Mouse Inscuteable Induces Apical-Basal Spindle Orientation to Facilitate Intermediate Progenitor Generation in the Developing Neocortex

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SUMMARY

Neurons in the mammalian neocortex arise from asymmetric divisions of progenitors residing in the ventricular zone. While in most progenitor divisions, the mitotic spindle is parallel to the ventricular surface, some progenitors reorient the spindle and divide in oblique orientations. Here, we use conditional deletion and overexpression of mouse Inscuteable (mInsc) to analyze the relevance of spindle reorientation in cortical progenitors. Mutating mInsc almost abolishes oblique and vertical mitotic spindles, while mInsc overexpression has the opposite effect. Our data suggest that oblique divisions are essential for generating the correct numbers of neurons in all cortical layers. Using clonal analysis, we demonstrate that spindle orientation affects the rate of indirect neurogenesis, a process where progenitors give rise to basal progenitors, which in turn divide symmetrically into two differentiating neurons. Our results indicate that the orientation of progenitor cell divisions is important for correct lineage specification in the developing mammalian brain.

INTRODUCTION

The mammalian brain is composed of thousands of neuronal subtypes. Neurons arise from a small set of progenitor cells that divide in a spatially and temporally controlled manner to generate the six-layered structure of a fully functional adult cortex (Caviness et al., 2009; Götz and Huttner, 2005; Pierani and Wassef, 2009; Rowitch and Kriegstein, 2010). How different fates are established in the daughter cells of these progenitors is poorly understood.

During early phases of mouse brain development (E9.0), the cortex consists of neuroepithelial progenitors (NEPs), which extend from the ventricular (apical) to the pial (basal) surface of the neural tube and divide symmetrically to amplify the progenitor pool. At the onset of neurogenesis (around E11.0), NEPs turn into so-called radial glial cells (RGCs) and adopt molecular and morphological characteristics of glial cells. RGCs are characterized by an apical fiber extending toward the ventricle and a basal fiber extending toward the pial surface (Caviness et al., 2009; Götz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009). RGCs occupy the most apical area of the cortex, called the ventricular zone (VZ). Their nuclei undergo a characteristic interkinetic nuclear migration where mitosis and S phase occur in the apical and basal areas of the VZ, respectively. RGCs give rise to the cortical neurons through two kinds of asymmetric divisions (Anthony et al., 2004; Malatesta et al., 2000; Noctor et al., 2001). Either, they divide into one RGC and another cell that migrates into the more basally located cortical plate (CP) where it differentiates into a neuron. Alternatively, RGCs generate one RGC and one intermediate progenitor cell (IPC). IPCs (also called basal progenitors [BPs] or nonsurface-dividing [NS-div] cells) lose their connection to the apical surface and reside in the cortical area between the VZ and intermediate zone (IZ) where they form the so-called subventricular zone (SVZ). IPCs undergo one to two rounds of symmetric division, generating either one or two pairs of neurons (Haubensak et al., 2004; Noctor et al., 2004), which can then populate all six layers of the cortex (Kowalczyk et al., 2009; Sessa et al., 2008). As the gene expression profiles of RGCs generating neurons are characteristically different from the ones generating IPCs (Pinto et al., 2008), the two modes of division seem to occur in distinct subpopulations of RGCs.

Whether or not the orientation of RGC divisions is relevant for neurogenesis has been a matter of intense debate. Early reports have demonstrated that vertical spindle orientation correlates with an asymmetric outcome in terms of daughter cell fates (Chenn and McConnell, 1995; Zhong and Chia, 2008), leading to models in which the unequal segregation of the apical and basal plasma membranes directs cell fate (Zhong and Chia, 2008). Consistent with this, mitotic spindles with vertical orientations are only found during the neurogenic phases of brain development (Haydar et al., 2003), while during the early expansion phase, keeping precise horizontal spindle orientation is crucial to maintain the neural progenitor pool (Fish et al., 2006; Yingling et al., 2008). The frequency of vertical divisions during the neurogenic phase, however, is too low to account for all divisions with asymmetric outcome (Chenn and McConnell, 1995; Haydar et al., 2003; Kosodo et al., 2004). This could be explained by the small size of the apical membrane domain of RGCs, such that even barely oblique mitotic spindles would give rise to cleavage planes that fail to bisect this domain resulting in its
asymmetric segregation (Kosodo et al., 2004). It has been demonstrated that increasing the rate of vertical divisions can affect progenitor cell number and location (Konno et al., 2008; Shitamukai et al., 2011). Functional evidence to demonstrate that either vertical or oblique spindle orientation is required for neurogenesis, however, remains to be established.

