

Ubiquitination of the GTPase Rap1B by the ubiquitin ligase Smurf2 is required for the establishment of neuronal polarity

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The development of a polarised morphology with multiple dendrites and a single axon is an essential step in the differentiation of neurons. The establishment of neuronal polarity is directed by the sequential activity of the GTPases Rap1B and Cdc42. Rap1B is initially present in all neurites of unpolarised neurons, but becomes restricted to the tip of a single process during the establishment of neuronal polarity where it specifies axonal identity. Here, we show that the ubiquitin ligases Smad ubiquitination regulatory factor-1 (Smurf1) and Smurf2 are essential for neurite growth and neuronal polarity, respectively, and regulate the GTPases Rho and Rap1B in hippocampal neurons. Smurf2 is required for the restriction of Rap1B to a single neurite. Smurf2 ubiquitinates inactive Rap1B and initiates its degradation through the ubiquitin/proteasome pathway (UPS). Degradation of Rap1B restricts it to a single neurite and thereby ensures that neurons extend a single axon.

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Introduction

Primary cultures of dissociated neurons are a well-established system to study the development of neuronal polarity and have facilitated the identification of signalling pathways essential for the first step in neuronal polarisation, the specification of axonal identity (Bradke and Dotti, 2000; Da Silva and Dotti, 2002; Wiggin *et al.*, 2005). The differentiation of hippocampal neurons can be subdivided into five stages (Dotti *et al.*, 1988). Unpolarised neurons initially form several equivalent neurites, which all have the potential to become an axon (stage 2). Neuronal polarity becomes apparent when

a single neurite is selected from these processes to become the axon (stage 3).

The establishment of neuronal polarity is initiated by phosphatidylinositol-3-kinase (PI3K). Production of phosphatidylinositol-3,4,5-trisphosphate leads to the activation of Akt/PKB (Akt) and inactivation of glycogen synthase kinase 3 β (GSK3 β ; Shi *et al.*, 2003; Jiang *et al.*, 2005; Yoshimura *et al.*, 2005; Yan *et al.*, 2006). This repression allows GSK3 β targets like collapsin response mediator protein-2 (CRMP2) to promote axon extension (Inagaki *et al.*, 2001; Zhou *et al.*, 2004; Yoshimura *et al.*, 2005). We have shown that the sequential activity of the GTPases Rap1B and Cdc42 is necessary and sufficient for the establishment of polarity in hippocampal neurons downstream of PI3K (Schwamborn and Püschel, 2004). Rap1B is initially present at the tips of all neurites of unpolarised early stage 2 neurons, but becomes restricted to a single neurite at late stage 2 (Schwamborn and Püschel, 2004). Rap1B is restricted to a single neurite of morphologically unpolarised neurons before the axon becomes distinguishable and other factors important for axon specification like the Par complex, phosphorylated atypical protein kinase C (aPKC), phosphorylated Akt, phosphorylated GSK3 β , APC, or CRMP2 are restricted to the axon of stage 3 neurons (Inagaki *et al.*, 2001; Shi *et al.*, 2003; Schwamborn and Püschel, 2004; Shi *et al.*, 2004; Jiang *et al.*, 2005; Yoshimura *et al.*, 2005; Yan *et al.*, 2006).

The redistribution of Rap1B is an essential step in the establishment of neuronal polarity. However, it is unclear how the restriction of Rap1B to a single neurite arises from an initially symmetric localisation at the tip of all neurites. One possible mechanism is the selective degradation of Rap1B. The UPS is the major route that targets proteins for degradation in eukaryotic cells (Glickman and Ciechanover, 2002). UPS-mediated destruction is essential not only for removing misfolded proteins but also for the regulation of many signalling pathways. Ubiquitination is catalysed by a cascade of three enzymes, the ubiquitin-activating E1, the ubiquitin-conjugating E2, and the ubiquitin-protein E3 ligase. The E3 enzyme is responsible for determining which proteins are selected for modification. In neurons, the UPS is present in axonal growth cones and has been implicated in the regulation of axon guidance, synapse formation, and neuronal plasticity, as well as neurodegenerative processes and regeneration (Campbell and Holt, 2001, 2003; DiAntonio and Hicke, 2004; Konishi *et al.*, 2004; van Roessel *et al.*, 2004; Nakata *et al.*, 2005). Here, we show that the UPS is required for the establishment of neuronal polarity. The HECT domain E3 ubiquitin ligases Smurf1 and Smurf2 coordinately regulate neurite extension and neuronal polarity through Rho and Rap1B, respectively. Smurf2 ubiquitinates inactive Rap1B and initiates its degradation through the proteasome. The degradation of Rap1B is essential to restrict it to a single neurite and to ensure that neurons extend only one axon.

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Results

Proteasome inhibitors induce supernumerary axons

To investigate how Rap1B is restricted to a single neurite, we analysed the effect of inhibiting the proteasome. Dissociated hippocampal neurons from E18 rat embryos were treated 16 h after plating with the proteasome inhibitor clasto-Lactacystin β -Lactone (Lactacystin) and analysed 48 h later by staining with the Tau-1 antibody as axonal marker and an anti-MAP2 antibody as a marker for minor neurites. Control neurons treated with the solvent DMSO acquired a normal polarity and extended a single axon (1.1 ± 0.1 axons per cell) that was positive for Rap1B and Tau-1 and 4 ± 0.6 minor

neurites (means \pm s.e.m.; $n = 150$; Figure 1A–C) as shown before (Schwamborn and Püschel, 2004). Treatment with Lactacystin induced the extension of supernumerary axons (3.5 ± 0.5 positive axons per cell; $n = 150$; Figure 1A and B; Supplementary Figure S1). The growth cones and in some cases also the shaft of these axons showed a strong Rap1B staining ($93 \pm 4\%$; $n = 70$) and were also positive for Par3 ($89 \pm 6\%$; $n = 70$; Figure 1A; Supplementary Figure S1C). Two different proteasome inhibitors, *N*-acetyl-leuciny-leucinylnorleucinal-H (ALLN) and MG-132, had the same effect (ALLN: 3.3 ± 0.3 axons per cell, $n = 150$; MG-132: 3.1 ± 0.7 , $n = 50$; Figure 1B–D; see Supplementary Figure S1D–I). Neurons treated with Lactacystin, ALLN, or MG-132

