Severe NDE1-mediated microcephaly results from neural progenitor cell cycle arrests at multiple specific stages

David J. Doobin¹,* Shahrnaz Kemal¹,* Tiago J. Dantas¹,* & Richard B. Vallee¹

Microcephaly is a cortical malformation disorder characterized by an abnormally small brain. Recent studies have revealed severe cases of microcephaly resulting from human mutations in the NDE1 gene, which is involved in the regulation of cytoplasmic dynein. Here using in utero electroporation of NDE1 short hairpin RNA (shRNA) in embryonic rat brains, we observe cell cycle arrest of proliferating neural progenitors at three distinct stages: during apical interkinetic nuclear migration, at the G2-to-M transition and in regulation of primary cilia at the G1-to-S transition. RNAi against the NDE1 paralogue NDEL1 has no such effects. However, NDEL1 overexpression can functionally compensate for NDE1, except at the G2-to-M transition, revealing a unique NDE1 role. In contrast, NDE1 and NDEL1 RNAi have comparable effects on postmitotic neuronal migration. These results reveal that the severity of NDE1-associated microcephaly results not from defects in mitosis, but rather the inability of neural progenitors to ever reach this stage.

¹Department of Pathology and Cell Biology, Columbia University, New York, New York 10032, USA. * These authors contributed equally to this work. Correspondence and requests for materials should be addressed to R.B.V. (email: rv2025@cumc.columbia.edu).
Autosomal-recessive primary microcephaly is a severe developmental condition characterized by small brain size and a substantial reduction in neuronal number, with the cerebral cortex most prominently affected. Laminar organization is often normal, though microcephaly can occasionally be accompanied by lissencephaly or other organization defects. A number of microcephaly genes have been identified, including ASPM, microcephalin/BRIT1, CDK5RAP2/Cep215, CENP/CPAP, SIL/STIL, WDR62, and NDE1. Several of these genes are associated with centrosome and/or mitotic function, suggesting that errors in neural progenitor cell proliferation contribute to disease pathology.

NDE1 was an early candidate gene for microcephaly as judged from mouse studies, and has subsequently been implicated in a particularly severe form of microcephaly and microsillencephaly in human patients. NDE1 and its paralogue, NDEL1, exhibit clear homology to Aspergillus NudE (Nuclear Distribution E) and function along with LIS1 in cytoplasmic dynein regulation. The NDE1 null mouse was reported to exhibit ectopic mitotic divisions accompanied by altered mitotic spindle orientation. Roles for NDE1 and NDEL1 in mitosis have been borne out by analysis of non-neuronal cells in vitro. However, human NDE1-associated microcephaly is much more severe than the forms of the disorder involving mitotic spindle assembly genes, such as ASPM and WDR62. This observation suggests that NDE1 might be involved in more than one aspect of neural progenitor proliferation.

In addition, patients with NDE1 mutations often exhibit microcephaly with lissencephalic features, suggesting potential roles for NDE1 during subsequent neuronal migration as well.

Mammalian neocortical development begins with the expansion of neuroepithelial cells within the neural tube, followed by formation of the layered neocortex. The apical-most region, which is adjacent to lateral ventricle and defined as the ventricular zone (VZ), is populated by the soma of radial glia progenitor (RGP) cells. These serve as stem cells, responsible for the production of all excitatory cortical neurons, most glial cells and adult stem cells. The RGP cells are highly elongated, with their apical and basal processes spanning the entire thickness of the developing neocortex. A hallmark of RGP cell behaviour is the cell cycle-linked oscillatory movement of the nucleus of RGP cells, termed interkinetic nuclear migration (INM). RGP mitosis occurs exclusively at the ventricle. The RGP nucleus then migrates basally during G1, progresses through S-phase in the upper portion of the VZ and then migrates apically during G2 toward the ventricle where the next mitotic division occurs.

Mitosis can be symmetric, resulting in self-renewal of the neural progenitor pool, or asymmetric, leading to one neural progenitor and either a post-mitotic neuron or an intermediate progenitor, each of which migrate away from the ventricle.

Our own studies revealed that knockdown of genes involved in apical INM prevent RGP nuclei from reaching the ventricle and undergoing mitosis. We found that apical migration is mediated by cytoplasmic dynein anchored to the nuclear envelope during G2, which carries the nucleus along a polarized microtubule network emanating from the apically anchored centrosome. Dynein is recruited to the Nuclear envelope by two G2-specific nuclear pore-mediated mechanisms in a Cdk1-dependent manner. The first mechanism is activated during early G2 and involves BicD2 binding to the nucleoporin RanBP2, whereas the second mechanism is activated during late G2 and depends on CENP-F binding the nucleoporin Nup133. On the basis of the restriction of nuclear-envelope NDE1/NDEL1 signal to late-G2 in HeLa cells, we envision that either NDE1, NDEL1, or both, might contribute to late apical nuclear migration in the developing brain, though this possibility has not been tested.

Both NDE1 and NDEL1 mRNAs and protein have been detected throughout the developing neocortex (Allen Developing Mouse Brain Atlas, http://developingmouse.brain-map.org). However, NDE1 is more highly expressed in areas of high proliferation, such as the VZ, whereas the highest levels of NDEL1 mRNA were detected in the cortical plate (CP), where neuronal migration takes place (Allen Developing Mouse Brain Atlas, http://developingmouse.brain-map.org). NDEL1 has not been implicated in microcephaly, but rather in later aspects of neuronal migration in animal models. These observations are consistent either with the different expression patterns for NDE1 and NDEL1 in the developing brain, or with differences in their function. Some evidence for NDEL1-specific roles has been reported in cultured cells, but the relative contributions of the two genes to neocortical development remains to be explored.

The current study was initiated to determine which NDE1 roles contribute most significantly to the extreme form of microcephaly seen in human patients. In addition, we investigated potential roles for NDEL1 during neocortical development, and tested the degree to which these two proteins can functionally compensate for one another. Through use of RNA interference (RNAi) against each paralogue, we find that both NDE1 and NDEL1 contribute to postmitotic neuronal migration. However, NDE1 inhibition alone causes a complete block of apical INM, contributing to a large reduction in mitotic index with no evidence for ectopic mitoses. We also find a potent block of the G1-to-S transition associated with primary cilia over-elongation after the co-depletion of NDE1 and NDEL1. Interestingly, NDE1 expression rescues most, but not all, of NDE1 functions, identifying a NDE1-specific role, after INM completion at a novel premitotic state. These results identify multiple new potential causes for microcephaly, and provide a basis for understanding the distinct pathogenic potential of NDE1 versus NDEL1.

