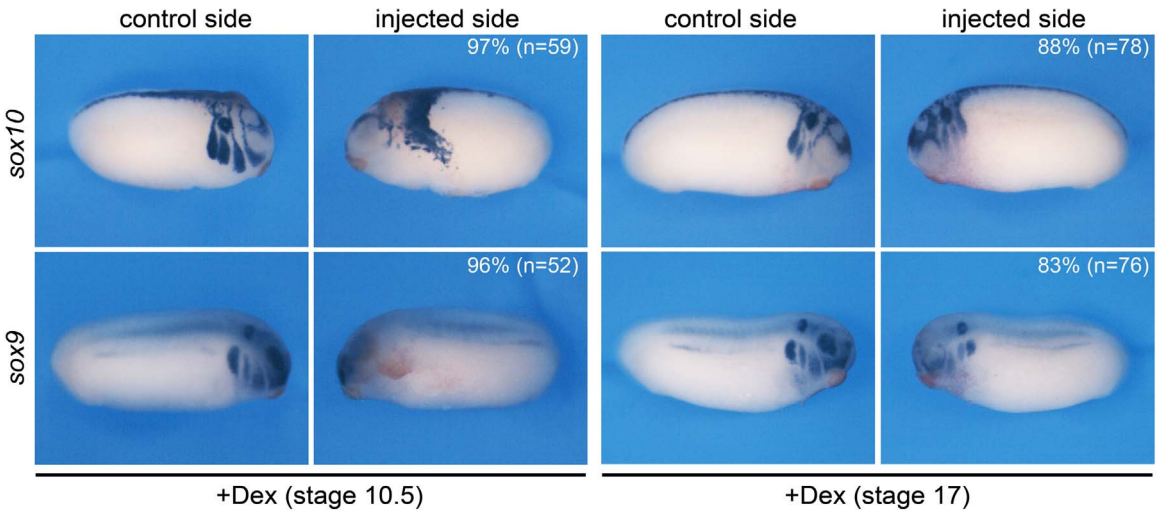

Fig. 6. Hes3 does not induce *de novo* a pigment cell differentiation program. Unilateral injection of *hes3GR* mRNA (500 pg) at the 2-cell stage followed by Dex treatment at the gastrula stage (NF stage 10.5) induced ectopic *sox10* expression, and repressed *sox9* expression at the tailbud stage. By contrast Dex treatment at the neurula stage (NF stage 17) had no impact on *sox10* or *sox9* expression. Lateral views, dorsal to top, anterior to right (control side) or anterior to left (injected side). The frequency of the phenotypes shown (%) and the number of embryos analyzed (n) are indicated in the upper right corner.

previously reported for Hes1, Hes3 and Hes5 in the developing mouse brain, for example (Hatakeyama et al., 2004).

Hes3 gain-of-function phenotype is reminiscent of the gain-of-function phenotype described for *Xenopus* Hairy2, now referred as Hes4 (Nichane et al., 2008). Unlike *Hes3*, *Hairy2* is expressed at the NPB (Tsuji et al., 2003), and Hairy2 mis-expression resulted in a loss of *sox10* and *snai2* expression early on, and a marked increased expression of these genes at later developmental stages. At these late stages *hairy2*-injected embryos had decreased skeletogenic (*sox9*) and melanocytic *(dct*) gene expression, while the expression of a marker for glial lineage, glial fibrillary acid protein (GFAP) was increased (Nichane et al., 2008). The early loss of *sox10* and its reactivation at later stage is a common feature of Hairy2 and Hes3 gain-of-function phenotypes, however the later phenotypes are distinct since only Hes3 is capable of promoting pigment cell differentiation, while Hairy2 promoted glial cell differentiation (Nichane et al., 2008). This conclusion was primarily based on the up-regulation of glial fibrillary acidic protein (GFAP) in Western blot of Hairy2-injected embryos. Recent genomic analyses indicate that during evolution a chromosomal rearrangement resulted in the loss of the GFAP gene in *Xenopus* and other anurans (Martinez-De Luna et al., 2017), therefore challenging these conclusions on the activity of Hairy2.

