Xenopus Id3 is required downstream of Myc for the formation of multipotent neural crest progenitor cells

William Light[1,*], Ann E. Vernon[1,*], Anna Lasorella[2], Antonio Iavarone[2] and Carole LaBonne[1,†]

[1]Department of Biochemistry, Molecular Biology and Cell Biology; and Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Evanston, IL 60208, USA
[2]Department of Neurology and Institute for Cancer Genetics, Columbia University, New York, NY 10032, USA
*These authors contributed equally to this work
†Author for correspondence (e-mail: clabonne@northwestern.edu)

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Summary

Neural crest cells, a population of proliferative, migratory, tissue-invasive stem cells, are a defining feature of vertebrate embryos. These cells arise at the neural plate border during a time in development when precursors of the central nervous system and the epidermis are responding to the extracellular signals that will ultimately dictate their fates. Neural crest progenitors, by contrast, must be maintained in a multipotent state until after neural tube closure. Although the molecular mechanisms governing this process have yet to be fully elucidated, recent work has suggested that Myc functions to prevent premature cell fate decisions in neural crest forming regions of the early ectoderm. Here, we show that the small HLH protein Id3 is a Myc target that plays an essential role in the formation and maintenance of neural crest stem cells. A morpholino-mediated 'knockdown' of Id3 protein results in embryos that lack neural crest. Moreover, forced expression of Id3 maintains the expression of markers of the neural crest progenitor state beyond the time when they would normally be downregulated and blocks the differentiation of neural crest derivatives. These results shed new light on the mechanisms governing the formation and maintenance of a developmentally and clinically important cell population.

Key words: *Xenopus,* Neural crest, Id3, Myc, Wnt, Slug, Stem cell

Introduction

Neural crest precursor cells arise at the lateral edges of the neural plate as early as mid-gastrulation (Knecht and Bronner-Fraser, 2001). Around the time of neural tube closure, these cells undergo an epithelial-mesenchymal transition (EMT) and migrate extensively throughout the embryo. Although migratory neural crest cells initially retain their stem cell-like characteristics, these cells will ultimately give rise to a large and diverse set of derivatives that includes most of the neurons and glia of the peripheral nervous system, melanocytes and the elements of the craniofacial skeleton – cell types that are fundamental to the establishment of the vertebrate body plan (Le Douarin and Kalcheim, 1999; Hall and Hörstadius, 1988).

Understanding how neural crest precursor cells are initially set aside in the early ectoderm is a matter of profound importance, both because the mechanisms involved may prove relevant to the development and maintenance of other stem cell populations, and because the formation of neural crest cells represents such a fundamental milestone in vertebrate evolution. During the past few years, significant progress has been made towards understanding this process. At the tissue level, induction of neural crest precursors at the lateral edges of the neural plate is thought to be mediated by signals emanating from the non-neural ectoderm, the non-axial mesoderm or both (reviewed by LaBonne and Bronner-Fraser, 1999). Although the precise molecular identities of these signals have yet to be fully elucidated, evidence from a number of model organisms suggests that members of the BMP, Wnt, FGF and Notch families all play essential roles in this process (reviewed by Heeg-Truesdell and LaBonne, 2004; Huang and Saint-Jeannet, 2004).

In particular, there is strong evidence that Wnt-family growth factors play a direct role in the induction of neural crest progenitor cells. In *Xenopus,* inhibition of Wnt signaling by a variety of means, including overexpression of a dominant-negative Wnt ligand, overexpression of the Wnt antagonist GSK3 or morpholino depletion of the Wnt receptor Frizzled7, blocks the earliest steps in the formation of this stem cell population (Saint-Jennet et al., 1997; LaBonne and Bronner-Fraser, 1998; Deardorff et al., 2001). Moreover, nuclear mediators of the Wnt signaling pathway, Lef/Tcf-family transcription factors, appear to bind to and directly regulate the promoter of *Slug*, an early marker of neural crest progenitors (Vallin et al., 2001). Importantly, although the requirement for Wnt signals during neural crest induction was first demonstrated in *Xenopus*, recent reports have provided strong evidence that Wnt proteins play an analogous role in both avian and zebrafish embryos (Garcia-Castro et al., 2002; Lewis et al., 2004).

Expression of the proto-oncogene *Myc* is one of the earliest responses to Wnt signaling at the neural plate border, and a recent report demonstrated that Myc function is required downstream of Wnt signals for the formation of neural crest precursor cells (Bellmeyer et al., 2003). Interestingly, Myc is

also a downstream target of Wnts in colorectal tumors and embryonic carcinoma cells (He et al., 1998; Willert et al., 2002), suggesting that the relationship between Wnt and Myc may be well conserved. Myc and the closely related proteins N-myc (Nmyc1) and L-myc are basic helix-loop-helix-zipper (bHLHZ) transcription factors. Together, Myc family proteins have been extensively studied for almost 25 years, and have been implicated in a plethora of essential cellular processes, including cell growth, cell proliferation, apoptosis and cellular differentiation (Grandori et al., 2000; Eisenman, 2001). The bHLHZ domain possessed by Myc family proteins mediates both their dimerization and DNA-binding activities, and Myc can bind to E box DNA sequences as a heterodimer with the small bHLHZ protein Max in order to regulate transcription. Because Myc misregulation is found in a high proportion of epithelial cancers and is often associated with aggressive and poorly differentiated tumors (Nesbit et al., 1999), a central focus of the Myc field of research is to understand the key transcriptional targets that mediate the downstream effects of this important regulatory protein.

One group of proteins that has been implicated as effectors of Myc-family proteins in a variety of cell types and cancers are the Ids (inhibitor of DNA binding). This family of small helix-loop-helix proteins comprises four related factors (Id1, Id2, Id3 and Id4) that function primarily by negatively regulating the DNA-binding ability of bHLH transcription factors (Ruzinova and Benezra, 2003). In addition, a subset of Id proteins (Id2 and possibly Id4) can also bind to Rb and the related pocket proteins p107 and p130 in a cell cycle-regulated manner, inhibiting the growth suppressive activities of these factors, and thus promoting entry into S phase (Lasorella et al., 2000). Recently, Id2 has been shown to be an important N-myc effector in neuroblastoma, a tumor that is derived from the neural crest stem cell population (Lasorella et al., 2002). This important finding suggested that Id proteins might also be key Myc targets during normal neural crest development.

In *Xenopus*, at least one Id family member, Id3, is expressed in neural crest precursors at a time consistent with a role as a Myc effector (Zhang et al., 1995; Liu and Harland, 2003). Using morpholino oligo-mediated depletion, we show here that Id3 is required downstream of Myc for the formation of neural crest stem cells at the neural plate border. In the absence of Id3, an excess of CNS progenitors forms in place of neural crest cells. Moreover, we show that the continued expression of Id proteins in the neural crest leads to the persistent expression of markers characteristic of multipotent neural crest progenitors, and blocks the differentiation of neural crest derivatives. These findings suggest that Id3 may play an important role in maintaining the neural crest stem cell population until the appropriate time for these cells to respond to signals directing their differentiation.

