Different Doublecortin (DCX) Patient Alleles Show Distinct Phenotypes in Cultured Neurons

EVIDENCE FOR DIVERGENT LOSS-OF-FUNCTION AND “OFF-PATHWAY” CELLULAR MECHANISMS**

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Edited by Velia Fowler

Doublecortin on the X-chromosome (DCX) is a neuronal microtubule-binding protein with a multitude of roles in neurodevelopment. In humans, DCX is a major genetic locus for X-linked lissencephaly. The best studied defects are in neuronal migration during corticogenesis and in the hippocampus, as well as axon and dendrite growth defects. Much effort has been directed at understanding the molecular and cellular bases of DCX-linked lissencephaly. The focus has been in particular on defects in microtubule assembly and bundling, using knock-out mice and expression of WT and mutant Dcx in non-neuronal cells. Dcx also binds other proteins besides microtubules, such as spinophilin (abbreviated spn; gene name Ppp1r9b protein phosphatase 1 regulatory subunit 9b) and the clathrin adaptors AP-1 and AP-2. Even though many non-sense and missense mutations of Dcx are known, their molecular and cellular defects are still only incompletely understood. It is also largely unknown how neurons are affected by expression of DCX patient alleles. We have now characterized several patient DCX alleles (DCX-R89G, DCX-R59H, DCX-246X, DCX-272X, and DCX-303X) using a gain-of-function dendrite growth assay in cultured rat neurons in combination with the determination of molecular binding activities and subcellular localization in non-neuronal and neuronal cells. First, we find that several mutants (Dcx-R89G and Dcx-272X) were loss-of-function alleles (as had been postulated) but surprisingly acted via different cellular mechanisms. Second, one allele (Dcx-R59H) formed cytoplasmic aggregates, which contained Hspa1B (heat shock protein 1B hsp70) and ubiquitinated proteins, trapped other cytoskeletal proteins, including spinophilin, and led to increased autophagy. This allele could thus be categorized as “off-pathway”/possibly neomorph. Our findings thus suggested that distinct DCX alleles caused dysfunction by different mechanisms.

Dcx4 is a neuronal microtubule (MT)-binding protein with many roles in neurodevelopment. In humans, DCX is a major locus for X-linked lissencephaly (1–3) presenting with cortical, hippocampal, and cerebellar defects and defects in major axon tracts (4–7). Much effort has thus been directed at understanding the molecular and cellular bases for this disease, with a particular focus on DCX-dependent defects in MT assembly and bundling. Experiments using a Dcx knock-out mouse, or double knockouts with the related genes Dclk1 and Dclk2 (8–14), or overexpression approaches (15) all argue strongly that Dcx does in fact play important roles in neurodevelopmental processes. The best studied defects are in neuronal migration in cortex (14, 16) and in hippocampus (17, 18). Axon and dendrite defects have also been described (6, 10, 17). For instance, dendrites in hippocampal pyramidal neurons are simplified in adult Dcx KO mice (17). Dendrite growth is also impaired in cortical neurons cultured from Dclk1/Dcx double knock-out embryos (8). Furthermore, short hairpin-mediated knockdown of Dcx in cultured rat neurons led to reduction of dendrite complexity (15, 19). These knock-out phenotypes are attributed to the required regulation of MTs by Dcx. The converse is also true; overexpression of Dcx increases dendrite complexity (15), further supporting a role for Dcx in modulating dendrite elaboration. Similarly, overexpression of Dclk1 increases dendrite complexity (20).

Dcx is also found in complexes with other proteins, including the actin-associated protein spinophilin (spn) (21), the clathrin adaptors AP-1 and AP-2 (22), and the cell adhesion molecule neurofascin (23), suggesting additional roles for Dcx. These interactions were mapped to regions C-terminal to the MT-binding sites (Fig. 1A). In fact, we previously discovered that Dcx promotes the endocytosis of neurofascin independent of MT binding (24), but dependent on binding to clathrin adaptors via the C-terminal YLPL motif.5 Among the known DCX

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*This work was supported by National Institutes of Health Grant R01NS081674 (to B. W.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

**This article was selected as a Paper of the Week.

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4 The abbreviations used are: Dcx, doublecortin on the X-chromosome; MT, microtubule; DIV, days in vitro; Lof, loss-of-function; spn, spinophilin/neurabinII; IP, immunoprecipitation; WB, Western blot; SBH, subcortical band heterotopia; LIS, lissencephaly.

5 C. C. Yap, L. Digilio, K. Kruczek, and B. Winckler., manuscript in preparation.
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patient alleles, some have mutations in the N-terminal MT-binding domain and lack MT binding, whereas others have truncated C termini and thus retain MT binding (Fig. 1A). It is not known whether both classes of DCX patient alleles impair dendrite growth equally.

The available knock-out mouse model for Dcx presents considerable challenges in terms of molecular characterization of DCX alleles. In particular, the phenotypes reported for Dcx KO mice are very subtle and often transient, presumably because of redundancy with Delk1 and Delk2 (8, 11, 14, 18). Double knock-out mice for Dcx and Delk1, however, do have profound neurodevelopmental defects, but they show poor viability and difficulty breeding (9, 10), making them a challenging and expensive model for carrying out Dcx mutant analysis. For these technical reasons, very little analysis is currently available about how DCX patient mutants behave in neurons. To circumvent these technical barriers, we decided to take advantage experimentally of the observation that Dcx can enhance dendrite growth when overexpressed (15). We thus used dendrite enhancement by Dcx expression as an assay for wild type Dcx function, using previously described MT binding-competent and MT binding-deficient mutants (25–27). All of these alleles were originally identified in patients with lissencephaly (2, 28, 29). We found that Dcx alleles that retain MT binding but lack the C-terminal regions (required for spn and AP adaptor binding) are defective for dendrite growth promotion (i.e. loss-of-function alleles) but to differing degrees. In particular, truncation mutants that retained MT and spn binding and lacked only the extreme C termini were less impaired than shorter truncation mutants that retained only MT binding. In addition, we found, surprisingly, that one of the mutations caused a cellular stress response in neurons through aggregate formation. These aggregates were ubiquitinated and were included in autophagosomes. Neurons thus likely up-regulated degradative responses to clear the aggregates. Failure to efficiently clear the aggregates may lead to eventual cellular dysfunction and even death. This allele thus engaged an “off-pathway” response that was not usually activated by Dcx and could be classified as “neomorph” by this criterion.

