Self-regulation of Stat3 activity coordinates cell-cycle progression and neural crest specification

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A complex set of extracellular signals is required for neural crest (NC) specification. However, how these signals function to coordinate cell-cycle progression and differentiation remains poorly understood. Here, we report in *Xenopus* a role for the transcription factor signal transducers and activators of transcription-3 (Stat3) in this process downstream of FGF signalling. Depletion of Stat3 inhibits NC gene expression and cell proliferation, whereas overexpression expands the NC domain and promotes cell proliferation. Stat3 is phosphorylated and activated in ectodermal cells by FGFs through binding with FGFR4. Stat3 activation is also modulated by Hair2 and Id3 proteins that, respectively, facilitate and disrupt Stat3-FGFR4 complex formation. Furthermore, distinct levels of Stat3 activity control *Hairy2* and *Id3* transcription, leading to Stat3 self-regulation. Finally, high Stat3 activity maintains cells in an undifferentiated state, whereas low activity promotes cell proliferation and NC differentiation. Together, our data suggest that Stat3, downstream of FGFs and under the positive and negative feedback regulation of *Hairy2* and *Id3*, plays an essential role in the coordination of cell-cycle progression and differentiation during NC specification.

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Introduction

The neural crest (NC) is a transient embryonic cell population that arises at the border of the neural plate and the non-neural ectoderm during gastrulation in vertebrates. As the neural tube closes, NC cells delaminate, migrate throughout the embryo and subsequently differentiate into a host of terminal cell types, including neurons and glia of the periph-

eral nervous system, craniofacial skeleton elements, smooth-muscle cells and melanocytes (Huang and Saint-Jeannet, 2004).

In *Xenopus*, NC fate determination integrates different environmental signals and occurs in two steps (LaBonne and Bronner-Fraser, 1998). Graded BMPs along the dorsal–ventral axis specify the ectoderm into neural plate, neural crest and epidermis (Mayor and Aybar, 2001). The intermediate BMP level, although required for NC induction, is, however, not sufficient. It sets up a competence zone for NC, but other signalling pathways are involved. The role of Wnt signals in NC induction is now well established (Saint-Jeannet *et al*, 1997; LaBonne and Bronner-Fraser, 1998). FGF signalling is also involved in NC induction (Monsoro-Burq *et al*, 2003). However, an excess of FGF signalling inhibits NC formation (Hong and Saint-Jeannet, 2007). In response to these extracellular signals, a set of genes encoding transcription factors called neural border specifiers, such as *Msx1*, *Pax3* and *Zic1*, defines a territory containing cells competent to form NC (Tribulo *et al*, 2003; Sato *et al*, 2005). In turn, these neural border transcription factors activate NC-specific genes like *Snail2* (Mayor *et al*, 1995), *FoxD3* (Sasai *et al*, 2001) or members of the Sox family (Spokony *et al*, 2002; Aoki *et al*, 2003) that specify NC fates.

Recent studies provide insights into the mechanisms by which extracellular signals interact to generate the NC and into the hierarchical relationships among neural border specifiers. FGF has been shown to induce NC through both *Msx1* and *Pax3* activities, while Wnt signals through *Pax3* activity (Monsoro-Burq *et al*, 2005). The transcriptional activation of the neural border *Zic1* is directly linked to BMP attenuation through a BMP inhibitory response module in its promoter (Tropepe *et al*, 2006). Some of the genes expressed at the neural plate border, such as the basic helix–loop–helix (bHLH) transcription factor *Hairy2* and the HLH factor *Id3* play a crucial role in cell-cycle progression and in the maintenance of cells in an undifferentiated state (Kee and Bronner-Fraser, 2005; Nagatomo and Hashimoto, 2007). *Hairy2* modulates *Id3* expression and *Id3* protein, by directly interacting with *Hairy2*, negatively regulates its activity (Nichane *et al*, 2008a, b). However, how extracellular signals are interpreted by NC to coordinate cell-cycle progression and differentiation is actually largely unknown.

Signal transducers and activators of transcription (Stat) proteins are transcription factors latent in the cytoplasm that are activated by many extracellular signals. Stats are activated by tyrosine phosphorylations (i.e., Tyr705 in Stat3) triggered by tyrosine kinase receptors (RTKs) or non-receptor tyrosine kinases such as Janus kinases (JAKs) and Src. The phosphorylated Stats then dimerize and translocate to the nucleus where they directly activate target genes (Levy and Darnell, 2002). *Stat3* is one family member whose loss in mice results in embryonic lethality (Takeda *et al*, 1997). Stat3 regulates cell proliferation and apoptosis in many tissues

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(Bromberg, 2001). It is essential for embryonic stem cell self-renewal (Niwa *et al*, 1998; Raz *et al*, 1999) and is aberrantly activated in many cancer cells (Bromberg *et al*, 1999). Its role in the development of the nervous system has only recently attracted attention. In the developing mouse neocortex, Stat3 has been shown to maintain neural precursor cells in an undifferentiated state (Yoshimatsu *et al*, 2006). In *Xenopus*, Stat3 is ubiquitously expressed in the early embryo (Nishinakamura *et al*, 1999). Recently, we found that Stat3 is required for Hairy2 DNA-binding-independent activities at the neural plate border (Nichane *et al*, 2008b). However, its role and mode of action during NC formation remain largely unknown. In this study, we show that Stat3 acts as an essential FGF-transducing signal. Furthermore, we provide evidence for a mechanism by which Stat3 self-regulates its own activation under the feedback regulation of Hairy2 and Id3. We also show that distinct levels of Stat3 activity promote the maintenance of cells in an undifferentiated state and their proliferation and differentiation into NC. Finally, we propose that the self-regulation of Stat3 is essential for the coordination of cell-cycle progression and differentiation during NC specification.

Results

Stat3 is essential for proliferation and survival of NC progenitors upstream of neural border specifiers

Based on our previous findings (Nichane *et al*, 2008b), we hypothesized that Stat3 may play an important role in the embryonic ectoderm as a regulator of NC specification. In *Xenopus*, Stat3 is expressed ubiquitously, as detected by *in situ* hybridization, from the one-cell stage to the end of gastrulation, and is later mainly restricted to the nervous system (Nishinakamura *et al*, 1999, data not shown). We first performed whole-mount immunostaining to detect the active form of Stat3 using a Stat3 phospho-tyrosine-705 (pTyr705-Stat3) antibody. In gastrula-to-neurula embryos, pTyr705-Stat3 was detected in ectodermal cells, including those of the NC region (Supplementary Figure S1). To investigate the role of Stat3 in NC development, we performed loss-of-function experiments using a morpholino antisense oligonucleotide (MO) that efficiently inhibits endogenous Stat3 mRNA translation (Figure 1A). As Stat3 is required for mesoderm induction (Ohkawara *et al*, 2004), the Stat3 MO was injected together with LacZ mRNA as a lineage tracer, in one animal blastomere of eight-cell-stage embryos to specifically deplete Stat3 protein in the ectoderm. We found that the expression of the NC marker *Snail2* was reduced in neurula injected embryos. Later, expression of *Snail2* and another NC marker, *Sox10*, was still reduced at tailbud stage. *MyoD* expression was not affected, indicating that the NC phenotype is not an indirect consequence of mesoderm perturbation. Injection of a control Stat3 MO containing seven mismatches (Stat3 MO7mis) did not significantly affect *Snail2* or *Sox10* expression (Figure 1B–E, and data not shown). The Stat3 MO phenotype could be rescued in dexamethasone (Dex)-treated embryos (stage 11.5–12) by co-injection of mRNA encoding a hormone-inducible, wild-type Stat3-GR construct lacking the Stat3 MO-recognition sites, indicating that the observed phenotype is specific (Supplementary Figure S2A–D).

