Protogenin prevents premature apoptosis of rostral cephalic neural crest cells by activating the $\alpha 5\beta 1$-integrin

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The bones and connective tissues of the murine jaws and skull are partly derived from cephalic neural crest cells (CNCCs). Here, we report that mice deficient of protegin (Prtg) protein, an immunoglobulin domain-containing receptor expressed in the developing nervous system, have impairments of the palate and skull. Data from lineage tracing experiments, expression patterns of neural crest cell (NCC) marker genes and detection of apoptotic cells indicate that the malformation of bones in Prtg-deficient mice is due to increased apoptosis of rostral CNCCs (R-CNCCs). Using a yeast two-hybrid screening, we found that Prtg interacts with Radil, a protein previously shown to affect the migration and survival of NCCs in zebrafish with unknown mechanism. Overexpression of Prtg induces translocation of Radil from cytoplasm to cell membrane in cultured AD293 cells. In addition, overexpression of Prtg and Radil activates $\alpha 5\beta 1$-integrins to high-affinity conformational forms, which is further enhanced by the addition of Prtg ligand ERdj3 into cultured cells. Blockage of Radil by RNA interference abolishes the effect of ERdj3 and Prtg on the $\alpha 5\beta 1$-integrin, suggesting that Radil acts downstream of Prtg. Prtg-deficient R-CNCCs display fewer activated $\alpha 5\beta 1$-integrins in embryos, and these cells show reduced migratory ability in in vitro transwell assay. These results suggest that the inside-out activation of the $\alpha 5\beta 1$-integrin mediated by ERdj3/Prtg/Radil signaling is crucial for proper functions of R-CNCCs, and the deficiency of this pathway causes premature apoptosis of a subset of R-CNCCs and malformation of craniofacial structures.

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Congenital craniofacial defects are the most common birth defects in human. Many craniofacial syndromes, such as the Collins, Robin and Di George syndromes, are associated with defects in neural crest (NC) development. In rodents, jaws and the skull vault are derived from the cephalic NC cells (CNCCs) and the head paraxial mesoderm cells. In the mouse embryo, the rostral CNCCs (R-CNCCs) originate from NC between diencephalon to rhombomere 2 (r2) and undergo an epithelial–mesenchymal transition at embryonic day 8.5 (E8.5), colonize the frontonasal primordium and branchial arches; bone formation; integrin

Abbreviation: BA, branchial arch; CM, conditioned medium; CNCCs, cephalic neural crest cells; C-CNCCs, caudal cephalic neural crest cells; E8.5, embryonic day 8.5; NC, neural crest; NCs, neural crest cells; non-TNCCs, trunk non-neural crest cells; Prtg, protegin; Prtg-f, full-length Prtg; Prtg-c, Prtg cytoplasmic domain; PrtgΔC, cytosolic domain-deleted Prtg; r2, rhombomere 2; RC, rostral cephalic; R-CNCCs, rostral cephalic neural crest cells; R-non-CNCCs, rostral non-cephalic neural crest cells; TNCCs, trunk neural crest cells; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

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a receptor by binding its ligand ERdj3.\textsuperscript{15} Although many activities of Prtg have been demonstrated in vitro and in chick embryos,\textsuperscript{15–17} roles of Prtg during mouse development are still unclear. In this study, we generated conventional Prtg knockout mutant mice. Defects of the craniofacial structure are observed in the neonatal Prtg mutants. We demonstrate that the defective skeletal phenotypes are due to abnormal apoptosis of R-CNCCs at E9–E10. The participating molecules involved in Prtg signaling include Radli and high-affinity conformational forms of the s5/1-integrin.

**Results**

**Generation of Prtg knockout mice.** A targeting vector that replaces exons 3–7 of the Prtg gene with the IRES-lacZ\textsuperscript{a} gene upon homologous recombination was generated (Figure 1a). Insertion of IRES-lacZ into the Prtg genome creates a premature termination of the Prtg protein and results in a peptide containing only the first 137 amino acids out of total 1192 amino acids. Germine transmission of the targeted allele was verified by Southern blotting (Figure 1b). No full-length Prtg (Prtg\textsuperscript{f}) protein is expressed in homozygous mice and about half of the amount of Prtg protein is present in heterozygous mice (Figure 1c). Results from immunofluorescence staining confirm that no Prtg protein is expressed in homozygous mice (Figure 1d).

