CCM3/PDCD10 stabilizes GCKIII proteins to promote Golgi assembly and cell orientation

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Summary
Mutations in CCM3/PDCD10 result in cerebral cavernous malformations (CCMs), a major cause of cerebral hemorrhage. Despite intense interest in CCMs, very little is known about the function of CCM3. Here, we report that CCM3 is located on the Golgi apparatus, forming a complex with proteins of the germinal center kinase III (GCKIII) family and GM130, a Golgi-resident protein. Cells depleted of CCM3 show a disassembled Golgi apparatus. Furthermore, in wound-healing assays, CCM3-depleted cells cannot reorient the Golgi and centrosome properly, and demonstrate impaired migration. Golgi disassembly after either depletion of CCM3 or dissociation of CCM3 from the GM130-GCKIII complex is the result of destabilization of GCKIII proteins and dephosphorylation of their substrate, 14-3-3ζ. Significantly, the phenotype induced by CCM3 depletion can be reverted by expression of wild-type CCM3, but not by disease-associated mutants. Our findings suggest that Golgi dysfunction and the ensuing abnormalities of cell orientation and migration resulting from CCM3 mutations contribute to CCM pathogenesis.

Key words: SOK1/YSK1, CCM, Mst

Introduction
Cerebral cavernous malformations (CCMs) affect over 0.5% of the population (Labauge et al., 2007). They are vascular lesions in the brain characterized by enlarged vessels (caverns) lined by endothelium, with no underlying smooth muscle or elastic tissue (Moriarity et al., 1999). Bleeding occurs frequently in these lesions, and can lead to headaches, seizures, focal neurological deficits and stroke (Moriarity et al., 1999; Zabramski et al., 1999).

The majority of CCMs are sporadic, but a significant minority have a genetic basis (Labauge et al., 2007). Three different CCM loci have been identified: CCM1/KRIT1 (Laberge-le Couteulx et al., 1999), CCM2/OSM (Liquori et al., 2003) and CCM3/PDCD10 (Bergametti et al., 2005). Mutations are inherited in heterozygosis and loss of the wild-type allele in endothelial cells induces the development of the malformations (akers et al., 2009; Pagenstecher et al., 2009). CCM1 and CCM2 are important in endothelial cell biology and vascular development (Hogan et al., 2008; Kleaveland et al., 2009; Whitehead et al., 2004; Whitehead et al., 2009). Biochemically, CCM2 is known to balance the activities of the small G proteins RhoA and cdc42 (Whitehead et al., 2009), probably through directed degradation of RhoA (Crose et al., 2009), whereas both CCM1 and CCM2 have been shown to form a complex that binds to the type I transmembrane receptor heart of glass (Kleaveland et al., 2009).

CCM3 has also been found in the CCM1-CCM2 complex, albeit only when one of the partners is overexpressed (Hilder et al., 2007; Stahl et al., 2008). Likewise, CCM3 has been found complexed to overexpressed phosphoprotein phosphatase type 2A (Goudreault et al., 2008), although the functional consequences of this interaction are not known.

The germinal center kinase III (GCKIII) subfamily of proteins consists of SOK1 (also referred to as YSK1 and STK25), Mst3 (also known as STK24) and Mst4 in mammals (Pombo et al., 2007; Ling et al., 2008). They belong to the Ste20 family of proteins, a large group of kinases that are characterized by a high degree of homology in their catalytic domain (Dan et al., 2001). The GCKIII kinases are involved in two important cellular processes: modulation of cell death and proliferation (Dan et al., 2002; Huang et al., 2002; Nogueira et al., 2008; Zhou et al., 2009), and regulation of cell migration (Lu et al., 2006; Preisinger et al., 2004). Specifically, Mst4 and SOK1 have been shown to associate with the cis-Golgi matrix protein GM130, where they play a role in determining the proper localization and morphology of the Golgi complex. SOK1 is also important for the invasion of cells into type I collagen and for cell migration in wound-healing assays, whereas overexpression of Mst4 seems to oppose these actions (Preisinger et al., 2004).

