

A Rap GTPase interactor, RADIL, mediates migration of neural crest precursors

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The neural crest (NC) is a highly motile cell population that gives rise to multiple tissue lineages during vertebrate embryogenesis. Here, we identify a novel effector of the small GTPase Rap, called RADIL, and show that it is required for cell adhesion and migration. Knockdown of *radil* in the zebrafish model results in multiple defects in NC-derived lineages such as cartilage, pigment cells, and enteric neurons. We specifically show that these defects are primarily due to the diminished migratory capacity of NC cells. The identification of RADIL as a regulator of NC migration defines a role for the Rap pathway in this process.

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During vertebrate development, precursor cells of various lineages undergo a complex set of morphogenetic movements to assume their final position in the embryo. Implementation of such movements depends on both the patterns set up by several morphogens such as Wnts, retinoic acid, bone morphogenic proteins (BMPs), and fibroblast growth factors (FGFs), as well as the ability of cells to respond to these signals by executing cell motion. Regulation of cell adhesion has emerged as a common theme in a number of settings where cellular precursors traverse large distances to assume their final differentiated state, but the underlying molecular mechanisms are poorly defined.

The zebrafish model offers an ideal experimental system for the evaluation of cell adhesion and cell motility

during embryogenesis. The neural crest (NC) cells, a transient population of multipotent progenitors arising at the lateral edge of the neural plate in vertebrate embryos, migrate extensively to give rise to a number of cell types such as the craniofacial skeleton, neurons and glia of the peripheral nervous system, and pigment cells (Le Douarin and Kalcheim 1999). Their migration requires active adhesion changes during both the epithelial-to-mesenchymal transition at the dorsal neural tube and their subsequent navigation along stereotypical pathways to their final position. The importance of NC migration during normal development is highlighted by the human constitutional defects associated with NC function, which often result in craniofacial abnormalities and account for up to 30% of all congenital birth defects (Jones and Trainor 2004). In some cases, specific genetic abnormalities have been identified, as in mutations in the adhesion/migration factor Ephrin B1 (*EFNB1*) in craniofrontonasal syndrome (Wieland et al. 2004), deletions of the migration-inducing gene *SLUG* in Waardenburg syndrome (WS2D) (Sanchez-Martin et al. 2002), and mutations in *GDNF* and its receptor *RET*, two important regulators of NC migration, in Hirschsprung disease (Edery et al. 1994; Ivanchuk et al. 1996; Iwashita et al. 2003). However, the majority of NC defects are unexplained at the molecular level.

The low-molecular-weight GTPase Rap is a member of the Ras GTPase superfamily encoding molecular switches that cycle between inactive GDP-bound and active GTP-bound states. Rap appears to have an evolutionarily conserved role in regulating adhesion and migration in a wide range of species, ranging from *Dictyostelium* to vertebrates (Bos et al. 2001; Caron 2003). However, defining the function of Rap in early vertebrate embryonic development is complicated by the presence of multiple Rap genes, and thus analysis of unique Rap interactors using a model organism may provide valuable information about this complex pathway.

In this study, we identify a novel downstream effector of Rap, RADIL (RA [Ras association] and DIL domains), which plays a critical role in cell adhesion and migration. Loss of Radil function in zebrafish embryos results in defects in NC migration. These observations point to a critical role for the Rap pathway in developmentally regulated cellular migration of NC and, more broadly, the role of cell adhesion in morphogenetic movements shaping the vertebrate embryo.

Results and Discussion

Interactions of RADIL with Rap

We initially identified RADIL in a screen for genes induced by an oncogenic translocation in a pediatric sarcoma (data not shown). While its role in cellular transformation appears to be modest, its domain architecture and effects on cellular migration prompted us to pursue its role in normal development. Human RADIL encodes a novel protein of 1075 amino acids, including RA, DIL, and PDZ domains (Supplementary Fig. S1). The RA domain is present in a number of downstream effectors of Ras and Rap, and interacts in vivo with these two GTPases, displaying varying degrees of specificity between them. The function of the DIL domain is un-

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known, but it is found in several cytoskeleton-associated proteins, such as myosin V. Finally, the PDZ domain at the C terminus represents a well-known protein–protein interaction platform. The domain architecture of RADIL is most closely related to AF6, an important regulator of cell adhesion that serves as an intermediary between membrane-bound proteins and actin cytoskeleton. In fact, RADIL has been described as AF6-like in a recent report characterizing the role of AF6 in cell adhesion (Zhang et al. 2005). However, in the absence of significant functional similarity between the two proteins, we propose the name RADIL, reflecting its domain architecture.