We report here an ex vivo overexpression approach as an experimentally efficient way for a first-pass screening of multiple DCX alleles in neurons. In particular, we dissected the cellular phenotypes of several DCX patient mutations in neurons and discovered that distinct DCX patient alleles caused dysfunction by different cellular mechanisms. This approach resulted in generating new hypotheses. The next step will be to take the insights generated and test their implications in a more physiological in vivo context in the future. In addition, more of the published alleles need to be screened in neuronal cultures to further delineate other possible cellular pathways in which DCX alleles are deficient or in which off-pathway events occur for some alleles.

Results

The functions of Dcx have been investigated on multiple levels, from biophysical (using purified proteins) to cellular (mostly in non-neuronal cells) to tissue level (using knock-out models). Taking mechanistic discoveries from an in vitro binding assay straight into the whole animal is, more often than not, impossible or not informative, especially for DCX, a human disease gene for which the KO mouse does not fully phenocopy the human pathology. One reason for this lack of phenocopy is likely the small size of the murine cortex, coupled to redundancy with other genes in the same family. Furthermore, because the DCX gene is on the X-chromosome, it cannot be determined from the inheritance pattern whether or not any given human DCX patient allele is loss-of-function. Therefore, important new insights might be gained by expression analysis of Dcx mutants in cultured neurons as an initial step. We thus decided to initiate an investigation of neuronal effects for a number of Dcx alleles that had previously only been studied in non-neuronal cells or in vitro. We chose to investigate a cellular process that was known to be affected by Dcx in cultured neurons, namely dendrite growth. Because we can read out the activity of WT Dcx with this assay by increased dendrite growth, we can ask which patient alleles are loss-of-function alleles and then correlate their molecular binding activities with their lack of or altered function.

C Terminus of Dcx Is Required for Dcx-mediated Increase in Dendrite Complexity—WT Dcx was previously shown to increase dendrite complexity in cultured neurons (15). Freshly dissociated rat cortical neurons from E18 embryos were electroporated prior to plating with plasmids encoding GFP (as control) or WT Dcx, and the number of dendrites intersecting a concentric circle (diameter of 30 μm) was counted at 5 days in vitro (DIV5) (Fig. 1B). This measurement corresponds to a simplified Scholl analysis because dendrites are still short at these times in culture. In agreement with previous reports, we found that expression of WT Dcx led to a significant increase in dendrite complexity compared with expressing GFP as a control (Fig. 1, C and D). The traced examples shown in Fig. 1C were chosen to represent the 50th percentile of each data set. We next asked whether the C terminus was necessary for Dcx-mediated dendrite complexity using the Dcx truncations Dcx-303X and Dcx-272X (Fig. 1A), which corresponded to published patient alleles. Dcx-272X expression did not increase dendrite complexity, in contrast to expression of WT-Dcx (Fig. 1, C and D). Dcx-303X was still partially active and increased dendrite complexity compared with Dcx-272X, but it was less potent than WT Dcx (Fig. 1, C and D). The Dcx C terminus was thus necessary for promoting dendrite growth.

DCX Patient Truncations DCX-303X and DCX-272X Associate with Microtubules—The MT-binding domain of Dcx has been mapped by multiple laboratories and resides in the DC repeat regions of the N terminus of Dcx (see Fig. 1A). A truncated Dcx (DC repeats 1 and 2: Dcx1–247) still bound to microtubules in vitro (27), and both Dcx-303X and Dcx-272X contain all of the DC repeats. To confirm in our own hands the ability of the Dcx C-terminal truncations to associate with microtubules inside cells, we used a cell-based detergent extraction assay, previously established by others (26, 27). WT Dcx has been established by multiple laboratories (including ours) to remain associated with MTs by this stringent cell-based assay. This assay is thus well suited to determine whether Dcx mutants are still principally able to associate with microtubules in cells (see “Experimental Procedures”).
We quantified the ability of the two truncation mutants to remain associated with microtubules in transfected cells as follows: compared with GFP as a negative control and WT Dcx as a positive control. COS cells were transfected with GFP, WT Dcx, Dcx-303X, or Dcx-272X. Lamin-cherry was co-transfected to mark transfected cells after detergent extraction. After 24 h, one coverslip was fixed directly (“unextracted”), and one coverslip was extracted in detergent in microtubule-stabilizing buffer BRB-80 (“extracted”) for each construct. The mean intensity of Dcx in transfected cells was then quantified. For GFP, ~90% of the GFP signal was extractable (Fig. 2, A, B, and F), whereas WT Dcx intensity was barely diminished by detergent extraction, demonstrating that WT Dcx remained bound to microtubule networks under these extraction conditions (Fig. 2, C and F). Similarly to WT Dcx, Dcx-303X (Fig. 2D) and Dcx-272X (Fig. 2E) were still clearly able to associate with MTs (Fig. 2F), again in agreement with previous observations that the microtubule-binding domain is entirely contained within the DC repeats (26, 27).

Spinophilin Binding Capacity Is Partially Impaired in Dcx-272X but Not Dcx-303X—Because Dcx-272X was not active in dendrite growth promotion even though it still associated with microtubules, we wondered whether binding to another protein via the more C-terminal domain (missing in the truncated Dcx-272X) might be required for full activity. The actin-bind-
also recovered in anti-Myc immunoprecipitations when Myc-spn was co-expressed in COS cells but not in controls lacking Myc-spn (Fig. 3A). When Dcx was expressed in COS cells, it co-localized strikingly with MTs but not with F-actin (phalloidin stain) (Fig. 3B). In contrast, when Dcx was co-expressed with spn in COS cells, Dcx was distributed on both MTs and F-actin (Fig. 3C). The boxed region of these quadruple-stained cells is shown in combinations of three channels in Fig. 3D to make the dual co-localization with distinct MTs (arrowhead) and F-actin (arrows) easier to appreciate.

