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A Unique CaMKII β Signaling Pathway at the Centrosome Regulates Dendrite Patterning in the Brain

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Abstract

The protein kinase calcium/calmodulin-dependent kinase II (CaMKII) predominantly consists of the α and β isoforms in the brain. Although CaMKII α functions have been elucidated, the unique catalytic functions of CaMKII β have remained unknown. Using knockdown analyses in primary neurons and in the rat cerebellar cortex in vivo, we report that CaMKII β operates at the centrosome in a CaMKII α -independent manner to drive dendrite retraction and pruning. We also find that the targeting protein PCM1 localizes CaMKII β at the centrosome. Finally, we uncover the E3 ubiquitin ligase Cdc20-APC as a novel centrosomal substrate of CaMKII β . CaMKII β phosphorylates Cdc20 at Ser51, which induces Cdc20 dispersion from the centrosome, thereby inhibiting centrosomal Cdc20-APC activity and triggering the transition from growth to retraction of dendrites. Our findings define a novel isoform-specific function for CaMKII β that regulates ubiquitin signaling at the centrosome and thereby orchestrates dendrite patterning, with important implications for neuronal connectivity in the brain.

The proper formation and morphogenesis of dendrites is fundamental to the establishment of neural circuits in the brain. Following exit from the cell cycle and migration to the appropriate location, post-mitotic neurons undergo carefully orchestrated steps in dendrite morphogenesis, including the generation and elaboration of extensive dendrite arbors followed by dendrite retraction and pruning^{1–2}. These steps in dendrite patterning are

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AUTHOR CONTRIBUTIONS

A.B. directed and coordinated the project. S.V.P., A.H.K., and Y.I. designed and performed in vivo experiments, biochemical assays, and morphological analyses. J.T.W.-G. prepared mass spectrometry samples and completed analyses of Cdc20 phosphorylation in S.P.G.'s laboratory. A.M. contributed molecular reagents. The manuscript was written by S.V.P. and A.B. and critically reviewed and commented on by all authors.

necessary for the accurate formation of neuronal circuitry. Defects in dendrite patterning may result in severe neurodevelopmental disorders³⁻⁴. Although the molecular basis of dendrite growth has been the subject of intense scrutiny⁵⁻⁸, the signaling mechanisms governing the transition from dendrite elaboration to pruning and retraction have remained poorly understood.

The calcium/calmodulin-dependent protein kinases (CaMKs) represent a critical link between the external environment and cellular responses in neurons. CaMKII is one of the major CaMKs in the brain, representing 1–2% of total brain protein⁹⁻¹¹. CaMKII exists as a holoenzyme composed of multiple subunits, which form a complex via their association domains¹²⁻¹⁵. Brain CaMKII predominantly consists of the α and β isoforms, which form heteromeric or homomeric complexes¹⁶⁻¹⁸. Previous studies have focused on the functions of CaMKII α ¹⁹⁻²². However, a specific role for CaMKII β as a protein kinase in the mammalian brain has remained unknown.

In this study, we report that CaMKII β has a distinct and specific function in the regulation of dendrite patterning in the mammalian brain. We identify a unique centrosomal targeting sequence (CTS) within the variable region of CaMKII β but not CaMKII α . The CTS mediates the specific interaction of CaMKII β with the centrosomal targeting protein pericentriolar material 1 (PCM1). Consequently, PCM1 localizes CaMKII β to the centrosome, where CaMKII β drives dendrite retraction and pruning independently of CaMKII α . Importantly, we uncover the ubiquitin ligase Cdc20-APC as the centrosomal substrate of CaMKII β . CaMKII β phosphorylates the APC coactivator Cdc20 at Ser51 in neurons, which induces Cdc20 dispersion from the centrosome, thereby inhibiting centrosomal Cdc20-APC activity and triggering a switch from growth to retraction of dendrites. Our findings define a unique CaMKII β pathway that regulates ubiquitin signaling at the centrosome to orchestrate dendrite patterning and hence the establishment of neuronal connectivity in the mammalian brain.

CaMKII β regulates dendrite patterning in mammalian neurons

To characterize the role of CaMKII β in neurons, we used granule neurons of the developing rodent cerebellar cortex. Granule neurons are the most abundant neurons in the brain and follow a highly typified spatial and temporal program of differentiation characteristic of neurons in the central nervous system, providing a robust system for studies of neuronal morphogenesis and connectivity²³⁻²⁵. Using an antibody that specifically recognizes CaMKII β , we first confirmed that the distinct isoforms of CaMKII β (β , β' , $\beta\epsilon$, $\beta'\epsilon$) are expressed in primary granule neurons, and protein levels increased with maturation (Fig. 1a).

To determine the function of CaMKII β in neurons, we employed a plasmid-based method of RNA interference (RNAi) to acutely knockdown CaMKII β expression²⁶. Expression of two shRNAs targeting distinct regions of CaMKII β reduced the expression of exogenous CaMKII β but not CaMKII α in COS cells (Fig. 1b), and robustly decreased the expression of endogenous CaMKII β but not CaMKII α in granule neurons as determined by immunoblotting and immunocytochemistry (Fig. 1c and Supplementary Fig. 1a–c). To

assess the role of CaMKII β in neuronal morphogenesis, we induced the knockdown of CaMKII β in granule neurons and examined axons and dendrites, which are easily identified based on their morphology and immunocytochemical markers^{27–28}. Remarkably, CaMKII β knockdown in granule neurons stimulated the elaboration of dendrites, which were characterized by longer primary dendrites and increased secondary and tertiary dendrite branching (Fig. 1d and Supplementary Fig. 1d). Accordingly, CaMKII β knockdown increased total dendrite length by up to 76% in granule neurons (Fig. 1e). In contrast, CaMKII β RNAi had little or no effect on axon length (Fig. 1f). In other experiments, CaMKII β RNAi had little or no effect on cell survival in granule neurons (Fig. 1g). Together, these results suggest that CaMKII β knockdown specifically stimulates the elaboration of dendrites in granule neurons.

To confirm that the CaMKII β RNAi-induced dendrite phenotype is the result of specific knockdown of CaMKII β , we performed a rescue experiment. We generated an expression plasmid encoding CaMKII β that is resistant to RNAi (CaMKII β -RES) (Fig. 1h). Expression of CaMKII β -RES, but not CaMKII β encoded by wild type cDNA (CaMKII β -WT), restored the typical appearance of dendrite arbors and reduced dendrite length and branching in the background of CaMKII β RNAi to that of control transfected neurons (Fig. 1i,j and Supplementary Fig. 1e). These data suggest that the CaMKII β RNAi-induced dendrite phenotype results from specific knockdown of CaMKII β rather than off-target effects of CaMKII β RNAi. In other experiments, expression of a constitutively active form of CaMKII β , in which the site of autophosphorylation Thr287 is replaced with the phosphomimetic residue aspartate (T287D CaMKII β), simplified dendrite arbors and profoundly reduced dendrite length and branching in granule neurons (Fig. 1k and data not shown). Thus, based on both inhibition of CaMKII β by rigorously-controlled RNAi and gain-of-function analyses, we conclude that CaMKII β restricts the extent of dendrite arborization and growth in granule neurons.

We next determined if the function of CaMKII β in dendrite patterning is generalizable in mammalian brain neurons. CaMKII β knockdown in hippocampal neurons substantially increased the number of secondary and tertiary dendrite branches and thereby increased dendrite length (Supplementary Fig. 2a–d). CaMKII β RNAi-induced dendrite elaboration in hippocampal neurons was reversed by CaMKII β -RES but not CaMKII β -WT, indicating the specificity of the CaMKII β RNAi-induced phenotype (Supplementary Fig. 2e,f). CaMKII β knockdown also stimulated dendrite elaboration in primary cerebral cortical neurons (Supplementary Fig. 3). These data suggest that CaMKII β inhibits the growth and arborization of dendrites in diverse populations of mammalian brain neurons.

To determine the cellular basis of CaMKII β regulation of dendrite patterning, we characterized the temporal dynamics of CaMKII β function in dendrite morphogenesis. We subjected individual granule neurons expressing constitutively active T287D CaMKII β and control vector-transfected granule neurons to time-lapse analyses. In control vector-transfected granule neurons, dendrites and their branches displayed dynamic elongation and retraction during 48 hours of analysis (Supplementary Fig. 4a,b), with an overall cumulative increase in total dendrite length (Supplementary Fig. 4c–e). In contrast, T287D CaMKII β -expressing granule neurons rarely had periods of dendrite growth and almost exclusively

displayed retraction of dendrites (Supplementary Fig. 4a,b), which cumulatively reduced total dendrite length (Supplementary Fig. 4c–e). Analyses of individual dendrites yielded similar results, with individual dendrites in T287D CaMKII β -expressing neurons also showing an increase in dendrite retraction events compared to individual dendrites in control granule neurons (Supplementary Fig. 4f–h). In a complementary line of experiments, time-lapse analyses of individual neurons and dendrites in control U6-transfected neurons and CaMKII β knockdown neurons revealed reduced periods of dendrite retraction and increased periods of dendrite growth, with an overall cumulative increase in total dendrite length, upon CaMKII β knockdown (Supplementary Fig. 4i–q). These data suggest that CaMKII β restricts the elaboration of dendrite arbors by triggering a switch from growth to active retraction of dendrites.

We next characterized the function of CaMKII β in regulating the minute dynamics of dendrite extension and retraction using live environment-controlled imaging analyses. Granule neurons expressing T287D CaMKII β or transfected with the control vector were imaged every ten minutes for a one hour period. In control neurons, dendrites had a large number of extension events with few retraction events (Supplementary Fig. 5a,b and Supplementary Video 1). In contrast, T287D CaMKII β -expressing neurons had numerous retraction events and few extension events (Supplementary Fig. 5a,b and Supplementary Video 1). In a complementary line of experiments, live imaging analyses revealed a significant increase in extension events and a significant reduction in retraction events in neurons upon CaMKII β knockdown (Supplementary Fig. 5c,d and Supplementary Video 2).

