Neuron, Vol. 48, 539–545, November 23, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.neuron.2005.09.030

# Mammalian Inscuteable Regulates Spindle Orientation and Cell Fate in the Developing Retina

Report

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## Summary

**During mammalian neurogenesis, progenitor cells can divide with the mitotic spindle oriented parallel or perpendicular to the surface of the neuroepithelium. Perpendicular divisions are more likely to be asymmetric and generate one progenitor and one neuronal precursor. Whether the orientation of the mitotic spindle actually determines their asymmetric outcome is unclear. Here, we characterize a mammalian homolog of Inscuteable (mInsc), a key regulator of spindle orientation in *Drosophila*. mInsc is expressed temporally and spatially in a manner that suggests a role in orienting the mitotic spindle in the developing nervous system. Using retroviral RNAi in rat retinal explants, we show that downregulation of mInsc inhibits vertical divisions. This results in enhanced proliferation, consistent with a higher frequency of symmetric divisions generating two proliferating cells. Our results suggest that the orientation of neural progenitor divisions is important for cell fate specification in the retina and determines their symmetric or asymmetric outcome.**

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## Introduction

During development of the mammalian nervous system a relatively small number of progenitor cells gives rise to a huge variety of different neurons and glial cells. How this diversity is achieved is still unclear. Recent results have suggested that oriented asymmetric cell divisions generating two different daughter cells are important for lineage diversity in the nervous system (Wodarz and Huttner, 2003; Zhong, 2003).

In the developing cerebral cortex, proliferating progenitor cells are mostly found in the ventricular zone (VZ), a pseudostratified neuroepithelium lining the lateral ventricles. During early neurogenesis, the progenitor pool increases exponentially by symmetric divisions generating two proliferating daughter cells (Rakic, 1995). Later, progenitors can also divide asymmetrically into one daughter progenitor and one differentiating neuron (Chenn and McConnell, 1995; Noctor et al., 2004). Finally, proliferation is terminated by symmetric divisions that generate two differentiating neurons (Miyata et al., 2001). Live imaging experiments in the vertebrate cortex have revealed a correlation between the orientation of progenitor divisions and their symmetric or asymmetric outcome: while horizontal divisions ("horizontal" refers to the orientation of the mitotic spindle) are symmetric, vertical divisions often generate two different daughter cells (Chenn and McConnell, 1995; Haydar et al., 2003). The cell fate determinant Numb localizes apically in dividing VZ progenitors and is therefore unequally distributed during vertical divisions only and might be responsible for their asymmetric outcome (Zhong et al., 1996). A similar correlation between the orientation of progenitor cell divisions and their symmetric or asymmetric outcome was found in the rat retina (Cayouette and Raff, 2003). Although these correlations are highly suggestive, the consequences of misorientation of progenitor divisions have not been analyzed. In fact, it is not clear whether the precise orientation of the mitotic spindle is important for mammalian development at all (Davidson, 1991).

In invertebrates, the molecular machinery for spindle orientation has been discovered. At the heart of this machinery is a *Drosophila* protein called Inscuteable (Insc) (Kraut et al., 1996). During early *Drosophila* embryogenesis, Insc is specifically expressed in epithelial cells of the procephalic neurogenic ectoderm (PNE). While other epithelial cells divide parallel to the embryo surface, these cells rotate their mitotic spindle into an apical-basal orientation. They divide asymmetrically into one daughter cell that remains epithelial and another daughter cell that comes to lie underneath the epithelium and gives rise to neurons. In *insc* mutants, spindle reorientation in the PNE does not occur. Conversely, *insc* can induce spindle reorientation when expressed in epithelial cells outside the PNE. Insc is a molecular linker that connects two conserved protein complexes. It localizes apically by binding to Par-3/Par-6/aPKC, a complex that is required for epithelial polarity and is expressed in all epithelial cells irrespective of their division plane (Schober

et al., 1999; Wodarz et al., 1999). Insc also interacts with the GoLoco domain protein Pins (Schaefer et al., 2000; Yu et al., 2000) and its binding partner Gαi (Schaefer et al., 2000). Pins and Gαi are basolateral in epithelial cells but are recruited to the apical side upon Insc expression. Pins can activate heterotrimeric G proteins in a receptor-independent way, and it is thought that polarized activation of heterotrimeric G proteins attracts one of the two spindle poles and thereby induces reorientation of the mitotic spindle (Betschinger and Knoblich, 2004).

