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Site-specific dephosphorylation of *doublecortin* (DCX) by protein phosphatase 1 (PP1)

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Mutations in *doublecortin* (DCX) cause X-linked lissencephaly (“smooth brain”) and double cortex syndrome in humans. DCX is highly phosphorylated in migrating neurons. Here, we demonstrate that dephosphorylation of specific sites phosphorylated by JNK is mediated by Neurabin II, which recruits the phosphatase PP1. During cortical development, the expression pattern of PP1 is widespread, while the expression of DCX and Neurabin II is dynamic, and they are coexpressed in migrating neurons. In vitro, DCX is site-specific dephosphorylated by PP1 without the presence of Neurabin II, this dephosphorylation requires an intact RVXF motif in DCX. Overexpression of the coiled-coil domain of Neurabin II, which is sufficient for interacting with DCX and recruiting the endogenous Neurabin II with PP1, induced dephosphorylation of DCX on one of the JNK-phosphorylated sites. We hypothesize that the transient recruitment of DCX to different scaffold proteins, JIP-1/2, which will regulate its phosphorylation by JNK, and Neurabin II, which will regulate its dephosphorylation by PP1, plays an important role in normal neuronal migration.

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Introduction

Mutations in *doublecortin* (DCX) cause X-linked lissencephaly (“smooth brain”) and double cortex syndrome in humans (des Portes et al., 1998; Gleeson et al., 1998). In mice, in utero reduction of DCX’s expression by RNAi has been shown to cause inhibition of neuronal migration (Bai et al., 2003). DCX is a microtubule-associated protein (MAP) that stabilizes microtubules (MTs) (Francis et al., 1999; Gleeson et al., 1999; Horesh

et al., 1999). The interaction with MTs is via an evolutionarily conserved Doublecortin (DCX) domain (Kim et al., 2003; Sapir et al., 2000; Taylor et al., 2000), where most missense mutations cluster. The effect of several of these mutations and the interaction of DCX with MTs studied in vitro and in transfected cells (Sapir et al., 2000; Taylor et al., 2000) demonstrated variable results. While the overexpression of some mutant DCX proteins stabilized MTs, the overexpression of others resulted in less stable MTs, thus suggesting that the dynamic regulation of MTs is an important factor in regulation of neuronal migration. The expression and phosphorylation of DCX are regulated during brain development (Francis et al., 1999; Gleeson et al., 1999). The phosphorylation of DCX by at least three different kinases has been demonstrated: JNK (Gdalyahu et al., 2004), Cdk5 (Tanaka et al., 2004), Protein Kinase A (PKA) and/or the MARK/PAR-1 family of protein kinases (Schaar et al., 2004). JNK phosphorylated DCX on at least three different sites: T321, T331, and S334 (Gdalyahu et al., 2004). Cdk5 phosphorylated DCX on S297 (Tanaka et al., 2004), and a different study suggested that S28 and S339 (which is identical to the S334 site phosphorylated by JNK, numbers differ in two DCX isoforms) are the major Cdk5 sites, with additional minor sites (Graham et al., 2004). PKA and/or the MARK kinase phosphorylated DCX on several sites, with the most significant one being S47 (Schaar et al., 2004). Since addition of low concentrations of okadaic acid, which inhibits PP2A (but not PP1), increased DCX’s phosphorylation, it has been suggested that this is the main phosphatase acting on DCX (Schaar et al., 2004). In vitro analysis indicated that DCX’s phosphorylation by Cdk5, PKA, and MARK reduced the affinity of DCX to MTs (Tanaka et al., 2004; Schaar et al., 2004). DCX is found in high concentrations in the cell soma, tips of neurites, and in growth cones of neurons (Friocourt et al., 2003; Gdalyahu et al., 2004; Schaar et al., 2004; Tanaka et al., 2004). Phosphorylation by Cdk5 apparently controls and localizes DCX to fine perinuclear MTs, but not to MT bundles in proximal processes (Tanaka et al., 2004). This specific localization suggested a unique role for

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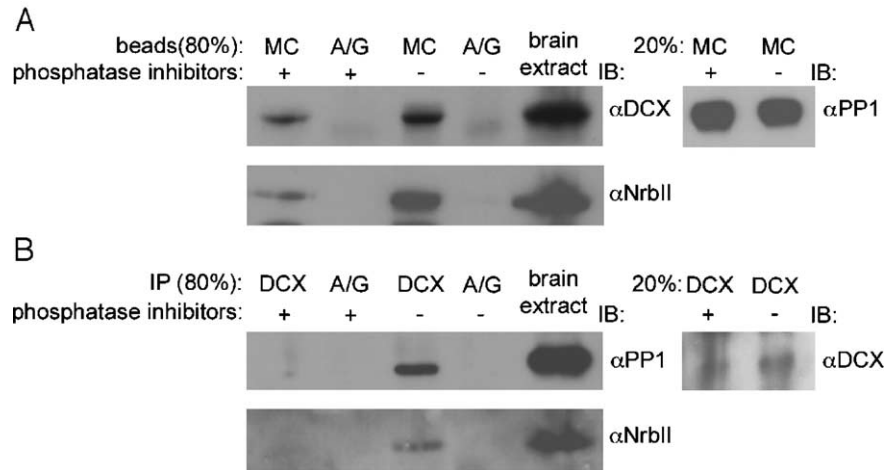


Fig. 1. DCX is found in a protein complex with Neurabin II and with PP1. (A) Brain extract (P6) was subjected to precipitation of PP1 using microcystin (MC) beads, which specifically binds the catalytic site of PP1 and inhibits enzymatic activity. A/G beads with anti-myc antibodies were used as a control. A cocktail of phosphatase inhibitors was added (+) or not (–) to the reaction samples. All samples were subjected to SDS-PAGE and Western blotted with the indicated antibodies. DCX and Neurabin II (NrbII) were detected in the MC precipitate only when phosphatase inhibitors were not included in the reaction. PP1 was detected in MC precipitates with and without phosphatase inhibitors. (B) Brain extract (P6) was subjected to immunoprecipitation of DCX using anti-DCX antibodies or A/G beads together with rabbit anti-mouse IgM as a control. PP1 and Neurabin II (NrbII) were detected in the DCX immunoprecipitate lacking phosphatase inhibitors. DCX was immunoprecipitated in the presence or absence of phosphatase inhibitors.

