The Interaction of mPar3 with the Ubiquitin Ligase Smurf2 Is Required for the Establishment of Neuronal Polarity

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The Par polarity complex consisting of the evolutionarily conserved proteins mPar3, mPar6, and aPKC regulates cell polarity in many cell types including neurons. Here we show that mPar3 is required for the establishment of neuronal polarity and links the Smurf2 to Kinesin-2. The HECT domain E3 ubiquitin ligase Smurf2 ensures that neurons extend only a single axon by initiating the degradation of inactive Rap1B through the ubiquitin/proteasome system. Its interaction with mPar3 is required to localize Smurf2 to growth cones and restrict Rap1B to the axon. Interfering with the binding of mPar3 to Kinesin-2 or Smurf2 to mPar3 and knockdown of mPar3 by RNAi disrupt the establishment of neuronal polarity through the failure to restrict Rap1B to a single neurite.

During the differentiation of hippocampal neurons, unpolarized cells initially form several equivalent neurites, all of which can have the potential to become an axon. Polarity is established when one of these processes is selected as the axon (stage 3) by a signaling pathway that includes PI3K,1 the phosphoinositide 3-kinase; aPKC, atypical PKC; mPar3, mammalian partitioning defective 3; and PKC, protein kinase C. Disruption of the sequential activity of the GTPases Rap1B and Cdc42 directs the establishment of polarity in hippocampal neurons downstream of PI3K (6). Initially, Rap1B is present in both unpolarized stage 2 neurons and polarized stage 3 neurons, but the appearance of mPar3 in all processes, mPar3 C terminus interacts with the Rac-specific guanine nucleotide-exchange factors (GEFs) STEF and Tiam1 to control tight junction assembly and to mediate the activation of Rac by Cdc42 through the Par complex to promote axon formation (5, 13, 14). In neurons, mPar3 and mPar6 are present in all processes of non-polarized stage 2 neurons and become restricted to the axon of polarized stage 3 neurons (6, 7). Overexpression of mPar3 or mPar6 disrupts neuronal polarity and induces the extension of several long Rap1B-negative processes instead of a single axon (4, 7). The mPar3/Smurf2 interaction is essential for the transport of mPar3 and aPKCs into neurites and the establishment of neuronal polarity.

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4 The abbreviations used are: PI3K, phosphoinositide 3-kinase; aPKC, atypical PKC; GSK3β, glycogen synthase kinase 3β; GST, glutathione S-transferase; HECT, homologous to E6-associated protein C terminus; MBP, maltose-binding protein; mPar3, mammalian partitioning defective 3; RNAi, RNA interference; shRNA, short hairpin RNA; Smurf2, Smad ubiquitination regulatory factor-2; UPS, ubiquitin/proteasome system; GEF, guanine nucleotide-exchange factors; HEK, human embryonic kidney; aa, amino acid; EGFP, enhanced green fluorescent protein; d.i.v., days in vitro.

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WITHDRAWN
mPar3 by RNA interference (RNAi) leads to the loss of endogenous Smurf2 from growth cones. The failure to restrict Rap1B to a single neurite results in the formation of supernumerary axons.

