NAP1 REGULATED CYTOSKELETAL DYNAMICS DURING CORTICAL NEURONAL DEVELOPMENT

YUKAKO YOKOTA

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Approved by:

Dr. Eva S. Anton

Dr. Richard E. Cheney

Dr. Kenneth A. Jacobson

Dr. Larysa H. Pevny

Dr. Benjamin D. Philpot

ABSTRACT

YUKAKO YOKOTA: Nap1 regulated cytoskeletal dynamics during cortical neuronal development
(Under the direction of Dr. Eva S. Anton)

Appropriate neuronal positioning and differentiation in the developing cerebral cortex requires dynamic regulation of neuronal cytoskeleton. Although the significance of cytoskeletal dynamics during the neuronal proliferation and migration in cerebral cortex is well established, the cytoskeletal regulators that choreograph the change in the neuronal cytoskeletal machinery from one that promotes oriented motility to one that facilitates stable laminar positioning and elaboration of axons and dendrites at the appropriate locations in the cerebral cortex remains unknown. We found that Nck associated protein 1 (Nap1), an adaptor protein that is thought to modulate actin nucleation, is selectively expressed in the cortical plate region of the developing cortex, where neurons terminate their migration and initiate their laminar specific differentiation. Inhibition of Nap1 disrupts neuronal differentiation. Premature expression of Nap1 in migrating neurons retards their migration and promotes their layer specific, post migratory differentiation. Nap1 mutant mice in which the function of the Nap1 gene has been disrupted by insertional mutagenesis display

profound neural tube formation and neuronal differentiation defects. Nap1 mutation disrupts lamellipodial formation and the ability to localize key actin cytoskeletal regulators such as WAVE1 to the protrusive edges of neurons where they are needed to elaborate process outgrowth. Together, these studies suggest that Nap1 may play an essential role in triggering the neuronal cytoskeletal changes underlying the post-migratory differentiation of cortical neurons, a critical step in functional wiring of the cerebral cortex.

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ABBREVIATIONS

aa	amino acid
Abi	Abl (Abelson tyrosine kinase) interactor
ASPM	abnormal spindle-like microcephaly associated protein
BAC	bacterial artificial chromosome
BDNF	brain derived neurotrophic factor
bp	base pair
CAD cell	catecholaminergic differentiated cell
Cdk5	cyclin dependent kinase 5
CGE	caudal ganglionic eminence
CNS	central nervous system
CP	cortical plate
Dcx	doublecortin
DISC1	Disrupted-in-Schizophrenia-1 gene
E	embryonic day
EM	electron microscope
FLIP	Filamin A interacting protein
FLNA	filamin A
HSPC300	Heat shock protein C 300
IP	immunoprecipitation
IZ	intermediate zone

JNK1	c-Jun N-terminal kinases
Kb	kilo bases
kD	kilodalton
KIF2A	Kinesin superfamily protein 2A
Lis1	lissencephaly 1 protein
MALDI	matrix assisted laser desorption/ionization
Map	microtubule associated protein
MGE	medial ganglionic eminence
MTOC	microtubule organizing center
MZ	marginal zone
Nap1	Nck associating protein 1
Nde1 (mNudE)	mouse homolog of NUDE, a nuclear distribution gene in *A. nidulans*
Ndel1 (NUDEL)	nuclear distribution element-like
NF	neural folds
nt	nucleotides
ORF	open reading flame
P	postnatal day
PDGF	platelet derived growth factor
PFA	paraformaldehyde
PH	Plekstrin Homology
PIR121	p53-inducible mRNA 121

PV	parvalbumin
RNAi	RNA interference
SEM	Scanning electron micrograph
SH3	Src homology 3
Sra-1	p140 Specifically Rac-associated protein-1 (mammalian homologue of PIR121)
SST	somatostatin
SVZ	subventricular zone
VZ	ventricular zone
WAVE	Wiskott-Aldrich syndrome protein (WASP)—family verprolin homologous protein 1
WB	Western blot
WT	wild type

CHAPTER I

BACKGROUND AND SIGNIFICANCE

Overview of cerebral cortex development

A sheet of neuroepithelial cells at the anterior end of neural tube give rise to the forebrain including cerebral cortex, hippocampus, and thalamus. During early stages of development, the neural precursor cells located at ventricular zone (VZ), the most inner layer surrounding the lumen of the neural tube, undergo a stereotype-pattern of inter-kinetic cell movement as they progress through the mitotic cycle. This leads to the generation of either new progenitor cells or postmitotic neuroblast that further differentiate into neurons. Neuronal layers in the cerebral cortex are generated in a precisely orchestrated inside-out pattern (Rakic, 1974; Altman, 1965). The final positioning of cortical neurons during development is achieved through a process of active migration of postmitotic neurons from the proliferative ventricular zone (VZ) and subventricular zone (SVZ) to their final sites of differentiation in the cortical plate (CP). The first cohort of post mitotic neurons migrate out of the proliferative ventricular zone to top of the emerging cerebral cortex to generate a layer of neurons called the preplate. The subsequent wave of neuronal migration splits the preplate into two layers, the superficial marginal zone and deeper subplate. Early generated neurons of the preplate and deeper layers migrate radially using somal

translocation, whereas later generated neurons utilize elongated radial glial guides to reach their target locations at the interface between CP and marginal zone (MZ) (Rakic, 1972; Sidman and Rakic, 1973; Hatten and Mason, 1990; Takahashi et al., 1990; Miyata et al., 2001; Nadarajah et al., 2001; Rakic, 2003).

Cortical interneurons migrate tangentially into the cerebral wall from the ganglionic eminence prior to radial migration to the cortical plate (deCarlos et al., 1996; Anderson et al., 1997; Ang et al., 2003). Most (~60%) of the cortical interneurons are derived from medial ganglionic eminence (MGE) and express either parvalbumin (PV) or somatostatin (SST). Some of the deeper layer cortical interneurons are also thought to arise from caudal ganglionic eminence (CGE). Irrespective of the type or origin, newly born neurons must migrate past previously generated neurons to the same address at the CP/MZ interface. At this interface, movement of neurons stops abruptly and cohorts of neurons begin to assemble into their respective layers. Even though radially migrating pyramidal neurons and tangentially migrating interneurons are generated in distinct proliferative niches and take different migratory routes into the cortex, neurons that are born at the same time share the same layer in the cerebral cortex. Once these neurons arrive at the final destination in the developing cerebral cortex, they initiate their final differentiation, extending axons and dendrites to form the characteristic patterns of synaptic connectivity.

Cytoskeletal dynamics during cortical development

Molecular signals that control the initiation, maintenance, and termination of migration ultimately have to be transmitted via membrane

receptors to neuronal cytoskeleton to effect distinct changes in neurons' migratory behavior. Dynamic regulation of neuronal cytoskeletal machinery in response to extracellular cues enables distinct changes in neuronal migration in the developing cerebral cortex (Cameron and Rakic, 1994; Rivas and Hatten, 1995). Molecular analysis of human migrational deficits strongly suggests that dynamic regulation of neuronal cytoskeleton leads to the sequential unfolding of the birth, migration, and placement of neurons in cortex. A microtubule associating protein (abnormal spindle-like microcephaly associated protein: ASPM) is expressed specifically in the VZ and is thought to modulate spindle activity of the neuronal progenitor cells to generate the appropriate number of post mitotic migrating neurons. Mutations in ASPM lead to microcephaly in humans (Bond et al., 2002). The significance of actin cytoskeleton in facilitating appropriate neuronal migration is evident in humans with periventricular heterotopia, where mutations in actin-binding protein filamin a (FLNA) results in failure of neuronal migration and accumulation of neuroblasts in the ventricular zone of cerebral cortex (Eksioglu et al., 1996; Fox et al., 1998; Moro et al., 2002; Sheen et al., 2001, Sarkisian et al., 2006). Proper expression of FLNA regulates the changes in cell shape from multipolar to bipolar state, needed for initiation of migration. Filamin A interacting protein (FLIP) is expressed in the VZ and is thought to degrade FLNA, thereby inhibiting premature onset of neuronal migration from the ventricular zone (Nagano et al., 2002, 2004). Another actin-associated protein that regulates initiation and maintenance of neuronal migration is non-muscle myosin II. The forward movement of the nucleus in migratory neurons is blocked by blebbistatin, a specific inhibitor of nonmuscle

myosin II. Accumulation of myosin II at the rear of migrating neurons and the resultant contraction of actomyosin is thought to play a prominent role in driving the forward translocation of the nucleus toward the centrosome (Bellion et al., 2005; Schaar and McConnell., 2005). Accordingly, mice with point mutation in Myosin IIB display wide range of abnormal neuronal migration (Ma et al., 2006).

Significant deficits in neuronal migration are also observed in following mutations in genes regulating microtubule cytoskeleton (Solecki et al, 2004). For example, Lis1 binds to microtubules, motor protein dynein, and related microtubule interactors such as dynactin, Ndel1 and Nde1 (Sapir et al, 1997; Efimov and Morris, 2000; Faulkner et al, 2000; Feng and Walsh, 2004; Kitagawa et al., 2000; Niethammer et al., 2000; Sasaki et al., 2000; Smith et al., 2000; Shu et al., 2004). During neuronal migration, this microtubule associated molecular network couples the centrosome and nucleus, resulting in the oriented translocation of nucleus (Shu et al., 2004; Solecki et al., 2004; Tanaka et al., 2004; Xie et al., 2003). In humans, mutation in Lis1 leads to Miller-dieker syndrome, a severe form of lissencephaly (Dobyns et al., 1993; Hattori et al., 1994). In mouse, truncation of Lis1 or partial loss of Lis1 leads to neuronal migrational defects and cortical disorganization (Hirotsune et al., 1998; Cahana et al., 2001). Loss of Lis1 leads to concentration of microtubules around the nucleus and failed dynein aggregation, whereas overexpression of Lis1 causes transport of microtubules to edges of the cell and aggregation of dynein and dynactin (Sasaki et al., 2000; Smith et al., 2000). Recently, Ndel1 and Lis1 were shown to interact with DISC1, a microtubule associating protein identified as a regulator of neuronal migration and as a strong susceptibility gene for schizophrenia (Millar et al., 2000; Kamiya et al., 2005).

Mutations in doublecortin (Dcx), another microtubule associated protein in migrating neurons, leads to X-linked lissencephaly (double cortex syndrome) in humans (des Portes et al., 1998; Gleeson et al., 1998, 1999). In these patients, neurons that migrated aberrantly are deposited in a broad band in subcortical layers. Dcx is critical for the stabilization of microtubule network (Francis et al., 1999; Gleeson et al., 1999; Horesh et al., 1999; Taylor et al., 2000). Dcx can associate with Lis1 and promote tubulin polymerization *in vitro* (Gleeson et al., 1999; Caspi et al., 2000; Feng and Walsh, 2001). Overexpression of Dcx results in aggregates of thick microtubule bundles resistant to depolymerization (Gleeson et al., 1999). Filamin1, Nde1 and Dcx are putative substrates for cyclin dependent kinase 5 (Cdk5) phosphorylation (Fox et al., 1998; Niethammer et al., 2000; Feng and Walsh, 2001; Tanaka, 2004). Cdk5 is expressed in migrating neurons and axonal growth cones of the developing cortex. Mice deficit with cdk5 and its subunit p35 and p39 display abnormal neuronal migration and placement in cerebral cortex (Ohshima et al., 1996; Chae et al., 1997; Gilmore et al., 1998; Kwon and Tsai, 1998; Nikolic et al., 1998; Ko et al., 2001). Mutations in other microtubule associating proteins such as Map 1/2 (Takei et al., 2000), Map 1b (Del Rio et al., 2004), KIF2A (Homma et al., 2003), tau (Dawson et al., 2001; Harada et al., 1994) and JNK1 (Chang et al., 2003) also lead to deficits in either neuronal migration or axon growth and guidance in the cerebral cortex.

Terminal phase of neuronal migration

Although the significance of cytoskeletal dynamics during the neuronal proliferation and migration is well established, the question of how the dynamic

regulation of neuronal cytoskeleton modulates the terminal phase of neuronal migration and leads to the final placement of neurons in cortex remains unknown. This final stage of neuronal migration is the least explored aspect of neuronal migration, in spite of its significance for genetics and acquired cortical malformations (Rakic, 1998; Rakic and Caviness, 1995; Olson and Walsh, 2002). The signal to terminate neuronal cell migration is thought to be provided either by the afferent fibers that migrating neurons encounter near their target location or by the ambient neuronal cell population that had already reached their final position (D'Arcangelo et al., 1995; Hatten and Mason, 1990; Ogawa et al., 1995; Sidman and Rakic, 1973). Alternatively, a change in the cell surface properties of the radial glial substrate at the top of the cortical plate may signal a migrating neuron to stop, detach, and differentiate. Translation of these signals to appropriately modify neuronal cytoskeleton critically influences the transition from migratory to post-migratory differentiation of cortical neurons.

To identify specific regulators of neuronal cytoskeleton during final phases of migration and neuronal placement, we looked for murine orthologues of *Drosophila* or *C.elegans* genes that are known to regulate distinct aspects of neuronal cell migration. Through this screening, we found that Nck associating protein 1 (Nap1), an adaptor protein that activates actin nucleation (Hummel et al., 2000, Bogdan and Klambt, 2003, Soto et al., 2002), is selectively expressed in the cortical plate regions of the developing cortex, where neurons terminate their migration and start differentiation.

Discovery of Nap1

Nap1 was first cloned as a binding protein to Src homology 3 (SH3) domain of Nck adaptor protein, with size of 125kDa contains multiple putative membrane binding domains (Baumgartner et al., 1995). Nap1 gene family is highly conserved through out evolution from *C.elegans* to human. Murine Nap1 is 40%, 60% and 99% identical in amino acid sequence level with *C.elegans*, *Drosophila* and human, respectively. In *C.elegans*, Nap1 homologue, GEX3 is shown to regulate cell migration and cell shape changes required for proper morphogenesis (Soto et al., 2002). In *Drosophila*, the Nap1 homologue, Kette is expressed uniformly during early embryogenesis, but becomes restricted to the nervous system during later stages of embryonic development (Baumgartner et al., 1995). The loss of Kette results in failure in axonal path finding as well as defective in glial migration. In both *C.elegans* and *Drosophila*, loss of Nap1 leads to embryonic lethality (Soto et al., 2002; Hummel et al., 2000; Bogdan et al., 2003). In human, Nap1 is expressed very broadly, but the strongest expression is observed in the brain. Interestingly, Nap1 was found to be down regulated in the patients with Alzheimer's disease, suggesting involvement in neuronal cell death. However, the underlying mechanism of Nap1 in this aspect is largely unknown (Suzuki et al., 2000).

Cellular function of Nap1

Nap1 mediates actin nucleation by forming a complex with WAVE, PIR121, Abi1/2 and HSPC300. Nap1 has been shown to directly interact with Abi1 and PIR121. Currently two models are proposed on how this complex activates

actin nucleation upon Rac activation. In the first model, the complex comprising Nap 1 modulates the activity of associated WAVE by dynamically relocalizing the complex to membrane edges upon Rac activation, causing actin nucleation and membrane protrusions (Stradal et al., 2004). In the alternative model, the Nap1 complex keeps WAVE inactive during resting state. Upon Rac activation, the complex releases WAVE and the liberated WAVE activates actin nucleation at the membrane edges (Cory and Ridle, 2002). Systematic, individual ablation of the expression of Nap1, WAVE, PIR121 and Abi1 by RNAi based approaches in *Drosophila* S2 Schneider cells and mammalian cell lines diminish Rac-induced actin remodeling and lamellipodia formation (Rogers et al., 2003, Kunda et al.,2003, Innocenti et al.,2004, Steffen et al.,2004).

A hypothesis of Nap1's role in cortical development

Despite these recent advances in our understanding of Nap1's cellular function and importance in invertebrate development, the role of Nap1 in the developing mammalian brain is largely unknown. The selective expression of Nap1 in the cortical plate region of the developing cerebral cortex, where neurons terminate their migration and begin to elaborate their layer specific characteristics leads us to hypothesize that Nap1 may play a role in the terminal phase of neuronal migration and the subsequent initiation of neuronal differentiation.

CHAPTER II

NAP1 EXPRESSION DURING THE DEVELOPMENT OF CEREBRAL CORTEX

Introduction

To investigate Nap1's functions during cortical development, we will map the spatial, temporal, and cellular pattern of Nap1 distribution in the developing mouse cerebral cortex. Determining the pattern of Nap1 expression in the developing cerebral cortex will be essential to evaluate if Nap1 is expressed in the right place at the right time to critically influence the transition from migratory to post-migratory differentiation of cortical neurons. This correlative study, establishing a link between the spatio-temporal pattern of expression of Nap1 with distinct aspects of neuronal migration and differentiation, is an essential step in the elucidation of functional significance of Nap1 molecule, initially in the cerebral cortex, and eventually in other laminar structures of the mammalian central nervous system.

Materials and methods

Historogy

For whole mount X-gal staining, embryos were fixed in 2% paraformaldehyde (PFA) and 0.1% gulteraldehyde in 0.1M phosphate buffer, and

then stained with β-galactosidase (Roche) according to the manufacture's protocol. For the slice X-gal staining, fixed brains were vibratome sliced to 50 μm thickness (Leica), mounted on the glass slide and proceeds for X-gal staining according to manufacture's instruction.

Immunohistochemistry

Primary neurons dissociated from E14 – E16 embryonic cerebral cortex were isolated and maintained in MEM (Gibco) with 10% BGS (Hyclone) and penicillin/streptomycin (Schmid et al., 2003). For the slices, E16 embryo was fixed in 4%PFA in 0.1M phosphate buffer, and then vibratome sliced to 50 μm thicknesses (Leica). Following antibody were used for immunostaining; anti-Tuj1 (1:500 mouse, Covance), anti-Tau1 (1:250 mouse, Sigma), anti-Map2 (1:250 mouse, Sigma), anti β-galactosidase (1:3000 rabbit, MP biomedicals), anti-Nap1 (rabbit, gift from Dr. Stradal, German Research Centre for Biotechnology). Appropriate Alexa-dye labeled secondary antibodies (Molecular Probes) or Cy2 and cy3 secondary antibody (Jackson ImmunoReseach) were used to detect primary antibody binding.

Western blot analysis

Cerebral cortex from various developmental stages were collected and lysed in lysis buffer (137mM NaCl, 50mM Tris pH7.4, 1mM EDTA, 10% glycerol, 1% NP40, 0.25% SDS and proteinase Inhibitor cocktail). Western blot analysis was performed with anti-Nap1 antisera (1:500, Anton lab).

In situ hybridization

Nap1 probe spans sequence #2281-3170 of the mouse Nap1 cDNA sequence. Nap1 cDNA was cut with EcoRV/EcoRI sites and cloned into pBluescript SK(-). After linearization with EcoRV, antisense transcripts were produced using T7 RNA polymerase. *In situ* hybridization was performed as previously described (Anton et al., 2004) at the UNC Neuroscience Center's *In situ* hybridization core facility.

Results

Developmental expression pattern of Nap1 in the cerebral cortex

To study the cytoskeletal dynamics underlying how neurons terminate their migration and start their final differentiation in the developing cerebral cortex, we mapped the embryonic cortical expression profiles of twenty five murine orthologues of *Drosophila* or *C. elegans* cytoskeleton related genes that are known to regulate distinct stages of neuronal migration or differentiation. Among the proteins screened, Nck associated protein 1 (Nap1) was selectively expressed in the differentiating neurons of the embryonic cerebral cortex.

In situ hybridization analysis indicates that Nap1 is primarily expressed in the cortical plate (CP) region of the embryonic cortex (E14-18), where neurons terminate their migration and begin their final, layer specific phenotypic differentiation (Fig.2.1A-C). The Nap1expresison in the CP region is prominent throughout the entire rostro-caudal region of the developing cerebral cortex (Fig. 2.1D-F). Identical expression pattern of Nap1 is evident in cortical sections from Nap1 indicator mice *(*$Nap1^{lacZ/+}$*)* in which β-gal expression is indicative of endogenous Nap1 expression pattern (Fig.2.2). Often, Nap1 expression is

strongest at the very top of the cortex (asterisk, Fig.2.2E), where migrating neurons initiate their post-migratory differentiation. Immunoblots of whole cell extracts of cortices from different embryonic ages indicate a pattern of increased Nap1 expression corresponding to increased levels of cortical neuronal differentiation (Fig. 2.2I). Co-immunolabelling with post- mitotic neuron specific Tuj-1 antibodies indicates that Nap1 is expressed specifically in cortical plate neurons, not by actively migrating neurons in the intermediate zone (Fig. 2.3A-C). Nap1 expression persists in postnatal cortical neurons as they differentiate and form mature synaptic connections (Fig.2.3 D-J). Co- immunolabelling with axonal and dendritic markers indicate that Nap1 is present in both axons and dendrites of differentiating cortical neurons. Prominent Nap1 expression is noticed in neuronal growth cones and in dendritic spine- like protrusions along neuritic shafts (Fig.2.3D-J). Together, these results indicate that during development Nap1 expression is induced in cortical neurons as they arrive in the cortical plate and initiate their post migratory differentiation, characterized by extension of processes and formation of functional connections.

Figure 2.1. Distribution of Nap1 in developing cerebral cortex.

(A- C) *In situ* hybridization mapping of Nap1 expression in E14, E16 and E18 cerebral cortex. Nap1 is specifically expressed in cortical plate region throughout the cortical development. (D-F) *In situ* hybridization mapping of Nap1 expression at E16 indicates prominent expression in the cortical plate region (arrowheads, D-F) throughout the entire rostro-caudal extent [rostral (D), middle (E), and caudal (F)] of the developing cerebral cortex (D-F). VZ-ventricular zone, IZ-intermediate zone, CP-cortical plate. Scale bar: *A-C, 250 μm, D-F,400μm.*

Figure2.1.

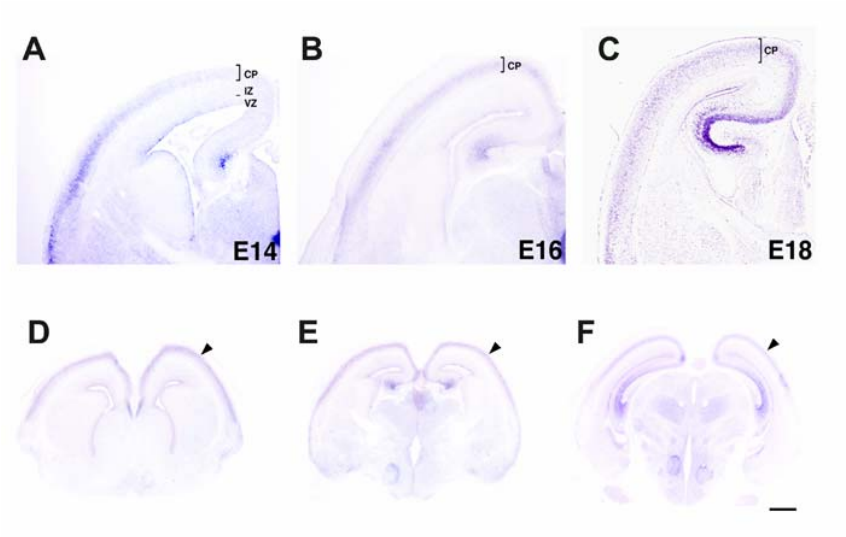


Figure 2.2. Nap1 distribution during cortical development.

(A, B) β-gal expression pattern in the embryonic cortex of mice heterozygous for Nap1 gene-trap insertion (A) is similar to that noticed with Nap1 *in situ* hybridization (B). Nap1 expression is prominent in the cortical plate (CP) region (arrowhead, A, B) where neurons terminate the migration and initiate their differentiation. (C- F) Cortical plate expression of Nap1 is noticed across the embryonic development at E14 [C], E16 [D], E18 [E], and P0 [F]. Asterisk indicates the strongest expression of Nap1 in the CP region. (G-H) Postnatally, at P7 [G] and P21 [H], Nap1 expression persists in all cortical neurons as they differentiate to form and maintain synaptic connections. (I) Immunoblot analysis of Nap1 expression in the developing cortex indicates that increase in Nap1 expression parallels increased neuronal differentiation. VZ-ventricular zone, IZ-intermediate zone, CP-cortical plate. Scale bar: *A-B, 300μm; C-E, 100 μm; F-H, 125μm.*

Figure2.2.

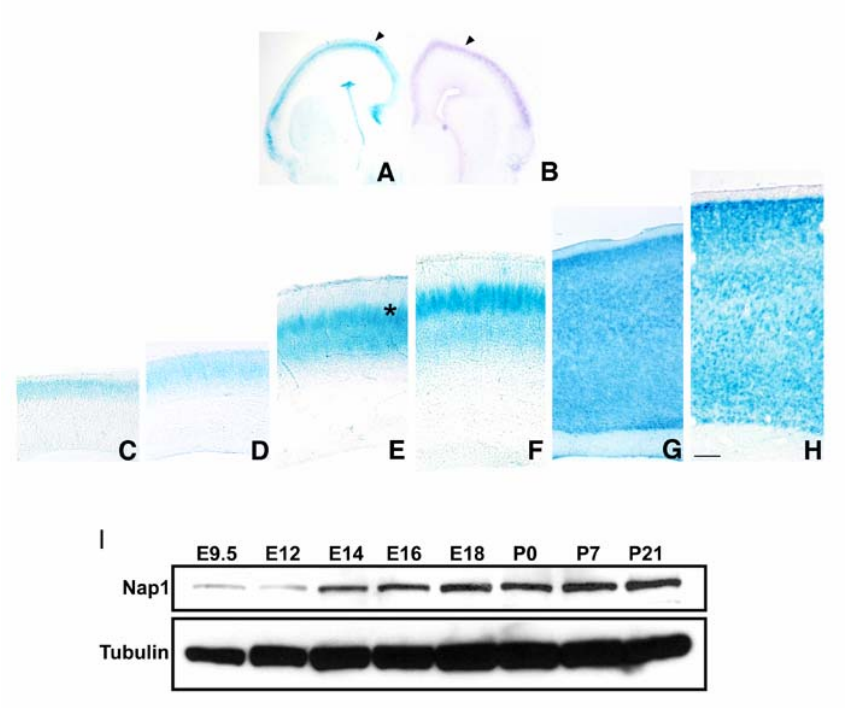
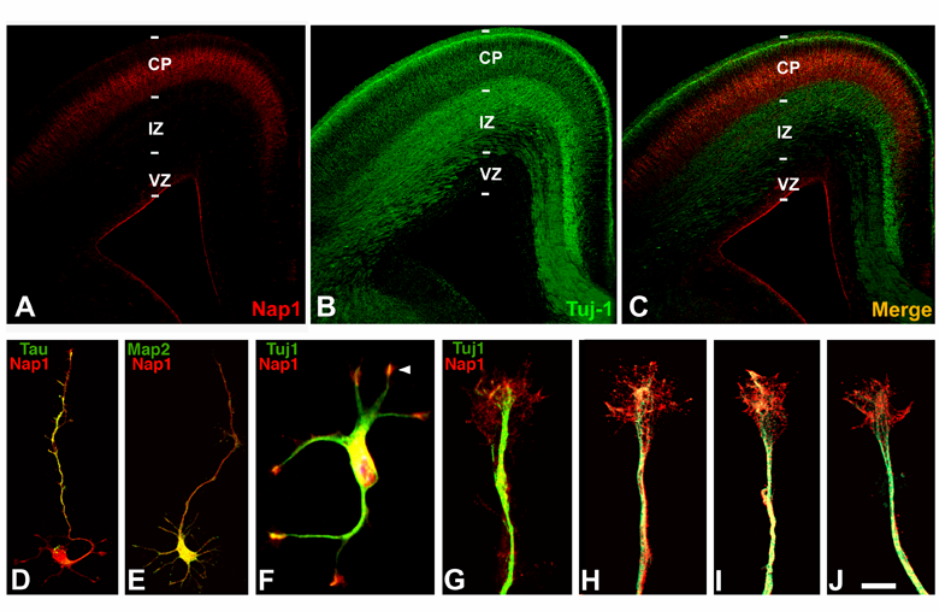


Figure 2.3. Immunohistochemical mapping of Nap1 expression.

(A-C) In E16 cortex, co- labeling with neuron specific Tuj-1 antibodies indicate that Nap1 (red) is specifically expressed in the cortical plate (CP) neurons, and not in the intermediate zone (IZ) region containing the migrating neurons. (D, E) Co- labeling of differentiating cortical neurons with axonal (Tau-1) and dendritic (Map2) markers, indicate that Nap1 is present in both axons (D) and dendrites (E). Yellow indicates co-labeled sites. (F) Nap1 is prominently expressed in the tips of cortical neurites (arrowhead, F). Panels G-J are higher magnification images of Nap1 expression at the leading edges of differentiating cortical neurons. Cortical neurons in panels F-J were co-labeled with Tuj-1 antibodies. Scale bar: *A-C, 250μm; D,E, 30 μm; F, 15 μm; G-J, 10 μm.*

Figure 2.3.


CP
IZ
VZ
A
Nap1
B
Tuj-1
C
Merge
Tau
Nap1
Map2
Nap1
Tuj1
Nap1
Tuj1
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Summary

Nap1 is distributed in the developing cerebral cortex in a manner indicative of its potential function in the transition from migratory to post-migratory differentiation of cortical neurons. This selective expression of Nap1 in the cortical plate supports our hypothesis that Nap1 may play a role in the terminal phase of neuronal migration and the subsequent initiation of neuronal differentiation.

CHAPTER III

THE ROLE OF NAP1 IN THE FINAL PLACEMENT AND DIFFERENTIATION OF NEURONS IN CEREBRAL CORTEX

Introduction

Specific expression of Nap1 in the CP region of the developing cerebral cortex where neurons' transition from migratory to post migratory state suggests that Nap1 may be critical for the termination of neuronal migration and the initiation of neuronal differentiation. To evaluate this hypothesis, we used gain and loss of function approaches to modify Nap1 function *in vitro* and *in vivo* during specific stages of the cerebral cortical development, and determined if the final placement and differentiation of cortical neurons were affected as a result. Specifically, we (a) ectopically induced Nap1 in migrating neurons using promoters that are active in post- mitotic, migratory neurons and determined if premature Nap1 induction in migrating neurons lead to premature initiation of neuronal differentiation, and (b) used shRNA mediated knockdown of Nap1 in cortical plate neurons to evaluate if their placement and differentiation is affected following Nap1 knockdown.

Materials and methods

Generation and characterization of Nap1 specific shRNA

The Nap1 unique target sequences, GCTCACCATCCTCAACGAC, GTTGCACACTGCACTTTCG, GTTCCTGAGTGAGAGCCTT, CCAGATTGCTGCAGCTTTG, and GGAATTCCTGGCGCTTGCA are located at 48-66 bp, 2031-2049 bp, 2547-2565 bp, 3111-3129 bp and 3168-3186 bp, respectively of Nap1 cDNA. As a negative control for each of the shRNA construct, 3 nt mutations were made in the respective targeting sequence (e.g., GCTTACCATTCTCAATGAC [control for 48-66bp target sequence]). The target sequence oligos and mutated target sequence oligos were subcloned into pCGLH vector (Fig. 3.1A, gift from Dr. Sestan, Yale University), which contains chicken β-actin promoter driven EGFP and H1 promoter for shRNA transcription. To test the efficiency of shRNA mediated Nap1 knockdown, targeting constructs were transfected into CAD cells or E16 primary cortical cells using Effectene (Qiagen) as per manufacturer's instructions. High transfection efficiency (>80% of total cells on the dish) was confirmed by EGFP expression. Four days later, cell extracts were immunoblotted with anti-Nap1, tubulin, or ErbB4 antibodies. Some of the transfected cortical neurons were fixed in 4% paraformaldehyde and immunolabeled with anti-Nap1 antibodies to further confirm that the Nap1 protein expression is diminished specifically in Nap1 shRNA expressing cells. Cortical neurons were also co-transfected with Nap1 shRNA (in pCRLH vector expressing RFP) and full length Nap1 –EGFP fusion plasmids. Four days later, shRNA expressing neurons (red) were evaluated for reduced GFP expression, compared to control shRNA expressing neurons.