DCX phosphorylated by Cdk5 during somal translocation. Overexpression of DCX in cerebellar neurons resulted in faster migration, while a point mutation in S297 (the site phosphorylated by Cdk5) or pharmacological inhibition of Cdk5 abolished this effect. The position of DCX in the growth cones, colocalizing with filamentous actin, required phosphorylation by

JNK (Gdalyahu et al., 2004). This localization suggested that DCX's phosphorylation by JNK might play an important role in neurite outgrowth, which is an important step in all modes of migration. Indeed, phospho- and unphospho-mimicry mutants of DCX (for the sites phosphorylated by JNK) exhibited a biological effect. Neurons overexpressing the phospho-mimicry

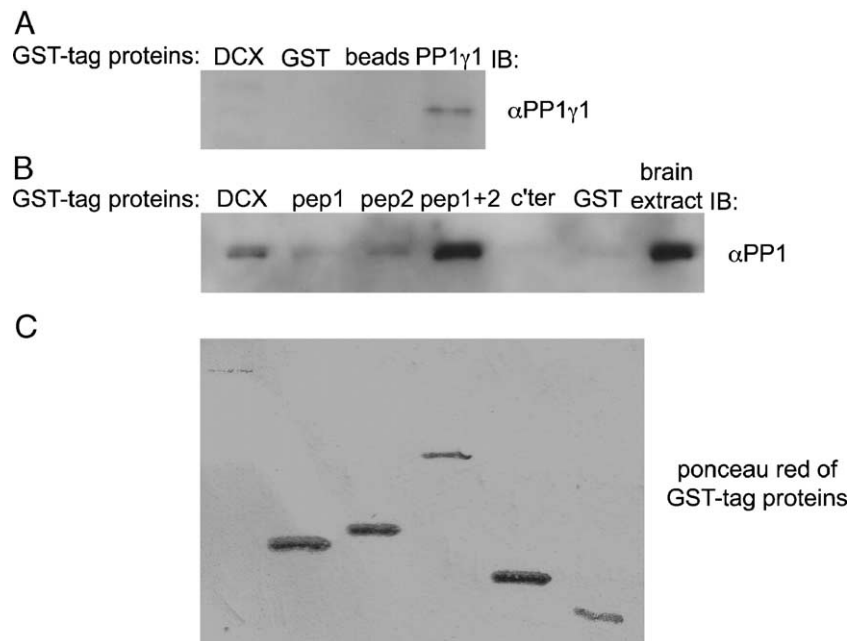


Fig. 2. PP1–DCX interaction required cellular mediators and an intact tandem DCX domain. (A) GST-DCX, GST, or glutathione agarose beads were incubated with recombinant PP1 γ 1; following incubation, the beads were washed, loaded on SDS-PAGE, and Western blotted. The blot was reacted with anti-PP1 antibodies, which detected the protein only in the control lane. (B) GST-tagged proteins DCX, pep1, pep2, pep1 + 2, C-terminus of DCX (c-ter), or GST was used to pull down PP1 from P3 brain extract. PP1 present in the extract (right lane) was pulled down by the full-length DCX and by the tandem DCX domain (pep1 + 2). (C) The recombinant proteins used in A and B were blotted and stained with Ponceau red, demonstrating the relative amounts of the proteins used in these assays, the amount of GST-DCX and GST-pep1 + 2 was slightly reduced in comparison with the other proteins.

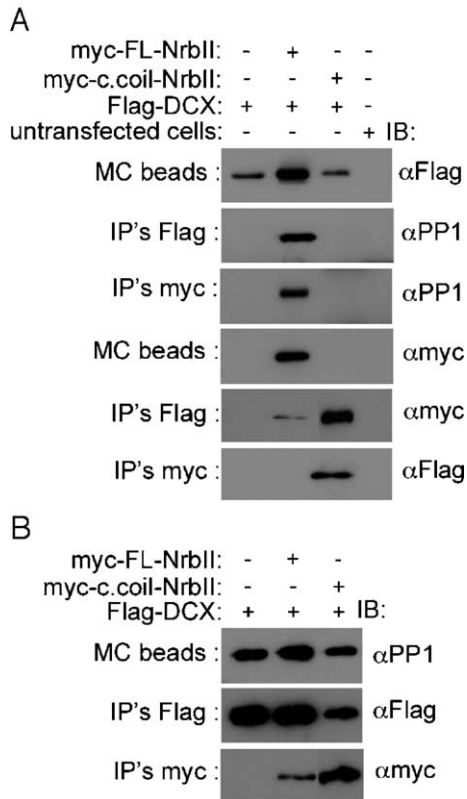


Fig. 3. Neurabin II is required for PP1–DCX interaction. (A) HEK293 cells were transfected with flag-DCX, myc-c.coil-Neurabin (I) and myc-full-length Neurabin II. The cell lysate was subjected to precipitation, using either microcystin (MC) beads, which bind PP1, or the indicated antibodies. 80% of the precipitant was subjected to SDS-PAGE and Western blotted with the indicated antibodies. (B) 20% of the precipitant was subjected to SDS-PAGE and Western blotted with the indicated antibodies.

mutants had more and longer neurites than those overexpressing the unphospho-mimicry mutants. Furthermore, neurons overexpressing the phospho-mimicry mutants moved faster and paused for a smaller fraction of the time than those expressing the unphospho-mimicry mutants (Gdalyahu et al., 2004).

Taken into consideration the importance of DCX's phosphorylation, we set to investigate whether DCX is dephosphorylated by PP1. This investigation was initiated since DCX interacts with Neurabin II (also known as spinophilin) (Tsukada et al., 2003). Neurabin II, an actin binding protein (Satoh et al., 1998), binds to PP1 (Allen et al., 1997). In addition, Neurabin II successfully mediates the interaction between DCX and actin (Tsukada et al., 2005). The coordinated and reciprocal action of serine/threonine (Ser/Thr) protein kinases and phosphatases produces transient phosphorylation, a fundamental regulatory mechanism for many biological processes. In this particular study, we investigated the importance of Neurabin II–DCX interaction in relation to DCX dephosphorylation by PP1 on sites phosphorylated by JNK. Immunoprecipitation studies from brain and mammalian cells demonstrated that Neurabin II is a critical factor in mediating DCX–PP1 interaction *in vivo*. Furthermore, we identified a novel RVXF motif in DCX that allows selective dephosphorylation of sites phosphorylated by JNK.