CCM3 has been shown to bind to GCKIII kinases, both in yeast two-hybrid assays (Ma et al., 2007; Rual et al., 2005) and in mammalian cells upon overexpression (Goudreault et al., 2008; Ma et al., 2007; Voss et al., 2007). The reported functional and biochemical consequences of this interaction are the modulation of the ERK pathway (Ma et al., 2007) and the phosphorylation of CCM3 by SOK1 (Voss et al., 2007).

In this work, we show for the first time that CCM3 is located on the Golgi apparatus, forming a complex with a GCKIII protein and GM130. CCM3 is important to maintain normal GCKIII protein levels and phosphorylation of the SOK1 substrate, 14-3-3ζ. As a consequence, cells with silenced CCM3 are impaired in their orientation and migration capabilities. These results link Golgi
We then asked what effect knockdown of CCM3 had on cell polarity and migration, because its SOK1 binding partner has been shown to regulate these events (Preisinger et al., 2004). To that end, we studied the effect of CCM3 knockdown on cell migration using a wound-healing assay. We found that cell migration was significantly impaired in cells depleted of CCM3 (Fig. 2B,C). This was true in both SaOS2 and HeLa cells (Fig. 2C), confirming that this is not a cell-specific effect.

The impaired migration correlated with the inability of cells depleted of CCM3 to reposition both the Golgi apparatus and the centrosome towards the leading edge of the wound when treated with serum. This was in distinct contrast to control siRNA-treated cells, which repositioned their Golgi apparatus and centrosome quite efficiently (Fig. 2D-G). Significantly, this was the case for SaOS2 cells transfected with siRNA or transduced with shRNA, and for HeLa cells, for which the effect was evident even 6 hours after initiation of the wound-healing assay (Fig. 2E for SaOS2 and supplementary material Fig. S2 for HeLa).

Golgi disassembly and reassembly are important for the repositioning of the organelle in wound-healing assays; the ERK pathway is believed to be important for the disassembly step (Bisel et al., 2008). As CCM3 has been reported to activate ERK in other cellular systems (Ma et al., 2007), we assessed ERK activity in CCM3-depleted cells. Unexpectedly, CCM3 depletion did not affect ERK activation by serum (Fig. 3A), suggesting that CCM3 regulates Golgi repositioning by means of an ERK-independent mechanism. Furthermore, in both serum-deprived and serum-stimulated cells, CCM3 knockdown increased the number of cells with a dispersed Golgi. This was irrespective of cell type or method of CCM3 knockdown employed (Fig. 3B,C), and was even apparent in normal proliferating cells (Fig. 4A,B). Furthermore, the cells with dispersed Golgi were the same as those that had undetectable CCM3 by immunofluorescence (supplementary material Fig. S3). The same Golgi phenotype was seen in HeLa cells (supplementary material Fig. S2), confirming that this is not a cell-specific phenomenon.

The dispersion seen after CCM3 knockdown could be due to the inability of the cell to reassemble the organelle after its disassembly. To test this hypothesis, we induced Golgi dispersion with the fungal metabolite Brefeldin A (BFA). BFA induced comparable dispersal of the Golgi apparatus in both control cells and cells depleted of CCM3. However, whereas a compact Golgi was discernible in most control cells within 90 minutes of washout of BFA, a high
percentage of cells with reduced CCM3 showed a dispersed tubuloreticular pattern even 180 minutes after washout of BFA (Fig. 4A, quantified in Fig. 4B and in supplementary material Fig. S2D for HeLa cells). Again, impaired Golgi reassembly was observed in all cell types tested and with both strategies of CCM3 knockdown. Furthermore, the effects of RNAi on Golgi reassembly were
dependent on CCM3 knockdown, because re-expressing CCM3 by transfection (Fig. 4C) rescued the effects of CCM3 shRNA on Golgi reassembly in both control cells and after BFA treatment (Fig. 4D). Likewise, expression of CCM3 also rescued both Golgi and centrosome orientation in the wound-healing assay (Fig. 4E for Golgi and data not shown for centrosome), and cell migration in the same assay (Fig. 4F).