**Prtg\textsuperscript{f}** mice have abnormality of craniofacial skeletons and cartilages. Heterozygous Prtg\textsuperscript{f/f} mice are morphologically normal and fertile. The mating between heterozygous Prtg\textsuperscript{+/−} mice produces Prtg\textsuperscript{−/−} homozygous neonates that are born with a normal Mendelian ratio, but have a higher mortality. In all, 44.4% of neonatal Prtg\textsuperscript{−/−} homozygous mice die within 72 h of birth. Another 11.1% of Prtg\textsuperscript{−/−} mice exhibit growth retardation and die before postnatal day 14; this is apparently due to malnutrition, which is revealed by smaller body sizes and delayed body-weight gain (data not shown). The remaining Prtg\textsuperscript{−/−} mutants survive to adulthood and are fertile. The progeny from mating between homozygous mice still exhibits the ~45% mortality rate within the first 3 days. We thus focused on finding the defects that are responsible for the death of the Prtg\textsuperscript{−/−} mutants within 72 h after birth.

As neonatal homozygotes have lower amounts or no milk in their stomachs (Figure 1f), we examined the enteric nervous system by measuring acetylcholinesterase activity in P1 gastrointestinal tract. There is no apparent difference of neuronal innervation in the intestine between the wild-type and Prtg\textsuperscript{−/−} mice (Figures 1g and h). Examination of the developing nervous system in E10.5 embryos by whole-mount staining using antibody against 165-kDa neurofilament reveals no abnormalities in the Prtg\textsuperscript{−/−} embryos (Figure 1j). The gross morphology of the cerebral cortex, hippocampus, eye, olfactory bulb, cerebellum and spinal cord in P1 Prtg\textsuperscript{−/−} mutants is normal by eosin and hematoxylin staining (data not shown).

Defective nasal structures including the palatal bones and/or nasal septum may bring about ingestion difficulty and/or a respiration deficiency, causing the death of the neonatal Prtg\textsuperscript{−/−} pups. Thus, we examined the cranial bones and cartilage of the P1 Prtg\textsuperscript{−/−} mutants with Alizarin red and Alcian blue staining. The Prtg\textsuperscript{−/−} mutants are found to have a shorter and thinner nasal septum (black-dotted circle in Figure 1l). In some severe cases, there is no nasal septum. Incomplete fusion of the basisphenoid bone is also observed in the Prtg\textsuperscript{−/−} knockout mice (arrow in Figure 1l). In addition, the palate of the Prtg\textsuperscript{−/−} mice is found to be thinner (arrow in Figure 1m). Furthermore, the Prtg\textsuperscript{−/−} mutants displayed a loss of branches of the ala temporalis and less mineralization of the parietal bone, temporal bone and supraoccipital bone (Figures 1p and r). The penetrance of skeletal defects ranges from 42.8 to 61.1% (Figure 1s). It is noted that all survived mutant pups contain normal nasal septum and palate when examined at P4, suggesting that the cause of perinatal death is likely due to defects of these two structures.

**Fewer rostral CNCCs are present in the Prtg\textsuperscript{−/−} mouse embryos.** The observed craniofacial defects of Prtg\textsuperscript{−/−} mutants are in structures that are developmentally derived from R-CNCCs and head paraxial mesoderm (Figure 1s). We conducted lineage tracing experiments by generating Prtg\textsuperscript{−/−} mutants with a Wnt1-Cre;R26R genetic background in which the NC-derived cells are marked with β-galactosidase.\textsuperscript{18,19} When Prtg\textsuperscript{−/−}; Wnt1-Cre;R26R embryos were examined at E10.5, fewer X-gal\textsuperscript{+} cells were detected in the diencephalon, mesencephalon and frontonasal primordium compared with those in the heterozygous embryos (arrowheads in Figures 2a and b, n = 6 for each genotype type). In addition, there are also fewer X-gal\textsuperscript{+} cells in dorsal midbrain, frontonasal process and upper jaw region...
of the E13.5 homozygous embryos (Figures 2c and d, n = 7 for each genome type). These results suggest that fewer R-CNCCs are present in Prtg<sup>−/−</sup> mutants. Notably, there is no defect in the X-gal-staining patterns of the dorsal root ganglia that develop from TNCCs in homozygous embryos.