Because MTs and F-actin occupy largely overlapping spaces in COS cells, visualizing Dcx pools on spn-containing F-actin structures was challenging. We thus determined whether Dcx was able to co-localize with spn in neurons where spn was greatly enriched in the actin-rich filopodia off the sides of dendrites and could thus be clearly distinguished from the MT-rich dendritic shaft. When Dcx was expressed with spn, Dcx strongly stained the dendrite shaft, but Dcx staining could also be observed in MT-poor, actin/spn-rich dendritic filopodia (Fig. 3E), suggesting that a subpopulation of Dcx was able to enter actin-rich surface structures. Dcx-272X also entered spn-rich dendritic filopodia (Fig. 3F). We were unable to clearly quantify whether it occurred to a similar extent as WT Dcx because expression levels varied from cell to cell after the transient transfections. We thus turned to a more quantifiable assay to determine the spn binding capacity of Dcx and the Dcx truncations, namely co-immunoprecipitations.

The binding site for spn on Dcx had been coarsely mapped previously. One paper reported no spn binding of Dcx-246X (21) and another reported that the second DC repeat (amino acids 170–260) is sufficient for binding (6). Also, a point mutation at position 57 (F57L) was shown to completely abrogate spn co-immunoprecipitation, as did mutations at positions 331 and 334 (32). The required Dcx sequences for spn binding are thus still not completely elucidated. Furthermore, Dcx-272X had not previously been tested for spn binding. Consistently less Myc-spn was immunoprecipitated with FLAG-Dcx-272X (Fig. 3G and H), indicating reduced binding (−50%) of spn to the truncated Dcx-272X. We then tested the spn binding of FLAG-Dcx-303X by co-immunoprecipitation. Dcx-303X showed no statistically significant decrease in spn co-immunoprecipitation compared with WT-Dcx (Fig. 3G and H). We note that the total expression levels of WT Dcx, Dcx-303X, and Dcx-272X were not different, indicating that the truncated C termini did not affect stability of the Dcx truncations. Our data contrasted somewhat with the conclusions of Bielas et al. (6), obtained with purified bacterially expressed fragments in vitro and who reported that spn binding ability was entirely contained within the N-terminal residues up to amino acids 260 (second DC repeat). In contrast, our data place important residues contributing to spn binding between residues 272 and 303. Our observations showed that the ability of Dcx truncations to support dendrite growth correlated with their ability to bind to spn and that MT binding was not sufficient.

**Dcx-246X Accumulates in the Nucleus and Is Unstable—**

Next, we generated Dcx-246X (Fig. 1A), also a patient allele, that was predicted to have no spn binding capacity based on previous reports (21) but to retain MT binding. We confirmed...
MT association of Dcx-246X (Fig. 4A). Unexpectedly, Dcx-246X was recovered at very low levels in the lysates that we had prepared for the purpose of immunoprecipitation, making it impossible for us to test spn binding by that assay. Because MTs usually are depolymerized in lysates prepared on ice, we wondered whether Dcx-246X remained associated with other Triton-insoluble material, such as nuclei or intermediate filaments, and was thus spun out and discarded in the pre-clear step. We therefore transfected HEK293T cells with FLAG-Dcx-246X, incubated them on ice to depolymerize MTs completely, and fractionated a soluble fraction (containing cytosolic protein, tubulin, and MT-associated proteins) and an insoluble fraction (containing nuclei and intermediate filaments) (Fig. 4B). As expected, tubulin was entirely found in the soluble fraction, and vimentin was found overwhelmingly in the pellet fraction. Unlike WT Dcx and FLAG-Dcx-272X, FLAG-Dcx-246X was expressed at low levels overall, and about 30% of it pelleted with nuclei and intermediate filaments into the insoluble pellet.

To assess the ability of Dcx-246X to associate with spn with an alternative approach, we expressed it in either COS cells or...
neurons, as in Fig. 3. In striking contrast to WT Dcx (shown in Fig. 3), Dcx-246X co-localized strikingly with MTs but not with spn/F-actin in extracted COS cells (Fig. 4C). The partitioning of Dcx-246X away from actin-rich structures was even more obvious in neurons where it was overwhelmingly excluded from actin/spn-rich protrusions off dendrite shafts (Fig. 4D). These results were consistent with the spn mapping to DCX previously published by Tsukada et al. (21).

When we evaluated the distribution of FLAG-Dcx-246X with MTs and spn/actin without extraction in COS cells (Fig. 4E), no co-localization with spn was apparent (see zoomed-in panels), but co-localization with MTs was extensive. Surprisingly, we observed accumulation of FLAG-Dcx-246X on or in the nucleus in many cells. Z-stacks through the cell revealed that FLAG-Dcx-246X appeared to be contained within the nucleus and not just peripherally associated with the nuclear
envelope (Fig. 4F). When we expressed Dcx-246X in neurons for 24 h, we frequently observed that FLAG-Dcx-246X accumulated in the nucleus as well (Fig. 4G), similar to our observations in unextracted COS cells.

When we tested whether Dcx-246X still supported dendrite growth, we could only find a total of two neurons expressing FLAG-Dcx-246X after 5 days of expression in three separate experiments (on six coverslips total). In contrast, we routinely recovered 200–1000 transfected cells per coverslip for WT Dcx. The low number of Dcx-246X expressing neurons could be due to the low levels of expression, as suggested by the observed instability of Dcx-246X in COS cell lysates. Alternatively, the low number of Dcx-246X expressing neurons could be due to their increased death. To distinguish between these two possibilities, we co-transfected Dcx-246X with soluble GFP. Because the co-transduction rate is >80%, we could determine whether few transfected neurons survived, suggesting death, or whether GFP-expressing neurons were plentiful but lacking Dcx-246X immunoreactivity, suggesting low expression. We recovered a large number of GFP-expressing neurons that were not immuno-positive for Dcx-246X. These observations argued that transfected cells were not overwhelmingly dying and that Dcx-246X was not stably expressed for 5 days. We note that in older neurons (transfected at DIV8 for 24 h; Fig. 4G) transfected neurons were detectable on each coverslip, albeit at much lower numbers compared with transfected WT Dcx. We conclude that Dcx-246X is an unstable protein.

