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Ndel1 palmitoylation: a new mean to regulate cytoplasmic dynein activity

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Regulated activity of the retrograde molecular motor, cytoplasmic dynein, is crucial for multiple biological activities, and failure to regulate this activity can result in neuronal migration retardation or neuronal degeneration. The activity of dynein is controlled by the LIS1–Ndel1–Nde1 protein complex that participates in intracellular transport, mitosis, and neuronal migration. These biological processes are subject to tight multilevel modes of regulation. Palmitoylation is a reversible posttranslational lipid modification, which can dynamically regulate protein trafficking. We found that both Ndel1 and Nde1 undergo palmitoylation *in vivo* and in transfected cells by specific palmitoylation enzymes. Unpalmitoylated Ndel1 interacts better with dynein, whereas the interaction between Nde1 and cytoplasmic dynein is unaffected by palmitoylation. Furthermore, palmitoylated Ndel1 reduced cytoplasmic dynein activity as judged by Golgi distribution, VSVG and short microtubule trafficking, transport of endogenous Ndel1 and LIS1 from neurite tips to the cell body, retrograde trafficking of dynein puncta, and neuronal migration. Our findings indicate, to the best of our knowledge, for the first time that Ndel1 palmitoylation is a new mean for fine-tuning the activity of the retrograde motor cytoplasmic dynein.

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Introduction

Regulated activity of the retrograde molecular motor, cytoplasmic dynein, is crucial for multiple biological activities ranging from mitosis to long-range neuronal transport. For example, mice lacking cytoplasmic dynein heavy chain exhibit early embryonic lethality (Harada *et al*, 1998), whereas abnormal neuronal transport is a common theme underlying pathogenesis of neurodegenerative diseases (for reviews, see Bruijn *et al*, 2004; Chevalier-Larsen and Holzbaur, 2006; Reiner *et al*, 2006; Stokin and Goldstein, 2006).

Dynein, a large multisubunit complex, belongs to one of the two families of microtubule motor proteins (for reviews, see Goldstein and Yang, 2000; Vallee *et al*, 2004). All dyneins contain the heavy chain, for ATPase and motor activities, and accessory subunits including light intermediate and light chains. The activity of dynein is regulated at multiple levels. For example, specific combinations of dynein isoforms may contribute to cargo specificity (Ha *et al*, 2008). Additional accessory proteins complexes, such as dynacin, allow dynein to bind a variety of cargoes, regulate dynein motor activity directly, and enhance dynein processivity (for reviews, see Schroer, 2004; Vallee *et al*, 2004). Finally, accumulating evidence suggest that the LIS1-containing protein complex is also involved in regulating cytoplasmic dynein motor activity. Deletions in the *LIS1* gene result in a severe human neuronal migration deficit known as lissencephaly (Reiner *et al*, 1993; Lo Nigro *et al*, 1997; Pilz *et al*, 1998). Protein dosage in this locus is crucial because both decreased and increased LIS1 protein levels affect brain development both in humans and in mice (Reiner *et al*, 1993; Hirotsune *et al*, 1998; Cahana *et al*, 2001; Bi *et al*, 2009).

LIS1 has been found to interact with several subunits of dynein and dynactin (Faulkner *et al*, 2000; Sasaki *et al*, 2000; Smith *et al*, 2000; Tai *et al*, 2002), as well as with the microtubule plus end-binding protein CLIP-170 (Coquelle *et al*, 2002), suggesting that it modulates dynein activity in more than one way. LIS1 interacts tightly with the evolutionary conserved NUDE proteins (Efimov and Morris, 2000). In mammals there are two NudE homologs: Nde1 and its related paralog Ndel1. Nde1 interacts with LIS1, several centrosomal proteins, and dynein light and intermediate chains (Feng *et al*, 2000; Feng and Walsh, 2004; Hirohashi *et al*, 2006a, b; Stehman *et al*, 2007). Ndel1 is found in complex with LIS1, and dynein heavy and intermediate chains (Sasaki *et al*, 2000; Niethammer *et al*, 2000a). LIS1, Ndel1, and Nde1 all participate in the dynein-mediated processes of intracellular transport (Liu *et al*, 2000; Sasaki *et al*, 2000; Smith *et al*, 2000; Niethammer *et al*, 2000b; Liang *et al*, 2004; Yamada *et al*, 2008), mitosis (Faulkner *et al*, 2000; Yan *et al*, 2003; Feng and Walsh, 2004; Tsai *et al*, 2005; Guo *et al*, 2006; Liang *et al*, 2007; Stehman *et al*, 2007; Vergnolle and Taylor, 2007), and neuronal migration (Hirotsune *et al*, 1998; Feng *et al*, 2000; Cahana *et al*, 2001; Feng and Walsh, 2004; Shu *et al*, 2004; Sasaki *et al*, 2005; Tsai *et al*, 2005, 2007; Grabham *et al*, 2007). These complex biological processes are subject to tight multilevel modes of regulation, in which reversible posttranslational modifications have an important role. For example, phosphorylation of Ndel1 is required for

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neuronal migration and function (Niethammer *et al*, 2000b; Toyo-oka *et al*, 2003; Taya *et al*, 2007), as well as for proper cell-cycle progression (Toyo-Oka *et al*, 2005; Mori *et al*, 2007).

In vitro studies have demonstrated that LIS1 can stimulate the ATPase activity of cytoplasmic dynein (Mesngon *et al*, 2006; Yamada *et al*, 2008). Furthermore, LIS1 suppressed the motility of cytoplasmic dynein on microtubules, whereas its interacting protein Ndel1 released the blocking effects of LIS1 (Yamada *et al*, 2008).

In vivo, injection of anti-LIS1 antibodies inhibited the anterograde transport of dynein to the tips of dorsal root ganglia neurons, thus suggesting that LIS1 may affect the activity of the anterograde motor kinesin in relation to dynein transport (Yamada *et al*, 2008).

Taking into consideration that reversible posttranslational modifications are likely to participate in the regulation of dynamic processes, we tested whether any of the LIS1–Ndel1–Nde1 complex proteins undergo palmitoylation. Palmitoylation is a reversible posttranslational modification, which has been shown to have an important role in the regulation of protein trafficking (Linder and Deschenes, 2007), and in the nervous system (Kang *et al*, 2008; for reviews, see Dunphy and Linder, 1998; Resh, 1999; El-Husseini Ael and Bredt, 2002; Washbourne, 2004). We found that both Ndel1 and Nde1 undergo palmitoylation on a conserved cysteine residue. We further identified the three palmitoylation enzymes DHHC2, DHHC3, and DHHC7, which are involved in this modification. Palmitoylation of Ndel1 reduced its interaction with cytoplasmic dynein resulting in reduced dynein activity measured by functional assays, including Golgi distribution, microtubule transport, ER-to-Golgi transport, transport to neurite tips, retrograde trafficking of dynein puncta in primary neurons, and aberrant pyramidal neuronal migration to the developing cortex. Our results suggest a new mode of regulating the activity of the molecular motor, cytoplasmic dynein.

Results

Nde1 and Ndel1 are palmitoylated proteins

We tested whether Ndel1/Nde1 or LIS1 undergo palmitoylation. *S*-palmitoylation is a reversible posttranslational modification, which involves a thioester-bound addition of the fatty acid palmitate to cysteine residues (for review, see Linder and Deschenes, 2007). In mammals there are 23 known palmitoylating enzymes called palmitoyl-acyl transferases (PAT) (Fukata *et al*, 2004) that share in common an Asp-His-His-Cys (DHHC)-cysteine rich domain that is essential for the catalytic activity. The 23 PATs (DHHC 1–23) were transfected to HEK293 cells and assayed (Fukata *et al*, 2006) for their capability to palmitoylate Ndel1 or Nde1 (Figure 1A and B, Supplementary Figure S1 is a longer exposure of 1B). This assay involves metabolic labelling of tritiated-palmitate to HEK293 cells transfected with the enzymes and the tested proteins. The results indicated that both proteins are palmitoylated, and the enzymes that are capable of palmitoylating Ndel1 and Nde1 are DHHC2, DHHC3, and DHHC7. Minor palmitoylation was noted with DHHC21. LIS1 was not palmitoylated using the same assay (data not shown). We next explored whether Ndel1 and Nde1 are palmitoylated *in vivo* in primary cortical neurons using a sensitive assay (Drisdel and Green, 2004; Drisdel *et al*, 2006). This assay involves an exchange of the endogenous palmitate-linked group with a biotin-labelled reagent. This reaction requires hydroxylamine-mediated cleavage of the palmitoyl-thioester bond, followed by a specific labelling with a sulfhydryl-reactive biotinylated reagent that is later identified by avidin-HRP (known as ABE, acyl-biotinyl exchange method). Our results indicate that both Ndel1 and Nde1 are palmitoylated *in vivo* in mouse cortical neurons (Figure 1C and D). In both cases, avidin–HRP labelled immunoprecipitated only Ndel1 (Figure 1C) or Nde1 (Figure 1D) when hydroxylamine hydrolysed the thioester as a neutral pH base, but not when it was not added. It has been previously demonstrated that a band shift of PSD95 can be noted after the addition of hydroxylamine (Fukata *et al*, 2004; Figure 1E). We noted a similar band shift of Ndel1 (Figure 1F), thus suggesting that a noticeable proportion of Ndel1 population is palmitoylated in CAD cells. Determination of the half-life of GFP–Ndel1 palmitoylation in an inducible DHHC7 HEK293 cell line was conducted using metabolic labelling of the cells with 17-octadecynoic acid (17-ODYA) overnight, followed by a chase with palmitic acid (Figure 1G and H). The half-life of Ndel1 palmitoylation was estimated to be 1.75 h ($R^2 = 0.985$). Ndel1 is a very stable protein and the protein half-life was estimated to be 24 h (Figure 1H and Supplementary data). In primary cortical neurons the half-life of Ndel1 palmitoylation was estimated to be 2.3 h, as determined by the relative reduction of the signal after incubation of the cortical cultures with the palmitate analogue 2-bromopalmitate (2-Br), which inhibits palmitoylation (Webb *et al*, 2000; Figure 1I and J).