To investigate fully the GTPase-binding preference of RADIL, we first cotransfected a Flag-tagged RADIL expression construct along with HA-tagged GTPases into AD293 cells. Immunoprecipitation using anti-Flag antibody, followed by immunoblotting with anti-HA antibody, clearly shows that RADIL preferentially binds to Rap, but not to any of the Ras family proteins (Fig. 1A). To test whether RADIL binding is dependent on the GTPase activation status, we cotransfected RADIL with constitutively active Rap (Rap E63) and dominant-negative (Rap N17) mutants (Fig. 1B). RADIL was effectively coimmunoprecipitated only with the constitutively active Rap mutant, suggesting that this interaction is dependent on the Rap-GTP state. Further demonstrating specificity, no interaction was observed with two additional GTPases—cdc42 and Rac (data not shown).

To investigate the role of RADIL in a mammalian tissue culture system, we knocked down endogenous *Radil* expression using lentiviral short hairpin RNAs (shRNAs) in a mouse breast epithelial cell line NMuMG. Given the well-described role of Rap and its effectors in cell adhesion, we performed short-term adhesion assays. Two independent hairpins achieved partial knockdown of *Radil* expression to ~40% of control cells, as determined by quantitative TaqMan PCR (qPCR) (Fig. 1D). *Radil* knockdown resulted in dramatic reduction of the adhesive properties of these cells to <10% of control cells (Fig. 1C), suggesting that RADIL is involved in modulating cellular adhesion.

To assess the role of RADIL in cellular migration, we also knocked down RADIL in a highly migratory human chondrosarcoma cell line, SW1353. Reduced *RADIL* expression (~50%) using two independent small interfering RNA (siRNA) duplexes (Fig. 1F), resulted in decreased (to ~40%) migratory capacity of these cells (Fig. 1E). RADIL therefore contributes to both cell adhesion and migration, properties that have been linked to Rap signaling. Ectopic overexpression of RADIL did not further enhance migration or adhesion of the parental cells used in either of these assays (data not shown), indicating that RADIL may be required, but is not the limiting component of these processes.

Recent evidence has suggested that some Rap effector molecules can interact with Rap GAPs, thus regulating the levels of active Rap-GTP through a direct feedback

