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Lis1 and Ndel1 influence the timing of nuclear envelope breakdown in neural stem cells

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Lis1 and Ndel1 are essential for animal development. They interact directly with one another and with cytoplasmic dynein. The developing brain is especially sensitive to reduced Lis1 or Ndel1 levels, as both proteins influence spindle orientation, neural cell fate decisions, and neuronal migration. We report here that Lis1 and Ndel1 reduction in a mitotic cell line impairs prophase nuclear envelope (NE) invagination (PNEI). This dynein-dependent process facilitates NE breakdown (NEBD) and occurs before the establishment of the bipolar spindle. Ndel1 phosphorylation is important for this function, regulating binding to both Lis1 and dynein. Prophase cells in the ventricular zone (VZ) of embryonic day 13.5 Lis1−/− mouse brains show reduced PNEI, and the ratio of prophase to prometaphase cells is increased, suggesting an NEBD delay. Moreover, prophase cells in the VZ contain elevated levels of Ndel1 phosphorylated at a key cdk5 site. Our data suggest that a delay in NEBD in the VZ could contribute to developmental defects associated with Lis1−Ndel1 disruption.

Introduction

Lis1 haploinsufficiency causes a severe developmental brain disorder in humans, type 1 lissencephaly (Cardoso et al., 2002; Mei et al., 2008). Homozygous loss of Lis1 or its binding partner, Ndel1, is thought to be lethal, as is the case in mice (Hirotsune et al., 1998; Sasaki et al., 2005). The C terminus of Lis1 binds to a microtubule motor, cytoplasmic dynein (Reiner et al., 1993; Sasaki et al., 2000), whereas the N terminus contains a LisH homodimerization domain (Kim et al., 2004). Between these domains is a coiled-coil region that provides flexibility to the Lis1 dimer (Tarricone et al., 2004). Ndel1 contains an N-terminal coiled-coil domain that interacts with Lis1 and an unstructured C terminus possessing five Ser/Thr-Pro (S/T-P) sites targeted by pro-directed kinases (Niethammer et al., 2000; Derewenda et al., 2007). Ndel1 also binds directly to dynein. In fact, Lis1 and Ndel1 are thought to associate with cytoplasmic dynein as a heterotetramer (Tarricone et al., 2004).

Cytoplasmic dynein is a multisubunit complex that contributes to processes requiring force generation. Dynein interacts with membranes through another multisubunit protein, dynactin, which also regulates dynein processivity (King and Schroer, 2000; Schroer, 2004). Although evidence that Lis1 and Ndel1 regulate dynein is accumulating, the precise mechanism is unclear. Lis1 modestly stimulates dynein’s ATPase function in vitro (Mesngon et al., 2006), and there is evidence for functional interactions in several organisms (Vallee and Tsai, 2006). Reduction of Lis1 in developing rat neurons inhibited interkinetic nuclear oscillations in the ventricular zone (VZ), reduced mitosis, and perturbed the acquisition of a bipolar morphology in the subventricular/intermediate zones (Tsai et al., 2005). In mice, reduction of Lis1, Ndel1, or dynein heavy chain (DHC) disrupted the link between the centrosome and the nucleus in migrating neurons (Shu et al., 2004). Lis1 overexpression could partly compensate for Ndel1 reduction, but not vice versa. Neither protein was able to rescue DHC deficiency (Shu et al., 2004; Sasaki et al., 2005). More recently, Lis1 reduction was shown to alter spindle orientation in neuroepithelial stem cells so that the cellular cleavage plane was biased toward asymmetrical cell divisions and neurogenesis (Yingling et al., 2008). It was hypothesized that this resulted in the early depletion of a progenitor pool.

