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Adaptor Protein Cerebral Cavernous Malformation 3 (CCM3) Mediates Phosphorylation of the Cytoskeletal Proteins Ezrin/Radixin/Moesin by Mammalian Ste20-4 to Protect Cells from Oxidative Stress*[S]

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Miguel Fidalgo[1,2], **Ana Guerrero**[2], **María Fraile, Cristina Iglesias, Celia M. Pombo, and Juan Zalvide**[3]

From the Department of Physiology and Centro Singular de Medicina Molecular y Enfermedades Crónicas (CIMUS), University of Santiago de Compostela, and the Instituto de Investigaciones Sanitarias (IDIS), 15705 Santiago de Compostela, Spain

Background: The adaptor protein cerebral cavernous malformation 3 (CCM3) is involved in cell death.
Results: Ezrin/radixin/moesin (ERM) proteins are phosphorylated after oxidative stress, and this requires CCM3 and the ERM kinase Mst4.
Conclusion: CCM3 is necessary for ERM protein phosphorylation after stress, which enhances survival.
Significance: This is a novel, functionally significant pathway that protects cells from death.

While studying the functions of CCM3/PDCD10, a gene encoding an adaptor protein whose mutation results in vascular malformations, we have found that it is involved in a novel response to oxidative stress that results in phosphorylation and activation of the ezrin/radixin/moesin (ERM) family of proteins. This phosphorylation protects cells from accidental cell death induced by oxidative stress. We also present evidence that ERM phosphorylation is performed by the GCKIII kinase Mst4, which is activated and relocated to the cell periphery after oxidative stress. The cellular levels of Mst4 and its activation after oxidative stress depend on the presence of CCM3, as absence of the latter impairs the phosphorylation of ERM proteins and enhances death of cells exposed to reactive oxygen species. These findings shed new light on the response of cells to oxidative stress and identify an important pathophysiological situation in which ERM proteins and their phosphorylation play a significant role.

The protein cerebral cavernous malformation 3 (CCM3), also called PDCD10 (programmed cell death 10) is the product of a gene whose mutation predisposes to the development of cerebral cavernous malformations (CCMs)[4], vascular lesions located in the central nervous system (1, 2). Biochemically, CCM3 is an adaptor protein that can bind to a variety of different proteins and protein complexes. CCM3 binds to proteins involved in cell to cell adhesion such as paxillin (3) and to membrane proteins such as the VEGF receptor 2 (4) and the orphan receptor heart of glass homolog 1 (HEG1) (5), in the latter case forming a complex with CCM1 and CCM2, two other proteins whose alteration has been related to cerebral cavernomas (6, 7). CCM3 can also bind to the germinal center kinase III (GCKIII) family of protein kinases (composed of Mst3, Mst4, and SOK1) (8–12), and this binding is important to prevent the development of cerebral cavernomas (13).

The GCKIII subfamily of proteins consists of SOK1 (also referred to as YSK1 and STK25), Mst3 (also known as STK24), and MASK/Mst4 in mammals (14, 15). 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 (16). The GCKIII kinases are involved in two important cellular processes: modulation of cell death and proliferation (17–20), and regulation of the cytoskeleton and Golgi morphology (11, 21, 22).

CCM3 has been found bound to GCKIII kinases on the cis face of the Golgi apparatus, forming a complex with the Golgi matrix protein GM130, and also as a part of a large cytoplasmic multiprotein subunit called STRIPAK, which includes phosphatase 2A, proteins involved in vesicular trafficking, and proteins that bind cytoskeletal elements (23, 24). CCM3 seems to be important for the shift of GCKIII kinases from the cis Golgi to the STRIPAK complex, as its silencing impairs the binding of GCKIII kinases to the STRIPAK complex and enhances their binding to GM130 (24).

