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The involvement of Rcc2 in mammalian neurogenesis

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THE INVOLVEMENT OF RCC2 IN MAMMALIAN NEUROGENESIS

by

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INVolVEMENT OF RCC2 IN MAMMILIAN NEUROGENESIS

LISA YANUSHEFSKI

ABSTRACT

Rcc2 is a Rac guanine nucleotide exchange factor recently identified as a principal signaling component of integrin adhesion complexes that also plays a central role in the completion of mitosis and cytokinesis. Rcc2 mRNA is enriched in a class of neural progenitors in the ventricular zone, short neural precursors. Although Rcc2 mRNA is present at high levels in the ventricular zone during neurogenesis, the impact of Rcc2 on cortical development has not been previously studied.

We used two methods to study the role of Rcc2 in vivo. First we isolated a portion of the upstream regulatory region of Rcc2 and used it to express a fluorescent protein. Additionally, we used an shRNA targeting Rcc2 to knockdown expression of Rcc2.

We found that the promoter region of Rcc2 labeled cells that were near the board of the ventricular zone and subventricular zone, and tended to be positive for Sox2 but not Tbr2, when compared to the general progenitor population. Progenitors electroporated with Rcc2 shRNA were closer to the ventricular surface than those with functioning Rcc2. Examination of the cell cycle in cells electroporated with Rcc2 shRNA indicated no difference to those with Rcc2.
We found that Rcc2 was active during neurogenesis in ventricular zone progenitors. Additionally, our analysis shows that Rcc2 may be involved in the migration of progenitors during neurogenesis. Further works needs to be done to further elucidate the role of Rcc2.
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<tr>
<td>aRG</td>
<td>apical radial glia</td>
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<tr>
<td>bRG</td>
<td>basal radial glia</td>
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<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>INM</td>
<td>interkinetic nuclear migration</td>
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<td>IPC</td>
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<td>N2a</td>
<td>Neuro2a</td>
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<td>PR</td>
<td>promoter region</td>
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<td>Rcc2</td>
<td>Regulator of Chromosome Condensation 2</td>
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<tr>
<td>SNP</td>
<td>short neural precursor</td>
<td></td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
<td></td>
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<tr>
<td>VZ</td>
<td>ventricular zone</td>
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Introduction

One major evolutionary development is the increasing complexity of the brain from simple neural systems in C. Elegans, to lissencephalic, or smooth, brains of mice, to the gyrencephalic, or folded, brains of primates. Although many mechanisms of neurogenesis are similar between different species, there are differences in neurogenesis as brain size and complexity increases. As brain complexity increases, multiple evolutionary procedures developed during neurogenesis to account for the larger size and emergence of diverse neural types. These include multiple progenitor types to increase the final number of neurons and scaffolding within the developing brain to allow for proper neural positioning. If the number of progenitors or their positioning and migration is disrupted, there are alterations in the final brain.

In mammals, nervous system tissue arises from the ectoderm. Part of the ectoderm forms the neural crest and then the neural tube. The neural tube is composed of neuroepithelial cells which divide at the surface of the ventricle and expand the number of proliferative cells. As development continues, the neuroepithelium transitions into the ventricular zone and the subventricular zone.

New aspects of the development of the mammalian neocortex are continually being discovered. The first ventricular zone progenitors discovered were apical radial glia (aRG) cells by Kölliker and His in the late 19th century (reviewed in Rakic, 2003). Scientists have been trying to identify the classes of
cells involved in neurogenesis and how these cells form the complex laminar
cortical structure. Initial experiments identified dividing cells, termed
spongioblasts, and radial fibers, although they were thought to be separate
structures. Rakic first identified the use of radial aRG fibers as a neural
migratory scaffold using electron microscopy in 1972 (Rakic, 1972). The radial
fibers are particularly important for gyrencephalic brains as the migratory path for
neurons is not linear. Neurons migrating along the same fibers form ontogenetic
columns (Rakic, 1988) as these neurons are frequently the progeny of the same
progenitor. Initially, it was thought that spongioblasts were a separate class of
cells from aRG. aRG were not definitively identified as self-renewing progenitors,
also known as stem cells, until dividing aRG were visualized using retroviral
labeling along with anatomical, immunohistochemical, and electrophysiology
evidence (Noctor et al., 2002).