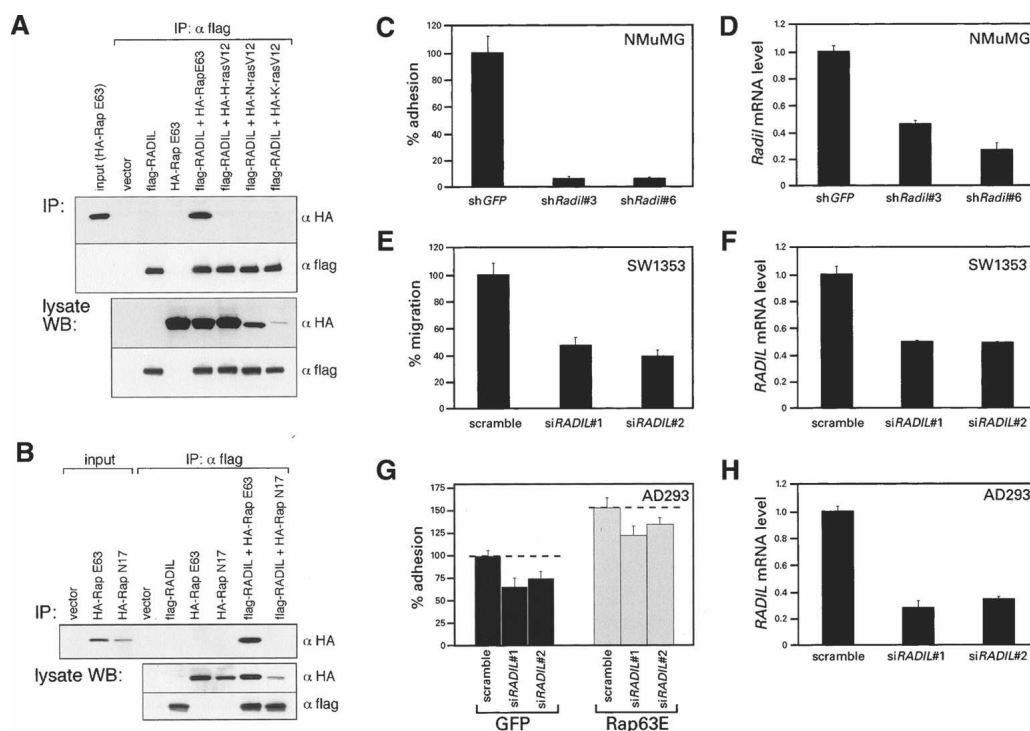


Figure 1. RADIL binds to activated Rap GTPase and is involved in cell adhesion and migration. (A) Immunoprecipitation of Flag-tagged RADIL with HA-tagged GTPases, showing preferential association of RADIL with Rap and not with H-ras, N-ras, or K-ras. (B) Immunoprecipitation of Flag-tagged RADIL with HA-tagged constitutively active Rap (RapE63), but not with dominant-negative (RapN17). (C) NMuMG cells with decreased *Radil* mRNA levels display defects in adhesion. (D) qPCR showing relative *Radil* mRNA levels. *Radil* knockdown was achieved in mouse NMuMG cells using two independent lentiviral hairpin constructs. (E) SW1353 cells with decreased *RADIL* levels display defects in migration. (F) qPCR showing relative *RADIL* mRNA levels. *RADIL* knockdown was achieved in human SW1353 cells using two independent siRNA duplexes. (G) RADIL is downstream from Rap. Adhesion defects in AD293 cells upon knockdown of endogenous *RADIL* expression cannot be fully suppressed by overexpression of constitutively active Rap63E, suggesting that RADIL is a downstream effector of Rap. (H) qPCR showing relative *RADIL* mRNA levels. *RADIL* knockdown was achieved in human AD293 cells using two independent siRNA duplexes.

loop (Su et al. 2003). Elucidation of epistatic relationships is therefore critical in ordering the pathway. We tested the effect of either overexpression of constitutively active RapE63 or knockdown of endogenous *RADIL* in the highly transfectable cell line AD293. Knockdown of *RADIL* (to ~30%) was achieved using two independent duplexes (Fig. 1H) and resulted in a significant decrease in cellular adhesion to ~70% of control cells (Fig. 1G). While expression of constitutively active Rap resulted in increased adhesion at baseline in this cell line, it did not abrogate the effect of *RADIL* knockdown, suggesting that *RADIL* may function downstream from Rap. As such, *RADIL* would be predicted not to affect the levels of the Rap-GTP active state. Indeed, overexpression of *RADIL* in AD293 cells did not affect the relative levels of Rap-GTP (data not shown). Together with the physical interaction between *RADIL* and the active GTP-bound Rap, these results suggest that *RADIL* may be a downstream effector of Rap.

RA domain proteins have been shown to bind to Ras GTPase and may display some promiscuity in their GTPase selection, particularly under *in vitro* conditions. To confirm that *RADIL* does not impinge on Ras-dependent signaling, we used two standard readouts for Ras function: focus formation in 3T3 cells and a Ras-dependent transactivation assay. Whereas titration of Ras-binding proteins can result in modulation of Ras-dependent output (Peterson et al. 1996; Shao and Andres 2000), increasing concentrations of *RADIL* did not affect the number of foci formed in 3T3 cells (data not shown). A standard Ras-responsive promoter was also unaffected by *RADIL* overexpression, either at basal or Ras-activated levels of reporter readout (Supplementary Fig. S2). Thus, *RADIL* appears to interact specifically with active Rap and does not modulate Ras signaling.

