A mouse homologue of *Drosophila pins* can asymmetrically localize and substitute for *pins* function in *Drosophila* neuroblasts

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Accepted 28 November 2002
Journal of Cell Science 116, 887-896 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00297

Summary

Asymmetric cell division is a fundamental mechanism used to generate cellular diversity in invertebrates and vertebrates. In *Drosophila*, asymmetric division of neuroblasts is achieved by the asymmetric segregation of cell fate determinants Prospero and Numb into the basal daughter cell. Asymmetric segregation of cell fate determinants requires an apically localized protein complex that includes Inscuteable, Pins, Bazooka, DmPar-6, DaPKC and Gαi. Pins acts to stabilize the apical complex during neuroblast divisions. Pins interacts and colocalizes with Inscuteable, as well as maintaining its apical localization. We have isolated a mouse homologue of *pins* (*Pins*) and characterized its expression profile. Mouse PINS shares high similarity in sequence and structure with Pins and other Pins-like proteins from mammals. *Pins* is expressed in many mouse tissues but its expression is enriched in the ventricular zone of the developing central nervous systems. PINS localizes asymmetrically to the apical cortex of mitotic neuroblasts when ectopically expressed in *Drosophila* embryos. Like Pins, its N-terminal tetratricopeptide repeats can directly interact with the asymmetric localization domain of Insc, and its C-terminal GoLoco-containing region can direct localization to the neuroblast cortex. We further show that *Pins* can fulfill all aspects of *pins* function in *Drosophila* neuroblast asymmetric cell divisions. Our results suggest a conservation of function between the fly and mammalian Pins homologues.

Key words: *Mouse Pins*, *pins*, Asymmetric cell division, Neuroblasts, Drosophila

Introduction

Asymmetric cell division in which a mother cell divides to generate two daughter cells of distinct sizes and fates contributes to the generation of cellular diversity in the *Drosophila* central (Spana and Doe, 1996; Skeath and Doe, 1998) and peripheral nervous systems (CNS and PNS) (Guo et al., 1996; Dye et al., 1998). Most neurons in the *Drosophila* CNS are derived from a layer of neural progenitor cells, neuroblasts (NBs) (Bossing at al., 1996). NBs arise from the neuroectoderm by delamination. Once delaminated, NBs enter mitosis and divide asymmetrically to generate a larger apical daughter, which remains as a NB and undergoes repeated divisions, and a smaller basal daughter which adopts a ganglion mother cell (GMC) fate and divides only once to give rise to two neurons/glia (reviewed by Goodman and Doe, 1993; Lu et al., 2000).

Previous studies have shown that *partner of insc (pins)* (Parmentier et al., 2000; Schaefer et al., 2000; Yu et al., 2000), together with *insc* (Kraut and Campos-Ortega, 1996; Kraut et al., 1996), *bazooka (baz)* (Kuchinke et al., 1998; Schober et al., 1999; Wodarz et al., 1999), *DmPar6* (Petronczki and Knoblich, 2001), *DaPKC* (Wodarz et al., 2000) and *Gαi* (Schaefer et al., 2001) form an apically localized protein complex that plays a crucial role in mediating NB asymmetric divisions. These studies have provided insight into how this apical protein complex might be assembled. During delamination NBs inherit apical-basal polarity cues through Baz/DaPKC/DmPar6, an evolutionarily conserved protein cassette that is already apically localized in epithelial cells of the neuroectoderm from which NBs arise. Insc, which can directly interact with Baz in vitro, is then recruited to this complex during delamination. Pins, which can directly interact with Insc, and Gαi, which can interact with Pins, then joins this complex. *pins* encodes a protein with seven tetratricopeptide repeats (TPR) at its N-terminal portion responsible for interaction with the asymmetric localization domain of Insc (Yu et al., 2000), and three GoLoco motifs (also known as GPR motif) at its C-terminal region capable of binding to the Gα subunit of heterotrimeric G proteins (Schaefer et al., 2000; Parmentier et al., 2000). Pins is required for the maintenance of Insc asymmetric localization and for Baz stability. Maternal and zygotic depletion of *pins* results in the cytoplasmic distribution of Insc during mitosis, similar to that observed in NBs derived from *baz* germ line clones (GLCs). In addition loss of *pins* function results in the drastic reduction in the intensity of Baz apical crescents.

The participation of all the apical components is required for correct mitotic spindle orientation along the apical basal axis,

as well as the asymmetric localization and segregation of the cell fate determinants Numb (Uemura et al., 1989) and Prospero (Pros) (Doe et al., 1991; Vaessin et al., 1991; Matsuzaki et al., 1992). Numb and Pros form cortical crescents at the basal side of mitotic NBs and segregate to the basal GMC daughter following cytokinesis (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995). Pros translocates into the nucleus of GMC where it activates GMC-specific gene expression and represses NB-specific gene expression (Doe et al., 1991; Vaessin et al., 1991). Numb can also be basally localized in mitotic GMCs and segregates preferentially to one of the post-mitotic GMC daughter cells (Buescher et al., 1998). Numb acts to antagonize Notch signaling by interacting with the intracellular domain of Notch (Frise et al., 1996; Skeath and Doe, 1998).

Several studies have shown that asymmetric cell divisions might be an evolutionarily conserved phenomenon in *Drosophila* and vertebrates. During ferret cortical neurogenesis, cortical precursors can undergo either symmetric or asymmetric cell divisions (McConnell, 1995). There is a strong correlation between the nature of a precursor division and the orientation of its cleavage plane. Symmetric divisions generating two precursor daughters tend to occur when cleavage planes are oriented perpendicular to the ventricular surface. By contrast, when cleavage planes are aligned parallel to the ventricular surface, cortical precursors tend to divide asymmetrically to generate a precursor and a neuron that migrates away to the cortical plate (Chenn and McConnell, 1995; Mione et al., 1997). Well-conserved homologues of Numb have been identified in mouse (Zhong et al., 1996), rat (Verdi et al., 1996) and chicken (Wakamatsu et al., 1999). When ectopically expressed in *Drosophila* NBs and sensory organ precursors (SOPs), numb is asymmetrically localized and can functionally substitute for *numb* function. Moreover, vertebrate numb homologues are asymmetrically localized in the ventricular zone progenitors of the developing mouse CNS and in neuroepithelial cells of the chick. Similar to its fly counterpart, asymmetrically localized numb physically interacts with the cytoplasmic region of mammalian notch and antagonizes notch signaling. Overexpression studies in mice and chicks suggest a role in neural differentiation and proliferation for these vertebrate numb homologues (Verdi et al., 1996; Wakamatsu et al., 1999). A putative mammalian homologue of *pros*, *Prox1*, has also been reported (Oliver et al., 1993; Wigle and Oliver, 1999).

We previously showed that *Drosophila* Pins shares high sequence homology with human LGN (Yu et al., 2000). It has also been reported that activator of G-protein signaling 3 (AGS3) (Takesono et al., 1999; Peterson et al., 2000), a rat homologue of *pins*, acts as a guanine dissociation inhibitor (GDI), inhibiting the rate of exchange of GDP for GTP by $G\alpha i_3$ (Bernard et al., 2001; De Vries et al., 2000; Natochin et al., 2000). However, how any of the mammalian Pins-related proteins might relate functionally to the fly Pins remains unclear. Here, we report the identification and characterization of a mouse homologue of *pins*. Mouse *Pins* encodes a protein that shares high homology with Pins. *Pins* mRNA is present from embryonic stage E11 onwards, in a variety of tissues except skeletal muscle. In the CNS, *Pins* transcript is associated with zones where proliferative cells are found. Like Pins (Yu et al., 2002), PINS can also physically interact with the asymmetric localization domain of Insc through its TPR (TPR3-TPR7). When expressed in *Drosophila* NBs, PINS localizes as an apical cortical crescent and can substitute for Pins functionally in all aspects of NB asymmetric division, such as Insc apical localization, Numb/Pros basal localization and apical/basal spindle orientation, as well as resolution of distinct sibling neuronal cell fates. Deletion analysis of PINS shows that, like Pins, its C-terminal GoLoco containing region specifies targeting to the membrane, whereas its N-terminal TPR further refines localization to the apical cortex. Our results show that *Pins* can be recognized by the *Drosophila* asymmetric division machinery and functionally replace *pins* for all aspects of NB asymmetric divisions.