We next performed gain-of-function studies using the above Stat3-GR construct. Stat3-GR overexpression in the

Figure 1 Stat3 regulates NC formation upstream of neural border specifiers. (A) Stat3 MO (10–15 ng per blastomere) but not Stat3 MO7mis (15 ng per blastomere) blocks endogenous Stat3 translation in animal cap explants. Western blot were performed using anti-Stat3 (top) and anti-GAPDH (bottom, loading control) antibodies. (B–N) Whole-mount *in situ* hybridization of embryos injected with a Stat3 MO (15 ng), a Stat3 MO7mis (15 ng) or Stat3-GR mRNA (200 pg) and analysed with indicated probes. (B–E) Stat3 depletion inhibits NC markers. *Snail2* is reduced in Stat3 MO (B, 62%, $n = 42$) but not in Stat3 MO7mis (C, 90% unaffected, $n = 30$)-injected neurula embryos. *Snail2* and *Sox10* are also reduced at tailbud stages (D, 56%, $n = 18$; E, 58%, $n = 26$). (F–H) Stat3 gain of function increases NC markers. Stat3-GR overexpression expands *Snail2* at neurula stage (F, 68%, $n = 25$). At tailbud stage, *Snail2* like *Sox10* is still expanded (G, 52%, $n = 21$; H, 57%, $n = 35$). Dex was added around stage 11.5–12. (I–N) Stat3 acts upstream of neural border specifiers. Stat3 MO injection reduces *Msx1* (I, 60%, $n = 20$), *Pax3* (J, 50%, $n = 14$) and *SoxD* (K, 34%, $n = 24$). Conversely, Stat3-GR overexpression increases *Msx1* (L, 45%, $n = 22$), *Pax3* (M, 56%, $n = 16$) and *SoxD* (N, 55%, $n = 18$). Neurula embryos are shown in dorso-anterior views with the injected side to the right. Tailbud embryos are shown in lateral views, with insets showing the control side.

ectoderm of embryos expanded *Snail2* and *Sox10* at neurula and tailbud stages, without affecting *MyoD* (Figure 1F–H and data not shown). As Stat3 must be phosphorylated at Tyr705

to become active, we also used a constitutively active Stat3-GR construct (Stat3CTC-GR; Bromberg *et al*, 1999) and compared its activity to wild-type Stat3-GR. At a low dose at which wild-type *Stat3*-GR had no effect, *Stat3CTC*-GR still efficiently expanded *Snail2*. Conversely, embryos injected with mRNA encoding a dominant-negative Stat3 mutant (Stat3YF-GR; Niwa *et al*, 1998) showed reduced *Snail2* and *Sox10* expression, like in Stat3 MO-injected embryos (Supplementary Figure S2E-I). Tyr705 phosphorylation is thus essential for Stat3 activity in NC.

To position Stat3 in the genetic cascade leading to NC specification, we analysed the effects of Stat3 gain- and loss-of-function on the earlier neural border specifier genes *Msx1*, *Pax3* and *SoxD* (Mizuseki *et al*, 1998; Monsoro-Burq *et al*, 2005; Sato *et al*, 2005). Stat3 depletion led to reduction of *Msx1*, *Pax3* and *SoxD*, whereas its overexpression upregulated their expression (Figure 1I-N).

As Stat3 plays an important role in cell proliferation and survival (Bromberg, 2001), we also examined the effect of Stat3 gain- and loss-of-function on cell cycle and cell death (Supplementary Figure S3). Analysis of the number of mitotic cells by immunostaining with an anti-pH3 antibody indicated that Stat3 depletion decreases cell proliferation in the ectoderm of neurula-stage embryos and that its overexpression increases the number of cycling cells. Through TUNEL assays, we observed that Stat3 knockdown increases the number of apoptotic cells. Due to the very low number of endogenous dying cells detectable by TUNEL in neurula-stage embryos, we were not able to determine whether conversely Stat3 overexpression inhibits apoptosis. The loss of cycling cells and the increase of cell death are not observed in Stat3 MO7mis-injected embryos and are rescued in the presence of Stat3-GR. Together, these data indicate that *Stat3* is required in NC progenitors upstream of the neural border specifiers and that it regulates cell proliferation and survival.

Stat3 is activated by FGF signals through FGFR4 in NC

Stat3 phosphorylation on Tyr705 is well known to occur through various cytokines and growth factors (Levy and Darnell, 2002). To identify the signals that activate Stat3 in the ectoderm of early embryos during NC induction, we first radially injected *Stat3* mRNA alone or together with mRNA coding for various FGF, Wnt and BMP ligands in four-cell-stage embryos. Animal caps were excised and analysed by western blot using a pTyr705-Stat3 antibody. An increased level of Stat3 phosphorylation was detected in caps overexpressing FGFs, but not Wnt8 or BMP4 (Figure 2A and Supplementary Figure S4A). Similar results were obtained using recombinant bFGF or eFGF proteins (not shown). The ability of FGF, Wnt and BMP ligands to activate Stat3 was also tested in embryos. *eFGF* injection, but not *Wnt8* or *BMP4*, increased the number of pTyr705-Stat3-positive cells in the ectoderm of gastrula- and neurula-stage embryos as revealed by whole-mount immunostaining (Supplementary Figure S4C and F, and data not shown). Among the four different FGF receptors (FGFRs) described in *Xenopus*, *FGFR4* is expressed at the right time and place in the ectoderm and in prospective NC during gastrulation and early neurulation (Hongo *et al*, 1999; Golub *et al*, 2000; Lea *et al*, 2009, and data not shown). To determine whether FGFR4 mediates Stat3 phosphorylation in ectodermal cells, we examined the level of active Stat3 in

