

MARCKS modulates radial progenitor placement, proliferation and organization in the developing cerebral cortex

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The radial glial cells serve as neural progenitors and as a migratory guide for newborn neurons in the developing cerebral cortex. These functions require appropriate organization and proliferation of the polarized radial glial scaffold. Here, we demonstrate in mice that the myristoylated alanine-rich C-kinase substrate protein (MARCKS), a prominent cellular substrate for PKC, modulates radial glial placement and expansion. Loss of MARCKS results in ectopic collection of mitotically active radial progenitors away from the ventricular zone (VZ) in the upper cerebral wall. Apical restriction of key polarity complexes [CDC42, β -catenin (CTNNB1), N-cadherin (CDH2), myosin IIB (MYO1B), aPKC ζ , LGL, PAR3, pericentrin, PROM1] is lost. Furthermore, the radial glial scaffold in *Marcks* null cortex is compromised, with discontinuous, non-radial processes apparent throughout the cerebral wall and deformed, bulbous, unbranched end-feet at the basal ends. Further, the density of radial processes within the cerebral cortex is reduced. These deficits in radial glial development culminate in aberrant positioning of neurons and disrupted cortical lamination. Genetic rescue experiments demonstrate, surprisingly, that phosphorylation of MARCKS by PKC is not essential for the role of MARCKS in radial glial cell development. By contrast, the myristoylation domain of MARCKS needed for membrane association is essential for MARCKS function in radial glia. The membrane-associated targeting of MARCKS and the resultant polarized distribution of signaling complexes essential for apicobasal polarity may constitute a critical event in the appropriate placement, proliferation and organization of polarized radial glial scaffold in the developing cerebral cortex.

KEY WORDS: Radial glia, Progenitors, Cerebral cortical development, MARCKS, Mouse, Laminal organization

INTRODUCTION

Radial glia (RG) function as a source of new neurons and provide an instructive scaffold for the radial migration and placement of neurons in the neocortex. Cell soma in the ventricular zone (VZ), an elongated radial process, and specialized adhesive contacts with both the ventricular and pial surfaces are the basic characteristics of a polarized radial glial cell (Schmechel and Rakic, 1979a; Schmechel and Rakic, 1979b). During cortical development, RG undergo interkinetic nuclear migration (INM) in the VZ before either symmetric or asymmetric divisions. Symmetric divisions of radial glia, prevalent during the earlier stages of corticogenesis, give rise to two daughter radial glial cells. By contrast, asymmetric divisions generate a daughter radial glial cell and a neuron or an intermediate precursor (Fishell and Kriegstein, 2003; Hartfuss et al., 2003; Malatesta et al., 2000; Miyata et al., 2001; Miyata et al., 2004; Noctor et al., 2001; Noctor et al., 2004). Once corticogenesis is complete, the radial glial progenitors transdifferentiate into astrocytes of the cortical parenchyma or ependymal cells, lining the ventricles of the mature brain (Merkle et al., 2004; Spassky et al., 2005).

The diverse functions of radial glia depend on dynamic regulation of the morphological and molecular polarity of these cells. Radial glial cells maintain a specialized apical membrane domain enriched

in protein members of the Par polarity complex [PAR6 (PARD6a – Mouse Genome Informatics), PAR3, aPKC ζ], PROM1 and CDC42 (Cappello et al., 2006; Hutterer et al., 2004; Manabe et al., 2002). Apical or basolateral restriction of β -catenin (CTNNB1), N-cadherin (CDH2), NUMB and NUMBL is also evident (Kadowaki et al., 2007; Rasin et al., 2007; Sestan et al., 1999; Takeichi, 2007; Zhong et al., 1996). The polarized expression of these proteins acts to regulate both the integrity of intercellular adhesion and the patterns of cell division of radial glia. Furthermore, a selective orientation and expression of microtubules and microtubule-associated proteins [e.g. LIS1 (PAFAH1B1), ASPM, DCLK1] in radial glia facilitate appropriate temporal regulation of cell division as well as radial process extension (Bond et al., 2002; Gleeson et al., 1999; Gleeson and Walsh, 2000; Rakic et al., 1996; Rivas and Hatten, 1995; Shu et al., 2006; Yingling et al., 2008). Lastly, the deployment of migration-modulating cell-surface molecules, such as astrotactin and SPARCL1, to distinct locations within the radial glial processes facilitates the proper radial migration and placement of cortical neurons (Anton et al., 1996; Gongidi et al., 2004; Patten et al., 2003; Patten et al., 2006; Rasin et al., 2007; Schmid et al., 2003). Although this polarized localization of specific signaling complexes to distinct subcellular domains within radial progenitors is essential for their function, little is known about the molecular regulators that govern this process.

Myristoylated alanine-rich C-kinase substrate protein (MARCKS), an actin-cross-linking protein and prominent cellular substrate of PKC, is enriched in embryonic radial progenitors. Its ability to modulate cytoskeleton, cell adhesion and cell polarity makes it an ideal candidate for regulating radial glial development (Blackshear et al., 1997; Calabrese and Halpain, 2005; Iioka et al., 2004; Matus, 2005; Myat et al., 1997; Zolessi and Arruti, 2001). MARCKS contains two highly conserved functional domains that regulate its activity:

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(1) an amino terminus consensus sequence for myristoylation, which facilitates association with lipid membranes; and (2) an effector domain phosphorylation site domain (PSD), which contains three serine residues that can be phosphorylated by PKC. Cooperation between these structural domains allows for the regulated localization of MARCKS to the plasma membrane. Membrane-localized MARCKS can serve as a docking station for calmodulin and several multivalent lipids, including phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidylserine (PS), as well as a site for bundling of F-actin filaments (Aderem, 1992a; Aderem, 1992b; Blackshear, 1993). The mechanistic link between the activities of MARCKS, PKC, calcium-calmodulin and PIP₂ allows for the selective targeting and regulation of numerous signal transduction complexes, especially members of the cell-polarity machinery such as CDC42, PAR3 and PAR6 (Hartwig et al., 1992; Sundaram et al., 2004; Swierczynski and Blackshear, 1995). The membrane-localized aPKC ζ can complex with PAR3 and PAR6 at apical adherens junctions to promote the establishment of apical-basal polarity and cell-fate determination via phosphorylation of GSK3 β and activation of CDC42 (Rolls et al., 2003; Suzuki et al., 2001; Wodarz et al., 2000; Yamanaka et al., 2001). Moreover, PIP₂ has recently been shown to complex with key components of the polarity machinery, including PTEN, CDC42 and aPKC ζ . The loss of aPKC ζ or PIP₂ localization causes apically confined signaling proteins to lose their polarized expression (Imai et al., 2006; Martin-Belmonte et al., 2007; Martin-Belmonte and Mostov, 2007). Together, these findings suggest that MARCKS is a potent upstream regulator of localization and function of cell-polarity complexes. We therefore investigated its function in development of polarized radial glial cells in the embryonic cerebral cortex.

Here, we demonstrate an essential role for MARCKS in the appropriate placement and proliferation of radial glial progenitor cells. Strikingly, disrupted MARCKS function leads to displacement of the radial progenitor niche, with proliferating, ectopically placed progenitors seen throughout the intermediate zone (IZ) and cortical plate (CP). *Marcks*^{-/-} radial glial scaffold is aberrantly organized, leading to defective neuronal layering and the formation of heterotopias. The apical localization of cell-polarity regulators such as aPKC ζ , PAR3, CDC42, β -catenin, LGL, myosin IIB, prominin and N-cadherin is lost in the *Marcks*^{-/-} radial progenitors. Surprisingly, these deficits are rescued with a form of MARCKS lacking a functional PSD, indicating that PKC phosphorylation of MARCKS is not essential for its function in RG. By contrast, the myristoylation moiety of MARCKS, essential for membrane targeting of this protein and its associated signaling complexes, is crucial for proper radial glial development. Together, these findings define a novel role for MARCKS and its ability to organize polarity complexes in the appropriate placement, proliferation and organization of radial glial cells in the developing cerebral cortex.