Effect of Nap1 in the post migratory differentiation

To determine the effect of Nap1 during post migratory differentiation of cortical neurons *in vitro*, dissociated E14 cortical neurons were transfected with either control, or Nap1 shRNA plasmids. Three days after transfection, neurons were fixed and immunolabeled with Tuj1. The total length, the number of primary, secondary, and tertiary branches of both axons and dendrites, and the number of dendrites on these neurons were measured as described in Schmid et al., 2004. Axons and dendrites were identified based on their morphology (Gaudilliere et al., 2004).

To determine the effect of Nap1 in post-migratory differentiation of cortical neurons *in vivo*, E15 embryos were electroporated with Nap1 or control shRNA (see *in utero* electroporation section and Gongidi et al., 2004 for detail), allowed to survive till postnatal day 2 or 17 and the patterns of dendritic and axonal morphology (i.e., length, numbers, branching patterns, orientation of apical processes, and the radial extent of basal dendrites) of control and Nap1 shRNA expressing neurons (GFP^+) in cerebral cortex was evaluated (Schmid et al., 2004; Anton et al., 2004). Neuronal differentiation index at P17 was measured as the percentage of area occupied by the GFP positive neurons per 25Kμm^2 total area. Neuronal position within the cortex was measured by binning the cortex to 10 equal bins and counting the number of neurons within the individual bins (Gongidi et al., 2004). In some experiments, cortices were removed from the embryos two days after electroporation, coronally sectioned, mounted on nucleopore membrane filters, and GFP labeled control or Nap1shRNA$^+$ neurons in the intermediate zone of the slices were repeatedly imaged using a Zeiss inverted microscope (attached to a confocal laser scanning system and a live cell

incubation chamber) for 2-3 hours (see *in vitro* electroporation section for detail). The rate of migration of the monitored cells was measured using LSM5 Pascal program (Zeiss).

In vitro electroporation

Lateral ventricles of E15-E16 mouse embryos were injected with 1.5 μl of a plasmid mixture, containing 4 μg/μl DNA (pCGLH-control EGFP, pCGLH-Nap1 shRNA) diluted 1:1 with mouse neuron nucleofector solution (Amaxa biosystems)/0.001% fast green, using a Parker Hannifin Picospritzer II. Immediately after injection, heads were subjected to electroporation (BTX/Genetronics) under the following conditions: LV mode, 70 Volts, 100 ms pulse length, 100 ms pulse interval, 8 pulses, unipolar [polarity]. Following electroporation, cortices were removed from the embryos, coronally sectioned (200 μm), mounted on nucleopore membrane filters, and cultured in MEM/10% FBS at 37°C/5% CO_2 for 24 hr. GFP labeled control or Nap1shRNA$^+$ neurons in the intermediate zone of the slices were repeatedly imaged using a Zeiss inverted microscope (attached to a confocal laser scanning system and a live cell incubation chamber) for 2-3 hours. The rate of migration of the monitored cells was measured using LSM5 Pascal program (Zeiss).

In utero electroporation

In utero electroporation was performed with E14-15 embryos. Pregnant female was anesthetized, embryos that remain attach to mother are exposed for injection and electroporation. High concentration (3~5 μg/μl) DNA was diluted

1:1 with mouse neuron nucleofector solution (Amaxa biosystems)/0.001% fast green, using a Parker Hannifin Picospritzer II. Immediately after injection, heads were subjected to electroporation (BTX/Genetronics) under the following conditions: LV mode, 28 Volts, 50 ms pulse length, 950 ms pulse interval, 5 pulses, unipolar [polarity]. Embryos were then removed after 48hr, 7days and 21days from surgery.

Neuronal differentiation analysis using cortical slice overlay assay

Control shRNA or Nap1-shRNA electroporated E16 cortical slices were maintained for 2 days in MEM as described above. EGFP expressing neurons were micro dissected from these slices, dissociated and seeded on to the 200 μm thick E16 cortical slices. After 3 days, slices were fixed and the extent of differentiation of control or Nap1 shRNA$^+$ neurons seeded on the CP region of the slice substrate was measured.

Nap1 knock down in cortical plate neurons

Nap1 shRNA was directly electroporated into cortical plate neurons by immersing intact E16 cerebra cortex into 5 μg/μl Nap1 shRNA plasmid diluted with 1:1 with mouse neuron nucleofactor solution for electroporation in the chamber electrode (Protech International Inc.). In some experiments, shRNA was injected beneath the skull, over the cortical surface, prior to electroporation (70V, 100ms duration, 100ms interval, 8 pulses, unipolar). These approaches enable the modification of Nap1 expression *in vivo* in cortical plate neurons only. Cortices were then sliced (200μm thick) and maintained in vitro for 3 days prior

to analysis of neuronal differentiation of control and Nap1 deficient neurons.

Ectopic induction of Nap1 in the developing cerebral cortex

To determine the effect of ectopic, premature induction of Nap1 in migrating neurons, NeuroD promoter-Nap1-IRES-EGFP or control NeuroD promoter-IRES-EGFP plasmids were *in utero* electroporated into E14-15 cerebral cortex (*in utero* electroporation section). Forty eight hours after electroporation, cerebral cortices were removed, vibrotome sliced, and immunolabeled with antibodies to markers that are normally expressed by neurons that are under going post migratory differentiation (i.e., Tbr-1, Brn-1) to evaluate if premature induction of Nap1 in migrating neurons induce premature differentiation as evidenced by premature layer specific molecular marker expression. The morphology of control or Nap1 expressing neurons in the intermediate zone was evaluated by immunolabeling them with anti-GFP antibodies to obtain higher magnification images of these neurons. The extent of migration of the control and Nap1 expressing neurons within the cerebral wall was analyzed by binning the cortex into 10 equal bins and count the number of neurons within the individual bins (Gongidi et al., 2004, and Schmid et al., 2004). In some experiments, intermediate zone containing GFP^+ control and Nap1 expressing neurons were microdissected, dissociated, and plated at a density of 500Kcells/ ml on laminin coated MatTek 35 mm glass bottom dishes. GFP^+ neurons were then repeatedly imaged using a Zeiss Pascal confocal microscope equipped with live incubation chamber. Some of these cultures were fixed in 4% paraformaldehyde after 7.5 hours *in vitro* and immunolabeled with neuron specific Tuj1 antibodies to

evaluate the morphological differentiation of control and Nap1 expressing neurons.

BDNF's effect on Nap1 expression

To examine if brain derived neurotrophic factor (BDNF) can induce Nap1 during neuronal differentiation, dissociated E16 cortical neurons (10^6 / ml) were treated with 1, 5, 10, 15, 25 ng/ml BDNF (Pepro Tech Inc.) for 48hrs. In some experiments, TrkB receptor bodies (R&D systems, 20 μg/ml) were added to BDNF to block BDNF activity. Changes in Nap1 expression level in these neurons were analyzed by immunoblotting. To examine if Nap1 function is essential for BDNF's effect on neuronal differentiation, dissociated E16 cortical neurons were first transfected with either control or Nap1 shRNA and then maintained in BDNF (25ng/ml) supplemented or normal media. Two days later, neurons were immunolabeled with neuron- specific Tuj1 antibodies to assess the extent of differentiation.

Results

Generation and characterization of Nap1 shRNA

To evaluate the effect of loss of function of Nap1 in cortical neuronal differentiation, we utilized shRNA mediated knockdown of endogenous Nap1 in cortical neurons. We generated shRNA constructs targeted to different mouse Nap1 specific regions. The candidate target sites were selected based on the following criteria: (1) target sequence were 19nt downstream of AAG, AAA or CAG/A sequences within Nap1 ORF, (2) GC content of the target sites were

between 30~70%, (3) target sites were not in the RNA loop structure, (4) no more than 4T s in the target sites, (5) the 19nt target sequence is absolutely specific to Nap1 (Taira et al., 2003). As a negative control for the shRNA constructs, 3nt mutations were made in each of the respective targeting sequences. The target sequence oligos and mutated target sequence oligos were subcloned into pCGLH vector, which contains chicken beta actin promoter driven EGFP and H1 promoter for shRNA transcription. Nap1 shRNA, but not control shRNA, specifically reduced Nap1 levels (Fig.3.1). Nap1 shRNA induced no changes in the expression levels of unrelated proteins such as tubulin or ErbB4. Furthermore, in neuronal cell lines (CAD) and embryonic cortical cells co-transfected with Nap1 or control shRNA (in pCRLH vector expressing RFP) and full length Nap1-EGFP fusion plasmids, Nap1 – EGFP expression was diminished only in Nap1shRNA expressing cells, but not in control shRNA expressing cells (Fig.3.2). Together, these studies confirm that Nap1 shRNA constructs can specifically suppress endogenous Nap1 protein expression.

Figure 3.1. ShRNA mediated knockdown of Nap1.

(A) Nap1 shRNA construct. Nap1 specific target sequence oligos and mutated target sequence oligos were subcloned into pCGLH vector, containing chicken β-actin promoter driven EGFP and H1 promoter for shRNA transcription. (B) Neuronal CAD cells were transfected with two different shRNA constructs specific to Nap1 and two corresponding control shRNA constructs (i.e., mutant shRNA). Nap1 shRNA specifically decreased Nap1 expression. Vector alone controls, untransfected cells, or control shRNA did not alter Nap1 expression.

Figure 3.1.

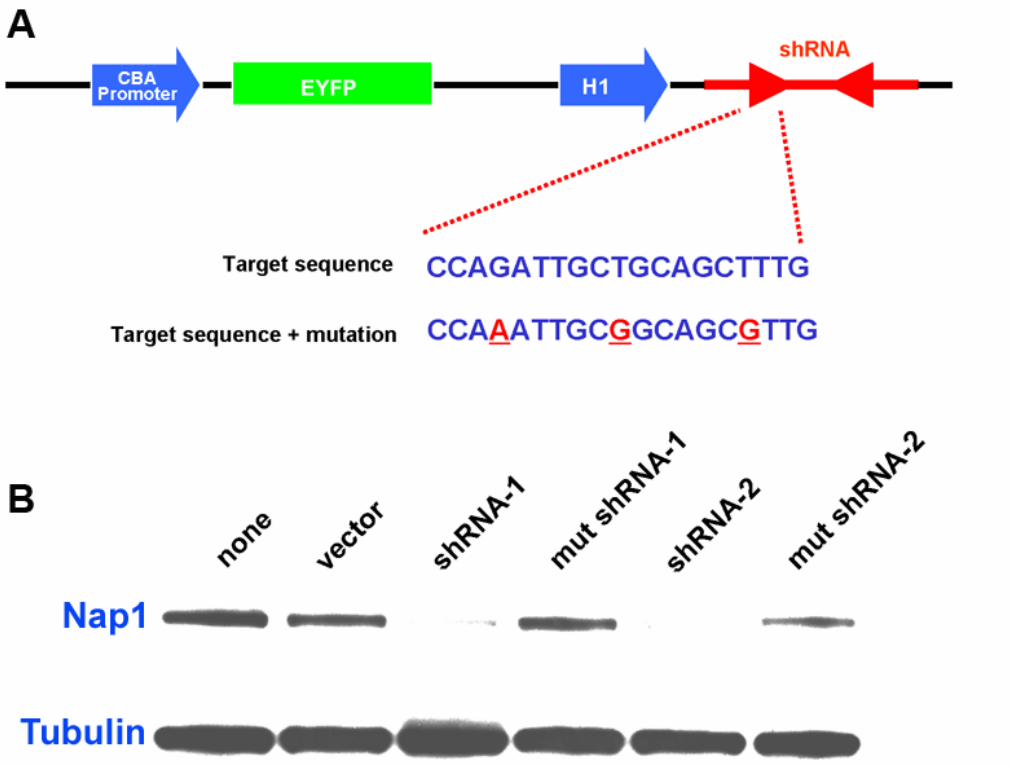
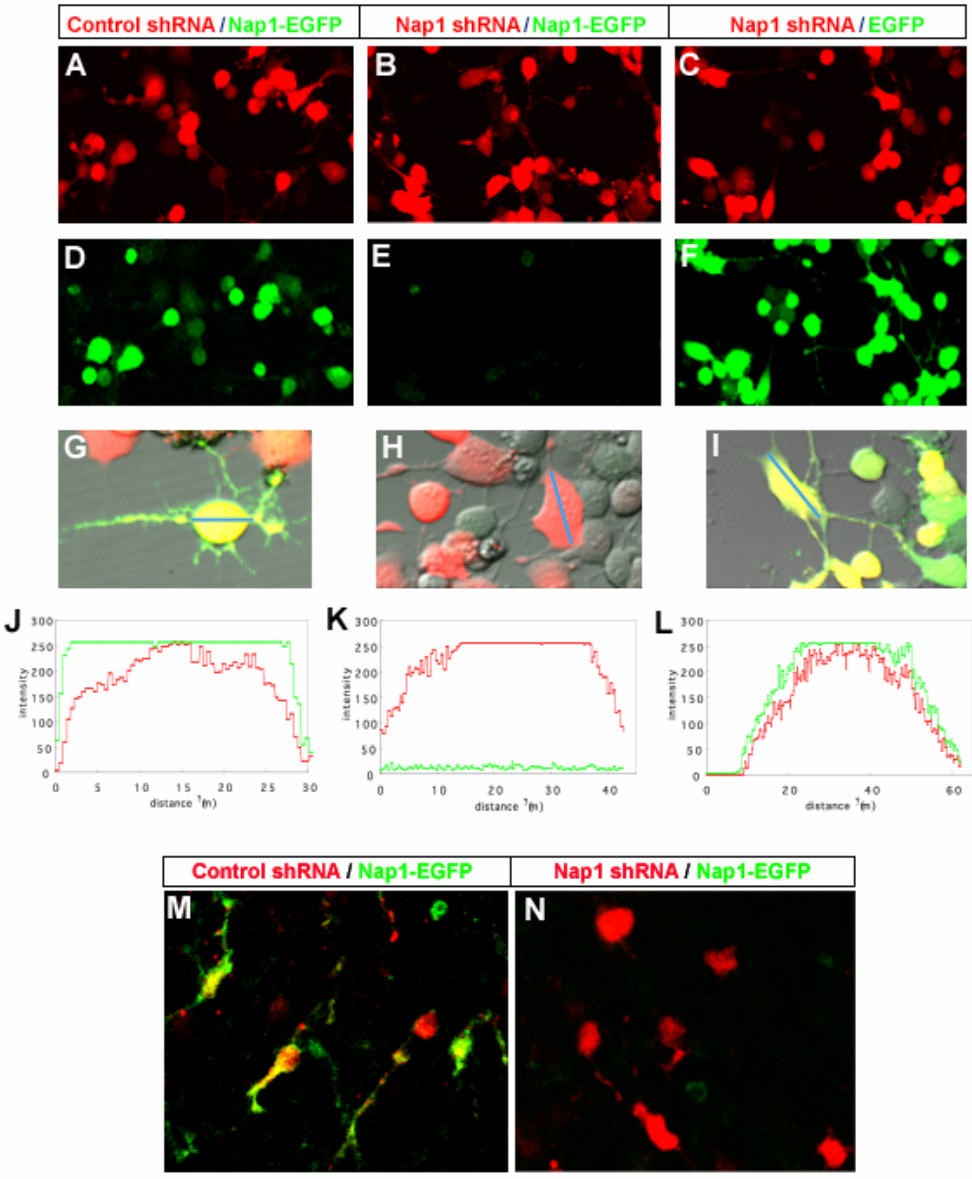

A
CBA Promoter
EYFP
H1
shRNA
Target sequence
CCAGATTGCTGCAGCTTTG
Target sequence + mutation
CCAAATTGCGGCAGCGTTG
B
none
vector
shRNA-1
mut shRNA-1
shRNA-2
mut shRNA-2
Nap1
Tubulin

Figure 3.2. ShRNA mediated knockdown of Nap1.

(A-L) CAD cells were co-transfected with Nap1-EGFP and control-shRNA (A,D,G,J) , Nap1-EGFP and Nap1-shRNA (B,E,H,K) or control EGFP and Nap1-shRNA (C,F,I,L). (G-H) High magnification merged images (RFP, EGFP and Phase light) of transfected CAD cells. Red and Green fluorescence intensity was measured across these cells (G-I, blue line). These relative fluorescence intensity measurements indicate that Nap1 shRNA specifically retards Nap1 expression (J-L). Cortical slices were electroporated with Nap1-EGFP and control-shRNA (M) or with Nap1-EGFP and Nap1-shRNA (N). Nap1 shRNA specifically suppresses Nap1 expression in cortical cells.

Figure 3.2.


Control shRNA / Nap1-EGFP
Nap1 shRNA / Nap1-EGFP
Nap1 shRNA / EGFP
A
B
C
D
E
F
G
H
I
J
K
L
intensity
distance (m)
Control shRNA / Nap1-EGFP
Nap1 shRNA / Nap1-EGFP
M
N

Defective neuronal differentiation following inhibition of Nap1 in vitro

To determine the effect of Nap1 in post-mitotic differentiation of cortical neurons *in vitro*, dissociated cortical neurons were transfected with either control or Nap1 shRNA. Three days later, neurons were immunolabeled with neuron specific Tuj1 antibody to assess the extent of differentiation. The total length, the number of primary, secondary, and tertiary branches of both axon and dendrites, and the number of dendrites on these neurons was quantified. Axons and dendrites were identified based on their morphology (Gaudilliere et al., 2004). Suppressing Nap1 expression in post mitotic cortical neurons significantly reduced their ability to elaborate characteristic axons and dendrites as indicated by the marked reduction in the extent, branching, and numbers of dendrites and axons (Fig. 3.3). Similar retardation of neuronal differentiation was also noticed when Nap1 deficient neurons were seeded on cortical plate region of E16 embryonic cortical slices, a relevant substrate where cortical neuronal differentiation normally occurs *in vivo* (Fig. 3.4). Furthermore, to specifically knockdown Nap1 during the final differentiation stage without affecting other aspects of cortical development, we modified Nap1 expression in CP neurons by electroporating Nap1 specifically into differentiating CP neurons. This approach targets only neurons that are in the CP and are in the process of initiating their differentiation. We observed retardation of neuronal differentiation when Nap1 expression was diminished in CP neurons (Fig.3.5). These observations indicate that Nap1 deficiency significantly impaired the ability of neurons to differentiate *in vitro*.

Figure 3.3. Suppression of Nap1 expression disrupts embryonic cortical neuronal differentiation.

(A-B) E14.5 cortical neurons were transfected with control (A) or Nap1 shRNA (B). Compared to control neurons, Nap1 deficient neurons displayed reduced axonal and dendritic growth and branching. (C) Quantification of neuronal differentiation defects in Nap1 deficient neurons. Asterisk indicates significant when compared with controls at $p<0.001$ (Student's t test). Scale bar: 20μm.

Figure 3.3.

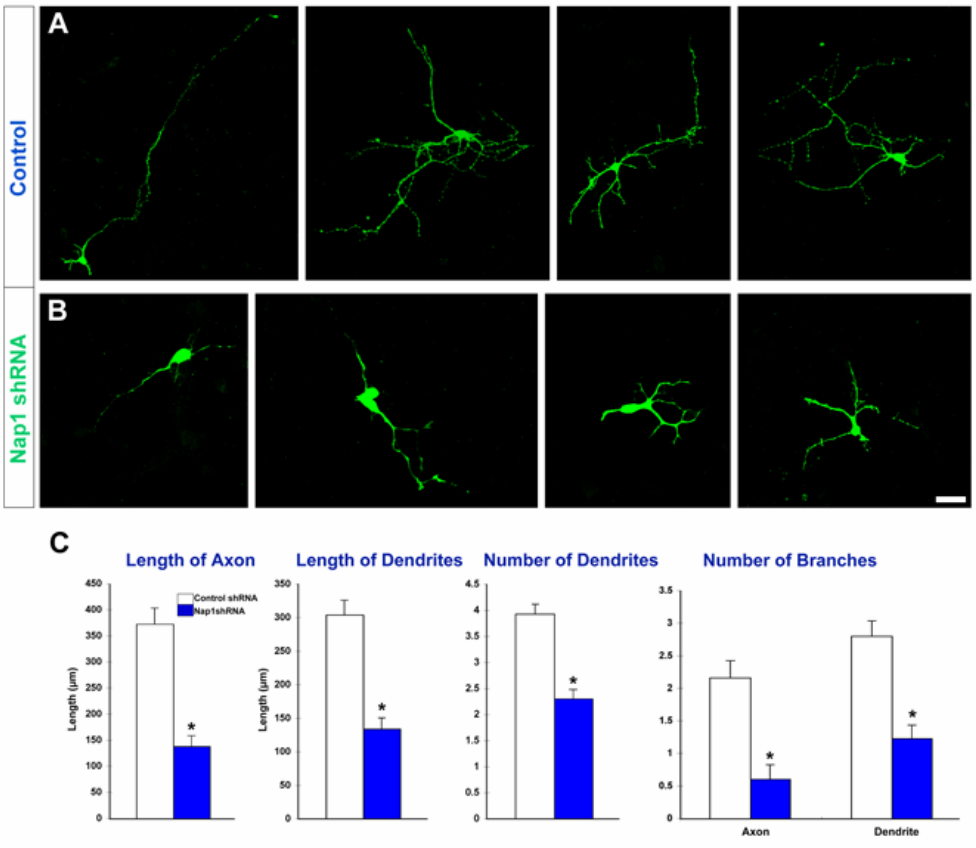

A
Control
B
Nap1 shRNA
C
Length of Axon
Length of Dendrites
Number of Dendrites
Number of Branches
Control shRNA
Nap1shRNA
Length (μm)
Axon
Dendrite

Figure 3.4. Suppression of Nap1 expression disrupts embryonic cortical neuronal differentiation in overlay assay.

(A) Schematic of cortical neuron overlay assay. Control or Nap1 shRNA – EGFP construct was electroporated into E16 cerebral cortex and slices were maintained for 2 days *in vitro*. $EGFP^+$ neurons from the IZ area were then micro-dissected, dissociated, seeded onto wild type, E16 cortical slices and cultured for 3 days. (B) $EGFP^+$ neurons differentiating on a cortical slice (red). (C-F) Compared to control neurons(C, D), Nap1 deficient neurons (E, F) display retarded neurite growth and branching. (G) Quantification of neuronal differentiation defects in Nap1 deficient neurons. Asterisk indicates significant difference when compare with control at $P<0.001$ (student t-test). Scale bar: 20μm.

Figure 3.4.

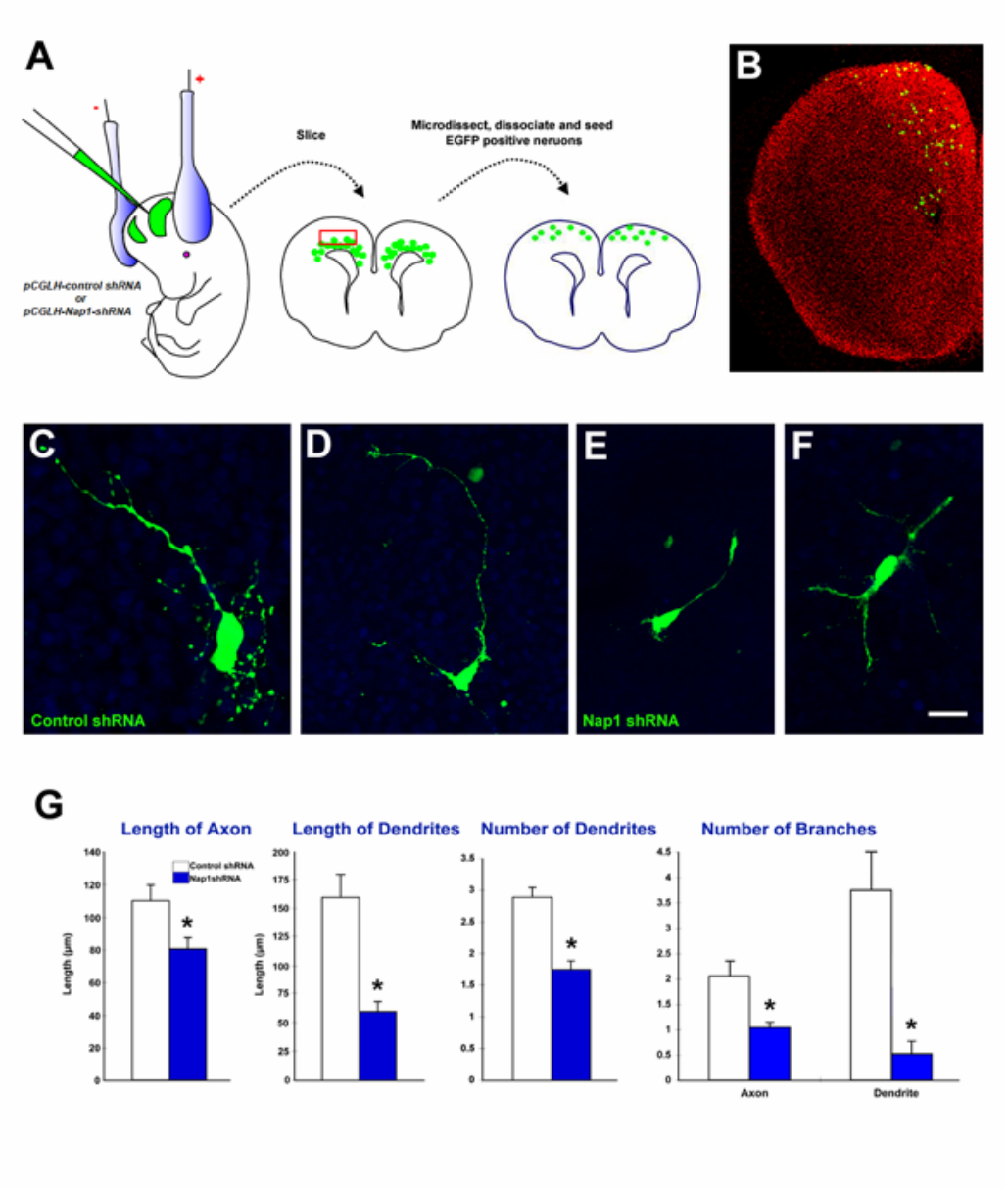
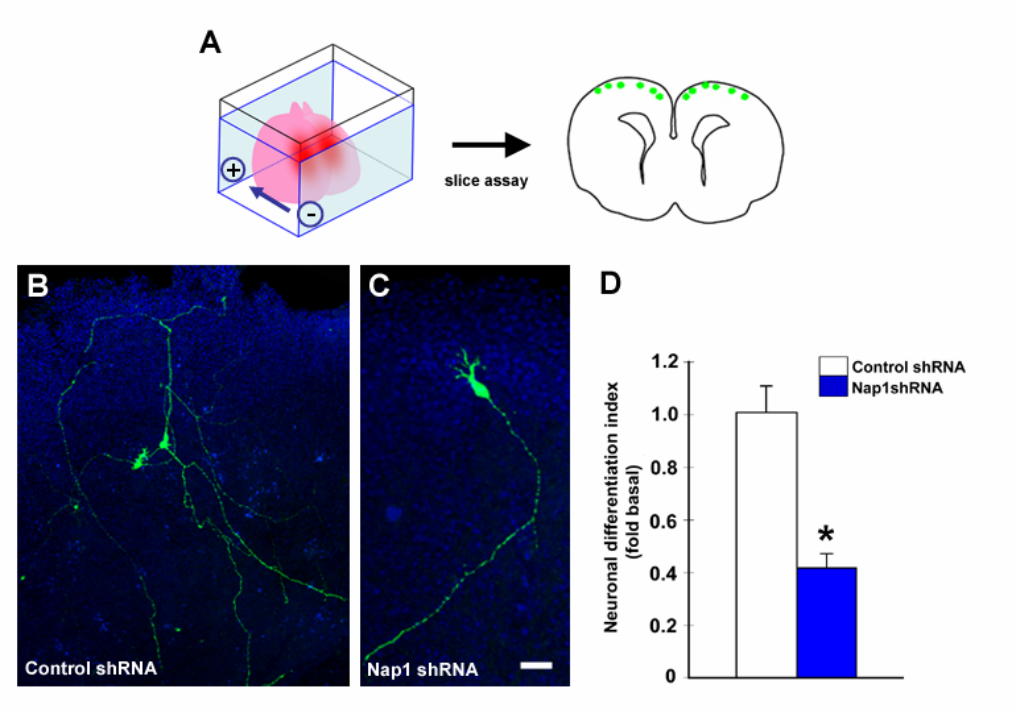

A
Slice
Microdissect, dissociate and seed
EGFP positive neruons
pCGLH-control shRNA
or
pCGLH-Nap1-shRNA
B
C
D
E
F
Control shRNA
Nap1 shRNA
G
Length of Axon
Length of Dendrites
Number of Dendrites
Number of Branches
Control shRNA
Nap1shRNA
Length (μm)
Axon
Dendrite

Figure 3.5. Suppression of Nap1 expression in cortical plate disrupts embryonic cortical neuronal differentiation.

(A) Schematic of pial injection of control or Nap1 shRNA constructs. shRNA constructs were injected above the pial surface followed by electroporation using a chamber electrode. This method preferentially targets differentiating CP neurons at the very top of the developing cortex. (B, C) Compare to control neurons, Nap1 deficient neurons displayed reduced growth and branching. (D) Analysis of the extent of neuronal differentiation indicates a substantial reduction in the complexity of neuronal processes growth and arborization in Nap1 deficient neurons. Asterisk indicates significant difference when compare with control at $P<0.001$ (student t-test). Scale bar: B 20 μm, C 15 μm.

Figure 3.5.