CaMKII β drives dendrite retraction and pruning in vivo

Having identified a critical function for CaMKII β in the control of dendrite patterning in primary neurons, we next determined the role of CaMKII β in dendrite morphogenesis in the intact developing cerebellar cortex. We first utilized rat organotypic cerebellar slices in which the architecture of the cerebellar tissue is preserved. Using a biolistics method of transfection, we induced CaMKII β knockdown in post-natal day six (P6) cerebellar slices²⁷. CaMKII β knockdown robustly stimulated dendrite elaboration in internal granule layer (IGL) granule neurons (Fig. 2a), substantially increasing the number of secondary and tertiary dendrite branches as well as total dendrite length (Fig. 2a,b and Supplementary Fig. 6a).

We next assessed CaMKII β function in the cerebellar cortex in the organism using an in vivo RNAi approach²⁸. We electroporated P3 rat pups with a CaMKII β RNAi plasmid that also expresses GFP (U6-CaMKII β i/CMV-GFP) or the corresponding control RNAi plasmid (U6/CMV-GFP) (Fig. 2c). Five days after electroporation, animals were sacrificed and cerebella were subjected to immunohistochemical analysis using the GFP antibody (Fig. 2d). At P8, IGL granule neurons in CaMKII β knockdown animals had longer dendrites with increased secondary and tertiary dendrite branching than IGL granule neurons in control animals (Fig. 2e). Morphometric analyses revealed a substantial increase in the number of secondary and tertiary dendrite branches and a nearly two-fold increase in total dendrite length in IGL neurons in CaMKII β knockdown animals compared to control animals (Fig. 2f and Supplementary Fig. 6b). CaMKII β knockdown had little or no effect on parallel fiber

patterning or the number of parallel fibers associated with IGL granule neurons (Supplementary Fig. 6c and data not shown). Together, these data reveal a physiological, cell-autonomous function for CaMKII β in restricting the elaboration of dendrite arbors in the cerebellar cortex in vivo.

Because CaMKII β triggered active retraction of dendrites in primary neurons (Supplementary Figs. 4 and 5), we reasoned that CaMKII β might also play a role in pruning of dendrite arbors in vivo, which follows the phase of dendrite growth and arborization¹. To test this possibility, we analyzed the effect of CaMKII β knockdown on dendrite morphogenesis in P12 rat pups in vivo. In control transfected animals, IGL granule neurons harbored few short dendrites with simplified arbors (Fig. 2g and Supplementary Fig. 6d–g), characteristic of the mature stage of dendrite differentiation in granule neurons. In addition, IGL granule neurons harbored dendritic claws (Fig. 2g and Supplementary Fig. 6h), which house synapses with afferent mossy fiber terminals and Golgi neuron axons^{1–2,29–32}, providing further evidence of dendrite maturation in control P12 animals. In contrast to control animals, IGL granule neurons in CaMKII β knockdown animals displayed longer, more branched dendrite arbors (Fig. 2g), with significantly increased total dendrite length ($78.3 \pm 3.0 \mu\text{m}$ in CaMKII β knockdown animals versus $43.8 \pm 1.5 \mu\text{m}$ in control animals; t-test, $p < 0.0001$), primary dendrite number (3.53 ± 0.10 in CaMKII β knockdown animals versus 3.01 ± 0.08 in control animals; t-test, $p < 0.005$) and secondary and tertiary dendrite branch number (1.13 ± 0.11 in CaMKII β knockdown animals versus 0.63 ± 0.07 in control animals; t-test, $p < 0.005$) (Supplementary Fig. 6d–g), suggesting that CaMKII β knockdown impairs dendrite pruning and blocks the differentiation of dendrites at the stage of exuberant arbors. Consistent with these observations, IGL granule neuron dendrites in CaMKII β knockdown pups had a significantly lower percentage of dendrites bearing claws ($31.9 \pm 2.2\%$ in CaMKII β knockdown animals versus $58.9 \pm 2.5\%$ in control animals; t-test, $p < 0.0001$) (Fig. 2g and Supplementary Fig. 6h). Together, these results suggest that CaMKII β plays an essential role in dendrite pruning at later stages of dendrite morphogenesis in the cerebellar cortex in vivo. Similar results were obtained in P10 cerebellar slices (Supplementary Fig. 6i,j), corroborating the conclusion that CaMKII β promotes dendrite pruning during later stages of dendrite development in the cerebellar cortex. Collectively, our findings suggest that CaMKII β regulates the patterning of dendrites throughout development in vivo.

CaMKII β functions independently of CaMKII α at the centrosome

The identification of a role for CaMKII β in the control of dendrite patterning in the mammalian brain led us to determine the molecular basis of CaMKII β function in neurons. We took advantage of the ability of CaMKII β -RES to rescue the CaMKII β RNAi phenotype to perform structure-function analyses of CaMKII β in the regulation of dendrite morphogenesis. In contrast to CaMKII β -RES, a catalytically inactive mutant of CaMKII β -RES in which the ATP binding site was disrupted (K43R CaMKII β -RES) failed to restrict the elaboration of dendrites in the background of CaMKII β RNAi in granule neurons (Fig. 3a), suggesting that CaMKII β requires the catalytic activity of the kinase to control dendrite patterning.

The requirement of the kinase domain in CaMKII β to inhibit growth and stimulate retraction of dendrites was intriguing given that CaMKII α , which harbors a very similar catalytic domain as CaMKII β , promotes dendrite growth and arborization^{27,33}. We therefore reasoned that CaMKII β but not CaMKII α might be localized at a subcellular site that endows CaMKII β specifically with the ability to phosphorylate local substrates and restrict the elaboration of dendrites. CaMKII β harbors an F-actin binding domain within the N-terminal portion of the variable region, but CaMKII β bound to F-actin appears to function independently of its catalytic domain as a scaffold protein that recruits CaMKII α to F-actin^{34–36}. Accordingly, deletion of the F-actin binding domain (Δ FABD) had little or no effect on the ability of CaMKII β -RES to restrict dendrite elaboration in the background of CaMKII β RNAi in granule neurons (Fig. 3b,c). We next focused on the C-terminal segment of the variable region (CTR ν) whose function has remained unknown. Remarkably, a CaMKII β -RES mutant in which the CTR ν was deleted (CaMKII β -RES Δ CTR ν) failed to restrict dendrite elaboration in granule neurons in the background of CaMKII β RNAi (Fig. 3b,c). These results suggest that the CTR ν is required for CaMKII β -regulation of dendrite patterning.

To characterize the function of the CTR ν within CaMKII β , we assessed whether the CTR ν localizes CaMKII β to a distinct subcellular site where CaMKII β controls dendrite patterning. A GFP-fusion protein of the CTR ν appeared as a perinuclear punctate signal that colocalized with the centrosomal protein pericentrin (Fig. 3d). In immuno-electron microscopy analyses, a pool of endogenous CaMKII β was present in the pericentriolar region in granule neurons (Supplementary Fig. 7a). In complementary biochemical fractionation experiments using granule neuron lysates, a pool of endogenous CaMKII β cofractionated with the centrosomal proteins γ -tubulin, Cdc20, and 14-3-3 ϵ (Fig. 3e). Importantly, endogenous CaMKII α did not appear in the centrosomal fraction despite the presence of CaMKII α in whole cell lysate (Fig. 3e). Together, these data demonstrate that the CTR ν represents a centrosomal targeting sequence (CTS) that localizes CaMKII β to the centrosome in neurons.

If the principal function of the CTS is to localize CaMKII β to the centrosome, forcibly targeting CaMKII β lacking the CTS to the centrosome by a different means should restore the ability of the CaMKII β mutant to inhibit dendrite growth and stimulate retraction. We therefore generated a chimeric protein in which we fused CaMKII β -RES Δ CTS at its N-terminus to the PACT domain of AKAP450, which localizes proteins to the centrosome (PACT-CaMKII β -RES Δ CTS) (Fig. 3b and Supplementary Fig. 7b)³⁷. Notably, addition of the PACT domain restored the ability of CaMKII β -RES Δ CTS to restrict the elaboration of dendrites in the setting of CaMKII β RNAi (Fig. 3c). In complementary experiments, replacing the variable region of CaMKII α with the CTS (CaMKII α -CTS) remarkably conferred CaMKII α with the ability to robustly restrict dendrite elaboration in the background of CaMKII β RNAi (Fig. 3b,f). Likewise, in contrast to wild type CaMKII α , expression of a PACT-CaMKII α fusion protein restricted dendrite elaboration in the background of CaMKII β RNAi (Fig. 3b,f). Collectively, these results suggest that the CTS endows CaMKII β with the ability to localize at the centrosome and control dendrite patterning.

The finding that CaMKII β regulates dendrite patterning from the centrosome, where CaMKII β but not CaMKII α is localized, suggested that centrosomal CaMKII β might function independently of CaMKII α in the control of dendrite morphogenesis. To test this hypothesis, we assessed the ability of a CaMKII β -RES mutant in which we removed the association domain (CaMKII β -RES Δ Assoc) to restrict dendrite elaboration in the background of CaMKII β RNAi. In control biochemical analyses, deletion of the association domain abrogated the interaction of CaMKII β with CaMKII α (Supplementary Fig. 7c). In morphology assays, deletion of the association domain remarkably had little or no effect on the ability of CaMKII β -RES to restrict the elaboration of dendrites in the background of CaMKII β RNAi in primary granule neurons and in cerebellar slices (Fig. 3g and Supplementary Fig. 7d–f), suggesting that the association domain is dispensable for CaMKII β control of dendrite patterning. Importantly, as with full-length CaMKII β , deletion of the CTS or mutation of the ATP binding site (K43R) blocked the ability of the CaMKII β Δ Assoc mutant to restrict the elaboration of dendrites (Fig. 3g, Supplementary Fig. 7d–f, and data not shown). In immunocytochemical analyses, although full-length GFP-CaMKII β was localized throughout the cell soma and processes (Supplementary Fig. 7g), CaMKII β Δ Assoc was enriched at the centrosome in granule neurons (Supplementary Fig. 7h). Deletion of the CTS blocked centrosomal enrichment of the CaMKII β Δ Assoc mutant (Supplementary Fig. 7h). Together, our data demonstrate that centrosomal CaMKII β functions independently of multimerization with CaMKII α to regulate dendrite patterning.