caps derived from embryos injected with increasing amounts of mRNA encoding a constitutively active FGFR4 (caFGFR4) or another FGFR (caFGFR1) (Umbhauer *et al*, 2000). As shown in Figure 2B, caFGFR4 induces a higher level of active Stat3 than caFGFR1. caFGFR4 appears thus to be more efficient than caFGFR1 in Stat3 phosphorylation. Similarly, *caFGFR4* overexpression increased Stat3 phosphorylation in embryos (Supplementary Figure S4D and G). Second, we imaged Stat3 nuclear translocation using a Stat3-GFP fusion protein in animal caps under the influence of constitutively active or dominant negative forms of FGFR4 or FGFR1. In control caps, Stat3-GFP was found in most cells equally distributed between the nucleus and cytoplasm. Some cells however showed Stat3-GFP accumulation in the nucleus, which most probably reflects activation by endogenous signals (Supplementary Figure S5A). In the presence of FGFs, but not Wnt8 or BMP4, Stat3-GFP was found predominantly in the nucleus in most cells (not shown). Stat3-GFP was also mainly nuclear in cells overexpressing *caFGFR4* but not in those overexpressing *caFGFR1*. Conversely, a dominant-negative form of FGFR4 (dnFGFR4; Hongo *et al*, 1999), but not dnFGFR1 (Amaya *et al*, 1991), reduced nuclear Stat3-GFP localization (Supplementary Figure S5B-E). We also imaged the subcellular localization of the constitutively active Stat3CTC-GFP and the dominant-negative Stat3YF-GFP mutants. Stat3CTC-GFP was found highly concentrated in the nucleus while Stat3YF-GFP was found exclusively in the cytoplasm. As expected, caFGFR4 was unable to induce nuclear translocation of Stat3YF-GFP, indicating that FGFR4 induces Stat3 nuclear translocation through Tyr705 phosphorylation (Supplementary Figure S5F-H). Third, we monitored Stat3 activation using the luciferase reporter pTATA-TK-Luc-4xM67 containing Stat-binding sites (Besser *et al*, 1999) in animal caps. Figure 2C and Supplementary Figure S4H show that FGFs, but not Wnt8 or BMP4, and caFGFR4, but not caFGFR1, activated the Stat reporter. Stat3YF-GR abolished reporter activation by caFGFR4, further indicating that FGFR4 increases transcription of the reporter through Stat3 Tyr705 phosphorylation. Fourth, to determine whether Stat3 interacts with FGFRs, co-immunoprecipitation (Co-IPs) using overexpressed tagged forms of the intracellular domains of FGFRs (FGFR-IC) and Stat3 were performed. Stat3 could be detected following FGFR4-IC immunoprecipitation, but not with FGFR1-3-IC (Figure 2D).

We next re-examined the role of FGFR1 and FGFR4 in NC. Supplementary Figure S6 shows that caFGFR4, but not caFGFR1, expanded NC. Conversely, interference with FGFR4, either with a dominant-negative FGFR4 construct (dnFGFR4) or with specific antisense MOs (FGFR4 MOs), inhibited NC. No such inhibitory effect was observed with dnFGFR1 or FGFR1 MOs, suggesting that FGFR4, but not FGFR1, has a role in the earliest steps of NC specification. We thus asked whether Stat3 is required downstream of FGFR4. As shown in Figure 2E-H, caFGFR4 was unable to induce *Snail2* and increase cell proliferation when co-injected with the Stat3 MO. No such inhibitory effect was observed with the Stat3 MO7mis. In contrast to caFGFR4, Wnt8 was still able to expand *Snail2* in Stat3-depleted embryos (Supplementary Figure S4I and J). Together, our results indicate that Stat3 is directly activated by FGF signals through FGFR4 binding and that it is required downstream of FGFR4 for NC formation.

Figure 2 Stat3 is activated by FGF signals through FGFR4. (**A**, **B**) Western blot analysis of animal caps derived from embryos injected with Stat3 (500 pg) alone or together with bFGF (FGF2), FGF3, eFGF (FGF4), FGF8a (100 pg each) or with caFGFR1 or caFGFR4 (500 pg and 1 ng) using anti-phosphorylated Tyr705 Stat3 (top) and anti-Stat3 (bottom) antibodies. A control western blot shows the level of overexpressed Myc-tagged caFGFR1 and caFGFR4. FGFs and caFGFR4 strongly induce Stat3 Tyr705 phosphorylation, while caFGFR1 has a modest effect. Note that caFGFR1 is expressed at a higher level than caFGFR4, although the same quantity of mRNA of both constructs was injected. (**C**) Luciferase assay using animal caps derived from embryos injected with a Stat reporter plasmid alone or together with caFGFR1 (500 pg and 1 ng), caFGFR4 (500 pg and 1 ng) or with caFGFR4 (1 ng) and Stat3YF-GR (500 pg). FGFR4, but not FGFR1, increases reporter activity. Stat3YF-GR abolishes reporter activation by caFGFR4. A control western blot shows the level of overexpressed Myc-tagged caFGFR1 and caFGFR4. (**D**) Co-IP using extracts from embryos overexpressing Stat3-HA and FGFR1-4 IC-Flag. Stat3 interacts with FGFR4-IC but not with the others FGFRs-IC. H indicates heavy chain. (**E**–**H**) Stat3 is required for NC downstream of FGFR4. Whole-mount *in situ* hybridization or pH3 immunostaining (arrows) of embryos injected with caFGFR4 (500 pg) together with Stat3 MO7mis (15 ng) or Stat3 MO (15 ng). *Snail2* and the number of proliferating cells are increased by caFGFR4 in Stat3 MO7mis-injected embryos (**E**, 65%, $n = 24$; **G**, 52%, $n = 29$), but is decreased in Stat3-depleted embryos (**F**, 57%, $n = 14$; **H**, 56%, $n = 27$). Neurula embryos are shown in dorso-anterior views, with the injected side to the right.

Hairy2 enhances Stat3 activation in NC by facilitating FGFR4–Stat3 interaction

Hairy2 is essential for NC proliferation downstream of FGFs and has DNA-binding-independent activities that require Stat3 (Nichane *et al*, 2008a, b). As FGFs also activates Stat3, we asked whether Stat3 and Hairy2 function together downstream of FGFR4 during NC specification. To approach this question, we first tested whether the Hairy2 protein complexes with Stat3. Co-IPs using overexpressed tagged constructs showed that Hairy2, but not other Hes factors such as Hes6, Hes2 or ESR10, specifically interacted with Stat3 (Supplementary Figure S7A). Endogenous Stat3 was also co-immunoprecipitated with overexpressed tagged Hairy2 (Figure 3A). We also asked whether Hairy2 interacts with the intracellular domain of FGFRs. Supplementary

Figure S7B shows that Hairy2 interacts with FGFR4-IC, but not with FGFR1-3-IC. Deletion analysis indicated that interaction of Hairy2 with Stat3 occurs through its Orange domain, while its binding with FGFR4 depends on its N-terminal region (Supplementary Figure S7C–E). Thus, Hairy2 binds to Stat3 and FGFR4 through separate domains.