Materials and Methods

Isolation of full-length mouse *Pins* cDNA

Initial database searches with the *pins* sequence identified two related expressed sequence tag (EST) clones: IMAGE:949074 and IMAGE:614560. The missing 5′ region was amplified in two rounds of 5′-RACE PCR, based on a marathon E11 mouse cDNA library (Clontech). The ‘full length’ mouse *Pins* cDNA contains 3355 nucleotides. Its sequence is identical to another sequence database entry for a mouse *Lgn* gene (BC021308; IMAGE: 5007832).

RNA and protein analyses

The cDNA from IMAGE:949074 corresponding to nucleotides 840-3355 of mouse *Pins* were labeled and used to probe northern blots containing 2 μg of poly(A)$^+$ RNA isolated from different stages of mouse embryos and a variety of mouse tissues (Clontech). For PINS detection, blots were incubated with pre-immune serum (1:2500) and purified anti-PINS antibody (1:2500), respectively, at 4° C overnight. Immunoreactive bands were visualized using horseradish peroxidase-coupled donkey anti-rabbit immunoglobulin G (IgG) secondary antibody and the ECL kit (Amersham).

GST fusion protein production and antiserum production

A polypeptide containing amino acids 478-672 of the mouse PINS coding region was expressed as a GST fusion protein in *Escherichia coli* and purified using glutathione sepharose (Pharmacia). Eluted GST-PINS protein was used to immunize rabbits. Purification of anti-PINS antibody was performed as follows: 200 μg of GST fusion protein was blotted on a membrane stripe. Following overnight incubation with serum, the stripe was washed with PBS and eluted by 1 M glycine (pH 2.5). Eluted antibody was neutralized using 1 M Tris (pH 8.5).

Immunofluorescence and in situ hybridization

E12.5 and E15.5 mouse embryos were dissected in ice-cold PBS, and fixed overnight in 4% paraformaldehyde in PBS. E15.5 brains were dissected before cryoprotection, performed overnight at 4°C in PBS/30% sucrose. Brains and embryos were quick frozen in OCT (Tissue-Tek) before sectioning (12 μm). For in situ hybridization on sections, a 1 kb *Pst*I fragment from *Pins* (nucleotides 1460-2460) subcloned into pBluescriptSK (Stratagene) was used as a template to generate sense and antisense RNA probes. Sections were rinsed once in PBS and pre-hybridized for 30 minutes at 65°C in a hybridization solution bath. They were then incubated overnight in an hybridization bath containing the probe. Hybridization procedure and buffer composition used were essentially as described by Strahle et al. (Strahle et al., 1994). For immunofluorescence, tissue sections were rinsed once in PBS and blocked for 15 minutes in 1% BSA in PBT

(0.25% Triton X-100 in PBS) and incubated with a rabbit polyclonal anti-Ki-67 antibody (1:500 dilution in PBT-3% BSA) overnight at 4°C. After three washes in PBT-0.1% BSA, an FITC-coupled goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch) was applied for 2 hours in PBT-3% BSA. To visualize DNA, Hoechst 33258 (Sigma) was added in the second of four washing baths (0.2 μg/ml in PBT). Sections were mounted in Vectashield mounting medium (Vector Labs).

Drosophila embryo collections, heat-shock induction and immunohistochemical staining were performed essentially as described (Tio et al., 1999; Yu et al., 2000). For most analyses, embryos were fixed in 4% paraformaldehyde for 15 minutes; for tubulin staining, fixations were carried out in 37% formaldehyde for 3 minutes. After staining, embryos were mounted in vectashield (Vector Labs). Primary antibodies used were rabbit anti-PINS (1:1000), anti-FLAG (M2, 1:2000, Sigma), rabbit anti-Pins (1:1000), rabbit anti-Insc (1:1000), anti-Miranda (1:1000, from F. Matsuzaki, RIKEN Center for Developmental Biology, Kobe, Japan), rabbit anti-NUMB (1:1000) and anti-Pon (1:1000) (from Y. N. Jan, Howard Hughes Medical Institute, UCSF, CA), mouse (1:15, from K. Zinn, Caltech, Pasadena, CA) and rabbit anti-EVE (1:2000 from M. Frasch, Mount Sinai School of Medicine, NY), mouse anti-Pros (1:5, from C. Q. Doe), rat anti-β-tubulin (1:15, Chemicon), mouse (1:2000, Promega) and rabbit anti-β-Gal (1:5000, Cappel). Secondary antibodies obtained from Jackson Laboratories were Cy3 conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-mouse and FITC-conjugated goat anti-rat. DNA was visualized by TO-Pro 3 (1: 5000, Molecular Probes).

Images were acquired and recorded using a Bio-Rad confocal microscope 1024 and processed using Adobe Photoshop.

Transposon construction, germline transformation and overexpression studies

The cDNA fragments encoding full-length mouse PINS, N-PINS (aa 1-369) and C-PINS (aa 366-672) were fused in-frame with a double-FLAG epitope at their respective C-terminal ends. The resultant DNA fragments were cloned into the *hs-Casper* transformation vector. Embryo injections used to generate germline transformants were performed essentially as described (Spradling, 1986). The expression of PINS, N-PINS and C-PINS in embryos was induced by a 15 minute heat shock at 34°C; after 1 hour recovery at 25°C in a moist chamber, embryos were processed for immunohistochemistry. Induced PINS, N-PINS and C-PINS possess two tandem FLAG epitopes at their C-terminus and were detected using either rabbit anti-PINS (1:1000 dilution) or monoclonal antibody M2 against FLAG (1:2000, Sigma). For rescue experiments, *hs-Pins*, $pins^{89}$/*TM3, Sb, Ubx-LacZ* male flies were crossed to $pins^{89}$/$pins^{89}$ females and embryos were collected and subjected to heat shock. Mutant embryos were identified with anti β-gal staining and analyzed for PINS, Insc, Miranda, and Pon localization. In parallel, the same collection of embryos were double stained with rabbit anti-β-Gal and anti-FLAG to ascertain that the observed non-β-Gal stained embryos were expressing the heat-induced transgene products. The heat shock regime used for the rescue of the RP2 duplication phenotype was previously described (Tio et al., 1999).

Yeast two-hybrid assay and protein-binding assays

Yeast two-hybrid assay and protein-binding assays were performed as described (Yu et al., 2000).

Results

A highly conserved mouse homologue of *Drosophila partner of inscuteable*

To isolate a mouse homologue of *Drosophila pins*, we searched the mouse EST database with the deduced amino acid sequence of fly *pins*. Two related EST clones, IMAGE:949074 and IMAGE:614560, showed significant homology to fly *pins* and were retrieved from Research Genetics Inc. Because both EST clones were not full-length and did not contain the entire coding region, we performed a 5′-RACE PCR using a marathon E11 mouse library (Clontech) to amplify the missing 5′-end fragment, which was subsequently ligated with IMAGE:949074 in order to construct the full-length cDNA of mouse *Pins*. The full-length mouse *Pins* cDNA comprises 3355 nucleotides, consistent with its mRNA size as judged from the northern blots (Fig. 2A,B). The full-length mouse *Pins* encodes a predicted open reading frame of 672 amino acids, containing seven N-terminal TPR and four C-terminal GoLoco motifs (Fig. 1A).

