Brief Communications

Antagonistic Effects of Doublecortin and MARK2/Par-1 in the Developing Cerebral Cortex

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Abnormal neuronal migration is manifested in brain malformations such as lissencephaly. The impairment in coordinated cell motility likely reflects a faulty mechanism of cell polarization or coupling between polarization and movement. Here we report on the relationship between the polarity kinase MARK2/Par-1 and its substrate, the well-known lissencephaly-associated gene doublecortin (*DCX*), during cortical radial migration. We have previously shown using *in utero* electroporation that reduced MARK2 levels resulted in multipolar neurons stalled at the intermediate zone border, similar to the phenotype observed in the case of DCX silencing. However, whereas reduced MARK2 stabilized microtubules, we show here that knock-down of DCX increased microtubule dynamics. This led to the hypothesis that simultaneous reduction may alleviate the phenotype. Coreduction of MARK2 and DCX resulted in a partial restoration of the normal neuronal migration phenotype *in vivo*. The kinetic behavior of the centrosomes reflected the different molecular mechanisms activated when either protein was reduced. In the case of reducing MARK2 processive motility of the centrosome was hindered, whereas when DCX was reduced, centrosomes moved quickly but bidirectionally. Our results stress the necessity for successful coupling between the polarity pathway and cytoplasmic dynein-dependent activities for proper neuronal migration.

Key words: **neuronal migration; DCX; MARK2/Par-1; lissencephaly; microtubules; *in utero* electroporation**

Introduction

Coordinated neuronal migration depends on development and maintenance of cell polarization. We have recently demonstrated a role for the polarity kinase MARK2/Par-1 in neuronal migration (Sapir et al., 2008). MARK2/Par-1 phosphorylates doublecortin (DCX) (Schaar et al., 2004), another microtubule-associated protein (MAP), mutations in which cause X-linked lissencephaly (Francis et al., 1999; Gleeson et al., 1999; Horesh et al., 1999). We hypothesize that interaction between MARK2/Par-1 and DCX may regulate cortical radial migration. These proteins are likely to affect microtubule dynamics, which are tightly balanced during neuronal migration.

MARK2/Par-1 plays an important role in regulation of cytoskeletal dynamics (Timm et al., 2006), in particular microtubule dynamics (Drewes et al., 1997; Drewes et al., 1998; Sapir et al., 2008). Following MARK2 phosphorylation its MAP substrates detach from tubulin polymers. Known substrates include tau, MAP-2/MAP-4, and DCX (Drewes et al., 1997; Schaar et al., 2004). DCX binds to microtubules in a unique position in between the protofilaments, and stabilizes them (Moores et al., 2004). Therefore, we raised the hypothesis that reduction in the cellular expression of DCX may affect microtubule dynamics. In addition, MARK2/Par-1 and DCX are suggested to play an important role in regulation of cell polarity. A genetic interaction between the dynein pathway and the polarity pathway is suggested by the fact that orthologs of LIS1, cytoplasmic dynein and DCX affect the first asymmetric cell division in *Caenorhabditis elegans* (Gönczy et al., 2000). LIS1 (Reiner et al., 1993) and DCX (des Portes et al., 1998; Gleeson et al., 1998), both of which are implicated in neuronal migration disorders, regulate centrosomal-nuclear coupling in a dynein-dependent pathway (Shu et al., 2004; Tanaka et al., 2004a; Tsai et al., 2005, 2007). Furthermore, reduction in MARK2 affects centrosomal movement in radially migrating neurons (Sapir et al., 2008). LIS1 and its interacting protein Ndel1 regulate Cdc42 (Kholmanskikh et al., 2003, 2006; Shen et al., 2008), which impacts a repertoire of targets including PAR-6, aPKC, PAR-3 and PAK5 (Govek et al., 2005). Based on the above observations, we hypothesized that MARK2/Par-1 and its substrate DCX may cooperate during neuronal migration as key node proteins joining the polarity and the dynein pathways.