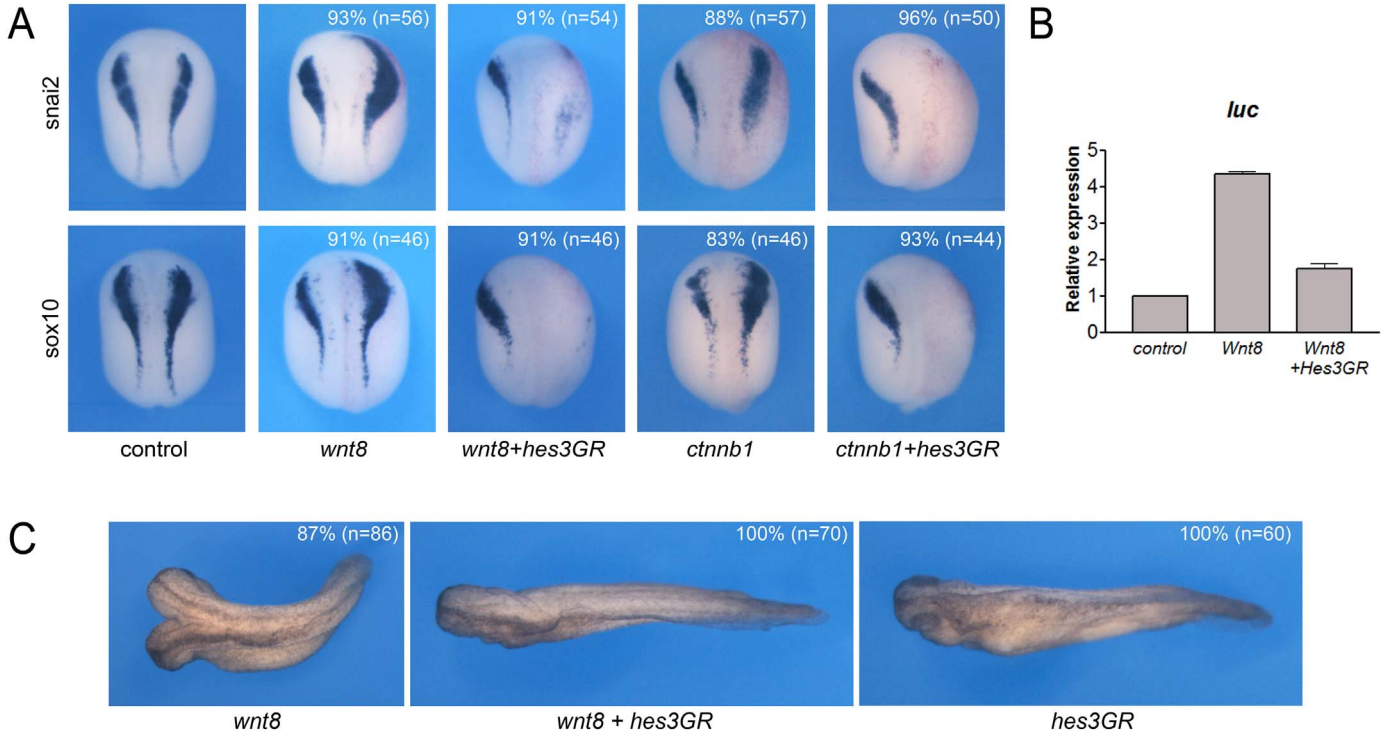

Fig. 7. Hes3 interferes with Wnt/β-catenin signaling. (A) Hes3GR blocks *snai2* and *sox10* induction by Wnt8 and Ctnnb1. Dorsal views, anterior to top. Injected side is to the right. (B) *wnt8* (200 pg mRNA) expression activates a TOP-FLASH reporter (10 pg DNA) construct in animal cap explants, this activity is inhibited by *hes3GR* mRNA coinjection (1 ng). (C) Ventral injection of *wnt8* mRNA (50 pg) in the marginal zone of 4-cell stage embryos induces secondary axis formation. Coinjection of *hes3GR* mRNA (500 pg) completely blocked axis duplication by Wnt8. Dorsal views, anterior to left. (A, C) The frequency of the phenotypes shown (%) and the number of embryos analyzed (n) are indicated in the upper right corner.