MATERIALS AND METHODS

Mice

Marcks mutant mice, PSD or myristoylation-domain-deficient mice were generated and genotyped as described earlier (Scarlett and Blackshear, 2003; Stumpo et al., 1995; Swierczynski et al., 1996). Mice were cared for according to animal protocols approved by the University of North Carolina and the National Institute of Environmental Health Sciences (NIEHS).

Immunohistochemistry

Cerebral cortical sections and cortical cells were immunolabeled as previously described (Schmid et al., 2003; Yokota et al., 2007b) with the following antibodies: anti-PAX6 (Iowa Hybridoma), anti-MYOIIB (Iowa Hybridoma), anti-phosphorylated vimentin (Abcam), anti-pericentrin (Abcam), anti-TBR2 (anti-EOMES – Mouse Genome Informatics)

(Abcam), anti-prominin-1 (Chemicon), anti-GLAST (anti-SLC1A3) (Chemicon), anti-TBR1 (Chemicon), anti-reelin (Chemicon), anti-SOX2 (Chemicon), anti-nestin (Chemicon), anti-BLBP (anti-FABP7) (Chemicon), anti- β -catenin (Sigma), anti-BrdU (Becton and Dickinson), anti-Ki67 (NovoCastra), anti-phosphorylated Histone 3 (PH3; Upstate/Millipore), anti-NUMB (Upstate/Millipore), anti-PAR3 (Upstate/Millipore), N-cadherin (Zymed), anti-BRN1 (anti-POU3F3) (Novus and gift of A. Ryan, McGill University), anti-MARCKS (Scarlett and Blackshear, 2003), and anti-LGL (gift of P. Brenwald, UNC-CH). Immunoreactivity was detected by incubation with appropriate Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch).

Analysis of PAX6 or TBR2-positive cell distribution and radial glial end-feet types

Ectopic PAX6⁺ or TBR⁺ cells within 10,000 μ m² of the upper cerebral wall were counted as previously described (Ghashghaei et al., 2006). To compare the thickness of PAX6⁺ and TBR2⁺ zones within the VZ/subventricular zone (SVZ) compartments, the ratio of the thickness of either PAX6⁺ or TBR2⁺ zones to the total thickness of the cerebral wall in the same location was measured and used as an index of cell-layer width. Cell counts and the thickness ratios were analyzed by two-way ANOVA with post-hoc Bonferroni *t*-tests.

Cell proliferation analysis

Mice were administered bromodeoxyuridine (BrdU, 100 mg/kg; Sigma) 1 hour before sacrifice. Cortices were then processed for anti-BrdU, anti-PH3 or anti-Ki67 immunolabeling. Immunopositive cells within the VZ or elsewhere in the cerebral wall were quantified as previously described (Ghashghaei et al., 2007; Ghashghaei et al., 2006; Gongidi et al., 2004). Mitotic cleavage orientation was measured as previously described (Feng and Walsh, 2004).

Cortical slice assay for neuron-radial glial interactions

To assess the interactions of migrating neurons with the radial glial scaffold, embryonic day 15.5 (E15.5) *Marcks*^{-/-} and wild-type (WT) cortices were electroporated with radial-glia-specific BLBP-promoter-driven DsRed2 (pBLBP-dsRed2) plasmids, and sliced on a vibratome (250 μ m thickness) as previously described (Schmid et al., 2004; Yokota et al., 2007a). GFP⁺ explants from the medial ganglionic eminence (MGE) of *Dlx5/6-CIE* E15.5 (Stenman et al., 2003) embryos were then microdissected and overlaid onto the MGE of the electroporated slices and maintained in DMEM/10% FBS for ~36 hours to allow for GFP⁺ neuronal migration into the cerebral wall. The interaction of GFP⁺ neurons with DsRed⁺ radial glial cells was repeatedly imaged at 10-minute intervals using a Zeiss live-cell-imaging laser-scanning microscope (Yokota et al., 2007a; Yokota et al., 2007b). Neuron-radial glial interaction (i.e. percentage of migrating neurons using the radial glia as a scaffold) and the rate of glial guided migration were measured as previously described (Yokota et al., 2007a).

RESULTS

Disrupted radial glial development in *Marcks* null cerebral cortex

MARCKS was widely expressed within the developing cerebral cortex. Enriched MARCKS expression was evident in the apical and basal ends of the cerebral wall, where radial progenitor cell soma and end-feet are located, respectively (Fig. 1A). MARCKS immunoreactivity was absent in *Marcks*^{-/-} cortex (Fig. 1B). Isolated radial glia in vitro, as well as actively dividing radial progenitors at the ventricular surface in vivo, expressed MARCKS (Fig. 1C,D). To examine the role of MARCKS in radial glial development and corticogenesis, we initially analyzed radial glia in *Marcks* null (*Marcks*^{-/-}) cortices at E15.5, when radial glia are actively proliferating and guiding migration. Labeling with a radial-glia-cell-specific anti-brain lipid-binding protein (BLBP) antibody indicated that, in the absence of MARCKS, the radial glial scaffold was severely disrupted. Strikingly, there was an ectopic band of BLBP⁺ progenitors outside the VZ. The ectopically displaced BLBP⁺ cells were seen