Next, the cysteine residue(s) undergoing *S*-palmitoylation were identified. Four Ndel1 cysteine residues were individually mutated (C203S, C273S, C293S, and C302S), and the mutated proteins were assayed for their capability to undergo palmitoylation (Figure 2A). The mouse Nde1 protein contains only one conserved cysteine residue, and it was mutated as well (C273S) (Figure 2B). The palmitoylation of both Ndel1 and Nde1 C273S mutant proteins was statistically significantly reduced using either DHHC2, DHHC3, or DHHC7, suggesting that this conserved site is the major site of palmitoylation on both proteins (Figure 2C and D). An alternative explanation will be that this site is required for palmitoylation of other sites. We conclude that both Ndel1 and Nde1 can be palmitoylated *in vitro* and *in vivo* by DHHC2, 3, and 7, and that C273 is important for palmitoylation.

Palmitoylation of Ndel1 affects its interaction with cytoplasmic dynein

Palmitoylation may affect numerous processes, including protein–protein interactions. Our analysis depicted a single lipid modification that is not sufficient for membrane attachment (for review, see Resh, 2006). Indeed, Ndel1 palmitoylation did not grossly increase its relative proportion in the membrane as the relative proportion of wild type or C273S Ndel1 localization in the membrane or in the cytosol did not change after expression of the palmitoylation enzyme. Furthermore, the relative proportion of endogenous or transfected Ndel1 did not significantly change after expression of wild-type or mutated DHHC7 (Supplementary Figures S2 and S3). Nevertheless, we do not exclude the possibility of fine changes in the interaction of Ndel1 and some membranal subpopulations. Ndel1 is known to complex with LIS1, cytoplasmic dynein, and neurofilaments. The main palmitoy-

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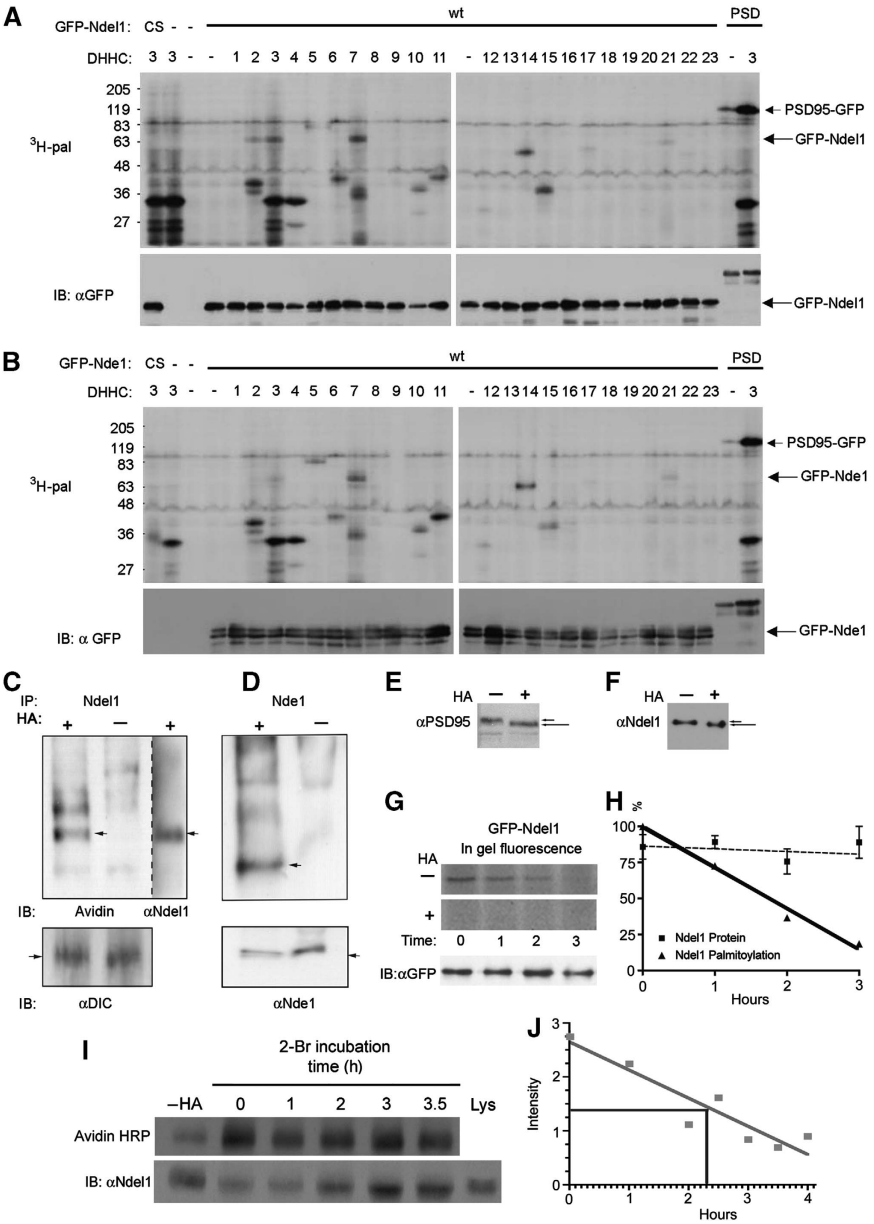

Figure 1 Ndel1 and Nde1 are palmitoylated proteins. (**A**, **B**) Radioactive palmitate was incorporated in HEK293 cells co-transfected with (**A**) wild-type GFP–Ndel1 or (B) GFP–Nde1 and each of the palmitoylation enzymes. (**C**, **D**) *In vivo* palmitoylation of Ndel1 and Nde1 using the ABE (acyl-biotinyl exchange) method. Brain proteins from P7 mice (**C**, **D**) were extracted in a buffer containing *N*-ethyl malamide. (**C**) Ndel1 or (**D**) Nde1 were immunoprecipitated using anti-Ndel1 or anti-Nde1 antibodies. Palmitate was removed by addition of hydroxylamine (controls without hydroxylamine). BMCC-Biotin binding to the free thioester group enabled detection of the palmitoylated population of proteins by avidin–HRP. The positions of palmitoylated Ndel1 or Nde1 are indicated by small arrows. The western blots using anti-Ndel1, anti-DIC, or Nde1 antibodies indicated the position of Ndel1 (**C**, top), and similar amount of immunoprecipitated DIC (**C**, bottom) or Nde1 (**D**, bottom) proteins. (**E**, **F**) A band shift is noted when (**E**) PSD95 from brain lysate or (**F**) Ndel1 from CAD cell lysate are separated on SDS–PAGE after addition of neutral pH hydroxylamine (without HA−, with HA + ,). Short arrows indicate the position of the nontreated bands, whereas the longer arrows indicate the positions of the hydroxylamine-treated bands. (**G**–**K**) Determination of Ndel1 palmitoylation half-life. (**G**, **H**) GFP–Ndel1 transfected in DHHC7 HEK293 inducible cell line, was metabolically labelled with 17-ODYA, followed by chase of palmitic acid. Ndel1 was immunoprecipitated and subjected to click chemistry, separated by SDS–PAGE, in-gel fluorescence was monitored (top panel), and the fluorescence was removed after HA treatment (middle panel). Similar amounts of Ndel1 were immunoprecipitated (lower panel). (**H**) Palmitoylation and protein half-life linear regression. The in-gel fluorescence intensity was normalized according to the amount of immunoprecipitated Ndel1 and plotted (data points are triangles and the linear regression is solid black line). Ndel1 protein half-life data (described in supplementary data) was ploted as well (data points are squares ± s.e.m. and the linear regression is a dashed line). Ndel1 is a very stable protein with an estimated half-life of about 24 h; Ndel1 palmitoylation half-life is about 1.7 h. (**I**, **J**) Half-life in cortical neurons. Cortical neurons were cultured in the presence of 2-bromopalmitate (2-Br) and palmitoylation at the indicated time points were determined by the ABE method. (**J**) The relative intensities after normalization with intensity of the immunoprecipitated protein were plotted against time and the half-life line is indicated.

lated cysteine in Ndel1 resides within the mapped interaction domain with cytoplasmic dynein in the C-terminal part of this protein (Sasaki *et al*, 2000), whereas the interaction with LIS1 has been localized to a different region within the coiled-coil domain (Derewenda *et al*, 2007; schematically presented in Figure 3A).