EXPERIMENTAL PROCEDURES

Plasmids—Expression vectors for Smurf2, RalGDS-RBD, mouse and human mPar3, and Par3–4N/2 were kindly provided by J. L. Wrana (University of Toronto), I. G. Macara (University of Virginia, Charlottesville), J. L. Bos (University Medical Centre, Utrecht), and K. Kaibuchi (Nagoya University), respectively. The pSHAG-1 vector (20) used for RNA interference was kindly provided by G. J. Hannon (Cold Spring Harbor Laboratory). Full-length Smurf2 and Smurf2 deletion constructs with the C2, WW, and HECT domain were generated by PCR by using the following primers: full length (aa 1–748): ATGTC TAACC CCGGA GCC and TCATT CCACA GCAAA TCCACA; C2 (aa 1–150), ATGTC TAACC CCGGA GCC and GTCCA CAACT TGTC TTCTGTG; WW (aa 129–350), GGACA GATAG TAGTA AGTCT TCAG and CTGTT GCTGT TTCTCA; HECT (aa 349–748), CAGCA AGTGG TATCG TTATG TCC and TCATT CCACA GCAAA TCCACA. The amplified sequences were cloned into pMAL-p2X (New England Biotechnology), pGEX-4T2, and pEGFP-C1 (Clontech), which was also used as a control vector. mPar3 deletion constructs comprising the CR1, PDZ1, PDZ2/3, and aPKC-binding region were generated by PCR using the following primers: CR1PDZ1 (aa 1–400), ATGAA AGTGA CCGTG TGCTT and GTGAA GCCCT GCACT GTTCA; PDZ1 (aa 200–400), AGCCT CCCGC GGGAT ACTA and GTGAA GCCCT GCACT GTTCA; PDZ2/3 (aa 398–697), ACGGT GCAGA GAGCA CCCC and GCTGG AAGAG GACTG GTCAGA; PDZ2 (aa 400–560), ACGGT GCAGA GAGCA CCCC and TGGCT CTGCA TTCAG TTCCC; PDZ3 (aa 560–780), AGCCA GATGC AGATTC CAAA and GCTGG AAGAG GACTG GTCAGA; aPKCBR (aa 700–1340), CTGCC CATTG AAACA GCGTT and TCAGG AATAG AAGGG CCTCC. The amplified sequences were cloned into pEGFP-C3 (Amersham Biosciences).

Neuronal Cultures and Transfection—Cultures of dissociated hippocampal neurons were prepared and transfected as described previously (6). Briefly, the hippocampus was dissected from E18 rat embryos, dissociated, and neurons plated onto glass coverslips coated with poly-ornithine (Sigma) at a density of 800,000 cells per coverslip (diameter 14 mm) in 24-well plates. Neurons were transfected 2 h after plating using Lipofectamine 2000 (Invitrogen) as described previously (6). After incubation for 2 h, the transfection medium was replaced by Neurobasal medium (supplemented with B27, 0.5 mM glutamine, and 100 units/ml penicillin/streptomycin; Invitrogen). The cells were detached after the transfection by moderate pipetting and replated onto new coverslips at a lower density (40,000–60,000 cells per coverslip in a 24-well plate). Neurons were fixed at 3 d.i.v. with 4% paraformaldehyde and 15% sucrose in phosphate-buffered saline for 20 min at 4 °C or (for staining with anti-Smurfl- and -Smurf2 antibodies) with methanol/acetone (1:1) for 20 min at −20 °C. To analyze the establishment of neuronal polarity, neurons were stained with the Tau-1 (as a marker for axons) and an anti-MAP2 antibody (minor neurites) and categorized as polarized when they formed a single axon (neurite longer than 90 μm and positive for Tau-1 in its distal part). Neurons were regarded as unpolarized when they did not extend an axon (no neurite was significantly longer than the others and not longer than minor neurites formed by polarized neurons, and none of the neurites showed strong Tau-1 staining). Neurons with an indeterminate phenotype extended one or more neurites between 45 and 90 μm (i.e., longer than normal minor neurites) with weak but detectable Tau-1 staining that was sometimes but not necessar-
il always stronger in the distal part or when the neurite was longer than 90 μm but did not show the characteristic Tau-1 staining pattern with a proximal to distal increase in staining intensity (21). Neurons with an indeterminate phenotype include cells that correspond to the categories “Stage 2–3” and “Multiple axon” used by Nishimura et al. (5). Hyperpolarized neurons formed two or more axons. Minor neurites were identified by staining with an anti-MAP2 antibody. To analyze the distribution of Rap1B and Smurf2, the immunofluorescence intensity was measured in growth cones. Background fluorescence intensity was determined for an identical cell-free area adjacent to the growth cone. A growth cone was scored as positive for Rap1B or Smurf2 when the fluorescence intensity was at least three times higher than background. To quantify the colocalization, puncta positive for Smurf2 and Kinesin-2 were counted. Puncta were defined as distinct, spatially limited fluorescence signals, at least 5 pixel in size that had intensity at least five times above background.