**CCM3 forms a complex with the cis-Golgi GCKIII proteins and GM130**

CCM3 has been found bound to the other CCM proteins, CCM2 and CCM1, at least upon overexpression (Hilder et al., 2007; Stahl et al., 2008; Voss et al., 2007), and depletion of CCM2 has been shown to affect cytoskeletal organization, probably through modulation of the small G proteins cdc42 and RhoA (Crose et al., 2009; Whitehead et al., 2009). Because small G proteins have been implicated in the regulation of centrosome relocation and directed cell migration (Nobes and Hall, 1999), the CCM3 phenotype could be mediated by their modulation. However, our results show that stress fiber formation was not affected in CCM3-depleted cells, either when serum deprived or when stimulated with serum. Moreover, when we measured the activity of cdc42 and RhoA in serum-deprived and serum-stimulated cells, we found no difference between control and CCM3-depleted cells (supplementary material Fig. S4). We concluded that the effect of CCM3 depletion was probably mediated by a mechanism other than modulation of small G proteins.

The localization of CCM3 on the Golgi is probably caused by its binding to a protein or proteins present there. CCM3 has been reported to interact with several different proteins and protein

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**Fig. 3. Lack of CCM3 results in Golgi disassembly.** In all cases, SaOS2 cells were serum starved overnight, and then treated with 10% serum for 90 minutes (serum) or left untreated (SD).

(A) Extracts from cells stably transduced with control or CCM3 shRNA were analyzed by immunoblotting using anti-p-ERK. Total ERK1,2 (ERK) was used as a loading control. (B) SaOS2 cells were analyzed by immunofluorescence confocal microscopy. The Golgi apparatus was visualized with antibodies against GM130 and Golgi 58K, and DNA was stained with Hoechst 33342. The arrows point to cells with dispersed Golgi. Scale bar: 10 μm. The lower panels show the dispersed Golgi area of a single cell (boxed region) at a higher magnification. (C) Quantification of Golgi dispersion of SaOS2 cells after transient transfection of control or CCM3 siRNA, and in stable cell populations with control or CCM3 shRNA. An average of 300 cells was counted per condition in each experiment. The average and standard deviation of three independent experiments are shown. *P<0.01.
complexes, among them the GCKIII family of kinases (Mst4, Mst3 and SOK1) (Goudreault et al., 2008; Ma et al., 2007; Rual et al., 2005; Voss et al., 2007). Two GCKIII family members (Mst4 and SOK1) have been found in the cis-Golgi network, and SOK1 has been shown to be involved in Golgi repositioning and cell migration (Preisinger et al., 2004). We found that all three GCKIII proteins are present in purified Golgi membranes. Surprisingly, Mst4 was less enriched than the other two proteins; this correlated with reduced binding of Mst4 to the Golgi protein GM130 (supplementary material Fig. S5). We confirmed that CCM3 and the GCKIII kinase SOK1 interact in mammalian cells when overexpressed (Fig. 5A). Moreover, Mst3 and Mst4 also bind to CCM3 (Fig. 5B). The interaction of CCM3 with SOK1, Mst3 or Mst4 is not an artifact of overexpression, because we detected endogenous CCM3 in immunoprecipitates of endogenous SOK1 and Mst4 (Fig. 5C). Furthermore, although endogenous CCM3 could not be detected in immunoprecipitates of Mst3, Mst3 was present in CCM3 immunoprecipitates (Fig. 5D). To identify the domain of SOK1 important for interaction with CCM3, we co-transfected CCM3 with full-length SOK1, the N-terminal kinase domain (SOK1ND) or the C-terminal regulatory domain (SOK1RD). CCM3 could be immunoprecipitated with full-length SOK1 and SOK1RD, but not with SOK1NC (Fig. 5E). Thus, the interaction between CCM3 and SOK1 is mediated by the C-terminal domain of the latter.