Two Pins homologs have been characterized in vertebrates: while AGS-3 is expressed only in certain tissues, LGN is ubiquitously expressed and shows higher sequence similarity with *Drosophila* Pins (Yu et al., 2003). Vertebrate homologs also exist for Par-3, Par-6, and aPKC (Ohno, 2001), but a functional vertebrate homolog of Insc has not yet been described. Here, we show that Inscuteable is functionally conserved in mammals and is required for correct orientation of the mitotic spindle in precursor cells of the rat retina. We use mammalian Inscuteable to show that misorientation of the mitotic spindle leads to proliferation and cell fate specification defects in the rat retina.

## Results

### Inscuteable Is Conserved in Evolution

Insc sequence homologs can be found in other insect species, like *Drosophila pseudoobscura* or *Anopheles gambiae* (see Figure S1 in the Supplemental Data available online). Using iterative BLAST searches (see the Supplemental Data), we could also define Insc-related genes in honeybee, pufferfish, chicken, mouse (Katoh, 2003), rats, and humans. Although the homology is highly significant within vertebrate and within insect species, conservation between these two groups is generally low. The vertebrate and invertebrate groups are connected by highly significant matches between the honeybee and pufferfish proteins, suggesting that the genes we identified represent a family of true orthologs. This is further supported by the fact that sequence homology is highest in a region that includes the asymmetry domain, a part of Insc required and sufficient for all functions (Knoblich et al., 1999) (Figure S1D). Homology searches indicate that this domain is part of a stretch of armadillo repeats, which often serve as interaction platforms for binding partners.

Since *Drosophila* Insc exists in a complex with Pins and Gαi, we analyzed the evolutionary conservation of the heterotrimeric complex between mInsc and its binding partners. In vitro translated mInsc binds to a GST-LGN fusion protein (Figure 1A). Conversely, in vitro translated LGN binds to bacterially expressed MBP-mInsc but not to a truncated form lacking the first 101 amino acids of the protein, which includes the part of mInsc homologous to the Pins binding region of *Drosophila* Insc (aa 302–459). To test whether mInsc can bind LGN in vivo, COS-7 cells were transfected with tagged versions of mInsc, LGN, and Gαi1. The three proteins (but not mInsc and Gαi1 alone) can be coimmunoprecipitated, suggesting that they form a protein complex analogous to the one described for *Drosophila* (Figure 1B).