The molecular machinery for spindle orientation during neurogenesis is best understood in *Drosophila* (Siller and Doe, 2009). In *Drosophila* neuroblasts, orientation of the mitotic spindle along the apical-basal axis is important for the asymmetric segregation of the cell fate determinants Numb (Hirata et al., 1995; Knoblich et al., 1995; Rhyu et al., 1994; Spana and Doe, 1995), Prospero (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995), and Brat (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006) into the basal daughter cell (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006), where these proteins prevent self-renewal and induce differentiation. In neuroblasts, the mitotic spindle is oriented by two protein complexes that assemble on its apical cell cortex. One complex consists of the PDZ domain proteins Par-3, Par-6, and the atypical protein kinase C (aPKC) while the other contains the GoLoco domain protein Pins, the heterotrimeric G protein subunit GzI, and the microtubule-binding protein Mushroom body defect (Mud). These two complexes are linked by an adaptor protein called Inscuteable (Insc). Insc can bind to both Pins (Schafer et al., 2000; Yu et al., 2000) and Par-3 (Schober et al., 1999; Wodarz et al., 1999) via multiple Armadillo repeats within its so-called asymmetry domain (Knoblich et al., 1999; Schafer et al., 2000). In insc mutants, mitotic spindles in neuroblasts are randomly oriented, leading to missegregation of cell fate determinants, and thus, cell fate defects in the developing nervous system. When Insc is ectopically expressed in epithelial cells, Pins and Mud are recruited from the basolateral to the apical cortex, and the mitotic spindle reorients from a horizontal into an apical-basal direction. Therefore, unlike all other components, Insc is not only required but also sufficient for spindle orientation along the apical-basal axis.

While the components of the *Drosophila* spindle orientation machinery are conserved in mammals, they have been studied mainly with regard to their roles in epithelial cell polarity (Goldstein and Macara, 2007), and most of them have additional functions in cell polarity or microtubule dynamics (Woodard et al., 2010). Mammalian Par-3, Par-6, and aPKC are important for spindle orientation, and—like their *Drosophila* counterparts—they are also required for epithelial apical-basal polarity. Pins has two mammalian homologs, AGS-3 and LGN (Sanada and Tsai, 2005; Yu et al., 2003). AGS-3 does not appear to be expressed in the brain at significant levels, and AGS3 knockout mice show no brain phenotype (Blumer et al., 2008). By contrast, LGN mediates planar spindle orientation in the developing brain (Konno et al., 2008; Morin et al., 2007), consistent with its role in mitotic spindle orientation during epithelial morphogenesis (Zheng et al., 2010), but is also required for microtubule aster formation and spindle morphology (Du et al., 2001), and regulates mitotic spindle orientation during epithelial morphogenesis (Zheng et al., 2010). Similarly, the mammalian Mud homolog NuMA has been shown to play a conserved role in the spindle orientation complex (Du and Macara, 2004) but has additional functions in organizing a bipolar mitotic spindle (Silk et al., 2009; Sun and Schatten, 2006).

Insc is conserved in vertebrates. Overexpression and RNAi studies have shown that the protein is involved in orienting the mitotic spindle in the rat retina (Zigman et al., 2005), and a similar function has been postulated in the mouse skin (Lechler and Fuchs, 2005). Moreover, in situ hybridization experiments showed that mouse *Inscuteable* (mInsc) is expressed in the developing neocortex at the time when the first neurons start to appear (Zigman et al., 2005). To test the role of mInsc in cortical development, we have generated conditional knockout and overexpression mice. We measure spindle orientation in 3D and show that the fraction of oblique divisions increases or decreases upon mInsc overexpression or deletion, respectively. We show that loss of mInsc leads to defects in neurogenesis and depletion of BPs, while mInsc overexpression has the opposite effect. Our data are consistent with a model in which oblique divisions preferentially give rise to BPs and, therefore, suggest a mechanism regulating the balance between direct and indirect neurogenesis during mouse brain development.

### RESULTS

**mInsc Is a Functionally Conserved Insc Homolog**

mInsc is expressed throughout the developing cortex during mid-neurogenesis (Figures 1A, 1B, and 1K) (Zigman et al., 2005). In the VZ, the protein is enriched at the spindle midzone in about 90% of the anaphase cells (yellow arrow in Figure 1C, and graph in Figure 1E). In 100% of the CP neurons, however, the protein is localized to the neuron cell body cytoplasm and concentrates on one side of the nucleus (yellow arrow in Figure 1D).

To test whether mInsc can functionally replace the *Drosophila* protein, we generated transgenic flies expressing C-terminally myc-tagged mInsc (mInsc::myc). When expressed in neuroblasts, mInsc::myc localizes into an apical crescent (Figures 1F and 1G). Like *Drosophila* Insc (Kraut et al., 1996), mInsc::myc can induce a reorientation of the mitotic spindle into an apical-basal orientation when ectopically expressed in epithelial cells (Figures 1H and 1I). Thus, mInsc is a functional homolog of *Drosophila* Insc.

**mInsc Conditional Gain- and Loss-of-Function Alleles**

To analyze the function of mInsc in mouse cortical development, we generated conditional loss-of-function and overexpression alleles (called *mInsc<sup>loxP</sup>* and *R26<sup>td</sup>*, respectively) (Figure 1J; see Figures S1A and S1B and Supplemental Experimental Procedures available online for details). Upon Cre recombination, the *R26<sup>td</sup>* line lost β-gal expression and showed strong and ubiquitous expression of mInsc::GFP (*R26<sup>mInsc::GFP</sup>* (Figure 1P). For brain-specific recombination we used NesCre<sup>δ</sup>, which expresses Cre in the forebrain of E8.5 embryos (Petersen et al., 2002). When combined with *mInsc<sup>loxP</sup>*; this line results in near-complete removal of mInsc from the cortex at E14.5 (Figures 1K and 1L). We call the recombined allele *mInsc<sup>δ</sup>*. Residual mInsc staining on the apical surface of the cortex (white arrow) is presumably due to truncated protein persistence or mosaic expression of Cre (Figure 1L). In addition we detected
some nonspecific antibody staining around blood vessels (yellow arrow) that is not due to the secondary antibody (Figures S1F and 1G). When combined with the R26KI allele, NesCre8 caused loss of β-gal expression (compare Figure 1M with 1N), and strong expression of the GFP fusion protein (Figures 1O and 1P) in the entire cortex at E14.5. Expression of the GFP fusion protein can also be detected as a 90 kDa band in immunoblots from E13.5 heads (Figure 1Q). This band is found in addition to the 60 kDa band from the endogenous protein in NesCre/+;R26KI/ki but not in NesCre/+ or R26KI/ki mice (Figure 1Q). Thus, we have generated functional tools for gain- and loss-of-function analysis of mInsc.