As both Stat3 and Hairy2 interact with FGFR4, we asked whether Stat3 and Hairy2 proteins exist in a complex together with FGFR4. Tagged Stat3 and FGFR4-IC were overexpressed in embryos with or without Hairy2 or the Hairy2ΔNterm mutant unable to bind FGFR4-IC. FGFR4-IC was immunoprecipitated and Stat3 and Hairy2 binding was monitored simultaneously. We found that Hairy2 and Stat3 are co-immunoprecipitated at the same time with FGFR4-IC. Strikingly, Stat3 binding to FGFR4 was increased in the

Figure 3 Hair2 enhances Stat3 activation in NC by facilitating FGFR4-Stat3 complex formation. (A) Endogenous Stat3 binds to overexpressed Hair2-HA but not to Notch ICD-HA in co-IPs. (B) Co-IP using extracts from embryos injected with Stat3-HA and FGFR4 IC-Flag with or without Hair2 Δ Nterm-HA or Hair2-HA. Hair2, but not Hair2 Δ Nterm, enhances Stat3 binding to FGFR4-IC. H and L indicate heavy and light chains, respectively. (C, D) Western blot analysis of animal caps derived from embryos injected with Stat3 (500 pg), eFGF (100 pg), Hair2 MOs (15 ng), Hair2-GR (500 pg) and Hair2 Δ Nterm-GR (500 pg), as indicated, using anti-phosphorylated Tyr705 Stat3 (top) and anti-Stat3 (bottom) antibodies. Hair2 cooperates with FGF to increase pTyr705 Stat3 level, while Hair2 knockdown or Hair2 Δ Nterm has the opposite effect. (E) Luciferase assay using animal caps derived from embryos injected with a Stat reporter plasmid alone or together with Hair2-GR (500 pg), Hair2 Δ Nterm-GR (500 pg), Hair2 MOs (15 ng) and caFGFR4 (1 ng), as indicated. Hair2 increases luciferase activity while Hair2 depletion or Hair2 Δ Nterm reduces it, even in presence of caFGFR4. (F-M) Hair2 and Stat3 cooperate in NC. Whole-mount *in situ* hybridization of tailbud embryos injected with Hair2-GR (100 pg), Stat3-GR (50 pg), Hair2 MOs (7.5 ng) and Stat3 MO (7.5 ng), alone or in combination as indicated (F-H, J-L). Sox10 is increased by co-injection of Hair2-GR and Stat3-GR mRNA (H, 59%, $n = 27$), but not when they are injected alone (F, 83%, unaffected, $n = 24$; G, 80%, $n = 26$). Conversely, Sox10 is decreased in embryos co-injected with sub-effective doses of Hair2 and Stat3 MOs (L, 67% $n = 24$), but not when they are injected alone (J, 86%, normal, $n = 21$; K, 93% $n = 30$). Tailbud embryos are shown in lateral views, with insets showing control sides. qPCR analysis of Sox10 expression in *in vitro* NC induced animal caps derived from embryos injected with Hair2-GR (25 pg per blastomere), Stat3-GR (25 pg per blastomere), Hair2 MOs (4 ng per blastomere) and Stat3 MO (4 ng per blastomere), alone or in combination as indicated (I, M). As in embryos, only coexpression of Hair2 and Stat3 or their simultaneous depletion affects Sox10. A control western blot shows in panel I that similar levels of Myc-Hair2-GR and Stat3-GR have been produced under each condition.

presence of Hair2 and reduced in the presence of Hair2ΔNterm (Figure 3B). In accordance with these data, Stat3–GFP and Hair2–GFP proteins were found to partially colocalize with a membrane-bound RFP (mRFP). Localization of Stat3–GFP and Hair2–GFP at the plasma membrane was slightly increased in the presence of FGFR4 (Supplementary Figure S8). Together, these data indicate that Stat3, Hair2 and FGFR4 form a ternary complex at the plasma membrane.

To determine the role of Hair2 in the complex, we first investigated whether the level of active Stat3 induced by FGF is affected by Hair2 gain and loss of function in animal caps. Overexpression of *Hair2–GR* increased the level of pTyr705-Stat3 induced by FGF in animal caps, while Hair2 depletion or *Hair2ΔNterm–GR* overexpression reduced its phosphorylation level to the basal level of control caps without FGFs (Figure 3C and D). Second, we examined the influence of Hair2 on the subcellular localization of Stat3–GFP in animal caps. We found that Hair2 enhances the number of cells with predominant nuclear localization of Stat3–GFP. Conversely, Hair2 depletion or *Hair2ΔNterm* reduced nuclear accumulation of Stat3–GFP. Hair2 MOs or *Hair2ΔNterm*, but not *Hair2* mRNA, co-injection also inhibited caFGFR4-induced nuclear translocation of Stat3–GFP (Supplementary Figure S9, and data not shown). Third, we monitored the effect of Hair2 on Stat3 activation in animal caps using the luciferase Stat reporter. Hair2–GR increased the reporter activity, while *Hair2ΔNterm–GR* or Hair2 depletion reduced it. *Hair2ΔNterm–GR* or Hair2, but not Hair2–GR, depletion also inhibited the activation of the Stat reporter by caFGFR4 (Figure 3E).

Finally, we wanted to know whether Hair2–Stat3 interactions are relevant in NC. Therefore, we first tested the ability of the *Hair2ΔNterm–GR* mutant to affect NC. In contrast to wild-type protein, *Hair2ΔNterm–GR* had no effect on *Snail2* and *Sox10* (not shown). Second, sub-effective doses of *Hair2–GR* and *Stat3–GR* mRNA were injected alone or together in embryos. While injection of *Hair2–GR* or *Stat3–GR* mRNA alone did not affect *Sox10*, co-injection of the two mRNA efficiently induced its expression (Figure 3F–H). Third, sub-effective doses of Hair2 MOs or Stat3 MO

were injected alone or together in embryos. Figure 3J–L shows that while the injection of Hair2 MOs or Stat3 MO alone did not affect *Sox10*, co-injection of the two MOs efficiently reduced its expression. Similar results were obtained in animal caps explants induced to form NC *in vitro* by injection of *noggin* and *Wnt8* mRNA (LaBonne and Bronner-Fraser, 1998; Figure 3I and M). Together, these results demonstrate that Hair2 enhances Stat3 activation during NC formation by facilitating FGFR4–Stat3 interaction.

Id3 negatively regulates Stat3 activation in NC by disrupting Stat3–Hair2–FGFR4 complex formation

As Hair2–Id3 antagonistic interactions play an essential role in NC (Nichane *et al*, 2008a), we tested whether Id3 gain and loss of function may also affect the level of active Stat3 induced by FGF in animal caps. We found that Id3 MO enhanced the level of pTyr705-Stat3 induced by FGF, while Id3–GR reduced it (Figure 4A and B). Consistent with this result, Stat3–GFP nuclear accumulation was reduced upon *Id3* overexpression and increased by its depletion in animal cap cells. Similarly, *Id3* but not Id3 MO co-injection blocked caFGFR4-induced nuclear translocation of Stat3–GFP (Supplementary Figure S10, and data not shown). We also found that in animal caps, Id3–GR reduced the activity of the Stat reporter, while Id3 depletion slightly increased it. Id3–GR also inhibited the activation of the Stat reporter by caFGFR4, while Id3 MO and caFGFR4 co-injection led to strong Stat-reporter activation (Figure 4C). Those negative effects of Id3 on Stat3 were not due to a reduction of Stat3 protein stability as determined by western blot analysis (Supplementary Figure S11A).