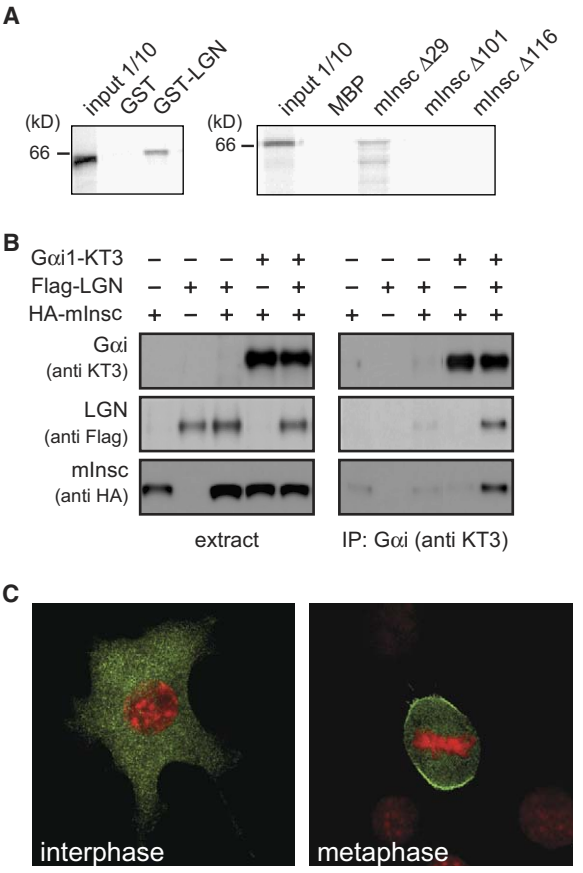


Figure 1. Identification of Mammalian Inscuteable

(A) In vitro translated mInsc protein (lacking aa 1–20) binds to GST-LGN but not to GST alone (left). In vitro translated LGN binds to MBP-mInscΔ29 but not to MBP-mInscΔ101, MBP-mInscΔ116, or MBP alone (right). (B) Immunoprecipitation of mInsc and LGN with Gαi1 in lysate of COS-7 cells transfected with Gαi1-KT3, Flag-LGN, and/or HA-mInsc. mInsc coimmunoprecipitates with Gαi1 only in the presence of LGN. (C) Subcellular localization of mInsc-GFP in NIH/3T3 cells during cell cycle.

Similar results were obtained for rat AGS-3, the second vertebrate homolog of Pins (data not shown).

To analyze the subcellular localization of mInsc, NIH/3T3 mouse fibroblasts were transfected with a functional GFP-tagged form of mInsc that retains the ability to bind to endogenous LGN (data not shown). During interphase, mInsc-GFP is uniformly cytoplasmic (Figure 1C). In mitosis, however, the protein translocates to the plasma membrane and often concentrates in the cortical area overlying the two spindle poles. This distribution is consistent with a function in spindle orientation and is similar to the one described for LGN (Yu et al., 2003).

### mInsc Expression Is Consistent with a Role in Spindle Orientation

To determine whether Insc is expressed in the nervous system at the time of asymmetric cell divisions, we performed in situ hybridizations of whole mouse embryos (Figure 2A). No mInsc expression was detected at E8, but at E10.5, the gene becomes strongly expressed in dorsal root ganglia and cranial ganglia. At E12.5, the expression pattern becomes more complex and includes

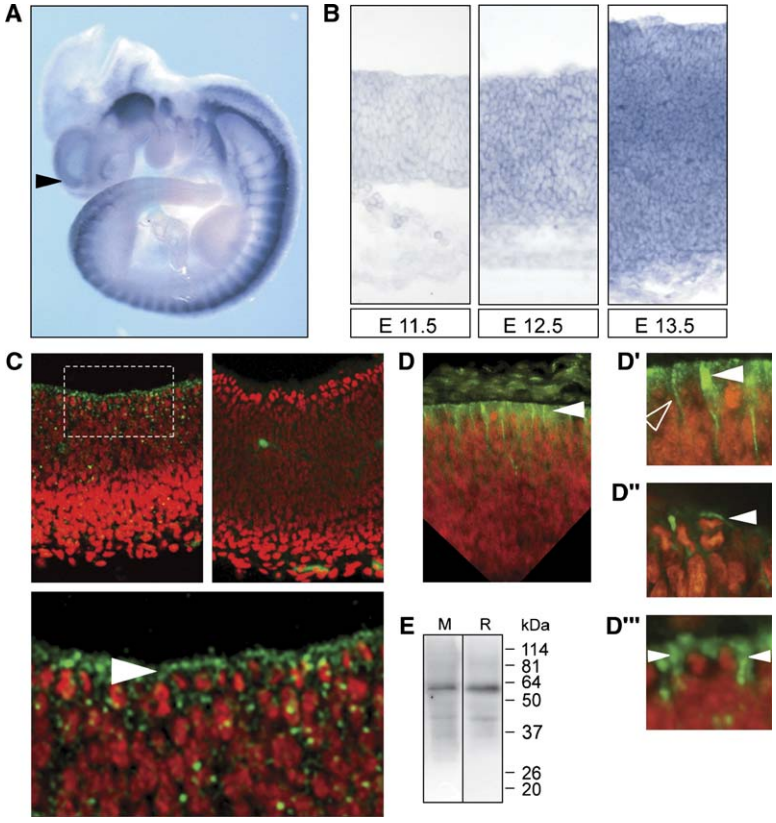


Figure 2. Expression Pattern and Localization of mInsc in the Developing Vertebrate Nervous System