The overall amino acid sequence of PINS is 49% identical to that of *Drosophila* Pins, 92% to that of human LGN, 60% to rat AGS3 and 45% to worm PINS (Fig. 1B). Mouse PINS is molecularly more similar to human LGN than rat AGS3, and another family of ESTs has since appeared in mouse databases, showing higher homology to rat AGS3 than to LGN (mouse AGS3). Hence, mouse PINS/LGN and m-AGS3/AGS3 may be paralogues formed by duplication after divergence of mammals and flies. The TPR of fly Pins have been shown to be required for binding to Insc, suggesting that mouse PINS might also be able to interact with fly Insc or an

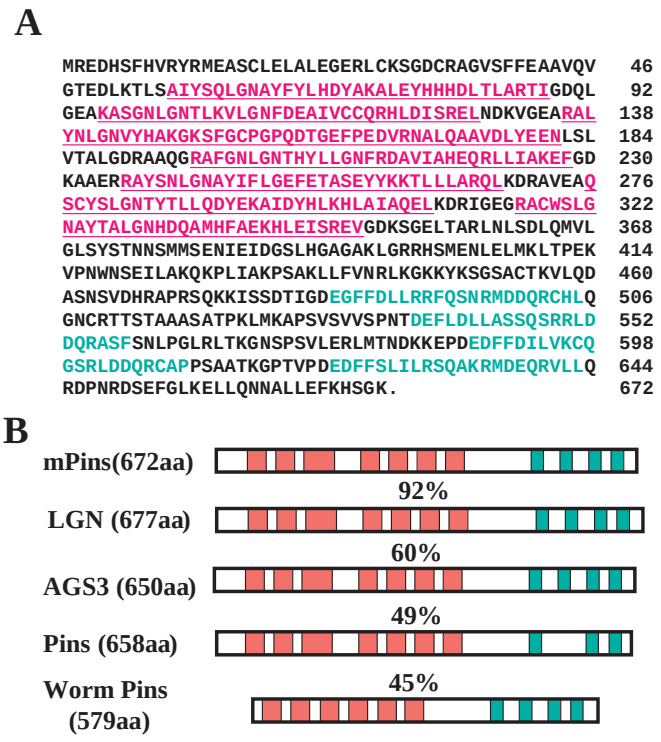

Fig. 1. Mouse PINS belongs to a highly conserved protein family. (A) The deduced amino acid sequence of mouse *Pins* derived from a 3.3 kb nucleotide sequence is shown (GenBank accession number AY081187). The seven TPR are in red and the four GoLoco motifs are in green. (B) The degree of amino acid identity between PINS and other homologues. The TPR and GoLoco repeats are highlighted. PINS shares 92% identity with human LGN, 60% identity with rat AGS3, 49% identity with fly Pins and 45% identity with worm PINS.

unidentified mouse homologue of Insc. PINS and LGN have four GoLoco motifs and they also share high homology at their C-terminal portion with Pins, suggesting that they may also be able to bind to the α-subunit of heterotrimeric G proteins. The overall homology among worm PINS, fly Pins, mouse Pins, rat AGS3 and human LGN may reflect functional conservation during evolution.

Mouse *Pins* is expressed widely in a variety of tissues and its expression in the developing CNS is enriched in regions showing high levels of proliferation

To examine the expression pattern of mouse *Pins*, we carried out a series of northern blots. The developmental northern blot analysis showed a major 3.3 kb transcript, detected from E11 to E17 embryos. No transcript was detected in E7 embryos (Fig. 2B). Multiple-tissue northern blot analysis revealed that mouse *Pins* mRNA was expressed in most adult tissues, including heart, liver, kidney, brain, lung, spleen and testis, but not in skeletal muscle (Fig. 2A).

pins plays an important role in the process of asymmetric division of neural progenitors in both the central and peripheral nervous systems of the fly. Given the sequence conservation between *pins* and *Pins*, we speculated that *Pins* might have a function in controlling some aspects of nervous system development in vertebrates as well, and studied its expression pattern during neurogenesis. In situ hybridization shows that *Pins* is expressed in the CNS of E12.5 mouse embryos. The expression is restricted, however, to a population of cells lying next to the ventricular zone (Fig. 3A-C). Three days later, at E15.5, the transcript is detected in the ventricular zone of the telencephalon and the roof of the diencephalon (Fig. 3F), with very little expression detected more caudally in the hindbrain and virtually no expression in the spinal cord (not shown). The ventricular distribution suggests that *Pins* could be restricted to mitotic progenitors. This would be consistent with the limited expression observed in the anterior brain at E15.5, when most neural progenitors are located in the developing cortex. We assessed whether *Pins* expression is limited to mitotic cells or is maintained for a certain period after cells exit the cell cycle and start to differentiate. *Pins* and Ki-67, a nuclear antigen specific for proliferating cells (Gerdes et al., 1983), were detected in adjacent sections of E12.5 embryos. The results show a restriction of *pins* transcript to cells in the proliferative region of the CNS (Fig. 3C-C″, 3G-H″). These results suggest that in the CNS, *Pins* is expressed in mitotic cells and absent from differentiating cells. Mouse *Pins* transcript was also detected in ganglia of the PNS (dorsal root ganglia, cranial sensory neurons and the sympathetic chain) at E12.5 (Fig. 3D,E).

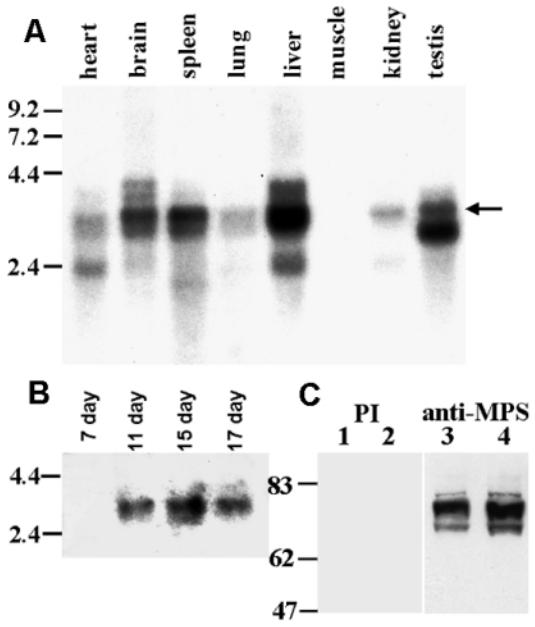

Fig. 2. Distribution and expression of mouse PINS in mouse adult and embryonic tissues. (A) Northern blot analysis of mouse *Pins* mRNA in a variety of mouse tissues. 2 μg of poly(A)$^+$ mRNA was used in each lane. Note that a major transcript of *Pins* can be seen as a 3.3 kb band. (B) *Pins* is expressed during embryogenesis. *Pins* mRNA of 3.3 kb is expressed from stage E11 onwards. (C) The PINS protein is expressed in liver and brain. 100 μg of protein extracts were used in each lane. A major 75 kDa protein band is detected in brain (lane 3) and liver (lane 4) by a purified anti-mouse PINS antibody while no immunoreactivity is detected with a pre-immune serum.

To determine the subcellular distribution of mouse *Pins* gene product, we raised a rabbit polyclonal antibody against the C-terminal half (aa 478-666) of PINS. In a western blot analysis, this purified antibody specifically recognized a protein of 75 kDa in liver and brain extracts, consistent with the predicted molecular weight of PINS, whereas the pre-immune serum did not (Fig. 2C). In parallel, polyclonal antibodies were raised against two specific peptides (aa 459-479 and aa 644-657), and an anti-LGN antibody directed against a peptide conserved between LGN and PINS was obtained (Blumer et al., 2002). Using this panel of antibodies, we could not achieve consistent results on mouse cryosections and were not able to conclusively address the question of PINS subcellular distribution in vivo.

Mouse PINS can interact with Insc in the yeast two-hybrid and GST pull-down assays

Because Pins interacts directly with Insc through its seven TPR (Yu et al., 2002), we tried to ascertain whether PINS also binds to Insc using yeast two-hybrid assays and in vitro binding assays. We confirmed that PINS interacts with Insc through its seven TPR; moreover, the minimal region including TPR3-7 is sufficient for its interaction with Insc (Fig. 4A).

The PINS-Insc protein interaction was further confirmed by GST pull-down assays. Mouse PINS is able to bind to all GST-Insc fusion proteins (full-length Insc, aa 1-859; Insc-5, aa 288-497; Insc-2, aa 258-578) containing the asymmetric localization domain of Insc (aa 288-497) but not to GST alone nor to N-terminal Insc (N-Insc, aa1-330), which lacks the asymmetric localization domain (Fig. 4B). Furthermore, N-PINS (aa 1-369) and TPR3-7 (aa 129-369) were pulled down by GST-Insc but not GST alone, whereas C-PINS (aa 366-672) could not bind to GST-Insc (Fig. 4C,D). These results indicate that the region TPR3-7 of PINS can interact directly with Insc and that PINS mimicks the Insc-binding properties of fly Pins.