Materials and methods

DNA constructs and embryological methods

XId3 cDNA was provided by G. Spore, XId2 cDNA was provided by T. Mohun, and human Id2 and Id3 cDNAs have been described (Lasorella et al., 2000). N- and C-terminally epitope tagged versions of these cDNA were generated by amplifying the coding regions of these proteins using low cycle-number PCR and a high fidelity polymerase (Tgo, Roche) and inserting them into the vectors pCS2-MycN and pCS2-MycC (provided by Robert Davis). All clones were confirmed by sequencing. All results shown are representative of at least two independent experiments. RNA for injection was produced in vitro from linearized plasmid templates using the Message Machine kit (Ambion). Collection, injection and in situ hybridization of *Xenopus* embryos was as described (LaBonne and Bronner-Fraser, 1998; Bellmeyer et al., 2003).

Morpholino assays

Morpholino oligos designed to target the translation-initiation site of Id3 (5′-ACCGCACTGGGCTGATGGCTTTCAT) were obtained from Gene Tools. To evaluate the effectiveness and specificity of these oligos in western blot experiments, embryos were injected with mRNA encoding either XId3, XId2 or H-Id3 at the two-cell stage and then subsequently re-injected with either Id3 MOs or with control MOs (5′-CCTCTTACCTCAGTTACAATTTATA) at dose of 5 ng/embryo. Embryos were collected at blastula or neurula stages, lysed in PBS/1%NP40 and extracts resolved by SDS PAGE. Following immunoblotting using α-myc (9E10) antibodies, labeled proteins were detected using HRP-conjugated secondary antibodies and enhanced chemiluminescence (Amersham). For embryo experiments, 5 ng of Id3 MO or 5 ng of control MO was injected into one animal blastomere at the eight-cell stage unless otherwise stated in the text. No interference with neural crest formation was ever observed in control MO-injected embryos, even at doses of up to 20 ng/embryo. MOs used to deplete Myc protein were as previously described (Bellmeyer et al., 2003).

Proliferation and TUNEL assays

For phosphohistone H3 detection, Id3 MO-injected embryos were fixed in formaldehyde at stage 13/14 and processed for β-gal activity. α-Phosphohistone H3 antibody (Upstate Biotechnology) was used at a concentration of 5 μg/ml; α-rabbit IgG-conjugated with alkaline phosphatase (Boehringer Mannheim) was used at 1:1000 and detected with BM Purple. For TUNEL assays, embryos injected with Id3 MOs or mRNA encoding Id3 were allowed to develop until stage 13/14. TUNEL staining was carried out using a protocol adapted from Hensey and Gautier (Hensey and Gautier, 1998) as described previously (Bellmeyer et al., 2003). Briefly, fixed embryos were rehydrated in PBT and washed in TdT buffer (Gibco) for 30 minutes. End-labeling was carried out at room temperature overnight in TdT buffer containing 0.5 μM digoxigenin-dUTP (Boehringer Mannheim) and 150 U/ml TdT (Gibco). Embryos were washed at 65°C in PBS/1 mM EDTA and detection of the digoxigenin epitope was carried out as for in situ hybridization. For Id3 depletion experiments, we calculated the percent total TUNEL-positive nuclei on the injected and control side of 115 embryos from four independent experiments. A nonparametric sign test was used to reject the null hypothesis that those values differed by more than 5% (P=0.001).

Western blot and chromatin immunoprecipitation (ChIP) assays

Western blots of 100 μg of whole cell lysates prepared from either adult human brain or the human glioma cell line T98G were developed using antibodies against Myc (9E10, Santa Cruz Biotechnology), Id3 (C-20, Santa Cruz Biotechnology) and α-tubulin (DM1A, Sigma-Aldrich). ChIP analysis was performed on logarithmically growing T98G cells. Cells were crosslinked with formaldehyde, chromatin was fragmented by sonication, and protein-DNA complexes were immunoprecipitated with either 4 μg of polyclonal anti-Myc antibody (N262, Santa Cruz Biotechnology) or 4 μg of normal rabbit immunoglobulins. Protein extracts (4 mg), diluted in 600 μl of RIPA buffer, were used for each immunoprecipitation. Precipitated DNA was analyzed by PCR using primers flanking the E-box located at position –2895 in the human *Id3* promoter. As a control for the Myc ChIP, we also analyzed the promoter of the LDL receptor gene (OLR1) that does not contain E-boxes and does not bind Myc

(Fernandez et al., 2003). The sequence of the primers used for PCR are: *Id3* promoter up, AGAGCGGAGCCAGAGCTCAGACATC; *Id3* promoter down, TGCTTCCAAGGGCTCCACTCTG; LDL Receptor (OLR1) up, ACTGCACCTGGCCAACTTTT; LDL Receptor (OLR1) down, TGCAAAGAAAAGAATACACAAAGGA.

Results

Expression of Id3 at the neural plate border is regulated by Myc

Based upon our published findings (Bellmeyer et al., 2003), we have speculated that Myc may be employed in the early embryonic ectoderm as a means of preventing premature cell fate decisions. Given that neural crest precursors and derivatives fail to form in Myc-depleted embryos (Bellmeyer et al., 2003), we have sought to identify the downstream targets that mediate Myc function in this important cell type. Interestingly, Id family proteins, which can function as negative regulators of cell differentiation, have recently been shown to be Myc effectors in neuroblastoma, a neural crest-derived pediatric tumor (Lasorella et al., 2002; Ruzinova and Benezra, 2003). In *Xenopus*, one Id family protein, *Id3*, is expressed in the early ectoderm in a pattern roughly reminiscent of the expression of *Myc* (Wilson and Mohun, 1995; Liu and Harland, 2003) (Fig. 1A). As a first step towards ascertaining whether Id3 plays an important role in neural crest formation, we sought to determine if the expression of this factor at the neural plate border is dependant upon Myc.

We previously described morpholinos directed at Myc transcripts that potently block translation of Myc protein in early *Xenopus* embryos (Bellmeyer et al., 2003). We used these morpholinos to deplete Myc from the early ectoderm, and then asked if Id3 expression at the neural plate border was altered as a consequence. Myc MOs were injected, together with β-galactosidase mRNA as a lineage tracer, into single cells of eight-cell stage embryos. This strategy allowed for the depletion of Myc protein in neural crest-forming regions of the ectoderm on one side of the embryo, with the other side of the embryo serving as an internal control. When injected embryos were examined by in situ hybridization at neurula stages, we found that Id3 expression was consistently diminished on the Myc-depleted side of the embryo, confirming that expression of *Id3* is at least partially dependent upon Myc function. However, given that *Id3* is initially expressed at low levels throughout the non-neural ectoderm prior to the onset of high expression at the neural plate border (Liu and Harland, 2003) (and not shown), we can not distinguish from these experiments whether Myc is required specifically for induction of high levels of *Id3* expression at the neural plate border, or alternatively for the maintenance and/or amplification of the earlier low level *Id3* expression in this region. Moreover, as *Id3* expression was never completely abolished in these experiments, (Fig. 1B), it appears likely that other upstream signals also play a role in the regulation of *Id3* expression at the neural plate border.