Two observations prompted us to explore another process that might be influenced by Lis1 pathways. The first was that...
dynein activity facilitated nuclear envelope (NE) breakdown (NEBD; Salina et al., 2002) by promoting prophase NE invagination (PNEI) in a microtubule-dependent manner, resulting in NE tearing. The second observation was a curious enrichment of Lis1 at the NE in prophase cells and in cells treated with nocodazole (Smith et al., 2000; Coquelle et al., 2002; Cockell et al., 2004). These observations suggest that Lis1 could function in neuroepithelial stem cells before its role in spindle orientation by contributing to dynein-dependent PNEI. We used in vivo and in vitro approaches to explore this possibility and found that both Lis1 and Ndel1 influence PNEI in cultured cell lines and in the developing mouse brain. Furthermore, we find that Ndel1 phosphorylation may be a key regulatory step and, as such, could fine tune the timing of NEBD.

**Results and discussion**

**Reducing Lis1 expression in COS-7 cells reduces PNEI**

As expected, Lis1 and Ndel1 accumulated at the NE and within invaginations in prophase cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200803071/DC1). PNEI can be quantified by examining prophase nuclei labeled with Hoechst dye and determining the percentage with invaginations (Fig. 1A). To determine whether Lis1 is important for PNEI, we transiently expressed a Lis1 short hairpin RNA (shRNA), which reduced Lis1 expression by 80% after 48 h (Fig. 1B). An shRNA for DHC reduced DHC expression by 70%. Scrambled sequences were used as negative controls. Lis1 shRNA reduced PNEI by ~40%, whereas DHC RNAi reduced PNEI by ~50% (Fig. 1B). The data support a model in which Lis1, like dynein, facilitates NEBD by promoting PNEI.

**Reducing Ndel1 expression in COS-7 cells reduces PNEI**

Earlier studies suggested that Lis1 and Ndel1 cooperate to regulate dynein (Yan et al., 2003; Shu et al., 2004), and Ndel1 accumulates at the prophase NE (Fig. S1, E and F; and Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200803071/DC1). Therefore, we anticipated that reducing Ndel1 expression might have a similar effect on PNEI. The Ndel1 shRNA reduced Ndel1 protein expression by ~50% in 48 h (Fig. 1C, top) and also reduced PNEI by ~50% (Fig. 1C). The phenotype was rescued by coexpression of human myc-tagged Ndel1, so the level of expression of wild-type Ndel1 from the cytomegalovirus promoter was apparently high enough to overcome shRNA expression driven by the U6 promoter. Interestingly, the phenotype was not rescued by coexpression of an Ndel1 construct with mutations in five key cdk phosphorylation sites (Ndel15A; Fig. 1C). Thus, Ndel1 phosphorylation could be a regulatory step in PNEI.

**Increasing Lis1 or Ndel1 expression in COS-7 cells increases PNEI**

We expected that transient overexpression of Lis1 or Ndel1 would block PNEI by disrupting normal stoichiometry of protein–protein interactions. Instead, we found that overexpression of Lis1 nearly doubled PNEI compared with controls (Fig. 1D). This was dependent on intact microtubules, as the increase was blocked by nocodazole (unpublished data). A mutant construct that expresses an N-terminal fragment of Lis1 containing the LisH domain reduced PNEI by 50%, suggesting that Lis1 dimers are likely to be used during dynein-dependent PNEI. This fragment did not accumulate at the prophase NE (unpublished data), so the redistribution of Lis1 to the NE may also depend on homodimerization. Overexpression of Ndel1 was found to have a similar effect on PNEI (Fig. 1E). This supports a model in which Ndel1 and Lis1 promote the microtubule-dependent invaginations attributed to dynein function (Beaudouin et al., 2002; Salina et al., 2002). Lis1 and Ndel1 expression may be elevated in prophase cells (Fig. S1, A and E), so transient overexpression of either protein may recapitulate or hasten a state conducive to PNEI.

**S/T-P phosphorylation sites in Ndel1 are important for stimulation of PNEI**

Interestingly, the phosphomutant Ndel15A did not induce PNEI. Normal PNEI was only modestly reduced by Ndel15A, so it did not significantly perturb endogenous Ndel1 function (Fig. 1F). S197, T219, and S231 are targets for cdk5 (Niethammer et al., 2000), whereas cdk1 targets S242 and T245 (Yan et al., 2003). Because cdk5 mainly functions in postmitotic neurons, we expected that a construct in which S/T→A mutations were restricted to the cdk5 sites (Ndel13A) would retain its ability to stimulate PNEI. However, this was not the case (Fig. 1F). Both cdk5 and the cdk5 activator, p35, were present at substantial levels in COS-7 cells (Fig. 1G), raising the intriguing possibility that cdk5 activity is important during PNEI.