The targeting protein PCM1 localizes CaMKII β at the centrosome

We next characterized the mechanism that localizes CaMKII β at the centrosome. In view of the importance of the centrosomal targeting sequence (CTS) in CaMKII β 's localization at the centrosome, we reasoned that the CTS might associate with a protein that targets CaMKII β to the centrosome. We therefore performed a yeast two hybrid assay using the CTS as bait and a human fetal brain library as prey to identify CTS-interacting proteins. In these assays, we identified the centrosomal targeting protein pericentriolar material 1 (PCM1) as an interactor of the CTS (data not shown). Importantly, in coimmunoprecipitation analyses in cells, PCM1 formed a complex with CaMKII β but not CaMKII α , and CaMKII β interacted with PCM1 in a CTS-dependent manner (Fig. 4a,b). In addition, endogenous PCM1 formed a complex with endogenous CaMKII β within centrosomal fractions of granule neurons (Fig. 4c).

To determine PCM1's role in targeting a pool of CaMKII β to the centrosome, we assessed the effect of PCM1 knockdown on the localization of CaMKII β Δ Assoc, which does not interact with CaMKII α and is enriched at the centrosome (Supplementary Fig. 7h). We found that knockdown of endogenous PCM1, achieved efficiently by two shRNAs targeting distinct regions of PCM1, led to the loss of centrosomal enrichment of CaMKII β Δ Assoc in neurons (Fig. 4d,e). These results suggest that PCM1 specifically localizes CaMKII β at the centrosome.

The requirement for PCM1 in localizing CaMKII β at the centrosome led us next to determine the functional role of PCM1 in dendrite morphogenesis. We found that PCM1 knockdown substantially increased dendrite arborization and growth in primary granule

neurons (Fig. 4f,g and data not shown). Importantly, the PCM1 RNAi-induced dendrite phenotype was reversed by expression of PCM1 encoded by an RNAi-resistant cDNA (PCM1-RES) but not wild type cDNA (PCM1-WT) (Fig. 4g,h and data not shown), indicating the specificity of the PCM1 RNAi-induced phenotype. These results suggest that like CaMKII β , PCM1 induces dendrite retraction. We also determined the effect of PCM1 knockdown on dendrite morphogenesis in the cerebellar cortex in vivo. We found that PCM1 knockdown triggered robust elaboration of IGL granule neuron dendrite arbors in P8 rat pups (Fig. 4i,j and Supplementary Fig. 8a). Analyses at later stages of dendrite development in P12 rat pups revealed that granule neuron dendrite arbors in PCM1 knockdown animals remained long and highly branched, containing substantially fewer dendritic claws as compared to control animals (Fig. 4k and Supplementary Fig. 8b–d). Thus, PCM1 knockdown phenocopied the effect of CaMKII β knockdown in impairing dendrite pruning and blocking dendrite differentiation at a stage of exuberant arbors in vivo. In other experiments, PCM1 knockdown suppressed the ability of activated CaMKII β to stimulate dendrite retraction in granule neurons (Supplementary Fig. 8e). Collectively, these results suggest that PCM1 is required for CaMKII β localization at the centrosome and hence the ability of CaMKII β to regulate the patterning of dendrites.

The ubiquitin ligase Cdc20-APC is a novel CaMKII β substrate

Since the catalytic activity of CaMKII β at the centrosome is required for regulating dendrite morphogenesis, we next sought to identify the protein substrates of centrosomal CaMKII β in neurons. We considered proteins that have established roles in dendrite development, localize to the centrosome, and contain a consensus sequence for CaMKII phosphorylation. Recently, the major mitotic E3 ubiquitin ligase Cdc20-APC has been identified as a critical mediator of dendrite growth that operates at the centrosome in post-mitotic neurons³⁸. We found that the N-terminus of the APC coactivator Cdc20 has several potential CaMKII sites, including Ser51 and Ser86 based on the CaMKII consensus sequence³⁹. Consistent with the possibility that CaMKII β might phosphorylate Cdc20, CaMKII β and Cdc20 formed a complex in 293T cells and neurons (Fig. 5a,b and Supplementary Fig. 9a).

Purified CaMKII catalyzed the phosphorylation of full-length and an N-terminal domain of Cdc20 (1–101) in vitro, and mass spectrometric analyses of phosphorylated Cdc20 revealed that CaMKII phosphorylated Cdc20 at Ser51, Ser84, and Ser86 (data not shown). Mutation analysis demonstrated that Ser86 and Ser51 contributed to CaMKII-mediated phosphorylation of Cdc20, while mutation of Ser84 to alanine did not significantly alter ³²P-ATP incorporation (Fig. 5c), suggesting that CaMKII β does not stoichiometrically phosphorylate Ser84 in vitro. Notably, Ser51 but not Ser86 is evolutionarily conserved from zebrafish to humans. We therefore generated an antibody to specifically recognize Cdc20 when phosphorylated at Ser51. The phospho-Cdc20 antibody recognized wild type Cdc20 but not the Ser51 mutant of Cdc20 when co-expressed with activated CaMKII β in cells (Fig. 5d). Immunoblotting experiments using the phospho-Cdc20 antibody revealed that endogenous Cdc20 is phosphorylated at Ser51 in granule neurons, and knockdown of CaMKII β reduced the level of endogenous Ser51-phosphorylated Cdc20 in neurons (Supplementary Fig. 9b). Together, these results indicate that endogenous CaMKII β

phosphorylates Cdc20 at Ser51 in neurons, raising the question of whether CaMKII β regulates the activity of Cdc20-APC at the centrosome to control dendrite patterning.

To determine the role of CaMKII β in regulating the ubiquitin ligase activity of centrosomal Cdc20-APC in neurons, we first utilized a Myc-PACT-securin-luciferase reporter, which is a validated readout of centrosomal Cdc20-APC activity in neurons³⁸. The reporter encodes the first 88 amino acids of the mitotic Cdc20-APC substrate securin fused to firefly luciferase. This portion of securin harbors the destruction box (D-box), an APC degron⁴⁰, and the PACT domain localizes the reporter to the centrosome³⁸. A mutant version of the reporter containing a mutation in the securin D-box (securin-DBM) serves as control⁴⁰. Thus, the ratio of securin to securin-DBM luciferase is inversely proportional to centrosomal Cdc20-APC activity³⁸. As reported, Cdc20 knockdown increased the relative levels of the securin-luciferase reporter in granule neurons (Fig. 5e)³⁸. In contrast, we found that CaMKII β knockdown reduced the relative levels of the securin-luciferase reporter in neurons (Fig. 5e), suggesting that CaMKII β inhibits centrosomal Cdc20-APC activity in neurons. CaMKII β knockdown also reduced levels of the centrosomal HLH protein Id1 (Fig. 5f), which is a physiological substrate of centrosomal Cdc20-APC in neurons³⁸. Collectively, these data suggest that CaMKII β inhibits Cdc20-APC activity at the centrosome and the consequent degradation of its substrates in neurons.

In view of Cdc20-APC's function in dendrite growth and arborization, the finding that CaMKII β inhibits the activity of Cdc20-APC at the centrosome in neurons suggested that CaMKII β might stimulate dendrite retraction via the regulation of centrosomal Cdc20-APC activity. Consistent with this prediction, in epistasis analyses, Cdc20 knockdown suppressed the effect of CaMKII β knockdown on dendrite elaboration in granule neurons (Fig. 5g). In addition, knockdown of the centrosomal Cdc20-APC substrate Id1 suppressed the ability of CaMKII β to induce dendrite retraction (data not shown). Together, these results suggest that CaMKII β stimulates dendrite retraction via inhibition of centrosomal Cdc20-APC activity in neurons.

Phosphorylation induces Cdc20 dispersion from the centrosome

We next assessed the role of Cdc20 phosphorylation at Ser51 in CaMKII β -regulated dendrite patterning. Expression of Cdc20-RES promotes dendrite growth and elaboration in the background of Cdc20 RNAi³⁸. We found that expression of a mutant Cdc20-RES protein in which Ser51 was replaced with the non-phosphorylatable residue alanine (S51A Cdc20-RES) stimulated dendrite growth more effectively than Cdc20-RES (Fig. 5h). In contrast, expression of a mutant Cdc20-RES protein in which Ser51 was replaced with phosphomimetic residue aspartate (S51D Cdc20-RES) failed to stimulate dendrite growth (Fig. 5h). Notably, expression of S51A Cdc20 but not wild type Cdc20 substantially impaired the ability of constitutively active CaMKII β to induce dendrite retraction (Fig. 5i). In other experiments, mutation of Ser84 or Ser86 had little or no effect on the ability of Cdc20-RES to drive dendrite elaboration (Supplementary Fig. 9c). Collectively, our results suggest that CaMKII β phosphorylates Cdc20 at Ser51 and inhibits centrosomal Cdc20-APC activity in neurons, thereby triggering a switch from dendrite growth and arborization to dendrite retraction.

We next determined the mechanism by which CaMKII β -induced phosphorylation of Cdc20 inhibits Cdc20-APC function at the centrosome in neurons. Because the localization of Cdc20 at the centrosome is critical for its function in dendrite growth and arborization³⁸, we asked whether CaMKII β phosphorylation of Cdc20 might interfere with the centrosomal localization of Cdc20. Strikingly, expression of wild type or constitutively active CaMKII β (T287D), but not the catalytically inactive K43R CaMKII β mutant or CaMKII α , induced dispersion of endogenous Cdc20 away from the centrosome in granule neurons (Fig. 6a,b and Supplementary Fig. 9d,e). Importantly, expression of CaMKII β had little or no effect on centrosomal structure in neurons as monitored by endogenous pericentrin or co-transfection with GFP-centrin (Supplementary Fig. 9e and data not shown). Conversely, CaMKII β knockdown reduced basal Cdc20 dispersion in granule neurons (Supplementary Fig. 9f), suggesting that endogenous CaMKII β inhibits the centrosomal localization of Cdc20 in neurons.