Id3 expression partially rescues neural crest formation in Myc-depleted embryos

If Id3 functions, at least in part, downstream of Myc, then it is important to determine whether Id3 function can compensate for the loss of Myc. We have previously demonstrated that Myc-depleted embryos can be rescued by injection of Myc transcripts that cannot be targeted by the morpholino; however, no rescue of neural crest formation can be achieved by Slug in this type of experiment (Bellmeyer et al., 2003). We therefore asked if expression of Id3 could rescue neural crest formation in Myc-depleted embryos. As previously reported, embryos injected with Myc MOs display greatly diminished or absent expression of the neural crest marker *Slug* at neural plate stages (Fig. 1C). By contrast, when such embryos were also injected with mRNA encoding Id3, a significant rescue of *Slug* expression was noted (Fig. 1D). This rescue was not complete, however, as *Slug* expression on the injected side was always

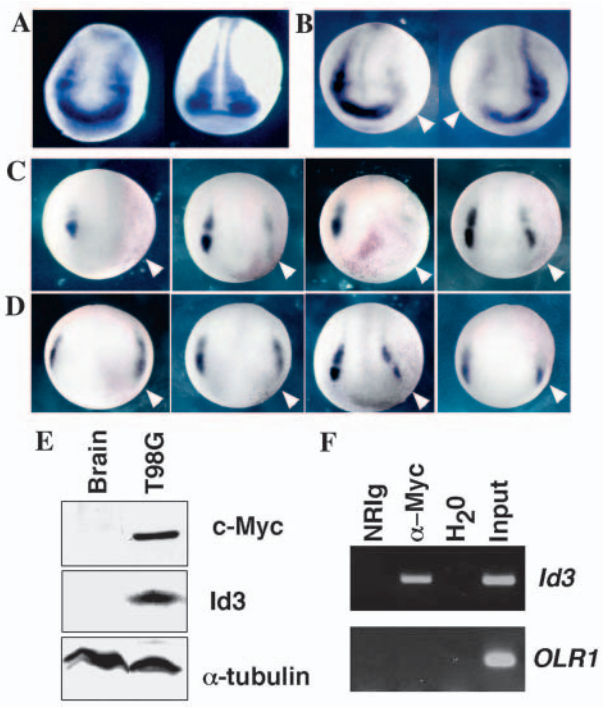

Fig. 1. Id3 is a Myc target in the neural crest. (A) In situ hybridization showing the expression of *Xenopus Id3* at stage 14 (left) and stage 19 (right). Strong expression is seen in both the neural crest-forming region of the neural plate border, and in the transverse neural fold, at open neural plate stages. (B) Following MO-mediated depletion of Myc protein, expression of *Id3* is greatly diminished in neural crest-forming regions on the injected side of the embryo (arrowheads). (C,D) Id3 rescue of Myc-depleted embryos. (C) In situ hybridization showing loss of *Slug* expression on Myc-depleted side of the embryo (arrowhead). (D) Expression of *Slug* is substantially rescued when mRNA encoding Id3 is subsequently injected into the Myc depleted side of the embryo. Red staining denotes expression of the lineage tracer β-galactosidase. Arrowheads indicate the injected side of the embryo. (E) Western blot analysis demonstrating that Myc and Id3 are co-expressed in the human glioma cell line T98G. Neither protein is expressed in adult human brain at detectable levels. (F) ChIP assay demonstrating a direct interaction between Myc and the Id3 promoter. Chromatin was extracted from logarithmically growing T98G cells and protein-DNA complexes were immunoprecipitated with a polyclonal antibody against Myc (*α-Myc*) or normal rabbit immunoglobulins (*NRIg*). Precipitated DNA was analyzed by PCR with primers from the *Id3* and, as negative control, the *OLR1* promoters. PCR was also performed in the absence of DNA (H_2O) and from 0.01% of the total protein-DNA complexes used in each immunoprecipitation (input).

weaker and more limited in expanse than on the control side of the embryo. Consistent with this observation, the later morphological development of Id3 rescued embryos was considerably less perturbed than that of embryos injected with only Myc MOs, but was never fully normal (not shown). These findings suggest that although Id3 can partially compensate for the loss of Myc, there are likely to be other essential downstream targets.

Id3 is a direct target of Myc

The rescue of Myc depletion by Id3 expression is a significant finding that indicates that Id3 plays an important role in neural crest formation downstream of Myc. Nevertheless, these findings do not conclusively demonstrate that Id3 is a direct Myc target gene. We therefore wished to use chromatin immunoprecipitation (ChIP) assays in order to determine if Myc interacts directly with the Id3 promoter in a relevant cell line.

In mammals, the expression of both Myc and Id3 is high in embryonic cells, typically extinguished in normal adult human tissues and frequently deregulated in a variety of human neoplasms. With respect to human neurectodermal tumors, malignant gliomas are among those most frequently associated with elevated Myc levels (Orian et al., 1992; Hirvonen et al., 1994). We therefore asked if expression of Myc in the human glioma cell line T98G might sustain elevated Id3 expression in these cells. Western blot analysis demonstrates that both Myc and Id3 are expressed at detectable levels in T98G cells, in contrast to adult human brain tissue (Fig. 1E), confirming that this is an appropriate cell line for ChIP analysis. Chromatin was extracted from logarithmically growing T98G cells, and protein-DNA complexes were immunoprecipitated using either a polyclonal antibody against Myc or Normal Rabbit Serum. Precipitated DNA was analyzed by PCR using primers derived from the *Id3* promoter, or from a promoter that lacks E box sequences (OLR1) as a negative control. Id3 promoter sequences were specifically amplified from the samples immunoprecipitated with antibodies to Myc (Fig. 1F), demonstrating that Myc does directly bind the *Id3* promoter in these cells.

Id3 is required for neural crest precursor formation

As ChIP analysis indicated that Id3 is a direct Myc target, we wished to determine if Id3 function is required for proper neural crest development. To this end, we designed morpholino oligos (MOs) to specifically deplete Id3 protein and demonstrated that these MOs effectively block translation of Id3 from exogenous mRNAs injected into early *Xenopus* embryos (Fig. 2A,B). By contrast, control MOs had no effect on Id3 translation (Fig. 2A). Importantly, although Id3 MOs were found to be strong inhibitors of *Xenopus* Id3 translation, they did not block the translation of human Id3 mRNAs, which lack the MO target sequence (Fig. 2B).

Once we determined that our Id3 MOs were potent tools for inhibiting Id3 function in early embryos, these MOs were injected, together with β-galactosidase mRNA, into single cells of eight-cell stage embryos. This allowed us to deplete Id3 protein in neural crest-forming regions of the ectoderm on one side of the embryo, with the other side serving as an internal control. When injected embryos were examined by in situ hybridization at neurula stages, expression of markers of neural

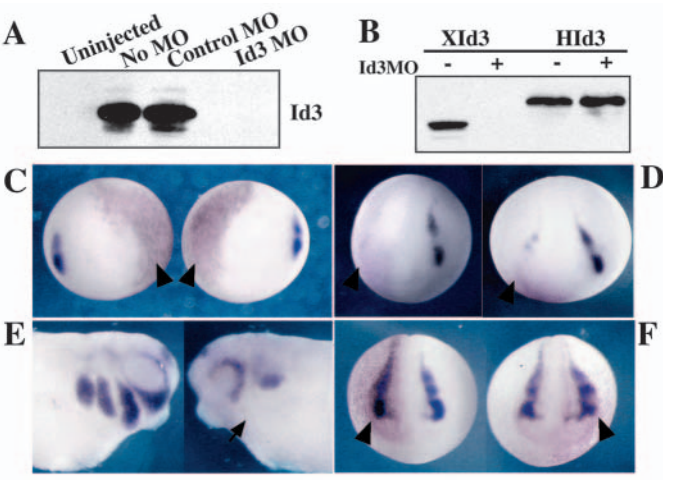

Fig. 2. Id3 is required for neural crest precursor formation. (A) Western blot of lysates prepared from control embryos or embryos injected with an epitope tagged Id3 and then further injected with control or Id3 MOs demonstrates successful Id3 depletion. (B) Western blot of lysates from injected embryos demonstrating that although Id3 MOs efficiently deplete *Xenopus* Id3 protein, they do not deplete the human Id3 used for rescue experiments. The size difference between *Xenopus* and human Id3 is primarily due to the presence of five rather than six Myc epitopes, respectively. (C,D) In situ hybridization of embryos injected with Id3 MOs shows loss of *Slug* (C) and *Sox10* (D) expression on the injected side of the embryo (arrowheads). (E) Id3-depleted embryos show reduced or absent migratory neural crest cells on the injected side (arrow), as visualized by *Twist* expression. (F) Loss of *Slug* expression in Id3-depleted embryos can be rescued by subsequent injection of human Id3, translation of which is not blocked by the MOs. Arrowhead indicates the injected side, red staining is from lineage tracer β-gal.