To further examine the role of phosphorylation, we increased or reduced cdk5 and cdk1 activity. The effect on PNEI was determined in cells with normal Ndel1 levels (Fig. 1H) and in cells overexpressing Ndel1 (Fig. 1I). Increasing cdk5 or cdk1 activity in the absence of Ndel1 overexpression did not alter PNEI. However, increasing the activity of both kinases almost doubled PNEI compared with controls. Furthermore, a significant inhibitory effect (50%) was observed when dominant-negative kinases (dncdk5 and dncdk1) were coexpressed. Neither dncdk5 nor dncdk1 was able to reduce PNEI when expressed individually, indicating that either (a) endogenous cdk5 or endogenous cdk1 is sufficient to allow normal PNEI to occur, or (b) the reagents incompletely inhibit their respective kinases. In fact, inhibiting cdk1 may actually increase PNEI (although this did not reach statistical significance), suggesting a complex relationship between the activity of this kinase and PNEI.

The aforementioned reagents produced somewhat different results when Ndel1 was also overexpressed. Cdk5 and cdk1 together enhanced PNEI over that stimulated by Ndel1. However, in this situation, the individual kinases were equally effective at stimulating PNEI. Expressing both dncdk5 and dncdk1 prevented PNEI induced by Ndel1 overexpression. However, PNEI was also inhibited when dncdk1 or dncdk5 was expressed individually, so each kinase alone is insufficient to fully phosphorylate ectopic Ndel1 at the appropriate sites to allow it to induce PNEI. Together, the data suggest that the activities of cdk5
proteins. We exposed Ndel1 to cdk5/p25 (p25 is a stable fragment of p35), cdk1/cyclin B1, or both. Cdk5/p25 caused a distinct Ndel1 banding pattern in Coomassie-stained gels (Fig. 2 B). Cdk1/cyclin B1 produced a modest band shift. The band pattern observed when both kinases were included was distinct from that produced by cdk5 alone, indicating that both kinases target Ndel1 in vitro. pS231Ab recognized Ndel1 phosphorylated in vitro by cdk5 or by cdk5 and cdk1 together but not by cdk1 alone (Fig. 2 B). Yan et al. (2003) demonstrated that an Ndel1 construct with S/T→E mutations bound more Lis1. In support of this, we find that Ndel1 phosphorylated in vitro by both cdk5 and cdk1 pulled down approximately fivefold more Lis1 than

and cdk1 can compensate for one another under some conditions. However, they also point to a dosage affect, i.e., the cell must have sufficient amounts of appropriately phosphorylated Ndel1 to carry out normal PNEI.

**Ndel1 phosphorylation impacts Lis1 and dynein binding**

Fig. 2 A shows a schematic of Ndel1 indicating the positions of cdk target residues. The pS231Ab antibody was generated against a peptide phosphorylated at S231, which is thought to be targeted by cdk5, not cdk1. To gain more insight into the role of Ndel1 phosphorylation, we turned to in vitro assays with purified

