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# Stage-Specific Control of Neural Crest Stem Cell Proliferation by the Small Rho GTPases Cdc42 and Rac1

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## SUMMARY

**The neural crest (NC) generates a variety of neural and non-neural tissues during vertebrate development. Both migratory NC cells and their target structures contain cells with stem cell features. Here we show that these populations of neural crest-derived stem cells (NCSCs) are differentially regulated by small Rho GTPases. Deletion of either *Cdc42* or *Rac1* in the NC results in size reduction of multiple NC target structures because of increased cell-cycle exit, while NC cells emigrating from the neural tube are not affected. Consistently, *Cdc42* or *Rac1* inactivation reduces self-renewal and proliferation of later stage, but not early migratory NCSCs. This stage-specific requirement for small Rho GTPases is due to changes in NCSCs that, during development, acquire responsiveness to mitogenic EGF acting upstream of both Cdc42 and Rac1. Thus, our data reveal distinct mechanisms for growth control of NCSCs from different developmental stages.**

## INTRODUCTION

The neural crest (NC) is a transient population of cells in higher vertebrates that, during embryonic development, emigrates from the dorsal neural tube to generate most of the peripheral nervous system and a variety of non-neural structures (Le Douarin and Dupin, 2003). Among fate-restricted cells, the NC harbors many cells termed neural crest stem cells (NCSCs) that display self-renewal capacity and multipotency in clonal cell culture assays. Intriguingly, very similar cells in terms of marker expression and potential have also been isolated from NC-derived structures during fetal development and even from the adult organism (Delfino-Machin et al., 2007). In vivo fate mapping revealed a lineage relationship between early, emigrating (e)NCSCs and NCSCs found at later stages (Wong et al., 2006). These findings suggest that a fraction of undifferentiated NCSCs must be maintained by self-renewal throughout development and postnatal stages, while other NC progeny undergo fate restriction, eventually exit the cell cycle, and differentiate. The mechanisms regulating NCSC maintenance and proliferation are only partially understood. In *Xenopus*, the transcriptional regulator Id3 is essential for NC formation from the neural plate and promotes proliferation and expansion of the NC domain (Kee and Bronner-Fraser, 2005). At similarly early stages of murine NC development, combinatorial Wnt and BMP suppress differentiation and, in the presence of FGF2, maintain mitotic eNCSCs (Kleber et al., 2005). However, NCSCs from sciatic nerves and dorsal root ganglia lose Wnt responsiveness, pointing to distinct mechanisms of growth control in different NCSC populations. At later stages, NC-derived stem and progenitor cells from the gut and the skin are maintained by endothelin 3 signaling or by the activity of the polycomb group transcriptional repressor Bmi-1 (Bondurand et al., 2006; Molofsky et al., 2003; Real et al., 2006), but the relatively mild or lineage-specific phenotypes of the respective mouse mutants do not support a general and indispensable role of these factors in NCSC expansion.

Cdc42 and Rac1 are ubiquitously expressed small Rho GTPases that act as molecular switches, cycling between an active GTP-bound and an inactive GDP-bound state (Jaffe and Hall, 2005). Rho GTPases play a central role in linking extracellular stimuli with the induction of multiple specific downstream effectors resulting in a variety of cellular responses. These include effects on cell polarity, migration, differentiation, proliferation, and apoptosis (Jaffe and Hall, 2005). Earlier studies have largely relied on pharmacological inhibition of Rho GTPases or on expression of dominant-negative or constitutively active forms. Although these studies have been fundamental for the understanding of basic Rho GTPase biology, these approaches are potentially nonspecific due to cross-activation or inhibition of other effectors. Moreover, being central molecules within a plethora of signaling pathways, Rho GTPase function can significantly vary from one cell type to another. To analyze a given Rho GTPase in a specific cell type in an in vivo setting, conditional gene targeting of specific Rho GTPases in transgenic mice has, therefore, become the method of choice (Wang and Zheng, 2007).

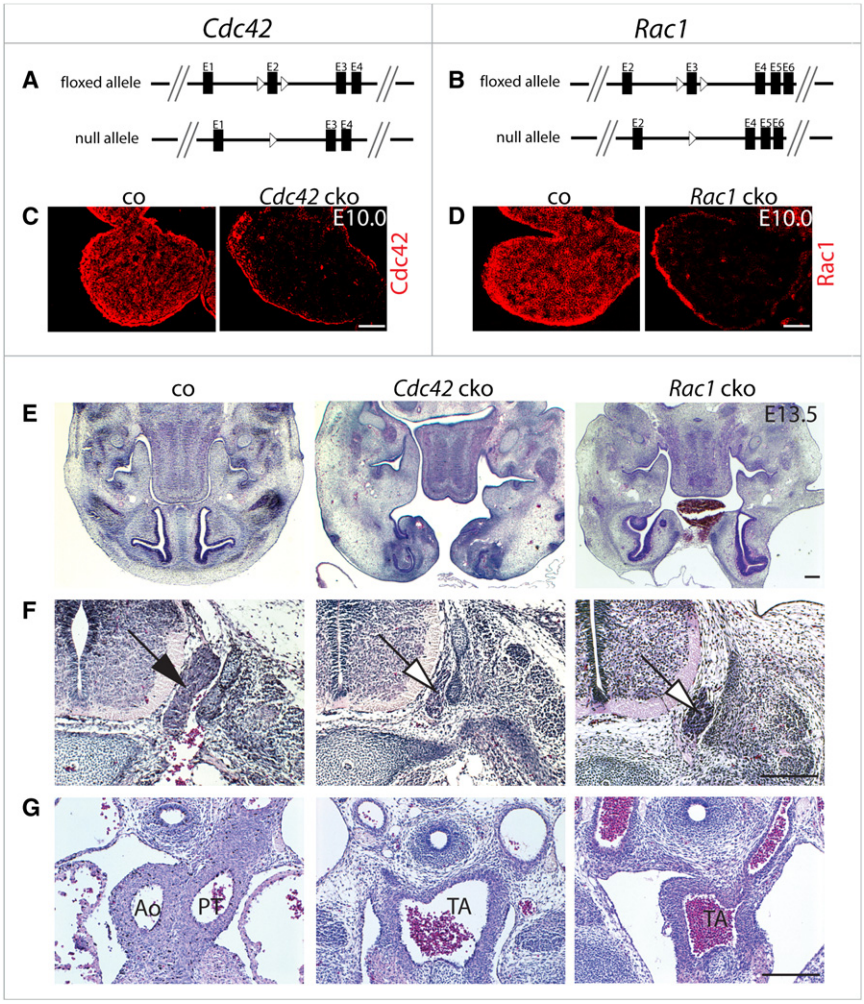


**Figure 1. Similar Developmental Defects in NC Derivatives of *Cdc42* and *Rac1 cko* Embryos**

(A and B) Exon 2 (E2) of the *Cdc42* locus and Exon 3 (E3) of the *Rac1* locus, respectively, are flanked by loxP sites (floxed allele) and deleted (null allele) in NC cells upon breeding with *Wnt1-Cre* mice.

(C and D) Loss of Cdc42 and Rac1 protein, respectively, in mutant NC cells populating pharyngeal arch 1 (PA1) at E10.0.

(E) Sagital median cleft in *Cdc42* and *Rac1* cko embryos shown on transverse sections at the level of the frontonasal process.

(F) Mutant DRG are present but appear reduced in size (white arrows) when compared to DRG in a control embryo (black arrow).

(G) Failure of aortico-pulmonary septum formation in the heart outflow tract of cko embryos. Scale bars: (C) and (D), 100 μm; (E)–(G), 200 μm. Ao, ascending aorta; PT, pulmonary trunk; TA, truncus arteriosus.

Knowledge about the roles of Rho GTPases in NC development is still limited. RhoV has been identified as an essential regulator of NC induction in *Xenopus* (Guemar et al., 2007). Recently, pharmacological inhibition of Cdc42/Rac1 was reported to affect migration and neurite extension of NC-derived cells in the enteric nervous system (ENS) (Stewart et al., 2007). Here, we performed gene ablation in the NC and find that *Cdc42* and *Rac1* are not required for maintenance, migration, and differentiation of NCSCs in the early phase of NC development. However, at later stages when NC cells have reached their initial targets, proliferation control becomes dependent on *Cdc42* and *Rac1*. Thus, Cdc42 and Rac1 are essential regulators of NC development that distinguish NCSCs from different stages.