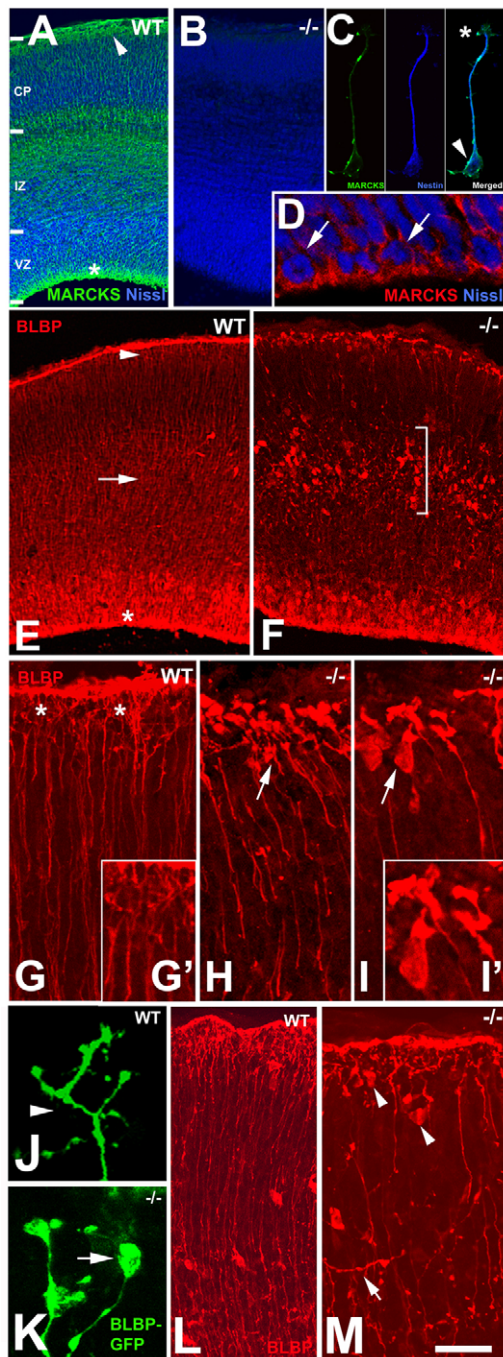


Fig. 1. Disruption in radial glial scaffold in *Marcks*^{-/-} cerebral cortex. (A–D) MARCKS is widely expressed in the E15.5 cerebral cortex (A, green). Prominent MARCKS expression is apparent in the apical and basal ends of the cerebral wall, where the radial progenitor cell soma (asterisk, A) and end-feet (arrowhead, A) are located. MARCKS immunoreactivity is absent in the *Marcks*^{-/-} brain (B). An isolated radial glial cell co-immunostained with anti-MARCKS and radial-glia-specific nestin (blue) antibodies indicates prominent MARCKS expression in the cell soma (arrowhead, C) and at the tips of the radial processes (asterisk, C). High magnification view of the VZ shows apical expression of MARCKS in actively dividing radial progenitors (arrows, D). Nuclei are counterstained with Nissl (blue) in A,B,D. (E–K) Anti-BLBP labeling outlines the normal radial glial scaffold in the wild-type (WT) cortex: radial glial cell soma in the VZ (asterisk, E), radial processes spanning the cerebral wall (arrow, E) and end-feet under the pial surface (arrowhead, E). In *Marcks*^{-/-} cortex, an ectopic band of BLBP⁺ cells is present in the IZ/CP of the cerebral wall, away from the VZ (bracket, F). Compared with the branched end-feet in control cortices (asterisks, G), radial glial end-feet are distorted and club-like in *Marcks*^{-/-} cortex (arrows; H,I). Insets (G',I') show end-feet from WT (G') and *Marcks*^{-/-} (I') cortex at high magnification. Labeling of radial glial end-feet using in utero electroporation of BLBP promoter-GFP plasmids into WT (J) and *Marcks*^{-/-} (K) cortices further illustrates this alteration [i.e. branched WT end-feet (arrowhead, J) versus club-like end-feet in *Marcks*^{-/-} radial glia (arrow, K)]. (L,M) As corticogenesis progresses (E18.5), the BLBP⁺ radial processes in WT cortices continue to expand across the extent of the cerebral wall (L), whereas MARCKS-deficient radial glia frequently display misoriented radial processes (arrow, M) and bulbous end-feet at or near the pial surface (arrowheads, M). Scale bar in M: 100 μ m for A,B; 30 μ m for D,L,M; 80 μ m for E,F; 20 μ m for G–I; 10 μ m for J,K. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.

throughout the IZ and CP (Fig. 1F, bracket). Compared with WT, fewer radial processes spanned the cerebral wall in *Marcks*^{-/-} mice (Fig. 1F). Further, in WT cortices, as radial glial end-feet reached the pial surface they fan out into thinly branched processes (Fig. 1G,G', asterisks), enabling proper interactions with the pial basement membrane. By contrast, end-feet of radial glial cells in the *Marcks*^{-/-} mice failed to branch appropriately and instead had a club-like, balled-up appearance as they reached the pial surface (arrow, Fig. 1H–I'). Electroporation of a radial-glia-specific BLBP-promoter-driven green fluorescent protein (GFP) plasmids into *Marcks*^{-/-} cortices also labels misshapen, GFP⁺ radial end-feet (Fig. 1K, arrow). The *Marcks*^{-/-} radial processes emanating from the VZ were discontinuous and

shorter, and frequently deviated from their radial course (Fig. 1E,F). An identical phenotype, with anomalous radial fibers, club-like end-feet, and ectopic radial progenitors was also observed in cortices immunolabeled with a different radial-glia-specific marker, the glutamate transporter GLAST (see Fig. S1 in the supplementary material).

As corticogenesis proceeded, the radial processes continued to expand across the extent of the cerebral wall. In E18.5 *Marcks*^{-/-} mice, the deficits in radial glial cells seen earlier in development were further exacerbated, with radial processes frequently turning perpendicular to, rather than extending radially towards, the pial surface (Fig. 1M, arrow). Furthermore, the disruption in the radial glial scaffold was also evident at the pallial/subpallial boundary. In WT cortices, a tightly concentrated band of radial fibers demarcated the boundary between dorsal and ventral cortex (see Fig. S1G in the supplementary material, arrow). In the *Marcks*^{-/-} cortices, however, fewer and shorter radial glial fibers were noticed in this region (see Fig. S1H in the supplementary material, asterisk). *Marcks* mutant mice died at birth, thus preventing postnatal analysis of radial progenitors. Taken together, these results indicate that MARCKS expression and function is required for the proper formation and maintenance of the radial glial scaffold.

MARCKS regulates the proliferation and placement of radial progenitors

Radial glia serve dual functions as neuronal precursors and as migratory guides. Radial progenitor ectopias in *Marcks*^{-/-} cortex suggest that loss of MARCKS leads to aberrant, ectopic placement

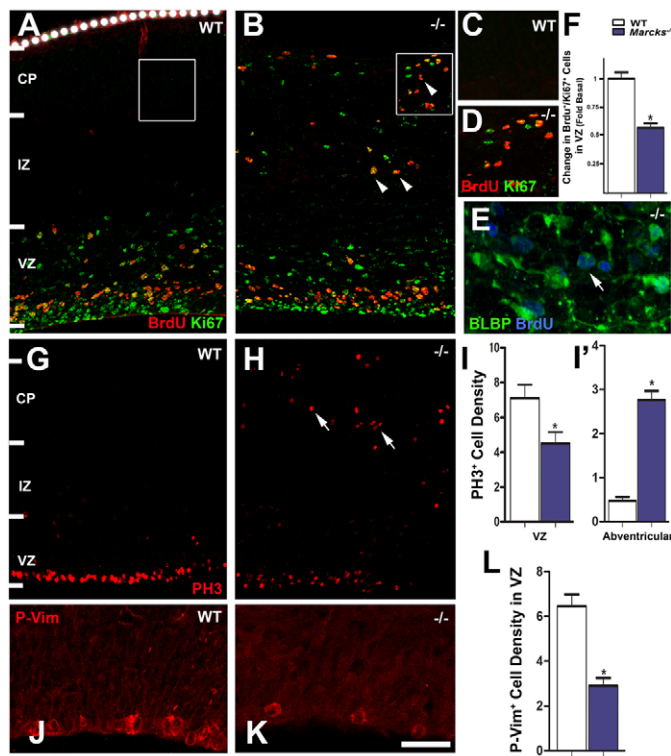


Fig. 2. MARCKS deletion leads to ectopic placement and proliferation of radial progenitors. (A,B) BrdU (red) and Ki67 (green) labeling of WT cortex (E15.5) indicates actively proliferating radial progenitors are primarily within the VZ/SVZ (A). By contrast, ectopic, mitotically active BrdU⁺/Ki67⁺ cells are found within the IZ/CP in *Marcks*^{-/-} cortex (arrowheads, B). (C,D) High magnification images of boxed regions of the CP in A and B, respectively, illustrate the ectopic presence of proliferating progenitors in *Marcks*^{-/-} cortex. (E) These ectopically proliferating cells are positive for BLBP (green), a marker for radial progenitors (arrow). BrdU labeling is blue. (F) BrdU and Ki67 co-labeling indicates that the fraction of actively cycling progenitors (BrdU⁺/Ki67⁺) in the VZ is reduced in *Marcks*^{-/-} cortex (blue bar). (G-L) Immunolabeling with PH3 (G,H) or anti-phospho-vimentin (P-Vim; J,K) antibodies reveals a uniform line of mitotically active, radial progenitors confined to the ventricular surface in WT cortex (G,J). In *Marcks*^{-/-} brains, fewer PH3⁺ and P-Vim⁺ cells are localized to the ventricular surface (H,I,K,L). M-phase, PH3⁺ progenitors are seen displaced throughout the IZ/CP in *Marcks*^{-/-} cortex (arrows, H). Quantification of the increase in ectopically placed [abventricular (IZ/CP)] PH3⁺ progenitors (I). Data shown are mean cell density (10⁴/mm²) ±s.e.m.; asterisks indicate significance when compared with controls at $P \leq 0.01$. Scale bar in K: 50 μ m for A,B,G,H; 40 μ m for C,D,J,K; 30 μ m for F. The dotted line in A indicates the pial surface. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.