crest precursor cells such as *Slug* (Fig. 2C) and *Sox10* (Fig. 2D) was significantly inhibited or abolished on the injected side. The expression of these markers was inhibited from the earliest stages they can be detected by in situ hybridization, indicating that Id3 depletion interferes with the induction rather than the maintenance of their expression. Moreover, all early neural crest markers examined, including *Slug*, *Sox10*, *Twist* and *Foxd3*, could be fully blocked in Id3-depleted embryos, indicating that no neural crest precursors form in the absence of Id3. Significantly, the effects of Id3 depletion phenocopy the effects of Myc depletion (Bellmeyer et al., 2003), and are specific as they can be rescued by injection of mRNA encoding human Id3, which cannot be targeted by the MOs (Fig. 2F).

Our previous findings have demonstrated that in the absence of Myc function, embryos form an excess of central nervous system (CNS) precursors at the expense of neural crest cells. Because Id3 appeared to be functioning downstream of Myc in neural crest formation, we next examined the expression of neural plate markers in Id3-depleted embryos. We found that the expression of both *Sox3* and *Opl* (*zic1*) was greatly expanded on the injected side of these embryos (Fig. 3A,B). The expanded expression of *Opl* is noteworthy, as this factor is often described as a neural crest marker. In reality, however, *Opl* expression marks a region of the early ectoderm that includes both neural crest and dorsal CNS precursors, and in our hands *Opl* expression is consistently expanded under

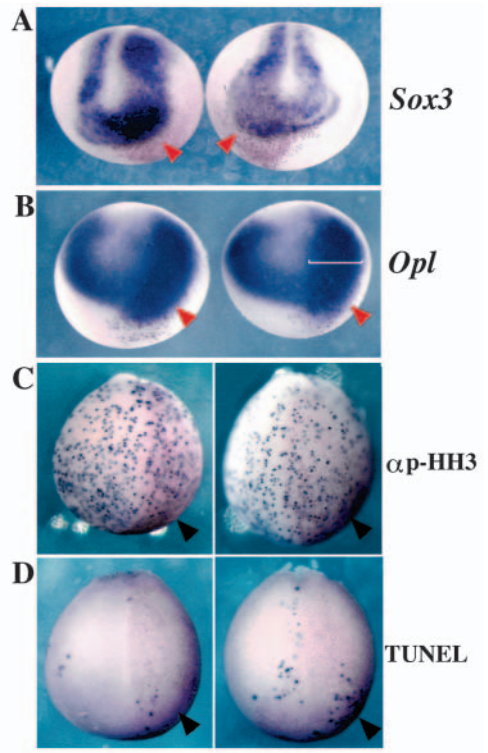

Fig. 3. Id3-depleted embryos form an excess of CNS progenitors. (A,B) In situ hybridization of Id3-depleted embryos. In the absence of Id3, the injected side of the embryo shows greatly expanded expression of *Sox3* (A, arrowhead), a marker of the proliferating multipotent CNS progenitor pool. Similarly, expression of *Opl*, a marker of early CNS and border cell fates is also greatly expanded (B). (C) Phosphohistone H3 immunocytochemistry of Id3-depleted embryos. No difference is seen in the number of actively cycling cells on the control versus Id3-depleted (arrowhead) side of the embryo. (D) Whole-mount TUNEL staining of Id3-depleted embryos. No change in the numbers of apoptotic cells is noted on the Id3-depleted side of the embryo (arrowhead) as compared with the control side. Red staining is lineage tracer β-gal.

conditions where neural crest precursors are lost. The expression of *Sox3*, by contrast, marks all proliferating multipotent CNS progenitors. The expansion of *Sox3* expression into neural crest-forming regions in Id3-depleted embryos indicates that, during normal development, Id3 plays a central role in the determination of neural plate versus neural plate border cell fates in the early embryonic ectoderm (Fig. 3A).

As some Id family members, and in particular Id2, have been implicated in the control of cell cycle progression in other systems (Hara et al., 1997; Lasorella et al., 2000; Lasorella et al., 2001), we next asked if the loss of neural crest precursors in Id3-depleted embryos could be explained by decreased cell proliferation. Such a mechanism seemed unlikely, however, given that the formation of neural crest precursors at the lateral edges of the neural plate has little dependence on cell proliferation, but instead results from a recruitment of surrounding ectoderm (Bellmeyer et al., 2003). Not surprisingly, therefore, when we examined the spatial distribution of cells immunoreactive to the mitosis marker phospho-histone H3 in the neural folds of Id3-depleted embryos, no apparent difference was noted in the numbers of actively cycling cells in regions of the neural folds that did or did not receive the MOs (Fig. 3C). We also examined whether the loss of neural crest precursors could be due to apoptosis, although the continued presence of large numbers of cells expressing β-gal throughout the Id3-depleted side of the embryo suggested that this mechanism was also unlikely. Id3-depleted embryos were reared to early neural plate stages when neural crest precursors should be forming, and apoptosis was assessed by whole-mount TUNEL staining. No significant difference in the numbers of TUNEL-positive nuclei was observed on the Id3-depleted side of the embryo when compared with the control side of the same embryo (Fig. 3D). Together, these data support a model in which Id3 directly influences cell fate decisions in the early embryonic ectoderm through its ability to modulate the activity of one or more transcription factors that control these decisions.

Id3 injected embryos show increased *Slug* expression

Given the clear importance of Id3 for neural crest precursor formation, we next wished to examine the consequences for neural crest development following upregulation of Id3. Although previous reports had suggested that Id3-, as well as Id2- or Id4-, injected embryos develop normally with no aberrant phenotype (Wilson and Mohun, 1995; Liu and Harland, 2003), the expression of neural crest markers was not specifically examined in those studies. Moreover, a recent report has suggested that Id family proteins are targets for N-terminal ubiquitination, and are rapidly turned over by the proteasome (Fajerman et al., 2004). This same study found that fusion of an epitope tag to the N terminus of Id2 was sufficient to stabilize that protein. Indeed, stabilization by N-terminal epitope tags is common for targets of N-end rule ubiquitination (Varshavsky et al., 2000). Based upon this information, we asked if an N-terminally epitope-tagged form of Id3 would be stably expressed in *Xenopus* embryos. We found that with multiple Myc tags fused to its N terminus, Id3 protein was indeed stably expressed, and could be detected by western blots through at least tailbud stages (Fig. 4A). Furthermore, this degree of stability was in marked contrast to that of an Id3 protein with multiple Myc tags fused to its C terminus (Fig. 4A).