Reduction in cellular DCX is expected to destabilize microtubules, while reduction in the cellular level of MARK2 should

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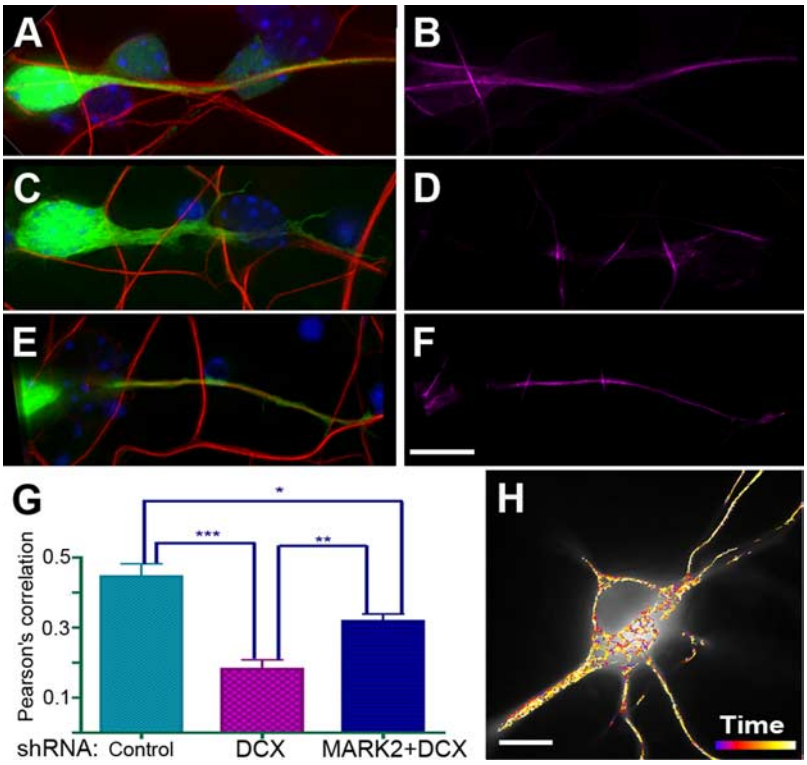

Figure 1. Reduction in DCX destabilizes microtubules *in vitro*. ***A–F***, Microtubule stability in cerebellar neurons. ***A***, ***C***, ***E***, Transfected neurons are labeled with GFP (green), immunostained with antidetyrosinated tubulin antibodies (red), and the nuclei labeled with DAPI (blue). ***B***, ***D***, ***F***, Colocalization between GFP and detyrosinated tubulin is shown in a false purple color. The treatments include control shRNA (***A***, ***B***), DCX shRNA (***C***, ***D***), and a combination of DCX and MARK2 shRNA (***E***, ***F***). Scale bar, ***F***, 10 μm. ***G***, Quantification of Pearson's correlation of the colocalization between GFP and detyrosinated tubulin was calculated using Imaris colocalization measurement tool and subjected to ANOVA statistical analysis. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; error bars indicate $\pm$SEM. ***H***, Hippocampal neurons transfected with indicated shRNA and EB3-GFP tracked using Imaris cell tracking system. A representative neuron with lines shown in different colors marking the advance of individual tracks over time. Scale bar, ***H***, 5 μm.

stabilize microtubules by alleviating its effect on DCX. Subsequently, coreduction of both proteins could potentially result in partial restoration of a normal migration pattern *in vivo*, as shown here. Reduction in MARK2 or DCX also affected processive centrosomal movement, a process subject to dynein regulation. The molecular mechanisms underlying nonprocessive motility differed in an essential manner. Whereas reduction in MARK2 resulted in significantly less motile centrosomes, reduction in DCX enhanced centrosomal movement in both forward and backward orientations, resulting however in reduced net processivity. These results demonstrate intercalation between the polarity pathway with the cytoplasmic dynein-mediated neuronal migration pathway and highlight the accurate coupling required for radial migration in the cerebral cortex.

Materials and Methods

Neuronal cultures and in utero *electroporation.* Hippocampal and cerebellar neurons were prepared as described previously (Brann et al., 2002; Gdalyahu et al., 2004) and transfected by the Amaxa protocol. Following electroporation, neurons were stained or imaged live as described previously (Sapir et al., 2008) and analyzed using the Delta-Vision system package, or Imaris software. In case of EB3-GFP transfections, eight cells with a total of 11,375 tracks were analyzed from control cells, and six cells with a total of 9145 tracks from DCX shRNA-treated cells. *In utero* electroporations were conducted as described previously (Sapir et al., 2008). Additional information is provided in supplemental material, available at www.jneurosci.org.