A
slice assay
B
Control shRNA
C
Nap1 shRNA
D
Control shRNA
Nap1shRNA
Neuronal differentiation index
(fold basal)
1.2
1.0
0.8
0.6
0.4
0.2
0
*

Defective neuronal differentiation following inhibition of Nap1 in vivo

To determine the effect of Nap1 in post-migratory differentiation of cortical neurons *in vivo*, E15 embryos were electroporated with Nap1 or control shRNA, allowed to survive till postnatal day 2 or 17, and the patterns of neuronal position, dendritic and axonal morphology of control and Nap1 shRNA expressing neurons were evaluated. Nap1 knockdown did not affect the positioning of neurons within the cortical plate. Quantitative analysis of neuronal position in the cortex indicates no difference between control and Nap1 shRNA expressing neurons (Fig. 3.6K). Furthermore, real time analysis of migration of Nap1 deficient and control neurons indicates that Nap1 knockdown did not affect neuronal migration. Control neurons migrated at an average rate of 21±2.8 μm/hr and Nap1 shRNA expressing cells migrated at a comparable rate of 19.2±2.4 μm/hr. However, Nap1 knock down significantly retarded all aspects of cortical neuronal differentiation *in vivo* (Fig. 3.6). Nap1 deficient neurons displayed significantly reduced axonal and dendritic process extension and branching. Additionally, the radial orientation of apical dendrites was disrupted in Nap1 deficient neurons (Vertical orientation of apical dendrites: control neurons = 3.07±0.55 °; Nap1 deficient neurons = 10±1.29°). The arborization pattern of apical and basal dendrites, as indicated by the ratio of horizontal versus ventral extension of apical and basal dendritic arbors, was also significantly retarded (control shRNA: 2.4±0.4 , Nap1 shRNA : 1.6±0.1; $P<0.01$ [Student t-test]). Furthermore, the terminal, post- migratory differentiation and maturation of cortical neurons in cerebral cortex is characterized by the elaboration of specialized dendritic protrusions essential for synaptic plasticity, i.e., dendritic

spines. We therefore analyzed the effect of Nap1 on dendritic spine morphology in the above cortical neurons. Nap1 deficiency profoundly retarded the dendritic spine density in these cortical neurons (Fig. 3.7). Together, these data demonstrate that Nap1 is critical for neuronal differentiation in the emerging cortical plate.

Figure 3.6. Knockdown of Nap1 disrupts embryonic cortical neuronal differentiation in vivo.

Cerebral cortices of E15.5 embryos were electroporated with control or Nap1 shRNA and differentiating neurons in the cortical plate were analyzed at post natal day 2 (A-L) or 17 (M-Q). (A-E) Cortical neurons expressing control shRNA displayed characteristic axons and dendrites (arrowheads, A, B) at their early stages of development. In higher magnification images of these cells (C-E), apical (arrowhead, D), basal dendrites (asterisk, D), and axon (arrow, D) are evident. (F-J) In contrast, Nap1 shRNA expressing neurons displayed significantly reduced axonal and dendritic growth and branching (arrowheads, F, G; H-J). (K) Analysis of neuronal position indicates no difference between control and Nap1 shRNA expressing neurons. (L) Quantification of dendritic numbers, length, and branches in control and Nap1 deficient neurons. (M-P) At post natal day 17, extensive dendritic arborization is evident in control neurons (arrows, L, M). Nap1 deficient neurons (arrowheads, N, O), however, displayed reduced dendritic growth and branching. (Q) Analysis of the extent of neuronal differentiation indicates a substantial reduction in the complexity of neuronal process growth and arborization in Nap1 deficient neurons. Asterisk indicates significant when compared with controls at $p<0.001$ (Student's t test). Scale bar: A-B, F-G, M-O 30 μm; C-E, H-J, 15μm.

Figure 3.6.

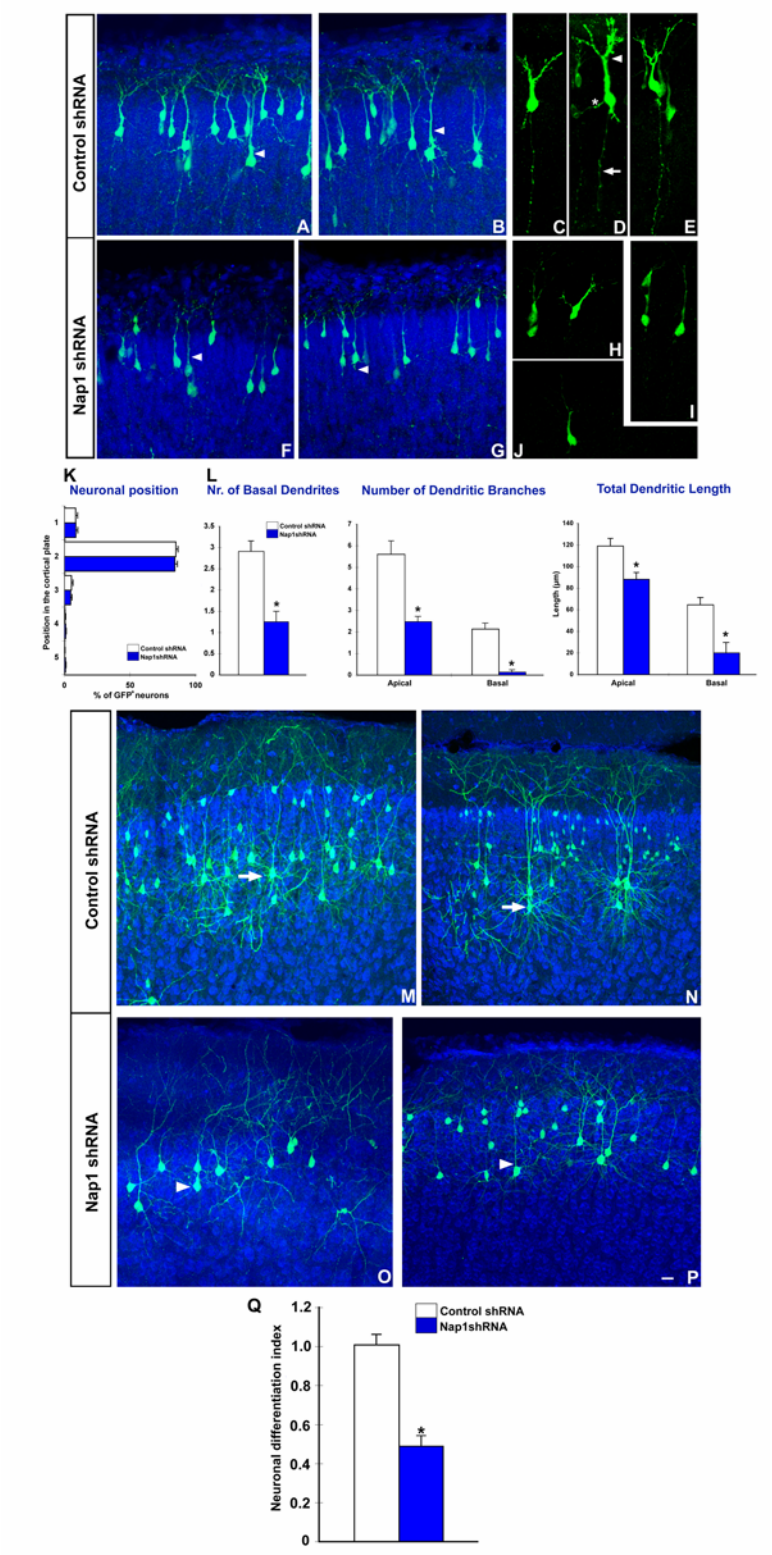
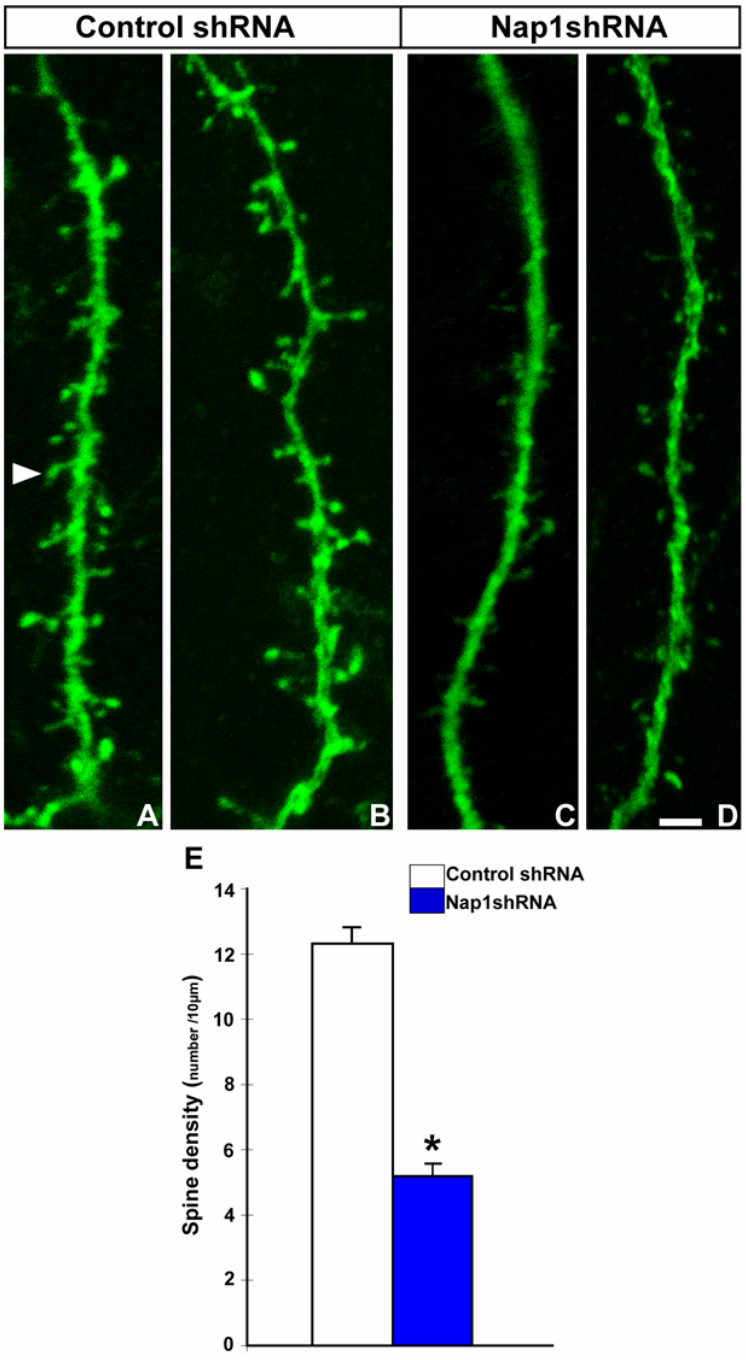

Control shRNA
Nap1 shRNA
A
B
C
D
E
F
G
H
I
J
K
Neuronal position
Position in the cortical plate
% of GFP+ neurons
Control shRNA
Nap1shRNA
L
Nr. of Basal Dendrites
Number of Dendritic Branches
Total Dendritic Length
Length (µm)
Apical
Basal
M
N
O
P
Q
Neuronal differentiation index
Control shRNA
Nap1shRNA

Figure 3.7. Reduced dendritic spine density in Nap1 deficient neurons.

(A-D) Dendritic spines (arrowhead, A) were imaged in control (A, B) and Nap1 shRNA (C, D) expressing neurons at post natal day 17. Nap1 knockdown significantly reduced the number of dendritic spines. (E) Quantification of dendritic spine density (number of spines/ 10μm length). Asterisk, significant when compared with controls at $p<0.001$ (Student's t test). Scale bar: 3μm.

Figure 3.7.


Control shRNA
Nap1shRNA
A
B
C
D
E
Control shRNA
Nap1shRNA
Spine density (number /10μm)
14
12
10
8
6
4
2
0
*

Ectopic expression of Nap1 promotes neuronal differentiation

If Nap1 expression normally facilitates neuronal differentiation, premature Nap1 induction in migrating neurons in the intermediate zone may lead to changes in migration and premature initiation of neuronal differentiation. To test this, we ectopically induced Nap1 in migrating neurons in the intermediate zone using NeuroD promoter, which is active in post- mitotic, migratory neurons (Fig. 3.8, Dr. F. Polleux [UNC Neuroscience Center], unpublished observations; Huang et al., 2000). By prematurely expressing Nap1 in migrating neurons before they arrive in the cortical plate, we asked if the induction of Nap1 will promote premature initiation of post migratory, differentiation state. Embryonic day 14 or 15 mouse cortices were *in utero* electroporated with either NeuroD promoter-Nap1-IRES-EGFP or control NeuroD promoter- IRES-EGFP plasmids. Forty eight hours after electroporation, cerebral cortices were removed, vibratome sliced, and immunolabeled with markers that are normally expressed by neurons that are under going post migratory layer specific differentiation in the cortical plate (e.g., Tbr-1[layer VI], Brn-1[layer II-IV]). Compared to controls, migration of Nap1 expressing neurons was significantly curtailed. Quantification of the extent of neuronal migration indicates that most of Nap1 expressing neurons are found in the lower intermediate zone, whereas control neurons migrate well into the upper IZ and CP (Fig. 3.9A, C, E, F). Importantly, control neurons in the intermediate zone display the characteristic morphology of migrating neurons, with leading and trailing processes (Fig.3.9 I, J). In contrast, Nap1 expressing neurons tend to have multiple long, branched processes, characteristic of differentiating neurons (Fig.3.9 K-P). Furthermore, majority of

Nap1 expressing neurons, but not control neurons, in the intermediate zone expressed molecular markers which are characteristically expressed by differentiating, post- migratory neurons in the cortical plate. When Nap1 was induced during early embryonic stages, significantly higher number of Nap1 expressing neurons in the IZ expressed Tbr-1, a T-domain transcription factor normally expressed in early generated glutaminergic cortical neurons (Fig.3.9B, G; Hevner et al., 2001). Similar induction of Brn-1, a POU- domain transcription factor normally expressed in upper layer cortical neurons (McEvilly et al., 2002; Sugitani et al., 2002), was evident when Nap1 was electroporated during late embryonic stages (Fig. 3.9D, H). Together, these observations suggest that ectopic Nap1 expression retards neuronal migration and promotes neuronal differentiation *in vivo*.

To further explore Nap1's role in neuronal differentiation, the intermediate zone containing control GFP or Nap1 over expressing neurons were micro dissected from the electroporated cortical slices, dissociated, plated at low density on laminin, and neuronal differentiation was monitored at different time points. We hypothesized that if Nap1 facilitates neuronal differentiation, we should notice rapid emergence of morphological differentiation in Nap1 expressing, but not control neurons. Immediately after attachment, both control and Nap1 expressing neurons display a smooth cell soma and are morphologically undifferentiated. However, within a few hours *in vitro*, in contrast to control neurons, Nap1 expressing neurons rapidly display signs of morphological differentiation as indicated by extension of multiple processes

(Fig.3.10). These *in vitro* observations further suggest that cell autonomous Nap1 expression promotes neuronal differentiation.

Figure 3.8. NeuroD promoter is active in post- mitotic, migratory neurons of the intermediate zone.

E15 mouse cortex was electroporated with NeuroD promoter-IRES-EGFP and imaged 24 hours later. GFP labeled post- mitotic neurons are visible in the intermediate zone. None were noticed in the proliferative ventricular zone. Cortical section was nuclear counterstained with bis benzimide (blue).

Figure 3.8.

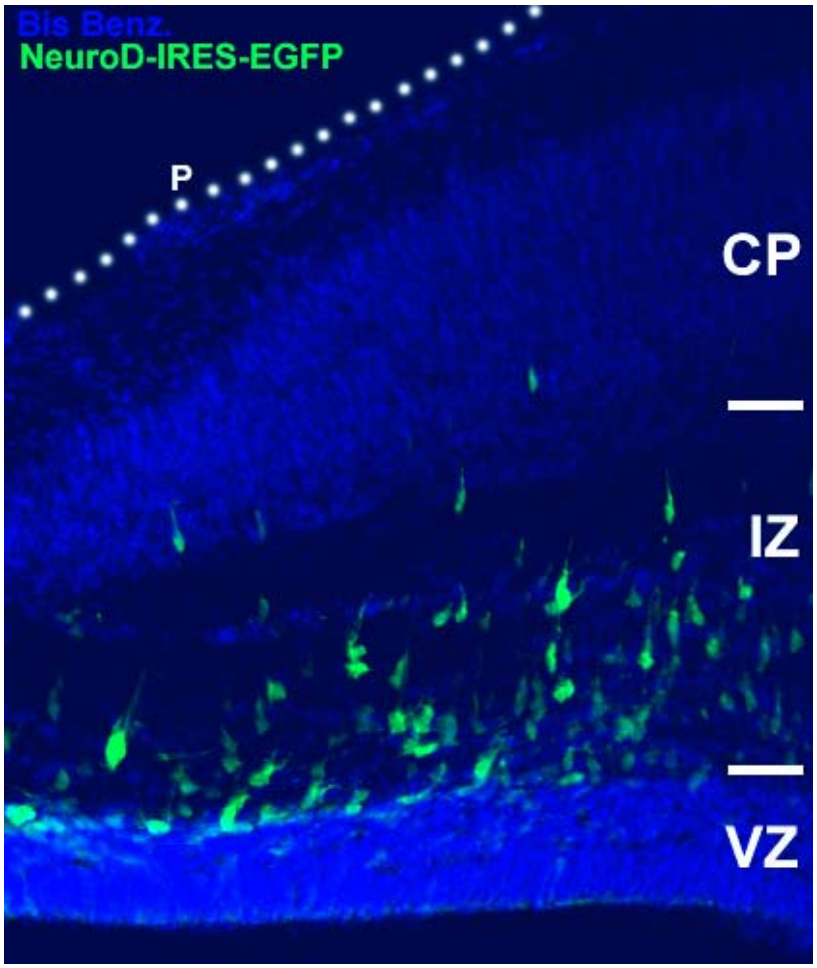


Figure 3.9. Ectopic expression of Nap1 in migrating neurons in the intermediate zone promotes premature neuronal differentiation.

NeuroD promoter is active in post- mitotic, migratory neurons of the intermediate zone. E14 or E15 embryonic cortices were electroporated with NeuroD promoter-Nap1-IRES-EGFP or NeuroD promoter -IRES-EGFP and the position of GFP^+ neurons and the expression of neuronal differentiation markers in GFP^+ neurons in the IZ were analyzed 48 hours later. Nap1 expression significantly retards the migration of neurons generated at E14 (A, E) or E15 (C, F). Significantly higher number of Nap1 expressing neurons in the IZ also express markers (Tbr1, Brn1) that are normally expressed by differentiating neurons in the CP (B, D, G, H). Higher magnification images of GFP immunolabeled neurons in the intermediate zone indicates that control neurons display the characteristic morphology of migrating neurons, with leading (arrow, I-J) and trailing processes (arrowhead, I-J), whereas Nap1 overexpressing neurons in the IZ tend to have multiple, branched processes (arrowheads, K-M), characteristic of differentiating neurons. (N-P) Quantification of neurons with multiple processes, total process length, and branch numbers suggest that premature expression of Nap1 in migrating neurons promotes premature differentiation of neurons. Data shown are mean ± SEM (n=6); asterisk, significant when compared with controls at $p<0.01$ (Student's t test). VZ-ventricular zone, IZ-intermediate zone, CP-cortical plate. Dotted lines in panels A and C indicate pial (top) and ventricular (bottom) surfaces.

Figure 3.9.

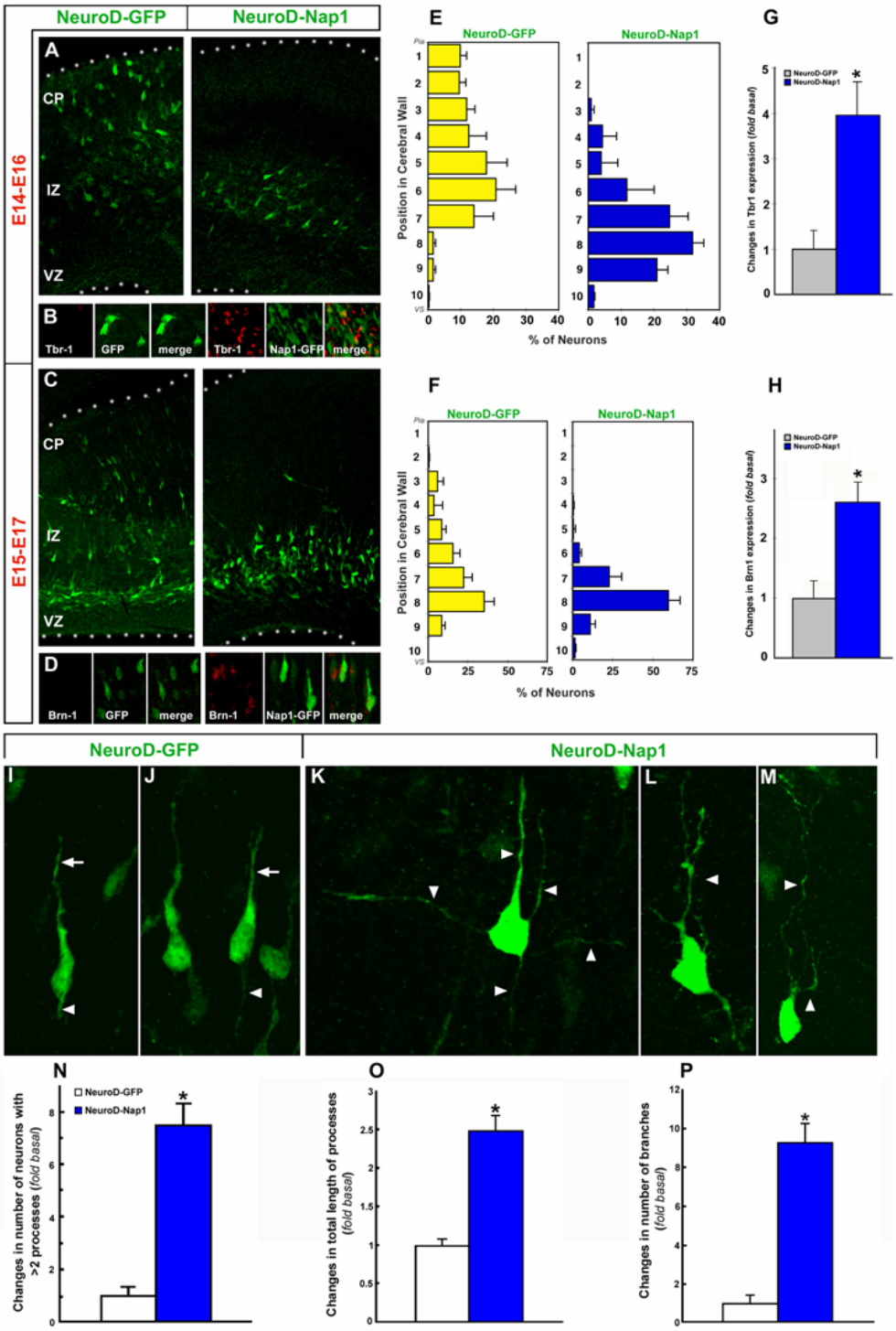
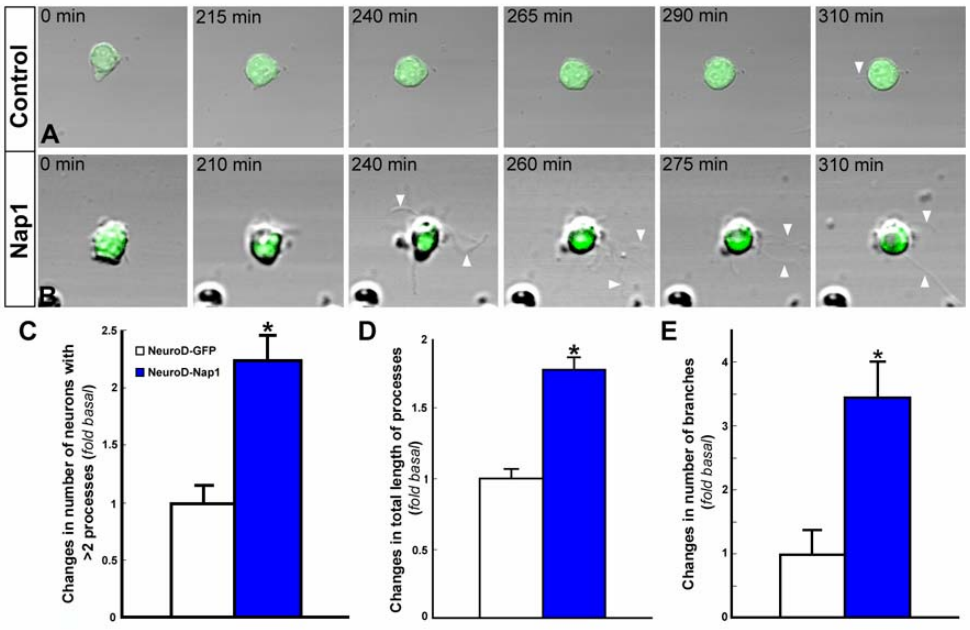

NeuroD-GFP
NeuroD-Nap1
E14-E16
E15-E17
A
B
C
D
CP
IZ
VZ
Tbr-1
GFP
merge
Nap1-GFP
Brn-1
E
F
G
H
Position in Cerebral Wall
% of Neurons
Changes in Tbr1 expression (fold basal)
Changes in Brn1 expression (fold basal)
I
J
K
L
M
N
O
P
Changes in number of neurons with >2 processes (fold basal)
Changes in total length of processes (fold basal)
Changes in number of branches (fold basal)

Figure 3.10. Nap1 over expression promotes neuronal differentiation.

(A, B) Dissociated, control GFP or Nap1 expressing neurons from the intermediate zone of embryos electroporated with Nap1-IRES-EGFP or EGFP were plated on laminin and repeatedly monitored for several hours. Phase light images of GFP expressing neurons were collected. Immediately after adhesion, both control (A) and Nap1 expressing neurons (B) have smooth, round cell bodies. However, in contrast to control neurons, Nap1 expressing neurons rapidly extend multiple processes (arrowheads), suggesting that Nap1 expression promotes neuronal differentiation. (C-E) Quantification of neuronal differentiation after 7.5 hours *in vitro* indicates that Nap1 expression promotes process extension and branching. Data shown are mean ± SEM (n=6); asterisk, significant when compared with controls at $p<0.01$ (Student's t test).

Figure 3.10.


Control
Nap1
0 min
215 min
240 min
265 min
290 min
310 min
A
0 min
210 min
240 min
260 min
275 min
310 min
B
C
NeuroD-GFP
NeuroD-Nap1
Changes in number of neurons with >2 processes (fold basal)
D
Changes in total length of processes (fold basal)
E
Changes in number of branches (fold basal)

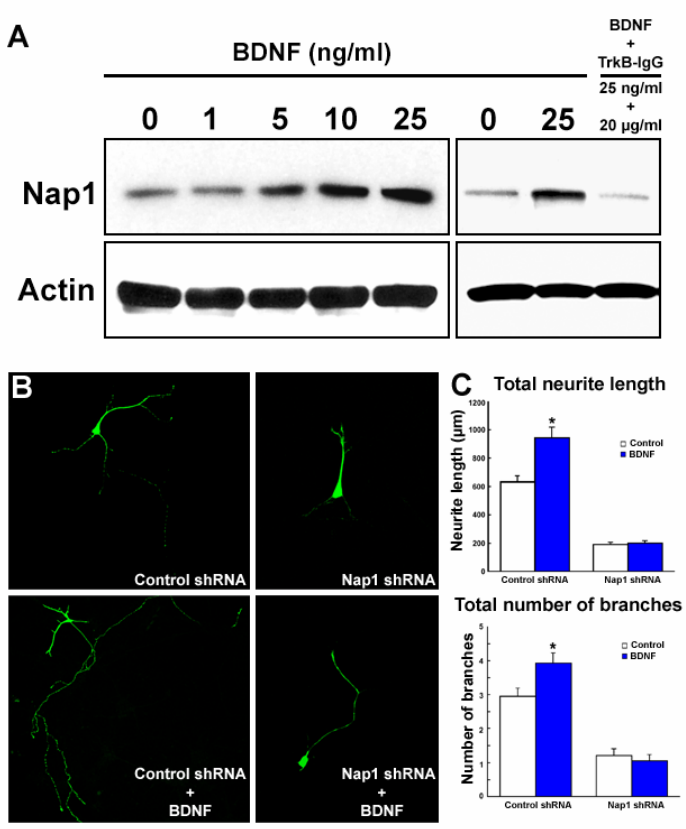
Induction of Nap1 by BDNF

What induces Nap1 in the differentiating neurons of the cortical plate? Context dependent activity of extracellular cues in the developing CP, such as brain derived neurotrpohic factor (BDNF), are required to trigger cortical neuronal differentiation (McAllister et al., 1996; Ghosh et al., 1994; Reichardt, 2006) and thus may induce Nap1 in cortical neurons. To examine this, dissociated E16 cortical neurons were treated with 1, 5, 10, 15, and 25 ng/ml BDNF for 48 hours and the level of Nap1 expression in these cells was analyzed by immunoblotting. BDNF induced a dosage dependent increase in Nap1 levels (Fig. 3.11A). This increase in Nap1 was abolished when BDNF activity was blocked with TrkB receptor bodies (TrkB-IgG, Cabelli et al., 1995; Fig. 3.11A). To examine if Nap1 function is essential for BDNF's effect on neuronal differentiation, dissociated E16 cortical neurons were first transfected with either control or Nap1 shRNA and then maintained in BDNF (25ng/ml) supplemented or normal media. Two days later, neurons were immunolabeled with neuron-specific Tuj1 antibodies to assess the extent of differentiation. BDNF induced neuritic growth and differentiation in control neurons, but BDNF effect was absent in Nap1 deficient neurons (Fig. 3.11 B, C). These data suggest that extracellular factors such as BDNF, which are known to play an essential role in the final post- migratory differentiation of neurons in cerebral cortex, can induce Nap1 expression in cortical neurons as they initiate their post- migratory terminal growth and differentiation in their appropriate laminar locations. Nap1 expression and function is critical to mediate the BDNF induced differentiation of cortical neurons.

Figure 3.11. BDNF induced Nap1 expression in embryonic cortical neurons.

(A) E14 cortical neurons were treated with different concentrations of BDNF (0-25ng/ml) for 2 days. Immunoblot analysis of Nap1 expression in these neurons indicates a dosage dependent effect of BDNF on Nap1 protein level. The effect of BDNF was abolished when BDNF activity was neutralized with TrkB-IgG. Immunblotting for actin indicates equal loading. (B) BDNF promotes growth and differentiation of embryonic cortical neurons expressing control, but not Nap1 shRNA. (C) Analysis of neuronal differentiation indicates that Nap1 deficiency significantly retards BDNF induced neurite growth and branching. Data shown are mean ± SEM (n=3); asterisk, significant when compared with controls at $p<0.01$ (Student's t test).

Figure 3.11.


A
BDNF (ng/ml)
BDNF + TrkB-IgG
25 ng/ml + 20 µg/ml
0 1 5 10 25 0 25
Nap1
Actin
B
Control shRNA
Nap1 shRNA
Control shRNA + BDNF
Nap1 shRNA + BDNF
C
Total neurite length
Neurite length (µm)
Control
BDNF
Control shRNA
Nap1 shRNA
Total number of branches
Number of branches

Discussion

Cytoskeletal regulation during neuronal migration and differentiation in the cerebral cortex

Molecular analysis of human cortical developmental deficits suggests that dynamic regulation of neural cytoskeleton determines distinct aspects of the generation, migration, and differentiation of neurons in cerebral cortex. During neurogenesis, a microtubule associating protein (abnormal spindle-like microcephaly associated protein: ASPM) is expressed specifically in the VZ and is thought to modulate the spindle activity of the neuronal progenitor cells, resulting in the generation of appropriate numbers of post mitotic neurons (Bond et al., 2002). As newly generated neurons exit the VZ and embark on their journey towards the CP, appropriate expression of actin- binding protein, filamin A (FLNA) regulates the initiation of migration (reviewed in Marin and Rubenstein, 2003; Mochida and Walsh, 2004, Ayala et al., 2007). Once neurons begin their migration, genes regulating microtubule cytoskeleton, including Lis1, doublecortin (Dcx), doublecortin like kinase (Dclk), Ndel1, MAP1b, MAP2, Tau, and mPAR6α, play an essential role in the maintenance of oriented neuronal motility (Solecki et al., 2004; Deuel at el., 2006; Koizumi et al., 2006; Shu et al., 2006; Tsai and Gleeson, 2005; Hatten, 2002). MAP1b, Tau, Filamin1, Nde1 and Dcx in migrating neurons are putative substrates for cyclin dependent kinase 5 (Cdk5), which together with its activating subunits, p35 and p39, functions to modulate normal neuronal migration in cerebral cortex (Tsai and Gleeson, 2005).

