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Review

The CCM3-GCKIII partnership

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Summary. Specific mutations in the *CCM3* gene predispose to the development of cerebral cavernous malformations, a special type of vascular lesions. This calls for an elucidation of the precise nature of the CCM3 protein and a deep understanding of its molecular regulation. In this review, we outline our current knowledge of the different CCM3 protein complexes. We focus on the GCKIII family of kinases as partners of CCM3 and discuss the functional consequences of this partnership, putting forward a putative model for the activation of these kinases.

Key words: Ste20 kinases, Cavernous malformations, Golgi apparatus, STRIPAK

Introduction

The protein Cerebral Cavernous Malformation 3 (CCM3), also known as Programmed Cell Death 10 (PDCD10) and TF-1 cell apoptosis-related protein 15 (TFAR15), was first identified as the product of an mRNA that was upregulated during apoptosis in hematopoietic cells (Wang et al., 1999), and was later shown to bind to at least two of the three members of the GCKIII family of Ste20 protein kinases in a double hybrid assay (Rual et al., 2005). CCM3 is a small protein with no clear sequence homology to any protein or domain of known function. It has attracted attention in the last few years because it is the product of a gene whose mutation predisposes to the development of a type of vascular lesions called cerebral cavernous malformations (CCM), in which some vessels of the Central Nervous System form abnormal structures, called caverns, where the endothelial cells have an aberrant shape and excessive permeability (Bergametti et al., 2005; Guclu et al., 2005). CCMs are among the most common vascular malformations of the brain, particularly in young people, and their main clinical manifestations are epileptic seizures and hemorrhagic stroke. Both sporadic and familial forms have been described, the proportion of the latter being close to 20% (Robinson et al., 1991). Three different genes can predispose to this pathology: CCM1/KRIT1, CCM2/OSM, and CCM3/PDCD10 (Labauge et al., 2007; Riant et al., 2010). As stated above, their involvement in a clinically important pathology has spurred significant interest in their biology and their involvement in CCM pathogenesis, which has been extensively reviewed (Labauge et al., 2007; Batra et al., 2009; Faurobert and Albiges-Rizo, 2010; Riant et al., 2010; Fischer et al, 2013). In this article, we will focus on the biochemistry and cell biology of CCM3 and its interacting partners, especially the GCKIII family of Ste20 kinases.

It has been proposed that CCM3 is an adaptor protein. Its crystal structure has been recently resolved (Ding et al., 2010; Li et al., 2010), and it has two distinct domains with a hinge between them (Fig. 1). In this review, we highlight advances in understanding the functional consequences of the interactions of CCM3 with different proteins, especially the GCKIII family of protein kinases, and we finish with a proposal of a possible pathway of activation of GCKIII proteins that

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involves CCM3 and other molecular partners.

The interacting partners of CCM3

GCKIII kinases

Perhaps the most stable partners of CCM3 are the serine threonine GCKIII kinases. Three protein members make up this family: namely SOK1 (also referred to as YSK1 and STK25), Mst3 (also known as STK24) and Mst4 (Pombo et al., 2007; Ling et al., 2008), and they belong to the superfamily of Ste20 proteins. These proteins were named after the *Saccharomyces cerevisiae* Ste20 proteins, which are involved in the yeast mating pathway and are considered mitogen-activated protein kinase kinase kinase kinases (MAP4K) (Pombo et al., 2007), although the members of the GCKIII family of proteins do not seem to activate MAP3 Kinases.

The N-termini of the GCKIII proteins comprise their catalytic domain, while their C-terminal part is considered regulatory. On the C-terminal part of Mst3 a functional nuclear export sequence (NES), a nuclear localization sequence (NLS) and a caspase cleavage site have been identified (Huang et al., 2002; Lee et al., 2004). Mst4 and SOK-1 also have an NLS and a caspase cleavage site, although the NES has still not been proven functional for those two proteins. The C- terminal part of GCKIII proteins also have a so-called WEF motif through which they bind to the scaffold protein MO25 (Filippi et al., 2011).

SOK-1 and Mst3 are activated by stress stimuli (Pombo et al., 1996; Huang et al., 2002), and this activation is important to drive the cells exposed to those insults to a programmed cell death. When cells are exposed to an intense enough stress and caspases are activated, Mst3 and SOK-1 are cleaved by these caspases and their previously inaccessible NLS sequences get exposed, driving their translocation (Huang et al., 2002; Nogueira et al., 2008). This relocation is likely to contribute to cell death even though the mediators of this outcome in the nucleus are still undefined. Interestingly, GCKIII kinases are also activated by stress without the need of caspase activation (Nogueira et al., 2008; Zhou et al., 2009; Costa et al., 2012), and they act in that case without entering the nucleus or being cleaved.