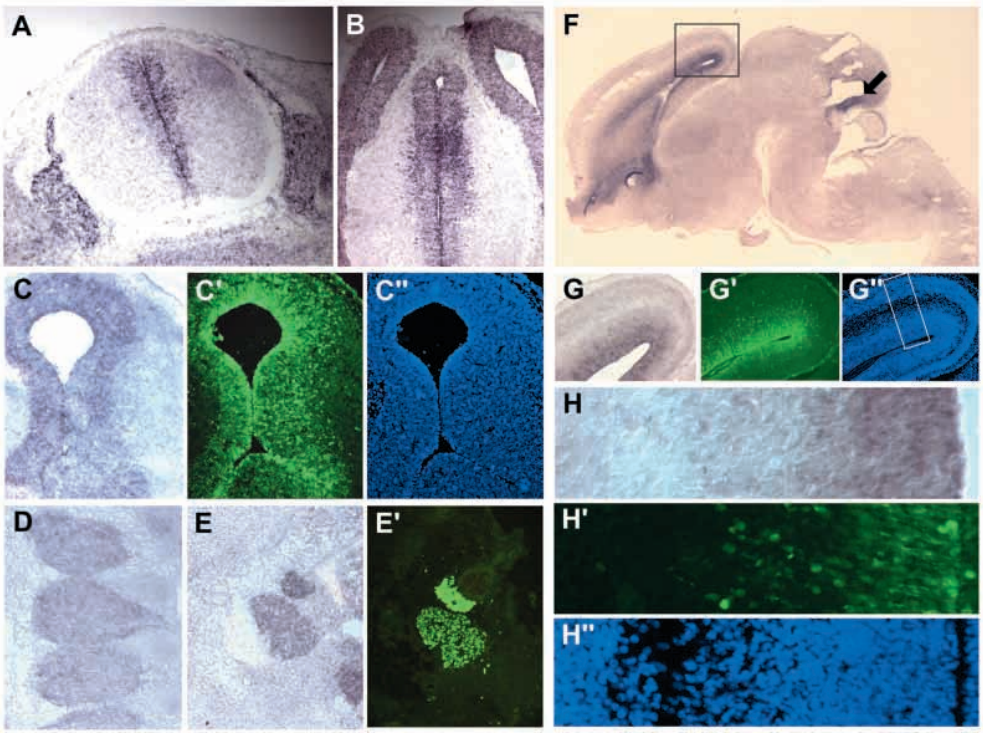

Fig. 3. Expression of mouse *Pins* in the developing nervous system. (A-C) Expression in the E12.5 embryonic CNS. *Pins* in situ hybridization on sagittal sections of an E12.5 embryo at the trunk level (A) and at the level of the midbrain and forebrain (B) shows expression restricted to the ventricular zone of the CNS. (C-C″) Transversal sections at the level of the right ventricle of the forebrain: *Pins* expression domain (C) corresponds to the proliferating cells of the ventricular zone, as revealed on an adjacent section by an anti Ki-67 staining (C′). (C″) Hoechst staining of all nuclei in the section seen in C′. Anterior is up. (D-E) *Pins* expression in the PNS at E12.5: dorsal root ganglia, seen on a parasagittal section (D), cranial sensory ganglia and sympathetic ganglia, seen on a transversal section (E), express *Pins* at E12.5. (E′) On a section adjacent to E, the cranial sensory complex (IX-X) and the superior cervical ganglion of the sympathetic chain (stronger signal) are visualized with an anti-Phox2a antibody (Tiveron et al., 1996). (F) Expression in the E15.5 brain: *Pins* is detected along the ventricular zone, with most expression seen in the forebrain. Some expression is also observed in the roof of the diencephalon (arrow) at the ventricular level. See G for a higher magnification of the boxed region. (G-G″) *Pins* expression is restricted to the proliferating zone: (G) *Pins*, (G′) Ki-67 in an adjacent section, (G″) Hoechst staining of the nuclei in the section seen in G′. (H-H″) higher magnification of sections shown in G-G″, as boxed in G″.

A

	FL-Insc
mPins	++
T1-7	++
T3-7	+
T4-7	-
T3-6	-
T4-6	-

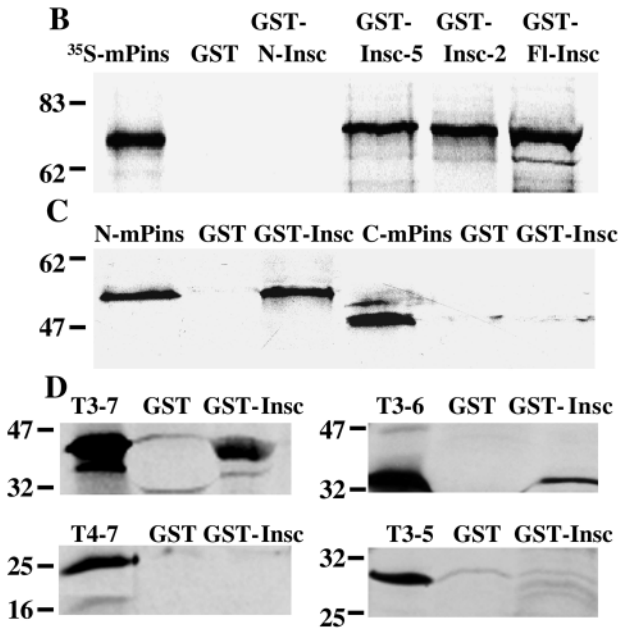

Fig. 4. Mouse PINS interacts with the asymmetric localization domain of *Drosophila* Insc through its TPR 3-7. (A) Yeast two-hybrid assays show that interaction between PINS and full-length Insc (FL-Insc) is mediated through the TPR repeats of Pins (T1-7 represents a construct containing all seven TPRs, T3-T7 represents a construct containing TPR 3 to 7, etc.). The interaction activities between Insc and various parts of PINS are semi-quantitated based on the time taken for colonies to turn blue in X-gal filter lift assay: ++, 30-90 minutes; +, >120 minutes; –, no significant staining. (B) PINS can interact specifically with the asymmetric localization domain of Insc (Insc-5, aa 288-497) as well as full-length Insc and Insc-2 (aa 258-578), which also contains the asymmetric localization domain, but not N-terminal Insc (N-Insc, aa 1-330) lacking this domain of Insc. In vitro translated [^{35}S]-labeled full-length PINS was incubated with sepharose 4B beads coupled to GST and various GST-Insc fusion proteins. PINS is able to bind to all GST-Insc fusion proteins (Fl-Insc, aa 1-859; Insc-5, aa 288-497; Insc-2, aa 258-578) containing the asymmetric localization domain of Insc (aa 288-497) but not to GST alone nor to N-terminal Insc (N-Insc, aa1-330), which lacks the asymmetric localization domain. (C,D) To further characterize this interaction, various [^{35}S]-labeled portions of PINS (N-PINS: aa 1-369; C-PINS: aa 366-672; T3-7: aa 129-369; T3-6: aa 129-315; T4-7: aa 182-369 and T3-5: aa 129-275) were incubated with sepharose 4b beads coupled to the full-length Insc GST fusion protein or coupled to GST alone. Like *Drosophila* Pins, N-PINS containing the TPR interacts with Insc, whereas C-PINS does not interact (C). The region TPR3-7 of PINS can be pulled down by Insc but TPR3-5 and TPR4-7 can not (D). Although a trace mount of TPR3-6 is pulled down by Insc, the region TPR 3-6 does not interact with Insc in yeast two-hybrid assay, suggesting that TPR7 is necessary for the interaction.