Using this stabilized form of Id3, we investigated the consequences of Id3 misexpression on neural crest development. mRNA encoding the N-terminally tagged Id3 was injected into one cell of two-cell stage *Xenopus* embryos, and the injected embryos were allowed to develop to stages at which the effects on neural crest markers could be assayed. In these experiments, we found that the Id3 protein translated from the injected mRNAs could be detected by western blots through at least tailbud stages (not shown). Forced expression of Id3 led to a modest expansion of neural crest precursor formation at neural plate stages, similar to what is observed following Myc overexpression (Fig. 4B,D; not shown). Somewhat surprisingly, a much more dramatic effect on *Slug* expression was observed when Id3-injected embryos were allowed to develop to migratory neural crest stages, a time during development when *Slug* expression normally becomes sharply downregulated.

Because the amount of excess *Slug* expression on the Id3-

injected side of the embryo was so much greater at stage 23 than at stage 13, it seemed highly unlikely that this change in phenotype could be explained by alterations in the rate of cell proliferation or survival. Nevertheless, to formally exclude these possibilities, we compared the numbers of mitotic and apoptotic cells on the control versus experimental side of embryos injected with mRNA encoding Id3. Injected embryos were reared to either stage 13 or stage 23 and then processed for whole-mount TUNEL labeling or anti-phospho-histone H3 immunocytochemistry. No significant differences in staining were observed at either of the stages examined (Fig. 4D,E; not shown). Taken together, these data led us to hypothesize that the excess *Slug* expression noted in Id3-injected embryos at stage 23 most probably reflected a failure to downregulate the expression of this gene after the onset of neural crest migration.

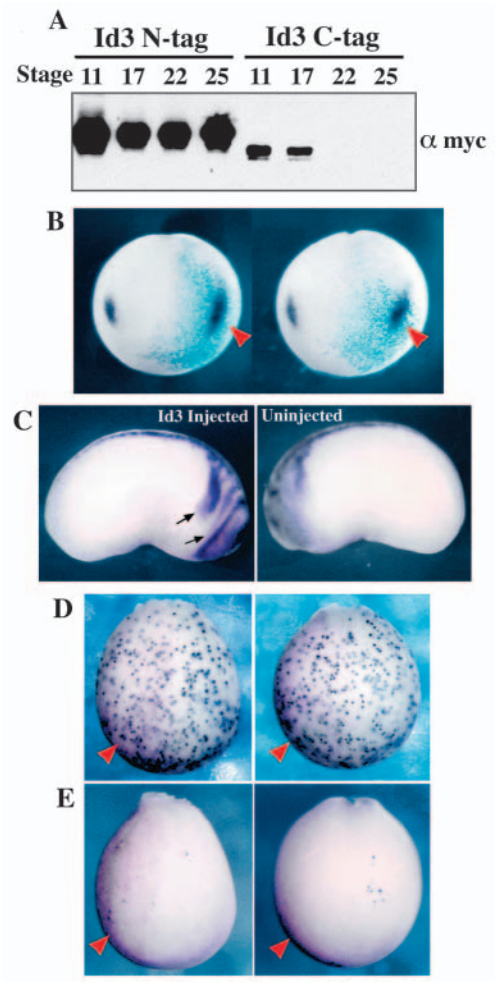

Fig. 4. (A) Western blot of whole embryo lysates prepared from progressively older embryos that were injected at the two-cell stage with mRNA encoding Id3 isoforms with epitope tags appended to either their N or C terminus. N-terminally tagged Id3 protein persists for a substantially longer period of development. (B) In situ hybridization of embryos injected with mRNA encoding the stabilized N-terminally tagged Id3 protein. Forced Id3 expression of Id3 leads to a modest increase in the expression of *Slug* in at the neural plate border on the injected side of the embryo (arrowhead). Cyan staining is lineage tracer β-gal. (C) Siblings of the embryos shown in B show substantially increased expression of Slug at migratory neural crest stages on the injected side of the embryo (left panel, arrows). At these stages expression of Slug has normally been downregulated in migrating neural crest cells, as seen on the control side of the embryo (right panel). (D) Phosphohistone H3 immunocytochemistry of Id3-injected embryos. No difference is seen in the number of actively cycling cells on the control versus Id3 injected (arrowhead) side of the embryo, indicating that the increase in Slug expression is unlikely to be secondary to an increase in cell proliferation. (E) Whole-mount TUNEL staining of Id3-injected embryos. No change in the numbers of apoptotic cells is noted on the Id3-depleted side of the embryo (arrowhead). Red staining is lineage tracer β-gal.

Id3 maintains expression of markers characteristic of multipotent neural crest progenitors

Slug is a zinc-finger-containing transcriptional repressor that plays a required role in both the formation of neural crest precursor cells and in the epithelial-mesenchymal transition that precedes the onset of neural crest migration (reviewed by Heeg-Truesdell and LaBonne, 2004). It is unclear why *Slug* expression is downregulated in neural crest cells soon after they become migratory or if this downregulation is in fact required for normal neural crest development. Indeed, a closely related and at least partially redundant factor, *Snail*, continues to be expressed by neural crest precursors throughout their migration.

In order to further explore the significance of the observed Id3-mediated failure to downregulate *Slug*, we examined the effects of Id3 activity on the expression of *Sox10*, a factor implicated in the regulation neural crest stem cell maintenance (Paratore et al., 2002; Kim et al., 2003). This HMG-family transcription factor is initially expressed in all multipotent neural crest stem cells (Fig. 5A). However, a short time after the onset of migration, at approximately stage 25, *Sox10* expression is turned off in most neural crest cells as they commit to form specific derivatives. Expression of *Sox10* persists only in the precursors of the peripheral glia and melanocytes (Fig. 5B), and is thought to play an instructive role in the development of those two neural crest derivatives (Mollaaghababa and Pavan, 2003).

When Id3-injected embryos were examined for *Sox10* expression at premigratory neural crest stages, little difference was seen in the expression of this gene on the control versus injected side of the embryo (not shown). In marked contrast, when *Sox10* expression was examined in siblings of these same embryos a short while later, at a time when the developmental potential of many neural crest cells has normally been at least partially restricted, we found that forced Id3 expression in neural crest cells led to a failure of these cells to downregulate *Sox10* expression. This phenomenon was particularly clear when *Sox10* expression was examined in cells migrating into the branchial arches, most of which would normally adopt cartilage fates. Although expression of *Sox10* is never maintained in this population of cells during normal development, high levels of expression were consistently noted on the Id3-injected side of the embryo, long after expression of this factor had been extinguished in the corresponding group of cells on the control side (compare Fig. 5C with 5D). To confirm that the persistence of *Sox10* expression represented a change in the specification state of these cells, and was not due to alterations in the number of cells present along the migratory pathway, we next examined the expression of *Twist* in sibling

embryos. In contrast to *Slug* and *Sox10*, *Twist* is a marker of neural crest precursors that is not downregulated by these cells as they begin to migrate and differentiate. Consistent with our hypothesis, we found no significant difference in the numbers of *Twist*-expressing cells on the control versus Id3-injected side of the embryo (compare Fig. 5E with 5F). Moreover, forced expression of Slug, which also causes an increase in neural crest progenitor cells (Fig. 5G,H), does not lead to the persistent expression of markers such as *Sox10* at migratory neural crest stages (compare Fig. 5I with 5J), in contrast to forced Id3 expression. Together, the above findings strongly support a model whereby forced expression of Id3 prevents neural crest cells from committing to form specific neural crest derivatives such as cranial facial cartilage, and instead maintains these cells in a multi-potent progenitor state.