Figure 1. **Lis1 and Ndel1 contribute to PNEI.** (A) COS-7 cell nuclei labeled with Hoechst. (left) A prophase nucleus before PNEI. (right) Nuclei with varying degrees of PNEI. (B, top) Lis1 shRNA (Lis1i) or dynein shRNA (DHCi) reduces protein expression by 48 h after transfection. (bottom) Relative to scrambled controls, the percentage of prophase cells with PNEI was reduced by Lis1i or DHCi RNAi. (C) Similarly, Ndel1 RNAi reduced Ndel1 expression and PNEI. This was prevented by coexpression of Ndel1 but not Ndel15A, which has S/T→A mutations in five cdk sites. (D) Increased PNEI was caused by full-length Lis1 (FL), whereas a Lis1 fragment [N term] reduced PNEI. (E) Nocodazole treatment for 13 h after transfection prevents Ndel1-induced PNEI. (F) Neither Ndel15A nor Ndel13A (S/T→A mutations in the first three cdk sites) increased PNEI. (G) COS-7 cells express cdk5 and p35. (H) Cells were transfected with cdk5/p25, cdk1/cyclin B1, or dominant-negative kinases (dn-cdk5 and dn-cdk1). (I) The kinases described in H were cotransfected with Ndel1. Data are the mean ± SD for three cultures. Significance was determined by Student’s t test. Error bars indicate ± SD. Bar, 10 μm.
Accumulation of Lis1 and Ndel1 at the prophase NE can occur in the absence of microtubules but requires an intact dynactin complex

unphosphorylated Ndel1 (Fig. 2 C). This was also true in coimmunoprecipitations (coIPs) of Ndel1 or Ndel15A co-expressed with active or dominant-negative kinases in COS-7 cells (Fig. 2 D). We anticipated that Ndel1 phosphorylation would have a similar impact on dynein binding but found instead that more unphosphorylated Ndel1 coimmunoprecipitated with purified bovine brain dynein (Fig. 2 E). Moreover, Ndel1 phosphorylation reduced the amount of Lis1 that coimmunoprecipitated with dynein, suggesting that phosphorylation not only strengthens Ndel1 binding to Lis1 but promotes the release of Lis1–pNdel1 complexes from dynein. Phosphorylation by either kinase alone was insufficient to reduce dynein binding (Fig. 2 F). Thus, although it is clear that Ndel1 phosphorylation impacts its interactions with the dynein motor machinery and PNEI, phosphorylation by individual kinases could have different biological consequences than phosphorylation by both kinases.

Accumulation of Lis1 and Ndel1 at the prophase NE can occur in the absence of microtubules but requires an intact dynactin complex

If dynein-dependent transport is important for relocation of Lis1 and Ndel1 to the NE, microtubule disruption should prevent their accumulation. However, prophase cells had typical NE enrichment of Lis1 3 h after exposure to nocodazole (Fig. 3 A) despite a complete loss of microtubules within minutes of drug addition. G2 cells also exhibited NE accumulation, and Ndel1 and dynein behaved similarly. A time course study indicated that nocodazole caused a transient increase in cells with Lis1–Ndel1–dynein enrichment at the NE and delayed NEBD (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200803071/DC1). A normal transient, microtubule-sparse state in G2 cells (Fig. S2 B) may be mimicked by microtubule disruption to allow accumulation of proteins at the NE.
respectively. Ponceau S and Coomassie staining of whole cell extract before nuclear isolation indicated that the samples had similar total protein levels (unpublished data). Substantially more Lis1 and dynein were present in nuclear extracts from cells expressing Ndel1. The pS231Ab labeled a band in this extract, but not in nuclear extract, from cells expressing Ndel15A. The pS231Ab band in the Ndel15A cytosolic extract may be endogenous Ndel1 that retained S231 phosphorylation in this short experiment (extracts were prepared 14 h after transfection). The biochemical and immunofluorescence data indicate that Lis1 and dynein recruitment to the NE is reduced if Ndel1 cannot become phosphorylated by cdk5 and cdk1. This is consistent with the finding that Ndel1 phosphorylation increases its binding to Lis1. However, in vitro phosphorylation by both kinases reduced Ndel1 binding to dynein. Coupled with the finding that Ndel15A blocks recruitment to the NE and is unable to induce PNEI, a model is emerging in which recruitment to the NE and induction of PNEI may involve sequential phosphorylation of different sites and/or cycles of phosphorylation/dephosphorylation.