Results

Common and distinct effects of NDE1 and NDEL1 RNAi. To address the specific roles of NDE1 or NDEL1 during neocortical development, we used in utero electroporation of embryonic day 16 (E16) rat brain to introduce plasmids expressing GFP alone or in combination with NDE1 or NDEL1 shRNAs. Reduction of protein levels (Supplementary Fig. 1A and Supplementary Fig. 8) and mRNA levels (Supplementary Fig. 1B) were confirmed in rat C6 glioma cells. Relative to control, NDE1 knockdown caused a nearly complete loss of transfected neurons from reaching the CP, which instead accumulated in the lower intermediate zone (IZ) and subventricular zone (SVZ; Fig. 1a,b). NDEL1 knockdown also prevented cells from reaching the CP, though some cells were observed in the upper IZ, and there was again a large relative accumulation in the lower IZ and SVZ (Fig. 1a,b). The effects of dual NDE1/NDEL1 knockdown were similar to those for NDE1 and NDEL1 alone (Fig. 1a,b).

To test the role of NDE1 compared with NDEL1 in RGP cells, we performed live imaging of brain slice preparations. Control RGP s exhibited normal apical nuclear migration, followed by mitosis at the ventricular surface, and subsequent basal migration (Fig. 2a, Supplementary Movie 1). In marked contrast, NDE1 knockdown severely inhibited INM. Although the RGP cells could initiate apical INM, the nuclei failed to migrate beyond the final 10 μm, never reaching the ventricular surface and entering mitosis (Fig. 2a, Supplementary Movie 2). This behaviour is consistent with a block in late apical nuclear migration during INM, as we have observed with knockdown of Nup133 or CENP-F. NDEL1 knockdown, in contrast, had no discernable effect on INM (Fig. 2a, Supplementary Movie 3). Intriguingly, the double knockdown of both NDE1 and NDEL1 prevented the
majority of RGP nuclei from initiating apical INM (Fig. 2a; Supplementary Movie 4). By live imaging, there was no evidence for increased apoptotic events among any of the knockdowns (Supplementary Movies 1–4).

Consistent with these results, fixed tissue analysis revealed that NDE1 knockdown caused a significant accumulation of RGP soma at 10–15 μm from the ventricle (Fig. 2b–d). However, NDE1/NDEL1 double knockdown caused an accumulation of RGP soma further than 30 μm from the ventricle (Fig. 2b–d). Although there is a small but significant difference in the RGP somal distributions between control and NDEL1 knockdown (Fig. 2c), this is not reflected when the groups of somal distances are examined at prescribed distances (Fig. 2d).

Our results, together, indicate that knockdown of either NDE1 or NDE1 inhibits postmitotic neuronal migration. NDE1 knockdown—but not NDEL1 knockdown—blocks the latter stage of apical INM. Nonetheless, combined NDE1 and NDE1 knockdown arrests RGP nuclei much further away from the ventricular surface than is observed for NDE1 knockdown alone, suggesting an unexpected synergistic function of the knockdown at a potentially earlier stage of the cell cycle.

Cell cycle effects of NDE1 and NDEL1 RNAi. To address this issue, and to determine how changes to INM progression may affect RGP cell proliferation and neurogenesis, we examined the effects of NDE1 and NDEL1 RNAi on cell cycle progression. By preventing nuclear migration to the ventricular surface of the developing brain, we predicted that NDE1 knockdown, but not NDEL1 knockdown, would prevent RGP cells from dividing, identifying a potential NDE1-specific microcephaly mechanism. To test for effects on mitosis, we stained sections from electroporated brains for phosphohistone-H3 (PH3) to mark mitotic cells. We observed a severe reduction in the mitotic index in both the NDE1 knockdown and NDE1/NDEL1 double knockdown conditions (Fig. 2e), but no effect from NDEL1 knockdown alone (Fig. 2e). We note that all mitotic events in control electroporated RGP s occurred at the ventricular surface. Importantly, we observed no examples of mitotic divisions of RGP cells away from the ventricular surface under any of the knockdown conditions.

The severe effect of NDE1/NDEL1 double knockdown on the distance of arrested RGP soma from the ventricle (Fig. 2b–d) led us to test whether these cells arrest at an earlier cell cycle stage. Staining for the G1 marker CyclinD1 revealed modest or undetectable increases, respectively, in the number of positive RGP nuclei in the NDE1 or NDEL1 shRNA conditions (Fig. 3a). However, combined NDE1/NDEL1 knockdown doubled the number of CyclinD1-positive cells (Fig. 3a), consistent with a potent G1 accumulation. To test this possibility further, we stained sections for bromodeoxyuridine (BrdU) following a 30 min in vivo pulse, which revealed a steep drop in S-phase cells in NDE1 and NDE1/NDEL1 double knockdown conditions (Fig. 3b). Staining for the proliferation marker Ki67 revealed no difference in the fraction of positive RGP cells between conditions (Supplementary Fig. 3A,B). Together, these results indicate a synergistic effect of NDE1/NDEL1 double knockdown on preventing G1-to-S transition, with no evidence of cell cycle exit.

To confirm that these knockdowns are indeed inducing a cell cycle arrest, and not merely a delay in cell cycle progression, we performed a series of BrdU pulses of varying durations. Intraperitoneal injections of BrdU every 3 h, for up to 24 h, revealed that nearly 100% of RGP s electroporated with control vector or NDEL1 knockdown were BrdU positive (Fig. 3c). In striking contrast, we saw only a small increase in the fraction of BrdU incorporating cells over longer pulse lengths in the NDE1 knockdown and NDE1/NDEL1 double knockdown conditions (Fig. 3c). Furthermore, this small increase is likely to result from the necessity of beginning BrdU pulses earlier (only 48 h after electroporation), when the shRNA knockdown is just starting to take effect.