Results

DCX, Neurabin II, and PP1 are found in the same protein complex

Previously, interactions between Neurabin II and DCX (Tsukada et al., 2003, 2005), or between Neurabin II and PP1, have been reported (MacMillan et al., 1999; Terry-Lorenzo et al., 2002). Here, the presence of the three proteins in the same complex was questioned. Microcystin is a potent inhibitor of PP1, and microcystin agarose beads are used for isolation of PP1 in its native conformation (Colbran et al., 2003). As expected, PP1 was pulled down from brain extract by microcystin beads, as well as Neurabin II and DCX (Fig. 1A). In a reciprocal way, detectable amounts of PP1 and Neurabin II were visible when DCX was immunoprecipitated (Fig. 1B). The addition of phosphatase inhibitors to the immunoprecipitations caused a reduction in the ability of all proteins to interact (Figs. 1A, B). Interestingly, DCX failed to precipitate Neurabin II when phosphatase inhibitors were added. This result may suggest that the DCX–NrbII–PP1 complex is sensitive to the phosphorylation status of the individual components. Many proteins that interact with PP1 contain a canonical PP1-binding sequence (R/KVXF/W) (Aggen et al., 2000; Cohen, 2002; Terrak et al., 2004). DCX contains such a sequence (KVRF, starting at amino acid 54). Taking this into

Table 1

DCX, PP1, Neurabin II, and JIP1 protein interactions

| | Bait (pAS2-1) | Prey (pACT2) | Growth on –His/–Ade |
|----|------------------|------------------|--------------------------|
| 1 | – | PP1 α | – |
| 2 | Dcx | PP1 α | – |
| 3 | Dcx | – | – |
| 4 | PP1 α | – | – |
| 5 | PP1 α | Dcx | – |
| 6 | – | Dcx | – |
| 7 | – | PP1 γ | – |
| 8 | Dcx | PP1 γ | – |
| 9 | PP1 γ | – | – |
| 10 | PP1 γ | Dcx | – |
| 11 | JIP1 | – | + (unspecific) |
| 12 | JIP1 | PP1 α | + (unspecific) |
| 13 | JIP1 | PP1 γ | + (unspecific) |
| 14 | – | JIP1 | – |
| 15 | PP1 α | JIP1 | – |
| 16 | PP1 γ | JIP1 | – |
| 17 | Neurabin II | – | – |
| 18 | Neurabin II | PP1 α | + (specific interaction) |
| 19 | Neurabin II | PP1 γ | + (specific interaction) |
| 20 | – | Neurabin II | – |
| 21 | PP1 α | Neurabin II | + (specific interaction) |
| 22 | PP1 γ | Neurabin II | + (specific interaction) |
| 23 | Neurabin II | Neurabin II-C.C. | + |
| 24 | Neurabin II-C.C. | Neurabin II | + |
| 25 | – | Neurabin II-C.C. | – |
| 26 | Neurabin II-C.C. | – | + |

Yeast cells were cotransformed with a “bait” vector and “prey”. Binding between the “bait” fusion protein and the “prey” fusion protein was assayed by nutritional selection on both histidine-dropout media and adenine-dropout media (as shown in the third column). No interaction was demonstrated between DCX and PP1 α / γ (rows 2, 5, 8, and 10) or JIP1 and PP1 α / γ (rows 12 and 13). Clear interaction between Neurabin II and PP1 α / γ (rows 15, 16, 18, and 19) and Neurabin II with Neurabin II-coiled-coil domain (row 20) was shown.

