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Crumbs 2 prevents cortical abnormalities in mouse dorsal telencephalon

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ABSTRACT

The formation of a functionally integrated nervous system is dependent on a highly organized sequence of events that includes timely division and differentiation of progenitors. Several apical polarity proteins have been shown to play crucial roles during neurogenesis, however, the role of Crumbs 2 (CRB2) in cortical development has not previously been reported.

Here, we show that conditional ablation of *Crb2* in the murine dorsal telencephalon leads to defects in the maintenance of the apical complex. Furthermore, within the mutant dorsal telencephalon there is premature expression of differentiation proteins. We examined the physiological function of *Crb2* on wild type genetic background as well as on background lacking *Crb1*. Telencephalon lacking CRB2 resulted in reduced levels of PALS1 and CRB3 from the apical complex, an increased number of mitotic cells and expanded neuronal domain. These defects are transient and therefore only result in rather mild cortical abnormalities. We show that CRB2 is required for maintenance of the apical polarity complex during development of the cortex and regulation of cell division, and that loss of CRB2 results in cortical abnormalities.

1. Introduction

During neurogenesis, radial glial progenitor cells within the neural epithelium undergo appropriate spatiotemporal proliferation, differentiation and migration in order to produce a functionally integrated nervous system with correct lamination and intercellular synaptic connectivity (Bystron et al., 2008; Geschwind and Rakic, 2013). Within the neuroepithelia, progenitor cells adhere to each other *via* subapically localized adherens junctions (Aaku-Saraste et al., 1996). Neuroepithelial cells undergo interkinetic nuclear migration and the nucleus translocates to the apical surface during mitosis (Chenn and McConnell, 1995). In contrast, intermediate progenitor cells that populate the subventricular zone (SVZ) undergo mitosis away from the ventricles. There are two evolutionarily conserved protein complexes that reside apical to these junctions: the PAR complex (consisting of PAR3-PAR6-aPKC) and the Crumbs (CRB) complex (consisting of either CRB-PALS1-PATJ or CRB-PALS1-MUPP1) (Assemat et al., 2008; Bulgakova and Knust, 2009). These protein complexes are important for regulation of cell division and proper development and layering of the cortex. Although for some proteins in these complexes, such as MPP3 and PALS1 (Kim et al., 2010; Dudok et al., 2013b) the function has been shown, the roles of some of the other apical proteins, such as CRB2 in cortical development still needs to be elucidated.

PAR3 and PAR6 maintain cortical neural progenitor cells in a proliferative state, preventing premature exit of the cell cycle and inhibiting neurogenic differentiation (Costa et al., 2008; Boroviak and Rashbass, 2011). Similarly, the CRB complex has also been implicated in murine cortical neurogenesis as removal of PALS1 causes premature withdrawal from the cell cycle and an excessive production of early-born post-mitotic neurons (Kim et al., 2010). Subsequent massive death of neurons, secondary to loss of essential cell survival signals in these mice leads to significant disruption throughout much of the cortex (Kim et al., 2010). Additionally, loss of the PALS1 interacting protein MPP3 results in loss of apical complex proteins and adherens junction proteins and affected neuronal

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migration, but without the massive cell death observed in *Pals1* conditional knockout (cKO) brains (Dudok et al., 2013b).

Furthermore, we have demonstrated that CRB2 is a novel regulator of early neural progenitors derived from mouse embryonic stem (ES) cells (Boroviak and Rashbass, 2011). In this *in vitro* model, CRB2 was upregulated at the onset of neuroepithelial specification and localized to the apical side of neural rosettes. We showed that ES cells with depleted CRB2 died at the onset of neural specification. Conversely, *Crb2* overexpression in undifferentiated ES cells increased proliferation and reduced terminal neural differentiation (Boroviak and Rashbass, 2011). Recent reports showed that mutations in *CRB2* cause nephrosis and cerebral ventriculomegaly in humans, suggesting alterations or defects in cortical development (Ebarasi et al., 2015; Slavotinek et al., 2015). Amino-acid variation were detected in CRB2 but not yet linked to eye disease (van den Hurk et al., 2005). Mutations in human *CRB1* result in Leber congenital amaurosis or retinitis pigmentosa (den Hollander et al., 1999, 2004; Richard et al., 2006). CRB1 is also expressed at low levels in the developing mouse cortex (den Hollander et al., 2002), but there are no clinical reports that mutations in human CRB1 result in cortical dysfunction.

To investigate the roles of CRB2 protein in cortical development, we used mouse models to assess the role of the CRB2 proteins during the development of the cortex. However, complete loss of CRB2 results in embryonic lethality by E12.5 (Xiao et al., 2011). Thus, it would not be possible to use these embryos to distinguish primary neurodevelopmental defects from effects that were secondary to the earlier gastrulation abnormalities. Therefore, to address our hypothesis we analyzed the developing dorsal telencephalon of murine embryos that had *Crb2* specifically ablated in this tissue on wild type genetic background as well as genetic background lacking *Crb1*. We demonstrate that the CRB2-depleted telencephalon undergoes premature neural differentiation and displays abnormalities in lamination. Yet, the effects observed are transient. Telencephalon lacking CRB2 resulted in disruption of apical polarity protein complexes, in partial reduced levels of adherens junction proteins, and a significant increase in cell division. As loss of CRB2 during development results in alterations in cerebral cortical development in mice, amino-acid variations in human *CRB2* might be associated with cortical dysfunction in information processing or neurodevelopmental disorders.

2. Materials and methods

Generation of the Crb2 *conditional knockout mice on wild type genetic background and background lacking* Crb1

All animal procedures were performed with permission of the animal experimentation committee (DEC) of the Royal Netherlands Academy of Arts and Sciences (KNAW), permit numbers NIN06-46, NIN08-41 and NIN12.93. *Crb2* floxed homozygous mice (*Crb2*$^{F/F}$ or *Crb2*) and *Crb1* homozygous mice on a wild type mixed genetic background (on 50% C57BL/6JOlaHsd and 50% 129/Ola) were as previously described (van de Pavert et al., 2004; Alves et al., 2013b). The Crb2 mice (on 50% C57BL/6JOlaHsd and 50% 129/Ola) were crossed with *Crb2*$^{F/+}$Emx1*Cre*$^{Tg/+}$ (B6.129S2-Emx1tm1(cre)Krj/J$^{+/-}$; Jackson lab) mice (on 50% C57BL/6JOlaHsd and 50% 129/Ola). These mice expressed *Cre* recombinase under the control of Emx1 in the developing neuroepithelium of cerebral cortex and hippocampus (Gorski et al., 2002). For homozygous *Crb2*$^{F/F}$ Emx1*Cre*$^{Tg/+}$ (*Crb2* cKO) experimental embryos we used heterozygous *Crb2*$^{F/+}$ Emx1*Cre*$^{Tg/+}$ embryos as controls. To generate *Crb2* cKO mice on a background lacking *Crb1* in the cortex (*Crb1*$^{-/-}$*Crb2*$^{F/F}$Emx1*Cre*$^{Tg/+}$ in short called *Crb1Crb2* cKO mice), we crossed *Crb1*$^{+/-}$*Crb2*$^{F/F}$Emx1*Cre*$^{Tg/+}$ mice (on 50% C57BL/6JOlaHsd and 50% 129/Ola background) with *Crb1*$^{-/-}$*Crb2*$^{F/F}$ (on 99.9% C57BL/6JOlaHsd background) mice to obtain control cortex expressing CRB2 on background lacking *Crb1* (*Crb1Crb2* control) and experimental cortex lacking CRB2 expressing CRB1 (*Crb1*$^{+/-}$*Crb2* cKO) and cortex lacking CRB2 on background lacking *Crb1* (*Crb1Crb2* cKO) (all three genotypes on 75% C57BL/6JOlaHsd and 25% 129/Ola genetic background). Previously, we did not observe changes in *Crb2* cKO phenotype or expression profile on mixed (50% C57BL/6JOlaHsd and 50% 129/Ola) or pure C57BL/6J genetic background (99.9% C57BL/6JOlaHsd) (Alves et al., 2013a,b). Mice were kept on a 12 h day/night cycle plus and were supplied with food and water *ad libitum*. DNA isolation and genotyping was performed as described previously (van de Pavert et al., 2004; Alves et al., 2013b). Mice had no mutations in the phosphodiesterase 6b (*pde6b*) or *Crb1* (*rd8*) genes. Animal care and use in this study was in accordance with protocols and approval of the animal care and use committee of the Royal Netherlands Academy of Arts and Sciences (KNAW; protocols DEC-NIN08.41 and DEC-NIN12.93).