## RESULTS

### Inactivation of *Cdc42* or *Rac1* in NC Cells

To assess the functions of *Cdc42* and *Rac1* in NC cells in vivo, we crossed mice homozygous for the floxed allele of either *Cdc42* or *Rac1* (Chrostek et al., 2006; Wu et al., 2006) with mice heterozygous for the respective allele, which additionally expressed the Cre recombinase under control of the *Wnt1* promoter (Danielian et al., 1998) (Figures 1A and 1B). *Wnt1-Cre*-mediated recombination efficiently deletes genes of interest in virtually the entire NC population (Lee et al., 2004; Wurdak et al., 2005). Efficiency of recombination was verified by genomic PCR, amplifying distinct fragments for wild-type, *floxed*, or *null* alleles of *Cdc42* and *Rac1*, respectively (Figure S1 available online). In *Wnt1-Cre/Cdc42*$^{lox/lox}$ and *Wnt1-Cre/Rac1*$^{lox/lox}$ embryos, the respective null allele was only observed in NC target tissue, whereas PCR amplification from nonrecombined tissue was negative for the *null* allele, demonstrating that expression of the Cre-recombinase was tissue-specific. Immunohistochemistry revealed significantly reduced expression of Cdc42 and Rac1 protein in recombined areas of the respective mutants (Figures 1C and 1D).

### *Cdc42* and *Rac1* Depletion Cause Multiple Defects in NC Derivatives

Both *Cdc42* and *Rac1* conditional knockout (cko) embryos were recovered up to embryonic day (E)13.5 with a Mendelian ratio, whereas litters from later developmental stages contained no mutant embryos (Figure S1). At E10.5, the gross morphology of *Rac1* cko embryos showed no obvious malformations. *Cdc42* cko embryos appeared also normal except for slight abnormalities in the mid-hindbrain region, where *Wnt1-Cre* is also expressed. These deficiencies appeared similar to those previously described for the developing central nervous system (CNS) lacking *Cdc42* (Cappello et al., 2006; Chen et al., 2006). At later stages than E10.5, the phenotypes of *Cdc42* and *Rac1* cko embryos appeared to be similar, with severe malformations in NC target tissues evident in both mutants (Figures 1E–1G). Craniofacial development was drastically impaired, as manifested by a facial cleft. Although initial palatal shelve formation appeared normal, palatal shelves developed poorly from E10.5 onward and failed to fuse. Constituents of the peripheral nervous

system (PNS), such as dorsal root ganglia (DRG) and other ganglia of the sensory-somatic and autonomic nervous system, were present but appeared all underdeveloped and reduced in size (Figure 1F; data not shown). During normal development, the heart outflow tract becomes divided into the aortic and pulmonary outflow vessels via formation of the aortico-pulmonary septum from the cardiac NC (Figure 1G). In contrast, the aortico-pulmonary septum failed to form in both mutants leading to a defect known as truncus arteriosus (TA), which is often seen upon impaired development of the NC cell pool that contributes to heart outflow tract formation (Wurdak et al., 2006).

### Emigration of NC Cells from Neural Tube to Initial Target Structures Appears Not to Be Affected by Loss of *Cdc42* and *Rac1*

As we observed abnormalities in all NC derivatives examined, *Cdc42* and *Rac1* are likely to fulfill general rather than lineage-specific roles in NC development. Rho GTPase function has been linked to cell migration and adhesion (Fukata et al., 2003). Therefore, we first investigated whether lack of *Cdc42* and *Rac1* might lead to defective NC cell migration. Upon *Wnt1-Cre*-mediated recombination of the ROSA26 Cre reporter allele (R26R) (Soriano, 1999), β-galactosidase is stably expressed in NC cells and their progeny, which allows in vivo fate mapping of control and mutant NC cells (Hari et al., 2002; Ittner et al., 2005). Using this system, we did not detect major migration deficits of both *Cdc42*- and *Rac1*-deficient NC cells at E10.5 in craniofacial areas, into the pharyngeal arches (PA), and in the trunk (Figures 2A and 2B). Moreover, at later stages, control and mutant NC cells have reached their initial targets, including nasofrontal structures, the eye, peripheral ganglia and nerves, and the proximal gut (Figures 2C–2F; data not shown). However, in the mutants, ganglia were smaller and distal parts of the gut lacked NC cell colonization, indicative for reduced numbers of NC-derived cells present in these structures (Simpson et al., 2007).

To confirm and quantify the migratory capacity of control and mutant NC cells at early stages of NC development, we generated NC cell explants in vitro from neural tubes isolated at E9.5 (Lee et al., 2004). In this defined cell culture system, the extent of NC outgrowth can be assessed 20 hr after initiation of the cultures. The explants formed from *Cdc42* and *Rac1* cko neural tubes were highly similar to their respective control explants. Quantification of the outgrowth area (Hari et al., 2002) did not reveal differences between control and cko explants (Figure S2) and the cell density per area was not changed in mutant explants (data not shown). Thus, impaired emigration from the neural tube is not a primary cause of the phenotype observed in *Cdc42* and *Rac1* cko embryos.

### *Cdc42* and *Rac1* Are Not Required for Differentiation of NC Cells

Apart from migration, failure of NC cells to properly differentiate might contribute to the *Cdc42* and *Rac1* cko phenotype. Possibly, loss of *Cdc42* and *Rac1* might interfere with cell-fate decisions in the NC population, favoring differentiation into a specific lineage at the expense of other fates. However, as apparently all examined NC-derived structures were affected, differentiation might rather be impaired at a global level than at the level of distinct lineages. Therefore, we investigated whether

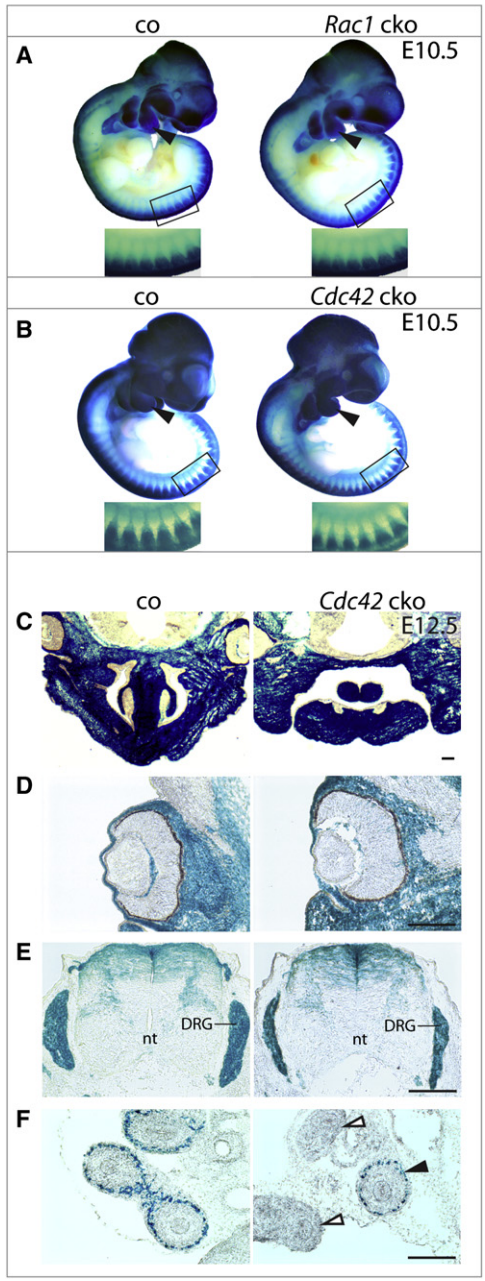


**Figure 2. *Cdc42* and *Rac1* Are Not Required for Migration of NC Cells to Initial Target Structures**

(A and B) In vivo fate mapping of NC cells in E10.5 cko and control (co) embryos. NC cells expressing β-galactosidase from the R26R Cre-reporter allele were visualized by whole-mount X-Gal stainings. *Cdc42* and *Rac1* cko embryos show normal distribution of NC cells. Arrowheads point to the first PAs populated by NC cells. Enlarged areas marked by boxes show presence of DRG and peripheral nerves.

(C–F) Histological sections of E12.5 *Cdc42* control and cko embryos stained with X-Gal show that NC cells have reached various targets such as nasofrontal structures (C), the eye (D), dorsal root ganglia (DRG, [E]), and proximal parts of the midgut ([F], arrowhead). Note DRG size reduction and lack of distal gut colonization (open arrowheads), pointing to reduced numbers of mutant versus control NC-derived cells in these structures. Similar histological data were obtained with Rac1 cko embryos (data not shown). Scale bars, 200 μm.

