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Transitin, a nestin-like intermediate filament protein, mediates cortical localization and the lateral transport of Numb in mitotic avian neuroepithelial cells

Yoshio Wakamatsu[1,2,*], Noriko Nakamura[3], Ju-Ahng Lee[4], Gregory J. Cole[4] and Noriko Osumi[3]

Neuroepithelium is an apicobasally polarized tissue that contains neural stem cells and gives rise to neurons and glial cells of the central nervous system. The cleavage orientation of neural stem cells is thought to be important for asymmetric segregation of fate-determinants, such as Numb. Here, we show that an intermediate filament protein, transitin, colocalizes with Numb in the cell cortex of mitotic neuroepithelial cells, and that transitin anchors Numb via a physical interaction. Detailed immunohistological and time-lapse analyses reveal that basal Numb-transitin complexes shift laterally during mitosis, allowing asymmetric segregation of Numb-transitin to one of the daughter cells, even when the cell cleavage plane is perpendicular to the ventricular surface. In addition, RNA interference (RNAi) knockdown of the transitin gene reveals its involvement in neurogenesis. These results indicate that transitin has important roles in determining the intracellular localization of Numb, which regulates neurogenesis in the developing nervous system of avian embryos.

KEY WORDS: Asymmetric cell division, Numb, Transitin, Nestin, Neuroepithelium, Chick

INTRODUCTION

Embryonic neuroepithelium (NE) is composed of elongated, apicobasally polarized epithelial cells, and contains neural stem cells that generate neurons and glial cells of the developing central nervous system, while simultaneously undergoing self-renewal. Studies in invertebrate systems suggest the involvement of asymmetric cell division in fate determination of neural stem cells. Fate determinants in *Drosophila*, such as Prospero and Numb, as well as a variety of adaptors and regulators, are asymmetrically localized in mitotic neuroblasts, according to the apicobasal polarity (for a review, see Roegiers and Jan, 2004). For example, basal daughter cells that receive Prospero and Numb differentiate into ganglion mother cells, whereas the apical daughter cell remains as a neuroblast. It is essential, therefore, that intracellular localization of the fate determinants and the plane of cell cleavage be coordinated.

In vertebrate nervous system development, previous studies have suggested that the plane of cell cleavage in mitosis could be correlated with the behavior of the daughter cells in NE (Chenn and McConnell, 1995; Cayouette et al., 2001). For example, in neuroepithelial cells (NE cells) of the new-born ferret neocortex region, when the cell cleavage plane is parallel to the ventricular surface, while the apical daughter cell appears to remain undifferentiated in the ventricular zone, the basal daughter cell rapidly migrates basally and may undergo neuronal differentiation (Chenn and McConnell, 1995). By contrast, cell division with the cleavage plane perpendicular to the ventricular surface results in a symmetric NE cell phenotype (Chenn and McConnell, 1995). Such observations, along with the asymmetric localization and segregation of fate determinants in *Drosophila* neurogenesis, implied that the mechanisms involved in the fate determination of daughter cells of neural stem cells in vertebrates might be similar to those in *Drosophila*. Consistent with this inference, in vertebrates, Numb protein localizes asymmetrically in mitotic NE cells (Zhong et al., 1996; Wakamatsu et al., 1999; Cayouette et al., 2001; Shen et al., 2002), as well as in mitotic cells of dorsal root ganglia (DRG) (Wakamatsu et al., 2000). Thus, depending on the orientation of cell cleavage, Numb can be inherited unevenly by daughter cells. It has been shown that Numb binds to the intracellular domain of Notch and antagonizes Notch activation (Guo et al., 1996; Wakamatsu et al., 1999). Thus, one of the functions of Numb is to regulate the differentiation of neural cells by modulating Notch signaling (for a review, see Cayouette and Raff, 2001). Consistently, conditional and non-conditional knockouts of *Numb* and numb-like in mice reveal severe defects in the maintenance of NE and in neurogenesis (Zhong et al., 2000; Zilian et al., 2001; Peterson et al., 2002; Peterson et al., 2004; Li et al., 2003).

In the developing central nervous system of avian embryos, Numb protein localizes in the basal cortex of the mitotic NE cells (Wakamatsu et al., 1999). As Numb is a cytoplasmic adapter protein, it must itself be anchored to cellular architectural components to localize asymmetrically in mitotic cells. In this study, we show that transitin, a type IV intermediate filament protein, colocalizes with Numb in the cortex of mitotic NE cells. Our biochemical assays and RNA interference (RNAi) analyses indicate that transitin associates directly with Numb, and provides an anchor site enabling Numb to localize in the cell cortex. These observations prompted us to follow the localization and segregation of transitin-EGFP in live NE cells, and we found that basally localized transitin moved laterally during mitosis. Thus, the lateral shift of transitin enables its asymmetric segregation to one of the daughter cells even when the cleavage plane is perpendicular to the ventricular surface. Consistent with the important role(s) of Numb in neurogenesis, transitin knockdown by RNAi promoted neuronal differentiation.

[1]Department of Developmental Neurobiology, Tohoku University, Graduate School of Medicine, Seiryo-machi 2-1, Aoba-ku, Sendai, Miyagi 980-8575, Japan. [2]PRESTO, Japan Science and Technology Corporation, Japan. [3]Department of Developmental Neuroscience, Center for Translational and Advanced Animal Research on Human Diseases, Tohoku University, Graduate School of Medicine, Seiryo-machi 2-1, Aoba-ku, Sendai, Miyagi 980-8575, Japan. [4]Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606, USA.

*Author for correspondence (e-mail: wakasama@mail.tains.tohoku.ac.jp)

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MATERIALS AND METHODS

Immunostaining

Immunostaining was performed as previously described (Wakamatsu et al., 1993; Wakamatsu et al., 1997; Wakamatsu et al., 2000). Polyclonal anti-chick Numb (Wakamatsu et al., 1999; Wakamatsu et al., 2000); monoclonal 7B3 (Henion et al., 2000) and EAP-300 anti-transitin (McCabe et al., 1992; Cole and Lee, 1997); and 16A11 anti-Hu (Marusich et al., 1994; Wakamatsu and Weston, 1997) antibodies were described previously. Anti-FLAG (M2, Invitrogen), anti-vimentin (VIM3B4, Roche), anti-γ-tubulin (GTU-88, Sigma), anti-phospho-vimentin (polyclonal, SantaCruz), anti-GFP (polyclonal, Chemicon), anti-neuron-specific type III β-tubulin (TuJ1, BAbCO) and anti-BrdU (BMC9318, Roche) were obtained commercially. TUNEL staining for detection of cell death was performed as described previously (Wakamatsu et al., 1998). For photographing and cell counts, individual cell was carefully examined on a standard fluorescence microscope (Axioplan 2, Zeiss) that was equipped with a cooled CCD camera (AxioCam, Zeiss) by changing the focal plane to ensure the examined cells were intact.