Though these observations clearly demonstrate that developmental stage specific expression and function of multiple cytoskeletal regulators critically

influence the generation and migration of neurons, the cytoskeletal changes or regulators essential to convert neurons that are engaged in oriented motility into neurons that are capable of extending axons and dendrites in the developing cortex are unclear. Our analyses show that Nap1 is a cytoskeletal regulator essential for this step in the developing cerebral cortex.

Suppression of Nap1 expression or mutations in Nap1 significantly retards neuronal differentiation (Fig. 3.3 – 3.7). Nap1 knockdown does not affect the migration or the placement of neurons. Cohorts of neurons that arrive in the cortical plate at the same time begin their morphological differentiation at the same time (Bayer and Altman, 1991; Miller, 1981). Thus the differences in differentiation noticed between control and Nap1 deficient neurons are unlikely due to delayed migration and resultant late initiation of differentiation by Nap1 deficient neurons. Furthermore, ectopic, premature induction of Nap1 in migrating cortical neurons retards their migration and promotes premature neuronal differentiation (Fig. 3.9, 3.10). Together, these observations suggest that expression of Nap1 is critical for the terminal, postmigratory differentiation of neurons in the developing cerebral cortex.

Regulation of Nap1 expression and function in the developing cortical plate

How does Nap1 modulate cortical neuronal differentiation? As a neuron undergoes terminal differentiation in distinct layers of the emerging cortical plate, it essentially transforms from a motile cell with a leading and trailing process, into one that is non-motile, but with multiple processes and branches. This requires generation of multiple membrane protrusive structures and

coordinated changes in actin and microtubule cytoskeleton in response to activity dependent, extracellular neuronal differentiation cues expressed in the developing cortical plate.

The growth and differentiation of cortical neurons rely on activity dependent neurotrophic factor (e.g., BDNF) signaling (McAllister et al., 1996; Ghosh et al., 1994; Reichardt, 2006). As such, the induction of Nap1, an essential cytoskeletal component of neuronal differentiation machinery, by BDNF in differentiating cortical neurons may also involve correlated neuronal activity. Selective expression of Nap1 in cortical plate neurons and the resultant formation of multimeric complexes (e.g., Nap1-WAVE1, Nap1-Cdk5) capable of distinct cytoskeletal regulation may usher in the cytoskeletal rearrangements that are essential to change the neuronal cytoskeletal machinery from one that promotes oriented motility to one that facilitates elaboration of axons and dendrites, and interconnectivity between appropriate synaptic partners.

CHAPTER IV

THE CELLULAR AND MOLECULAR MECHANISMS UNDERLYING NAP1 FUNCTION DURING CORTICAL NEURONAL DIFFERENTIATION

Introduction

During the development of cerebral cortex, we found that Nap1 plays an essential role in the timely differentiation of neurons. As neurons arrive in the cortical plate, neuronal cytoskeleton has to be modified from one that promotes cell motility to one that promotes neurite growth and differentiation. How Nap1 influences this cytoskeletal transition is unknown. We therefore sought to determine some of the cellular and molecular mechanisms underlying Nap1's functions during cortical neuronal differentiation. To explore this question, we determined the domains of Nap1 that are critical for its function, generated and characterized a Nap1 mutant mouse model, studied the significance of Nap1 in relevant cellular functions such as process extension and motility, evaluated the functional dynamics of Nap1 associated molecular complexes such as WAVE1, and identified novel Nap1 interactors in the developing cerebral cortex.

Materials and methods

Generation and characterization of Nap1 fragments

To characterize the domains of Nap1 that are critical for its function,

serial deletion fragments of Nap1 were subcloned into pCMV-EGFP plasmid (Clonetech) to generate Nap1 fragments fused to EGFP (pCMV-ΔNap1-EGFP). Cos7 cells or E9.5 neuroepithelial cells were transfected with Nap1 fragments to analyze the cellular localization of these EGFP tagged constructs. Nap1 deletion fragments were also subcloned into pCIG-IRES-EGFP plasmids and used to transfect wild type or mutant cells, alone or in combination with Nap1 shRNA, to analyze the effect of Nap1 deletion fragments.

Immunohistochemistry

Primary neuroepithelial cells from E9.5 telencephalon was isolated and maintained in DMEM with 10% FBS and penicillin/streptomycin (Schmid et al., 2003). The following antibodies were used: anti-cortactin (mouse, 4F11, Upstate), anti- WAVE1 (rabbit, gift from Dr. S. Kwak, Wyeth Laboratories), anti-Nestin (Iowa Hybridoma Bank), anti- acetylated tubulin (mouse, Sigma), anti-tyrosinated tubulin (mouse, Sigma), anti-Tuj1 (mouse, Covance), anti β-galactosidase (rabbit, MP biomedicals), anti-Nap1 (rabbit, gift from Dr. Stradal, German Research Centre for Biotechnology), anti-Mena (rabbit, gift from Dr. Gartler, MIT) and anti-RC2 (Iowa Hybridoma Bank). Appropriate Alexa-dye labeled secondary antibodies (Molecular Probes) or Cy2 and cy3 secondary antibody (Jackson ImmunoReseach) were used to detect primary antibody binding. For phalloidin staining, either an Alexa 488-phalloidin (Molecular Probes) or a TRITC-phalloidin (Sigma) was used.

Generation of Nap1 mutant mice

Two independent ES cell lines, XE133 and XE68, containing identical insertions in the Nap1 locus were obtained (BayGenomics). These insertions utilized the pGT1lxf gene trap vector, which contains an En2 splice acceptor flanked by a 5′ lox71 and 3′ loxP site, and a gene fusion, β-geo, between β-galactosidase (β-gal) and the neomycin resistance gene (Mitchell et al., 2001). RT-PCR of the ES cells was used to confirm the presence of the insertion. Briefly, 5 μg total RNA isolated from ES cells using the Trizol method was reverse transcribed with MMLV reverse transcriptase using oligo 78 (5′-TAATGGGATAGGTTACGT-3′). PCR was then performed using cDNA derived from XE133 or XE68 ES cells as a template with the following primers: oligo 79 (common) 5′-AGTATCGGCCTCAGGAAGATCG-3′ and XE133FP1 5′-CTGTATACAAACTGGTACTTGG-3′ or XE68FP1 5′-AGACAGCCATGGAGAACCAACC-3′. To confirm that the XE133 and XE68 ES cells contained a single insertion site, Southern blotting was performed using genomic DNA isolated from ES cells digested with EcoRV and probed with a β-galactosidase specific [α-^{32}p] dCTP labeled DNA probe. ES cells harboring gene trap insertions were used to generate chimeric mice by blastocyst injection, and germline transmission was achieved by mating male chimeric mice to wild-type C57Bl6 females. Additionally, F2 heterozygous males were outcrossed to wild type CD1 females; however, genetic background did not affect embryonic viability or phenotype. These mice were generated in collaboration with Dr. Pevny.

Northern blot analysis and Nap1 mutant genotyping

Total RNA was isolated from E9.5 embryos using Trizol (Invitrogen) and analyzed by Northern blotting using αP^{32} dCTP DNA probes for Nap1 and β-actin. Genomic insertion site of the pGT1lxf gene trap vector was determined by sequence analysis of nested PCR products. PCR genotyping analysis of WT allele generates a 710 b.p. product using the following primers: Nap1-FPa (common) 5′-CTGTCTGCCTGCCTCTGCTTTTTG-3′ and Nap1-RP2a 5′-CCCACCCCCCAGGGTTTGAGTAGAG-3′, mutant allele generates 325 b.p. product using Nap1-FPa and pGT1lxf-RPa 5′-CCACCCTGGGGTTCGTGTCCTAC-3′.

Characterization of Nap1 mutant neuroepithelial cells

Telencephalic neuroepithelium of E9.5 embryos were dissociated in DMEM with 10% FBS and penicillin/streptomycin, maintained on laminin coated coverslips for 4 days to permit differentiation, fixed in 4% PFA, and immunolabeled with RC2, Tuj1 and anti-nestin antibodies. Images of differentiated and undifferentiated neuroepithelial cells were obtained with a Zeiss Pascal confocal microscope.

In some experiments, small pieces of telencephalon tissue were cultured as explants on laminin coated glass bottom 35mm dishes in DMEM/ 10% FBS. Time lapse images of neuroepithelial cells migrating out from the explants were obtained using Zeiss Pascal live cell imaging system. The rate and direction of migration were analyzed using Zeiss image browser. After 4 days *in vitro*, explants were immunostained with various cell-type specific markers as

described earlier.

For scanning EM, neuroepithelial cells were washed with warm PBS for 2-3 times, fixed in 3% glutaraldehyde in 0.15M sodium phosphate buffer, pH7.4, and submitted to UNC EM facility.

Live imaging of neural tube development in Nap1 mutants

E8.5-9.5 embryos from Nap1$^{lacZ/LacZ}$, ACTB-EGFP or Nap1$^{wt/wt}$, ACTB-EGFP mice were immobilized with a mix of artificial cerebrospinal fluid and 1% low melting point agarose on a MatTek 35 mm glass bottom dish, immersed in OptiMEM/10% FBS media, and placed in a live incubation chamber attached to a Zeiss Pascal confocal microscope. The apposing neural folds of the head region of the embryos were repeatedly imaged every 3 minutes for up to 2 hours.

PDGF assays

For PDGF treatment, telencephalic neuroepithelial cells were cultured in DMEM (Gibco) without serum for 12 hrs, treated with 10ng/ml PDGF-BB for 15 min, then fixed in 4% PFA and processed as above for immunofluorescence with phalloidin or anti- cortactin antibodies. In some experiments, PDGF (10 μg/ml) coated heparin beads were placed adjacent to cells and they were repeatedly imaged at 1-5 minute intervals (Suetsugu et al., 2003) with Zeiss confocal microsope. The rate of membrane extension towards the beads as well as the number of cells responding to PDGF with lamellipodial formation was measured.

WAVE1 protein tracking

To characterize the movement of WAVE1 protein in normal and Nap1 disrupted cells, WAVE1 (gift from Dr. Terada, University of Texas Southwestern) and Kaede (MBL Co.) were subcloned into pCAGS plasmid to generate WAVE1 fused to Kaede. E9.5 neuroepithelial cells from wild type and Nap1 mutant cells were transfected with WAVE1-Kaede (green). Some of the wild type cells were also co-transfected with Nap1 shRNA and WAVE- Kaede. Twenty four hours later, localized spots in transfected cells were photoconverted with a 200m.sec pulse of UV laser (351-364nm) attached to a Leica SP2 Laser Scanning confocal microscope. The movement of converted WAVE1-Kaede (red) was evaluated by time lapse imaging of WAVE1 green/red fluorescence. The relative changes in WAVE1 fluorescence intensity in the photoconverted regions of the cells were measured using Zeiss LSM image browser and image J program.

Screening of Nap1's binding partner in the developing cerebral cortex

Anti-Nap1 antisera, anti-actin or anti-tubulin were added to extracts prepared from E16 telencephalon lysed in Buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 5% glycerol, 1% TritonX100, 1%, NP-40, 1%, protease inhibitor cocktail and phosphatase inhibitor) for immunoprecipitation. Non-immune serum was used as controls. Antibody-Antigen complexes were captured with Protein A Sepharose beads (Zymed). Beads were then washed five times in lysis buffer, suspended in PBS, and submitted for MALDI mass-spec analysis at UNC Proteomics Core Facility. Purified actin and tubulin were purchased from Cytoskeleton Inc. to biochemically evaluate Nap1's association

with actin and tubulin.

Results

Functional domains of Nap1

Having established the functional significance of Nap1 in cortical neuronal differentiation, we sought to determine the domains of Nap1 that are critical for its function. Initially, we generated serial deletion fragments of Nap1 fused to EGFP(pCMV-ΔNap1-EGFP), transfected Cos7 cells with Nap1 fragments, and analyzed the cellular localization of different Nap1 fragments(Fig. 4.1). The full length Nap1 localized to the membrane edges (arrowheads, Fig. 4.1B). Deletion of one putative membrane association domain at C-terminus (1019 a.a. fragment) did not alter the localization to the membrane edges. However, deletion of all four membrane association domains in the C-terminal region lead to the association of the deleted Nap1 (910 a.a. and 898 a.a. fragments, Fig. 4.1D, E) with acetylated tubulin positive, stable microtubules (Fig 4.2). Shorter deletion fragments either associate with membrane edges (707 a.a. and 480 a.a. fragments, Fig. 4.1 F, G) or were diffusely distributed through out the cell (315 a.a. and 67 a.a. fragments, Fig. 4.1 H, J). Intriguingly, a 200 a.a fragment that contains one membrane association domain from the N-terminal region, localized to the microtubule organizing center (Fig. 4.1 I). This serial deletion analysis suggest that distinct domains of Nap1 may play a role in the specific targeting or association of Nap1 to distinct cellular compartments (i.e., membrane edges, microtubules, or microtubule organizing center) during neuronal development. Surprisingly, Nap1, in addition to its previously suggested role in actin dynamics,

may also be capable of modulating microtubule cytoskeleton.

Figure 4.1. Serial deletion analysis of Nap1.

(A-J) Serial deletion fragments of Nap1 fused to EGFP (pCMV-ΔNap1-EGFP) were transfected into Cos7 cells to analyze the cellular localization of different Nap1 fragments. Full length Nap1 localized at the membrane edges (B, arrowheads). Deletion of the C-terminal region up to 898aa leads to the association of these Nap1 fragments (910 and 898aa) with microtubules (D, E). (F-J) Shorter deletion fragments either associate with membrane edges (arrowheads; F, G) or were diffusely distributed through out the cell (H, J). A 200 a.a fragment from the N-terminal region (I), localized to microtubule organizing center (MTOC). Arrows indicate the length of the respective Nap1 fragment. Dark gray vertical lines indicate putative membrane association domains of Nap1.

Figure 4.1.

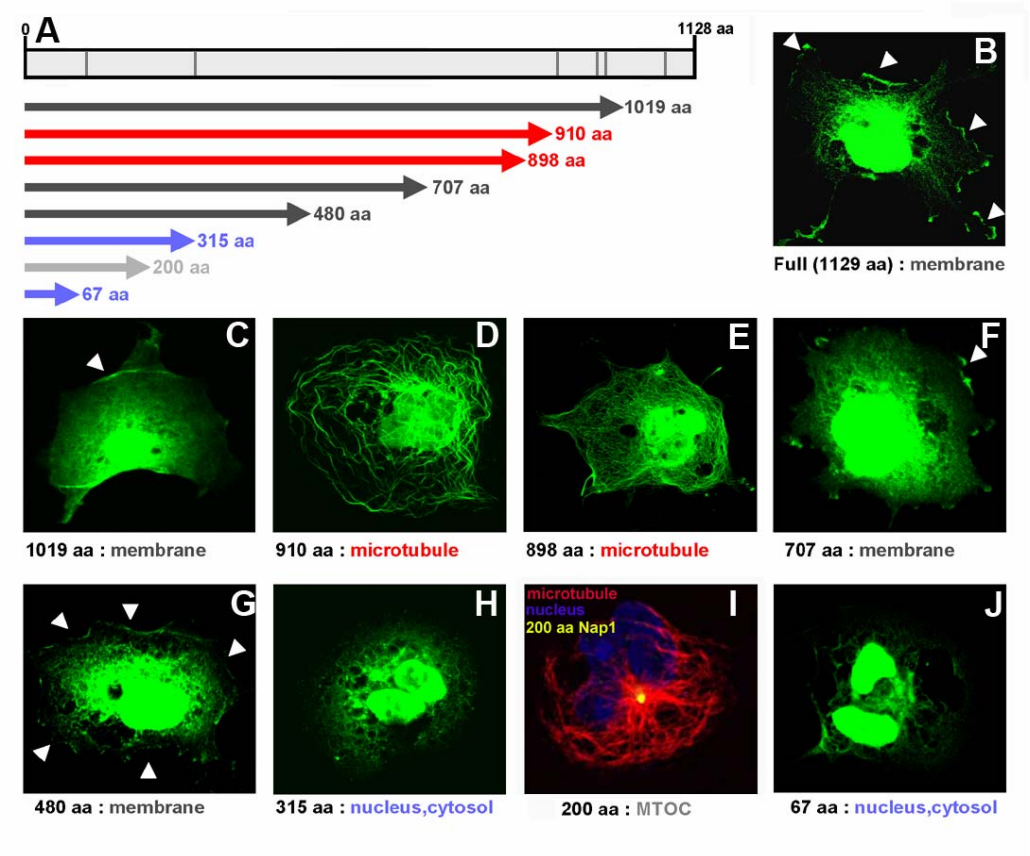
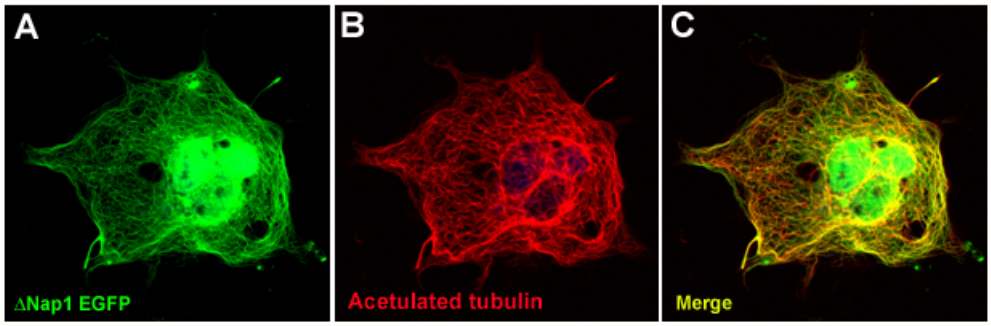

A
0
1128 aa
1019 aa
910 aa
898 aa
707 aa
480 aa
315 aa
200 aa
67 aa
B
Full (1129 aa) : membrane
C
1019 aa : membrane
D
910 aa : microtubule
E
898 aa : microtubule
F
707 aa : membrane
G
480 aa : membrane
H
315 aa : nucleus,cytosol
I
microtubule
nucleus
200 aa Nap1
200 aa : MTOC
J
67 aa : nucleus,cytosol

Fig.4.2 C-terminal deletion of Nap1.

(A-C) Deletion of the C-terminal region (11228-898aa) leads to the association of this Nap1 fragment with stable, acetylated microtubules.

Fig4.2.



Defective neuronal differentiation in Nap1 mutant mice

Since Nap1's localization on membrane edges might be critical for its function to induce process outgrowth during neuronal differentiation in the cortical plate, we generated a Nap1 mutant mouse line from ES cells in which the function of the Nap1 gene has been disrupted by insertional mutagenesis with β-geo reporter (Leighton et al., 2001; BayGenomics), resulting in the deletion of Nap1 C-terminal region essential for the membrane localization of Nap1. The insertion site was mapped to the intronic region flanked by exons 24 and 25 (Fig. 4.3A). The resulting protein is thus a fusion containing the N-terminal 898 amino acids (a.a.) of Nap1 (ΔC Nap1), fused to the 1291 a.a. of the β-geo reporter (Fig. 4.3A, B). Northern analysis with a Nap1 probe demonstrates the presence of wild type Nap1 transcript in the wild type, but not in the homozygote embryos (Fig. 4.3D). Immunoblot analysis confirms the absence of wild type Nap1 protein and the presence of a mutant Nap1-ΔC-βgal fusion protein in homozygous mutants [$Nap1^{lacZ/lacZ}$] (Fig. 4.3C).

Analysis of litters derived from heterozygous crosses demonstrate that by embryonic day 8.5 (E8.5), Mendelian ratios of wild type, heterozygous and homozygous embryos are detected; however, there was a drastic decrease in the number of homozygotes by E10.5, and no live $Nap1^{lacZ/lacZ}$ embryos were found at E11.5. Phenotypically normal $Nap1^{lacZ/lacZ}$ embryos could be recovered through E7.5, but embryos recovered from E8.5-E10.5 had varying degrees of morphological abnormalities ranging from severe neurulation defects to complete resorption. The most common phenotype observed in $Nap1^{lacZ/lacZ}$ E10.5 embryos is the strikingly open, undulating neural folds, which remain unfused

along most of the rostral extent of the embryo, up to the mid spine (Fig. 4.3E). Wild type littermate controls at this stage display complete closure of the cranial neural tube and spinal cord (Fig. 4.3F, G). 12.4% of heterozygote mice show an open neural tube phenotype; however most heterozygous animals survive to adulthood (Fig. 4.3H). Histological analysis of these mutant heterozygous animals at E9.5 demonstrated dramatic abnormalities in the telencephalic neuroepithelium (Fig. 4.3I, J). Apical localization of actin filaments in neuroepithelial cells during neural tube closure is essential to complete this process (Ybot-Gonzalez and Copp, 1999; Copp et al., 2003a, b; Rakeman and Anderson, 2006). Analysis of actin distribution with phalloidin labeling indicate that, in contrast to wild type embryos, apical accumulation of actin filaments needed for neural tube closure is severely disrupted in Nap1 mutants (Fig. 4.3H''). To evaluate the neural tube defect further, we crossed Nap1 heterozygotes to ACTB-EGFP mice, expressing EGFP in all tissues under chicken β-actin promoter, to generate $Nap1^{lacZ/+}$, ACTB-EGFP mice. These mice were intercrossed to generate $Nap1^{lacZ/lacZ}$, ACTB-EGFP embryos. Live confocal imaging of apposing neural folds in the head region of these embryos indicate that the movement of neuroepithelial cells towards midline needed for neural tube closure is disrupted in Nap1 mutants (Movie 4.1-4.3). To further investigate the motility of these neuroepithelial cells, we evaluated the orientation and speed of migration of wild type and Nap1 mutant neuroepithelial cells. We confirmed that these cells dissociated from the telencephalon of E9.5 embryos are nestin positive neuroepithelial cells (Fig 4.4). Dissociated neuroepithelial cells were seeded on laminin coated coverslips to generate monolayers of cells. After 24 hrs, a wound

was made by scraping with a micropipette tips (diameter: 0.5 mm). Nap1 mutant cells filled the wound width faster than wild type cells (Fig 4.5A). Even though the rate of migration was faster in Nap1 mutant cells (WT: 20.17 ±1.35 μm/hr, Nap1: 34.17±1.71 μm/hr) the orientation of their migration was random and often away from the wound space, when compare to wild type cells (Fig.4.5 B, C). This random, fast movement of Nap1 mutant neuroepithelial cells *in vitro* is indicative of potentially disrupted neuroepithelial cell movement during neural tube closure in Nap1 mutant embryos.

Figure 4.3. Generation and characterization of Nap1 mutant mice.

(A) Genomic structure of Nap1 locus on mouse chromosome 2, and insertion of gene trap vector pGT1lxf (BayGenomics) between exons 24 and 25. Wild type Nap1 protein is approximately 125 kD, whereas mutant truncated-fusion protein is approximately 241 kD. (B) Brain extract from 3-week postnatal wild type (WT/WT) and heterozygous ($Nap1^{LacZ/WT}$) mice was subjected to immunoprecipitation (IP) followed by Western blot (WB) analysis with an anti-β-galactosidase antibody. Truncated Nap1-β-gal fusion protein is expressed in $Nap1^{LacZ/wt}$, but not in WT brains. Similar analysis using Rosa 26 brain extract is shown as a control. (C) Extract from E9.5 homozygous ($Nap1^{LacZ/LacZ}$) and wild type mice were similarly subjected to immunoprecipitation followed by Western blotting with a C-terminal specific anti-Nap1 antibody to demonstrate the absence of wild-type Nap1 protein in homozygous mutant embryos. (D) Total RNA from wild type, heterozygous and homozygous embryos were analyzed by Northern blot using a Nap1 C-terminal probe, demonstrating the lack of wild type transcripts in mutants. β-actin is shown as a loading control. (E) Scanning electron micrograph (SEM) of the dorsal surface of a E9.5 $Nap1^{LacZ/LacZ}$ mutant embryo, where the neural folds (NF) remain unfused and wavy along the entire length of the anteroposterior axis. Anterior is to the left. (E') Anterior view of the cranial neural folds, which are completely open and curled over at the edges. White arrow indicates ventral midline. (F, G) SEM of the dorsal surface (F) and anterior view (G) of the neural tube in a wild type E9.5 embryo. The neural folds have completely closed, and the surface appears smooth. (H) SEM of the dorsal surface of an E10.5 embryo, which displays a neurulation defect. The luminal

surface is completely exposed, and the edges of the neural folds have curled outward. (H′) β-gal expression in Nap1$^{LacZ/WT}$ embryos indicates Nap1 expression in the developing neural tube (arrow). (H′′) E9.5 wild type and mutant embryos were labeled with phalloidin and the midline region of anterior neural tube areas were imaged. Actin accumulation in the apical region of neuroepithelial cells is evident in wild type (arrows), but not Nap1 mutant (arrowhead) embryos. (I) H&E stained section of a wild type E9.5 embryo. Note the closed cranial neural tube (*). (J) Section from an E9.5 heterozygous animal, which demonstrates a neural tube defect. Note the neural folds which appear to curl outward, and remain unfused (*).

Figure 4.3.

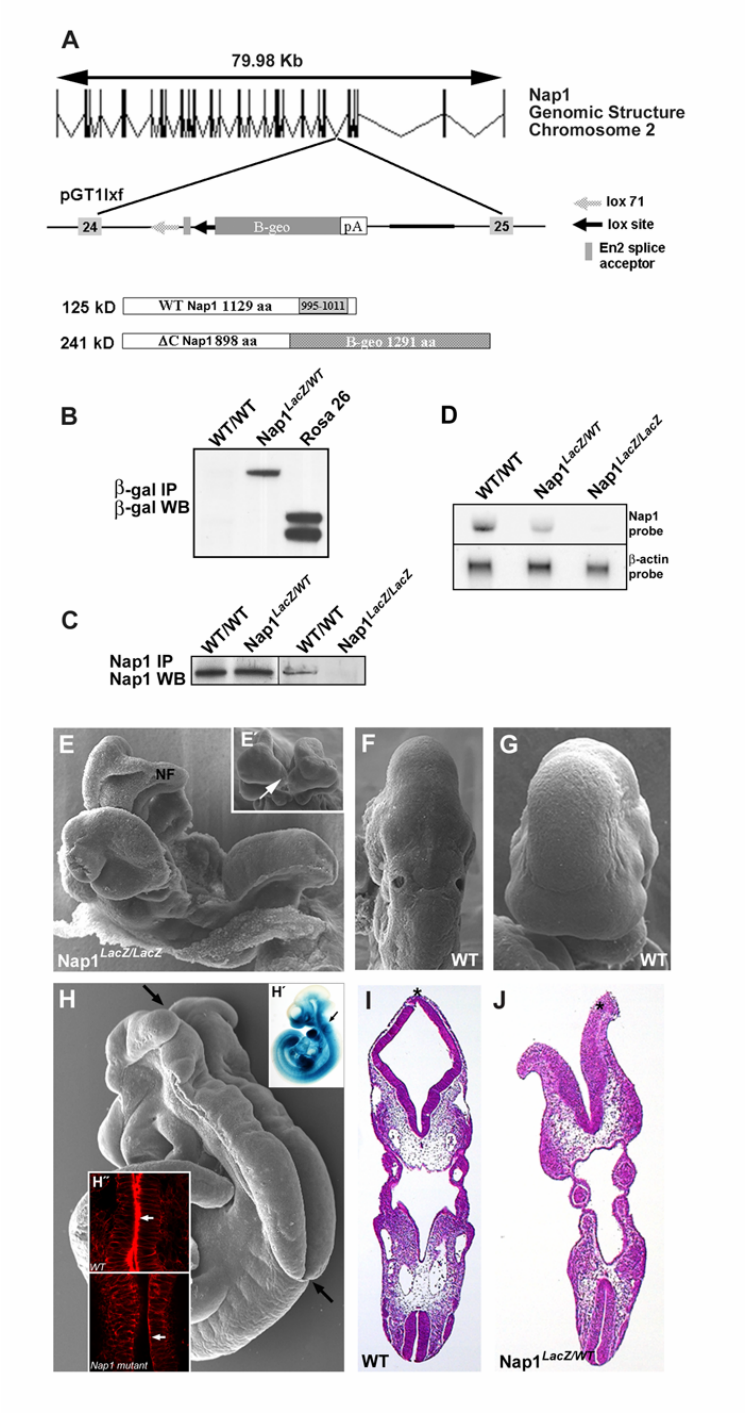

A
79.98 Kb
Nap1
Genomic Structure
Chromosome 2
pGT1lxf
24
B-geo
pA
25
lox 71
lox site
En2 splice
acceptor
125 kD
WT Nap1 1129 aa
995-1011
241 kD
ΔC Nap1 898 aa
B-geo 1291 aa
B
WT/WT
Nap1LacZ/WT
Rosa 26
β-gal IP
β-gal WB
D
WT/WT
Nap1LacZ/WT
Nap1LacZ/LacZ
Nap1
probe
β-actin
probe
C
WT/WT
Nap1LacZ/WT
WT/WT
Nap1LacZ/LacZ
Nap1 IP
Nap1 WB
E
NF
E´
Nap1LacZ/LacZ
F
WT
G
WT
H
H´
H´´
WT
Nap1 mutant
I
WT
J
Nap1LacZ/WT

Figure 4.4. Nestin positive telencephalic neuroepithelial cells.

Dissociated cells from the telencephalic neuroepithelium of E9.5 embryos were labeled with anti- nestin antibodies (red), phalloidin (green), and nuclear marker, bis benzimide (blue).

Fig 4.4.

