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The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity

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The establishment of a polarized morphology is an essential step in the differentiation of neurons with a single axon and multiple dendrites. In cultured rat hippocampal neurons, one of several initially indistinguishable neurites is selected to become the axon. Both phosphatidylinositol 3,4,5-trisphosphate and the evolutionarily conserved Par complex (comprising Par3, Par6 and an atypical PKC (aPKC) such as PKCλ or PKCζ) are involved in axon specification. However, the initial signals that establish cellular asymmetry and the pathways that subsequently translate it into structural changes remain to be elucidated. Here we show that localization of the GTPase Rap1B to the tip of a single neurite is a decisive step in determining which neurite becomes the axon. Using GTPase mutants and RNA interference, we found that Rap1B is necessary and sufficient to initiate the development of axons upstream of Cdc42 and the Par complex.

The establishment of a polarized morphology and the functional specialization of different cellular compartments are essential steps in the differentiation of neurons. However, the initial signals that establish a cellular asymmetry and the pathways that subsequently translate this asymmetry into the development of multiple dendrites and a single axon are largely unknown[1,2]. Primary cultures of dissociated hippocampal neurons are widely used as a model system to elucidate the molecular mechanisms that establish the asymmetric organization of cellular structures[1,3]. Shortly after plating, hippocampal neurons first extend lamellipodia (stage 1) and subsequently several processes, which are initially indistinguishable (minor neurites, stage 2) until one of them becomes the axon (stage 3)[3,4]. Differences in actin dynamics are thought to have an important role in this process. Destabilization of actin filaments by cytochalasin D or bacterial toxin B is sufficient to induce the formation of multiple axons[5]. The molecular nature of the symmetry-breaking signal and the negative feedback that blocks the formation of additional axons once an axon has been specified remain to be elucidated.

The tripartite Par complex has an essential, evolutionarily conserved role in determining polarity in many cell types and is also involved in the specification of axons in hippocampal neurons[6–8]. Binding of active Rac or Cdc42 to the Par6/aPKC complex enhances the activity of aPKCs (PKCλ and PKCζ), suggesting that aPKC regulation by GTPases is one function of this complex[6,9–11]. The initial steps that lead to the recruitment of the Par complex remain to be identified. In addition to the restricted localization of the Par3/Par6 complex at the tip of the developing axon, phosphatidylinositol 3-kinase (PI3K) activity is essential for axon specification[8]. The axonal localization of the PI3K product PI(3,4,5)P3, as revealed by the distribution of phosphorylated Akt (P-Akt), suggests that similar to its role in chemotaxis, this pathway may also be involved in the establishment of neuronal polarity[8,12].

To elucidate the initial events that determine which neurite becomes the axon and restrict the Par complex to a single neurite, we investigated the role of the GTPases Rap1B and Cdc42 in the establishment of neuronal polarity. Here we show that Rap1B is specifically localized to the tip of the growing axon in cultures of hippocampal neurons and acts upstream of Cdc42 and the Par complex to determine axonal identity. Rap1B determines which neurite will become the axon and directs the recruitment of Cdc42, which is essential for the formation of molecularly distinct dendrites and axons.

RESULTS

Rap1B is localized at the tip of the axon

Dissociated hippocampal neurons from E18 rat embryos initially extend several processes of similar length (stage 2) that are indistinguishable until one neurite begins to elongate rapidly to become the axon (stage 3)[4]. The remaining minor neurites begin to differentiate into dendrites at 4 days *in vitro* (4 d.i.v., stage 4)[4]. At 1–2 d.i.v. (stage 2), Rap1B was found in the cell body and either in the tips of all neurites or restricted to a single neurite and absent from the remaining processes (**Fig. 1a–e**). At 1 d.i.v., 73.4 ± 4.1% (mean ± s.e.m.) of the neurons contained Rap1B in all neurites, whereas at 2 d.i.v., before axons were visible, it was restricted to a single growth cone in 81.2 ± 5.2% of the cells ($n = 150$ for each stage in three independent experiments). Only a small minority showed an intermediate distribution with Rap1B in more than one but not all neurites (1 d.i.v., 8 ± 1.3%, 2 d.i.v., 3 ± 0.9%; $n = 150$ for each stage, 3 experiments).

By contrast, a gradual shift in the distribution of Cdc42 was observed when we compared early (1 d.i.v.) and late (1.5–2 d.i.v.) neurons at stage 2. In early stage 2, the majority of neurons showed Cdc42 staining in the tips of all neurites (**Fig. 1e**; 65 ± 2.4%; $n = 120$ neurons, 3 experiments). Cdc42 became more restricted in late stage 2