Far-western blotting and immunoprecipitation

cDNA fragments of transitin and Numb were subcloned into pET41 vectors [for glutathione S-transferase (GST) fusion, also contain 2×His tags and an S tag, Novagen] or pMAL vectors (for MBP fusion, New England Biolabs). GST-fusions of Numb were purified with a B-PER kit (Pierce). Anti-GST (z5, SantaCruz) and anti-MBP (New England Biolabs) were obtained commercially. Far-western blotting was performed as described (Wakamatsu et al., 1999).

For co-immunoprecipitation assay, GST-Numb(158-582) and Mal-Transitin(328-816) [Mal-Trans(328-816)] were purified with glutathione- and maltose-affinity columns, respectively. Purified proteins were mixed in a binding buffer [50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl, 10% glycerol, 0.5 mg/ml BSA, 5 mM β-mercaptoethanol] at 4°C for 1 hour. Anti-GST rabbit antibody was added and the mixture was further incubated at 4°C for 1 hour. Finally, protein A resin was added to the mixture and the suspension was incubated at 4°C for 1 hour. After extensive washing in phosphate-buffered saline (PBS), the resin was collected by a brief centrifugation, and the resin was suspended in PBS. Bound proteins were eluted in SDS-PAGE sample buffer at 90°C for 10 minutes. Elutes were subjected to standard SDS-PAGE.

Electroporation into chick embryonic spinal cord

Expression vectors of N-terminally FLAG-tagged transitin were constructed in pyDF30 (Wakamatsu and Weston, 1997). pEGFP-N1, pEGFP-F and pd1EGFP were obtained from BD-Clontech. pRCAS(B) was a gift from Koji Tamura (Tohoku University, Sendai, Japan). For sustained, low levels of *Trans(1-327)-d1EGFP* expression, *Trans(1-327)* was connected in frame to *d1EGFP* (unstable mutant of *EGFP* with the attached PEST sequence), and subcloned into pRCAS(B) for a relatively weak promoter activity of the long terminal repeat (LTR), and genome integration. Electroporation into the neural tube of chick embryos was performed basically as described (Funahashi et al., 1999; Suzuki et al., 2006). For a knockdown of transitin, corresponding RNA (sense: 5′-CCCAUUGCAAUGAGCCAGGTT-3′, antisense: 5′-CCUGGCUCAUUGCAAUGGGTT-3′) was generated and annealed (Custom siRNA Synthesis, Takara), and injected into the lumen of the neural tube, along with the *EGFP* expression vector, for electroporation. For a short hairpin (sh)RNA-mediated knockdown of transitin, DNA sequence, corresponding to transitin cDNA 289-309 (5′-CCAGGG-ACAACCTGTATGAGG-3′), was inserted to pSilencer (Ambion) to generate pSilencer-*Trans289*. For negative control experiments, a mutated sequence (5′-CCAGGTACCACCTGGATAAGG-3′; mutations are underlined) was used for construction of pSilencer-*Trans289m*. BrdU pulse-labeling was performed as previously described (Wakamatsu et al., 2000).

Time-lapse imaging of cultured chick brain slices

Embryonic day (E)3 chick forebrain was electroporated with expression vectors of RCAS(B)-*Trans(1-327)-d1EGFP* and pDsRed2-nuc (purchased from Clontech, carrying a red fluorescent protein gene, *DsRed2*, fused to a nuclear localization signal of Large T antigen), E5 transfected telencephalon were sliced to 200 μm thickness with vibratome (MICROSLICER DTK-3000W, D.S.K.), embedded in a collagen gel, and Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 10% fetal calf serum and 5% horse serum was overlaid. Slices were observed in a CO_2 incubating chamber at 38°C set on an inverted fluorescence microscope (Axiovert, Zeiss), with a cooled CCD camera (AxioCam). Normal rate of NE cell proliferation and differentiation, as well as normal neuronal layer formation, were confirmed by BrdU uptake and immunostaining with anti-Hu antibody after 24 hours of culture (data not shown).

RESULTS

In vivo localization of Numb and transitin

During a search for Numb-associated proteins, we discovered that immuno-reactivity to a monoclonal antibody, 7B3 (Henion et al., 2000), showed an overlapping intracellular localization with Numb in interphase processes and in the basal cortex of mitotic NE cells in various regions of the developing central nervous system (Fig. 1A,B), as well as in the cortex of mitotic cells in the developing DRG (Fig. 1C). Because 7B3 antibody has been shown to recognize transitin, an intermediate filament protein (Henion et al., 2000), we tested another monoclonal antibody (EAP-300), which was independently raised against transitin (McCabe at al., 1992; Cole and Lee, 1997). This antibody gave identical immunoreactivity in mitotic NE cells (data not shown), thereby confirming the asymmetrical localization of transitin. Detailed observation of Numb and transitin staining revealed a faithful colocalization throughout the M phase of NE cells (Fig. 2A).

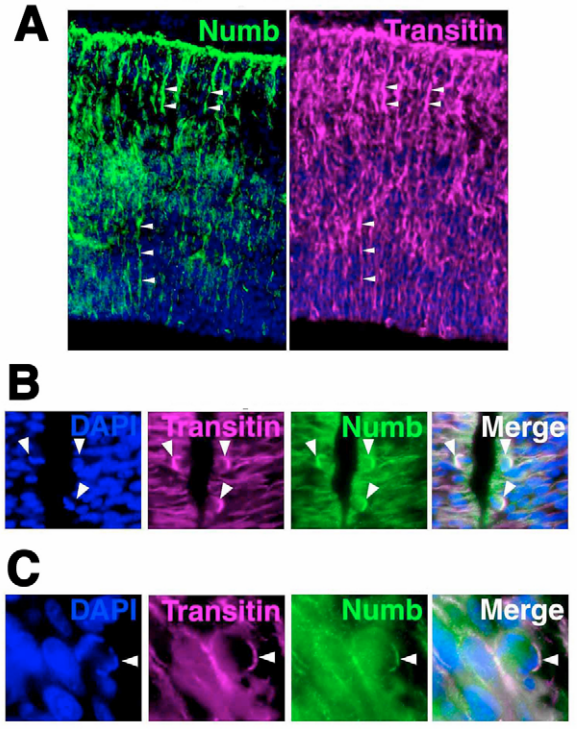

Fig. 1. Numb and transitin colocalize in developing chick neural tissues. Blue is DAPI nuclear staining. (**A**) Transverse section of an embryonic day (E)5 optic tectum, stained with anti-Numb (green) or 7B3 anti-transitin (purple). Arrowheads indicate radial fibers of neuroepithelial cells. (**B**) Transverse section of an E4 spinal cord. Three mitotic figures are indicated by arrowheads. Numb (green) and transitin (purple) colocalize in the basal side of mitotic cells. (**C**) Transverse section of E4 dorsal root ganglia. A mitotic cell in anaphase is indicated by an arrowhead. Numb and transitin colocalize in a part of the cell cortex.