of the ventricular progenitors within the cerebral wall. We therefore sought to determine if MARCKS plays a role in regulating proliferation and expansion of radial glial progenitors in the developing cerebral cortex. To investigate this, mice were injected with BrdU, which labels cells during the S-phase of the cell cycle, and sacrificed 1 hour after injection. This short pulse of labeling with BrdU enabled us to test if the ectopic neural progenitors seen in the *Marcks*^{-/-} cortex were indeed actively proliferating at their current locations as opposed to undergoing mitosis elsewhere in the VZ prior to migration to ectopic locations. BrdU incorporation confirmed that ectopic, BLBP⁺ radial progenitors within the IZ and

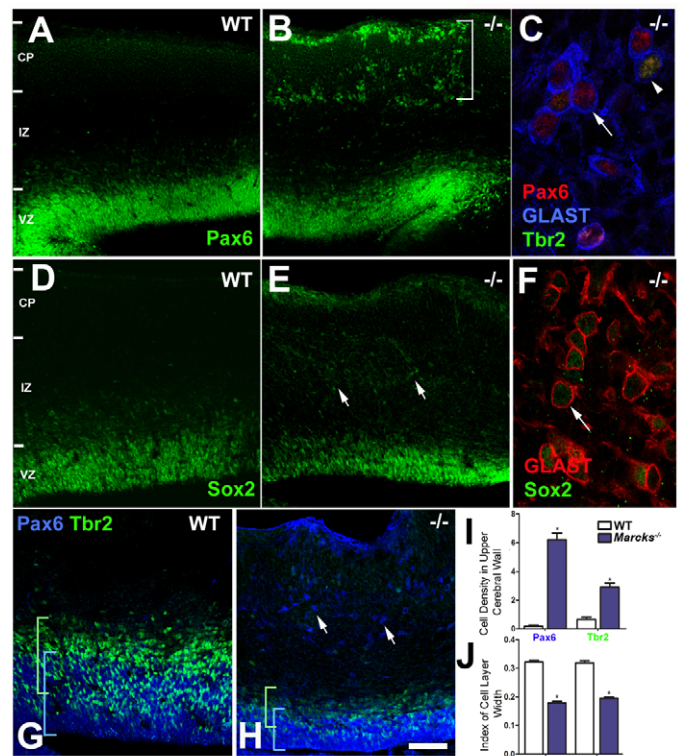


Fig. 3. Molecular identity of ectopic progenitors in *Marcks*^{-/-} cortex. (A-F) Immunolabeling of E15.5 radial progenitors in WT (A,D) and *Marcks*^{-/-} (B,E) cortex with anti-PAX6 and anti-SOX2 antibodies illustrates the presence of ectopic PAX6⁺ (bracket, B) and SOX2⁺ (arrows, E) progenitors within the IZ/CP in *Marcks*^{-/-} cortex. These ectopic PAX6⁺, TBR2⁺ and SOX2⁺ progenitors within the IZ/CP co-label with radial-glia-specific anti-GLAST antibodies (arrows, C,F; arrowhead in panel C indicates a GLAST⁺ cell labeled with both PAX6 and TBR2). (G,H) Radial and intermediate progenitors in WT and *Marcks*^{-/-} cortex were immunolabeled with anti-PAX6 and anti-TBR2 antibodies. Compared with WT (G), PAX6⁺ and TBR2⁺ cells are displaced away from the VZ/SVZ region in *Marcks*^{-/-} cortex (arrows, H). (I,J) Quantification of PAX6⁺ and TBR2⁺ cell density (10⁴/mm²) in the upper cerebral wall indicates the extent of ectopic placement of progenitors in *Marcks*^{-/-} cortex (I). The width of PAX6⁺ and TBR2⁺ cell layers in the VZ is significantly reduced in *Marcks*^{-/-} cortex (compare brackets in G and H; quantified in J). Data shown are mean ±s.e.m.; asterisk indicates significance when compared with controls at $P \leq 0.01$. Scale bar in H: 100 μ m for A,B,D,E; 20 μ m for C,F; 50 μ m for G,H; 75 μ m for I. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.

CP in *Marcks*^{-/-} cortex were actively proliferating (Fig. 2A-D,E). These ectopic BrdU⁺ cells also co-labeled with Ki67, a non-G1 cell-cycle marker. BrdU⁺/Ki67⁺ cells represent actively cycling progenitors, thus indicating the actively proliferating nature of these ectopic progenitors.

In the VZ of *Marcks*^{-/-} cortex, the number of actively cycling BrdU⁺/Ki67⁺ progenitors was significantly reduced (Fig. 2A,B,F). Both BrdU⁺ alone and Ki67⁺ alone cell densities in the VZ were also reduced in the absence of MARCKS (BrdU: $-26.3 \pm 0.8\%$; Ki67: $-28.4 \pm 1.1\%$). Further analysis of mitotically active radial progenitors with M-phase markers, PH3 or phosphorylated vimentin

(Pixley et al., 1984) confirmed the reduction in radial progenitor proliferation in the VZ and ectopic progenitor placement following MARCKS inactivation (Fig. 2G-L).

Although the disruption of VZ organization in *Marcks*^{-/-} cortex precludes comprehensive analysis of spindle orientation in the VZ, measurement of cleavage orientation in proliferating anaphase progenitors immediately adjacent to the ventricular surface indicated disrupted mitotic orientation in the *Marcks*^{-/-} progenitors (see Fig. S2 in the supplementary material). A reduction in vertical cleavage plane and an increase in horizontal cleavage plane was noticed in the absence of MARCKS (see Fig. S2 in the supplementary material). Vertical cleavage plane is suggestive of potential symmetric division, whereas horizontal cleavage is indicative of asymmetric division (Feng and Walsh, 2004). This potential shift towards asymmetric proliferation of *Marcks*^{-/-} progenitors may further help deplete the radial progenitor pool in the VZ of *Marcks*^{-/-} cortex and is consistent with reduced radial-process density noticed in the *Marcks*^{-/-} cortex. However, it is important to note that cleavage orientation is not always a reliable indicator of symmetric vs. asymmetric divisions (Gotz and Huttner, 2005). Additional analyses based on live imaging of progenitor proliferation and cell identity of daughter cells will be needed to elucidate the exact nature of the function of MARCKS in symmetric or asymmetric progenitor divisions.

To clarify the molecular identity of ectopically placed progenitors in *Marcks*^{-/-} cortex, we immunolabeled these cells with anti-GLAST, PAX6, SOX2 or TBR2 antibodies. Antibodies to the transcription factors PAX6 and SOX2 typically labeled apical radial progenitors (Fig. 3A,D, respectively), whereas the anti-TBR2 antibody labeled the intermediate progenitor cells (IPC) within the SVZ that are generated from radial progenitors (Fig. 3G). In the absence of MARCKS, these cell populations appeared to exist outside their native VZ/SVZ confines (Fig. 3A,B,D,E,G-I). PAX6⁺

and SOX2⁺ radial progenitors were seen throughout the IZ and CP, with a substantial number of these normally VZ restricted progenitors displaced all the way to the pial surface in the *Marcks*^{-/-} cortex (bracket, Fig. 3B, and arrows, Fig. 3E, respectively). Of the ectopic progenitors, 73.3±2.7 and 19.9±2.5% were PAX6⁺ or TBR2⁺, respectively. Ectopic PAX6⁺ and SOX2⁺ cells also co-immunolabeled with radial-glia-specific anti-GLAST antibodies (arrows, Fig. 3C,F, respectively). Moreover, the levels of PAX6⁺ and TBR2⁺ cells retained within the VZ and SVZ appeared to be reduced in *Marcks*^{-/-} cortex, possibly as a result of the ectopically displaced cells. The average thickness of both the PAX6⁺ and TBR2⁺ progenitor zones in the VZ/SVZ (Fig. 3G,H, brackets) was reduced by nearly 50% in the *Marcks*^{-/-} cortex (Fig. 3J). The overall density of PAX6⁺ and TBR2⁺ cells was also reduced in *Marcks*^{-/-} cortex (PAX6, -27.3±1.3%; TBR2, -27.5±1.6%). These results indicate a novel role for MARCKS in the appropriate placement and proliferation of radial progenitors in the developing cerebral cortex. Disruption of MARCKS function leads to aberrant, reduced proliferation and ectopic placement of progenitors in the upper reaches of the developing cerebral wall, away from the normal radial progenitor niches.