Immunohistochemical analysis

Timed matings were set up and the morning that the vaginal plug was discovered after mating was designated E0.5. Pregnant females were killed by CO_2/O_2 and cervical dislocation and the embryos dissected. Embryonic heads and brains were dissected away from the bodies (which were used for genotyping). Tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 3 h at 4 °C and cryoprotected in 15% and 30% sucrose in PBS and then embedded in Tissue-Tek O.C.T. compound (VWR) prior to 15 μm coronal cryostat sections being cut. Sections were incubated in primary antibody overnight at 4 °C solution in 0.1% Triton-X100/PBS with 5% heat inactivated donkey serum. The following primary antibodies were used: CD133 (1:250; Abcam), CRB1 (1:200) (van de Pavert et al., 2004), CRB2 (1:250; Custom made (Boroviak and Rashbass, 2011)), CRB3 (1:500, obtained from Dr. A. Le Bivic, Developmental Biology Institute of Marseille Luminy, Marseille, France), Cre (1:250; Covance), cleaved Caspase 3 (1:200, Cell signaling), CUX1 (1:250, Santa Cruz), Ki67 (1:100, BD Biosciences), N-cadherin (1:100; BD Biosciences), Nectin (1:500; MBL), Nestin (1:300; Abcam), PALS1 (1:200; Abcam, 1:500; Proteintech), PAR3 (1:200; Millipore), Phospho Histone H3 (pH3, 1:500; Millipore), Reelin (1:100; Millipore), SOX2 (1:250; Millipore), TBR1 (1:200; Abcam), TBR2 (1:500; Abcam), TUJ1 (1:500; Covance). Fluorescent secondary antibodies were used according to manufacturer's instructions (Jackson Immunolaboratories). DAPI (4,6-diamidino-2phenylindole, Molecular Probes) or TO-PRO3 was used to visualize nuclei (see legends of Figures). Slides were mounted in Vectashield (Vector Laboratories) or Mowiol and glass coverslips were sealed with nail varnish. Images were captured using Zeiss CLSM 510 confocal microscope or Zeiss Apotome microscope with Axioimager. Images were processed using ImageJ (NIH, http://rsb.info.nih.gov/ij), Photoshop CS4 (Adobe) and Bridge CS4 (Adobe).

BrdU injections and cell countings

For BrdU injections plugged female mice were injected peritoneally with 200 μl 1 mg/ml BrdU in 0.9% NaCl and killed 30 min or 24 h after BrdU injection after which the embryos were obtained and processed as described above. For anti-BrdU immunohistochemistry, slides were incubated in PBS 0.3% Triton X-100 followed by incubation in 2 M HCl for 45 min and subsequently washed with 10 mM sodium citrate after which immunohistochemistry was performed as described above. For anti-Ki67 immunohistochemistry, antigen retrieval was first performed by boiling slides in 10 mM

sodium citrate buffer after which acid treatment was performed as described above. For quantification of pH3 and BrdU cells in E12.5 cortex, cells were manually counted per 100 μm cortex. Cells within 3 cell nuclei of the apical membrane where considered to be apical, whereas cells outside this region where considered basal. For the cell proliferation experiments 3 embryos/group were used for the cell countings. Statistical analysis was performed using one-way ANOVA with the Bonferroni correction. Data are shown as average ± standard error of the mean (SEM) and $p < 0.05$ was considered statistically significant.

3. Results

Cre mediated deletion results in loss of CRB2 expression in the dorsal telencephalon

During cortical neurogenesis, CRB2 is expressed on the apical surface of the ventricular zone (VZ) progenitors of the dorsal telencephalon in wild type embryos (Fig. 1) (Kim et al., 2010). To determine whether CRB2 played a crucial role in cortical development, we examined homozygous *Crb2*$^{F/F}$Emx1*Cre*$^{Tg/+}$ (*Crb2* cKO) and control heterozygous *Crb2*$^{F/+}$Emx1*Cre*$^{Tg/+}$ (control) embryos on wild type background with *Crb2* deleted in the cortical progenitors of the dorsal telencephalon (Gorski et al., 2002). Control littermates showed a CRB2 expression profile during development similar to the wild type embryos (Fig. 2A–C). In contrast, *Crb2* cKO embryos had no detectable apical CRB2 expression in the dorsal telencephalon by E12.5 (Fig. 2A'–C'). The *Crb2* cKO animals survived into adulthood and did not display any overt morphological or behavioural defects after birth (data not shown). Since in the Emx1*Cre* line Cre recombinase is not expressed in the ventral telencephalon, we studied the ventral expression of CRB2 in *Crb2* cKO embryos. This revealed that in control cortex, the level and localization of CRB2 is comparable between dorsal and ventral telencephalon (Fig. 2D and E). In contrast, in *Crb2* cKO embryos there is a clear loss of CRB2 from the dorsal telencephalon, whereas levels and localization of CRB2 in the ventral telencephalon is comparable to control embryos (Fig. 2D' and E'). This shows that there

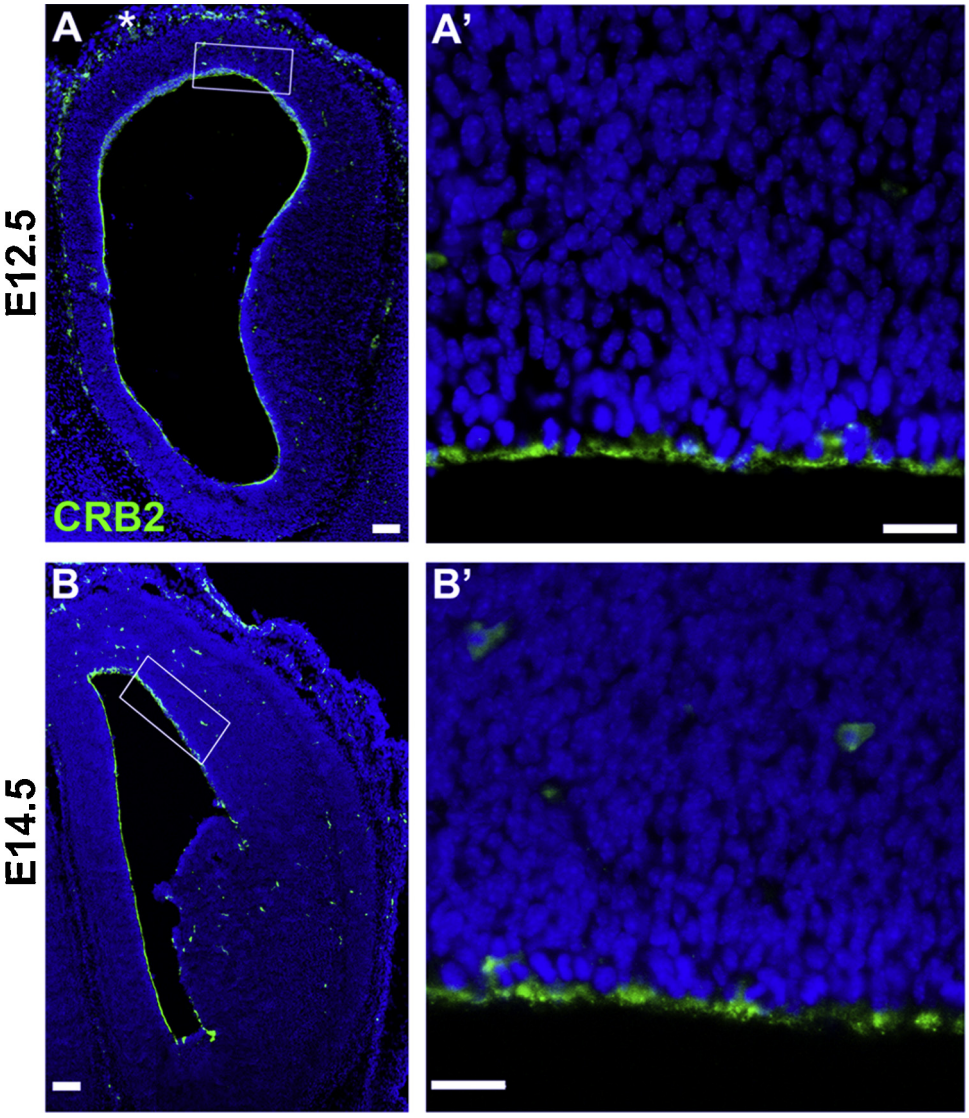

Fig. 1. CRB2 protein localization in wild-type mouse embryos. Coronal sections through the telencephalon of E12.5, and E14.5 mouse embryos immunostained for CRB2. (A–B) CRB2 is apically enriched in the telencephalon at E12.5 (A) and E14.5 (B). (A′–B′) Higher magnification images of sections nearby the sections indicated in the boxed areas in A–B. Some ectopic CRB2-positive cells seem randomly localized in the cortex, they did not colocalize with the antibodies used in this study. *Indicates non-specific staining because the staining was still present in *Crb2* cKO cortex (data not shown). Nuclei counterstained with DAPI (blue). Scale bars: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

