SUPPLEMENTAL INFORMATION

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

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SUPPLEMENTAL MATERIALS AND METHODS

Zebrafish husbandry
Fish were raised and kept under standard laboratory conditions (Westerfield 1993). Embryos were staged as previously described (Kimmel et al. 1995). Tg(foxD3:GFP) fish have been previously described and were a generous gift from Dr. D. Gilmour (Gilmour et al. 2002).

Zebrafish embryo stains
In situ hybridization conditions have been previously described (Thisse et al. 1993). Probes used have also been previously described: foxD3 (Kelsh et al. 2000), sox10 (Dutton et al. 2001), crestin (Luo et al. 2001), dlx2a (Akimenko et al. 1994), dct (Lister et al. 1999), xdh (Parichy et al. 2000), mbp (Brosamle and Halpern 2002). Zebrafish radil in situ probe corresponds to nucleotides 1659–2552 in the full-length radil cDNA and was cloned into pGEM-T Easy vector (Promega). Staining with anti-Hu antibody has been previously described (An et al. 2002). Alcian Blue cartilage staining protocol has been previously described (Kimmel et al. 1998). TUNEL staining was done using the ApopTag peroxidase in situ apoptosis detection kit from Chemicon following the manufacturer’s instructions.

Zebrafish full-length radil
Zebrafish EST (IMAGE:7996116) corresponds to a full-length radil ORF. Since the EST does not contain upstream stop codons of the putative translational start, we performed 5’RACE PCR. We isolated approximately 400 bp of additional sequence (novel exon 1 and full sequence of exon 2) with several upstream stop codons, strongly suggesting that the EST represents a full-length ORF clone. Composite full-length radil cDNA sequence is available in GenBank (EU031809).

Zebrafish primers for the RT-PCR analysis of splice MOs
radil.ex4d.F: GTTCTGCCGCAGCCTCAGTGAAACC
radil.ex4d.R: GGTATTAGCATCAGAGGGTCTTTGTAC
radil.ex14d.F: AAAGGACCGTACGGCCTGGGAATGG
radil.ex14d.R: CAGACGAATCAAATCCACAGCACTC
gapdh.F: CCATGTTTGTCACTGGGTGTCAAACCATG
gapdh.R: AGGAATTACTTTGGCCTACAGCCTTGCC

In vivo time-lapse microscopy
Embryos were anaesthetized with Tricaine and embedded in 0.5% low-melt agarose containing PTU to inhibit melanophore pigment accumulation. Images were taken on a Zeiss LSM5 PASCAL confocal microscope. Multiple Z-sections, every 1µm, were taken every 10 minutes for 4h. Images and movies represent signal reconstruction across multiple sections.

Expression plasmids
Human EST (IMAGE:4827628) represents a full-length RADIL clone. The expression construct was developed by PCR cloning of the ORF into pCMV-Tag2B vector resulting
in N-terminally flag-tagged RADIL. HA-tagged human Rap1A plasmids (RapE63 and RapN17) were a generous gift from Vijay Yajnik. HA-tagged human H-, N-, K-ras plasmids were a generous gift from Kun-Liang Guan. All plasmid constructs were verified by sequencing.

**Tissue culture**

All lines were propagated in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin/streptomycin and maintained at 5% CO₂ at 37°C. Additionally, media for NMuMG cells contained 10 µg/ml insulin.

**Immunoprecipitation (IP) and Western blotting**

AD293 cells, an adherent variant of human HEK293 cells (Stratagene), were plated at a density of 7x10⁵ per 10 cm dish one day prior to transfection. Transient transfection was carried out with 6 µg of total plasmid DNA/dish using Fugene 6 transfection reagent (Roche). Expression of transfected genes was analyzed 24 h post transfection. Cells were washed with phosphate-buffered saline and disrupted with cold lysis buffer: 150 mM NaCl, 1% NP40, 50 mM Tris (pH 8.0), 1 mM EDTA, 1X protease inhibitor cocktail (Complete™ EDTA-free, Roche). Cell lysates were cleared by centrifugation at 14,000 rpm for 15 min at 4°C. 10 µg of M2 anti-flag antibody (Sigma) was added to the cleared lysates and incubated overnight. 30µl of Protein G agarose bead suspension (Roche) was added for 2h at 4°C. Finally, beads were washed three times with 1 ml of cold lysis buffer. Bound proteins were eluted with 2X SDS sample buffer and loaded onto 4-15% SDS-PAGE gel (ReadyGel, Bio-Rad). For immunoblotting analysis, proteins were transferred onto Immobilon PVDF membrane (Millipore). The HA-tagged GTPases were detected using rabbit anti-HA antibody (H6908, Sigma) and visualized with Western Lightning Plus chemiluminescence kit (Perkin Elmer).