To further understand the mechanism responsible for the G1-to-S arrest seen in the NDE1/NDEL1 double knockdown condition, we began to consider cellular processes necessary for this transition. NDE1 has been reported to be involved in primary cilia resorption in cultured non-neuronal cells, with NDE1 inhibition resulting in ciliary over-elongation. Proper initiation of ciliary resorption was required, in turn, for G0- or G1-to-S-phase progression in proliferating ciliated cells. To test whether the G1 accumulation we see in NDE1/NDEL1 double knockdown might reflect a related mechanism, we co-electroporated control and shRNA-encoding plasmids with the ciliary marker Arl13B-mCherry, which reliably labels primary cilia, and examined primary cilia length. NDE1 knockdown caused moderate elongation of RGP cell primary cilia (Fig. 4a,b). In contrast, the combined NDE1/
Figure 2 | NDE1 knockdown blocks apical nuclear migration and potently reduces the mitotic index. (a) Live-imaging montage of GFP-expressing RGP cells at E19 with a control empty vector expressing GFP alone, or shRNAs to NDE1, NDEL1 or both genes along with a GFP reporter. Representative tracings from multiple RGP cells for each condition are shown at right. Montage panels are shown at 30 min intervals (Supplementary Movies 1–4). (b) Representative images of the VZ from the electroporated brains stained for the mitotic marker phosphohistone-H3 (PH3). Arrowheads mark soma of PH3+/GFP+ RGP cells. Dashed line represents the ventricular surface. (c,d) Measurements of the distance between the bottom of the nucleus and the ventricular surface, corresponding to the apical process length, across the various conditions. NDE1 knockdown shifted the apical process length distribution towards shorter distances, with a significant accumulation of RGPs with an apical process of 0–15 μm. NDE1/NDEL1 double knockdown, however, shifted the apical process length distribution to larger distances, with a significant accumulation of RGPs with an apical process of 30–45 μm. Each dot represents an individual apical process length measurement for one electroporated RGP cell. (e) Effect of RNAi on RGP cell mitotic index, measured as the number of electroporated RGP cells positive for PH3 divided by the total number of electroporated RGP cells. All mitotic figures of RGP cells were located at the ventricular surface, and NDE1 knockdown, as well as NDE1/NDEL1 double knockdown, caused a strong reduction in the mitotic index. Data presented as scatterplot in c with bars representing the median ± the interquartile range, and as mean ± s.e.m. in d and e. Kolmogorov-Smirnov test for non-parametric distributions used in c (*P < 0.05, n = 1,012–1,073 RGP cells). Unpaired t-test used in d and e (*P < 0.05, n = 3 embryonic brains from different mothers). Scale bar, 10 μm.
NDE1 knockdown caused a doubling of primary cilia length compared with controls (Fig. 4a,b). The NDE1/NDEL1 double knockdown also had the greatest effect on increasing primary cilia length in cultured retinal epithelia cells (Supplementary Fig. 5A,B), confirming the synergistic effect of NDE1 and NDEL1 knockdown in this process. To test for a causative role of the primary cilia in the G1-to-S arrest, we knocked down IFT172—a component of the anterograde IFT (intraflagellar transport) complex critical for primary cilia assembly—to inhibit ciliogenesis in RGP s (Supplementary Fig. 4B). Although IFT172 knockdown itself had little effect on the fraction of RGPs in G1, it rescued the G1-to-S block seen in NDE1/NDEL1 double knockdown, as judged by the decrease in the number of CyclinD1-positive RGPs (Fig. 4c,e). Furthermore, analysis of soma position revealed that the majority of RGPs displayed an INM phenotype similar to NDE1 knockdown alone, suggesting that RGPs now progressed through G1 into S-phase and were arrested during apical INM in G2 (Fig. 4c,d). These results further
support the necessity of either NDE1 or NDEL1 in initiating resorption of the primary cilia to allow for transition from G1 into S-phase.

Cross rescue reveals shared and unique functions. To define the relative functions of NDE1 and NDEL1 further, in addition to ensuring RNAi specificity, we performed rescue experiments using RNAi-resistant NDE1 and NDEL1 tagged with mCherry (Supplementary Fig. 1C). NDE1 or NDEL1 overexpression each largely rescued the neuronal migration defect seen in knockdown of either NDE1 or NDEL1 (Fig. 5a–c). Although NDEL1 overexpression only partially rescued the neuronal migration defect seen in NDE1 knockdown, these results suggest that each parologue alone is sufficient for postmitotic migration. Live-imaging of RGP cells further revealed that RNAi-resistant NDE1 fully rescued NDE1 RNAi-inhibited apical INM, mitosis, and subsequent basal migration (Fig. 6a, Supplementary Movies 5 and 6). In addition, fixed imaging results demonstrated full rescue of somal positioning (Fig. 6c) as well as mitotic index (Fig. 6d), both in NDE1 RNAi and NDE1/NDEL1 double knockdown cells (Fig. 6e–g; Supplementary Fig. 6). These results indicate that NDE1 alone is sufficient for INM and cell cycle progression in RGPs.

We also tested for functional complementation of the two paralogues by overexpressing NDEL1 in NDE1 knockdown RGPs. Live imaging revealed that NDEL1 overexpression fully rescues apical INM, but, surprisingly, the nuclei remained at the ventricle for hours without entering mitosis (Fig. 7a, Supplementary Movie 7). Fixed imaging confirmed the distribution of RGP soma was radically altered by NDEL1 overexpression in NDE1 knockdown RGPs, with nearly all RGP nuclei located adjacent to the ventricle (Fig. 7b,c). Surprisingly, a similar effect on somal distribution was seen with NDEL1 overexpression alone in wild-type rat brain (Fig. 7b,c).

NDE1 and NDEL1 are known to be critical during the progression of mitosis in non-neuronal cells, with inhibition of either paralogue resulting in prometaphase–metaphase arrest17–19. Therefore, it was surprising that RGP cells overexpressing NDE1 alone, or in combination with NDE1 shRNA, were nearly all negative for PH3 (Fig. 7b), with the
mitotic index reduced to a level comparable to that resulting from NDE1 knockdown (Fig. 7d). The nuclear envelope in these ventricular surface-arrested cells was also observed to remain intact, as determined by staining for lamin-associated protein 2 (LAP2; Supplementary Fig. 7B). DNA remained uncondensed, as judged by DAPI staining, and centrosomes remained unseparated at the RGP apical endfoot 25,44 (Supplementary Fig. 7A). Similar results were obtained when RNAi-resistant NDEL1 was used to rescue the NDE1/NDEL1 double knockdown (Fig. 7e–g).