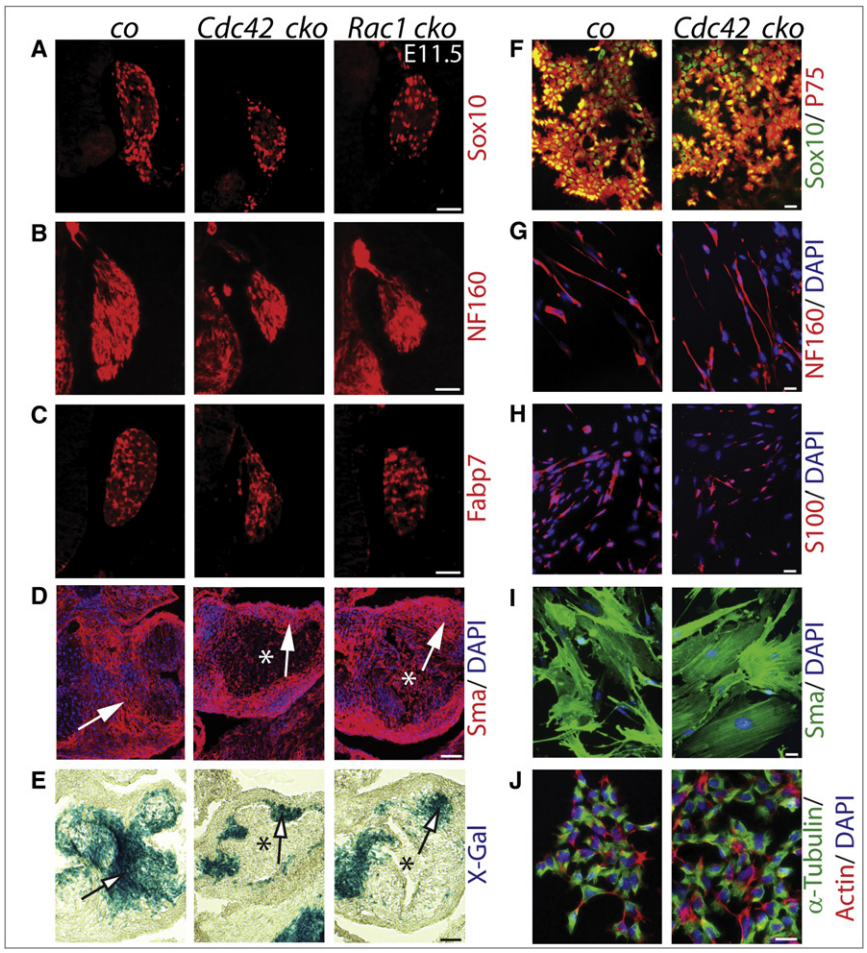


**Figure 3. Loss of *Cdc42* and *Rac1* Does Not Interfere with Differentiation of NC Cells**

(A) Expression of Sox10 in cells within the DRG of control and cko embryos.

(B) Presence of neuronal differentiation in mutant DRG.

(C) Satellite glia in DRG express the early glial marker Fabp7.

(D and E) Although aortico-pulmonary septa in the mutants fail to form, cardiac NC-derived cells in the outflow tract region of the heart have generated smooth muscle cells expressing Sma (arrows). Near adjacent sections were stained with X-Gal for localization of cardiac crest cells revealed by in vivo fate mapping using the R26R Cre-reporter allele. Blood cells are marked with an asterisk (*).

(F) *Cdc42*-deficient eNCSCs express the NCSC markers Sox10 and P75 after outgrowth from the neural tube.

(G–J) Normal differentiation of *Cdc42*-deficient eNCSCs into neuronal (G), glial (H) and smooth muscle (I) cell types upon induction of differentiation. *Cdc42*-deficient eNCSCs show normal cytoskeletal morphology (J). Scale bars: (A)–(E), 50 μm; (F)-(J), 10 μm.

different neural and non-neural cell types were present in the mutant NC tissues. Evidently, loss of *Cdc42* or *Rac1* was compatible with overt neuronal differentiation, such as in DRG and sympathetic ganglia (Figure 3B, data not shown). Early glial differentiation was marked by the presence of fatty acid binding protein 7 (Fabp7) in both control and mutant satellite glia (Figure 3C). Furthermore, mutant NC-derived cells in the outflow tract region of the heart stained positive for smooth muscle actin, indicating that smooth muscle formed correctly (Figures 3D and 3E). In addition to these differentiation markers, presence of Sox10-positive cells was seen in DRG and peripheral nerves (Figure 3A). Sox10 is expressed in NC stem and progenitor cells and in the peripheral glial lineage. All markers investigated were present at a comparable level to respective control animals in correlation to the size of the evaluated tissues. Furthermore, there were no signs of ectopic differentiation into a specific lineage.

To further assess whether multipotency of NC cells was impaired, we generated NC explants from *Cdc42* cko and control embryos in vitro. 20 hr after initial plating and outgrowth from the neural tube, NC explants were highly homogeneous for the NCSC markers Sox10 and P75 (Figure 3F), but negative for differentiation markers (Paratore et al., 2001; data not shown). Upon induction of differentiation over several days, mutant NC cells showed normal differentiation into neuronal, glial, and smooth muscle cell types (Figures 3G–3I). Moreover, no abnormalities were found in the cytoskeletal morphology of mutant eNCSCs (Figure 3J). Taken together, we did not detect any signs of reduced or altered differentiation in *Cdc42*- and *Rac1*-deficient NC cells in vivo and of differentiation of *Cdc42*-deficient NC cells into diverse derivatives in vitro. We conclude that *Cdc42* and *Rac1* do not influence the capacity of NCSCs to acquire multiple different cell fates and to undergo initial steps of differentiation.

## *Cdc42* and *Rac1* Are Essential for Mitotic Activity and Cell-Cycle Control in NC Target Structures

The broad phenotypes in mutant NC derivatives are consistent with the hypothesis that loss of *Cdc42* and *Rac1* both lead to a depletion of the NC cell pool. Accordingly, *Cdc42* and *Rac1* might play a role in promoting proliferation or they might be required for survival of NC cells. Assessing apoptosis within NC tissues at E10.5 and E11.5, however, did not reveal signs of impaired survival in *Cdc42* or *Rac1* cko embryos (data not shown). Several reports have implicated Rho GTPases in cell-cycle control (Narumiya and Yasuda, 2006). Thus, *Cdc42* and *Rac1* might be required for proliferation of NC cells. A reduction in mitotic activity would lead to a general loss of NC cells, reducing the overall cell pool size necessary for proper formation of NC derivatives. To investigate this possibility, we applied an antibody against phospho-histoneH3 (pH3) to examine proliferation in PA1 and -2 of *Cdc42* and *Rac1* cko embryos that expressed β-galactosidase as a NC lineage label. pH3 is detected during G2 to M transition of the cell cycle when