The formation of neural crest derivatives is blocked in Id3-expressing cells

If Id3 functions to maintain neural crest cells in a progenitor state then, consequentially, it should have a significant inhibitory effect on the formation of neural crest derivatives. We therefore characterized the effects of Id3 on the differentiation of two neural crest-derived cell types, melanocytes and cartilage. Embryos were injected in either one or both cells at the two-cell stage with mRNA encoding the N-terminally tagged Id3, and then reared to stages when melanocyte formation could be assessed. Embryos injected unilaterally showed a significant decrease in the number of melanocytes on the Id3 expressing side of the embryo (Fig. 6A). During normal development, a significant fraction of melanoblasts migrate to the side of the embryo contralateral to where they initially formed. This contralateral migration can make it difficult to assess the full extent of the block to melanocyte formation in unilaterally injected embryos. We therefore carried out experiments in which embryos were injected bilaterally, and here we observed profound deficits in melanocyte number relative to sibling control embryos (Fig. 6B,C). Moreover, these Id3-injected embryos also had reduced or absent dorsal fins, a structure whose formation is neural crest-dependant (Fig. 6B,C).

Similar results were obtained when *Sox9*, a marker of cartilage cell differentiation, was examined. Post-migratory neural crest cells in the branchial arches express *Sox9* as they commit to form chondrocytes. However, expression of this marker was inhibited in neural crest cells expressing Id3, indicating that cartilage formation had been blocked (Fig. 6D,E). By contrast, forced Id3 expression had no discernable effect on *Sox9* expression in the developing ear (Fig. 6D,E, red arrows). In addition to its profound effects on neural crest development, Id3 expression also affects the differentiation of other ectodermally derived cell types. For example, expression of *N-tubulin* in differentiating CNS-derived primary neurons was inhibited in Id3-injected embryos (Fig. 6F). Interestingly, however, the most frequently affected cells were the primary sensory neurons, or Rohon Beard cells, a cell type with close evolutionary and molecular ties to the neural crest. Loss of these cells was confirmed by examining the expression of the sensory neuron marker *Brn3b* (Fig. 6G). The effects of Id3 on ectoderm-derived cell types are specific and direct, as the development of the underlying mesoderm is unperturbed in these experiments (Fig. 6H and not shown).

Promotion of neural crest progenitor fate is a conserved activity of Id family proteins

The ability of Id3 to maintain neural crest cells in a multi-potent stem cell-like fate has important implications for both normal neural crest development and for the formation of neural crest-derived tumors. Indeed, one Id family member, Id2, functions downstream of N-myc during the development

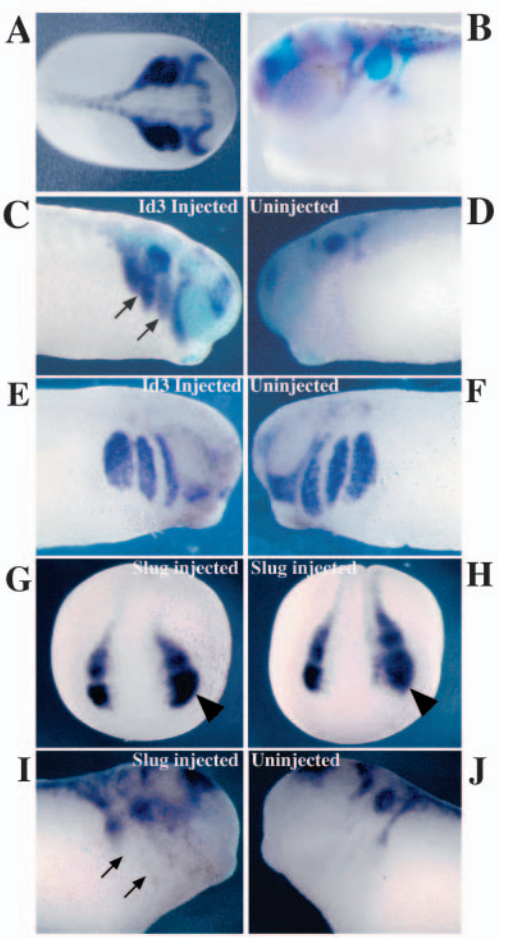

Fig. 5. (A) In situ hybridization showing *Sox10* expression just after the onset of neural crest migration. At these stages, *Sox10* marks the entire neural crest stem cell population. (B) Double in situ hybridization showing expression of *Sox10* (cyan) and *N-tubulin* (magenta) at stage 28. *Sox10* expression at these stages is restricted to glia in the peripheral ganglia and CNS (aqua) as well as to melanoblasts (not shown). (C,D) Forced expression of Id3 leads to the persistence of *Sox10* expression in neural crest cells migrating to the pharyngeal pouches on the injected side of the embryo (C, arrows). At this stage, expression of *Sox10* has normally been downregulated in all neural crest cells except those committing to a glial or melanocyte fate, as seen on the uninjected side of the embryo (D). (E,F) No significant difference is seen in the number of migratory neural crest precursor cells following forced Id3 expression, as visualized by comparing expressing of *Twist* on the Id3-injected (E) versus uninjected (F) side of the embryo. (G,H) Overexpression of Slug leads to expanded expression of markers of neural crest precursor cells on the injected side of the embryo (arrowheads) at neural plate stages. (I,J) In contrast to forced Id3 expression, Slug expression does not lead to persistent expression of *Sox10* in migrating neural crest cells (arrows indicate location of migratory neural crest cells).

of neuroblastomas, which are cancers of neural crest progenitor cells (Maris and Matthay, 1999). Id2 has been more extensively studied than has Id3, and importantly, it is thought to have some activities distinct from Id1 and Id3. All four Id family proteins can act as naturally occurring dominant-negative transcription factors by forming non-functional heterodimers with targeted proteins and prevent those proteins from binding DNA. However, Id2, and possibly Id4, can also bind to Rb-family pocket proteins and block their cell cycle-inhibitory activities, thus promoting entry into S phase (Lasorella et al., 2001).

Because Id2, rather than Id3, has been closely linked to neuroblastoma, it was important to determine if the ability of Id3 to promote neural crest progenitor fate was a conserved function of Id-family proteins or was an activity unique to Id3. To this end, we asked if an N-terminally tagged form of *Xenopus* Id2 could phenocopy the effects of Id3. Indeed, we found that forced Id2 expression led to the persistent expression of *Sox10* in later stage embryos in a manner indistinguishable from what we had observed for Id3 (Fig. 7A,B). Because the functional differences between Id2 and Id3 have only been examined for the mammalian proteins, we confirmed this finding by repeating the experiment with N-terminally epitope tagged forms of Human Id2 and Id3. Again, we found that forced expression of either of these proteins resulted in maintenance of a gene expression profile consistent with the persistence of a precursor state (Fig. 7C-F; not shown). Because Id3 is unable to bind Rb-related proteins, the general ability of Id family proteins to promote a neural crest progenitors fate is most probably due to their ability to inhibit the DNA binding activity of one or more proteins involved in neural crest cell fate diversification.