Disruption of *Radil* function in zebrafish

To study the *in vivo* function of *RADIL* in vertebrate development we cloned a full-length zebrafish cDNA. Zebrafish *radil* encodes a protein of 1125 amino acids with 49% identity and 64% similarity to human *RADIL*. The level of *radil* expression in zebrafish is regulated over the course of 7 d of development (Fig. 2A). Very low but detectable levels of *radil* transcript are present in one-cell embryos, suggesting the presence of maternally deposited transcripts and the levels of *radil* increase upon the onset of zygotic transcription (around the 1000-cell stage), eventually peaking at 72 h post-fertilization (hpf). To investigate the spatial distribution of *radil* transcripts, we performed *in situ* hybridization using *radil*-specific probe (Supplementary Fig. S3). The expression pattern appeared to be ubiquitous, and enriched in the anterior part of the embryos.

To investigate the consequences of abrogation of *radil* function in zebrafish, we used an antisense morpholino (MO) knockdown approach. A number of specific defects affecting craniofacial cartilage, enteric neurons, cranial ganglia, glial cells of the lateral line, as well as iridophore and xanthophore pigment cells were evident (Fig. 2C–H; Supplementary Fig. S4). Other lineages, such as melanophores and dorsal root ganglia, appeared to be unaffected or only marginally affected (Supplementary Fig. S4). These effects were specific to *radil* gene knockdown as judged by several criteria. Three independent MOs (one targeting the translational start, ATG MO, and the other

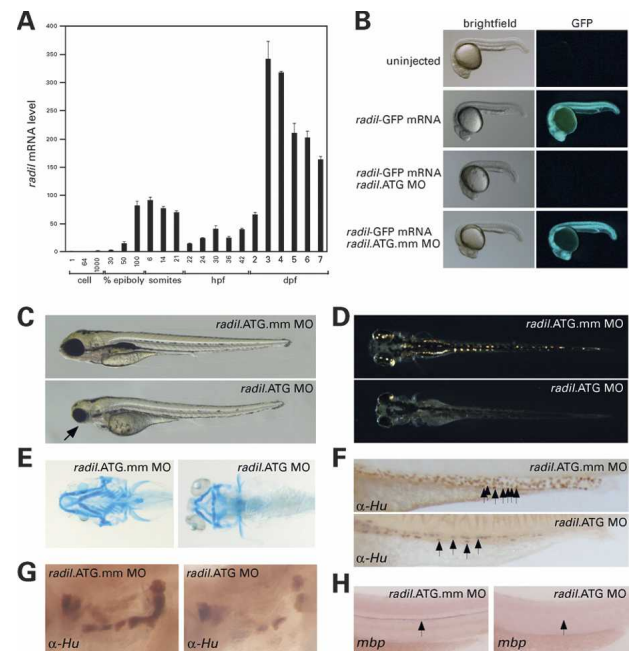


Figure 2. Characterization of *radil* knockdown phenotypes. (A) qPCR showing relative *radil* mRNA levels during early zebrafish embryogenesis. (B) Specificity of *radil*.ATG MO. Translational fusion of the first 25 nucleotides of *radil* ORF to GFP is efficiently suppressed by the ATG MO, but not by the mismatch control MO. Embryos at 24 hpf are shown. (C) Morphology of *radil* knockdown embryos at 4 dpf, compared with the mismatch control-injected embryos. Arrow points to the jaw defect. (D) Iridophore pigmentation defects in *radil* knockdown embryos at 4 dpf compared with the mismatch control-injected embryos. (E) Craniofacial defects in *radil* knockdown embryos at 7 dpf compared with the mismatch control-injected embryos. Cartilage was stained with Alcian Blue. (F) Enteric neuron defects in *radil* knockdown embryos at 4 dpf compared with the mismatch control-injected embryos, visualized with anti-Hu antibody staining. Arrows point to individual enteric neurons, highlighting the decreased numbers in *radil* morphants. (G) Cranial ganglia defects in *radil* knockdown embryos at 4 dpf compared with the mismatch control-injected embryos, visualized with anti-Hu antibody staining. (H) Significant reduction in the glial cells of the lateral line in *radil* knockdown embryos at 3 dpf compared with the mismatch control-injected embryos, visualized by *in situ* hybridization with *mbp* (*myelin basic protein*) probe.