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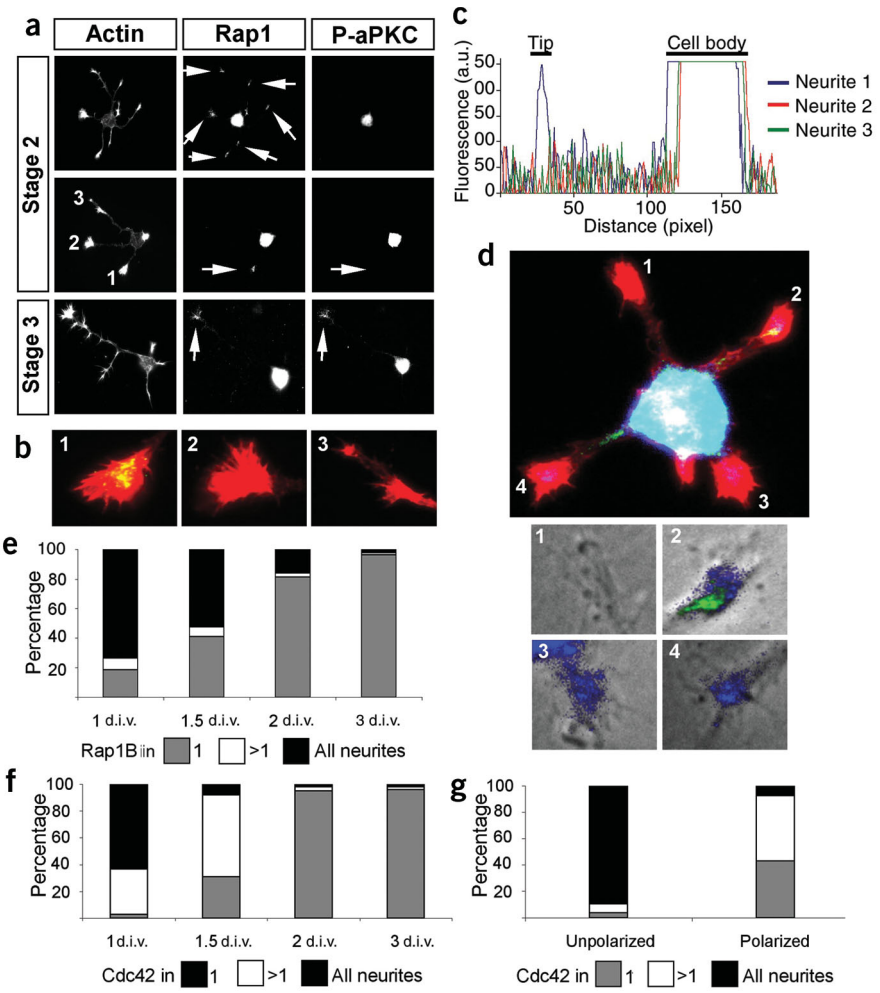

Figure 1 Polarized distribution of Rap1B. (**a**, **b**) Hippocampal neurons were fixed at stage 2 (1.5 d.i.v.) or stage 3 (2.5 d.i.v.) and stained with phalloidin-rhodamine (red), anti-Rap1 (green) and anti-P-aPKC (blue) (arrows: staining at the tip of neurites). (**b**, **c**) Higher magnification of the growth cones (**b**) and profiles of Rap1 immunofluorescence (**c**, arbitrary units) in the neurites marked 1–3 in **a**. (**d**) Hippocampal neurons were fixed at stage 2 and stained with phalloidin-rhodamine (red), anti-Rap1 (green) and anti-Cdc42 (blue). Higher magnifications of the growth cones marked 1–4 are shown as overlays of phase contrast and immunofluorescence pictures. (**e**–**g**) Hippocampal neurons were fixed at 1 d.i.v. (early stage 2), 1.5 d.i.v., 2 d.i.v. (late stage 2) or 3 d.i.v. (stage 3) and stained with anti-Rap1 and anti-Cdc42. The percentage of neurons (mean ± s.e.m.) with Rap1B (**e**) or Cdc42 (**f**) in one neurite (1; gray bars), in more than one but not in all neurites (>1; white bars) or in all neurites (all; black bars) is shown. (**g**) The percentage of unpolarized (Rap1B in all neurites) or polarized neurons (Rap1B in one neurite) that contained Cdc42 in one (hatched bars), in more than one but not all (white bars) and in all neurites (black bars) is shown.

(n = 103 neurons, 3 experiments) when 61 ± 3.1% of the cells contained Cdc42 in some but not all neurites at 1.5 d.i.v. The loss of Rap1B from all but one neurite preceded the redistribution of Cdc42 (**Fig. 1d**,**f**,**g**). At 1 d.i.v. (stage 2; n = 120 neurons, 3 experiments) the majority of neurons (89.5 ± 10.3%) with an unpolarized distribution of Rap1B in all neurites also showed Cdc42 staining in all growth cones. Only 7.4 ± 2.9% of the neurons contained Cdc42 in all neurites, and 49.4 ± 6.1% contained Cdc42 in several but not all neurites when Rap1B was restricted to a single growth cone (**Fig. 1g**). In stage 3 neurons (3 d.i.v.; n = 82 neurons, 3 experiments), Rap1B (in 96.3 ± 9.3% of cells) and Cdc42 (in 96.0 ± 4.1% of cells) were localized exclusively to the tip of the axon (**Fig. 1a**,**e**,**f**; **Supplementary Fig. 1** online). The specific localization of Rap1B in the axon is not a consequence of rapid neurite growth. When the extension of axons was blocked by a low concentration of the microtubule stabilizing drug taxol[13], Rap1B was nevertheless restricted to a single neurite (**Supplementary Fig. 2** online).