Mouse PINS is asymmetrically localized as an apical crescent when ectopically expressed in *Drosophila* NBs

Because PINS can interact with Insc, we assessed whether the cellular machinery responsible for the localization of Pins in *Drosophila* NBs can recognize and localize PINS. Transgenic fly lines carrying P-element insertions containing full-length PINS, N-PINS (aa 1-369) and C-PINS (aa 366-672) under the control of the *hsp70* promoter were generated. Those various PINS constructs were tagged at their extreme carboxyl termini with a double-FLAG epitope and were detected in the transgenic animals with anti-FLAG antibody following induction. No expression was detected in the transgenic embryos lacking heat-shock induction (Fig. 5C), whereas PINS is ubiquitously expressed in embryos treated with 15 minute heat shock (Fig. 5F). The subcellular localization of ectopically expressed full-length PINS resembles that of the endogenous fly Pins in NBs, forming an apical cortical crescent during mitosis (Fig. 5F). By contrast, neither N-PINS nor C-PINS were asymmetrically localized in NBs when overexpressed in a wild-type (WT) background. N-PINS was cytoplasmic, whereas C-PINS was cortical in mitotic (metaphase) NBs, showing identical localization patterns to their equivalent counterparts from fly Pins (Fig. 5I,L) (Yu et al., 2002). These results show that the protein localization machinery of *Drosophila* NBs can recognize full-length PINS as if it were the endogenous *Drosophila* Pins.

Surprisingly, although PINS and fly Pins show only 32% identity at their C-terminus, the anti-PINS antibody, which was raised against the C-terminal region, can recognize the endogenous fly Pins in

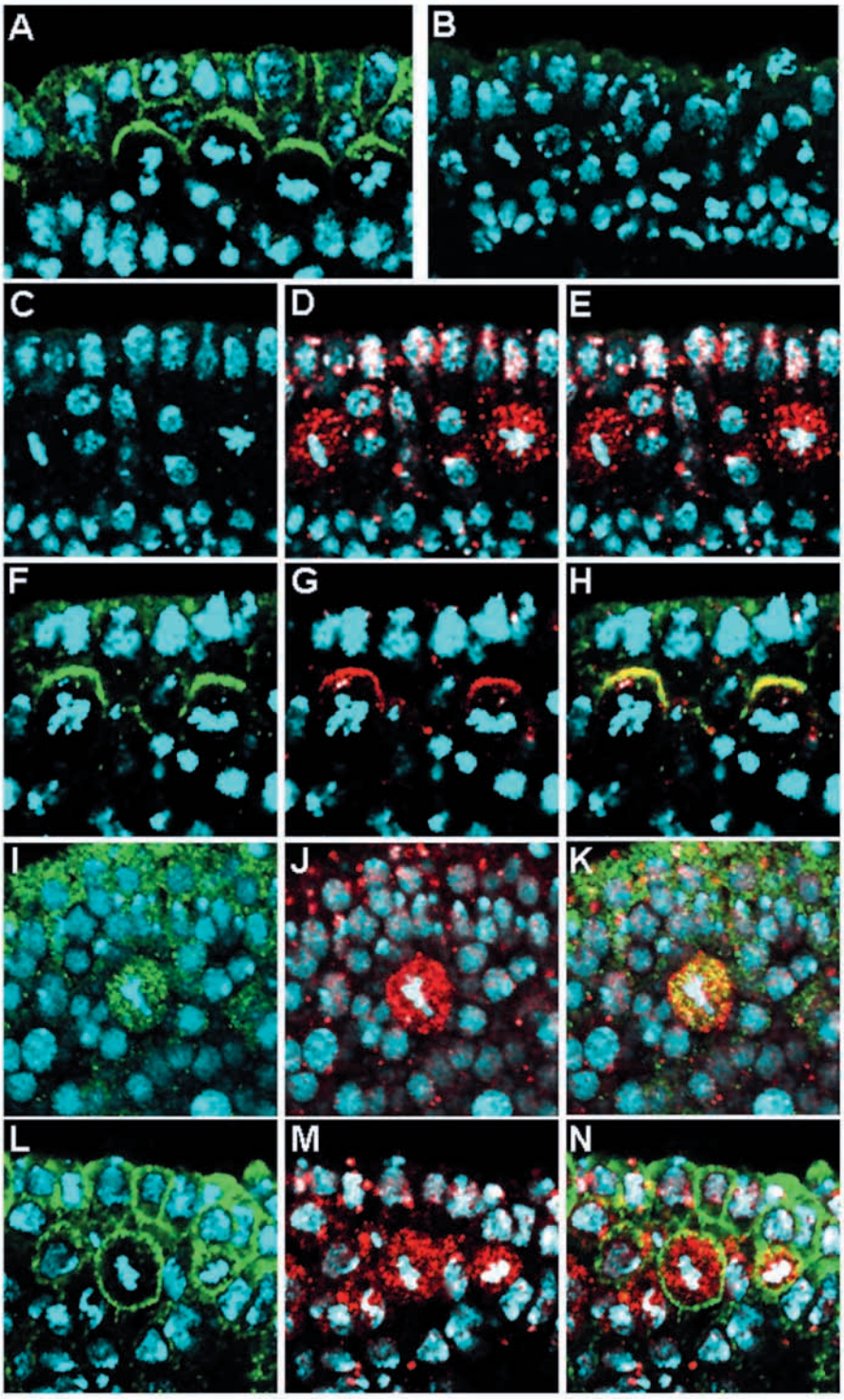

Fig. 5. Ectopically expressed mouse PINS can restore apical localization of Insc in mitotic NBs. Anti-PINS antibody can recognize endogenous fly Pins in WT mitotic NBs (A), whereas in *pins* mutant NBs no signal can be detected (B). Pins is in green and DNA is in cyan. So, although the C-terminal regions of fly Pins and PINS show 33% identity, the anti-PINS raised against the C-terminal region can cross-react with endogenous fly Pins. (C-N) NBs from embryos lacking both the maternal and zygotic components of *pins* which, in addition, also carry a transgene in which a full-length mouse *Pins* cDNA is placed under the control of the *hsp70* promoter are shown. (C-E) Without heat-shock induction, no PINS protein can be detected in *pins* mutant background (C) and Insc (red) is cytoplasmic (D); (E) is the merged image of (C) and (D). (F-H) With heat-shock induction, PINS can be detected as an apical crescent in *pins* mutant mitotic NBs using anti-FLAG staining (F); endogenous Insc can be recruited onto the apical cortex (G); in the merged image (H) it can be seen that Insc and PINS are colocalized as apical crescents in mitotic NBs. (I-K) Heat-shock-induced N-terminal PINS shows cytoplasmic localization (I) and cannot restore Insc apical localization (J) in *pins* mutant NBs. In the merged image (K) it can be seen that both Insc and N-terminal PINS are localized to the cytoplasm. (L-N) C-terminal PINS is cortically localized in NBs (L),whereas endogenous Insc is still cytoplasmic (M); (N) shows a merged image of (L) and (M).

wild-type *Drosophila* embryos (Fig. 5A). In *Drosophila pins*-mutant embryos, anti-PINS staining did not show any immunoreactivity (Fig. 5B), indicating that this cross-reaction is specific to fly Pins.

Mouse *Pins* can fulfill all aspects of *Drosophila pins* function in neuroblast asymmetric divisions

Because PINS can interact physically with Insc and can localize as an apical cortical crescent when ectopically expressed in the *Drosophila* NBs, we wondered whether PINS could functionally substitute for *Drosophila* Pins. To determine whether *Pins* can function in flies, the phenotypes associated with flies lacking both maternal and zygotic *pins* function were scored following *hsp70*-mediated expression of full-length PINS, N-PINS and C-PINS, respectively.

In *pins* null mutants, Insc asymmetric localization is disrupted and the protein becomes cytoplasmic in dividing NBs. As expected, in transgenic *pins* mutant embryos without heat shock, Insc localization is cytoplasmic in dividing NBs (Fig. 5D). However, in *pins* mutant embryos with one copy of hs-*Pins* (full length) subjected to 15 minute heat shock and 1 hour recovery, the ectopically expressed full-length PINS was detected as an apical crescent using anti-FLAG antibody (Fig. 5F) and recruited Insc back to the apical cortex (Fig. 5G). By contrast, neither N-PINS (cytoplasmic; Fig. 5I) nor C-PINS (uniformly cortical; Fig. 5L) could form an apical crescent or restore Insc apical crescent in transgenic *pins* mutants (Fig. 5J,M). These results provide the first indication that full-length PINS can be recognized by the protein localization machinery of *Drosophila* NBs and that it can functionally mimic fly Pins.