New classes of progenitor cells are being identified, and current
knowledge of neural progenitors includes aRG and short neural precursors
(SNPs) (Gal et al., 2006) in the ventricular zone (VZ) along with intermediate
progenitor cells (IPCs) (Miyata et al., 2004) and basal radial glia (bRG) (Reillo et
al., 2011) in the subventricular zone (SVZ) (Figure 1a). aRG are self-renewing
progenitors while the other progenitors have limited proliferations before
committing to a neurogenic fate. The specific progeny of each division is
influenced by the plane of division, where a symmetrical division with the apical
contact split evenly will produce a combination of aRG and SNPs, and
asymmetrical divisions produce a ventricular progenitor and a neuron or basal progenitor (Haydar et al., 2003, Noctor et al., 2004) (Figure 1b). Regulation of the plane of division is necessary for maintaining the population of neural progenitors (Konno et al., 2008). The plane of division is effected by multiple factors, including integrin complexes (Streuli, 2009). During early and mid-neurogenesis, aRG produce neurons or neural progenitors, but during late neurogenesis they switch to an astrogenic producing population (Voigt, 1989).

Neural progenitors have unique expression of genes which are responsible for a variety of functions within the developing neocortex. Some of these markers are commonly used for immunohistochemistry to differentiate types of cells. Common markers of ventricular progenitors are transcription factors which include Sox2 and Pax6 and IPCs are labeled with Tbr2. Additionally there are DNA promoter regions of genes which are specifically expressed in selected neural types which can be used with fluorescent markers to identify progenitors. Ventricular zone progenitors are labeled with Tα1 (Gal et al., 2006), aRG with BLBP and GLAST (Hartfuss et al., 2001), and IPCs with Tbr2 (Pontious et al., 2008).

It is unclear why so many different progenitor types have evolved and what effect the morphology of the processes has on their function and neurogenesis. One of the prevailing theories is that an abundance of progenitors increases the final neural population. There is limited space along the ventricular
surface in which progenitors can divide, and expansion in the SVZ, where cells can divide away from the ventricle, expands the proliferative zone (Kriegstein et al., 2006). This theory is supported by increased numbers of certain progenitor types, such as bRG, in gyrencephalic brains along with a larger SVZ (Lui et al., 2011). Certain progenitor types are altered in neurodevelopmental disorders. For example, the SNP population is reduced in a model of Down’s syndrome (Tyler and Haydar, 2013).

The apical and basal processes of neural progenitors play an important role in neurogenesis, yet aRG are the only class of cells with both an apical and basal process. SNPs lack a basal process, bRG lack an apical process, and IPCs have a multipolar morphology without contacts to either the ventricle or pial surface (Figure 1). The apical contact with the ventricle has a primary cilium which allows for signaling molecules within the cerebrospinal fluid (CSF) to affect ventricular zone neurogenesis (Lehtinen and Walsh, 2011). This apical process also contains the centrosome and is necessary for interkinetic nuclear migration (INM) which allows cells to divide at the surface of the ventricle (Götz and Huttner, 2005). In the VZ, aRG and SNPs divide at the surface of the ventricle. INM allows the nuclei of cells to cycle through different positions in the ventricle allowing a large number of progenitors to divide at the ventricular surface. While in S-phase, aRG and SNPs are in the top of the VZ, move toward the surface in G2, go through mitosis at the surface, and return to the top of the VZ during G1. There have been several proposed reasons for the necessity of progenitors to
divide at the ventricular surface. A centrosome is located in the apical contact with the ventricle, which requires the cell body to move toward the ventricular surface for mitotic spindle assembly (Chenn et al., 1998). Additionally, there are signaling gradients throughout the ventricular zone. As the progenitors move toward the ventricular surface they are exposed to different signals which can affect the neurogenic and proliferative potential of the cells (Del Bene, 2011). The basal to apical movement of progenitors is mediated by microtubule and actin based motors (Kosodo et al., 2011; Schenk et al., 2009). The apical to basal movement may be driven by the displacement of the cell nuclei (Kosodo et al., 2011).