One possible mechanism by which Id3 may affect Stat3 is through direct protein–protein interactions. Through Co-IPs using overexpressed tagged proteins, we found that Id3 interacts with Stat3 (Supplementary Figure S11B). Endogenous Stat3 protein also interacts with overexpressed tagged Id3 in embryos (Figure 4D). As (i) both Id3 and Hair2 proteins bind to Stat3 and (ii) Id3, which antagonizes Hair2 activity, binds as Stat3 to the Orange domain of Hair2 (Nichane *et al*, 2008a), we asked whether Id3 and Hair2 compete for Stat3 binding. To answer this question, we

Figure 4 Id3 negatively regulates Stat3 activation in NC by disrupting Stat3–Hair2–FGFR4 ternary complex. (A, B) Western blot analysis of animal caps derived from embryos injected with *Stat3* (500 pg), *eFGF* (100 pg), Id3 MO (20 ng) and *Id3–GR* (500 pg), as indicated, using anti-phosphorylated Tyr705 Stat3 (top) and anti-Stat3 (bottom) antibodies. Id3 depletion enhances Stat3 Tyr705 phosphorylation by FGF, while Id3 inhibits it. (C) Luciferase assay using animal caps derived from embryos injected with a Stat3 reporter alone or with *Id3–GR* (500 pg), Id3 MO (20 ng) and caFGFR4 (1 ng), as indicated. Id3, even in presence of caFGFR4, reduces the level of Stat reporter activity, while Id3 depletion enhances it. Note that co-injection of Id3 MO and caFGFR4 mRNA activates the Stat reporter more strongly than caFGFR4 alone. (D) Endogenous Stat3 interacts with overexpressed Id3–HA but not with XNAP/Nrarp–HA in co-IP. (E) Co-IP using extracts from embryos injected with *Stat3–Flag* and *Id3–HA*, alone or together with increasing doses of *Hair2–HA*. *LacZ* was used to keep constant the amount of injected mRNA. Stat3–Flag was immunoprecipitated and binding of Id3–HA and Hair2–HA to Stat3 was analysed. Note that binding of Id3 and Hair2 to Stat3 is mutually exclusive. (F) Co-IP using extracts from embryos overexpressing *Stat3–HA* and *FGFR4–IC–Flag*, with or without *Id3–HA* or *Hair2–HA*. Id3, in contrast to Hair2, decreases the amount of Stat3 co-immunoprecipitated with FGFR4–IC. H and L indicate heavy and light chains, respectively. (G–P) Id3 inhibits Stat3 function in NC. Whole-mount *in situ* hybridization of embryos injected with *Stat3–GR* (200 pg, G, H, M–O or 500 pg, I, J) alone or together with *Id3–GR* (500 pg, H, J) or with an Id3 MO (20 ng, N) or Hair2 MOs (15 ng, O) and analysed with *Sox10* or *Delta1*. qPCR analysis of *Sox10* and *Delta1* in *in vitro* NC induced animal caps derived from embryos injected with *Stat3–GR* (100 pg per blastomere) alone or together with *Id3–GR* (100 pg per blastomere), an Id3 MO (10 ng per blastomere) or Hair2 MOs (7.5 ng per blastomere), and harvested at the indicated stages (K, L, P). Controls western blots show the level of Stat3–GR and Flag–Id3–GR produced under each condition in panels K, L, P. Id3 blocks Stat3's ability to expand *Sox10* and induce *Delta1* in embryos and in *in vitro* NC induced explants. Respective expansions/inductions: G, 54%, *n* = 35; H, 16%, *n* = 28; I, 67%, *n* = 15; J, 0%, *n* = 19. Id3 knockdown enhances the Stat3 overexpression phenotype. *Stat3–GR*, which alone has no effect (M, 80% unaffected, *n* = 26), strongly upregulates *Delta1* (N, 58%, *n* = 19) in the presence of the Id3 MO in embryos and in *in vitro* NC induced explants. No such effect was observed upon co-injection of Hair2 MOs (O, 92% unaffected, *n* = 12). Neurula embryos are shown in dorso-anterior views, with the injected side to the right, or in lateral views, with insets showing the control sides.

overexpressed tagged Stat3 and Id3 in embryos in the presence of increasing levels of Hairy2. Binding of Id3 and Hairy2 to immunoprecipitated Stat3 was monitored simultaneously. Figure 4E shows that increased binding of Hairy2 to

Stat3 reduces the amount of Id3 co-immunoprecipitated with Stat3, indicating that the binding of Hairy2 and Id3 to Stat3 is mutually exclusive. We also tested whether Id3 interacts with FGFRs. We found that Id3 specifically interacts with FGFR4-

IC but not with the other FGFRs-IC (Supplementary Figure S11C). In accordance with this observation, we found that an Id3-GFP fusion protein partially colocalizes at the plasma membrane with mRFP in animal caps (Supplementary Figure S8). We then asked whether Id3 binding to FGFR4 affects Stat3-FGFR4 interaction. Figure 4F shows that the amount of Stat3 associated with FGFR4 was, in contrast to *Hairy2*, decreased by Id3.

To test whether Id3 regulates Stat3 function during NC formation, we first examined the consequence of the coexpression of *Id3-GR* on the function of Stat3-GR. We found that Id3-GR blocks the expansion of *Snail2* and *Sox10* by Stat3-GR as well as its ability to induce one of its direct target, *Delta1* (Yoshimatsu *et al*, 2006), in embryos and in *in vitro* NC induced explants (Figure 4G–L and Supplementary Figure S12A–C). Based on these data, one prediction is that Id3 depletion may increase Stat3 activity. Indeed, injection of Id3 MO with a sub-effective dose of *Stat3-GR* mRNA led to upregulation of *Delta1* and *SoxD* in embryos and in *in vitro* induced NC explants. Injection of this same dose of *Stat3-GR* mRNA alone or together with *Hairy2* MOs did not increase *Delta1* or *SoxD* expression (Figure 4M–P and Supplementary Figure S12D–G). Stat3 was however unable to expand *Snail2* and *Sox10* in Id3-depleted embryos, suggesting that Id3 is absolutely required for NC development downstream of Stat3 (not shown). Together, these results indicate that Id3 negatively regulates Stat3 activation in NC by disrupting Stat3-Hairy2-FGFR4 ternary complex formation.