Rap1B accumulation in a single growth cone preceded axonal localization both of phosphorylated Akt/PKB (P-Akt) and Par3 (**Fig. 1a**; **Supplementary Fig. 1** online). P-Akt and Par3 were found in all neurites at stage 2, but they were restricted to the axonal tip in stage 3 neurons[8]. The Par complex mediates the activation of aPKC by Cdc42[6,9–11]. An antibody specific to active PKCζ and PKCλ phosphorylated on residues T410 or T403 (P-aPKC) revealed the presence of P-aPKC at the tip of axons in stage 3 neurons only, but never in undifferentiated neurites (**Fig. 1a**). Rap1B was found exclusively at the tip of the axon once it could be identified by Tau-1, Par3 and P-aPKC staining at 3 d.i.v., and it remained axon-specific at least until 5 d.i.v. (**Fig. 1a**; **Supplementary Fig. 1** online). We therefore conclude that Rap1B restriction to a single neurite marks the future axon in late stage-2 neurons. This suggests that the concentration of Rap1B in a single growth cone is a decisive step in determining which neurite becomes the axon that precedes localization of Cdc42 and the Par complex to a single neurite.

Rap1B and Cdc42 induce supernumerary axons

To test whether Rap1B or Cdc42 is sufficient to initiate the formation of an axon, we transfected hippocampal neurons (2 h after plating) with expression vectors for enhanced green fluorescent protein (EGFP)–tagged, constitutively active Rap1BV12 or for hyperactivated Cdc42L28, and then we analyzed them at 2.5 d.i.v. (**Fig. 2**; **Supplementary Fig. 3** online). Cdc42L28 is a variant of Cdc42 that autonomously cycles between the GTP- and GDP-bound states and differs from constitutively active Cdc42V12 in its ability to transform cells[14]. Neurons transfected with a vector for EGFP formed 1.1 ± 0.1 axons and 5.4 ± 0.3 minor neurites per cell at 2.5 d.i.v. (stage 3) (**Fig. 2a**,**b**; n = 78 neurons, 5 experiments). Active Rap1BV12 induced the formation of supernumerary axons at the expense of minor neurites (3.7 ± 0.2 axons, 1.1 ± 0.3 minor neurites per cell, n = 64, 5 experiments). Expression of Rap1BV12 after polarization at 3 d.i.v. did not result in the formation of multiple axons and did not affect the length of dendrites (analyzed at 5 d.i.v.; **Supplementary Fig. 4** online). Cdc42L28 also induced supernumerary axons but had a less pronounced effect than Rap1BV12 and did not significantly reduce the number of minor neurites (**Fig. 2a**,**b**; 2.1 ± 0.2 axons per cell, n = 58, 5 experiments). By contrast, neurons expressing Cdc42V12 did not extend any

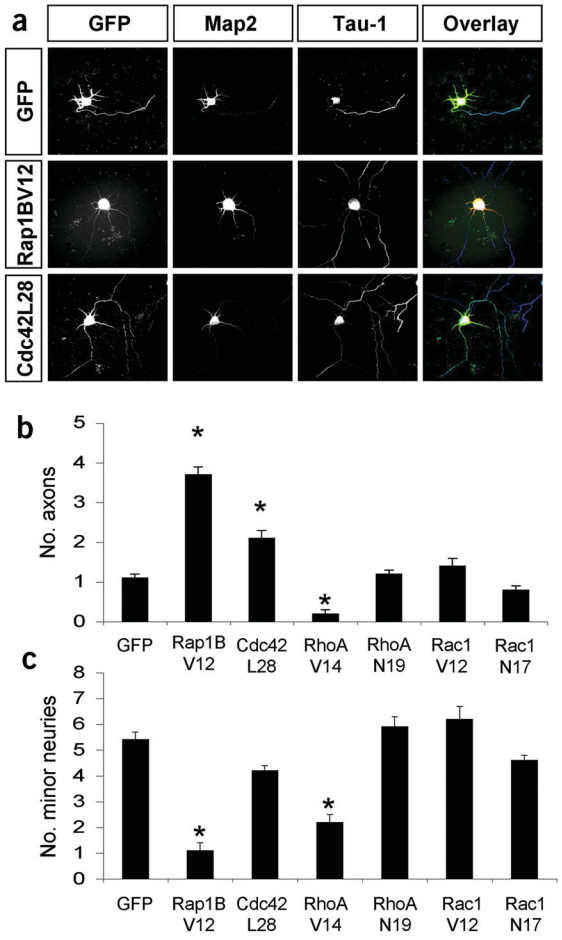

Figure 2 Induction of supernumerary axons by Rap1B and Cdc42. (**a**–**c**) Hippocampal neurons were transfected 12 h after plating with expression vectors for EGFP, EGFP-Rap1BV12, -Cdc42L28, -RhoAV14, -RhoAN19, -RhoAV12 or -RhoAN17 (**a**, green fluorescence) and analyzed at 2.5 d.i.v. by staining with Tau-1 (**a**, blue; axonal marker) and anti-MAP2 (**a**, red, dendritic marker). The development of neuronal polarity was analyzed by counting the number of axons (**b**) and minor neurites per cell (**c**) (mean ± s.e.m.; $*P < 0.001$ compared to EGFP).

neurites (data not shown). This suggests that cycling of Cdc42 between the GDP- and the GTP-bound forms is essential for its function in neuronal polarity, similarly to yeast Cdc42p[15,16].