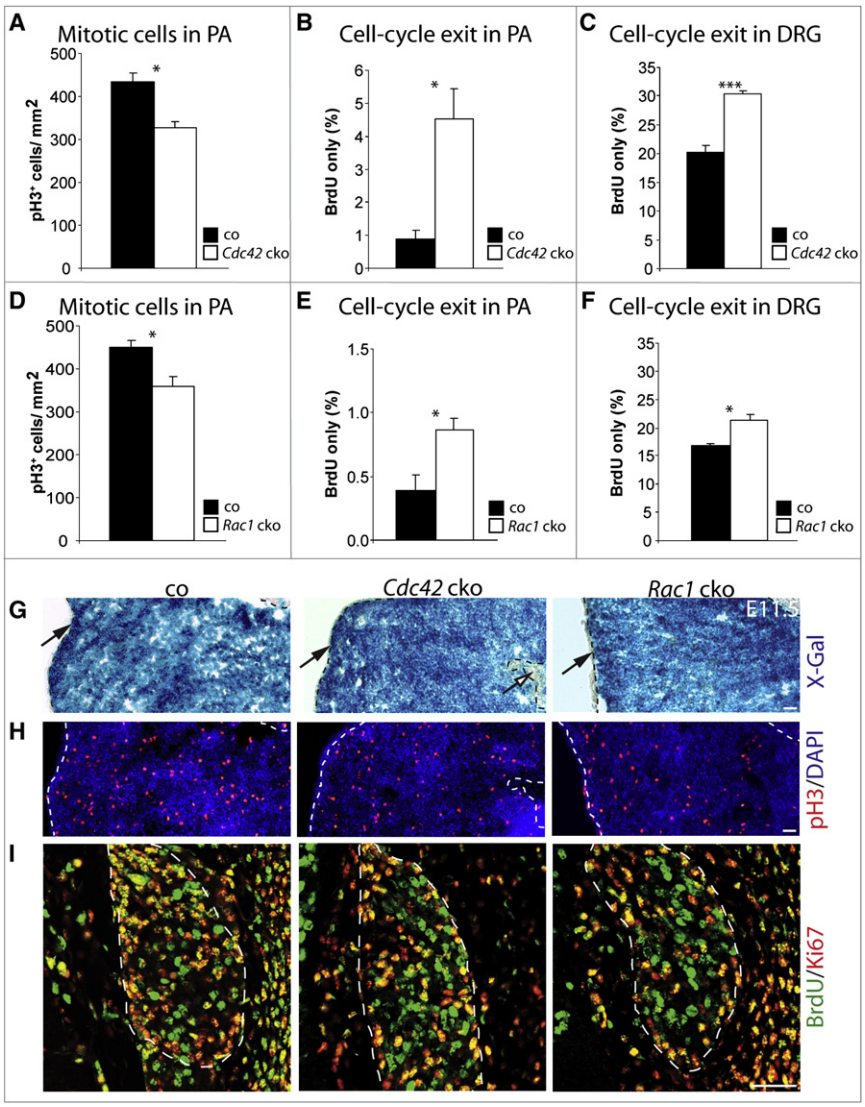


**Figure 4. Reduced Mitotic Activity and Increased Cell-Cycle Exit in NC-Derived Structures of *Cdc42* and *Rac1* cko Embryos at E11.5**

(A and D) Reduced numbers of mitotic cells in mutant PA1 and -2 as detected by pH3 staining at E11.5. (G and H) PA1 tissue within quantified regions is largely X-Gal positive, demonstrating the neural crest origin of the vast majority of cells. Black and white arrows mark pharyngeal ectoderm and core mesoderm. These tissues are not NC-derived and were excluded from the quantification. (B and E) Cell-cycle exit was quantified 24 hr after a BrdU pulse. Cells having exited the cell cycle show incorporation of BrdU but no expression of the proliferation marker Ki67. NC cells that have populated PA1 and -2 show an increase in cell-cycle exit at E11.5. (C,F, and I) Cells in mutant DRG show significantly increased cell-cycle exit at E11.5. Values represent mean ± SEM. Scale bars, 50 μm. *$p < 0.05$; ***$p < 0.005$.

chromatin condensation occurs. Strikingly, while there was no statistically significant difference at E10.5 (Figures S3A and S3D), we saw a reduction in the number of pH3-positive cells by 24.5% ($p < 0.05$) in NC-derived tissue in *Cdc42* mutants and by 19.6% ($p < 0.05$) in *Rac1* mutants at E11.5 (Figures 4A, 4D, 4G, and 4H). As cell density per area was unchanged (Figure S4), these results demonstrate a reduced mitotic activity of mutant NC progenitor cells. Next, we examined whether loss of *Cdc42* or *Rac1* induced premature exit from the cell cycle in NC cells. To this end, a BrdU pulse was given 24 hr before analysis, followed by quantification of the relative number of cells that had incorporated BrdU into their DNA. BrdU-positive cells that did not express the proliferation marker Ki67 anymore have exited the cell cycle at the time point of analysis. Apart from the PA1 and -2, we examined the DRG of control and cko embryos in order to analyze non-neural as well as neural derivatives of NC origin. At E10.5, no significant changes in cell-cycle exit were found in DRG and in the PAs of *Cdc42* and *Rac1* cko embryos (Figures S3B, S3C, S3E, S3F, and S3G). In contrast, we observed a drastic increase in cell-cycle exit at E11.5 in both mutants: the number of DRG cells that had exited the cell cycle was increased by 50.1% ($p < 0.005$) in *Cdc42* and by 27.5% ($p < 0.05$) in *Rac1* mutants (Figures 4C, 4F, and 4I). In PA1 and -2, overall numbers of cell-cycle exit were comparatively low. However, the increase in cell-cycle exit amounted to 401.1% ($p < 0.05$) in *Cdc42* and 123.1% ($p < 0.05$) in *Rac1* mutants, respectively (Figures 4B and 4E).

Taken together, we observed a reduction of mitotically active cells in both mutants, accompanied by a significant increase in cell-cycle exit. Interestingly, these changes did not occur early in NC development, as mutant embryos at E10.5 showed no major alterations. This suggests that during early stages of NC development, NC cell proliferation might occur independently of *Cdc42* and *Rac1*. These small Rho GTPases apparently turn into crucial regulators of proliferation, however, after NC-derived cells have reached their initial targets.

## Stage-Specific EGF Responsiveness Regulating Small Rho GTPase-Dependent Self-Renewal and Proliferation

Knowledge of the mitogen acting upstream of Cdc42 and Rac1 might be necessary to understand how NC cells become dependent on these small Rho GTPases over time. In the CNS, expression of epidermal growth factor receptor (EGFR) increases during development, mediating a transition from mitotic FGF to EGF responsiveness in neural stem and progenitor cells (Ciccolini, 2001; Lillien and Raphael, 2000). To assess whether similar sequential changes occur during neural crest development, we first monitored the spatiotemporal expression of EGFR in vivo. At E9.5, EGFR expression was generally low, and Sox10-positive neural crest cells that migrated away from the neural tube did not display EGFR expression (Figure S5A). At E10.5, EGFR

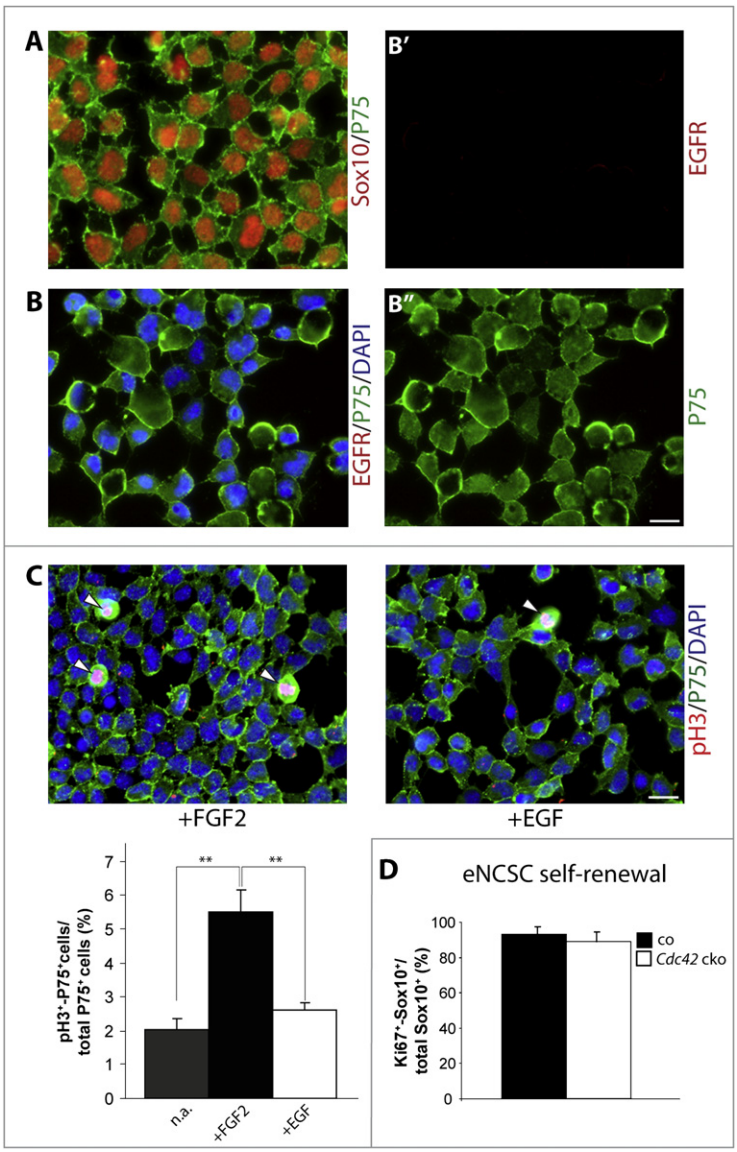


**Figure 5. Lack of EGF Responsiveness in eNCSCs**

(A and B) eNCSC explants were generated by outgrowth from E9.5 neural tubes. eNCSCs homogeneously express the NCSC markers Sox10 and P75 (A and B″), but do not express EGF receptor (EGFR) (B and B′). (B) is an overlay of the stainings shown in (B′) and (B″).

