

# Requirement of dendritic Akt degradation by the ubiquitin–proteasome system for neuronal polarity

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**A**symmetric distributions of activities of the protein kinases Akt and glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) are critical for the formation of neuronal polarity. However, the mechanisms underlying polarized regulation of this pathway remain unclear. In this study, we report that the instability of Akt regulated by the ubiquitin–proteasome system (UPS) is required for neuron polarity. Preferential distribution in the axons was

observed for Akt but not for its target GSK-3 $\beta$ . A photo-activatable GFP fused to Akt revealed the preferential instability of Akt in dendrites. Akt but not p110 or GSK-3 $\beta$  was ubiquitinated. Suppressing the UPS led to the symmetric distribution of Akt and the formation of multiple axons. These results indicate that local protein degradation mediated by the UPS is important in determining neuronal polarity.

## Introduction

The creation of a precise morphology in which a neuron generates multiple dendrites and one long axon is essential for the formation of neuronal circuitry. The establishment of axon–dendrite polarity is an important feature of neurons (Craig and Banker, 1994). The primary cultured hippocampal neuron is an established model for the characterization of neuronal polarity (Dotti et al., 1988). Cultured hippocampal neurons extend several minor neurites after plating, which remain indistinguishable in stages 1 and 2, after which one of them develops into an axon at stage 3. In contrast, the others develop into dendrites (Dotti et al., 1988; Craig and Banker, 1994). Local activity of the phosphatidylinositol (PI) 3-kinase–Akt–glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) pathway is required for both the establishment and maintenance of neuron polarity in these neurons (Shi et al., 2003, 2004; Arimura et al., 2004; Menager et al., 2004; Jiang et al., 2005; Yoshimura et al., 2005). A recent study suggested that polarized growth occurs before neurites are formed (de Anda et al., 2005). PI 3-kinase is activated at the tip of the newly specified axon to stimulate Akt kinase (Shi et al., 2003; Menager et al., 2004). Activated Akt then phosphorylates and inactivates GSK-3 $\beta$ , turning neurites to axons (Shi et al., 2003, 2004;

Arimura et al., 2004; Menager et al., 2004; Jiang et al., 2005; Yoshimura et al., 2005). Furthermore, active Akt is found in the soma and axon terminus but not in other neurites, and the expression of constitutively active Akt leads to the formation of multi-axons (Shi et al., 2003; Jiang et al., 2005). Therefore, activation of Akt in the axon is critical for axon formation (Jiang et al., 2005). However, the mechanism through which the asymmetrical activation of Akt is established remains unknown.

Protein degradation by the ubiquitin (Ub)–proteasome system (UPS) is important for the regulation of many cellular functions, including cell cycle, growth, and polarity (Obin et al., 1999; Wang et al., 2003; Hegde, 2004; Bryan et al., 2005; Ozdamar et al., 2005). In response to various stimuli, the UPS, which involves the sequential action of Ub-activating enzymes (E1), Ub-conjugating enzymes (E2), and Ub ligases (E3), can be activated, resulting in the conjugation of Ub to the lysine residues of proteins (Glickman and Ciechanover, 2002; Hegde, 2004). Those proteins tagged with poly-Ub are then degraded by the proteasome complex.

Because Akt stability in different types of cells is regulated by the UPS (Kim and Feldman, 2002; Martin et al., 2002; Adachi et al., 2003; Riesterer et al., 2004; Rusinol et al., 2004), it is possible that the asymmetrical activation of Akt is caused by its selective distribution mediated by the UPS. In this study, we have examined the role of the UPS in neuronal polarity and found that selective degradation of Akt by the UPS in dendrites is required for generating neuronal polarity.

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Abbreviations used in this paper: GSK-3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; myr, myristoyl; PA, photoactive; PI, phosphatidylinositol; Ub, ubiquitin; UPS, Ub–proteasome system; WT, wild type.

The online version of this article contains supplemental material.

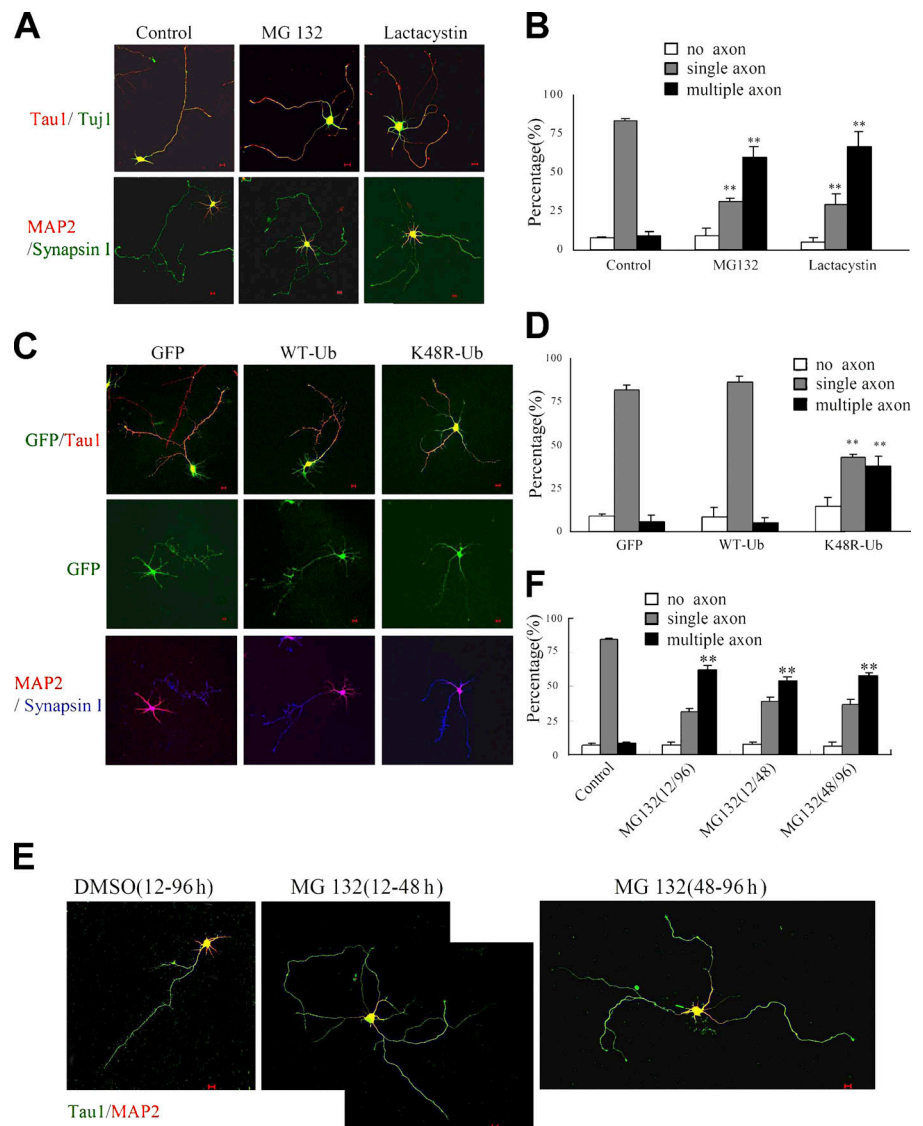
## Results

### The UPS is required for both the establishment and maintenance of neuronal polarity

To test whether the UPS is involved in neuronal polarity, we first examined the effect of UPS inhibition on axon–dendrite specification in cultured hippocampal neurons. As shown in Fig. 1 (A and B), UPS inhibition by MG132 and lactacystin, two agents known to inhibit the proteasome, led to the loss of neuron polarity and formation of multiple axons. The percentages of neurons with no axon, a single axon, or multiple axons were  $7.33 \pm 1.15$ ,  $83.33 \pm 1.15$ , and  $9.33 \pm 2.31\%$ , respectively, in neurons treated with DMSO, whereas the percentages were  $9.00 \pm 4.58$ ,  $31.33 \pm 2.31$ , and  $59.67 \pm 6.81\%$ , respectively, in neurons treated with MG132 ( $n = 100$ ; three experiments; Fig. 1 B). Similarly, lactacystin dramatically reduced the number of neurons with a single axon and increased the number of neurons with multiple axons (Fig. 1 B). Furthermore, expressing K48R-Ub, a dominant-negative form of Ub known to