Thus, NDEL1 overexpression can compensate for NDE1 during G1-to-S progression and apical INM, but excess NDEL1 induces a premitotic arrest in RGP cells even after the nucleus has reached the ventricular surface, preventing them from entering mitosis.

BicD2 expression reinforces a novel G2-to-M arrest. Our lab previously found that overexpression of BicD2, the limiting protein for dynein recruitment to the nuclear envelope during early G2 (ref. 25), can rescue RNAi knockdown for genes involved in either the early- or late-G2 dynein recruitment pathways. This resulted in restoration of apical INM as well as subsequent mitosis 25. Importantly, BicD2 is only targeted to the nuclear envelope during G2, providing an experimental means to restore nuclear envelope dynein recruitment exclusively during this cell cycle stage 31.

In the current study, overexpression of BicD2 along with NDE1 shRNA fully rescued apical INM, though, in this case, nuclei accumulated at the ventricle for prolonged periods of time without detectable signs of mitotic entry (Fig. 8a, Supplementary Movie 8). Consistent with these results, the majority of RGP soma in fixed brain sections were located at the ventricular surface (Fig. 8b,c) with intact nuclear envelope, uncondensed DNA and unseparated apical centrosomes (Supplementary Fig. 7A,B). Altogether, these results provide further evidence for the requirement of NDE1 during late-G2 dynein recruitment to the nuclear envelope, and reveal a new NDE1-specific role after completion of

Figure 5 | NDE1 or NDEL1 overexpression largely rescues the neuronal migration defects seen after knockdown of either protein. To test for RNAi rescue and functional complementation, embryonic rat brains were co-electroporated at E16 with shRNA to the various conditions with cDNA encoding RNAi-resistant proteins for self-rescue, and standard cDNA for cross-rescue. All the analyses were done at E20. Quantification of the amount of electroporated cells migrating into the cortical plate (CP) is shown at right. (a) Overexpression of NDE1 and NDEL1 produced no significant change in the amount of neurons migrating into the CP. (b) NDE1 knockdown with overexpression of RNAi-resistant NDE1 rescued neuronal migration into the CP. Overexpression of NDEL1 during NDE1 knockdown partially rescued neuronal migration into the CP. (c) Overexpression of RNAi-resistant NDEL1 or NDE1 with NDEL1 knockdown rescued the neuronal migration into the CP, even increasing the fraction of electroporated cells in the CP. Data are presented as mean ± s.e.m., and unpaired t-tests used for all comparisons, ∗P < 0.05, n = 3 embryonic brains from different mothers. Scale bar, 50 μm.
NDE1 KD + NDE1 OE

Figure 6 | RNAi-resistant NDE1 overexpression rescues all defects seen in radial glia progenitors across knockdown conditions. RNAi-resistant NDE1 was co-electroporated into embryonic rat brains with a GFP control empty vector or along with NDE1 shRNA or NDE1 and NDEL1 shRNAs at E16, and analysed at E20. (a) Restoration of apical interkinetic nuclear migration, mitosis and subsequent basal migration of progeny measured by live imaging. Arrowheads marks the radial glia progenitor (RGP) of interest. Montage panels are shown at 30 min intervals. Full movie can be found in Supplementary Material (see Supplementary Movie 5), as well as an additional movie that more clearly displays the two-daughter cell progeny (Supplementary Movie 6). (b) Representative images of RGPs stained for PH3 within the VZ in various specified co-expression conditions. Arrowheads mark mitotic electroporated RGPs. Dashed line indicates ventricular surface. (c) Soma position of RGPs with RNAi-resistant NDE1 overexpressed during NDE1 knockdown indicates that the somal positioning distribution is rescued. (d) Overexpression of RNAi-resistant NDE1 with NDE1 knockdown also rescues the mitotic index. (e) Representative image of NDE1/NDEL1 double knockdown with overexpression of RNAi-NDE1, stained for PH3. Arrowheads mark mitotic electroporated RGPs. Dashed line indicates the ventricular surface. (f,g) Overexpression of RNAi-resistant NDE1 with double NDE1/NDEL1 knockdown rescues the distribution of RGP nuclei in the VZ and restores the mitotic index of RGP cells. Data are presented as scatterplot in c and f with bars representing the median ± the interquartile range, and as mean ± s.e.m. in d and g. Kolmogorov–Smirnov test for non-parametric distributions used in c and f (*P<0.05, n = 428–474 RGP cells in c and n = 224–260 RGP cells in f). Unpaired t-test used in d and g (*P<0.05, n = 3 embryonic brains from different mothers). Scale bars, 10 µm. Also see Supplementary Fig. 6.

apical INM but before mitotic entry. Notably this NDE1 function cannot be rescued by overexpression of NDEL1 or BicD2. Intriguingly, when both NDE1 and NDEL1 were knocked down and BicD2 overexpressed, the vast majority of RGP nuclei remain arrested far from the ventricular surface in a CyclinD1 positive state (Fig. 8e–i), similar to the double NDE1/NDEL1 knockdown condition without BicD2 overexpression. The mitotic index was again severely reduced (Fig. 8e,g). As BicD2 overexpression acts solely during G2, these results further confirm that most RGP cells subjected to NDE1/NDEL1 double knockdown were indeed arrested during G1 rather than G2, as suggested earlier by the CyclinD1 and primary cilia results.
Discussion

NDE1 and NDEL1 are each involved in neocortical development, though genetic analysis in rodents and human patients have revealed distinct phenotypic and disease causing potential for the two paralogues. Direct analysis of the functional similarities and differences between the two genes remains very limited, making an understanding of the factors leading to these developmental abnormalities challenging. Although NDE1, in particular, has been implicated in a severe form of human microcephaly, the specific underlying mechanism is uncertain. We find here that NDE1 and NDEL1 can substitute for each other in diverse aspects of neurogenesis and neuronal migration. Our data, however, reveal a strongly predominant role for NDE1 in RGP cell behaviour. We identify three distinct
non-mitotic stages of RGP cell cycle progression that are susceptible to reduction in NDE1 or both NDE1/NDEL1, each of which should lead to a marked decrease in neurogenesis (Fig. 9). These observations should help resolve the mechanisms responsible for NDE1-associated microcephaly and other forms of the disease.