RNA Interference — For the knockdown of mPar3 by RNAi the following target sequences were selected for the generation of shRNA vectors based on the pSHAG-1 plasmid (20): TCAGCCTCATCCAGCAGGCGGTGACCCGC (Par3 RNAi) and TCAGCCTTATCGAGTACGGCTGGGTGCCC (Par3 RNAiMut) and an RNAi construct described previously (22).

Immunofluorescence — The following antibodies were used: Tau-1 (Chemicon, MAB3420; dilution 1:1000) and anti-MAP2 (Chemicon, AB5622; 1:200), anti-Rap1 (BD Biosciences, 610195; 1:500), anti-Smurfl (Abgent, AP2104b; 1:500), anti-Smurf2 (Abgent, AP2105b; 1:500), anti-Kinesin-2 (Abcam, ab24626; 1:500), and Alexa-conjugated secondary antibodies (Molecular Probes; 1:1000). A Zeiss LSM 510 confocal laser-scanning microscope was used to analyze the colocalization of Kinesin-2 and Smurf2. Neuronal morphology was analyzed using the WASABI software (Hamamatsu), ImageJ 1.33u (NIH), and Adobe Photoshop. The length of axons and dendrites was determined using the Spot software (Diagnostic Instruments). To analyze the colocalization of Smurf2 and Kinesin-2, Pearson coefficient and Mander overlap coefficient were calculated using ImageJ 1.33u and the intensity correlation analysis module (NIH). The Student’s t test was used to test statistical significance.

FIGURE 2. The PDZ domains 2 and 3 of mPar3 interact with the Smurf2 HECT domain. A and C, deletion constructs of Smurf2 (A) and mPar3 (C) are shown schematically. The numbers refer to the position in the amino acid sequence. B and D,пар3 and Smurf2

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**Immunoprecipitation, Pull-down assays, and Western Blot—**Pull-down assays were performed as described (19). GST or MBP fusion proteins were immobilized on glutathione-Sepharose (Amersham Biosciences) or amylase coupled to agarose beads (New England Biotechnology), respectively. After washing the beads with lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 10 mM MgCl₂, 4 mM EDTA, 10% glycerol, 1% Triton X-100, and phenylmethylsulfonyl fluoride), they were incubated with lysates from transfected HEK 293 T cells, washed, and bound proteins eluted with sample buffer. HEK 293 T cells were lysed 48 h after transfection with lysis buffer (2% Triton X-100 and complete protease inhibitor mixture (Roche) in phosphate-buffered saline) for 30 min at 4 °C.

To study the direct interaction of mPar3 and Smurf2 bacterially expressed MBP-Smurf2 was immobilized on amylase beads (New England Biotech). After four washes with binding buffer (200 mM Tris, pH 7.4, 250 mM NaCl, and 1 mM dithiothreitol), the beads were incubated with bacterial lysates containing GST fusion proteins of different mPar3 domains. After five washes with pull-down buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 1.5 mM MgCl₂, 5 mM EDTA, 10% glycerol, 0.1% Triton X-100, and complete protease inhibitor mixture (Roche) in phosphate-buffered saline), bound proteins were eluted with sample buffer.

