[Cell Cycle 5:9, 976-983, 1 May 2006]; ©2006 Landes Bioscience

Report

Common and Divergent Roles for Members of the Mouse DCX Superfamily

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Original manuscript submitted: 02/21/06
Manuscript accepted: 03/18/06

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/abstract.php?id=2715

KEY WORDS

doublecortin, microtubules, protein family, cytoskeleton, actin, intracellular localization

ACKNOWLEDGEMENTS

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NOTE

Supplementary Material can be found at:
http://www.landesbioscience.com/journals/cc/coquelleCC5-9-sup.pdf

ABSTRACT

The doublecortin-like (DCX) domains serve as protein-interaction platforms. DCX tandem domains appear in the product of the X-linked doublecortin (*DCX)* gene, in retinitis pigmentosa-1 (*RP1)*, as well as in other gene products. Mutations in the human *DCX* gene are associated with abnormal neuronal migration, epilepsy, and mental retardation; mutations in *RP1* are associated with a form of inherited blindness, while *DCDC2* has been associated with dyslectic reading disabilities. Motivated by the possible importance of this gene family, a thorough analysis to detect all family members in the mouse was conducted. The DCX-repeat gene superfamily is composed of eleven paralogs, and we cloned the DCX domains from nine different genes. Our study questioned which functions attributed to the DCX domain, are conserved among the different members. Our results suggest that the proteins with the DCX-domain have conserved and unique roles in microtubule regulation and signal transduction. All the tested proteins stimulated microtubule assembly in vitro. Proteins with tandem repeats stabilized the microtubule cytoskeleton in transfected cells, while those with single repeats localized to actin-rich subcellular structures, or the nucleus. All tested proteins interacted with components of the JNK/MAP-kinase pathway, while only a subset interacted with Neurabin 2, and a nonoverlapping group demonstrated actin association. The sub-specialization of some members due to confined intracellular localization, and protein interactions may explain the success of this superfamily.

INTRODUCTION

The existence of a shared defined protein domain among different proteins is usually accompanied with common functional activities. Although multiple algorithms define protein domains based on amino acid similarity, the exact definition of which functions are shared still needs to be demonstrated experimentally. The focus of our study is the evolutionary conserved doublecortin (DCX) domain.[1,2] Mutations in the X-linked gene DCX result in lissencephaly in males, and abnormal neuronal positioning (subcortical band heterotopia) in females.[3,4] The first function attributed to the DCX domain, which appears in tandem in DCX, was its interaction with the microtubule (MT) cytoskeleton.[5-7] This activity was detected also in a related gene product DCLK,[8,9] and in RP1 which contains two DCX domains.[10] RP1 is the protein product of retinitis pigmentosa-1 (*RP1)*.[11,12] Mutations in this gene result in progressive blindness, not only in humans, but also in a mouse model.[13] Recently, an additional member of this protein family, doublecortin kinase 2, has been described, and found to posses MT binding activities.[14] The structure of the DCX domain is a ubiquitin-like fold,[15] different from that of classical MAPs. DCX is embedded in fenestrations localized between protofilaments from which MTs are built.[16] This is a previously uncharacterized MAP binding site, ideal for microtubule stabilization.

DCX is not only a unique MAP, but it also interacts with multiple additional proteins (reviewed in refs. 17 and 18). Notably, DCX interacts with two scaffold proteins neurabin 2 and JIP1/2. Neurabin 2 connects DCX to actin microfilaments.[19,20] In addition, neurabin 2 binds to protein phosphates 1 (PP1), thereby regulating site-specific dephosphorylation of DCX (Shmueli et al., in press). JIP1/2 (JNK Interacting Protein 1 or 2) acts as a scaffold for both DCX and one of the kinases that phosphorylates it: JNK (cJun-N-terminal Kinase).[21] The phosphorylation state of DCX on JNK sites affects neuronal motility in vitro. In addition, JIP1 connects DCX to the molecular motor conventional kinesin, which mobilizes DCX in neurons.[21] We hypothesized that the selective recruitment of DCX to either a neurabin 2 complex or a JIP1 complex is important in regulating its activity.[22]

Although it is clear that the DCX-domain proteins are an important group of proteins, the characterization of the group as a whole is still lacking. So far no studies have addressed the possible common functions among them. Here, we have undertaken to study proteins interactions, MAP activity, and the subcellular localization of nine of the superfamily members. Our study highlights common and unique features for various members of this superfamily, illustrating their widespread functional importance.

MATERIALS AND METHODS

Database homology search. Our database similarity search initiated with an NCBI blast search using the sequence of mouse doublecortin protein (NP_034155.2). In addition, all mouse proteins with the DCX domain were retrieved from the CDD (Conserved Domain DB). In a similar fashion, DCX proteins from the EBI InterPro db, which were not detected in CDD were extracted and added.

Plasmid construction. Sequences of the primers used in this study are found in Supplementary Fig. 1. The ORF of *BAC26042* was amplified by PCR from the RIKEN clone 4732421G10, accession number XM_489891.1 (RIKEN® FANTOM™ CLONES, K.K. Dnaform, Ibaraki, Japan). The N-terminus of *Rp1* (amino acids 1-262, encompassing both DCX repeats), was amplified from the IMAGE clone 2123311, (Invitrogen Life Technologies, Paisley, Great Britain). The ORF of *Dcdc2* was amplified by PCR from the IMAGE clone 6306251. A DNA fragment encoding the N-terminus of DCDC2B (amino acids 1-83) was amplified by PCR from the RIKEN clone E030003B04. A DNA fragment from DCLK2 (amino acids 41-295) was amplified by RTPCR from RNA extracted from E12 mouse brain. A DNA fragment encoding the N-terminus of RP1L1 (amino acids 31-261) was amplified by RTPCR from RNA extracted from P12 mouse eye. A region of FLJ46154 (amino acids 226-500) was amplified by RTPCR from RNA extracted from adult mouse eye. PCR fragments were digested by EcoRI and XhoI (or EcoRI and SalI for FLJ46154), and cloned in pBluescript II KS(-) (Stratagene, USA), peGFP-C2 (Clonetech, USA), pcDNA3 (Invitrogen, USA) PCR-modified in order to encode the FLAG tag, and pGEX4T1 (Pharmacia, USA) at the EcoR1 and XhoI restriction sites. All PCR products and all translation phases of the fusion proteins were verified by sequencing. Vectors encoding FLAG-DCX, FLAG-DCLK, GFP-DCLK, DCX-DsRed1, GST-DCXpep1+2, GST-DCLK and GST-DCL were described previously.[1,5,8,23] Vectors encoding Myc-JNK2-MKK7 and Myc-JIP1/2 were described elsewhere.[21] The plasmid encoding the coiled-coil domain of Neurabin II was received from Dr. Miki Tsukada. GST fusion peptides were expressed in BL21 lysE strain of *Escherichia coli*, and purified using glutathione-agarose beads (Sigma, Israel).