We find that NDE1 RNAi causes substantial numbers of RGP nuclei to become arrested during apical INM. This effect blocks

Figure 8 | BicD2 overexpression rescues apical nuclear migration but not entry into mitosis in radial glia progenitors depleted of NDE1. cDNA for full-length BicD2 was co-electroporated into embryonic rat brains with a GFP control empty vector or along with NDE1 shRNA or NDE1 and NDEL1 shRNAs at E16, and analysed at E20. (a) The overexpression of BicD2 in radial glia progenitors (RGPs) lacking NDE1 restores apical migration, though the soma accumulate at the ventricle for hours without any evidence of mitosis. Montage panels are shown at 30 min intervals. Full movie can be found in Supplementary Movie 8. (b) Representative images of BicD2 overexpression on both a wild-type and NDE1 knockdown background with staining for PH3. Arrowheads mark mitoses in electroporated cells. Dashed line indicates ventricular surface. (c) BicD2 overexpression did not alter the somal distribution of control RGP cells but caused the vast majority of NDE1 knockdown RGP soma to accumulate at the ventricular surface. (d) Despite the accumulation of RGP soma at the ventricle in NDE1 knockdown with BicD2 overexpression, the mitotic index remained reduced. (e) Representative image of RGP cells with BicD2 overexpression along with double NDE1/NDEL1 knockdown, stained for PH3. Dashed line indicates ventricle. (f,g) Overexpression of BicD2 with NDE1/NDEL1 double knockdown fails to rescue the somal distribution pattern or mitotic index of double NDE1/NDEL1 knockdown RGP cells. (h,i) The same ratio of RGP nuclei were positive for CyclinD1 whether or not BicD2 was overexpressed along with the double NDE1/NDEL1 knockdown, indicating the prominence of the G1-to-S block in the double knockdown, and the G2 specificity of the BicD2 rescue strategy. Arrowheads mark electroporated RGP nuclei positive for CyclinD1. Dashed line indicates ventricle surface. Data are presented as scatterplot in c and f with bars representing the median ± the interquartile range, and as mean ± s.e.m. in d,g and i. Kolmogorov–Smirnov test for non-parametric distributions used in c and f (*P < 0.05, n = 407–591 RGP cells in c and n = 421–487 RGP cells in f). Unpaired t-test used in d,g and i (*P < 0.05, n = 3 embryonic brains from different mothers). Scale bars, 10 μm. Also see Supplementary Fig. 7.
the RGP soma from reaching the ventricular surface, preventing these cells from ever entering mitosis\textsuperscript{25}. The distance from the ventricular surface at which the NDE1 knockdown nuclei arrest is similar to that seen for CENP-F and Nup133 knockdown, the two upstream factors in the late-G2 pathway for recruitment of cytoplasmic dynein to the nuclear envelope\textsuperscript{25,31,34}. Though definitive G2-immunohistochemical markers are lacking, our live imaging results strongly support G2 arrest, and a failure to enter mitosis at the ventricular surface. Analysis of a NDE1 null mouse, however, revealed ectopic mitotic figures\textsuperscript{6}, which we do not see in either NDE1, NDEL1, or combined knockdown RGP cells. Conceivably, the residual mitoses in the NDE1 null mouse could be associated with intermediate progenitor cell locations within the SVZ, or result from compensatory upregulation of NDE1 in the NDE1 null mouse.

In examining functional redundancy between NDE1 and NDEL1, we uncovered another premitotic NDE1-specific role during cell cycle progression. According to our data, NDE1 is required to act following completion of apical INM to avoid a block in G2-to-M transition. This arrest occurs before histone-H3 phosphorylation, nuclear envelope breakdown, DNA condensation, centrosome separation and centrosome release from the apical endfoot. Importantly, NDEL1 was unable to compensate for NDE1 knockdown at this stage. In fact, overexpression of NDEL1 induced a similar phenotype, suggesting a dominant-negative effect, perhaps involving formation of heterodimers with NDE1 (ref. 46). By utilizing BicD2 overexpression, we were also able to force NDE1 knockdown RGP cells to complete INM, yet these cells remained unable to enter mitosis. This stands in contrast to BicD2 rescue of CENP-F knockdown in RGP cells, which were able to complete apical INM and enter mitosis\textsuperscript{25}, and suggests an as-yet unidentified, spatially sequestered apical signal responsible for RGP mitotic entry. There has been one prior report of a NDE1 mutation leading to a partial increase in the G2 population of non-neuronal cells\textsuperscript{2}. How these observations may relate to ours is uncertain. Nonetheless, our data point to a NDE1-specific role in the G2-to-M transition, separate from its role in the late-G2 pathway of recruiting dynein to the nuclear envelope and identify an underlying difference between the two paralogues.

Primary cilia have been implicated in cell cycle progression in a number of cell lines and developing tissue\textsuperscript{38,40,47,48}. Even though the primary cilia membrane has been shown to be retained for most of the RGP cell cycle\textsuperscript{23}, previous reports have provided evidence that resorption of primary cilia needs to be initiated for ciliated cells to transition into S-phase\textsuperscript{38,40}. NDE1, in particular, has been implicated in this process in ciliated non-neuronal cell lines and during development of the zebrafish head\textsuperscript{38,49}. A similar block in the G1-to-S transition in RGP cells was observed in our experiments, accompanied by the overelongation of primary cilia. An additionally intriguing aspect of our observations was that this arrest was exacerbated by the double knockdown of NDE1/NDEL1, unlike apical INM inhibition, which was readily apparent when only NDE1 was knocked down. This enhanced G1/S block with double knockdown suggests that each paralogue alone can contribute to initiating ciliary resorption in RGP, and rescue of this phenotype by inhibiting ciliogenesis indicates that dysregulation at the primary cilia is causal of the G1/S block in the NDE1/ NDEL1 double knockdown in RGP cells.