S231 is phosphorylated in prophase neural stem cells

Although both cdk5 and cdk1 activity in COS-7 cells induced Ndel1 phosphorylation at S231, inhibition of endogenous cdk5

Dynein’s interaction with the NE is disrupted by overexpression of dynactin subunits (Salina et al., 2002). Dynactin colocalizes with Lis1 at the NE in nocodazole-treated cells (Fig. S2 D). Interestingly, overexpression of the p50 dynactin subunit prevented Lis1 redistribution to the NE in response to nocodazole (Fig. S2 E), indicating that Lis1 traffics to the NE with dynein. Lis1 colocalized at the NE with an ER marker (anti-protein disulfide isomerase; Fig. S2 C) but not with a Golgi or lysosomal marker (GM130; Lysotracker red; not depicted), so reorganization of the ER may contribute to accumulation of dynein machinery at the prophase NE.

Ndel1 phosphorylation influences the accumulation of Lis1 and dynein at the NE

When expressed in COS-7 cells, Ndel1 and, to a lesser extent, Ndel15A accumulated at the NE in response to nocodazole (Fig. 3 B). Comparing mean pixel intensities along lines ending at the NE confirmed this impression, as did comparison of pixel intensities around the NE and in the proximal cytoplasm (Fig. 3 D). Expression of Ndel15A substantially reduced dynein or Lis1 redistribution to the NE (Fig. 3 B–F). This was confirmed biochemically by analyzing Western blots of nuclear extracts from nocodazole-treated cells (Fig. 3 G). In this experiment, Ndel1 or Ndel15A was coexpressed with cdk5/cdk1 or dncdk5/dncdk1, respectively. Ponceau S and Coomassie staining of whole cell extract before nuclear isolation indicated that the samples had similar total protein levels (unpublished data). Substantially more Lis1 and dynein were present in nuclear extracts from cells expressing Ndel1. The pS231Ab labeled a band in this extract, but not in nuclear extract, from cells expressing Ndel15A. The pS231Ab band in the Ndel15A cytosolic extract may be endogenous Ndel1 that retained S231 phosphorylation in this short experiment (extracts were prepared 14 h after transfection). The biochemical and immunofluorescence data indicate that Lis1 and dynein recruitment to the NE is reduced if Ndel1 cannot become phosphorylated by cdk5 and cdk1. This is consistent with the finding that Ndel1 phosphorylation increases its binding to Lis1. However, in vitro phosphorylation by both kinases reduced Ndel1 binding to dynein. Coupled with the finding that Ndel15A blocks recruitment to the NE and is unable to induce PNEI, a model is emerging in which recruitment to the NE and induction of PNEI may involve sequential phosphorylation of different sites and/or cycles of phosphorylation/dephosphorylation.

S231 is phosphorylated in prophase neural stem cells

Although both cdk5 and cdk1 activity in COS-7 cells induced Ndel1 phosphorylation at S231, inhibition of endogenous cdk5
p231 labeled the NE in prophase cells and in nocodazole-treated COS-7 cells (Fig. 4, B and C). Cdk5 function is critical during brain development, so we examined whether pS231Ab staining was present in neural stem cells in the developing VZ. Parasagittal sections of embryonic day 13.5 mouse brains were processed for pS231Ab immunofluorescence (Fig. 4 D). Mitotic cells identified by Hoechst staining and proximity to the ventricular surface were labeled by pS231Ab, suggesting that the timing of S231 phosphorylation is coordinated with mitotic entry. Some pS231Ab-labeled cells appeared to be in prophase based on a lamin-associated protein 2 (LAP2) pattern, so phosphorylation by cdk5 at S231 can occur at the appropriate time to contribute to PNEI and NEBD (Fig. 4 E).