CCM3 is also called programmed cell death 10 (PDCD10) on the basis of its up-regulation during apoptosis in hematopoietic cells (1). Thus, CCM3 is likely to be important in cell death. However, its specific role is not clear, as it has been reported to have both prosurvival functions (9) and proapoptotic effects (25–27). We report here the effect of CCM3 on cell survival after oxidative stress. CCM3 is essential for the activation of the GCKIII kinase Mst4 after oxidative stress. Furthermore, Mst4

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[1] Present address, Black Family Stem Cell Institute, Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine.
[2] Fellows from the Ministerio de Ciencia e Innovación.
[3] To whom correspondence should be addressed: Department of Physiology, Laboratory of Cell Signalling and Cancer Research, School of Medicine, University of Santiago de Compostela, San Francisco s/n, 15705 Santiago de Compostela, A Coruña, Spain. Tel.: 34-981-582-658; Fax: 34-981-574-145. E-mail: juan.zalvide@usc.es.
[4] The abbreviations used are: CCM, cerebral cavernous malformation; GCKIII, germinal center kinase III; STRIPAK, striatin-interacting phosphatase and kinase; NAC, *N*-acetyl-L-cysteine; ST, staurosporine; PBN, *N*-tert-butyl-α-phenylnitrone; PI, propidium iodide; ERM, ezrin-radixin-moesin; pERM, phospho-ezrin (Thr-567) radixin (Thr-564) moesin (Thr-558).

phosphorylates ERM proteins to protect cells from death, and inactivation of CCM3 impairs this phosphorylation, resulting in enhanced susceptibility to oxidative stress.

EXPERIMENTAL PROCEDURES

Antibodies, Plasmids, and Drugs—The antibodies used in this study were rabbit polyclonal CCM3 (Proteintech Group); mouse monoclonal β-tubulin (clone B-5–1-2) (Sigma-Aldrich); mouse monoclonal anti-cytochrome *c* (catalog no. 556433) (BD Biosciences); mouse monoclonal GAPDH 6C5 (catalog no. CB-1001) (Calbiochem); goat polyclonal GM130 (catalog no. sc-16268); goat polyclonal SOK1 (catalog no. sc-6865), goat polyclonal MST3 (catalog no. sc-21400), goat polyclonal MST4 (catalog no. sc-7150), rabbit polyclonal ERK2 (catalog sc-154), rabbit polyclonal phospho-p38 (Thr-180/Tyr-182) (catalog no. sc-17852), and mouse monoclonal HA probe (catalog no. sc-7392) (Santa Cruz Biotechnology); rabbit monoclonal Mst4 (catalog no. 2049-1) (Epitomics); rabbit polyclonal ezrin/radixin/moesin (catalog no. 3142), rabbit polyclonal phospho-ezrin (Thr-567)/radixin(Thr-564)/moesin(Thr-558) (catalog no. 3141), rabbit polyclonal p38 (catalog no. 9212), rabbit polyclonal SAPK/JNK (catalog no. 9258), mouse monoclonal phospho-SAPK/JNK (Thr-183/Tyr-185) (catalog no. 9255), mouse monoclonal phospho-ERK1/2 (Thr-202/Tyr-204) (catalog no. 4370), rabbit polyclonal phospho-Akt (Ser-473) (catalog, no. 9271), rabbit polyclonal Mst3 (catalog no. 3723), rabbit polyclonal Mst4 (catalog no. 3822), and rabbit monoclonal cleaved caspase 3 (catalog no. 9661) (Cell Signaling Technology, Inc.); mouse monoclonal SOK1 clone 1G6 (Abnova); rabbit polyclonal 14–3-3ζ-phospho Ser-58 (catalog no. PA1-4612) (Affinity BioReagents); and mouse monoclonal β catenin (BD Biosciences). The secondary antibodies used were goat anti-rabbit Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 546 (Molecular Probes), and goat anti-rabbit HRP and goat anti-mouse HRP (Pierce).

All plasmids were constructed using standard molecular biology techniques.