inhibit the UPS (Antonelli et al., 1999), markedly reduced the number of neurons with a single axon and increased the number of neurons with multiple axons, whereas expressing a control vector or the wild-type (WT) Ub did not affect neuron polarity ( $n = 100$ ; three experiments; Fig. 1, C and D). UPS inhibition also increased the number of axons and extended or maintained the mean length of axons (Fig. S1, A–D; available at <http://www.jcb.org/cgi/content/full/jcb.200511028/DC1>). These results suggest that the UPS is critical for the formation of neuronal polarity.

To test whether the UPS is required for both the establishment and maintenance of neuronal polarity, we treated neurons with MG132 before (from 12 to 48 h after plating) or after (from 48 to 96 h) the establishment of neuronal polarity (most of the neurons have established polarity 48 h after plating; Fig. S1 E). As shown in Fig. 1 ( $n = 100$ ; three experiments; E and F), treatment with MG132 before or after the establishment of neuronal polarity dramatically increased the number of neurons with multiple axons. These results indicate that the UPS is also required for maintenance of neuronal polarity. To further support



**Figure 1. UPS is required for neuronal polarity.** (A) 12 h after plating, neurons treated with DMSO (control), MG132, and lactacystin for 84 h were stained with antibodies against Tuj1 (neuron marker; green), Tau1 (axon marker; red; top), MAP2 (dendrite marker; red) and synapsin I (axon marker; green; bottom). (B) Quantification of the effects of MG132 and lactacystin on neuronal polarity. (C) Neurons transfected with GFP and pcDNA3, wild-type (WT), or K48R-Ub before plating were fixed at the fourth day in culture and stained with the indicated antibodies. (D) Quantification of polarity defects of the neurons transfected with the indicated constructs before plating. (E) Neurons treated with MG132 from 12 to 48 or from 48 to 96 h after plating were fixed at the 96 h in culture and stained for MAP2 (red) or Tau1 (green). (F) Quantification of the effects of MG132 on neuron polarity in different periods. 0.15  $\mu$ M MG132 and 0.3  $\mu$ M lactacystin. \*\*,  $P < 0.001$  versus control. Error bars represent SEM. Bars, 10  $\mu$ m.

this notion, we transfected neurons with WT-Ub or K48R-Ub 48 h after plating. The expression of K48R-Ub but not WT-Ub led to the formation of multiple axons in the neurons in which the polarity had been previously established ( $n = 100$ ; three experiments; Fig. S1, F and G). Although these axons were Tau1 and synapsin I positive, it is not clear whether they were functional. We then examined the uptake and release of FM4-64 dye, which is an assay used to show the functionality of axons in synaptic vesicle recycling (Jiang et al., 2005). As shown in Fig. S1 (H and I), FM4-64 dye was taken up only by axon termini but not by dendrites in the neurons treated with DMSO or transfected with WT-Ub. Moreover, in the presence of 45 mM KCl, the dye was taken up by the axons formed after treatment with MG132 or after transfection with K48R-Ub. The dye was then released in the presence of 90 mM KCl (Fig. S1, H and I). Therefore, these axons were functional in synaptic vesicle recycling. Collectively, these results suggest that UPS was required for both the establishment and maintenance of neuronal polarity.

#### Akt but not p110 or GSK-3 $\beta$ is degraded by the UPS in neurons

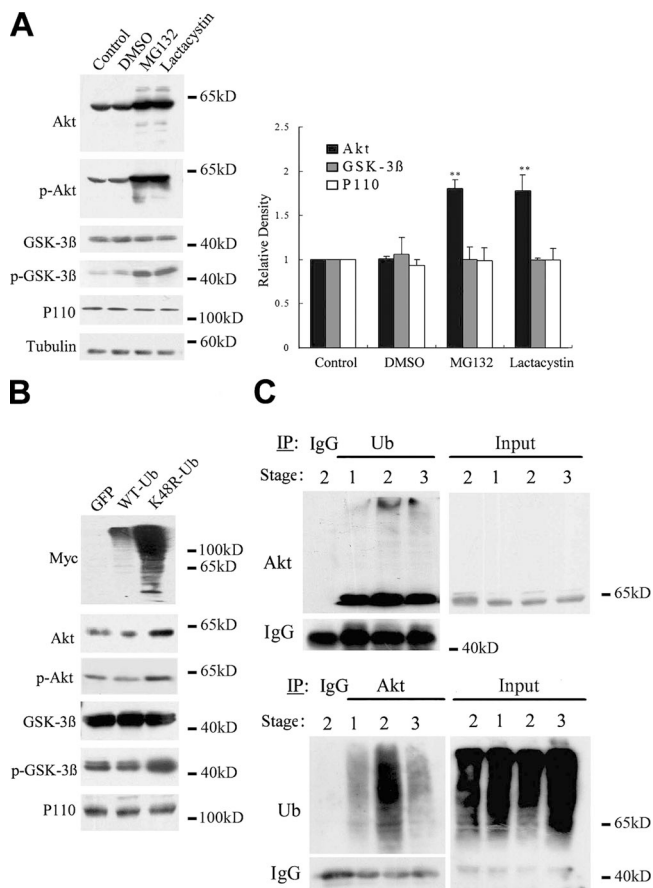
To identify the molecules degraded by the UPS during the formation of neuronal polarity, we examined the protein levels for P110 (a subunit of PI 3-kinase), Akt, the phosphorylated form of Akt (p-Akt), GSK-3 $\beta$ , and the phosphorylated form of GSK-3 $\beta$  (p-GSK-3 $\beta$ ) in the neurons treated with MG132 and lactacystin from 12 to 48 h after plating. As shown in Fig. 2 A, protein levels of Akt in the treated cultures were greatly increased as compared with those in the control. Similarly, in the neurons treated with MG132 and lactacystin, the levels of p-Akt and p-GSK-3 $\beta$  were also markedly increased as compared with those in control cultures (Fig. 2 A). However, the two inhibitors did not affect the protein levels of P110 or GSK-3 $\beta$  (Fig. 2 A). Consistent with these results, the protein levels and activity of Akt but not those of P110 and GSK-3 $\beta$  were greatly enhanced by transfection with K48R-Ub (Fig. 2 B). The aforementioned results point to the possibility that Akt is degraded by the UPS during the formation of neuronal polarity.

It has been known that ubiquitination of a protein is a critical step for its degradation by the UPS. We then examined whether Akt was ubiquitinated in the stage 1–3 neurons. As shown in Fig. 2 C, Akt was found in complexes precipitated by the antibody against Ub, and the levels of Akt in these complexes were increased in stage 2 neurons compared with those in stage 1 and 3 neurons (Fig. 2 C). In contrast, P110 and GSK-3 $\beta$  were not ubiquitinated in stages 1–3 (Fig. S1 J). Furthermore, treatment with MG132 or lactacystin from 12 to 24 h after plating greatly increased the level of the ubiquitinated form of Akt (Fig. S1 K). These results indicate that Akt was ubiquitinated and that the level of the ubiquitinated form of Akt was increased in stage 2 neurons and by UPS inhibition. This possibility was further supported by the reciprocal immunoprecipitation experiments in which complexes that were immunoprecipitated by anti-Akt antibody were Western blotted with anti-Ub antibody (Fig. 2 C). Together, these results suggest that Akt but not P110 and GSK-3 $\beta$  is

preferentially degraded by UPS during the formation of neuronal polarity.