Reduced PNEI and delayed NEBD in the VZ of developing Lis1+/− mice

We were interested in whether Lis1 deficiency had an impact on PNEI or the timing of NEBD in the mouse VZ. To examine this, parasagittal sections of embryonic day 13.5 Lis1+/− mouse brains were immunostained for histone H3, which labels all stages of mitosis, and LAP2, which labels the NE (Fig. 5, A and B). As observed by others, mitotic cells were mispositioned in the Lis1+/− brains (Gambello et al., 2003). All H3-positive cells were analyzed in multiple sections from three animals of each genotype to determine whether there were differences in mitotic stage or in the number of prophase cells with invaginations. Mitotic cells were easily identified by H3 staining, and mitotic stage was easily determined by LAP2 staining and chromatin arrangement (Fig. 5 C). The percentage of H3-positive cells with intact NEs was significantly higher in Lis1+/− animals relative to wild-type littermates (prophase; Fig. 5 E). Of these, the percentage with clear PNEI was reduced in the mutant animals (Fig. 5 D), as was the percentage of prometaphase cells (Fig. 5 E), raising the possibility that NEBD is delayed in response to Lis1 deficiency. Upon examination of Lis1 immunofluorescence in the VZ, we found that prophase cells were more intensely labeled than surrounding cells, so Lis1 is present at the appropriate time to regulate dynein-dependent PNEI in the developing brain (Fig. 5 F).

The simplest explanation of this Lis1+/− data is that Lis1 deficiency causes an NEBD delay in the VZ. Beaudouin et al. (2002) estimated that an NEBD delay was caused by microtubule disruption in living normal rat kidney (NRK) cells, which normally complete NEBD 10 min after the appearance of invaginations. After exposure to 1 μM nocodazole, the same process required 15 min. This represents a 5-min delay or a 50% increase in the duration of NEBD, which could have serious consequences in vivo. Our data do not allow us to estimate the duration of the delay caused by Lis1 deficiency. Upon examination of Lis1 immunofluorescence in the VZ, we found that prophase cells were more intensely labeled than surrounding cells, so Lis1 is present at the appropriate time to regulate dynein-dependent PNEI in the developing brain (Fig. 5 F).

(by dnecd5) blocked cdk1-stimulated S231 phosphorylation, whereas inhibition of endogenous cdk1 (by dnecd1) did not prevent cdk5-stimulated S231 phosphorylation (Fig. 4 A). One possible explanation for this is that phosphorylation of distinct sites by cdk1 increases S231 phosphorylation by cdk5 in the cell.

Several experiments indicated that pS231A could be used to label S231 phosphorylated by cdk5 in fixed cells (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200803071/DC1).
brains were removed and fixed in 4% paraformaldehyde for 24–48 h.

were dissected. Tails were genotyped at the time of harvesting. Embryonic type males were anesthetized 13.5 d after observing plugs, and embryos fer to these animals as CA) and have been described previously (Hirotsune et al., 1998). We re-

T. Wynshaw-Boris (University of California, San Francisco, San Francisco, mice heterozygous for a null Pafah1b1-neo allele were provided by

Antibodies

Lis1 and Ndel1 rabbit polyclonal antibodies have been extensively character-

Materials and methods

Cell lines

NKR and green monkey kidney cells (COS-7) were used as indicated. All cell lines were maintained in DME supplemented with 10% fetal calf se-

Mouse brain sections

Pafah1b1-neo mice heterozygous for a null Lis1 allele were provided by

Fixed brains were dehydrated, embedded in paraffin, and sectioned into 8-μm-thick parasagittal sections.

Microscopy and immunofluorescence

COS-7 and NRK cells were plated onto 12-mm glass coverslips in 24-well plates. Coverslips were mounted on glass slides using Prolong Gold Anti-
fade (Invitrogen). For Lis1, p150glued, and HA immunofluorescence, cells were fixed in 3% paraformaldehyde followed by permeabilization with

Microscopy and immunofluorescence

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fade (Invitrogen). For Lis1, p150glued, and HA immunofluorescence, cells were fixed in 3% paraformaldehyde followed by permeabilization with

Mouse brains

Sections were stained on glass slides in a humidified chamber. Sections were dewaxed and permeabilized with a 100% ice-cold methanol or