(A) Whole mount *mInsc* in situ hybridization showing mInsc expression in cerebral cortex (arrowhead), cranial ganglia, and dorsal root ganglia in E10.5 mouse embryo. (B) *mInsc* in situ hybridization on sagittal sections of lateral ventricle at E11.5, E12.5, and E13.5 embryos shows increased mInsc RNA expression in mouse developing cerebral cortex. The ventricular surface is shown up. (C) Immunofluorescence staining of *mInsc* along the VZ (left) and immunoreactivity after blocking with mInsc-MBP peptide (right). Bottom panel shows higher magnification of boxed region. mInsc is concentrated on the apical surface (arrowhead). (D) mInsc protein expression in embryonic rat retina. Inscuteable is localized to the apical side (arrowhead) of the neuroepithelium. Progenitors in interphase (D′) and mitosis (D″ and D‴) are shown at high magnification. (D″) mInsc localizes to the apical side of vertically dividing cell. (D‴) Horizontally dividing cell with mInsc concentrated at poles (arrowheads) and apical cortex. (E) Anti-mInsc detects a single strong band in immunoblot of mouse (M) and rat (R) brain lysates.

tissues inside and outside the nervous system (data not shown). mInsc expression is also detected in the developing retina and forebrain. In situ hybridization of cortical sections reveals weak mInsc expression at E11.5 that increases over time and becomes strong at E13.5 (Figure 2B). Interestingly, this expression pattern correlates with the fraction of vertical divisions in the developing telencephalon which peaks at E14 (Haydar et al., 2003). To analyze the subcellular distribution of mInsc, we generated a specific antibody against the MBP-tagged C-terminal part of the protein that detects a single strong band of the predicted molecular weight in mouse brain extracts (Figure 2E). The immunoreactivity can be competed by preincubation of the antibody with bacterially expressed mInsc protein (data not shown). In mouse embryo sections at E12.5, the antibody primarily stains dorsal root and cranial ganglia, and the overall staining pattern correlates well with the in situ pattern in consecutive sections (data not shown). In the E12.5 developing cortex, however, immunostaining is primarily observed on the apical side of the cells in the VZ (Figure 2C). This may indicate translational control of mInsc protein expression. Such a translational control mechanism has been described in *Drosophila* where Insc translation is regulated by Abstrakt, an RNA-binding protein conserved in vertebrates (Irion et al., 2004).

### mInsc Is Required for Spindle Orientation in the Rat Retina

To address the function of mInsc in mammalian nervous system development, we used the rat retina explants and monitored the division planes of neural progenitors in real time. Retroviral infection allowed us to both unambigously determine cleavage orientation of a single progenitor cell and perform a clonal analysis of its progeny (Cayouette and Raff, 2003). mInsc is almost completely conserved between mice and rats (Figure S1), and the mInsc antibody specifically detects mInsc in extracts of E19 rat brains (Figure 2E). During retinal development, mInsc protein is detected primarily on the apical side of the retinal neuroepithelium (Figure 2D). In interphase, the protein is found in the apical and basal processes (Figure 2D′, open arrowhead). When cells move apically, mInsc concentrates in the apical half of the cell body (Figure 2D′, arrowhead). During mitosis, mInsc localizes into a distinct apical crescent in some progenitor cells that divide vertically (Figure 2D″). However, in the progenitors dividing with the mitotic spindle oriented horizontally it localizes to the apical and also lateral cell cortex (Figure 2D‴). This localization is strikingly similar to the *Drosophila* protein.

To investigate the requirement of mInsc for spindle orientation in neural progenitors, we knocked down mInsc by a retrovirus expressing a short hairpin RNA corresponding to a part of the mInsc coding region. The construct strongly reduces Insc expression in fibroblasts, thus demonstrating its feasibility for Insc loss-of-function experiments (Figure 3C). E19–E20 retinal explants were infected with GFP-expressing control RNAi or mInsc RNAi retroviruses. Orientation of progenitor divisions was determined by live imaging of dividing GFP-labeled progenitor cells in retinal explant cultures. Examples of vertical and horizontal divisions are shown in Figure 3A. In control explants, almost 50% of the progenitor divisions were within 30° of the apical basal axis. Upon mInsc RNAi, however, almost 40% of the divisions