To confirm that there are losses of R-CNCCs in the absence of Prtg protein, we analyzed expression patterns in E9–E10 embryos of several NCC markers, which are also critical for the development of craniofacial structures by whole-mount in situ hybridization. In wild-type embryos, the expression of
Twist1 is detected in the maxilla and mandible of BA1. A drastic decrease in Twist1 expression in BA1 is detected in Prtg−/− mutants (Figure 2f). Diminished Lhx7 in the frontonasal process and the maxilla of BA1 is also observed in Prtg−/− mutant embryos (arrows in Figure 2h). To examine whether C-CNCCs are defective in Prtg−/− mutant embryos, we analyzed expression of Msx2, a marker for NCCs derived from posterior midbrain to rhombencephalon NC. A decrease in Msx2 expression is detected in the maxilla and mandible of Prtg−/− mutant embryos at E9.5 (white arrows in Figure 2i), expression of Msx2 in BA2 and BA3 is the same between wild-type and Prtg−/− mice (red arrows in Figures 2i and j). Analysis of Dlx5 expression also indicates that the development of BA2 and BA3 is normal in the Prtg−/− embryos. Expression of Fgf8 in anterior neural ridge (black arrows) and BA1 epithelium (white arrows) is the same between wild-type and Prtg−/− embryos (arrows in Figures 2o and p).
Prtg is involved in the survival but not proliferation of rostral CNCCs. Fewer R-CNCCs present in Prtg<sup>−/−</sup> embryos could be due to cell death and/or decreased cell proliferation. To discriminate these possibilities, we conducted a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to detect whether there is a change in cell survival in Prtg<sup>−/−</sup> embryos. In the rostral cephalic sections, there are 18 ± 2.6% more TUNEL<sup>+</sup> cells in E9.0 Prtg<sup>−/−</sup> embryos, 43 ± 2.2% more TUNEL<sup>+</sup> cells in E9.5 Prtg<sup>−/−</sup> embryos and 66 ± 15% more TUNEL<sup>+</sup> cells in E10.0 Prtg<sup>−/−</sup> embryos compared with Prtg<sup>/+</sup> embryos (Figures 3a–k). The increase in TUNEL<sup>+</sup> cells is spatially restricted to rostral cephalic region and temporally to the period between E9.0 and E10.0. We also performed a 6-h BrdU labeling for cell proliferation analysis in E8.5 and E9.5 Prtg<sup>−/−</sup> embryos during the time when expression of the Prtg protein is at its highest. There is no decrease of cell proliferation rate in rostral and caudal cephalic/trunk sections at either time points (Figure 3l, and data not shown).

To verify that the increased TUNEL<sup>+</sup> cells are NCCs, mouse embryos with a Wnt1-Cre;CAT-EGFP<sup>flox</sup> genetic background are used. Apoptotic cells are revealed by cleaved caspase 3 (cCasp3<sup>+</sup>) staining. Colocalization of cCasp3<sup>+</sup> and EGFP<sup>+</sup> staining indicates that the apoptotic cells are NCCs. At E9.5, there is a 95 ± 22% more cCasp3<sup>+</sup>/EGFP<sup>+</sup> R-CNCCs in the Prtg<sup>−/−</sup> mutant embryos compared with heterozygous embryos (Figures 4a–h and y). No increase of cell death is observed in TNCs of Prtg<sup>−/−</sup>;Wnt1-Cre;CAT-EGFP<sup>flox</sup> embryos (Figures 4i–l and y). Furthermore, apoptosis among non-NCCs (cCasp3<sup>−/−</sup>/EGFP<sup>−/−</sup>) cells along the entire body axis is the same between heterozygous and homozygous embryos (Figure 4y). These results reveal that the increase in apoptotic cells observed in the rostral cephalic region of the Prtg<sup>−/−</sup> embryos during E9–E10, as shown in Figure 3, are likely to involve R-CNCCs. Note that no increase of apoptotic cells are observed in dorsal neural tube (Figure 3b), suggesting that Prtg dose not affect delamination of CNCCs from the neural tube.