Altered apical polarity and placement of radial progenitors in the *Marcks* null cortex

The aberrant proliferation and ectopic placement of radial progenitors in the *Marcks*^{-/-} cortex suggest that MARCKS-deficient progenitors may have lost their ability to maintain proper apicobasal polarity and adherens-junctional complexes at the ventricular surface. Recent studies have shown that apical or basolateral localization and activity of CDC42 and NUMB/NUMBL regulates appropriate radial progenitor differentiation (Cappello et al., 2006; Rasin et al., 2007). Furthermore, the activity of β -catenin, localized to the apical adherens junction, modulates the appropriate expansion

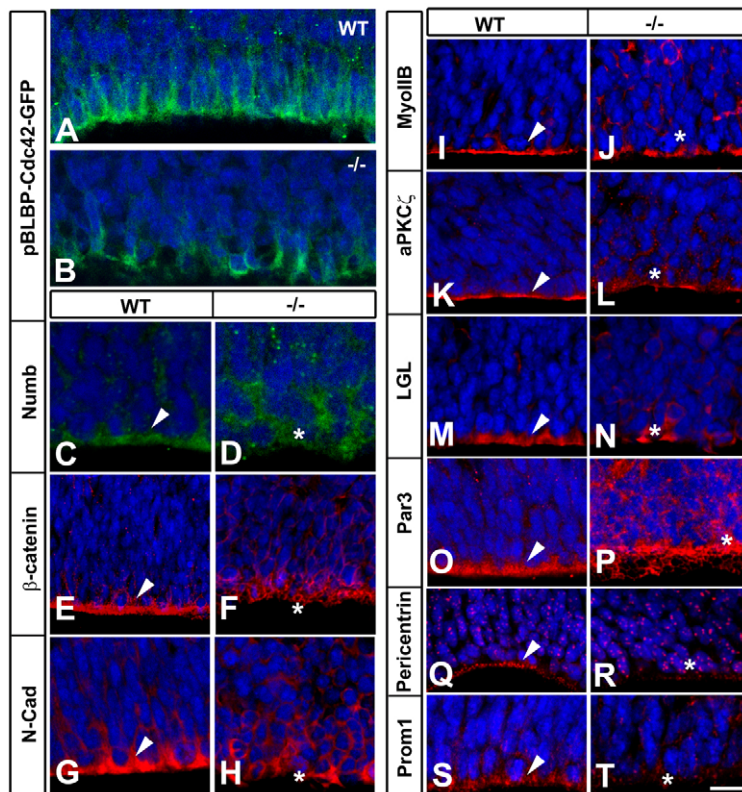


Fig. 4. Changes in the expression of apical polarity markers in *Marcks*^{-/-} radial progenitors. (A,B) CDC42-GFP expression in WT (A) and *Marcks*^{-/-} (B) radial progenitors shows that normal apical localization of CDC42-GFP is disrupted in *Marcks* null radial progenitors (B). (C-T) Immunolabeling of E15.5 cortices with anti-NUMB (C,D), β -catenin (E,F), anti-N-cadherin (N-cad; G,H), anti-myosin IIb (MyoIIb; I,J), anti-aPKC ζ (K,L), anti-LGL (M,N), anti-PAR3 (O,P), anti-pericentrin (Q,R) and anti-prominin1 (PROM1; S,T) antibodies demonstrates a loss of polarized localization of these proteins away from the apical surface of the *Marcks*^{-/-} radial progenitors (compare areas indicated by arrowheads and asterisks). Punctate labeling with anti-pericentrin (Q-R) indicates isolated centrosomes. Nuclei are counterstained with Nissl (blue). Scale bar in T: 10 μ m for A-D; 20 μ m for E-T.

of the radial progenitor population (Chae et al., 2004; Wrobel et al., 2007). We therefore examined changes in expression and localization of CDC42, NUMB and β -catenin in the *Marcks*^{-/-} cortex. To evaluate the apical localization of CDC42 in radial progenitors, WT and *Marcks*^{-/-} cortices were electroporated with a BLBP-promoter-driven CDC42-GFP (pBLBP-CDC42-GFP) plasmid. CDC42-GFP localization reflected endogenous CDC42 localization (Etienne-Manneville and Hall, 2003). CDC42 in *Marcks* null progenitors did not localize prominently to the apical surface as in WT progenitors. Instead, CDC42 expression was seen mainly in more basolateral domains of the radial progenitor cell soma (Fig. 4A,B). Normal apical localization of NUMB was also disrupted in MARCKS-deficient progenitors. Diffuse NUMB localization was evident in these progenitors (Fig. 4C,D). Similarly, immunolabeling for β -catenin revealed that *Marcks* null progenitors lose the polarized localization of β -catenin to the apical surface, with aberrant localization of β -catenin seen throughout the radial progenitor cell soma (Fig. 4E,F).

To further investigate the potential role of MARCKS in maintaining apical polarity of radial progenitors, we immunolabeled *Marcks*^{-/-} cortices with a battery of markers for apical cell polarity. Immunolabeling with antibodies for N-cadherin, the apical-junctional complex protein; MYO1B, a modulator of the actin cytoskeleton localized to the apical cell surface; and cell-polarity proteins aPKC ζ , LGL, PAR3, pericentrin and prominin 1 revealed disrupted apical localization in MARCKS-deficient cortex (Fig. 4G-T). These findings demonstrate that a loss of MARCKS impairs appropriate polarized localization of crucial cell-polarity signals in radial progenitors. This failure of radial progenitors to maintain appropriate apical cell polarity and proper contact with the ventricular surface can disrupt the apical adherens-junction complexes of radial progenitors, leading to the detachment and displacement of radial progenitors from the VZ into the IZ/CP in *Marcks*^{-/-} cortex.

Loss of MARCKS impairs radial-glia-guided migration and laminar organization of neurons

In addition to functioning as neuronal precursors, radial glia also facilitate glial-guided migration and laminar organization of neurons. To determine if the function of the radial glial scaffold as a

neuronal migratory guide is compromised in the absence of MARCKS, we assayed its ability to support neuronal migration. We first labeled radial glia in E15.5 WT and *Marcks*^{-/-} cortices with BLBP-promoter DsRed2 (pBLBP-dsRed2) plasmid electroporation. Next, GFP⁺ explants from the MGE of *Dlx5/6-CIE* mice, in which cortical interneurons express GFP, were overlaid onto slices of electroporated cortices. The GFP⁺ neurons from the MGE migrated into the slices, allowing for the visualization of individual neuron-radial glial interactions (Fig. 5A-C). Live imaging of these interactions indicated that significantly fewer GFP⁺ neurons utilized the radial glial fibers as a scaffold for migration in the *Marcks*^{-/-} cortex (Fig. 5D). Moreover, the rate of glial-guided neuronal migration was significantly reduced on *Marcks*^{-/-} radial glia (Fig. 5B,C,E). These findings demonstrate that, in addition to modulating the proliferation and placement of radial progenitors, MARCKS can influence the ability of radial glia to function as migratory guides in the developing cerebral cortex.