Stat3 controls *Hairy2* and *Id3* transcription in an opposite and dose-dependent manner

Hairy2 positively regulates Stat3 activity while Id3 negatively regulates it. How is the balance of the activity of these two proteins with antagonistic roles on Stat3 achieved to allow correct NC development? To answer this question, we thus investigated the effect of Stat3 gain and loss of function on *Hairy2* and *Id3* expression. First, we observed that Stat3 depletion reduces *Hairy2* and that, in contrast, it increases *Id3* expression (Figure 5A and B). As *Id3* is a direct target of BMP and Notch signalling (Reynaud-Deonauth *et al*, 2002; Von Bubnoff *et al*, 2005), we also examined *BMP4* and *Delta1* expression in Stat3 MO-injected embryos. Figure 5C and D shows that Stat3 depletion increases *BMP4* and reduces *Delta1*. This phenotype may be a consequence of *Hairy2* inhibition, as similar effects have been observed in *Hairy2* MOs-injected embryos (Nichane *et al*, 2008a,b). Second, we injected increasing amounts of *Stat3-GR* mRNA into embryos and analysed *Hairy2*, *Id3*, *BMP4* and *Delta1* expression. Figure 5E and F shows that a low dose of *Stat3-GR* expands *Hairy2* and reduces *Id3*. At the highest dose used, *Hairy2* activation was not observed anymore and *Id3* was rather increased. Quantification of mRNA levels in *in vitro* induced NC explants confirmed a switch from *Hairy2* to *Id3* activation upon increasing the doses of *Stat3-GR* (Figure 5I and J). *BMP4* was repressed at low doses of *Stat3-GR* in both embryos and in *in vitro* NC induced explants. Such a repression was not observed at higher doses, which may be due to the inability of Stat3 at this dose to increase *Hairy2* (Figure 5G and K). *Delta1* was progressively upregulated in response to increasing doses of *Stat3-GR* (Figure 5H and L). Interestingly, whereas *Id3* upregulation was only observed at the highest dose of *Stat3-GR*, *Delta1* was already efficiently induced at

the intermediate dose, suggesting that *Id3* upregulation might be a consequence of *Delta1* induction. To test this hypothesis, a high dose of *Stat3-GR* mRNA was co-injected together with mRNA encoding a dominant-negative form of Delta1 (*Delta1^{stu}*) or with a Delta1 MO in embryos. Supplementary Figure S13 shows that interfering with Delta1 inhibits Stat3's ability to increase *Id3* transcription. Together, these experiments indicate that Stat3 controls *Hairy2* and *Id3* transcription in an opposite and dose-dependent manner. Low Stat3 activity levels induce *Hairy2*, which results in *BMP4* and *Id3* repression. At higher levels, *Hairy2* is no longer increased and *Delta1* is induced, which in turn upregulates the Notch target *Id3*.

Distinct levels of Stat3 promote the maintenance of NC in an undifferentiated state and their progression through proliferation and differentiation

As the results above suggest that Stat3 activity is finely controlled by *Hairy2* and *Id3*, we hypothesized that its level of activity may be important for correct NC formation. To test this hypothesis, we injected increasing amount of *Stat3-GR* mRNA in embryos and analysed the expression of *Snail2* at neurula stage and *Sox10* at tailbud stage. Both *in vivo* and *in vitro*, a low dose of *Stat3-GR* expanded *Snail2* and *Sox10*. At intermediate dose, *Snail2* was inhibited at neurula stage, while *Sox10* was expanded at tailbud stage, suggesting that the expression of NC markers is only transiently repressed by Stat3 at neurula stage. At a higher dose of *Stat3-GR*, both *Snail2* and *Sox10* were reduced. In contrast, increasing amount of *Stat3-GR* mRNA induced a progressive upregulation of *SoxD* (Figure 6A–F). No change was observed on neural and epidermal markers, suggesting that the effects observed are not due to tissue conversion (Supplementary Figure S14). These results suggest that high Stat3 activity level maintains prospective NC cells in an undifferentiated and non-specified state, and that low Stat3 activity level is required for expression of NC markers.

Increasing evidence suggest that differentiation is tightly coupled and coordinated to cell cycle. We, thus, further investigated the effect of increasing doses of Stat3 on NC by studying its effects on cell cycle. We first analysed cell proliferation in the ectoderm of embryos, using pH3 immunostaining, in response to the same increasing amounts of *Stat3-GR* mRNA used above. At low dose, Stat3-GR efficiently promoted cell proliferation. At higher doses, an only modest increase of the number of cycling cells was observed (Figure 6G). No change in apoptosis was observed at the different *Stat3-GR* doses used (Figure 6H). These data suggest that high levels of Stat3 activity may slow down the cell cycle. To further test this hypothesis, we analysed the effects of increasing amounts of Stat3-GR on the expression of cell-cycle regulators in *in vitro* NC induced explants. Figure 6I shows that *CyclinD1*, a G1-phase regulator, was progressively upregulated in response to increasing amounts of Stat3-GR, which is consistent with previous data showing that *CyclinD1* is a direct target of Stat3 (Leslie *et al*, 2006). Interestingly, the CyclinD1-binding partner *CDK4* (Vernon and Philpott, 2003) was only efficiently upregulated by a low dose of *Stat3-GR* (Figure 6J). Conversely, the cell-cycle inhibitor *p27Xic1* (Su *et al*, 1995) was progressively upregulated in response to increasing doses of *Stat3-GR* (Figure 6K). Thus, high levels of Stat3 slow down the cell cycle, while

Figure 5 Stat3 controls *Hairy2* and *Id3* transcription in an opposite and dose dependent manner. (A–D) Whole-mount *in situ* hybridization of neurula embryos injected with a Stat3 MO (15 ng) and analysed with the indicated markers. Stat3 depletion reduces *Hairy2* (A, 59%, $n = 17$) and *Delta1* (D, 46%, $n = 26$), and increases *Id3* (B, 65%, $n = 20$) and *BMP4* (C, 43%, $n = 28$). Neurula embryos are shown in dorso-anterior views, with the injected side to the right. (E–L) Different doses of Stat3-GR have distinct effects on *Hairy2*, *Id3*, *BMP4* and *Delta1* expression. Embryos were injected with increasing amounts of Stat3-GR mRNA (100, 200 and 500 pg) and analysed at neurula stage by whole-mount *in situ* hybridization with the indicated probes. For each marker, the percentage of embryos with normal, reduced or increased expression at different doses of Stat3 used is shown (E–H). qPCR analysis of *Hairy2*, *Id3*, *BMP4* and *Delta1* expression in *in vitro* NC induced animal caps derived from embryos injected with increasing amount of Stat3-GR (50, 100 and 250 pg per blastomere). A control western blot shows that increasing levels of the Stat3-GR protein have been produced (I–L). In embryos and in *in vitro* NC induced explants, *Hairy2* is induced and *BMP4* is repressed at a low dose of Stat3. In contrast, *Delta1* and *Id3* are upregulated at higher doses.

lower levels increase cell proliferation. Together, these observations suggest that distinct levels of Stat3 promote the maintenance of cells in an undifferentiated state and their progression through proliferation and NC differentiation.