To assess whether CaMKII β stimulates Cdc20 dispersion independently of CaMKII α in neurons, we tested the effect of deletion of the association domain on the ability of CaMKII β to induce endogenous Cdc20 dispersion in granule neurons. Remarkably, the CaMKII β Δ Assoc mutant protein induced Cdc20 dispersion as effectively as CaMKII β (Fig. 6c), suggesting that the association domain is dispensable for CaMKII β -induced dispersion of Cdc20 in neurons. Importantly, deletion of the CTS in CaMKII β Δ Assoc diminished its ability to induce Cdc20 dispersion (Fig. 6c). Together, our results suggest that the protein kinase CaMKII β functions at the centrosome independently of CaMKII α to drive Cdc20 dispersion in neurons.

To determine whether the regulation of Cdc20 localization by CaMKII β is relevant to dendrite morphogenesis, we first compared dendrite length in granule neurons displaying centrosomal enrichment of Cdc20 with neurons in which Cdc20 was dispersed. Neurons with dispersed endogenous Cdc20 had substantially shorter dendrites compared to neurons with centrosomally localized Cdc20 ($35.8 \pm 10.2 \mu\text{m}$ in neurons with dispersed Cdc20 versus $105.5 \pm 9.9 \mu\text{m}$ in neurons with centrosomal Cdc20; t-test, $p < 0.01$) (Fig. 6d and Supplementary Fig. 9g). In other experiments, dispersion of endogenous Cdc20 in granule neurons increased with maturation (Supplementary Fig. 9h), suggesting that Cdc20 dispersion correlates temporally with CaMKII β function in dendrite retraction and pruning. If Cdc20 dispersion from the centrosome is critical for CaMKII β -induced dendrite retraction, then forcibly localizing Cdc20 to the centrosome should override the CaMKII β response. Consistent with this prediction, we found that a Cdc20 protein fused to the centrosome localizing PACT domain (PACT-Cdc20) suppressed the ability of T287D CaMKII β to promote dendrite retraction in granule neurons (Fig. 6f). In control experiments, although expression of T287D CaMKII β induced Cdc20 dispersion, T287D CaMKII β failed to disperse PACT-Cdc20 (Fig. 6e), suggesting that PACT-Cdc20 is insensitive to CaMKII β regulation. These results suggest that Cdc20 dispersion from the centrosome is critical for CaMKII β regulation of dendrite patterning.

We next assessed the role of CaMKII β -induced Cdc20 phosphorylation at Ser51 in the control of Cdc20 localization at the centrosome. In immunocytochemical analyses using the phospho-Cdc20 antibody, we found that endogenous Ser51-phosphorylated Cdc20

immunoreactivity had a broader distribution beyond the centrosome in granule neurons, suggesting that the dispersed pool of Cdc20 is phosphorylated at Ser51 (Fig. 6g). Importantly, expression of activated CaMKII β increased the number of neurons harboring phosphoSer51-Cdc20 immunofluorescence (Supplementary Fig. 9i), whereas knockdown of endogenous CaMKII β but not CaMKII α reduced the number of neurons with Ser51-phosphorylated Cdc20 (Supplementary Fig. 9j). To directly test the importance of Ser51 phosphorylation in Cdc20 dispersion, we characterized the effect of mutations of Ser51 on Cdc20 localization in neurons. We found that the non-phosphorylatable S51A Cdc20 mutant protein appeared to be more enriched at the centrosome than wild type Cdc20, while the phosphomimetic S51D mutant appeared to be less enriched at the centrosome (Fig. 6h). Quantification of the Cdc20 immunofluorescence signal using linescan analyses revealed that the S51A Cdc20 signal peaked at the centrosome and displayed a more limited distribution outside the centrosome (Fig. 6i). By contrast, S51D Cdc20 had a widened centrosomal peak with a broader non-centrosomal signal (Fig. 6h,i). In other experiments, expression of T287D CaMKII β robustly induced dispersion of wild type Cdc20 but not S51A Cdc20 from the centrosome in granule neurons (Fig. 6j), suggesting CaMKII β induces Cdc20 dispersion in a Ser51 phosphorylation-dependent manner. Importantly, addition of the PACT domain to S51D Cdc20 restored the ability of S51D Cdc20 to stimulate dendrite growth and elaboration, suggesting that the function of Ser51 phosphorylation is to localize Cdc20 away from the centrosome (Supplementary Fig. 9k). These data suggest that CaMKII β -induced phosphorylation of Cdc20 at Ser51 triggers its dispersion from the centrosome, leading to the inhibition of centrosomal Cdc20-APC activity and the promotion of dendrite retraction in neurons. Collectively, we have elucidated a centrosomal CaMKII β signaling pathway that controls the patterning of dendrite arbors with important consequences on the establishment of neuronal connectivity in the mammalian brain (see model in Supplementary Fig. 10).

DISCUSSION

In this study, we have discovered the first catalytic function of CaMKII β in the mammalian brain. Remarkably, CaMKII β operates at the centrosome in a CaMKII α -independent manner to drive dendrite retraction and pruning. CaMKII β localizes at the centrosome by forming a complex with the centrosomal trafficking protein PCM1. Accordingly, PCM1 is required for CaMKII β -regulation of dendrite morphogenesis and the pruning of dendrites in postnatal rat pups *in vivo*. We have also identified the ubiquitin ligase Cdc20-APC, which is enriched at the centrosome in neurons, as a novel substrate of CaMKII β . CaMKII β phosphorylates Cdc20 at Ser51 and thereby triggers the dispersion of Cdc20 from the centrosome, culminating in the inhibition of centrosomal Cdc20-APC activity and consequent dendrite retraction. Collectively, our findings define a novel isoform-specific function of CaMKII β that regulates ubiquitin-dependent protein degradation at the centrosome and thereby orchestrates dendrite patterning in mammalian brain neurons.

The identification of an unexpected function for CaMKII β in dendrite retraction and pruning bears significant ramifications for our understanding of the major protein kinase CaMKII. As the predominant CaMKII isoforms in the brain, CaMKII α and CaMKII β form holoenzyme complexes in neurons^{16–18}. However, nearly all of the reported functions of

CaMKII have focused on CaMKII α in homomeric or heteromeric complexes^{19–22}. Accordingly, CaMKII β has been previously relegated to a largely redundant role within CaMKII α heteromeric complexes or as a scaffold recruiting CaMKII α to specific cellular locales such as dendritic spines^{34–36}. Our finding that CaMKII β operates at the centrosome in a CaMKII α -independent manner unveils a biological function for CaMKII β homomeric complexes. Thus, rather than simply contributing to CaMKII α complexes, CaMKII β homomers harbor a major biological function at the centrosome as drivers of dendrite retraction and pruning and consequent neuronal connectivity in the mammalian brain. During brain development, CaMKII β expression peaks at earlier time points relative to CaMKII α ⁴¹. Therefore, it will be interesting to determine in future studies whether centrosomal CaMKII β homomeric complexes might also contribute to earlier aspects of neuronal development in which centrosomal signaling is thought to play a critical role, including neuronal polarization and migration^{42–43}. Beyond the nervous system, it will be important to identify the role of CaMKII β homomers in cellular homeostasis and development in other organ systems.

Elucidation of the CaMKII β /Cdc20 signaling link at the centrosome provides a mechanism by which CaMKII β promotes dendrite retraction and pruning. By inducing the phosphorylation of Cdc20 and inhibiting Cdc20-APC ubiquitin ligase activity at the centrosome, CaMKII β triggers a switch from dendrite growth and elaboration to dendrite retraction and pruning. These findings suggest that the centrosome may represent a critical signaling platform that integrates diverse cellular signals to determine the phase of dendrite morphogenesis in neurons. In view of the fundamental role of centrosome signaling in diverse systems from the control of cell polarity and migration to ciliary morphogenesis to vesicular transport⁴³, it will be interesting to explore whether centrosomal CaMKII β phosphorylation of Cdc20, or of other substrates yet to be identified, might contribute to these diverse biological processes.

The identification of the CaMKII β /Cdc20 signaling link also advances our understanding of the regulation of the major ubiquitin ligase Cdc20-APC. CaMKII β phosphorylates Cdc20 at Ser51, inducing dispersion of Cdc20 from the centrosome and inhibiting centrosomal Cdc20-APC activity. The phosphorylation-dependent control of the subcellular localization of Cdc20 represents an entirely new mode of regulation of the ubiquitin ligase Cdc20-APC. Interestingly, CaMKII β had little or no effect on Cdc20 binding to core APC subunits, the *in vitro* ubiquitin ligase activity of Cdc20-APC, or Cdc20 polyubiquitination (data not shown), highlighting the significance of Cdc20 dispersion as a critical cellular mechanism for regulating centrosomal Cdc20-APC activity. Future research should explore whether Cdc20 has additional roles outside of the centrosome, bearing in mind the exciting possibility that Cdc20 dispersion might be part of a developmental program that coordinates dendrite patterning with other steps in the establishment of neuronal connectivity.

Although CaMKII β and CaMKII α are similar in their catalytic, autoregulatory, and association domains, they diverge in their variable region⁴¹. We have identified a centrosomal targeting sequence (CTS) in the unique variable region of CaMKII β , thus providing spatial specificity for CaMKII β function. Further, we have found that the CTS mediates the interaction of CaMKII β specifically with the centrosomal targeting protein

PCM1. Although PCM1 targets structural proteins to the centrosome⁴⁴, our findings suggest that PCM1 may also drive the centrosomal localization of signaling proteins. Notably, in addition to interacting with CaMKII β , endogenous PCM1 formed a complex with endogenous Cdc20 in neurons and stimulated CaMKII β phosphorylation of Cdc20 (data not shown). These observations suggest that beyond recruiting CaMKII β to the centrosome, PCM1 may also operate as a scaffold protein that organizes the CaMKII β /Cdc20 signaling pathway at the centrosome.