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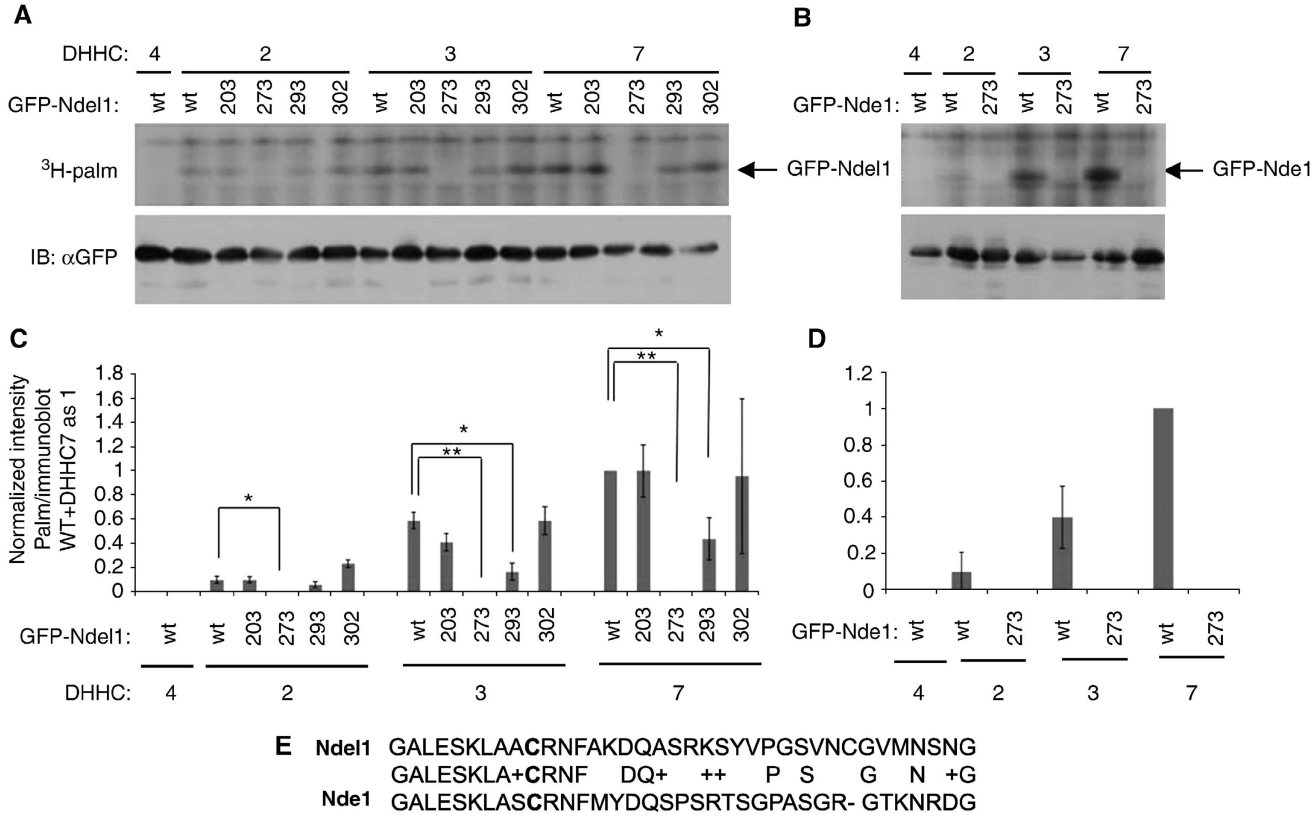

Figure 2 Identification of Ndel1 and Nde1 palmitoylated site. (**A**, **B**) Ndel1 (**A**) and Nde1 (**B**) are palmitoylated on cys 273 by DHHC2, 3, and 7. HEK293 cells co-transfected with wild-type GFP–Ndel1, or GFP–Nde1 or mutated on the indicated cysteines together with the indicated palmitoylation enzymes DHHC2, 3, and 7, were labelled with [^{3}H]-palmitate. The cell lysates separated by SDS–PAGE were subjected to fluorography and expression levels of GFP–proteins were monitored by immunoblotting with an anti-GFP antibody. (**C**, **D**) The autoradiograms in (**A**, **B**) were subjected to quantification demonstrating that when Cys 273 is mutated, palmitoylation decreased. (**E**) Sequence comparison (BLAST) between Ndel1 and Nde1 of the amino acids surrounding Cys 273.

We used immunoprecipitation to test whether Ndel1 palmitoylation affects its interaction with cytoplasmic dynein or with LIS1 in transfected cells. Palmitoylated Ndel1 (co-expressed with its palmitoylation enzyme DHHC7) exhibited reduced interaction with the cytoplasmic dynein complex (Figure 3B) when dynein was co-immunoprecipitated with Ndel1. In contrast, the unpalmitoylatable C273S-mutated version did not exhibit the observed reduction in interaction with the dynein complex, confirming that this effect is due to palmitoylation of Ndel1. The interaction between Ndel1 and LIS1 was not affected by palmitoylation, or by the C273 point mutation. Using metabolic labelling and click chemistry, we could demonstrate that Ndel1 palmitoylation is reduced in the presence of DHHC7-DN or when C273 is mutated (Figure 3C). Similar reduction in the interaction between Ndel1 and DIC was noted when Ndel1 was co-immunoprecipitated with DIC. In this case palmitoylation of Ndel1 was reduced in HEK293 cells expressing inducible DHHC7 after treatment with the well-characterized inhibitor of palmitoylation 2-Br (Jennings *et al*, 2009; Figure 3D). Unlike Ndel1, Nde1 co-immunoprecipitation with the dynein complex was not affected by palmitoylation (Figure 3E). Similar amounts of immunoprecipitated Nde1 in the presence or absence of the palmitoylation enzyme DHHC7 were observed. Nevertheless, cys 273 has an important role in mediating the binding of Nde1 to cytoplasmic dynein, as Nde1 C273S exhibited a 5.6-fold reduced interaction with DIC (paired Student's *t*-test, $P = 0.0004$) regardless of the presence of the palmitoylation enzyme (Figure 3E). The difference between the behaviour of Nde1 and Ndel1 in that respect may be due to slight differences in the sequence of the amino acids surrounding C273 (Figure 2E). Amino acids in that region may also contribute to the interaction with dynein. Structural analysis of the complexes involving Nde1 or Ndel1 and dynein are likely to provide better insight into this issue. The interaction between Nde1 and LIS1 also was not affected by palmitoylation, and not by introduction of the C273S mutation (data not shown). Therefore, despite the high sequence similarity (72.6%) between Ndel1 and Nde1, our results uncovered a clear functional difference between these two paralog proteins.

The idea that Nde1 and Ndel1 have non-overlapping functions in regard to palmitoylation-dependent dynein interactions, prompted us to carry out a phylogenetic analysis for Nde1 and Ndel1 from invertebrates and vertebrates (Supplementary Figure S4). The analysis revealed a clear distinction between the groups of Nde1 and Ndel1 proteins in vertebrates, whereas invertebrates (represented here by two different species of *Drosophila*) contain a single Nde1 protein. Interestingly, the possible divergence between the Protostomia (invertebrates) and the Deuterostomia (vertebrates) was estimated to occur 700–520 Mya (Erwin and Davidson, 2002) or between 580 and 520 Mya (Halanych, 2004). Therefore, it is likely that as sequences diverged, unique functionalities were adopted for the two proteins.