mInsc Controls Oblique and Vertical Spindle Orientations in the Developing Cortex

Previous RNAi experiments have suggested that the function of mInsc in controlling the orientation of neural precursor divisions is conserved from Drosophila to mice (Zigman et al., 2005). We therefore asked whether mInsc controls the orientation of mitotic spindles in RGCs and, if so, whether the loss- and gain-of-function alleles influence spindle orientation and lineage specification in the developing brain.

Various conflicting reports exist on the wild-type orientation of mitotic spindles in RGCs (Chenn and McConnell, 1995; Haydar et al., 2003; Konno et al., 2008). In these reports, spindle orientations were measured relative to a line representing the ventricular surface. As this methodology neglects spindle orientations in Z direction (out of the focal plane) and is therefore imprecise due to the curved apical surface of the ventricle, we used 3D image reconstruction and computational analysis to obtain more precise measurements. E11.5 and E13.5 embryos were stained for β-tubulin (β-Tub), α-tubulin (α-Tub), and phosphorylated Histone H3 (PH3) to mark centrosomes, mitotic spindles, and mitotic chromatin, respectively. Cell outlines were determined from the α-Tub staining. Embryonic brains were paraffin embedded, and individual anaphase RGCs were reconstructed in 3D from confocal stacks of coronal brain sections (Figures 2A–2C; Figures S2A–S2C; asterisks in Figures 2A and 2B point at centrosomes). Using the Imaris 3D visualization software, we then defined the position of the two centrosomes and placed five points at different positions along the apical surface of the 3D-rendered cell. These points were used to determine the best-fitting plane by orthogonal distance regression and to calculate the angle φ between
a vector connecting the two dots marking the centrosomes, and the normal vector of the plane, marking the apical surface. The angle $\alpha$ of the spindle orientation was calculated as $90^\circ$ minus the angle $\phi$ (Figure 2D).

Using this procedure, we determined the division angle of radial glia cells from NesCre/+ (ctrl), NesCre/+:mInsc/GFP (cko), and NesCre/+;R26minsc::GFP/ (cki) mice at both E11.5 and E13.5. At E11.5, RGCs divide in a planar orientation with mitotic spindles oriented in parallel to the ventricular surface (angles less than $30^\circ$), consistent with previous observations (Haydar et al., 2003; Konno et al., 2008; Kosodo et al., 2004). At this stage, division angles in cko and cki mice are not significantly different from controls (Figure 2E; Table S1). Although we cannot exclude that Cre recombination is not efficient in early stages, this suggests that mInsc is not functional at early stages of neurogenesis.

At E13.5, however, 63% of the mitotic spindles in control embryos are at angles between $0^\circ$ and $30^\circ$, while $33%$ are between $30^\circ$ and $60^\circ$. Consistent with previous reports, we found that vertically oriented mitotic spindles (between $60^\circ$ and $90^\circ$) are rare (Haydar et al., 2003) and are not seen in more than 3% of all mitotic cells (Figure 2F, and blue bar in Figure 2G). In cko mice, however, the vast majority of mitotic spindles ($95\%$) were between $0^\circ$ and $30^\circ$, oblique divisions ($30^\circ < 0 < 60^\circ$) were strongly reduced ($5\%$), and vertical spindles were never seen (Figure 2F, red bar in Figure 2G; Table S1). Upon overexpression of mInsc in cki mice, however, the fraction of oblique and vertical divisions was significantly increased (63%) (Figure 2F, green bar in Figure 2G; Table S1). Thus, like in Drosophila, mInsc is required and sufficient for orienting mitotic spindles along the apical-basal axis. Importantly, the mInsc spindle orientation phenotype is different from the one observed in LGN knockout mice and LGN knockdown in chicken spinal cord (Konno et al., 2008; Morin et al., 2007), where spindle orientation is randomized, and the number of horizontal spindles is actually decreased.

We also tested the subcellular localization of mInsc::GFP in R26mInsc::GFP/ RGCs at E14.5 (Figure 2H). In 100% of the interphase progenitors, mInsc::GFP is cytoplasmic. When nuclei are close to the ventricular surface in preparation for mitosis, the protein concentrates in the apical stalk as well as on the basal side of the RGC body (yellow arrow in Figures 2H and 2I, and yellow bar in Figure 2M). In about 80% of mitotic cells, mInsc concentrates in an apical crescent (orange arrow in Figures 2H and 2J, and green bar in Figure 2M) that enlarges in prometaphase while the basal staining disappears (green arrow in mInsc concentrates in the apical stalk as well as on the basal side of the RGC body (yellow arrow in Figures 2H and 2I, and yellow bar in Figure 2M). In about 80% of mitotic cells, mInsc concentrates in an apical crescent (orange arrow in Figures 2H and 2J, and green bar in Figure 2M) that enlarges in prometaphase while the basal staining disappears (green arrow in
In anaphase, finally, mInsc becomes symmetric again and is distributed on both sides of about 90% of anaphase RGCs and in the spindle midzone (blue arrow in Figures 2H and 2L, and blue bar in Figure 2M). Taken together, these data demonstrate that mInsc is an important regulator of spindle orientation in the developing mouse brain.