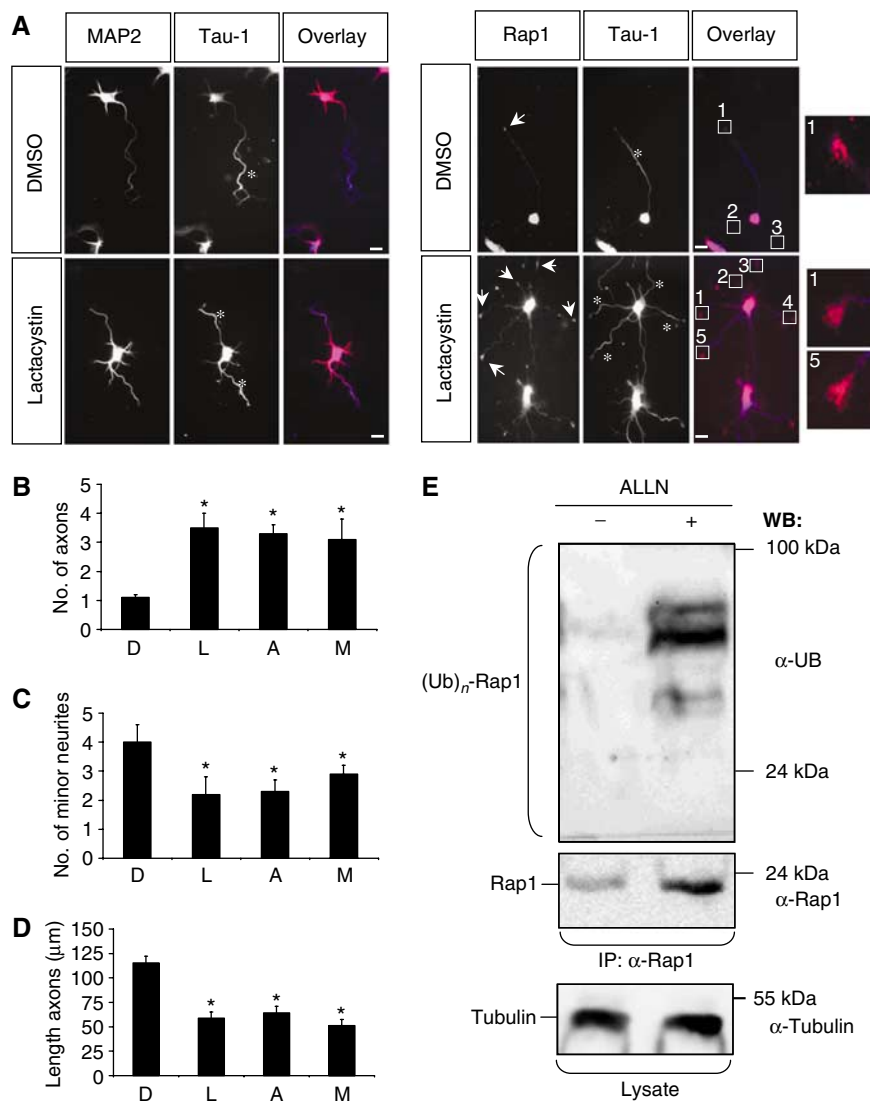


Figure 1 Inhibition of the proteasome disrupts neuronal differentiation. (A–D) Hippocampal neurons were cultured in the presence of solvent (DMSO), 1.5 μ M clasto-Lactacystin β -Lactone (Lactacystin), 40 μ M ALLN, or 1.5 μ M MG-132 (all dissolved in DMSO) for 48 h and analysed at 3 d.i.v. (stage 3) by staining with an anti-MAP2 antibody (A; red), the Tau-1 (blue), and an anti-Rap1 (red) antibody. Lactacystin induced the formation of multiple axons (asterisks) whose growth cones were all positive for Rap1B (A, arrows). Insets show higher magnifications of the marked growth cones. Axons identified by Tau-1 immunoreactivity are marked by asterisks. The scale bar is 12 μ m. (B–D) The effect of Lactacystin (L), ALLN (A), or MG-132 (M) was analysed by determining the number of axons (B) or minor neurites (C) per cell and the length of axons (E) (means \pm s.e.m.; * $P < 0.001$ compared to control (DMSO (D)); three independent experiments). (E) Hippocampal neurons were incubated overnight with solvent (DMSO, –) or 40 μ M ALLN (+). Rap1B was precipitated from cell lysates using an anti-Rap1 antibody (IP) and analysed by Western blot using anti-ubiquitin, anti-Rap1, anti- α -tubulin, and the Tau-1 antibodies (WB). Luminescence signals from Western blots were collected for 1 s (s) and 30 min to detect poly-ubiquitinated Rap1B visible after proteasome inhibition. Staining for tubulin confirmed the loading of comparable amounts of protein. The slight increase in the amount of α -tubulin upon inhibition of the proteasome is consistent with the formation of supernumerary axons.

showed a significant reduction in the number of minor neurites (Lactacystin: 2.2 ± 0.6 minor neurites per cell, $n = 150$; ALLN: 2.3 ± 0.4 , $n = 150$; MG-132: 2.9 ± 0.3 , $n = 50$; Figure 1C), indicating that the supernumerary axons were produced at the expense of minor neurites. In addition, the length of axons was reduced by 50% compared with controls (DMSO: $116 \pm 7 \mu\text{m}$, $n = 70$; Lactacystin: $59 \pm 6 \mu\text{m}$, $n = 50$; ALLN: $64 \pm 7 \mu\text{m}$, $n = 70$; MG-132: $51 \pm 6 \mu\text{m}$; $n = 50$; Figure 1D). The length of minor neurites was not affected (Supplementary Figure S1D). The induction of supernumerary axons suggested a misregulation of Rap1B. To test if it is a target for the UPS, Rap1B was immunoprecipitated from cultured hippocampal neurons and shown to be ubiquitinated by staining the Western blot with an anti-ubiquitin antibody (Figure 1E; Supplementary Figure S1J). Taken together, these results demonstrate that the proteasome is required for the normal development of neuronal polarity.

Smurf2 initiates degradation of inactive Rap1B

Smurf1 and Smurf2 were originally identified as ubiquitin ligases that interact with Smad proteins (Zhu *et al*, 1999; Kavsak *et al*, 2000). Smurf1 regulates cell polarity and motility in tumour cells, where it is recruited to the leading edge by PKC ζ to ubiquitinate RhoA (Wang *et al*, 2003). We tested if Smurf1 or its closely related homologue Smurf2 is involved in the regulation of Rap1B. Wild-type Smurf2, but neither Smurf1 nor catalytically inactive Smurf2, decreased the level of Rap1B after coexpression in HEK 293T cells (Figure 2A). The proteasome inhibitors ALLN (Figure 2A) and clasto-Lactacystin β -Lactone (data not shown) blocked this effect.

In immunoprecipitation and GST pull-down assays, catalytically inactive Smurf2 (Smurf2C716A) (Wang *et al*, 2003) interacted with the dominant-negative Rap1BN17 mutant, nucleotide-free, and GDP-bound Rap1B, whereas preloading with the non-hydrolysable GTP analogue GTP γ S inhibited the interaction (Figure 2B and C; Supplementary Figure S2A and B). As reported before for Smurf1 (Wang *et al*, 2003), no interaction was detectable by immunoprecipitation when wild-type Rap1B was coexpressed with Smurf2C716A. Smurf1 but not Smurf2 bound inactive RhoA, as described previously (Wang *et al*, 2003) (data not shown). Coexpression with Smurf2 markedly increased the amount of ubiqui-

tin-conjugated Rap1B in HEK 293T cells, whereas no Rap1B ubiquitination was observed after expression of inactive Smurf2 (Figure 2D). To verify that Smurf2 catalysed the ubiquitination of Rap1B, bacterially expressed proteins were purified and used for an *in vitro* ubiquitination assay (Figure 2E and F). Both full-length Smurf2 and the isolated Smurf2 HECT domain but not Smurf2C716A mediated the ubiquitination of Rap1B *in vitro*. Ubiquitination was observed only after preincubation of Rap1B with GDP but not with GTP γ S (Supplementary Figure S2F). Thus, Rap1B is a direct target for the E3 ligase Smurf2 that selectively modifies inactive Rap1B.

Localisation of Smurf2 in neurons

In most stage 2 neurons, Smurf2 was found in the cell body and at the tips of most or all neurites (Figure 3A and B). At stage 3, the majority of the neurons still contained Smurf2 in most or all growth cones. Smurf1 showed a similar distribution at both stages (Figure 3B, and data not shown). The specificity of the anti-Smurf1 and anti-Smurf2 antibodies was confirmed by immunofluorescence (Supplementary Figure S5A). Smurf2 is localised not only to the cell body and the growth cones but is also present in neurite shafts, where it displays a punctate staining pattern (Figure 3A).

Only inactive Rap1B is a target for Smurf2-mediated ubiquitination. Therefore, we investigated the distribution of active GTP-bound Rap1B during neuronal polarisation *in situ* using the Rap1-binding domain (RBD) of RalGDS fused to GST that selectively binds active Rap1 (Reedquist and Bos, 1998) as described before (Lafuente *et al*, 2004). Hippocampal neurons were fixed at different stages and incubated with GST-RalGDS-RBD. The distribution of GST-RalGDS-RBD was visualised with an anti-GST antibody and the fluorescence intensity normalised to cell volume based on labelling with 5-chloromethylfluorescein diacetate (CMFDA). At stage 2, the majority of neurons showed a strong staining indicating active Rap1B at the tip of a single neurite and lower levels of Rap1B-GTP in the remaining processes ($52 \pm 4\%$; $n = 75$; Figure 3C–E). Fewer neurons showed comparable RalGDS-RBD staining that did not differ significantly in intensity in several ($17 \pm 2\%$) or all neurites ($31 \pm 2\%$). In stage 3 neurons, active Rap1B was found almost exclusively in the axonal growth cone ($84 \pm 6\%$ of the