Throughout neurogenesis, the cell cycle length increases, from 8.1 hours at e11 to 18.4 hours at e16. This increase is primarily due to an increased length in G1 (Takahashi et al., 1995). Increasing the cell cycle length can cause cells to exit the cell cycle (Calegari and Huttner, 2003). Additionally, different classes of progenitors, such as RG and SNPs, have different cell cycle lengths (Stancik et al., 2010). The length of G1, which is when progenitors move away from the ventricular surface, is intrinsic to cell age and type.

Progenitors interact with extracellular matrix through integrins, which are membrane bound heterodimers with an α and β subunit. They bind to extracellular laminin and fibronectin and can induce signaling pathways that can affect cell survival, proliferation, and differentiation. Blocking β integrin signaling
disrupts the structure of the VZ and causes more cells to divide away from the ventricular surface (Loulier et al., 2009).

A member of the integrin signaling complex is Regulator of Chromosome Condensation 2 (Rcc2). In human cells, the protein Rcc2 is also known as telephase disk protein of 60 kDA (TD-60). Rcc2 binds with cortactin (Grigera et al., 2012), and regulates Rac1 and adenosine 5’-diphosphate ribosylation factor 6 (Humphries et al., 2009). Rcc2 binds to nucleotide free Rac1 but not GDP bound Rac1 (Mollinari et al., 2003). Rcc2 is expressed specifically in late G2 and mitosis and is aligned with microtubules along the equatorial plane in dividing cells (Figure 2a) (Andreassen et al., 1991). Suppression of Rcc2 arrests cells in G2/M phase and leads to a disorganized spindle (Mollinari et al., 2003).

Cortactin is a cytoplasmic protein that can promote polymerization of actin and is often localized in the growth cone of migrating cells and in the equatorial plane of dividing cells (Du et al., 1998 and Grigera et al., 2012). Cortactin is localized in a similar pattern to Rcc2 during cell division, and Rcc2 is present during immunoprecipitation of cortactin, indicating that they may be in the same complex during mitosis.

Rcc2 was identified in a fibronectin signaling pathway between Arf6 and Rac1 (Figure 2b). Integrins interact with fibronectin to induce intracellular signaling. When Rcc2 was knocked down in cell culture, the time to activation of Arf6 was decreased. Rac1 activity was also increased following Rcc2
knockdown. Rac1 activity is associated with random migration, while inhibition of Rac1 increased continuous directional migration (Figure 2c) (Pankov et al., 2005). Knockdown of Rcc2 did not alter the total distance traveled by MEF cells on fibronectin; however the direction of movement was less persistent. The increased activity of Arf6 and Rac1 were dependent on fibronectin signaling, indicating that Rcc2 is activated by fibronectin through the integrin signaling complex causing inhibition of Arf6 and Rac1 (Humphries et al., 2009).

One regulator of Rcc2 is miR-29c; miR-29c decreases the levels of both Rcc2 mRNA and protein by binding to the 3'UTR. In gastric carcinoma, miR-29c is downregulated, and Rcc2 is upregulated. Alternatively, when miR-29c precursor is introduced to cells, Rcc2 mRNA and protein levels are decreased. To examine the effect of Rcc2 on cell proliferation, Rcc2 knockdown caused a decrease in cell viability and proliferation. MiR-29c suppresses growth of tumors, which may be mediated by Rcc2 (Matsuo et al., 2013).

Overall, the previous literature indicates that Rcc2 is active during mitosis and downregulation of Rcc2 causes uncoordinated cell migration and a decrease in cell proliferation. We hypothesized that we would label a neural progenitor population enriched in SNPs and knockdown of Rcc2 would alter the migration and proliferation of neural progenitors.

Methods
Selection of Rcc2

Previously, two neural populations, aRG and all ventricular zone progenitors, were labeled using pGlast+ and pTa1+ respectively and separated using FACS to examine mRNA expression using a microarray. The mRNA expression levels for these two populations were compared and sorted by differential expression levels. From this list, genes were selected that had a twofold higher expression in the pTa1+ population. We screened using published in situ data for expression in the ventricular zone (VZ) and subventricular zone (SVZ). Bioinformatics were used to determine the strength and size of putative promoter regions (PR) and ideal candidates were identified. From this information we focused on genes that have strong PR with localized VZ expression. We examined the role of Rcc2 during neurogenesis due to its increased expression in ventricular zone, and involvement in processes associated with neurogenesis.