**siRNA and shRNA knockdowns**

The siRNA duplexes targeting human RADIL were purchased from Invitrogen. Sense strands with dTdT overhangs are listed in 5’ to 3’ direction:

- siRADIL#1: CCAUCGAGCUCCUGUACUUdTdT
- siRADIL#2: CCAGCAGUGCGUCUACUAUdTdT

Control siRNA was a scramble sequence with no homologies in the human genome (Qiagen):

Scramble: UUCUCCGAACGUGACACGUdTdT

siRNA duplexes were transfected using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Briefly, cells were plated in the afternoon and transfected the next day with siRNA duplexes at a final concentration of 40 nM for 5 hours, followed by change of culture medium. The transfection was repeated on day 2 using the same conditions. On the third day, cells were either harvested for evaluation of mRNA knockdown efficiency or used for adhesion and migration assays.

The shRNA hairpins against mouse Radil were cloned into a lentiviral pLKO.1puro vector as AgeI/ EcoRI fragments. Regions targeted are listed in 5’ to 3’ direction:

- shRadil#3: GCAGAGGATCATGACACTGAT
- shRadil#6: CCTCACGTTGAGGACACCTTT

Control shRNA was designed to target GFP, a gene not expressed endogenously.
Lentiviruses were packaged using standard protocols. NMuMG target cell transduction and puromycin selection were done using standard protocols.

**Adhesion assays**
Cells were trypsinized, spun down, and resuspended in serum-free media. Cells were plated on precoated 96-well plates from BD Biosciences. NMuMG cells were plated on fibronectin-coated plates at 2x10^5/well for 1h. AD293 cells were plated on Collagen I-coated plates at 6x10^4/well for 1h. Subsequently, non-adhering cells were removed by a vigorous shake. Wells were washed three times with 250 ul of PBS and finally fixed in 5% paraformaldehyde. For quantitation of adhesion, plates were stained using the fluorescent nucleic acid stain SYTO60 (Molecular Probes, Eugene, OR) at 1:8000 dilution in PBS. Quantitation was done by measuring the absorption at 700 nm using the Odyssey Imaging System (LI-COR, Lincoln, NE). The relative percentage of adhesion was obtained by normalizing to adhesion of control siRNA- or shRNA-treated cells. To carefully control for the number of cells plated, cells were counted using a hemocytometer and the final input was determined by MTS cell viability assay following manufacturer’s recommendations (Promega).

**Migration assays**
Cells were trypsinized, spun down, and resuspended in serum-free media. SW1353 cells were plated at 3x10^4/migration chamber. Chambers were stained with Crystal Violet and quantified after cells were allowed to migrate for 18 hours. Input cell number was controlled similarly to adhesion assays.

**RNA manipulations**
For cell culture experiments, RNA was extracted using an RNEasy kit from Qiagen. Zebrafish RNA was extracted using Trizol reagent (Invitrogen). cDNA synthesis was performed using SuperScript II Reverse Transcriptase from Invitrogen.

**qPCR**
The sequences of the PCR primer pairs and fluorogenic probes (all listed from 5’ to 3’) used for quantification mRNA abundance:

**Human set:**
- hRADIL_F, CCAGCAGAAATGCCCACTCT
- hRADIL_R, CGATTCCCTCGAGCCTGTGA
- hRADIL_probe, VIC-ATGCAGAGCATGGAGG-MGBNFQ
- hGAPDH_F, GGTGGTCTCCTCTGACTTCAACA
- hGAPDH_R, GTGGTCGTTGAGGGCAATG
- hGAPDH_probe, VIC-AACCACCTCCACCTTTGACGCTG-TAMRA

**Mouse set:**
- mRADIL_F, CCTGCTCAAGATCTCCAAAAGG
- mRADIL_R, GCCTGCTTTTCCGCTAGTTCT
- mRADIL_probe, VIC-CACTGTCTGGGAGAAGA-MGBNFQ
- mGAPDH_F, GCTACACTGCCGACTTCAACA
- mGAPDH_R, GAAGGTGGAAGAGTGGGAGTTG
mGAPDH_probe, VIC-CCTGCGACTTCAAC-MGBNFQ