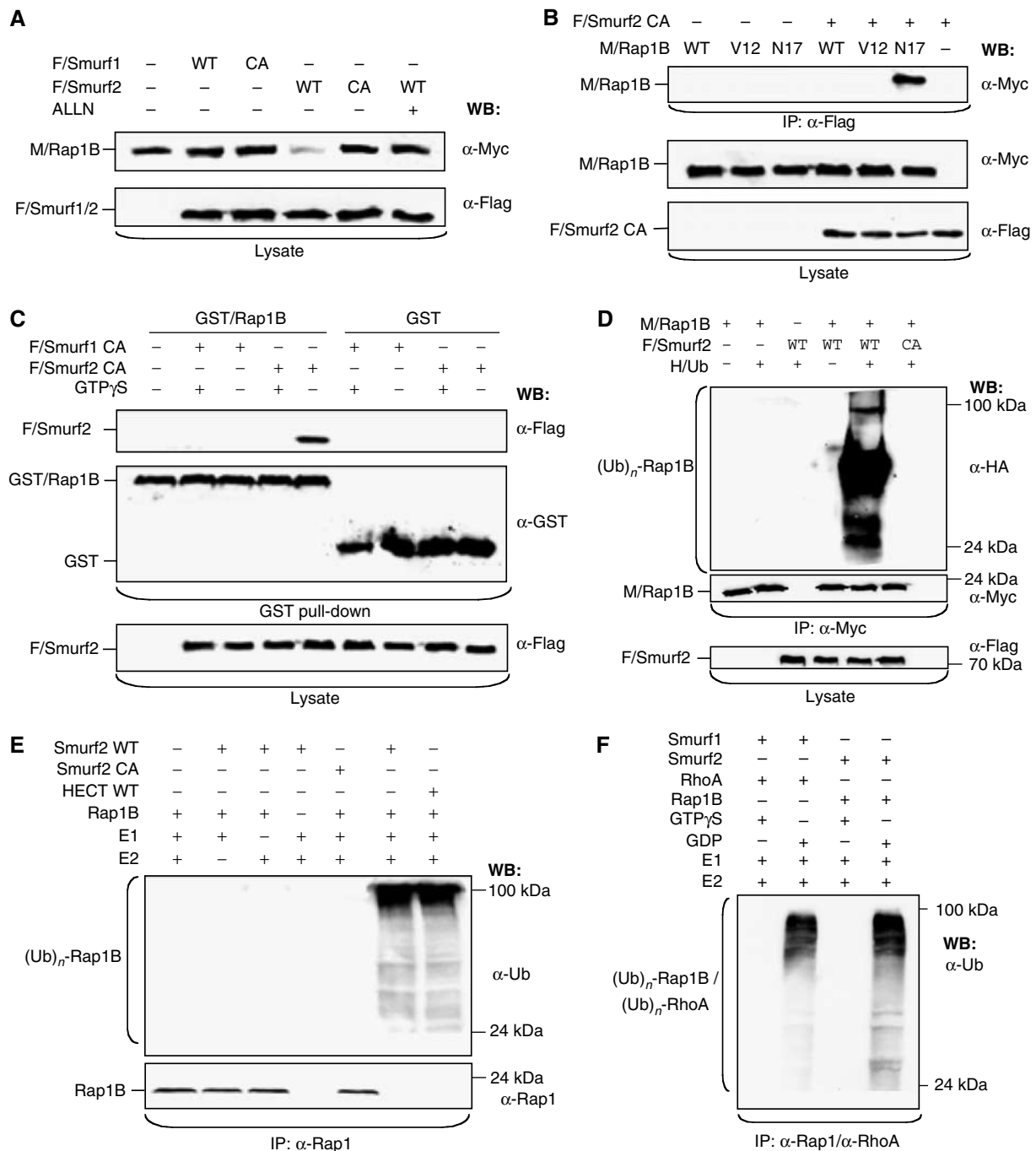
Figure 2 Smurf2 ubiquitinates Rap1B. (A) HEK 293T cells were transfected with expression vectors for wild-type (WT) or catalytically inactive (CA: Smurf1C699A or Smurf2C716A) Flag-tagged Smurf1 (F/Smurf1) or Smurf2 (F/Smurf2) and myc-tagged Rap1B (M/Rap1B) as indicated. ALLN (40 μM) was added 16 h before cells were lysed as indicated and protein expression in cell lysates analysed by Western blot using anti-myc and anti-Flag antibodies. (B) HEK 293T cells were transfected with expression vectors for Flag-Smurf2C716A and WT or mutant myc-Rap1B (constitutively active: V12; dominant-negative: N17). Smurf2C716A was precipitated from cell lysates using the anti-Flag antibody (IP) and Rap1B detected by Western blot (WB) using the anti-myc antibody. The expression of equivalent amounts of protein was confirmed by Western blot. (C) HEK 293T cells were transfected with expression vectors for Flag-Smurf1C699A or Flag-Smurf2C716A and the cell lysates incubated with bacterially expressed GST or GST-Rap1B coupled to glutathione-Sepharose and preloaded with 0.7 mM GTP γ S (+) as indicated. Bound Smurf2 (top panel) was detected in Western blots using an anti-Flag antibody. The expression of equivalent amounts of Smurf2 and GST-Rap1B or GST was confirmed by Western blot. (D) HEK 293T cells were transfected with vectors for HA-tagged ubiquitin (HA/Ub), myc-Rap1B (M/Rap1B), and WT or catalytically inactive Flag-Smurf2 (CA), incubated overnight with 40 μM ALLN, and Rap1B was immunoprecipitated with an anti-myc antibody. Ubiquitin-conjugated Rap1B ((Ub) $_n$ -Rap1B) and Rap1B were detected with anti-HA and anti-myc antibodies, respectively. Flag-Smurf2 and myc-Rap1B expression was confirmed by Western blot of cell lysates using anti-Flag and anti-myc antibodies, respectively. (E) Bacterially expressed and purified Rap1B and WT or catalytically inactive Smurf2 (CA) or the HECT domain of Smurf2 were used for an *in vitro* ubiquitination assay after combining the indicated proteins. Recombinant ubiquitin, ubiquitin-activating enzyme (E1), and UbcH5c (E2) were added to the reaction mixture as indicated. After performing the *in vitro* ubiquitination, Rap1B was immunoprecipitated from the samples (IP) using an anti-Rap1 antibody. Poly-ubiquitinated Rap1B ((Ub) $_n$ -Rap1B) and Rap1B were detected by Western blot using anti-ubiquitin and anti-Rap1B antibodies. (F) Bacterially expressed and purified Rap1B, RhoA, Smurf1, and Smurf2 were used for an *in vitro* ubiquitination assay after combining the indicated proteins. Rap1B and RhoA were preloaded with 0.7 mM GDP or 0.7 mM GTP γ S. Recombinant ubiquitin, ubiquitin-activating enzyme (E1), and UbcH5 (E2) were added to the reaction mixture as indicated. After performing the *in vitro* ubiquitination, Rap1B and RhoA were immunoprecipitated from the samples (IP) using an anti-Rap1 or an anti-RhoA antibody. Ubiquitinated GTPases ((Ub) $_n$ -Rap1B/(Ub) $_n$ -RhoA) were detected by Western blot using an anti-ubiquitin antibody.

neurons; $n = 75$; Figure 3C–E). The specificity of the staining was confirmed in COS 7 cells treated with the Epac activator 8-(4-chloro-phenylthio)-2'-*O*-methyladenosine-3'-5'-cyclic monophosphate (8-CPT) (Enserink *et al*, 2002), and by incubating neurons with bacterially expressed GST (Supplementary Figures S3A–C). Thus, the growth cones of stage 2 neurons show quantitative differences in the level of Rap1B-GTP, and in the majority a single growth cone contained a high level of active Rap1B.

Smurf2 regulates axon specification

To test whether Smurf2 is involved in the establishment of neuronal polarity, we transfected hippocampal neurons with expression vectors for EGFP and wild-type or catalytically

inactive Smurf2 (Smurf2C716A). The establishment of neuronal polarity was analysed at stage 3 (3 d.i.v.). Control neurons transfected with a vector for EGFP formed a single axon (1.1 ± 0.2 axons per cell) with an average length of $121 \pm 13 \mu\text{m}$ and 5.4 ± 0.2 minor neurites with an average length of $26 \pm 4 \mu\text{m}$ ($n = 84$; Figure 4; Supplementary Figure S4). Overexpression of wild-type Smurf2 led to loss of neuronal polarity. The majority of neurons did not form an axon as demonstrated by a significant reduction in the number of axons per cell (0.3 ± 0.2 ; $n = 67$), whereas minor neurites were not affected (5.1 ± 0.5 per cell; $n = 67$). Unlike Smurf1 (see below), wild-type Smurf2 had no effect on minor neurites. The length of axons or minor neurites (axons: $117 \pm 13 \mu\text{m}$; minor neurites: $35.0 \pm 2.2 \mu\text{m}$; $n = 67$) and the



number of minor neurites (5.1 ± 0.5) were not changed significantly compared with controls. The specificity of the effect for axons indicates that Smurf2 regulates the development of polarity and not neurite growth in general.

Expression of Smurf2C716A had a dominant-negative effect. Transfected neurons developed supernumerary axons (2.0 ± 0.1 per cell; $n = 59$; Figure 4; Supplementary Figure S4) and showed a significant reduction in the number of minor neurites (3.1 ± 1.0 per cell; $n = 59$), indicating that these axons were formed at the expense of minor neurites. The extension of multiple axons suggests that Smurf2C716A interferes with the degradation of Rap1B. The persistence of Rap1B that would normally be removed transforms minor neurites into axons.

Smurf2 is required for the establishment of neuronal polarity

To confirm that Smurf2 is required for neuronal polarisation, we suppressed endogenous Smurf2 by RNA interference (RNAi) using expression vectors for small hairpin RNAs (shRNAs). The efficiency of the shRNAs was confirmed by cotransfecting HEK 293T cells with expression vectors for Smurf1 or Smurf2 and the corresponding shRNAs (Figure 5A). As control for their specificity, we used vectors for shRNAs that contained two mismatches to the target sequence (Huppi *et al*, 2005) (RNAiMut) that abrogated the effect of the shRNAs. The efficiency of RNAi was also confirmed in transfected hippocampal neurons by immunofluorescence (Supplementary Figure S5).

Transfection of the RNAi vector (pSHAG-1 without insert) or the control vectors (Smurf1RNAiMut and Smurf2RNAiMut) did not change the number of axons or minor neurites per cell (0.9 ± 0.3 axons and 4.9 ± 0.5 minor neurites per cell; $n = 72$, Figure 5B and D). Suppression of Smurf2 resulted in the formation of supernumerary axons (2.1 ± 0.3 axons per cell; $n = 39$; Figure 5B and D) as observed for dominant-negative Smurf2C716A. The axonal identity of the neurites was also confirmed by staining with anti-synapsin I (Supplementary Figure S5U) and anti-Par3 antibodies. $80 \pm 10\%$ ($n = 36$) supernumerary axons were positive for Par3, similar to the values for controls (pSHAG-1: $84 \pm 9\%$, $n = 62$; Smurf2-RNAi: $80 \pm 10\%$, $n = 36$; Smurf2-RNAi-Mut: $71 \pm 9\%$, $n = 31$). These supernumerary axons were functional for synaptic vesicle recycling as shown by uptake of the dye FM4-64 (Supplementary Figures S5V and W). The number and length of minor neurites were not changed (Supplementary Figures S5C–F). An RNAi vector directed against a different target sequence in the *Smurf2* mRNA had the same effects (Supplementary Figures S5G–L). Analysis of neurons at 6 d.i.v. confirmed the formation of multiple axons after a knockdown of Smurf2 (Supplementary S5M–P). Rap1B was present in all supernumerary axons after knockdown of Smurf2 in $74 \pm 7\%$ ($n = 61$) of the transfected neurons (Figure 5C and D). In addition, increased Rap1 staining was also observed along the shaft of these axons. Incubation with RalGDS-RBD confirmed that the supernumerary axons contained active Rap1B (Supplementary Figure S6). Consistent with the observation that a knockdown of Rap1B after neuronal polarity is already established did not affect axon specification (Schwamborn and Püschel, 2004), suppression of Smurf2 after 3 d.i.v. had no effect on the number of axons (Supplementary Figures S5Q and R). By

contrast, inhibition of the proteasome resulted in the formation of supernumerary axons (Supplementary Figures S5S and T), suggesting that the UPS is required also to maintain normal polarity and may regulate a different set of proteins at this stage, such as CRMP2 (Wiggin *et al*, 2005).