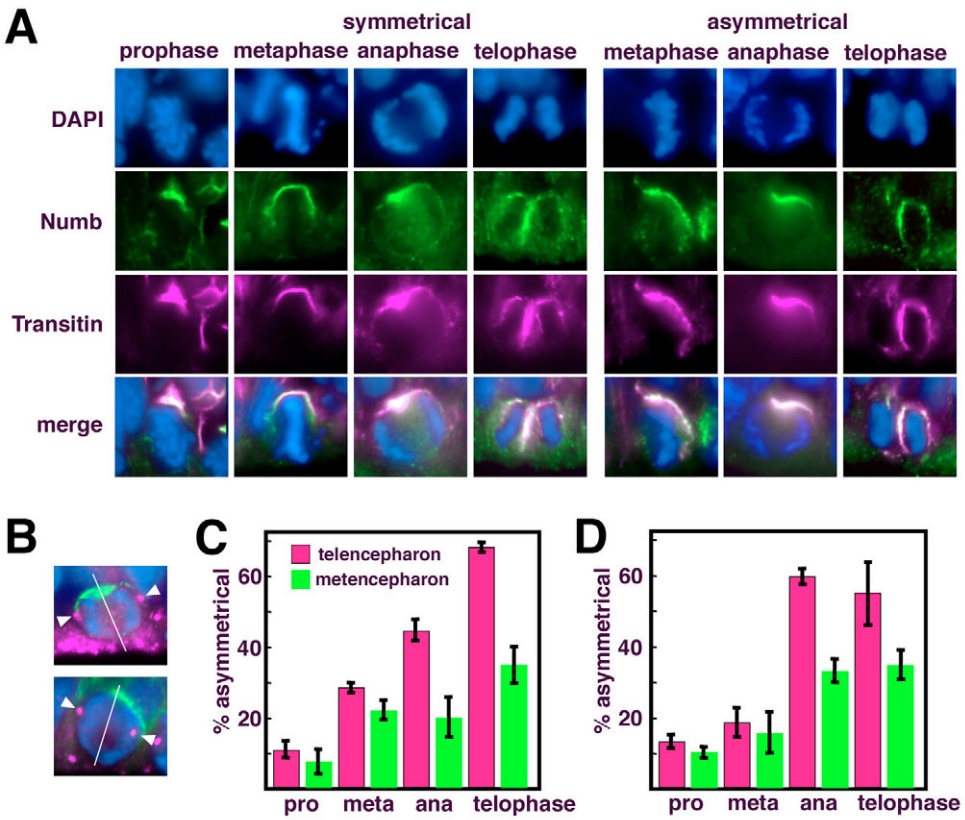

Fig. 2. Colocalization and segregation of Numb and transitin in mitotic neuroepithelial cells. (**A**) Cortical colocalization of Numb and transitin. Pallium region of an embryonic day (E)5 chick telencephalon was stained with anti-Numb (green), 7B3 anti-transitin (purple) and DAPI nuclear dye (blue). Various phases of mitosis are shown. The apical ventricular surface is towards the bottom of each panel. Although Numb and transitin localize in the basal side of the prophase cell, both symmetric and asymmetric localization/segregation are observed in meta-, ana- and telophase. (**B**) Symmetric (above) and asymmetric (below) localization of Numb in relation to the spindle pole in anaphase neuroepithelium (NE) cells. Anti-γ-tubulin staining (purple, arrowheads) is overlaid with anti-Numb (green) and DAPI (blue) staining. The predicted cleavage plane is indicated by a white line. (**C**,**D**) The proportion of asymmetrically localized Numb (C) and transitin (D) in mitotic NE cells of E5 telencephalic pallium (purple) and ventral metencephalon (green). Three embryos (approximately 300 mitotic NE cells/embryo) were examined to obtain each bar.

For asymmetrically localizing factors to be segregated unevenly to daughter cells after cell division, the localization of such factors and the cleavage orientation will have to be coordinated. In this paper, we classified the orientation of the cleavage plane (and metaphase plate) into three groups, according to Sanada and Tsai (Sanada and Tsai, 2005). Thus, cells with vertical cleavage have a mitotic spindle parallel to the ventricular surface (60-90°), cells with horizontal cleavage have spindle that are perpendicular to the surface (0-30°), and intermediate cells have spindle in between (30-60°). In the case of mammalian neocortical region (Chenn and McConnell, 1995; Sanada and Tsai, 2005), a significant proportion of mitotic NE cells showed a horizontal and intermediate cleavage plane (e.g. approximately 20% and 30%, respectively, in E15 mouse cortex) (Sadana and Tsai, 2005). We thus examined the orientation of the cleavage plane of E4 and E5 telencephalon (pallium region), and of E4 metencephalon (ventral half) of chick embryos because active neurogenesis appeared to occur at these stages, based on the expression of neuronal markers. Transverse sections were double-stained with DAPI (chromosomes) and anti-γ-tubulin antibody for spindle poles (data not shown, but see Fig. 2B). With a significant contrast to the proportion observed in mammals (Chenn and McConnell, 1995; Sanada and Tsai, 2005), approximately 14% and 5% of mitotic NE cells showed an intermediate and a horizontal cleavage plane, respectively, and more than 80% of mitotic NE cells were classified as vertical. Although a slightly angled cleavage plane could be sufficient to unevenly segregate fate determinants localized in the narrow apical endfoot, if any are localized there (see Kosodo et al., 2004), basal localization of Numb and transitin in the cell cortex seemed to be too wide. However, both Numb and transitin were often asymmetrically segregated to one of the daughter cells in telophase (Fig. 2A). Thus, the localization of Numb and transitin was further examined in relation to the cleavage plane and to the stages of M phase in mitotic NE cells of E4 pallium and ventral metencephalon (Fig. 2B-D). In both cases, the proportion of NE cells with asymmetrically-localized Numb and transitin increased in later M phase (approximately 60% and 35% of telophase NE cells in the telencephalon and metencephalon, respectively, see also Fig. 2C,D), suggesting basal Numb and transitin were relocated laterally during the M phase. Interestingly, the proportion of asymmetrically localized Numb and transitin is higher in the telencephalon than that in the metencephalon (Fig. 2C,D), which might have reflected upon the rate of neurogenesis at the stages examined.

Physical interaction between Numb and transitin

Because Numb and transitin faithfully colocalized in the cell cortex, these proteins might form a complex. To test the physical interaction of Numb and transitin, we first performed far-western blotting analysis (Wakamatsu et al., 1999). Because of the large size of transitin (Yuan et al., 1997), pieces of transitin cDNA were subcloned into bacterial expression vectors (Fig. 3A) to obtain GST-fusion of the transitin recombinants. Expression of GST-transitin fusions was confirmed by Coomassie Blue staining (Fig. 3B) and by western blotting with anti-GST antibody (data not shown). Membrane-transferred fusion proteins were overlaid with lysates prepared from *Numb*-transfected NIH3T3 cells. Binding of Numb to a fragment of a linker sequence between the rod and the heptad repeat domains of transitin (Yuan et al., 1997), Trans(328-816) (Fig. 3B), was detected by anti-Numb antibody. Because this experiment did not show whether the binding of Trans(328-816) and Numb was direct or indirect, we performed a similar assay with a recombinant MBP-fusion of Trans(328-816) and recombinant GST-fusion of Numb, and a series of Numb deletion mutant proteins (Fig. 3C). Anti-GST antibody detected the binding of Numb to Trans(328-816) (Fig. 3D), suggesting that the interaction of