As a part of craniofacial skeletal defects exhibited in P1 Prtg<sup>−/−</sup> mutants is derived from the head paraxial mesoderm lineage, we conducted TUNEL experiments using Mesp1-Cre;CAT-EGFP<sup>flox</sup> mice<sup>18</sup> to examine effects of Prtg protein on mesoderm derivatives. It was found that few apoptotic cells are paraxial mesoderm-derived cells in rostral cephalic region (white arrows in Figures 4m–t and z) and trunk region (Figures 4u–x and z) of either E10 Prtg<sup>−/−</sup>.
Figure 4  Increase of apoptotic R-CNCCs in E9.5 embryos. (a–l) Transverse sections of E9.5 Prtg⁺⁻⁻;Wnt1-Cre;CAT-EGFP⁺lox and Prtg⁻⁻⁻; Wnt1-Cre; CAT-EGFP⁺lox embryos were labeled with the antibody against cCasp3. (a–h) At the rostral cephalic level. cCasp3⁺/EGFP⁺ cells are marked with white arrows and cCasp3⁺/EGFP⁻ cells are marked with white arrowheads. (c, e, g) and (d, f, h) are higher magnifications of the white boxes in (a) and (b). (i–l) At the trunk level. (k) and (l) are higher magnifications of the white boxes in (i) and (j). (m–x) Transverse sections of E10 Prtg⁺⁻⁻;Mesp1-Cre;CAT-EGFP⁺lox and Prtg⁻⁻⁻; Mesp1-Cre;CAT-EGFP⁺lox embryos were subjected to TUNEL assay. (m–t) At the rostral cephalic level. (u–x) At the trunk level. White arrows mark TUNEL⁺/EGFP⁺ cells. (o, q, s) and (p, r, t) are higher magnifications of the white boxes in (m) and (n). (w) and (x) are higher magnifications of the white boxes in (u) and (v). Scale bar, 600 μm for (a, b), (i, j, m, n, u, v); 125 μm for C–H; 250 μm for (k, l); 100 μm for (o–t) and (w, x). (y) Quantification of the apoptotic cells in Prtg⁺⁻⁻;Wnt1-Cre;CAT-EGFP⁺lox and Prtg⁻⁻⁻;Wnt1-Cre;CAT-EGFP⁺lox embryos at levels of rostral cephalic and trunk regions. All sections were examined for EGFP and cCasp3 staining. The numbers of the cCasp3⁺ cells per field were counted. The results are shown as the mean ± S.E.M. (n = 4; *P < 0.05, by Student’s t-test). R-non-CNCCs: rostral non-cephalic NCCs; non-TNCCs: trunk non-NCCs. (z) TUNEL⁺/EGFP⁺ cells in Prtg⁺⁻⁻;Mesp1-Cre;CAT-EGFP⁺lox and Prtg⁻⁻⁻;Mesp1-Cre;CAT-EGFP⁺lox embryos were quantified and shown as the mean ± S.E.M. (n = 4). RC: rostral cephalic level.
Mesp1-Cre;CAT-EGFP^{flox} or Prtg^{−/−};Mesp1-Cre;CAT-EGFP^{flox} embryos. Furthermore, the earliest time point that defective alkaline phosphatase activity, an early marker of osteogenesis, can be detected in parietal bone is at E15.5 (data not shown). As the defect occurs long after Prtg ceases to be expressed at E10, we reason that it is a consequential effect due to the loss of R-CNCCs.

Prtg protein interacts with Radil. We used the Prtg cytoplasmic domain (Prtg-c) as a bait to search molecules that mediate signaling of Prtg protein in a yeast two-hybrid assay. Among 1.5 × 10^6 independent clones, 58 DNA fragments were found to interact with Prtg-c. One of these, Radil, shows the strongest interaction with Prtg-c (Figure 5a). As the knockdown of Radil, a Rap1 GTPase interactor,
interrupts migration of NCCs and results in their apoptosis in zebrafish, 24 we thus focused on Radil as a putative Prtg downstream binding partner. Immunoprecipitation was performed using HEK293T cell lysates containing overexpressed Flag-tagged Prtg-c and Myc-tagged Radil. The anti-Flag immune complex was then subjected to western blotting. Radil is co-precipitated with Prtg-c (Figure 5b). Moreover, Radil interacts with Prtg-f but not with full-length Punc (Punc-f) (Figure 5c). The extracellular domain of Punc has the highest identity (43.8%) with that of Prtg. 28 The interaction between Prtg and Radil is mediated through the cytoplasmic domain of Prtg, as cytosolic domain-deleted Prtg (PrtgΔC) cannot associate with Radil (Figure 5c, lane 3).

We also analyzed interaction between Prtg and Radil at the cellular level. When overexpressed alone, Radil is localized in a cytoplasmic pool in HEK293T cells (Figure 5d and Ahmed et al. 26). Overexpression of Prtg-f, but not PrtgΔC nor Punc-f, leads to the translocation of Radil to the plasma membrane where it colocalizes with Prtg-f (Figures 5e–g). In addition, when sections of E9.0 Prtg+/−; Wnt1-Cre; CAT-EGFPflox embryos are examined with antibody against Radil, Radil is clustered as punctate particles concentrated along cell–cell junctions in R-CNCCs (Figures 5h and i); however, Radil is somewhat evenly distributed in Prtg-null R-CNCCs (Figures 5j and k), further supporting that Radil is recruited to plasma membrane by Prtg.