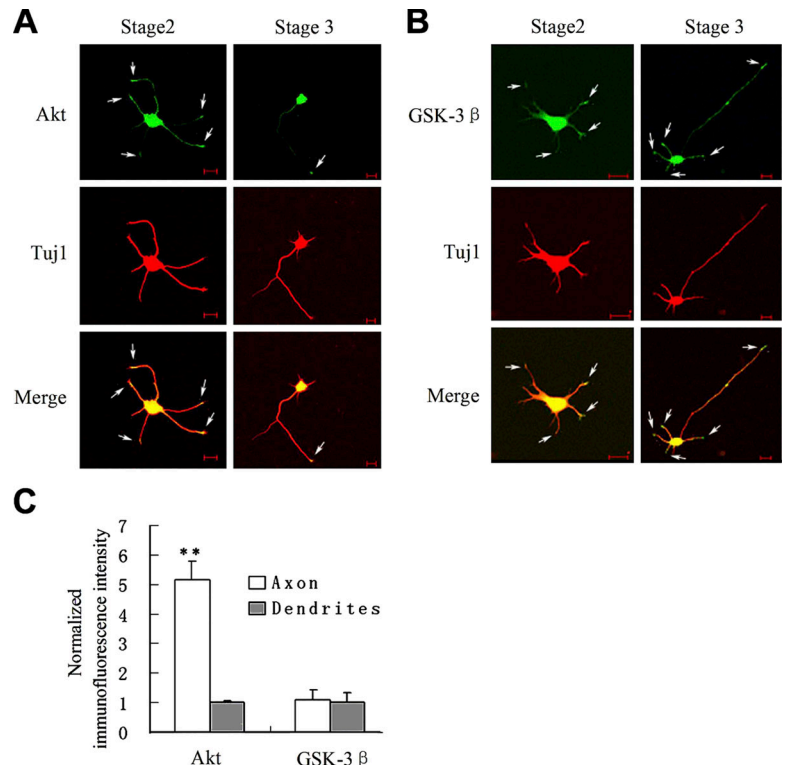
#### Akt is present in axon tips but not in dendrites of stage 3 neurons

We then examined the localization of Akt and GSK-3 $\beta$  by immunostaining. Akt was present in both the soma and multiple minor processes of stage 2 neurons. In stage 3 neurons, however, Akt was preferentially found in the soma and axonal tips (Fig. 3 A). In contrast, GSK-3 $\beta$  was found in all neurites in both stage 2 and 3 neurons (Fig. 3 B). Statistical analysis of the density of immunofluorescence obtained through scanning the tips of >90 neurons in stage 3 revealed that the density (normalized by the mean density in dendritic tips) of Akt in axonal tips was five times higher than that in dendritic tips, whereas the density of GSK-3 $\beta$  between axonal and dendritic tips was not different (Fig. 3 C). Moreover, the densities of p-Akt and p-GSK-3 $\beta$  in



**Figure 2. Akt is degraded by UPS.** (A) Total cell lysates isolated from the neurons treated with DMSO, MG132, and lactacystin from 12 h after plating were collected at 48 h in culture and Western blotted with indicated antibodies. Quantification of the densities of Akt, GSK-3 $\beta$ , and P110 are shown in the right panel. Data represent the mean  $\pm$  SEM (error bars) of three independent experiments. \*\*,  $P < 0.001$  versus control. (B) Western blot of total cell lysates at 2 d in culture isolated from the neurons transfected with GFP, myc-tagged WT-Ub, or K48R-Ub before plating. (C) Stage-dependent ubiquitination of Akt. Total cell lysates isolated from neurons growing in stages 1 (6 h in culture), 2 (18 h in culture) and 3 (48 h in culture) were immunoprecipitated with anti-Ub (top) or anti-Akt (bottom) antibodies, and the immunocomplexes were Western blotted with antibodies against Akt or Ub. IgG served as a control.

Figure 3. **Asymmetric distribution of Akt at stage 3.** (A and B) Immunostaining of neurons at stages 2 and 3 with anti-Akt (A) and -GSK-3 $\beta$  (B) antibodies. Arrows indicate the presence of Akt or GSK-3 $\beta$  protein in the neurite tips. (C) Quantification of the normalized fluorescence intensity of Akt and GSK-3 $\beta$  in axonal and dendritic tips of neurons at stage 3 ( $n = 90$ ). \*\*,  $P < 0.001$  versus dendrites. Error bars represent SEM. Bars, 10  $\mu$ m.



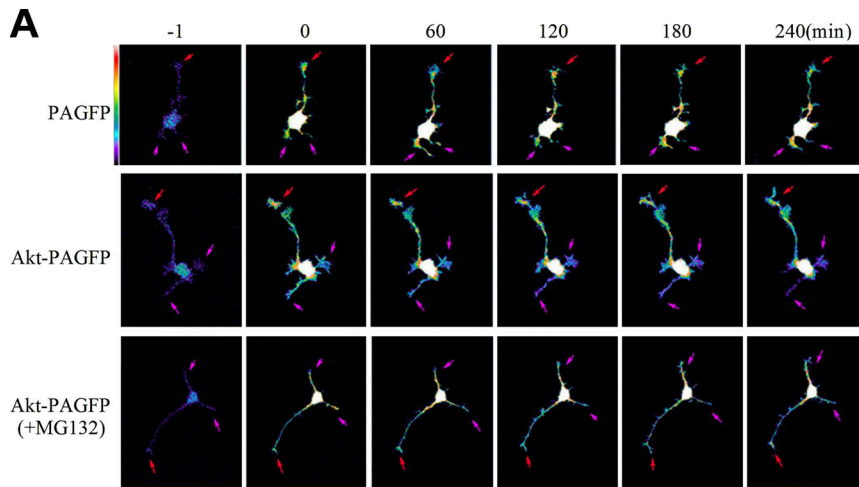
the axonal tips were four to six times higher than those in dendritic tips (Fig. S2, A–C; available at <http://www.jcb.org/cgi/content/full/jcb.200511028/DC1>). Therefore, the distribution of Akt was changed from symmetric to asymmetric as neurons progressed from stage 2 to 3. This shift from a symmetric to asymmetric distribution of Akt points to the possibility that Akt was selectively degraded in the dendrites.