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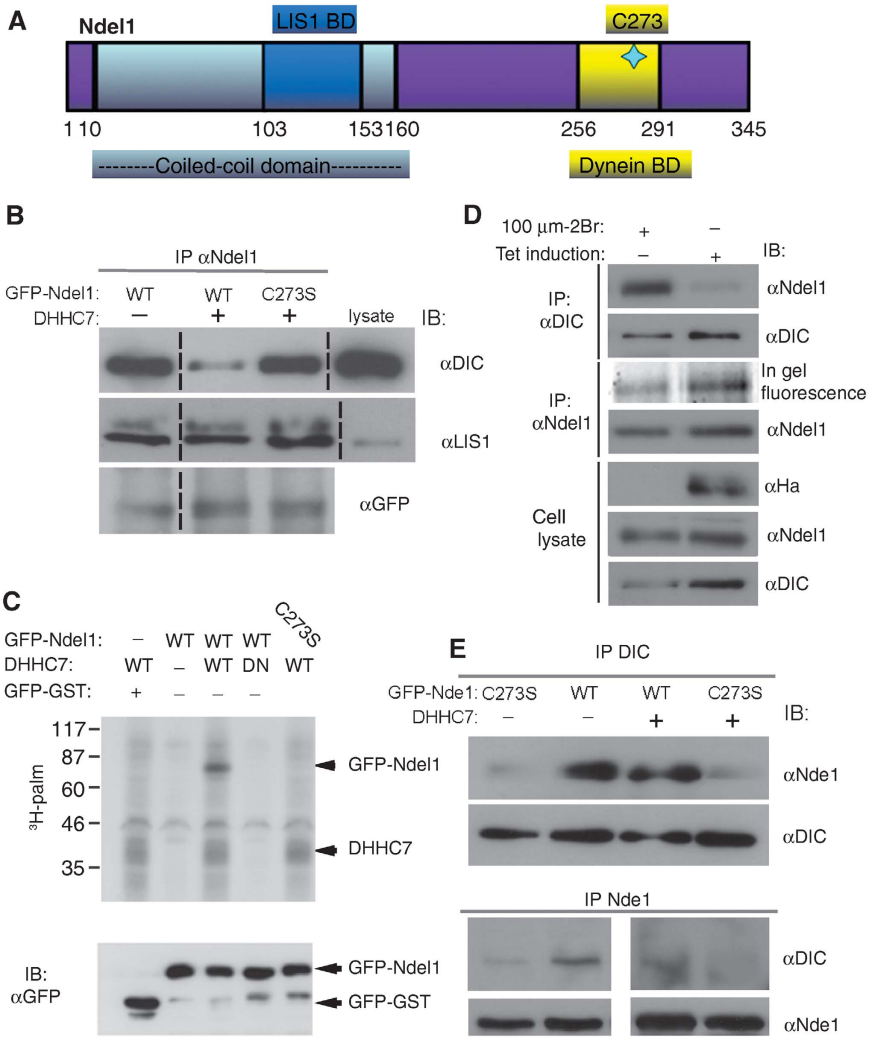

Figure 3 The effect of Ndel1 or Nde1 palmitoylation on their interaction with dynein intermediate chain. (**A**) Schematic presentation of Ndel1 and its binding domains. The coiled-coil domain (amino acids 10–160, light grey) contains the mapped binding domain with LIS1 (amino acids 103–153, blue). The dynein-binding domain (amino acids 256–291, yellow) contains the palmitoylated site C273. (**B**) HEK293 cells were transiently transfected with GFP–Ndel1wt/C273S with or without HA–DHHC7. Ndel1 or DIC were immunoprecipitated. The interaction between Ndel1 and DIC is reduced when Ndel1 is palmitoylated (second lane from the left side). In the absence of the palmitoylation enzyme (DHHC7) or when Ndel1 cannot undergo palmitoylation (C273S), the interaction is increased. (**C**) Introduction of DHHC7 DN or Ndel1 C273S reduces Ndell palmitoylation as evident by ^{3}H-palmitate incorporation. Similar amounts of the proteins were expressed evident by anti-GFP western blot. (**D**) Reduction in Ndel1 palmitoylaiton increases its interaction with DIC. More Ndel1 is co-immunoprecipitated with DIC after 2-Br treatment (upper row), similar amounts of DIC were immunoprecipitated and similar amount of Ndel1 were present in the lysates. Nevertheless, Ndel1 palmitoylation was decreased after 2-Br treatment evident by in gel fluorescence. The induction of DHHC7 in the HEK293 cell line was evident by western blot with the anti-HA tag. (**E**) The interaction between Nde1 and DIC is unaffected by palmitoylation. However, the interaction of mutant Nde1 (C273S) with DIC is diminished. The result is seen by both reciprocal immunoprecipitations: IP of DIC (top panel) and IP of Nde1 (bottom panel).

Palmitoylation is a reversible modification; therefore, a plausible hypothesis is that the differential interaction of palmitoylated versus unpalmitoylated Ndel1 with cytoplasmic dynein is a new switch mechanism to modulate the activity of the molecular motor. This hypothesis was tested directly by monitoring the activity of cytoplasmic dynein using several cellular assays using different cells. The levels of Ndel1 and the palmitoylation enzymes vary in the different cells (Supplementary Figure S5). Ndel1 is highly expressed in the rat brain and in CAD cells; it is expressed at moderate levels in NIH3T3 cells and at very low levels in COS7 cells and HEK293 cells. DHHC7 expression is very low in NIH3T3 cells and HEK293 cells, and is better expressed in CAD, COS7, and brain lysate, whereas DHHC3 is expressed at high levels in NIH3T3 cells. Therefore, in some cases, we needed to transfect both Ndel1 and the palmitoylation enzyme to observe an effect. We demonstrated that the Ndel1 is palmitoylated when it is co-transfected with the palmitoylation enzyme and its palmitoylation is significantly reduced when Ndel1 (C273S) was co-transfected with the wild-type enzyme or when wild-type Ndel1 was co-transfected with the DN-enzyme in HEK 293 cells and COS7 cells (Figure 3C and Supplementary Figure S6).

Cytoplasmic dynein and Golgi structure

Several lines of evidence suggest that the perinuclear position of the Golgi is driven by cytoplasmic dynein-mediated transport. In cells lacking cytoplasmic dynein heavy chain (DHC),

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the Golgi is dispersed (Harada *et al*, 1998) and injection of anti-DHC2 antibody also led to dispersion of the Golgi complex (Vaisberg *et al*, 1996). In addition, depletion of cytoplasmic dynein prevented the centrosomal localization of exogenously applied Golgi-derived vesicles in partially permeabilized cells (Corthesy-Theulaz *et al*, 1992). Finally, overexpression of the dynamitin subunit of the dynactin complex, which dissociates the dynein complex, also led to the dispersion of the Golgi complex among other cellular phenotypes (Burkhardt *et al*, 1997). Similarly, the activity of Ndel1 and LIS1 in regulating dynein-mediated perinuclear clustering of the Golgi apparatus was demonstrated in *Lis1*- and *Ndel1*-null mouse embryonic fibroblasts (Sasaki *et al*, 2005). In tissue culture cells, this activity has been demonstrated using either Ndel1 mutants defective in LIS1 or DHC binding or by the silencing of Ndel1 using siRNA (Liang *et al*, 2004).

The compactness of the juxtanuclear Golgi complex was measured in NIH3T3 cells by immunostaining with anti-Mannosidase II (an integral Golgi enzyme) antibodies (Figure 4). Palmitoylated Ndel1 significantly induced Golgi dispersion (Figure 4C–E) without any obvious effects on the microtubule cytoskeleton (data not shown). In addition, a portion of Ndel1 clearly localized to the Golgi (Figure 4A and C). In contrast, when endogenous palmitoylation is reduced by expression of DHHC7-DN, the size of the Golgi is not changed (Figure 4A, B and E). Similarly, palmitoylated Nde1 or expression of individual components Ndel1, Nde1, or the palmitoylation enzyme DHHC7 alone had no effect on Golgi distribution (Figure 4E). These results are consistent with our previous findings, suggesting that strong interaction of unpalmitoylated Ndel1, but not Nde1, with cytoplasmic dynein is required for the perinuclear positioning of the Golgi.

Transport of short microtubules mediated by cytoplasmic dynein

Transport to the cell periphery of short microtubule fragments synthesized at the centrosome is mediated by cytoplasmic dynein (Ahmad *et al*, 1998; Abal *et al*, 2002). The effect of Ndel1 palmitoylation status on this transport was assayed by following short microtubules nucleated at the centrosome in a 'pulse-chase' regime. After treatment with nocodazole, which disassembled existing microtubules, nocodazole was washed away and cells were recovered for 3 min to allow reassembly of microtubules. Finally, cells were treated with low concentrations of vinblastine, which inhibits microtubule polymerization, but does not cause micortubule disassembly (Ahmad and Baas, 1995). Our results indicate that when Ndel1 is palmitoylated, less microtubule fragments reached the cell periphery (Supplementary Figure S7A–C, H). In addition, under these experimental conditions, substantial peri-surface fluorescence is evident for the Ndel1. Moreover, addition of unpalmitoylated Ndel1 to these cells enhanced cytoplasmic dynein activity, indicated by the marked increase in the level of microtubule fragments that reach the cell periphery (Shu *et al*, 2004; Supplementary Figure S7G). However, transfection of Nde1 did not affect this activity (Shu *et al*, 2004; Supplementary Figure S7I), neither in the presence of the palmitoylation enzyme, nor in case of cys 273 mutation (Supplementary Figure S7D–F, I). It is noteworthy that when C273 Ndel1 palmitoylation site is mutated, its activity did not differ from that of the unpalmitoylated wild-type Ndel1 (Supplementary Figure S7H). We concluded that palmitoylation of Ndel1 reduced the efficacy of dynein-mediated transport of short microtubules to the cell periphery.