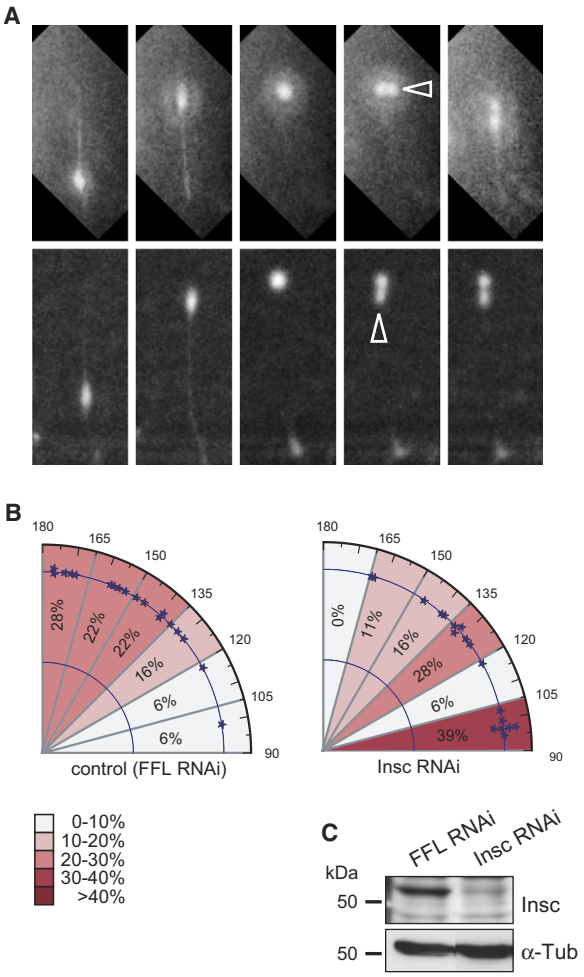


Figure 3. Inscuteable Is Required for Correct Orientation of Rat Retinal Progenitor Cell Divisions

(A) Representative horizontal (top) and vertical (bottom) divisions during time-lapse recordings of dividing retinal progenitors. (B) Orientation of divisions of retinal precursor cells (90°, mitotic spindle horizontal; 180°, mitotic spindle vertical) infected with GFP expressing control RNAi (left) or mInsc RNAi (right) retroviruses. n = 18 for each. (C) mInsc RNAi efficiency analyzed by immunoblotting with mInsc antibody in lysates of NIH/3T3 cells upon infection with control and mInsc-RNAi retroviruses.

were within 15° to the retinal surface; however, a large part of the divisions had an oblique orientation (Figure 3B). We conclude that mInsc is required for the correct orientation of vertical progenitor divisions in the rat retina.

### mInsc RNAi Leads to Proliferation and Cell Fate Specification Defects

We used mInsc RNAi to test the consequences of spindle misorientation for proliferation and cell fate specification in the rat retina. Retinal explants were infected at day E20, and the progeny of GFP-expressing infected cells were analyzed at P10, when proliferation in the retina has largely ceased and progenitor cells have differentiated. Control infected cells rarely generate more than four daughter cells. Upon mInsc RNAi, however, a significant fraction of clones had five, six, or even seven GFP-positive cells (Figure 4A). Such large clones can either be generated when progenitor cells divide into two daughter cells that both continue to proliferate and undergo more than one round of subsequent divisions or by a failure to exit the cell cycle. However, mInsc RNAi has no effect on proliferation when applied at P2 (Figure 4B), a stage when a large proportion of retinal progenitor cells exit the cell cycle. To test whether the increase in proliferation is paralleled by a decrease in differentiating cells, we analyzed the frequency of different retinal cell types among the progeny of retrovirally infected cells. Cell types were identified by their morphology and location within the retinal cell layers. Identification of bipolar cells was confirmed by staining with the trancription factor Islet-1, a neuronal differentiation marker (Figure 4D). The major cell type generated at E21 are rod photoreceptor cells (Figure 4C). Upon mInsc RNAi, the number of photoreceptor cells is significantly reduced while the number of later-differentiating bipolar neurons is increased (Figure 4C). To additionally exclude the possibility that the cell fate change upon mInsc RNAi is not due to mislocalization of the cell fate determinant Numb, we have analyzed Numb localization in mInsc RNAi infected retinal progenitors. As shown in Figures S2A and S2B, apical asymmetric Numb localization is not affected by the mInsc RNAi, showing that the cell fate change is due to mInsc disruption. Taken together, these data suggest that the change in spindle orientation caused by the depletion of mInsc protein increases the proliferative potential of retinal progenitor cells and causes a transformation of photoreceptor cells into bipolar neurons.