Expression of the isolated catalytic domain of Smurf2 (EGFP-Smurf2-HECT) had the same effect as expressing full-length Smurf2 (Figure 6A and B). The shRNA directed against Smurf2 targets a region within the first 200 nucleotides of the coding sequence and is unable to suppress expression of EGFP-Smurf2-HECT. Thus, coexpression of wild-type EGFP-Smurf2-HECT should block the induction of supernumerary axons after suppression of Smurf2. Indeed, we observed a reversal of the phenotype induced by the knockdown of Smurf2 and a loss of polarity (0.5 ± 0.1 axons; 4.6 ± 0.6 minor neurites; $n = 41$; Figure 6A and B). Thus, Smurf2 is required for the regulation of Rap1B during the establishment of neuronal polarity.

Rap1BR5 is protected against ubiquitination

Two lysine residues (K6 and K7) are essential for the ubiquitination of RhoA (Ozdamar *et al*, 2005). Rap1B contains a single lysine at a homologous position (K5; Supplementary Figure S7A). Mutation of K5 to arginine (Rap1BR5) results in a strong reduction in, but not complete elimination of, the ubiquitination by Smurf2 (Figure 6C). As described for the corresponding RhoA mutation (Ozdamar *et al*, 2005), Rap1BR5 was not degraded after coexpression of Smurf2 (Figure 6C), showing that K5 is essential for Rap1B ubiquitination. Expression of wild-type Rap1B in hippocampal neurons had no effect on neuronal polarity (1.1 ± 0.2 axons per cell; $n = 62$) and did not block the loss of axons resulting from Smurf2 coexpression (Figure 6D and E). By contrast, expression of Rap1BR5 induced the formation of multiple axons (1.9 ± 0.2 axons per cell; $n = 41$; Figure 6D and E), whereas it had no effect on minor neurites (Supplementary Figure S7D). The identity of supernumerary axons was confirmed by staining with an anti-synapsin I antibody (Supplementary Figure S7E). Supernumerary axons induced by Rap1BR5 were functional for synaptic vesicle recycling as shown by uptake of the dye FM4-64 (Supplementary Figures S7F and G). This effect was also observed after coexpression with Smurf2 (1.8 ± 0.3 axons per cell; $n = 35$; Figure 6D and E). The disruption of neuronal polarity by Rap1BR5 is unlikely to result from complex formation with and inhibition of endogenous Smurf2 as the interaction of Rap1BR5 with Smurf2CA was reduced compared with wild-type Rap1B (Supplementary Figure S7B). The activity of Rap1BR5 was not increased compared with Rap1B after expression in HEK 293T cells (Supplementary Figure S7C). Thus, a mutation that blocks Rap1B degradation by the UPS prevents the inactivation of Rap1B by Smurf2 and mimics GTPase activation.

Smurf1 regulates neurite extension

In contrast to Smurf2, expression of Smurf1 had no significant effect on the number of axons (1.2 ± 0.2 ; $n = 58$) or minor neurites per cell (6.2 ± 0.9 ; $n = 58$; Figure 4). However, the length of both axons and minor neurites was significantly increased (axons: $217 \pm 21 \mu\text{m}$; minor neurites: $63 \pm 5 \mu\text{m}$; $n = 58$; Supplementary Figure S4). Catalytically inactive Smurf1C699A blocked neurite growth and reduced the num-

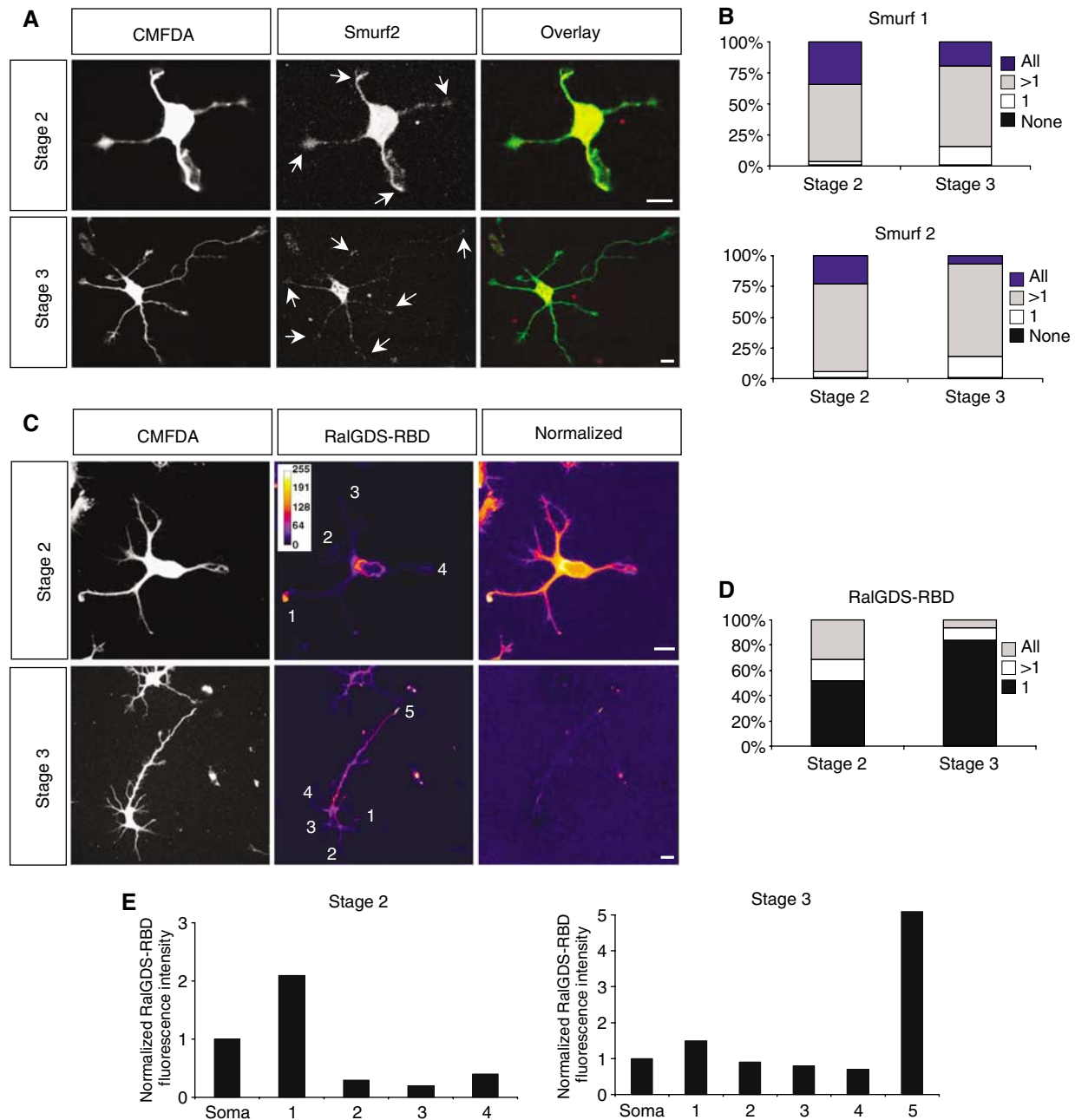


Figure 3 Distribution of Smurf1 and Smurf2 and active Rap1B during neuronal differentiation. (A, B) Hippocampal neurons were fixed at stage 2 or 3 and stained with anti-Smurf1 (not shown) or anti-Smurf2 antibodies (A, red). Neurons were analysed by confocal microscopy. Projections of z-stacks that contained the complete cell are shown. The immunofluorescence signals were normalised for cell volume by labelling with CMFDA (A, green). The percentage of cells that contain Smurf1 (B, top) or Smurf2 (B, bottom) in all, several (>1; the axon and one or more minor neurites in stage 3), one (1; the axon in stage 3), or none of the neurites is shown. Arrows indicate staining at the tips of neurites. At stage 2, 71 ± 5% of the cells contained Smurf2 in more than one but not all neurites, 23 ± 3% in all neurites, 5 ± 2% of the neurons showed Smurf2 in a single and 1 ± 1% in none of the neurites (*n* = 75; three experiments). At stage 3, Smurf2 was restricted to the axon in 17 ± 3% of the cells (1), 7 ± 1% contained Smurf2 in all growth cones and 75 ± 5% in the axon and one or more minor neurites in addition (>1; *n* = 150; three independent experiments). For each growth cone, the normalised fluorescence intensity was calculated as the ratio of the fluorescence intensities of Smurf1 or Smurf2 and CMFDA in the growth cone. A growth cone was scored as positive for Smurf1 or Smurf2 if the value for the relative fluorescence intensity was at least three times higher than the value for the background. Scale bars are 12 µm. (C–E) Hippocampal neurons were fixed at stage 2 or 3, incubated with bacterially expressed GST-RalGDS-RBD that specifically binds Rap1B-GTP, and bound GST-RalGDS-RBD detected *in situ* with an anti-GST antibody. The immunofluorescence signals were normalised for cell volume by labelling with CMFDA. The intensity of fluorescence (RalGDS-RBD) and the normalised fluorescence intensity (normalised) were colour-coded. The normalised fluorescence intensity was calculated as the ratio of the fluorescence intensities of RalGDS-RBD and CMFDA in the growth cone. Blue indicates weak staining, white strong labelling. The distribution of RalGDS-RBD binding sites, indicating active Rap1B, was analysed by determining the percentage of cells that showed binding in all, several (>1; the axon and one or more minor neurites in stage 3), or one (1; the axon in stage 3) of the neurites (D). (E) The normalised immunofluorescence intensity (arbitrary units) in the growth cones marked in (C) is shown. A growth cone was scored as positive for active Rap1B when the normalised fluorescence intensity was at least double the value of that in the soma.