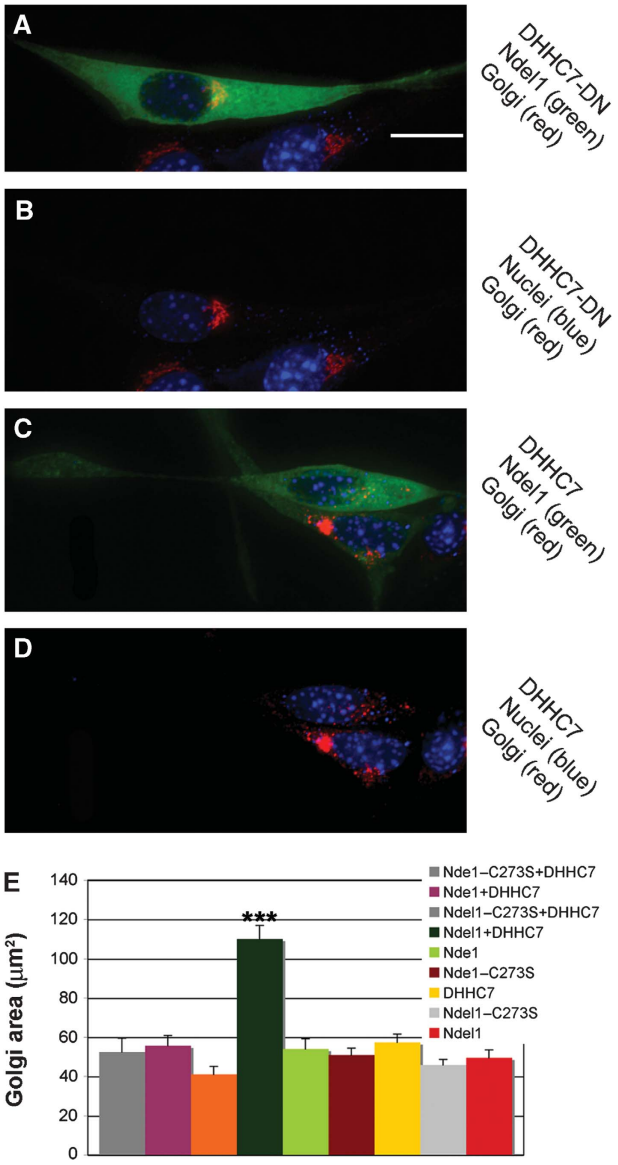

Figure 4 Palmitoylated Ndel1 reduces cytoplasmic dynein activity resulting in dispersed Golgi. NIH3T3 cells were transfected with all combinations of GFP–Ndel1 (wild type or C273S) with DHHC7 WT or C160S mutant (DHHC7-DN), or combinations of GFP–Nde1(wild type or C273S) and/or DHHC7 (wild type or DN). (**A**, **B**) When Ndel1 palmitoylation is low in the presence of DHHC7-DN, (**B**) the Golgi immunostained with anti-mannosidase II antibodies, is compact (**A**, merged picture). (**C**, **D**) When Ndel1 is palmitoylated (**D**), in the presence of DHHC7, the Golgi is more distributed (**D**, merge **C**). (**E**) The Golgi area in the indicated transfected cells was measured and subjected to statistical analysis. Co-transfection of Ndel1 and the palmitoylation enzyme DHHC7 increased the area of the Golgi (ANOVA, $P<0.0001$, $n=21$). Error bars indicate s.e.m. value. Size bar is 15 μm.

Dynein-mediated VSVG transport

The viral glycoprotein VSVG is synthesized in the ER and then transported towards the Golgi complex along microtubules by using the activity of the retrograde motor cytoplasmic dynein (Presley *et al*, 1997). It has been reported that the

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temperature-sensitive fluorescent VSVG is rapidly translocated into the Golgi region after a 40–32°C temperature shift. Activation of cytoplasmic dynein is expected to result in a compact fluorescent VSVG localization during a shorter time period. This was clearly visualized in cells transfected with Ndel1 versus control cells (Supplementary Figure S8A–C versus D–F, and G). In this system, palmitoylation had also a clear effect on the activity of the retrograde motor (Supplementary Figure S9). When Ndel1 is palmitoylated, the rate in which the mutant VSVG protein is translocated to the Golgi is markedly reduced (Supplementary Figure S9A–C, and G). In contrast, Ndel1 mutated in the main palmitoylation site displayed a significant increase in VSVG translocation (Supplementary Figure S9G). In conclusion, palmitoylated Ndel1 reduced the activity of the retrograde motor protein cytoplasmic dynein, and thereby inhibited ER-to-Golgi mobilization of VSVG.

Transport to neurite tips and retrograde trafficking of dynein puncta

The localization of LIS1 and Ndel1 at the tips of neurites is determined by a balance between molecular motors working in different orientations: cytoplasmic dynein and kinesin (Taya *et al*, 2007). To test whether palmitoylation of Ndel1 affects the balance between cytoplasmic dynein and kinesin activities, we quantified the relative immunoreactivity fluorescence intensity of Ndel1, LIS1, and Nde1 in the neurite tips of differentiated CAD cells in comparison with the relative immunoreactivity fluorescence intensity found at the connection of the neurite to the cell body. Most differentiated CAD cells exhibited relatively higher intensities of LIS1 and Ndel1 at the neurite tips 57 ± 0.07% ($n = 49$) and 84 ± 0.05% ($n = 39$), respectively (Figure 5A, C and I). The ddition of the palmitoylation inhibitor 2-Br, significantly reduced the relative localization of LIS1 and Ndel1 at the neurite tips (8.5 ± 3.3% ($n = 70$, $P < 0.0001$) and 62 ± 7.3% ($n = 45$, $P = 0.02$) respectively, Figure 5B, D and I). Therefore, as expected, this treatment led to enhanced Ndel1 and LIS1 mobilization in the retrograde direction towards the cell body. In contrast, the localization of Nde1 at the tips was not altered after the inhibition of palmitoylation (data not shown).

Overexpression of the dominant-negative form of GFP–DHHC7 significantly reduced the relative position of Ndel1 at the neurite tips, from 51 ± 6.1% ($n = 68$) to 27 ± 6% ($n = 55$, $P = 0.006$) in comparison with overexpression of this enzyme (Figure 5E, F, and I). In agreement with previous results, the localization of Nde1 was not affected by the overexpression of either DHHC7 or DN-DHHC7 (the relative frequency of Nde1 at the tip was 71 ± 7.3% ($n = 39$) versus 55 ± 7.7% ($n = 42$)).

Next, we tested the effect of expressing the Ndel1 palmitoylation mutant on the localization of LIS1. In concord with the assays described above, the enhancement of cytoplasmic dynein activity by the palmitoylation mutant led to a reduction of the relative amount of LIS1 localized in neurite tips in comparison with wild-type Ndel1 (Figure 5G–I). In these cells, the relative proportion of cells expressing more LIS1 in the tips was decreased in a significant way from 53 ± 7% ($n = 41$) to 23 ± 5% ($n = 52$, $P = 0.002$). Expression of either wild-type or the C273S mutant of Nde1 did not affect LIS1 localization at the tips of neurites in differentiated CAD cells

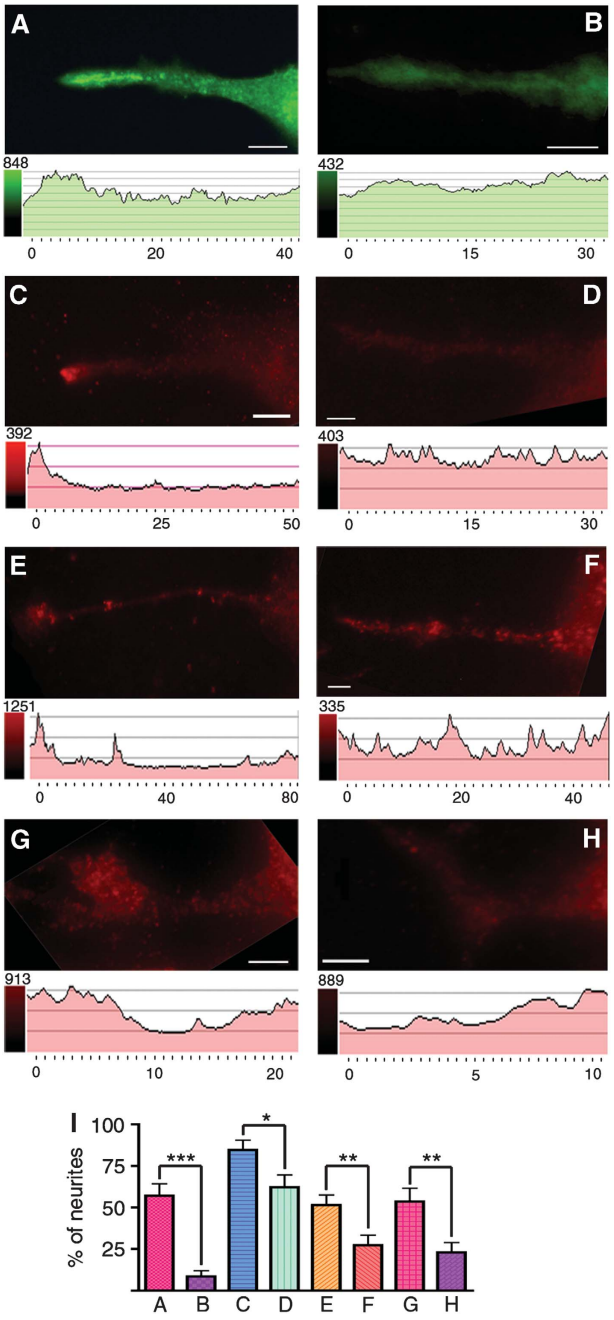

Figure 5 Ndel1 palmitoylation shifts the cellular localization of LIS1 and Ndel1 towards neurite tips. Below each of the neurite images, there is line histogram demonstrating the relative fluorescence intensity of the immunostained protein along the neurite. Differentiated CAD cells were treated with methanol (as a control) (**A**, **C**) or with 7.5 mM 2-Br (**B**, **D**) for 3.5 h, and then fixed and stained with anti-LIS1 antibodies (**A**, **B**) or anti-Ndel1 (**C**, **D**). WT or the dominant-negative form of GFP–DHHC7-transfected CAD cells were stained with anti-Ndel1 antibodies (**E**, **F**). WT or the C273S mutated form of GFP–Ndel1-transfected CAD cells were stained with anti-LIS1 antibodies (**G**, **H**). (**I**), The percentage of neurites exhibiting relatively higher intensity of fluorescence at the tip was plotted and subjected to Student's *t*-test, significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

(data not shown). We conclude that Ndel1 palmitoylation leads to reduced dynein activity that may disturb the balance between the activities of the plus and minus end motors, resulting in the accumulation of LIS1 and Ndel1 at neurite tips.