Since glial-guided neuronal migration determines neuronal positioning in cortex, we next assayed if laminar organization of neurons is compromised in *Marcks*^{-/-} cortex. Labeling with antibodies to the transcription factors BRN1 and TBR1 typically delineates upper and lower cortical layers, respectively, in the developing cerebral cortex. Anti-TBR1 antibodies labeled differentiated pyramidal neurons enriched in deeper layers, whereas anti-BRN1 antibodies labeled neurons of layers 2-4 (Fig. 6A). Compared with controls, TBR1⁺ and BRN1⁺ neurons in the *Marcks*^{-/-} brain were present outside their normal laminar positions, with no clear delineation between cortical layers (Fig. 6B,C). The disrupted migration of these neurons leads to cobblestone lissencephaly. These ectopic outpocketings in pial surface consist of both TBR1⁺ and BRN1⁺ neurons (Fig. 6B,C). These heterotopias were more prevalent in medial cortex and always appeared above regions with disorganized, intermixed cortical layers. The disruption in cortical layering in the *Marcks*^{-/-} cortex progressively worsened during later stages of cortical development (Fig. 6E,F). Labeling of Cajal-Retzius (CR) neurons at E12.5 with anti-reelin antibodies indicated no obvious deficits in the initial placement of marginal zone CR neurons, which are thought to arrive into the developing cortex in a radial-glia-independent manner (see Fig. S3 in the

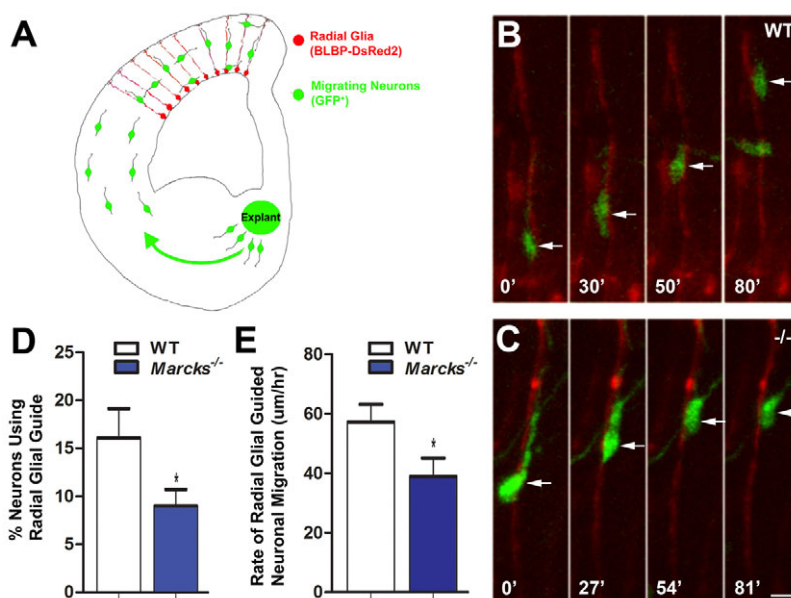


Fig. 5. *Marcks* deletion disrupts radial glia-guided migration.

WT and *Marcks*^{-/-} E15.5 cortices were electroporated with BLBP promoter-dsRed2 plasmids to label radial glia (red). (A) GFP⁺ explants from the medial ganglionic eminence of *Dlx5/6-CIE* mice were then overlaid onto electroporated slices to enable neuronal migration on radial glial processes. Time-lapse imaging of these assays was used to monitor radial glia-neuron interactions. (B) Illustration of a migrating neuron (arrow, green) along a WT radial glial process (red). Time elapsed between observations is indicated in minutes. (C) Illustration of the altered migration of a neuron (arrow, green) on *Marcks*^{-/-} radial processes (red). (D) Quantification of radial glia-neuron interactions in these assays indicates that fewer neurons initiate contact with and utilize MARCKS-deficient radial glial processes as migratory guides. (E) Compared with WT controls, the average rate of radial-glia-guided migration is significantly lower on *Marcks*^{-/-} radial glia. Data shown are mean \pm s.e.m.; asterisk indicates significance when compared with controls at $P \leq 0.01$. Scale bar in C: 7.5 μ m for B,C.

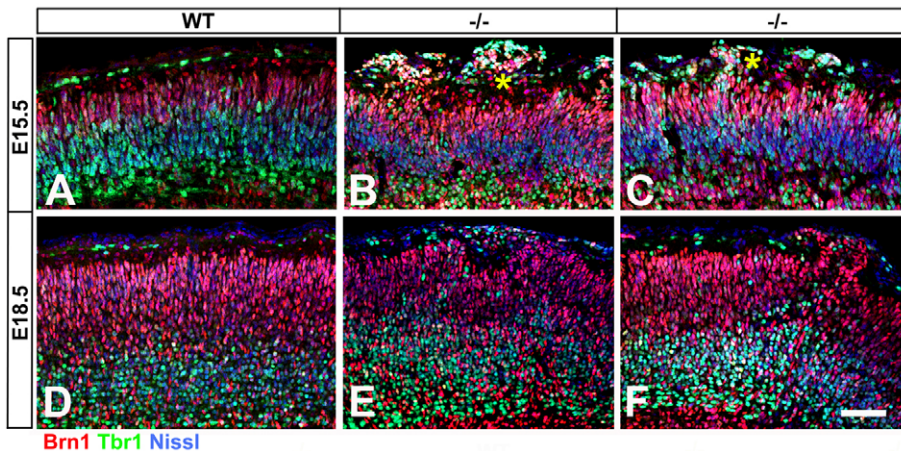


Fig. 6. Defective neuronal lamination in MARCKS-deficient cortex.

(A) Immunolabeling with anti-BRN1 (green) and anti-TBR1 (red) antibodies demarcates distinct layers in E15.5 WT CP. (B,C) BRN1⁺ and TBR1⁺ neurons are intermixed and laminar organization is disrupted in *Marcks*^{-/-} cortex (asterisks). (D-F) This neuronal layering defect persists at later developmental stages (E18.5; compare D with E and F). Scale bar in F: 50 μ m for A-F.

supplementary material). Together, these observations suggest that disrupted radial glial scaffold contributes to defective neuronal migration and layer formation in the *Marcks*^{-/-} neocortex.

Myristoylation-mediated targeting of MARCKS to the plasma membrane, but not phosphorylation by PKC, is essential for MARCKS function in radial progenitors

How does MARCKS affect the development of radial progenitors in the cerebral cortex? MARCKS serves as the predominant cellular substrate of PKC and can bind to the plasma membrane via its myristoylated N-terminus to form PIP₂-containing signaling complexes. We thus sought to determine the domains of MARCKS that are crucial for its function in the developing cerebral cortex. Mutations were made to either the PSD, containing multiple serine residues known to be phosphorylated by PKC, or the N-terminal sequence, which encodes a myristoylation moiety (Fig. 7A) (Scarlett and Blackshear, 2003; Swierczynski and Blackshear, 1996). Transgenic lines expressing either the PSD-mutant or the myristoylation (Myr) mutant MARCKS protein were crossed into a *Marcks* null background to determine if either of these domain-specific MARCKS mutant proteins could rescue the *Marcks* null phenotype in the developing cerebral cortex. The different *Marcks* mutant alleles were expressed at levels comparable to the normal *Marcks* allele. The PSD mutant rescued the aberrant placement of BLBP⁺ and PAX6⁺ progenitors in the IZ/CP (Fig. 7B-D,F-H), disrupted apical expression of cell polarity complexes such as β -catenin (Fig. 7J-L) and defects in cortical lamination (Fig. 7N-P). This suggests that the PKC phosphorylation domain of MARCKS is not crucial for its function in developing cortex. By contrast, myristoylation (Myr) mutant MARCKS failed to rescue any of the defects, including the ectopic placement of BLBP⁺/PAX6⁺ progenitors (Fig. 7B,C,E-G,I), disrupted apical localization of β -catenin (Fig. 7J,K,M) and aberrant cortical lamination (Fig. 7N-O,Q). Previous studies using non-cell-type-specific Hematoxylin-Eosin staining failed to detect this role of the myristoylation moiety (Swierczynski et al., 1996). Here, using multiple cell-type-specific tools for examination of cortical lamination, cell proliferation and polarity, we found that the targeted myristoylation of MARCKS was indeed crucial for cortical development. Together, these findings indicate that anchoring of MARCKS to the plasma membrane via its N-terminus myristoylation moiety and the resultant formation of signaling complexes, but not MARCKS phosphorylation via PKC, is crucial for the appropriate proliferation, placement and differentiation of radial glia.