Electron microscopy. Samples were prepared using tubulin in PEM buffer (100 mM PIPES pH 6.9, 1 mM $MgSO_4$ and 1 mM EGTA), at a concentration of 20 μM (according to the monomer, around 1.1 $mg.ml^{-1}$) with or without the addition of 0.5 or 2 μM of indicated proteins and incubated in the presence of 2 μM GTP (sodium salt, Sigma, Israel) for 30 min at 37°C. Four to eight microliters of each sample was applied to a carbon-coated copper 400-mesh electron microscope grid, which had been previously glow-discharged for 30 sec. to render the carbon surface hydrophilic. The sample was then stained with two successive 10 μl drops of uranyl acetate stain (1% in water). The grid was blotted after 1 minute. Samples were examined with an FEI T12 electron microscope operating at 120 kV. Images were acquired on a SIS MegaviewIII CCD camera.

Cell culture. Culturing of COS-7 and HEK-293 cells were performed as described previously.[24,25]

Antibodies. Primary antibodies used in this study were anti α-tubulin (clone DM1A, Sigma, Israel), anti-actin (clone sc-40, Santa Cruz, USA), anti-FLAG (M2 clone from Sigma, Israel) and anti-myc (E910 clone from Sigma, Israel). In addition, anti-DCX antibodies,[1] anti-DCLK antibodies,[8] and anti-DCLK2 antibodies were used for Western blots. A synthetic peptide corresponding to the unique C terminus of *Mus musculus* DCLK2 (aa 741–756) was conjugated to KLH (keyhole limpet hemocyanin) and used as immunogen in rabbit. Rabbit antiserum was used in a 1:250 dilution for Western blot analysis. Secondary antibodies used for the immunofluorescence studies were made in goat and conjugated to CY3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or ALEXA-488 (Molecular Probes). Secondary antibodies used for the immunoblots were peroxidase-conjugated AffiPure Goat anti-mouse or anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Immunostaining. Fourty-eight hours after transfection, COS-7 cells were fixed in 3% paraformaldehyde (Sigma, Israel), 0.05% glutaraldehyde (MERCK, Germany) in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2mM magnesium acetate, pH6.9). They were washed three times 5 min in phosphate-buffered saline (PBS) and permeabilized for 25 min in 0.1% Triton X-100 in PBS. The cells were then treated twice with 1 $mg.ml^{-1}$ $NaBH_4$ in PBS, for 10 min, washed three times 5 min in PBS, blocked in PBS/BSA (PBS containing 0.1% BSA) and labeled with the monoclonal antibody DM1A raised against α-tubulin, or stained with rhodamine- or Alexa-488-phalloidine (Sigma, Israel) for 1 hour at 37°C (humid atmosphere). After washing 3 times 10 min in PBS/BSA, in the case of tubulin staining, the cells were incubated with the secondary antibody for 45 min at 37°C (humid atmosphere). Chromosomes were stained with 0.1 $\mu g.ml^{-1}$ DAPI in PBS (Sigma, Israel) for 5 min. Coverslips were mounted using Immu-Mount (Thermo Shandon, USA). All antibodies were diluted in PBS/BSA. All images are maximal-intensity projections of X/Y optical section stacks acquired by a BioRad confocal microscope.

Immunoprecipitation assay. Immunoprecipitation was preformed from transfected-HEK293 cells using FLAG-M2 beads (Sigma, Israel) or A/G beads (Santa Cruz, USA) along with the c-myc 9E10 monoclonal antibody. Cells were cotransfected with each of the FLAG-doublecortin proteins and each of the myc-tagged-DCX-interacting proteins that we investigated (see hereafter). Transfected-cell extracts were prepared in IP buffer (Tris-HCl 50 mM, pH 6.8 at room temperature, NaCl 0.15 M and Triton X-100 1%) supplemented with protease-inhibitors cocktail (Sigma, Israel). The samples were separated by SDS-PAGE and immunoblotted with the suitable combination of antibodies [anti-FLAG (M2 clone) or anti-myc (E910 clone)].

GST pull-down assay. P1 mouse brain extract was prepared in T-T buffer (20 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA and 0.1% Triton X-100), supplemented with protease-inhibitors cocktail (Sigma, Israel). 1.5 mg protein-containing-brain extract were incubated with 10 μg of GST-fusion proteins, at 4°C for 3 hours. Glutathione beads (15 μl, Sigma, Israel) in 30 μl of T-T buffer were added to the protein mixture and rotated for 30 min at room temperature. After three washes with T-T buffer, 3X sample buffer was added and beads were boiled and ran on SDS-PAGE gel. Immunoblot was performed with anti α-tubulin and anti actin antibodies.

Tubulin and MAPs purification. Brains from P7 ICR pups were retrieved and homogenized in cold PEM buffer (100 mM Pipes, 2 mM EGTA, 1 mM $MgSO_4$) containing 10% Glycerol, 1 mM ATP and 1 mM PMSF) and ultracetrefuged (100,000 x g) to obtain a clear lysate. To the lysate extra ATP (1 mM) GTP (1 mM) and Glycerol (30%) were added before incubation at 34°C for 45 minutes. Polymerized MTs were pelleted at 100,000 x g at 34°C and the pellet was resuspended in cold PEM containing 2 mM GTP, homogenized and depoymerized further by incubation of ice. Tubulin was spun at 100,000 before loading onto a phosphocellulose column (Pharmacia). MAPs were eluted from the column using 0.4M NaCl.