Several studies have shown that the GTPases Rac and Rho regulate the extension of neurites[1,17–22]. In order to investigate whether Rho and Rac are also involved in the establishment of neuronal polarity, we tested the effect of dominant-negative and constitutively active Rho and Rac (**Fig. 2b,c**; **Supplementary Table 1** and **Supplementary Fig. 3** online; 4 experiments). Consistent with previous results[19,23], RhoAV14 reduced the number and length of both axons and minor neurites. Whereas RhoAN19, Rac1V12 and Rac1N17 did not significantly change the number of axons or minor neurites, RhoA and Rac1 had antagonistic effects on neurite extension (**Supplementary Fig. 3b,c** online). RhoAV14 and Rac1N17 decreased and RhoAN17 and Rac1V12 increased the length of neurites (**Supplementary Fig. 3b,c** online). Thus, Rho and Rac regulate the extension of neurites irrespective of their identity. The temporal order of their appearance in the axon suggests that Rap1B, Cdc42 and the Par complex act sequentially in a hierarchical pathway that determines axonal identity. If this is true, then axons induced by active Rap1B or Cdc42 should be positive for Par3 and aPKC. The growth cones of all supernumerary axons induced by Rap1BV12 and Cdc42L28 contained Par3, P-aPKC and P-Akt (**Fig. 3** and data not shown). The presence of P-Akt indicates that both GTPases probably can activate PI3K as described for Rho GTPases in neutrophil-like HL-60 cells[24,25]. Thus, active Rap1B is sufficient to transform neurites extended by unpolarized neurons into axons when expressed before overt polarization.

Rap1B and Cdc42 are required for neuronal polarity

To test whether Rap1B and Cdc42 are required for axon specification, we cotransfected neurons with expression vectors for small hairpin RNAs (shRNA) to suppress Rap1B or Cdc42 expression by RNA interference (RNAi) and EGFP to mark the transfected cells[26]. Staining with antibodies showed a complete loss of Rap1B or Cdc42 from the growth cones and only a weak residual signal in the cell body of transfected cells that was visible only at longer exposure times (**Fig. 4a**). Transfection of the control RNAi vector (pSHAG-1 without insert) did not change the number of axons or minor neurites per cell (**Fig. 4b–d**; 1.2 ± 0.1 axons, 4.9 ± 0.4 minor neurites, n = 69, 4 experiments). Knockdown of Rap1B or Cdc42 by RNAi led to a complete loss of polarity (**Fig. 4a–d**). The majority of neurons did not form any axons, and the number and length of minor neurites did not significantly change (Rap1B: 0.2 ± 0.1 axons, 5.1 ± 0.3 minor neurites, n = 82; Cdc42: 0.4 ± 0.1 axons, 4.8 ± 0.4 minor neurites per cell, n = 78, 4 experiments) (**Supplementary Tables 1** and **2** and **Supplementary Fig. 3** online). Expression of an shRNA directed

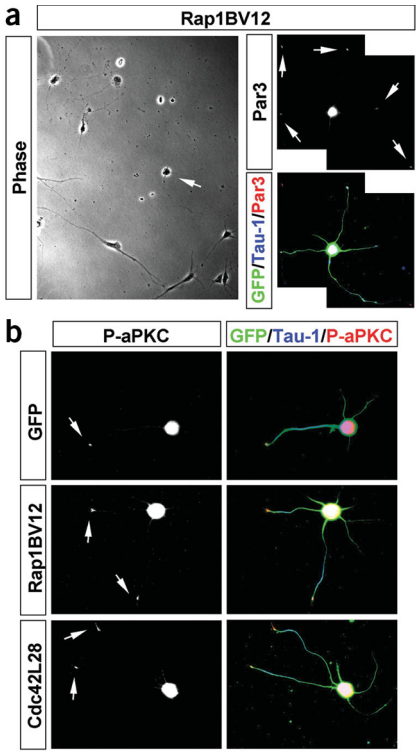

Figure 3 Distribution of axonal markers after expression of GTPases. (**a**, **b**) Hippocampal neurons were transfected with expression vectors for EGFP or EGFP-tagged GTPases (green) and analyzed at 2.5 d.i.v. by staining with Tau-1 (blue), anti-Par3 (**a**, red) and anti-aPKC (**b**, red).