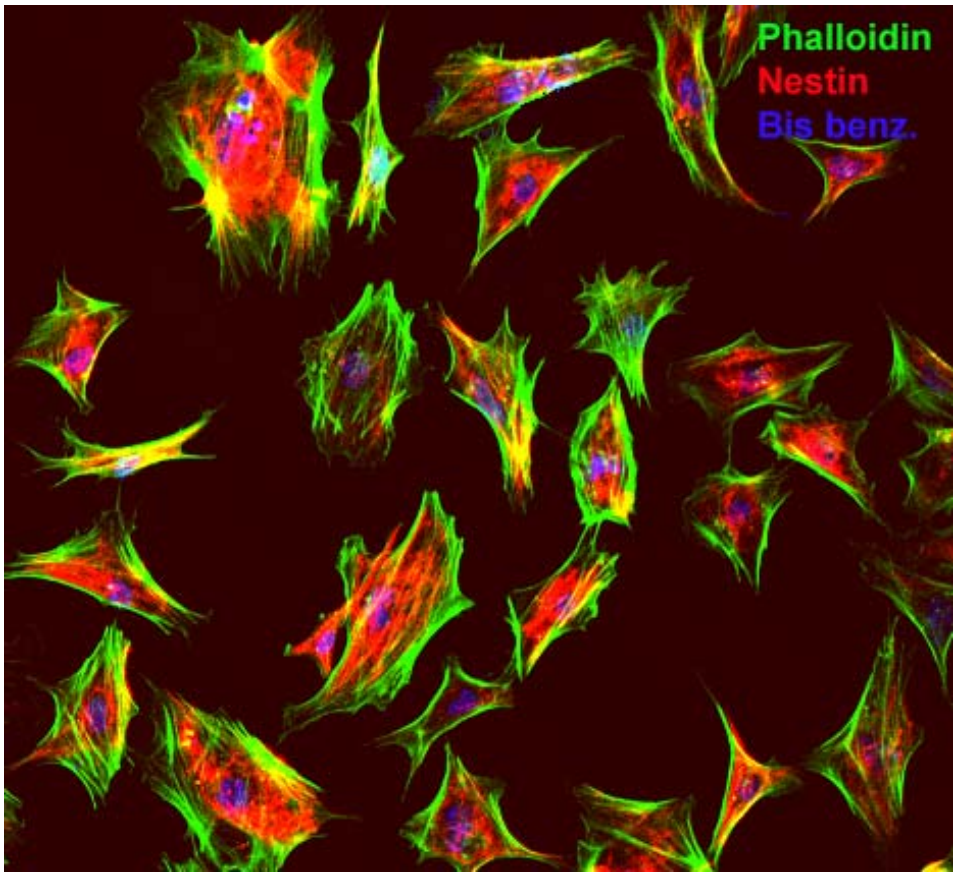
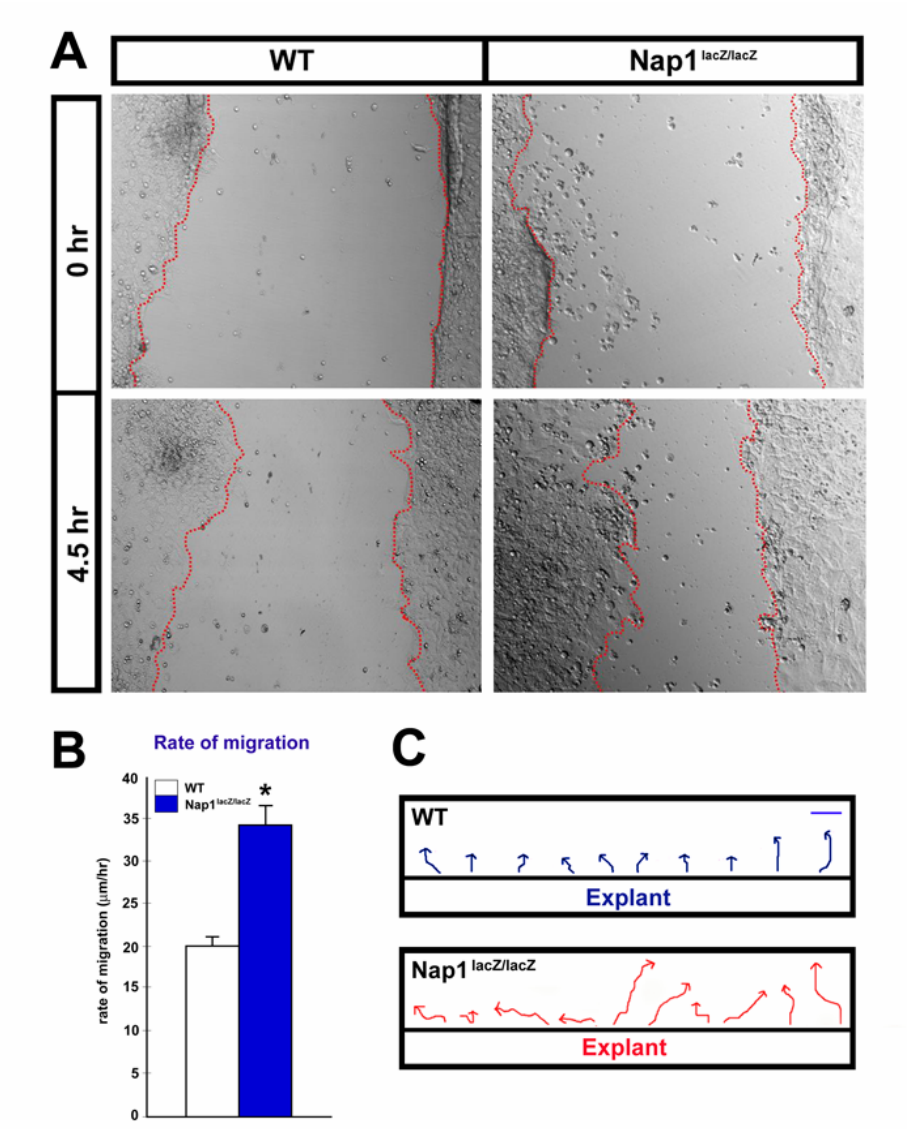


Figure 4.5. Effect of Nap1 in neuroepithelial cell motility.

(A) Scratch-wound assay of wild type and Nap1 mutant neuroepithelial cell monolayers from E9.5 embryos. Monolayers of neuroepithelial cells were cultured for 24 hrs followed by scratch-wound with pipette tips. Phase contrast images of neuroepithelial cells were obtained at 0 hr and 4.5 hr after wounding. Nap1 mutant cells filled the wound space faster than wild type cells. (B) Quantification of the rate of migration of neuroepithelial cells. Nap1 mutant cells migrate significantly faster than wild type cells. Asterisk indicates significant when compared with controls at $p<0.01$ (Student's t test). (C) Orientation of neuroepithelial cells migrating out from the neuroepithelial cell explants were traced for 1hr period. Most of the wild type cells migrate perpendicularly to the explants, whereas Nap1 mutant cells migrate in multiple random orientations. Scale bar: 10 μm.

Figure 4.5.


A
WT
Nap1 lacZ/lacZ
0 hr
4.5 hr
B
Rate of migration
WT
Nap1 lacZ/lacZ
*
rate of migration (μm/hr)
40
35
30
25
20
15
10
5
0
C
WT
Explant
Nap1 lacZ/lacZ
Explant

Nap1's role in neuroepithelial cell differentiation

The early embryonic lethality prevents the use of this mouse model to study Nap1's role in cortical neuronal differentiation *in vivo*. However, to understand how Nap1 may influence neuronal differentiation, wild type and Nap1 mutant E9.5 telencephalic neuroepithelial cells, which eventually give rise to radial glia and cortical neurons, were cultured for 4 days to allow for the generation of RC2 positive radial glia and classIII β-tubulin (Tuj-1) positive neurons *in vitro*. Nap1 mutant neuroepithelial cells are defective in their ability to give rise to radial glia and neurons. Wild type neurons display the characteristic differentiated phenotype with elongated axons, dendrites, growth cones, and dendritic spines (Fig. 4.6 A, B). In contrast, Nap1 mutant neurons are severely defective in their ability to differentiate and extend axons and dendrites (Fig. 4.6 C-F). Instead, Nap1 mutant neurons extend short, ill-defined stumps of neurites. These data further support the hypothesis that Nap1 plays an essential role in the final phenotypic differentiation of cortical neurons. Intriguingly, Nap1 may also play a role in the differentiation of radial glia, which divides asymmetrically to give rise to post- mitotic daughter neurons (Fig 4.7). Consistent with this possibility, Nap1 mutant explant cultures contained 12.8 % fewer neurons than the wild type explants (% of Tuj1 + neurons- WT: 29.50±3.67%; Nap1 mutant: 16.70±2.31%)

Figure 4.6. Defective neuronal differentiation in Nap1 mutants.

Telencephalic neuroepithelial cells from wild type and Nap1 mutant mice were maintained *in vitro* to generate neurons. Compared to wild type neurons (A, B, arrows), Nap1 mutant neurons (C) are severely defective in their ability to extend axons and dendrites. Higher magnification images of Nap1 mutant neurons (D, E) illustrate the ill- defined, short, stubby processes extended by these mutant neurons (arrowheads, D-E). (F) Assessment of neuronal differentiation (i.e., % of neurons with processes greater than 20μm length) indicates drastic deficits in Nap1 mutant neurons. Scale bar: A-C, 20 μm; D-E, 7μm.

Figure 4.6.

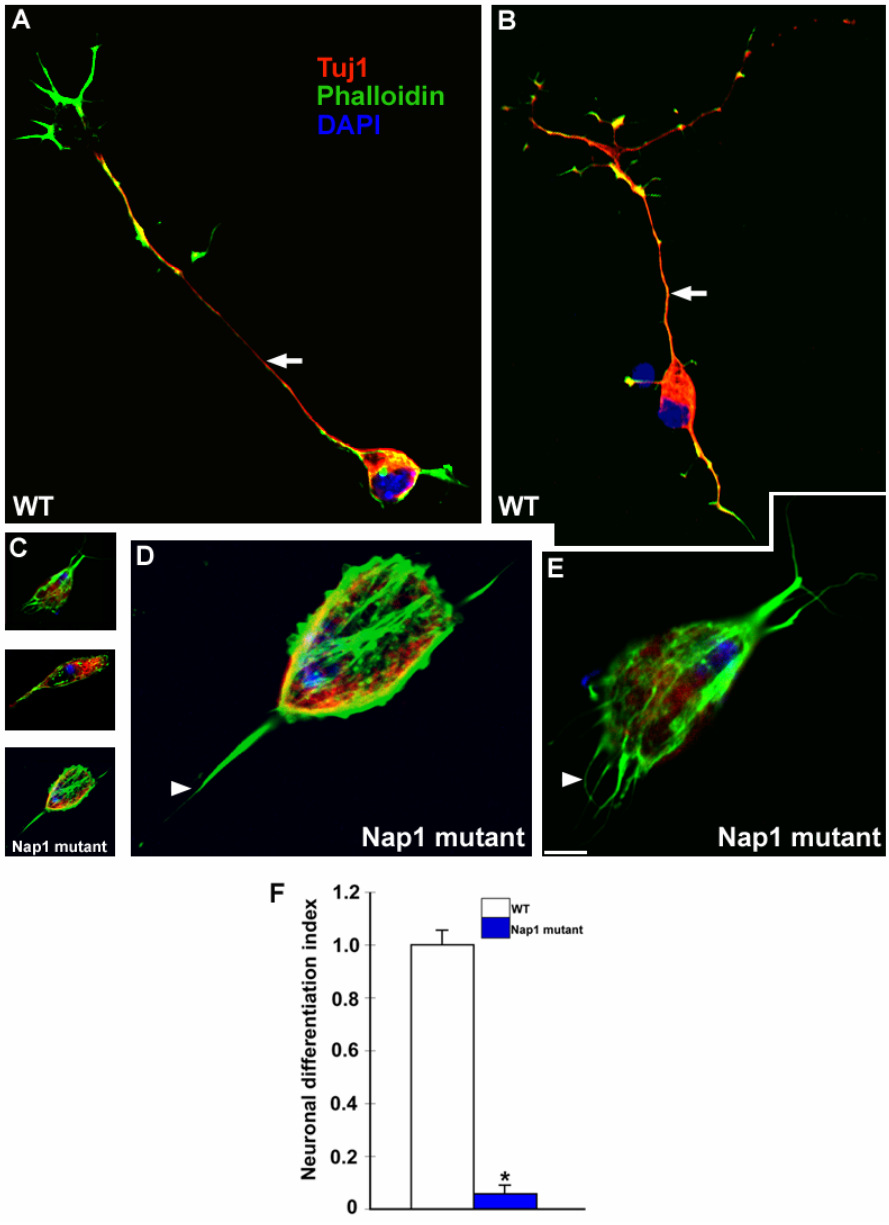
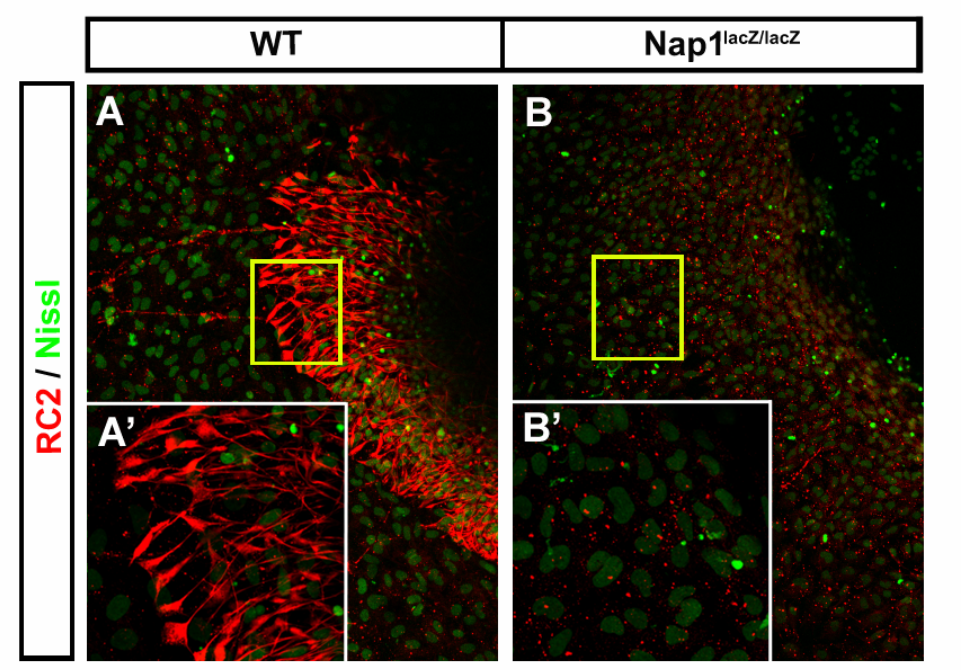

A
Tuj1
Phalloidin
DAPI
WT
B
WT
C
Nap1 mutant
D
Nap1 mutant
E
Nap1 mutant
F
Neuronal differentiation index
1.2
1.0
0.8
0.6
0.4
0.2
0
WT
Nap1 mutant
*

Figure 4.7. Defective Radial glial differentiation in Nap1 mutants.

E9.5 telancephalic neuroepithelium was cultured as explants for 4 days *in vitro* to generate RC2 positive radial glial cells. (A)Wild type explants contained RC2 positive radial glial cells with characteristic elongated processes. In contrast, a severe deficit in RC2 positive radial glial cells was evident in Nap1 mutant explant cultures. (A′, B′) High magnification images of outlined regions of panels A and B, respectively.

Figure 4.7.



Nap1's role in cytoskeletal organization

To evaluate the cellular mechanisms underlying Nap1's role in neuronal differentiation, we first analyzed the cytoskeletal organization of Nap1 mutant neuroepithelial cells. Since the early embryonic lethality of Nap1 mutants precludes the use of neurons, we used the telencephalic neuroepithelial cells that give rise to neurons in these studies. A significant disruption in actin cytoskeletal organization was noticed in Nap1 mutant cells. In contrast to wild type cells, actin filaments preferentially formed actin bundles around the cell cortex of the Nap1 mutant cells (Fig.4.8A, B). 94.6(±1.5) % of the mutant cells have actin filaments arrayed around the edges of the cells, compared to 2.96(±1.6) % of the wild type cells. Furthermore, the ratio of tyrosinated tubulin (i.e., newly polymerized tubulin) to acetylated tubulin (aged, stable form of tubulin) increases in Nap1 mutants and the acetylated tubulin fibers often form circular mesh works in Nap1 mutants. shRNA mediated knock down of Nap1 also disrupted tubulin structure (Fig.4.8C-F).

Importantly, Nap1 mutant cells are mostly devoid of lamellipodia (Fig.4.8 A,B, Fig4.9). Live imaging of wild type and Nap1 mutant neuroepithelial cells indicate that wt cells display no defects in lamellipodial formation or activity, whereas Nap1 mutants are devoid of lamellipodia, and instead extend long, spiky processes which resemble flaccid filopodia (Movie 4.4 and 4.5). In Nap1 mutant cells, immunoreactivity for cortactin, a marker of peripheral lamellipodia, is absent from the cell periphery, demonstrating that indeed these cells lack lamellipodia (Fig.4.9 A-F). This was further confirmed by immunoreactivity for Mena (Fig. 4.9 G-K), which localizes to active actin nucleation edges (Fig. 4.9 I).

To investigate if protrusive structures, undetectable by immunohistochemical methods, at the dorsal cell surface of Nap1 mutant cells, we analyzed the neuroepithelial cells using scanning electron microscopy (Fig.4.10). In contrast to wild type cells with lamellipodial structures at the periphery of the cells (Fig. 4.10 A-C), Nap1 mutant cells contain spiky, filopodial-like extensions in the peripheral surface (Fig. 4.10 D-G). No dorsal lamellipodial extensions were evident in Nap1 mutants. C-terminal deleted Nap1 (pCIG-ΔNap1-IRES-EGFP) overexpressing wild type cells displayed a disrupted actin cytoskeletal organization and lamellipodial formation. ShRNA mediated knockdown of Nap1 in wild type cells also disrupted actin cytoskeletal organization and lamellipodial formation (Fig.4.11 A,B,E). Conversely, expression of full length Nap1 (pCIG-Full Nap1-IRES-EGFP) rescued the Nap1 mutant phenotype (Fig.4.11 C-E).

Figure4.8. Actin and microtubule cytoskeletal organization is disrupted in Nap1 mutants.

(A-B) Dissociated cells from the telencephalic neuroepithelium of E9.5 wild type and Nap1 mutant embryos were stained with phalloidin (green) and deoxyribonuclease (red). Phalloidin staining indicates F-actin, whereas deoxyribonuclease labels free actin monomers. Compared to wild type cells (A), actin filaments (green) accumulate at the edges of Nap1 mutant cells (B). (C, D) Immunolabeling of wild type and mutant cells with anti- acetylated tubulin antibodies, indicates disrupted acetylated tubulin organization in Nap1 mutants. Compared to the orderly array of stable microtubules in the wild type cells (C, white arrowhead), acetylated microtubule strands appear to be disrupted and form concentric rings in Nap1 mutant cells (D, blue arrowhead). Similar disruptions in acetylated tubulin organization is also evident in Nap1 shRNA expressing cells (E). Immunoblot analysis of acetylated tubulin and tyrosinated tubulin in wild type and Nap1 mutant telencephalon indicates that Nap1 mutation reduced the level of stable, acetylated microtubules (blue arrowhead, F).

Figure 4.8.

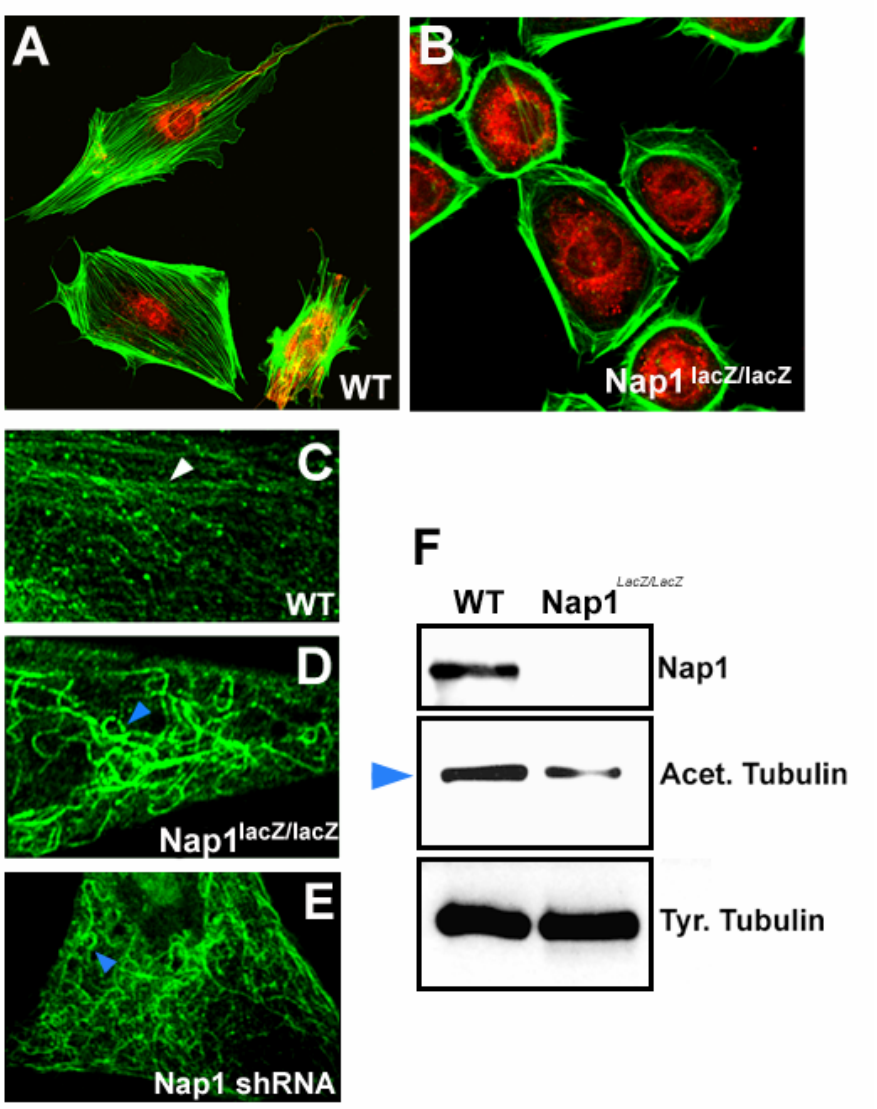

A
WT
B
Nap1 lacZ/lacZ
C
WT
D
Nap1lacZ/lacZ
E
Nap1 shRNA
F
WT
Nap1LacZ/LacZ
Nap1
Acet. Tubulin
Tyr. Tubulin

Figure 4.9. Analysis of lamellipodial formation in Nap1 mutant cells.

Primary neuroepithelial cells from wild type (A-C) and Nap1 mutant embryos (D-F) were labeled with phalloidin (green) and anti- cortactin antibodies (red). Wild type cells generate normal lamellipodia as seen by cortactin immunoreactivity and phalloidin staining (arrowheads, B), whereas cells from Nap1 mutant cells generate abundant spiky protrusions (E), but not many lamellipodia. (G-J) Similar deficits in lamellipodia were also noticed when control and mutant cells were labeled with phalloidin (green) and anti- Mena antibodies (red). Arrowheads (G,H) indicate lamellipodia. (K) Quantification of cells with lamellipodia indicates a 90 % reduction in lamellipodial formation in Nap1 mutant cells. Number of cells/ group> 3000. Data shown are mean ± SEM; asterisk, significant when compared with controls at $p<0.001$ (Student's t test).

Figure 4.9.

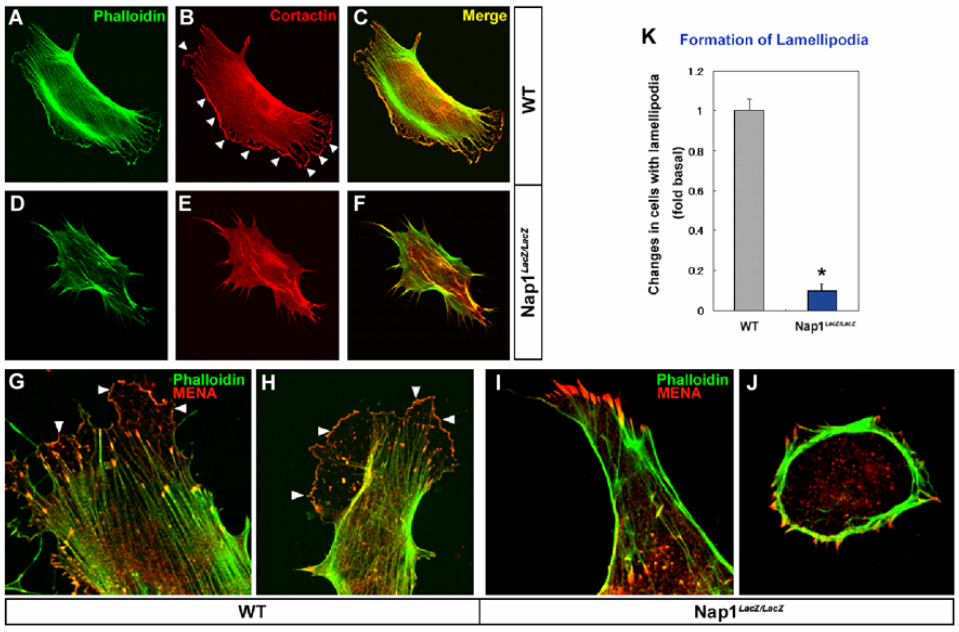

A
Phalloidin
B
Cortactin
C
Merge
WT
D
E
F
Nap1LacZ/LacZ
K
Formation of Lamellipodia
Changes in cells with lamellipodia (fold basal)
1.2
1
0.8
0.6
0.4
0.2
0
*
WT
Nap1LacZ/LacZ
G
Phalloidin
MENA
H
I
Phalloidin
MENA
J
WT
Nap1LacZ/LacZ

Figure 4.10. Scanning EM analysis of wild type and Nap1 mutant neuroepithelial cells.

(A-C) Scanning EM of a E9.5 WT neuroepithelial cell. High magnification view of peripheral lamellipodial structure in outlined areas are shown in panels B and C. (D-G) Nap1 mutant cells lack peripheral or dorsal lamellipodia and instead, extend long filopodial like spikes from the peripheral edges.

Figure 4.10.

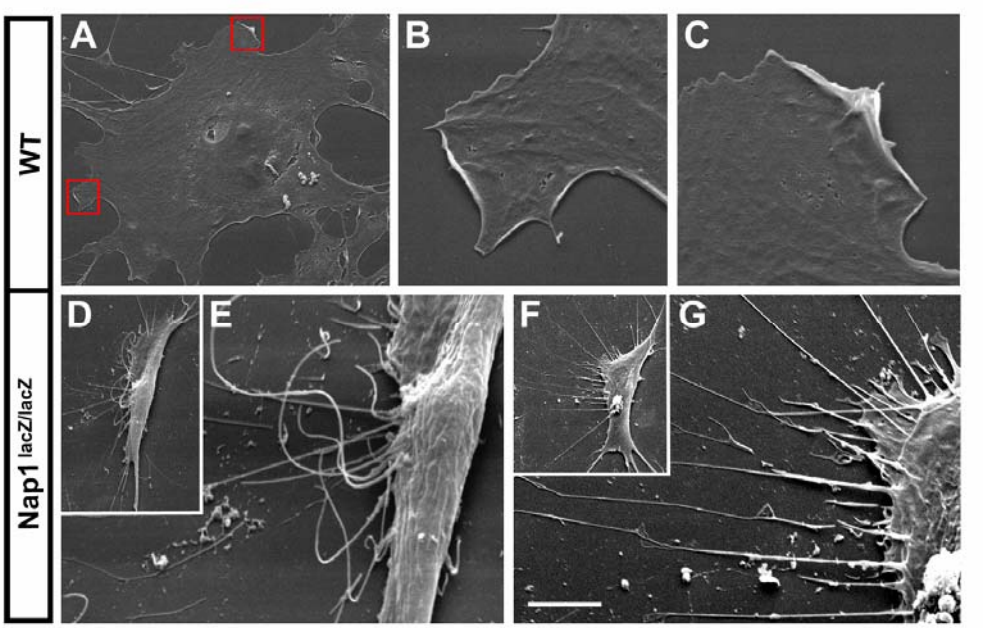
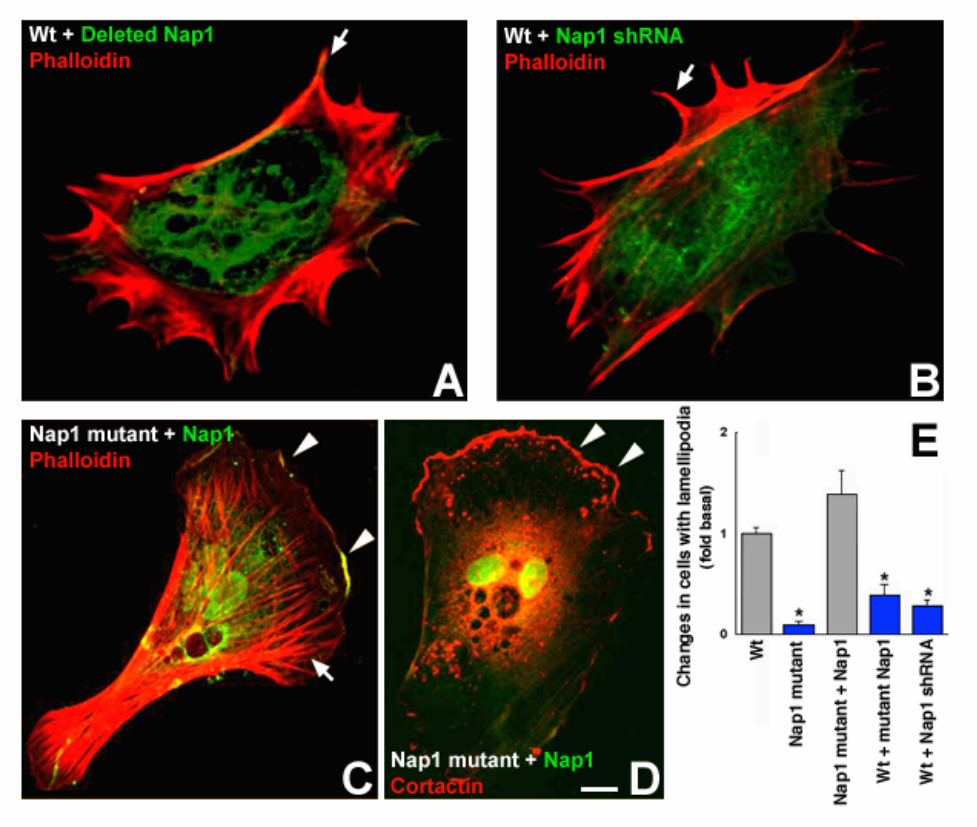


Figure 4.11. Rescue of Nap1 effect.

Expression of C-terminal deleted Nap1 (A) or Nap1 shRNA (B) in wild type neuroepithelial cells leads to disorganized actin (red), spiky process extension (arrows, A, B), and loss of lamellipodia (E). Expression of full length Nap1 rescues the mutant phenotype. Actin stress fibers (arrow, C) and lamellipodia (arrowheads, C and D; E) seen in wild type cells are evident in rescued mutant cells. (E) Quantification of Nap1 effect. Asterisk, significant when compared with controls at $p<0.001$ (Student's t test). Scale bar: 5μm

Figure 4.11.