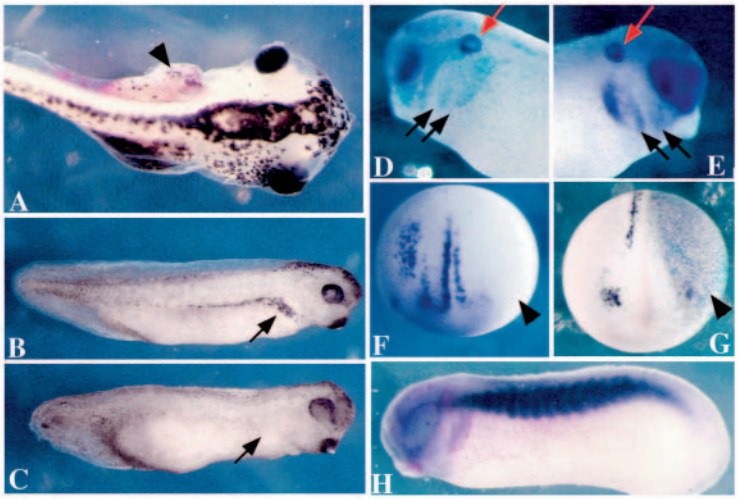

Fig. 6. Neural crest derivatives fail to differentiate in Id3-injected embryos. (A) Forced Id3 expression leads to the formation of substantially fewer melanocytes on the injected side (arrowhead) of embryos that were injected in one cell at the two-cell stage. (B,C) Embryos injected bilaterally with Id3 mRNA (C) show more dramatic deficits in melanocyte formation (arrows) and also have reduced or absent dorsal fins as compared to sibling control embryos (B). (D) Following forced expression of Id3, neural crest cells that populate the branchial arches fail to give rise to chondrocytes, as visualized by *Sox9* expression (black arrows) on the injected (D) versus control (E) side of the embryo. Expression of *Sox9* in the developing ear (red arrows) is unperturbed by Id3 expression. (F) The differentiation of *N-tubulin*-expressing primary neurons is also inhibited on the Id3-injected side of the embryo (arrowhead). (G) Rohon-Beard sensory neurons, as marked by expression of *brn3*, are particularly sensitive to Id3 (injected side marked by arrowhead and β-gal staining, blue). (H) The effects of Id3 in these experiments are directly on the ectoderm. In situ hybridization for muscle actin expression shows that the underlying mesoderm is unperturbed on the injected side of the embryo.

Expression of Id3 does not irreversibly block differentiation of melanocytes

If Id3 blocks the differentiation of neural crest derivatives as a consequence of maintaining these cells in a multipotent progenitor state, rather than by fundamentally altering their potential, then its effects should be reversible. In other words, once released from the inhibitory influence of Id3, neural crest cells should regain their ability to respond to signals directing the formation of specific neural crest derivatives, assuming that these signals are still being transmitted at that point in development. To determine if this is the case, we injected decreasing doses of Id3, under the assumption that protein turnover and dilution effects would result in cells being released from the inhibitory effects of Id3 at progressively earlier times in their development. We chose to examine the development of melanocytes in these experiments, as this is one of the last neural crest lineages to segregate.

Significantly, we were able to identify a dose of Id3 that caused embryos to develop with a significant excess, rather than a deficit of melanocytes (Fig. 8C) relative to sibling control embryos (Fig. 8A) or those injected with higher doses of Id3 (Fig. 8B). Importantly, embryos injected with either a high or low dose of Id3 displayed persistent expression of *Sox10* at stage 26 (not shown), indicating that in both cases the neural crest cells had retained expression of markers of the multipotent progenitor state for some period of time longer than would occur during normal development. Our interpretation of these findings is that once the ectopically provided Id3 protein had turned over to an extent sufficient to allow neural crest cell differentiation, these newly responsive progenitor cells responded to endogenous signals that dictated melanocyte formation. Moreover, because at earlier time points in their development neural crest progenitor cells had been blocked from responding to signals dictating alternative fates, a greater number of these cells were available to commit to a melanocyte fate than would be the case during normal development.

Discussion

A minimum of four distinct signaling pathways, Wnt, BMP, FGF and Notch, are believed to play important roles in the initial formation of neural crest precursor cells at the neural plate border. Each of these signals has also been implicated in the induction or patterning of the adjacent neural plate. Moreover, later in neural crest development, Wnt-, BMP-, FGF- and Notch-mediated signals also play instructive roles in directing the formation of specific neural crest derivatives. For example, BMP treatment directs neural crest stem cells to develop as autonomic neurons (Shah et al., 1996), while

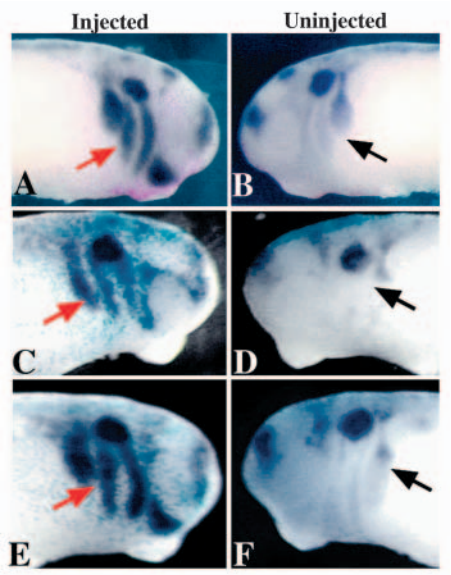

Fig. 7. Promotion of neural crest progenitor fate is a conserved activity of Id family proteins. (A,B) Expression of an N-terminally tagged form of *Xenopus* Id2 phenocopies the effects of Id3, leading to the persistent expression of *Sox10* (red arrows) on the injected (A) versus control (B) side of the embryo. (C-F) Expression of N-terminally epitope tagged forms of human Id2 (C versus D) and human Id3 (E versus F) had identical effects.

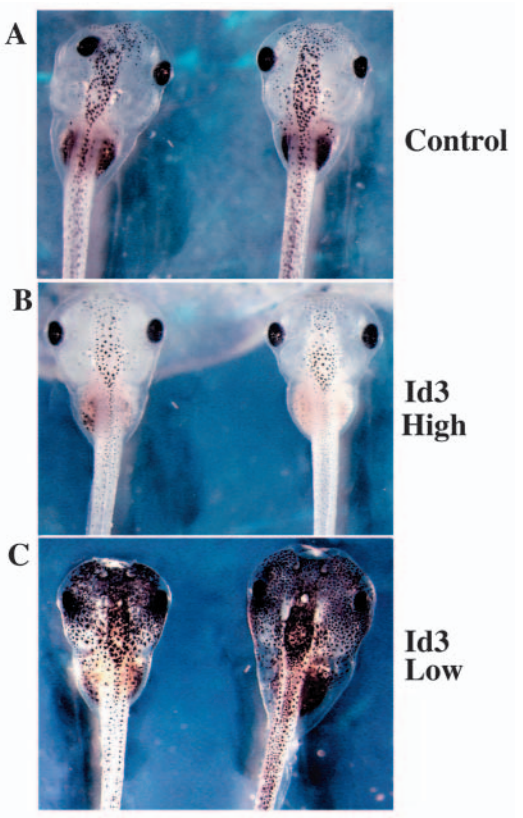

Fig. 8. The ability of Id3 to block the differentiation of neural crest derivatives is reversible. Control embryos at stage 40 (A) display substantially more melanocytes than do sibling embryos injected with high doses of Id3 (B). In marked contrast, when embryos are injected with lower doses of Id3 in order to allow cells to recover from the effects of Id3 at an earlier time point, a profound increase in the numbers of melanocytes is observed (C). These results demonstrate that although forced expression of Id3 maintains neural crest cell in a progenitor state for a prolonged period of time, it does not irreversibly alter their ability to give rise to neural crest derivatives.

canonical Wnt signals have been shown to be essential mediators of both melanocyte and sensory neuronal development (Lee et al., 2004; Lewis et al., 2004). Indeed, this paradigm of reiterated signaling is a common one in developmental biology. Owing to the relatively small cadre of available growth factor families, the same signals must by necessity be repeatedly deployed in order to mediate the enormous number of signaling events that underlie the specification of different germ layers, tissues and cell types. Given how frequently the same extracellular signals are used, however, and because the same signal may elicit vastly different responses at different times in the development of a cell population such as the neural crest, the embryo must employ strategies to prevent cells from responding prematurely or inappropriately to such signals.