#### Dendritic Akt is degraded

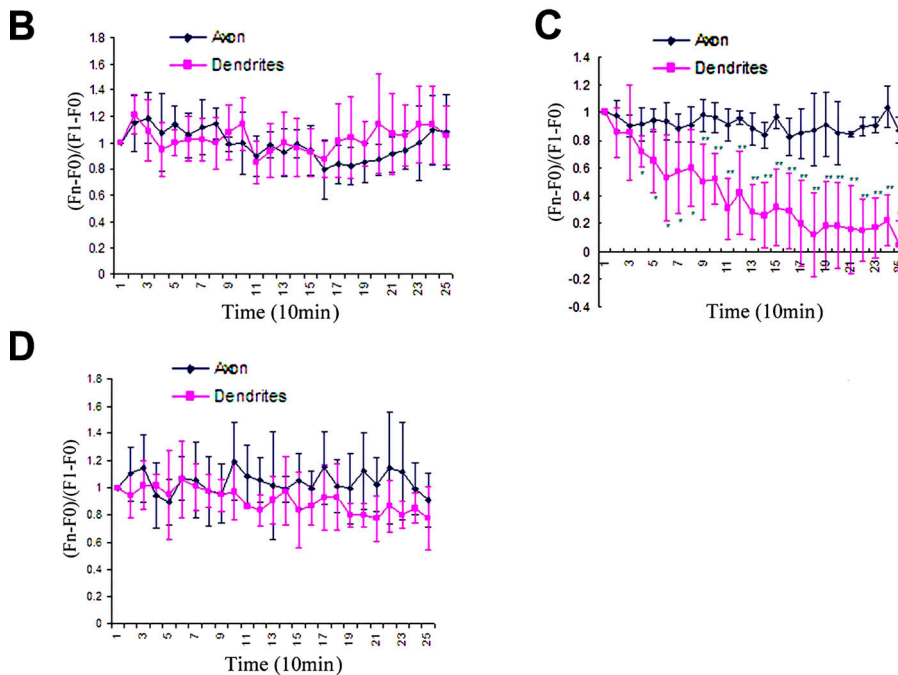
To directly observe whether Akt is degraded in dendrites, we transfected neurons with Akt–photoactive (PA) GFP, which encodes Akt fused to a PAGFP (Fig. S2 D; Patterson and Lippincott-Schwartz, 2002). The intensities of fluorescence in neurons transfected with either Akt-PAGFP or PAGFP were monitored for 4 h after photoactivation, which was initiated 36 h after transfection. The relative intensities of fluorescence between axonal and dendritic tips in the neurons expressing PAGFP were similar ( $P > 0.05$ ;  $n = 10$ ; Fig. 4, A and B). In contrast, in the neurons expressing Akt-PAGFP, the fluorescence intensity in the dendrites was much weaker than that in axons (Fig. S2 E), and the relative intensity of fluorescence in dendritic tips after photoactivation gradually decreased compared with that in axonal termini ( $n = 10$ ; Fig. 4, A and C). At the end of observation, the relative intensity of the fluorescence for Akt-PAGFP in axonal tips was 21 times higher than that detected in dendritic tips ( $n = 10$ ; Fig. 4, A and C). Moreover, treatment with MG132 prevented the decrease in the intensity of Akt-PAGFP in dendrites ( $n = 10$ ; Fig. 4, A and D). Together with the immunostaining results, these findings indicate that preferential degradation of dendritic Akt was mediated by the UPS.

#### UPS inhibition inhibits maintenance of the asymmetrical distribution of Akt/p-Akt in stage 3 neurons

We then examined whether UPS inhibition affects Akt/p-Akt localization. As shown in Fig. 5 A and Fig. S3 A (available at <http://www.jcb.org/cgi/content/full/jcb.200511028/DC1>), treatment with MG132 and lactacystin led to the presence of Akt/p-Akt in all neurites and consequently converted these neurites into axons. The effects of these inhibitors on the redistribution of Akt/p-Akt was further confirmed by transfection experiments. Expressing K48R-Ub resulted in the presence of p-Akt/Akt in most of the neurites, whereas expressing WT-Ub did not affect the distribution of these molecules (Fig. 5 B and Fig. S3 B). In contrast, the distribution of GSK-3 $\beta$  was not affected by MG132 or by expressing K48R-Ub to inhibit the UPS (Fig. S3, C and E). However, inhibiting the UPS resulted in the expression of p-GSK-3 $\beta$  in the terminals of most neurites (Fig. S3, D and F). Because p-GSK-3 $\beta$  is a downstream target of p-Akt, these results suggested that the redistribution of p-GSK-3 $\beta$  from an asymmetric to symmetric distribution was caused by a similar change in the distribution of p-Akt after inhibition of the UPS. To examine whether local inhibition of the UPS affects neuronal polarity, we applied MG132 to a neurite in a stage 2 neuron and stained the neuron with antibodies against Tau1 or Akt. As shown in Fig. S3 G, the neurite treated with MG132 for 4 h was enriched in Akt staining and became longer and Tau1 positive, indicating that this neurite was turned into an axon in response to local inhibition of the UPS. Therefore, UPS inhibition prevented neurite terminals from losing p-Akt/Akt and p-GSK-3 $\beta$ , resulting in the formation of multiple axons.



**Figure 4. Dendritic Akt is selectively degraded.** (A) Time-lapse images of neurons transfected 36 h with PAGFP and Akt-PAGFP and displayed at 60-min intervals after photoactivation (photoactivation at 0 min). Dendritic reduction in Akt-PAGFP (purple arrows) was observed 30 min after photoactivation and persisted until the end of observation (240 min). Red arrows indicate axons, and purple arrows indicate dendrites. (B–D) Quantification of the normalized intensity of PAGFP (B), Akt-PAGFP (C), and Akt-PAGFP + MG132 (D). Every unit on the x axis is 10 min. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus axons. Error bars represent SEM.

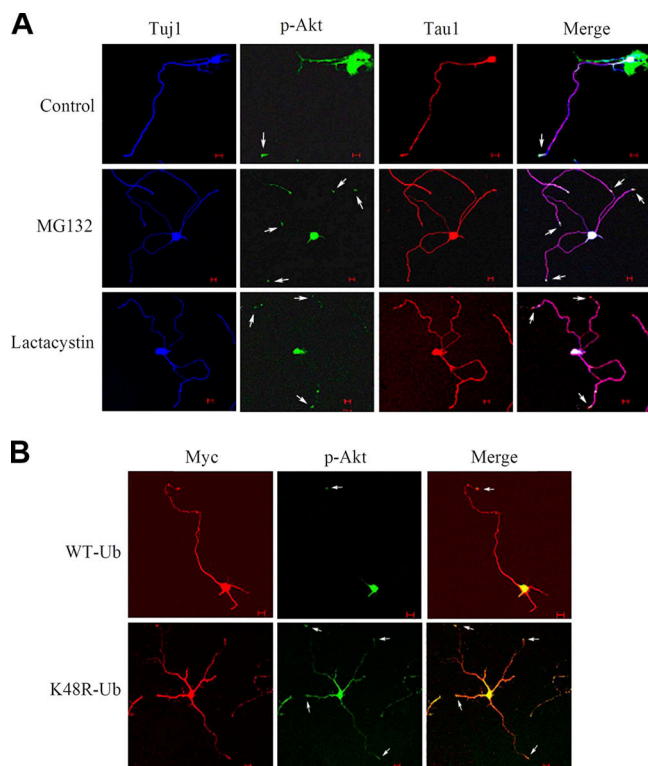


### Inhibition of Akt activity results in its degradation

It has been known that protein degradation can be regulated by posttranslational modifications (Glickman and Ciechanover, 2002; Hegde, 2004). The phosphorylation state of Akt affects its stability in different cell types (Kim and Feldman, 2002; Martin et al., 2002; Adachi et al., 2003; Riesterer et al., 2004; Rusinol et al., 2004). To test whether Akt phosphorylation regulates its degradation by the UPS in hippocampal neurons, we examined the ubiquitination state of Akt in stage 2 neurons in response to the treatment with wortmannin, a PI 3-kinase inhibitor also known to suppress Akt phosphorylation. Wortmannin treatment greatly increased the level of ubiquitinated Akt (Fig. 6, A and B). The neurons treated extended only several short processes (Fig. 6 C), which is consistent with a previous study (Menager et al., 2004). Moreover, the protein levels of Akt (Fig. 6 C) and p-Akt (Fig. S4 A, available at <http://www.jcb.org/cgi/content/full/jcb.200511028/DC1>) in all neurite terminals

were reduced to undetectable levels. Similarly, p-GSK-3 $\beta$  was absent from all neurites after the application of wortmannin (Fig. S4 A). However, the distribution of GSK-3 $\beta$  was not affected (Fig. 6 C). Treatment with MG132 reversed the wortmannin-induced Akt loss from neurite terminals, but this treatment reversed neither the loss of p-Akt from neurite terminals (Fig. 6 C and Fig. S4 A) nor the effect of wortmannin application on polarity (Fig. S4 B), indicating that Akt activity is required for axon formation. Together, these results suggest that the inhibition of Akt phosphorylation results in its degradation mediated by the UPS.