The domain of SOK1 that binds to CCM3 (amino acids 333 to 426) is C terminal with respect to its binding site for GM130 (amino acids 270 to 302) (Nogueira et al., 2008; Preisinger et al., 2004) and does not overlap with it. This is compatible with SOK1 binding to GM130 and CCM3 simultaneously. To determine if such a trimeric complex can exist in cells, we transfected epitope-tagged versions of SOK1 and CCM3 in HEK293 cells (Fig. 5F). SOK1 was recovered in immunoprecipitates of endogenous GM130 with the same efficiency as when transfected alone or with CCM3. By
contrast, whereas CCM3 could be detected in the GM130 immunoprecipitate, its recovery was greatly increased when SOK1 was co-transfected. Furthermore, both SOK1 and GM130 were readily detected in immunoprecipitates of endogenous CCM3 in SaOS2 cells (Fig. 5G). These results demonstrate that CCM3 forms a complex with GM130 and strongly suggest that SOK1 (and probably other GCKIII proteins) bridge this interaction.

**Effects of CCM3 depletion on GCKIII proteins**

The CCM3-interacting proteins SOK1 and Mst4 are important for Golgi assembly and cell orientation. Thus, we asked whether CCM3 could modulate their activities. Depletion of CCM3 induced inhibition of SOK1 kinase activity, both in HeLa and in SaOS2 cells (Fig. 6A). This inhibition correlated with downregulation of the levels of SOK1 and the other two GCKIII proteins, Mst3 and Mst4. This was not a non-specific effect of RNAi, because it was reversed by the expression of CCM3 (Fig. 6B). We studied further the mechanism of this downregulation of GCKIII proteins and found that depletion of CCM3 did not induce changes in SOK1 mRNA (Fig. 6C). We then asked whether downregulation could be due to destabilization of the proteins. Thus, we assessed the decay of Mst3 after protein synthesis inhibition with cycloheximide (CHX) (as Mst3 is the only GCKIII protein we could consistently detect by western blot in shCCM3 cells). Depletion of CCM3 induced a marked shortening of the half-life of the Mst3 protein (13.9 hours in control cells versus 5.5 hours in CCM3-depleted cells; Fig. 6D).

To gain further insight into the mechanism of destabilization of GCKIII proteins in the absence of CCM3, we treated cells with the proteasome inhibitor MG132. Mst3 accumulated significantly in cells depleted of CCM3 but not in control cells, confirming that Mst3 degradation in the absence of CCM3 is mediated through the proteasome (Fig. 6E). Consistently, when we assessed the amount of ubiquitylated SOK1 in CCM3-depleted cells, we found that it was at least as much as in cells with normal amounts of CCM3, despite the reduced amount of SOK1. This was the case both in untreated cells and in cells treated with the proteasome inhibitor.
We concluded that degradation of GCKIII proteins is mediated at least in part by ubiquitylation and degradation through the proteasome, and that this is enhanced in the absence of CCM3. To assess whether the stabilization of the kinase component of the GM130-GCKIII complex by CCM3 plays a crucial role in Golgi assembly and cell migration, we expressed all possible combinations of GCKIII proteins in CCM3-depleted cells. All combinations tested resulted in an increase in Golgi assembly, with the expression of the three proteins inducing levels of Golgi assembly indistinguishable from that of control cells (Fig. 7A).