For immunoprecipitation, brains from 5–10 E18 rat embryos were lysed in 2% Triton X-100, 137 mM NaCl, 2.7 mM KCl, 1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, and phenylmethylsulfonyl fluoride for 1 h at 4 °C and insoluble components removed by centrifugation. For immunoprecipitation, the following antibodies were used: anti-mPar3 (Upstate; 07–264, 1:1000), anti-GST (Amersham Technology; 2272, 1:1000), anti-Smurf1 (Abgent; 1:1000), anti-Smurf2 (Abgent; 1:1000), anti-Myc (Cell Signaling Technology; 2272, 1:1000), anti-EGFP (BabCo; MMS-11P, 1:1000), anti-mPar3 (Upstate; 07–330, 1:500), and horseradish peroxidase-coupled secondary antibodies (MoBiTec, Göttingen; E80305, 1:2000), anti-EGFP (BabCo; MMS-11P, 1:1000), anti-Myc (Cell Signaling Technology; 2272, 1:1000), anti-EGFP (BabCo; MMS-11P, 1:1000), and horseradish peroxidase-coupled secondary antibodies (MoBiTec, Göttingen; E80305, 1:2000).

**RESULTS**

**mPar3 Interacts Directly with Smurf2**—The HECT domain of Smurf1 and Smurf2 is responsible for initiating the destruction of Rap1B during the establishment of neuronal polarity (15). Smurf2 displayed a punctate staining pattern in neurites (Fig. 1A), which resembled the distribution of mPar3 and Kinesin-2 (4). To investigate whether Smurf2 might be transported via a kinesin-dependent mechanism like mPar3 we analyzed the distribution of Smurf2 and Kinesin-2 (4). To verify the colocalization of Smurf2 and Kinesin-2, Pearson coefficient (0.6 ± 0.02; n = 6) and Mander overlap coefficient (0.72 ± 0.03; n = 6) were calculated for several axons including both growth cone and shaft. The Mander overlap coefficient provides a measure for the proportion of signals that coincide in the red and green channel over their total intensity. A value of 1 indicates complete colocalization. The value determined for both coefficients confirmed that Smurf2 and Kinesin-2 colocalized to a large extent. The colocalization raises the possibility that Smurf2, like Smurf1 (19, 23), interacts with the Par complex. To investigate the association with the Par complex, Smurf1, Smurf2, or Kinesin-2 was immunoprecipitated from lysates of E18 rat brain (Fig. 1B). Smurf3 coprecipitated not only with Kinesin-2, PKCε, and Smurf1 as shown previously (4, 19) but also with Smurf2. These results show that Smurf2 and mPar3 form a complex in the developing brain. Interestingly, the precipitation of Smurf2 led to the copurification of Smurf1 and vice versa suggesting that Smurf1 and...
-2 may be present in a complex together with mPar3, PKCζ, and Kinesin-2.

To characterize the interaction of mPar3 and Smurf2 in more detail, we used a pull-down assay with GST- and MBP-fusion proteins of Smurf2 and Myc-tagged mPar3 expressed in HEK 293 T cells (Fig. 2, A and C). Full-length MBP-Smurfl2 specifically associated with mPar3 (Fig. 2B). This interaction was independent of ubiquitin ligase activity as shown by the binding of mPar3 to catalytically inactive MBP-Smurfl2 C716A. Pull-down assays with individual domains of Smurf2 and mPar3 identified the HECT domain of Smurf2 and mPar3 fragment comprising the third PDZ domain (Par3-PDZ3) as the sequences that mediated this interaction (Fig. 2, B and D). The absence of an interaction with the aPKC-binding region of mPar3 (aPKCBR) indicates that Smurf2, unlike Smurf1, does not associate with mPar3 through aPKC. To confirm their direct interaction, full-length Smurf2 and individual domains of mPar3 were expressed in bacteria as MBP- and GST-fusion proteins, respectively, and used for a pull-down assay (Fig. 3). Specific interaction of Smurf2 with PDZ3 but not of the other tested mPar3 fragments. The GST domain of Smurf2 only binds to the third PDZ domain of mPar3.