Antibodies

Lis1 and Nde11 rabbit polyclonal antibodies have been extensively character-

or neurons. BP cells divide at the basal surface of the VZ (away from the ventricle) and are more restricted in their potential, primarily giving rise to neurons. There is evidence that G2 is of longer duration in BP cells, which are more likely to produce neurons, than in NE or RG cells (Calegari et al., 2005). Interestingly, Lis1 loss causes increased neurogenesis at the expense of the stem cell pool (Yingling et al., 2008). Although this has been correlated with altered cleavage plane orientation, our data raise the intriguing possibility that the defect originates even earlier in response to delayed NEBD, which could drive cells toward the BP fate and division within the sub-VZ. Our newly established link between Ndel1 phosphorylation and PNEI could allow precise control over the timing of NEBD by coordinated stimulation of cdk1 and cdk5 in response to intrinsic and extrinsic cues.
Phosphoepitope-specific antibodies in the flowthrough were purified on a phosphopeptide column. The cdk5 mouse monoclonal antibody (DC17) was provided by L.-H. Tsai (Massachusetts Institute of Technology, Cambridge, MA). The following antibodies were used (Santa Cruz Biotechnology, Inc.): DCC (74.1 mouse monoclonal), pS/T (N19 rabbit polyclonal), histidine tag (Histag, mouse monoclonal), myc (9E10 mouse monoclonal), and HA (rabbit polyclonal and mouse monoclonal). The histone H3 antibody was purchased from Millipore. Mouse monoclonal antibodies against pS0, pS150, and p21 were purchased from BD Biosciences. The anti-r-tubulin antibody and the anti-PDI mouse monoclonal antibodies were purchased from Sigma-Aldrich and Assay Designs, respectively.

Visualizing Ndel1-GFP in living cells
COS-7 cells were synchronized in G0 by growing at confluence for 3 d, replated at low density on 25-mm glass coverslips, and transfected with an expression construct designed to express full-length Ndel1 with an N-terminal EGFP tag. Control cultures were transfected with cEGFP-only constructs. The next day, coverslips were transferred into fresh medium containing 25 mM Hepes, pH 7.4, in a water-heated custom-built microscope stage warmed to 37°C. Cells expressing a relatively low level of EGFP were selected for imaging using an inverted microscope (TE2000; Nikon) and image acquisition software (DeltaVision; Applied Precision, LLC) coupled to an air-cooled charged-device camera. Fluorescent images were acquired every 2 min for 90 min using a Plan-100x/1.6 oil immersion objective.

Constructs and transfections
Complementary hairpin sequences for Ndel1 (276–294 bp; GCAGGCTC-CAGTGTIAAGA), DYNCH11 (9,753–9,771 bp; GAAGGTCATGAGCATCACGCAAGA, and Lis1 (1,062–1,080 bp; GAGTTGTGGCTGATGACAAG) were synthesized and cloned into pSilencer under control of the U6 promoter (version 2.0; Ambion; Shu et al., 2004). To generate HA-Lis1 and HA-NLIS1, full-length murine, Lis1, and a Lis1 fragment encoding amino acids 1–87 were subcloned into a pCruzHA vector (Santa Cruz Biotechnology, Inc.). Myc-tagged Ndel1 constructs (Ndel1, Ndel1123, and Ndel15A), untagged Ndel1 point mutants (S231A, S242A, and T245A), Ndel1-GFP, cdk5, pS25, and dndc5 constructs have been described previously (Smith et al., 2005). Donor-RABP2s A33R mutation (a putative ATP site) and inhibits endogenous kinase activity by sequestering cdk5 activity (van den Heuvel and Harlow, 1993), was also obtained from Addgene. The dncdk5 and dncdk1 constructs are well characterized and have been used in hundreds of published papers from many laboratories. A pS0 construct was provided by T.A. Schroer (The Johns Hopkins University, Baltimore, MD) and has been described previously (Quinney et al., 1999). Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s directions. Transfection efficiencies were generally 90%.

PNL analysis in COS-7 cells
Randomly cycling cells were transfected with the indicated constructs for the indicated times. Digital images of cell phases were obtained from fixed cultures and analyzed for PNEI. The presence or absence of nuclear invaginations was readily observed after Hoechst staining. A nucleus was considered PNEI positive if invaginations were deeper than 2 μm. Kidney-shaped nuclei without clear invaginations were not considered PNEIpositive. Control cultures were transfected with EGFP-only constructs. For co-IP with Ndel1, equal protein loading as determined by Western blotting.