Quantitative measurements. The colocalization program in the SoftWorx suite was used to determine the degree of colocalization and the Pearson coefficient of correlation was calculated. The program generates a new product image of two channels after subtracting a threshold value for each.

Neuronal migration following *in utero* electroporation was analyzed from at least three different embryos and at least eight different sections. Sections were stained with DAPI, and the different brain areas were identified. Comparison between different treatments of the ratio of GFP-positive cells reaching the superficial area of the cortical plate (CP) was analyzed using one-way ANOVA, and Tukey's post test was performed using GraphPad Prism version 4.00 for Macintosh (GraphPad Software).

To evaluate centrosomal displacement, centrosomal locations were extracted by the transfected centrin fluorescence. In cases where the centrioles split, each individual centriole was analyzed. The route taken by each centrosome was computationally rotated to a horizontal orientation, and the displacement along the axis was calculated for successive time points. Frames were recorded at 2 min intervals.

Results

The hypothesis that reduction of DCX may result in impaired microtubule stability was tested first by immunostaining of transfected primary cerebellar and hippocampal neurons (Fig. 1). Cotransfection was performed with small hairpin RNA directed against DCX or a control shRNA or a combination of DCX and MARK2 shRNA and an expression plasmid for GFP as a marker. Cells were stained using antidetyrosinated α-tubulin antibodies, a marker of stabilized microtubules (Fig. 1*A–F*). Microtubule stabilization was reduced in DCX shRNA-treated neurons (Fig. 1, compare *C*,*D*, *A*,*B*). This was evaluated by comparing the degree of colocalization of GFP with the immunolabeled detyrosinylated tubulin between DCX shRNA- and control shRNA-transfected cells. The degree of colocalization was quantified by the Pearson coefficient of correlation (Fig. 1*G*) (in primary cerebellar neurons Student's t test $p < 0.0001$, mean $\pm$ SEM of control 0.44 ± 0.035, $n = 14$, mean $\pm$ SEM of DCX shRNA 0.18 ± 0.02, $n = 23$) and was found to be statistically significant. Similar results were obtained in cultured hippocampal neurons (data not shown). We have previously demonstrated that MARK2 reduction increased microtubule stability (Sapir et al., 2008). Therefore, we postulated that coreduction of DCX and MARK2 may result in increased stability of microtubules in comparison with the single reduction of DCX. Coreduction of DCX and MARK2 resulted in an observed increase in microtubule stability (Fig. 1, compare *E*,*F*, *C*,*D*). The degree of colocalization was quantified as described above, and the increase in microtubule stability in comparison with DCX shRNA treatment was found to be statistically significant, although it was still lower than control levels (Fig. 1*G*) (ANOVA analysis, DCX shRNA vs the double treatment $p < 0.01$, control vs the double treatment $p < 0.05$, DCX shRNA vs control $p < 0.001$). These results suggest that coreduction of DCX and MARK2 converges at the level of microtubule stability.

We next examined microtubule dynamics in live cells by mea-

suring the duration of EB3-GFP residence on microtubule tips (Fig. 1*H*) in cultured hippocampal neurons. Each track in the EB3 movie is encoded by a rainbow color representing time. Neurons transfected with DCX shRNA exhibited less stable microtubules implied by the shorter duration of EB3 on the tips of microtubules (Student's *t* test $p < 0.0001$, mean ± SEM of control 7.364 ± 0.11, $n = 11{,}373$, mean ± SEM of DCX shRNA 6.398 ± 0.11, $n = 9143$). An inverse correlation was noted for the rate of microtubule polymerization; microtubules polymerized faster in the absence of DCX than in control cells (Student's *t* test $p < 0.0001$, mean ± SEM of control 0.3406 ± 0.002, $n = 11{,}373$, mean ± SEM of DCX shRNA 0.3974 ± 0.002, $n = 9143$). Collectively, the data are consistent with altered microtubule growth and stability, indicating that DCX may be an important regulator of microtubule dynamics.