Wt + Deleted Nap1
Phalloidin
A
Wt + Nap1 shRNA
Phalloidin
B
Nap1 mutant + Nap1
Phalloidin
C
Nap1 mutant + Nap1
Cortactin
D
E
Changes in cells with lamellipodia (fold basal)
2
1
0
Wt
Nap1 mutant
Nap1 mutant + Nap1
Wt + mutant Nap1
Wt + Nap1 shRNA
*
*
*

PDGF induced lamellipodial formation in neuroepithelial cells

To further test the role of Nap1 in lamellipodial formation, we tested the ability of wild type, Nap1 mutant, or Nap1 shRNA expressing cells to form lamellipodia in response to PDGF. PDGF activates the Rac pathway and induces the formation of lamellipodia as well as ring ruffles (Krueger et al., 2003). Serum starved wild type and Nap1 disrupted cells were challenged with 10 ng/ml PDGF to assess their ability to form lamellipodia (Fig. 4.12). 36% of wild type cells produced lamellipodia in response to PDGF treatment, but only 1.7% or 3.2% of the Nap1 mutant or Nap1 shRNA cells, respectively, formed lamellipodia under the same conditions (Fig. 4.12). Nap1 disrupted cells are deficient in their ability to generate both dorsal and peripheral lamellipodial ruffles, two different types of lamellipodia noticed in cells undergoing active process extension (Suetsugu et al., 2003; Abercrombie et al., 1970). Furthermore, when wild type and Nap1 mutant cells were presented with PDGF coated beads, wild type cells readily extended lamellipodia in the direction of the beads, whereas Nap1 mutant cells failed to respond similarly. (Fig.4.13, Movie 4.6 and 4.7). Together, these data demonstrate that Nap1 protein is essential for the formation and activity of lamellipodia.

Figure 4.12. Nap1 disrupted cells do not generate lamellipodia in response to PDGF treatment.

Wild type cells lack lamellipodia when maintained in serum-free media (A), but initiate peripheral lamellipodia (arrowheads, B, C) and dorsal ruffles (arrow, C) after PDGF treatment (B, C). Nap1 mutant cells also do not generate lamellipodia when maintained in serum-free media (D), but they are unable to generate lamellipodia in response to PDGF treatment (E). Cortactin immunoreactivity (green) was used to label lamellipodia. Phalloidin staining (red) indicates F-actin. Nap1 shRNA expressing cells also do not respond to PDGF (F, G). Quantitation of the response of cells to PDGF treatment, expressed as percentage of cells (>900 cells/group) with peripheral lamellipodia (F) or dorsal ruffles (G) under each of the conditions shown. Nap1 disruption severely retards induced lamellipodial formation and activity.

Figure 4.12.

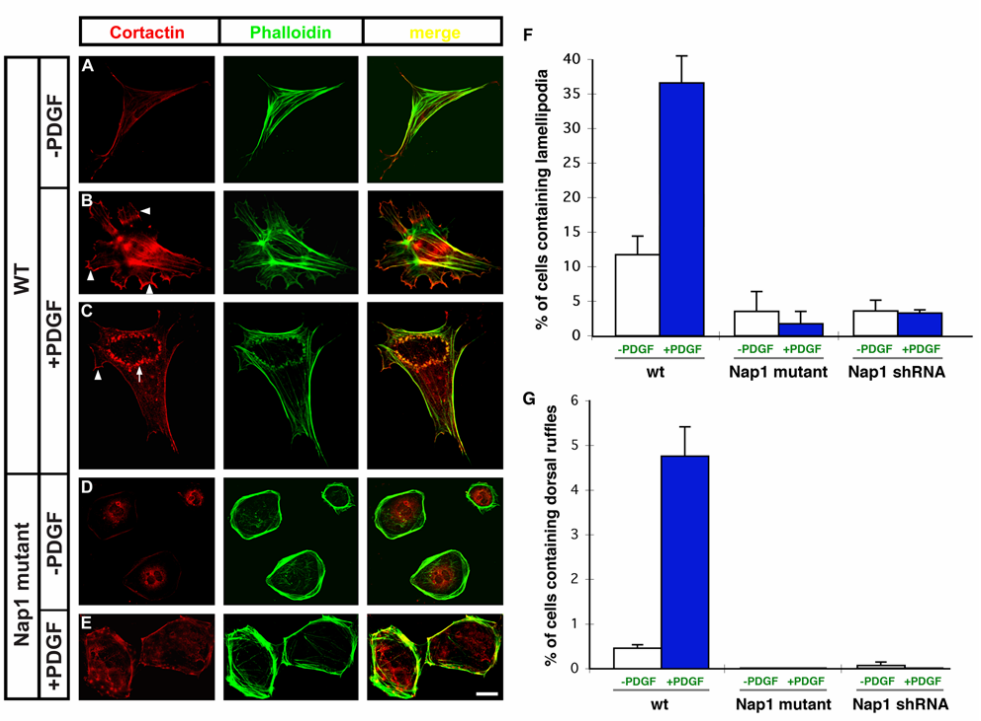
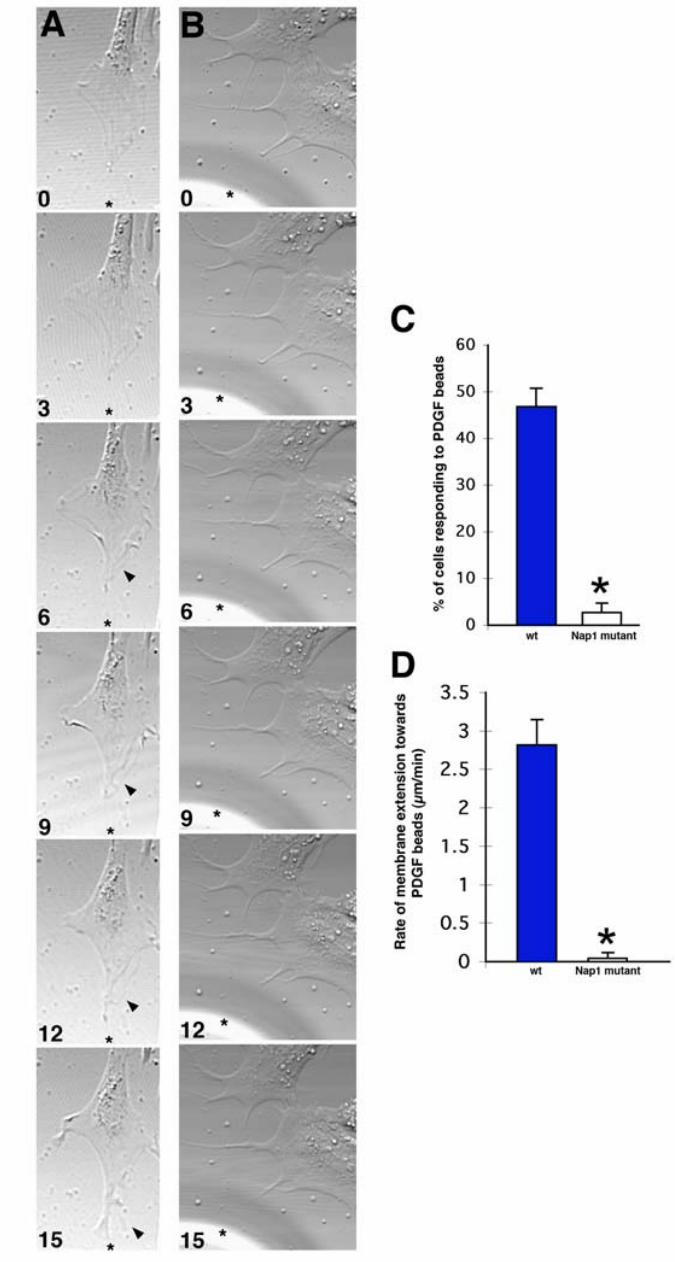

Cortactin
Phalloidin
merge
WT
-PDGF
+PDGF
Nap1 mutant
-PDGF
+PDGF
A
B
C
D
E
F
% of cells containing lamellipodia
40
35
30
25
20
15
10
5
0
-PDGF +PDGF
wt
Nap1 mutant
Nap1 shRNA
G
% of cells containing dorsal ruffles
6
5
4
3
2
1
0

Figure 4.13. PDGF stimulation of Nap1 mutant cells.

(A-B) PDGF coated beads were placed adjacent to wild type (A) and Nap1 mutant cells (B) and repeatedly imaged at 5-10 minute intervals using a Zeiss inverted microscope attached to a live cell incubation chamber. Wild type neuroepithelial cells rapidly extended lamellipodia towards PDGF bead (arrowhead, A). In contrast, Nap1 mutant cells did not respond to PDGF beads (asterisk, B). (C, D) Quantitation of the response of cells to PDGF treatment, expressed as percentage of cells responding to PDGF beads (C) and the rate of membrane extension towards the beads (D). n=3. Data shown are mean ±SEM; asterisk, significant when compared with controls at $p<0.01$ (Student's t test).

Figure 4.13.


A
B
0
3
6
9
12
15
C
% of cells responding to PDGF beads
60
50
40
30
20
10
0
wt
Nap1 mutant
D
Rate of membrane extension towards PDGF beads (μm/min)
3.5
3
2.5
2
1.5
1
0.5
0
wt
Nap1 mutant

Effect of Nap1 on intracellular dynamics of WAVE1

Lamellipodial activity and active, multiple membrane protrusions are essential steps in the initiation of neurite growth that occurs as neurons transform from migratory to post- migratory differentiation state in cerebral cortex. It is thought that during lamellipodial formation, Rac1 activation triggers active WAVE1 [(WASP (Wiskott–Aldrich syndrome protein)-family verprolin homologous protein1] complex to localize to membrane protrusions, causing actin nucleation in the protrusive edges of motile cells. The regulation of the subcellular localization of WAVE1 plays an important role in the functional activity of WAVE1 (Eden et al., 2002, Stradal et al., 2004). Nap1, which forms a complex with WAVE1, is hypothesized to play a role in the functional status or subcellular targeting of WAVE1. Given the aberrant lamellipodial phenotype of the Nap1 disrupted cells, we tested the effect of Nap1 on WAVE1 localization. Biochemical analysis of WAVE1 expression in Nap1 mutants indicates that Nap1 mutant cells maintain the expression of WAVE1. We then immunolabelled wild type and Nap1 mutant cells with anti-WAVE1 antibodies and analyzed the pattern of WAVE1 localization. In contrast to wild type cells, WAVE1 does not localize to the membrane ruffles at the leading, protrusive edges of the Nap1 mutant cells (Fig. 4.14A). Similar deficits in WAVE1 localization are also evident in Nap1 shRNA expressing cells (Fig.4.14B). This deficit was rescued by the re-expression of full length Nap1 (Fig.4.14B). To determine if Nap1 is essential to appropriately target WAVE1 to protrusive edges, we analyzed WAVE1 protein movement in wild type, Nap1 mutant, or Nap1 shRNA expressing cells. We generated WAVE1 fused with Kaede, a photoconvertible fluorescent protein which can be spectrally changed

from green to red with UV light (Ando et al., 2002). Both wild type and Nap1 mutant cells were transfected with WAVE1-Kaeda. Some of the wild type cells were also co- transfected with WAVE1-Kaede and Nap1 shRNA. Localized green to red conversion of WAVE1 in transfected cells was induced with a 200msec. pulse of UV light. Time lapse analysis of the movement of photoconverted WAVE1 (red) indicates that in wild type cells WAVE1 gets targeted to and move towards protrusive membrane edges, whereas in Nap1 disrupted cells WAVE1 movement to membrane edges is severely retarded, thus confirming the essential role of Nap1 in the appropriate cellular localization of WAVE1 (Fig.4.15A,B; Movies 4.8 – 4.10). Targeting of unrelated proteins such as PH-Akt to membrane edges was not affected in Nap1 deficient cells.

Figure 4.14. Defective localization of WAVE1 to protrusive membrane edges in Nap1 mutant cells.

(A) Wild type and mutant telencephalic neuroepithelial cells were labeled with phalloidin (green) and anti-WAVE1 antibodies (red). In wild type cells, WAVE1 predominantly localizes to lamellipodial membrane edges (arrowhead, A), whereas in Nap1 mutant cells, WAVE1 localization to lamellipodial protrusion is mostly absent. (B) Quantification of cells with WAVE1 localization on membrane edges indicates a significant deficit in Nap1 mutant or Nap1 deficient cells. Expression of C-terminal deleted Nap1 or Nap1 shRNA in wild type neuroepithelial cells leads to disrupted WAVE1 localization. This deficit can be rescued by expression of full length Nap1. Data shown are mean ±SEM; asterisk, significant when compared with controls at $p<0.01$ (Student's t test).

Figure 4.14.

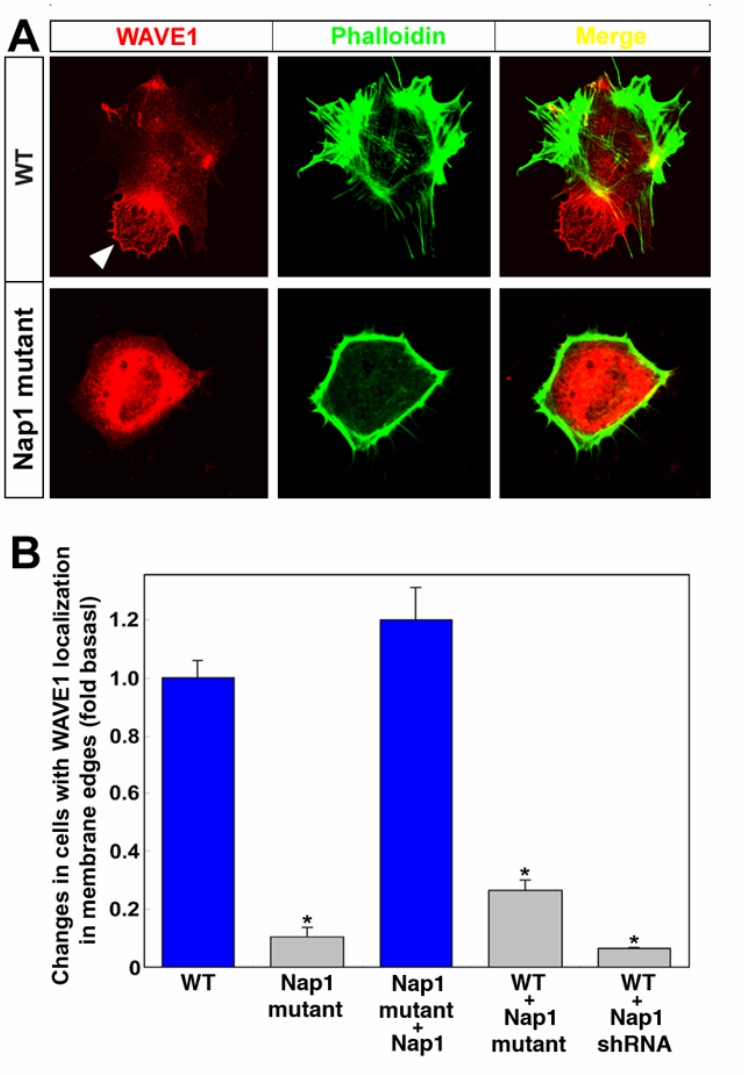
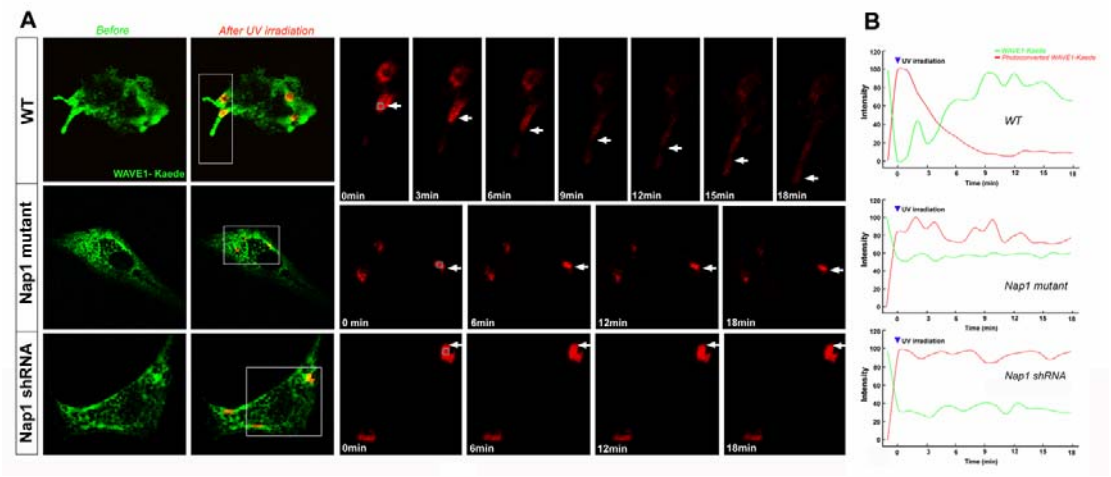

A
WAVE1
Phalloidin
Merge
WT
Nap1 mutant
B
Changes in cells with WAVE1 localization in membrane edges (fold basasl)
0
0.2
0.4
0.6
0.8
1.0
1.2
*
*
*
WT
Nap1 mutant
Nap1 mutant + Nap1
WT + Nap1 mutant
WT + Nap1 shRNA

Figure 4.15. Analysis of defective localization of WAVE1 to protrusive membrane edges in Nap1 mutant cells using photoconvertable protein,Kaede.

(A,B) Tracking of WAVE1 localization in Nap1 disrupted cells. Wild type, Nap1 mutant, or Nap1 shRNA expressing cells were transfected with WAVE1- Kaede (green). After localized photoconversion with a UV laser, time-lapse images of photoconverted WAVE1- Kaede (red) were obtained. In wild type cells, WAVE1 actively moved towards the protrusive edges of the cells (arrows [wt panels], A; Movie 4.8). In contrast, WAVE1 movement is significantly retarded in Nap1 mutants (arrows [Nap1 mutant panels], A; Movie 4.9) and in Nap1 knockdown cells (arrows [Nap1 shRNA panels], A; Movie 4.10). Time after photoconversion is indicated in minutes. (B) Measurement of relative fluorescent intensities of WAVE1-green and WAVE1-red in defined areas within the photoconverted spots indicate that in wt cells both types of WAVE1 trafficked normally, whereas in Nap1 mutants or Nap1 shRNA expressing cells, movement of WAVE1 is highly restricted. Also see Movie files 4.8, 4.9, 4.10.

Figure 4.15.


A
Before
After UV irradiation
WT
WAVE1- Kaede
Nap1 mutant
Nap1 shRNA
0min
3min
6min
9min
12min
15min
18min
0 min
6min
12min
18min
0min
6min
12min
18min
B
UV irradiation
WT
Nap1 mutant
Nap1 shRNA
Intensity
Time (min)

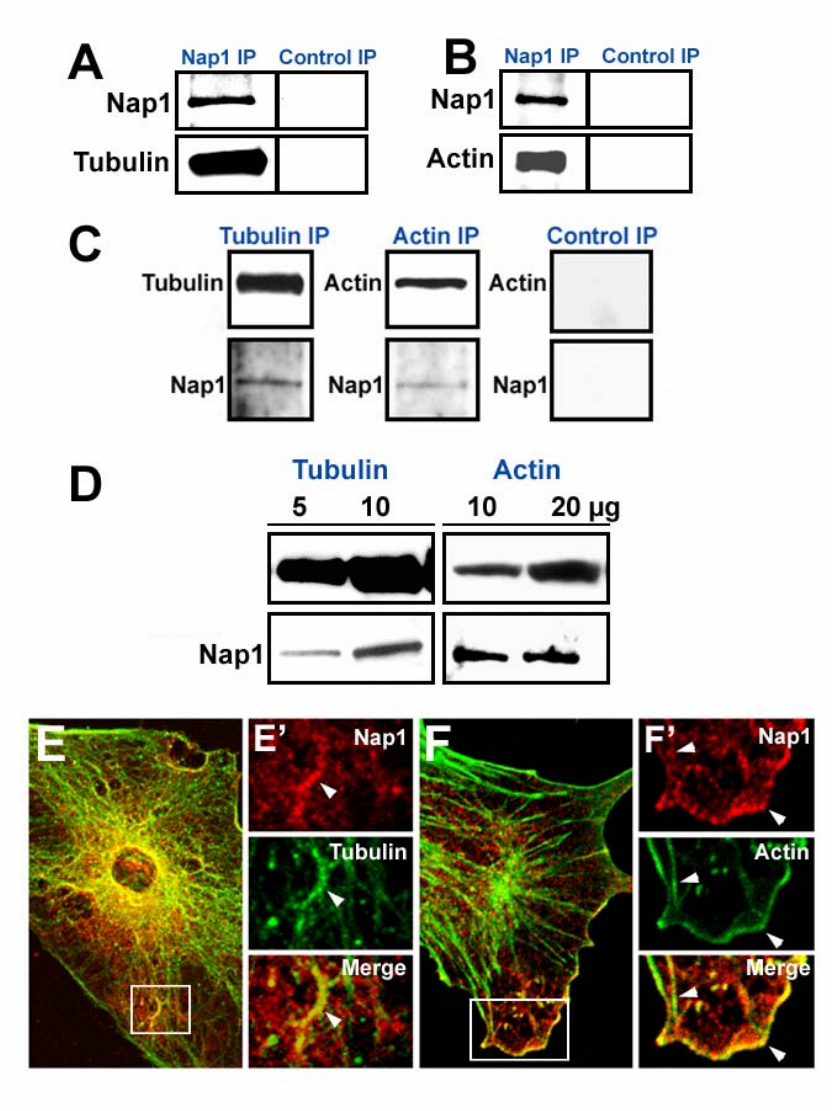
Nap1's association with actin and tubulin

To identify Nap1 interacting proteins in the developing cerebral cortex, Nap1 associated proteins were immunoprecipitated from cortical lysates with anti-Nap1 antibody linked protein A beads. MALDI-TOF mass spectrometry analysis of coprecipitated proteins consistently identified actin and tubulin in Nap1 immunoprecipitates. Furthermore, probing of Nap1 immunoprecipitates with anti tubulin or actin antibodies indicate that both tubulin and actin co-immunoprecipitate with Nap1 (Fig. 4.16 A,B). Conversely, Nap1 is detected in tubulin or actin immunoprecipitates (Fig. 4.16 C). Nap1 was also detected in purified tubulin and actin (Fig. 4.16 D). In neuroepithelial cells, Nap1 co-localized with microtubules and actin filaments (Fig. 4.16 E,F). Together, these observations suggest that Nap1 associates with both tubulin and actin in the developing cerebral cortex.

Figure 4.16. Association of Nap1 with actin and tubulin in the developing cerebral cortex.

To determine Nap1 – tubulin/actin association, cortical extracts were immunoprecipitated (IP) with Nap1 antibodies and immunoblotted with tubulin/actin antibodies, or vice versa. (A, B) Tubulin and actin was found in Nap1 immunoprecipitates. (C) Conversely, Nap1 was detected in tubulin or actin immunoprecipitates. Immunoprecipitation with control antibodies do not coprecipitate Nap1, actin, or tubulin. Nap1 was also found to be associated with purified tubulin and actin (D). (E,F) When neuroepithelial cells were co-immunolabeled with anti-Nap1 and tubulin antibodies, Nap1 was co-localized with microtubules (E, arrow heads E') and actin filaments (F, arrow heads F'). Higher magnification images of outlined areas of (E) and (F) are shown in (E') and (F'), respectively.

Figure 4.16.


A
Nap1 IP
Control IP
Nap1
Tubulin
B
Nap1 IP
Control IP
Nap1
Actin
C
Tubulin IP
Actin IP
Control IP
Tubulin
Actin
Actin
Nap1
Nap1
Nap1
D
Tubulin
Actin
5
10
10
20 µg
Nap1
E
E'
Nap1
Tubulin
Merge
F
F'
Nap1
Actin
Merge

Discussion

Nap1's function during neural tube development

Nap1 mutation in Nap1$^{lacZ/lacZ}$ mice clearly disrupts neural tube formation (Fig.4.2; Rakeman and Anderson, 2006). Although small amounts of normally spliced Nap1 can apparently be generated in Nap1 gene trap- insertion mutants (Rakeman and Anderson, 2006; Leighton et al., 2001) and Nap1 C-terminal deleted protein may have gain of function effects, similar neural tube defect was also noticed following loss of function missense mutation in the evolutionarily conserved L17P residue at the Nap1 N-terminal (Rakeman and Anderson, 2006). Normally, primary neural tube closure is initiated at the hindbrain/cervical boundary and proceeds in both rostral and caudal directions. Brain closure also depends on secondary closure events initiated at the midbrain/forebrain boundary and at the rostral tip of the forebrain (Copp et al., 2003). In Nap1 mutants, neural tube is open along most of the rostro-caudal extent (Fig.4.2), indicating a failure of normal neural tube closure events.

Completion of the neural tube closure depends on lamellipodial protrusions from the apical cells of the apposing neural folds. Interdigitation of these lamellipodial protrusions from across the midline facilitates cell- cell recognition and adhesion, leading to the fusion of the neural folds and the formation of neural tube (Copp et al., 2003; Geelen et al., 1979). The defective lamellipodial activity in Nap1 mutant neuroepithelial cells and the resultant changes in neuroepithelial cell movement (Figs4.8 – 4.10, Movies 4.4 - 4.5) may have disrupted this process essential for neural tube closure in Nap1 mutants. Furthermore, the abnormal migration pattern observe in neuroepithelial cell may

also contribute to the deficit in neuronal tube closure defect (Fig. 4.4).

Cellular and molecular mechanisms underlying Nap1 function

Nap1's ability to promote neuronal differentiation may depend on its ability to appropriately target or control the functional status of associated components of cytoskeletal machinery essential for neuronal process elaboration and maintenance.

Nap1 is a member of the WAVE complex. Nap1, which interacts directly with Sra1/PIR121 (which binds to GTP-bound Rac1) and Abi1 (which binds the SH3 domain of Nck) , forms a tetrameric complex containing Sra1/PIR121, Abi1/2, and HSPC300 to regulate WAVE1 activity (Kitamura et al., 1996; Kitamura et al., 1997; Kobayashi et al., 1998; Hummel et al., 2000; Soto et al., 2002; Yamamoto et al., 2001; Eden et al., 2002). WAVE1, in contrast to the related WASP proteins, which are autoinhibitory and are activated by binding GTP-bound Cdc42 to participate in the formation of filopodia, is constitutively active and acts downstream of Rac1 to initiate lamellipodia formation (Biyasheva et al, 2004; Blagg and Insall, 2004; Corey and Ridley, 2003; Machesky et al., 1999; Miki et al., 1998; Nakagawa et al., 2001; Innocenti et al., 2004; Kunda et al., 2003; Rogers et al., 2003; Rohatgi et al., 2000; Steffan et al., 2004). Nap1 containing WAVE complex regulates the functional status and cellular targeting of WAVE1. Upon activation of Rac and resultant changes in WAVE complex, WAVE1 binds and activates Arp2/3, leading to actin polymerization and branched actin filament formation at protrusive membrane edges and subsequent lamellipodial extension (Blanchoin et al., 2000; Eden et al., 2002; Gautreau et al., 2004; Bogdan and

Klambt, 2003; Stradal et al., 2004; Innocenti et al., 2004; Kunda et al., 2003; Millard et al., 2004; Rogers et al., 2003; Steffan et al., 2004; Svitkina and Borrisey, 1999).

The localization of WAVE1 at the edges of extending processes is essential to drive the localized activation of Arp2/3 complex and actin polymerization at the protrusive edges (Hahne et al., 2001; Nakagawa et al., 2001). Of the three highly homologous members of WAVE proteins (WAVE1-3), only WAVE1's expression is limited to the developing brain (Dahl et al., 2003; Sossey-Alaoui et al., 2003). Loss of WAVE1 function disrupts cerebral cortical development and functions such as learning and memory (Dahl et al., 2003; Soderling et al., 2003, 2007). Nap1 appears to be essential not only for the targeting of WAVE1 to the membrane, but also for the stability of WAVE1 (Rakeman and Anderson, 2006; Steffen et al., 2004). Reduction in WAVE protein levels were noticed in Nap1 mutants or Nap1 shRNA expressing melanoma cells (Steffen et al., 2004; Rakeman and Anderson, 2006). Although, how the decreased stability of WAVE1 affects its cellular targeting remains to be elucidated, the lack of WAVE targeting to the protrusive edges, not the reduced WAVE levels, appears to underlie the lamellipodial defects in Nap1 deficient cells (Steffen et al., 2004). Nap1 deficiency also disrupts the membrane localization of other WAVE complex components Sra1 and Abi1 (Steffan et al., 2004). In addition to being regulated by Nap1-Abi1/2-PIR121-HSPC300 complex (Echarri et al., 2004; Eden et al., 2002; Gautreau et al., 2004; Grove et al., 2004; Innocenti et al., 2004; Rakeman and Anderson, 2006; Steffen et al., 2004), WAVE1 can also be phosphorylated by Cdk5. Cdk5 can thus down modulate WAVE1's ability to activate Arp2/3 dependent actin polymerization (Kim et al., 2006). Interestingly, the decrease in dendritic

spines following phosphorylation of Cdk5 sites in WAVE1 is similar to that noticed in Nap1 deficient neurons (Fig. 3.7). Furthermore, Cdk5 and its regulatory subunit, p35, can form a complex with PIR121, Nap1, and WAVE1 (Kim et al., 2006). Inactivation of Nap1 disrupts not only WAVE1 function (Fig.4.14, 4.15), but may also inappropriately activate formins (Insall and Jones, 2006, Rakeman and Anderson, 2006). Thus, Nap1 by acting as a nodal point member of multiple complexes regulating the functional status of key cytoskeletal regulators such as WAVE1, may coordinate the cytoskeletal rearrangements needed to transform neurons from a migratory to post migratory differentiation state.

Cytoskeletal regulation in differentiating cortical neurons: Nap1's role

Post- migratory, differentiating cortical neurons undergo extensive neurite growth and guidance to generate the appropriate axon- dendritic architecture and connectivity. In general, microtubule polymerization is thought to drive neurite growth and elongation, whereas actin polymerization is critical for the ability of neurite growth cones to respond to guidance cues in the environment. Coordination of both actin and microtubule dynamics is essential for the differentiating cortical neurons to form and maintain appropriate patterns of connections in the embryonic cortex (Dent and Kalil, 2001; Marsh et al., 1984; Strasser et al., 2004; Rochlin et al., 1999). Nap1's ability to organize actin cytoskeleton and regulate microtubule stability places it in a unique position to influence both microtubule and actin dynamics during this process. In the neuronal growth cone, retrograde flow in filopodial actin bundles also moves

microtubules rearwards, indicating that microtubules may be structurally linked to actin (Schaefar et al., 2002, review by Rodriguez et al., 2003). Though the exact nature of actin- microtubule cross talk and coordination during cortical neuronal differentiation is yet to be fully elucidated, induction of Nap1 may influence neuronal cellular domains such as actin arcs or axon branch points, where actin and microtubules were found to modulate each other's organization and function during neuronal extension (Dent and Kalil, 2001; Schaefer et al., 2002). Elucidating how Nap1 differentially associates with and modulates the organization of actin and microtubule compartments in differentiating neurons will be essential to further delineate Nap1's significance during corticogenesis.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTION

Summary

The selective expression of Nap1 in the cortical plate region of the developing cortex, where neurons terminate their migration and initiate their laminar specific differentiation, lead us to hypothesize that Nap1 may mediate the changes in the neuronal cytoskeletal machinery from one that promotes oriented motility to one that facilitates stable laminar positioning and elaboration of axons and dendrites at the appropriate locations in the developing neocortex. Does Nap1 allow for the timely initiation of the differentiation program in cortical neurons? We addressed this question in several different ways: (1) developmental mapping of Nap1 expression indicates that Nap1 is selectively expressed in cortical plate neurons as they start to differentiate. It is not expressed in migrating neurons, (2) inactivation of Nap1 retards neuronal differentiation *in vitro* and *in vivo*, (3) inactivation of Nap1 does not affect neuronal migration or positioning. Nap1 inactivated neurons arrive appropriately at the cortical plate. However, they do not differentiate properly when they get there, (4) Ectopic Nap1 expression in migrating neurons (using Neuro-D promoter, which is active in post- mitotic, migratory neurons), retarded neuronal migration and promoted expression of markers normally expressed in

differentiating cortical neurons. These Nap1 expressing neurons also display morphological characteristics typical of differentiating neurons [i.e., multiple process growth and branches], (5) Ectopic Nap1 expression cell autonomously promotes neuronal differentiation *in vitro*, (6) Nap1 is induced by BDNF, a potent regulator of cortical neuronal differentiation. BDNF's effect depends on Nap1 expression, (7) Nap1 mutant mice in which insertional mutagenesis was used to disrupt Nap1 gene function display dramatic neural tube defects and severe deficits in neuronal differentiation, (8) Nap1 can interact with and modulate both actin and microtubule cytoskeletal organization, and (9) Nap1 mutation disrupts lamellipodial formation and the ability to localize key actin cytoskeletal regulators such as WAVE1 to the protrusive edges of neurons where they are needed to elaborate process outgrowth. The most parsimonious conclusion we can draw from these observations obtained through complimentary gain and loss of function experimental approaches is that Nap1 plays an essential role in the timely differentiation of neurons once they get to the cortical plate, thus enabling the completion of the functional wiring of the cerebral cortex.