DISCUSSION

In this study, we demonstrate that appropriate radial glial placement, proliferation and organization in the developing cerebral cortex depends on MARCKS signaling. In the absence of MARCKS, radial progenitors were displaced into the IZ/CP and proliferated there aberrantly. Furthermore, *Marcks*^{-/-} radial glia lost their uniquely polarized morphology, with their cell soma frequently failing to anchor to the ventricular surface, their elongated radial process deviating from its radial course to the pial surface and the end-feet devoid of their characteristic branched phenotype. Deletion of MARCKS leads to a mis-localization of signaling complexes (CDC42, aPKC ζ , PAR3, LGL, N-cadherin) essential for radial progenitor polarity. The disruption in apical polarity and aberrant basal end-feet activity following MARCKS deletion may underlie the misplacement of radial progenitors in the *Marcks*^{-/-} neocortex. Further, disrupted radial glial scaffold organization and impaired glial-guided migration culminates in aberrant cortical lamination and cobblestone lissencephaly in *Marcks*^{-/-} cortex (Fig. 8). Genetic rescue experiments indicate that the myristoylated membrane-targeting domain of MARCKS, but not the PKC-phosphorylation domain, is crucial for the function of MARCKS in radial glia. Targeting of MARCKS to the plasma membrane may enable it to serve as an adaptor for the subcellular anchoring of signaling complexes necessary for appropriate radial glial progenitor placement, proliferation and function.

MARCKS and radial glial organization

Radial glia cells are essential for the proper construction of the mammalian brain. They function as a source of neurons and neurogenic intermediate precursors and serve as permissive and instructive guides for the migration and placement of neurons (Gotz et al., 2002; Rakic, 2003; Sessa et al., 2008). These diverse functions of radial glia depend on their ability to maintain structural and molecular polarity. The uniquely polarized morphology of radial glial cells, with a cell soma anchored to the ventricular surface and an elongated basal radial process spanning the cerebral wall, enables their functions (Ayala et al., 2007; Gotz and Huttner, 2005; Kriegstein and Gotz, 2003; Rakic, 2003).

The establishment, maintenance and function of the radial glial scaffold is facilitated through the regulation of apicobasal polarity in these cells. The restricted apical or basolateral expression of CDC42 or NUMB/NUMBL, respectively, act to regulate the integrity of intercellular adhesion and patterns of proliferation of radial glia (Cappello et al., 2006; Rasin et al., 2007). Similarly, a selective deployment of cell surface receptors to the basal radial glial

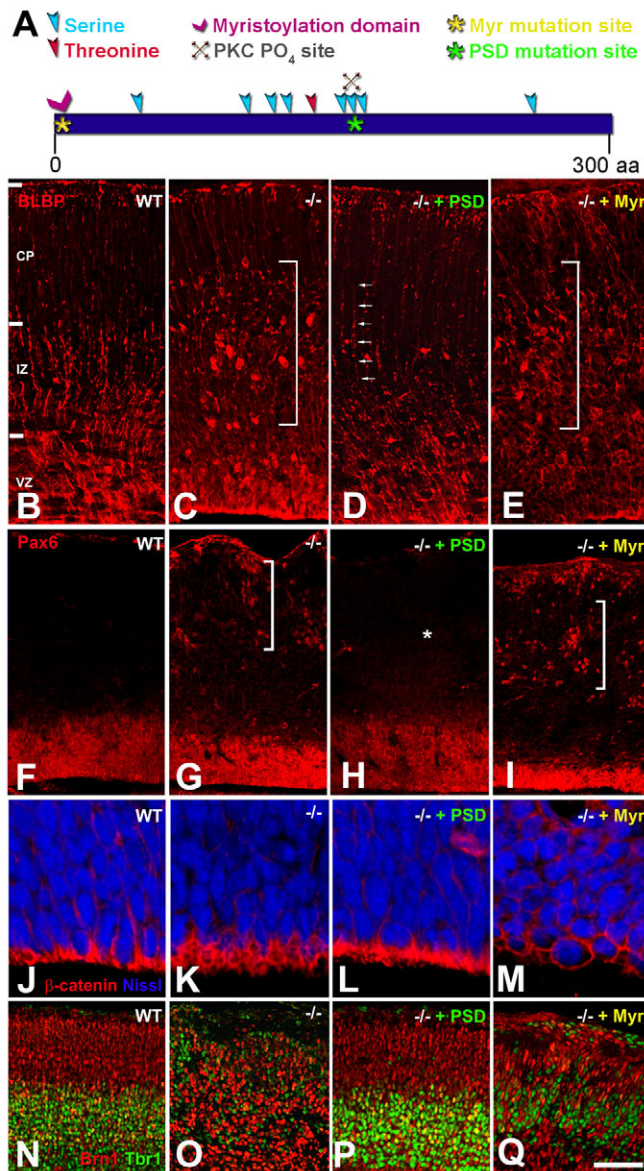


Fig. 7. Myristoylation domain of MARCKS is crucial for its function. (A) A schematic of full-length MARCKS. Transgenic lines expressing MARCKS with a mutated PSD or myristoylation moiety (Myr) were crossed onto a *Marcks*^{-/-} background to determine the functionally crucial domains of MARCKS. Regions mutated in the transgenic lines are indicated (asterisks). (B,C) Immunolabeling with an anti-BLBP antibody indicates a normal radial glial scaffold in WT cortex (B) and a disrupted radial glial scaffold with ectopic BLBP⁺ radial progenitors in *Marcks*^{-/-} cortex (bracket, C). Anti-PAX6 labeling also indicates ectopic placement of radial progenitors in *Marcks*^{-/-} cortex (F,G; bracket). The PSD-domain mutant MARCKS is able to rescue the ectopic BLBP⁺ (D) and PAX6⁺ (H) radial progenitor distribution in the upper IZ/CP. Arrows in D point to radial processes in *Marcks*^{-/-} + PSD cortex as seen in WT controls. Similarly, the asterisk in H indicates a lack of ectopic PAX6⁺ cells in *Marcks*^{-/-} + PSD cortex. Moreover, PSD-domain mutant MARCKS also rescues the disrupted apical localization of β -catenin (J-L), and the cortical lamination defects (N-P). The Myr-domain mutant MARCKS, however, fails to rescue the ectopic placement of BLBP⁺ (bracket, E) or PAX6⁺ (bracket, I) progenitors, the loss of apical-restricted expression of β -catenin in the VZ (M), or cortical lamination defects (Q). Scale bar in Q: 80 μ m for B-E; 40 μ m for F-I; 100 μ m for K-N; 20 μ m for O-R; 70 μ m for S-V. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.

end-feet allow for proper interaction with the pial basement membrane. GPR56, an adhesion G-protein-coupled receptor protein preferentially expressed by the radial glial end-feet, facilitates interaction with the pial basement membrane. Mutations in GPR56 result in a cobblestone-like lissencephaly, in which neurons overmigrate out of the cortical parenchyma, likely due to abnormal anchoring of glial end-feet to a defective pial basement membrane (Li et al., 2008). Similar defects were also seen in β 1 integrin mutant brains (Graus-Porta et al., 2001). These studies do clearly indicate that the structure and function of radial glia and the resultant construction of the cerebral cortex depends on selective targeting of key signaling complexes to apical or basal cellular domains of radial glia.