In vivo, reducing DCX by *in utero* electroporation resulted in a pronounced inhibition of neuronal migration in comparison with control ($p < 0.0001$) (Fig. 2, compare *B*, *A*). The phenotype was similar to that of a previous report (Bai et al., 2003). Notably, shRNA reduction of MARK2 had a similar effect (Fig. 2*C*). We noted the presence of a typical band of neurons stalled at the intermediate zone (IZ) border in the cortical plate, where it has been demonstrated that multipolar neurons accumulate (Bai et al., 2003; Sapir et al., 2008). Coreduction of MARK2 and DCX resulted in a partial but significant improvement in neuronal migration in comparison with the individual reduction in either DCX or MARK2 (Fig. 2*D*,*E*) (statistical analysis by ANOVA, coreduction in comparison with DCX shRNA $p < 0.001$, coreduction in comparison with MARK2 shRNA $p < 0.05$). Most strikingly, there was a notable decrease in the number of neurons stalled at the IZ boundary. These findings suggest that DCX may be an important MARK2 substrate in the developing cortex.

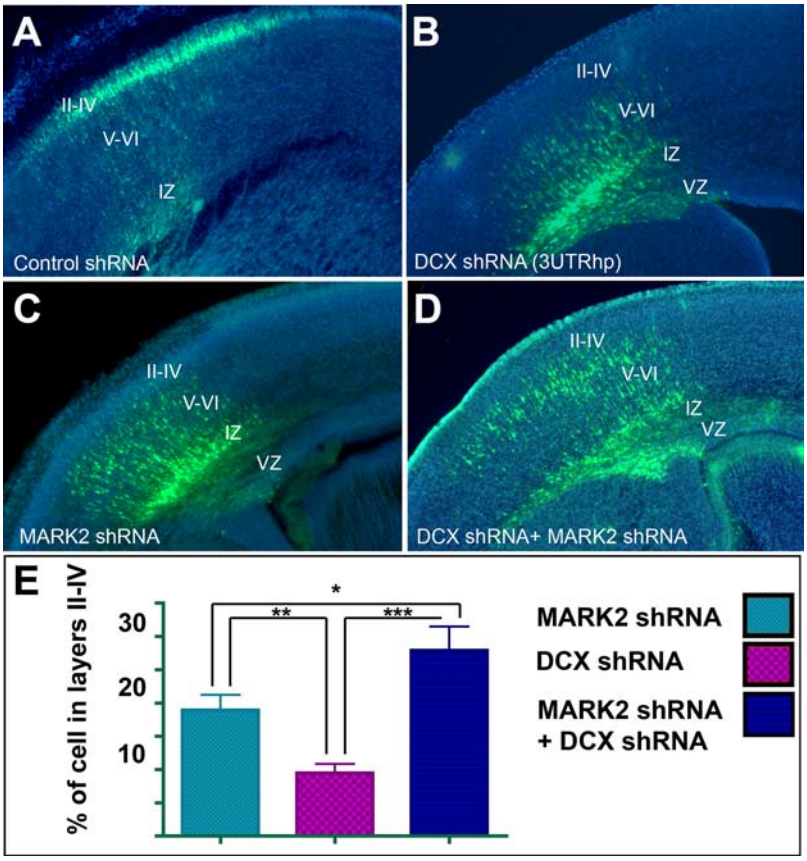

Figure 2. Reduction in MARK2 partially rescues DCX migration phenotype *in vivo*. Brains electroporated at embryonic day 14.5 (E14.5) *in utero* with the indicated shRNA and analyzed at E18.5. ***A***, A brain section electroporated with control shRNA. Most labeled cells reach the superficial layers of the cortex. ***B***, Reduction in DCX inhibits neuronal migration. ***C***, Reduction in MARK2 results in cell accumulation in the IZ border. ***D***, Coreduction in MARK2 and DCX partially rescues the migration phenotype in comparison with the individual genes reduced. ***E***, Quantification of cells reaching the superficial part of the CP from at least four brains expressing either DCX shRNA, or MARK2 shRNA, or both DCX and MARK2 shRNA ± SEM, statistical analysis by ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