Figure 3. Cortical Neuronal Number Is Affected in Adult Mutant Brains

(A–D) Nissl staining of 2-month-old mice from wild-type (A), full knockout (B), conditional knockout (C), and conditional knockin (D).

(E–P) Expression analysis for molecular markers identifying layers II–IV (Satb2) (E, I, and M), layers III and V (FoxP1) (F, J, and N), and layer VI (FoxP2) (G, K, and O), and Cux-1 (H, L, and P) in adult control (E–H), cko (I–L), and cki (M–P) adult brains.

(Q) Quantification of the alteration of upper cortical neurons and deep cortical neurons in mlnsc mutant mice, with respect to control cortices. *p < 0.05; **p < 0.01; ***p < 0.001; Welch two-sample t test (n = 3 for each genotype).

Figures 2H and 2K). In anaphase, finally, mlnsc becomes symmetric again and is distributed on both sides of about 90% of anaphase RGCs and in the spindle midzone (blue arrow in Figures 2H and 2L, and blue bar in Figure 2M). Taken together, these data demonstrate that mlnsc is an important regulator of spindle orientation in the developing mouse brain.
**Figure 4. mInsc Mutant Mice Show Altered Cerebral Cortex Thickness and Altered Neurogenesis**

(A–C and E–G) Cresyl violet staining of the anterior (ant) and medial (med) coronal sections of E14.5 control (A and E), conditional knockout (B and F), and conditional knockin (C and G) cortices showing the alteration of the cortical thickness. lge, lateral ganglionic eminence.

(D) Schematic representation of the embryonic forebrain. Red lines show the level at which the coronal sections were taken.

(N–S) Immunostaining with Nestin and Tuj1 of the anterior (ant) and medial (med) sections of E14.5 control (N and O), conditional knockout (P and Q), and conditional knockin (R and S) cortices.
mlnc Function Is Essential for Proper Formation of the Adult Cortical Layers

Both NesCre/+; mlnsc<sup>fl/fl</sup> and NesCre/+; R26<sup>ki/ki</sup> mice survive to adulthood, although NesCre/+; R26<sup>ki/ki</sup> animals frequently show epileptic crisis. Despite their viability, however, both mutants show clearly visible and reproducible defects in cortical organization (Figure 3). Nissl staining of brains from 2-month-old animals shows that cortical thickness is reduced in NesCre/++; mlnsc<sup>fl/fl</sup> mice and increased in NesCre/++; R26<sup>ki/ki</sup> mice (Figures 3A–3D). Very similar brain defects are observed in mlnsc<sup>−/−</sup> L3 mice (Figure 3B), indicating that the time of onset of NesCre8 expression is not relevant for the strength of the phenotype.

The different layers of the developing cortex can be recognized by their unique cell density and morphology in Nissl stains. Analysis of the various mlnsc alleles reveals that layer organization is not dramatically affected in NesCre/++; mlnsc<sup>fl/fl</sup> mice (Figure 3C) while in NesCre/++; R26<sup>ki/ki</sup> mice, layer IV is barely recognizable and seems to be fused with layer V (Figure 3D). In addition, GFAP staining indicates an alteration of the white matter layer thickness (Figure S3). To further characterize these adult brain phenotypes, we used layer-specific markers. We used FoxP2 as a marker for layer VI, FoxP1 as a marker for layers III and V, Satb2 as a marker for layers II–IV (Britanova et al., 2008; Ferland et al., 2003), and Cux1 as a marker for layers III–IV (Nieto et al., 2004).

The number of Satb2-positive cells is reduced in the NesCre/++; mlnsc<sup>fl/fl</sup> mice (Figures 3E and 3I), while in the NesCre/++; R26<sup>ki/ki</sup> mice, their number is increased (Figure 3M). Similar results were obtained using Cux1 antibody as a marker of the upper layers (Figures 3H, 3L, and 3P). The nuclear marker FoxP1 is present in two stripes corresponding to layers III and V (Figure 3F). Cell density is strongly reduced in NesCre/++; mlnsc<sup>fl/fl</sup> mice (Figure 3J) while the number of cells in these layers is significantly increased in NesCre/++; R26<sup>ki/ki</sup> mice (Figure 3N). Finally, layer VI of the adult cortex, which is the first to be formed during embryogenesis, is also affected in both mutant conditions, as shown by immunostaining with the Foxp2 antibody (Figures 3G, 3K, and 3O). A quantitive analysis of the cells positive for the various layer-specific markers (Figure 3Q) confirms this phenotypic analysis. These data demonstrate that mlnsc levels are important for neurogenesis and suggest that vertical orientation of the mitotic spindle is relevant for cortical development.