ERdj3/Prtg/Radil signaling promotes cell migration by inducing cell surface expression of the high-affinity α5β1-integrin. As Radil was shown to control migration of NCCs in zebrafish, 24 we examined whether Prtg facilitates cell migration by recruiting Radil in the transwell migration assay using cultured AD293 cells. Ectopic expression of Radil or Prtg promotes 39 ± 5% and 45 ± 7% more cell migration, respectively (Figure 6a, bars 2 and 3). Overexpression of Radil and Prtg together in AD293 cells produces a synergistic effect that results in 107 ± 13% increase in cell migration (Figure 6a, bar 4). When conditioned medium (CM) containing ERdj3 (ERdj3-CM) is added to the lower chamber of the transwell, 309 ± 69% more cell migration is detected in AD293 cells overexpressing both Prtg and Radil (Figure 6a, bar 10). Effects of ERdj3 and Prtg on AD293 cell motility are completely abolished when Radil is knocked down by RNA interference (Figure 6b, bar 6 versus bar 8).

A recent study shows that Radil regulates adhesion of cultured cells on fibronectin by activating β1-integrin. 26 Furthermore, knockdown of α5-integrin triggers apoptosis of CNCCs. 13 We thus utilized cell cytometry to examine whether increased expression of high-affinity β1- and α5-integrin subunits on the cell surface is the underlying mechanism responsible for cell mobility induced by ERdj3/Prtg/Radil signaling. Overexpression of either Prtg or Radil increases presentation of high-affinity β1- and α5-integrin subunits on the plasma membrane (Figures 6c–f). An addition of ERdj3-CM to AD293 cells overexpressing both Prtg and Radil increases cell surface expression of high-affinity β1/integrin and α5-integrin to about three times of the control level (Figures 6d and f, bar 8 versus bar 1). Note that the extent of increased expression of the activated β1- and α5-integrin subunits induced by various treatments of ERdj3, Prtg and Radil match well to the levels of cell motility induced by these treatments. We further demonstrated that overexpressed Prtg is able to co-precipitate endogenous β1/integrin in AD293 cells in an immunoprecipitation assay (Figure 6g).

Expression of the high-affinity α5β1-integrin decreases in Prtg-null R-CNCCs. We further interrogate levels of high-affinity α5β1-integrins on R-CNCC cell membrane in Prtg+/−; Wnt1-Cre; CAT-EGFPflox mutant embryos by immunofluorescence staining. About 35% less of immunofluorescence intensity of the activated β1-integrin subunit are detected in E9.0 Prtg-null R-CNCCs and rostral non-CNCCs; however, no change of the activated β1/integrin subunit expression is detected in Prtg-deficient TNCCs and non-TNCCs (Figures 7a–h, q). Expression of the activated α5-integrin subunit in E9.0 R-CNCCs, but not rostral non-CNCCs, also decreases 20% in the absence of Prtg protein (Figures 7i–p and q). Note that amounts of total β1- and α5-integrin subunits do not change in Prtg+/− mutant embryos (Figure 7r). To confirm there are fewer high-affinity α5β1-integrins in Prtg-null R-CNCCs, we examined their cell mobility using the in vitro transwell assay. When dissociated cells isolated from E9.0 embryos were put to test, there were 45% fewer Prtg-null R-CNCCs migrating through the membrane than Prtg heterozygous R-CNCCs. In contrast, only 22% fewer Prtg-null rostral non-CNCCs migrate through the membrane than the Prtg heterozygous rostral non-CNCCs (Figure 7s). No difference in migration ability is detected in Prtg-null TNCCs and non-TNCCs. These results suggest that decreased expression of both high-affinity α5- and β1-integrin subunits might exacerbate the deficiency of integrin activity in Prtg-null R-CNCCs.
Discussion

NCCs are a transient population of cells that undergo epithelial-to-mesenchymal transition along the anteroposterior body axis. Depending on their origins in the neural tube, NCCs migrate and populate in cranial, cardiac, trunk and enteric domains. 3 In each domain, these cells enter into various tissues and become cell types that match the needs of that particular tissue by interacting with neighboring cells.
Here we report that an immunoglobulin family member, Prtg, is involved in the survival of R-CNCCs that contribute to the osteogenic and chondrogenic cells in the craniofacial structures. In Prtg\textsuperscript{-/-} embryos, increased cell death is detected in R-CNCCs during E9–E10 (Figure 8a).

Later in development, the loss of these cells delays the development of mesoderm-derived mesenchymal cells. These direct and indirect effects manifest the craniofacial phenotypes that we detect in Prtg\textsuperscript{-/-} mutant mice at birth. We further provide in vitro evidence that Prtg recruits Radil to...
the plasma membrane, and subsequently changes the
structure of the α5/1-integrin to high-affinity conformation,
and facilitates cell migration (Figure 8b). In the absence of
Prtg proteins, fewer Radil proteins are translocated to
the membrane, and consequently fewer α5/1-integrins are
activated to high-affinity conformation in Prtg−/− embryos;
thus, a subset of R-CNCCs undergoes premature apoptosis.
As Radil associates with Rap1, and talin is the converging
point in the inside-out integrin activation,26–28 ERdj3/Prtg/ Radil signaling pathway may also involve Rap1 and talin
to activate α5/1-integrin (Figure 8b).