Immunoprecipitation
For co-IP with DIC, equimolar Lis1 and histidine-tagged Ndel1 were incubated with and without dynein in BrB80 buffer (80 mM Pipes, 1 mM EGTA, and 1 mM MgCl2) at 37°C for 10 min. Proteins were exposed to DIC mouse monoclonal antibody, 74.1, and conjugated to agarose beads (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C. Precipitated proteins were analyzed by Western blotting. For co-IP with Lis1, equimolar from Millipged Ndel1 and untagged Lis1 were incubated with or without the His-tag antibody for 1 h at 4°C. Protein A-Sepharose was added to each sample for 1 h at 4°C. After washing beads, precipitated proteins were analyzed by Western blotting.

Nocodazole treatment
Cells were exposed to 10 μM nocodazole (EMD) for 3–5 h and processed as described in the Microscopy and immunofluorescence section. Co-IP was performed with anti-His tag antibody.

Preparation of crude nuclear fractions
Transfected COS-7 cells were exposed to 10 μM nocodazole for 3 h, incubated in 20 vol of cold hypotonic buffer (10 mM Hepes, pH 7.4, 2 mM MgCl2, 25 mM KO, 1 mM DTT, and 1 mM PMSF) and incubated on ice for 10 min. The nuclei were then pelleted by centrifugation at 1,000 g for 10 min. 50% of the nuclear fraction from each set of transfected cultures was loaded onto an SDS-PAGE gel and immunoblotted to estimate the ratio of soluble to nuclear pools of endogenous DIC and Lis1 and exogenous Ndel1. Equal protein loading was confirmed by Ponceau S staining of the membrane and Coomassie blue staining of samples loaded on another gel.

Immunofluorescence intensity analysis
One set of COS-7 cell cultures was transfected with myc-tagged Ndel1, and a second set was transfected with myc-tagged Ndel15A. After 12 h, cells were exposed to nocodazole for 5 h and fixed and processed for Myc, Lis1, and DIC immunofluorescence. Multiple same-exposure three-channel digital images were acquired for each set of cultures (FITC for Lis1 and dynactin, Texas red for Myc, and DAPI to label nuclei). Each image contained 5–10 transfected cells. Differences in fluorescence intensity at the NE were measured in two ways. (1) Pixel intensities were determined along 3-μm lines that ended within the NE region. The first pixel was set to an intensity of 10, and the remaining pixels were normalized accordingly. The mean intensities were calculated for 50 lines. (2) The mean of Myc pixel intensities of 10–30 points in the cytoplasm far from the NE was compared with mean pixel intensities of 10–30 points in the cytoplasm near the NE. Pixel intensities were obtained using ImageJ software (National Institutes of Health).

Analysis of S231 phosphorylation by Western blotting
Transfected cells were lysed in 150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8. The protein concentrations were determined using protein assay concentrate (Bio-Rad Laboratories), and equal amounts of proteins were separated on polyacrylamide SDS gels, transferred to polyvinylidene difluoride membranes, and immunoblotted with pS231A and other antibodies according to standard protocols. The blots were exposed to autoradiography film for a sufficient time to observe and minimize the saturation of bands.

Online supplemental material
Fig. S1 shows that Lis1 and Ndel1 accumulate at the prophase NE, where they colocalize with dynein and dynactin. Fig. S2 shows that dynactin integrity is important for Lis1 redistribution to the NE in response to nocodazole. Fig. S3 shows characterization of pS231A, a phosphospecific Ndel1 antibody. Video 1 shows Ndel1-GFP accumulating at the NE. Video 2 shows that GFP does not accumulate at the NE. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200803071/DC1.

Lis1+/− mice were provided by A. Wynshaw-Boris, and shRNA constructs were provided by L.-H. Tsai.
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