mPar3 Links Smurf2 to KIF3A and Is Required for the Localization of Smurf2 to Growth Cones—mPar3 directly binds to the Kinesin-2 subunit KIF3A that is required for its transport into growth cones (4). The direct interaction suggests that mPar3 links Smurf2 to KIF3A. To test this possibility, KIF3A and mPar3 were co-expressed in HEK 293 T cells and GST-Smurfl2 used for a pull-down assay. KIF3A bound to GST-Smurfl2 only upon co-expression with mPar3 (Fig. 4A). To test whether the interaction with mPar3 mediated the transport of Smurf2 into neurites, we expressed isolated interaction domains to competitively inhibit the formation of a complex (4). Co-expression of mPar3 fragment comprising PDZ domains 2 and 3 or of the HECT domain strongly reduced the formation of a complex between mPar3 and Smurf2 (Fig. 4, C and D). Blocking KIF3A-dependent transport or the association of KIF3A and mPar3 by expression of fragments from KIF3A or mPar3 that mediate this interaction prevent the localization of mPar3 and aPKC to growth cones and results in the failure to specify an axon (4). To test if the interaction with mPar3 was required for the localization of Smurf2 to growth cones, we

FIGURE 4. mPar3 links Smurf2 to KIF3A. A and B, bacterially expressed GST-Smurfl2 (Sm2) was coupled to glutathione-Sepharose beads and incubated with lysates of HEK 293 T cells transfected with vectors for Myc-tagged mPar3 and KIF3A as indicated. Bound proteins and comparable amounts of Myc-mPar3 and KIF3A in cell lysates were detected by Western blot (WB) using anti-Myc, -KIF3A, and -GST antibodies. KIF3A bound to GST-Smurfl2 only upon co-expression of mPar3 (A). KIF3A did not affect the interaction of Smurf2 and mPar3 (B). C and D, Myc-mPar3, Flag-Smurfl2, and GFP, GFP-mPar3-PDZ23 (P23) or -Smurf2-HECT (HECT) were co-expressed in HEK 293 T cells and Myc-mPar3 or Flag-Smurfl2 immunoprecipitated as indicated. Immunoprecipitated proteins and comparable amounts of proteins in cell lysates were detected by Western blot using anti-Myc (C), anti-Flag (D), and anti-GFP antibodies. Co-expression of GFP-PDZ23 (C) or GFP-HECT (D) blocked the interaction of Smurf2 and mPar3.
Smurf2 staining was observed in the axonal growth cone of 3 d.i.v., which contained Smurf2 in most neurons at this stage.

Knockdown of mPar3 Blocks Smurf2 Transport and Neuronal Polarization—To confirm that mPar3 is required for the localization of Smurf2 to growth cones, we suppressed endogenous mPar3 by RNAi using an expression vector for a small hairpin RNA (shRNA) (20) that specifically targets mPar3 isoforms containing the CR1 domain. The efficiency of the anti-mPar3 shRNA was confirmed by Western analysis of HEK 293 T cells cotransfected with expression vectors for EGFP, EGFP-tagged Par3–4N/2, Par3-PDZ2, Par3-PDZ3, Smurf2-HECT (HECT), Smurf2-HECT-C716A (HECT CA), and a shRNA directed against mPar3 (Par3 RNAi), or vectors for the anti-Par3 shRNA and human Myc-Par3 (Par3-RNAiMut) (Fig. 5; supplemental Figs. S1 and S2). As a control for the specificity of the RNAi vector, we constructed a shRNA that contained two mismatches to the target sequence (RNAiMut) that abrogated the knockdown of mPar3 (supplemental Fig. S1) (24). When mPar3 was suppressed by RNAi, only 20 ± 4% of the neurons (n = 45) showed Smurf2 staining in the growth cone of the longest neurite, whereas Smurf2 was not affected by expression of the inactive mPar3-RNAiMut (Fig. 5B; Par3-RNAiMut, 73 ± 7%, n = 71). Co-expression of human mPAR3 with the anti-mPar3 shRNA vector rescued the loss of endogenous mPar3 (Fig. 5; supplemental Fig. S3, F and H). 76 ± 6% (n = 30) of the neurons showed Smurf2 staining in the growth cone of the longest neurite (Fig. 5B).