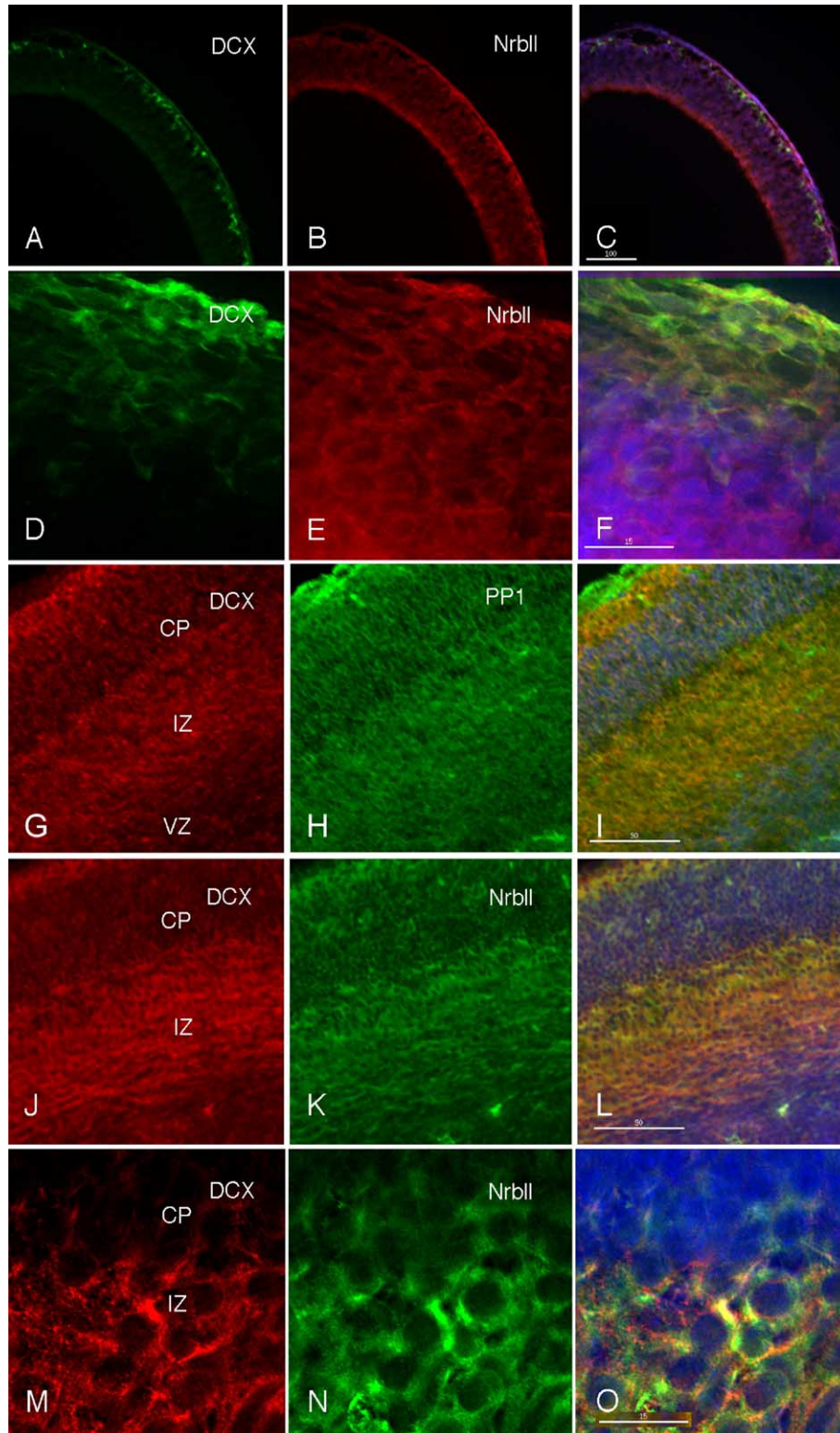


Fig. 4. Expression of DCX, Neurabin II, and PP1 in the developing brain. (A–F) E10.5 mouse brain sections were immunostained with anti-DCX antibodies (A, low magnification, D, high magnification) and anti-Neurabin II antibodies (B, low magnification, E, high magnification), and DAPI was used to label nuclei (merge C, low magnification F, high magnification). (G–I) E15.5 mouse brain sections were immunostained with anti-DCX antibodies (G) and anti-PP1 antibodies (H), and DAPI was used to label nuclei (merge, I). Note the ubiquitous expression of PP1. (J–O) E15.5 mouse brain sections were immunostained with anti-DCX antibodies (J, low magnification, M, high magnification) and anti-Neurabin II antibodies (K, low magnification, N, high magnification), and DAPI was used to label nuclei (merge, L, low magnification, O, high magnification). Pictures were taken using Deltavision RT microscope system, pictures taken at high magnification (60 \times) as Z stacks were deconvolved, and the pictures presented in panels D–I and M–O are a single Z section. Scale bars: C, 100 μ m, F, I, O 15 μ m, L, 90 μ m.

consideration, the possible direct interaction between these two proteins was tested.

Neurabin II is a critical factor for DCX–PP1 interaction

The interaction between recombinant DCX and PP1 γ 1 protein was tested using GST-pull-down assay (Fig. 2A). No recombinant PP1 γ 1 was pulled down using GST-DCX or GST. When a brain extract was used for a similar assay (Fig. 2B), endogenous PP1 was pulled down. It should be noted that brain extract contains endogenous Neurabin II, which we have shown to be present in a complex with DCX and PP1 (Fig. 1). The interaction between DCX and PP1, mediated by Neurabin II, required the intact tandem DCX domain (pep1 + 2) (Fig. 2B). The individual DCX domains (pep1 or pep2) were not sufficient to fully reconstitute this interaction (Fig. 2B). The question whether Neurabin II is necessary for the interaction between DCX and PP1 was investigated using HEK293 cells, which lack endogenous Neurabin II. As predicted, in the absence of Neurabin II, Flag-DCX and PP1 did not co-precipitate (Fig. 3A). Furthermore, the addition of full-length NrbII facilitated the interaction between DCX and PP1 (Fig. 3A). Although considerable amounts of Flag-DCX were precipitated with NrbII-c. coil domain, no PP1 was detected (Fig. 3A). This is readily explained by the lack of PP1 binding site in the NrbII-c. coil domain.

To strengthen the above observations, a two-hybrid assay was conducted. The results (Table 1) demonstrate that Neurabin II-c. coil domain is capable of interacting with the full-length Neurabin II protein in vivo (Table 1, row 20). Confirming published results (Terry-Lorenzo et al., 2002), the interaction domain of NrbII with PP1 is not within the c. coil domain and only full-length Neurabin II interacts with PP1 γ 1 (Table 1, row 15.16). Furthermore, our results indicate that JIP1 does not interact with PP1 or with NrbII,

whereas we have previously demonstrated the interaction between DCX and JIP1 (Gdalyahu et al., 2004). Next, the question when and where do these interactions exist was raised.

DCX and Neurabin II are expressed in migrating neurons

PP1 is known to be ubiquitously distributed and involved in regulation of a broad range of unrelated cellular functions as cell-cycle progression and muscle relaxation (Bollen, 2001; Cohen, 2002). The ubiquitous expression of PP1 is reflected in immunostaining of E15.5 cortical sections (Figs. 4G–I) and of primary hippocampal neurons (Figs. 4D–F). The expression of PP1 was very low in E10.5 cortexes (data not shown), suggesting some developmentally controlled expression. In the developing brain, Neurabin II and DCX were found to be expressed in a regulated manner (Figs. 4A–F, J–O). In cortices of E10.5 embryos, DCX was expressed only in few cells in the marginal zone (Figs. 4A–F), while Neurabin II was more widely expressed. At E15.5, both DCX and Neurabin II were highly expressed in the intermediate zone, where neurons are undergoing active migration (Figs. 4J–O). The expression of Neurabin II was in a gradient, with higher expression in the upper part of the intermediate zone close to the boundary between the intermediate zone and the cortical plate. Higher magnification revealed partial colocalization between Neurabin II and DCX (Figs. 4M–O).

In primary hippocampal neurons, a partial colocalization between DCX and Neurabin II was observed (Figs. 5A–C). Neurabin II localizes to subcellular domains rich in filamentous-actin, whereas the localizations of PP1 and DCX were widespread. Therefore, the observed colocalization of PP1 with DCX was more pronounced (Figs. 5D–F). Next, the possible dephosphorylation of DCX by PP1 was examined.

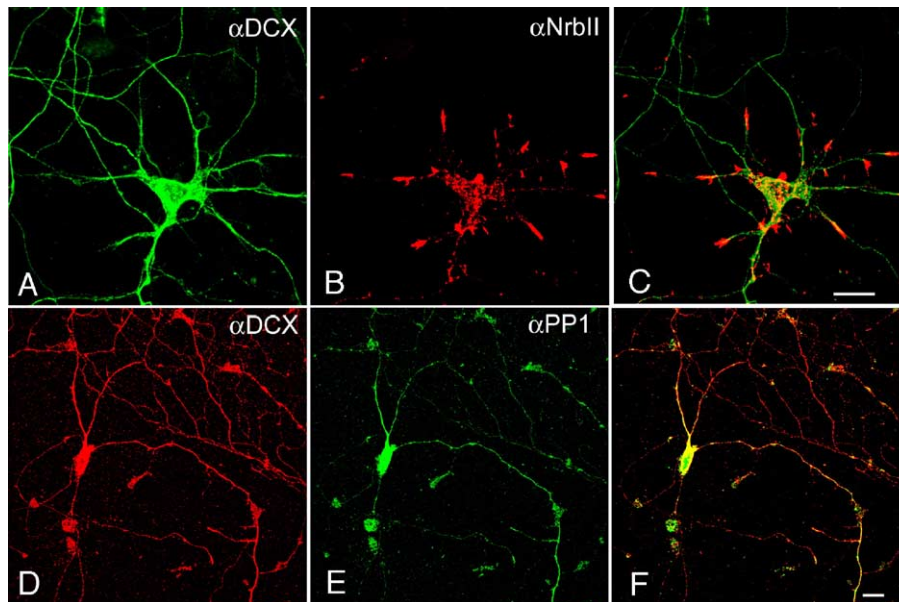


Fig. 5. Intracellular localization of DCX, Neurabin II, and PP1. Primary hippocampal neurons (7 DIV) were immunostained with anti-DCX antibodies (A, D) and anti-Neurabin II (NrbII) (B) or anti-PP1 (E). Note only partial colocalization between DCX and Neurabin II (C), which the colocalization between DCX and PP1 is much more striking (F). All images are maximal-intensity projections of *X/Y* optical section stacks acquired by a BioRad confocal microscope. Scale bar is 15 μ m.