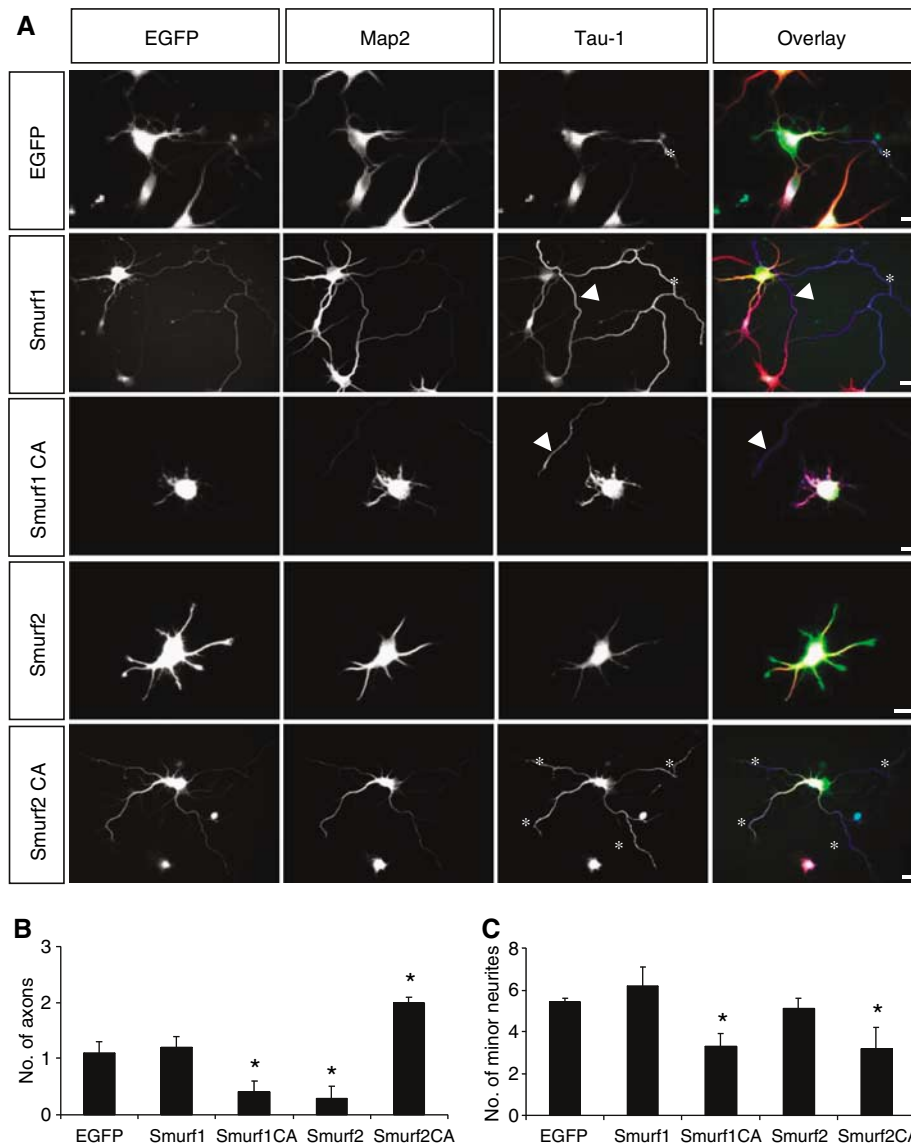


Figure 4 Expression of Smurf1 and Smurf2 disrupts neuronal differentiation. (A–C) Hippocampal neurons were transfected 2 h after plating with expression vectors for EGFP or EGFP and Smurf1, Smurf1C699A, Smurf2, or Smurf2C716A (A, green). Transfected cells were analysed at 3 d.i.v. by staining with the Tau-1 (blue; asterisks) and anti-MAP2 antibodies (red). The development of neuronal polarity was analysed by counting the number of axons (B) and minor neurites (C) per cell (means \pm s.e.m.; * $P < 0.001$ compared to EGFP; five independent experiments). Axons extended by untransfected cells are marked by arrowheads. Scale bars are 12 μ m.

ber of axons (0.4 ± 0.2 ; $n = 61$) and minor neurites per cell (3.3 ± 0.6 ; $n = 61$). The effects of Smurf1 and Smurf1C699A were very similar to those of dominant-negative or constitutively active RhoA, respectively (Schwamborn and Püschel, 2004). Thus, manipulating Smurf1 activity probably interferes with the regulation of Rho and thereby affects the extension of neurites irrespective of their identity.

Knockdown of Smurf1 by RNAi resulted in a phenotype distinct from that of suppressing Smurf2 and had the same effect as dominant-negative Smurf1C699A. Transfected neurons showed a reduction in the number of axons and minor neurites per cell (0.3 ± 0.4 axons and 3.1 ± 0.7 minor neurites per cell; $n = 41$; Figure 5B and D). The length of minor neurites was not changed significantly (Supplementary Figures S5C–F). After knockdown of Smurf1, Rap1B was still restricted to a single neurite, although this was much shorter than a normal axon (Figure 5C). The signal for Rap1B was

often reduced in these neurons, possibly reflecting a negative feedback resulting from increased Rho activity, which may involve PTEN (Shi *et al*, 2003; Schwamborn *et al*, 2004; Li *et al*, 2005). The persistence of Rap1B in a single neurite is consistent with the conclusion that Smurf1 does not regulate neuronal polarity but neurite growth. These results show that Smurf1 performs a function distinct from that of Smurf2. Whereas Smurf2 is required for the establishment of neuronal polarity, Smurf1 regulates neurite extension through Rho.

Rap1B activation and Smurf2-dependent degradation

Our results show that both increasing and decreasing Smurf2 activity disrupt neuronal polarity. Although only inactive Rap1B is a substrate for Smurf2, increasing Smurf2 expression in neurons is able to initiate the complete degradation of Rap1B. A high concentration of Smurf2 most likely increases