Although signaling and cell-intrinsic mechanisms that promote dendrite growth have been the subject of substantial interest^{5–8}, the master regulatory mechanisms that govern the developmental transition from dendrite elaboration to dendrite pruning in the mammalian brain have remained to be elucidated. The identification of centrosomal CaMKII β signaling as a mechanism that restricts dendrite elaboration and promotes dendrite pruning suggests that pathways that actively drive dendrite retraction have evolved to sculpt dendrite arbors and thus establish accurate neuronal circuits. Since abnormalities in dendrite development represent prominent pathological features in mental retardation and autism spectrum disorders^{4,45–46}, it will be interesting to determine if deregulation of centrosomal CaMKII β signaling contributes to the pathogenesis of neurodevelopmental disorders of cognition.

ONLINE METHODS

Antibodies

Monoclonal CaMKII β and CaMKII α antibodies (Zymed), polyclonal rabbit phosphoThr287-CaMKII β antibody (Badrilla), polyclonal rabbit CaMKII α , CaMKII β , and ERK 1/2 antibodies (Cell Signaling), rabbit BAD, mouse Myc, mouse Actin, rabbit Cdc20, rabbit Id1, and rabbit SnoN antibodies (Santa Cruz Biotechnology), mouse Flag, γ -tubulin, Calbindin, and MAP2 antibodies (Sigma), mouse α -tubulin, mouse HA (clone 16B12), and rabbit pericentrin antibodies (Covance), rabbit and mouse GFP antibodies (Invitrogen), mouse beta-galactosidase antibody (Promega), and rat HA (clone 3F10) antibody (Roche) were purchased. Rabbit 14-3-3 ϵ antibody was a generous gift from Dr. Alastair Aitken (University of Edinburgh). Rabbit PCM1 antibody has been described⁴⁴. Rabbit phosphoSer51-Cdc20 antibody was generated by injection of rabbits with the phosphopeptide CAANRSHpSAGRTPG (amino acids 44–57 in rat Cdc20) and purified by standard affinity purification at Cell Signaling Technology.

Plasmids

Rat CaMKII β and CaMKII α cDNA was a gift from Dr. Tobias Meyer (Stanford) and was cloned into pcDNA3 to produce Myc epitope-tagged and Flag epitope-tagged CaMKII β expression plasmids and into pEGFP-C1 (Clontech) to produce an N-terminal GFP-tagged CaMKII β expression plasmid. C-terminal GFP-tagged chicken PCM1 in pEGFP-N1 (Clontech) has been described⁴⁴. GFP-centrin was a gift from Dr. Karl Munger (Harvard).

shRNA plasmids were produced by insertion of the following oligonucleotides into pBS/U6 or pBS/U6-CMV-GFP: U6-CaMKII β .1: 5'-GT CCG ACG CTG TGT CAA GCA CAAGTAAAC AGC TTG ACA CAG CGT CGG ACC TTTTGTG; U6-CaMKII β .2: 5'-GCA GCT AAG ATC ATT AAC ACG CAAGTAAAC GGT GTT AAT GAT CTT AGC TGC

CTTTTTG; U6-PCMIi.1: 5'-CTT GAA GCT CTA ATG GCT GAA CTTTTTG; U6-PCMIi.2: GGA GCA CAT GGA TGA AGT ATG CTTTTTG; U6-Cdc20i and U6-Id1i have been described³⁸.

Mutations in CaMKII β and other expression plasmids were performed using standard protocols and confirmed by sequencing. F-actin binding domain and C-terminal variable region deletion constructs were generated by deletion of the F-actin binding domain and C-terminal variable region (consisting of the linker and C-terminal segment) as described^{36,41}. CaMKII β -RES Δ Assoc and CaMKII β -RES Δ CTS/Assoc were created by standard PCR subcloning of CaMKII β lacking the association domain and CaMKII β lacking the association domain and C-terminal variable region^{36,41} into expression constructs.

Primary neuron cultures and transfection

Primary cerebellar granule neurons were prepared from P6 rat pups and transfected via a modified calcium phosphate protocol as described²⁸. To avoid the possibility that morphological effects of RNAi or protein expression were a result of changes in cell survival, we included the expression plasmid encoding the anti-apoptotic protein Bcl-x1 in all neuronal transfections. The CaMKII β RNAi-induced dendrite phenotype was identical in the presence or absence of Bcl-x1 expression (data not shown). Hippocampal and cerebral cortical neuron cultures were prepared from E18 rat embryos as described⁴⁷ and transfected using a modified calcium phosphate protocol.

Cerebellar slice cultures and in vivo electroporation

P6 and P10 rat cerebella were prepared as described²⁷. Individual neurons in P6 and P10 slices were transfected after two or four days, respectively, using biolistics (Helios gene gun, Bio-Rad) as described²⁷. Four days after transfection, slices were subjected to immunohistochemical analyses.

All experiments using live animals have been approved by the HMS Standing Committee on Animals and strictly conform to their regulatory standards. In vivo electroporation of P3 Sprague-Dawley rat pups was performed as described²⁸. Five or nine days after electroporation (P8 or P12, respectively), animals were euthanized, and cerebella harvested. Coronal sections of cerebella (40 μ m) were prepared and subjected to immunohistochemistry with the GFP and Calbindin antibody and the DNA dye bisbenzimidazole (Hoechst 33258).

Time-lapse analyses of dendrite morphogenesis

For initial time-lapse analyses, granule neurons were plated on etched coverslips (Bellco) and transfected after one day in vitro (DIV1). Beginning at DIV2, individual neurons were monitored by noting their position on the grid and obtaining sequential images of that position over the 48 hour period of analysis. Neurons were randomly selected. For CaMKII β knockdown experiments, granule neurons were similarly plated but transfected on DIV0. Beginning at DIV2, CaMKII β knockdown and control U6-transfected neurons were imaged.

For live confocal imaging analyses, granule neurons were plated on glass-bottom multi-well plates (MatTek) and transfected as described for initial time-lapse analyses. Beginning at DIV2, live imaging was performed using a PerkinElmer Life and Analytical Sciences UltraVIEW spinning-disk confocal system with a Nikon Ti-E Perfect-Focus microscope. An environment-controlled chamber maintained the neurons at 37°C, 5% CO₂. Images were acquired and analyzed using Volocity software (PerkinElmer Life and Analytical Sciences). A 20x objective was used in combination with an automated stage to capture images over 48 hours. Neurons were chosen randomly and followed by saving their coordinates on the motorized stage. Dendrite extension and retraction events were defined as events where dendrites extended or retracted by greater than 2 μm over the one hour period analyzed.

Immunocytochemistry

For visualization of centrosomal proteins, neurons were fixed in absolute methanol for ten minutes at -20°C and subjected to immunofluorescence analysis after blocking and staining with the indicated antibodies according to standard protocols. For other immunocytochemistry experiments, neurons were fixed in 4% paraformaldehyde for 20 minutes at room temperature and analyzed as described²⁸. Cells were counted as having dispersed Cdc20 if there were multiple discrete Cdc20 puncta (three or greater) or if puncta of Cdc20 immunofluorescence localized away from the centrosome based on GFP-centrin or endogenous pericentrin.

Immuno-electron microscopy analyses

Granule neurons were fixed in 4% paraformaldehyde with 0.025% glutaraldehyde and 5 μg/mL Taxol in Brinkley Buffer 1980 (80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA) for ten minutes at 37°C. Neurons were permeabilized in 0.1% Triton X-100 in BRB80 with 5 μg/mL Taxol and immunostained with CaMKIIβ or control (IgG) antibody overnight. Samples were then incubated with 5 nm gold-conjugated Protein A, sectioned using an ultramicrotome (Reichert), and collected on coated copper grids. Sections were visualized using a JEOL 1200EX transmission electron microscope.

Centrosomal fractionation of primary neuronal lysates

Centrosomal fractions from granule neurons or cortical neurons were isolated as described⁴⁸. Briefly, neurons were treated with 2 μg/mL nocodazole and 1 μg/mL cytochalasin D at 37°C for one hour, washed sequentially in cold 1X PBS, 0.1X PBS + 8% sucrose (w/w), and 8% sucrose, and then lysed in a hypotonic lysis buffer with a protease inhibitor cocktail (leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and pepstatin) and NaF. Homogenates were centrifuged at 1500 g for 10 min, and PIPES pH 7.2 (final concentration 10 mM) was added to the post-nuclear supernatant. The post-nuclear fraction was treated with 2 μg/mL DNase I for 30 min at 4°C and then layered on top of a discontinuous sucrose gradient (70%/50%/40% sucrose (w/w) in 10 mM PIPES pH 7.2, 0.1% Triton X-100, and 0.1% β-ME) and subjected to ultracentrifugation at 20,000 rpm in a SW-60 rotor (Beckman) for 1.5 hr. Fractions were collected from the bottom of the tube and analyzed by immunoblotting.

Immunoprecipitation analyses

Cells were lysed in 150 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% NP40 containing protease inhibitors. Lysates were briefly pre-cleared with a combination of Protein A/G sepharose beads and then incubated with either the appropriate antibody or antibody-conjugated beads overnight. For non-conjugated antibodies, the antibody-protein complexes were immunoprecipitated with Protein A/G beads. Immunoprecipitated proteins bound to beads were washed several times, and lysates were analyzed by SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting analysis.

Purification of GST-Cdc20 fusion proteins and in vitro kinase assays

GST-Cdc20 constructs were expressed in *E. coli*, and purified via solubilization in buffer containing 500 mM NaCl, 20 mM Tris pH 7.5, 0.2 mM EGTA, 0.2 mM EDTA, and protease inhibitors. Purified GST-Cdc20 proteins were incubated with CaMKII purified from rat forebrain (Calbiochem) for ten minutes at 37°C in the presence of 10 mM HEPES pH 7.5, 10 mM MgCl₂, 1 mM Na₃VO₄, 0.5 mM CaCl₂, 2.5 mM dithiothreitol, 10 µg/mL calmodulin, 200 µM ATP (containing trace ³²P-γ-ATP). Reactions were terminated by addition of SDS sample buffer, and samples were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. Radioactivity was visualized by Phosphorimager analysis.