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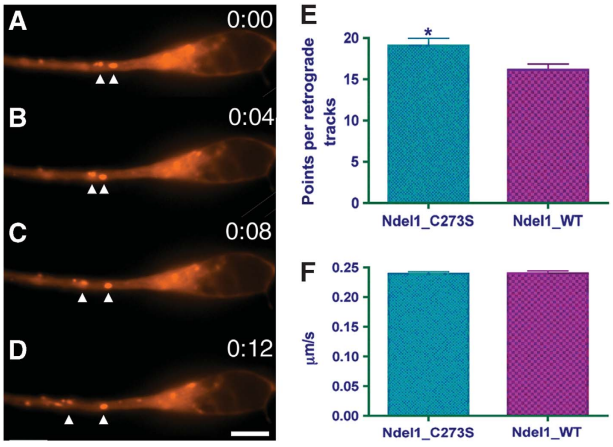

Figure 6 Unpalmitoylated Ndel1 increases the processivity of DIC–mCherry puncta retrograde tracks. Primary cerebellar neurons were transfected with either GFP–Ndel1 wild type or C273S mutant and DIC–mCherry. The red fluorescent puncta were tracked using Imaris software. (**A–D**) DIC–mCherry puncta were identified and their movements in the axon were tracked. In each time-lapse (here 4-s intervals are shown), the position of each puncta was compared with its previous position. Arrowheads mark trackable puncta. Retrograde and anterograde motility was defined according to the movement to or from the cell body. (**E**) The number of points in each retrograde track was plotted and the differences between neurons transfected with Ndel1 C273S and Ndel1 were significant $P = 0.0107$. (**F**) Expression of Ndel1 C273S did not change the velocity in the retrograde direction.

The aforementioned experiments provided us with indirect evidence regarding DIC activity. To monitor DIC activity directly, we transfected primary cerebellar neurons with DIC-1B–mCherry and either GFP–Ndel1 or GFP–Ndel1(C273S), and monitored the movements of the fluorescent dynein puncta within the axons (Ha *et al*, 2008). DIC puncta were tracked using the Imaris program (Supplementary Movies 1 and 2 are examples of DIC–mCherry puncta in neurons transfected with GFP–Ndel1 or GFP–Ndel1 (C273S), respectively). In each time frame, the position of the puncta were subtracted from the previous time frame and the relative orientation in relation to the cell body was determined (Figure 6A–D). We monitored DIC-1B–mCherry puncta over time and noticed that we could monitor them over more time frames. The processivity of DIC-1B–mCherry puncta increased in a significant manner when the unpalmitoylatable form of Ndel1 (C273S) was present; the number of points per track increased to 19.07 ± 0.9, $n = 280$ versus 16.12 ± 0.7, $n = 314$; $P = 0.0107$ (Figure 6E). Thus, Ndel1 (C273S) resulted in dynein containing particles that moved during a longer time interval. Nevertheless, there was no change in the average retrograde velocity (Figure 6F).

Ndel1 palmitoylation and neuronal migration

The functional connection between cytoplasmic dynein and Ndel1 during neuronal migration is well documented (Sasaki *et al*, 2000, 2005; Niethammer *et al*, 2000b; Shu *et al*, 2004). Therefore, we investigated the expression pattern of the palmitoylation enzymes: DHHC2, DHHC3, and DHHC7 displayed in the genepaint database (http://www.genepaint.org) (Visel *et al*, 2004). No signal for the minor Ndel1 palmitoylation enzyme DHHC21 (see Figure 1) was noted in the developing embryo. DHHC2, DHHC3, and DHHC7 are expressed in several tissues (Supplementary Figure S10). Interestingly, a pronounced expression of DHHC7 was noted in the cortical plate (CP) of the developing brain cortex, whereas DHHC2 was stronger in the ventricular zone (VZ). The expression pattern of DHHC3 was relatively uniform. These results are consistent with the idea that DHHC7/3/2 can regulate the palmitoylation of Nde1/Ndel1 in the developing brain. To further pursue this idea, we tested whether Ndel1 palmitoylation affects neuronal migration *in vivo* using *in utero* electroporation (Figure 7). The expression of DHHC7-DN reduced the proportion of neurons that reached the superficial layers of the cortical plate in comparison with control (compare Figure 7E with J. The quantification of the relative proportion of cells in the different bins are shown in panel K). The expression of Ndel1, which activates cytoplasmic dynein, inhibited neuronal migration (Figure 7B, G and K). The expression of Ndel1 C273S, which may activate cytoplasmic dynein, indeed impaired neuronal migration (Figure 7C, H and K). Finally, the expected inhibition of the retrograde motor by DHHC7 expression caused a significant retardation of neuronal migration (Figure 7D, I and K). We, therefore, conclude that a tight balance of dynein activity is required for proper neuronal migration and that enhanced inhibition or, to a lesser extent, increased activation of the molecular motor impairs this process.

Next we tested the significance of reduction of each of the three palmitoylation enzymes in the developing brain. The efficacy of each of the designed shRNA sequences was verified in ectopically transfected HEK293 cells (Supplementary Figure S11). The efficacy of reducing endogenous DHHC3 was also tested using immunostaining of transfected primary neurons (Supplementary Figure S11). Knock down of each enzyme independently, reduced the proportion of neurons that reached the superficial layers of the cortical plate in comparison with control (Figure 8A–D). This reduction was statistically significant as tested by ANOVA (Figure 8F). The most pronounced effect of knock down of a single enzyme on radial migration was noted with reduced levels of DHHC7, although combined reduction of all three enzymes exhibited further reduction (Figure 8D–F). To further verify that these effects are indeed due to the reduction in the endogenous levels of the palmitoylating enzymes, silent mutations were introduced in DHHC7 allowing resistance to the corresponding shRNA (Supplementary Figure S11). The addition of this DHHC7-resistant form rescued the DHHC7 shRNA-induced migration phenotype (Figure 8G–I). The improvement in neuronal migration was significant as assayed by paired *t*-test (Figure 8I). In addition, enzyme specificity was explored by trying to rescue knock down of DHHC7 by DHHC3 or knock down of DHHC2 by DHHC7. In both cases, there was no significant improvement in neuronal migration. Collectively, these observations indicate that Ndel1 palmitoylation enzymes control proper neuronal migration during brain development.

Discussion

Ndel1 and Nde1

Our results demonstrate, to the best of our knowledge, for the first time, palmitoylation on conserved cysteines of the two Nde paralogs. Significantly, in contrast to Ndel1 palmitoylation, which impinges on the activity of the motor itself, the