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The above results show that GCKIII proteins can rescue the Golgi phenotype induced by CCM3 depletion and suggest that CCM3 induces its effects through binding to this family of proteins on the cis face of the Golgi. We reasoned that, if this were the case, dissociation of CCM3 from GCKIII proteins should have the same effect as CCM3 depletion. Thus, we disrupted the GCKIII-CCM3 interaction by overexpressing SOK1RD, the C-terminal domain of SOK1 that binds CCM3. As expected, SOK1RD could bind to endogenous CCM3 (Fig. 7B, lower panels). Importantly, expression of SOK1RD clearly reduced the amount of CCM3 bound to endogenous SOK1 (Fig. 7B, middle and lower panels). Consistent with the binding of CCM3 to GCKIII proteins being important for its effects, levels of SOK1 were also greatly reduced (Fig. 7B, upper panels). Furthermore, the percentage of cells with disassembled Golgi was significantly higher in cells expressing SOK1RD than in control cells (Fig. 7C).

Having established that the effects of CCM3 on Golgi assembly are mediated through its binding and modulation of GCKIII proteins, we asked what event downstream of these kinases could be important for this effect. 14-3-3ζ has been shown to be phosphorylated at serine 58 by SOK1, both in untreated cells and after oxidative stress (Preisinger et al., 2004; Zhou et al., 2009). Consistent with 14-3-3ζ being a target of GCKIII proteins, its phosphorylation at serine 58 was diminished in CCM3-depleted cells (Fig. 8A). More significantly, transfection of the 14-3-3ζ phosphorylation-mimicking mutant S58D (but not wild-type 14-3-3ζ or the non-phosphorylatable S58A mutant) rescued the phenotype of CCM3 knockdown (Fig. 8B). Thus, the effects of CCM3 on Golgi assembly are mediated at least in part through 14-3-3ζ phosphorylation at serine 58.
Clinically relevant CCM3 mutants cannot rescue the CCM3 depletion phenotype

If the cellular phenotype of CCM3 depletion we have described is relevant to CCM pathogenesis, then clinically relevant CCM3 mutants should not be able to rescue it. Most CCM3 mutations are deletions of the whole gene or premature stop codons that result in highly defective truncated proteins. However, two different mutations in codons 196 and 203 have been described. Although both result in premature stop codons, the mutated genes encode proteins that are close in length to the wild-type protein (212 residues) (Fig. 9A) (Bergametti et al., 2005; Liquori et al., 2006). When we expressed these mutants, we found that their levels of expression were similar to that of wild-type CCM3 (Fig. 9A). Strikingly, the mutants were unable to rescue the defect in Golgi assembly induced by CCM3 depletion (Fig. 9B); this correlated with the inability of both mutants to bind to SOK1 efficiently (Fig. 9C) or to restore SOK1 expression to normal levels (Fig. 9A). The fact that mutants of CCM3 that lead to CCM formation were unable to rescue the Golgi phenotype induced by the depletion of wild-type CCM3 suggests that this phenotype is central to the pathophysiology of cavernous malformations. Finally, the inability of the CCM3 mutants to bind SOK1, to restore SOK1 levels to normal or to rescue the Golgi phenotype is consistent with the function of CCM3 in Golgi assembly being mediated by its binding to, and stabilization of, SOK1 and other GCKIII proteins.

Discussion

CCMs are vascular lesions in which endothelial cells form large caverns instead of normal capillaries. Because of the nature and location of the lesion, direct biochemical and cellular study of these abnormalities has been difficult. However, the realization that some CCMs have a genetic basis, and the identification of three CCM loci, has allowed the modeling of the disease at both the whole-animal and cellular level by inactivation of the relevant genes. Studies inactivating CCM1 and CCM2 have shown that these genes are important for cytoskeletal regulation. Accompanying evidence indicates that their products interact with endothelial transmembrane receptors, which suggests that endothelial cell shape and signaling might underlie at least some forms of the disease. However, no study showing a similar function for CCM3 has been reported so far.