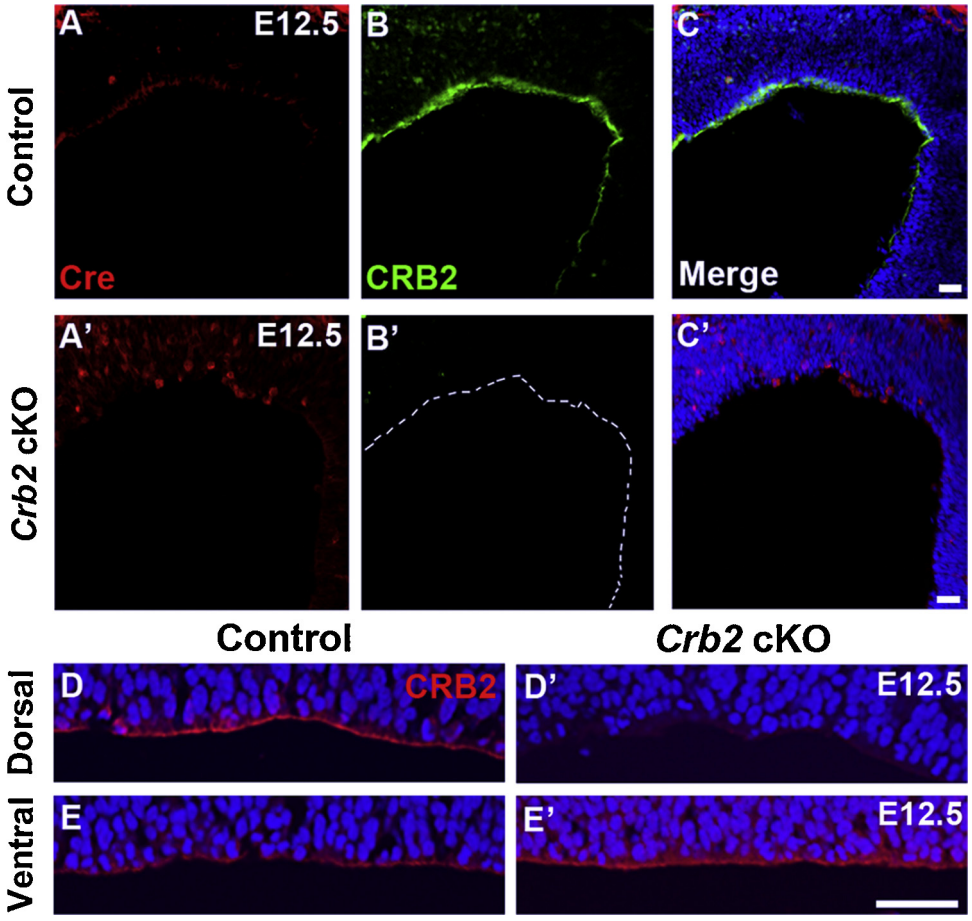

Fig. 2. Loss of CRB2 in dorsal telencephalon of *Crb2* cKO cortices. Coronal sections through the telencephalon of E12.5 control and cKO littermate embryos immunostained as indicated. (A–C) CRB2 protein is apically localized in the cortex of control embryos. (A'–C') CRB2 expression is completely lost in the cKO cortex. Dotted line in B' outlines apical surface of the cortex. In the ventral telencephalon of control cortex level and localization of CRB2 is comparable between dorsal and ventral telencephalon (Fig. 2D and E). In contrast, in *Crb2* Emx1*Cre* cKO embryos, in which Cre is expressed in the dorsal, but not the ventral, telencephalon, there is a clear loss of CRB2 from the dorsal telencephalon, whereas levels and localization of CRB2 in the ventral telencephalon is comparable to control embryos (Fig. 2D' and E'). Nuclei counterstained with DAPI or TO-PRO3 (blue). Scale bars: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

is a specific loss of CRB2 from the dorsal telencephalon in Emx1*Cre* *Crb2* cKO embryos.

Loss of CRB2 affects the neural progenitor cell population

To investigate the consequences of loss of CRB2 in cortical progenitor cells, the expression profile of SOX2 (Bylund et al., 2003), Nestin (Lendahl et al., 1990), Prominin (Marzesco et al., 2005) and TBR2 (Sessa et al., 2008) was analyzed in both control $Crb2^{F/+}$ Emx1$Cre^{Tg/+}$ and *Crb2* cKO littermates. At E12.5, SOX2 positive progenitors with clear nuclear staining were observed in the VZ of the control telencephalon (Fig. 3A). In contrast very few cells in the *Crb2* cKO cortex showed distinct nuclear localization of SOX2 (Fig. 3A'). At E14.5 the staining for SOX2 in the *Crb2* cKO dorsal telencephalon did not appear to be different from the controls. (Fig. 3B, B'). At E17.5 the control telencephalon had some SOX2 positive cells lining the ventricles however the majority was dispersed within the SVZ (Fig. 3C). In contrast, at this stage there were no SOX2 positive cells detected in the dorsal telencephalon of *Crb2* cKO mutants although SOX2 positive cells were still present in the lateral region (Fig. 3C').

Expression of Prominin (CD-133) an apical neuroepithelial stem cell marker (Marzesco et al., 2005) was completely lost in the *Crb2* cKO dorsal telencephalon at E14.5 whilst it was still present in the controls (Fig. 3D, D'). Nestin staining was also disturbed in the mutant cortex, so that its expression was markedly reduced at E12.5 compared to the control littermates (Fig. 3E, E'). At E14.5 the Nestin positive fibres were disorganized and significantly reduced in the mutant telencephalon compared to the controls (Fig. 3F, F'). By E17.5, Nestin expression was specifically restricted to a population of cells close to the ventricle in the control (Fig. 3G). In contrast, Nestin expression was completely downregulated in the mutant (Fig. 3G').

Combined the SOX2, Prominin and Nestin data implied that there were significant alterations in the apical neural progenitor population in the mutant. Therefore, the expression pattern of TBR2 was analyzed to determine whether the intermediate progenitor population was also affected. At E12.5, TBR2 was expressed in the SVZ of the control cortex (Fig. 3H). Interestingly, in the *Crb2* cKO cortex TBR2 positive cells were detected not only in the SVZ but also within the VZ (Fig. 3H'). Taken together these results indicate that conditional deletion of CRB2 from the cortex leads to a depletion of the apical neural progenitor pool and a concomitant increase in the basal progenitor pool.

Analysis of Crb2 *cKO cortex on genetic background lacking* Crb1

Previously, we have generated *Crb1* KO and *Crb2*Chx10*Cre* cKO, *Crb2*Crx*Cre* and *Crb2*Pdgfra*Cre* mice (van de Pavert et al., 2004; Alves et al., 2013b, 2014) and analyzed *Crb2*Chx10*Cre* cKO retinas on genetic background lacking *Crb1* (Pellissier et al., 2013). As removal of CRB2 in the developing cortex resulted in a clear