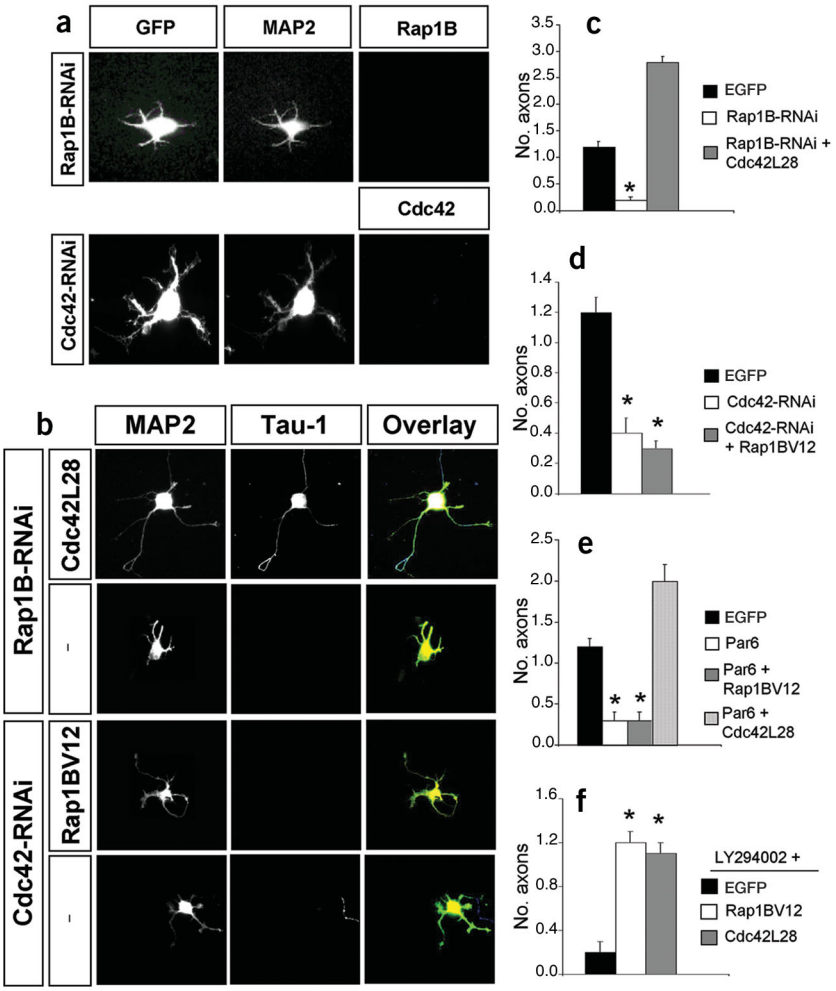

Figure 4 Rap1B and Cdc42 act sequentially. (**a**) Neurons were cotransfected with expression vectors for anti-Rap1B or anti-Cdc42 shRNAs and EGFP at 12 h after plating and stained with anti-MAP2, anti-Rap1 or anti-Cdc42 at 2.5 d.i.v. (**b**–**d**) Neurons were transfected with pSHAG-1 (–) or expression vectors for anti-Rap1B or anti-Cdc42 shRNAs and EGFP, EGFP-Rap1BV12 or EGFP-Cdc42L28 (green). (**e**) Cultures were transfected with expression vectors for EGFP or EGFP-Par6c and Rap1BV12 or Cdc42L28. (**f**) Cultures were transfected with expression vectors for EGFP, EGFP-Rap1BV12, or EGFP-Cdc42L28 and incubated with 100 μM LY294002 for 54 h. The number of axons formed per cell was determined at 2.5 d.i.v. after staining with Tau-1 and anti-MAP2 (means ± s.e.m.; $*P < 0.001$, compared to EGFP).

against Rap1A, the closest homolog of Rap1B, had no effect on neuronal polarity (data not shown). Knockdown of Rap1B after the establishment of polarity did not affect the number of axons or the length of dendrites (**Supplementary Fig. 4** online).

Destabilization of actin filaments by cytochalasin D is sufficient to induce the formation of multiple axons (**Fig. 5a**)[5]. Rap1B, P-Akt, P-aPKC and Par3 were present in the tips of all axons after treatment with cytochalasin D (**Fig. 5b**). The induction of multiple axons by cytochalasin D required Rap1B and was completely prevented when Rap1B was suppressed by RNAi in cytochalasin D–treated neurons (**Fig. 5c**; EGFP: 4.3 ± 0.2, n = 57 neurons; Rap1B-RNAi: 0.6 ± 0.1 axons per cell, n = 61 neurons; 3 experiments; $P < 0.001$, compared to EGFP). These results confirmed the central role of Rap1B in the specification of axonal properties.

Rap1B acts upstream of Cdc42 and the Par complex

The loss- and gain-of-function approaches showed that Rap1B and Cdc42 are both necessary and sufficient for axon specification. To establish that Rap1B depends on Cdc42 to induce axons, we tested whether the loss of polarity resulting from the knockdown of Rap1B could be rescued by expressing an activated form of Cdc42. Co-transfection of an expression vector for EGFP-Cdc42L28 with the anti-Rap1B RNAi construct restored the ability to extend axons and induced the development of multiple axons (**Fig. 4b–d**; 2.8 ± 0.1 axons, n = 70, 4 experiments). By contrast, EGFP-Rap1BV12 could not rescue the loss of polarity that resulted from the absence of Cdc42 (0.3 ± 0.05 axons per cell, n = 90, 4 experiments). These results support the idea that Rap1B acts upstream of Cdc42.

As described previously[8], the expression of EGFP-Par6c in hippocampal neurons resulted in the absence of axons, but it did not affect the number and length of minor neurites (**Fig. 4e**; **Supplementary Tables 1** and **2** and **Supplementary Fig. 4** online; 0.3 ± 0.1 axons per cell, n = 65, 4 experiments). The disruption of neuronal polarity by Par6c depended entirely on its ability to interact with Cdc42 through the Cdc42/Rac interactive binding (CRIB) motif, as the Par6cΔCRIB deletion mutant had no effect on axon specification or the distribution of Cdc42 or P-aPKC (**Supplementary Methods**, **Supplementary Table 1** and **Supplementary Fig. 5** online; and data not shown). Rap1BV12 was not able to reverse the loss of polarity resulting from the uniform distribution of Par6c (0.3 ± 0.1 axons per cell, n = 70, 4 experiments). By contrast, Cdc42L28 restored a polar phenotype and, in some cases, also induced supernumerary axons (2.0 ± 0.2 axons per cell, n = 71, 4 experiments). These results confirmed that Par6 acts downstream of Rap1B.