In this paper, we show that CCM3 is important for Golgi assembly and for centrosome and Golgi orientation in wound-healing assays. Independently of its role in directed cell migration, the adequate positioning of centrosome and Golgi has been proposed to reflect the capability of the cell to polarize (Yadav et al., 2009); therefore, lack of polarizing ability is likely to be an important consequence of CCM3 inactivation. The two functions, cell orientation and Golgi assembly, are likely to be related, as the Golgi apparatus needs to disassemble and reassemble to change its position within the cell when the cell undergoes reorientation, and the integrity and position of the Golgi complex are known to be important for this orientation and directed migration (Vinogradova et al., 2009; Yadav et al., 2009). The effects of CCM3 on the Golgi apparatus are also likely to be linked to CCM pathogenesis, as two different clinically important mutants cannot rescue the effects of CCM3 knockdown on the Golgi.

Strong circumstantial evidence points to the Golgi apparatus as the crucial site of action of the GCKIII-CCM3 complex. The effects caused by CCM3 depletion are compatible with Golgi disassembly being the primary effect. In addition, GCKIII proteins and 14-3-3ζ phosphorylation are important for CCM3 functions, and these proteins have been localized to the Golgi (Preisinger et al., 2004; this article). Although there might be a pool of CCM3 that is not associated with the Golgi apparatus, it is clear that, independently of its location, CCM3 exerts the effects we report here through the binding and modulation of GCKIII proteins and the phosphorylation of 14-3-3ζ. Importantly, CCM3 does not overly affect the activity of the small G proteins Rho and cdc42, or the actin cytoskeleton, which suggests that it is not acting in concert with CCM1 and CCM2.

The relevant biochemical effect of CCM3 with respect to GCKIII proteins seems to be to stabilize them, because, in the absence of CCM3, both the levels and kinase activity of all three GCKIII proteins are downregulated due to destabilization of the proteins. Given that this facilitated degradation occurs through a proteasome- and ubiquitin-dependent pathway, CCM3 is likely to exert its function by protecting GCKIII proteins from ubiquitin ligation. Whether it directly impairs the action of an E3 ligase or inhibits a modification that marks GCKIII proteins for ubiquitylation will be the subject of future studies that will require the identification of the relevant E3 ligase.

The identification of the specific GCKIII protein or proteins that are important for CCM3 action will also require future research. We show here that only the expression of all three family members (SOK1, Mst3 and Mst4) fully restores normal Golgi assembly, but dissection of the contribution of each GCKIII protein to the phenotype induced by CCM3 depletion will require inhibition, not overexpression, of each individual kinase. However, SOK1 is the most likely GCKIII protein to be important in this respect. First, SOK1, but not the other two family members (Mst3 and Mst4), has been shown to be involved in Golgi assembly and cell orientation (Preisinger et al., 2004). Second, our results show that phosphorylation of 14-3-3ζ at serine 58 is an important downstream event for the CCM3 phenotype and SOK1 is the only GCKIII protein that has been shown to phosphorylate 14-3-3ζ.

Phosphorylation of 14-3-3ζ at serine 58 is an important consequence of GCKIII activity. Because of the pleiotropic nature of 14-3-3 proteins, it is difficult to pinpoint the mechanism through which this modification can be linked to Golgi assembly and cell orientation. However, it should be noted that 14-3-3ζ has been shown to bind to proteins important for cell polarity, such as par3 (Hurd et al., 2003). Intriguingly, besides its role in cell polarity, par3 is associated with tight junctions and is important for their assembly (Anderson and Van Itallie, 2008). Importantly, one of the characteristic features of the cells that form cerebral cavernomas is their aberrant tight junctions, which result in a leaky blood-brain barrier (Clatterbuck et al., 2001).

It is possible that a defect in cell polarity might also underlie the pathogenesis of other CCM syndromes. For instance, cells deficient in CCM2 have low activity of cdc42, a master regulator of cell polarity (Whitehead et al., 2009). Because depletion of CCM3 affects cell orientation without having a detectable effect on the activity of cdc42, different CCM proteins might be inducing the same or a very similar cellular effect through different mechanisms.