GCKIII kinases also play important roles in non stressed cells. Thus, their catalytic activity is important for Golgi organization and positioning, cell polarity and migration (Preisinger et al., 2004; Fidalgo et al., 2010). Interestingly, the effect over Golgi morphology has morphological consequences in the setting of polarity of both neuronal and non-neuronal cells. For example, the Francis Barr group has shown that SOK1 is important in Golgi positioning in fibroblasts, through the phosphorylation of the 14-3-3ζ (Preisinger et al., 2004). Curiously, it is also through the phosphorylation of the 14-3-3ζ proteins that SOK-1 regulates at least part of its effects on cell death when not activated by caspases (Zhou et al., 2009). Lastly, SOK-1 has been shown to regulate Golgi morphology and axon initiation in neurons through its interaction with the LKB1 kinase and the golgin GM130 (Matsuki et al., 2010).

Furthermore, GCKIII kinases are required for brush border formation of epithelial cells. The groups of Hans Clevers and Johannes L Bos have identified that the LKB1-STRAD-MO25 kinase complex influences the asymmetric distribution of cellular components by the triggering of a signaling pathway involving Mst4 to induce brush border formation through the phosphorylation of Ezrin (ten Klooster et al., 2009; Gloerich et al., 2012). Phosphorylation of Ezrin by Mst4 has also been shown to be significant in the context of cell survival, as cells depleted of Mst4 and subsequently with low levels of phosphorylated Ezrin are more susceptible to die by necrosis than cells with normal levels of Mst4 (Fidalgo et al., 2012).

Another member of the GCKIII family, Mst3 has been shown to perform two apparently unrelated roles in the biology of the cell. This kinase can inhibit cell migration affecting phosphorylation of paxillin, probably through phosphorylation and inactivation of the tyrosine phosphatase PTP-PEST (Lu et al., 2006). Also, it can promote cell cycle progression by phosphorylation of NDR kinases (Stegert et al., 2005).

The GCKIII proteins have also been related to human pathologies other than CCMs, such as Alzheimer' s disease, Parkinson' s disease (PD) and type 2 diabetes. SOK-1 knockdown reduces Tau phosphorylation in embryonic neurons (excessive Tau phosphorylation promotes the formation of neurofibrillary tangles which are evident in diseased neurons in the brains of Alzheimers' s patients at autopsy) (Matsuki et al., 2012). Mst3 and SOK-1 have also been proposed as putative LRRK2 substrates and interacting proteins in an unbiased proteomic approach (mutations in LKKK2 are the most prevalent cause of autosomal dominantly inherited PD) (Zach et al., 2010). Finally, it has recently been observed that in the skeletal

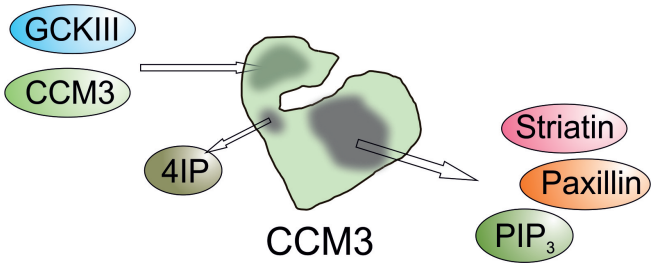

Fig. 1. Interactions of CCM3. A scheme of the CCM3 protein is shown together with the surfaces through which it interacts with other molecules. There are three patches involved in these inter actions. GCKIII proteins or a second CCM3 molecule can bind to the N-terminal lobe. Striatin, paxillin, PI(3,4,5)P3, and possibly other molecules can bind to the C-terminal domain of CCM3, and inositol-(1,3,4,5)-P4 (4IP) binds to a distinct place on the hinge of the molecule.

muscle of type 2 diabetic patients SOK-1 levels are significantly higher than compared with people with normal glucose tolerance and that SOK-1 regulates the expression of relevant metabolic enzymes (Nerstedt et al., 2012).