PI3K activity is required for neuronal polarization[8]. Pharmacological inhibition of PI3K in hippocampal neurons by LY294002, 6–8 h after plating, led to a complete loss of axons, whereas the number of neurites was not affected (**Fig. 4f**; 0.2 ± 0.1 axons per cell, 5.5 ± 0.5 neurites per cell, n = 78, 4 experiments). Inhibition of PI3K may block axogenesis by interfering with the activation of GTPases. Therefore, we tested whether expression of active GTPases was able to compensate for the absence of PI(3,4,5)P3. The restoration of a polar morphology by Rap1BV12 and Cdc42L28 indicates that PI(3,4,5)P3 acts upstream of Rap1B and Cdc42 (**Fig. 4f**; Rap1BV12: 1.2 ± 0.1, n = 75; Cdc42F28L: 1.1 ± 0.1 axons per cell, n = 72; 3 experiments). Expression of these GTPases did not induce supernumerary axons when PI3K was inhibited, indicating that PI(3,4,5)P3 production is required for the efficient induction of multiple axons.

DISCUSSION

Here we show that the localization of Rap1B to a single growth cone is an essential step in inducing hippocampal neurons to form an

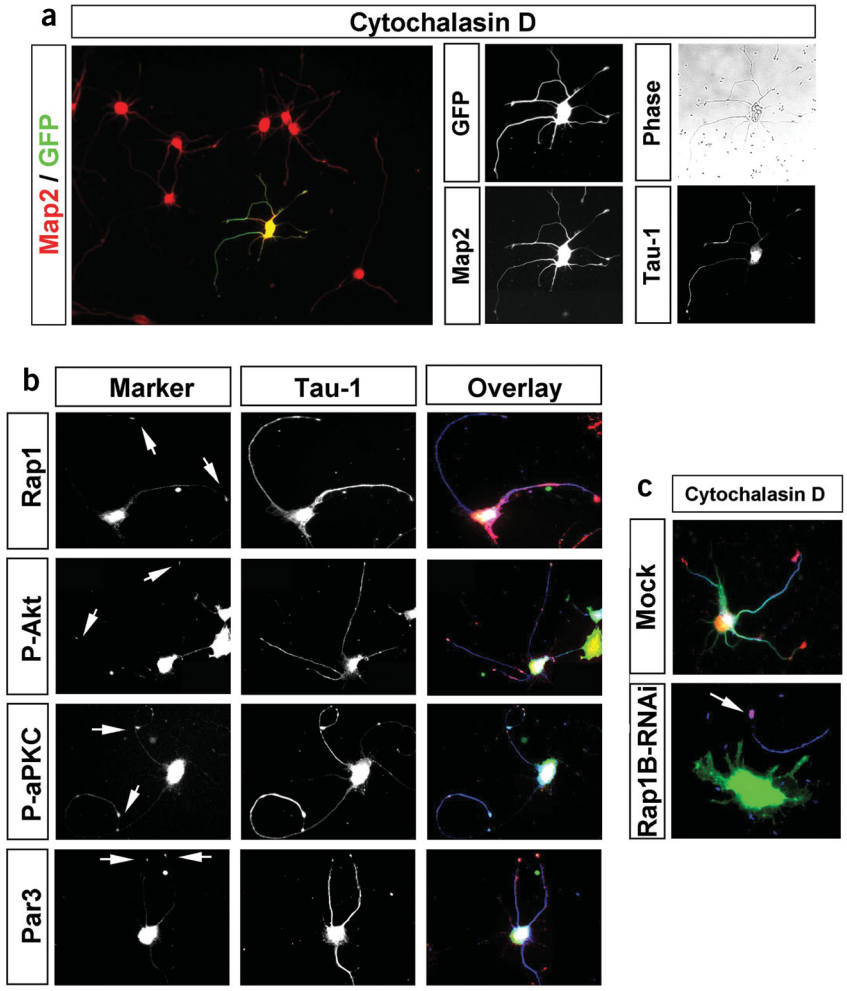

Figure 5 The induction of multiple axons by actin depolymerization depends on Rap1B. (**a**) Hippocampal neurons were transfected with pEGFP-N1 (green), incubated with 1 μM cytochalasin D for 54 h, and stained at 2.5 d.i.v. with Tau-1 (blue) and anti-MAP2 (red). Cytochalasin D induced the formation of multiple axons. Treatment with solvent (chloroform) had no effect (not shown). (**b**) Hippocampal neurons were incubated with 1 μM cytochalasin D for 54 h and stained at 2.5 d.i.v. with phalloidin-rhodamine (red), Tau-1 (blue) and anti-Rap1, anti-P-Akt, anti-P-aPKC or anti-Par3 (green). (**c**) Neurons were transfected with pEGFP-N1 (green) and pSHAG-1 (Mock) or an expression vector for the anti-Rap1B shRNA, and treated with 1 μM cytochalasin D for 54 h. After staining with Tau-1 (blue) and anti-Rap1 (red), the number of axons formed per cell was determined. The arrow marks a Rap1B-positive axon tip of an untransfected neuron.