(C) Following outgrowth from the neural tube, wild-type eNCSCs were challenged with either FGF2 or EGF. The number of P75-expressing mitotic eNCSCs in response to these factors was quantified by pH3 staining. While FGF2 led to an increase in mitotic cells, EGF-treated mitotic cells remained at levels comparable to nontreated control cells (n.a.). White arrowheads mark pH3-positive nuclei.

(D) eNCSCs were expanded and maintained in the presence of Wnt1, BMP2, and FGF2. Numbers of cells expressing the stem cell marker Sox10 and the proliferation marker Ki67 are comparable in *Cdc42* mutant (*Cdc42* cko) and control (co), demonstrating that self-renewal and proliferation of the early population of NCSCs are not affected. Values represent mean ± SEM. Scale bars, 10 μm. $^{**}p < 0.01$.

expression—although still relatively weak and heterogeneous—became apparent in DRG, the gut, and PA1 (Figures S5B–S5D). Strikingly, at E11.5, EGFR was very strongly and broadly expressed in DRG, the gut, and PA1. Moreover, Sox10-positive cells in DRG and the ENS coexpressed EGFR. To corroborate these findings on the cellular level, explant cultures of NCSCs were prepared from distinct developmental stages (Experimental Procedures). Both E9.5 neural tube and E11.5 organotypic gut explant cultures consisted of undifferentiated cells that expressed the NCSC markers Sox10/P75 (Figure S6; Paratore et al., 2001). Moreover, 99.6 ± 0.6% of all P75-positive cells in gut explants also expressed α4-integrin (data not shown), which identifies these cells as gut NCSCs (gNCSCs) (Bixby et al., 2002; Kruger et al., 2003). In agreement with the data obtained in vivo, eNCSCs from E9.5 neural tube explants did not express EGFR (Figures 5A and 5B). In contrast, virtually all P75-positive cells in explants of E11.5 organotypic gut cultures expressed EGFR (Figures 6A and 6B), demonstrating that, unlike eNCSCs, gNCSCs express EGFR. To assess whether stage-specific EGFR expression correlated with factor responsiveness, eNCSCs and gNCSCs were exposed to FGF2 or EGF in defined culture conditions. In eNCSCs from neural tube explants, FGF2, but not EGF, promoted proliferation, as assessed by quantification of mitogenic P75-positive cells (Figure 5C). In contrast, gNCSCs were responsive to both mitogenic FGF2 as well as EGF (Figure 6C). Thus, EGF is a stage-specific growth factor during NC development.

Since EGF is one of the factors that can lead to Cdc42 and Rac1 activation (Fanger et al., 1997; Sini et al., 2004), we tested whether EGF-mediated proliferation in gNCSCs might involve Cdc42 and/or Rac1. To this end, we measured the relative number of mitotic cells in explants of gNCSCs derived from either control, *Cdc42* cko, or *Rac1* cko E11.5 gut. Strikingly, proliferation of both *Cdc42*- and *Rac1*-deficient P75-positive cells was reduced to levels found in the absence of EGF (Figure 6C), demonstrating that EGF-induced proliferation requires *Cdc42* and *Rac1*. In contrast, FGF2—that, unlike EGF, is a mitogen for both eNCSCs and gNCSCs—promoted proliferation independently of *Cdc42* and *Rac1*. Thus, increased cell-cycle exit and altered proliferation in *Cdc42* and *Rac1* mutants in vivo (Figure 4) can be explained by small Rho GTPase-dependent EGF signaling active at later, but not early, stage NC development.

The above experiments reveal a mitogenic role of EGF/small Rho GTPases in cultures containing NCSCs, but they do not directly address whether actual stem cell self-renewal is controlled by this signaling cascade. At early stages of NC development, combinatorial Wnt and BMP signaling, in the presence of FGF2, suppresses differentiation and maintains self-renewing multipotent eNCSCs from neural tube explants (Kleber et al., 2005). As expected from our in vivo analysis (Figure 2) and given that these early stage cells are not responsive to EGF (Figure 5), deletion of *Cdc42* and *Rac1*, respectively,

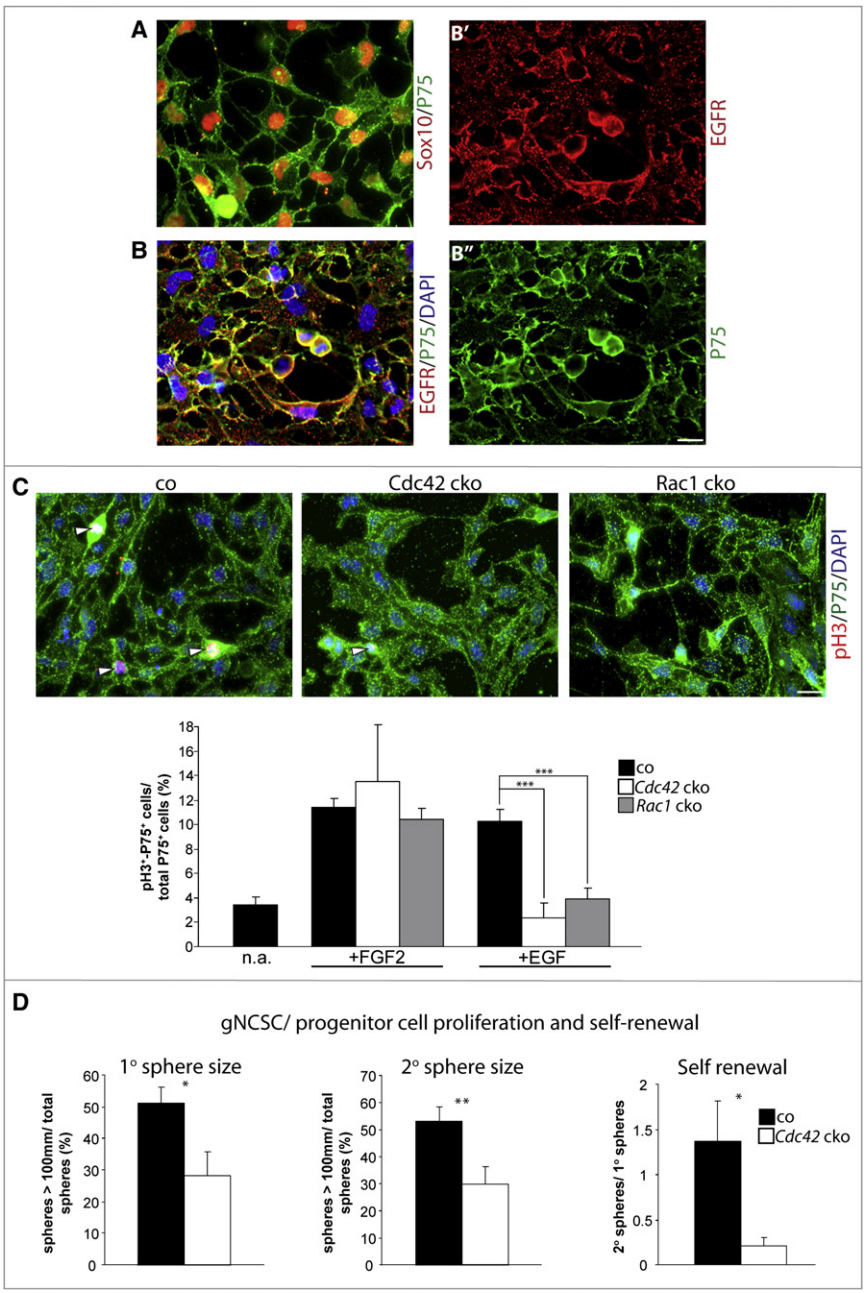


**Figure 6. EGF Acts via Cdc42 and Rac1 to Promote Cell Proliferation of Gut NC Stem and Progenitor Cells**

gNCSC explants were generated by outgrowth from E11.5 gut. In addition to the NCSC markers Sox10 and P75 (A and B″), explant cells homogeneously express the EGF receptor (EGFR; [B] and [B′]). (B) is an overlay of the stainings shown in (B′) and (B″). (C) Following outgrowth from the gut, control (co), *Cdc42* cko, and *Rac1* cko enteric NC cells were challenged with either FGF2 or EGF. The number of P75-expressing mitotic gNCSCs in response to these factors was quantified by pH3 staining. FGF2 induced proliferation in both control and mutant cells. However, EGF induced proliferation only in control, but not *Cdc42*- and *Rac1*-deficient gNCSCs. Pictures for treatment with EGF are shown. White arrowheads mark pH3-positive nuclei. (D) Control and *Cdc42*-deficient enteric spheres were generated from E12.5 guts. The percentage of spheres with a diameter larger than 100 μm is lower in mutants (1° sphere size, 2° sphere size), indicating reduced proliferation. In addition, the self-renewal capacity is significantly reduced in mutants. Values represent mean ± SEM. Scale bars, 10 μm. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