Discussion

Stat3 mediates FGF signalling through FGFR4 in NC

By gain and loss of function in *Xenopus*, we provide the first evidence for a role of Stat3 in NC during vertebrate development. We showed that Stat3 is essential for proliferation and survival of NC progenitors, and that it functions upstream of the neural border specifiers. We found that Stat3 is activated

in early ectodermal cells by FGFs, and that it is essential downstream of FGF signals for NC specification and proliferation. In mouse neural progenitors, Stat3 has been also found to be activated by FGF signals (Yoshimatsu *et al*, 2006). However, the mechanism underlying Stat3 activation in the mouse neocortex has not been investigated. We found that during gastrulation Stat3 activation in the ectoderm occurs through specific binding to the FGFR4 receptor, which induces its phosphorylation at Tyr705 and translocation to the nucleus. Our results thus establish Stat3 as a new intracellular effector of FGF signals in the *Xenopus* embryonic ectoderm, in addition to the well known PI3K/Akt, MAPK and PLC γ pathways (Eswarakumar *et al*, 2005). They also

Figure 6 Stat3 has dose-dependent effects on NC and cell-cycle markers. (A–F) Different doses of Stat3-GR have distinct effects on NC markers. Embryos were injected with increasing amounts of Stat3-GR mRNA (100, 200 and 500 pg) and analysed by whole-mount *in situ* hybridization for *Snail2* and *SoxD* at neurula stage and *Sox10* at tailbud stage. For each marker, the percentage of embryos with normal, reduced or increased expression at different doses of Stat3 used is shown (A–C). qPCR analysis of *Snail2*, *Sox10* and *SoxD* expression in *in vitro* NC induced animal caps derived from embryos injected with increasing amounts of Stat3-GR (50, 100 and 250 pg per blastomere) (D–F). In embryos and in *in vitro* NC induced explants, Stat3-GR increases *Snail2* and *Sox10* at low doses and inhibits their expression at higher doses. *SoxD* is progressively upregulated upon increasing the dose of Stat3-GR. (G–K) Dose-dependent effects of Stat3 on cell cycle. Percentage of embryos injected with increasing amounts of Stat3-GR (100, 200 and 500 pg) showing increased pH3 (G) or TUNEL (H) staining. Stat3-GR efficiently promotes cell proliferation only at low dose. No change in the number of apoptotic cells is observed at the different doses of Stat3-GR tested. qPCR analysis of *CyclinD1*, *CDK4* and *p27XIC1* in *in vitro* NC induced animal caps derived from embryos injected with increasing amounts of Stat3-GR (50, 100 and 250 pg per blastomere) (I–K). *CDK4* is efficiently induced only at a low dose of Stat3. In contrast, *p27XIC1* is induced at a high dose.

provide the first evidence for a direct link between FGF and Stat3 during the earliest steps of NC specification. In agreement with our data, *FGFR4*, among the different FGFRs, is strongly and broadly expressed in the ectoderm during gastrulation and early neurulation at the time of NC specification (Hongo *et al*, 1999; Golub *et al*, 2000; Lea *et al*, 2009, and data not shown) and is required for NC formation. Such a link between Stat3 and a specific receptor also helps to understand how FGFs through various receptors and intracellular effectors mediate their distinct cellular responses. Stat3 is probably, however, not the only mediator of FGFR4 in NC, given that a constitutively active Stat3 could not rescue FGFR4 inhibition (not shown). It is also possible that FGFs may be required upstream but also downstream of Stat3 during NC development. The reiterative use of other signals in NC such as Wnts has been already reported (Sato *et al*, 2005).

Hairy2 and Id3 function as positive and negative feedback regulators of Stat3 activation in NC

Our results have demonstrated an unexpected ‘scaffolding’ role of the bHLH protein Hairy2 in the selective recruitment of a ubiquitous factor (Stat3) to a specific receptor (FGFR4). Through this mechanism, Hairy2 functions as a positive regulator of Stat3 activation in NC. Several experiments support this view: (1) Hairy2 acts downstream of FGF signalling and requires Stat3 for its DNA-binding-independent activities (Nichane *et al*, 2008a,b); (2) Hairy2 increases Stat3 phosphorylation, nuclear accumulation and transcriptional activity; (3) Hairy2 binds through distinct domains to Stat3 and FGFR4 and facilitates Stat3–FGFR4 interaction; (4) Hairy2 and Stat3 colocalize at the plasma membrane with FGFR4 and (5) Hairy2 and Stat3 cooperate *in vivo* during NC formation. In accordance with our results, Hes1, the orthologue of Hairy2, has been also shown to associate with Stat3 in the developing mouse central nervous system, facilitating Jak2 phosphorylation of Stat3 (Kamakura *et al*, 2004).

Id3 is a natural dominant-negative HLH regulator that plays a role in NC proliferation (Kee and Bronner-Fraser, 2005) and interacts with Hairy2 (Nichane *et al*, 2008a). Strikingly, our results indicate that Id3 is a novel negative regulator of Stat3, in addition to the SOCS and PIAS proteins (Levy and Darnell, 2002), and that it attenuates FGF signals in NC: (1) Id3 decreases Stat3 phosphorylation, nuclear accumulation and transcriptional activity; (2) Id3 interacts with Stat3 and competes with Hairy2 for binding to Stat3; (3) Id3 interacts with FGFR4 and decreases the amount of Stat3 associated with FGFR4 and (4) Id3 overexpression and depletion affects Stat3 activities during NC development.

Evidence presented here shows that *Hairy2* and *Id3* transcription is regulated in an opposite manner by distinct levels of Stat3 activity. Low levels of Stat3 activity resulted in *Hairy2* upregulation and *Id3* repression. In contrast, at higher Stat3 levels, *Hairy2* was no longer activated and *Id3* was upregulated through Delta1. Based on our data, we propose a model of regulation of Stat3 activity downstream of FGF/FGFR4 signalling (Figure 7). In our model, Hairy2 and Id3 function as positive and negative regulators of Stat3 activity in NC. By upregulating the transcription of these two regulators, the positive one (Hairy2) at low dose and the negative one (Id3) at high dose, Stat3 indirectly self-regulates its activation in a feedback loop. As an excess of FGFs inhibits NC formation

Figure 7 A model of Stat3 self-regulation in NC. Stat3, downstream of FGF signalling, self-regulates its activity through threshold-dependent activation of a positive (Hairy2) and a negative (Id3) feedback loop. The black arrows indicate transcriptional regulation and blue arrows direct protein-protein interactions.

(Hong and Saint-Jeannet, 2007), this mechanism of self-regulation is thus crucial for correct response of NC to a precise level of FGFs.

Stat3 controls cell-cycle progression and differentiation of NC

Mechanisms coupling cell cycle and cell fate are important for correct development (Cremisi *et al*, 2003; Miller *et al*, 2007). Our data support a role for Stat3 in the coordination of cell-cycle progression and NC differentiation. While high levels of Stat3 activity maintain prospective NC cells in an undifferentiated and non-specified state, low levels of Stat3 activity promote cell division and expression of NC markers. In Supplementary Figure S15 we show that, downstream of FGFs, the sequential regulation of Stat3 activity through positive and negative feedback regulation of Hairy2 and Id3 is important for coordination of cell-cycle progression and NC specification. Our model suggests that during gastrulation, in response to FGF signals, neural border cells are early on maintained in an undifferentiated and unspecified state by high Stat3 and Hairy2 activity. Later, as the level of Stat3 activity progressively increases, Id3 becomes upregulated and downregulates Stat3 and Hairy2 activity, thus allowing cells to proliferate and differentiate. In accordance with this model, *Hairy2* at the neural border is detected before *Id3* and NC-specific markers (Tsuiji *et al*, 2003; Kee and Bronner-Fraser, 2005). Moreover, epistatic experiments suggest that Stat3 is required both in unspecified and differentiated NC. Indeed, a constitutively active Stat3 (Stat3CTC-GR) could not overcome NC inhibition in Hairy2- and Id3-depleted embryos and co-injection of Id3-GR and/or Hairy2-GR could not rescue the Stat3 MO phenotype (not shown). In accordance to our results, the relative strength of Stat92E, the only Stat homologue in *Drosophila*, has recently been shown to regulate self-renewal or differentiation in renal and nephric stem cells (Singh *et al*, 2007). The protooncogene c-Myc has been also shown to control the balance between hematopoietic stem cell (HSC) self-renewal and differentiation. Low levels of c-Myc ensure self-renewal of long-term HSCs, while high c-Myc level induces differentiation towards short-term HSCs. Those cells become transient amplifying cells, which rapidly expand through proliferation while continuing to differentiate