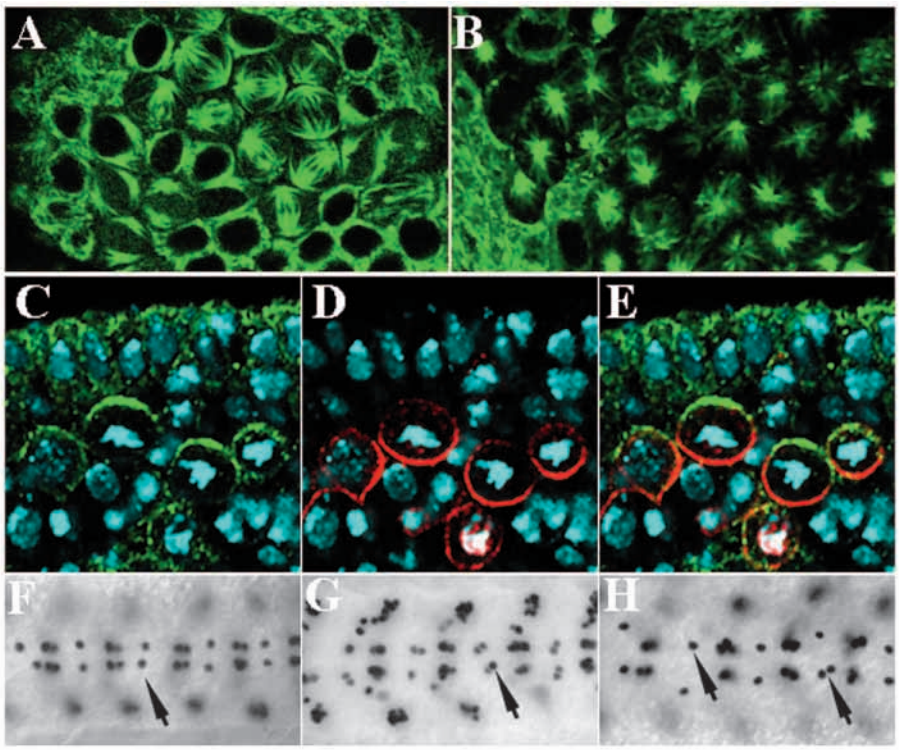

Fig. 6. Mouse *Pins* fulfills all aspects of the *Drosophila pins* function. (A,B) mitotic spindle in the cells of mitotic domain 9 is visualized by anti-β-tubulin staining. In *pins* null mutants, mitotic spindles fail to reorient by 90° as in WT and consequently are all aligned parallel to the surface of the embryo (A). Ectopically expressed mouse PINS in *pins* mutant embryos can restore this 90^0 spindle reorientation in cell of mitotic domain 9, causing the mitotic spindles to be aligned perpendicular to the surface of the embryo as in WT (B). (C-E) In *pins* mutant embryos, Miranda localization is defective in the form of uniform cortical localization or misplaced cortical crescents (Schaefer et al., 2000; Yu et al., 2000; data not shown); ectopic expression of PINS results in its apical cortical localization (PINS apical crescent in green, C) and can also restore basal cortical localization of Miranda (basal crescents in red, D) in mitotic NBs. Panel E is a merged image of panels C and D. (F-H): One Eve-expressing RP2 neuron can be found at a characteristic position in each WT hemisegment (arrow, F); RP2 neurons are duplicated in a high proportion of hemisegments in *pins* mutants embryos (G); expression of PINS protein in *pins* mutant embryos can restore the WT situation in the great majority of hemisegments (H).

It has been shown that, although the mitotic spindle of WT mitotic domain 9 cells undergo a 90° rotation to orient perpendicular to the surface of the embryo, this rotation fails to occur in the absence of *pins* function and, consequently, the spindles are aligned parallel to the embryo surface (Yu et al., 2000) (Fig. 6A). Ectopically expressed full-length PINS in *pins* mutant embryos is able to restore this 90° spindle rotation in the cells of mitotic domain 9 (Fig. 6B) due to its ability to recruit Insc to the apical cortex and stabilize the apical complex. By contrast, expressing either N-PINS or C-PINS in *pins* mutants does not restore this 90° spindle reorientation (data not shown).

In *pins* mutants, proteins that normally localize as basal cortical crescents in mitotic NBs at metaphase, such as Mir/Pros and Pon/Numb, are mislocalized either as randomly placed cortical crescents or are localized throughout the cell cortex (Yu et al., 2000). However, the introduction of PINS into *pins* mutants, which localizes as an apical crescent (Fig. 6C), allows the basal proteins such as Miranda to localize normally (Fig. 6D). Finally, *pins* mutant embryos show defects in the resolution of alternative sibling cell fates. These defects are most easily seen by following the GMC4-2a>RP2/RP2sib sublineage. In *pins* mutant embryos, RP2sib adopts the fate of its sibling, the RP2 neuron, resulting in the duplication of the Even-skipped (Eve) positive RP2 neurons in 60% of the hemisegments (Fig. 6G) (Yu et al., 2000). Moreover, a small proportion of the GMC4-2a cells are also mis-specified, resulting in the loss of Eve-expressing RP2 neurons in 15% of the hemisegments. However, ectopic expression of PINS in *pins* mutant embryos results in a significant rescue of these cell-fate transformations (Fig. 6H) (only 6% of hemisegments show RP2 duplication, n=180), whereas neither N-PINS nor C-PINS could mediate this rescue (data not shown).

Taken together, these results show that *Pins* can functionally

substitute for fly *pins* and apparently fulfill all aspects of its function in neuroblast asymmetric divisions.

Discussion

We have characterized a mouse homologue of *Drosophila pins*. The mouse and *Drosophila* Pins proteins share significant amino acid homology and similar functional domains over the entire length of the coding region. Mouse *Pins* expression shows a wide distribution in most tissues including the nervous system, where its expression is enriched in proliferative regions like the ventricular zone. Our results show that PINS is able to interact with the asymmetric localization domain of *Drosophila* Insc through its TPR (TPR3-7). When ectopically expressed in NBs, PINS localizes to the apical cortex as a crescent and rescues all the defects associated with NB asymmetric divisions caused by the loss of *pins* function.

Two mouse homologues of Pins exist

Database searches of the mouse genome with the fly Pins amino acid sequence identified EST clones that encode two Pins-like proteins with varying homologies to Pins. The mouse protein showing a higher percentage of homology to *Drosophila* Pins is referred to as PINS. PINS shows a higher level of homology to human LGN than to rat AGS3 (Fig. 1). The second mouse protein is more closely related to AGS3 than to LGN and is therefore referred to as mouse AGS3 (IMAGE:5720527 and IMAGE:5685096). Hence, there are at least two homologues of *Drosophila* Pins in mouse, PINS and mouse AGS3. Similarly, the human genome project also identifies two Pins-like sequences, LGN (previously identified by Mochizuki et al. (Mochizuki et al., 1996)) and AGS3 (previously identified in the rat by Takesono et al. (Takesono et al., 1999)). Hence, PINS/LGN and mouse AGS3/AGS3 appear to be paralogues, formed by duplication after divergence of mammals and flies.

The two Pins-like proteins identified in the mammalian genomes have different features. In situ hybridization of mouse *Pins* and *Ags3* showed a distinct distribution in the neural tube: *Pins* is enriched in a layer of cortical precursors, whereas *Ags3* is uniformly distributed in the neural tube, suggesting distinct roles for these proteins during neurogenesis (X. Morin, unpublished). This is reminiscent of the localization profiles of mouse *numb* and *numb-like* in the neural tube of the mouse embryo (Zhong et al., 1997).

Asymmetry machinery of *Drosophila* NBs can recognize mouse PINS

We have shown that mouse PINS interacts specifically with the asymmetric localization domain of *Drosophila* Insc via its TPR region. This is identical to what we observed with fly Pins (Yu et al., 2002), and suggests that they are structurally similar. Ectopically expressed PINS colocalizes with endogenous fly Pins and forms an apical crescent in NBs. Moreover, ectopically expressed N-terminal PINS and C-terminal PINS show cortical and cytoplasmic localization, respectively, in NBs, equivalent to the data we obtained in our previous domain dissection analyses of fly Pins (Yu et al., 2002). These observations suggest that PINS achieves its asymmetric localization by using the same mechanism as fly Pins (see below).