**Dcx Alleles Dcx-R89G and Dcx-R59H Do Not Associate with MTs and Are Loss-of-Function Alleles**—We then made two Dcx alleles that are reportedly deficient for MT binding, Dcx-R89G and Dcx-R59H. As expected, Dcx-R89G staining is lost after detergent extraction (Fig. 5, A and B). Dcx-R59H also showed greatly reduced MT association (Fig. 5B). We noted that in many cells Dcx-R59H could be observed in detergent-insoluble aggregates (see below), and it was usually the fluorescence associated with these aggregated puncta that contributed the remaining Dcx-R59H intensity in the measurements of extracted cells, rather than Dcx-R59H remaining clearly associated with a microtubule network. We did not observe such aggregates with Dcx-R89G.

We then tested whether Dcx-R89G and Dcx-R59H were functional with respect to increasing dendrite complexity (as in Fig. 1). Neither Dcx-R89G nor Dcx-R59H increased dendrite complexity compared with WT Dcx control (Fig. 5C). We noted one striking difference between Dcx-R89G and Dcx-R59H in the dendrite growth experiments, namely that we observed more than an order of magnitude fewer Dcx-R59H expressing neurons after 5 days, compared with WT Dcx and Dcx-R89G. This was not due to problems with the plasmid per se because we observed comparable numbers of transfected COS cells for all the plasmids used, including Dcx-R59H.

To more fully characterize the molecular binding properties of these two patient alleles, we also tested them for spn binding by co-immunoprecipitation. We saw somewhat lower levels of complex formation for Dcx-R89G (Fig. 5, D and E), but the difference was not statistically significant compared with WT Dcx binding. The failure of Dcx-R89G to promote dendrite growth was therefore likely due to its inability to bind MTs. We could not determine the capacity of Dcx-R59H to bind to Myc-spn because Dcx-R59H showed extremely high levels of nonspecific binding to anti-Myc-agarose-conjugated beads (probably due to aggregation), which made interpretation of co-immunoprecipitations dubious. Our data so far suggest that Dcx loss-of-function can result from loss of MT binding (Dcx-R89G) or loss of spn binding (Dcx-R59H) and that different DCX patient alleles thus act via different cellular loss-of-function mechanisms. In addition, our data demonstrate that neither MT binding nor spn binding of Dcx are sufficient for full function.

To corroborate the immunoprecipitation experiments, we then tested whether association of Dcx-R89G with spn could be demonstrated in cells. We thus repeated the COS cell detergent extraction (shown in Fig. 5A) except that Myc-spn was co-transfected with Dcx-R89G. Dcx-R89G was now detergent-resistant (Fig. 5F) and remained prominently associated with spn and F-actin. The Myc-spn co-expression was thus sufficient to impart detergent resistance onto Dcx-R89G (compare Fig. 5, A–F), consistent with its lacking MT binding but retaining spn binding. Consistent with these observations, Dcx-R89G also entered actin-rich, MT-poor spines in dendrites of transfected neurons (Fig. 5G).

**MT Binding-deficient R59H Patient Allele Forms Cytoplasmic Aggregates**—As noted previously, Dcx-R59H was present in detergent-resistant cytoplasmic aggregates in detergent-resistant COS cells (Fig. 6A). When Myc-spn was co-expressed with Dcx-R59H in COS cells and then detergent-extracted, much of the Dcx-R59H remained detergent-resistant in clear alignment

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**FIGURE 4. Dcx-246X accumulates in the nucleus and is unstable.** A, quantification of MT association of Dcx-246X in detergent-extracted COS cells (details as in Fig. 2). B, HEK293T cells were transfected with WT FLAG-Dcx, FLAG-Dcx-246X, or FLAG-Dcx-272X. Cells were incubated on ice to disassemble microtubules and then lysed in detergent. The washed insoluble pellet (containing nuclei and intermediate filaments) and the soluble fraction were collected and probed for FLAG, tubulin (tub) and vimentin (vim). All of the tubulin was found in the soluble fraction, and vimentin was found in the pellet. All of the Dcx proteins with the exception of Dcx-246X were completely soluble in the absence of assembled microtubules. Dcx-246X, however, was found at lower levels overall and about one-third of it pelleted with nuclei and intermediate filaments. C, COS cells were transfected with Myc-spn and Dcx-246X, and cells were extracted in detergent in microtubule stabilizing buffer. Dcx-246X was detergent-resistant and localized prominently with microtubules but not with spn/actin. Single channels are shown in the zoomed-in panels below. D, co-expression of Dcx-246X (red) and Myc-spn (green) in neurons. Map2 is counterstained in blue. Dcx-246X is restricted to the microtubule-dense dendrite shaft and does not enter spn-enriched protrusions off the dendrite shaft. E, COS cells were transfected with GFP-spn (green) and FLAG-Dcx-246X (red) and fixed without detergent extraction. Dcx-246X was found prominently on microtubules but not on spn-decorated actin structures. Small panels on the right show each individual channel of a zoomed in region as well as tracings for easier comparison. In addition, prominent nuclear association is seen in many cells. F, COS cells were transfected with FLAG-Dcx-246X and lamin-cherry and imaged with structured illumination microscopy (Zeiss Apotome), taking Z stacks. The bottom and middle sections of the Z-stack are shown. Dcx-246X is found on cytoplasmic microtubules but also diffusely inside the nucleus. The lamin-cherry channel was used to segment the voxel space corresponding to the nucleus of a different cell. Dcx-246X could be seen clearly contained within the nuclear voxel space. G, DIV8 neurons were transfected with GFP (green) and FLAG-Dcx-246X (red) and counterstained for Map2 (blue). About half of the transfected cells show prominent nuclear accumulation of DCX-246X with only faint staining of dendrites.
with spn/actin-rich structures (Fig. 6B). Dcx-R59H can thus still bind to spn but tends toward formation of insoluble cytosolic aggregates.

When we expressed Dcx-R59H in DIV8 neurons for shorter times (24 h), expressing neurons were easily found, but in many of them (about 50%) Dcx-R59H was in cytoplasmic aggregates in dendrites and soma (Fig. 6C). Some of these aggregates appeared to contain Map2 and actin (Fig. 6D, panels 2 and 4, arrows), although the majority of Map2 and F-actin was still distributed normally in soma and dendrites. To better visualize whether expression of Dcx-R59H affected the distribution of the actin cytoskeleton, we co-transfected mRuby-LifeAct (Fig. 6F).