proteins were examined for their *in vitro* dephosphorylation by PP1 γ 1 (Fig. 6C). A striking difference in the dephosphorylation of the two proteins was revealed with anti-phospho-DCX S331, T334 antibodies. The F57L mutated DCX protein remained phosphorylated in comparison with the wild-type protein (Fig. 6C), although some (and probably non-specific) dephosphorylation occurred. Therefore, the existence of the conserved KVRF motif is likely to

play a role in targeting of the enzyme (PP1 γ 1) to the studied substrate (DCX).

PP1 γ 1 dephosphorylates DCX on T331, S334 *in vivo*

Neurabin II is known to dimerize through its C-terminal coiled-coil domain (Nakanishi et al., 1997; Satoh et al., 1998; Oliver et

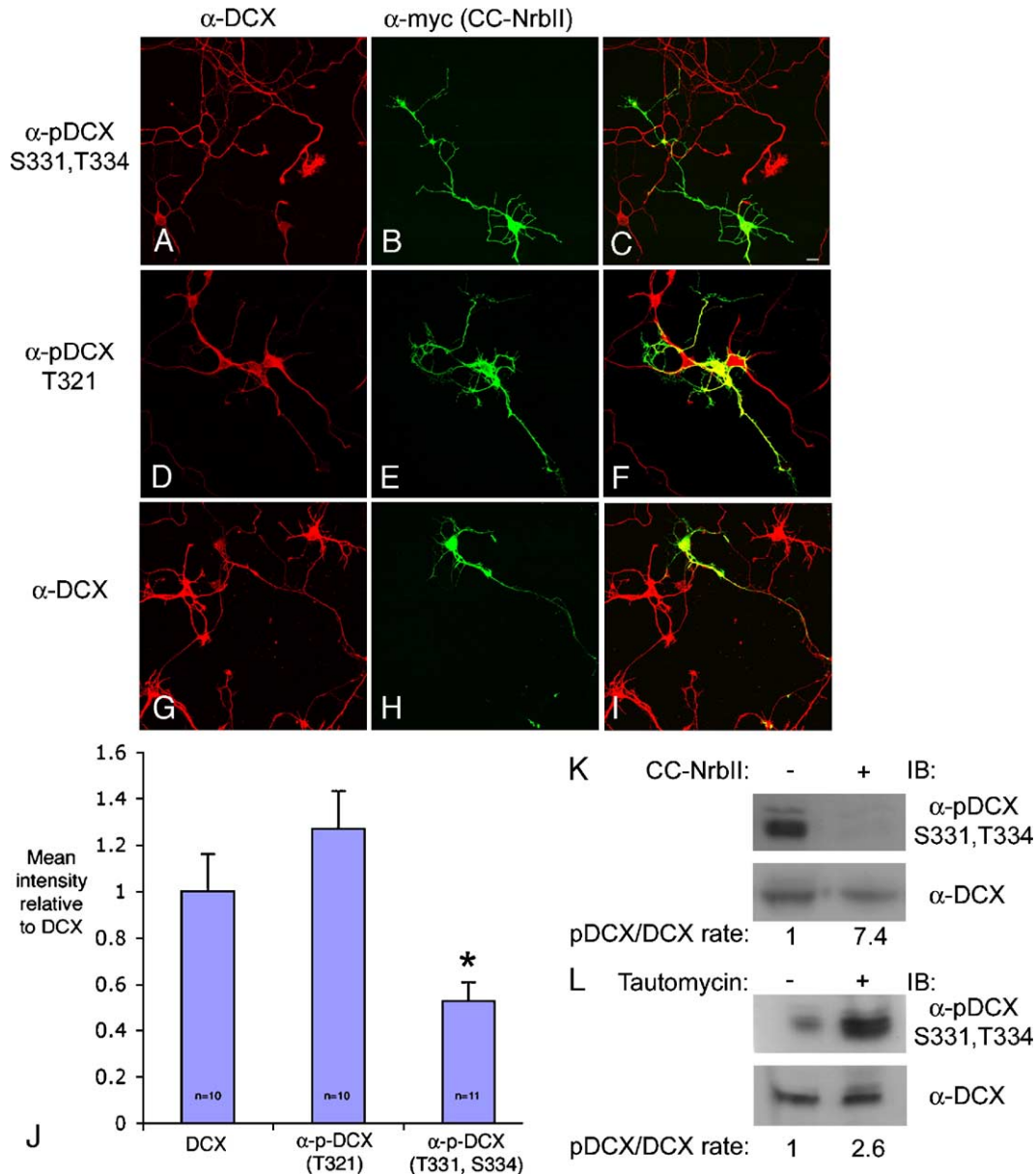


Fig. 7. Overexpression of the myc-tagged coiled-coil domain of Neurabin II specifically reduced DCX phosphorylation on specific sites. Neurons (3 DIV) were transfected with the myc-tagged coiled-coil domain of Neurabin II (CC-NrbII) and then fixed and immunostained with the anti-myc and a panel of three anti-DCX antibodies (anti-phospho-DCX S331, T334, anti-phospho-DCX T321, and anti-DCX) 6 days later (9 DIV). Representative neurons are shown (A–I). All images are maximal-intensity projections of X/Y optical section stacks acquired by a BioRad confocal microscope. Scale bar is 15 μ m. (J) Quantification of the results; at least 10 neurons were analyzed in a quantitative way. A region of interest was defined for each neuron in the myc channel. The mean pixel intensity was calculated, and the ratio between DCX staining and myc staining was calculated. Data were corrected for the background and different intensities of the specific antibodies using other immunostainings. The ratio of staining with anti-DCX versus anti-myc was arbitrary defined as 1. The reduction of staining using anti-phospho-DCX S331, T334 was significant $P = 0.021$ using Welch t test. (K) Cortical neurons were transfected with myc-coiled-coil Neurabin II or left untransfected. Two days post-transfection, cells were collected and lysates were subjected to SDS-PAGE. Blotting the membranes with anti-DCX as well as with anti-p-DCX revealed a seven-fold decrease in DCX phosphorylation on T331, S334 following transfection. (L) P1 mice brain extract was treated with tautomycin (100 μ M final concentration), or with DMSO as a control, for 30 min at 30°. Samples were subjected to SDS-PAGE. Blotting the membranes with anti-DCX as well as with anti-p-DCX revealed a 2.6-fold increase in DCX phosphorylation on T331, S334 following tautomycin treatment.