two targeting splice donor sites of exons 4 and 14) resulted in similar phenotypes (Supplementary Table S1). The exon 4 donor splice site MO resulted in the use of a cryptic splice donor site within exon 4 and is predicted to produce an in-frame deletion of 57 amino acids within the Radil protein (Supplementary Fig. S5). The exon 14 donor splice site MO resulted in the incorporation of an intron and premature Radil protein truncation (Supplementary Fig. S6). No phenotypes were observed upon injection of three corresponding mismatch MOs at the same concentrations. Furthermore, injection of increasing concentrations of *radil*.ATG MO resulted in progressively more severe phenotypes demonstrating a dose-response relationship (Supplementary Fig. S7). Consistent with the specificity of these MOs, translational fusion of the first 25 nucleotides of *radil* to GFP was efficiently suppressed by the ATG MO but not by the mismatch control MO (Fig. 2B).

The cell types affected by *radil* knockdown point to a defect with early NC development. The initial induction

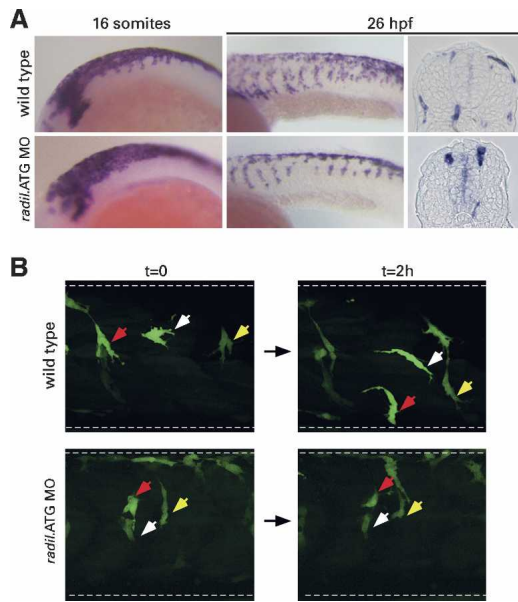


Figure 3. *radil* knockdown results in defects of NC migration. (A) Defect in early NC migration in *radil* knockdown embryos, as visualized by in situ hybridization with *crestin* marker. Embryos were fixed at the 16-somite stage and 26 hpf. Sections were taken at the 26-hpf time point, halfway through the yolk extension. (B) Defect in early NC migration in *radil* knockdown embryos, as visualized using the *foxD3:GFP* transgenic line. Panels shown represent frames taken from Supplementary Movies 1 and 2. Movies were started at ~24 hpf and 31 hpf for wild-type and *radil* morphants, respectively. The dotted white lines mark the top of the embryo and the edge of the yolk extension. Arrows (white, yellow, and red) identify individual cells and their respective changes in position over time.

of NC is not affected in the *radil* morphant embryos at the six-somite stage (Supplementary Fig. S8), as indicated by robust expression of two early NC markers *foxd3* and *sox10*. Subsequent migration of these cells, however, showed profound defects. We used the pan-NC marker *crestin* to follow cell migration (Fig. 3A; Supplementary Fig. S12). At the 16-somite stage, wild-type embryos clearly show migrating NC cells, but *radil* morphant embryos have significant impairment with no NC cell migration evident at this stage. At the 26-hpf stage, *crestin*-positive cells in wild-type embryos have reached the yolk extension, whereas *radil* morphants have fewer *crestin*-positive cells, some of which have not migrated as far, while others have remained in their original positions at the neural keel. To demonstrate the migration defects at single-cell resolution, we took advantage of the (*foxD3:GFP*) transgenic line, in which NC cells are tagged and can be monitored by real-time imaging (Gilmour et al. 2002). Knockdown of *radil* resulted in defects in gross cell migration compared with wild-type embryos (Fig. 3B; Supplementary Movies 1, 2).