What are the mechanisms that enable radial glial cells to maintain their specialized structural and molecular polarity? Although much work has focused on uncovering the function and composition of various signaling complexes crucial for regulating radial glial polarity (Cappello et al., 2006; Rasin et al., 2007; Yokota et al., 2009), little is known about the mechanisms that govern the subcellular localization of these signaling complexes. In this study, we identified MARCKS as a potential regulator of targeted localization of polarity-related signaling complexes. For example, specialized apical junctional complexes, enriched in the protein N-cadherin, anchor radial progenitors to the ventricular surface. Formation of these apical-junctional complexes is thought to provide spatial cues for the subsequent recruitment of polarity-related signaling cues, including the PAR3/PAR6/aPKC ζ complex, to the apical surface (Nelson, 2003). There is an enriched MARCKS localization at the apical aspects of radial glial progenitors, overlapping with the expression domains of Par complex proteins (aPKC ζ and PAR3), CDC42 and β -catenin. CDC42 co-immunoprecipitates with MARCKS (data not shown). Although the exact nature of the interaction of MARCKS with each of these complexes is yet to be fully elucidated, disruption in MARCKS expression clearly leads to a loss in the polarized subcellular expression of each of these proteins (Fig. 4). Furthermore, restricted expression of NUMB, regulated indirectly via LGL and directly via aPKC ζ -dependent phosphorylation, modulates asymmetric cell division and morphological polarity of radial glia (Betschinger et al., 2003; Hutterer et al., 2004; Rasin et al., 2007; Smith et al., 2007; Zilian et al., 2001). Loss of MARCKS also disrupts the polarized localization of NUMB in radial progenitors. The widespread disruption in the subcellular localization of crucial cell-polarity signaling complexes in the absence of MARCKS may thus induce changes in radial progenitor proliferation within the VZ. The altered proliferation noticed in the embryonic cortex is likely to include both symmetrically and asymmetrically proliferating radial glia. Disruption in the known association of MARCKS with membrane cytoskeletal components, such as F-actin, may further undermine the proliferative process. Moreover, mislocalization of crucial polarity/junctional proteins following MARCKS disruption could lead to a weakening of apical intercellular adhesion. This disruption in apical adherens junctions, coupled with disrupted basal end-feet – pial basement membrane links in MARCKS-deficient radial progenitors, could in turn trigger the release of these progenitors from the VZ and their misplacement into the upper IZ and CP.

Although MARCKS is expressed in neuroepithelium as early as E8.5 (Blackshear et al., 1996; Blackshear et al., 1997), the primary organization of the neuroepithelium and the initial formation of radial glia appears to be unperturbed in non-exencephalic *Marcks*^{-/-} cortex. This suggests that *Marcks*^{-/-} radial glial disruption is not an indirect result of an earlier neuroepithelial disruption.

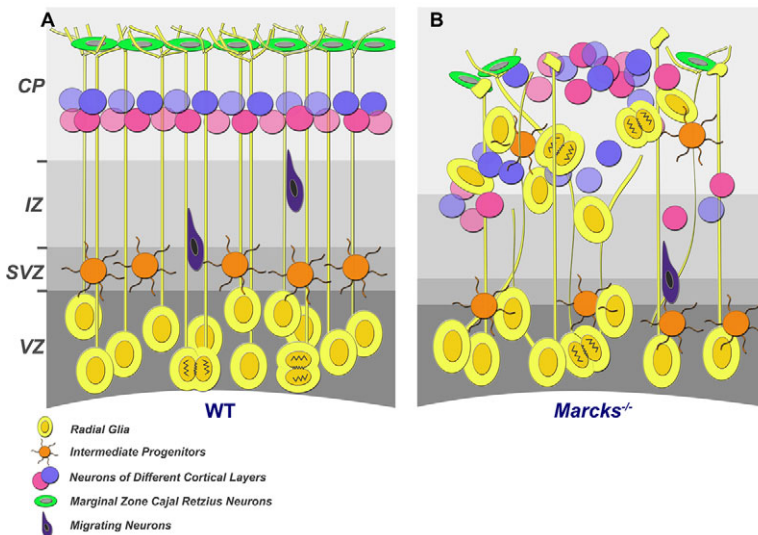


Fig. 8. Role of MARCKS in radial glial organization and cortical development. (A) MARCKS facilitates the proper placement and organization of the radial glial scaffold. This is essential for the laminar organization of neurons in the neocortex. (B) Loss of MARCKS leads to ectopic placement of radial progenitors within the IZ/CP, a disorganized radial glial scaffold with abnormal glial end-feet and process orientation, and aberrant cortical lamination. MARCKS may serve as an anchor for the polarized spatial localization of crucial signaling complexes within radial glial progenitors, and thus promotes proper radial glial development and cortical organization. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

Functional domains of MARCKS and their relevance in radial glial development

MARCKS is a major substrate for PKC, and the phosphorylated form of MARCKS shows overlapping polarized expression with PKC (Blackshear et al., 1996). As PKC is essential for the regulation of polarity and fate determination of neural progenitors (Ghosh et al., 2008), disruption in the mechanistic link between MARCKS and this signaling pathway would seem like a plausible means for regulating radial glial polarity. PKC mediated-phosphorylation of MARCKS occurs within the highly conserved PSD, which consists of three unique serine residues. A second conserved domain, the amino terminus myristoylation site, facilitates association of MARCKS with lipid membranes. When unphosphorylated, the PSD domain binds calmodulin with high affinity, crosslinks F-actin and binds to multivalent lipids, including PIP₂ and PS (Blackshear et al., 1992; Hartwig et al., 1992; Sundaram et al., 2004). Phosphorylation of the PSD domain decreases the electrostatic interaction of MARCKS with the plasma membrane, thus facilitating release of MARCKS from the membrane (Park et al., 2008; Swierczynski and Blackshear, 1995; Swierczynski and Blackshear, 1996). F-actin crosslinking activity of MARCKS is also reduced following phosphorylation (Arbuzova et al., 2002). Phosphorylated MARCKS translocates either to the cytosol or nucleus, where it has been shown to co-localize with a diverse array of cellular complexes, including components of the secretory pathway as well as γ -tubulin (Park et al., 2008). Surprisingly, we found that phosphorylation by PKC is not crucial for the role of MARCKS in radial glial cell polarization. Rather it is the integrity of the myristoylation domain that appears to be crucial for mediating the role of MARCKS in radial glial cell function. Disrupting the MARCKS membrane association appears to disrupt radial progenitor cell polarity.

How then does MARCKS act to regulate radial glial cell polarity? Our findings raise the possibility that, aside from serving as a substrate for PKC, MARCKS acts in radial progenitors as a scaffold or anchor, allowing for the membrane localization of crucial signaling complexes. Membrane-associated MARCKS is known to serve as docking site for various protein signaling complexes including calmodulin, PIP₂ and PS, as well as a site for bundling of F-actin filaments (Aderem, 1992b; Blackshear, 1993; Hartwig et al., 1992; Sundaram et al., 2004; Swierczynski and Blackshear, 1995). Regulation of MARCKS myristoylation therefore provides a

mechanistic link between the activities of calmodulin, PIP₂ and MARCKS. PIP₂ has been shown to complex with components of the polarity machinery, including PTEN, CDC42 and aPKC ζ (Janetopoulos and Devreotes, 2006; Marin-Vicente et al., 2008; Martin-Belmonte et al., 2007). PTEN, in turn, can recruit PAR3 to the plasma membrane, allowing for an additional mechanism for the targeted localization of the Par polarity complex (Feng et al., 2008). This ability of MARCKS to serve as a potential anchor for the spatial restriction of a network of crucial signaling complexes within radial glial progenitors may thus serve to promote radial glial form and function and the resultant cortical organization (Fig. 8).

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/17/2965/DC1>

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