Smurf2 initiates the removal of Rap1B from all but a single neurite and ensures that Rap1B specifies only a single axon (15). Therefore, we investigated whether interfering with the interaction of mPar3 and Smurf2 prevented the restriction of Rap1B to a single growth cone (Fig. 6; supplemental Fig. S3, E–I). Rap1B staining was observed exclusively in the axonal growth cone of 89 ± 3% of the cells (n = 71), whereas only 15 ± 3% (n = 71) of control neurons expressing EGFP had multiple Rap1B positive growth cones. As a consequence of displacing Smurf2 from growth cones by interfering with the interaction between KIF3A and mPar3 (Par3–4N/2 expression) or between mPar3 and Smurf2 (Par3-PDZ3), neurons showed Rap1B staining not blocked the binding of KIF3A to mPar3 or between mPar3 and Smurf2 by expressing the isolated domains that mediated these interactions (Par3–4N/2, Par3-PDZ3, and Smurf2-HECT; Fig. 5; supplemental Fig. S3, E–I). We investigated the presence of endogenous Smurf2 in the growth cone of the longest neurite at 3 d.i.v., which contained Smurf2 in most neurons at this stage. Smurf2 staining was observed in the axonal growth cone of 78 ± 6% of control neurons expressing EGFP (n = 52). Expression of Par3–4N/2 displaces endogenous mPar3 from KIF3A (4) and leads to the loss of Smurf2 from growth cones. Smurf2 was detectable in the growth cone of the longest neurite only in a minority of cells (Fig. 5B; 27 ± 4%, n = 50). When the interaction of Smurf2 and mPar3 was blocked by Par3-PDZ23 (data not shown), Par3-PDZ3, Smurf2-HECT, or Smurf2-HECT-CA, the percentage of neurons with Smurf2 staining in growth cones was also strongly reduced (Fig. 5B; Par3-PDZ3, 35 ± 4%, n = 32; Smurf2-HECT, 23 ± 2%, n = 43). Expression of PDZ2 as a control had no effect (77 ± 5%, n = 33). Thus, the localization of Smurf2 to growth cones depends on the interaction of KIF3A with mPar3 and mPar3 with Smurf2 indicating that mPar3 links Smurf2 to KIF3A.
only in the axon but in multiple growth cones (Fig. 6B; Par3−4N/2, 67 ± 7% of the neurons show Rap1B in multiple growth cones, n = 65; Par3-PDZ3, 55 ± 8%, n = 38). PDZ2 had no effect (11 ± 7%, n = 32). Expression of the isolated wild type Smurf2 HECT domain that is sufficient to bind mPar3 and target Rap1B for degradation resulted in the loss of endogenous Smurf2 from growth cones (Fig. 6, A and B) and reduced the number of neurons with a Rap1B-positive growth cone to 10 ± 7% (n = 49). By contrast, expression of the catalytically inactive Smurf2-HECT C716A had a dominant negative effect. It displaced endogenous Smurf2 from growth cones and thereby blocked the degradation of endogenous Rap1B. As a consequence, the number of neurons containing Rap1B in several neurites was increased compared with EGFP expression (Fig. 6, A and B; 64 ± 6%, n = 51). A knockdown of mPar3 by RNAi also prevented the restriction of Rap1B to a single neurite and resulted in the presence of Rap1B in multiple neurites (i.e. in two or more neurites) in 59 ± 8% (n = 51) of the cells. The Par3-RNAiMut control showed no changes in Rap1B distribution compared with controls expressing only EGFP (Fig. 6B; Par3-RNAiMut, 21 ± 4% neurons with Rap1B in multiple axonal growth cones, n = 38). A second RNAi construct, directed against mPar3 (Par3 RNAi), gave the same results (Fig. 6B). Co-expression of human mPAR3 with the mPar3 shRNA vector rescued endogenous mPar3. Only 11 ± 7% (n = 32) of the neurons showed Rap1B staining in multiple growth cone (Fig. 6, A and B; supplemental Fig. S3, H and I). Thus, blocking the interaction of Smurf2 with mPar3 or suppressing mPar3 by RNAi prevents the restriction of Rap1B to a single neurite.