Construction of plasmids

We used PCR to isolate a 2.7 kb portion of the upstream regulatory region of Rcc2 using Phusion polymerase and added SacI and KpnI restriction enzyme sites to the 5’ and 3’ end of the sequence. This regulatory sequence was cloned upstream of the coding region of a destabilized zsGreen (dsZsGreen) which has a half-life of 2 hours in vivo. Plasmids were purified using an endofree Maxi kit.
(Qiagen) for in utero electroporation surgeries. Confirmation of cloning was achieved using PCR and enzyme digests with internal sites.

shRNA sequences were generated by putting the mRNA sequence of Rcc2 into the shRNA sequence designer from clontech. Two potential shRNA sequences were selected and sense and antisense oligonucleotides with SalI and BglII overhangs were ordered from IDT and annealed together and cloned into an expression plasmid, CAG-RFP-H1-shRNA (CRLH-shRNA). An shRNA against luciferase (luc) was used as a control. Neuro2A (N2a) cells were grown in 1:1 fortified DMEM to OPTI-MEM with 10% FBS and 1% penicillin and streptomycin. 1 million cells were transfected using Kit V for the Amaxa Nucleofector using the suggested settings. Cells were plated either in culture dishes or on coverslips. 48 hours after transfection, cells were rinsed three times in cold sterile PBS then collected in RIPA lysis buffers with inhibitors. Lysates were placed on ice for 45 minutes then spun down at 13,000 rpm at 4C for 20 minutes. The supernatant was collected and the protein concentration was measured. Protein samples were prepared with 20 µg of protein, 4x dye, and 10x reducing agent to a total of 30 µL and denatured for 10 minutes at 70C. Samples were run on a gradient gel at 150V for 90 minutes. Transfer to a nitrocellulose membrane occurred overnight at 30V at 4C. Membranes were probed for transfer quality using Ponceau S. Membranes were washed in TBST followed by block in 5% milk in TBST for 1 hour. Rcc2 antibody (Bethyl, Cat No. IHC-00155) was used at a concentration of 1:5000 overnight in 5% milk in TBST.
Following three washes in TBST, Goat anti Rabbit horseradish peroxidase secondary was used at a concentration of 1:1000. Chemiluminescent images were collected using Dura substrate (Thermo, 34076) and the Gel Logic 2200 imaging system. Membranes were washed overnight in TBST and reprobed for beta actin as a control for overall protein levels. Cultures grown on cover slips were fixed using 4% PFA and stained for Rcc2 (1:50) and DAPI.

The Amaxa Nucleofector resulted in an 80-90% transfection rate of the N2a cells as determined by counting the number of RFP+/DAPI+ out of all DAPI+ cells. The western blot and staining showed a partial knockdown with Rcc2 shRNA1 and an almost complete knockdown of Rcc2 shRNA2 (Figure 3). Staining revealed little Rcc2 protein in cells that were transfected with CRLH-Rcc2 shRNA2 and high levels of Rcc2 protein in non-transfected cells. For all following experiments, Rcc2 shRNA2 was used, and is referred to as Rcc2 shRNA.

In utero electroporation

Pregnant CD1 dams at embryonic day 14.5 (E14.5) were anesthetized with a ketamine/xylazine mixture. Using aseptic techniques, the uterine horns were exposed using a midline laparotomy. IUE was performed with 3 µg/µL of pRcc2-dsZsGreen, a ubiquitous promoter driving a cyan fluorescent protein (CAG-CFP), CRLH-Luc, and/or CRLH-Rcc2 shRNA. One to two µL of DNA were injected into the lateral ventricle of each embryo. The anode of an electrode was
placed over the injected hemisphere of the embryo. Four pulses 50 µs separated by 950 µs of 35V at e14.5 was applied with the anode on the dorsal side of the brain. During the surgery, embryos were kept moist with warm physiological saline. After all desired embryos were electroporated, they will be returned to the abdomen, which was be filled with warm physiological saline. The abdominal wall and skin were sutured shut. Triple antibiotic ointment was applied over the stitches. Yohimbine was given to reverse Xylazine anesthesia along with Buprenex for analgesia.