Several lines of evidence support the view that PINS, when overexpressed in *Drosophila* NBs, can functionally substitute for fly Pins. First, like the C-terminal rgion of fly Pins, C-terminal PINS is localized at the cell cortex of WT NBs, and its overexpression in Pins$^-$ embryos can lead to the generation of two equal-sized NB daughter cells (F. Yu, unpublished). Second, Insc can interact with both PINS and fly Pins through their seven TPR in vitro, suggesting that, like fly Pins, PINS is able to form a complex with Insc to orient asymmetric cell divisions of NBs. Third, the N-terminal regions of both PINS and fly Pins are insufficient to localize to the apical cortex; the C-terminal regions by themselves are uniformly localized to the cortex, but they do not localize apically. These observations indicate that both fly Pins and PINS mediate their apical localization in two steps: membrane targeting mediated by the C-terminal region and apical recruitment involving the N-terminal portion. Finally, the introduction of PINS can rescue all aspects of asymmetric division defects seen in *pins* mutant NBs.

What is the role of mouse *pins* in vertebrates?

We showed in a previous study that *Drosophila* Pins plays a crucial role in asymmetric cell divisions of NBs (Yu et al., 2000). It is asymmetrically localized as a crescent in NBs, GMCs, muscle progenitor cells (F. Yu, unpublished) and sensory organ precursors (Bellaiche, 2001). *Drosophila* Pins is also expressed in most other embryonic and larval tissues, where it is distributed around the lateral cell cortex. Interestingly, in the developing CNS, mouse *Pins* shows a restricted expression pattern. It is restricted to zones of proliferation and is absent from differentiating post-mitotic cells. However, to date, we have not been able to observe asymmetric localization of PINS by immunostaining of either tissue culture cells or the developing ventricular zone of E12.5 mouse embryos (data not shown). This apparent difference of their localization modes between fly Pins and mouse PINS may reflect non-conservation of the asymmetry machinery between *Drosophila* and mouse. In this regard, many components known to be asymmetrically localized in *Drosophila* – for example Insc, *partner of numb* (*pon*), and *miranda* – do not have apparent orthologues in mammals. The homologue of *pros, prox 1*, albeit present in mammals, plays a distinct role. Although the mouse counterpart of *Drosophila* Numb shows asymmetric localization in cortical precursors, the mode of its asymmetric localization is probably different, as the mammalian counterpart of *Drosophila pon*, which directs asymmetric localization of Numb in *Drosophila*, does not appear to exist. Finally, *insc* seems to be unique to flies and absent from mammals and nematodes.

Pins and Pins-related proteins share in common their ability to bind Gα proteins and exert a GDI function, thereby inhibiting the exchange of GDP-bound for GTP-bound forms of Gα. Although present in different tissues, Pins and Pins-related proteins may mediate their functions by regulating G-protein activity. It has been reported recently that human LGN localizes to the cytoplasm at interphase and subsequently to the mitotic spindle during mitosis, and interacts with NuMA and regulates mitotic spindle organization (Du et al., 2001).

Another study reports that human LGN localizes to the nucleus at interphase and the midbody during cytokinesis in cultured cells (Blumer et al., 2002).

It has been reported that, in addition to asymmetric localization in dividing precursors, mouse NUMB is also localized to the Golgi region, in particular endosomes, clathrin-coated pits and vesicles. NUMB can interact with the endocytic machinery, α-adaptin and Eps15. A dominant negative form of NUMB inhibits clathrin-mediated endocytosis, suggesting a role for NUMB in endocytosis (Santolini et al., 2000). Whether PINS functions in this process remains to be determined. Heterotrimeric G proteins are known to be involved in protein trafficking, particularly endocytosis (reviewed by Ferguson, 2001), and a role for PINS in this process seems to be reasonable. PINS might act as a GDI to regulate G signaling, which, in turn, regulates protein trafficking. Further studies are required to understand the various functions of *Pins* during mouse development.

We thank C. Q. Doe, M. Frasch, Y. N. Jan, F. Matsuzaki, S. Lanier and K. Zinn for providing antibodies and/or flies; Fumio Matsuzaki for advice on anti-FLAG staining; Ong Chin Tong for help with embryo injections; Chai Ling Lee and Fock Sion Hing for outstanding technical assistance; and the Bloomington stock center for efficiently providing stocks. F.Y. would like to thank Hongyan Wang for critically reading the manuscript. X.Y. is an adjunct staff at the Department of Anatomy, National University of Singapore. This work was supported by the Institute of Molecular and Cell Biology, Singapore and the Wellcome Trust, UK.

References

Bellaiche, Y., Radovic, A., Woods, D. F., Hough, C. D., Parmentier, M. L., O'Kane, C. J., Bryant, P. J. and Schweisguth, F. (2001). The partner of Inscuteable/Discs-large complex is required to establish planar polarity during asymmetric cell division in *Drosophila*. *Cell* **106**, 355-366.

Bernard, M. L., Peterson, Y. K., Chung, P., Jourdan, J. and Lanier, S. M. (2001). Selective interaction of AGS3 with G-proteins and the influence of AGS3 on the activation state of G-proteins. *J. Biol. Chem.* **276**, 1585-1593.

Blumer, J. B., Chandler, L. J. and Lanier, S. M. (2002). Expression analysis and subcellular distribution of the two G-protein regulators AGS3 and LGN indicate distinct functionality. Localization of LGN to the midbody during cytokinesis. *J. Biol. Chem.* **277**, 15897-15903.

Bossing, T., Udolph, G., Doe, C. Q. and Technau, G. (1996). The embryonic CNS lineages of *Drosophila melanogaster*: I. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Dev. Biol.* **179**, 41-64.

Buescher, M., Yeo, S. L., Udolph, G., Zavortink, M., Yang, X., Tear, G. and Chia, W. (1998). Binary sibling neuronal cell fate decisions in the *Drosophila* embryonic central nervous system are non-stochastic and require *inscuteable* mediated asymmetry of ganglion mother cells. *Genes Dev.* **12**, 1858-1870.

Chenn, A. and McConnell, S. K. (1995). Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. *Cell* **82**, 631-641.

De Vries, L., Fischer, T., Tronchere, H., Brothers, G. M., Strockbine, B., Siderovski, D. P. and Farquhar, M. G. (2000). Activator of G protein signaling 3 is a guanine dissociation inhibitor for Galpha i subunits. *Proc. Natl Acad. Sci. USA* **97**, 14364-14369.

Doe, C. Q., Chu-LaGraff, Q., Wright, D. M. and Scott, M. P. (1991). The prospero gene specifies cell fates in the *Drosophila* central nervous system. *Cell* **65**, 451-464.

Du, Q., Stukenberg, P. T. and Macara, I. G. (2001). A mammalian Partner of inscuteable binds NuMA and regulates mitotic spindle organization. *Nature Cell. Biol.* **3**, 1069-1075.

Dye, C., Lee, J., Atkinson, R., Brewster, R., Han, P. and Bellen, H. J. (1998). The *Drosophila sanpodo* gene controls sibling cell fate encodes a Tropomodulin homolog, an actin/tropomyosin associated protein. *Development* **125**, 1845-1856.

Ferguson, S. S. (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.* **53**, 1-24.

Frise, E., Knoblich, J. A., Younger-Shepherd, S., Jan, L. Y. and Jan, Y. N. (1996). The *Drosophila* Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage. *Proc. Natl. Acad. Sci. USA* **93**, 11925-11932.

Gerdes, J., Schwab, U., Lemke, H. and Stein, H. (1983) Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int. J. Cancer* **15**, 13-20.

Goodman, C. S. and Doe, C. Q. (1993). Embryonic development of the *Drosophila* central nervous system. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez-Arias), pp. 1091-1131. New York: Cold Spring Harbor Laboratory Press.

Guo, M., Jan, L. Y. and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron* **17**, 27-41.

Hirata, J., Nakagoshi, H., Nabeshima, Y. and Matsuzaki, F. (1995). Asymmetric segregation of the homeodomain protein Prospero during *Drosophila* development. *Nature* **377**, 627-630.

Knoblich, J. A., Jan, L. Y. and Jan, Y. N. (1995). Asymmetric segregation of Numb and Prospero during cell division. *Nature* **377**, 624-627.