did not affect self-renewing proliferation of eNCSCs (Figure 5D; Table S1). Indeed, neither the percentage of Sox10-expressing eNCSCs nor the number of mitotically active cells was diminished in mutant stem cell cultures (Table S1). Unlike for early stage eNCSCs, factors instructively promoting stem cell maintenance at later stages of NC development remain to be identified (Kleber et al., 2005). Nevertheless, the self-renewal capacity of later stage NCSCs can be monitored in nonadhesive sphere cultures by serial sphere passaging (Molofsky et al., 2003). Therefore, to investigate the role of *Cdc42* in gNCSC self-renewal, we generated ENS spheres from control and cko embryos at E12.5. As the embryonic gut might contain additional types of sphere-forming cells not related to the NC, we tracked NC-derived cells using the *R26R* reporter allele so that spheres formed from recombined NC cells could be identified based on X-Gal staining. In agreement with fewer NC-derived cells being present in cko NC target structures (Figure 2), the number of primary spheres generated from *Cdc42* cko guts (sphere frequency) was reduced by 62.3% ($p < 0.005$) as compared to control spheres. Importantly, upon subcloning the amount of mutant secondary spheres that formed from recombined primary spheres was decreased by 84.6% ($p < 0.05$), demonstrating a drastically decreased self-renewal capacity of gNCSCs (Figure 6D). Moreover, while control spheres could be further subcloned, mutant ENS cells did not form tertiary spheres (data not shown). While sphere passaging reflects stem cell self-renewal, the sphere size is indicative of progenitor cell proliferation. Average sphere size was reduced in mutant spheres (primary spheres: control 137 ± 24 μm, mutant 89 ± 36 μm; secondary spheres: control 150 ± 50 μm, mutant 92 ± 17 μm). In particular, spheres with a diameter larger than 100 μm were reduced by 45.0% in primary and by 43.6% in secondary mutant spheres, reflecting reduced proliferation of ENS stem and progenitor cells in the absence of *Cdc42* (Figure 6D), confirming the data obtained with adhesive cko gut explant cultures (Figure 6C). ENS sphere formation from *Rac1*-deficient ENS cells was equally reduced, and spheres appeared smaller in size, too (data not shown), pointing to a similar requirement of

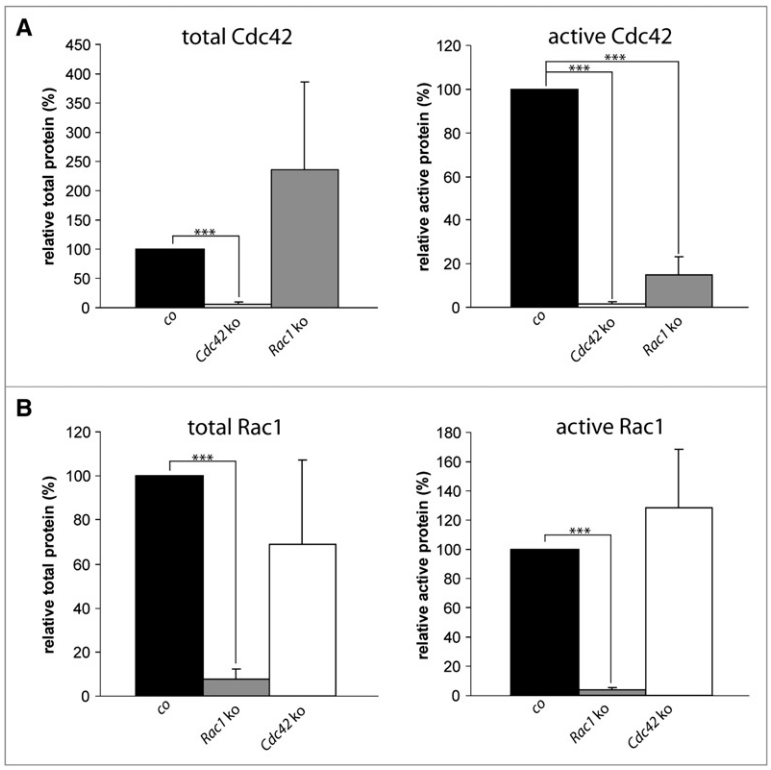


**Figure 7. Cdc42 and Rac1 Appear to Act in a Common Pathway, with Rac1 Being an Upstream Activator of Cdc42**

Western blots and pull-down assays using GST-PAK-CD constructs were done with lysates from Adenovirus-*Cre*-infected ENS spheres. Infected spheres were wild-type (co) or carried two floxed alleles for *Cdc42* (*Cdc42* ko) and *Rac1* (*Rac1* ko), respectively.

(A) Total Cdc42 was significantly reduced in *Cdc42* ko as compared to control lysates (left panel). Levels of active GTP-bound Cdc42 were reduced in both *Cdc42* ko and *Rac1* ko as compared to control (right panel).

(B) Total levels of Rac1 were significantly reduced in lysates from *Rac1* ko (left panel). Activity of Rac1 was significantly lower in *Rac1* ko, but not in *Cdc42* ko, as compared to control (right panel). ***$p < 0.005$.

*Cdc42* and *Rac1* in gNCSC self-renewal and progenitor cell proliferation.

### Cdc42 and Rac1 Appear to Share a Common Pathway, with *Rac1* Being Required for Cdc42 Activation

The similar phenotypes of *Cdc42* and *Rac1* cko embryos in vivo and the indistinguishable roles of these small Rho GTPases in EGF-dependent cell-cycle regulation of gNCSCs suggests that Cdc42 and Rac1 might involve overlapping signaling pathways. To address this issue, we first compared alterations in downstream signaling events upon *Cdc42* versus *Rac1* mutation in the NC by examining the expression profile of several key regulators of cell-cycle dynamics. Quantitative real-time RT-PCR (qRT-PCR) was carried out with tissue from PA1 and -2 of *Cdc42* and *Rac1* cko and control embryos at E11.5. While expression of CyclinD1 and other regulators was not changed, $P21^{Cip1}$ expression levels were significantly increased in both mutants (Figure S7), suggesting that Cdc42 and Rac1 might act as suppressors of $P21^{Cip1}$ in NC-derived tissue. qRT-PCR analysis also revealed that loss of *Rac1*, but not of *Cdc42*, resulted in increased expression of Pten and c-Myc that have been associated with regulation of stem cell self-renewal (Groszer et al., 2006; Wilson et al., 2004). Although for technical reasons we were unable to confirm on the protein level the ~1.5-fold induction of $P21^{Cip1}$ mRNA levels in the cko embryos, the overall data are consistent with the idea that Cdc42 and Rac1 might act in NC cells through partially overlapping downstream signaling targets. To directly assess whether Cdc42 and Rac1 pathways overlap, we examined levels of active (i.e., GTP-bound) forms of Cdc42 and Rac1 in the respective mutants. To this end, gNCSCs carrying two floxed alleles of either *Cdc42* or *Rac1* were expanded in sphere cultures in the presence of EGF, followed by adenovirus-Cre-mediated gene deletion. *Cdc42* ablation resulted in loss of total Cdc42 protein and of active Cdc42, as expected, whereas Rac1 activation was unchanged in *Cdc42*-deficient as compared to wild-type cells (Figure 7). Intriguingly, however, loss of *Rac1* caused reduced activation of both Rac1 as well as Cdc42, indicating that Cdc42 activation occurs downstream of Rac1. Thus, activation of Cdc42 and Rac1 appears to rely on overlapping pathways, likely explaining the similar phenotypes found upon *Cdc42* and *Rac1* inactivation in the NC.