To further characterize the NC defects, we undertook a more detailed analysis of craniofacial development (Supplementary Fig. S9). Expression of *dlx2a*, which at 24 hpf is expressed in migratory NC that forms pharyngeal arches, was mildly affected in *radil* morphants, particularly in the hyoid and branchial arches. However, *dlx2a* expression by 48 hpf clearly showed significant reduction of mandibular and hyoid arches and absence of branchial arches in *radil* morphants. Similarly, the staining pattern of *gsc*, a marker of early chondrocytes,

showed a dramatic reduction in *radil* morphants at 48 hpf. Together, these findings are consistent with a progressive defect in NC migration, resulting in insufficient numbers of migrating NC cells populating the pharyngeal pouches, which leads to loss of early chondrocyte markers and craniofacial cartilage defects observed at 7 dpf (Fig. 2).

The migration defect in NC precursors in *radil* morphants is associated with a reduced number of NC derivatives at later stages of development. To determine whether these cells have an increased rate of apoptosis, we measured cell death in *radil* morphants using TUNEL staining. Knockdown of *radil* was associated with a dramatic increase in apoptosis compared with the uninjected controls, as well as the mismatch control MO-injected embryos (Fig. 4; Supplementary Fig. S10). To distinguish between NC cell apoptosis and failure of migration as the primary cause of developmental defects in *radil* morphants, we suppressed cell death using overexpression of the zebrafish *bcl2* anti-apoptotic gene (Liu et al. 2003; Langenau et al. 2005). Injection of the *radil* ATG MO together with *bcl2* mRNA led to efficient rescue of apoptosis measured at 24 hpf (Fig. 4), as well subsequent time points until 5 dpf (Supplementary Fig. S11). However, these embryos showed no rescue of cartilage, enteric neurons, or iridophore defects (Fig. 4). Similar findings were observed when apoptosis was suppressed using functional inactivation of p53 by coinjecting *radil*.ATG MO with p53 MO (Robu et al. 2007; data not shown). Furthermore, *bcl2* mRNA coinjection did not rescue the migration defect of *crestin*-positive cells at 24 hpf (Supplementary Fig. S12). Thus, the induction of apoptosis and migration defects can be uncoupled in *radil* morphant embryos, providing evidence that migration defects may be primarily responsible for the phenotypes observed in these embryos, which is consistent with our findings in mammalian cells showing that RADIL expression is required for normal cell adhesion and migration.

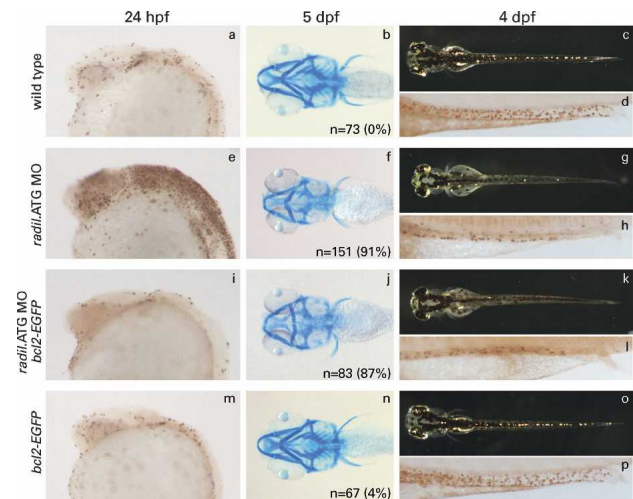


Figure 4. Apoptosis is not the primary cause of the abnormalities of NC-derived lineages in *radil* morphants. (a,e,i,m) Knockdown of *radil* in zebrafish results in the onset of apoptosis, as shown by TUNEL assay. *bcl2* overexpression can rescue apoptosis in *radil* morphants at 24 hpf, but does not rescue craniofacial (b,f,j,n), iridophore (c,g,k,o), or enteric neuron (d,h,l,p) defects later in development. Sample size (n) and the percentage of embryos with craniofacial abnormalities are indicated.