To our knowledge, the members of the GCKIII family of Ste20 kinases were the first proteins known to interact with CCM3. The first evidence came when Rual et al performed a high throughput double hybrid analysis in yeast, in which they detected interaction of CCM3 (PDCD10 in the article) with both STK24 and STK25 (Rual et al., 2005). The specificity of this detection (no other protein was found to interact with CCM3 or with the kinases) suggested that this might be a high affinity and stable interaction, as we now know is the case. The interaction was soon detected in mammalian cells (Ma et al., 2007; Voss et al., 2007) and our group validated it for the endogenous proteins (Fidalgo et al., 2010). The N-terminal domain of CCM3 binds to the C-terminal non catalytic domain of GCKIII proteins (Fidalgo et al., 2010; Voss et al., 2009; Zheng et al., 2010). That same N-terminal part can mediate CCM3 homodimerization, and interestingly the crystal structure of pure CCM3 shows the protein forming dimers (Li et al., 2010). However, careful binding studies have concluded that the CCM3-GCKIII heterodimer (at least the CCM3-Mst4 complex) has a much higher affinity than both CCM3 and Mst4 homodimers, which can also form (Ceccarelli et al., 2011). Thus, although homodimers of CCM3 and Mst4 (and probably other GCKIII proteins) possibly form at specific times or places in the cell, most of the complexes between CCM3 and the GCKIII kinases are likely to be in a heterodimeric form.

The CCM complex

CCM3 (cerebral cavernous malformation 3) derives its name from its involvement in the development of cerebral cavernomas. The products of the other two genes whose mutation also results in these vascular lesions, CCM1/KRIT1 and CCM2/OSM, bind to each other and form the so-called CCM complex (Zawistowski et al., 2005; Zhang et al., 2007), at least part of which is bound to the orphan receptor HEG1 (homolog of the zebrafish gene *heart of glass*) in endothelial cells (Kleaveland et al., 2009; Gingras et al., 2012). HEG1, CCM1 and CCM2 are all necessary for correct endothelial development, both in zebrafish and in mice (Mably et al., 2003, 2006; Whitehead et al., 2004, 2009; Hogan et al., 2008; Kleaveland et al., 2009; Boulday et al., 2009, 2011). They are important in several aspects of endothelial cell biology, such as regulation of different types of cell-cell junctions, endothelial permeability and barrier function, cytoskeletal organization, cell polarity, and differentiation (Whitehead et al., 2004; Glading et al., 2007; Glading and Ginsberg, 2010; Lampugnani et al., 2010; Stockton et al., 2010; Wustehube et al., 2010). Alterations of the HEG1-CCM complex are suspected to be central in the pathogenesis of cerebral cavernomas, although the exact mechanism connecting them to the disease is still the subject of investigation.

When the CCM1, CCM2 and CCM3 genes were identified, one of the first questions asked was whether they could interact with each other. Indeed, the C-terminal domain of CCM3 can bind to CCM2 and be part of the CCM complex (Hilder et al., 2007; Voss et al., 2007, 2009; Stahl et al., 2008; Li et al., 2010). This binding is likely to be important for endothelial biology, as loss of CCM3 also affects endothelial biology in a manner similar (although not identical) to loss of the other genes (Zheng et al., 2010; Zhu et al., 2010; Chan et al., 2011; He et al., 2010). The connection of CCM3 with the CCM complex has been strengthened by studies in a paralog of CCM2 called CCM2L. CCM2L can bind to CCM1 but not to CCM3, and thus it disrupts the CCM1-CCM2-CCM3 complex. Overexpression of CCM2L has the same phenotypic effects in endothelial cells as loss of CCM2 in mice (Zheng et al., 2012), although the situation may be more complicated in zebrafish (Rosen et al., 2013). Surprisingly, binding of CCM3 to CCM2 is not easy to detect, and usually at least one of the components has to be overexpressed to clearly see the interaction. This suggests that, regardless of its relevance for CCM pathogenesis, the CCM3-CCM2 complex is a minor proportion of all the CCM3 present in the cell at any given time. One possibility could be that this complex is dynamically unstable.

Other molecules

The C-terminus of CCM3 has been found to bind to a plethora of different molecules, mostly located at different domains of the cell membranes. Based on its three-dimensional structure -structurally similar to focal adhesion targeting (FAT) domains- it was predicted and shown to bind to Leucine-Aspartate repeat motifs (LD motifs), such as those of the scaffolding protein paxillin. This interaction has been seen *in vitro*, but several pieces of evidence suggest that CCM3 can also bind to paxillin or other LD motif-containing proteins *in vivo*: the affinity of CCM3 binding to the LD motifs of paxillin is similar (although lower) than that of FAK, a *bona fide* paxillin interactor; and endogenous CCM3 and paxillin colocalize in some cells, specifically in pericytes (Li et al., 2011). CCM3 can also bind to gamma-protocadherins in neurons through which it influences cell survival (Lin et al., 2010), and to the VEGF receptor VEGFR2, affecting its endocytosis (He et al., 2010). Moreover, CCM3 can bind molecules other than proteins. Through a specific pocket on the hinge of the protein, it can bind to inositol-(1,3,4,5)-P4 (Ding et al., 2010), and through its FAT domain to phosphatidylinositol-(3,4,5)-P3 (Dibble et al., 2010).