**mPar3-dependent Smurf2 Localization Is Required for Neuronal Polarity**—To investigate the consequences of preventing the localization of Smurf2 to growth cones for neuronal polarity, we analyzed the effect of Par3−4N/2, Par3-PDZ-3, and the anti-mPar3 shRNA on axon specification (Fig. 7). Interfering with the function of mPar3 in Smurf2 localization should prevent the restriction of axonal identity to a single neurite. The majority of control neurons expressing EGFP showed normal polarity with a single Tau-1 positive axon and several MAP2 positive minor neurites (Fig. 7B; 78 ± 7%, n = 84). As described before (4), interfering with the KIF3A-dependent transport of mPar3 by Par3−4N/2 expression affected axon specification and results in the extension of neurites that lack a clear axonal character. 33 ± 5% (n = 57) of the neurons formed a single or multiple processes that were longer than minor neurites but shorter than axons (Fig. 7C; 76 ± 40 μm; n = 57; the difference to control was not significant because of the large variation in neurite length). Because these neurites were neither axons that fulfilled all criteria (length and Tau-1
immunoreactivity) nor minor neurites, we categorized the phenotype of these neurons as indeterminate to indicate that these cells differed from unpolarized neurons but neither developed a normal polarity nor formed multiple axons (see “Experimental Procedures” for details). In addition to the $30 \pm 6\%$ polarized neurons with a single axon, Par3–4N/2 expression also induced a significant number of neurons with multiple axons ($28 \pm 4\%; n = 57$). Blocking the interaction of mPar3 and Smurf2 by Par3-PDZ3 had a similar effect and increased the percentage of cells with an indeterminate phenotype ($54 \pm 5\%, n = 34$).

Suppression of mPar3 by RNAi had an effect similar to that of expressing Par3-PDZ3 and reduced the percentage of neurons with normal polarity ($19 \pm 4\%, n = 40$). Knockdown of mPar3 led to an increase in the number of neurons with neurites that had axonal characteristics but did not show a normal Tau-1 staining pattern and were shorter than normal axons (indeterminate phenotype: $48 \pm 6\%$ of cells; $48 \pm 6 \mu m; n = 40$). This phenotype probably reflects the multiple functions of mPar3 in axon specification. mPar3 is required for the transport and function of aPKCs (4, 10–12, 25) as well as for the regulation of Rac GEFs like STEF or Tiam1 (5, 14). The majority of neurons expressing the control construct Par3-RNAiMut showed normal polarity ($73 \pm 8\%, n = 34$). Co-expression of human mPAR3 with the anti-mPar3 shRNA vector rescued the loss of endogenous mPar3 to a large extent. $54 \pm 1\% (n = 37)$ of the neurons formed a single axon (Fig. 7B). The reduction in the percentage of neurons with a single axon in comparison to controls (EGFP and Par3-RNAiMut) probably results from the disruption of neuronal polarity in cells where the amount of exogenous human mPar3 is too high (4, 7). Thus, mPar3 is required for neuronal polarity and an important aspect of its function is the localization of Smurf2 to growth cones.
DISCUSSION