## DISCUSSION

This report provides the first evidence for a crucial involvement of two small Rho GTPases, Cdc42 and Rac1, in NC development. Using mice with a deletion of either *Cdc42* or *Rac1* in the NC, we show that proliferation and the balance between mitotically active and quiescent cells is *Cdc42* and *Rac1* dependent. Importantly, growth control during NC development is stage specific: while self-renewing proliferation of eNCSCs is *Cdc42* and *Rac1* independent, NC stem and progenitor cells, after having reached their initial targets, require these small Rho GTPases for cell-cycle control. These developmental changes are associated with altered responsiveness of NC cells to mitogenic growth factors: eNCSCs emigrating from the neural tube are first FGF2, but not EGF, responsive. At later stages, however, NC cells as analyzed in the ENS start to express the EGF receptor and acquire EGF responsiveness. Thereby, EGF, but not FGF2, acts through Cdc42/Rac1 to promote proliferation. Thus, although endowed with a similar set of stem cell features, early and late NCSCs can be distinguished based on different mechanisms regulating proliferation.

### Proliferation Control in NC Stem and Progenitor Cells

So far, knowledge on how NC cell proliferation is controlled has been limited. In *Xenopus*, the helix-loop-helix transcription factor Id3 acts as a cell-intrinsic regulator of NC cell proliferation and survival (Kee and Bronner-Fraser, 2005). However, in contrast to what we observed for *Cdc42* and *Rac1* in mice,

Id3 in *Xenopus* appears to be involved already at the stage of NC induction. In mice, several *Id* genes with overlapping functions exist, but single and double knockout mice display milder phenotypes than obtained upon loss of Id3 function in *Xenopus.* Thus, a direct implication of *Id* genes in *Cdc42*- and *Rac1*-dependent NC proliferation appears unlikely. With respect to extracellular regulators of early NC cell proliferation, FGF2 promotes cell-cycle progression in neural tube explant cultures, as shown here (Figure 5), and the combined activities of Wnt and BMP in the presence of FGF2 allows expansion of multipotent eNCSCs by symmetrical cell division (Kleber et al., 2005). The latter involves canonical Wnt signaling via its downstream signaling component β-catenin (S.B.-M. and L.S., unpublished data), which, in astrocytes and skin progenitor cells, can be regulated by Cdc42 (Etienne-Manneville and Hall, 2003; Wu et al., 2006). In NC cells, however, β-catenin activity is only observed during early stages (Kleber et al., 2005), while it decreases in postmigratory structures such as DRG that are affected in *Cdc42* and *Rac1* cko embryos. Moreover, we demonstrate that Wnt, BMP, and FGF2 are able to support eNCSC self-renewal even in the absence of *Cdc42* or *Rac1* (Figure 5; Table S1). Thus, FGF2 and Wnt/β-catenin signaling in early NC cells is not associated with Cdc42/Rac1-mediated cell-cycle control.

At later stages, FGF2 is still a mitogenic signal for NC cells. However, unlike eNCSCs, later stage NC stem and progenitor cells also become sensitive to mitogenic EGF due to upregulation of the EGFR (Figure 6; Figure S5). This is reminiscent of developmental changes occurring during CNS development, when neural stem cells switch from FGF2 to FGF2/EGF responsiveness (Ciccolini, 2001; Lillien and Raphael, 2000). Intriguingly, while FGF2-induced proliferation remains Cdc42/Rac1-independent during NC development, the mitogenic response of late stage gNCSCs to EGF can be abolished by either *Cdc42* or *Rac1* inactivation. Thus, EGF can be placed upstream of both Cdc42 and Rac1 in regulating cell-cycle progression of NC cells that have reached their initial targets.

The analogous proliferation phenotypes obtained in vivo and in cell culture upon *Cdc42* and *Rac1* deletion in the NC raises the possibility that, downstream of EGF, Cdc42 and Rac1 share a signaling pathway to activate common targets. Indeed, the expression of $P21^{Cip1}$ was similarly upregulated in NC tissue from *Cdc42* and *Rac1* cko embryos, while the expression of several other cell-cycle regulators remained unaffected in both mutants (Figure S7). Conceivably, Cdc42 and Rac1 act through regulation of $P21^{Cip1}$ to control cell-cycle progression of late stage NC cells. However, *Rac1*, but not *Cdc42*, deletion resulted in increased levels of Pten and c-Myc expression, indicating that Rac1 might have a somewhat broader activity spectrum than Cdc42. This is consistent with our finding that Rac1 appears to act upstream of Cdc42: levels of active Rac1 were unchanged in *Cdc42*-deficient NC cells, while active Cdc42 levels were reduced in the *Rac1* ko (Figure 7). Such cross-activation between *Cdc42* and *Rac1* has previously been established in fibroblasts, although with reverse hierarchical order (Czuchra et al., 2005). Thus, Cdc42 and Rac1 can functionally interact, which in the NC appears to involve Cdc42 activation by Rac1 in a shared pathway mediating mitogenic EGF signaling.

In addition to EGF, several other receptor tyrosine kinases can signal through Rho GTPases. Of these, PDGFRα plays a role in cranial and cardiac NC development, which has, however, not been attributed to the control of proliferation (Tallquist and Soriano, 2003). ErbB2/3 activation by the growth factor neuregulin (NRG) promotes gliogenesis in NC cells and supports Schwann cell proliferation and differentiation. Interestingly, NRG-dependent Schwann cell proliferation is mediated by Cdc42, but not Rac1, pointing to distinct roles of these molecules in Schwann cell development (Benninger et al., 2007). The cell-lineage-specific function of NRG and the differential activation of Cdc42 versus Rac1 by NRG make it improbable that the effects described in our study involve NRG signaling. Finally, integrin signaling can also act as an activator of Cdc42 and Rac1 (Schwartz and Shattil, 2000). However, ablation of *integrinβ1* in the NC led to a milder phenotype than observed in *Cdc42* and *Rac1* cko embryos, in that mutant mice were viable up to 3 weeks of age with developmental abnormalities restricted to the PNS (Pietri et al., 2004). In sum, we cannot exclude that mitogenic Cdc42 and Rac1 are activated by distinct local signals in different NC derivatives. Such signals might, for instance, modulate mitogenic EGF and contribute to the distinct extent of cell-cycle exit observed in mutant PA versus DRG (Figure 4). However, the broad phenotypes observed upon small Rho GTPase inactivation, similarly affecting virtually all NC derivatives, rather, speak for a general mechanism of Cdc42- and Rac1-mediated growth control at later stages of NC development. According to this model, EGF and Rac1/Cdc42 form a signaling cascade controlling the pool size of the NC population after its emigration from the neural tube, although this has to be confirmed by conditional inactivation of EGF signaling in NC derivatives.

Although Cdc42 and Rac1 have been implicated in migration control in other systems (Fukata et al., 2003), a cellular phenotype in the cko embryos became apparent only after E10.5, and emigration from the neural tube was apparently not affected in the absence of these small Rho GTPases. The in vivo situation was recapitulated in vitro by mutant eNCSCs that did not display any overt migration deficiencies in neural tube explant cultures. These findings are in agreement with recent reports demonstrating normal migration ability of *Cdc42* and *Rac1* null mutant fibroblasts, respectively (Czuchra et al., 2005; Vidali et al., 2006). Nevertheless, we cannot exclude a role of Rac1/Cdc42 in migration, such as during morphogenesis of craniofacial structures at later stages of NC development or in the full-length colonization of the gut during ENS formation (Stewart et al., 2007). Indeed, distal gut portions were not or less populated by NC-derived cells in mutants as compared to the control (Figure 2), which—in addition to defective gNCSC proliferation and maintenance—could be associated with migration deficits. Of note, however, proliferation and directed migration are intricately linked during ENS development, with proliferation being the key mechanism driving invasion of the gut by NC cells (Simpson et al., 2007). Thus, small Rho GTPases might elicit associated functions in migration and proliferation. Importantly, the amenability of appropriate culture systems allowed us to demonstrate on the cellular level that Rac1 and Cdc42 regulate NCSC proliferation cell autonomously and independently of migration.