FIGURE 5. Dcx alleles Dcx-R89G and Dcx-R59H do not associate with MTs and are loss-of-function alleles. A and B, in-cell MT association was assessed as in Fig. 2 for Dcx-R89G and Dcx-R59H. Dcx-R89G was completely detergent-extracted (A). Quantification is shown in B for 50–60 cells. C, cortical neurons in culture were electroporated prior to plating with Dcx or Dcx mutants (as in Fig. 1), and the number of intersecting dendrites with a circle at 30 μm diameter was determined 5 days later (DIV5). Three independent experiments were carried out in separate cultures, and one representative experiment is shown in C for WT Dcx, Dcx-R89G, and Dcx-R59H. The number of cells quantified in this experiment was as follows: 58 cells for GFP, 61 cells for WT Dcx, 34 cells for Dcx-R59H, and 48 cells for Dcx-R89G. Only WT Dcx led to a statistically significant increase in dendrite complexity compared with GFP controls (using Mann-Whitney test, p < 0.0001). The same outcomes were obtained in all three independent experiments. D and E, Myc-spn was co-transfected into COS cells together with WT Dcx (1st lane), or Dcx-R89G (2nd lane). The average of four independent immunoprecipitation experiments is shown in E. Error bar indicates S.E. One representative experiment is shown in D from the same blot with an intervening irrelevant lane removed. F, COS cells were transfected with Myc-spn and Dcx-R89G and cells extracted in detergent in microtubule-stabilizing buffer. Unlike A, Dcx-R89G was detergent-resistant in the presence of co-expressed Myc-spn and localizes prominently to spn/actin structures. G, Dcx-R89G (red) was able to associate with spn/actin-rich protrusions (green) off the dendrite shaft in neurons.
FIGURE 6. Dcx-R59H patient allele forms cytoplasmic aggregates enriched in spn, HSP70, ubiquitin, and autophagosome proteins. A, Dcx-R59H forms detergent-resistant cytoplasmic aggregates in COS cells. Panel 1 is a merged image of the individual channels shown in panels 2–4. B, in COS cells co-expressing Myc-spns, Dcx-R59H becomes detergent-resistant and co-localizes strikingly with F-actin and spn. This suggests that Dcx-R59H retains spn binding. B, panel 1 is a merged image of the individual channels shown in panels 2–4. C and D, Dcx-R59H forms cytoplasmic aggregates in DIV8 neurons that contain some MAP2 and actin (C). D shows single channel close-ups of soma for Dcx-R59H (panel 1), Map2 (panel 2), and phalloidin (blue in panels 3 and 4). Arrows point at location of Dcx-R59H aggregates. E, Ruby-LifeAct (red) decorates F-actin rich structures at the tip and off the shaft of dendrites (panel E). This pattern is not disrupted by Dcx-R59H containing cytoplasmic aggregates (panel E’, green), but each aggregate contains some Ruby-LifeAct. Map2 is counterstained in blue. F, localization of Myc-spns (red) in DIV8 neurons in the absence of Dcx-R59H (panel 1), in the presence of non-aggregated Dcx-R59H (panel 2), and in the presence of aggregated Dcx-R59H (panel 3). Dcx-R59H localizes to spn-enriched dendritic protrusions when not aggregated (panel 2) but disrupts the off-shaft localization of Myc-spns when aggregated, pulling it completely into cytoplasmic aggregates in the dendritic shaft (panel 3). Map2 is counterstained in blue. G, DIV8 neurons were transfected with Dcx-R59H (green) and fixed 24 h later. They were counterstained with antibodies to endogenous Map2 (blue) and hsp70 (red) (panel 1), endogenous spn (blue) and ubiquitin (red) (panel 2), and endogenous p62 (red) and ubiquitin (blue) (panel 3). G, panels 4 and 5, to visualize autophagosomes, neurons were transfected with Dcx-R59H (green) and RFP-LC3 (red) and fixed after 24 h. Cells were additionally stained against endogenous ubiquitin (blue) (panel 4) or endogenous p62 (blue) (panel 5). Arrows point at Dcx-R59H containing cytoplasmic aggregates.
Cellular and Molecular Pathology of Different DCX Alleles

6E) or Myc-spn (Fig. 6F). Aggregates of DCX-R59H were highly co-localized with Ruby-LifeAct, arguing that the aggregates included F-actin, but the overall distribution of Ruby-LifeAct was still normal, and it distributed prominently to actin-rich protrusions off the dendrite shaft (Fig. 6E). As shown above, Myc-spn was found in dendritic protrusion off the shaft as well as in dendrite tips (Fig. 6F, panel 1). In those neurons where Dcx-R59H was not found in aggregates, Dcx-R59H greatly co-localized with Myc-spn in actin-rich dendritic protrusions, consistent with its ability to bind spn (Fig. 6F, panel 2). The Myc-spn distribution appeared undisturbed in these cells. In those neurons where Dcx-R59H was found in aggregates, Myc-spn localization was profoundly changed and co-localized completely with Dcx-R59H aggregates in the dendrite shaft (Fig. 6F, panel 3). Virtually none of the Myc-spn remained in actin-rich dendritic protrusions off the shaft, indicating that Dcx-R59H sequestered most, if not all, Myc-spn in the cytoplasmic aggregates in the dendrite shaft. Of all the Dcx allelic we tested (WT, Dcx-303X, Dcx-272X, Dcx-R89G, and Dcx-R59H), Dcx-R59H was the only one that formed aggregates and disrupted the distribution of other cytoskeletal elements. Dcx-R59H thus has off-pathway effects and might manifest as an “off-pathway/neomorph” allele at the subcellular level.

Cytoplasmic Aggregates of Dcx-R59H Contain hsp70, Ubiquitin, and Markers of Autophagosomes—Cytoplasmic aggregates of misfolded proteins are a common finding in multiple neurodegenerative disease. All cells, including neurons, possess cellular machinery to recognize and dispose of misfolded cytoplasmic proteins. Many misfolded proteins are recognized by hsp70, tagged with ubiquitin, and ultimately degraded by the proteasome or in autophagosomes to maintain proteostasis and health. We therefore asked whether cytoplasmic aggregates of Dcx-R59H contained hsp70, ubiquitin, or markers of autophagosomes (p62 and LC3) (Fig. 6G). We observed clear co-localization of Dcx-R59H aggregates with hsp70 and ubiquitin (Fig. 6G, panels 1 and 2). Cytoskeletal components (Fig. 6G, MAP2, panel 1, and spn, panel 2) were found in the same aggregates. These observations argue that when Dcx-R59H misfolded in neurons, it was recognized by the hsp70/ubiquitination machinery and tagged for degradation. Co-localization of Dcx-R59H aggregates was also apparent with p62 and LC3 (Fig. 6G, panels 3–5) but not with all of the aggregates. Neurons expressing Dcx-R59H aggregates thus contain autophagosomes filled with ubiquitinated proteins and Dcx-R59H. In addition, neurons containing Dcx-R59H aggregates showed disruption of normal cytoskeletal organization that might contribute to neuronal dysfunction for this particular allele.