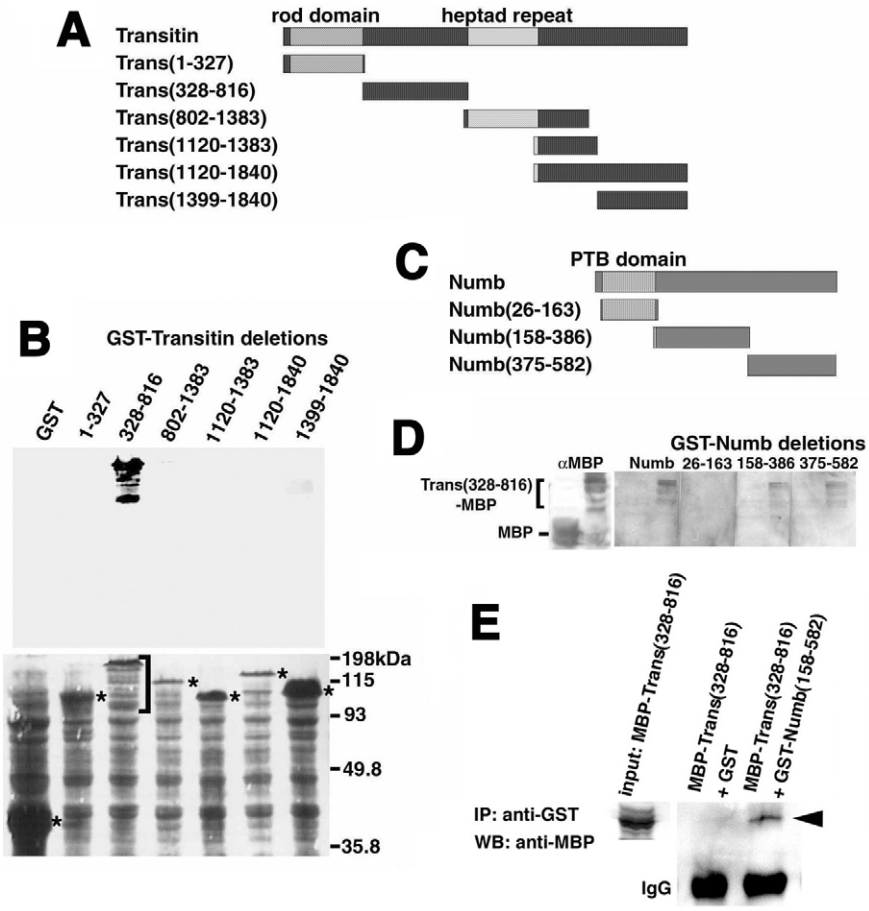

Fig. 3. Numb binds to transitin. (**A**) Structure of the chick transitin protein and of its deletion mutants. (**B**) Upper panel shows the binding of Numb that was extracted from chick *Numb*-transfected NIH3T3 cells to one GST-fused transitin deletion protein (328-816), but not to bacterially expressed GST (containing His tags and an S tag, see Materials and methods) or to the other GST-fusion of transitin deletions that were blotted. Bound Numb is detected by anti-Numb antibody. Lower panel shows Coomassie Brilliant Blue (CBB) protein staining of SDS-PAGE gel. Positions of recombinant proteins are indicated by asterisks and a square bracket. (**C**) Structure of the chick Numb protein and of its deletion mutants. (**D**) Direct binding of Numb to Trans(328-816). Whole extracts of MBP- and MBP-Trans(328-816)-fusion expressing bacteria were separated on SDS-PAGE, blotted on a membrane and incubated with purified GST-fusions of Numb. Bound GST-Numb fusions were detected by anti-GST antibody. Expression of MBP and MBP-Trans(328-816)-fusion was confirmed by anti-MBP antibody. (**E**) Transitin and Numb bind directly in a liquid-phase assay. Purified MBP-Trans(328-816) and GST-Numb(158-582) were mixed and pulled down by anti-GST antibody. Immunoprecipitated (IP) MBP-Trans(328-816) was detected by western blotting (WB) with anti-MBP antibody. MBP-Trans(328-816) used for this assay is indicated as 'input' in the left panel, detected by western blotting with anti-MBP antibody. IP, immunoprecipitated; PTB, phospho-tyrosine-binding domain; WB, western blotting.

transitin and Numb was direct. Furthermore, deletions in the *Numb* construct (Fig. 3C) revealed the interaction to be via the carboxy sequence of Numb and not the phospho-tyrosine binding (PTB) domain (Fig. 3D). The direct binding of Trans(328-816) and Numb was further confirmed by co-immunoprecipitation assay (Fig. 3E, see also Materials and methods). Thus, affinity-purified Trans(328-816)-MBP and GST-fusion of the C-terminal half of Numb [Numb(158-582)] were pre-incubated in solution, and GST-Numb(158-582) was immunoprecipitated with anti-GST antibody. Co-precipitated Trans(328-816)-MBP could be detected with anti-MBP antibody.

Transitin as an interface between cell membrane and Numb

Colocalization in vivo, and binding in vitro, both suggested that transitin forms a complex with Numb in vivo. To test whether Numb provided an anchor in the cell for transitin, or vice versa, misexpression studies were performed in chicken NE (Fig. 4). First, overexpression of *Numb* had no effect on the basal localization of endogenous transitin in mitotic NE cells (Fig. 4B), suggesting that Numb did not function as an anchor for transitin. Overexpression of the Numb-binding sequence of transitin, Trans(328-816), showed unexpected results (Fig. 4C). We found endogenous levels of Numb increased significantly without altering endogenous transitin localization. Numb was observed throughout the cytoplasm of mitotic cells, along with transgene-derived Trans(328-816), suggesting a stabilization of Numb. Because this mis-localization of Numb could be explained just by the increased levels of endogenous Numb, we hypothesized that overexpression of the rod domain of transitin, Trans(1-327), which lacked the Numb-binding sequence but could form intermediate filaments, might dilute the endogenous transitin along with its Numb-binding site in the cytoplasm. In prophase and metaphase cells with low levels of *Trans(1-327)* expression, Trans(1-327) colocalized basally with endogenous transitin and Numb (Fig. 4D), suggesting that transitin initially localized asymmetrically as filaments. Moreover, as predicted, in cells with high levels of Trans(1-327) expression, the basal localization of Numb was no longer observed (Fig. 4D).