The precise function of primary cilia in radial glia remains unresolved. Knockout mice for KIF3A or IFT88, proteins integral for cilia formation, have largely normal brain development\textsuperscript{50,51}, and we detected no difference in CyclinD1 labelling or somal distribution in our IFT172 knockdown conditions alone. Nonetheless, lack of primary cilia is very different from dysregulation of the ciliary growth cycle and cilia overelongation, which may explain why development occurs largely normally without primary cilia, while aberrations in primary cilia length/resorption may block cell proliferation. This view is further supported by a report of a block to RGP cell cycle progression caused by knockdown of TcTex1, which also affects ciliary resorption\textsuperscript{40}. Nevertheless, it remains to be resolved whether NDE1 and NDEL1 are playing a role in recruiting specific regulators of the primary cilia, whether they facilitate retrograde intraflagellar transport, or whether they play an entirely separate role in primary cilia regulation/signalling.

Microcephaly arises from impaired proliferation of the progenitor cells, resulting either from defects in S-phase, or more commonly, in mitotic progression\textsuperscript{1}. The current study, however, reveals three alternative mechanisms associated with a severe microcephaly-causing gene, NDE1, and is the first to link aberrations in apical INM to human disorders of corticogenesis. Relative to rodents, development of the human neocortex relies on an expanded outer subventricular zone containing many additional RGP\textsuperscript{52–54}. All of these, however, must derive from apical RGP in the ventricular zone that must undergo INM to successfully proliferate. Both NDE1 and NDEL1 are known to serve critical functions during mitosis, in particular during spindle alignment\textsuperscript{17} and chromosome segregation\textsuperscript{16,18,19}. Although NDE1 is likely required for these functions in RGP, our data show that acute NDE1 knockdown arrests RGP before mitotic entry. Our data, therefore, indicate that the inability to reach mitosis is an equally or more important cause of microcephaly than an arrest within mitosis itself.

Patients with NDE1 mutations exhibit altered cortical lamination, in addition to reduced brain size\textsuperscript{2,7–9}, which raises the question of whether defects in proliferation are solely accountable for the laminar deficits seen by magnetic resonance imaging. In addition to the three major cell cycle roles for NDE1 identified in RGP proliferation, NDE1 and NDEL1 were each shown to be crucial for the migration of the postmitotic neuronal precursors into the cortical plate. Thus, these changes in postmitotic migration likely explain the subsidiary cortical laminar dysplasia seen in many of the patients with NDE1 microcephaly\textsuperscript{2,7}.
In utero electroporation. Plasmids were transfected by intraventricular injection into the lateral ventricle of rat embryos. Under light anesthesia from ketamine (75–95 mg kg\(^{-1}\)), a 16-gauge needle was introduced into the cranium of rats at embryonic day 16 (E16) and electroporated. In brief, an incision (75–95 mg kg\(^{-1}\)) was made in the skin, and the skull was exposed. The embryos were anesthetized with ketamine (75–95 mg kg\(^{-1}\)) and xylazine (5 mg kg\(^{-1}\)). Upon proper anesthetic induction, a laparotomy at the site of the previous incision was performed and the uterus exposed. The embryos were excised from the uterus, the brains dissected out and placed overnight in 4% paraformaldehyde (PFA) fixative dissolved in PBS at 4°C. Following fixation, the brains were embedded in 4% agarose in the presence of PFA and sliced in 20 μm coronal sections. The sections were blocked in blocking solution containing PBS and 0.3% Triton X-100 supplemented with 3% normal donkey serum for 1 h. Primary antibodies were incubated overnight in blocking solution at 4°C, sections were washed 3× in PBS and secondary antibodies and blocking solution were incubated for 2 h at room temperature. The sections were mounted on slides using Aqua-Poly/Mount (Polysciences, Inc.) and imaged using a Nikon confocal microscope. The slices were then washed in 0.1 M sodium borate for 10 min and then PBS, before antibody incubation.

Cell cultures and western blotting. Rat C6 glioma cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and maintained at 37°C with 5% CO\(_2\). Transfection with siRNAs against NDE1 and NDEL1 was performed using a Lonza nucleofector kit V and an Amaxa Nuclefector according to the manufacturer’s instructions. The cells were collected 72 h later and sorted using fluorescence-activated cell sorting (FACS) to isolate GFP-positive cells. The collected cells were lysed on ice in RIPA buffer containing DTT and a protease inhibitor cocktail (Sigma). Purified cell lysates were loaded on a polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked in PBS with 5% non-fat dry milk for 1 h and incubated with primary antibodies against NDE1 (Abnova, H00054820-M01, 1:1,000 dilution), NDEL1 (81569, Sigma) and β-tubulin (Sigma: 1:2,000 dilution) for 2 h at room temperature, and incubated with secondary LI-COR antibodies followed by incubation with fluorscense-conjugated secondary antibodies.