Discussion

Cortical malformations are recovered in the human populations at a surprisingly high rate and can be familial or arise spontaneously de novo (1–3, 28). One manifestation of cortical malformation is subcortical band heterotopia (SBH) and lissencephaly (LIS; smooth brain). A recent extensive study on human patients with SBH found that 100% of familial SBH is caused by mutations in the X-linked gene DCX. For de novo mutations, DCX mutations account for 50–80% of cases (28). Both DCX-linked SBH (in females) and DCX-linked LIS (in males) are likely caused by neuronal migration defects, but axon outgrowth and guidance defects have also been reported. In the hippocampus, lamination defects as well as dendritic defects are found in the Dcx null mouse (17), and the hippocampus is affected in some of the human patients (4). For patients with SBH and LIS, intellectual disability and epilepsy are common and are likely due to a combination of misplaced neurons in the cortex and in the hippocampus, defects in major axon tracts (such as the corpus callosum), and connectivity problems due to misplaced neurons and defective dendrite growth. Even though many non-sense and missense mutations of DCX are known and more are continuously added to the list, the molecular and cellular defects for most of them are still only incompletely understood.

In this work, we analyzed and correlated the cellular and molecular characteristics of several DCX patient alleles. Our work led to the following conclusions and new insights. 1) Loss-of-function of Dcx can arise from either loss of MT binding or loss of C-terminal binding regions. 2) Sequences in the C terminus of Dcx (past the MT-binding domains contained in the DC repeats) are required for full Dcx function. These C-terminal sequences are necessary for full spn binding, AP-2 binding, as well as contain several regulatory phospho-sites. 3) Because Dcx-303X shows only partial loss-of-function, absence of the phospho-sites (past residue 303) does not give strong phenotypes for dendrite elaboration. 4) Dcx-246X is an unstable protein that aberrantly accumulates in the nucleus. 5) One patient’s alleles (DCX-R59H) form aggregates that activate autophagy. Neurons containing these aggregates show disrupted cytoskeletal components. We thus suggest that Dcx-R59H can act as a cellular off-pathway/possibly neomorph allele.

Our findings lead to the suggestion that cellular pathology caused by different DCX patient alleles are due to either loss-of-function (Lof) or off-pathway/potentially neomorph mechanisms (summarized in Table 1). Given the recent discoveries of the importance of Dcx and Dclk1 in regeneration (33), a full understanding of the importance of different domains of Dcx has translational significance in addition to the relevance for neurodevelopment and for neurodevelopmental disorders.

DCX Lof Alleles for Dendrite Growth Can Map To MT Binding or to C-terminal Binding Domain—We identified several DCX patient alleles that show complete (DCX-R89G and DCX-272X) or partial (DCX-303X) loss-of-function phenotypes when assayed for Dcx-mediated increases in dendrite complexity (Table 1). We then carried out binding analysis as well as localization studies to further characterize the molecular defects of these alleles. Dcx-R89G is deficient in MT binding, and its inability to support dendrite growth thus argues decisively that MT binding by DCX is required. Because both Dcx-272X and Dcx-303X show robust MT association in cells, their inability to support increased dendrite growth is not due to loss of MT association but demonstrates that MT association is not sufficient for Dcx function. Rather, binding interactions or regulatory phosphorylation sites (or both) contained in the C terminus of Dcx are required as well for full Dcx function. One candidate for an additional necessary binding partner of Dcx is spn, which we show here to bind less efficiently to Dcx-272X.
Summary of phenotypes of DCX alleles and proposed mechanism of pathology

| DCX allele | Molecular phenotypes | Subcellular phenotypes | PhenoType Lof/Gof for dendrites | pathological mechanism proposed |
|------------|----------------------|------------------------|-------------------------------|-------------------------------|
| WT         | MT binding, yes      | None                   | Wild type                     | NA                            |
| 303X       | MT binding, yes      | None noted             | Lof/hypomorph                 | Interactions with extreme C terminus needed (AP-2, phosphorylation) |
| 272X       | MT binding, yes      | None noted             | Lof/amorph                    | Full spn binding needed       |
| 246X       | MT binding, yes      | Nuclear accumulation, low expression | No cells expressed the protein | Protein is unstable. Nuclear accumulation might also have negative effects on cells |
| R89G       | MT binding, no       | Present in actin-rich protrusions, but not on MTs | Lof/amorph                   | MT binding required, spn binding not sufficient |
| R59H       | MT binding, no       | Cytoplasmic aggregates consistent with misfolding, increased autophagy in many cells. Disruption of actin, Map2 and spn | Off-pathway/possibly neomorph | Loss of MT binding. In addition, other cytoskeletal elements are disrupted, which might cause additional "off-pathway" pathology |

We propose that the partial loss of spn binding contributes to the Lof phenotype of Dcx-272X. In contrast, Dcx-303X still binds normally to spn as well as to MTs but is lacking the clathrin adaptor AP-binding site (W146C) as well as a number of regulatory phosphorylation sites (34, 35). Dcx-303X behaves as a hypomorph in our assay, suggesting that AP binding might be additionally required for full Dcx function. We previously showed that Dcx promotes the endocytosis of a cell adhesion molecule (24), but further work is needed to explore which endocytic cargo might require Dcx for trafficking to support dendrite elaboration.