**Signaling involved in the survival of R-CNCCs.** Two
possible, but not exclusive, scenarios may explain how cell
death program is initiated in a subset of Prtg-deficient
R-CNCCs as observed in this study. First, it had been shown
that the α5/1-integrin supports survival of cultured cells
grown on fibronectin by upregulating Bcl-2 expression.29
Signaling molecules, including Shc, FAK, Ras, PI3K and Akt,
are required for the increase of bcl-2 transcription induced by
α5/1-integrin activity.30 It is thus likely that extracellular
matrix molecules in Prtg−/− embryos cannot transmit
survival signals for Prtg-deficient R-CNCCs due to insuffi-
cient amounts of the high-affinity α5/1-integrin on plasma
membrane of these cells. Alternatively, the migration defect
may be the cause for R-CNCC death. In the absence of Prtg,
sufficient exclusion of high-affinity α5/1-integrin interrupts
migration of a subset of R-CNCCs along their migratory
pathways toward frontonasal primordium and BA1, thus
preventing their access to the local survival factors, such as
BMP2, BMP4 and FGF8.22,31 Results that 45% fewer
Prtg-null R-CNCCs migrate through the transwell membrane
(Figure 7s) lend some support to the latter possibility. In
addition, Smolen et al.32 showed that the blockage of
apoptosis is not able to prevent craniofacial defects observed
in zebrafish after knocking down Radil, suggesting that
effects of Radil are primarily on migration. Owing to many
cells migrating at the same time in mouse E8.5–E9.0

![Figure 8](https://example.com/figure8.png)

**Figure 8** A proposed model of ERdj3/Prtg signaling for craniofacial bone formation. (a) R-CNCCs populate at fontonasal primordium and BA1 during E9–E10 (pink dots). Upon loss of Prtg, increased apoptosis of a subset of R-CNCCs (marked as *) occurs. (b) Stimulation of Prtg by ERdj3 in R-CNCCs recruits Radil, Rap1 and talin to form a complex, which induces conformational changes of the α5/1-integrin. If expression of the high-affinity α5/1-integrin reaches above a threshold, full migratory and survival signaling for R-CNCCs ensues.

![Figure 7](https://example.com/figure7.png)

**Figure 7** Lower levels of the activated α5/1-integrin are expressed in the Prtg-null R-CNCCs. Transverse sections of Prtg−/−;Wnt1-Cre;CAT-EGFPlox and Prtg−/−;Wnt1-Cre;CAT-EGFPlox were labeled with the antibody against activated j/1- (a–h) or activated j/5-integrin (i–p). At the cephalic level (a–d), expression of the activated j/1-integrin in Prtg−/− embryos is decreased in R-CNCCs and rostral non-CNCCs (R-non-CNCCs) (b, d) than in those in Prtg−/− embryos (a, c). No difference between Prtg−/− and Prtg−/− embryos is observed in TNCCs and trunk non-NCCs (e–h). Expression of the activated α5-integrin is decreased in Prtg−/− R-CNCCs (j), but not in R-non-CNCCs, TNCCs and non-TNCCs (I, n, p). Scale bar, 10 μm. (q) Quantification of the mean fluorescence intensities of the activated j/1- and α5-integrin subunits in each cell in Prtg−/−;Wnt1-Cre;CAT-EGFPlox and Prtg−/−;Wnt1-Cre;CAT-EGFPlox embryos. Fluorescence intensities of more than 400 cells of each group were measured, and mean intensity was then calculated. Data (mean ± S.E.M.) were then normalized against those of Prtg−/− embryos (n = 4; * P < 0.05, by Student’s t-test). (r) Expression of j/1- and α5-integrin subunits in embryos was analyzed by western blots. (s) Quantitative results of the cell mobility assay. Cells from different regions of Prtg−/−;Wnt1-Cre;CAT-EGFPlox or Prtg−/−;Wnt1-Cre;CAT-EGFPlox embryos were dissociated and subjected to transwell migration assay. Ratios of cells migrating through membrane were calculated and further normalized to the Prtg−/− groups and were shown as the mean ± S.E.M. (n = 3; * P < 0.05, by Student’s t-test).
embryos and only a part of R-CNCCs is affected by Prtg deficiency, we were not able to resolve these two possibilities in this study. Finding a new way to label only a few R-CNCCs in mouse embryos and performing direct comparison of migration rates of R-CNCCs in control and Prtg−/− embryos will be needed to solve the issue.