In summary, our findings demonstrate that clinically relevant mutations of CCM3 are unable to rescue the phenotypes that result from knockdown of CCM3. These abnormalities are likely to play a role in the pathogenesis of cerebral cavernomas and, if confirmed, this could set the stage for corrective therapeutic strategies aimed at CCM3.
The antibodies used in this study are: rabbit polyclonal C3M (ProteinTech Group); rabbit polyclonal GM130 CB-1008 (Calbiochem); mouse monoclonal FLAG M2, mouse monoclonal Golgi 58K clone 58K-9, mouse monoclonal α-tubulin clone B-5-1-2 and mouse monoclonal γ-tubulin clone GTU-88 (Sigma-Aldrich); goat polyclonal C3M (sc-67908), goat polyclonal SOKI (sc-6865), goat polyclonal MST3 (sc-21400), goat polyclonal MST4 (sc-7150), goat polyclonal GM130 (sc-16268), mouse monoclonal anti-phospho-ERK (sc-7383) and mouse monoclonal HA probe (sc-7392) (SantaCruz Biotechnology); rabbit polyclonal ERK1-2 4695, rabbit polyclonal MST3 3723 and rabbit polyclonal MST4 3822 (Cell Signaling Technology); mouse monoclonal SOKI clone 1G6 (Abnova); mouse monoclonal cytokeratin c clone 7H8.2C12 (Becton Dickinson Biosciences); rabbit polyclonal GFP (ab290) (Abcam); and rabbit polyclonal 14-3-3-γ-phospho-Ser58 (PAI-4612) (Affinity BioReagents). The secondary antibodies used are: donkey anti-goat AlexaFluor488, goat anti-mouse AlexaFluor488 (Molecular Probes); goat anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories); goat anti-rabbit horseradish peroxidase (HRP), goat anti-mouse HRP (Pierce).

All plasmids were constructed using standard molecular biology techniques. The oligonucleotides designed for the different mutants were purchased from Thermo Scientific (sequence available upon request).

For actin staining, cells grown on glass slides were fixed for 15 minutes in PFA 2%, permeabilized with 0.1% Triton X-100, and incubated with the indicated antibodies followed by fluorescent secondary antibodies. DNA was stained with Hoechst33342. The coverslips were mounted in aqueous medium with anti-fading agents (Gel-Mount) and examined by fluorescent microscopy.

Transfections, immunoprecipitation and immunoblotting HEK293 cells were transfected using the calcium phosphate protocol. SaOS2 cells were transfected using the cell line nucleofector kit with Nucleofector II from Amazax Biosystems following manufacturer’s instructions.

Kinase assays
Extractions were immunoprecipitated with anti-SOK1 antibody and kinase assays were performed as described (Pombo et al., 1996). Briefly, immune complexes were collected with protein G-sepharose beads. Beads were washed three times in lysis buffer, three times in LiCl buffer (500 mM LiCl, 2 mM DTT, 100 mM Tris-HCl pH 7.4) and three times in assay buffer (20 mM MOPS pH 7.2, 2 mM EGTA, 10 mM MgCl2, 0.1% Triton X-100, 1 mM DTT). Kinase assays were started by the addition of myelin basic protein (MBP) and [γ32P]ATP (100 μM, 3000-9000 c.p.m./pmol). After 5 minutes at 30°C, the kinase reactions were stopped with Laemmli sample buffer. Following SDS-PAGE and autoradiography, the bands corresponding to the substrate were cut out of the gel and radioactivity was determined by liquid scintillation counting in a 1414 liquid scintillation counter (Wallac-Winspex).


A

SD

Serum

shControl

shCCM3

B

Fold difference

(Cdc42 and RhoA Activation Signal)

shControl SD

shCCM3 SD

shControl serum

shCCM3 serum

Cdc42

RhoA