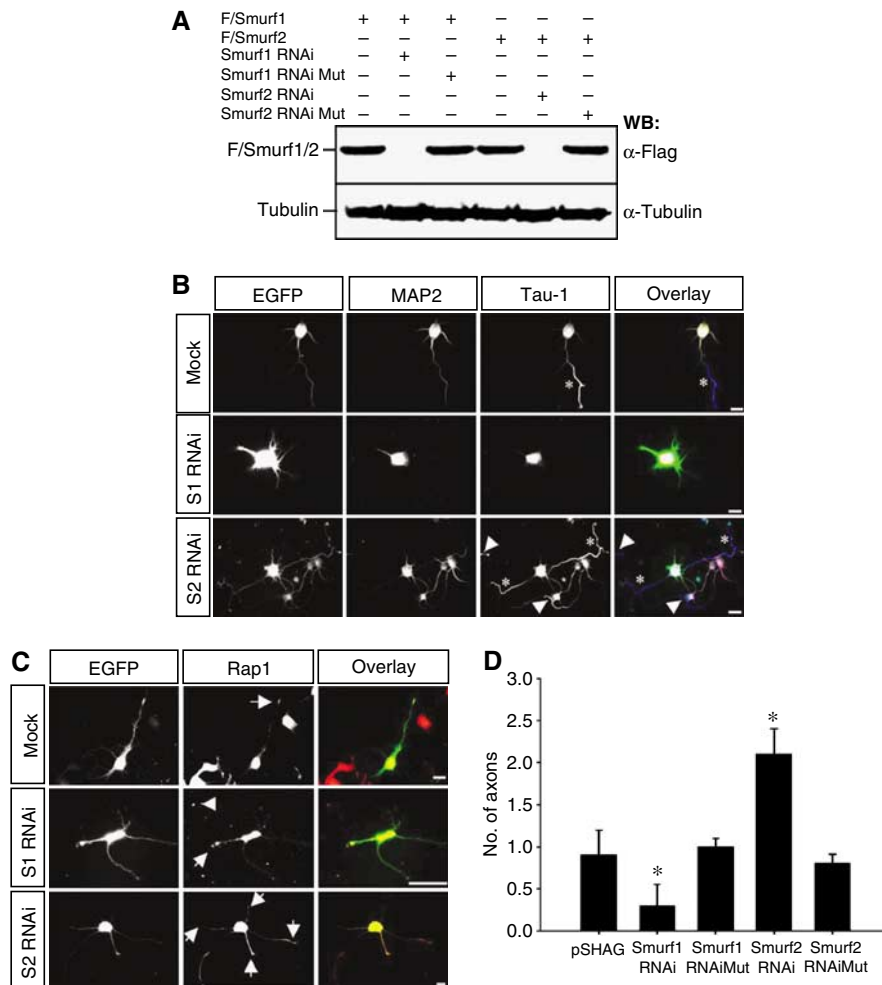


Figure 5 Smurf2 knockdown induces supernumerary axons. **(A)** HEK 293T cells were transfected with Flag-tagged Smurf1 (F/Smurf1) or Smurf2 (F/Smurf2) and expression vectors for shRNAs directed against Smurf1 (S1 RNAi) or Smurf2 (S2 RNAi) as indicated. As control, Flag-Smurf1 or Flag-Smurf2 was cotransfected with expression vectors for inactive shRNAs (Mut). Smurf1 and Smurf2 expression was determined by Western blots of cell lysates using an anti-Flag antibody. The loading of equivalent amounts of protein was confirmed by using an anti- α -tubulin antibody. **(B, D)** Hippocampal neurons were transfected with pSHAG-1 (Mock) or expression vectors for shRNAs directed against Smurf1 (S1 RNAi) or Smurf2 (S2 RNAi) and EGFP (B, green). As control, constructs for inactive shRNAs (Mut) were used. **(B)** Transfected cells were analysed at 3 d.i.v. (stage 3) by staining with the Tau-1 (blue) and anti-MAP2 antibodies (red). Arrowheads mark the soma or axon of untransfected neurons. The development of neuronal polarity was analysed by counting the number of axons **(D)** and minor neurites (Supplementary Figure S4C) per cell (mean \pm s.e.m.; * $P < 0.001$ compared to mock; $n = 3$ independent experiments). **(C)** To analyse the effect of a Smurf1 or Smurf2 knockdown, neurons were stained with an anti-Rap1 antibody. In control transfections, $82 \pm 7\%$ of the neurons ($n = 53$; three experiments) contained Rap1B in a single neurite (arrow). After knockdown of Smurf1, $71 \pm 6\%$ of the neurons still showed Rap1B in a single neurite (arrow, $n = 47$). Rap1B was present in all supernumerary axons after knockdown of Smurf2 (arrows). Rap1 immunoreactivity in the soma or in axons of untransfected cells is marked by arrowheads in **(B)** and **(C)**. Scale bars are $12 \mu\text{m}$.

the probability that Rap1B is ubiquitinated while cycling between the active and inactive states. This suggests that the amount of Smurf2 determines the extent of Rap1B activation that is required to protect it from degradation (Figure 7B). To test this hypothesis, we transfected HEK 293T cells with a constant amount of expression vector for Rap1B and increasing amounts of the Smurf2 vector. Rap1B was activated by increasing concentrations of the Epac activator 8-CPT (1.1 – $8.8 \mu\text{M}$; EC_{50} : $2.2 \mu\text{M}$). Coexpression of Smurf2 without addition of 8-CPT led to the complete degradation of Rap1B (Figure 7A). Addition of $2.2 \mu\text{M}$ 8-CPT protected Rap1B from degradation. When the amount of the Smurf2 expression vector was increased, a much higher concentration of 8-CPT ($8.8 \mu\text{M}$) was required to protect Rap1B from degradation. These results suggest that the

concentration of Smurf2 determines at which level of activity Rap1B is protected from degradation.

Discussion

Our results show for the first time that ubiquitination and proteolytic degradation of GTPases are essential for the establishment of neuronal polarity. Initially, Rap1B is present in all growth cones until it becomes restricted to a single neurite (Schwamborn and Püschel, 2004). However, it is unclear how this restriction is achieved. Pharmacological inhibition of the proteasome induced the formation of multiple axons, which were shorter than normal. The phenotype of these neurons suggests that the misregulation of Rho and Rap1B resulting from a block of their degradation is a major

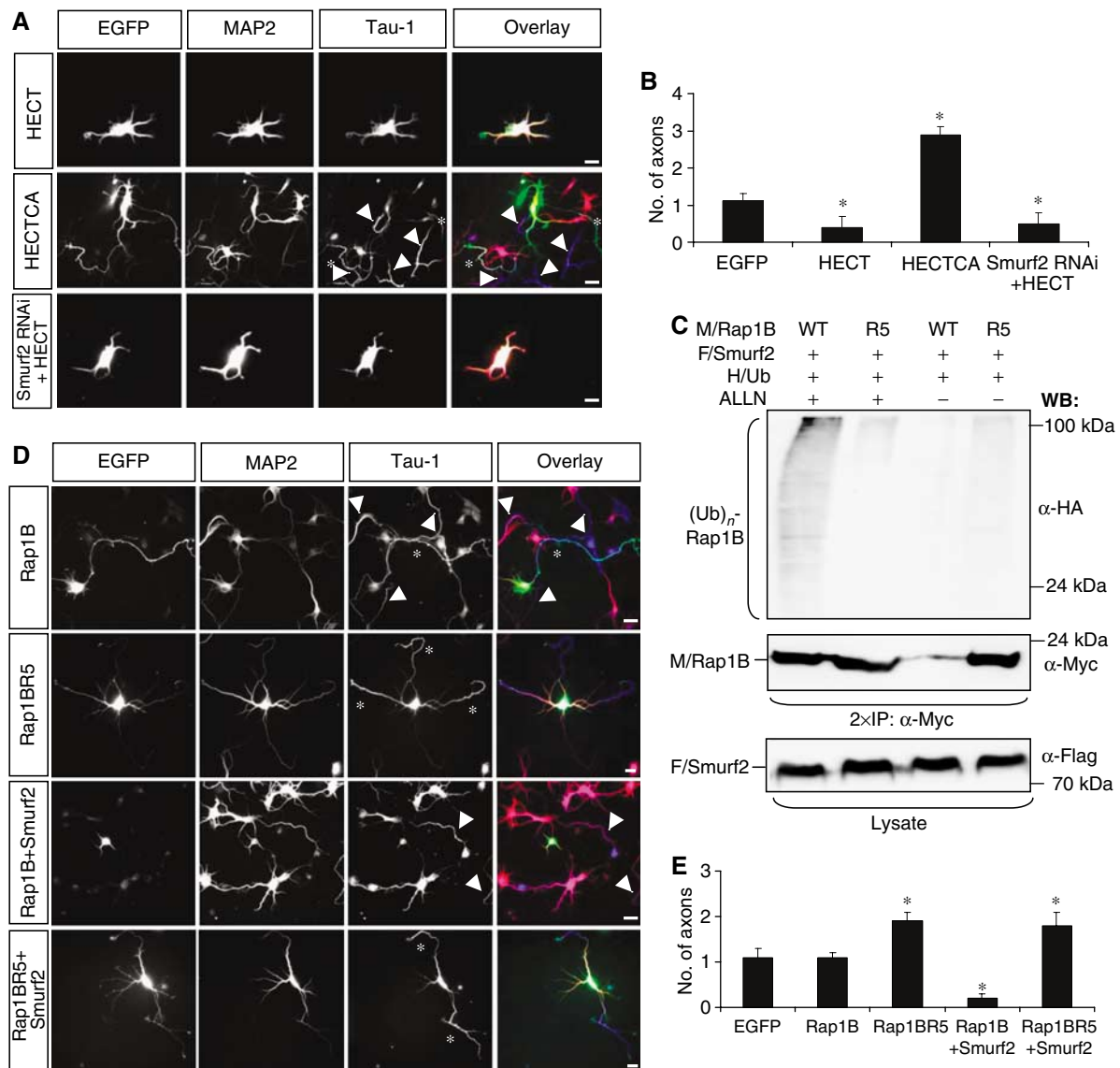


Figure 6 Mutation of a single lysine residue blocks Smurf2-dependent inhibition of Rap1B. (A, B) Hippocampal neurons were transfected 2 h after plating with expression vectors for EGFP-Smurf2-HECT (HECT), -Smurf2-HECT C716A (HECTCA), or EGFP-Smurf2-HECT and an shRNA directed against Smurf2 (A, green). Scale bars are 12 μ m. (C) HEK 293T cells were transfected with vectors for HA-tagged ubiquitin (HA/Ub), myc-Rap1B (M/Rap1B, WT) or myc-Rap1BR5 (R5), and Flag-Smurf2 (F/Smurf2), incubated overnight with 40 μ M ALLN, and Rap1B immunoprecipitated with an anti-myc antibody. Ubiquitin-conjugated Rap1B ((Ub)_n-Rap1B) and Rap1B were detected with anti-HA and anti-myc antibodies, respectively. Flag-Smurf2 and myc-Rap1B expression was confirmed by Western blot of cell lysates using anti-Flag and anti-myc antibodies. (D, E) Hippocampal neurons were transfected 2 h after plating with expression vectors for Rap1B, Rap1BR5, Rap1B and EGFP-Smurf2, or Rap1BR5 and EGFP-Smurf2 (D, green). Transfected cells were analysed at 3 d.i.v. by staining with the Tau-1 (blue; asterisks) and anti-MAP2 antibodies (red). (B, E) The development of neuronal polarity was analysed by determining the number of axons per cell (means \pm s.e.m.; **P* < 0.001 compared to EGFP; *n* = 3 independent experiments). Axons extended by untransfected cells are marked by arrowheads in (A) and (D).

contributor to this effect. The extension of multiple axons reflects the failure to limit Rap1B activity whereas the reduction of axon length may be attributed at least in part to increased Rho signalling. Inhibiting the proteasome probably also affects the degradation of additional factors involved in axon specification and extension. Consistent with the effects of inhibiting the UPS, interfering with the activity of the E3 ligases Smurf1 and Smurf2 leads to defects in neurite growth and axon specification, respectively. A similar observation was reported recently after submission of this manuscript, showing that an unknown E3 ligase restricts Akt to the axon by initiating the degradation of inactive Akt in minor neurites

(Yan *et al*, 2006). The restriction of Rap1B to a single neurite (stage 2) precedes that of Akt (stage 3), suggesting that the removal of Akt from minor neurites depends on the prior specification of axonal identity.