The largest proportion of CCM3 resides in a complex called STRIPAK (Goudreault et al., 2008). This multiprotein assembly contains the protein phosphatase 2A catalytic subunit, together with scaffolding and

regulatory subunits (PP2A A and striatins, respectively) and several other proteins related to membrane or cytoskeleton biology. CCM3 binds to Striatin 3 through its FAT C-terminal domain, leaving its N-terminus free to bind to a GCKIII protein (Kean et al., 2011).

CCM3 as an adaptor protein for GCKIII kinases

CCM3 has been found in several different places in the cell, including the cellular membrane, the cytoplasm, the cytoplasmic cis face of the Golgi apparatus, and some still-to-be-defined structures in the cell periphery (Fidalgo et al., 2010, 2012; He et al., 2010; Lin et al., 2010, 2011; Kean et al., 2011). In almost all cases, CCM3 is bound to at least one GCKIII protein. CCM3 usually binds the GCKIII proteins through its N-terminal domain while it anchors the dimer to the rest of the structure where they sit through its C-terminal domain. This has been shown for the STRIPAK complex and the CCM1/CCM2 ensemble (Zheng et al., 2010; Kean et al., 2011), and we hypothesize that this configuration may also be true for other molecules that bind to the C-terminus of CCM3, especially membrane proteins such as VEGFR2, paxillin, protocadherins, and possibly also for phosphatidylinositols. This capability of CCM3 to bridge GCKIII proteins to a variety of other molecules may be important to target the kinases to specific molecular complexes/locations in the cell. Indeed, when CCM3 is depleted by RNAi techniques, the GCKIII kinases are no longer targeted to the STRIPAK complex, where a large proportion of them are under normal circumstances (Kean et al., 2011). Further, the GCKIII proteins are destabilized and their levels fall precipitously, making it unlikely that they can be part of the different complexes where they are normally found (Fidalgo et al., 2010).

Some binding partners of the GCKIII kinases do not seem to need CCM3 as an adaptor. The absence of CCM3 does not affect the GCKIII proteins that are located on the cis face of the Golgi. In fact, their amounts may even go up when CCM3 is not available to target the kinases to other places in the cell (Kean et al., 2011). This is consistent with the mechanism by which the CCM3/GCKIII dimer is targeted to the Golgi apparatus. Here, it is the GCKIII kinase, and not CCM3, the partner which binds to the rest of the structure. All three GCKIII proteins can directly bind to the golgin GM130, and they do so through a stretch of aminoacids at the end of their catalytic domain. CCM3 in turn binds to the C-terminus of the kinase forming a ternary complex with GM130 (Preisinger et al., 2004; Fidalgo et al., 2010). While it will be interesting to test whether the C-terminus of CCM3 is engaged in any interaction while the protein is on the Golgi, this does not seem to be important for the targeting of the kinase to GM130. There is at least another relevant interaction of the GCKIII kinases in which CCM3 does not seem to have an influence, or at least no influence of CCM3 has been described. That is their binding to the scaffolding protein MO25 (Filippi et al., 2011; Fuller et al., 2012; Mehellou et al., 2013), which can activate the kinase as we will see below.

Functional consequences of CCM3 and GCKIII partnerships

The binding of the CCM3-GCKIII dimer to STRIPAK seems to inhibit the kinase through dephosphorylation in residues of its activating loop. Since most of the GCKIII proteins reside in the STRIPAK complex at any given moment, this ensemble apparently acts as a store of inactive GCKIII kinases which are ready to be activated when the phosphatase activity of STRIPAK is inhibited. This would explain the phosphorylation and activation of these proteins after okadaic acid treatment at doses known to inhibit PP2A (Fuller et al., 2012). Further, the activation of all three GCKIII proteins by oxidative stress might be related to the sensitivity of PP2A to that stimulus (Foley et al., 2004), something that will be worth exploring in the future. There is also the possibility that the GCKIII kinases can still phosphorylate molecules of the STRIPAK complex, but this has not been described so far.