al., 2002), this same domain (but non-overlapping) mediates its interaction with DCX (Tsukada et al., 2003). Therefore, a construct containing the myc-tagged C-terminal coiled-coil domain including the final 207 amino acids of Neurabin II (CC-NrbII) was prepared. The overexpressed protein interacts with DCX (illustrated also in Fig. 3) and with Neurabin II (Table 1, row 20), thus enabling *in vivo* recruitment of PP1. This construct lacks Neurabin II actin binding domain, therefore it will be expressed in a ubiquitous manner. In transfected primary hippocampal neurons, the CC-NrbII was expressed all over the neuron as evident by immunostaining with anti-myc antibodies (Figs. 7B, E, H). Transfected neurons were subjected to immunostaining with a panel of anti-DCX antibodies phospho-specific T331, S334, or T321 and general anti-DCX antibodies. Only in the case of immunostaining with the phospho-specific T331, S334 antibodies the recognition was markedly reduced (Figs. 7A–C, J). The immunostaining of transfected neurons with the phospho-specific T321 antibodies was slightly increased (Figs. 7D–F, J), while immunostaining with anti-DCX antibodies was used as baseline staining (Figs. 7G–J). To further control this experiment, neurons were transfected with an additional coiled-coil construct containing both a GFP and an myc tag (data not shown) or GFP. Again, the immunoreactivity was reduced only in the presence of the coiled-coil Neurabin II (but not with GFP) and immunostaining with phospho-specific T331, S334 antibodies.

Quantitative biochemical assays complemented the immunostaining results. A marked decrease in DCX (T331,S334) phosphorylation was visible in primary neuronal cultures transfected with the c. coil domain, but not in mock-transfected neurons (Fig. 7K). Furthermore, a specific PP1 inhibitor, tautomycin, significantly increased DCX's phosphorylation on the same sites (Fig. 7L), suggesting that DCX is an *in vivo* PP1 substrate. In the brain, Neurabin II selectively interacts with PP1 γ 1 (Carmody et al., 2004). Collectively, these results suggest that the site specificity of PP1 is maintained *in vivo*.

Discussion

DCX–PP1

DCX is a key player in regulating the migration of post-mitotic neurons, and its activity is regulated by transient protein phosphorylation (Reiner et al., 2004). We have proposed that phosphorylation–dephosphorylation cycles on specific sites may serve as a molecular switch in determining the modes of neuronal migration (Reiner et al., 2004). Here, specific dephosphorylation on DCX's amino acid residues S331, T334 by PP1 has been demonstrated both *in vitro* and *in vivo*. The phosphorylation of these sites is important for neurite outgrowth and for motility of neurons (Gdalyahu et al., 2004). Although direct physical interaction between DCX and PP1 γ 1 has not been demonstrated, the enzyme is capable of dephosphorylating specifically S331, T334, and not other sites, both *in vivo* and *in vitro*, thus suggesting a transient weak interaction, typical for enzymes and substrates. Most of the known PP1 cellular regulatory subunits interact via a conserved “R/KVXF/W” motif frequently preceded by basic residues (Cohen, 2002). Indeed, we identified such a motif within DCX (KVRF, initiating at amino acid 54), which is likely to mediate its selective dephosphorylation by PP1 *in vitro*.

An apparent difference of enzyme specificity towards two sites of the substrate (S331, T334 versus T321) was detected by reactivity of phospho-specific antibodies. It may be explained by the local conformation of the substrate and/or its relative exposure to the enzyme. The determination of sequence specificities of protein phosphatases is not well known. Protein phosphatases are believed to recognize higher order structure in substrates in addition to the primary sequence surrounding the phospho-serine or phospho-threonine. Peptide studies with PP1 have revealed a preference for basic residues N-terminal to the phospho-serine (Bartleson et al., 2003). Interestingly, the S331, T334 site is preceded by several basic residues, while the T321 site is not, suggesting an importance for the primary sequence surrounding the site.

There are multiple examples of site specificity of different phosphatases and a few for PP1. A tyrosine phosphatase PTP ϵ caused reduced phosphorylation of Src at Y527 and indirectly increased phosphorylation via auto-phosphorylation of Src at Y416 (Gil-Henn and Elson, 2003). The Tau protein, which is hyperphosphorylated in Alzheimer's disease, is the substrate of multiple phosphatases. Among the sites dephosphorylated, pT212 was shown to be only the substrate for PP1, and not of other phosphatases (Rahman et al., 2005). Site-specific dephosphorylation by PP2A and PP1 was demonstrated using two phosphorylated sites on smooth muscle myosin light chain (Nomura et al., 1992). *In vivo*, additional levels of regulation are conferred upon PP1 activity, for example, its interaction with inhibitor-1, a potent phosphatase inhibitor (Connor et al., 1998a,b; Endo et al., 1996, 1997) and, as will be discussed below, its interaction with Neurabin II.

DCX–Neurabin II–PP1

In vivo Neurabin II targets PP1 to DCX. It has been recently demonstrated that dimerization of Neurabin I, and of Neurabin II, via the C-terminal coiled-coil domains inhibits filopodial outgrowth and, as such, the interaction of these proteins with filamentous actin. Overexpression of the actin-binding domains of Neurabin I, and II, promoted filopodia (Terry-Lorenzo et al., 2005). Therefore, overexpression of the C-terminal coiled-coil domain is likely to mobilize endogenous Neurabin II from its actin-binding sites and enables positioning of PP1 in close proximity with DCX. As shown in our study, this results in specific dephosphorylation of DCX. Moreover, Neurabin II plays a key role in mediating this interaction. The interaction between Neurabin II and PP1 depends on the RVXF motif in Neurabin II (Hsieh-Wilson et al., 1999; Gibbons et al., 2005). In the developing brain, Neurabin II is coexpressed with DCX in migrating neurons at E15.5, whereas at E10.5 there is much less colocalization between the two proteins. The particular colocalization of Neurabin II and DCX suggested a potential role for Neurabin II in mediating DCX functions in migrating neurons. In addition to targeting PP1, Neurabin II also mediates the interaction between DCX and actin microfilaments (Tsukada et al., 2005). The best characterized activity of Neurabin II (or spinophilin) is its function in dendritic spines (Feng et al., 2000). Neurabin I has been implicated to play a role in spine formation as well (Terry-Lorenzo et al., 2005). A role for DCX in dendritic spines has not been described up to now. So far, no neuronal migration phenotype has been observed in Neurabin II knockout mice (Feng et al., 2000). Nevertheless, the brain size of the knockouts was smaller than that of the wild type, most apparent in the hippocampus. Taken our results into consideration, it may be worth to investigate *in vivo* a possible role for Neurabin II in neuronal migration.