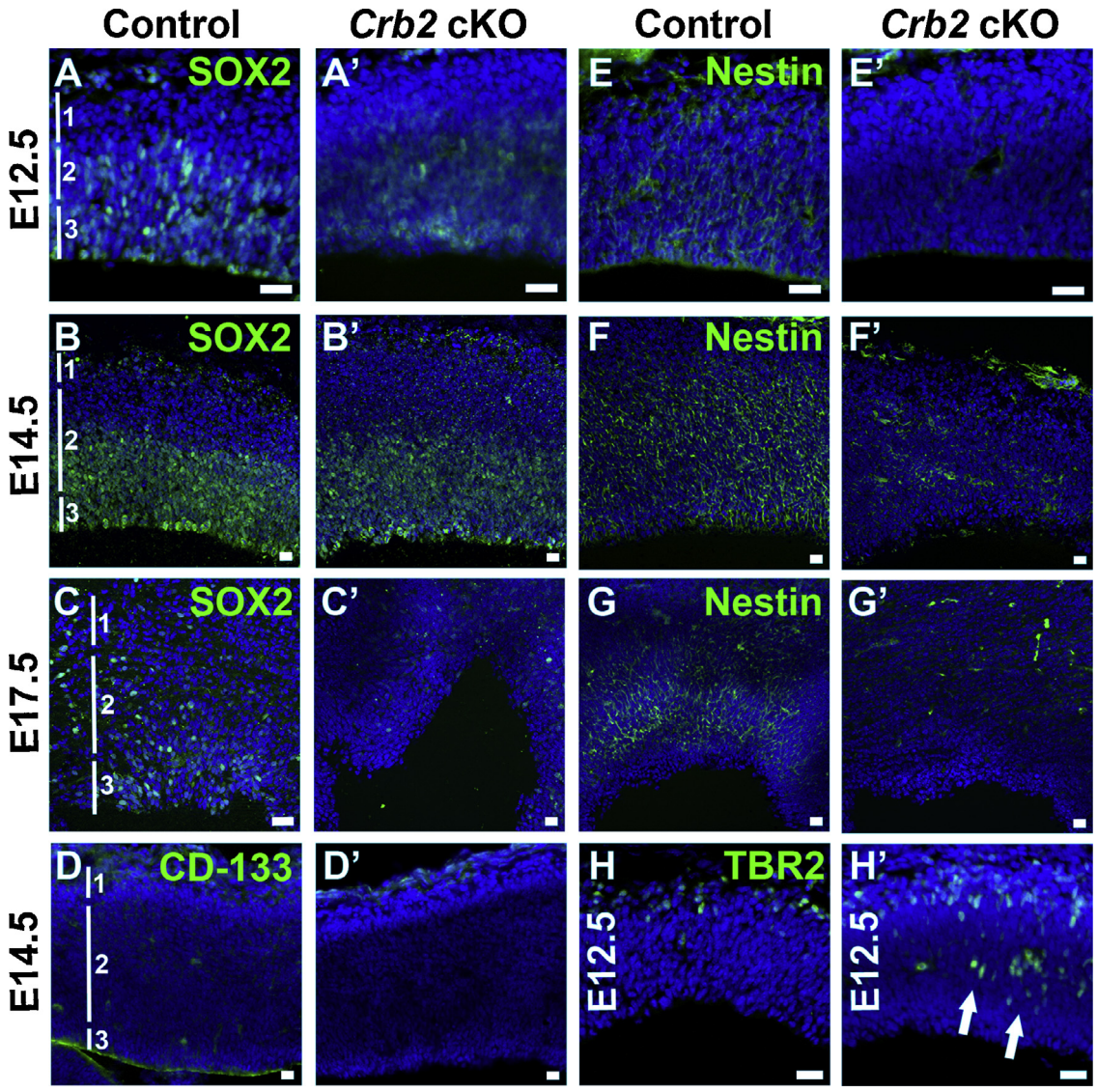

Fig. 3. Depletion of CRB2 affects the neural progenitor population in the dorsal telencephalon. Coronal sections through the telencephalon of control and cKO littermate embryos immunostained as indicated. (A–C) SOX2 is predominantly expressed in the VZ and in the SVZ neural progenitors in the control embryos. (A'–C') In the *Crb2* cKO embryos, SOX2 staining is similar to control at E12.5 and E14.5 and decreased at E17.5. (D–D') Expression of apical neural stem cell protein CD-133 is completely lost in the *Crb2* cKO cortex. (E–G) In the control, Nestin staining spans the cortex at E12.5 and E14.5. At E14.5 Nestin immunoreactivity is restricted to a specific population of cells in the dorsal telencephalon. (E'–G') In the cKO cortex, Nestin expression is significantly disrupted at all three embryonic stages. (H, H') In the control, $TBR2^{+}$ intermediate progenitors are detected in the SVZ and preplate. In the *Crb2* cKO cortex, mislocalised $TBR2^{+}$ cells are detected in the VZ (arrows in H'). Nuclei counterstained with DAPI (blue). Lines denoted with 1, 2 and 3 indicate the CP (1), IZ (2) and the SVZ/VZ (3) respectively. Scale bars: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phenotype in the developing cortex in embryos but no detectable morphological phenotype in newborn pups, we analyzed *Crb2* cKO cortex on genetic background lacking *Crb1* (*Crb1Crb2* cKO, expressing Cre recombinase), and as control homozygous $Crb2^{F/F}$ floxed cortex on genetic background lacking *Crb1* (*Crb1Crb2* control, not expressing Cre recombinase). Although CRB1 is predominantly expressed in the developing retina, in the developing cortex there are low levels of *Crb1* transcripts (den Hollander et al., 2002). Therefore, we aimed to investigate whether *Crb2* cKO cortex on genetic background lacking *Crb1* result in a more severe cortical phenotype than on wild type background. Immunohistochemistry for CRB2 in the developing cortex at E12.5 and E14.5 showed expression of CRB2 at the apical membrane in cortex expressing CRB2 (*Crb1Crb2* control; Fig. 4A–D) compared to cortex lacking CRB2 ($Crb1^{+/-}$*Crb2* cKO and *Crb1Crb2* cKO; Fig. 4A'–D' and A"–D"). Immunohistochemistry for CRB1 in the developing cortex at E12.5 and E14.5 showed that there was a clear loss of the low levels of CRB1 protein from the apical membrane in cortex lacking *Crb1* (*Crb1Crb2* control and *Crb1Crb2* cKO; Fig. 4A–D and A"–D") compared to cortex expressing CRB1 ($Crb1^{+/-}$*Crb2* cKO; Fig. 4A'–D').

CRB2 is required for maintenance of the apical complex proteins

To investigate the effect that *Crb2* deletion had on the expression of apical polarity components, the expression of two proteins – PALS1, a member of the Crumbs complex, and PAR3, a member of the PAR complex, were analyzed. In the control littermate embryos on wild type genetic background, PALS1 was expressed in the apical domain of the VZ cells at E12.5 and E14.5 (Fig. 4E and F) in a similar manner as that described by others (Kim et al., 2010). In contrast, in the *Crb2* cKO embryos on wild type genetic background, the expression of PALS1 was barely detectable at both these stages (Fig. 4E' and F'). Consistent with previously published data (Manabe et al., 2002; Bultje et al., 2009), PAR3 expression was

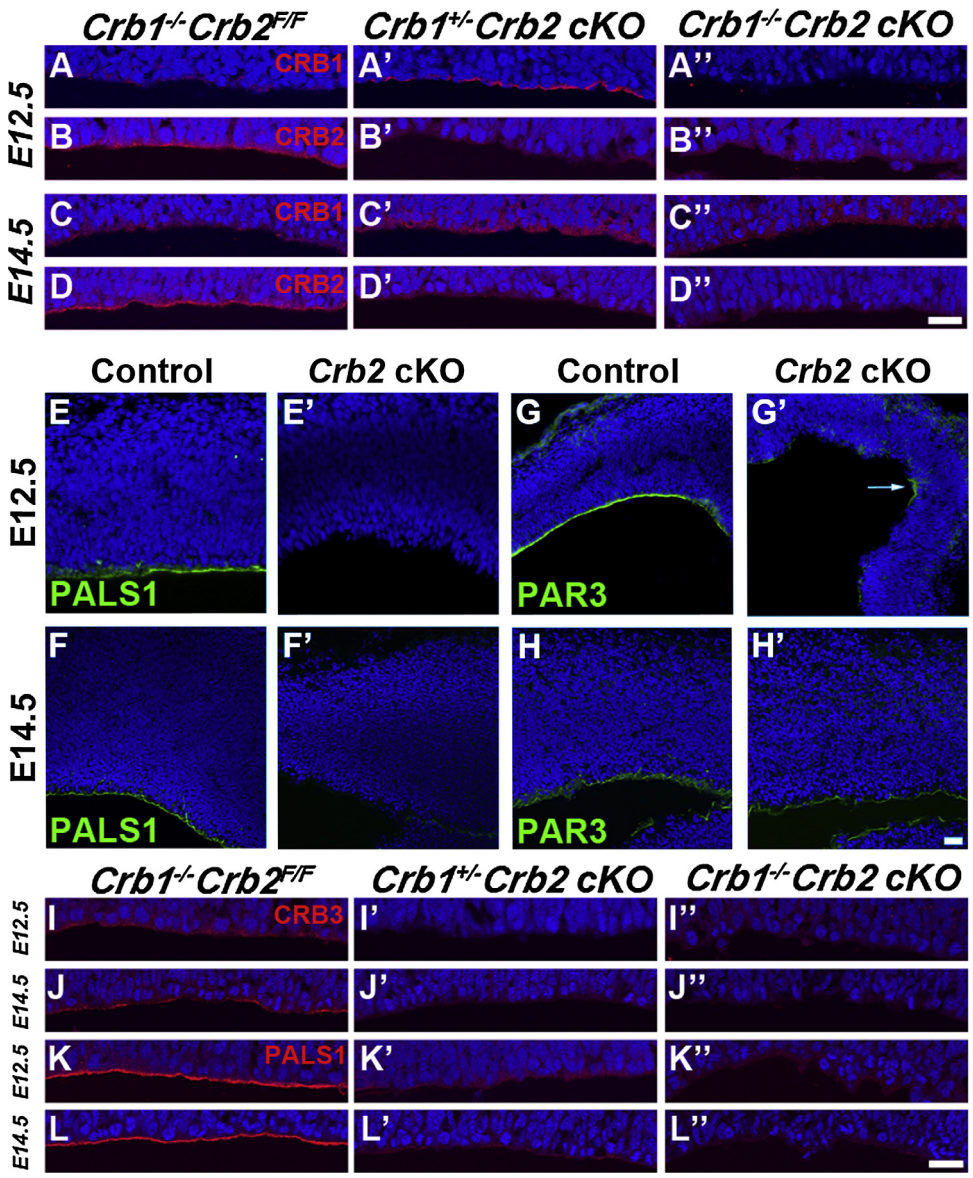

Fig. 4. Deletion of *Crb2* by Emx1*Cre* in the dorsal telencephalon affects localization of apical polarity proteins. In E12.5 cortex, CRB1 in *Crb1*$^{+/-}$*Crb2* cKO cortex (A'), which is absent in *Crb1Crb2* control (A) and *Crb1Crb2* cKO (A") cortex. (B–B") CRB2 in *Crb1Crb2* control (B) cortex, which is markedly reduced in *Crb1*$^{+/-}$*Crb2* cKO (B') and *Crb1Crb2* cKO (B") cortex. (C–D") CRB1 and CRB2 localization and level in E14.5 cortex. (E, F) PALS1 localization in control cortex at E12.5 and E14.5. (E', F') In cKO cortex, PALS1 expression is barely detected at both stages. (G, H) PAR3 in control cortex at E12.5 and E14.5. (G', H') PAR3 localization in *Crb2* cKO cortex. Arrow in G' indicates PAR3 expression in the lateral cortex. (I–I") CRB3 localization in E12.5 *Crb1Crb2* control cortex (I). In contrast, upon removal of CRB2 (I', I") there is reduced CRB3 expression. (J–J") CRB3 in E14.5 control cortex (J), which is like E12.5 strongly reduced in *Crb1*$^{+/-}$*Crb2* cKO (J') and *Crb1Crb2* cKO (J") cortex. (K–L") Loss of PALS1 upon removal of CRB2 (K', K") but not of CRB1 (K) in E12.5 cortex. Additionally, in E14.5 cortex the reduced PALS1 expression is even more prominent upon removal of CRB2 (L', L") but not of CRB1 (L). Nuclei counterstained with DAPI or TO-PRO3 (blue). Scale bars: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