The binding of GCKIII proteins to the cis Golgi protein GM130 was proposed to activate the kinases when first described. *In vitro* binding of GM130 and SOK1 was shown to result in SOK1 auto-phosphorylation in thr174 on its activation loop (Preisinger et al., 2004). More recently, this simplistic view has been called into question. The activation of full length GCKIII proteins (as opposed to their activation by caspases) encompasses at least two phosphorylation events, one on their activation loop, and a second one on their C-terminus (Thr328 in human Mst3), which is dependent on the former and the result of autophosphorylation. Fully phosphorylated Mst3 dissociates from GM130 and associates to the scaffolding protein MO25 (Fuller et al., 2012), an interaction known to enhance GCKIII activity (Filippi et al., 2011). These two apparently contradictory results lead to the interesting possibility that binding to GM130 may be an intermediate state between inactive GCKIII proteins (those bound to the STRIPAK complex) and fully active kinases. Further work is required to determine if this is indeed the case and, if so, whether binding to GM130 is a necessary step in GCKIII activation in the absence of active caspases.

GCKIII proteins have an intimate relation with the MO25/STRADα/LKB1 complex. *In vitro* binding of MO25α to nonphosphorylated GCKIII kinases can increase their activity moderately (Filippi et al., 2011). Also, overexpression of the MO25 partner STRADα results in the phosphorylation of the cytoskeletal adaptor Ezrin by Mst4. Despite this, STRADα does not change the phosphorylation of the activation loop of Mst4 (ten Klooster et al., 2009), which suggests that its effect is either downstream or independent of this phospho-

rylation event. The results of Fuller et al (Fuller et al., 2012), which show that only fully phosphorylated Mst3 can bind to MO25, suggest that the former may be the case. If so, it could be predicted that MO25 would activate GCKIII proteins pre-phosphorylated in their activation loop much more efficiently than their non-phosphorylated counterparts. Intriguingly, MO25 can activate the equivalent phosphomimetic mutants of the related Ste20 kinases SPAK and OSR1 about 100 fold, despite the fact that its binding affinity to the wild type proteins is even lower than its affinity towards GCKIII proteins (Filippi et al., 2011).

Although CCM3 is generally assumed to bridge active GCKIII proteins to different structures in the cell, and to be important for their function, this has not been formally proved. However, these structures sometimes contain substrates of the kinases, and lack of CCM3 inhibits their phosphorylation and the location of GCKIII kinases in the structure. Even when a substrate has not been identified in a given structure, CCM3 is still important for the activities of GCKIII proteins. Thus, both STK25 and CCM3 knockdown result in Golgi morphology alterations and underphosphorylation of the adaptor protein 14-3-3zeta (Preisinger et al., 2004; Fidalgo et al., 2010). Also, CCM3 can bind to the focal adhesion complex, where the Mst3 substrate PTP-PEST resides (Lu et al., 2006). CCM3 is also important for Mst4 translocation to the cell periphery and phosphorylation of ERM proteins after oxidative stress (Fidalgo et al., 2012), and mediates cell proliferation promoted by Mst4 in other settings (Ma et al., 2007). More relevant to cerebral cavernous malformation development, mutations of CCM3 and GCKIII proteins give rise to similar (although not identical, as we have said before) phenotypes in zebrafish endothelial cell development as CCM1 and CCM2, and this correlates with enhanced Rho activity in all cases (Zheng et al., 2010). Given that CCM3 targets GCKIII proteins to the CCM1/CCM2 complex, this is interpreted as GCKIII proteins playing an important role in CCM development. Whether GCKIII kinases are the effectors of CCM3 in other settings where this adaptor protein binds to different cell structures, such as VEGFR2, protocadherins or phosphoinositides will need further investigation. However, overall the results from many different labs support the idea that CCM3 is important for GCKIII activation and targeting to their place of action and, conversely, that GCKIII proteins are likely to be important effectors of CCM3.