We then examined whether the effect of wortmannin on Akt/p-Akt was caused by its action on neurons at stage 2. As shown in Fig. S4 (C and D), treatment of stage 2 neurons with wortmannin for 2 h greatly increased the level of ubiquitinated Akt and led to the absence of Akt from all neurite terminals. Therefore, the inactivation of Akt increased its degradation in the neurons, leading to the inhibition of neurite growth.



**Figure 5. UPS inhibition disrupts the asymmetric distribution of p-Akt.** (A) Neurons treated with MG132 and lactacystin from 12 h after plating were fixed at 96 h in culture and stained for Tuj1, Tau1, and p-Akt. (B) Neurons transfected with WT-Ub and K48R-Ub before plating were fixed at 96 h in culture and stained with anti-myc and -p-Akt antibodies. Arrows indicate the presence of p-Akt in axon tips. Bars, 10  $\mu$ m.

This possibility was further supported by the observation that local inhibition of PI 3-kinase/Akt affected neurite growth. As shown in Fig. S4 (E and F), local application of wortmannin to a neurite of a stage 2 neuron reduced Akt protein levels in this neurite and, consequently, inhibited its growth.

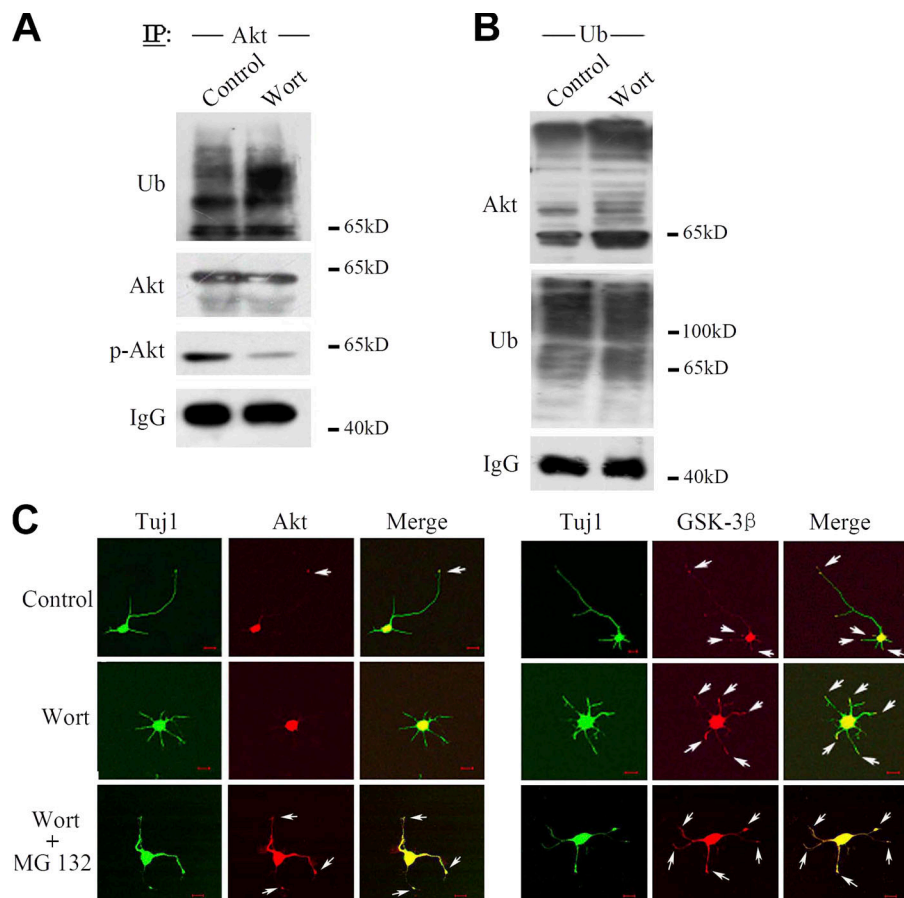
To directly study the effect of inhibiting Akt phosphorylation on its degradation in live cells, we monitored the changes in the fluorescence intensity of Akt-PAGFP in neurons in response to wortmannin. As shown in Fig. 7 (A and B), the relative fluorescence intensity found in the axons between the neurons treated with wortmannin and those treated with DMSO was similar ( $P > 0.05$ ;  $n = 10$ ). In contrast, wortmannin treatment in the first 80 min after photoactivation greatly reduced the relative intensity of fluorescence in dendrites (Fig. 7, A and C). After 2 h, the relative intensity of fluorescence in dendritic tips in both control and wortmannin-treated groups was  $\sim 20\%$  of the intensity found immediately after photoactivation. Immunostaining with Akt/p-Akt antibodies further revealed that treatment with wortmannin for 2 h markedly decreased the level of p-Akt in the neuron but did not affect the levels of Akt in axon terminals (Fig. S4 G). Because wortmannin inhibits PI 3-kinase to suppress Akt phosphorylation, our results suggested the possibility that the inhibition of Akt phosphorylation accelerates its degradation in dendrites but does not affect its protein level in axons. Together, our results suggest that the inactive form of Akt was preferentially degraded in dendrites.

### Expressing constitutively active Akt destroys the polarized distribution of Akt and disrupts neuronal polarity

To test whether the activated form of Akt is resistant to the degradation mediated by the UPS (Fig. S5 A, available at <http://www.jcb.org/cgi/content/full/jcb.200511028/DC1>), we transfected the neurons with WT Akt and myristoyl (myr)-Akt, a constitutively active form of Akt (Kohn et al., 1996; Cong et al., 1997), and examined the ubiquitination of Akt 2 d later. As shown in Fig. 8 A, WT Akt was highly ubiquitinated, whereas myr-Akt was barely ubiquitinated. These results further suggested that the active form of Akt in neurons is resistant to degradation mediated by the UPS. We then examined whether the expression of myr-Akt affects the polarized distribution of Akt to induce the formation of multiple axons. Neurons transfected with GFP-pcDNA3 or GFP-myristoyl-Akt were stained with an antibody against Akt 4 d after plating (Fig. S5 B). In the neurons expressing GFP, Akt was found mostly in the soma and axon terminals. In contrast, in the neurons expressing myr-Akt, Akt was found in the entire cell (Fig. S5 B). Moreover, the percentages of neurons with no axon, a single axon, and multiple axons were  $4.67 \pm 2.31$ ,  $81.67 \pm 2.89$ , and  $13.67 \pm 0.58\%$ , respectively, in the neurons transfected with GFP. In contrast, the percentages were  $5.33 \pm 3.06$ ,  $55.33 \pm 4.58$ , and  $39.33 \pm 4.51\%$ , respectively, in the neurons transfected with WT Akt and  $5.67 \pm 2.08$ ,  $26.33 \pm 7.77$ , and  $67.67 \pm 10.21\%$ , respectively, in neurons treated with myr-Akt ( $n = 100$ ; three experiments; Fig. 8, B–D). Additionally, myr-Akt was more potent than WT Akt to induce the formation of multiple axons in the neurons after the initial establishment of polarity ( $n = 100$ ; three experiments; Fig. 8, E and F). These results demonstrated that the expression of constitutively active Akt eliminated the polarized distribution of Akt and disrupted neuronal polarity.