enriched at the apical surface of the VZ progenitors in the control embryos at E12.5 and E14.5 (Fig. 4G and H). Similar to PALS1 expression, the expression of PAR3 at the apical membrane was also reduced in the *Crb2* cKO cortex at E12.5 (Fig. 4G'). Whereas there was a difference for PAR3 staining at E12.5 in the *Crb2* cKO cortex, at E14.5 there was no detectable difference (Fig. 4H'). Next, we analyzed the expression of apical complex proteins in the *Crb2* cKO cortex on genetic background lacking *Crb1*. CRB3 is expressed at the apical membrane in the wild type cortex (Srinivasan et al., 2008). At E12.5, CRB3 was apically localized in cortex expressing CRB2 lacking *Crb1* (*Crb1Crb2* control; Fig. 4I), with higher levels of CRB3 at the apical membrane at E14.5 (Fig. 4J). Cortex lacking CRB2 on background lacking *Crb1* (*Crb1Crb2* cKO) or cortex lacking CRB2 expressing CRB1 (*Crb1*$^{+/-}$*Crb2* cKO) showed reduced levels of apical CRB3 at E12.5 and E14.5 (Fig. 4I'–J"). Next, we investigated the localization of PALS1 at the apical cortical membrane. At E12.5, similar as CRB3, PALS1 was apically localized in cortex expressing CRB2 lacking *Crb1* (*Crb1Crb2* control; Fig. 4I and J). Cortex lacking CRB2 on background lacking *Crb1* (*Crb1Crb2* cKO) or cortex lacking CRB2 expressing CRB1 (*Crb1*$^{+/-}$*Crb2* cKO) showed reduced levels of apical PALS1 at E12.5 and E14.5 (Fig. 4K'–L"). These data strongly suggest that CRB2 is required for the maintenance of apical polarity proteins such as PALS1, CRB3 and PAR3.

CRB2 is required for maintenance of adherens junctions

To determine if loss of *Crb2* also has an effect on localization of junction-associated proteins, the expression pattern of N-cadherin

(Kadowaki et al., 2007) and ZO-1 (Aaku-Saraste et al., 1996) were analyzed. At E12.5, N-cadherin expression was enriched in the apical domain of the VZ progenitors of control embryos on wild type genetic background (Fig. 5A). However, in the *Crb2* cKO dorsal telencephalon at wild type genetic background, the apical expression of N-cadherin was perturbed and instead N-cadherin was expressed in a diffuse manner within the VZ as well as weakly within the SVZ (Fig. 5A'). At E14.5, N-cadherin was still detected at the apical surface and within the SVZ of control embryos (Fig. 5B). However, by this stage there was no N-cadherin expression within the *Crb2* cKO dorsal telencephalon (Fig. 5B'). In contrast to the reduced levels of N-cadherin at the apical region, ZO-1 appeared more restricted at the apical region in the *Crb2* cKO telencephalon (Fig. 5C, C'). These data imply that the junctional proteins N-cadherin and ZO-1 require apical CRB2 expression for correct localization.

Next, we examined the *Crb2* cKO telencephalon on genetic background lacking *Crb1*. Immunohistochemistry for the adherens junction protein β-catenin showed that at E12.5, cortex lacking CRB2 on background lacking *Crb1* (*Crb1Crb2* cKO) or cortex lacking CRB2 expressing CRB1 (*Crb1*$^{+/-}$*Crb2* cKO) showed no obvious differences in levels of β-catenin compared to cortex expressing CRB2 lacking *Crb1* (*Crb1Crb2* control; Fig. 5D–D"), but β-catenin localization seemed to be partially redistributed more basally in the cortex lacking CRB2. In contrast, at E14.5 there was a partial reduction and redistribution of β-catenin in cortex lacking CRB2 expressing CRB1 (*Crb1*$^{+/-}$*Crb2* cKO) compared to cortex expressing CRB2 on background lacking Crb1 (*Crb1Crb2* control; Fig. 5E, E'), and a prominent decrease and partial redistribution in β-catenin levels in the cortex lacking CRB2 on background lacking *Crb1* (*Crb1Crb2* cKO; Fig. 5E").

We observed an expression pattern similar to β-catenin for the adherens junction protein p120 catenin. At E12.5, there is no difference in the level and localization of p120 catenin in telencephalon lacking CRB2 on background lacking *Crb1* (*Crb1Crb2* cKO) or cortex lacking CRB2 expressing CRB1 (*Crb1*$^{+/-}$*Crb2* cKO) compared to telencephalon expressing CRB2 on background lacking *Crb1* (*Crb1Crb2* control; Fig. 5F–F"). In contrast, in E14.5 cortex there

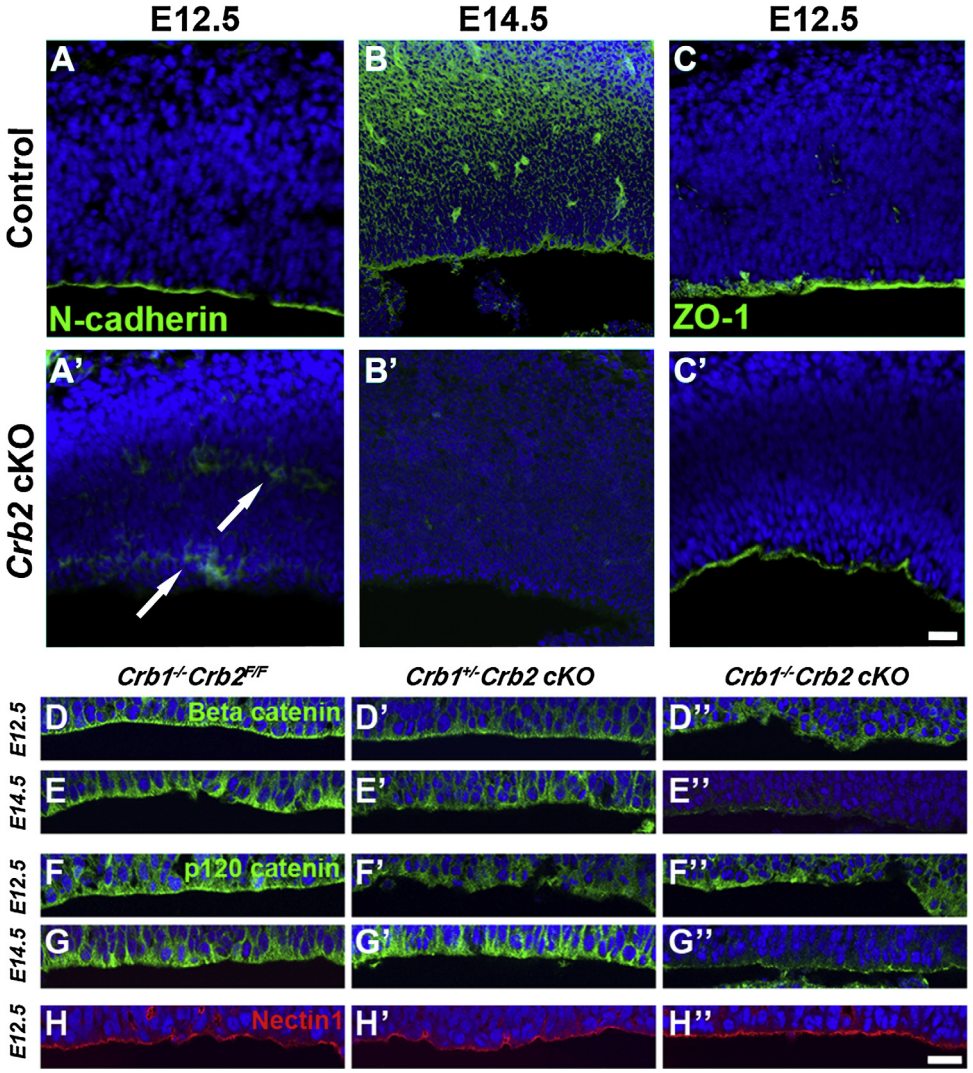

Fig. 5. Loss of CRB1 and CRB2 results in reduced levels of adherens junction proteins. (A, B) N-cadherin is apically enriched in the control at E12.5. At E14.5, N-cadherin is apically localized and also detected in the SVZ. (A', B') In the cKO cortex, there is a significant reduction in N-cadherin expression at both E12.5 and E14.5. Arrows in A' show residual N-cadherin expression in the cKO cortex at E12.5. (C, C') Apical expression of ZO-1 is unaffected in the cKO cortex. (D–D") At E12.5 there is some reduction in level of β-catenin in *Crb1Crb2* cKO cortex. (E–E") However, at E14.5 there is a clear reduction of β-catenin expression from *Crb1Crb2* cKO (E") but not from *Crb1*$^{+/-}$*Crb2* cKO (E') cortex. (F–F") At E12.5 there is no difference in levels of p120 catenin at the apical membrane. (G–G") In contrast, at E14.5 there is a clear reduction of p120 catenin expression in *Crb1Crb2* cKO (G") but not from *Crb1*$^{+/-}$*Crb2* cKO (G') cortex. (H–H") Nectin1 at the apical membrane is not affected by loss of CRB1 and CRB2. Nuclei counterstained with DAPI or TO-PRO3 (blue). Scale bars: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

is a dramatic reduction in the apical expression of p120 catenin in cortex lacking CRB2 on background lacking *Crb1* (*Crb1Crb2* cKO; Fig. 5G") compared to cortex expressing CRB2 on background lacking *Crb1* (*Crb1Crb2* control) and cortex lacking CRB2 expressing CRB1 (*Crb1*$^{+/-}$*Crb2* cKO; Fig. 5G–G'). The transmembrane adherens junction protein Nectin1 showed no difference between the different cortices (Fig. 5H–H"). Overall, the data shows that loss of *Crb2* affects apical localization of adherens junction proteins N-cadherin, β-catenin and ZO-1, whereas the apical localization of Nectin1 was unaffected.