Kraut, R. and Campos-Ortega, J. A. (1996). *inscuteable*, a neural precursor gene of *Drosophila*, encodes a candidate for a cytoskeleton adaptor protein. *Dev. Biol.* **174**, 65-81.

Kraut, R., Chia, W., Jan, L. Y., Jan, Y. N. and Knoblich, J. A. (1996). Role of *inscuteable* in orienting asymmetric cell divisions in *Drosophila*. *Nature* **383**, 50-55.

Kuchinke, U., Grawe, F. and Knust, E. (1998). Control of spindle orientation in *Drosophila* by the Par-3-related PDZ-domain protein Bazooka. *Curr. Biol.* **8**, 1357-1365.

Lu, B., Jan, L. and Jan, Y. N. (2000). Control of cell divisions in the nervous system: symmetry and asymmetry. *Annu. Rev. Neurosci.* **23**, 531-556.

Matsuzaki, F., Koizumi, K., Hama, C., Yoshioka, T. and Nabeshima, Y. (1992). Cloning of the Drosophila prospero gene and its expression in ganglion mother cells. *Biochem. Biophys. Res. Commun.* **182**, 1326-1332.

McConnell, S. K. (1995). Constructing the cerebral cortex: neurogenesis and fate determination. *Neuron* **15**, 761-768.

Mione, M. C., Cavanagh, J. F., Harris, B. and Parnavelas, J. G. (1997). Cell fate specification and symmetrical/asymmetrical divisions in the developing cerebral cortex. *J. Neurosci.* **17**, 2018-2029.

Mochizuki, N., Cho, G., Wen, B. and Insel, P. A. (1996). Identification and cDNA cloning of a novel human mosaic protein, LGN, based on interaction with G alpha i2. *Gene* **181**, 39-43.

Natochin, M., Lester, B., Peterson, Y. K., Bernard, M. L., Lanier, S. M. and Artemyev, N. O. (2000). AGS3 inhibits GDP dissociation from galpha subunits of the Gi family and rhodopsin-dependent activation of transducin. *J. Biol. Chem.* **275**, 40981-40985.

Oliver, G., Sosa-Pineda, B., Geisendorf, S., Spana, E. P., Doe, C. Q. and Gruss. P. (1993). Prox 1, a prospero-related homeobox gene expressed during mouse development. *Mech. Dev.* **44**, 3-16.

Parmentier, M. L., Woods, D., Greig, S., Phan, P. G., Radovic, A., Bryant, P. and O'Kane, C. J. (2000). Rapsynoid/partner of Inscuteable controls asymmetric division of larval neuroblasts in *Drosophila*. *J. Neurosci.* **20**, RC84 [Online].

Peterson, Y. K., Bernard, M. L., Ma, H., Hazard, S., III, Graber, S. G. and Lanier, S. M. (2000). Stabilization of the GDP-bound conformation of Gialpha by a peptide derived from the G-protein regulatory motif of AGS3. *J. Biol. Chem.* **275**, 33193-33196.

Petronczki, M. and Knoblich, J. A. (2001). DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in *Drosophila*. *Nat. Cell. Biol.* **3,** 43-49**.**

Santolini, E., Puri, C., Salcini, A. E., Gagliani, M. C., Pelicci, P. G., Tacchetti, C. and di Fiore, P. P. (2000). Numb is an endocytic protein. *J. Cell Biol.* **151**, 1345-1352.

Schaefer, M., Shevchenko, A. and Knoblich, J. A. (2000). A protein complex containing Inscuteable and the Galpha-binding protein Pins orients asymmetric cell divisions in *Drosophila*. *Curr. Biol.* **10**, 353-362.

Schaefer, M., Petronczki, M., Dorner, D., Forte, M. and Knoblich, J. A. (2001). Heterotrimeric G proteins direct two modes of asymmetric cell division in the *Drosophila* nervous system. *Cell* **107**, 183-194.

Schober, M., Schaefer, M. and Knoblich, J. A. (1999). Bazooka recruits Inscuteable to orient asymmetric cell divisions in *Drosophila* neuroblasts. *Nature* **402**, 548-551.

Skeath, J. B. and Doe, C. Q. (1998). Sanpodo and Notch act in opposition to Numb to distinguish sibling neuron fate in the *Drosophila* CNS. *Development* **125**, 1857-1865.

Spana, E. P. and Doe, C. Q. (1996). Numb antagonises Notch signaling to specify sibling neuron cell fate. *Neuron* **17**, 21-26.

Spana, E. P. and Doe, C. Q. (1995). The prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in *Drosophila*. *Development* **121**, 3187-3195.

Spradling, A. C. (1986). P-element mediated transformation. In *Drosophila: A Practical Approach* (ed. D. B. Roberts), pp. 175-197. Oxford, IRL Press.

Strahle, U., Blader, P., Adam, J. and Ingham, P. W. (1994). A simple and efficient procedure for non-isotopic in situ hybridization to sectioned material. *Trends Genet.* **10**, 75-76.

Takesono, A., Cismowski, M. J., Ribas, C., Bernard, M., Chung, P., Hazard, S., III, Duzic, E. and Lanier, S. M. (1999). Receptor-independent activators of heterotrimeric G-protein signaling pathways. *J. Biol. Chem.* **274**, 33202-33205.

Tio, M., Zavortink, M., Yang, X. and Chia, W. (1999). A functional analysis of *inscuteable* and its roles during *Drosophila* asymmetric cell divisions. *J. Cell. Sci.* **112**, 1541-1551.

Tiveron, M. C., Hirsch, M. R. and Brunet, J. F. (1996). The expression pattern of the transcription factor Phox2 delineates synaptic pathways of the autonomic nervous system. *J. Neurosci.* **16**, 7649-7660.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L. Y. and Jan, Y. N. (1989). numb, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell* **58**, 349-360.

Vaessin, H., Grell, E., Wolff, E., Bier, E., Jan, L. Y. and Jan, Y. N. (1991). *prospero* is expressed in neuronal precursors and encodes a nuclear protein that is involved in the control of axonal outgrowth in *Drosophila*. *Cell* **67**, 941-953.

Verdi, J. M., Schmandt, R., Bashirullah, A., Jacob, S., Salvino, R., Craig, C. G., Amgen EST Program, Lipshitz, H. D. and McGlade, C. J. (1996). Mammalian numb is an evolutionarily conserved signaling adapter protein that specifies cell fate. *Curr. Biol.* **6**, 1134–1145.

Wakamatsu, Y., Maynard, T. M., Jones, S. U. and Weston, J. A. (1999). NUMB localizes in the basal cortex of mitotic avian neuroepithelial cells and modulates neuronal differentiation by binding to NOTCH-1. *Neuron* **23**, 71-81.

Wigle, J. T. and Oliver, G. (1999). Prox1 function is required for the development of the murine lymphatic system. *Cell* **98**, 769-778.

Wodarz, A., Ramrath, A., Grimm, A. and Knust, E. (2000). *Drosophila* atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J. Cell Biol.* **150**, 1361-1374.

Wodarz, A., Ramrath, A., Kuchinke, U. and Knust, E. (1999). Bazooka provides an apical cue for Inscuteable localization in *Drosophila* neuroblasts. *Nature* **402**, 544-547.

Yu, F., Morin, X., Cai, Y., Yang, X. and Chia, W. (2000). Analysis of *partner of inscuteable*, a novel player of *Drosophila* asymmetric divisions, reveals two distinct steps in inscuteable apical localization. *Cell* **100**, 399-409.

Yu, F., Ong, C. T., Chia, W. and Yang, X. (2002). Membrane targeting and asymmetric localization of *Drosophila* partner of inscuteable are discrete steps controlled by distinct regions of the protein. *Mol. Cell. Biol.* **22**, 4230-4240.

Zhong, W., Feder, J. N., Jiang, M. M., Jan, L. Y. and Jan, Y. N. (1996). Asymmetric localization of mammalian homolog during mouse cortical neurogenesis. *Neuron* **17**, 43-53.

Zhong, W., Jiang, M. M., Weinmaster, G., Jan, L. Y. and Jan, Y. N. (1997). Differential expression of mammalian Numb, Numblike and Notch1 suggests distinct roles during mouse cortical neurogenesis. *Development* **124**, 1887-1897.