## Discussion

While the orientation of cell division is important for invertebrate development, it has been suggested that vertebrates develop differently and rely entirely on cell migration and diffusible morphogens (Davidson, 1991). Although the significance of the mitotic spindle orientation for cell fate determination has been anticipated for a long time (Martin, 1967), only more recent live imaging techniques in the developing mammalian brain have allowed clearer correlations between the orientation of a division and the fate of the resulting daughter cells. In the ventricular zone of the developing ferret (Chenn and McConnell, 1995) or the developing mouse brain (Haydar et al., 2003) as well as in the rat retina (Cayouette and Raff, 2003), progenitor divisions along the vertical axis are more likely to generate two different daughter cells, while parallel divisions are usually symmetric. Whether this is just a correlation or actually reflects a determining role of spindle orientation for daughter cell specification was unclear. In this study, we use the mammalian homolog of Insc as a tool to influence spindle orientation in vertebrates. We show that mInsc depletion ablates vertical mitotic spindle orientations in retinal progenitors and leads to defects in cell fate specification and proliferation. Our results demonstrate that spindle orientation not only predicts but actually determines the fate of the two daughter cells.

We favor a model in which vertically oriented retinal progenitor divisions are important for generating differentiating daughter cells at the expense of proliferating

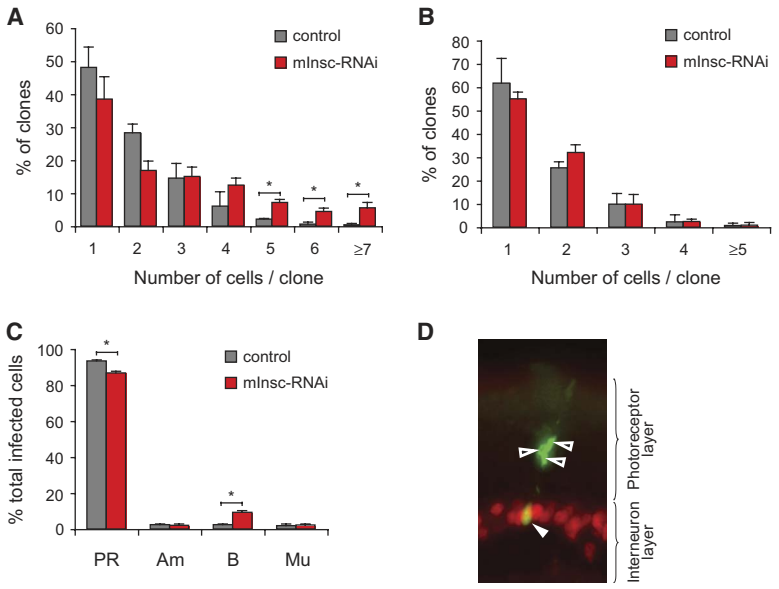


Figure 4. Clonal and Lineage Analysis of Rat Retinal Cells upon mInsc RNAi

(A) Rat retinal progenitor cells infected at E20 with mInsc RNAi retroviruses give rise to larger clones. (B) Infection of retinal progenitors at P2 results in no change in clone size upon mInsc RNAi. (C) Lineage analysis of retinal explants infected with mInsc RNAi and GFP-control retroviruses at E20 reveals a reduction in photoreceptor number and increase in the percentage of bipolar cells in the absence of Inscuteable. PR, photoreceptor cells; Am, amacrine cells; B, bipolar neurons; Mu, Müller glia. In all panels, data represent the average of three independent experiments analyzed for each retrovirus. Error bars represent standard deviations of three independent experiments. Asterisks (*) mark statistically significant differences (Student's t test: $p < 0.05$). (D) Example retinal explant infected with the mInsc RNAi retrovirus stained at P10 for Islet-1 (red). GFP is in green. Clone containing three photoreceptor cells (empty arrowheads) and one bipolar cell (filled arrowhead).

progenitors. Upon mInsc RNAi, cells that would normally differentiate into photoreceptor cells change their fate and remain proliferating progenitor cells instead. When they differentiate at later stages, they are more likely to adopt the later bipolar neuron fate. In contrast to bipolar cells, the number of Müller glia is not increased, although they also differentiate at a late stage. Müller glia are generated in small numbers and formally, our data suggest that their number is regulated independently of progenitor pool size. Although this model provides an attractive explanation of our data, we cannot exclude that the clone size and cell fate phenotypes are actually unlinked. For example, progenitor cells that normally generate one bipolar cell via an asymmetric division might be forced to divide symmetrically upon mInsc knock-down and thereby give rise to two bipolar cells instead.