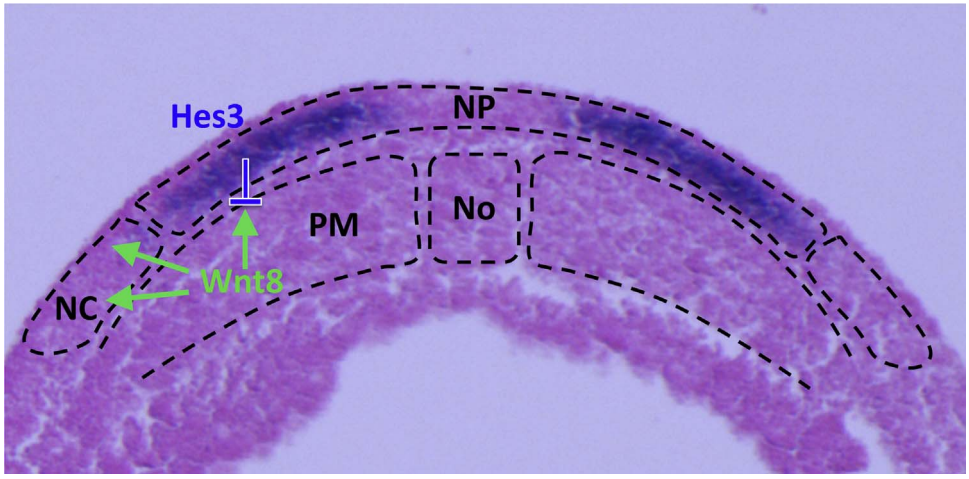

Fig. 8. Model for the establishment of the NP/NC boundary by Hes3. Hes3 expression at the lateral margin of the NP restricts the response to Wnt8 to establish the NP/NC boundary. The different embryonic territories on this transverse section of a stage 15 embryo (Fig. 2S) are outlined. NC, neural crest; No, notochord; NP, neural plate; PM, paraxial mesoderm.

Importantly, neither factor is capable of inducing *sox10* expression *de novo* at later stage (Fig. 6; Nichane et al., 2008), suggesting that Hairy2 and Hes3 mis-expression after NC specification are likely to maintain NC progenitors in an undifferentiated state. The up-regulation of the early NPB genes *pax3* and *zic1* in Hes3-injected embryos (Fig. 3A) is consistent with the view that Hes3 maintains NC progenitor cells as immature precursors. This activity is comparable to that of mouse Hes1, which plays a crucial role in preventing differentiation and maintaining/expanding embryonic neuronal progenitor cells in the brain (Ohtsuka et al., 2001). It is important to point out that because Hes3 is not expressed in the NC this is unlikely to be its normal function. However, mis-expression of Hes3 in the NC territory may assume a function similar to that of Hairy2/Hes4, a Hes gene expressed at the NPB (Glavic et al., 2004; Nichane et al., 2008).

While several Hes family members act as effectors of the Notch pathway (Kageyama et al., 2007), Hairy2 has been proposed to mediate both BMP attenuation and FGF activation during NC induction (Nichane et al., 2008). Our results indicate that Hes3 act as a negative regulator of Wnt/β-catenin signaling. This was demonstrated in three assays, (i) NC induction by Wnt8 and β-catenin, (ii) Wnt8-mediated activation of a TOP-FLASH reporter construct, and (iii) axis duplication by Wnt8. Furthermore the induction of *snai2* and *sox10* by Wnt8 in neuralized animal cap explants was also significantly repressed by Hes3 expression (Fig. 2). The difference in the modulation of these signaling pathways may explain the divergent outcome in Hairy *vs.* Hes3 mis-expression experiments with regard to activation of the melanocytic lineage. A role for Wnt/β-catenin signaling in melanocyte specification from NC progenitors is well documented and conserved across species (Dorsky et al., 1998, 2000; Dunn et al., 2000; Jin et al., 2001; Hari et al., 2012). The activation of this differentiation program is consistent with our observations implicating Hes3 as a negative regulator of Wnt/β-catenin signaling. While Hes3 expression initially prevents NC progenitors differentiation, as Hes3 protein levels subside overtime, it is likely that the sensitivity of these progenitors to endogenous Wnt ligands is restored, leading to the activation of a pigment cell differentiation program.

Altogether these observations suggest that the normal function of Hes3 at the edge of the NP is to restrict the response to mesoderm-derived Wnt ligands, a mechanism by which Hes3 participates in the establishment of a sharp boundary of gene expression at the NPB (Fig. 8). Experiments are underway to determine the molecular mechanisms by which Hes3 interferes with Wnt/β-catenin signaling intracellularly.

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