mlnc Regulates Embryonic Cortical Development

To determine the developmental origin of those cortical phenotypes, we analyzed cortical development in mlnsc knockout and overexpression mice (Figure 4). E11.5 brain sections from control, conditional knockout, germline-transmitted knockout, and conditional knockin show no difference in the expression of nestin, Tbr1 (neurons), or Tbr2 (intermediate progenitors) (Figure S4), indicating that mlnsc has no obvious role in early neurogenesis. We then stained coronal sections of E14.5 control and mutant brains with cresyl violet (Figures 4A–4J). We examined comparable sections at anterior and medial levels. No major structural abnormalities were detected at this stage, but NesCre/++; mlnsc<sup>fl/fl</sup> brains were smaller than controls (Figures 4A and 4B). The lateral ventricles were enlarged, and overall cortical thickness as well as the protrusion of the lateral ganglionic eminence into the ventricles were reduced (Figures 4A, 4B, 4E, and 4F). NesCre/++; R26<sup>ki/ki</sup> brains, on the other hand, exhibited increased cortical thickness (Figure 4A, 4C, 4E, and 4G). These alterations in cortical thickness were observed in both central and lateral regions (refer to the scheme in Figure 4K). Quantification of those phenotypes showed that cortical thickness was reduced by around 20% both medially and laterally in NesCre/++; mlnsc<sup>fl/fl</sup> mice, while it was increased by about 20% in the medial region and by around 40% in the central and lateral regions in NesCre/++; R26<sup>ki/ki</sup> mice (Figure 4L). A more detailed examination of these phenotypes revealed that the alterations in cortical thickness are largely due to changes in the IZ and the CP while the VZ is almost unaffected (Figures 4H–4J). In NesCre/++; mlnsc<sup>fl/fl</sup> mice the thickness of the IZ and CP were reduced by about 25% and 40% (Figure 4M) while in NesCre/++; R26<sup>ki/ki</sup> mice, both layers were thicker, up to more than three times (Figure 4M).

To test whether these alterations were due to changes in cellular composition, we stained E14.5 NesCre/++, NesCre/++; mlnsc<sup>fl/fl</sup>, and NesCre/++; R26<sup>ki/ki</sup> embryos for Nestin, BPLP, and TuJ1, which label neural progenitors and neurons, respectively (Menezes and Luskin, 1994; Yachnis et al., 1993). While Nestin staining of NesCre/++; mlnsc<sup>fl/fl</sup> mice does not reveal any obvious abnormalities, RGCs in NesCre/++; R26<sup>ki/ki</sup> brains showed an alteration in the radial organization of the RGC fibers (Menezes and Luskin, 1994) (Figures 4N–4P, Figure S5). TuJ1 staining revealed that neurons in the IZ and CP of NesCre/++; mlnsc<sup>fl/fl</sup> brains are reduced while in NesCre/++; R26<sup>ki/ki</sup> brains the area occupied by those neurons is enlarged, consistent with the observed increase in cortical thickness (Figures 4Q–4S). In NesCre/++; mlnsc<sup>fl/fl</sup> brains, however, TuJ1<sup>+</sup> neurons in the VZ were rarely found (arrow and inset in Figure 4R), while in NesCre/++; R26<sup>ki/ki</sup> brains they seemed to be more abundant (arrow and inset in Figure 4S). Together, these data indicate that changes in spindle orientation do affect neurogenesis in the developing cortex, with consequent alteration of its thickness, although the number of RGCs is not strongly affected (Figure 5R).
**Figure 5. mlnsc Mutation Affects CP Development and Neural Progenitor Positioning**

(A–C) Immunohistochemistry for Tbr1 on E14.5 control (A), conditional knockout (B), and conditional knockin (C) cortices reveals a defective neuronal production leading to a reduction in CP thickness in the conditional knockout (B) and to a CP thickening in conditional knockin (C) embryos.

(D–G) Immunohistochemistry for Pax6 on E14.5 control (D), conditional knockout (E), conditional knockin (F), and heterozygous germline-transmitted knockin (G) showing the aberrant positioning of neural progenitors in knockin cortices (F and G) compared to the control (D). White arrows in (G) point to groups of Pax6+ cells in the IZ/CP.
mlnc Levels Affect Neurogenesis and Neural Progenitor Localization
To characterize the initial defects in mlnc-deleted or -overexpressing mice, we used markers for CP neurons. CP neurons are the first recognizable layer of the developing neocortex, and are identified by the expression of Map2 and the transcription factor Tbr1 (Fujimori et al., 2002; Hevner et al., 2001). At E14.5, the number of Tbr1* cells is reduced by almost half in NesCre++; mlnsc<sub>fl/fl</sub> brains while in NesCre++; R26<sub>ki/ki</sub> mice the number of these cells is significantly increased (Figures 5A–5C and 5P). Staining for Map2 shows similar alterations in CP neurons in the two genotypes (Figures 5H–5J).

To test whether the decrease in neurogenesis occurs at the expense of cortical progenitor cells, we used the nuclear RGC marker Pax6 (Figures 5D–5F and 5L–5N) (Götz et al., 1998). Although the high density of Pax6* nuclei in the VZ makes it impossible to obtain precise quantitative measurements, we did not find any striking changes in the number of Sox2+ VZ progenitors in NesCre++; mlnsc<sub>fl/fl</sub> or in NesCre++; R26<sub>ki/ki</sub> mice (Figure 5R). However, vertical spindle reorientation in NesCre++; R26<sub>ki/ki</sub> mice leads to the frequent generation of Pax6* progenitors that are located outside the VZ, in the IZ, or the CP (Figures 5F and 5N). The number and frequency of those cells were increased even more when Cre recombination was induced in the germine of R26<sub>ki/+</sub> mothers using MoreCre. In E14.5 embryos from those mothers (named R26<sub>mlnsc::GFP/+</sub>), clusters of Pax6+ cells were frequently seen in the IZ, and Pax6+ cells were present even in the CP where they replaced differentiating neurons forming gaps in the Map2+ layer of cells (Figures 5G and 5K, arrows, and Figure 5O). Quantitative analysis revealed that Pax6+ cells were six times more abundant in the IZ and three times more abundant in the CP of R26<sub>mlnsc::GFP/+</sub> mice when compared to NesCre++; R26<sub>ki/ki</sub> (Figure 5Q). These observations are consistent with previous in utero electroporation experiments (Konno et al., 2008), although the previous conclusion that endogenous mlnc does not orient mitotic spindles in the mouse cortex (Fish et al., 2008; Konno et al., 2008) is clearly not supported by our data.