How does mInsc orient mitotic spindles? In *Drosophila*, Insc is thought to act by polarizing G protein signaling and thereby attracting astral microtubules to the apical cell cortex. In vertebrates, overexpression of heterotrimeric G proteins causes oscillations of the mitotic spindle (Du and Macara, 2004), suggesting that G protein activity—like in flies—regulates the attachment of astral microtubules to the cell cortex. The mammalian Pins homolog LGN is present in the mouse ventricular zone (Yu et al., 2003) and might activate G proteins. Although the existing LGN antibodies did not allow us to determine its subcellular localization in mouse brain (data not shown), mInsc might act by recruiting LGN to the apical and lateral cell cortex, resulting in polarized G protein activation. This model predicts that it is the asymmetric distribution of mInsc in vertically dividing progenitors rather than its presence that influences spindle orientation. Consistently, mInsc overexpressed in fibroblasts is without consequence (data not shown).

While this report was under revision, the identification of mouse Inscuteable has been described independently (Lechler and Fuchs, 2005). When expressed as a GFP fusion protein, mInsc colocalizes with LGN and Par-3 in mitotic basal cells of developing mouse epidermis. These cells divide asymmetrically and give rise to basal and suprabasal cells with mitotic spindles perpendicular to the basement membrane. The same mechanism of spindle reorientation could therefore lead to specification of different cell types in the developing retina and to stratification in the epidermis.

The identification of mInsc provides a unique tool to analyze the importance of oriented divisions in various other vertebrate tissues. For example, it was proposed that adult mammalian neural stem cells divide asymmetrically along the apical basal axis (Johansson et al., 1999). It will be interesting to test whether spindle orientation is essential for asymmetric stem cell divisions as well. If so, this will be an important factor in exploiting the regenerative capacity of these cells.

## Experimental Procedures

### Cell Culture

NIH/3T3 and COS-7 cells were cultured under standard conditions. Retroviral-mediated gene transfer was performed by using the Phoenix packaging cell line as described previously (Kondo and Raff, 2000).

### Retinal Explant Culture

Timed pregnant Sprague-Dawley rats were purchased from Vienna Medical University. E19–E20 and P2 rat retinal explants were prepared and infected with retroviruses as previously described (Cayouette et al., 2001). For clonal analysis, the explants were infected at E20 or P2 and kept 12 days in culture. Clones derived by each infected retinal progenitor cell were scored and analyzed for the cell number and cell composition on cryosections of fixed retinal explants. Cells were identified on the basis of their morphology and position within the retina.

### Plasmid Constructs

DNA oligos for shRNA silencing were designed by RNAi oligo retriever (http://katahdin.cshl.org/RNAi/html/rnai.html). CTACAACCC CACCTTCCTTGGCTATGATGAAGCTTGATCATAGTCAGGGGAGGT GGGGTTGTAGTCCTTTTTT as Oligo A and GATCAAAAAAGGACTA CAACCCCACCTCCCCTGACTATGATCAAGCTTCATCATAGCCAAG GAAGGTGGGGTTGTAGC as OligoB were used for the mInsc-RNAi (targeting the Inscuteable region conserved in mouse and rat Inscuteable). Oligonucleotides were cloned into pSHAG vector (Invitrogen), and the U6-shRNA cassette was transferred to the Gateway

compatible retroviral vector pMSCV-PGK-GFP (provided by Beug H.). Controls were empty pBird retrovirus (Tang et al., 2001) (Figures 4A and 4C) or pMSCV-PGK-GFP retrovirus containing firefly luciferase RNAi oligos (Paddison et al., 2002) (Figures 3B, 3C, and 4B). The mInsc-EGFP retrovirus was constructed by cloning the 1.7 kb mInsc cDNA into the pLEGFP-N1 vector (Clontech). GST-LGN was a gift from Ian G. Macara. Human Gαi1, mInsc, and human LGN were cloned into pcDNA3.1 vectors with KT3 (PPEPET), HA, Flag, or *myc* epitope tags, respectively, as described (Kimple et al., 2004).