Mass Spectrometry Analyses

The Coomassie stained band corresponding to the putative Cdc20 protein was excised from the gel and destained using 50% CH₃CN/50 mM NH₄HCO₃. Gel was dehydrated with 100% CH₃CN, followed by rehydration with 2 M Urea/25 mM Tris buffer, pH 8.8, containing 25 ng/µl of the endopeptidase Lys-C. In gel digestion was performed overnight at 37°C. LC-MS/MS data were obtained using an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher). The sample was loaded onto a pulled fused silica microcapillary column (125 µm, 15 cm) packed with C₁₈ reverse-phase resin (Magic C18AQ; Michrom Bioresources). Peptides were separated using an Agilent 1200 series binary pump across a 50 min linear gradient of 4–25% CH₃CN in 0.125% HCOOH at a flow rate of 800 nL/min. In each data collection cycle, one full MS scan (300–1500 m/z) was acquired in the Orbitrap (6 × 10⁴ resolution, automatic gain control (AGC) target of 10⁶), followed by 10 data-dependent MS/MS scans in the LTQ (AGC target, 10⁴; threshold 5 × 10³) using the 10 most abundant ions and electron transfer dissociation (ETD) for fragmentation. We employed a reaction time of 69 ms and an anion target value of 2 × 10⁵ reagent ions. SEQUEST was used to search MS/MS spectra for rat Cdc20 sequence to identify Cdc20 phosphopeptides. The search parameters used for post-translational modification included 79.96633 Da on serine, threonine, and tyrosine for phosphorylation.

Luciferase reporter assays

The Myc-PACT-securin-luciferase or Myc-PACT-securin DBM-luciferase plasmids were transfected with the indicated RNAi plasmid or control U6 vector in granule neurons as described³⁸. Four days later, neurons were treated with cycloheximide (100 µg/mL) for four hours, and lysates were subjected to luciferase measurements using luminometry (Promega). Luminescence values obtained with securin-luciferase transfection were divided by those of

securin DBM-luciferase (Centrosomal Securin/Securin DBM), yielding a value inversely proportional to centrosomal Cdc20-APC activity.

Analysis of neuronal morphology and imaging

To analyze the axonal and dendritic morphology of primary neurons in culture, in slices, and in vivo, images of individual neurons were captured randomly in a blinded manner on a Nikon Eclipse TE2000 epifluorescence microscope using a digital CCD camera (Diagnostic Instruments). SPOT software was used to measure individual process length by tracing. Axons and dendrites were identified in transfected neurons based on morphology and selective expression of MAP2 and Tau1 in dendrites and axons, respectively (data not shown). Total dendrite length was determined by summing the lengths of all dendrite processes measured from a single neuron. To analyze dendrite morphology in vivo, granule neurons residing in the IGL were selected for morphometry. Percent parallel fiber association was determined by counting the number of parallel fibers and cell bodies present in a specific region of a section in consecutive sections in a blinded manner as described⁴⁹. To analyze cell survival, neurons were transfected with the β -galactosidase expression plasmid and subjected to immunocytochemistry using the β -galactosidase antibody and the DNA dye bisbenzimidazole (Hoechst 33258). Cell survival was scored by assessment of process fragmentation and nuclear condensation.

Confocal images were collected using an Olympus IX81 microscope with FluoView1000 scanning confocal unit (taken with a 40X/0.90 NA Olympus UPlanSApo or 60X/1.42 NA Oil Olympus PlanApoN objective). Labeled neurons were excited at 405 nm, 488 nm and 559 nm, and emission was collected at 425–475 nm, 500–545 nm, and 575–675 nm for Hoechst, Alexa Fluor-488, and Cy3, respectively.

Linescan analyses

Images taken from transfected neuron cohorts using identical acquisition parameters were subjected to linescan analyses. The immunofluorescent signal was analyzed using Olympus Fluoview-1000 image analysis software by taking pericentrin signal as the location of the centrosome and tracing GFP-Cdc20 signal and quantifying immunofluorescence intensity along the line. Linescan intensity plots from individual neurons were aligned at the centrosomal peak (defined as zero on the x-axis) and were combined to generate an average for the cohort. Plots were thresholded at the level of background signal. Intensity values along the traced line are reported as a percentage of the maximum centrosomal peak signal.

Statistics

All analyses were completed from a minimum of three independent experiments. The number of cells contributing to each condition was equally distributed across independent experiments. Statistical analyses were performed with GraphPad Prism 4.0. All histograms are presented as mean + SEM unless otherwise noted. The Student's t-test was utilized for comparisons in experiments with two sample groups. In experiments with more than two sample groups, analysis of variance (ANOVA) was performed followed by Bonferroni's post-hoc test. For non-parametric data with more than two sample groups, the Kruskal-Wallis test was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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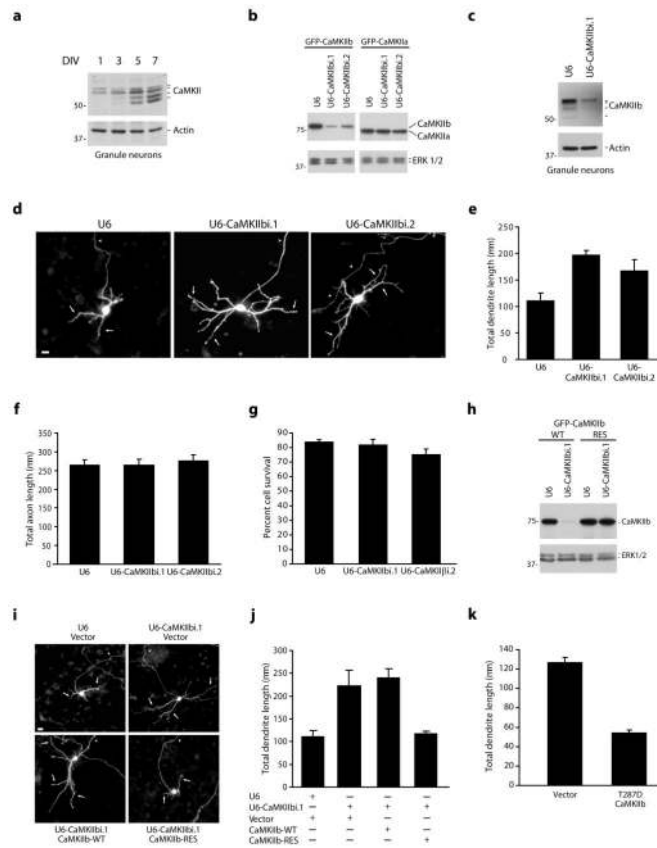


Figure 1. CaMKII β restricts the elaboration of dendrites

a, Lysates of granule neurons were immunoblotted with the indicated antibodies. DIV = days in vitro. Full-length blots for all immunoblotting analyses are presented in Supplementary Fig. 11. **b**, Lysates of COS cells transfected with GFP-CaMKII β or GFP-CaMKII α together with the CaMKII β RNAi or control U6 plasmid were immunoblotted with the indicated antibodies. **c**, Lysates of granule neurons electroporated with the CaMKII β RNAi or control U6 plasmid were immunoblotted with the indicated antibodies. **d**, Granule neurons transfected with a CaMKII β RNAi or control U6 plasmid together with GFP were subjected to immunocytochemistry using the GFP antibody. In all images of neuronal morphology, arrows and arrowheads indicate dendrites and axons, respectively. Scale bar = 10 μ m. **e**, Total dendrite length for granule neurons treated as in (d) was quantified and is presented as mean + SEM. Total dendrite length was significantly increased in CaMKII β knockdown neurons compared to control U6-transfected neurons (ANOVA; $p < 0.005$). 270 neurons were measured. **f**, Granule neurons were analyzed as in (d). Total axon length was not significantly different in CaMKII β knockdown neurons and control U6-transfected neurons. **g**, Granule neurons were transfected with a CaMKII β RNAi or control U6 plasmid and analyzed for cell survival. Cell survival was not significantly different in CaMKII β knockdown neurons and control U6-transfected neurons. **h**, Lysates of COS cells transfected with GFP-CaMKII β -WT or GFP-CaMKII β -RES together with the CaMKII β RNAi or control U6 plasmid were immunoblotted with the indicated antibodies. **i**, Granule neurons transfected with the CaMKII β RNAi or control U6 plasmid together with the expression plasmid encoding CaMKII β -WT, CaMKII β -RES, or control vector and GFP

were analyzed as in (d). Scale bar = 10 μm . **j**, Total dendrite length for granule neurons treated as in (i) was quantified. CaMKII β -RES, but not CaMKII β -WT, significantly reduced total dendrite length compared to control vector in the background of CaMKII β RNAi (ANOVA; $p < 0.005$). 360 neurons were measured. **k**, Granule neurons transfected with constitutively active T287D CaMKII β or control vector were analyzed as in (d). Total dendrite length was significantly reduced in T287D CaMKII β -expressing neurons compared to control vector-transfected neurons (t-test, $p < 0.0005$). 180 neurons were measured.