Microtubule assembly. Tubulin from calf brain was purified as described previously.[26] The assembly rate of tubulin to form polymers was monitored using a light scattering assay.[27,28] Purified tubulin was diluted in PEM buffer to a final concentration of 20 μM (according to the monomer, around 1.1 $mg.ml^{-1}$). GST-recombinant proteins were concentrated using Vivaspin columns (Vivascience, Germany) in EB buffer (100 mM Tris-HCl, 120 mM NaCl, 10% glycerol). 0.5 or 2 μM recombinant proteins were added to the tubulin in a volume of EB buffer corresponding to 10% of the final volume, so that 1% glycerol is reached. Absorbance was measured at 350 mm at 1 min intervals in a Uvicon spectrophotometer equipped with temperature controlled cells. Switching the temperature to 37°C induced assembly, and to 4°C the disassembly.

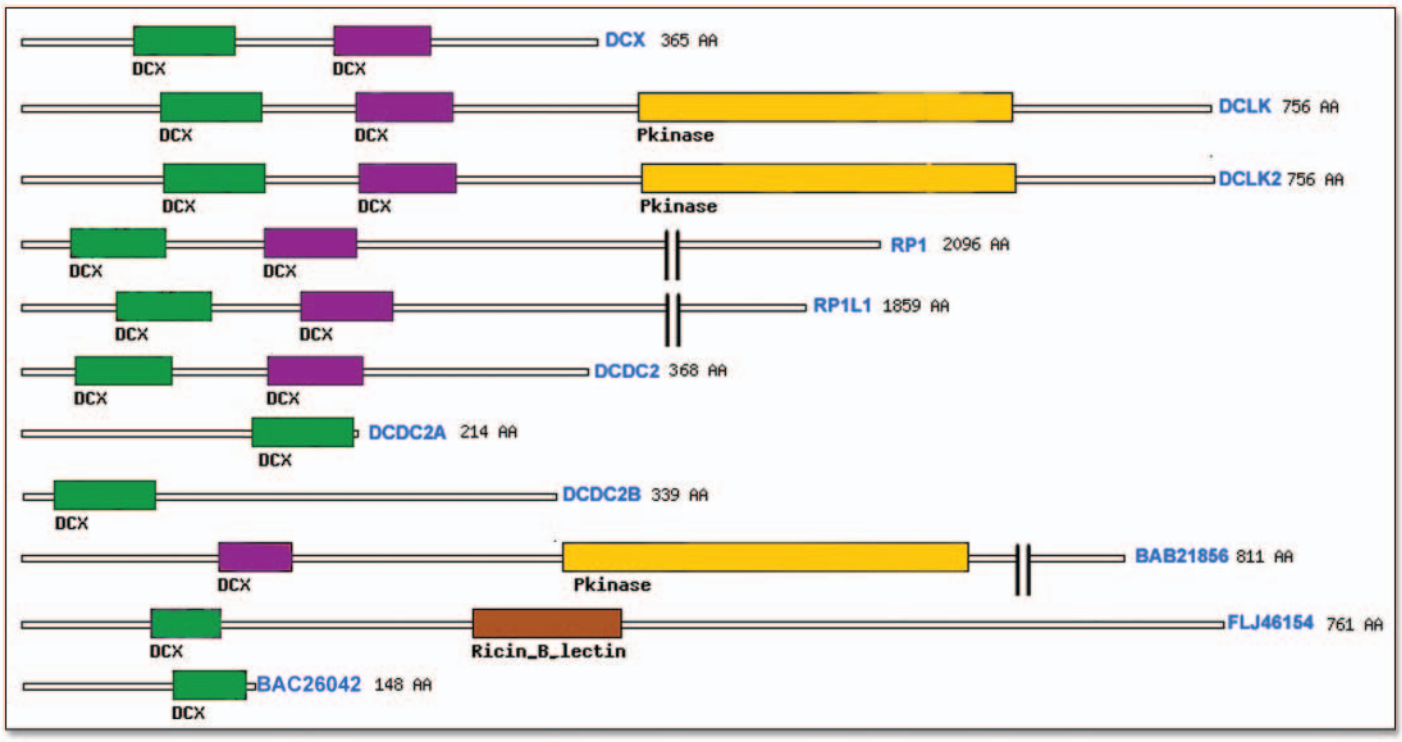

Figure 1. Schematic presentation of the mouse DCX superfamily. DCX domains most similar to the N-terminal part of DCX are labeled in light grey, whereas those most similar to the C-terminal are marked in dark grey. Additional protein domains noted are protein kinase (Pkinse) and lectin binding (Ricin_B_lectin).

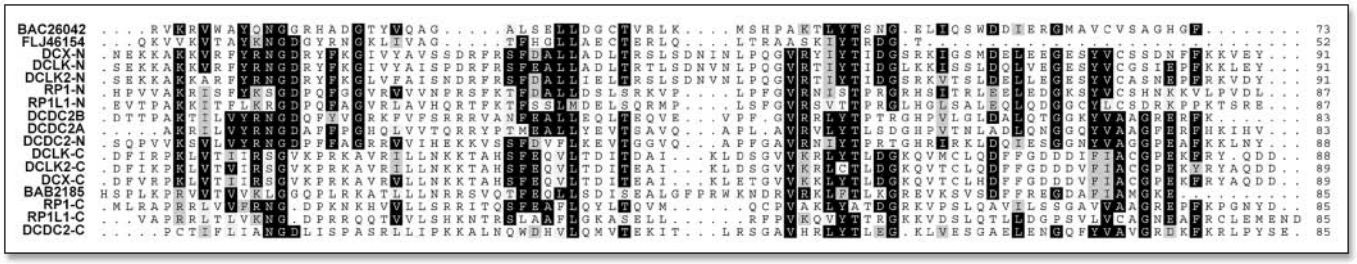

Figure 2. Multiple alignment of the 17 individual DCX domains. ClustalW program was used to create a multiple alignment, the graphic output was generated using GCG prettybox program.