### Discussion

The PI 3-kinase pathway plays instructive roles in cell polarization and migration during chemotaxis (Funamoto et al., 2002; Iijima et al., 2004) and also regulates axon–dendrite specification in hippocampal neurons (Shi et al., 2003; Menager et al., 2004). Activation of PI 3-kinase at the terminals of neurites triggers a sequential response, including the stimulation of Akt, GSK-3 $\beta$ , and CRMP-2, leading to the formation of neuronal polarity (Shi et al., 2003, 2004; Jiang et al., 2005; Yoshimura et al., 2005). We report that Akt degradation within dendrites by the UPS is required for both the establishment and maintenance of neuronal polarity. Specifically, local instability of Akt/p-Akt in dendrites mediated by the UPS led to axon–dendrite polarity. Several findings support this conclusion. First, Akt was increasingly ubiquitinated in neurons grown from stage 1 to 2, whereas p110 and GSK-3 $\beta$  were not ubiquitinated (Fig. 2 C and Fig. S1 J). Second, Akt was present in the axon but not in the dendrites of stage 3 neurons (Fig. 3). Third, UPS inhibition restored the presence of Akt in all neurites and destroyed neuronal polarity (Fig. S3). Lastly, disruption of polarized Akt distribution by the expression of constitutively active Akt prevented the formation of neuronal polarity (Fig. 8). Thus, the preferential localization

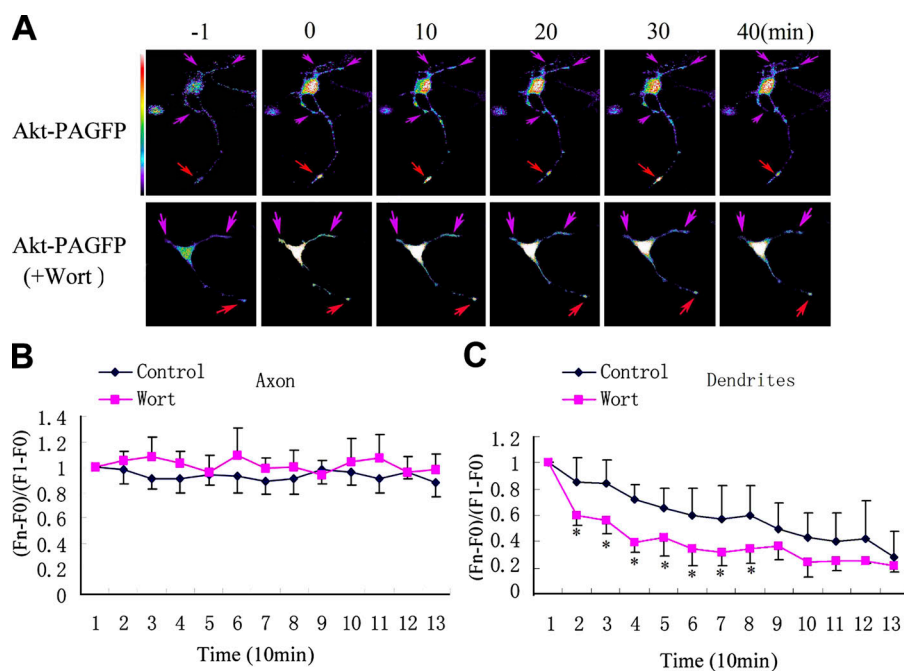


**Figure 6. Inhibiting Akt activity increases its ubiquitination and leads to its absence from neurites.** (A and B) Total cell lysates isolated from neurons treated with wortmannin from 12 h after plating were collected at 48 h in culture and immunoprecipitated with anti-Akt (A) or -Ub (B) antibodies, and the immunocomplexes were Western blotted with antibodies against Ub or Akt. (C) Effect of wortmannin (Wort) treatment 12–48 h after plating in the presence or absence of MG132 on the distribution of Akt (left) and GSK-3 $\beta$  (right). 200 nM wortmannin and 0.15  $\mu$ M MG132. Arrows indicate the presence of Akt or GSK-3 $\beta$  protein in the neurite tips. Bars, 10  $\mu$ m.

of Akt to the axon controls the local activity of the Akt–GSK-3 $\beta$  pathway, leading to the formation of neuronal polarity.

It has been known that the polarity of epithelial cells (Mv1Lu cells) is regulated by the UPS (Wang et al., 2003;

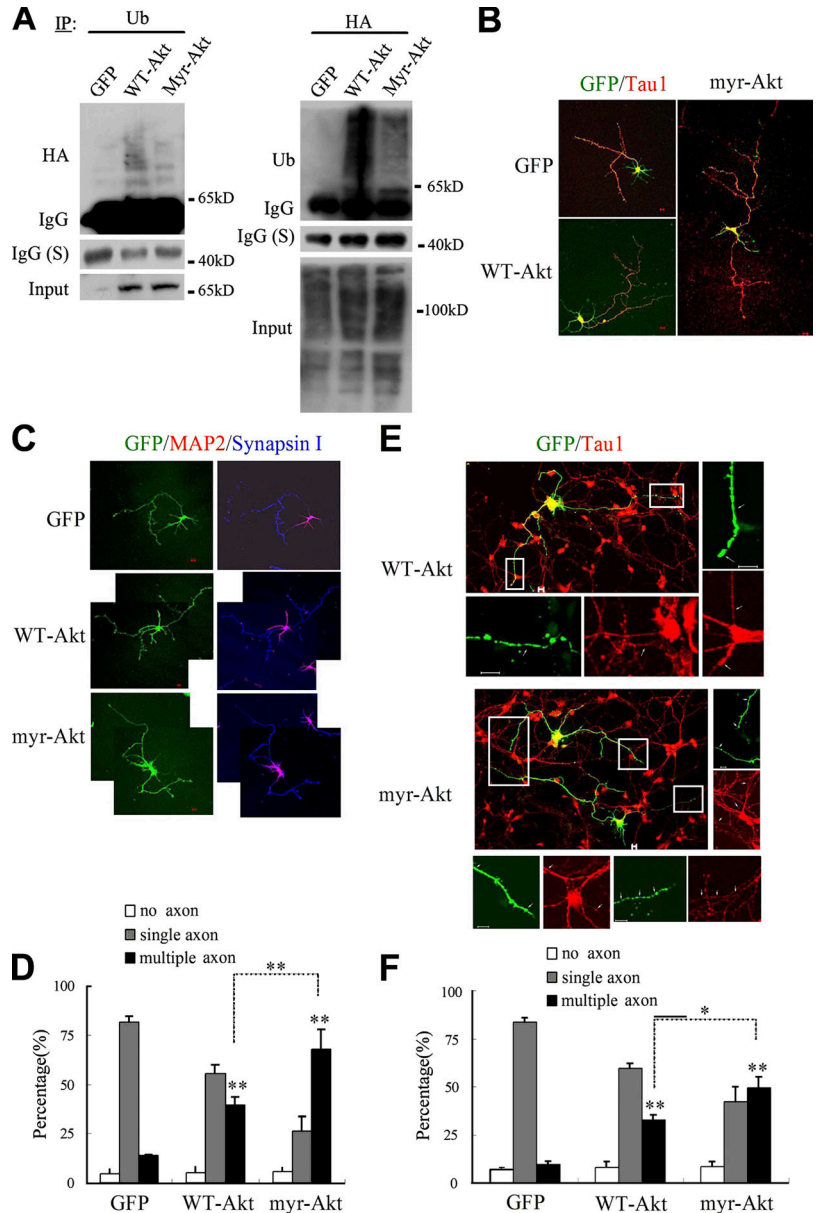
Bryan et al., 2005; Ozdamar et al., 2005). We have shown that neuronal polarity is also controlled by the UPS. Furthermore, in these epithelial cells, the overexpression of Smurf1, an Ub ligase, induces the specific degradation of RhoA to