CRB2 is required for restricting cell division in the ventricular zone

To determine if loss of apical CRB2 expression influenced the location of mitotic cells, the expression profile of pH3 (phosphorylated Histone H3) a late G2/mitotic phase marker was analyzed. In control cortex expressing CRB2 on background lacking *Crb1* (*Crb1Crb2* control), the majority of pH3$^+$ mitotic cells are localized at the apical membrane, whereas only a minor proportion of mitotic cells have a more basal localization (Fig. 6A and D; total 5.86 ± 0.17, apical 5.09 ± 0.15, basal 0.77 ± 0.07 pH3$^+$ cells/100 μm cortex, $n = 3$ embryos/group, 4–10 images/embryo). Quantification of mitotic cells in cortex lacking CRB2 expressing CRB1 (*Crb1*$^{+/-}$*Crb2* cKO) revealed that the total number of mitotic cells is increased compared to control, due to increased number of apically localized mitotic cells, whereas the number of basally localized mitotic cells was not different (Fig. 6B and D; total 7.34 ± 0.26, apical 6.25 ± 0.23 ($p < 0.01$), basal 1.09 ± 0.11 pH3$^+$ cells/100 μm cortex, $n = 3$ embryos/group, 4–10 images/embryo). Interestingly, in cortex lacking CRB2 on background lacking *Crb1* (*Crb1Crb2* cKO) the number of mitotic cells is increased even further, and the number of basally localized mitotic cells is significantly increased (Fig. 6C and D; total 9.31 ± 0.29, apical 7.65 ± 0.23 ($p < 0.0001$), basal 1.66 ± 0.15 ($p < 0.05$) pH3$^+$ cells/100 μm cortex, $n = 3$ embryos/group, 4–10 images/embryo).

In the retina, loss of both *Crb1* and *Crb2* results in increased cell proliferation (Pellissier et al., 2013). Therefore, to investigate whether *Crb2* cKO cortex on a background lacking *Crb1* affect cell proliferation, we performed BrdU injections in pregnant female mice to determine the rate of cell division in embryonic cortices.

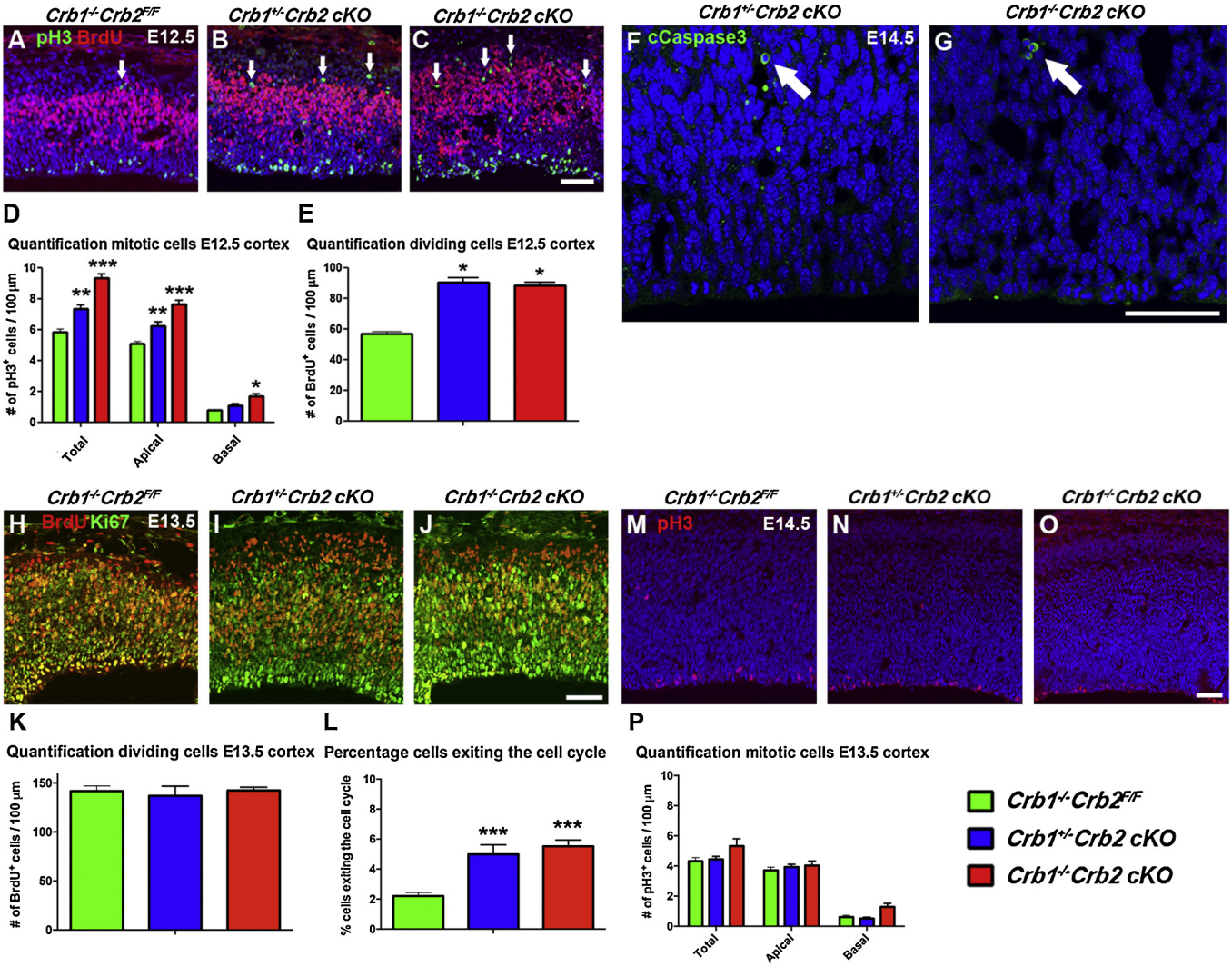

Fig. 6. CRB1 and CRB2 are transiently required to restrict proliferation. (A–C) In *Crb1Crb2* control cortex (A) the majority of mitotic cells are localized at the apical membrane, with only occasionally a basally localized mitotic cell (arrow). In contrast, both in *Crb1*$^{+/-}$*Crb2* cKO (B) and in *Crb1Crb2* cKO (C) cortex, there are more apically localized mitotic cells and in *Crb1Crb2* cKO also more basally localized mitotic cells (arrows). (D) Quantification of mitotic cells in *Crb1*$^{+/-}$*Crb2* cKO cortex. (E) Quantification of BrdU$^+$ cells shows that in both *Crb1*$^{+/-}$*Crb2* cKO and in *Crb1Crb2* cKO cortex there is a nearly two-fold increase in cell proliferation. (F, G) Occasionally there is some apoptosis observed in *Crb1*$^{+/-}$*Crb2* cKO (F) and in *Crb1Crb2* cKO (G) cortex. (H–L). Removal of CRB2 on wild type and on background lacking *Crb1* results in increased percentage of BrdU$^+$/Ki67$^-$ cells compared to total pool of BrdU$^+$ cells. (M–P) In E14.5 cortex, there is no difference anymore in mitosis. Nuclei counterstained with TO-PRO3 (blue). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$. Scale bars: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Thirty minutes of BrdU exposure in E12.5 embryos showed that cortex lacking both CRB2 and CRB1 (*Crb1Crb2* cKO) and cortex lacking CRB2 expressing CRB1 (*Crb1*$^{+/-}$*Crb2* cKO) displayed an increased rate of cell division compared to control cortex expressing CRB2 on background lacking *Crb1* (*Crb1Crb2* control; Fig. 6A–C, E; *Crb1Crb2* control 58.2 ± 1.1, *Crb1*$^{+/-}$*Crb2* cKO 88.9 ± 2.7, *Crb1Crb2* cKO 88.3 ± 2.3 BrdU$^+$ cells/100 μm cortex, $n = 3$ embryos/group, 3–4 images/embryo, $p < 0.05$).

Surprisingly, although we found increased cell division in E12.5 cortex lacking CRB2 on background lacking *Crb1* (*Crb1Crb2* cKO), we did not observe an increase in cortical thickness (data not shown). We reasoned that this could be attributed to increased apoptosis or an increase in the percentage of cells exiting the cell cycle. First, we investigated the possibility of increased apoptosis in the mutant cortex by performing immunohistochemistry for the marker for apoptosis cleaved Caspase 3 (cCaspase3). Although at E12.5 and E14.5 we found sporadic apoptosis in both cortex lacking CRB2 expressing CRB1 (*Crb1*$^{+/-}$*Crb2* cKO) and cortex lacking CRB2 on background lacking *Crb1* (*Crb1Crb2* cKO) cortex, the cell death could clearly not compensate for the increased cell division observed in the mutant cortex (Fig. 6F, G and data not shown).