RESULTS

Identification of mouse proteins containing a DCX domain. The mouse proteome was searched for sequences similar to that of the DCX domain resulting in a total of 11 proteins containing one or two DCX repeats. A schematic presentation of the protein products is shown (Fig. 1). In addition to DCX domains, three proteins contained kinase domains, and one contained a ricin lectin site, which is predicted to bind carbohydrates.[29]

Since several of the proteins contain a single DCX domain, we conducted analysis for the seventeen individual DCX domains. Closer inspection of the protein sequences of the DCX domains by multiple alignment, revealed several interesting features (Fig. 2). The sequences of the N-terminal DCX domain in proteins containing a tandem domain are more similar to each other than to the C-terminal DCX domain of the same protein. Most of the proteins with a single DCX domain are more similar to those from the N-terminal group and one is more similar to the C-terminal group.

The presence of the DCX domain in multiple different gene products raised the question whether they sharing the same functions. Full length cDNA's or fragments containing the corresponding DCX domains from nine different gene products were cloned in different expression vectors (details in methods). Additional information regarding the chromosomal positions of these genes, their accession numbers, and the number of exons are found in (Supplementary Table 1).

DCX-domain proteins and Mts. DCX is best known for its MAP activity. The effects of the DCX repeat proteins on MT assembly rate were assayed in vitro using light scattering assay (Figs. 3A, C, D). This assay is based on an increase in optical scattering as microtubules polymerize, measured as an effective optical density. Most DCX proteins enhanced MT polymerization, albeit at different levels (Fig. 3A), suggesting the formation of cold resistant MT structures. Normal polymerized MT morphology was confirmed by subjecting the reactions containing the recombinant proteins to negative staining (see electron microscope images of MT assembled in the presence of FLJ46154 (Fig. 3B), other reactions are not shown). FLJ46154 did not induce MT bundling as could be expected with a single DCX domain. DCDC2 and the tandem DCX domains of RP1 induced MT polymerization albeit at lower levels (Fig. 3C). An additional protein examined, DCL (a normal product of the DCLK gene lacking the kinase domain)[30] allowed the formation of MT that exhibited high stability to the cold (Fig. 3D).

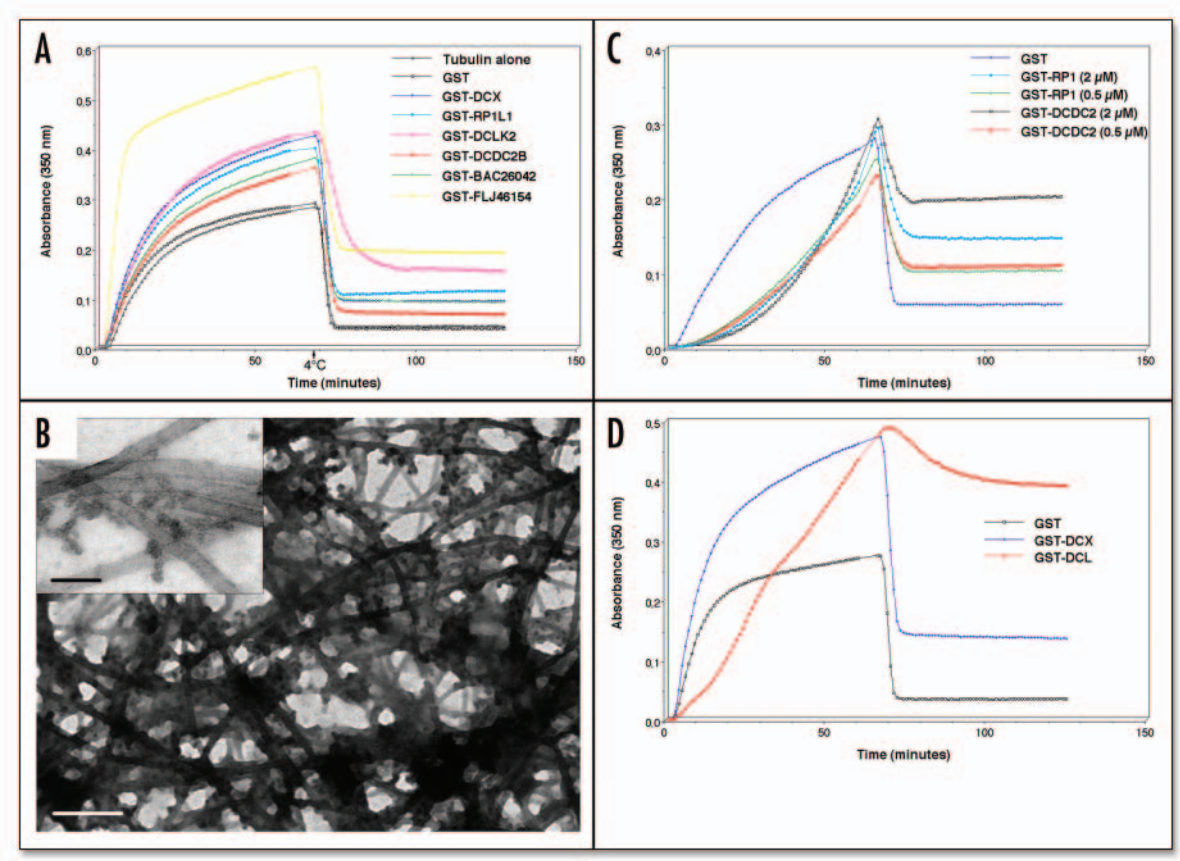

Figure 3. DCX-motif peptides increase microtubule assembly rate in vitro. The assembly rate of tubulin was measured by turbidity assay as described in methods. The GST-fusion proteins included (A) GST, DCX, BAC26042, FLJ46154, DCLK2, RP1L1, and DCDC2B at 2 μM. (C) GST, RP1, DCDC2 (0.5 or 2 μM). (D) GST, DCX, DCL (2 μM). (B) Electron microscopy analysis of MTs in the presence of FLJ46154. A dense mesh of MTs was visible using low magnification, individual MTs can be observed using a higher magnification (small insert).

Electron microscopy of MTs assembled in the presence of DCL did not reveal any abnormal structures or bundling (data not shown).