Presence of a redundant gene(s) that substitutes loss of Prtg. Expression of Prtg protein in mesoderm begins as early as E7 and is present in almost all cells between E8.25 and E9.5, except in the notochord and differentiating cardiac cells.15 When levels of Prtg mRNA are perturbed by an RNA interference approach in stage 6 chick embryos, ingestion of paraxial mesoderm precursor cells is impaired.17 If levels of Prtg mRNA are perturbed at stage 9, precocious neuronal differentiation in the developing chick neural tube occurs.18 Furthermore, using a mouse mandible organ culture, Takahashi et al.18 showed that knockdown of Prtg mRNA in tooth germ causes an arrest of the tooth development. These results suggest that Prtg may have roles in migration of primitive mesoderm cells, neuronal differentiation and tooth germ formation. Thus, it is unexpected that no embryonic lethality, no abnormality in the nervous system and no defects in dental tissue could be detected in Prtg−/− mutants. Further studies are required to find out why the discrepancy occurs and to search for the redundant gene compensating the loss of Prtg during development.

Materials and Methods

Animals. Adult C57BL/6J mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan, ROC). CAT-EGFPflox mice were described previously.32 R26R transgenic mice were kindly provided by Ting-Fen Tsai. Wnt1-Cre mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). Mesp1-Cre mice were obtained from RIKEN BioResource Center (Ibaraki, Japan).32 All mice were handled according to the university guidelines and experiments were approved by the National Yang-Ming University Animal Care and Use Committee. For the timed pregnancies, mice were set up in the late afternoon and when plugs were detected the next morning, this was designated as E0.5.

Materials. Expand high-fidelity Taq DNA polymerase, deoxynucleotides, 4′,6-diamidino-2-phenylindole (DAPI), the in situ Cell Death Detection kit (TUNEL), reagents used in the in situ hybridization were obtained from Roche (Indianapolis, IN, USA). Taq DNA polymerase was purchased from Promega (Madison, WI, USA). The primers used in the PCRs were synthesized by MBiol (Taipei, Taiwan). All other chemicals, unless otherwise specified, were purchased from Sigma (St. Louis, MO, USA).

Vector construction. The various Prtg plasmids had been constructed previously.15 The mouse open reading frames of Punc and Radl were amplified by PCR using primers listed in Supplementary Table S1 and cloned to eEF1-Myc-His. The plasmids containing Dlx5, Fgf8, Lhx7 and Mesp2 for in situ hybridization were provided by J Rubenstein (Department of Psychiatry, UCSF, San Francisco, CA, USA), Y-T Yan (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan), V Fachnis (National Institute for Medical Research, Medical Research Council, London, UK) and Y-H Liu (Department of Biochemistry and Molecular Biology, Kenneth R. Norris Cancer Hospital and Institute, Los Angeles, CA, USA), respectively. Other probes were generated by PCR using E9.5 mouse cDNA as template and cloned in pCRII-TOPO (Invitrogen, Grand Island, NY, USA). DNA fragments containing Radl sequences and scramble sequences (Supplementary Table S1) were cloned into pUH-puro-SIBR to give rise to siRadl and siS. All plasmids were verified by restriction enzyme mapping and/or sequencing.

Antibodies. Mouse anti-Prtg monoclonal antibody (PRTG2, 1 μg/ml) has been described previously.15 Mouse anti-β-tubulin antibody (E7, 1 : 10 000), mouse anti-neurofilament antibody (2H3, 1 : 900) and anti-Myc antibody (9E10, 1 : 5000) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA). Rabbit monoclonal antibody against cCasp3 (S1A1E, 1 : 400) was purchased from Cell Signaling (Danvers, MA, USA). Mouse anti-Flag M2 monoclonal antibody (1 : 1000) and rabbit anti-Flag polyclonal antiseraum (1 : 1000) were purchased from Sigma. Mouse anti-activated αs-integrin antibody (SNAKAS1, 10 μg/ml) was from Dr. MU Humphries. Rabbit anti-αs-integrin antibody (1 : 1000) was from Chemicon (Billerica, MA, USA). Rat anti-β1-integrin antibody (9EG7, 10 μg/ml) was from BD Bioscience (San Jose, CA, USA). Rabbit anti-β1-integrin antibody (ab52971, 1 : 1000) was from Abcam (Cambridge, MA, USA). Rabbit anti-Radil antibody (1 : 300) was from Abgent (San Diego, CA, USA).