Figure 5.1. Neuronal positioning and differentiation in the developing cerebral cortex.

Neurons migrate radially and tangentially into the developing cortical plate. A fundamental problem in cortical development is to understand how neurons choreograph their transition from migratory to post- migratory, layer- specific differentiation state at the appropriate locations in the cerebral cortex. Characterization of cytoskeletal mechanisms underlying this process will be essential to understand how the patterned positioning and connectivity of neurons emerge in the cerebral cortex. In this study, we analyzed this process using Nap1 signaling in cortical plate as a molecular model and demonstrated that Nap1 may play an essential role in triggering the neuronal cytoskeletal changes underlying the post-migratory differentiation of cortical neurons, a critical step in functional wiring of the cerebral cortex.

Fig 5.1.

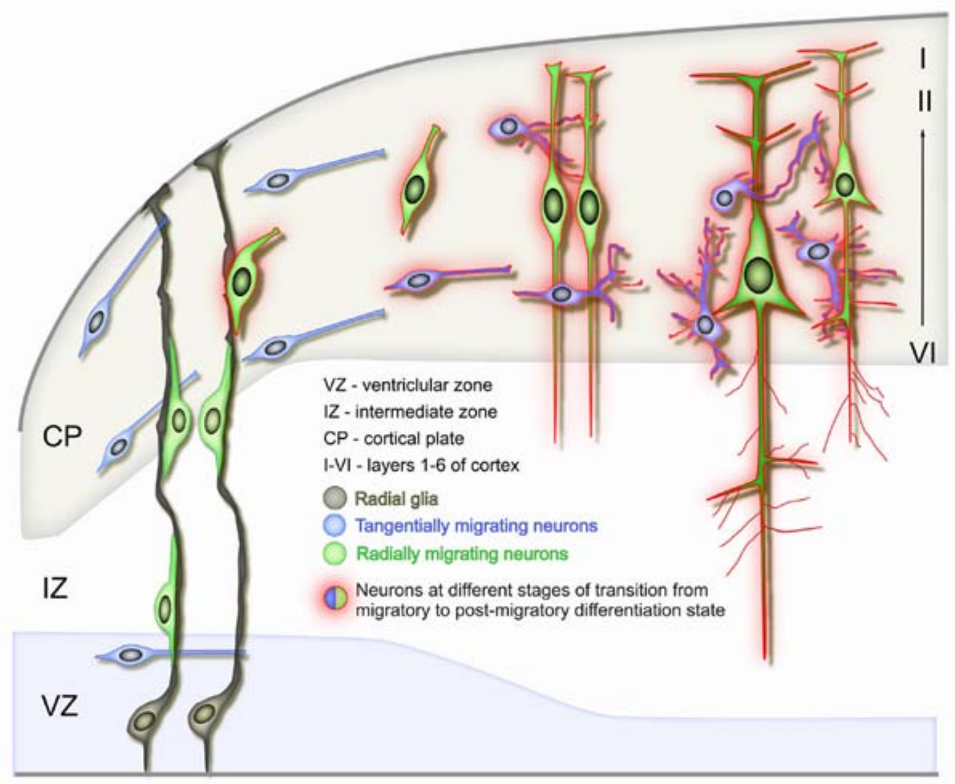
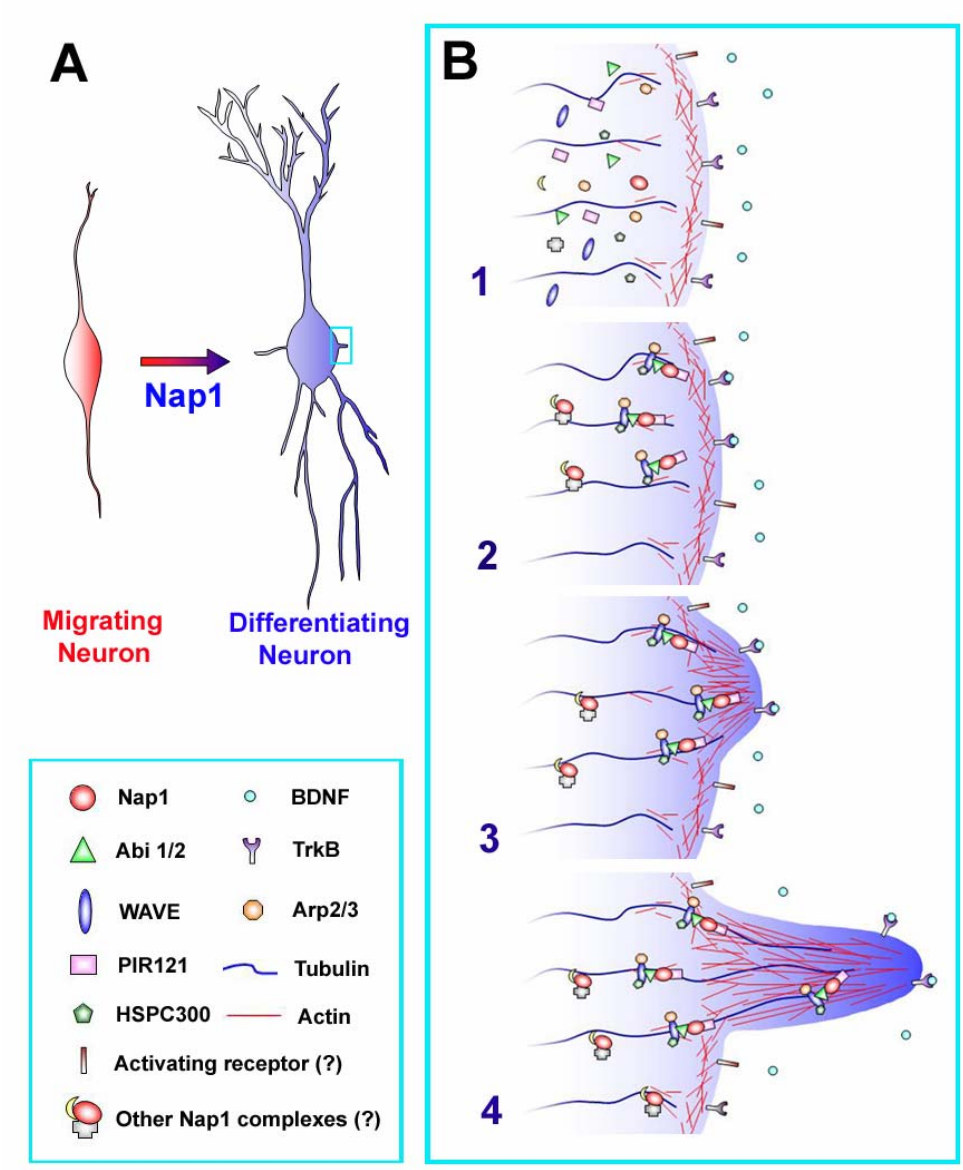

I
II
VI
CP
IZ
VZ
VZ - ventriclular zone
IZ - intermediate zone
CP - cortical plate
I-VI - layers 1-6 of cortex
Radial glia
Tangentially migrating neurons
Radially migrating neurons
Neurons at different stages of transition from migratory to post-migratory differentiation state

Figure 5.2. Model of Nap1 mediated initiation of axonal and dendritic process extension.

(A) Nap1 is expressed in neurons as they terminate their migration and initiate their differentiation. (B) Extracellular differentiation signals such as BDNF or other yet to be characterized cell surface receptor activation may promote the induction of Nap1 expression (1). Induction of Nap1 and the resultant formation of complexes such as Nap1/WAVE1/Abi/PIR121/HSPC300 or others (e.g. Nap1/cdk5/p35/WAVE1) may facilitate multiple membrane protrusions and processes out growth (2). Activated Nap1 complexes may coordinate actin and microtubule dynamics at the protrusive edges of differentiating neurons (3), resulting in the extension of multiple dendritic and axonal processes (4). How Nap1 affects or coordinates both microtubule and actin cytoskeletal dynamics during this process remains to be fully elucidated.

Fig 5.2.


A
Nap1
Migrating Neuron
Differentiating Neuron
Nap1
Abi 1/2
WAVE
PIR121
HSPC300
Activating receptor (?)
Other Nap1 complexes (?)
BDNF
TrkB
Arp2/3
Tubulin
Actin
B
1
2
3
4

Although the role of Nap1 in cortical neuronal differentiation is clear, several fundamental questions regarding Nap1 function remains to be fully delineated: (1) What are the critical functional domains of Nap1?, (2) How does Nap1 interact with both actin and microtubule?, (3) What is the role of Nap1 complex formation in its function?, (4) What are the regulatory elements of Nap1?, (5) What is the role of Nap1 in cerebral cortical function? These questions could be explored using the following approaches.

What are the critical functional domains of Nap1?

Using Nap1 recombinant fragments, we will assay if any of the Nap1 domains can rescue the deficits in lemellipodial formation, actin/microtubule organization, cell motility, and the cellular localization of critical Nap1 complex members such as WAVE1 in Nap1 deficient cells. This will also help determine if distinct Nap1 domains have distinct cellular functions. The Nap1 domains that appear to be functionally critical in these assays will then be evaluated for their role in cortical neuronal differentiation *in vitro*. Nap1 domains that significantly influence cortical neuronal differentiation *in vitro* can be further tested *in vivo* in embryonic cortex using electroporation based assays described earlier.

How does Nap1 interact with both actin and microtubule?

To evaluate the significance of Nap1- actin/ microtubule interactions, we will generate His6 tagged Nap1 and Nap1 fragments (i.e., domains that were identified as functionally critical in experiments described earlier). We will first determine if each of the Nap1 fragments can bind to purified tubulin or actin

using established binding assays (Taylor, 2000; Weber et al., 2004). Full length and Nap1 fragments will also be assessed for their ability to polymerize actin or tubulin using respective actin or tubulin polymerization assays (Cytoskeleton Inc.). Furthermore, the changes in stability of microtubules (MT) noticed in Nap1 deficient cells may have resulted from decreased stabilization of MT or increased destabilization of MT. MT nucleation may also have been affected in the absence of Nap1. Evaluation of microtubules after nocodazole treatment, quantification of MT nucleation/growth rate using live imaging of EB1-GFP labeled MT plus- ends, and live imaging of MT with β-tubulin-GFP could be used to study these possibilities. Considering the emerging evidence for crosstalk between the actin and microtubule cytoskeletons during neuronal differentiation (Strasser et al., 2004), the ability of Nap1 to influence actin nucleation, and the evidence for Nap1 regions capable of associating with microtubules, these studies should help to determine the nature of Nap1's effect on actin/ microtubule cytoskeletal dynamics and the domains of Nap1 essential for these interactions.

What is the role of Nap1 complex formation in its function?

Nap1 can form complexes with WAVE-Abi1/2-PIR121-HSPC300 (Echarri et al., 2004; Eden et al., 2002; Gautreau et al., 2004; Innocenti et al., 2004; Rakeman and Anderson, 2006; Steffen et al., 2004), and with Cdk5 and its regulatory subunit, p35 (Kim et al., 2006). Nap1's ability to be part of distinct complexes may enable it to differentially coordinate cytoskeletal rearrangements during distinct stages of neuronal development. Thus it is important to determine whether there are developmental changes in the ability of Nap1 to form specific complexes,

whether formation of different complexes lead to different effects on neuronal development, whether different domains of Nap1 are involved in the formation of specific complexes, and how critical mediators of neuronal development such as growth factors (e.g., BDNF) or activity may influence Nap1 complex formation.

To identify Nap1 associated complexes that are critical for its cellular function in cerebral cortex, we will use nanoelectrospray tandem mass spectrometry (UNC proteomics core facility) to analyze Nap1 co-immunoprecipitated proteins from different stages of embryonic cortex or from neurons treated with BDNF or activity inhibitors. Though the initial focus will be on analyzing the significance of known Nap1 complexes during cortical neuronal development, any novel Nap1 interactors identified in these studies will also be tested for their role in specific Nap1 functions during cortical neuronal differentiation as described earlier. Once we identify Nap1 associated complexes in embryonic cortex, we will determine if the formation of these complexes are altered in Nap1 over expressing or Nap1 knockdown cells. We will use Nap1 fragments to determine which regions of Nap1 are critical for Nap1 complex formation, if different regions of Nap1 form different associations, and whether they correlate with functional differences noticed with Nap1 fragments in studies outlined earlier.

The immunoprecipitation analysis of Nap1 complexes, studies on the role of distinct Nap1 domains in Nap1 complex formation, and corresponding cell functional analysis will help identify if Nap1 forms different types of complexes and whether formation of different Nap1 complexes leads to different functional

outcomes during cortical neuronal development. Although the mass spectrometry based screen of Nap1 associated molecules in embryonic cortex is likely to identify both direct and indirect interactors of Nap1, alternate approaches such as yeast two hybrid screen for Nap1 interactors will also be useful.

What are the regulatory elements of Nap1?

How is Nap1 selectively induced in the cortical plate? What are the transcription factors that may regulate Nap1's expression in the CP region? Differentiating cortical neurons selectively express specific transcription factors (e.g. Brn1/2, Ctip2, Cux1, Emx1, ER81, Etv1, FoxO1, FoxP1/2, Klf6, Lhx2/5, LMO4, Otx1, Png1, Satb2, SCIP, Sox5, Tbr1, Tst1, Zfp312, etc.). The presence of binding sites for these transcription factors in Nap1 promoter or the identification of evolutionally conserved domains within this region will help identify potentially critical Nap1 regulatory elements. Once identified, the role of these domains in selective expression of Nap1 in the CP can be tested using Nap1 promoter analysis in transgenic mouse models.

What is the role of Nap1 in cerebral cortical function?

The continued expression of Nap1 in differentiated neurons in the postnatal brain and its expression in regions other than the cerebral cortex suggest it may have other functions in the brain. Furthermore, the early embryonic lethality of Nap1 C-terminal deletion mice suggests that *in vivo* analysis of Nap1 function during cerebral cortical development requires

conditional *Nap1* gene targeting. Therefore, to study cell type specific role of Nap1 in the developing and mature brain, we are currently generating a floxed Nap1 mouse. *Nap1* gene encodes a protein of 1131 amino acids from 31 exons that span 80.6 kb of genomic DNA on chromosome 2. To conditionally delete *Nap1* gene, LoxP sites were targeted to flank the third and eighth exons of *Nap1* gene. We choose this approach for the following reason. It will result in out of frame translation after exon 2, thus resulting in the removal of functionally critical membrane association domains and other highly conserved regions of Nap1. Targeting of shorter segments (e.g., exons 2-3) will result in- frame splicing of downstream exons and thus generate truncated Nap1 protein with potential dominant negative functions (Fig.5.3).

The Nap1 targeting vector was electroporated into E14 ES cell line, a line known to exhibit enhanced capacity for germ line transmission. Following positive and negative selection, nearly 1000 colonies were expanded and screened by PCR and Southern blot analysis for appropriately targeted ES cells, which had undergone homologous recombination (Fig.5.3 B, C). The genomic locations used for generating DNA probes and PCR primers are indicated (Figure. 5.3). Currently, we have identified one positive clone through PCR and southern blot screening, but we'll continue to screen for more positive clones. Homologously recombined ES cells will be tested *in vitro* for efficient Flpe and Cre mediated recombination prior to blastocyst (C57Bl/6) injection and generation of germline transmitting chimeric mice. Mice carrying gene-trap venus allele will be crossed with *Actin-Flpe* transgenic mice (*Tg*ActFlpe: Jackson

Lab) in order to delete the gene-trap Neo cassette from the targeted allele, thus generating progeny with floxed Nap1 gene.

We will mate "floxed" Nap1 mice with transgenic mice that express Cre recombinase under the control of various neuronal subtype and developmental stage specific promoters. To maximize the number of neurons exhibiting a complete loss of Nap1 function, the floxed Nap1 mice will be mated with the Cre mice in a background heterozygous for the null allele of Nap1. This will result in transgenic mice deficient in Nap1 expression in specific promoter – active cortical neuronal cells. It will still be possible to follow the fate of these cells because they will continue to express EYFP from the reporter allele. Initially we will use SynapsinI – Cre to inactivate Nap1 in cortical plate neurons, since it is active in post - mitotic cortical neurons from E13 onwards (Zhu et al., 2001). However, we will also use other Cre lines such as Parvalbumin-Cre (obtained from Dr. S. Arber), Nex-Cre (obtained from Dr. K-A. Nave), Dlx5/6- Cre (Stenman et al., 2003). Cre reporter analysis indicates that Parvalbumin-Cre is widely expressed in the post- mitotic cortical plate interneurons from P0. NEX-Cre is expressed in differentiating cortical pyramidal neurons from embryonic day 14 (Beggs et al., 2003, Schwab et al., 2000), whereas Dlx 5/6-Cre is expressed only in the developing interneurons (Stenman et al., 2003). By inactivating Nap1 in distinct neuronal cell types and at different stages of neuronal development (e.g., pre-migratory or post- migratory stage), we hope to delineate the diverse roles of Nap1 in neocortical function.

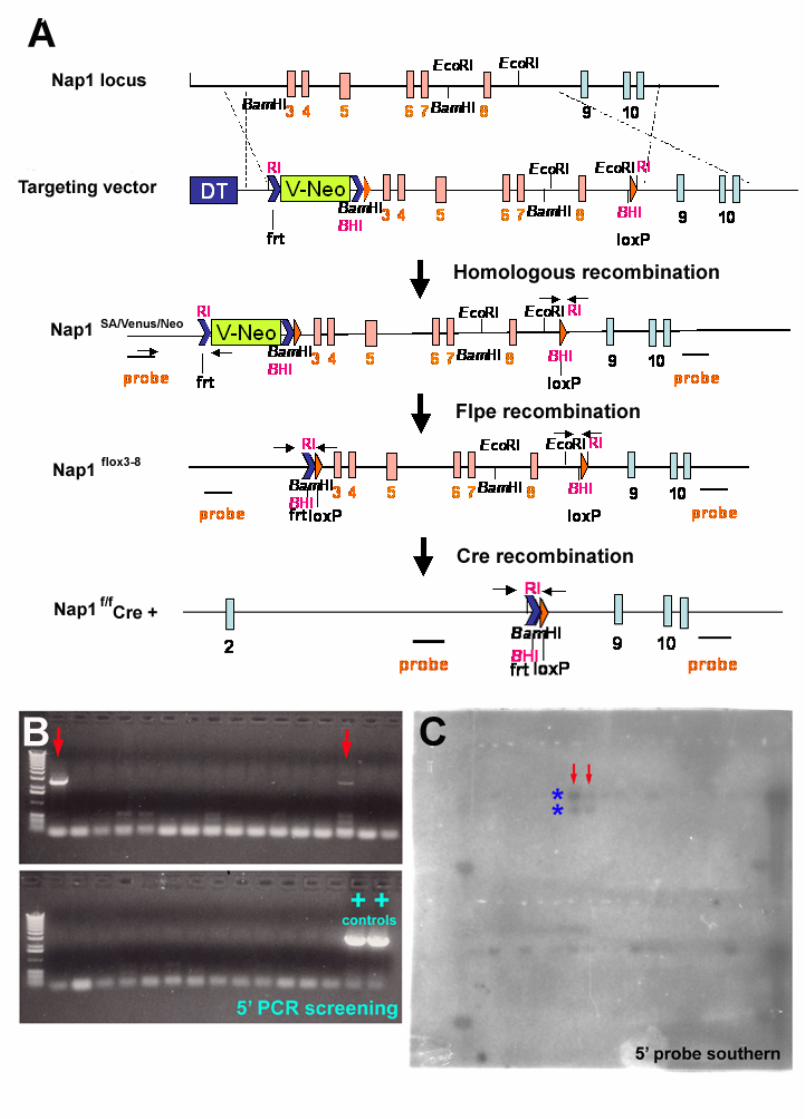
The crux of the significance of this study lies in the observation that coordinated migration and placement of neurons in the developing cerebral

cortex depends on dynamic regulation of neuronal cytoskeleton. The activities of various extracellular cues and cell surface signaling receptors known to regulate distinct aspects of neuronal migration have to induce specific changes in neuronal cytoskeleton to engender the patterned neuronal movement, placement, and differentiation. The change in the neuronal cytoskeletal machinery from one that promotes oriented motility to one that facilitates stable laminar positioning, and elaboration of axons and dendrites is a critical step in the emergence of functional organization of neurons in cerebral cortex. What are the cytoskeletal regulators essential to affect this transition? Here we show that selective induction of Nck associated protein 1 (Nap1) and the resultant changes in cytoskeletal dynamics is essential for the terminal, post- migratory differentiation of neurons in the developing cerebral cortex. Use of Nap1 signaling as a molecular model to further characterize the cytoskeletal mechanisms controlling the final stages of neuronal differentiation will be essential to elucidate the basic principles guiding the emergence of neuronal laminar architecture and connectivity in the cerebral cortex.

Figure 5.3. Strategy for floxing the Nap1 $^{exon3\sim8}$ showing the targeting construct and the targeted Nap1 locus.

(A) Targeting vector contains selection cassettes encoding neomysin phosphotransferase (neo) and diphtheria toxin (DT). Neo and DT are used as positive/negative selection markers for successful homologous recombination. The Neo cassette flanked by two frt sites will be removed by Flpe recombinase. Cre recombinase will be used to carry out site specific recombination between the two loxP sites, thus eliminating the expression of the Nap1 gene. The genomic locations of regions used for probes (—) and PCR primers (→) used to verify homologous recombination and Flpe excision are indicated. (B) Two ES cell clones (red arrows) with appropriate homologous recombination (2 kb product) as detected by PCR is shown. (C) The PCR results were confirmed with southern blotting analysis. DNA was digested with EcoRI and two positive clones (red arrow) show 13.6 Kb wild type band and 7.6 Kb recombined genomic DNA.

Figure 5.3.


A
Nap1 locus
EcoRI
EcoRI
BamHI
3 4
5
6 7
BamHI
8
9
10
Targeting vector
DT
RI
V-Neo
frt
BamHI
BHI
3 4
5
6 7
BamHI
8
EcoRI
EcoRI
RI
BHI
loxP
9
10
Homologous recombination
Nap1 SA/Venus/Neo
RI
V-Neo
probe
frt
BamHI
BHI
3 4
5
6 7
BamHI
8
EcoRI
EcoRI
RI
BHI
loxP
9
10
probe
FIpe recombination
Nap1 flox3-8
RI
probe
BamHI
BHI
frt
3 4
5
loxP
6 7
BamHI
8
EcoRI
EcoRI
RI
BHI
loxP
9
10
probe
Cre recombination
Nap1 f/f Cre +
RI
2
probe
BamHI
BHI
frt
loxP
9
10
probe
B
+ +
controls
5' PCR screening
C
5' probe southern

MOVIE LEGENDS

Movie 4.1 – 4.3. Defective neural tube closure in Nap1 mutant embryos.

Midline region of apposing neural folds in the head region of Nap1$^{lacZ/llacZ}$, ACTB-EGFP or Nap1$^{wt/wt}$, ACTB-EGFP E8.5 embryos was repeatedly imaged at 3 minute intervals using a Zeiss inverted microscope attached to a live cell incubation chamber. The movement of neuroepithelial cells towards midline needed for neural tube closure occurs in wild type embryos (Movei 4.1, arrow indicates midline), but not in Nap1 mutants (Movie 4.2). Lower magnification imaging of mutant embryos (imaged from back of the head region) shows that the apposing cephalic neural folds, instead of fusing together, separate in opposite direction in Nap1 mutants (Movie 4.3). Time length: Movie 4.1(1.3hr.), 4.2(1hr.), 4.3(1.2hrs).

Movie 4.4, 4.5. Defective lamellipodial formation in Nap1 mutant cells.

E9.5 neuroepithelial cells from wild type and Nap1 mutant telencephalon was repeatedly imaged at 1 minute intervals using a Zeiss inverted microscope attached to a live cell incubation chamber. These images were compiled as AVI movie files to illustrate the differences in lamellipodial activity between wild type (Movie 4.4) and Nap1 mutant cells (Movie 4.5). Wild type cells elaborate active lamellipodia (arrow, Movie 4.4) whereas Nap1 mutants are unable to generate any lamellipodia and instead extend spiky protrusions (arrow, Movei 4.5). Time length= 60minutes.

Movie 4.6, 4.7. PDGF stimulation of Nap1 mutant cells.

PDGF coated beads were placed adjacent to wild type and Nap1 mutant cells and repeatedly imaged at 5-10 minute intervals using a Zeiss inverted microscope attached to a live cell incubation chamber. Wild type neuroepithelial cells rapidly extended lamellipodia towards PDGF bead (Movei 4.6). In contrast, Nap1 mutant cells did not respond to PDGF beads (Movie 4.7).

Movie 4.8 – 4.10. WAVE1 movement in wild type and Nap1 disrupted cells. Areas of photoconverted WAVE1-Kaede (red) were time-lapse imaged every minute using a Leica SP2 laser scanning confocal microscope for 18 minutes. These images were compiled as AVI movie files to illustrate the differences in WAVE1 protein movement between wild type (Movie 4.8) and Nap1 mutant (Movie 4.9) or Nap1 shRNA expressing cells (Movie 4.10). WAVE1 moved normally to protrusive edges of the wild type cells, whereas WAVE1 movement is disrupted in Nap1 mutant or knockdown cells.

REFERENCES

Abercrombie, M., Heaysman, J.E., and Pegrum, S.M. (1970). The locomotion of fibroblasts in culture. II. "Ruffling." *Exp. Cell Res* 60, 437–444.

Altman, J and G.D. Das (1965). Post-natal origin of microneurons in the rat brain. *Nature* 207, 953-6

Anderson, S.A., Eisenstat, D.D., Shi, L., Rubenstein, J.L. (1997). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. *Science* 278, 474-6.

Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A. (2002). An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc Natl Acad Sci U S A* 99, 12651-6.

Ang, E.S. Jr, Haydar, T.F., Gluncic, V., Rakic, P. (2003). Four-dimensional migratory coordinates of GABAergic interneurons in the developing mouse cortex. *J Neurosci*. 23, 5805-15.

Anton E.S., Ghashghaei H.T., Weber J.L., McCann C., Fischer T.M., Cheung I.D., Gassmann M., Messing A., Klein R., Schwab M.H., Lloyd K.C., and Lai, C. (2004). Receptor tyrosine kinase ErbB4 modulates neuroblast migration and placement in the adult forebrain. *Nat Neurosci* 7, 1319-28.

Ayala R., Shu T., and Tsai L. H. (2007). Trekking across the brain: the journey of neuronal migration. *Cell* 128:29-43.

Baumgartner, S., Martin, D., Chiquet-Ehrismann, R., Sutton, J., Desai, A., Huang, I., Kato, K. and Hromas, R. (1995). The HEM proteins: a novel family of tissue-specific transmembrane proteins expressed from invertebrates through mammals with an essential function in oogenesis. *J Mol Biol* 251, 41-9.

Bayer, S. A., and Altman, J. (1991). Development of the cortical plate. In *Neocortical Development.* (Eds. Bayer, S. A., and Altman, J.), Raven Press, NY.

Beggs, H.E., Schahin-Reed, D., Zang, K., Goebbels, S., Nave, K.A., Gorski, J., Jones, K.R., Sretavan, D., Reichardt, L.F. (2003). FAK deficiency in cells contributing to the basal lamina results in cortical abnormalities resembling congenital muscular dystrophies. *Neuron* 40, 501-14.

Bellion,A., Baudoin, J.P., Alvarez, C., Bornens, M., Metin, C. (2005). Nucleokinesis in tangentially migrating neurons comprises two alternating phases: forward migration of the Golgi/centrosome associated with centrosome splitting and myosin contraction at the rear. *J Neurosci* 25, 5691-9.

Biyasheva, A., Svitkina, T., Kunda, P., Baum, B. and Borisy, G. (2004). Cascade pathway of filopodia formation downstream of SCAR. *J Cell Sci* 117, 837-48.

Bladt, F., Aippersbach, E., Gelkop, S., Strasser, G. A., Nash, P., Tafuri, A., Gertler, F. B. and Pawson, T. (2003). The murine Nck SH2/SH3 adaptors are important for the development of mesoderm-derived embryonic structures and for regulating the cellular actin network. *Mol Cell Biol* 23, 4586-97.

Blagg, S. L. and Insall, R. H. (2004). Solving the WAVE function. *Nat Cell Biol* 6, 279-81.

Blanchoin, L., Amann, K.J., Higgs, H.N., Marchand, J.-B., Kaiser, D.A., and Pollard, T.D. (2000). Direct observation of dendritic actin filament networks nucleated by Arp2/3 complex and WASP/Scar kinases. *Nature* 404, 1007–1011.

Bogdan, S. and Klambt, C. (2003). Kette regulates actin dynamics and genetically interacts with Wave and Wasp. *Development* 130, 4427-37.

Bond J., Roberts E., Mochida G.H., Hampshire D.J., Scott S., Askham J.M., Springell K., Mahadevan M., Crow Y.J., Markham A.F., Walsh C.A., and Woods C.G. (2002). ASPM is a major determinant of cerebral cortical size. *Nat Genet* 32, 316-20.

Cabelli R.J., Shelton D.L., Segal R.A., and Shatz C.J. (1997). Blockade of endogenous ligands of trkB inhibits formation of ocular dominance columns. *Neuron* 19, 63-76.

Cahana, A., Escamez, T., Nowakowski, R.S., Hayes, N.L., Giacobini, M., von Holst, A., Shmueli, O., Sapir, T., McConnell, S.K., Wurst, W., Martinez, S., Reiner, O. (2001). Targeted mutagenesis of Lis1 disrupts cortical development and LIS1 homodimerization. Proc Natl Acad Sci U S A. 98, 6429-34.

Cameron, R.S., Rakic, P. (1994). Identification of membrane proteins that comprise the plasmalemmal junction between migrating neurons and radial glial cells. *J Neurosci*. 14, 3139-55.

Caspi, M., Atlas, R., Kantor, A., Sapir, T., Reiner, O. (2000). Interaction between LIS1 and doublecortin, two lissencephaly gene products. *Hum Mol Genet* 9, 2205-13.