Results

Activity of Rcc2 promoter region

We decided to examine the activity of the cloned Rcc2 promoter region during the peak of neurogenesis, at e14.5. Online in situ databases showed mRNA expression of Rcc2 at e14.5, so there was evidence that the promoter would be active during this time. We co-electroporated CAG-CFP and pRcc2-dsZsGreen together and collected the embryos 24 hours later. The CFP+ and dsZsGreen+ cells were examined for their location along the ventricular surface, in the VZ, in the overlap between the VZ and SVZ, and above the SVZ. Additionally the progenitors were classified with PH3, Sox2, and Tbr2 (Figure 4a, b). A higher proportion of the dsZsGreen+ were located at the top portion of the VZ (Figure 4c) and were positive for Sox2, which indicates that at 24 hours (Figure 4d), pRcc2 labels ventricular zone progenitors in S-phase. The pRcc2+
cells were also examined for morphological characteristics. There was a range of morphologies observed in the labeled cells, including SNPs, aRG, IPCs, and bRG. There were several observed pairs of cells which may have been daughter cells of the same division which were SNPs and bRG, which could suggest a method of generating both types of cells from a single aRG progenitor. However, the Rcc2 promoter produced inconsistent results between surgeries which made conclusions about the promoter activity difficult.

Knockdown of Rcc2

We examined the role of Rcc2 during neurogenesis by knocking down expression of Rcc2. The two shRNAs that were used were luciferase as a control and the Rcc2 shRNA that was validated in N2a cells. All surgeries were done with one uterine horn electroporated with the control plasmid and the other with the Rcc2 knockdown plasmid. Experiments were done with control and knockdown from the same litter. The distance of electroporated cells from the ventricle was measured along with their identity for Sox2, Tbr2, and PH3 (Figure 5a). We collected embryos 24 hours after IUE in order to examine alterations in the ventricular zone and subventricular zone prior to a high proportion of cells becoming post mitotic. The number of electroporated cells that were also Tbr2+ and Sox2+ was not altered (Figure 5b). However, when the location of the electroporated cells was taken into consideration, there were alterations in the knockdown population. In the overall electroporated population and in the Sox2+
population, cells electroporated with Rcc2 shRNA were closer to the ventricular surface (Figure 5c, d). There is a similar trend in the Tbr2+ population but it is not significant (Figure 5e). This indicates that Rcc2 is not involved in the fate determination of neural progenitors from ventricular zone progenitors to intermediate progenitors since the proportion of cells was not altered. Additionally, there are more PH3+/RFP+ cells closer to the ventricular surface in the Rcc2 knockdown, which is significantly increased 20-40 µm away from the surface at the ventricle. Typically, PH3+ cells are located along the ventricle or within the subventricular zone, so the presence of mitotic cells within the ventricular zone but not along the ventricle could indicate a problem in interkinetic nuclear migration.

After 48 hours more electroporated cells have left the ventricular zone and are becoming neurons. Investigating the effects of Rcc2 knockdown at this time point gives information about migration and the neurogenic potential of the progenitors. Similar to the surgeries from e14.5-e15.5, the 48 hour surgery had similar overall numbers of Sox2+ and Tbr2+ progenitors. The localization of RFP+ cells is also altered due to knockdown of Rcc2 after 48 hours. There are more Sox2+/RFP+ cells close to the ventricular surface, and the distribution of Tbr2+ cells is closer to the ventricle. There are also fewer RFP+ cells that are negative for both Sox2 and Tbr2 away from the ventricle, which indicates that either post mitotic cells are being generated later or there are complications with migration (Figure 6).
Additionally, at e16.5 there were fewer PH3+ cells 60-80 µm away from the surface of the ventricle (Figure 7). This range is part of the SVZ where IPCs typically divide.

There were no changes in cell death as measured by cleaved caspase 3 (Figure 8) or the number of electroporated cells per section indicating that there are no significant differences in cell death or division.

Cell cycle

Looking at 24 and 48 hours after IUE at e14.5 indicated that there were deficits with the location of cells electroporated with Rcc2 shRNA. However from that information we cannot definitively conclude what is the cause of the alterations. Once again, we performed IUE surgeries at e14.5, and collected embryos 24, 26, 28, 30, 32, and 34 hours after IUE. The brains were stained for PH3 to measure the mitotic divisions and get an insight into the cell cycle length due to knockdown of Rcc2. The distance to the ventricle was also measured since cells undergo interkinetic nuclear migration and move toward the ventricle surface in order to divide.