We have previously suggested that Myc, which is strongly expressed at the neural plate border from mid-gastrula stages, may play an essential role in preventing premature cell fate decisions in this region of the embryonic ectoderm. This hypothesis was based upon studies showing that morpholino-mediated depletion of Myc leads to a loss of neural crest precursors and derivatives via a mechanism independent of changes in cell proliferation, apoptosis or growth (Bellmeyer et al., 2003). In the absence of Myc, excess CNS progenitors form in place of the neural crest. When considering the mechanisms by which Myc may act in eliciting these effects, we took note of what was known about Myc targets in neural crest-derived cancers.

Neuroblastomas, which are among the most common solid tumors of childhood, are of particular significance to our studies because these tumors are derived from the multipotent neural crest progenitor population, and because they are frequently associated with misregulation of MYC. Significantly, recent studies have implicated ID2 as an important MYC target. ID2 expression was found to be upregulated in neuroblastoma-derived cell lines and tumors with N-myc amplification (Lasorella et al., 2000; Lasorella et al., 2002; Raetz et al., 2003), while MYC was shown to bind directly to the ID2 promoter in fibroblasts and epithelial cells using chromatin immunoprecipitation (ChIP) assays (Lasorella et al., 2000; Siegel et al., 2003). Our findings that Myc can also bind directly to the Id3 promoter are consistent with the results of a recent genomic screen for high affinity Myc targets (Fernandez et al., 2003). Moreover, it has been reported that viral misexpression of Id2 leads to excess neural crest production in avian embryos (Martinsen and Bronner-Fraser, 1998), providing a link between Id proteins and the neural crest.

Ids, a family of four (Id1-Id4) naturally occurring dominant-negative transcription factors that mediate their effects primarily through their highly conserved HLH domain (Benezra et al., 1990; Norton, 2000), are thought to be

important regulators of a range of epithelial cell types (Coppe et al., 2003). These proteins have been shown to bind to, and prevent DNA binding by, transcription factors of the bHLH, Pax and ETS families (Benezra et al., 1990; Yates et al., 1999; Roberts et al., 2001). In addition Id2, and possibly Id4, can bind to and interfere with Rb-family tumor suppressors (Rb, p107 and p130, referred to as 'pocket proteins'), and in this way are proposed to influence both cell cycle progression and tumorigenesis (Lasorella et al., 2000). Id2 has been shown to act as a substrate for cyclin A-dependent Cdk2 activity, and phosphorylation of Id2 on serine 5 may alter its ability to bind and inhibit specific target proteins (Hara et al., 1997).

Although in *Xenopus* expression of *Id2* in neural crest cells is not observed until migratory stages, another Id family member, *Id3,* is expressed in the early ectoderm in a pattern roughly reminiscent of the expression of *Myc* (Wilson and Mohun, 1995) (Fig. 1A). We find here that Id3 is a Myc target that plays an essential role in the formation and maintenance of neural crest stem cells. Embryos in which Id3 has been depleted develop without neural crest cells, and in their place an excess of CNS progenitors form. These findings phenocopy the effects of Myc depletion (Bellmeyer et al., 2003). Moreover, expression of Id3 can significantly rescue the expression of neural crest markers in Myc-depleted embryos. Together, these findings suggest a model in which Myc and Id3 may regulate cell fate decisions in the early embryonic ectoderm by preventing neural plate border cells, such as the neural crest, from prematurely responding to the signals that are patterning the adjacent ectoderm.

Following their emigration from the neural tube, early migratory neural crest cells initially retain their stem cell-like characteristics, including the potential to contribute to the sensory neuronal, autonomic neuronal, glial, smooth muscle and ectomesenchymal lineages (Baroffio et al., 1991; Paratore et al., 2002; Kim et al., 2003). However, these cells soon begin responding to signals that direct their development into specific neural crest derivatives, as evidenced by the downregulation of pan-neural crest markers expressed by the early progenitor population, and the expression of markers characteristic of specific differentiating lineages. We find that enforced misexpression of Id3 in the migratory neural crest population maintains the expression of markers characteristic of the progenitor state, and delays or prevents the differentiation of neural crest derivatives. For example, Id3-expressing cells sustain robust expression of *Sox10*, a factor that has itself been implicated in the maintenance of stem cell identity (Paratore et al., 2002; Kim et al., 2003), and *Slug*, an important regulatory protein expressed by all neural crest precursor cells (LaBonne and Bronner-Fraser, 2000), long beyond the time that most neural crest cells on the control side of the embryo have downregulated these factors. Importantly, Id3 expression does not appear to irreversibly alter the potential of these cells. Once their pool of Id3 has turned over, neural crest cells are capable of responding to signals that direct the formation of specific derivatives such as melanocytes. These findings suggest a model in which Id family proteins expressed in the neural crest progenitor pool help control the timing with which these cells respond to differentiative signals during normal development. We cannot formally rule out an alternative explanation of our findings, however, in which Id3 dictates the outcome of neural crest cell fate determination in a dose-dependant fashion. Future studies might profitably explore whether controlling the timing of release from Id3 activity can lead to excess recruitment of neural crest progenitors to fates other than melanocytes.

Mechanistically, we find that the effects of up- or downregulating Id3 are independent of changes in the rates of proliferation or apoptosis. Although Id family members are known to have some divergent activities, we find that both Id2 and Id3 can maintain neural crest cells in a multipotent progenitor state. In contrast to Id2 and Id4, Id3 is unable to bind to Rb-family tumor suppressors (Lasorella et al., 2001), making it unlikely that Rb-related mechanisms can explain the effects of forced Id3 expression. Instead, Id3 probably exerts its effects by binding to, and inhibiting the activity of, one or more bHLH, Pax or Ets-family transcription factors necessary for neural crest diversification. A key challenge for the future will be to identify the physiologically relevant Id3 targets in normally developing neural crest cells. Moreover, determining if such factors are also Id targets in neuroblastomas will be an important avenue of investigation.

Beyond neuroblastomas, a number of other clinically significant cancers, including melanomas and neuro-epitheliomas, are neural crest derived. In light of these links, it is noteworthy that, in addition to Myc and Id proteins, other regulators of early neural crest development, including Slug, Snail, Twist and Sip1, are also misregulated in human cancers (Batlle et al., 2000; Rosivatz et al., 2000; Yang et al., 2004) (reviewed by Heeg-Truesdell and LaBonne, 2004). These striking molecular parallels suggest that by determining the mechanisms by which factors such as Id3 regulate the normal development of neural crest cells, we will also gain important insight into their related roles in tumorigenesis.

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