Testing the effect of DCX-domain proteins in cells is expected to be more indicative of their physiological functions. The expression of the tandem DCX domain is required to stabilize MTs in transfected cells.[1] Therefore, this specific group of proteins was tested in transfected COS-7 cells using GFP fusion proteins (Fig. 4), or Flag-tagged proteins (data not shown). Among the tested proteins, DCX, DCLK and RP1 are known MAPs[5-10] (Fig. 4A1–8, B1–8 and D1–8). Here we show that the DCX domains of DCLK2 (Fig. 4C1–8), and RP1L1 (Fig. 4E1–8) also localize along MTs. In the case of DCDC2 MT bundles were observed in some cells (Fig. 4F1–8), while most cells exhibited nuclear and nucleolar localization (Supplementary Fig. 2), thus suggesting a tight regulation of DCDC2 subcellular localization. DCDC2 and the DCX domains of RP1 and RP1L1, gave rise to thinner MT bundles than the other proteins (compare Fig. 4F1–8, D1–8 and E1–8), suggesting a gradation of MT affinity among the DCX domain proteins. Differences in the cellular distribution of MT bundles were also observed. While overexpressed DCX, DCLK, DCLK2 and RP1L1 provoked the distribution of variable MT bundles, RP1 and, to some extent, DCDC2 induced MTs in concentric circles around the nucleus (Fig. 4).

The relative stability of MTs in transfected cells was examined by treating cells with 33 μM nocodazole, which usually results in disruption of the MT cytoskeleton (untransfected cells in Fig. 4 and Supplementary Fig. 2). MT bundles exhibited variable degrees of resistance to nocodazole treatment, however no correlation was observed between the initial thickness of the bundles and their stability. DCDC2, RP1 and RP1L1, that induced thinner bundles, stabilized MTs with the same efficacy as DCX, DCLK and DCLK2 (compare Fig. 4F1–8, D1–8 and E1–8 to Fig. A1–8, B1–8 and C1–8). Proteins with a single DCX repeat did not offer nocodazole protection as expected (data not shown).

DCX proteins and the nucleus. Two DCX repeats are required for MT interaction and bundling,[1] indeed proteins expressing a single DCX repeat did not localize along tubulin polymers (Fig. 5). A unifying and prominent feature among the different DCX repeat proteins is their apparent nuclear localization. Two exceptions were noted with DCLK, and DCX where only their Flag-tagged versions were observed also in the nucleus (data not shown), but not the fluorescent-tagged versions. The observed nuclear accumulation was high with the DCX region of RP1, BAC26042, DCDC2B, in comparison with others (Fig. 4D1–8, Fig. 5E–H and A–D). Moreover, DCDC2, DCDC2B and the DCX region of RP1L1 (Supplementary Fig. 2A–D, Fig. 5A–D and Fig. 4E1–8) accumulate in nucleoli, assuming new functions for the DCX domain. Furthermore, DCDC2B showed an intriguing nuclear localization, superimposing with condensed chromatin (DAPI stain) (Fig. 5A–D) in numerous overexpressing cells. Apparent nuclear localization of the DCX domain of FLJ46154 was observed as well (Fig. 5I–T).

DCX and the cytoskeleton. DCX has been previously shown to interact directly with MTs. Nevertheless, some results implicated an interaction of DCX with the actin cytoskeleton, directly or via Neurabin 2.[19,20] Interestingly, BAC26042 and, to some extent the DCX domains of DCLK2 localized to the cell periphery, at sites reminiscent of lamellipodia (Fig. 5E–H and Fig. 3C1–4). In the case of FLJ46154 (Fig. 5I–T), some localization to the cell periphery and to actin stress fibers (colocalizing with red-phalloidine) was noted (Fig. 5I–L and Q–T). In addition, prominent localization of this protein was noted in the bridge of the midbody structure (Fig. 5M–P), where actin is known to localize. This suggests a possible involvement of FLJ46154 in cytokinesis.