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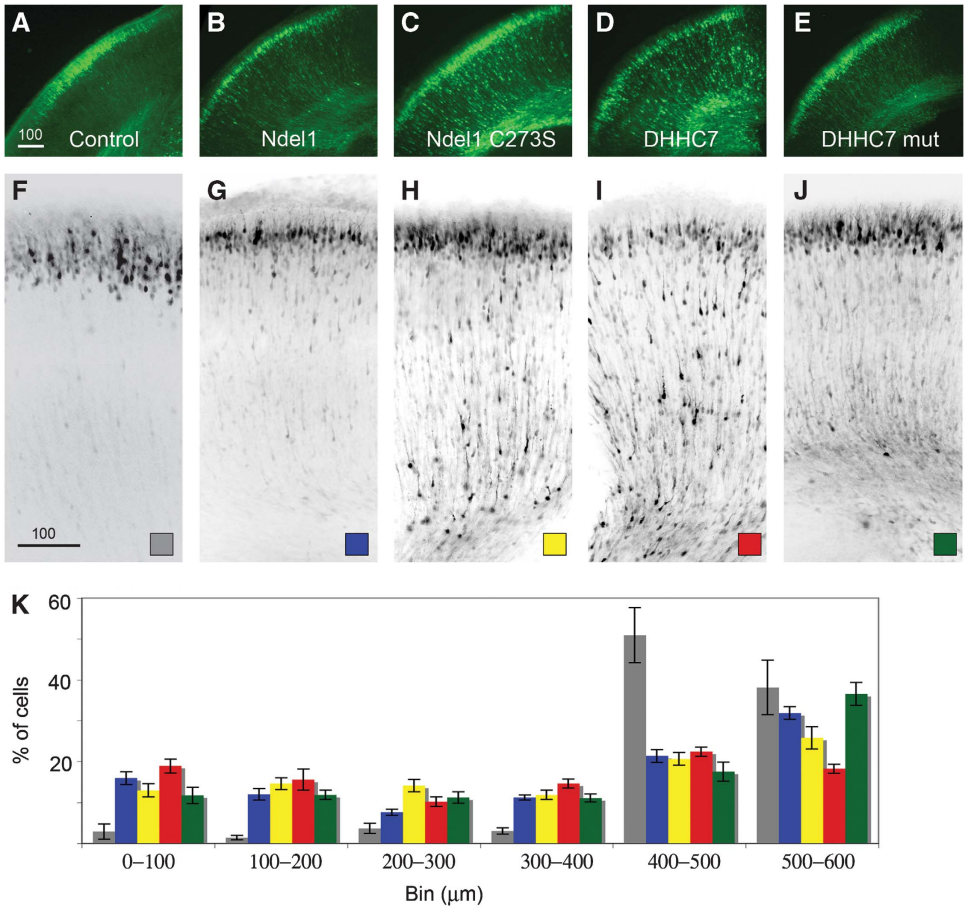

Figure 7 Palmitoylation of Ndel1 affects neuronal migration. E14.5 embryos were electroporated *in utero* with plasmids expressing (left to right) (**A**, **F**) control; (**B**, **G**) Ndel1; (**C**, **H**) Ndel1 C273S; (**D**, **I**) DHHC7; or (**E**, **J**) DHHC7-DN lacking enzymatic activity together with a GFP-expressing plasmid. Brains were fixed, sectioned, and pictured 4 days after electroporation. (**K**) Sections from at least four different brains were analysed by counting the number of neurons found in six individual bins of equal sizes, and compared their relative percentage in each bin by ANOVA. Ndel1 (blue) differed significantly from the control (grey). DHHC7-DN (green) exhibited reduced migration in comparison with control migration (grey) with most neurons found in the superficial layer of the cortical plate. Expression of DHHC7 (red) significantly impaired neuronal migration, with a similar number of neurons found among different bins. Expression of Ndel1 C273S (yellow) inhibited the neuronal migration in a moderate but significant manner.

palmitoylation of Nde1 (Ndel1's paralog) on the conserved cysteine had no effect on the measured dynein-mediated activities. Although the significance of Nde1 palmitoylation is a matter of future studies, the apparent divergent regulatory activities of the two similar proteins have an evolutionary basis reflected in their differential interaction with dynein subunits. Nde1 interacts with dynein light chain (Feng *et al*, 2000; Stehman *et al*, 2007) and DIC (Stehman *et al*, 2007). Ndel1 was also reported to interact with the intermediate chains, but also with light intermediate chains (Niethammer *et al*, 2000b) and cytoplasmic dynein heavy chain (Sasaki *et al*, 2000; Niethammer *et al*, 2000b), including two identified binding domains, one of which in the P1 loop (Sasaki *et al*, 2000). Similarly, the Nde paralogs also vary in their perspective roles in targeting of dynein to kinetochores (Vergnolle and Taylor, 2007).

A new mode of regulating cytoplasmic dynein

Our results indicate a new mean for regulating the activity of the molecular motor cytoplasmic dynein through the reversible palmitoylation of Ndel1. The regulation of the molecular motor is rather complex. Dynein heavy chain has at least two different regions involved in regulation of the ATPase enzymatic activity; the stalk and the C-terminal domain (for review, see Vallee and Hook, 2006). In addition, the transient interaction of dynein light chains (Makokha *et al*, 2002; Nyarko *et al*, 2004; Vallee *et al*, 2004), regulated by phosphorylation (Song *et al*, 2007), affects the structure of the dynein intermediate chain (Benison *et al*, 2006). The phosphorylation of the dynein intermediate chain also regulates its binding to dynactin (Vaughan *et al*, 2001) that in turn is involved in regulation of dynein processivity (Schroer, 2004). Furthermore, dyenin is regulated by interacting proteins such as dynactin (for reviews, see Schroer, 2004; Vallee *et al*, 2004). Interestingly, the palmitoylation of the dynein intermediate chain has been recently reported after a proteomics screen for neuronal palmitoylation substrates (Kang *et al*, 2008). However, the identity of the enzymes responsible for this modification and its functional significance is of yet unknown. The possibility that Ndel1 and cytoplasmic dynein are simultaneously co-modified will be a topic of future studies.

Palmitoylation is similar to phosphorylation in respect to its reversible modulation. Palmitoylation of Ndel1 affected

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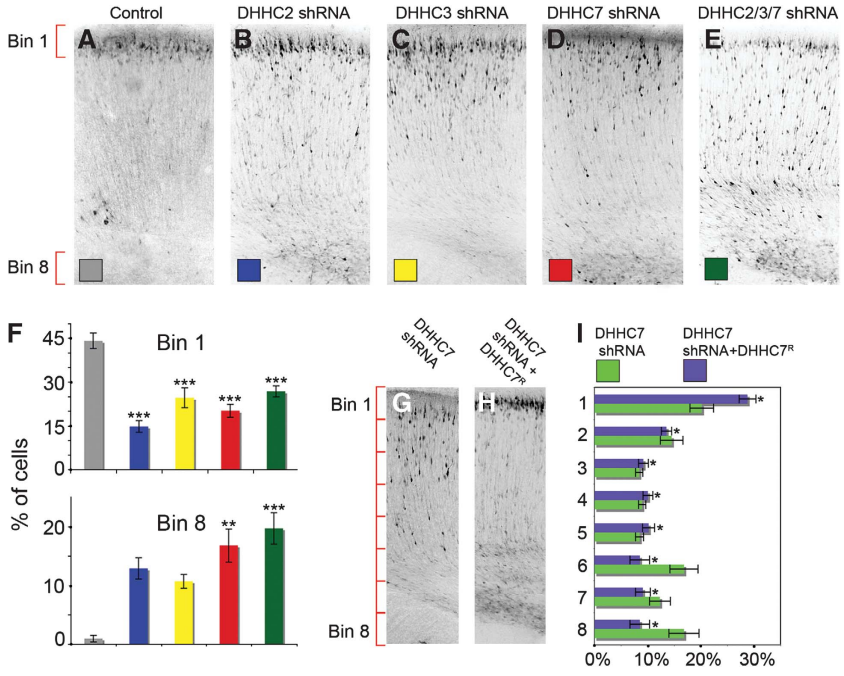

Figure 8 Reduction in Ndel1 palmitoylation enzymes inhibits neuronal migration. (**A**–**E**) E14.5 embryos were electroporated *in utero* with GFP expression plasmids and either (**A**) global control shRNA, (**B**) DHHC2 shRNA, (**C**) DHHC3 shRNA, (D) DHHC7 shRNA, or (**E**) a combination of DHHC2,3, and 7 shRNA. Brains were analysed 4 days after electroporation, and the position of GFP-labelled neurons was quantified in each of eight bins, in which bin 1 marked the most superficial area and bin 8 the area close to the ventricular zone. (**F**) The percentage of cells in each bin were calculated and compared by ANOVA. Each of the individual shRNA and the mixture of all three shRNA reduced the percentage of cells reaching the most superficial layer, ($***P<0.001$) (**F**, bin 1). The effect of DHHC7 and the mixture of all three shRNA was most obvious in bin 8 ($**P<0.01$, $***P<0.001$) (**F**). Among the individual shRNA, the effect of DHHC7 was most pronounced. (**G**–**I**) The specificity of DHHC7 shRNA was verified by a rescue experiment. The percentage of neurons stalled in their migration route was significantly decreased when a DHHC7 expression plasmid harbouring silent mutations and resistant to the shRNA was introduced ($*P=0.032$) (compare **G** and **H**). (**I**) The results were subjected to paired *t-test*.