The E3 ubiquitin ligase Smurf2 initiates the restriction of Rap1B to a single neurite by targeting inactive Rap1B for degradation (6, 15). Our results reveal a new role for mPar3 in the establishment of neuronal polarity upstream of Rap1B. The interaction of Smurf2 and mPar3 is required for the localization of Smurf2 to growth cones and the development of neurons with a single axon. Smurf2 and Kinesin-2 colocalize in neurites and growth cones of hippocampal neurons and can be co-immunoprecipitated from lysates of embryonic brain. mPar3 links Smurf2 to KIF3A by a direct interaction of its third PDZ domain with the Smurf2 HECT domain. Expressing the mPar3 and Smurf2 domains that mediate the interaction of KIF3A and mPar3 or mPar3 and Smurf2 as competitive inhibitors prevent the localization of Smurf2 to growth cones. This results in the failure to restrict Rap1B to a single neurite and the formation of multiple long neurites with an indeterminate character that do not show a Tau-1 staining pattern typical for axons. Suppression of mPar3 by RNAi has the same effect. Because KIF3A is required for the transport of mPar3 into neurites and mPar3 links Smurf2 to KIF3A, our results suggest that Kinesin-2 mediates the transport of Smurf2. Rap1B is restricted to a single neurite concomitant with the appearance of mPar3 in all growth cones (6). Our results suggest that mPar3 initiates the degradation of Rap1B by targeting Smurf2 to all growth cones, where it ubiquitinates inactive Rap1B. Although Rap1B is present initially in all neurites of stage 2 neurons, its extension into axons (4, 7). Our results show that mPar3 is required for the establishment of neuronal polarity. After the suppression of mPar3 by RNAi, neurons lack a single axon and instead form multiple long neurites of similar length similar to the effect of overexpressing mPar3 (4, 5, 7). These neurites can be categorized as axons by their length but do not show the Tau-1 staining pattern typical for axons. A similar defect is observed when interfering with the transport of mPar3, blocking the function of STEF, or inhibiting the interaction of mPar3 and Smurf2 (4, 5, and this manuscript). The indeterminate phenotype resulting from the disruption of mPar3 function probably reflects its multiple roles both upstream and downstream of Rap1B. mPar3 is required upstream of Rap1B to restrict Rap1B to a single neurite by initiating its destruction through the UPS and downstream of Rap1B and Cdc42 to promote axon growth through Rac and aPKC (5, 7). Thus, although the suppression of mPar3 function or a block of its transport into neurites prevents the degradation of inactive Rap1B and promotes the specification of multiple axones, these neurites do not acquire all axonal characteristics probably because of a failure to stimulate Rac and aPKC that also depends on mPar3.

A recent genetic analysis of the Drosophila Par3, Par6, and aPKC homologs came to the conclusion that they are not required for the establishment of neuronal polarity in the mushroom body (27). The Par complex members responsible for specification in these neurons were shown to promote the growth of a single axon and extend only a single axon. Our results indicate that normal hippocampal neurons display a single axon during their migration from the multipolar morphology already during their migration from the ventricular zone (30).

mPar3 and Smurf2

FIGURE 7. Interfering with mPar3 function disrupts neuronal polarity. A, hippocampal neurons were transfected 2 h after plating with expression vectors for EGFP, EGFP-tagged Par3-A4N, Par3-PDZ, Par3-PDZ, Smurf2-HECT (H7C), Smurf2-HECT-C716A (H7C CA), an shRNA directed against mPar3 (Par3 RNAi), or vectors for the anti-Par3 shRNA and human Myc-Par3 (RNAn + h Par3) (green). Transfected cells were analyzed at 3 d.i.v. by staining with the Tau-1 (blue) and anti-MAP2 antibodies (red). Scale bar, 40 μm. B, the development of neuronal polarity was analyzed by counting the number of unpolarized neurons (no axon, blue), polarized neurons (single axon, red), neurons with an indeterminate phenotype (multiple long but Tau-1 negative axons, yellow), or neurons with multiple axons (green) (see under “Experimental Procedures” for details of the categorization). C, the average length of the longest neurite is shown (means ± S.E.; *, p < 0.001 compared with EGFP).
absence of aPKC and lack of Rac activity but also to defects in the regulation of the GTPases Rap1B and Rho and affects both axon specification and neurite extension.

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