DCX has been shown to complex with dynein. The lack of *Lis1* resulted in an increase in the distance between the nucleus and the centrosome; expression of DCX in neurons lacking one *Lis1* allele compensated for the observed phenotype (Tanaka et al., 2004a). We have previously shown that reduction in MARK2 levels suppressed movement of centrosomes in migrating cortical neurons, resulting in heterotropic multipolar cells at the IZ boundary. Therefore we investigated the dynamic behavior of the centrosome in migrating neurons with reduced DCX. Distinct modes of centrosomal motility during radial neuronal migration can be identified in time-lapse movies of the brain slices (Fig. 3*A–G*; supplemental movies 1–4, available at www.jneurosci.org as supplemental material). Control centrosomes labeled by centrin-RFP move continuously in a forward direction (Fig. 3*A*,*B*,*G*), as reported previously (Tsai et al., 2007; Sapir et al., 2008). In the presence of DCX shRNA, in contrast, centriole splitting and random motility of centrosomes were evident in many cells (Fig. 3*C*,*D*,*G*; supplemental movies 1,2, available at www.jneurosci.org as supplemental material). In several cases the centrosome traveled from the leading edge all the way to the rear of the cell. This extraordinary motility has not been previously observed in migrating cortical neurons. Interestingly, coexpression of MARK2 shRNA with DCX shRNA suppressed the nonoriented centrosomal movement observed with DCX shRNA alone (Fig. 3*E*,*F*,*G*; supplemental movies 3,4, available at www.jneurosci.org as supplemental material). Centrosomal motility in the coreduction condition was slightly increased compared with MARK2 shRNA alone. This result is in agreement with the partial rescue phenotype (Fig. 2).

Individual cells from each treatment were subjected to a more detailed analysis to characterize the centrosome movements (Fig. 4*A–E*). Centrosomes in the control neurons moved in the most processive manner, and the net distances covered were the largest (Fig. 4, compare *A*, *B–D*). Their mean velocity of 24 μm h^{-1} agrees with previously published measurements (Fishell and Hatten, 1991; Rio et al., 1997; Bovetti et al., 2007). A histogram of the displacements measured between frames (120 s intervals) shows a Gaussian distribution peaked at 1 μm/frame. DCX shRNA-treated centrosomes showed a much wider distribution of displacements (Fig. 4*E*, red), with tails extending to very large positive (toward the pial surface) and negative [toward the ventricular zone (VZ)] values. The ratio between the total path traveled