mlnc Levels Affect Proliferation and BP Generation
Changes in cortical thickness and neuronal differentiation observed in mlnc mutant and mlnc-overexpressing brains could be due to alterations in the position of mitotic cells and/or in RGC proliferation. In order to distinguish between these possibilities, we first stained E14.5 sagittal brain sections with anti-PH3 to look at proliferative cells in both VZ and SVZ.

Possibilities, we first stained E14.5 sagittal brain sections with Ki67. In this experiment, the fraction of Ki67+ BrdU+ cells within the total BrdU-positive population can be used as an indicator of cell cycle exit of progenitors. We found no significant differences in NesCre++; mlnsc<sub>fl/fl</sub>, in NesCre++; R26<sub>ki/ki</sub>, or in R26<sub>mlnsc::GFP/+</sub> mice (Figure 6J), indicating that mlnc has no strong effect on average cell cycle length both in apical and BPs.

The altered proliferation pattern could be due to a difference in position or fate of the dividing cells. In wild-type animals, proliferation basal to the VZ is due to IPCs, which can be specifically marked by staining for Tbr2 (Figure 6K) (Englund et al., 2005). In NesCre++; mlnsc<sub>fl/fl</sub> mice, the number of Tbr2* cells is reduced (Figure 6L) while this number is increased in NesCre++; R26<sub>ki/ki</sub> brains (Figure 6M). This effect can be enhanced by germline recombination of the R26<sup>ki</sup> allele in R26<sub>mlnsc::GFP/+</sub> mice (Figure 6N). Quantification of these phenotypes confirms this observation (Figure 6O). Interestingly, the extra BPs are no longer confined to the SVZ but frequently found in the more basal parts of the cortex. Thus, modifying spindle orientation changes the frequency with which RGCs give rise to intermediate progenitors. As proliferation and cell cycle exit rates of RGCs do not change in the mutant conditions, we can exclude that this is a consequence of alterations in RGC proliferation. Therefore, we postulate that spindle orientation influences the fate that RGC daughters assume after division.

To obtain more direct evidence for the proposed lineage changes, we used in utero electroporation (Figures 7A–7R). For this we electroporated a construct expressing RFP into brains of E14.5 control, knockout, and embryos from R26<sub>ki/ki</sub> males crossed to NesCre++; R26<sub>ki/ki</sub> females. We used NesCre<sup>+</sup>; R26<sub>ki/ki</sub> embryos in order to avoid the observed massive ectopic location of apical and BPs.

Long-term time-lapse experiments during mid-late neurogenesis show that apical progenitors undergo only one division in
Figure 6. Proliferation and Cell Cycle Analysis

(A–C) PH3⁺ cell number in M phase residing in the SVZ region is affected in the conditional knockout (B), and in the conditional knockin (C) E14.5 brains, compared to the control (A).
24 hr (Noctor et al., 2004). In order to look at the fate of the daughter cells after one division of apical progenitors, embryos were collected 1 day after electroporation. RFP+ cells are found in the VZ and IZ of brains from control, knockout, and knockin embryos (Figures 7B, 7E, 7H, 7K, 7N, and 7Q). While the elec-tporated RFP+ cells have migrated beyond the basal border of the Pax6 expression zone in control and knockout animals, the RFP+ cells are located right at the edge of this expression zone in the mInsc-overexpressing animals (compare Figures 7C and 71 with Figure 7F). To determine the identity of those cells, we used the BP marker Tbr2. In control and mutant brains, Tbr2 is expressed in a subset of the RFP+-electroporated cells. In control animals, Tbr2 is expressed in 23% of the RFP+-electroporated cells while this fraction is reduced to about 10% in NesCre+/; mInsc<sup>fl/fl</sup> embryos. In mInsc-overexpressing animals, in contrast, the BP marker is expressed in over 50% of the electroporated cells (determined as the number of Tbr2<sup>+</sup>, RFP+ cells divided by the total number of RFP+ cells, Figure 7T). As the percentage of Pax6<sup>+</sup>/RFP<sup>+</sup> progenitor cells among all electroporated (RFP+) cells does not change (Figure 7S), these results indicate that a re-orientation of the mitotic spindle along the apical-basal axis causes RGCs to preferentially generate intermediate progenitors after division.

Taken together, our data reveal that spindle orientation along the apical-basal axis is mediated by mInsc and is important for promoting neurogenesis. Apical-basal divisions are more likely to give rise to intermediate progenitors, and this effect may be responsible for the increased rates of neurogenesis observed upon mInsc overexpression.