**Figure 7. Akt degradation in dendrites is accelerated by inhibiting its activity.** (A) Time-lapse images of neurons transfected with Akt-PAGFP before plating, treated with DMSO or wortmannin, and photoactivated 36 h after transfection were displayed at 10-min intervals after photoactivation (photoactivation at 0 min). Reduction of Akt-PAGFP in dendrites of the neurons treated with wortmannin 10 min before photoactivation was faster than that in the control. Red arrows indicate axons, and purple arrows indicate dendrites. (B and C) Quantification of the normalized intensity of Akt-PAGFP in axons (B) and dendrites (C). Every unit on the x axis is 10 min. \*,  $P < 0.05$  versus control. Error bars represent SEM.

**Figure 8. Overexpression of constitutively active Akt inhibits its degradation and abolishes neuronal polarity.**

(A) Total cell lysates of neurons transfected with the indicated constructs before plating were immunoprecipitated after 48 h in culture with antibodies against Ub (left) or HA tag (right). The immunocomplexes were Western blotted with anti-HA or -Ub antibodies. IgG(S), IgG in short exposure time. (B and C) Neurons transfected with GFP-pcDNA3, GFP-WT-Akt, or GFP-myr-Akt before plating were fixed after 96 h in culture and stained for Tau1 (B) or MAP2 and synapsin I (C). (D) Quantification of polarity defects in neurons transfected with the indicated constructs before plating. \*\*,  $P < 0.001$  versus GFP or WT-Akt. (E) Neurons were transfected with GFP-pcDNA3, GFP-WT-Akt, and GFP-myr-Akt at 48 h in culture and stained for Tau1 (red) 5 d in culture. Boxed areas of stained axons are shown at higher magnifications in adjoining panels (GFP, green; Tau1, red). Arrows indicate that the neurites in the neurons labeled with GFP were Tau1 positive. (F) Quantification of polarity defects in neurons transfected with the indicated constructs after the establishment of polarity. \*\*,  $P < 0.001$  versus GFP; \*,  $P < 0.05$  versus WT-Akt. Bars, 10  $\mu\text{m}$ .



affect cell polarity. Smurf1 is recruited by PKC $\zeta$  to cell protrusions, where it controls the local level of RhoA to regulate cell polarity and protrusion formation (Wang et al., 2003). It is plausible that a specific Ub ligase, which controls Akt degradation, may be recruited to dendritic tips, leading to local Akt degradation.

In neurons and other cell types, Akt can be degraded by the UPS in response to different stimuli (Kim and Feldman, 2002; Martin et al., 2002; Adachi et al., 2003; Riesterer et al., 2004; Rusinol et al., 2004). Furthermore, depending on cell type, both active Akt and Akt may be degraded. An important finding in this study is that blocking Akt phosphorylation by wortmannin promoted its degradation (Figs. 6 and 7), and inhibiting the UPS resulted in the maintenance of Akt activity in neurite terminals, which consequently became axons (Fig. 5). Consistent with this, the inhibition of a receptor tyrosine kinase, which activates PI 3-kinase, inhibits the establishment of neuronal

polarity (Shi et al., 2003). We also found that in dendrites of the neurons expressing Akt-PAGFP, Akt degradation was accelerated by inhibition of its phosphorylation. However, in axons, the level of Akt was not affected by wortmannin after the establishment of polarity (Fig. 7). Although the explanation of the differential response to wortmannin of Akt in axons and dendrites is not clear, a possible interpretation is that an E3 ligase specific for Akt may be selectively activated in dendrites but not in axons.

It is important to note that there was more ubiquitinated Akt in stage 2 than that in stage 1 and 3 neurons (Fig. 2 C). Stage 2 is a critical period for the initiation of neuron polarity. In this stage, all neurites exchange phases of elongation and retraction, and all neurites have the potential to become axons (Dotti et al., 1988; Craig and Banker, 1994). During this frequent alternation, the growth and retraction of the Akt level in the neurite terminals may be rapidly regulated by the UPS.



After the establishment of neuronal polarity, Akt was enriched in axon terminals but not in dendrites, and disruption of the asymmetrical distribution of Akt by UPS inhibition or by Akt activation throughout the cell led to the formation of multiple axons (Fig. 8, E and F; and Fig. S1, F and G). Therefore, the polarized distribution of Akt as a result of its localized degradation in dendrites as compared with axons was also required to maintain neuronal polarity.

It has been shown that protein transport also affects protein localization to regulate neuronal polarity (Jareb and Banker, 1997). It is therefore possible that dendritic instability of Akt arises not from local degradation but through differential protein transport. However, several lines of evidence indicate that dendritic instability of Akt was not caused by the inefficiency of Akt transportation. First, Akt but not P110 and GSK-3 $\beta$  was ubiquitinated (Fig. 2 C and Fig. S1 J), and blocking Akt phosphorylation increased its ubiquitination (Fig. 6). Second, UPS inhibition restored Akt/p-Akt presence in all neurites and suppressed the formation of neuronal polarity (Fig. 5 and Fig. S3, A and B). Third, activation of Akt-PAGFP in whole cells did not reveal the differential transportation of Akt between axons and dendrites (Fig. 4 A). Lastly, the expression of constitutively active Akt prevented the polarized distribution of Akt and inhibited the formation of neuronal polarity (Fig. 8).

Some polarity decision molecules such as mPar3/mPar6/aPKC and Rap1B/Cdc42 are present in most neurites in stage 2 and are then redistributed exclusively to axons in stage 3 neurons during the formation of neuron polarity (Schwamborn and Puschel, 2004; Shi et al., 2003, 2004). The stage-dependent change in the distribution of these molecules similar to that of Akt is important for the establishment of neuronal polarity. However, it is unclear how their localizations are regulated. We found that mPar3 and aPKC were ubiquitinated during the formation of neuronal polarity (Fig. S5 D), suggesting that regulation of their localized redistributions may be through the same mechanism that results in the redistribution of Akt.

In the dendrites of mature hippocampal neurons, local degradation of postsynaptic proteins mediated by the UPS plays an important role in synaptic plasticity and spine morphology (Pak and Sheng, 2003; Hegde, 2004). An important implication of our findings is that local protein degradation mediated by the UPS is also essential for the establishment of normal morphology in early stages of neuronal development. In summary, Akt degradation in dendrites mediated by UPS was required for neuronal polarity, and this degradation was regulated by its phosphorylation state.