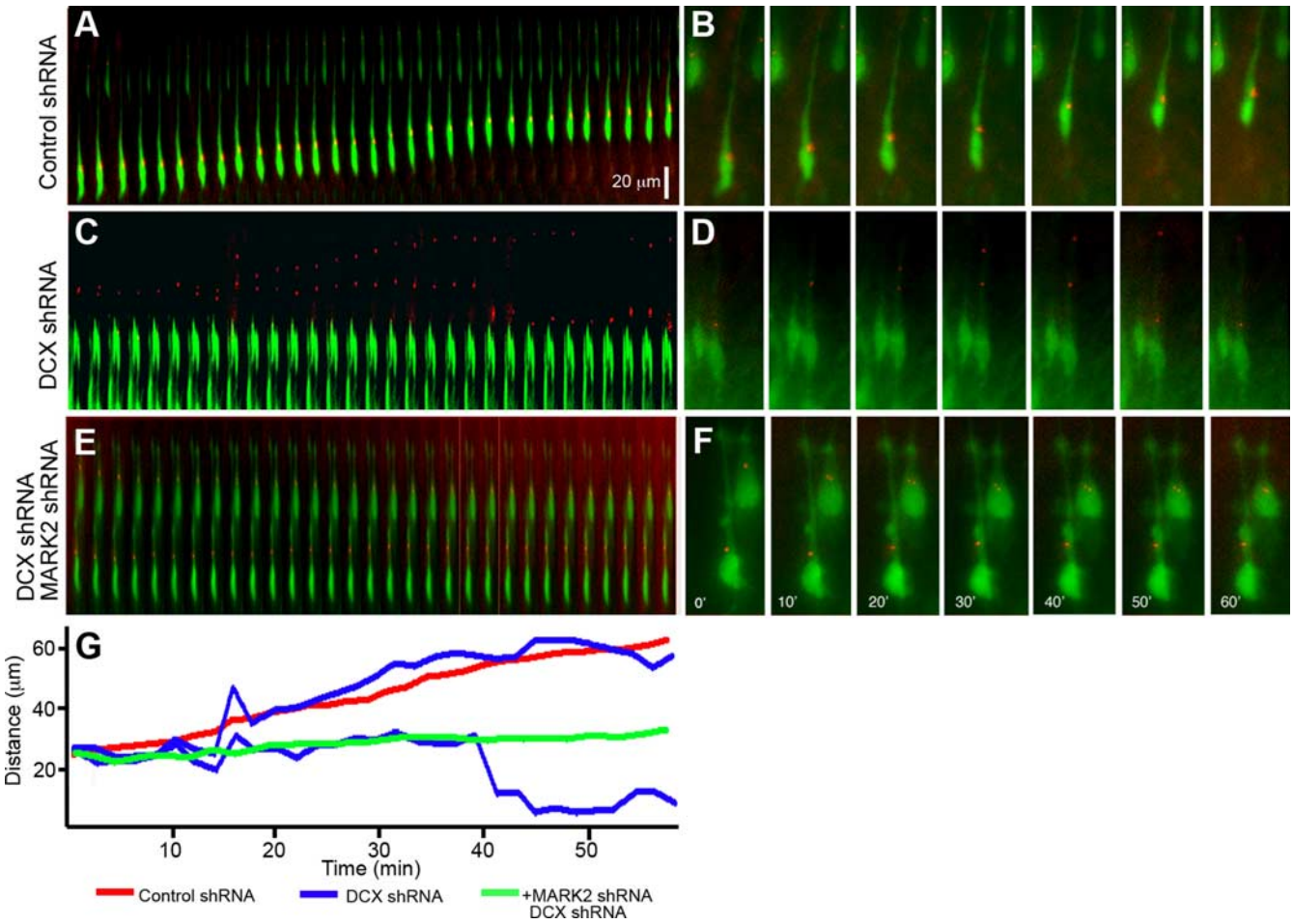

Figure 3. Analyses of centrosomal motility in organotypic brain slices from *in utero* electroporated brains. ***A–F***, Time-lapse fluorescence microscopy of migrating cells in live brain slices. Cells expressing GFP with indicated shRNA; centrin-RFP (red) marks one centrosome or two separate centrioles. ***A***, ***B***, Control shRNA, continuous centrosomal movement preceding the nucleus; ***B***, images from ***A*** every 10′. ***C***, ***D***, DCX shRNA-treated cells show uncoupled movement of centrioles. ***D***, Centrosome split is visible in selected images from ***C***. ***E***, ***F***, Partial rescue of centrosomal stationary behavior is achieved by treatment with both MARK2 shRNA and DCX shRNA. ***F***, Images taken every 10′. ***G***, The location of the individual centrosome/centriole is each frame is plotted against time.

and the net distance that was eventually covered by each centrosome differed greatly between control and DCX shRNA-treated cells. In the control shRNA-treated cells the value was 1.1 ± 0.1, whereas in DCX shRNA-treated cells it was 10.6 ± 3.9, suggesting that the net distance traveled was smaller due to the strong tendency to reverse direction and thereby move in both orientations. The mean displacement of MARK2 shRNA-treated centrosomes was the lowest, and it differed significantly from the other groups. Cotransfection of MARK2 and DCX shRNA almost eliminated the mean displacement of the centrosomes in the negative direction. Although it differed from the MARK2 shRNA group, it did not reach control levels, again consistent with a partial rescue of the normal phenotype.

Examining the data more closely, in control cells 72% of the measured centrosome displacements were between 1 and 2 μm, corresponding to speeds of 0.5–1.0 μm min^{-1} (Fig. 4*E*, blue). The direction of movement was identical to that of the cells. In DCX shRNA-treated cells 8.9% of the centrosome displacements were oriented toward the ventricle. The fraction of events occurring at speeds exceeding 1 μm min^{-1} reached 21%, compared with only 11% in the control group. In MARK2-treated cells, 98% of displacements were measured at 1 μm or less (Fig. 4*E*, yellow), indicating a net speed of 0.0–0.5 μm min^{-1} over the frame interval. Cotransfection by MARK2 and DCX shRNA led to an increase in the fraction of displacements >2 μm from 1.8% to 5.0% (Fig. 4*E*, green). A small number of backward events were recorded in cotransfected cells, 1.5% versus none in the case of MARK2 shRNA alone. The fraction of backward events was very small compared with the case of DCX shRNA alone, consistent with the effect of MARK2 in moderating the phenotype of reduced cellular DCX. In summary, the detailed analysis demonstrates that introduction of both types of shRNA improves several specific parameters that are negatively affected by either individual shRNA.