Taken together with previous results (Shi *et al*, 2003; Nishimura *et al*, 2004; Schwamborn and Püschel, 2004), our data suggest the following model (Figure 7B). Initially, Rap1B is present at the tips of all neurites (early stage 2). However, only a single growth cone contains a high level of active Rap1B-GTP, whereas most neurites show low levels of active Rap1B in the majority of stage 2 neurons. The differences in the amount of GTP-bound Rap1B between

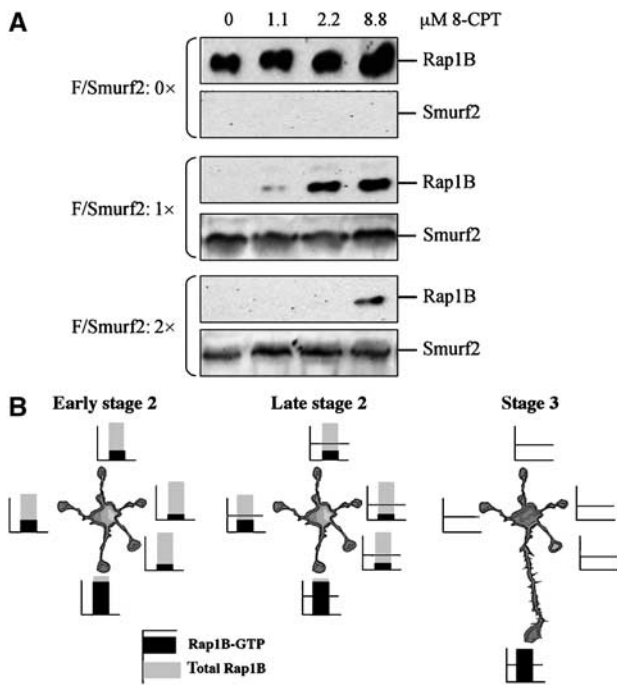


Figure 7 The concentration of Smurf2 determines the extent of Rap1B activation required to protect it from degradation. **(A)** HEK 293T cells were cotransfected with a constant amount of an expression vector for myc-tagged Rap1B (M/Rap1B) and increasing amounts of a vector for Flag-tagged Smurf2 (F/Smurf2) as indicated. 8-CPT was added at the indicated concentrations to activate endogenous Epac 16 h before cell lysates were analysed by Western blot using anti-myc and anti-Flag antibodies. **(B)** In early stage 2 neurons, comparable amounts of Rap1B protein are found in all neurites (Schwamborn and Püschel, 2004). However, one neurite contains a higher amount of active Rap1B than the other neurites, reflecting intrinsic differences between these neurites (Bradke and Dotti, 1997; Da Silva *et al*, 2005; de Anda *et al*, 2005). In late stage 2 neurons, Smurf2 is transported into all neurites and initiates the degradation of Rap1B in those where Rap1B activity is below the threshold defined by the local concentration of Smurf2. The degradation of Rap1B removes it from all but a single neurite, which becomes the axon. Active Rap1B together with Smurf1 reduces Rho activity to allow the rapid extension of the axon (Yamada *et al*, 2005).

neurites probably result from the differential activity of Rap1B GEFs and/or GAPs. PI3K acts upstream of Rap1B (Schwamborn and Püschel, 2004) and may activate Rap1B GEFs (Bos *et al*, 2001; Cullen and Lockyer, 2002) to initiate axon specification. However, PI3K activity as visualised by the distribution of active Akt initially is present in all neurites at stage 2 when active Rap1B is already restricted to a single neurite (Shi *et al*, 2003; Schwamborn and Püschel, 2004). Thus, PI3K activity alone does not explain the preferential activation of Rap1B in a single neurite. It remains an open question how the accumulation of different amounts of Rap1B-GTP arises in the growth cones of stage 2 neurons. The unequal distribution of Rap1B-GTP may reflect intrinsic differences between neurites as suggested by the asymmetric localisation of the membrane ganglioside sialidase Neu3 or the role of the centrosome in axon specification (Bradke and Dotti, 1997; Da Silva *et al*, 2005; de Anda *et al*, 2005).

Without the removal of Rap1B from all minor neurites, even a low level of active Rap1B is sufficient to induce axons, as shown by the phenotype of neurons after suppression of

Smurf2 by RNAi. Smurf2 initiates the restriction of Rap1B to a single neurite by targeting GDP-bound or nucleotide-free Rap1B for degradation in growth cones with low levels of active Rap1B. The removal of Rap1B confines the specification of axonal identity to a single neurite. This conclusion is based on the following evidence. First, inhibition of the UPS results in the formation of multiple axons that are positive for Rap1B and Par3. Second, only inactive nucleotide-free or GDP-bound Rap1B interacts with Smurf2 and is ubiquitinated by it *in vitro* and in cells. Ubiquitination of Rap1B initiates its degradation via the UPS. Third, overexpression of Smurf2 in neurons leads to a loss of axons whereas catalytically inactive Smurf2C716A blocks Rap1B degradation and induces supernumerary axons. Fourth, suppression of Smurf2 by RNAi results in the failure to restrict Rap1B to a single neurite, the accumulation of Rap1B at the tips of neurites, and the formation of multiple axons. Fifth, a single amino-acid exchange that interferes with ubiquitination results in a Rap1B mutant that is resistant to inhibition by Smurf2 and disrupts neuronal polarity after expression in hippocampal neurons.

The effects of manipulating the activity of Smurf2 suggest that it sets a local threshold for the activity of Rap1B that has to be surpassed to prevent its destruction (Figure 7B). This function of Smurf2 depends on the cycling of Rap1B between the active and inactive states. Rap1B is protected against destruction when only a small proportion is GDP-bound or nucleotide-free and, therefore, the probability that it is ubiquitinated is low. An increase in ubiquitin ligase activity or a reduction in the amount of active Rap1B increases the likelihood that it is ubiquitinated and degraded before it is reloaded with GTP. The continuing destruction of Rap1B eventually leads to its complete removal from a growth cone. Rap1B is completely degraded if the proportion of active Rap1B in a growth cone is below the threshold level defined by Smurf2. Thus, the activation of Rap1B determines which neurite becomes the axon whereas Smurf2 prevents that neurites with low amounts of active Rap1B also acquire an axonal identity. Thereby, Smurf2 limits neuronal polarisation and insures that only a single neurite is specified as an axon. This mechanism ultimately results in the transformation of quantitative differences in the amount of active Rap1B between individual growth cones into a qualitative one (axonal identity) and ensures that only a single axon is extended.

Upon the specification of the axon, its rapid extension is initiated, which requires a reduction in the level of Rho activity. In fibroblasts, Smurf1 promotes the local degradation of Rho and regulates cell polarity and protrusion (Wang *et al*, 2003). The effects of expressing wild-type or dominant-negative Smurf1 and its knockdown by RNAi demonstrate that Smurf1 is required for neurite extension. Probably together with RhoGAPs (Yamada *et al*, 2005), Smurf1 promotes axon elongation by ubiquitinating inactive Rho and targeting it to the proteasome. Thus, Smurf1 and Smurf2 coordinately regulate the activity of Rho and Rap1B. Restriction of Rap1B to a single neurite specifies the formation of a single axon, whereas the rapid extension of the axon is facilitated by the degradation of Rho. The local regulation of GTPases by the UPS may, therefore, be a general mechanism to establish cell polarity. Our results highlight the importance of the UPS for regulating the activity of GTPases and show that the effect of activating Rap1B critically depends on the concentration of

Smurf2. Smurf2 sets a threshold that determines the level of Rap1B activation required to protect it from degradation.

Materials and methods

Plasmids

Expression vectors for wild-type and mutant Smurf1 and Smurf2 (Wang *et al*, 2003), HA-ubiquitin (Treier *et al*, 1994), RalGDS-RBD (Franke *et al*, 1997), HA-Epac (de Rooij *et al*, 1998), Rap1BN17, Rap1BV12 (Lou *et al*, 2002), and pSHAG-1 (Paddison *et al*, 2002) were kindly provided by JL Wrana (University of Toronto), D Bohmann (University of Rochester), JL Bos (UMC Utrecht), T Imamura (JFCR Cancer Institute, Tokyo), Danny Altschuler (University Pittsburgh), and GJ Hannon (Cold Spring Harbor Laboratory). Full-length Smurf2 and Smurf2 deletion constructs containing the HECT domain (aa 349–748) were generated by PCR and cloned into pGEX-4T2 (Amersham) or pEGFP-C1 (Clontech) that was also used as a control vector. The coding sequence of Rap1B was amplified from mouse brain cDNA to generate pBK-myc-Rap1B, pBK-EGFP-Rap1B, and pGEX-Rap1B. The R5 mutation was introduced into Rap1B by PCR.