The requirement of transitin for the basal localization of Numb was further confirmed by knockdown of transitin with RNAi (Fig. 4E,F). Double-stranded (ds)RNA, corresponding to the transitin mRNA sequence (see also Materials and methods), was electroporated into the dorsal neural tube of E2 chicken embryos, along with an expression vector of *EGFP* to visualize transfected cells. At 16 hours after transfection, compared with neural tube cells transfected with *EGFP* alone or with *EGFP* and mutated dsRNA, endogenous transitin immunoreactivity was clearly reduced in the neural tube cells co-transfected with *EGFP* and transitin double-strand RNA (Fig. 4E). Under this condition, localization of Numb in the basal and basolateral cortex of *EGFP*-transfected prophase and metaphase NE cells was examined (Fig. 4F). Transfection of *EGFP* alone or its co-transfection with mutated RNA showed no difference in the localization of Numb. Thus, 94.7% (n=95) of *EGFP*-transfected cells and 93.1% (n=72) of cells co-transfected with mutated dsRNA revealed clear basal or basolateral cortical localization of Numb. By contrast, transitin gene knockdown resulted in a severe reduction of basal or basolateral Numb

immunoreactivity in the cell cortex (Fig, 4E), and only 31.6% of transfected mitotic NE cells (n=80) showed such localization of Numb. These observations suggested that, in mitotic NE cells of chick embryos, transitin localized in the basal cortex and provided anchor sites for Numb.

Dissociation of intermediate filament structure during mitosis

Transitin was initially identified as an intermediate filament-associated protein that was co-purified with classical intermediate filaments such as vimentin and desmin (Breckler and Lazarides, 1982). In fact, full-length transitin required either vimentin or desmin to participate in stable intermediate filament formation, unless its C-terminal tail was removed. Because vimentin is expressed in NE and is colocalized in the radial processes of interphase NE cells (Fig. 5A), we compared the intracellular localization of vimentin and transitin in mitotic NE cells. vimentin colocalized basally with transitin from prophase to metaphase (Fig. 5A,B). However, from anaphase, basal localization of vimentin was no longer observed and, instead, punctate vimentin immunoreactivity was observed in the cytoplasm (Fig. 5B). This observation contrasted with the persistent cortical localization of transitin throughout mitosis (Fig. 5C,D, lower panels). It has been shown previously that intermediate filament proteins, such as vimentin, are phosphorylated in M phase, and such phosphorylation leads to the disassembly of the intermediate filaments (Ando et al., 1989; Goto et al., 1998). Consistently, phosphorylated vimentin was observed in M-phase NE cells (Fig. 5C,D upper panels). Interestingly, phospho-vimentin (Ser39) was seen in the basal cortex and in the cytoplasm (Fig. 5C), whereas phospho-vimentin (Ser72) was detected throughout the cytoplasm from prophase to metaphase (Fig. 5D). Phosphorylation of vimentin and dissociation from the basal cortex suggested that, in the basal cortex of mitotic NE cells, transitin no longer participates in the rigid intermediate filament structure and that transitin-Numb complexes may be present in a more flexible form in the cell cortex.

Dynamic movement of transitin in mitosis

As described above, endogenous Numb-transitin complexes appeared to be relocated during mitosis. Therefore, we were motivated to examine the orientation of cell cleavage and the movement of Numb-transitin during mitosis of NE cells in live embryos. As indicated above, FLAG-tagged Trans(1-327) could localize in the basal cortex of mitotic NE cells when the expression level was relatively low (Fig. 4D), probably by participating in intermediate filament structure. We generated an expression vector of *d1EGFP*-fusion of *Trans(1-327)* (see Materials and methods), and found that Trans(1-327)-d1EGFP fusion proteins faithfully colocalized in the basal cortex of mitotic NE cells with endogenous transitin (Fig. 6A) and Numb (Fig. 6B). We then transfected this construct into the dorsal telencephalon (pallium region) of E3 chick embryos, and slices of the transfected telencephalon were prepared 2 days later. The localization of Trans(1-327)-d1EGFP was then examined in slice culture (see Materials and methods). An expression vector of *DsRed2* with a nuclear localization signal (pDsRed2-nuc, see Materials and methods) was sometimes co-transfected, allowing chromosomes on the metaphase plate and the cleavage orientation to be observed, when co-expressed. Out of 53 mitotic NE cells examined, only one of them revealed a nearly horizontal cleavage plane (Table 1). Consistent with the observation in fixed tissues (see above), in 52 mitotic cells, the cleavage plane was almost vertical (Table 1). In 18 cases of the 52 mitotic cells vertically dividing, Trans(1-327)-d1EGFP was not actively transported, and was segregated evenly to both of the

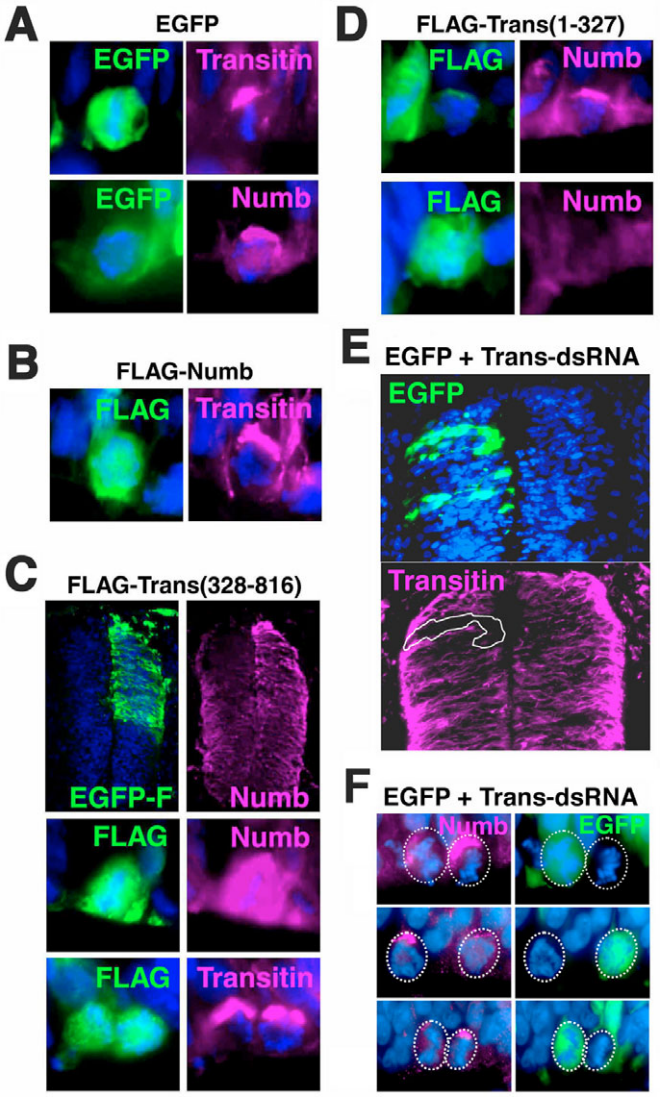

Fig. 4. Effects of transitin deletions in transfected neuroepithelial cells. Transfected spinal cord was examined 1.5 days after transfection. (**A**) *EGFP* transfection does not affect the basal localization of transitin (purple, upper panels) or Numb (purple, lower panels) in mitotic neuroepithelial cells. (**B**) Overexpression of FLAG-Numb has no effect on transitin localization. (**C**) Transfection of FLAG-Trans(328-816) increases Numb expression (low magnification in upper panels and high magnification in middle panels). EGFP-F shows a transfected area. No effect on transitin localization was observed (lower panels). (**D**) Weakly expressed FLAG-Trans(1-327) colocalizes with Numb in the basal cortex (upper panels), whereas high levels of FLAG-Trans(1-327) disrupts Numb localization (lower panels). (**E**,**F**) RNAi-mediated depletion of transitin causes a loss of basal Numb crescent. A mixture of *EGFP* expression vector and double-stranded (ds)RNA corresponding to the transitin gene sequence was electroporated into a developing neural tube. Transfected cells (green) show little staining for anti-transitin (E, outlined). (F) Transfected mitotic neuroepithelium (NE) cells (green) possess little anti-Numb immunoreactivity, whereas untransfected neighboring mitotic cells have apparent basal Numb crescent. Circled areas show individual cells. (A-F) Basal is up, except for low-magnification pictures of spinal cord in C. Blue is DAPI nuclear staining.