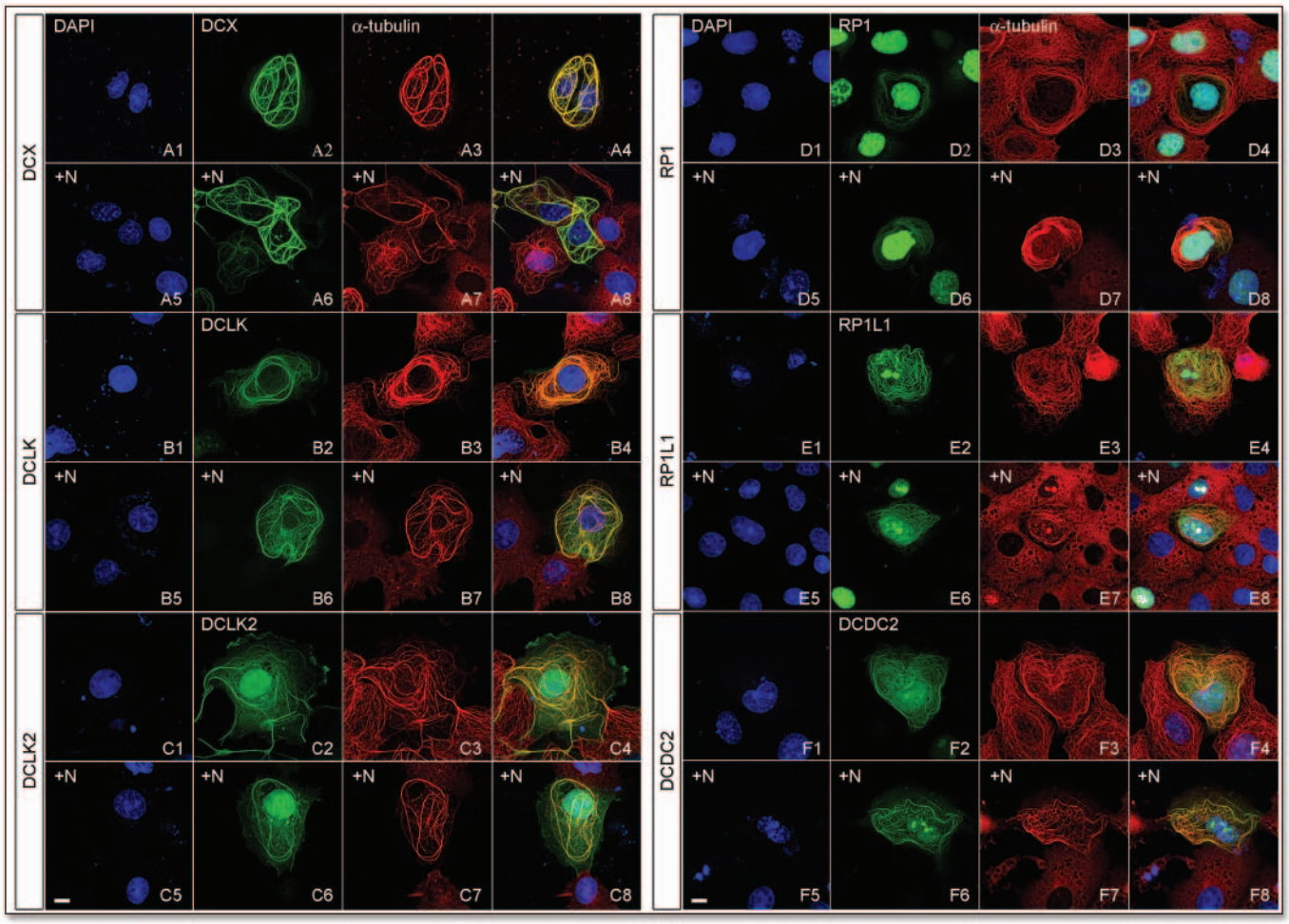

Figure 4. Overexpression of DCX motif proteins affects microtubule organization. DCX (A1–A8), the tandem DCX domains of DCLK (B1–B8), DCLK2 (C1–C8), RP1 (D1–D8), RP1L1 (E1–E8), and full-length DCDC2 (F1–F8) were overexpressed as fluorescent-fusion proteins; each set of transfected cells was subjected to nocodazole treatment ("+N", A5–A8, B5–B8, C5–C8, D5–D8, E5–E8 and F5–F8). Cells were fixed 48 hr following transfections and immunostained using anti-α-tubulin antibodies (DM1a, Sigma; A3, A7, C3, C7, D3 D7, E3 and E7), and stained with DAPI (A1, A5, B1, B5, C1, C5, D1, D5, E1, E5, F1 and F5). Pictures A2, A6, B2, B6, C2, C6, D2, D6, E2, E6, F2 and F6 show the GFP signal. All images are maximal-intensity projections of X/Y optical section stacks acquired by a BioRad confocal microscope. Scale bar is 10 μm.

The possible association of recombinant DCX proteins with actin and tubulin in a newborn brain extract was assayed (Fig. 6A). All the DCX-motif proteins tested were capable of pulling down tubulin subunits, albeit at different levels. The capability of pulling-down actin differed significantly; positive results were obtained for DCX, DCLK2, RP1 (weak), RP1L1 (weak), BAC26042, FLJ46154 and DCDC2B. No actin was observed in the case of pull-down by DCLK, and DCDC2. In addition, we examined whether DCX proteins are detected in the MAP fraction for P7 mice (Fig. 6B). This experiment was possible only for proteins for which we developed antibodies for; DCX, DCLK, and DCLK2. Indeed, these three proteins were enriched in the MAP fraction, as have been previously reported for DCX[7] and DCLK.[8,9]

The DCX domain and protein-protein interactions. As mentioned above, DCX interacts with the JIP1/2 scaffold protein, and with JNK. The domains of interactions were mapped within DCX to either conserved DCX domains, and within JIP to the PID (protein interaction domain), suggesting that the DCX domains function as a platform for protein-protein interactions. In addition, we tested possible interactions with neurabin 2, a scaffold for actin and PP1. The interactions were investigated by cotransfecting HEK293 cells with Flag-tagged-DCX-domain proteins and myc-tagged interacting protein (JIP1, JIP2, SH3-PID domain of JIP1, which is highly similar to that of JIP2, kinase-dead JNK2, and the coiled-coil domain of neurabin 2). The assays involved reciprocal coimmunprecipitations using myc- or Flag-antibodies (Fig. 7). Strikingly, a common feature was noted among all the DCX domain superfamily tested, which is their ability to interact with JIP1 and JIP2. The interaction domain is including the PID part of JIP1. Only in the case of DCDC2B, a unidirectional interaction was observed, whereas in the case of all other proteins the coimmunoprecipitation assays worked in both directions. The interaction with JNK was less conspicuous; DCX, DCLK2, FLJ46154 and BAC26042 interacted with JNK in both directions, whereas DCLK and DCDC2B interacted with JNK in one direction. In addition, the interaction with JNK was negative for RP1, RP1L1 and DCDC2. The immunoprecipitation of DCDC2 was conducted under more stringent conditions, due to low extraction efficacy of the DCDC2 protein, which is found mainly in the nucleus. Thus the results obtained with DCDC2 may not be conclusive. The interaction with neurabin 2 was positive only for the most related members of this superfamily; DCX, DCLK, and DCLK2. Positive and negative controls for the immunoprecipitation experiments were conducted in parallel (Supplementary Fig. 3), using Flag-tagged DCX (as a positive control), protein A/G beads as negative control, and Flag-tagged C-terminal region of DCX, lacking the DCX repeats (as a negative control).