dynein-mediated intracellular transport of several cargoes; it reduced the compactness of the Golgi that is redistributed in the cell during mitosis. In addition, transport of short microtubule fragments, and dynein-mediated transport of mutant VSVG was enhanced with overexpression of Ndel1, but was reduced when Ndel1 was palmitoylated. Furthermore, Ndel1 and LIS1 are also cargoes of cytoplasmic dynein, their intracellular localization is shifted towards the cell body when Ndel1 palmitoylation is reduced. There is no precedence in which palmitoylation of a dynein-interacting protein is known to affect its interaction with dynein and as such to affect dynein activity. Huntingtin undergoes palmitoylation by its interacting protein HIP14 (DHHC-17) (Huang *et al*, 2004), and this activity is important for its proper trafficking and function (Ohyama *et al*, 2007). In addition, Huntingtin interacts directly with dynein intermediate chain and is suggested to facilitate dynein-mediated vesicle motility (Caviston *et al*, 2007). In addition, it has been demonstrated that Huntingtin phosphorylation functions as a bimodular switch in regulating the direction of vesicle transport (Colin *et al*, 2008). Although a relationship between Huntingtin palmitoylation and dynein-mediated activity has not been demonstrated, it is attractive to speculate that this interaction may also be regulated by the status of Huntingtin palmitoylation. In another system, a different posttranslational modification of the RNA-binding protein, La, by sumoylation in sensory neurons was found to regulate the directionality of its transport (van Niekerk *et al*, 2007). Sumoylated La preferentially interacts with cytoplasmic dynein that transports it retrogradely, whereas sumoylation-incompetent La shows only anterograde transport. However, the La RNA chaperone protein has not been shown to affect dynein activity as observed in case of Ndel1. Importantly, *S*-nitrosylation of MAP1b was shown to be involved in axonal response to nitric oxide (Stroissnigg *et al*, 2007) and this modification resulted in a change in MAP1b-binding affinity to microtubules. In agreement with other data suggesting that MAP1b or other related proteins inhibit dynein activity (Ding *et al*, 2006; Jimenez-Mateos *et al*, 2006), a model has been proposed in which *S*-nitrosylated MAP1b consequently inhibits dynein activity, leading to axonal retraction. Thus suggesting that a signalling cascade initiated by nitric oxide results in inhibition of dynein activity.

It has been postulated that reversible palmitoylation has an important role in the nervous system (for reviews, see El-Husseini Ael and Bredt, 2002; Washbourne, 2004). Our results emphasize that Ndel1 palmitoylation is of great significance, particularly, in neuronal cells. Our *in vivo* treatments indicated that either reduced or increased Ndel1 palmitoylation resulted in impaired neuronal migration. The effect was much more pronounced when the molecular motor was inhibited. So far neuronal migration inhibition has been demonstrated in cases in which the activity of the molecular motor is reduced; reduction in dynein heavy chain, LIS1, (Tsai *et al*, 2007) Ndel1 (Shu *et al*, 2004), or overexpression of dynamitin, or dominant-negative LIS1 (Tsai *et al*, 2005). To the best of our knowledge, this is the first example demonstrating that tilting the balance of molecular motor activity results in neuronal migration retardation. Our experimental findings are summarized in the simplistic model, in which

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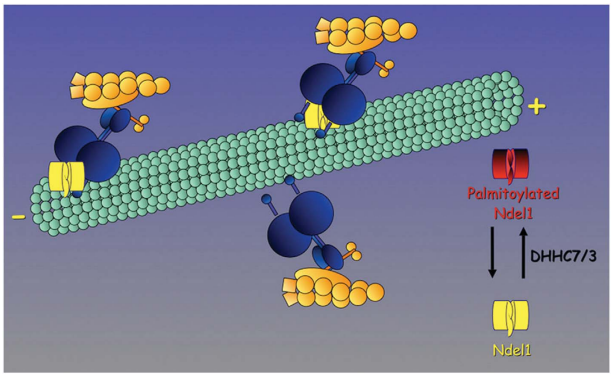

Figure 9 A schematic model demonstrating the effect of palmitoylation of Ndel1 on cytoplasmic dynein activity. The molecular motor, cytoplasmic dynein (blue) moves towards the minus ends of microtubules (light green). The large dynactin complex (orange) is attached to dynein. Ndel1 can be found either palmitoylated (red) after the activity of DHHC7 or DHHC3, or depalmitoylated (yellow). When Ndel1 is depalmitoylated it binds better to the motor domain of dynein and promotes its activity.

Ndel1 can be found in two states (Figure 9). Ndel1 can be found palmitoylated (red) after the activity of either DHHC7 or DHHC3, or depalmitoylated (yellow), by yet unknown depalmitoylation enzymes. In the depalmitoylated form, Ndel1 binds better to the retrograde motor and stimulates its activity. When Ndel1 is palmitoylated, the molecular motor works less effectively and transport is reduced. Our findings are likely to impact all known dynein-mediating activities, from mitosis of the single cells to long-range neuronal transport processes that are affected in cases of neuronal degeneration diseases.

Materials and methods

Palmitate labelling in HEK293 cells

Palmitate labelling was performed as described previously (Fukata *et al*, 2004, 2006). Briefly, transfected HEK293 or COS7 cells were labelled with 0.25 mCi/ml [^{3}H]palmitic acid (Perkin-Elmer) for 4 h. Cells were washed with PBS, scraped with SDS–PAGE sample buffer with 10 mM DTT, and boiled for 2 min. For fluorography, SDS–PAGE gels with protein samples were treated with Amplify (GE Healthcare) for 30 min, dried under vacuum, and exposed at −80°C.

Labelling of palmitoylated Ndel1 and Nde1 in cortical neurons

E15 cortices was dissected and cortical neurons were dissociated into MEM medium supplemented with 6% glucose, GlutaMAX, 5% horse serum, 5% fetal calf serum, B27, and gentamycin. Briefly, cells were homogenized in lysis buffer (PBS supplemented with 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 μg/μl aprotinin, 1 μg/μl pepstatin A and 10 μg/μl leupeptin, with 40 mM of *N*′-ethylmaleimide (E3876, Sigma)). The cell lysates were passed through a 21-gauge needle and left for 30 min on ice. The cell lysates were centrifuged at 4°C for 15 min at 13 000 r.p.m. The cleared supernatants were transferred to new Eppendorf tubes and then the tubes were rotated at 4°C overnight. Proteins were then immunoprecipitated with specific antibodies using A/G beads, after IP an additional blocking was conducted by incubating the beads for 1 h at RT in PBS supplemented with 40 mM of *N*′-ethylmaleimide. The beads were then washed with PBS followed by removal of palmitate by addition of 1 M hydroxylamine (pH 7.5) (55458, Fluka), whereas the control was incubated with PBS for 1 h at RT. After this incubation, the beads were washed thrice in PBS and then incubated with 320 μM EZ-Link BMCC-Biotin (21900, PIERCE). Proteins were separated by SDS–PAGE and blotted with ExtrAvidin-peroxidase (E2886, Sigma) or reacted with specific antibodies.

Palmitoylation half-life in mouse cortical neurons

E15 cortices was dissected and cortical neurons were dissociated into MEM medium supplemented with 6% glucose, GlutaMAX, 5% horse serum, 5% fetal calf serum, B27, and gentamycin. Neurons ($\sim 5 \times 10^6$) were incubated at 37°C with 10 μM 2-Br for the indicated times. Cells were collected by centrifugation and washed once with ice cold PBS. Palmitoylation of Ndel1 was detected as described previously (Drisdel and Green, 2004; Drisdel *et al*, 2006), with some modifications. Briefly, cells were homogenized in lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 μg/μl aprotinin, 1 μg/μl pepstatin A and 10 μg/μl leupeptin) supplemented with 20 mM of *N*′-ethylmaleimide (E3876, Sigma). Proteins were precipitated by methanol–chloroform method and dissolved into 4% SDS, 50 mM Tris (pH 7.4), 5 mM EDTA with 10 mM *N*′-ethylmaleimide, followed by a 10-min incubation at 37°C with addition of lysis buffer with 1 mM *N*′-ethylmaleimide. Proteins were immunoprecipitated with specific antibodies. Palmitate was removed by addition of 1 M hydroxylamine (55458, Fluka), whereas the control was incubated with 1 M Tris–HCl (pH 7.4). Both the treated protein and the control were incubated with 100 μM EZ-Link BMCC-Biotin (21900, PIERCE). Proteins were separated by SDS–PAGE and blotted with ExtrAvidin-peroxidase (E2886, Sigma) or with specific antibodies. After blotting and exposure, the baseline was subtracted from the relative intensities and results were subjected to a linear regression analysis. The results fitted a linear regression model ($r^2 = 0.85$). The calculated half-life was derived from the deduced line (presented in Figure 1H). Additional repetitions agreed with the calculated half-life.

Data regarding plasmids, antibodies, cell culture, transfections, immunoprecipitation, immunostaining, metabolic labelling using 17-ODYA and click chemistry, protein half-life, microtubule transport, and image analysis are detailed in the supplementary data.

Statistical analysis

The statistical analysis used included Student's *t*-test (parametric or non-parametric) in comparison of two populations using the InStat program. In case of multiple comparisons ANOVA analysis was used. Linear correlation curves were used for half-life determination. The later two analyses were conducted using Prism version 4 for Macintosh (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

In utero electroporation

In utero electroporations were conducted as previously described (Sapir *et al*, 2008a, b). Brains were fixed, sectioned, and pictured 4 days after electroporation. Sections from at least four different brains were analysed by counting the number of neurons found in indicated number of individual bins of equal sizes, and their relative percentage in each bin was compared by ANOVA. Pictures were taken from 60-μm thick vibrotome sections using Applied Precision DeltaVision microscopy. Animal protocols were approved by the Weizmann Institute IACUC.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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