Discussion

This study highlights a possible role of centrosomal processivity in successful radial migration in the developing cerebral cortex. Radial migration involves tight coordination between cell polarity and the cytoskeleton. This is reflected in centrosomal motility, which requires the activity of molecular motors and cytoskeletal integrity. In addition, regulation of polarity is important for coupling of the centrosome and the nucleus. The roles of MARK2/Par-1 in cell polarity are well established (Biernat et al., 2002; Pellettieri and Seydoux, 2002; Macara, 2004). DCX is a MAP that regulates microtubule stability (Francis et al., 1999; Horesh et al., 1999). Since DCX is phosphorylated by MARK2, and this phosphorylation induces the release of DCX from microtubules, we reasoned that these two proteins could couple between the polarity and motility pathways that maintain proper neuronal migra-

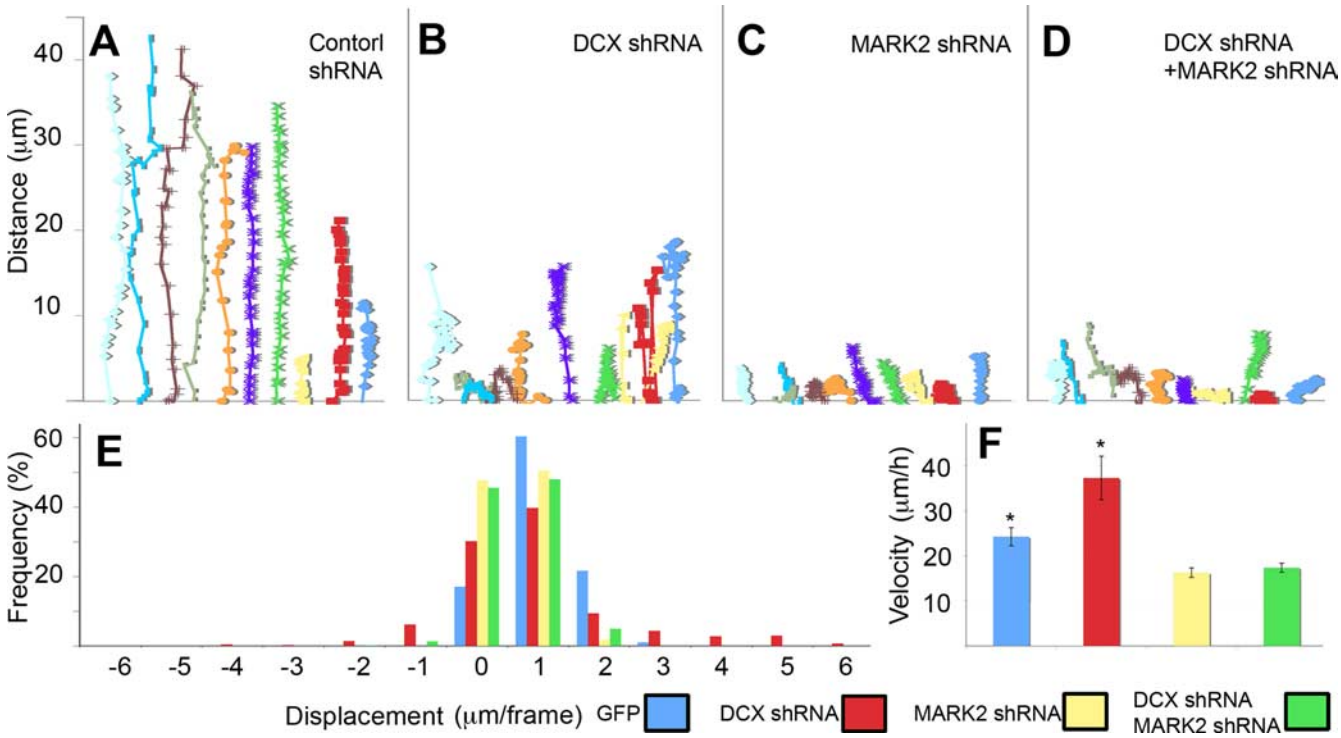

Figure 4. Analyses of centrosomal motility in organotypic brain slices from *in utero* electroporated brains. ***A–D***, Centrosomal tracks of control and indicated shRNA treated cells in live brain slices. Centrosomes were visualized using centrin-RFP. The location of the individual centrosomes in each frame is presented: control shRNA (***A***), DCX shRNA (***B***), MARK2 shRNA (***C***), DCX and MARK2 shRNA (***D***). ***E***, The relative displacement of each centrosome was plotted and binned according to the distance moved and the direction. The movement in the direction of the pial surface was plotted positive, while the movement toward the VZ was negative. Cells treated with DCX shRNA moved the most in both directions (red histograms), whereas MARK2 shRNA-treated cells (yellow histograms) moved the least. GFP control cells (blue histograms) moved the most in the positive orientation (1–2 μm per frame). A slight improvement is noticed in the double treated cells (green histograms). ***F***, The calculated average velocities subjected to statistical analysis. MARK2 shRNA-treated cells (yellow histogram) were significantly slower than all the other groups; a statistically significant improvement was detected in MARK2 and DCX shRNA-treated cells (green histogram). Control centrosomes (blue histogram) moved somewhat slower (not statistically different) than DCX shRNA centrosomes (red histogram). *$p < 0.05$; error bars indicate $\pm$SEM.