Therefore, next we determined percentage of cells exiting the cell cycle after a 24 h BrdU exposure in E13.5 embryos. We performed immunohistochemistry for BrdU and Ki67 and quantified the number of BrdU$^+$/Ki67$^-$ cells compared to the total number of BrdU$^+$ cells, which represents the population of cells that have exited the cell cycle. This revealed that the number of BrdU$^+$ cells is not different (Fig. 6H–K; *Crb1Crb2* control 141.7 ± 5.3, *Crb1*$^{+/-}$*Crb2* cKO 136.8 ± 9.9, *Crb1Crb2* cKO 142.3 ± 3.4 BrdU$^+$ cells/100 μm cortex, $n = 2$–3 embryos/group, 2–4 images/embryo). However, *Crb2* cKO cortex and *Crb2* cKO cortex lacking *Crb1* showed an increased number of progenitor cells exiting the cell cycle (Fig. 6H–J, L; *Crb1Crb2* control 2.20 ± 0.24%, *Crb1*$^{+/-}$*Crb2* cKO 4.99 ± 0.63%, *Crb1Crb2* cKO 5.52 ± 0.42%, ($p < 0.001$) $n = 2$–3 embryos/group, 2–4 images/embryo).

Additionally, to further explore whether the increased cell division is observed throughout the development of the cortex, or is only transient, we quantified mitotic cells in E14.5 cortex.

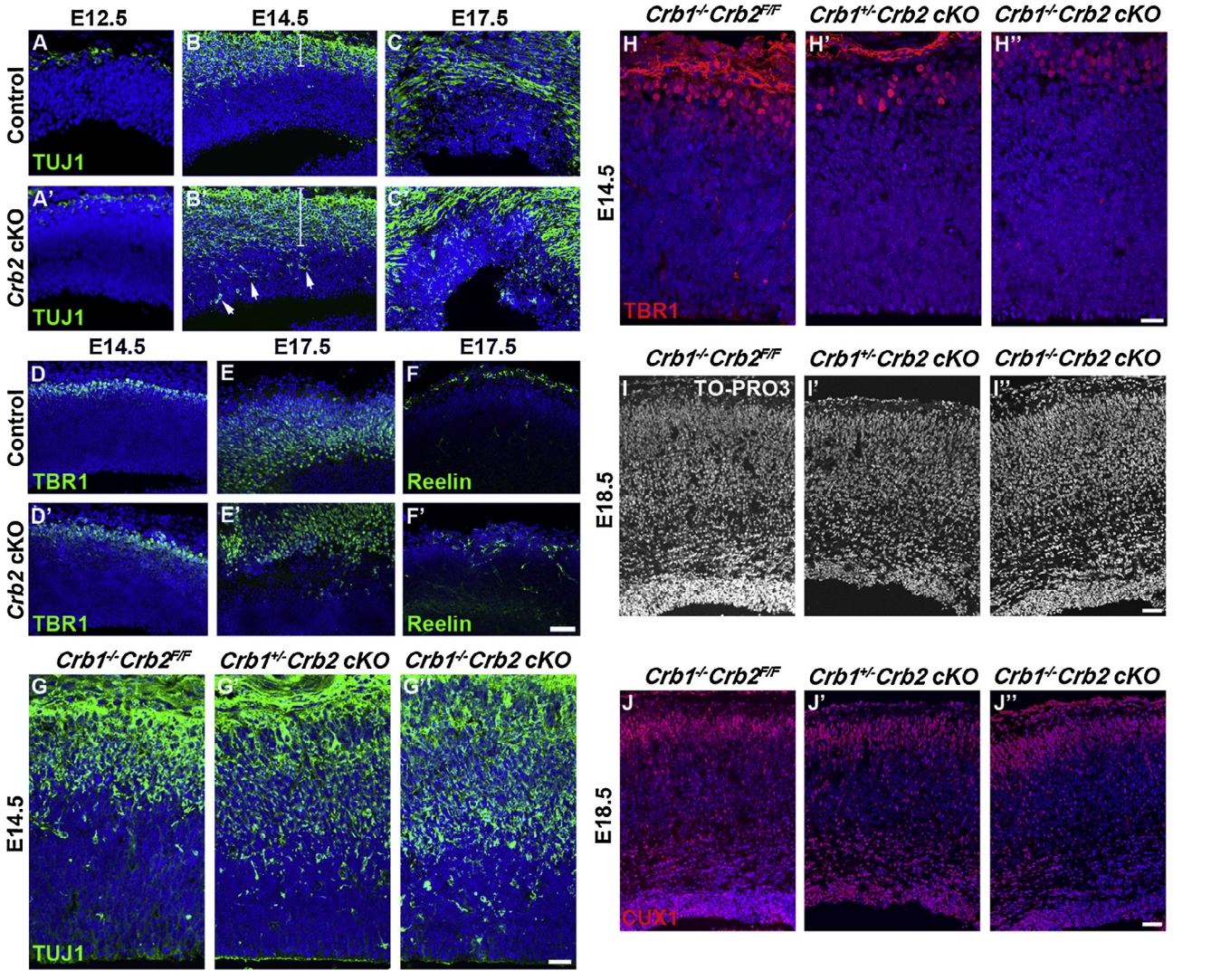

Fig. 7. Mislocalisation of neurons in the *Crb2* cKO cortex. (A–A') TUJ1$^+$ neurons are observed in the upper SVZ in both control and *Crb2* cKO cortex at E12.5. The lines in Figure B and B' indicate the thickness of the Tuj1-positive layer. (B–C') TUJ1$^+$ neurons are predominantly observed in the upper layers of the control cortex. In the *Crb2* cKO cortex, there is a marked increase in TUJ1$^+$ neurons in the VZ and the SVZ (arrows in B'). (D, E) TBR1$^+$ post-mitotic neurons in control brain. (D'–E') In the *Crb2* cKO brain at E17.5, TBR1$^+$ cells are mislocalized. (F, F') In control cortex, Reelin$^+$ Cajal-Retzius cells are localized in the marginal zone. In the *Crb2* cKO brain, the defined layer of Reelin$^+$ cells is disrupted and Reelin$^+$ cells are aberrantly localized. (G–G") in E14.5 cortex the area occupied by TUJ1$^+$ neurons is increased in *Crb1*$^{+/-}$*Crb2* cKO and *Crb1Crb2* cKO cortex. (H–H") However, TBR1$^+$ cell pattern seemed to be not different between the different genotypes. (I–J") In E18.5 cortex, lamination (I–I") as well as localization of CUX1$^+$ late-born neurons (J–J") is unaffected upon loss of CRB1 and/or CRB2. Nuclei counterstained with DAPI or TO-PRO3 (blue). Scale bars: 20 μm in G" and H", 50 μm in F', I" and J". (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

This showed that the number of mitotic cells, both apically as well as basally localized mitotic cells, is not significantly different, strongly suggesting that the increased cell division we observed at E12.5 is only a transient effect in *Crb2* cKO cortex lacking *Crb1* (Fig. 6M–P; *Crb1Crb2* control total 4.32 ± 0.23, apical 3.71 ± 0.21, basal 0.61 ± 0.12; $Crb1^{+/-}Crb2$ cKO total 4.44 ± 0.2, apical 3.93 ± 0.18, basal 0.51 ± 0.09; *Crb1Crb2* cKO total 5.32 ± 0.48, apical 4.03 ± 0.3, basal 1.29 ± 0.23 $pH3^+$ cells/100 μm cortex, $n = 2$ embryos/group, 2–7 images/embryo). Thus, although at E12.5 loss of CRB1 and CRB2 results in increased progenitor cell proliferation in the cortex, this effect is only transient and is compensated for by increased exit from the cell cycle.

Loss of CRB2 causes precocious neural differentiation and mild abnormalities in cortical lamination

In order to determine whether loss of CRB2 affected terminal neural differentiation, expression of TUJ1 and TBR1, markers for (early) post-mitotic neurons, was analyzed. At E12.5 there was no significant alteration in the early neural protein TUJ1 in the *Crb2* cKO cortex compared to the $Crb2^{F/+}$ Emx1$Cre^{Tg/+}$ control cortex (Fig. 7A, A'). However, at E14.5 there seemed to be an expansion of the TUJ1 positive neuronal domain in the *Crb2* cKO cortex compared to the control (Fig. 7B, B'). Furthermore, mislocalised TUJ1 positive neurons were also observed in the VZ of the mutant telencephalon (Fig. 7B'). By E17.5, there was a notable increase in TUJ1 positive neurons in all layers of the cortex in both control and mutant littermates (Fig. 7C, C').

To further investigate the lamination and spatial pattern of neural differentiation in the *Crb2* cKO brains, the expression of two cortical neuronal proteins, TBR1 a marker for post-mitotic neurons (Bulfone et al., 1995) and Reelin which marks Cajal-Retzius cells in the superficial marginal zone of the cortical plate (D'Arcangelo et al., 1995), were analyzed. At E14.5 there was no significant difference in TBR1 expression profile between *Crb2* cKO mutants and their control littermates (Fig. 7D, D'). At E17.5, TBR1 was expressed in the upper layers of the cortex and in the subplate of the control cortex (Fig. 7E). In the *Crb2* cKO cortex, TBR1 expressing cells were detected in the same regions as in the controls (Fig. 7E'). However, a few TBR1 positive cells were also detected outside their normal expression domain (Fig. 7E'). At the same stage, the Reelin positive cells were expressed in a compact laminar pattern in the controls (Fig. 7F). In contrast, in the *Crb2* cKO dorsal telencephalon, Reelin labelled cells were detected in a disorganized fashion at the marginal zone (Fig. 7F').