RNA isolation and quantitative RT–PCR analysis. C6 cells were transfected with NDE1/NDEL1 siRNA plasmids and rescue constructs using an Am MAX Nucleofector as described for western blot. Seventy-two hours following transfection, GFP fluorescent cells were sorted using a FACS Aria Cell Sorter (BD). Positive cell lysates, mRNA to cDNA synthesis and quantitative PCR were performed using the Power SYBR Green Cells-to-Ct Kit (Applied Biosystems). cDNA samples were amplified using quantitative PCR using an ABI 7900 HT quantitative PCR machine. Primers were designed to have TMs of about 60 degrees and to generate amplicons of 70 to 200 base pairs, separated by at least one intron. Three replicates were done for each condition per experiment and the experiments were performed in triplicates. Relative RNA levels were assessed by the comparative cycle (C\(_o\)) method and values were normalized against β-actin and Gapdh expression levels. The primers used in this study were: β-actin FW: 5’-CCCGCGGTGATACACAGTTCTTCT-3’; β-actin RV: 5’-GCATCCATCCTGGAACACT-3’; Gapdh FW: 5’-CAACCTTCTCCATTGTTCAAGC-3’; Gapdh RV: 5’-GGACCCAGCTCTTCTGATG-3’. Antibodies. Antibodies used in this study were mouse monoclonal against phosphohistone H3 (Abcam, ab14955, 1:500 dilution), phospho-vimentin (Millipore, 05-774, 1:250 dilution), centrin3 (Abnova, H80001070-M01, 1:250 dilution), gluamylinated tubulin (Addiagon, GT335, 1:250 dilution), rabbit polyclonal against CyclinD1 (Thermo Scientific, RM-9104, 1:250 dilution), Goat (Santa Cruz, sc-30135, batch #2615, 1:250 dilution), Lamin-associated protein 2 (Santa Cruz, sc-28541, 1:500 dilution), thrbl (Abcam, ab31940, 1:500 dilution), Axl3 (ProteinTech, 17711-1-AP, 1:250 dilution), and rat monoclonal against Sema4D (Abcam, ab23036, 1:200 dilution). Donkey fluorophore-conjugated secondary antibodies (Jackson Labs, 1:500 dilution) were used together with DAPI (4′,6-diamidino-2-phenylindole, Thermo Scientific, D62248, 1:1,000 dilution), NDEL1 (Abnova, H00054820-M01, 1:1,000 dilution) and β-tubulin (Sigma; 1:2,000) were used for immunoblotting. To develop in a LI-COR system, fluorochrome secondary antibodies were acquired from Invitrogen (dilution 1:1,000) and Rockland (dilution 1:10,000) to use for western blotting.
sections were imaged using a × 60 1.42 N.A. oil objective or a × 10 0.40 N.A. air objective. All drawings were composed using Inkscape open source software. All images were analyzed using ImageJ software (NIH, Bethesda, MD, USA). Distance and primary cilia length measurements were also performed using this software. Live-imaging movies were constructed on ImageJ at 12 fps., with each frame representing a 10 min progression in real time. Tracings were made by measuring the distance from the ventricular surface to the bottom of the soma—the same as was used for fixed imaging analysis—every 20 min, corresponding to every two frames in the movies. All statistical analysis was performed using Prism (GraphPad Software, La Jolla, CA, USA). For fixed analysis of distances of RGP soma from the ventricle (apical process length), distributions were represented as scatterplots with bars showing the median and interquartile range. This was done to provide a more comprehensive representation of the data, since all conditions failed the D’Agostino & Pearson omnibus normality test. Due to the nonparametric nature of these distributions, Kolmogorov-Smirnov tests were used for all comparisons of experimental conditions to the corresponding control condition. The ROUT method was used to identify any outliers, none of which were found across the conditions. Measurements of the distance between RGP nuclei and ventricular surface were made from three different embryonic rat brains, each from a different mother. For mitotic index, CycD1 ratio, BrdU ratio, K67 ratio and the fraction of electroporated cells reaching the cortical plate, all data are plotted as the mean with standard error of mean. Measurements were all made from at least three different embryonic rat brains across at least two separate operations per condition. Animals from all successful operations were included in the analysis. For the index measurements, at least 100 RGP cells were counted from at least three slices per brain. For results with Gaussian distributions, comparisons were made first using analysis of variance tests to determine whether there was a significant difference, followed by unpaired t-tests to compare individual experimental conditions to the appropriate control condition. For all non-Gaussian distributions, a Kruskal–Wallis test was used first, followed by Kolmogorov–Smirnov tests or Dunn’s multiple comparison tests for direct comparison of individual conditions. For all analyses, significance was accepted at the level of P < 0.05.

Data availability. The authors declare that all relevant data supporting the findings of this study are either provided in the Article and Supplementary files or available from the authors on request.