## Materials and methods

### Materials

The following primary antibodies were used: mouse monoclonal antibody against Ub; goat anti-Tau1 or -P110; rabbit anti-PKC $\xi$  (C-20; Santa Cruz Biotechnology, Inc.); rabbit antibody against GFP; synapsin and mouse monoclonal anti-GFP, -Tuj1, -MAP2, or -Tau1 antibodies (Chemicon); rabbit anti-p-Akt (Ser473), p-GSK-3 $\beta$  (Ser9), anti-Akt, or -GSK-3 $\beta$  antibodies (Cell Signaling Technology); mouse anti-myc or -HA antibodies (Sigma-Aldrich); and rabbit anti-Par3 antibody (Upstate Biotechnology). The following secondary antibodies were used: donkey Cy5-, FITC-, and Rhodamine Red-X-conjugated antibodies against mouse, rabbit, or goat

IgGs (Jackson ImmunoResearch Laboratories) and Texas red- or FITC-conjugated and AlexaFluor488- or -546-conjugated goat anti-mouse or rabbit IgGs (Invitrogen). HRP-conjugated anti-mouse or rabbit secondary antibodies and all materials for Western blotting were purchased from GE Healthcare. FM4-64 dye was obtained from Invitrogen. MG132, lactacystin, and all other reagents were purchased from Sigma-Aldrich.

### Neuronal culture and transfection

Rat primary hippocampal neurons were prepared as previously described (Shi et al., 2003). In brief, hippocampi dissected from embryonic day (E) 18 rats were digested with a mixture of proteases at 37°C for 15 min and dissociated with a pipette in MEM containing Earle's salts with 15% FBS, 0.5% glucose, 1 mM sodium pyruvate, and 25  $\mu$ M glutamine. Neurons were plated onto glass coverslips coated with poly-D-lysine at a density of 100–200 neurons/mm<sup>2</sup>. Neuronal cultures were incubated at 37°C with 5% CO<sub>2</sub>. After 1 h, the medium was changed to neurobasal medium (with B27 supplement and 0.5 mM glutamine). Before the establishment of polarity, neurons were transfected with different constructs using nucleofector (Rat Neuron Nucleofector Kit; Amaxa Biosystems). After the establishment of polarity (48 h after plating), neurons were transfected using the calcium phosphate. In brief, the constructs were mixed with 250 mM CaCl<sub>2</sub> and an equal volume of 2 $\times$  Hepes-buffered saline (274 mM NaCl, 10 mM KCl, 1.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 15 mM D-glucose, and 42 mM Hepes, pH 7.06). The DNA-calcium complex was incubated for 20 min and added to the neurons in DME without glutamine. After transfection, neurons were washed three times with DME, incubated for 1 h, and transferred to the original medium for 3 d. His6-myc-WT-Ub and His6-myc-K48R-Ub constructs were gifts from E. Burstein (University of Michigan Medical School, Ann Arbor, MI). HA WT-Akt, K179M Akt, and myr-Akt constructs were gifts from A. Bellacosa (Fox Chase Cancer Center, Philadelphia, PA). PAGFP-N1 construct was a gift from J. Lippincott-Schwartz and G.H. Patterson (National Institute of Child Health and Human Development, Bethesda, MD).

### Immunocytochemistry

Neurons cultured on coverslips were washed three times with PBS and fixed with 4% PFA in PBS containing 0.4% sucrose at 4°C for 30 min. The fixed neurons were washed, incubated with 0.5% Triton X-100 in PBS for 5 min, and blocked with 10% FBS in PBS for 1 h at room temperature. Neurons were probed with the primary antibodies at 4°C overnight and washed three to six times with 0.05% Tween-20 in PBS. They were then incubated with the secondary antibodies at room temperature for 1 h and washed three to six times with 0.05% Tween-20 in PBS. All antibodies were diluted with PBS containing 10% FBS. Axons are defined as Tau1-positive/MAP2-negative neurites with a mean length of >120  $\mu$ m 4 d after plating or neurites with a length twice that of other neurites at 2 d in culture (Shi et al., 2003). Dendrites are defined as MAP2-positive/Tau1-negative neurites.

### Image acquisition and quantification

Images were acquired by fluorescent microscopes (LSM510 Axiovert 200M [Carl Zeiss Microimaging, Inc.]; or E600 FN NeuroLucida system [Nikon]). Neuronal morphology was analyzed using the Physiology software of LSM510 (Carl Zeiss Microimaging, Inc.). In the PAGFP experiments, neurons were cotransfected with PAGFP (or Akt-PAGFP) and RFP before plating. Photobleaching was performed using a two-photon microscope (LSM510 META NLO Axioskop 2 FS MOT; Carl Zeiss Microimaging, Inc.) at 37°C with 5% CO<sub>2</sub> (Patterson and Lippincott-Schwartz, 2002). Images were acquired every 10 min, and each cell was observed for 4 h. Data analysis was performed using MetaMorph software (Molecular Devices). Protein levels of Akt in the axonal and dendritic tips were estimated by normalizing the intensity of PAGFP/RFP fluorescence, which was calculated according to the equation  $(F_n - F_0)/(F_1 - F_0)$ , in which F<sub>0</sub> is the PAGFP/RFP fluorescence 1 min before photoactivation, F<sub>1</sub> is the PAGFP/RFP fluorescence right after photoactivation, and F<sub>n</sub> is the PAGFP/RFP fluorescence of  $(n - 1) \times 10$  min.

### Immunoprecipitation and immunoblotting

Total proteins were extracted using radioimmunoprecipitation buffer (25 mM Tris-HCl, pH 7.4, 150 mM KCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS), and protein concentrations were measured using a protein assay kit (Bio-Rad Laboratories). Immunoprecipitation was conducted by incubation of the cell extracts with primary antibodies (1:50) overnight at 4°C. Protein G or A Sepharose was then added and incubated for 4 h. The immunocomplexes were collected and washed with radioimmunoprecipitation buffer. For Western blots, proteins were denatured by boiling in sample buffer for 5 min, separated on 6–10% SDS PAGE,

and transferred to polyvinylidene difluoride membrane. After blocked with 5% fat-free milk in PBS, the polyvinylidene difluoride membrane was probed with the indicated primary antibodies and HRP-conjugated secondary antibodies. The bands were visualized with an ECL system (GE Healthcare). The densities of the bands were determined by ImageQuant software (GE Healthcare).

#### FM4-64 dye recycling

Neurons were incubated with 10  $\mu$ M FM4-64 and 45 mM KCl for 1 min and washed with normal medium for 15 min. FM4-64 fluorescence was observed using an inverted microscope (LSM510 Axiovert 200M; Carl Zeiss MicroImaging, Inc.). Neurons were imaged again after destaining in 90 mM KCl for 5 min.

#### Local perfusion

Neurons on coverslips were perfused locally using a micropipette (tip opening of <1  $\mu$ m) pointed to a specific region of the neuron (Zheng et al., 1994). Perfusion medium contained culture medium with 200 nM DMSO/wortmannin or 0.15  $\mu$ M MG132. The micropipette was positioned near one neurite, and local perfusion was then performed for a period of 2–4 h using the PM8000-B eight-channel pressure injector system (positive pressure of 2 psi was applied at 2 Hz; World Precision Instruments). The images were acquired by microscopes (TE2000E; Nikon) at one image/2 min. The data were analyzed by MetaMorph software.

#### Statistics

Statistical analysis was conducted using the *t* test. Group differences resulting in *P* values of <0.05 were considered statistically significant.

#### Online supplemental material

Fig. S1 shows that UPS inhibition affects both the establishment and maintenance of neuron polarity. Fig. S2 shows the asymmetric distribution of p-Akt and p-GSK-3 $\beta$  in stage 3 neurons. Fig. S3 shows that UPS inhibition disrupts the asymmetric distribution of Akt and p-GSK-3 $\beta$ . Fig. S4 shows the effects of wortmannin on neurite growth, Akt degradation, and distribution. Fig. S5 shows that expressing myr-Akt disrupts the asymmetric distribution of Akt, and mPar3 and aPKC are ubiquitinated. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.2005111028/DC1>.

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