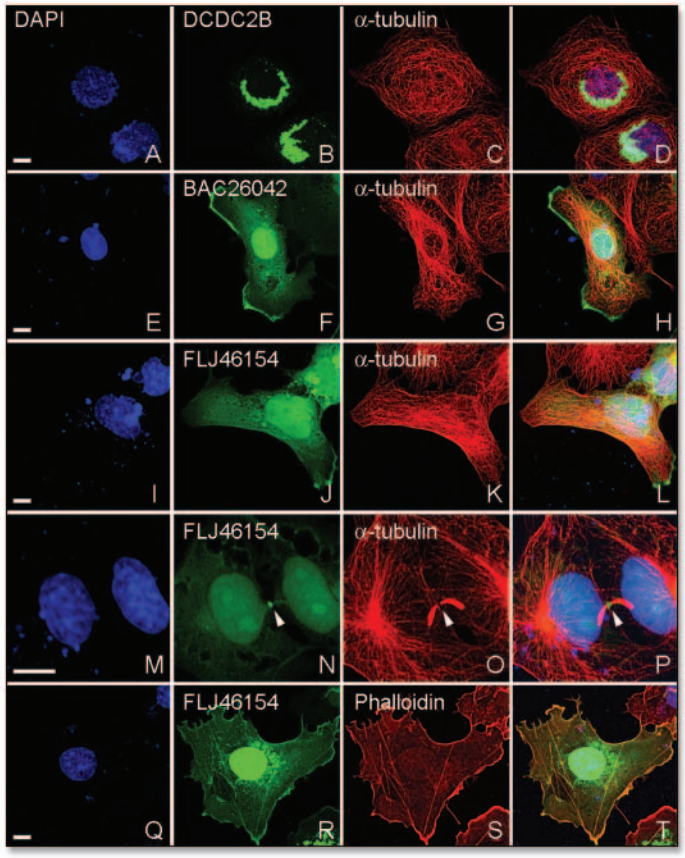

Figure 5. Overexpression of proteins with a single DCX repeat. DCDC2B (A–D), BAC26042 (E–H) and FLJ46154 (I–T) were overexpressed as GFP-fusion proteins, cells were fixed 48 hr following transfections and immunostained using anti-α-tubulin antibodies (DM1a, Sigma), and stained with DAPI (A–P), or stained with rhodamine-phalloidine and DAPI (Q–T). Note that no obvious overlap with microtubules is seen. Possible colocalization with actin was noted for BAC26042 and FLJ46154 (E–H and I–T respectively). It is worth noting that, during cytokinesis, the highest GFP-FLJ46154 signal (N) was detected at the midbody (M–P, arrows). All images are maximal-intensity projections of X/Y optical section stacks acquired by a BioRad confocal microscope. Scale bar is 10 μm.

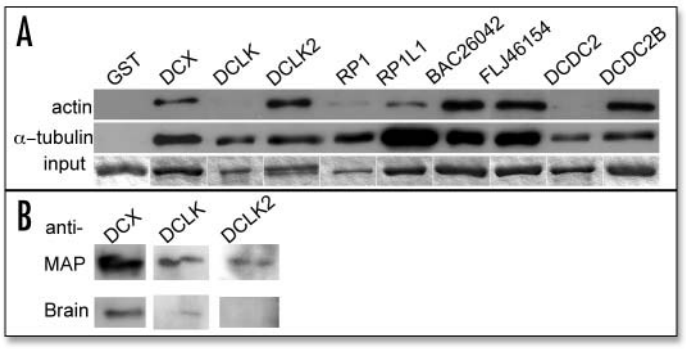

Figure 6. (A) Brain extracts (1.5 mg) were incubated with 10 μg of purified GST- recombinant proteins (1/15 of the input is shown in the bottom panel as Coomassie Blue stain). The pulled-down proteins were run on SDS-PAGE and immunoblots were performed with either anti-actin antibodies (top panel) or anti- α-tubulin (middle panel) antibodies. (B) DCX, DCLK, and DCLK2 are enriched in the MAP fraction. Phosphocellulose purified tubulin from P7 mouse brain or MAPs dissociated from a phosphocellulose column loaded with depolymerized tubulin following 0.4 M NaCl washes were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

DISCUSSION

Our current study has allowed evaluating the common and diverse functions of nine out of eleven members of the DCX superfamily of proteins. Two unifying functional aspects were detected in proteins with single and tandem repeats; all influenced MT polymerization, and all interacted with the JNK scaffold proteins, JIP1 and JIP2, thus, at least a subset of these proteins are likely to mediate signal transduction pathways. Although all the DCX-motif proteins affected MT assembly, their effect was not uniform. Most of the proteins assisted in MT polymerization, albeit at different levels. Our previous results indicated that the first DCX-repeat of DCX can promote MT assembly in a very efficient manner,[1] and here a single repeat existing in FLJ4615 was sufficient and very efficient in this activity. Since a single DCX domain is sufficient for promoting MT assembly in vitro, the different proteins may participate in regulation of MT dynamics in vivo. This may be an interesting topic for future studies. In cells, all of the tandem DCX-domain superfamily of proteins interacted with and stabilized MTs. This is clearly a function, which is common for the tandem repeat proteins, thus removal of a domain alters the property of the protein, and increases the functional flexibility of the family.

Our studies suggest a possible involvement of DCX proteins in mitosis and this has been previously documented in the case of DCX,[31] and DCLK.[32] The first study demonstrated that ectopic expression of DCX in HEK293 cells perturbed the mitotic machinery, caused abnormal spindle orientation, and impaired mitotic progression.[31] The second study demonstrated that DCLK regulated the formation of bipolar mitotic spindles and the proper transition from prometaphase to metaphase during mitosis in HEK293 cells and neuronal progenitors. In cultured cortical neural progenitors, DCLK RNAi disrupted the structure of mitotic spindles and the progression of M phase, causing an increase of cell-cycle exit index and an ectopic commitment to a neuronal fate.[32] Interestingly, the *C. elegans* ortholog of DCLK, ZYG-8 has been found to be important for assembly of astral microtubules.[33] The mutant ZYG-8 phenotype was observed with several alleles including mutations in the DCX domain. Thus, suggesting, that regulation of the MT-based mitotic spindle may represent a conserved function among DCX-domain proteins. Questioning the roles of the individual members of the DCX-superfamily in mitosis and differentiation remains to be an interesting open issue.

Among the tandem DCX-domain proteins, DCDC2 expression in most cells is confined to the nucleus, therefore it may be interesting to question its mitotic role. Recently, *DCDC2* has been suggested as a candidate gene for reading disabilities.[34,35] Furthermore, reducing DCDC2 expression in the developing brain inhibited neuronal migration.[34] It will be interesting to investigate whether the endogenous protein retains a nuclear localization in migrating neurons.

Nuclear localization among the transfected proteins was common to most DCX domain proteins, with the exceptions of BAC26042 and FLJ46154. The route in which these proteins enter the nucleus is unknown. Using several cellular localization prediction programs (for example: http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl), no canonical nuclear localization signals were detected. Nevertheless, it should be noted that the DCX domain is basic, including lysine and arginine residues, which may participate in a noncanonical nuclear localization signal.