In the retina, loss of *Crb1* and *Crb2* results in an increase in retinal thickness, a clear disruption of layering integrity followed by massive retinal disorganization and eventually retinal degeneration (Pellissier et al., 2013). To investigate whether in the cortex a similar phenotype occurred, and whether loss of both CRB1 and CRB2 results in a more dramatic lamination defect than loss of CRB2 only, we first investigated the pattern of TUJ1 positive neurons. This revealed that similar to *Crb2* cKO (see Fig. 7A–C'), both in cortex lacking CRB2 expressing CRB1 ($Crb1^{+/-}Crb2$ cKO) and in cortex lacking CRB2 on background lacking *Crb1* (*Crb1Crb2* cKO) the area occupied by $TUJ1^+$ cells seems to be increased, with no difference between cortex lacking CRB2 expressing or lacking *Crb1* (Fig. 7G–G"). Next, we analyzed the localization of $TBR1^+$ neurons in the different cortices. Similar to the phenotype observed in *Crb2* cKO cortex, at E14.5 the pattern of $TBR1^+$ neurons seemed to be not different between cortex expressing CRB2 on background lacking *Crb1* (*Crb1Crb2* control) and cortex lacking CRB2 expressing CRB1 ($Crb1^{+/-}Crb2$ cKO) and cortex lacking CRB2 on background lacking *Crb1* (*Crb1Crb2* cKO) (Fig. 7H–H"). Finally, we investigated whether gross overall architecture of the cortex would be affected in *Crb2* cKO cortex lacking *Crb1*. To this end we performed nuclear staining on E18.5 cortex. This showed that loss of CRB2 and/or CRB1 seems to not affect late stage cortical layering (Fig. 7I–I"). Furthermore, the distribution of the late-born $CUX1^+$ neurons is not affected by the removal of CRB2 and/or CRB1 (Fig. 7J–J"). Overall, these results indicate that in the absence of CRB2 there is premature and mislocalized differentiation of neurons and subtle abnormalities in cortical lamination although gross cortical morphology is unaffected. However, this phenotype is not exacerbated in *Crb2* cKO lacking *Crb1* compared to *Crb2* cKO on wild type genetic background.

4. Discussion

Recent reports showed that mutations in human *CRB2* cause nephrosis and cerebral ventriculomegaly in humans, suggesting alterations or defects in cortical development (Ebarasi et al., 2015; Slavotinek et al., 2015). To investigate the functional requirement of CRB2 in mice we used Emx1*Cre* to specifically ablate *Crb2* in the dorsal telencephalon. We studied the loss of CRB2 on a wild type genetic background as well as loss of CRB2 on genetic background lacking *Crb1*. Our results indicate that CRB2 plays an important role in recruiting other proteins to the apical and junctional domain. Furthermore, absence of CRB2 results in premature neural differentiation and abnormal lamination indicating that *Crb2* does play a role in cortical neural development. Additionally, to investigate whether there could be functional redundancy between CRB2 and other Crumbs proteins, we made mutant mice lacking both *Crb1* and *Crb2* in the dorsal telencephalon. In these cortices, levels of PALS1 and CRB3 at the apical membrane are reduced and progenitor cell division increased at E12.5 and E14.5, but the overall lamination of the cortex is grossly unaffected from E18.5.

Crb2 cKO cortex and *Crb2* cKO cortex lacking *Crb1* showed decreased expression of polarity proteins of the Crumbs and PAR complexes. Removal of CRB2 results in reduced levels of both PALS1 and CRB3. Removal of CRB2 on genetic background lacking *Crb1* results in further reduction of adherens junctions proteins N-cadherin, ß-catenin, p120. It has been shown previously that PALS1 is an intracellular binding partner of CRB2 (Kim et al., 2010); therefore, the reduced levels of PALS1 expression in the *Crb2* cKO cortex was not unexpected. Conditional removal of *Pals1* leads to premature neural differentiation, massive apoptosis of precocious neurons and subsequently ablation of the entire neocortex (Kim et al., 2010). Intriguingly, the massive cell death phenotype observed in Emx1*Cre Pals1* cKO embryos (Kim et al., 2010) was not observed in *Crb2* cKO on wild type genetic background or *Crb2* cKO cortex lacking *Crb1* despite the absence of apically enriched PALS1. Recently it was shown that conditional ablation of MPP3, an interacting partner of PALS1 affects apical expression of PALS1 but does not mimic the gross death phenotype observed in *Pals1* cKO mice (Dudok et al., 2013a,b). It is plausible that the timing of *Pals1* ablation, together with its effect on interacting proteins influences cell survival and that this temporal sequence of events is different between the *Pals1* cKO and *Crb2* cKO and *Crb2* cKO lacking *Crb1* embryos. The reduced levels of PAR3 expression after CRB2 deletion is in agreement with previous studies in mammalian epithelial cell lines that have reported a direct interaction between the Crumbs and PAR complexes (Hurd et al., 2003). Surprisingly, apical expression of PAR3 was restored in the *Crb2* cKO cortex by E14.5. It is therefore possible that two separate mechanisms regulate PAR3 localization at different stages of neural development: the earlier mechanism is CRB2 dependent whilst at later stages a CRB2 independent mechanism is in place. Alternatively, progenitors at different developmental stages may have different requirements for setting up polarity protein complexes. An in depth understanding of the context-dependent dynamics of cell polarity pathways in the manifestation of neural progenitor polarity will help determine

how polarity proteins effect specialized and diverse functions during neocortical development.

Given the important roles for polarity proteins during neural development, it is highly plausible that the switch in cell fate to generate intermediate progenitors/neurons instead of maintaining an apical progenitor-state in the *Crb2* cKO cortex may stem from the disruption of the apical domain. The absence of apical components may lead to inadequate tethering of the cells to the VZ, thereby, exposing these cells to different extrinsic cues and subsequently affecting their fate. It has been reported that intact cell junctions are a fundamental prerequisite for normal neural progenitor cell proliferation in *Drosophila* (Lu et al., 2001). However, the presence of intact apical junctions is not an absolute requirement in regulation of vertebrate neurogenesis. For instance, conditional knockout of aPKCλ disrupted adherens junctions yet failed to have an impact on neurogenesis (Imai et al., 2006). Conversely, in MALS triple knockout mutant embryos, adherens junctions were unaffected but significant defects in proliferation of neural progenitors were observed (Srinivasan et al., 2008). Taken together, this suggests that the *Crb2* cKO phenotype cannot be solely attributed to loss of apical junctional components.

Mislocalization of Reelin and TBR1 positive cells in the absence of CRB2 suggests that secondary to defects in cell polarity, lamination is also mildly affected in the mutant developing cortex. Reelin is crucial for the inside-out layering of the cortex (Caviness, 1982) and mislocalization of Reelin positive cells in the *Crb2* cKO cortex could in turn affect the precise localization of layer-specific neurons in the cortex. One possible explanation is that absence of apical CRB2 expression renders cells unable to respond appropriately to extrinsic guidance cues and subsequently affects their spatial localization.

One key question that needs to be addressed is how CRB2 links signalling events during neurogenesis to regulate neuronal output. Crumbs is the only known apical polarity protein to have an extracellular domain and therefore it is tempting to speculate that it is an ideal candidate for the transduction of signals originating at the luminal surface to the neural progenitor cells. The Notch signalling cascade is essential for maintenance of progenitor pools and in the control of neurogenesis in the developing and adult brain. Inactivation of Notch signalling results in depletion of the progenitor population and induces precocious neural differentiation. On the other hand, activation of Notch signalling keeps the neural stem cells in a progenitor state and thereby maintains the progenitor pool (Bertrand et al., 2002; Kageyama et al., 2008; Kopan and Ilagan, 2009). A potential interaction between Crumbs and Notch was initially reported in *Drosophila* (Herranz et al., 2006). Recently it was reported that in zebrafish, the Crumbs–Notch pathway is important for restriction of mitosis to apical surface and also in the maintenance of neuroepithelial polarity. Crumbs proteins were shown to directly interact with the extracellular domain of Notch and inhibit its activity (Ohata et al., 2011). These studies suggest that the interaction between Notch signalling cascade and Crumbs is evolutionarily conserved and that Crumbs is part of a negative feedback loop during Notch signalling. Based on this it can be predicted that the conditional removal of CRB2 would lead to ectopic activation of Notch signalling. Interestingly, the phenotype observed in *Notch1*, *Hes1* and *Hes5* loss of function mutant brains is remarkably similar to that of *Crb2* cKO phenotype, which is the loss of progenitor pools and precocious neural differentiation (Ohtsuka et al., 1999; Yoon and Gaiano, 2005; Mizutani et al., 2007; Pellissier et al., 2013). In the developing brain, CRB2 and Notch may positively regulate each other and CRB2 could directly bind to extracellular domain of Notch and sequester progenitor cells from neural differentiation signals. Overall, the above-proposed mechanisms are not mutually exclusive and CRB2 may act *via* the concerted action of several interacting proteins and interplay of signaling pathways.

Acknowledgments

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