Targeted disruption of the mouse Prtg gene. The targeting vector was linearized and electroporated into 129/Sv ES cells. G418-resistant ES colonies obtained by homologous recombination were selected by Southern blot hybridization. Two correctly targeted ES cell lines and one Neo pop-out ES cell line were injected into blastocysts and the resultant chimeras bred with C57BL/6J mice. Genotyping was performed by Southern blotting and/or PCR using the primers listed in Supplementary Table S1. Heterozygous F1 mice were backcrossed with C57BL/6J mice and mice after congenic N12 were used in the study.

Histological analysis, X-gal staining and whole-mount in situ hybridization. Embryos were dissected and fixed with 4% paraformaldehyde at 4 °C for 20 min (for X-gal staining), or overnight (for in situ hybridization and histological staining). For tissue sections, embryos were further soaked in 30% sucrose for overnight and then cryostat sectioned at 12 μm. For the histology, frozen sections were stained with hematoxylin–eosin using standard procedures. For whole-mount X-gal staining, the embryo were processed for β-galactosidase activity as described.15 The in situ hybridization procedure was performed as described previously.28 The protocols of staining with Alizarin red to reveal bone and Alcian blue to reveal cartilage were published previously.26

Detection of cell death. Transverse sections of embryos were cut at 12 μm and subjected to TUNEL assay and DAPI staining. Alternatively, sections were labeled with antibodies against cCasp3. The numbers of TUNEL+ and cCasp3+ cells were counted manually.

Yeast two-hybrid analysis. The rat Prtg-c (amino acids 966–1193) was cloned into the vector pBTM-116 as a bait. An E7 mouse embryo cDNA library fused to the GAL4 activation domain (Clontech) was used as the prey library. Other procedures have been described previously.15

Cell culture, protein extraction, immunoprecipitation and Western blotting. Previously described procedures were followed for these experiments.15

Migration assay. Human AD293 cells were transfected with different plasmids using calcium phosphate method and cultured for 2 days in 10% FBS/DMEM at 37 °C in a 5% CO2 incubator. Cells were then detached from culture dishes using 1 μM EDTA and resuspended in 0.5% FBS/DMEM medium. A total of 1 × 105 cells was seeded to the migration chamber (Millicell with 8 μm pore size; Millipore, Billerica, MA, USA); the lower chambers contained CM collected from pEF1- or pERdj3-transfected HEK293 cells. The experiments were performed for 24 to 72 h after transfection. Duration of migration lasted 4 h and migratory cells were stained with Hoechst 33258 and quantified by counting four random fields.

For detection of primary cell mobility, tissues from rostral cephalic region and trunk region were dissected from E9.0 (15–16 somite stage) embryos and dissociated with collagenase (0.75 mg/ml) at 37 °C for 5 min.16 Dissociated cells were filtered through 40 μm cell strainers (BD Falcon, San Jose, CA, USA). A total of 3 × 105 cells were seeded to the upper chamber coated with fibronectin. Duration of migration lasted 5 h. EGFP+ and EGFP− cells in the upper chambers and bottom chambers were fixed and stained with DAPI, and quantified under a fluorescence microscope. The ratio of migration is defined as cell numbers of the bottom chambers divided by the total cell numbers in both chambers and normalized to that of control embryos.
Flow cytometry. AD293 cells were dissociated with trypsin, washed two times with PBS, re-suspended in DMEM containing 0.1% BSA and 20 mM HEPES, pH 7.4. Cells were then recovered at room temperature for 30 min. Cells were incubated with anti-ph1-integrin (9E6G) or anti-ph5-integrin (SN6A51) antibody at room temperature for 1 h. For positive control, 2 mM Mn²⁺ was added when cells were incubated with primary antibodies. Cells were washed three times with PBS and incubated with Alexa-488-conjugated donkey anti-rat IgG or Alexa-488-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, Baltimore, PA, USA) at room temperature for 45 min. After three washes in PBS, cells were fixed in 4% paraformaldehyde at room temperature for 15 min and re-suspended in PBS. Flow cytometry measurement was taken in a FACSCalibur flow cytometer using the CellQuest Pro software (BD Biosciences) for data acquisition and analysis. Integrin activation was expressed as the ratio of the mean of fluorescence to the Mn²⁺-treated group.

Conflict of Interest
The authors declare no conflict of interest.

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Author contributions
Y-CW, H-CJ, Y-HW and M-JF conceived and designed the experiments. Y-CW, H-CJ, Y-HW, W-CY, Y-LL, S-FL and C-L conducted experiments. Y-CW and M-JF wrote the manuscript.

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