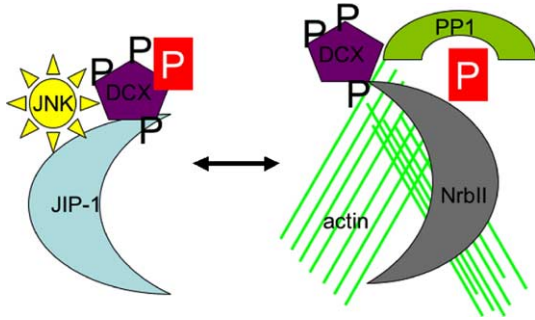


Fig. 8. A schematic model describing the dynamic interactions of DCX with two scaffold proteins (JIP-1/2 and Neurabin II). The interaction with JIP-1/2 facilitates the phosphorylation of DCX on several sites (left side), while the interaction with Neurabin II enables its dephosphorylation on a specific site (indicated in red), but not on other sites.

This study has allowed us to identify specific dephosphorylation of DCX by PP1 through its interaction with the scaffold protein Neurabin II (Fig. 8). This site is phosphorylated by JNK, while DCX is bound to a different scaffold protein JIP-1. In the growth cone, JNK-phosphorylated DCX is enriched in the filamentous actin-rich region. Therefore, in a phosphorylation–dephosphorylation cycle, DCX bound to JIP-1, phosphorylates by JNK, dissociates, and binds to Neurabin II bound to actin, which will mediate its dephosphorylation by PP1.

Experimental methods

Cell culture and transfection

HEK293 cells

293-T cells were grown in DMEM medium (Gibco, Auckland, New Zealand) supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Culture was split using standard trypsinization procedures. Transfections of plasmid DNA were carried out routinely using calcium phosphate precipitation (Graham and van der Eb, 1973), using 10 μ g of plasmid per 9 cm plate.

Hippocampal neurons

Primary hippocampal culture was described previously (Brann et al., 2002). Neurons 3 days in vitro (DIV) were transfected as described previously (Craig, 1991) with removal of DNA mixture after 70 min, or using the Amexa[®] electroporation protocol, fixed, and stained 6 days later. For biochemical measurements, neurons were lysed 2 days after transfection in immunoprecipitation buffer supplemented with protease inhibitor cocktail. Sixty five micrograms of untransfected and transfected neuron cell lysate samples were subjected to SDS-PAGE.

Plasmids and antibodies

Plasmids

Myc-JNK2-MKK7 (kinase active and kinase dead) was received from Dr. Kerkhoff, University of Würzburg (Otto et al., 2000). Flag-DCX, GST tag FL, pep1, pep2, pep1 + 2, and c-ter of DCX were described previously (Hoesh et al., 1999; Sapir et al., 2000). GST-DCX was mutated using PCR-based site-directed mutagenesis with the following primers (and their reverse complement primers): F57L: 5' -AAGCCAAGAAGGTACGTTA-TACCGCAATGGGGACCGCTA-3', Myc-FL-NrbII and myc-coiled-coil-NrbII constructs (containing the 207 C-terminal amino acids of Neurabin II) were received from Dr. Miki Tsukada, PP1 γ 1-pTACTAC expression vector

was received from Drs. Shirish Shenolikar, Richard Honkanen, and David Roadcap (Zhang et al., 1992).

Antibodies

DCX phospho-specific polyclonal antibodies, rabbit anti-GST-DCX, and mouse anti-DCX monoclonal antibodies were described in (Gdalyahu et al., 2004); goat anti-DCX antibodies IgG (SC-8066), mouse monoclonal anti-PP1 IgG_{2b} (SC-7482), goat anti-NrbII antibodies IgG (SC-14774), and mouse anti-myc antibodies (SC-40) were from Santa Cruz, CA, USA. Mouse anti-LIS1 were described in Sapir et al. (1997). Rabbit anti-mouse IgG was a gift from Prof. Eshhar Zelig. The sheep AP-PP1 γ 1 antibodies (#154) were a gift from Dr. Brian Wadzinski. Peroxidase-conjugated antibodies used for immunoblotting included; goat anti-mouse IgG (H + L), goat anti-rabbit IgG (H + L) (Jackson ImmunoResearch, PA, USA); rat anti-mouse Ig_k light-chain (BD Pharmingen, NJ, USA); rabbit anti-sheep IgG (H + L) (Kirkegaard and Perry Laboratories, Maryland, USA); donkey anti-goat IgG (H + L) (Santa Cruz, CA, USA). Secondary antibodies used for immunostaining are Cy3-conjugated donkey anti-goat IgG (H + L), Cy2-conjugated donkey anti-mouse IgG (H + L), Cy2-conjugated donkey anti-rabbit IgG (H + L), Cy3-conjugated goat anti-rabbit IgG (H + L), and fluorescein (FITC)-conjugated affinitypure goat anti-rabbit IgG (H + L); these were from Jackson ImmunoResearch, PA, USA. Alexa Fluor[®] 488 goat anti-mouse IgG (H + L) was from Molecular Probes, OR, USA.

Immunostaining

Neurons

Neurons were fixed and stained after 7–10 DIV as described (Schwarz and Futerman, 1996); coverslips were mounted with Vectashield or HardSet (Vector, CA) and examined using a BioRad confocal microscope.

Brain sections

E10.5 and E15.5 embryos for immunostaining were perfused using 4% PFA and postfixed in 4% PFA for 30 min. Embryos were cryoprotected in 20% sucrose–PBS, and coronal sections (20 μ m) were collected on slides. Sections were rehydrated with PBS-T (1 \times PBS, 0.1% Triton X-100), blocked in PBS-T 10% FCS, and labeled in 4 $^{\circ}$ C for ON with different antibodies. Slides were washed with PBS-T and incubated with secondary antibodies for 1 h RT washed with PBS-T and mounted using Xylene with DAPI (4',6'-diamidino-2-phenylindole) (Sigma, Rehovot, Israel). Data were analyzed using a Deltavision RT microscope.