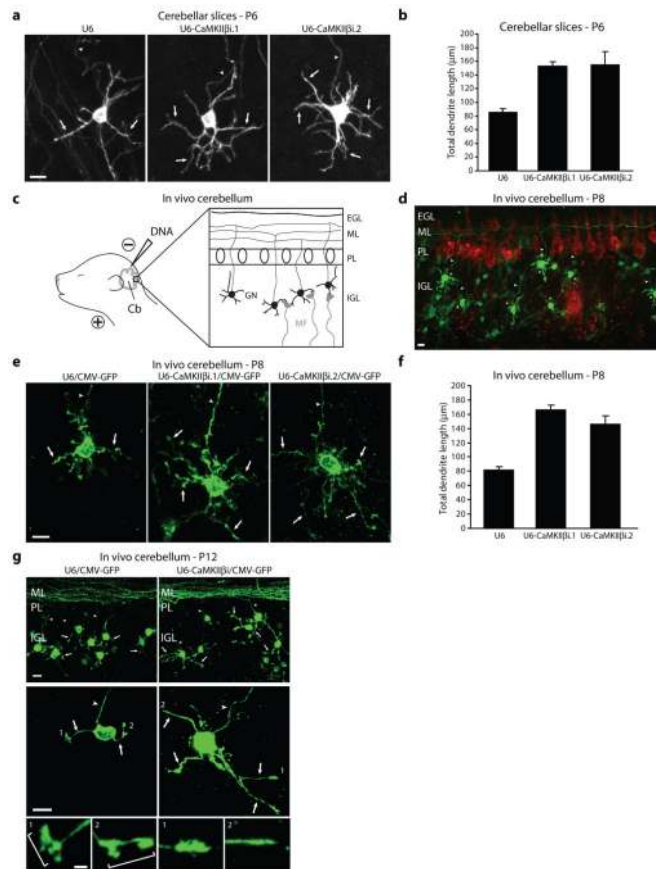


Figure 2. CaMKII β regulates dendrite patterning in vivo

a, Post-natal day six (P6) rat cerebellar slices transfected by a biolistics method with the CaMKII β RNAi or control U6 plasmid together with GFP were subjected to immunohistochemistry using the GFP antibody. Scale bar = 10 μ m. **b**, CaMKII β knockdown neurons had significantly longer dendrites compared to control U6-transfected neurons (ANOVA, $p < 0.0001$). 199 neurons were measured. **c**, Schematic of in vivo electroporation approach. **d**, Rat pups electroporated in vivo with a U6-CaMKII β /CMV-GFP RNAi or control U6/CMV-GFP plasmid were sacrificed five days after electroporation and cerebella were subjected to immunohistochemistry using the GFP and Calbindin antibody. Representative control-transfected granule neurons are shown. Scale bar = 10 μ m. **e**, Rat pups were electroporated as in (d) and representative neurons for each condition are shown. Scale bar = 10 μ m. **f**, IGL granule neurons analyzed as in (d) were subjected to morphometric analysis. Total dendrite length was significantly increased in IGL granule neurons in CaMKII β knockdown animals compared to control U6 animals (ANOVA, $p < 0.0001$). 266 neurons were measured. **g**, Rat pups electroporated in vivo with the U6-CaMKII β /CMV-GFP RNAi or control U6/CMV-GFP plasmid were sacrificed nine days after electroporation and cerebella were analyzed as in (d). (*Top*) Representative cerebellar sections from each condition are shown. Scale bar = 10 μ m. (*Bottom*) Representative IGL granule neurons for each condition are shown. Scale bar = 10 μ m. Inset: Zoomed view of dendritic tips of individual neurons. Scale bar = 2.5 μ m. Bracket identifies dendritic claws.

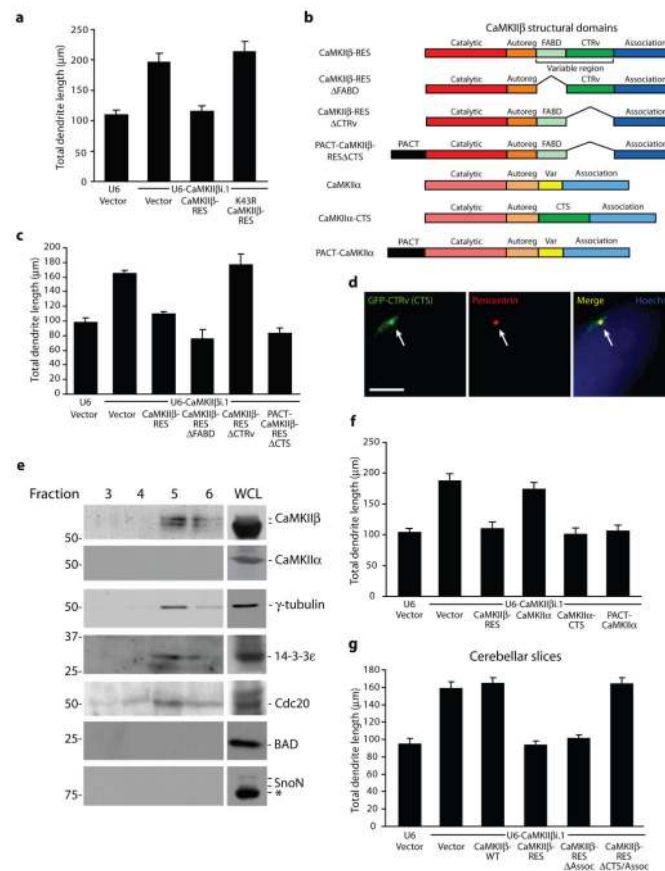


Figure 3. CaMKII β operates at the centrosome to specifically control dendrite patterning

a, Granule neurons transfected with the CaMKII β RNAi or control U6 plasmid together with CaMKII β -RES, K43R CaMKII β -RES, or control vector and GFP were analyzed as in Fig. 1d. CaMKII β -RES, but not K43R CaMKII β -RES, significantly reduced total dendrite length compared to control vector in the background of CaMKII β RNAi (ANOVA; $p < 0.0001$). 390 neurons were measured. **b**, Schematic of major structural domains of CaMKII β . **c**, Granule neurons transfected with the CaMKII β RNAi or control U6 plasmid together with CaMKII β -RES, CaMKII β -RES Δ FABD, CaMKII β -RES Δ CTrv, PACT-CaMKII β -RES Δ CTS, or control vector and GFP were analyzed as in (a). CaMKII β -RES, CaMKII β -RES Δ FABD, and PACT-CaMKII β -RES Δ CTS, but not CaMKII β -RES Δ CTrv, significantly reduced total dendrite length compared to control vector in the background of CaMKII β RNAi (ANOVA; $p < 0.0001$). 660 neurons were measured. **d**, Granule neurons transfected with GFP fused to the C-terminal variable region (GFP-CTRv) were subjected to immunocytochemistry using the GFP and pericentrin antibody. Arrows indicate colocalization of GFP-CTRv with pericentrin. Scale bar = 5 μ m. **e**, Fractions isolated from a granule neuron centrosome preparation were immunoblotted using the indicated antibodies. Asterisk indicates non-specific band. WCL, whole cell lysate. **f**, CaMKII β -RES, CaMKII α -CTS, and PACT-CaMKII α , but not CaMKII α , significantly reduced total dendrite length compared to control vector in the background of CaMKII β RNAi in granule neurons (ANOVA; $p < 0.0001$). 600 neurons were measured. **g**, CaMKII β -RES and CaMKII β -RES Δ Assoc, but not CaMKII β -WT or CaMKII β -RES Δ Assoc/CTS, significantly reduced

granule neuron total dendrite length compared to control vector in the background of CaMKII β RNAi in cerebellar slices (ANOVA; $p < 0.0001$). 479 neurons were measured.

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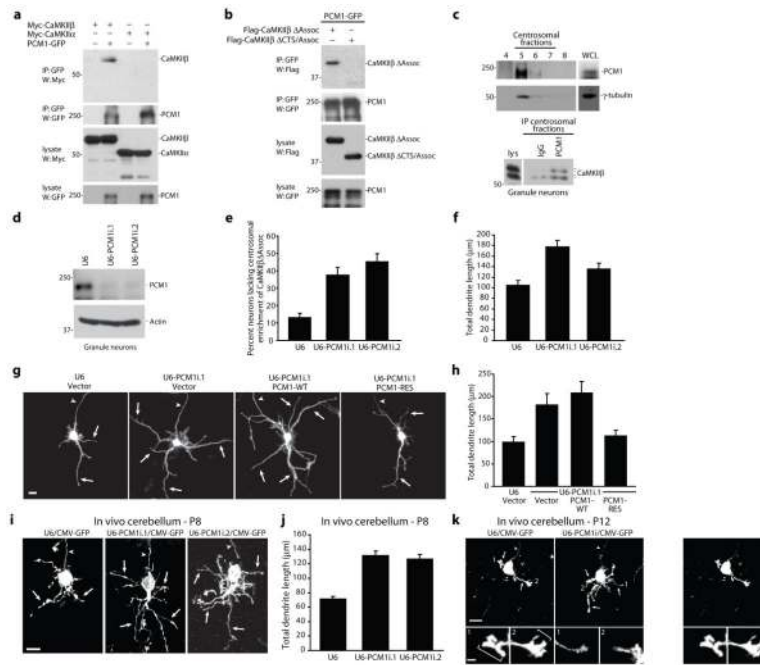


Figure 4. The centrosomal targeting protein PCM1 localizes CaMKII β at the centrosome
a, Lysates of 293T cells transfected with Myc-CaMKII β or Myc-CaMKII α together with PCM1-GFP or control vector were immunoprecipitated using the GFP antibody and immunoblotted with the indicated antibodies. **b**, Lysates of 293T cells transfected with Flag-CaMKII β Δ Assoc or Flag-CaMKII β Δ CTS/Assoc together with PCM1-GFP were immunoprecipitated using the GFP antibody and immunoblotted with the Flag or GFP antibody. **c**, Fractions isolated from a granule neuron centrosome preparation were immunoblotted using the PCM1 or γ -tubulin antibody. Pooled centrosomal fractions were immunoprecipitated with the PCM1 or control (IgG) antibody and immunoblotted with the CaMKII β antibody. **d**, Lysates of granule neurons electroporated with the PCM1 RNAi or control U6 plasmid were immunoblotted with the indicated antibodies. **e**, Granule neurons transfected with a PCM1 RNAi or control U6 plasmid together with GFP-CaMKII β Δ Assoc were analyzed as in Fig. 3d. The percentage of neurons lacking centrosomal enrichment of GFP-CaMKII β Δ Assoc was significantly increased in PCM1 knockdown neurons compared to control U6-transfected neurons (ANOVA, $p < 0.005$). 394 neurons were analyzed. **f**, Total dendrite length was significantly increased in PCM1 knockdown neurons compared to control U6-transfected neurons (ANOVA; $p < 0.0001$). 180 neurons were measured. **g**, Granule neurons transfected with the PCM1 RNAi or control U6 plasmid together with PCM1-WT, PCM1-RES, or control vector and GFP were analyzed as in (f). Scale bar = 10 μ m. **h**, PCM1-RES, but not PCM1-WT, significantly reduced total dendrite length compared to control vector in the background of PCM1 RNAi (ANOVA; $p < 0.0001$). 240 neurons were measured. **i**, Rat pups electroporated in vivo with a U6-PCM1i/CMV-GFP RNAi or control U6/CMV-GFP plasmid were sacrificed at P8 and analyzed as in Fig. 2d. Scale bar = 10 μ m. **j**, Total dendrite length was significantly increased in IGL granule neurons in PCM1 knockdown animals compared to control U6 animals (ANOVA, $p < 0.0001$). 270 neurons were measured. **k**, Rat pups electroporated in vivo with the U6-PCM1i/CMV-GFP RNAi or

control U6/CMV-GFP plasmid were sacrificed at P12 and analyzed as in Fig. 2g. Scale bar = 10 μm . Inset: Scale bar = 2.5 μm .