Neuronal cultures and transfection

Cultures of dissociated hippocampal neurons were prepared and transfected as described previously (Schwamborn and Püschel, 2004; Schwamborn *et al*, 2006). 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. After the neurons attached (2 h after plating), they were transfected using Lipofectamine 2000 (Invitrogen) as described. After an incubation for 2 h, the transfection medium was replaced by Neurobasal medium (supplemented with B27, 0.5 mM glutamine, and 100 U/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 (PBS) for 20 min at 4°C or (for staining with anti-Smurf1 and anti-Smurf2 antibodies) with methanol/acetone (1:1) for 20 min at –20°C. For immunofluorescence, hippocampal neurons were permeabilised with 0.1% Triton X-100 in PBS, blocked with 10% FCS in PBS (blocking buffer), and incubated with primary and secondary antibodies in blocking buffer. To analyse the establishment of neuronal polarity, neurons were stained with the Tau-1 (as a marker for axons) and an anti-MAP2 antibody (minor neurites). Processes longer than 50 µm and showing Tau-1 immunoreactivity in their distal segments that increases from proximal to distal were counted as axons, and MAP2-positive neurites longer than one-cell diameter and shorter than 50 µm as minor neurites. To analyse neuronal morphology and normalise immunofluorescence signals to cell volume, Cell Tracker Green (CMFDA, 5-chloromethylfluorescein diacetate; Molecular Probes) was used according to the manufacturer's instructions.

RNA interference

For the knockdown of Smurf1 and Smurf2 by RNAi, the following target sequences were selected for the generation of shRNA vectors based on the pSHAG-1 plasmid (Paddison *et al*, 2002): TGCCACT CAA CCGACACTGT GAAAACAC (Smurf1), TGCCACTCAAC TGA CACTGTGA AGAACAC (Smurf1Mut), ACAATGGTG CAACATGTGG ACAGTCTTC (Smurf2), ACAATGGCG CAACATGTGAA CAGTCTTC (Smurf2Mut). The pSM2 (Silva *et al*, 2005) plasmid with the target sequence CTGTATTCCATGGACATTA (plasmid HP_580232; Open Biosystems) was used as a second shRNA vector for the knockdown of Smurf2 (Smurf2 shRNA-2).

Immunofluorescence

The following antibodies were used: Tau-1 (Chemicon; #MAB3420; dilution 1:400), anti-MAP2 (Chemicon; #AB5622, 1:500), anti-GFP (BabCo; #MMS-119P, 1:5000), rabbit anti-Par3 (Upstate; #07-330, 1:400), monoclonal anti-Rap1 (BD Biosciences; #610195, 1:200), polyclonal anti-Smurf1 (#AP2104a, 1:400) and anti-Smurf2 (Abgent; #AP2105a, 1:400), and Alexa-conjugated secondary antibodies (Molecular Probes; 1:1000). The specificity of the Smurf1 and Smurf2 antibodies was confirmed in Western blots (not shown) and

immunofluorescence (Supplementary Figure S5). The anti-Rap1 antibody recognises both Rap1A and Rap1B (95% amino-acid sequence identity) but not other GTPases. Hippocampal neurons express mainly Rap1B (Schwamborn and Püschel, 2004). DMSO, ALLN, clasto-Lactacystin β-Lactone, and MG-132 (Calbiochem) were directly added to neuronal cultures 16 h after plating to a final concentration of 30, 40, 1.5, and 1.5 µM, respectively (Wang *et al*, 2003).

To analyse the distribution of Smurf1 and Smurf2, the normalised immunofluorescence intensity in the growth cones was calculated as the ratio of Smurf1 or Smurf2 fluorescence intensity and cell volume as measured by the CMFDA signal. Background fluorescence intensity was determined by the same procedure for an identical cell-free area adjacent to the growth cone. A growth cone was scored as positive for Smurf1 or Smurf2 when the relative fluorescence intensity was at least three times higher than background.

For the *in situ* detection of active Rap1B (Lafuente *et al*, 2004), hippocampal neurons were fixed, permeabilised with 0.1% Triton X-100 in PBS, blocked with 10% FCS in PBS, and incubated overnight at 4°C with bacterially expressed GST or GST-RalGDS-RBD in PBS with 10% FCS and 20 mM free glutathione (Sigma). GST fusion proteins were affinity-purified on glutathione-Sepharose beads (Amersham). The distribution of bound GST fusion protein was revealed by immunofluorescence using an anti-GST antibody (Amersham; #27-4577-01, 1:1000). To analyse the distribution of active Rap1B quantitatively, the normalised immunofluorescence intensity in the growth cones was calculated as the ratio of RalGDS-RBD fluorescence intensity and cell volume as measured by the CMFDA signal. As a reference, the corresponding value measured in the soma (excluding the nucleus) was normalised to one. A growth cone was scored as positive for active Rap1B when the normalised immunofluorescence intensity of RalGDS-RBD was at least double the value in the cell soma.

A Zeiss LSM 510 confocal laser scanning microscope with the LSM Imager software (Zeiss) was used to analyse the distribution of Smurf1, Smurf2, and active Rap1B. For all other images, a Zeiss AxioScope equipped with a SPOT CCD camera and the SPOT 4.1 software (Diagnostic Instruments) were used. The same settings (exposure time, gain, binning) and the same post-processing operations were always used to analyse the distribution of a specific protein. During post-processing with Adobe Photoshop, the brightest pixel in a picture was set as white, the darkest pixel as black, and intermediate values were distributed proportionally.

Neuronal morphology was analysed using the WASABI software (Hamamatsu), ImageJ 1.33u (NIH), and Adobe Photoshop. The development of neuronal polarity was analysed as described previously (Schwamborn *et al*, 2006). The length of axons and dendrites was determined using the Spot software (Diagnostic Instruments). The Student's *t*-test was used to test statistical significance.

Pull-down assays

Pull-down assays were performed as described (Wang *et al*, 2003). GST fusion proteins were immobilised on glutathione-Sepharose (Amersham). Bacterially expressed GST-Rap1B was preloaded with 0.7 mM GDP or GTPγS (Sigma). After washing the beads with lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, 10 mM MgCl₂, 4 mM EDTA, 10% glycerol, 1% Triton X-100, and Complete protease inhibitor cocktail (Roche)), they were incubated with lysates from transfected HEK 293T cells, washed, and bound proteins eluted with sample buffer.

Immunoprecipitation and Western blot

HEK 293T cells were lysed 48 h after transfection with lysis buffer 1 (2% Triton X-100 and Complete protease inhibitor cocktail (Roche) in PBS) for 30 min at 4°C. To analyse the interaction with GTPases, the lysis buffer was supplemented with 10 mM MgCl₂. For immunoprecipitation of ubiquitinated proteins from neuronal cultures, 2 × 10⁵ hippocampal neurons from E18 rat embryos were plated per 10-cm dish. At 24 h after plating, solvent (DMSO) or ALLN (40 µM final concentration) was directly added to the medium. After culture for 24 h, neurons were lysed in lysis buffer 2 (1% SDS in PBS) for 30 min at room temperature. Subsequently, the lysate was diluted 1:10 with PBS. Ubiquitinated proteins were precipitated for 4 h at 4°C with an anti-Rap1 antibody and overnight with Protein-G agarose. To analyse Rap1B5 ubiquitination

(Figure 6C), cells were lysed in lysis buffer 1, ubiquitinated proteins precipitated, bound proteins eluted by boiling in 1% SDS, and the immunoprecipitation repeated with the same antibody.

For immunoprecipitation and Western blots, the following antibodies were used: anti-Flag M2 (Sigma; #F1804, 1:2000), anti-myc (CST; #2272, 1:1000), anti-Smurf1, anti-Smurf2 (Abgent; #AP2104a, #AP2105a, 1:1000), anti-GST (Amersham; #27-4577-01, 1:5000), anti-HA (Roche; #1867423, 1:500), anti-ubiquitin P4D1 (Santa Cruz; #sc-8017, 1:400), anti- α -tubulin (Sigma; #T6199, 1:2000), and HRP-coupled secondary antibodies (MoBiTec, Göttingen). Rap1B was activated by addition of 8-CPT (Tocris). Western blots were analysed using the LAS-1000 luminescent image analyser (Fujifilm) and the WASABI software (Hamamatsu).

In vitro ubiquitination

Bacterially expressed GST-Smurf2 (wild type and C716A), wild-type GST-HECT, and GST-Rap1B were purified using glutathione-Sepharose (Amersham) in TLB buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, 10 mM MgCl₂, 4 mM EDTA, 10% glycerol, 1% Triton

X-100, Complete protease inhibitor cocktail (Roche)). Ubiquitin, ubiquitin-activating enzyme (E1), and UbcH5c (E2) were obtained from BostonBiochem. The *in vitro* ubiquitination was performed as described previously (Wang *et al*, 2003) and detected using the anti-ubiquitin antibody P4D1 (Santa Cruz).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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