References
1. Barkovich, A. J., Guerini, R., Kuzniecky, R. L., Jackson, G. D. & Dobyns, W. B. A developmental and genetic classification for malformations of cortical development: update 2012. Brain 135, 1348–1369 (2012).
2. Alkuraya, F. S. et al. Human mutations in NDE1 cause extreme microcephaly with liencephaly. Am. J. Hum. Genet. 88, 536–547 (2011).
3. Thornton, G. K. & Woods, C. G. Primary microcephaly: do all roads lead to microlyssencephaly? Nat. Genet. 15, 583–594 (2007).
4. Noctor, S. C., Martinez-Cerdeño, V., Ilic, L. & Kriegstein, A. R. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat. Neurosci. 7, 136–144 (2004).
5. Gao, P. et al. Deterministic progenitor behavior and unitary production of neurons in the neocortex. Cell 159, 775–788 (2014).
6. Feng, Y. & Walsh, C. A. Mitotic spindle regulation by Nde1 controls cerebral migration: update 2012. Brain 135, 1348–1369 (2012).
7. Liang, Y. et al. Nudel modulates kinetochore association and function of cytoplasmic dynein in M phase. Mol. Biol. Cell 18, 2656–2666 (2007).
8. Stehman, S. A., Chen, Y., McKenney, R. J. & Vallee, R. B. Nudel and NudE are required for mitotic progression and are involved in dynein recruitment to kinetochores. J. Cell Biol. 178, 583–594 (2007).
9. Vergnolle, M. A. S. & Taylor, S. S. Cenp-F links kinetochores to Ndel1/Nde1/Lis1/dynein microtubule motor complexes. Curr. Biol. 17, 1173–1179 (2007).
10. Kriegstein, A. & Alvarez-Buylla, A. The glial nature of embryonic and adult neural stem cells. Annu. Rev. Neurosci. 32, 149–184 (2009).
11. Efimov, V. P. & Morris, N. R. The LIS1-related NUDF protein of Aspergillus a central component of the centrosome. J. Cell Biol. 190, 107–119 (2011).
12. McKenney, R. J., Vershinin, M., Kunwar, A., Vallee, R. B. & Gross, S. P. LIS1 and cytoplasmic dynein. Neuron 523–535 (2011).
13. Niethammer, M. Nudel and NudE as regulators of cytoplasmic dynein activity. EMBO J. 29, 107–119 (2009).
14. Bradshaw, N. J., Hennah, W. & Soares, D. C. NDE1 and NDEL1: twin neurodevelopmental proteins with similar ‘nature’ but different ‘nurture’. Biomol. Concepts 4, 447–464 (2013).
15. Kim, S. et al. Nde1-mediated inhibition of ciliogenesis affects cell cycle re-entry. Nat. Cell Biol. 13, 351–360 (2011).
16. Jackson, P. K. Do cilia put brakes on the cell cycle? Nat. Cell Biol. 4, 357–365 (2002).
17. Li, A. et al. Deterministic progenitor behavior and unitary production of neurons in the neocortex. Cell 159, 775–788 (2014).
18. Tsai, J.-W., Lian, W.-N., Kemal, S., Kriegstein, A. R. & Vallee, R. B. KIF1A inhibition immortalizes brain stem cells but blocks BDNF-mediated neuronal migration. Nat. Neurosci. 19, 253–262 (2016).
19. Splinter, D. et al. Bicaudal D2, dynein, and kinesin-1 associate with nuclear pore complexes and regulate centrosome and nuclear positioning during mitotic entry. PLoS Biol. 8, e1000350 (2010).
20. Bully, S. et al. A Nup133-dependent NPC-anchored network tethers centrosomes to the nuclear envelope in prophase. J. Cell Biol. 192, 855–871 (2011).
21. Feng, Y. et al. LIS1 regulates CNS laminination by interacting with mNudE, a central component of the centrosome. Neuron 28, 665–679 (2000).
22. Shmueli, A. et al. Nde1 palmitoylation: a new mean to regulate cytoplasmic dynein activity. EMBO J. 29, 107–119 (2009).
23. Bragg, S. K., Hennah, W. & Soares, D. C. NDE1 and NDEL1: twin neurodevelopmental proteins with similar ‘nature’ but different ‘nurture’. Biomol. Concepts 4, 447–464 (2013).
24. Kim, S. et al. Nde1-mediated inhibition of ciliogenesis affects cell cycle re-entry. Nat. Cell Biol. 13, 351–360 (2011).
25. Jackson, P. K. Do cilia put brakes on the cell cycle? Nat. Cell Biol. 13, 340–342 (2011).
26. Li, A. et al. Ciliary transition zone activation of phosphorylated Tctex-1 controls ciliary resorption, S-phase entry and fate of neural progenitors. Nat. Cell Biol. 13, 402–411 (2011).
27. Bangs, F. K., Schrode, N., Hadjantonakis, A.-K. & Anderson, K. V. Lineage specificity of primary cilia in the mouse embryo. Nat. Cell Biol. 17, 113–122 (2015).
28. Paridaen, J. T. M., Wilsch-Brauninger, M. & Huttner, W. B. Asymmetric inheritance of centrosome-associated primary cilium membrane directs ciliogenesis after cell division. Cell 155, 333–344 (2013).
29. Ezratty, E. J. et al. A role for the primary cilium in notch signaling and epidermal differentiation during skin development. Cell 145, 1129–1141 (2011).
30. Spear, P. C. & Erickson, C. A. Apical movement during interkinetic nuclear migration is a two-step process. Dev. Biol. 370, 33–41 (2012).
31. Youn, Y. H., Pramparo, T., Hirotsune, S. & Wynshaw-Boris, A. Distinct dose-dependent cortical neuronal migration and neurite extension defects in Lis1 and Nde1 mutant mice. J. Neurosci. 29, 15520–15530 (2009).
46. Bradshaw, N. J. et al. PKA phosphorylation of NDE1 is DISC1/PDE4 dependent and modulates its interaction with LIS1 and NDEL1. J. Neurosci. 31, 9043–9054 (2011).
47. Kim, S. & Tsiokas, L. Cilia and cell cycle re-entry: more than a coincidence. Cell Cycle 10, 2683–2690 (2011).
48. Hu, W. F. et al. Katanin p80 regulates human cortical development by limiting centriole and cilia number. Neuron 84, 1240–1257 (2014).
49. Maskey, D. et al. Cell cycle-dependent ubiquitylation and destruction of NDE1 by CDK5-FBW7 regulates ciliary length. EMBO J. 34, 2424–2440 (2015).
50. Insolea, R., Bazzi, H., Shao, W., Anderson, K. V. & Shi, S.-H. Cortical neurogenesis in the absence of centrioles. Nat. Neurosci. 17, 1528–1535 (2014).
51. Tong, C. K. et al. Primary cilia are required in a unique subpopulation of neural progenitors. Proc. Natl. Acad. Sci. USA 111, 12438–12443 (2014).
52. Hansen, D. V., Liu, J. H., Parker, P. R. L. & Kriegstein, A. R. Neurogenic radial glia in the outer subventricular zone of human neocortex. Nature 464, 554–561 (2010).
53. Fietz, S. A. et al. OSVZ progenitors of human and ferret neocortex are epithelial-like and expand by integrin signaling. Nat. Neurosci. 13, 690–699 (2010).
54. Dehay, C., Kennedy, H. & Kosik, K. S. The outer subventricular zone and primate-specific cortical complexification. Neuron 85, 683–694 (2015).
55. Baffet, A. D. et al. Cellular and subcellular imaging of motor protein-based behavior in embryonic rat brain. Methods Cell Biol. 131, 349–363 (2016).

Acknowledgements
We thank Drs Alexandre Baffet and Daniel Hu for the critical reading of the manuscript, the members of the Vallee lab, Drs Hynek Wichterle, Franck Polleux, Ellen Ezratty, Luis Oliveira and Gregg Gandersen for technical expertise and feedback, and Drs. Kathryn Anderson and Iain Cheeseman for providing reagents. This project was supported by NIH HD40182 to R.B.V., NINDS F30NS095577 to D.J.D. and an AHA/ASA 15POST2508068 postdoctoral fellowship to T.J.D.

Author contributions
Conceptualization was done by R.B.V., D.J.D, S.K. and T.J.D.; methodology was by D.J.D., S.K. with T.J.D. for primary cilia-related experiments and qRT–PCR; data acquisition was done by D.J.D. with T.J.D. for primary cilia-related experiments; writing—original draft by D.J.D. and S.K.; writing—review and editing by D.J.D., T.J.D., S.K. and R.B.V.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Doobin, D. J. et al. Severe NDE1-mediated microcephaly results from neural progenitor cell cycle arrests at multiple specific stages. Nat. Commun. 7:12551 doi: 10.1038/ncomms12551 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2016