It is likely that a subset of the mammalian DCX proteins shown to interact with JIP and JNK may be novel JNK substrates, and phosphorylation controls their endogenous activity. Furthermore,

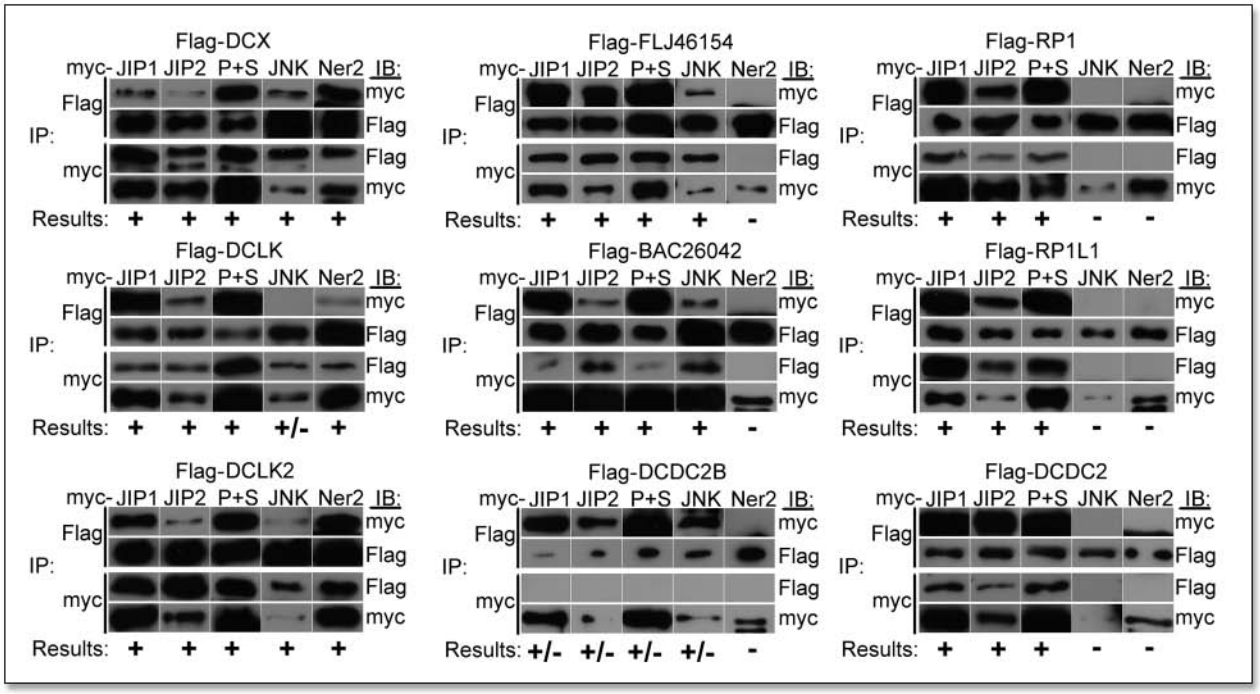

Figure 7. Testing the interactions of the DCX motif superfamily with a portion of DCX interacting proteins by coimmunoprecipitation. FLAG-tagged-DCX domains or full-length proteins were expressed in HEK293 cells together with each of the tested myc-tagged partners (JIP1, JIP2, PID and the SH3 domains of JIP1 marked as P+S, kinase dead JNK2 marked as JNK, and the coiled-coil domain of neurabin II marked as Ner2). Coimmunoprecipitation was done with either anti-FLAG antibodies or with anti-myc antibodies. The efficacy of the coimmunoprecipitation by reacting the immuno-blot with the antibody used for immunoprecipitation (15% of the immuno-precipitate), and the coimmunoprecipitation was tested by reacting the immuno-blot with the antibody directed against the tag of the coexpressed protein (85% of the immuno-precipitate).

the interaction of all the tested proteins with JIP1/2 suggests a conserved and important role in mediating signal transduction. Substantiating this hypothesis are recent experiments which indicated that RP1 plays a role in regulating JNK signaling cascades in the eye.[36]

A distinct set of proteins interacted with the scaffold protein neurabin 2. Positive interactions were observed with DCX, DCLK, and DCLK2, closely related family members, but not with other DCX-domain containing gene products. Future studies aimed at solving the structure of the different DCX proteins in association with their binding partners may assist in identification of key amino acid residues, and structural elements, involved in this interaction. Neurabin 2 is a scaffold protein and binds to PP1[37] as well as to actin.[38] Therefore, it may have been possible to speculate that neurabin 2 mediates interactions of DCX proteins with actin. However, such a correlation was not observed. Actin was pulled down from a brain extract using recombinant DCX, DCLK2, RP1 (weak), RP1L1 (weak), BAC26042, FLJ46154 and DCDC2B. No actin was observed in the case of pull-down by DCLK, and DCDC2. However, although neurabin 2 regulates the interaction of DCX with actin, DCX has the capability of directly interacting with actin and affecting its polymerization.[19,20] It is possible that additional DCX proteins interact directly with actin, alternatively additional mediators and regulators may exist.

In summary, the DCX-domain superfamily of proteins has proven to be a very successful family whose members are involved in signal transduction and cytoskeletal regulation, some of which are already implicated in and pathological and developmental processes.

Acknowledgements

We thank Prof. Shmuel Pietrekovski, and Dr. Arlette Fellous for helpful discussions. The work has been supported in part by the Fritz Thyseen Stifung Foundation, the Israeli Science Foundation (grant no. 270/04), Foundation Jérôme Lejeune, Minerva foundation with funding from the Federal German Ministry for Education and Research, the German-Israeli collaboration grant Gr-1905, the Kekst center, the David and Fela Shapell Family Center for Genetic Disorders Research. O.R. is an Incumbent of the Berstein-Mason professorial chair of Neurochemistry. F.C. was supported by a Post-Doctoral Fellowship from the Association pour la Recherche sur le Cancer (Villejuif, France), and by the Sir Charles Clore Post-Doctoral Fellowship, the Weizmann Institute of Science (Rehovot, Israel).

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