tion. Reduction of DCX resulted in dissociated centrioles and hypermotile centrosomes, but a loss of processivity and spatial coordination with the nucleus. Reduction in immunostaining with anti-detyrosinated tubulin under the same conditions suggests that these effects may be explained by a loss of microtubule stability. In contrast, MARK2 reduction had the opposite effect, strongly impairing centrosome motility. The lack of centrosomal motility has been tied to dynein activity in the other systems (Tsai et al., 2007). Both treatments blocked neuronal migration *in vivo* and led to a build-up of cells at the IZ border. When the two shRNA treatments were applied simultaneously, the suppression of cell migration and that of centrosome motility were both significantly alleviated. Because DCX's phosphorylation by MARK2 releases it from microtubules (Schaar et al., 2004), an antagonistic mechanism of cellular control via MARK2 regulation of DCX can be suggested. Consequently, the removal of both elements should at least partly restore the normal phenotype. Therefore the results in this work support a coupling mechanism between cell polarity and microtubule stability via these two proteins.

Opposing effects of DCX and MARK2 have also been noted in regulation of polarity in hippocampal neurons. Reduction of DCX inhibited dendrite development (Cohen et al., 2008), whereas reduction of MARK2 stimulated their development (Terabayashi et al., 2007). A correlation between polarity and centrosome behavior has been observed in case of manipulating the levels of the key polarity protein PAR-6 which resulted in impaired migration of primary cerebellar neurons (Solecki et al., 2004). Inhibition of the cell polarity factors GSK3β or PKCζ in migrating olfactory neurons resulted in impairment of centrosome reorientation and of process stabilization (Higginbotham et al., 2006). Slow motility of the centrosome and impaired coupling between the centrosome and the nucleus have been observed previously when the activity of the microtubule-associated molecular motor cytoplasmic dynein was suppressed, either directly or by interference with accessory proteins such as LIS1 and Ndel1 (Shu et al., 2004; Tsai et al., 2007). DCX is a substrate of multiple kinases, including Cdk5 (Tanaka et al., 2004b), JNK (Gdalyahu et al., 2004) and MARK2 (Schaar et al., 2004), which are all involved in regulation of neuronal migration. Of particular interest is the role of protein phosphatase 1 (PP1) in centrosomal splitting, since this phosphatase is capable of dephosphorylating DCX in a site-specific manner (Shmueli et al., 2006; Bielas et al., 2007). DCX and MARK2 are capable of mediating a cross talk between the microtubule and actin cytoskeletons (Tsukada et al., 2003, 2005; Matenia et al., 2005; Johne et al., 2008). In summary, neuronal migration coupled to seamless motility of the polarized centrosome requires a tight balance of factors involved in regulation of molecular motors and the cytoskeleton.

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