Concluding remarks

In this study, we describe an effector of Rap, termed RADIL, which displays a preference for Rap-GTP binding and modulates Rap-dependent processes including cell adhesion and migration. To gain *in vivo* insight into the function of RADIL, we used the zebrafish model system and identified the highly motile NC lineage as being particularly affected by *radil* knockdown. Defects in such a diverse set of differentiated cells as cartilage, iridophores, and enteric neurons suggest an early defect at a level of undifferentiated precursor NC cells. Using a combination of approaches, we showed that impaired adhesive/migratory properties of NC cells may underlie the observed phenotypes.

Rap has been strongly implicated in the regulation of cellular adhesion in multiple biological settings (Bos 2005). However, given the multiplicity of Rap functions as well as genetic redundancy of the Rap family in most organisms, studies of individual Rap regulators and effectors have been most informative in elucidating specific Rap functions during development. While the mouse knockout of AF6 resulted in early embryonic lethality due to abnormal cell–cell junctions (Zhadanov et al. 1999), other Rap effector knockouts have shown very specific phenotypes: For instance, RapL knockout mice display defects only in adhesion and migration of lymphocytes and dendritic cells (Katagiri et al. 2004). Given that the unifying theme of Rap function studies appears to be the regulation of cadherin- and integrin-based adhesion, *in vivo* studies are critical in providing the relevant physiological context.

Modulation of adhesion has emerged as an important player in early morphogenetic cell movements (Solnica-Krezel 2006). In the first study to globally abrogate Rap function in zebrafish, defects in convergence and extension movements during gastrulation, which involve dorsoventral narrowing (convergence) and anterioposterior lengthening (extension) of embryonic tissues, were recently observed (Tsai et al. 2007). This strong effect on early embryo morphogenetic movements makes the assessment of later roles of Rap quite challenging. However, a role for Rap and adhesion modulation is likely in later developmental processes, such as the NC migration, as suggested by the significant defects observed in *radil* morphant embryos.

Modulation of cellular adhesion through integrins and cadherin has been shown to have a profound impact on NC migration. Conditional deletion of $\beta 1$ integrin by Ht-PA-Cre, expressed specifically in NC cells at the start of migration, leads to severe Hirschsprung-like phenotypes in mice (Breau et al. 2006). *Cadherin-11* in mouse and *Xenopus* and *Cadherin-7* in chick are specifically expressed only in the migratory NC and not in the earlier premigratory NC. However, forced overexpression of *Cadherin-11* in *Xenopus* and *Cadherin-7* in chick results in failure of NC migration (Nakagawa and Takeichi 1998; Borchers et al. 2001). Therefore, a complex picture emerges where NC migration is dependent on spatial, as well as temporal, regulation of NC cell adhesion. The identification of RADIL provides a link between NC migration and a well-known adhesion mediator, Rap, revealing another key component of this important biological pathway. Future identification of RADIL-interacting proteins is likely to elucidate a more complete molecular framework for RADIL function in regulating cell adhesion and migration.