DISCUSSION

**Spindle Orientation and Cortical Development**

To address the role of nonplanar spindle orientation in cortical development, we have generated a conditional deletion of mInsc. Unlike Drosophila Pins, Par-3, Par-6, and aPKC, Insc has a single, clearly defined mammalian homolog (Katoh, 2003; Lechler and Fuchs, 2005; Zigman et al., 2005). In Drosophila embryos, Insc is exclusively expressed in asymmetri-cally dividing cells, and no functions other than asymmetric cell division have been reported. This is different for other proteins involved in this process. The apical Par proteins are also involved in epithelial polarity and cell migration. Mutating centrosomal proteins like Asp (Aspm in mice) (Fish et al., 2006, 2008) or Cnn (CDK5RAP2 in mice) (Barrera et al., 2010) might affect signaling pathways by disrupting primary cilia and will influence centrosome asymmetry, which was proposed to be important in cortical neurogenesis (Wang et al., 2009). Mutating dynein-binding proteins like Lis1 causes defects in spindle morphology and cell migration (Yingling et al., 2008). Therefore, our mInsc knockout and mInsc-overexpression mice are partic-ularly specific tools to analyze spindle orientation.

The spindle orientation defects we observe in mInsc-deficient mice are different from the one previously reported for LGN, the mouse homolog of the Insc-binding partner Pins. In LGN knock-outs, the orientation of the mitotic spindle is randomized while lack of mInsc causes almost all mitotic spindles to assume a planar orientation. This is in agreement with the functions re-ported for the two genes in flies and explains why the two genes have different effects on cortical neurogenesis (Konno et al., 2008; Shitamukai et al., 2011) (and this study).

**Nonplanar Divisions Preferentially Generate Intermediate Progenitors**

Our results suggest that intermediate progenitors are more likely to arise from oblique or horizontal divisions (in which the spindle is oriented oblique or vertical, respectively). First, increasing or decreasing mInsc expression elevates or reduces the number of neurons, respectively. At the same time, both the total number of apical progenitors and the number of mitotic cells in the VZ remain constant. Second, mInsc levels affect the number of Tbr2-positive intermediate progenitors and the number of cells dividing outside the VZ. And finally, apical pro-genitors labeled by electroporation of RFP-expressing plasmids are more likely to give rise to Tbr2-positive intermediate progeni-tors when mInsc levels are increased. We propose a model in which mInsc influences spindle orientation and thereby regu-lates the balance between direct and indirect neurogenesis (Figure 8). Whether or not mInsc is required for generating all or most BPs is not clear. It is remarkable that the terminal forebrain phenotype of mInsc mice is similar to the one observed for Tbr2, in which intermediate progenitors are essentially absent (Arnold et al., 2008; Sessa et al., 2008); in both cases, thickness of the CP is reduced by about 40%. While the outer layers are more affected in Tbr2<sup>-/-</sup> mice, however, NesCre+/;mInsc<sup>fl/fl</sup> mice show similar defects across all layers. This could be explained if intermediate progenitors initially form through a spindle orien-tation-dependent mechanism, but later neurogenesis can also proceed through a partially redundant, mInsc-independent mechanism.
Figure 7. Intermediate Progenitor Production Is Affected in mInsc Mutant Mice

(A–R) RFP electroporation in E14.5 control, knockout, and heterozygous conditional knockin embryos, killed 24 hr later, and double stained with Pax6 (A–I) or Tbr2 (J–R) reveals a preference in the production of intermediate progenitors in the conditional heterozygous knockin after RGC division.

(S and T) Quantitative analysis of the number of RFP+/Pax6+ versus total RFP+ (S), and RFP+/Tbr2+ versus total RFP+ (T) (n = 3 for each genotype). (S) cki and cko p value = 1; (T) cko p value = 0.003; cki p value = 1.2e-11. n.s., not significant. ***p < 0.001.
Inscuteable Regulates Intermediate Progenitors

Figure 8. Model
Spindle orientation influences the choice between direct and indirect neurogenesis. In wild-type (A), divisions adopting a horizontal mitotic spindle give rise to neurons using preferentially direct neurogenesis, whereas divisions with oblique and vertical spindle orientations generate neurons preferentially through indirect neurogenesis. In mInsc knockout brains (B), the divisions with oblique and vertical spindle orientation are depleted, and as a result, RGC cells divide asymmetrically giving rise to neurons through direct neurogenesis, and the total number of neurons produced is reduced. In mInsc knockin brains (C), divisions adopting an oblique or vertical spindle orientation are increased. As a result, RGCs generate more intermediate progenitors, amplifying the neuron production. RG, radial glia; N, neurons; IP, intermediate progenitors.

How could asymmetry be established during progenitor divisions? Quite likely, mInsc-independent direct neurogenesis and mInsc-dependent indirect neurogenesis might use different mechanisms. Both the narrow apical plasma membrane domain and the basal process that connects progenitors to the pial surface should be inherited by only one daughter cell during oblique or vertical division. As BPs do not maintain their connection to the apical surface (Götz and Huttner, 2005; Miyata et al., 2004) but do contain a basal process, the asymmetric inheritance of those structures could contribute to intermediate progenitor formation. For example, intermediate progenitors could simply move out of the VZ after S phase because they are not attached to the apical surface. Alternatively, apically localized proteins could perform a more direct signaling role. It has been proposed that the asymmetric inheritance of Par3 can activate Notch signaling in one daughter cell of an apical progenitor (Bultje et al., 2009). As levels of Notch signaling are lower in intermediate progenitors and decreasing levels of Notch signaling promotes the formation of intermediate progenitors (Mizutani et al., 2007; Pierfelice et al., 2011), one could imagine that the loss of Par3 during an oblique division establishes BP fate in one of the two daughter cells. Alternatively, the basal process could carry certain signaling molecules, whose asymmetric inheritance alters daughter cell fate (Schwamborn et al., 2009).