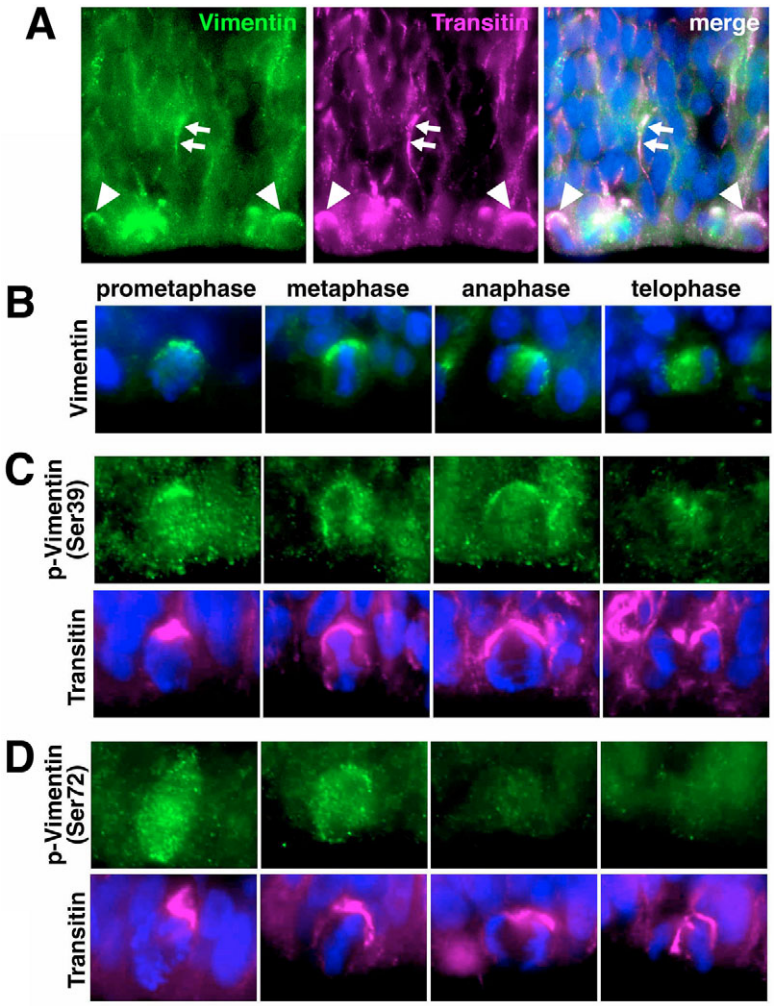

Fig. 5. Basal localization of vimentin is dispersed in mitosis, whereas transitin persists in the cell cortex. Blue is DAPI nuclear staining. Basal is up. (**A**) Colocalization of vimentin (green) and transitin (purple) in radial fibers of neuroepithelium (NE) cells (arrows) and in the basal cortex of mitotic NE cells (arrowheads) is observed. (**B**) Non-filamentous vimentin staining (green) is observed basally in prometaphase, but dissociated from the cortex in ana- and telophase. (**C**,**D**) Phosphorylated vimentin is found abundantly in early M phase. Transitin persists in the cortex throughout M phase (lower panels). In the late phase of mitosis, transitin is preferentially segregated in one of the two daughter cells, despite the cell cleavage plane being parallel to the apicobasal axis.

daughter cells (Table 1). In the remaining 34 cases, the fusion protein appeared to shift laterally from late metaphase to early anaphase (Fig. 6C, and see Movie 1 in the supplementary material), during which time Trans(1-327)-d1EGFP would be allowed to segregate to one of the daughter cells (Fig. 6C). However, unlike endogenous transitin, the asymmetric cortical localization of Trans(1-327)-d1EGFP was gradually lost during anaphase, suggesting a disassembly of intermediate filament (see above). Interestingly, we occasionally observed that oblique metaphase plate orientation rotates along with Trans(1-327)-d1EGFP during mitosis of NE cells (Fig. 6C), as observed in the mouse cortex (Sanada and Ysai, 2005).

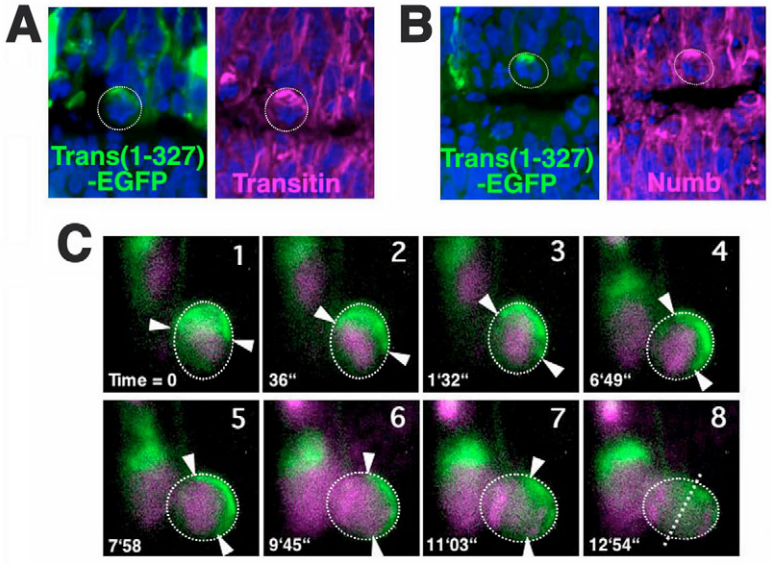

Fig. 6. Dynamic lateral transport of Trans(1-327)-d1EGFP in mitotic neuroepithelial cells. Blue is DAPI nuclear staining. Basal is up. (**A**,**B**) Trans(1-327)-d1EGFP (green) basally colocalizes with endogenous transitin (A) and Numb (B) in mitotic neuroepithelium (NE) cells (outlined). (**C**) Time-lapse analysis of sliced, cultured neuroepithelium co-transfected with *Trans(1-327)-d1EGFP* (green) and *DsRed2-nuc* (purple), indicating chromosomes. Cell shape is outlined. The border of Trans(1-327)-d1EGFP localization is indicated by arrowheads. A broken straight line in panel 8 indicates the cleavage plane. 'Time=0' indicates the start point of observation.