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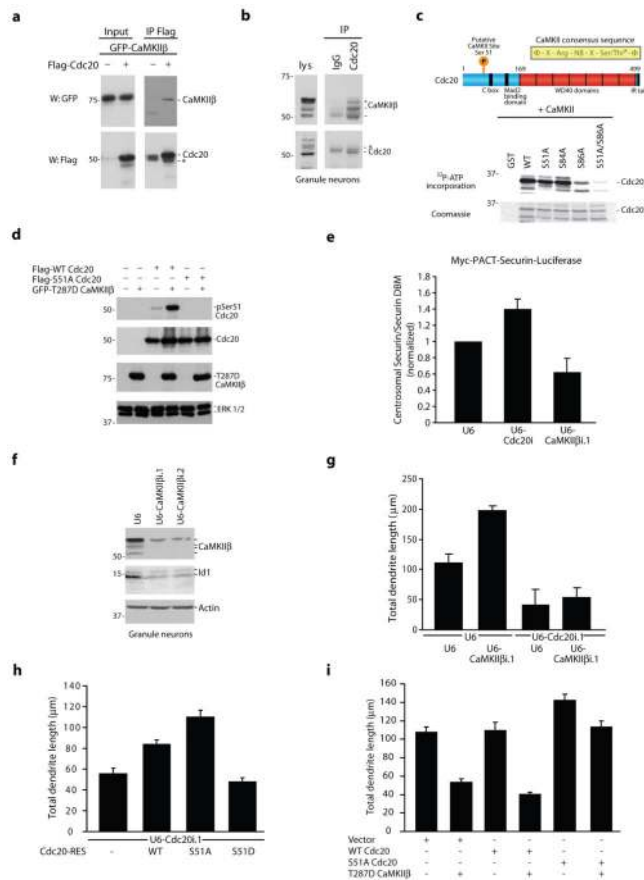


Figure 5. CaMKII β phosphorylates Cdc20 at Ser51 and thereby inhibits centrosomal Cdc20-APC activity in neurons

a, Lysates of 293T cells transfected with GFP-CaMKII β together with Flag-Cdc20 or control vector were immunoprecipitated using the Flag antibody and immunoblotted with the indicated antibodies. **b**, Lysates of granule neurons were immunoprecipitated with the Cdc20 or control (IgG) antibody and immunoblotted with the indicated antibodies. Asterisk indicates IgG heavy chain. **c**, (*Top*) Schematic of CaMKII consensus sequence. (*Bottom*) Recombinant WT and S51A, S84A, S86A, and S51A/S86A mutants of an N-terminal region of Cdc20 (1–101) fused to GST were incubated in vitro with purified CaMKII. **d**, Lysates of 293T cells transfected with Flag-WT Cdc20 or Flag-S51A Cdc20 together with GFP-T287D CaMKII β or control vector were immunoblotted with the indicated antibodies. **e**, Granule neurons were transfected with the Cdc20 RNAi, CaMKII β RNAi, or control U6 plasmid together with Myc-PACT-securin-luciferase or Myc-PACT-securin-DBM-luciferase and analyzed by luminometry. The relative amount of the centrosomal securin-luciferase reporter was significantly increased in Cdc20 knockdown neurons and significantly reduced in CaMKII β knockdown neurons compared to control U6-transfected neurons (Kruskal-Wallis; $p < 0.01$, $n = 4$). **f**, Lysates of granule neurons electroporated with the CaMKII β RNAi or control U6 plasmid were immunoblotted with the indicated antibodies. **g**, CaMKII β knockdown significantly increased total dendrite length compared to control, and Cdc20 RNAi significantly reduced total dendrite length in granule neurons in the presence or absence of CaMKII β RNAi (ANOVA, $p < 0.0001$). 420 neurons were measured. **h**,

Expression of S51A Cdc20-RES, but not S51D Cdc20-RES, significantly increased total dendrite length compared to control vector or WT Cdc20-RES in the background of Cdc20 RNAi in granule neurons (ANOVA, $p < 0.0001$). 428 neurons were measured. **i**, Expression of T287D CaMKII β significantly reduced total dendrite length compared to control in the background of control vector or WT Cdc20, but not in the background of S51A Cdc20 (ANOVA, $p < 0.0001$). 540 neurons were measured.

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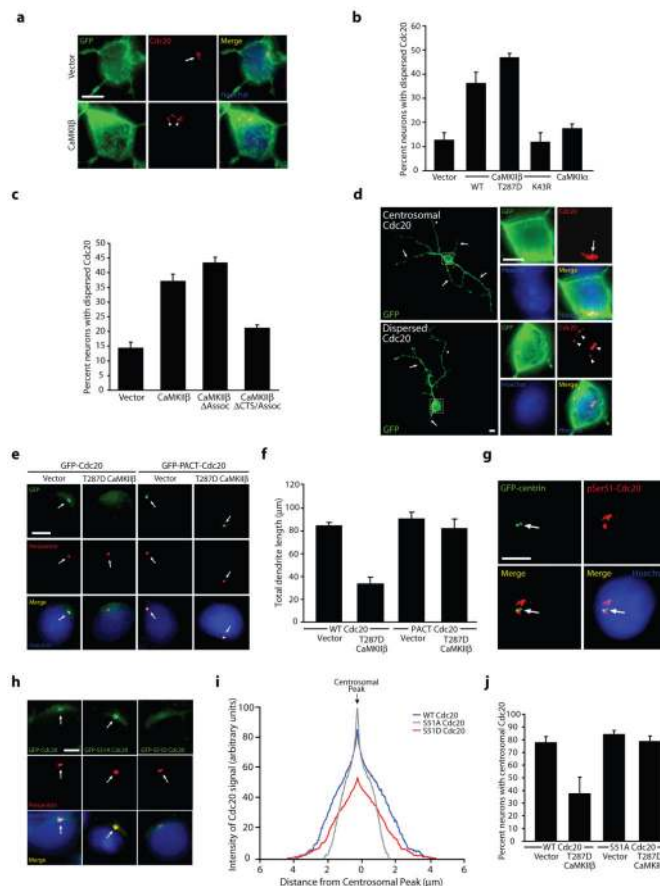


Figure 6. CaMKII β -induced phosphorylation of Cdc20 promotes Cdc20 dispersion from the centrosome, leading to the restriction of dendrite elaboration

a, Granule neurons transfected with CaMKII β or control vector together with farnesylated GFP (fGFP) were subjected to immunocytochemistry using the GFP or Cdc20 antibody. Arrows indicate Cdc20 localized to the centrosome, while arrowheads denote dispersed Cdc20. Scale bar = 5 μ m. **b**, The percentage of neurons displaying dispersed endogenous Cdc20 was significantly increased in T287D CaMKII β - and WT CaMKII β -expressing neurons, but not K43R CaMKII β - or CaMKII α -expressing neurons, as compared to control vector-transfected neurons (ANOVA; $p < 0.0001$). 622 neurons were analyzed. **c**, The percentage of neurons displaying dispersed endogenous Cdc20 was significantly increased in CaMKII β - and CaMKII β Δ Assoc-expressing neurons, but not CaMKII β Δ CTS/Assoc-expressing neurons, as compared to control vector-transfected neurons (ANOVA; $p < 0.0001$). 360 neurons were analyzed. **d**, Granule neurons transfected with T287D CaMKII β together with fGFP were analyzed as in (a). Arrows indicate dendrites, while dashed box indicates the magnified region in inset images. Scale bar = 5 μ m. **e**, Granule neurons transfected with GFP-Cdc20 or GFP-PACT-Cdc20 together with T287D CaMKII β or control vector were analyzed as in Fig. 3d. Arrows indicate the location of the centrosome, which is labeled with pericentrin. Scale bar = 5 μ m. **f**, Total dendrite length was significantly reduced in T287D CaMKII β -expressing neurons compared to control-transfected neurons in the background of Cdc20 (ANOVA, $p < 0.0005$), but not in the background of PACT-Cdc20. 360 neurons were measured. **g**, Granule neurons transfected with GFP-centrin were

subjected to immunocytochemistry using the GFP or phosphoSer51-Cdc20 antibody. Arrowheads indicate phosphoSer51-Cdc20 immunoreactivity. Scale bar = 5 μ m. **h**, Granule neurons transfected with GFP-Cdc20, GFP-S51A Cdc20, or GFP-S51D Cdc20 were analyzed as in (e). Scale bar = 5 μ m. **i**, Granule neurons treated as in (h) were analyzed by linescan analysis. The location of the centrosome was identified based on pericentrin immunoreactivity. S51A Cdc20 had enhanced signal at the centrosome and reduced cytosolic signal compared to WT Cdc20. In contrast, S51D Cdc20 had reduced centrosomal signal and broader non-centrosomal signal compared to WT Cdc20 (based on peak centrosomal signal intensity, ANOVA, $p < 0.0001$). 270 neurons were analyzed. **j**, Expression of T287D CaMKII β significantly reduced the percentage of neurons with centrosomally-enriched WT Cdc20 compared to control vector, but had no effect on the centrosomal localization of S51A Cdc20 (ANOVA; $p < 0.01$). 361 neurons were analyzed.