If there was a difference in the cell cycle length due to Rcc2 knockdown, we would expect to see differences in the number of PH3+ cells and RFP+ positive cells along the ventricular surface. 26 hours after IUE, knockdown of Rcc2 decreased the number of PH3+/RFP+ cells. There were no significant
differences in the number of surface mitotic divisions due to Rcc2 knockdown. At
24 and 30 hours after surgery, there were more PH3+/RFP+ cells 20-40 μm
away from the surface of the ventricle (Figure 9). This an atypical location for
divisions, since mitotic events usually occur at the surface of the ventricle or in
the subventricular zone. The SVZ has less PH3+ cells due to Rcc2 knockdown
32 hours after IUE surgery.

Similar to 24 and 48 hours after IUE, the RFP+ cells with knocked down
Rcc2 were closer to the ventricle than cells with functioning Rcc2 at 26, 28, and
30 hours after IUE. There were no differences in the number of RFP+ cells in the
first 40 μm from the surface of the ventricle. At 24, 32, and 34 hours after IUE,
the distribution of electroporated cells is similar.

Conclusion

Rcc2 is thought to be involved with numerous processes within cells,
including mitosis and migration. Since the role of Rcc2 has not been investigated
in neurogenesis, we first examined when a portion of the upstream regulatory
region of Rcc2 was active during neurogenesis. While we did find a sparse
labeling in multiple cell types, it was not consistent between experiments. This
may be because we used a version of zsGreen which was destabilized so it only
had a half-life of 2 hours in vivo. Therefore, if it was only active during a
particular time in the cell cycle, there could be differences in expression due to the cells being in different portions of the cell cycle. The labeling ranged from only a few cells to almost ubiquitous labeling. An explanation would be there were a few promoter sites could have random errors while growing and copying the plasmids, which could cause differences between experiments. In particular, we saw almost no labeling 48 hours after IUE with pRcc2-dsZsgreen, which could indicate that either the electroporated cells were not in the right part of the cell cycle of parts or the promoter region were getting silenced. From the experiments conducted, the reason for inconsistent labeling is not clear. However, we were able to conclude that the promoter was active at least part of the time and decided to continue examining the role of Rcc2 by knocking down expression using shRNA plasmids. In order to test activity of the Rcc2 promoter, we would need to perform time lapse imaging to examine activity throughout the cell cycle.

One of the first questions we wanted to answer was if knockdown of Rcc2 affected the balance of neural progenitors in the developing neocortex. We found that the numbers of ventricular zone progenitors and intermediate progenitors was not altered. However in this experiment, we found that the positioning of neural progenitors was altered after knockdown of Rcc2, with the cells lacking Rcc2 remaining closer to the ventricular surface.
The cause for the alterations in location was not clear from the two time points. One possible explanation was the cell cycle length was altered in knockdown which would change the time that progenitors were leaving the ventricular zone and the time at which they migrated to the ventricular surface. Alternatively, migration could be altered due to knockdown of Rcc2 and the interaction with the extracellular matrix through integrin signaling.

In order to test this, we examined the effect of Rcc2 through multiple time points to see if the cell cycle length was altered. If the cell cycle length was altered, we expected to see similar patterns of labeled cells but at different time points. Instead, what we saw was similar patterns at certain time points, although at other time points the cells with knocked down Rcc2 were closer to the ventricle. At a few time points, there were more PH3+ cells in bins which we would not typically expect to see PH3+ cells. This indicates that there could be alterations in the migration of cells when expression of Rcc2 is reduced. Experiments knocking down Rcc2 in cell culture found that directional migration was reduced, although the overall distance moved was the same. Neural progenitors undergo directional movement when they undergo interkinetic nuclear migration and leave the ventricular zone; deficits in directional movement would cause accumulation of cells close to the ventricle.

There are no changes in the total number of cells, which supports the idea that the cell cycle length is not altered. Prior reports indicated that Rcc2 was
required for progression through G2/M phase, which we did not observe in our experiments. Some of these indicated that Rcc2 was present specifically in the equatorial plane of dividing cells; in our stains of a mouse neuroblastoma cell line, Rcc2 appeared to be present throughout the cells, and was excluded from the chromosomes during cell division. Therefore in neural cells, the role of Rcc2 could be different from that of alternate cell lines. Some of the prior experiments were also done on human cell lines; therefore Rcc2 could have different roles in mouse and human lines. The different results could also be a result of using different cell lines, since the role of \textit{Rcc2} in neurogenesis has not been examined previously. To further examine the role of \textit{Rcc2}, experiments would have to be done to compare between species, and between different classes of cells in the same species.