Zebrfish set:
zfRadil_F, TTCAGTGCACGGAGGATAACAC
zfRadil_R, GCCGGGACTTGCATTGG
zfRadil_probe, VIC-CGTCTGACAGTTCTTAG-MGBNFQ
zfGAPDH_F, CCAAGGCTGTAGGCAAAGTAATTC
zfGAPDH_R, GGACACGGAAGGCCATACC
zfGAPDH_probe, VIC-CTCAATGGCAAGCTTA-MGBNFQ

All samples were done in triplicate and the relative RADIL expression level was derived by standardizing the input to control, GAPDH.
SUPPLEMENTAL REFERENCES


### SUPPLEMENTAL TABLE

**Table S1. Summary of morpholino knockdown studies**

<table>
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<th>morpholino</th>
<th>amount injected</th>
<th># affected</th>
<th>% affected</th>
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<tbody>
<tr>
<td><em>radil</em>.ATG</td>
<td>4 ng</td>
<td>347 / 389</td>
<td>89.2</td>
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<td>5 ng</td>
<td>295 / 320</td>
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<td>3 ng</td>
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<td>3.6</td>
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<tr>
<td></td>
<td>5 ng</td>
<td>356 / 598</td>
<td>59.5</td>
</tr>
<tr>
<td></td>
<td>6 ng</td>
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<td>0</td>
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<tr>
<td></td>
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<td>6.9</td>
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</table>

Embryos were scored as affected based on the visual inspection of craniofacial and iridophore defects at 5 dpf.
SUPPLEMENTAL FIGURE LEGENDS

**Figure S1.** Schematic illustration of the domain structure of RADIL and AF6. Abbreviations: RA, Ras-association domain; DIL, diluted; PDZ, domain present in PSD-95, Dlg, and ZO-1/2.

**Figure S2.** (A-C) RADIL expression does not affect a Ras-responsive promoter. (A) The promoter used responds to increasing doses of K-rasV12. (B) Expression of RADIL, or an irrelevant protein, LRRC15, does not affect the luciferase signal at basal levels of promoter activation. (C) Expression of RADIL does not affect the K-rasV12 mediated activation of the promoter.

**Figure S3.** Zebrafish *radil* is ubiquitously expressed. Expression pattern of *radil* was monitored in 24 hpf embryos using *in situ* hybridization with a *radil*-specific probe (antisense) and compared to the control probe (sense).

**Figure S4.** Analysis of additional NC lineages in *radil* morphants. (A) Melanophore pigment cells where analyzed at 48 hpf using either brightfield images or *in situ* hybridization using * dct* (*dopachrome tautomerase*), marker of melanophores. (B) Xanthophore pigment cells were analyzed at 24 hpf using *xdh* (*xanthine dehydrogenase*) *in situ* hybridization analysis. (C) Dorsal root ganglia (DRG) were analyzed at 4 dpf using anti-*Hu* antibody. Stronger staining in the neural tube is consistently seen in *radil* morphants.

**Figure S5.** Exon 4 splice donor morpholino characterization. (A) Schematic diagram illustrating the position of the morpholino (red line) targeting splice donor site of exon 4. Diagonal lines underneath the exons illustrate the usage of a cryptic splice donor site within exon 4. (B) Splice morpholino results in the inhibition of correct exon 4-5 splicing and in the usage of a cryptic splice donor site within exon 4. RT-PCR analysis was conducted at 72 hpf showing a correlation between MO doses resulting in phenotypes (5ng and 8ng) and the presence of a novel aberrant PCR product. Sub-phenotypic dose (3 ng) results in significant amount of wild type PCR product remaining. (C) Predicted effect of splice morpholino on Radil protein sequence. The shorter RT-PCR product was cloned and sequenced. Conceptual protein translation predicts an internal 57 amino acids deletion after the RA domain.