axon. Subsequently, Cdc42, Par3 and P-Akt are localized to the Rap1B-positive growth cone, before active aPKC is detectable at the tip of the axon in stage-3 neurons (**Supplementary Fig. 6** online). Cdc42 reorganizes the cytoskeleton and intracellular trafficking to allow the rapid extension of the axon through the Par complex, Rho GTPases and probably additional effectors as well[1,2,27]. The signal that establishes the initial asymmetry and initiates the concentration of Rap1B in a single neurite probably involves the activation of PI3K[8]. As active Rap1B and Cdc42 are able to induce the formation of axons in the presence of the PI3K inhibitor LY294002, PI3K acts upstream of Rap1B (**Supplementary Fig. 6** online) and may be required primarily to recruit Rap1B GEFs[28,29]. However, PI(3,4,5)P3 production, as revealed by P-Akt accumulation, initially is detectable at the tips of all neurites and persists there after Rap1B is restricted to a single process. Therefore, it is likely that PI(3,4,5)P3 production depends on a positive feedback involving activation of PI3K by Rap1B or Cdc42[24,25,28,29]. As Rap1B is lost from neurites, PI(3,4,5)P3 synthesis and P-Akt decline at a slower rate than the removal of the GTPases. Notably, expression of active Rap1B and Cdc42 did not result in the extension of multiple axons when PI3K was inhibited, suggesting that PI(3,4,5)P3 production is involved in the induction of supernumerary axons.

Rap1B belongs to the Ras subfamily of GTPases and fulfills several essential functions in multicellular organisms. Despite extensive studies of Rap1 GTPases, their physiological function is still not fully understood[28,29]. Initially, Rap1 was thought to act mainly as an antagonist of Ras. More recently, a Ras-independent function to regulate cell adhesion has emerged. Our results provide evidence for a novel function of Rap1B in the establishment of neuronal polarity and show that Rap1 may serve a general role as a positional signal and organize cell architecture. The requirement for Rap1B in the establishment of polarity is similar to the function of the Rap1 homolog Bud1p/Rsr1p in determining the position of the incipient bud site in *Saccharomyces cervisiae*[30–32]. However, unlike its homolog Bud1p/Rsr1p, Rap1B is absolutely required for polarization, and its elimination prevents axon formation. The similarity between the pathway that selects a single neurite to become an axon and that which determines the localization of the bud site in *S. cerevisiae* suggests that Rap1B and Cdc42 represent highly conserved modules regulating the establishment of cell polarity.

In young neurons, endogenous Cdc42 is present in most neurites, whereas in stage-3 neurons, it is found exclusively in the axon. The redistribution of Cdc42 is slower than that of Rap1B, which indicates that it may be lost from Rap1B-negative processes while retained in the growth cone of the future axon. The loss of axons after knockdown of Cdc42 by RNAi confirms that Cdc42 is essential for neuronal polarization. The establishment of polarity in *S. cerevisiae* requires a positive feedback loop that, starting from a uniform distribution, results in the local concentration of Cdc42p and requires the cycling of Cdc42p between the GTP- and GDP-bound forms[16,32]. Deletion of *cdc42* results in isotropic growth and the inability to form a daughter bud[32]. A hyperactivated, fast-cycling Cdc42p mutant, however, forms multiple buds at random positions, indicating that Cdc42p is required not only for bud emergence and maintenance of growth, but also to prevent the formation of more than one bud[15]. The ability of Cdc42L28, but not Cdc42V12, to induce supernumerary axons implies that, like in the budding yeast, active Cdc42 is restricted to a single neurite in a process that requires GTPase cycling (**Supplementary Fig. 6** online).

In contrast to Rap1B and Cdc42, which are required to select a single neurite to become the axon and extend rapidly, Rho and Rac antagonistically regulate the extension of both axons and minor neu-

rites. Increasing Rho or decreasing Rac activity resulted in a reduction of neurite length, whereas decreasing Rho or increasing Rac activity had the opposite effect, as already observed in other types of neurons[1,17–22,33]. Consistent with previous results[23], expression of RhoAV14 led to a reduction in the number of axons and minor neurites. However, we do not think that the effect of RhoAV14 on the number of axons per cell indicates a role in the establishment of polarity. RhoAV14 affected axons and neurites irrespective of their identity, whereas dominant-negative RhoAN19 had no significant effect on the number of neurites.

Par3, Par6 and aPKCs, as well as their homologs in other organisms, form an evolutionarily conserved complex that regulates cell polarity in epithelial cells, embryonic blastomeres, neuronal precursors and neurons[6,8,34]. In neurons, Par3 is present in multiple neurites of nonpolarized stage-2 neurons and becomes restricted to the axon in stage-3 neurons[7,8]. The localization of Rap1B to a single neurite precedes the restriction of Par3 and active aPKC to the axon. Thus, the distribution of Rap1B is the earliest molecular asymmetry described so far in neurons. Supernumerary axons induced by Rap1B mutants and cytochalasin D were all positive for Par3 and aPKC, indicating that Par6 and aPKC act downstream of Rap1B. Active Rac or Cdc42 stimulate PKC activity through binding to the Par6/aPKC complex[6,9–11]. One function of Rap1B may be to localize activation of aPKC to the tip of the prospective axon by regulating Cdc42 and the Par complex. Pharmacological inhibition of aPKC prevents neuronal polarization. Localized PKCζ activity induced by the recruitment of the Par6/PKCζ complex is also required for all aspects of cell polarity in astrocytes[9]. However, overexpression of Par6c resulted in a block of axon formation, suggesting that the Par complex has a more complex role. The available data[6–8,10] indicate that Cdc42 and the Par complex are mutually dependent for their function or act in parallel pathways. Thus, the precise function of the Par complex in the establishment of neuronal polarity and its relationship to Cdc42 remain to be elucidated.