Other work previously suggested that MT binding is not sufficient for all roles of Dcx. For example, a patient mutation in the linker between the two DC repeats (W146C) binds MTs but not the MT motor KIF1A. Dcx-146C fails to rescue normal in vitro morphogenesis after Dcx knockdown (9), suggesting important functions for Dcx in regulating MT motors in addition to MT polymerization and bundling. We note that Dcx null neurons initially stall in a multipolar morphology after leaving the ventricular zone and show defects in taking on the normal bipolar morphology of migratory neurons (14, 36). This early morphological transition is distinct from dendrite formation of neurons at DIV5, the process we are analyzing in this work. We also note that the dendrite defect is apparent in culture and thus likely not a consequence of aberrant migration and neuronal positioning, as has been suggested for dendrite morphology defects in the hippocampus (17).

Another example is Dcx-S47R. The Dcx-S47R mutant still binds to MTs but does not support migration of neural progenitors in an in vitro migration assay (37) and mislocalizes to the soma in neurons (9). The Ser-47 site is phosphorylated by PKA and has been implicated in regulating actin via binding of the GEF Asef2 (37). A role for spn was not tested for this mutant. Dcx-272X does not restore normal actin distribution in Dcx null cultured neurons (10), again suggestive for a role of the spn binding interaction and/or additional C-terminal sequences playing important functional roles. Interestingly, Dcx-303X shows complete loss-of-function in terms of migration from an in vitro aggregate of cerebellar granule neurons (31), although in our work we see only a partial defect for dendrite growth. It might thus be the case that clathrin adaptor binding is differentially required for distinct cellular Dcx-mediated processes (migration versus dendrite growth). This possibility will be further examined in the future.

On the flip side, our experiments also demonstrate that binding interactions mediated by the C terminus are not sufficient for full Dcx function in the absence of MT binding. Dcx-R89G can still bind spn (Fig. 5) as well as the clathrin adaptor AP-2. It also still contains all phospho-sites, but it nevertheless does not support the increase in dendrite complexity we see for WT Dcx. These observations suggest a model for multifunctional roles of Dcx, including (but not limited to) MT binding, spn binding, and/or clathrin adaptor binding (Table 1).

**Patient Allele DCX-R59H Causes Off-pathway Effects at the Cellular Level**—Our experiments furthermore clearly demonstrate that not all DCX patient alleles are Lof or hypomorph alleles. DCX-R59H not only does not support increased dendrite complexity but additionally leads to cytoplasmic aggregates. Consistent with our interpretation that Dcx-R59H misfolds and aggregates, the Arg-59 side chain was shown to be buried, and a substitution at Arg-59 (R59L) resulted in changed folding (25). We propose that neurons expressing Dcx-R59H show manifestations of activated degradative pathways aimed at degrading Dcx-R59H aggregates. The aggregates are positive for hsp70, consistent with misfolded protein, become ubiquitinated, and many of them contain LC3 and p62, indicative of autophagosome formation. We speculate that neurons expressing Dcx-R59H up-regulate stress pathways to increase autophagy of accumulated Dcx-R59H and its increased clearance. In addition, it is possible that the aggregates have some detrimental side effects because aggregates of Dcx-R59H contain several components of the cytoskeleton, such as actin and Map2. Most strikingly, however, they accumulate spn, a known

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C. C. Yap, unpublished observations.

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**TABLE 1**

Summary of phenotypes of DCX alleles and proposed mechanism of pathology

Gof is gain-of-function; NA is not applicable, and ND is not determined.
binding partner of Dcx. We speculate that Dcx-R59H might thus impair the functions of other cytoskeletal elements as an off-pathway effect. Recent characterization of DCX mutations identified Dcx-R59H as one of the more severe alleles in patients (28) and one that is only identified de novo. Interestingly, a small percentage of patients (~15%) show microcephaly (28). A subset of DCX alleles might thus cause some excess cell death in patients, but more work will be required to explore this idea.

**Experimental Procedures**

**Reagents**

**Antibodies**—Anti-DCX (rabbit polyclonal against N terminus) (catalog no. 4604, lot 3, Cell Signaling) recognizes a single band at 45 kDa from fetal rat brain. Rabbit polyclonal, against the C terminus (catalog no. 18724, lot GR55971-1, Abcam), recognizes a single band at 45 kDa from mouse brain lysate. Rabbit polyclonal (anti-FLAG F7425, lot 064M475V) and mouse polyclonal (catalog no. F1804, lot SLB4607V) were from Sigma. Anti-GFP, mouse (catalog no. A11120, lot 877587), was from Molecular Probes. Rabbit monoclonal (2956S, lot 4, from Cell Signaling), recognizes a single band at 27 kDa from HCC827 cell lysate. GFP booster was from Chromotek. Anti-tubulin mouse DM1a was from (Sigma). Rat monoclonal (catalog no. y01/34, lot G0714) was from Santa Cruz Biotechnology. Anti-vimentin rabbit BS-0756R (lot 9A23M41, 1:5000 WB) was from Bioss. Anti-c-Myc mouse 9E10 (catalog no. SC-40, lot B0614) and rabbit polyclonal A-14 (catalog no. SC789, lot H0114) were from Santa Cruz Biotechnology. Agarose-conjugated goat polyclonal was from Bethyl Laboratories. F-actin phalloidin 647 (catalog no. A22287) was from Molecular Probes. Anti-Map2 chicken antibody (RRID AB_2138173) was from EnCor Biotechnology. This antibody shows a single band on Western blottings from rat and mouse brain lysates (EnCor Biotechnology). Anti-ubiquitin (P4D1) mouse (catalog no. 610607, lot 4114835, BD Biosciences) showed a single band at 70 kDa on WB from HeLa cell lysate. GFP-spinophilin was from Ora Bloom (Fordham University); myc-spinophilin was from Lawrence Brass (University of Pennsylvania); and mcherry was from Clontech.

**Generation of DCX Mutants**

**Point Mutations**—Point mutations were introduced using site-directed QuikChange mutagenesis kit from Stratagene. All mutations were confirmed by sequencing. Multiple versions of WT and mutant Dcx constructs were created and fused with either GFP, Myc, or FLAG tags. No differences in behaviors were apparent, and the most convenient tag was used in different experiments.

**Neuronal Cultures**—Neuronal cultures were prepared from E18 rat hippocampi, as approved in the Institutional ACUC protocol 3422. Hippocampi from all pups in one litter were combined and thus contained male and female animals. Cells were plated on poly-1-lysine-coated coverslips and incubated with DMEM and 10% horse serum. After 4 h, the cells were transferred into serum-free medium supplemented with B27 (Gibco) and cultured for 9–12 DIV. Transfections were carried out with Lipofectamine 2000. Alternatively, neurons were electropropated (BTX Harvard apparatus) after dissociation and then cultured for 5 days.