**Figure S6.** Exon 14 splice donor morpholino characterization. (A) Schematic diagram illustrating the position of the morpholino (red line) targeting splice donor site of exon 14. (B) Splice morpholino results in the inhibition of correct exon 14-15 splicing and in the incorporation of the intervening intron. PCR amplification of the exon 14-15 junction showing longer PCR product in injected embryos. Analysis was conducted at 48 hpf.
(C) Predicted effect of splice morpholino on Radil protein sequence. The longer PCR product was cloned and sequenced. Conceptual protein translation predicts premature termination due to a stop codon in the incorporated intron.

**Figure S7.** Dose-response relationship of *radil*.ATG MO. Increasing doses of the *radil*.ATG MO result in increasing severity of phenotypes (jaw phenotype shown).

**Figure S8.** Induction of early NC is unaffected in *radil* morphants. 6-somite (6S) stage embryos were stained for *foxD3* and *sox10*, markers of early NC cell population.

**Figure S9.** Further analysis of the craniofacial skeleton development. (A) *In situ* hybridization analysis using *dlx2a*, which marks the migratory chondrocytic NC cells. Analysis was done at 24 hpf. (B) *In situ* hybridization analysis using *dlx2a* at 48 hpf. (C) *In situ* hybridization analysis using *gsc*, which at 48hpf marks early chondrocytes.

**Figure S10.** Knockdown of *radil* triggers induction of apoptosis. TUNEL assays were conducted on 24 hpf embryos injected with ATG or the mismatch control morpholino. While modest elevation of the TUNEL staining was observed with the control morpholino over the uninjected controls, it was dramatically lower compared to the ATG morpholino.

**Figure S11.** *bcl2* overexpression significantly rescues apoptosis in *radil* morphants throughout the course of the 5-day experiment. TUNEL assays were conducted at indicated times. Embryos at 24 hpf (same as shown in Figure 4) are shown again for the purpose of time course completeness.

**Figure S12.** *bcl2* overexpression does not significantly rescue the migration defect of *crestin*-positive cells. (A) Wild type embryo stained with *crestin in situ* marker illustrating the quantification scheme employed. The distance traveled by *crestin*-positive cells was divided the whole height of the embryo (excluding the yolk extension). Quantification was carried out at three positions (I, II, III) along the length of the yolk extension. (B) Quantitative analysis of *crestin*-positive cell migration showing no significant rescue by *bcl2* mRNA coinjection. (n) represents the number of individual embryos analyzed in each category. Red bars represent averages. Two-tailed Student’s t-test was used to conduct pair-wise comparisons of the data. **, indicates p<1x10^-10; *, indicates p>0.01; no asterisk indicates p>0.1. (C) Representative images of the embryos analyzed in (B).

**Movie S1.** NC migration in wild type (*foxD3:GFP*) embryos. Trunk NC cells were followed as they migrated ventrally towards the yolk extension. Images were taken every 10 minutes for 4h, starting at approximately 24 hpf.

**Movie S2.** NC migration in *radil* morphants, visualized using the (*foxD3:GFP*) transgenic line. Trunk NC cells were followed as they migrated ventrally towards the
yolk extension. Images were taken every 10 minutes for 4h, starting at approximately at 31 hpf.
A

B

C

Smolen_Figure S2.
radil (antisense)

24 hpf

radil (sense)

24 hpf
A

**brightfield**

**dct**

B

**xdh**

C

**α-Hu**

*radil(ATG).MM MO*
A

exon 4

exon 5

B

wild type  8 ng  5 ng  3 ng  water

radil

gapdh

C

Full length:

| RA | DIL | PDZ |

Internal 57 amino acids deletion due to splice MO:

| RA | DIL | PDZ |
A

exon 14  exon 15

B

wild type  Splice MO  wild type  Splice MO

radil  gapdh

C

Full length:

RA  DIL  PDZ

Truncation due to splice MO:

RA  DIL
wild type

radil.ATG 1 ng

radil.ATG 2 ng

radil.ATG 3 ng

radil.ATG 4 ng

radil.ATG 5 ng

Smolen_Figure S7
sox10

foxD3

wild type  radil.ATG MO

Smolen_Figure S8.
A  wild type  radil.ATG MO
dlx2a

B  wild type  radil.ATG MO
dlx2a

c  wild type  radil.ATG MO
gsc
A

B

1. wild type (n=28)
2. radil.ATG (n=37)
3. radil.ATG + bcl2 mRNA (n=32)
4. bcl2 mRNA (n=37)

C

**wild type**
**radil.ATG**
**bcl2 mRNA**
**radil.ATG + bcl2 mRNA**