Phenotypic comparison of *radil* morphants with known NC mutants could also yield insight into the pathways where Radil might be involved. During early neural development multipotent NC cells restrict their fate potential into ectomesenchymal and nonectomesenchymal pools (Kelsh and Eisen 2000). Ectomesenchymal NC derivatives include craniofacial skeleton and fin mesenchyme, while nonectomesenchymal NC derivatives include pigment cells, neurons, and glia. Analysis of phenotypes in zebrafish *sox10* mutants has been clearly shown to affect only the nonectomesenchymal NC lineages (Kelsh and Eisen 2000; Dutton et al. 2001). In contrast, *radil* morphants display more broad defects, suggesting that both pools of NC precursors are affected. Zebrafish *tfap2a* mutants display phenotypes in both NC precursor pools, but the primary defect appears to be in early patterning of NC subpopulations (Knight et al. 2003; Barrallo-Gimeno et al. 2004). While NC migration appears to be a major defect in *radil* morphants, *tfap2a* mutants display modest if any aberrant cell migration. Consistent with the patterning defect in *tfap2a* mutants, embryos display absence of entire prechondrogenic NC streams that populate pharyngeal arches at 24 hpf. In contrast, expression of *dlx2a* in *radil* morphants is only modestly affected at 24 hpf, but dramatically reduced at 48 hpf, arguing for a later role of Radil compared with Tfap2a. Zebrafish *foxd3* mutants exhibit a complex phenotype with defects in NC specification, survival, and migration (Lister et al. 2006; Stewart et al. 2006). Given the substantial phenotypic overlap with *radil* morphants and the involvement of Foxd3 in NC migration, we tested whether *radil* expression was affected *foxd3* mutants. While no difference in *radil* expression was observed (data not shown), it remains possible that other Foxd3 target genes may functionally interact with Radil.

A common phenotype frequently observed in the NC mutants is the induction of apoptosis. Functional abrogation of several classes of molecules can lead to NC apoptosis, including adhesive molecules ($\alpha 4\beta 1$ integrin), signaling molecules (*Fgf8*, *Fgfr1*), and transcription factors (*foxd3*, *tfap2a*, and *foxi*) (Abu-Issa et al. 2002; Nissen et al. 2003; Trokovic et al. 2003; Barrallo-Gimeno et al. 2004; Stewart et al. 2006). Collectively, these studies suggest that disruption of NC specification, migration, survival, or differentiation, can lead to increased cell death. However, in most cases the causal relationship between apoptosis and any of the above steps is unclear. The analysis of *radil* knockdown is particularly interesting in this context, in that its effects appear linked to cell migration, as opposed to NC specification, and that suppression of apoptosis in *radil* morphants by overexpressing the anti-apoptotic gene *bcl2* or knocking down p53 function fails to rescue the appropriate development of craniofacial cartilage or other NC-derived lineages. Thus, our data suggests that adhesion/migration defects underlie the phenotypes observed, and apoptosis may be a secondary result of failure of cells to establish their appropriate developmental context.

Taken together, genetic evidence strongly suggests that defects in NC migration can lead to abnormalities in NC-derived lineages. In this report we provide evidence for the role of the Rap pathway in NC migration, raising the possibility that Rap regulators and effectors may be candidates for genes involved in human neurocristopathies.

Materials and methods

MOs

All MOs were ordered from GeneTools. MOs used were as follows: *radil*.ATG, GGCAGATGAGGAACCGTAGAACATG; *radil*.ATG.mm, GGCACATcAGGAAGCGTAcAAGATG; *radil*.ex4d, ATTTTCGGCTGACCTGTTGGTTGTAG; *radil*.ex4d.mm, ATTTaGGCTcACCTcTTGaTTcTAG; *radil*.ex14d, CAGCACACTCACAAAGTCCATCGA; *radil*.ex14d.mm, CAcCagACACTCagAAGTcGATgGA; p53, GCGCCATTGCTTTGCAAGAATTG (Robu et al. 2007). Mismatches in the control MOs are indicated in lowercase.

MOs were dissolved in 1× Danieau's buffer and injected at indicated concentrations (Table 1) into the yolk of one-cell stage embryos in a 1-nL volume. All of the analyses presented were conducted with embryos injected with 4 or 5 ng of *radil*.ATG MO.

bcl2 overexpression

bcl2-EGFP mRNA was synthesized as described previously (Liu et al. 2003). Two-hundred picograms of mRNA were injected per embryo. For the *bcl2* rescue of apoptosis experiment, *radil*.ATG MO was injected at 4 ng per embryo.

Further information of about materials and methods is provided in the Supplemental Material.

Acknowledgments

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