Table 1. Summary of time-lapse observation of the cell cleavage plane and movement of Trans(1-327)-d1EGFP

Cleavage plane* Localization†	Horizontal Symmetric	Horizontal Asymmetric	Vertical Symmetric	Vertical Asymmetric
Number of cells	0	1	18	34

*The cleavage plane of mitotic neuroepithelial cells was classified as 'horizontal' or 'vertical' to the ventricular surface.
†Transport pattern of Trans(1-327)-d1EGFP was classified as 'symmetric' or 'asymmetric' when the fusion protein was either statically localized in the basal cortex or was laterally transported towards one of the sister cells, respectively.

Involvement of transitin in neurogenesis

Taken together, the fact that Numb-transitin complexes seemed to be segregated asymmetrically in a significant proportion of mitotic NE cells, and that Numb is a fate determinant, transitin might be involved in the fate determination by controlling the segregation of Numb. Thus, the effect of transitin RNAi on cell fate was examined (Fig. 7). Because the effect of transitin dsRNA seemed to not last for long enough to examine neurogenesis, an expression vector of transitin short hairpin RNA (shRNA) was constructed for RNAi (pSilencer-*Trans289*), along with an expression vector carrying a mutated sequence (pSilencer-*Trans289m*) for negative control experiments. Such vectors were co-transfected into the newly formed neural tube with *EGFP*, and we examined, at a dorsoventrally intermediate level, BrdU uptake for proliferation, TUNEL staining for cell death and neuronal marker expression for differentiation (Fig. 7). Transfection of *Trans289* resulted in a significant decrease of 7B3 immunoreactivity (Fig. 7A), confirming a successful knockdown of transitin, whereas *Trans289m* showed no significant change in transitin expression, as expected (data not shown). *Trans289* transfection also caused a decrease in Numb level in the basal processes of NE cells (Fig. 7B), supporting the idea that the association with transitin stabilizes Numb (see above), although the cytoplasmic pool of anti-Numb immunoreactivity was not significantly affected (Fig. 7B).

Compared with embryos transfected with an empty pSilencer or *Trans289m* as negative controls, the proportion of BrdU-positive cells was significantly reduced at 24 hours after transfection of the *Trans289* shRNA expression vector (Fig. 7C,D). Knockdown of transitin also increased cell death, revealed by TUNEL staining both 24 and 48 hours after transfection (Fig. 7E), but a similar increase of cell death was observed even when *Trans289m* was transfected (Fig. 7E), suggesting that

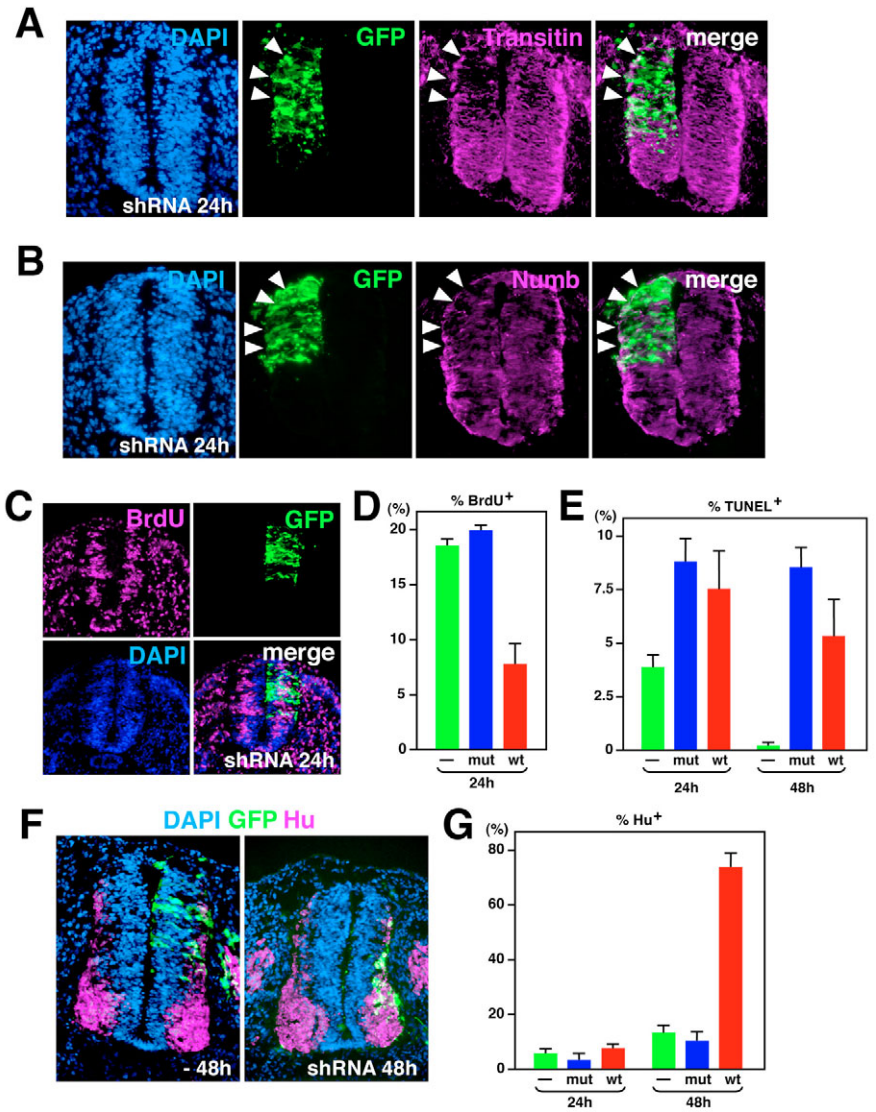

Fig. 7. Effects of transitin knockdown by shRNA on neuroepithelium cell fate. (**A**) Transfection with pSilencer-*Trans289* shRNA expression vectors successfully knockdowns transitin expression. At 24 hours after electroporation the transfected area, revealed by the expression of co-transfected *EGFP*, shows weak expression of transitin (purple; arrowheads). (**B**) *Trans289*-transfection results in a downregulation of Numb expression (purple). The reduction of anti-Numb immunoreactivity is evident in the basal processes of neuroepithelium (NE) cells. (**C**) BrdU uptake of neural tube cells co-transfected with *EGFP* and pSilencer-*Trans289*. Little overlap of GFP-fluorescence and anti-BrdU immunostaining is observed. (**D**) The proportion of BrdU-GFP double-positive neural tube cells co-transfected with *EGFP* and empty pSilencer (–; green), or pSilencer carrying mutated transitin sequence (*Trans289m*, mut; blue), or pSilencer carrying the wild-type transitin sequence (*Trans289*, wt; red), 24 hours after transfection. (**E**) Proportion of TUNEL-GFP double-positive neural tube cells co-transfected with *EGFP* and pSilencer (–), *Trans289m* (mut), or *Trans289* (wt) 24 and 48 hours after transfection. (**F**) Neuronal differentiation of transfected neural tube cells co-transfected with *EGFP* and *Trans289* (wt) 48 hours after transfection. Whereas most empty pSilencer-transfected cells remain Hu-negative in the ventricular zone (left panel), *Trans289*-transfected cells migrate basally, express Hu and intermingle with untransfected neurons (right panel). (**G**) The proportion of Hu-EGFP double-positive neural tube cells co-transfected with *EGFP* and pSilencer (–), *Trans289m* (mut), or *Trans289* (wt) 24 and 48 hours after transfection. Five embryos (approximately 200-300 cells/embryo) were examined to obtain each bar in D,E,G. Error bars indicate standard deviations.