We investigated the role of Rcc2 in the developing mouse neocortex. We found that knockdown of Rcc2 altered the localization of progenitors in the neocortex, which resulted in progenitors and post-mitotic progeny to be closer to the ventricular surface. In our experiments, it appears that the alterations found may be attributed to alterations in migration, however further experiments need to be done to determine the cause of this phenomenon.
Figure 1: (a) Schematic of the known types of progenitors in the developing brain. Radial glia (blue) self renewing and form short neural precursors (red), basal radial glia (green), and intermediate progenitor cells (yellow). All of these cell types can create neurons (black). Common markers for labeling the cells with *in utero* electroporation and immunohistochemistry are noted. (Tyler and Haydar, 2013). (b) The progeny resulting from a division is affected by the inheritance of the apical contact (Götz and Huttner, 2005)
Figure 2: (a) Rcc2 is thought to be a part of the integrin signaling complex and acts as an inhibitor of Arf6 and Rac1 (Danen, 2009). (b) Staining HeLa cells with an antibody against TD-60 showed localization to the equatorial plane in dividing cells and to the centromeres in pro-metaphase (Mollinari et al., 2003). (c) Knockdown of Rcc2 in MEF cells reduced directional migration causing a reduction in distance moved from the starting location (Humphries et al., 2009).
Figure 3: Knockdown of Rcc2 shRNA in N2a cells for 48 hours causes moderate reduction of RCC2 with shRNA1 and almost complete reduction with shRNA2. (a) Cells with functioning RCC2 show RCC2 staining throughout the cells. For Rcc2 shRNA2, only untransfected cells show significant RCC2 staining. (b) Western blot of cell lysates collected from transfected cells shows partial and almost complete knockdown for Rcc2 shRNA1 and shRNA2 respectively.
Figure 4: CAG-CFP and pRcc2-dsDsZsgreen were electroporated into CD-1 dams which were pregnant at e14.5. (a) Embryos were collected 24 hours later and stained for Sox2, Tbr2, and PH3. (a’) pRcc2+ cell that is positive for Sox2. (a’’) pRcc2+ cell that is positive for Tbr2. (b) Electroporated cells were placed into bins based on their location. The majority of pRcc2+ cells were in the overlapping region between the upper ventricular zone and subventricular zone. (b’ (c) Compared to cells labeled by a ubiquitous promoter, more pRcc2+ cells were labeled by Sox2, and less were labeled by Tbr2 or PH3.
Figure 5: (a) shRNA constructs for Luciferase or Rcc2 were electroporated into embryos at e14.5 and collected at e15.5. (b) There were no significant differences in the number of Sox2+ and Tbr2+ labeled RFP+ cells. (c) The localization of the entire RFP+ population was shifted closer to the ventricle due to knockdown of Rcc2. (d) Additionally there were more Sox2+/RFP+ cells closer to the ventricle when Rcc2 is knocked down. (e) There were no significant differences in the Tbr2+ electroporated population, but there was a trend for the control cells to be further away from the ventricle.
Figure 6: (a) e14.5 embryos were electroporated with CRLH plasmids with either luciferase or Rcc2 shRNA and stained for Sox2 and Tbr2. (b-e) Closed circles are luciferase control and open circles are Rcc2 shRNA. Y-axis is percentage of total RFP+ cells. (b) Sox2+ only progenitors lacking Rcc2 are closer to the ventricular surface. (c) Tbr2+ only progenitors with Rcc2 are further from the ventricle.
Figure 7: 48 hours after knockdown of Rcc2, there are fewer PH3+ cells 60-80 μm away from the surface of the ventricle.
Figure 8: There were no changes in cleaved caspase 3 activity 24 or 48 hours after knockdown of Rcc2 at e14.5.
Figure 9: There are an increased number of PH3+/RFP+ cells 20-40 µm away from the surface of the ventricle 24 and 30 hours after IUE surgery at e14.5.
Works Cited


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