## Cdc42 and Rac1 Functions Are Cell-Type and Stage Specific

Although small Rho GTPases have previously been implicated in regulating stem cells other than NCSCs, they do not execute a universal stem cell function. Rather, they show functional diversity depending on the type of Rho GTPase, the cell type, and the time of action. In the CNS, deletion of *Cdc42* leads to progenitor cell depletion, but this is an effect secondary to gradual loss of polarity protein complex and adherens junctions (Cappello et al., 2006). As a consequence, mutant cells fail to self-renew at the apical site of the neuroepithelium and, therefore, acquire a neurogenic fate. Such a mechanism unlikely contributes to the phenotypes described in our study, as expression of polarity protein complex was unaltered in *Cdc42*- or *Rac1*-deficient postmigratoy NC derivatives (data not shown). In the hematopoietic system, *Rac1* is required for the retention of hematopoietic stem cells and progenitors (HSC/Ps) in their microenvironmental niche, but an effect on proliferation was only observed in vitro and not in vivo (Cancelas et al., 2005). Deletion of *Cdc42* in HSC/Ps also led to impaired retention, but mutant HSC/Ps showed increased cell-cycle entry, thus increasing the number of HSC/Ps (Yang et al., 2007). Apparently, in the hematopoietic system, the function of *Cdc42* in cell-cycle control is the inverse of its role in the NC. In skin progenitor cells, *Rac1* plays a very different role from *Cdc42*. Similar to its role in late stage NCSCs, *Rac1* is crucial for maintenance of skin stem cells present in the hair follicle, but possibly not of interfollicular epithelial stem cells (Benitah et al., 2005; Castilho et al., 2007; Chrostek et al., 2006). In contrast, unlike in NCSCs, *Cdc42* controls differentiation of progenitor cells into hair follicle cells (Wu et al., 2006). In sum, there is increasing evidence that the function of a given small Rho GTPase is specific for the type of stem and progenitor cells (Wang and Zheng, 2007). The present study now also reveals stage-specific roles of small Rho GTPases in stem cells. During NC development, *Cdc42* and *Rac1* activities mark a transition in NCSC growth control, acting as critical cell-cycle regulators of virtually the entire pool of NC cells after emigration from the neural tube. Similarly, NCSCs from different developmental stages display intrinsic differences in their responsiveness to fate-promoting cues (Bixby et al., 2002; Kleber et al., 2005). However, while signals inducing differentiation are also differentially interpreted depending on the location, the control of proliferation appears to be more general in late stage NC stem and progenitor cells, leading to broad developmental defects in NC derivatives upon *Cdc42* and *Rac1* inactivation.

## EXPERIMENTAL PROCEDURES

### Generation of cko Mice

Animals homozygous for the floxed *Cdc42* allele ($Cdc42^{lox/lox}$) (Wu et al., 2006) were mated with mice heterozygous for the floxed allele that additionally carried the *Wnt1-Cre* transgene (Danielian et al., 1998) ($Wnt1\text{-}Cre/Cdc42^{lox/wt}$). Offspring with a $Wnt1\text{-}Cre/Cdc42^{lox/lox}$ genotype were termed *Cdc42 cko* embryos, while littermates lacking the *Wnt1-Cre* transgene or carrying a wild-type *Cdc42* or *Rac1* allele did not exhibit any overt phenotype, thus serving as control animals. For in vivo fate mapping of recombined cells, the R26R allele (Soriano, 1999) was bred into $Cdc42^{lox/lox}$ mice, which were mated with $Wnt1\text{-}Cre/Cdc42^{lox/wt}$ mice to obtain *Cdc42* cko and control embryos that expressed β-galactosidase upon Cre-mediated recombination. The same breeding strategy was used for $Rac1^{lox/lox}$ animals (Chrostek et al., 2006) to obtain *Rac1 cko* and control embryos. Genotyping was done by PCR on genomic DNA.

### Histochemistry, Immunofluorescence, and X-Gal Staining

Hematoxylin-eosin stainings (H&E) were done on paraffin sections. Immunofluorescence stainings were done on paraffin (7 μm) or cryo (10–12 μm) sections. For all antibody stainings on sections, except for Cdc42, Rac1, and Sma, antigen retrieval was done with 10 mM citrate buffer in a steam cooker device at 110°C for 5 min. Fixation was done with 4% paraformaldehyde (PFA) for sections, and 4% formaldehyde for cells in PBS for 10 min at room temperature with the following exceptions: fixation for Cdc42 staining on cryosections was with prechilled 4% PFA for 10 min on ice. Fixation for Rac1 stainings on cryosections was with prechilled 4% PFA/2% acetic acid in PBS for 10 min followed by prechilled ethanol:acetic acid (95:5) for 2 min, all on ice. Antibodies and substrates used for stainings are given in Table S2. BrdU incorporation was done according to the manufacturer's instructions (Roche). X-Gal (Applichem) stainings were done according to standard protocols.

### Cell Culture

eNCSC explant cultures from E9.5 trunk neural tubes were done as described (Lee et al., 2004). Migratory capacity of eNCSCs in vitro (migration index) was determined as described (Hari et al., 2002). Cultures were incubated in defined medium (DM), supplemented with 10 ng/ml FGF2. For eNCSC maintenance experiments, medium was switched after 20 hr to DM with 10 ng/ml FGF2, 200 ng/ml Wnt1, and 50 ng/ml BMP2. To assess growth factor response of eNCSCs, medium was withdrawn after 16 hr, cells were starved for 2 hr in DM, and subsequently pulsed with DM plus 50 ng/ml FGF2 or 50 ng/ml EGF, respectively, for additional 2 hr. gNCSC explant cultures were obtained from isolated E11.5 guts. Culture conditions were similar to eNCSC explant cultures with the following exceptions: cultures were incubated for 24 hr in DM with 10 ng/ml FGF2, 10 ng/ml EGF, and 10 ng/ml GDNF. To assess growth factor response of gNCSCs, medium was withdrawn after 24 hr, and cells were starved for 2 hr in DM and subsequently pulsed with DM plus 50 ng/ml FGF2 or 50 ng/ml EGF, respectively, for an additional 3 hr. gNCSC spheres were grown from E12.5 control and mutant ENS cells. Isolated guts were dissociated into single cells with 1 mg/ml collagenase type I (Worthington) dissolved in HBSS w/o Ca and Mg. Selective growth of NC-derived cells was accomplished with NC-specific sphere (NCS) medium and verified by visualization of the lineage marker β-galactosidase. NCS medium contained DMEM-F12, 20 ng/ml FGF2, 20 ng/ml IGF1, 20 ng/ml EGF, 10 ng/ml GDNF (all Peprotech), 1% N2 supplement, 2% B27, 1% Penicillin/Streptomycin (all Invitrogen), 50 μM 2-mercaptoethanol, and 15% chicken embryonic extract. Poly(2-hydroxyethyl methylacrylate) (Sigma) was used to coat culture plates to eliminate adhesion of cells to the plastic surface. To assess self-renewal, secondary spheres were cultured from defined numbers of cells obtained from dissociated primary spheres. The average number of cells/primary spheres was used to calculate the ratio between secondary and primary spheres.

### Quantitative Real-Time RT-PCR

Total RNA from E11.5 PA1/2 cells was isolated with the TRIZOL protocol (Invitrogen) according to the manufacturer's instructions, and 2 μg was used for each cDNA synthesis. cDNA synthesis was performed using Ready-To-Go You-Prime First-Strand Beads (Amersham). The same amount of cDNA was used for each PCR reaction. qRT-PCR was performed in a Lightcyler (Applied Biosystems) using Master SYBR Green kit (ABgene). Each result was normalized by the housekeeping 18S gene expression. The primer pairs used for each PCR can be found in Table S3.

### Rac1-GTP and Cdc42-GTP Pull-Down Assays

gNCSC spheres were grown from E12.5 wild-type, $Cdc42^{lox/lox}$, and $Rac1^{lox/lox}$ guts as mentioned above. Adenovirus-*Cre*-mediated recombination was done by infecting single cell suspensions of secondary spheres. Infected cells were cultured to form tertiary spheres. Medium was washed off 4 days after infection and replaced with virus-free NCS medium for additional 2 days. GST-PAK-CD constructs were provided by J. Collard (The Netherlands Cancer Institute, Amsterdam, Holland). Rac1 and Cdc42 activities were measured as described (Benninger et al., 2007).

### Statistical Analysis

Each experiment was performed with at least three independent samples. Statistical significance was tested with the unpaired Student's t test.

## SUPPLEMENTAL DATA

The Supplemental Data include seven figures and three tables and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00054-X.

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