CCM3-GCKIII interactions and kinase activation

Based on what we know about the relationship of CCM3 with GCKIII proteins and with other complexes, we would like to put forward a possible model for caspase-independent GCKIII activation, which is exposed in Fig. 2. According to this, most of the CCM3-GCKIII dimers in the cell would be bound to the STRIPAK complex (Goudreault et al., 2008). CCM3 would bridge the complex to STRIPAK through Striatin (Kean et al., 2011), and GCKIII proteins would be dephosphorylated (Gordon et al., 2011) and thus, probably inactive. In this model, STRIPAK would be an inhibitor of GCKIII but also a reservoir of inactive GCKIII proteins ready to be activated when needed. Even in unstimulated cells, a pool of the CCM3-GCKIII dimers is complexed through its kinase subunit with the GM130, which in turn is bound to the cis Golgi apparatus (Preisinger et al., 2004; Fidalgo et al., 2010). The kinase here would be phosphorylated in its activation loop (Preisinger et al., 2004). We do not know whether there is a significant amount of interchange between these two pools of CCM3-GCKIII. However, the idea that STRIPAK- and GM130-bound dimers are in dynamic equilibrium is consistent with the STRIPAK complex being a ready-to-use reservoir of GCKIII proteins. Upon the presence of an activating stimulus, GCKIII proteins would dissociate from GM130 and bind

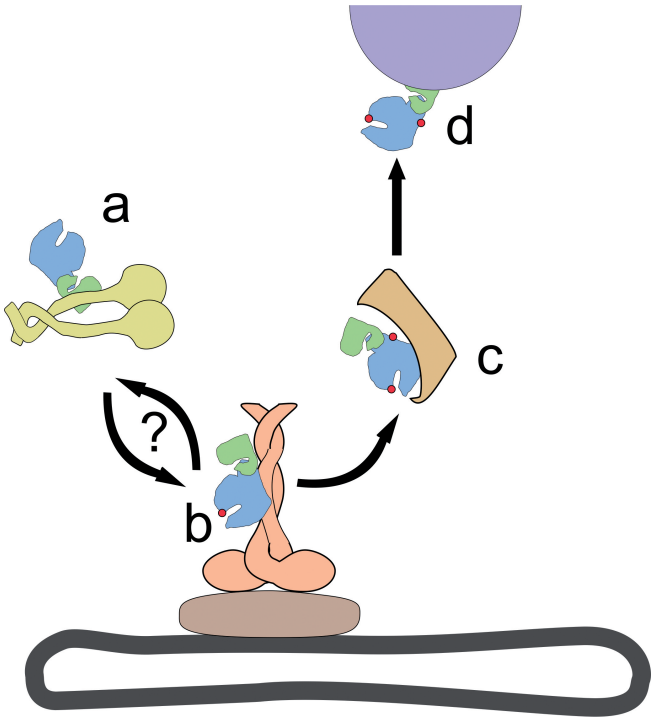

Fig. 2. A proposal for caspase-independent activation of GCKIII proteins. According to this, most of the CCM3 (green)-GCKIII (blue) dimer in unstimulated cells would reside in the STRIPAK complex, bound to the striatin subunit of PP2A and with the kinase in an unphosphorylated, inactive form (a). This would be in dynamic equilibrium with a smaller pool bound to the Golgin GM130 on the cytoplasmic face of the cis side of the Golgi apparatus, where the activation loop of the GCKIII kinase would be phosphorylated, possibly by autophosphorylation (b). Upon activation, the dimer would dissociate from GM130 and bind to the scaffold Mo25, in a process in which the kinase would acquire a second phosphorylation in its C-terminus (c). Finally, the CCM3 would dock the completely phosphorylated kinase to the target cell (d).

to MO25, probably in a transient manner (Filippi et al., 2011; Mehellou et al., 2013), and during this switch or prior to it they would acquire a second phosphorylated residue in its C-terminal domain (Thr328 in Mst3) (Fuller et al., 2012). We have chosen to depict CCM3 bound to GCKIII even though there is no evidence for or against its presence in this complex. Finally, the active GCKIII would dock to different cellular structures to phosphorylate its substrates.

This mechanism of kinase activation would have several advantages for the cell. First, as said above, it would provide a pool of inactive but readily activatable kinases. The involvement of GM130 would also result in enrichment of active kinases in a specific place of the cell, especially if it is polarized. This cell asymmetry in GCKIII activation is also underscored by the involvement of Mo25 in their activation, which is part of a complex important in many aspects of cell polarization.

In summary, GCKIII proteins and the CCM3 adaptor protein form a close partnership and we are just beginning to glimpse its regulation and functions. Further work is required to fully determine its functional consequences. However, it is increasingly clear that the GCKIII/CCM3 dimer has an important role in the regulation of this family of kinases, which involves both purely biochemical mechanisms and important cell biology elements. Study of these mechanisms will help us understand not only the biology of CCMs, but also other important functions of GCKIII kinases.

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