### Time-Lapse Imaging

A Zeiss 200M inverted microscope equipped with a custom-made motorized xyz stage, $CO_2$ controled incubator (37ºC, 5% $CO_2$), and 25× objective was used for time-lapse imaging with a CCD video camera (Proper Scientific). Images were aquired on multiple positions every 5 min for 24–48 hr. Time-lapse experiments were analyzed using Metamorph software. Each time-stack of images, representing one position, was reconstructed into a stack. The angle of the mitotic spindle was determined by drawing a line along the axis of the mitotic spindle and a line along the migrational pathway of cells prior to division. The resulting data were analyzed using Origin 6.1 (OriginLab).

### Immunofluorescence and Antibodies

Mouse embryos were dissected in cold PBS and fixed in 4% paraformaldehyde, frozen in O.C.T compound (Sakura), and stored at −70ºC. Retinal explants were fixed in 4% paraformaldehyde or 10% trichloroacetic acid (TCA) and frozen as described before (Cayouette et al., 2001). Frozen tissue sections of 10–15 μm were used for in situ and antibody stainings. Rabbit anti-mouse Inscuteable antibody was generated against an MBP fusion of mInsc lacking amino acids 1–198 and affinity purified against the antigen. For Figures 2D–2D′′′, a tyramide signal amplification kit (TSA™ kit #12, Molecular Probes) was used after incubation with primary antibody. Anti-Islet-1 (T. Jessell) was obtained from the Developmental Studies Hybridoma Bank. Goat polyclonal NUMB and rabbit polyclonal GFP antibody were purchased from Abcam. Vectashield with DAPI (Vector Labs) was used to visualize DNA and mount the sections. Images were recorded on a Zeiss LSM510 confocal microscope and processed with Adobe Photoshop.

### In Situ Hybridization

Whole-mount RNA in situ hybridization was performed as described in Wilkinson and Nieto (1993), and in situs on O.C.T. (Sakura) sections were performed as described previously (Neubuser et al., 1995), with minor modifications. A DIG-labeling kit (Roche) was used to synthesize RNA probes that were detected with alkaline phosphatase-coupled anti-DIG antibodies by using BM purple (Boehringer Mannheim). 1.7 kb of mInsc cDNA in pBluescript II KS+ was used as a template.

### In Vitro and Immunoprecipitation Assays

MBP- and GST-fusion proteins were expressed under standard conditions and immobilized onto amylose or gluthation linked agarose beads (Sigma), respectively. For binding and washing, binding buffer (PBS/0.1% NP40) was used. In vitro translations were performed using the TnT Coupled Reticulocyte Lysate System (Promega). 20 μl of fusion protein coupled to the respective beads were mixed with 100 μl binding buffer and 20 μl in vitro translated 35S-methionine containing protein, incubated for 2 hr at 4ºC, and washed for 30 min with the binding buffer. Transfections of COS-7 cells, immunoprecipitations, and Western blots were performed as previously described (Kimple et al., 2004).

### Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/48/4/539/DC1/.

### Acknowledgments

We want to thank Ciara Gallagher, Maria Novatchkova, Sarah K. Bowman, Masakazu Yamazaki, and Maria Pia Postiglione for critically reading the manuscript; Karin Paiha and Pawel Pasierbek for help with microscopy; Christine Jolicoueur for technical assistance; Hartmut Beug, Ian Macara, and the Developmental Studies Hybridoma Bank for reagents. Work in J.A.K.'s lab is supported by the Austrian Academy of Sciences and the Austrian Research Fund (FWF). Work in M.C.'s lab is funded by the Canadian Institute of Health Research. Work in D.P.S.'s lab is funded by NIH grants R01 GM062338 and P01 GM065533. Work in B.A.B.'s lab is funded by NIH grant NEI R01 EY11310.

Received: July 7, 2005
Revised: September 6, 2005
Accepted: September 28, 2005
Published: November 22, 2005

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