Proteins

GST-tagged protein purification

GST plasmids, representing different DCX fragments, were transfected into BL21(DE3)RIL bacteria (Stratagene, La Jolla, CA), which were grown in LB at 30 $^{\circ}$ C to an optical density of 0.7–0.8. Induction of protein expression was carried out using 0.2 mM IPTG for 4 h. Protein was extracted in NETN buffer (0.5% Nonidet P-40, 0.1 M NaCl, 1 mM EDTA, 20 mM Tris–HCl, pH 8.0) with sonication. The soluble fraction was bound to glutathione agarose beads (Sigma, Rehovot, Israel) for 1 h, cleaned by centrifugation above 20% sucrose–NETN, and washed extensively in NETN buffer before elution from column in 10 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 10 mM glutathione, 10% glycerol. For kinase assays, GST-DCX protein was kept conjugated to glutathione agarose beads.

PP1 γ 1 purification

The phosphatase was purified according to a protocol received from Dr. Shirish Shenolikar. The PP1 γ 1 expression plasmid was transfected into BL21(DE3)RIL bacteria (Stratagene, La Jolla, CA), which were grown in pre-chilled LB with ampicillin, 1 mM MnCl₂, and 50 μ M IPTG at 15 $^{\circ}$ C until OD reached 0.6–0.8. Cells were harvested and resuspended in 50 mM Tris pH 7.5, 0.1 mM EGTA, 1 mM MnCl₂, 0.1% β ME with protease inhibitors (1 mM PMSF, 1 mM benzamidine). Cells were lysed in a French press, and a cleared lysate was loaded (at \sim 3 ml/min) onto a

5 ml heparin Sepharose column (Pharmacia) pre-equilibrated in low-salt buffer (20 mM Tris base pH 7.4, 0.1 mM EDTA, 2 mM MnCl₂, 0.2% 2-ME, 0.01% Brij 35, and 20% glycerol (V/V)). The column was then washed with 20–50 ml low-salt buffer, and protein elution from column was done with 0–100% linear gradient of high-salt buffer (low-salt buffer with 1 M NaCl). Fractions were assayed for phosphatase activity using *p*-nitrophenylphosphate (pNPP) solution (50 mM MES pH 7, 0.5 mg/ml BSA, 2 mM pNPP).

Immunoprecipitation

Immunoprecipitation was performed from ICR mice (P6) brain extracts prepared in IP buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100) supplemented with protease inhibitors (Sigma, Rehovot, Israel). One milligram of brain extract was incubated with indicated antibodies for 2 h at 4°C. Following this, 10 µl (bed volume) of protein A/G agarose (Santa Cruz, San Diego, CA) or microcystin agarose beads (#16-147, Upstate, NY) preblocked in IP buffer supplemented with 10 mg/ml BSA (Sigma, Rehovot, Israel) was added to each sample for additional 2 h. Immunoprecipitated proteins were pelleted by centrifugation, washed three times with IP buffer, eluted by addition of SDS-PAGE sample buffer, boiled for 2 min, and analyzed on SDS-PAGE.

HEK293 cells immunoprecipitation was similar to immunoprecipitation from brain extract, with an additional step of preclearing the cell lysate on A/G beads for 1 h at 4°C.

GST pull-down assay

Mouse P3 brain extract was prepared in T-T buffer (20 mM Tris–HCL pH 8, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100), supplemented with protease inhibitors (Sigma, Rehovot, Israel). Recombinant protein (PP1γ1) or brain extract (1 mg) was incubated with 10 µl of GST-tagged proteins at 4°C for 3 h. Glutathione beads (10 µl bed volume) preblocked in T-T buffer supplemented with 10 mg/ml BSA (Sigma, Rehovot, Israel) were added to the protein mixture and rotated for 30 min at RT; after four washes with T-T buffer, 2× sample buffer was added, and the beads were boiled and subjected to SDS-PAGE gel analysis.

Yeast two-hybrid assay

Yeast two-hybrid assay was performed as described by Sweeney et al. (2000).

Kinase and phosphatase assays

293-T cells were transfected with Myc-JNK2-MKK7 (kinase active and kinase dead). After 48 h, cells were washed in PBS, collected by scraping, pelleted, and resuspended in 2× kinase reaction buffer (100 mM β-glycerophosphate, 4 mM DTT, 40 mM MgCl₂, 2 mM sodium orthovanadate, 2 mM benzamidine, 2 mM EDTA, 2 mM EGTA) supplemented with protease inhibitors cocktail (Sigma, Rehovot, Israel). Cells were sonicated 3 times for 10 s every 1 min and were precipitated by centrifugation at 13,000 rpm for 15 min at 4°C. One milligram of supernatant (~100 µl) was incubated with 150 µM ATP (for a cold reaction) or 20 µM ATP supplemented with 2.5 µCi [³²P]ATP (for the radioactive reaction) along with 20 µl packed volume of GST-DCX beads for 1.5 h or 30 min at 30°C, respectively. Beads were washed three times with 2× reaction buffer, the fourth wash was with 1× PP1 reaction buffer (50 mM HEPES, 5 mM dithiothreitol, 0.1 mM Na₂EDTA, 0.025% Tween-20, 1 mM MnCl₂, adjusted to pH 7 with 10 M NaOH). Beads were resuspended in 2× PP1 reaction buffer brought to final volume of 100 µl containing PP1γ1 protein and incubated at 30°C for the times detailed in the legends to figures. Beads were washed once with 1× PP1 reaction buffer, and the conjugated proteins were eluted by addition of SDS-PAGE sample buffer, boiled for 2 min, and analyzed on SDS-PAGE followed by immunoblotting and/or autoradiography.

Tautomycin treatment

Brain extract was prepared from ICR P1 mice using immunoprecipitation buffer supplemented with protease inhibitor cocktail. After extraction, the extracts were centrifuged at high speed for 10 min. Tautomycin

(A.G. Scientific, San Diego, CA) was added to the brain extract at a final concentration of 100 µM. Another tube with brain extract was supplemented with DMSO as a control. After 30 min at 30°C, sample buffer was added to the samples, boiled, and subjected to SDS-PAGE.

Quantification

Normalized intensity values were derived by dividing the luminosity of the phospho-specific bands or those of the autoradiogram bands, with the luminosity of the related total (αDCX) bands subtracting the related background band. Analysis of covariance was used to examine the effect of antibody and time and to compare the rate of change over time for the two antibodies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mcn.2006.01.014.

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