**COS and HEK293 cells** were maintained in DMEM + 10% fetal bovine serum, and all transfections were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

**Immunocytochemistry**

Cells were fixed in 2% paraformaldehyde, 3% sucrose, PBS in 50% conditioned medium at room temperature for 30 min and quenched in 10 mM glycine/PBS for 10 min. The fixation conditions used do not introduce holes into the overwhelming majority of cells (30). Coverslips were then blocked in 5% horse serum, 1% BSA, PBS ± 0.05% Triton X-100 for 30 min. Antibodies were diluted in 1% BSA/PBS and incubated for 1–2 h. Coverslips were mounted in Prolong Gold mounting medium and viewed on a Zeiss Z1-Observer with a ×40 objective (EC Plan-Neofluar 40×/0.9 polarization, working distance = 0.41). ApoTome structured illumination was used for most images for better resolution. Images were captured with the Axiocam 503 camera using Zen software (Zeiss) and processed identically in Adobe Photoshop. No non-linear image adjustments were performed. Imaris software (Bitplane) was used for determining Pearson coefficients and 3D voxel representations.

**Detergent Extractions**—Detergent extractions of live cells were carried out in BRB80 buffer (80 mM Pipes, 1 mM MgCl2, 1 mM EGTA, pH 6.8) with 0.3% Triton X-100 at 37 °C for 3 min (24, 25, 26). Cells were washed twice in BRB80 buffer and fixed.

**Soluble/Insoluble Protein Sample Preparation**—Low passage 293 cells plated overnight at 80% confluency in 10% FBS DMEM were transfected with 8 μg of FLAG-Dcx WT, Dcx-246X, Dcx-R89G, or Dcx-R59H and 14 μl of Lipofectamine 2000. After 48 h, adherent cells were washed with ice-cold PBS, and lysed/scraped off the plate with a rubber policeman into 1 ml of IP buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 1% Nonidet P-40, 1 mM PMSF, 1 mM DTT, 1 × Halt protease + phosphatase inhibitor mixture). Cell lysates were recovered from the dish and mechanically lysed with three passages.
through a 28-gauge needle syringe. Lysates were rotisserie for 1 h at 4 °C, followed by centrifugation at 21,000 relative centrifugal force for 20 min at 4 °C. Supernatants were removed, diluted into 4X Laemmli sample buffer, and boiled. Pellets were washed with 500 μl of IP buffer, rotisserie for 30 min, and centrifuged at 21,000 relative centrifugal force for 20 min at 4 °C. The supernatant was aspirated, and the pellet was dissolved in 2× Laemmli buffer. Equivalent samples of both soluble and insoluble protein sample were used for Western blotting.

**Immunoprecipitations**—One 10-cm culture dish of COS7 cells per sample were transfected with either 7.5 μg of FLAG-Dcx construct alone or 5 μg of Myc-spinophilin plus 5 μg of FLAG-Dcx construct with 2.5 μl of Lipofectamine 2000/μg of DNA. Cells were generally split in the morning and then transfected about 6 h later. Media were changed the next day. Cells were lysed on day 2 in lysis buffer (20 mM Hepes, pH 7.4, 2 mM EDTA, 2 mM EGTA, 100 mM NaCl, 10 mM NaF, 1 mM PMSF (added just before use)), 1% Triton X-100, protease inhibitor mixture used at 1× (11 873 580 001, Roche Applied Science). Lysates were pre-cleared with control agarose beads (Pierce control-agarose resin (Thermo Scientific 26150)) overnight and then incubated with goat anti c-Myc-agarose 0.45 μg/μl (S-190-104). Beads were washed several times and then boiled and separated on polyacrylamide gel. After transfer to nitrocellulose, blots were probed for Myc-spinophilin with c-Myc (9E10, Santa Cruz Biotechnology catalog no. sc-40 (mouse, 1:2000)) and FLAG-Dcx (Sigma catalog no. F7425 (rabbit, 1:2000)) followed by LiCOR fluorescent secondary antibodies (1:20,000) and imaged on a LiCOR Odyssey fluorescence imager. Internal data produced by LiCOR showed that their Odyssey infrared imaging system (used in this study) has a greater than 4000-fold linear range compared with 250-fold for chemiluminescence. For immunoprecipitations, band intensity determined from the LiCOR was corrected to the input (total lysate) and then normalized to WT Dcx.

**Dendrite Quantification**—Dendrites were quantified on images of transfected cells fixed on DIV5 and counterstained with Dcx antibody. Because dendrites were short at this time point, a simplified Scholl analysis was used where the number of intersecting dendrites was counted at two concentric rings only, with a diameter of 30 μm and one of 60 μm. The 30-μm measurements are reported in the figure.

**Statistical Analysis**—Statistical analysis was carried out using Graphpad Prism software. Data sets were first evaluated as non-parametric. Non-parametric data sets were analyzed with the Wilcoxon signed-rank test, and parametric data sets were analyzed with Student’s t test, and parametric data sets were analyzed with the parametric test. Co-immunoprecipitations were quantified with the Odyssey infrared imaging system (used in this study) has a greater than 4000-fold linear range compared with 250-fold for chemiluminescence. For immunoprecipitations, band intensity determined from the LiCOR was corrected to the input (total lysate) and then normalized to WT Dcx.

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**Statistical Analysis**—Statistical analysis was carried out using Graphpad Prism software. Data sets were first evaluated as parametric versus non-parametric. Non-parametric data sets were analyzed using Mann-Whitney U test, and parametric data sets were analyzed with Student’s t test or analysis of variance followed by post hoc test. For dendrite measurements, three repeats using independent cultures were quantified with >50 cells counted per condition. The exact number of cells is indicated in the figure legend for each graph. For co-immunoprecipitations, statistical analysis of the Dcx-spinophilin co-IP data was carried out with Graphpad Prism software. For each replication the individual lanes on the Western blots were corrected to the expression levels in the cell lysates and then normalized to the WT control. Significance was evaluated in Prism using the Wilcoxon signed-rank test.
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