overexpression of shRNA caused non-specific cell death. Expression of neuron-specific type III β-tubulin (TuJ) (Lee et al., 1990) and Hu RNA-binding protein 16A11 (Marusich et al., 1994; Wakamatsu and Weston, 1997) was also examined both 24 and 48 hours after transfection (Fig. 7F,G). Although no significant change in neurogenesis ahead of schedule was observed 24 hours after transfection of *Trans289*, most transfected cells expressed Hu (Fig. 7F) and TuJ1 (data not shown), and intermingled with untransfected neurons in the neuronal layer (Fig. 7F). Thus, transitin knockdown inhibited proliferation and promoted neuronal differentiation.

DISCUSSION

Cell cleavage plane and asymmetric cell division

It has been described previously that, in the mammalian cortical region, a significant proportion of mitotic NE cells shows horizontal and intermediate cleavage planes (Chenn and McConnell, 1995; Sanada and Tsai, 2005), although it remains to be seen whether such angled cell cleavage will indeed result in the uneven segregation of fate determinants. In avian brains, more than 80% of NE cells show a vertical cleavage plane, similar to that in the neural retina (Silva et al., 2002). Although the cleavage plane and the fate of daughter cells has not yet been correlated in avian systems, because Numb and transitin can be segregated asymmetrically even with vertical cleavage, the rate of asymmetric cell division can be underestimated in experiments if only the cleavage plane is used as an indicator of asymmetric cell division.

Transitin anchors Numb to the basal cortex

Previously, we have reported that Numb localizes in the basal cortex of mitotic NE cells (Wakamatsu et al., 1999). In this study, we show that the intermediate filament protein transitin provides an anchor site for Numb in the basal cortex of mitotic NE cells. How transitin initially localizes to the basal cortex of prophase NE cells is not clear, but it was previously reported that transitin mRNA is preferentially transported to the basal processes of interphase NE cells (Lee and Cole, 2000). It is therefore possible that locally translated transitin in the basal processes may be transported to the cortex prior to mitosis. Consistent with this idea, in our time-lapse analysis we often observed that Trans(1-327)-d1EGFP in the basal process moved apically prior to the M phase (Y.W. and N.N., unpublished observation).

The regulatory mechanisms of asymmetric cell division have been extensively studied in *Drosophila* nervous system development, and Numb localizes asymmetrically in mitotic neural cells (for a review, see Roegiers and Jan, 2004). Despite many similarities in vertebrates and invertebrates in the regulatory mechanism of development, however, chick and *Drosophila* now appear to have some differences, because the genome project of *Drosophila* showed that this widely used experimental animal does not have cytoplasmic intermediate filaments (Rubin et al., 2000). Even in vertebrates, the molecular machinery to control Numb localization does not appear to be conserved, because mouse Numb localizes in the apical side of NE cells (Zhong et al., 1996), whereas avian Numb localizes in the basal cortex (this study) (see also Wakamatsu et al., 1999). Nestin is the closest relative of transitin, because they are categorized in the same intermediate filament subclass due to the sequence homology in their rod domain, and because they are expressed in NE cells and muscle precursors (for a review, see Herrmann and Aebi, 2000). However, the sequence of the C-terminal tail, which is responsible for Numb-transitin association, is not conserved in nestin (Y.W., unpublished data), and, more importantly, nestin is not asymmetrically localized in mitotic NE cells (YW, unpublished observation). Although it is not known whether nestin is involved in neurogenesis, for reasons mentioned above, nestin does not seem to directly regulate Numb localization in mouse NE cells.

Lateral transport of transitin in mitosis

In this study, we show that, even if the vertical cleavage plane would result in a horizontal cell division, such cells can still segregate Numb-transitin complexes asymmetrically, because these components, anchored within the basal cortex, shift laterally in late M phase, and thereby allow preferential segregation into one of the two daughter cells. It remains to be studied how the lateral transport of Numb-transitin complexes is regulated. Because, in a third of NE divisions, Trans(1-327)-d1EGFP still remained in the basal cortex and segregated symmetrically, some unknown mechanism(s) probably determines whether the lateral transport during M phase is initiated. It is of note that, at the early phase of mitosis in NE cells, vimentin is phosphorylated, which probably leads to the dissociation of the intermediate filament structure. It has been shown that such phosphorylation-dependent dissociation of intermediate filaments in the M phase is important for cytokinesis (Ando et al., 1989; Goto et al., 1998) and that Aurora B activity, which is strictly regulated during M phase, is involved in this process (Goto et al., 2003). Thus, dissociation of rigid intermediate filament structure of vimentin-transitin by phosphorylation of vimentin in the early M phase may permit the transport of transitin. Such dissociation of intermediate filament in M phase is consistent with the fact that cortically-localized Trans(1-327)-d1EGFP becomes cytoplasmic in the late M phase.

Transitin in neurogenesis

As mentioned above, *Numb* has been shown to regulate neurogenesis in mouse embryos, both positively and negatively (Zhong et al., 2000; Zilian et al., 2001; Peterson et al., 2002; Peterson et al., 2004; Li et al., 2003). The cause of such discrepancy is unclear, but changes in the expression of Numb isoforms during development (Bani-Yaghoub et al., 2007) might explain the differences observed between mouse knockout lines, at least in part. In any case, the requirement of transitin for the proper intracellular localization of Numb suggests the involvement of transitin in the neurogenesis of avian embryos. Consistently, transitin knockdown causes a depletion of NE cells by reducing proliferation and promoting neuronal differentiation, although how the reduction of transitin expression causes such a phenotype remains elusive. One possibility is that, because transitin stabilizes Numb (see above), transitin knockdown may lead to the reduction of Numb protein, which would otherwise inhibit neurogenesis. This idea is consistent with the observation that *Numb*-knockout mice show precocious neurogenesis (Zhong et al., 2000; Peterson et al., 2002; Peterson et al., 2004). Alternatively, by losing the transitin anchor, a release of functional Numb in the cytoplasm may promote neurogenesis, possibly by inhibiting Notch signaling. This idea is consistent with the decreased neurogenesis observed in certain *Numb*-knockout mouse lines (Zilian et al., 2001; Li et al., 2003). Because a recent study (Zhou et al., 2007) suggests that *Numb* may also influence neurogenesis independently of Notch signaling, it seems important to knockdown *Numb* in avian system in order to compare the phenotype with that of mouse knockouts. Nevertheless, transitin is unique, because no other intermediate filament protein has been shown to regulate neurogenesis.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/13/2425/DC1

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