METHODS

Neuronal culture and transfection. The cDNA for mouse Par6c was amplified by PCR from postnatal day 0 (P0) brain cDNA and cloned into pEGFP-N1 (Clontech). Rap1BV1, Cdc42L28, GFP-RhoAV14, GFP-RhoAN19, GFP-Rac1V12, GFP-Rac1N17 and mycPar3 were kindly provided by S.J. Shattil (The Scripps Research Institute), R.A. Cerione (Cornell University), M.R. Philips (New York University School of Medicine) and I.G. Macara (University of Virginia), respectively. Rap1BV12 and Cdc42L28 were cloned into pEGFP-N1 that was also used as control vector. The pSHAG-1 vector[26], used for RNA interference, was kindly provided by G.J. Hannon (Cold Spring Harbor Laboratory). Cultures of dissociated hippocampal neurons were prepared as described previously[8,35]. Briefly, hippocampi from embryonic day 18 (E18) rats were dissected, incubated with papain for 20 min at 37°C and dissociated by pipetting in plating medium (Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum, 0.5 mM glutamine and 100 U/ml penicillin/streptomycin; Invitrogen). Neurons were plated onto glass coverslips coated with poly-ornithine (Sigma) at a density of 200,000 cells per coverslip and cultured at 37°C and 5% CO_2. After neurons attached to the substrate (around 6 h after plating) the medium was changed to Neurobasal medium with B27 supplement, 0.5 mM glutamine and 100 U/ml penicillin/streptomycin (Invitrogen). Then, 12 h after plating, neurons were transfected using Lipofectamine 2000 (Invitrogen) following published protocols[36]. After 60 h in culture, neurons were fixed with 4% paraformaldehyde and 15% sucrose in phosphate-buffered saline (PBS) for 20 min at 4°C and processed for immunohistochemistry.

RNAi. Knockdown of Rap1B and Cdc42 was achieved by RNAi following published methods using the pSHAG-1 vector[26]. The following oligonucleotides were used: Rap1B: TTCTGACCTT GTTCCTTGGG GACAACTCGA AGCTTGGGGT TGTTCCTAAG GAACAGGGTC AGAACCTTTT TTT and ATCAAAAAAA GGTTCTGACC CTGTTCCTTA GGAACAACCC CAAGCTTCGA GTTGTCCCCA AGGAACAAGG TCAGAACG; Cdc42: TCTGTGGATA ACTTAACGGT CGTAGTCTGA AGCTTGAGAC TACGACTGTT AAGTTGTCTA CGGACAGTTT TTT and GATCAAAAAA CTGTCCGTAG ACAACTTAAC AGTCGTAGTC TCAAGCTTCA GACTACGACC GTTAAGTTATC CACAGACG.

Immunofluorescence. The Tau-1 and anti-MAP2 antibodies (Tau-1 and anti-MAP2) were obtained from Chemicon; anti-P-aPKC (cat. no. 9378) and anti-P-Akt (cat. no. 9277) were from Cell Signaling Technology; rabbit anti-Par-3 was from Upstate Biotechnology; monoclonal anti-Rap1 and anti-Cdc42 were from BD Biosciences; polyclonal anti-Cdc42 was from Santa Cruz Biotechnology; Alexa-594, Alexa-488, and Alexa-350-conjugated secondary antibodies and phalloidin-rhodamine were from Molecular Probes. The antibody to Rap1 recognizes both Rap1A and Rap1B (95% amino acid sequence identity), but not Rap2. Rap1 immunoreactivity in neurons was almost completely eliminated by the knockdown of Rap1B by RNAi (**Fig. 4a**), showing that hippocampal neurons express only the Rap1B protein. Cytochalasin D, LY294002 and taxol (Calbiochem) were directly added to neuronal culture medium 6–8 h after plating to a final concentration of 1 μM, 100 μM and 10 μg/ml, respectively, as described previously[5,8,13]. Neuronal morphology was analyzed using a Zeiss Axiophot microscope equipped with a Hamamatsu CCD camera, and analyzed using WASABI software (Hamamatsu) and Adobe Photoshop. The stage of neuronal differentiation was determined following published criteria[4]. Processes that were at least twice as long as the other processes and showed Tau-1 immunoreactivity in their distal segments but no MAP2 staining were counted as axons; MAP2-positive neurites longer than one cell diameter were counted as minor neurites[37]. The development of neuronal polarity was analyzed by determining the number of axons and minor neurites formed per cell. Neurons that did not extend an axon were classified as unpolarized. The Student's *t*-test was used to test statistical significance.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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