Chae, T., Kwon, Y.T., Bronson, R., Dikkes, P., Li, E., Tsai, L.H. (1997). Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. *Neuron* 18, 29-42.

Chang, L., Jones, Y., Ellisman, M.H., Goldstein, L.S., Karin, M. (2003). JNK1 is required for maintenance of neuronal microtubules and controls phosphorylation of microtubule-associated proteins. *Dev Cell* 4, 521-33.

Copp, A. J., Greene, N. D. and Murdoch, J. N. (2003a). Dishevelled: linking convergent extension with neural tube closure. *Trends Neurosci* 26, 453-5.

Copp, A. J., Greene, N. D. and Murdoch, J. N. (2003b). The genetic basis of mammalian neurulation. *Nat Rev Genet* 4, 784-93.

Cory G.O., and Ridley A.J. (2002). Cell motility: braking WAVEs. *Nature* 418(6899),732-3

D'Arcangelo, G., Miao, G.G., Chen, S.C., Soares, H.D., Morgan, J.I., Curran, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature* 374, 719-23.

Dawson, H.N., Ferreira, A., Eyster, M.V., Ghoshal, N., Binder, L.I., Vitek, M.P. (2001). Inhibition of neuronal maturation in primary hippocampal neurons from tau deficient mice. *J Cell Sci* 114, 1179-87.

Dahl, J. P., Wang-Dunlop, J., Gonzales, C., Goad, M. E., Mark, R. J. and Kwak, S. P. (2003). Characterization of the WAVE1 knock-out mouse: implications for CNS development. *J Neurosci* 23, 3343-52.

de Carlos, J.A., Lopez-Mascaraque, L., Valverde, F. (1996). Dynamics of cell migration from the lateral ganglionic eminence in the rat.*J Neurosci*. 16, 6146-56.

Del Rio, J.A., Gonzalez-Billault, C., Urena, J.M., Jimenez, E.M., Barallobre, M.J, Pascual, M., Pujadas, L., Simo, S., La Torre, A., Wandosell, F., Avila, J., Soriano, E. (2004). MAP1B is required for Netrin 1 signaling in neuronal migration and axonal guidance. *Curr Biol* 14, 840-50.

Dent, E.W., and Kalil, K. (2001). Axon branching requires interactions between dynamic microtubules and actin filaments. *J. Neurosci.* 21, 9757-9769.

des Portes, V., Francis, F., Pinard, J.M., Desguerre, I., Moutard, M.L., Snoeck, I., Meiners, L.C., Capron, F., Cusmai, R., Ricci, S., Motte, J., Echenne, B., Ponsot, G., Dulac, O., Chelly, J., Beldjord, C. (1998) doublecortin is the major gene causing X-linked subcortical laminar heterotopia (SCLH). *Hum Mol Genet* 7, 1063-70.

Deuel T.A., Liu J.S., Corbo J.C., Yoo S.Y., Rorke-Adams L.B., and Walsh C.A. (2006). Genetic interactions between doublecortin and doublecortin-like kinase in neuronal migration and axon outgrowth. *Neuron* 49, 41-53.

Dobyns, W.B., Reiner, O., Carrozzo, R., Ledbetter, D.H. (1993). Lissencephaly. A human brain malformation associated with deletion of the LIS1 gene located at chromosome 17p13. JAMA 270, 2838-42.

Echarri, A., Lai, M. J., Robinson, M. R., and Pendergast, A. M. (2004). Abl interactor 1 (Abi-1) wave-binding and SNARE domains regulate its nucleocytoplasmic shuttling, lamellipodium localization, and wave-1 levels. *Mol Cell Biol* 24, 4979-93.

Eden, S., Rohatgi, R., Podtelejnikov, A. V., Mann, M. and Kirschner, M. W. (2002). Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. *Nature* 418, 790-3.

Efimov, V. P. and Morris N. R. (2000). The LIS1-related NUDF protein of Aspergillus nidulans interacts with the coiled-coil domain of the NUDE/RO11 protein. *J Cell Biol* 150, 681-8.

Eksioglu, Y.Z., Scheffer, I.E., Cardenas, P., Knoll, J., DiMario, F., Ramsby, G., Berg, M., Kamuro, K., Berkovic, S.F., Duyk, G.M., Parisi, J., Huttenlocher, P.R., Walsh, C.A. (1996). Periventricular heterotopia: an X-linked dominant epilepsy locus causing aberrant cerebral cortical development. *Neuron* 16, 77-87.

Faulkner, N.E., Dujardin, D.L., Tai, C.Y., Vaughan, K.T., O'Connell, C.B., Wang, Y., Vallee, R.B. (2000). A role for the lissencephaly gene LIS1 in mitosis and cytoplasmic dynein function. *Nat Cell Biol* 2, 784-91.

Feng, Y. and C. A. Walsh (2004). Mitotic spindle regulation by Nde1 controls cerebral cortical size. *Neuron* 44, 279-93.

Fox, J.W., Lamperti, E.D., Eksioglu, Y.Z., Hong, S.E., Feng, Y., Graham, D.A., Scheffer, I.E., Dobyns, W.B., Hirsch, B.A., Radtke, R.A., Berkovic, S.F., Huttenlocher, P.R., Walsh, C.A. (1998). Mutations in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia. *Neuron* 21, 1315-25.

Francis, F., Koulakoff, A., Boucher, D., Chafey, P., Schaar, B., Vinet, M.C., Friocourt, G., McDonnell, N., Reiner, O., Kahn, A., McConnell, S.K., Berwald-Netter, Y., Denoulet, P., Chelly, J. (1999). Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. *Neuron* 23, 247-56.

Gaudilliere B., Konishi Y., de la Iglesia N., Yao G.,and Bonni A. (2004). A CaMKII-NeuroD signaling pathway specifies dendritic morphogenesis. *Neuron* 41, 229-41.

Gautreau, A., Ho, H. Y., Li, J., Steen, H., Gygi, S. P., and Kirschner, M. W. (2004). Purification and architecture of the ubiquitous Wave complex. *Proc Natl Acad Sci U S A* 101, 4379-83.

Geelen, J. A., and Langman, J. (1979). Ultrastructural observations on closure of the neural tube in the mouse. *Anat Embryol (Berl)* 156:73-88.

Ghosh A., Carnahan J., and Greenberg, M. E. (1994). Requirement for BDNF in activity-dependent survival of cortical neurons. *Science* 263:1618-23.

Gilmore, E.C., Ohshima, T., Goffinet, A.M., Kulkarni, A.B., Herrup, K. (1998). Cyclin-dependent kinase 5-deficient mice demonstrate novel developmental arrest in cerebral cortex. *J Neurosci* 18, 6370-7.

Gleeson, J.G., Allen, K.M., Fox, J.W., Lamperti, E.D., Berkovic, S., Scheffer, I., Cooper, E.C., Dobyns, W.B., Minnerath, S.R., Ross, M.E., Walsh, C.A. (1998). Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. *Cell* 92, 63-72.

Gleeson, J.G., Minnerath, S.R., Fox, J.W., Allen, K.M., Luo, R.F., Hong, S.E., Berg, M.J., Kuzniecky, R., Reitnauer, P.J., Borgatti, R., Mira, A.P., Guerrini, R., Holmes, G.L., Rooney, C.M., Berkovic, S., Scheffer, I., Cooper, E.C., Ricci, S., Cusmai, R., Crawford, T.O., Leroy, R., Andermann, E., Wheless, J.W., Dobyns, W.B., Walsh, C.A., (1999). Characterization of mutations in the gene doublecortin in patients with double cortex syndrome. *Ann Neurol* 45, 146-53.

Gleeson, J.G., Lin, P.T., Flanagan, L.A., Walsh, C.A. (1999). Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron* 23, 257-71.

Gongidi V., Ring C., Moody M., Brekken R., Sage E.H., Rakic P., and Anton E.S. (2004). SPARC-like 1 regulates the terminal phase of radial glia-guided migration in the cerebral cortex. *Neuron* 41, 57-69.

Grove, M., Demyanenko, G., Echarri, A., Zipfel, P. A., Quiroz, M. E., Rodriguiz, R. M., Playford. M., Martensen, S. A. , Robinson, M. R., Wetsel, W. C., Maness, P. F., and Pendergast, A. M. (2004). ABI2-deficient mice exhibit defective cell migration, aberrant dendritic spine morphogenesis, and deficits in learning and memory. Mol Cell Biol. 24,10905-22.

Hahne, P., Sechi, A., Benesch, S., and Small, J. V. (2001). Scar/WAVE is localised at the tips of protruding lamellipodia in living cells. *FEBS Lett.* 492: 215-220.

Harada, A., Oguchi, K., Okabe, S., Kuno, J., Terada, S., Ohshima, T., Sato-Yoshitake, R., Takei, Y., Noda, T., Hirokawa, N. (1994). Altered microtubule organization in small-calibre axons of mice lacking tau protein. *Nature* 369, 488-91.

Hatten, M.E. and Mason C.A. (1990). Mechanisms of glial-guided neuronal migration in vitro and in vivo. *Experientia* 46, 907-16

Hatten, M.E. (2002). New directions in neuronal migration. Science. 297:1660-3.

Hattori, M., Adachi, H., Tsujimoto, M., Arai, H., Inoue, K. (1994). Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase. *Nature* 370, 216-8.

Hevner R.F., Shi L., Justice N., Hsueh Y., Sheng M., Smiga S., Bulfone A., Goffinet A.M., Campagnoni A.T., and Rubenstein J.L. (2001). Tbr1 regulates differentiation of the preplate and layer 6. *Neuron* 29, 353-66.

Hirotsune, S., Fleck, M.W., Gambello, M.J., Bix, G.J., Chen, A., Clark, G.D., Ledbetter, D.H., McBain, C.J., Wynshaw-Boris, A. (1998). Graded reduction of Pafah1b1 (Lis1) activity results in neuronal migration defects and early embryonic lethality. *Nat Genet* 19, 333-9.

Homma, N., Takei, Y., Tanaka, Y., Nakata, T., Terada, S., Kikkawa, M., Noda, Y.,

Hirokawa, N. (2003). Kinesin superfamily protein 2A (KIF2A) functions in suppression of collateral branch extension. *Cell* 114, 229-39

Horesh, D., Sapir, T., Francis, F., Wolf, S.G., Caspi, M., Elbaum, M., Chelly, J., Reiner, O. (1999) Doublecortin, a stabilizer of microtubules. *Hum Mol Genet* 8, 1599-610.

Huang, H-P., Liu, M., El-Hodiri, H. M., Chu, K., Jamrich, M., and Tsai, M. J. (2000). Regulation of the pancreatic islet- specific gene *BETA2* (*neuroD*) by Neurogenein 3. *Mol Cell Biol* 20, 3292-3307.

Hummel, T., Leifker, K., and Klambt, C. (2000). The Drosophila HEM-2/NAP1 homolog KETTE controls axonal pathfinding and cytoskeletal organization. *Genes Dev* 14, 863-73.

Innocenti, M., Zucconi, A., Disanza, A., Frittoli, E., Areces, L. B., Steffen, A., Stradal, T. E., Di Fiore, P. P., Carlier, M. F., and Scita, G. (2004). Abi1 is essential for the formation and activation of a WAVE2 signalling complex. *Nat Cell Biol* 6, 319-27.

Insall, R.H., and Jones, G. E. (2006). Moving matters: signals and mechanisms in directed cell migration. *Nat Cell Biol* 8, 776-9.

Kamiya, A., Kubo, K., Tomoda, T., Takaki, M., Youn, R., Ozeki, Y., Sawamura, N., Park, U., Kudo, C., Okawa, M., Ross, C.A., Hatten, M.E., Nakajima, K., Sawa, A. (2005). A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development. *Nat Cell Biol* 7, 1167-78.

Kim Y., Sung J.Y., Ceglia I., Lee K.W., Ahn J.H., Halford J.M., Kim A.M., Kwak S.P., Park J.B., Ho Ryu S., Schenck A., Bardoni B., Scott J.D., Nairn A.C., and Greengard P. (2006). Phosphorylation of WAVE1 regulates actin polymerization and dendritic spine morphology. *Nature* 442, 814-7

Kitagawa, M., Umezu, M., Aoki, J., Koizumi, H., Arai, H., Inoue, K. (2000) Direct association of LIS1, the lissencephaly gene product, with a mammalian homologue of a fungal nuclear distribution protein, rNUDE. *FEBS Lett* 479, 57-62.

Kitamura, T., Kitamura, Y., Yonezawa, K., Totty, N. F., Gout, I., Hara, K., Waterfield, M. D., Sakaue, M., Ogawa, W., and Kasuga, M. (1996). Molecular cloning of p125Nap1, a protein that associates with an SH3 domain of Nck. *Biochem Biophys Res Commun* 219, 509-14.

Kitamura, Y., Kitamura, T., Sakaue, H., Maeda, T., Ueno, H., Nishio, S., Ohno, S., Osada, S., Sakaue, M., Ogawa, W. et al. (1997). Interaction of Nck-associated protein 1 with activated GTP-binding protein Rac. *Biochem J* 322 , 873-8.

Ko, J., Humbert, S., Bronson, R.T., Takahashi, S., Kulkarni, A.B., Li, E., Tsai, L.H. (2001). p35 and p39 are essential for cyclin-dependent kinase 5 function during neurodevelopment. *J Neurosci* 21, 6758-71.

Kobayashi, K., Kuroda, S., Fukata, M., Nakamura, T., Nagase, T., Nomura, N., Matsuura, Y., Yoshida-Kubomura, N., Iwamatsu, A., and Kaibuchi, K. (1998). p140Sra-1 (specifically Rac1-associated protein) is a novel specific target for Rac1 small GTPase. *J Biol Chem* 273, 291-5.

Koizumi H., Tanaka T., and Gleeson J.G. (2006). Doublecortin-like kinase functions with doublecortin to mediate fiber tract decussation and neuronal migration. *Neuron* 49, 55-66.

Krueger, E. W., Orth, J. D., Cao, H., and McNiven, M. A. (2003). A dynamin-cortactin-Arp2/3 complex mediates actin reorganization in growth factor-stimulated cells. *Mol Biol Cell* 14, 1085-96.

Kunda, P., Craig, G., Dominguez, V., and Baum, B. (2003). Abi, Sra1, and Kette control the stability and localization of SCAR/WAVE to regulate the formation of actin-based protrusions. *Curr Biol* 13, 1867-75.

Kwon, Y. T. and L. H. Tsai (1998). A novel disruption of cortical development in p35(-/-) mice distinct from reeler. *J Comp Neurol* 395, 510-22.

Leighton P.A., Mitchell K.J., Goodrich L.V., Lu X., Pinson K., Scherz P., Skarnes W.C., and Tessier-Lavigne, M. (2001). Defining brain wiring patterns and mechanisms through gene trapping in mice. *Nature* 410, 174-9.

Ma, X., Kawamoto, S., Uribe, J., Adelstein, R.S. (2006). Function of the neuron-specific alternatively spliced isoforms of nonmuscle myosin II-B during mouse brain development. *Mol Biol Cell* 17, 2138-49

Machesky, L. M., Mullins, R. D., Higgs, H. N., Kaiser, D. A., Blanchoin, L., May, R. C., Hall, M. E., and Pollard, T. D. (1999). Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex. *Proc Natl Acad Sci U S A* 96, 3739-44.

Marin O., and Rubenstein J. L. (2003). Cell migration in the forebrain. *Annu Rev Neurosci* 26, 441-83.

Marsh, L., and Letourneau, P.C. (1984). Growth of neurites without filopodial or lamellipodial activity in the presence of cytochalasin B. *J. Cell Biol.* 99, 2041-2047.

McAllister, A. K., Katz, L. C., and Lo, D. C. (1996). Neurotrophin regulation of cortical dendritic growth requires activity. *Neuron*. 17:1057-64.

McEvilly R.J., de Diaz M.O., Schonemann M.D., Hooshmand F., and Rosenfeld M.G. (2002). Transcriptional regulation of cortical neuron migration by POU domain factors. *Science* 295, 1528-32.

Miki, H., Suetsugu, S., and Takenawa, T. (1998). WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *Embo J* 17, 6932-41.

Millard, T. H., Sharp, S. J., and Machesky, L. M. (2004). Signalling to actin assembly via the WASP (Wiskott-Aldrich syndrome protein)-family proteins and the Arp2/3 complex. *Biochem J* 380, 1-17.

Millar, J.K., Wilson-Annan, J.C., Anderson, S., Christie, S., Taylor, M.S., Semple, C.A., Devon, R.S., Clair, D.M., Muir, W.J., Blackwood, D.H., Porteous, D.J. (2000). Disruption of two novel genes by a translocation co-segregating with schizophrenia. *Hum Mol Genet* 9, 1415-23.

Miller, M. (1981). Maturation of rat visual cortex. I. A quantitative study of Golgi-impregnated pyramidal neurons. *J. Neurocytol.* 10, 859-878.

Mitchell, K. J., Pinson, K. I., Kelly, O. G., Brennan, J., Zupicich, J., Scherz, P., Leighton, P. A., Goodrich, L. V., Lu, X., Avery, B. J., Tate, P., Dill, K., Pangilinan, E., Wakenight, P., Tessier-Lavigne, M., and Skarnes, W. C. (2001). Functional analysis of secreted and transmembrane proteins critical to mouse development. *Nat Genet* 28, 241-9.

Miyata T, Kawaguchi A, Okano H, Ogawa M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* 31, 727-41.

Mochida G.H., and Walsh, C.A. (2004). Genetic basis of developmental malformations of the cerebral cortex. *Arch Neurol* 61, 637-40.

Moro, F., Carrozzo, R., Veggiotti, P., Tortorella, G., Toniolo, D., Volzone, A.,

Guerrini, R. (2002). Familial periventricular heterotopia: missense and distal truncating mutations of the FLN1 gene. *Neurology* 58, 916-21.

Nadarajah, B., Brunstrom, J.E., Grutzendler, J., Wong, R.O., Pearlman, A.L. (2001) Two modes of radial migration in early development of the cerebral cortex. *Nat Neurosci*. 4, 143-50.

Nagano, T., Yoneda, T., Hatanaka, Y., Kubota, C., Murakami, F., Sato, M. (2002). Filamin A-interacting protein (FILIP) regulates cortical cell migration out of the ventricular zone. *Nat Cell Biol* 4, 495-501.

Nagano, T., Morikubo, S., Sato, M. (2004). Filamin A and FILIP (Filamin A-Interacting Protein) regulate cell polarity and motility in neocortical subventricular and intermediate zones during radial migration. *J Neurosci* 24, 9648-57.

Nakagawa, H., Miki, H., Ito, M., Ohashi, K., Takenawa, T., and Miyamoto, S. (2001). N-WASP, WAVE and Mena play different roles in the organization of actin cytoskeleton in lamellipodia. *J Cell Sci* 114, 1555-65.

Niethammer, M., Smith, D.S., Ayala, R., Peng, J., Ko, J., Lee, M.S., Morabito, M., Tsai, L.H. (2000). NUDEL is a novel Cdk5 substrate that associates with LIS1 and cytoplasmic dynein. *Neuron* 28, 697-711.

Nikolic, M., Chou, M.M., Lu, W., Mayer, B.J., Tsai, L.H. (1998). The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity. *Nature* 395, 194-8.

Ohshima, T., Ward, J.M., Huh, C.G., Longenecker, G., Veeranna, Pant, H.C., Brady, R.O., Martin, L.J., Kulkarni, A.B. (1996). Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. *Proc Natl Acad Sci U S A* 93, 11173-8.

Ogawa, M., Miyata, T., Nakajima, K., Yagyu, K., Seike, M., Ikenaka, K., Yamamoto, H., Mikoshiba, K. (1995). The reeler gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. *Neuron* 14, 899-912.

Olson, E.C. and Walsh C.A. (2002). Smooth, rough and upside-down neocortical development. *Curr Opin Genet Dev* 12, 320-7.

Rakic, P (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. J Comp Neurol 145, 61-83

Ralic, P (1974). Neurons in rhesus monkey cisual cortex: systematic relation between time of origin and eventual diposition. *Science* 183, 425-7

Rakic, P. and V. S. Caviness, Jr. (1995). Cortical development: view from neurological mutants two decades later. *Neuron* 14, 1101-4.

Rakic, P. (1998). Images in neuroscience. Brain development, VI: radial migration and cortical evolution. *Am J Psychiatry* 155, 1150-1.

Rakic, P. (2003). Developmental and evolutionary adaptations of cortical radial glia. *Cereb Cortex* 13, 541-9

Rakeman, A. S., and Anderson, K. V. (2006). Axis specification and morphogenesis in the mouse embryo require Nap1, a regulator of WAVE-mediated actin branching. *Development* 133, 3075-83.

Reichardt, L. F. (2006). Neurotrophin-regulated signalling pathways. *Philos Trans R Soc Lond B Biol Sci.* 361:1545-64.

Rivas, R. J. and Hatten, M.E. (1995). Motility and cytoskeletal organization of migrating cerebellar granule neurons. *J Neurosci* 15, 981-9.

Rochlin, M.W., Dailey, M.E., and Bridgman, P.C. (1999). Polymerizing microtubules activate site-directed F-actin assembly in nerve growth cones. *Mol. Biol. Cell* 10, 2309-2327.

Rodriguez, O.C., Schaefer, A.W., Mandato, C.A., Forscher, P., Bement, W.M., Waterman-Storer, C.M. (2003). Conserved microtubule-actin interactions in cell movement and morphogenesis. *Nat Cell Biol* 5, 599-609.

Rogers, S. L., Wiedemann, U., Stuurman, N., and Vale, R. D. (2003). Molecular requirements for actin-based lamella formation in Drosophila S2 cells. *J Cell Biol* 162, 1079-88.

Rohatgi, R., Ho, H. Y. and Kirschner, M. W. (2000). Mechanism of N-WASP activation by CDC42 and phosphatidylinositol 4, 5-bisphosphate. *J Cell Biol* 150,

1299-310.

Sapir, T., Elbaum, M., Reiner, O. (1997). Reduction of microtubule catastrophe events by LIS1, platelet-activating factor acetylhydrolase subunit. EMBO J. 16, 6977-84.

Sarkisian, M.R., Bartley, C.M., Chi, H., Nakamura, F., Hashimoto-Torii, K., Torii, M., Flavell, R.A., Rakic, P. (2006). MEKK4 signaling regulates filamin expression and neuronal migration. *Neuron* 52, 789-801.

Sasaki, S., Shionoya, A., Ishida, M., Gambello, M.J., Yingling, J., Wynshaw-Boris, A., Hirotsune, S. (2000). A LIS1/NUDEL/cytoplasmic dynein heavy chain complex in the developing and adult nervous system. *Neuron* 28, 681-96.

Sheen,V.L., Dixon, P.H., Fox, J.W., Hong, S.E., Kinton, L., Sisodiya, S.M., Duncan, J.S., Dubeau, F., Scheffer, I.E., Schachter, S.C., Wilner, A., Henchy, R., Crino, P., Kamuro, K., DiMario, F., Berg, M., Kuzniecky, R., Cole, A.J., Bromfield, E., Biber, M., Schomer, D., Wheless, J., Silver, K., Mochida, G.H., Berkovic, S.F., Andermann, F., Andermann, E., Dobyns, W.B., Wood, N.W., Walsh, C.A. (2001). Mutations in the X-linked filamin 1 gene cause periventricular nodular heterotopia in males as well as in females. *Hum Mol Genet* 10, 1775-83.

Schaar, B.T., McConnell, S.K. (2005). Cytoskeletal coordination during neuronal migration. *Proc Natl Acad Sci U S A* 102, 13652-7.

Schaefer A.W., Kabir N., and Forscher, P. (2002). Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. *J Cell Biol* 158,139-52.

Schmid R.S., McGrath B., Berechid B.E., Boyles B., Marchionni M., Sestan N., and Anton, E. S. (2003). Neuregulin 1-erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex. *Proc Natl Acad Sci U S A* 100, 4251-6.

Schmid, R.S., Shelton, S., Stanco, A., Yokota, Y., Kreidberg, J.A., and Anton, E. S. (2004). alpha3beta1 integrin modulates neuronal migration and placement during early stages of cerebral cortical development. *Development* 131, 6023-31

Schwab, M.H., Bartholomae, A., Heimrich, B., Feldmeyer, D., Druffel-Augustin, S., Goebbels, S., Naya, F.J., Zhao, S., Frotscher, M., Tsai, M.J., Nave, K.A. (2000). Neuronal basic helix-loop-helix proteins (NEX and BETA2/Neuro D) regulate

terminal granule cell differentiation in the hippocampus. *J Neurosci* 20, 3714-24.

Shu, T., Tseng, H.C., Sapir, T., Stern, P., Zhou, Y., Sanada, K., Fischer, A., Coquelle, F.M., Reiner, O., and Tsai, L.H. (2006). Doublecortin-like kinase controls neurogenesis by regulating mitotic spindles and M phase progression. N*euron* 49, 25-39.

Shu, T., Ayala, R., Nguyen, M.D., Xie, Z., Gleeson, J.G., Tsai, L.H. (2004). Ndel1 operates in a common pathway with LIS1 and cytoplasmic dynein to regulate cortical neuronal positioning. *Neuron* 44, 263-77.

Sidman, R.L. and Rakic, P. (1973). Neuronal migration,with special reference to developing human brain: a review. *Brain Res* 62, 1-35

Smith, D.S., Niethammer, M., Ayala, R., Zhou, Y., Gambello, M.J., Wynshaw-Boris, A., Tsai, L.H. (2000). Regulation of cytoplasmic dynein behaviour and microtubule organization by mammalian Lis1. *Nat Cell Biol* 2, 767-75.

Soderling, S.H., Langeberg, L.K., Soderling, J.A., Davee, S.M., Simerly, R., Raber, J., and Scott, J.D. (2003). Loss of WAVE-1 causes sensorimotor retardation and reduced learning and memory in mice. *Proc Natl Acad Sci U S A* 100,1723-8.

Soderling, S. H., Guire, E. S., Kaech, S., White, J., Zhang, F., Schutz, K., Langeberg, L. K., Banker, G., Raber, J., and Scott, J. D. (2007). A WAVE-1 and WRP signaling complex regulates spine density, synaptic plasticity, and memory. *J Neurosci* 27, 355-65.

Solecki D.J., Model L., Gaetz J., Kapoor T.M., and Hatten M.E. (2004). Par6alpha signaling controls glial-guided neuronal migration. *Nat Neurosci* 7, 1195-203.

Sossey-Alaoui, K., Head, K., Nowak, N., and Cowell, J. K. (2003). Genomic organization and expression profile of the human and mouse WAVE gene family. *Mamm Genome* 14, 314-22.

Soto, M. C., Qadota, H., Kasuya, K., Inoue, M., Tsuboi, D., Mello, C. C., and Kaibuchi, K. (2002). The GEX-2 and GEX-3 proteins are required for tissue morphogenesis and cell migrations in C. elegans. *Genes Dev* 16, 620-32.

Steffen, A., Rottner, K., Ehinger, J., Innocenti, M., Scita, G., Wehland, J., and Stradal, T. E. (2004). Sra-1 and Nap1 link Rac to actin assembly driving lamellipodia formation. *EMBO J* 23, 749-59.

Stenman, J., Toresson, H., Campbell, K. (2003). Identification of two distinct progenitor populations in the lateral ganglionic eminence: implications for striatal and olfactory bulb neurogenesis. *J Neurosc* 23,167-74.

Stradal, T.E., Rottner, K., Disanza, A., Confalonieri, S., Innocenti, M., and Scita, G. (2004). Regulation of actin dynamics by WASP and WAVE family proteins. *Trends Cell Biol* 14, 303-11.

Strasser,,G. A., Rahim, N. A., VanderWaal, K. E., Gertler, F. B., and Lanier, L. M. (2004). Arp2/3 is a negative regulator of growth cone translocation. *Neuron* 43,81-94.

Suetsugu, S., Yamazaki, D., Kurisu, S., and Takenawa, T. (2003). Differential roles of WAVE1 and WAVE2 in dorsal and peripheral ruffle formation for fibroblast cell migration. *Dev Cell* 5, 595-609.

Sugitani, Y., Nakai, S., Minowa, O., Nishi, M., Jishage, K., Kawano, H., Mori, K., Ogawa, M., and Noda T. (2002). Brn-1 and Brn-2 share crucial roles in the production and positioning of mouse neocortical neurons. *Genes Dev* 16, 1760-5.

Suzuki, T., Nishiyama, K., Yamamoto, A., Inazawa, J., Iwaki, T., Yamada, T., Kanazawa, I., and Sakaki, Y. (2000). Molecular cloning of a novel apoptosis-related gene, human Nap1 (NCKAP1), and its possible relation to Alzheimer disease. *Genomics* 63, 246-54.

Svitkina, T.M., and Borisy, G.G. (1999). Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. *J. Cell Biol.* 145, 1009–1026.

Taylor, K.R., Holzer, A.K., Bazan, J.F., Walsh, C.A., Gleeson, J.G. (2000). Patient mutations in doublecortin define a repeated tubulin-binding domain. *J Biol Chem* 275, 34442-50.

Taira, K., Miyagishi, M., Kawasaki, H., Akashi, H. (2003). RNAi experimental protocol. *Youdo sha* [Japanese]

Takahashi, T., Misson, J.P., Caviness, V.S. Jr. (1990) Glial process elongation and branching in the developing murine neocortex: a qualitative and quantitative immunohistochemical analysis. *J Comp Neurol.* 302, 15-28.

Takei, Y., Teng, J., Harada, A., Hirokawa, N. (2000). Defects in axonal elongation

and neuronal migration in mice with disrupted tau and map1b genes. *J Cell Biol* 150, 989-1000.

Tanaka, T., Serneo, F.F., Higgins, C., Gambello, M.J., Wynshaw-Boris, A., Gleeson, J.G. (2004). Lis1 and doublecortin function with dynein to mediate coupling of the nucleus to the centrosome in neuronal migration. *J Cell Biol* 165, 709-21.

Taylor, K.R., Holzer, A.K., Bazan, J.F., Walsh, C.A., Gleeson, J.G. (2000). Patient mutations in doublecortin define a repeated tubulin-binding domain. *J Biol Chem* 275, 34442-50.

Tsai, L. H., and Gleeson, J. G. (2005). Nucleokinesis in neuronal migration. *Neuron* 46:383-8.

Weber, K.L., Sokac, A.M., Berg, J.S., Cheney, R.E., Bement, W.M. (2004). A microtubule-binding myosin required for nuclear anchoring and spindle assembly. *Nature* 431, 325-9.

Xie, Z., Sanada, K., Samuels, B.A., Shih, H., Tsai, L.H. (2003). Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. *Cell* 114, 469-82.

Yamamoto, A., Suzuki, T., and Sakaki, Y. (2001). Isolation of hNap1BP which interacts with human Nap1 (NCKAP1) whose expression is down-regulated in Alzheimer's disease. *Gene* 271, 159-69.

Ybot-Gonzalez P., and Copp, A. J. (1999). Bending of the neural plate during mouse spinal neurulation is independent of actin microfilaments. *Dev Dyn* 215, 273-83.

Zhu, Y., Romero, M.I., Ghosh, P., Ye, Z., Charnay, P., Rushing, E.J., Marth, J.D., Parada, L.F. (2001). Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain. *Genes Dev* 15, 859-76.