(Wilson *et al*, 2004). Interestingly, the gradient of c-Myc activity during HSC differentiation inversely correlates with the gradient of Stat3 activity that regulates NC specification. As c-Myc is required for NC development (Bellmeyer *et al*, 2003), it will be interesting to determine how it controls, together with Stat3, NC proliferation and differentiation.

Possible role of Stat3 in other ectodermal tissues

FGF growth factors are well-characterized mitogenic signals. They also play many roles in ectodermal cell-fate decisions such as during neural induction (Delaune *et al*, 2005), placode development (Ahrens and Schlosser, 2005) and posteriorization of the CNS (Ribisi *et al*, 2000). However, how ectodermal cells respond to graded FGF signals to coordinate cell-cycle progression and specific differentiation programmes, remains unanswered. As the active form of Stat3 is broadly detected in the entire ectoderm of early embryos, Stat3 could have a more general role as a mediator of FGF signals in the coordination of ectodermal AP and DV patterning with cell-cycle progression. Indeed, our preliminary results indicate that Stat3 depletion reduces in embryo placodal (*Six1*, *Pax8*), pan-neural (*Sox2*) and posterior neural (*HoxB9*) markers (Supplementary Figure S16A-D). These results also support our view that the Stat3 MO phenotype in NC is not due to tissue conversion, but rather due to modulation of cell proliferation and survival. Conversely, overexpression of Stat3-GR induces those markers in embryos and in animal caps (Supplementary Figure S16E-L). Identification of other tissue-restricted, Stat3-interacting factors that regulate its phosphorylation or its binding to specific target genes will be required to better understand how Stat3 functions in the ectoderm during vertebrate development.

Materials and methods

MOs and embryo manipulations

The following antisense MOs (Genetools) were used:

FGFR1a MO (5'-AGGGACCTTCCGGAGAACATCCCAA-3'),
FGFR1b MO (5'-AGGGACATTCGGAGAACATCCCAA-3'),
FGFR4a MO (5'-GATCCAGACATGACTGGTGCCTATG-3') and
FGFR4b MO (5'-GATCCAGACATGGCTGGCGCGTATG-3').

Hairy2a MO, Hairy2b MO, Delta1 MO (Nichane *et al*, 2008a), Id3 MO (Kee and Bronner-Fraser, 2005), Stat3 MO and Stat3 MO7mis (Ohkawara *et al*, 2004; Nichane *et al*, 2008b) were previously described. *Xenopus* embryo injections, animal cap explant dissections, vibratome section and *in situ* hybridization all followed standard procedures (Sive *et al*, 2000). Embryos were injected at the eight-cell stage in one animal blastomere. Nuclear *lacZ* mRNA (100–250 pg per blastomere) was used as lineage tracer. GR constructs were induced by addition of Dex around stage 11.5–12 (10 μ M) (Sigma). All injections were performed at least three times and the indicated effects correspond to one representative experiment.

Whole-mount immunostaining, proliferation and TUNEL assay

Whole-mount immunostaining was performed with bisected embryos to allow optimal antibody penetration. Briefly, embryos were rehydrated, washed in TBST, blocked over 2 h in TBST/5% BSA and incubated with a pTyr705-Stat3 primary antibody (1/300) overnight (O/N) at 4°C. Embryos were then washed 4 $\times$ over 1 h in TBST/0.2% BSA, blocked over 1 h in TBST/5% BSA and incubated with an anti-rabbit IgG secondary antibody coupled with AP (1/500) O/N at 4°C. After extensive washes (4 $\times$ over 1 h and O/N at 4°C), embryos were stained with NBT/BCIP in AP buffer. TUNEL assays and phospho-histone H3 immunostainings were performed as previously described (Kee and Bronner-Fraser, 2005) using a secondary goat anti-rabbit IgG antibody coupled to AP (Chemicon, 1/500). Rhodamine dextran (RLDx) (Molecular Probes)

was used as lineage tracer and embryos were sorted left-right injected side before pH3 immunostaining or TUNEL assay.

Western blot, co-IP and recombinant protein

Western blot analysis and co-IP using embryos (50/sample) or animal caps (20/sample) were performed as described (Nichane *et al*, 2008a). Anti-Flag (M2; Sigma), anti-Myc (clone 9E10; Sigma) and anti-HA (clone HA-7; Sigma) primary antibodies were revealed with a goat anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (Jackson Immunosource, at 1/30 000). Stat3 (C-20, at 1/1000; Santa Cruz) and pTyr705-Stat3 (Cell Signaling, at 1/1000) primary antibodies were detected with protein A-peroxidase (Sigma, at 1/10 000). GAPDH was used as loading control (Abcam, at 1/5000). For detection of pTyr705-Stat3, animal caps dissected at stage 9 and treated directly with Dex were cultured until stage 11.5–12. Samples were lysed in RIPA buffer supplemented with Phospho Stay Solution (Novagen), PMSF (Bio-Rad) and protease inhibitor cocktail (Roche) as previously described (Fuentesalba *et al*, 2007). Purified recombinant mouse bFGF (FGF2) and human eFGF (FGF4) proteins (R&D Systems) were added to the animal caps directly after dissection and used at 20- to 50-ng/ml range.

qPCR analysis

NC induced animal cap explants derived from embryos radially injected at the animal pole with *noggin* and *Wnt8* mRNA (50 pg each per blastomere), with or without experimental mRNAs, were dissected at stage 9. Dex was added at stage 11.5–12 and animal caps (20 per sample) were cultured until the indicated stage (16 or 28). Total RNA was extracted using the RNeasy mini kit (Qiagen). cDNA was synthesized using the iScript cDNA synthesis kit

(Bio-Rad). qPCR was performed using a Gene Amp TM PCR system 9600 (Perkin Elmer Biosystems) and a qPCR core kit for SYBR Green I (Eurogentec). Samples were normalized to GAPDH. All qPCR experiments were performed in duplicate and repeated at least three times. Error bars represent s.d.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author Contributions: MN conceived and designed the experiments. MN and XR performed the experiments. MN and EB analysed the data. MN and EB wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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