How could mInsc act on a molecular level? In Drosophila, the expression of Insc recruits Pins to the apical cortex and acts as a molecular switch for spindle orientation. In the mouse cortex, however, progenitor cells seem to express equal levels of mInsc regardless of division orientation. It has been demonstrated that horizontal spindle orientation in epithelial cells depends on aPKC-mediated phosphorylation of LGN (Hao et al., 2010). Assuming that a similar mechanism regulates horizontal spindles in the cortex, mInsc could simply inhibit this pathway by binding to the aPKC/Par-3/Par6 complex and thereby promote non-planar orientation of the mitotic spindle. In this model, the role of mInsc would not be to instruct apical-basal orientation in a binary manner but to introduce imprecision and cause a degree of stochasticity in the orientation of progenitor divisions. This would explain why mInsc expression levels do not decide on the orientation of individual progenitor divisions, but overall changes of mInsc expression have a strong influence on the fraction of cells that divide in a nonplanar fashion.

Spindle Orientation and Cortical Evolution
It has been proposed that changes in spindle orientation have influenced cortical evolution (Bond et al., 2002; Fish et al., 2008; Zhang, 2003). The gene Aspm, which is required for correct orientation of early proliferative neuroepithelial divisions (Fish et al., 2008), has evolved adaptively in primates suggesting a functional alteration during primate evolution. Aspm is localized at spindle poles and is particularly important for correct planar orientation of symmetric progenitor divisions (Fish et al., 2008). It has been proposed that adaptations in Aspm function have increased the fidelity and number of early symmetric divisions, thereby increasing progenitor pools, neuron number, and brain size (Ponting and Jackson, 2005). Given that mInsc regulates spindle orientation, it could have a similar role in primate evolution. In fact, intermediate progenitors play an important role in cortical evolution: in primates these cells can generate many more than two neurons, thus amplifying the total number of neurons arising from one ventricular progenitor. Human and ferret brains contain a population of outer subventricular zone (OSVZ) progenitors that have been attributed a key role in amplifying neuron numbers (Fietz et al., 2010; Hansen et al., 2010). Live-imaging experiments have suggested that spindle orientation is crucial for establishing this cell population (Shitamukai et al., 2011; Wang et al., 2011). Given that mInsc is a key regulator of intermediate progenitor formation, it could regulate OSVZ progenitor formation as well. In this case, characterization of evolutionary changes in the mInsc locus and a functional analysis in the human brain might yield important information on how this unique cell population has arisen in evolution.
EXPERIMENTAL PROCEDURES

Primary Antibodies
Primary antibodies used were: rabbit anti-mln5 (1:100; Zigman et al. [2005]); mouse anti-β-gal (Promega); chicken anti-GFP (1:500; Abcam); rabbit anti-Sattb2 (1:500; Abcam); rabbit anti-Foxp1 (1:500; Abcam); rabbit anti-Foxp2 (1:500; Abcam); mouse anti-TuJ1 (1:500; Sigma-Aldrich); rabbit anti-nestin (1:500; Becton Dickinson); rabbit anti-Pax6 (1:300; Covance); rabbit anti-Tbr1 (1:500; Abcam); mouse anti-Map2 (1:500; Chemicon); rabbit anti-Tbr2 (1:500; Abcam); rabbit anti-PH3 (1:500; Upstate); and mouse anti-PH3 (1:500; Cell Signaling). Secondary antibodies were conjugates of Alexa Fluor 488, Alexa Fluor 568, and Alexa Fluor 647 (1:500; Invitrogen). DAPI (4′,6-diamidino-2-phenylindole) was used as nuclear counterstaining. Slices were washed with PBS and mounted in Fluorescent Mounting Medium (DakoCytomation). Images were recorded using a Zeiss Axiovert 200 M confocal microscope.

3D Reconstruction and Angle Determination
Fifteen micron coronal sections of E11.5 and E14.5 embryonic brains paraffin embedded were stained with mouse anti-β-gal (1:1000; Sigma-Aldrich), and rabbit anti-PH3 (Upstate), using the staining protocol described in the Supplemental Experimental Procedures. Z stacks with an interval of 0.5 μm were taken using a Zeiss Axiovert 200 M confocal microscope.

After 3D reconstruction of the confocal stacks of a dividing cell with the Imaris software, five points were placed arbitrarily at different positions of the 3D-rendered plane ventricular surface, and two points were placed at the positions of the two centrosomes. The coordinates of the five points were used to determine the best-fitting plane by orthogonal distance regression.

The angle φ between the vector connecting the two points marking the centrosomes and the normal vector of the regression plane was calculated, and 90° minus the angle φ was used as the division angle α. All calculations were done using the R programming environment.

In order to have an estimate of the upper limit of error for the division angle calculation, each of the five points was in turn left out for determining the best-fitting plane. Thereby, five planes determined by just four points were received, and the angles for these were determined as well as the standard deviation (SD) of the angles. The median of the SDs over all angle determination was 6.4°.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.09.022.

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