Hydrogen peroxide (H_2O_2), *N*-acetyl-L-cysteine (NAC), staurosporine (ST), and 2′,7′-dichlorofluorescein diacetate were purchased from Sigma-Aldrich. *N*-tert-butyl-α-phenylnitrone (PBN), 2,2′-azobis(2-methylpropionamidine) dihydrochloride, and propidium iodide were purchased from Calbiochem.

siRNA and shRNA-mediated Knockdown—All chemically synthesized siRNAs were purchased from Dharmacon (siRNA CCM3 ON-TARGETplus and siRNA control ON-TARGETplus) and transfected using a cell line nucleofector kit with a nucleofector II from Amaxa Biosystems. Stable cell populations with silenced CCM3, SOK1, Mst3, Mst4, or control were obtained via selection after lentiviral transduction using MISSION® lentiviral non-target shRNA control transduction particles or MISSION® lentiviral shRNA transduction particles against human CCM3, SOK1, Mst3, or Mst4 from Sigma-Aldrich (TRC). shRNAs sequences are available upon request.

RT-PCR Analysis—RNA was extracted using TRIzol reagent (Invitrogen). RT-PCR was performed by standard procedures using adequate PCR amplification primers (sequence available upon request). PCR products were separated by gel electrophoresis and visualized by ethidium bromide staining.

Cell Culture and Treatments—Hek293 cells, SaOS2 cells, MCF10A cells (ATCC), and primary endothelial human coronary artery endothelial cells (European Collection of Cell Cultures) were grown as recommended.

Treatment with H_2O_2 (Sigma-Aldrich) was at a concentration of 500 μM at the times specified in each individual experiment in KRH medium (115 mM NaCl, 3.6 mM KCl, 1.3 mM KH_2PO_4, 25 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgCl_2$) at 37 °C. When required, 25 mM NAC or 20 mM PBN were added for 30 min before H_2O_2 treatment.

Immunoprecipitation, Immunoblotting, and Kinase Assays—Western blotting and immunoprecipitations were performed by standard procedures, after preparation of extracts in lysis buffer (20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 1 mM DTT, 400 μM PMSF, 2 μM pepstatin, 2.3 μg/ml aprotinin, 2 μM leupeptin). Detection of cytochrome *c* release was performed as in Ref. 19. All Western blot analyses were replicated at least two times to ensure reproducibility.

For kinase assays, extracts were immunoprecipitated with goat polyclonal SOK1 antibody, goat polyclonal Mst3 antibody, or goat polyclonal Mst4 antibody, and kinase assays were performed as described (28).

Fluorescence and Image Analysis—For immunofluorescence, cells were cultured on polylysine-covered coverslips and fixed for 15 min in paraformaldehyde 4%, permeabilized for 10 min in PBS with 0.25% Triton X-100 (or fixed in 50% methanol-50% acetone for endogenous Mst4), preincubated for 30 min with 1% BSA in PBS with 0,25% Triton X-100, and incubated with the indicated antibodies followed by fluorescent secondary antibodies. DNA was stained with Hoechst 33342. The coverslips were mounted in aqueous medium with anti-fading agents (gel/mount). Confocal images were collected using a Leica confocal microscope equipped with a high grade color corrected plan apochromat lens for confocal scanning ×63/1.32 objective. Leica Confocal Software was used for acquisition and analysis. Images are combinations of optical sections taken in the *z* axis at 0.5-μm intervals. For all microscope photographs, Adobe Photoshop software was used to cut, resize, and mount the photographs in the figures.

Determination of Cell Viability—Unless stated otherwise, cell viability was determined by trypan blue exclusion assay. Briefly, cells were stained with trypan blue solution (0.08%) (Sigma-Aldrich) at specific times after treatment with H_2O_2 (500 μM) or ST (50 nM) for 5 h. Dead cells (blue) *versus* live cells were counted under a microscope. Cell viability is expressed as the percentage of dead cells.

For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assay, cells were seeded in a 96-well plate (25.000 cells/cm^2) for 24 h and then exposed to various concentrations of ST for 5 h. At the end of the incubation with the drug, the cells were incubated in 100 μl of a 0.5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) at 37 °C for 4 h and lysed in 100 μl of the solubilization solution (0.01 M HCl, 10% SDS) at 37 °C

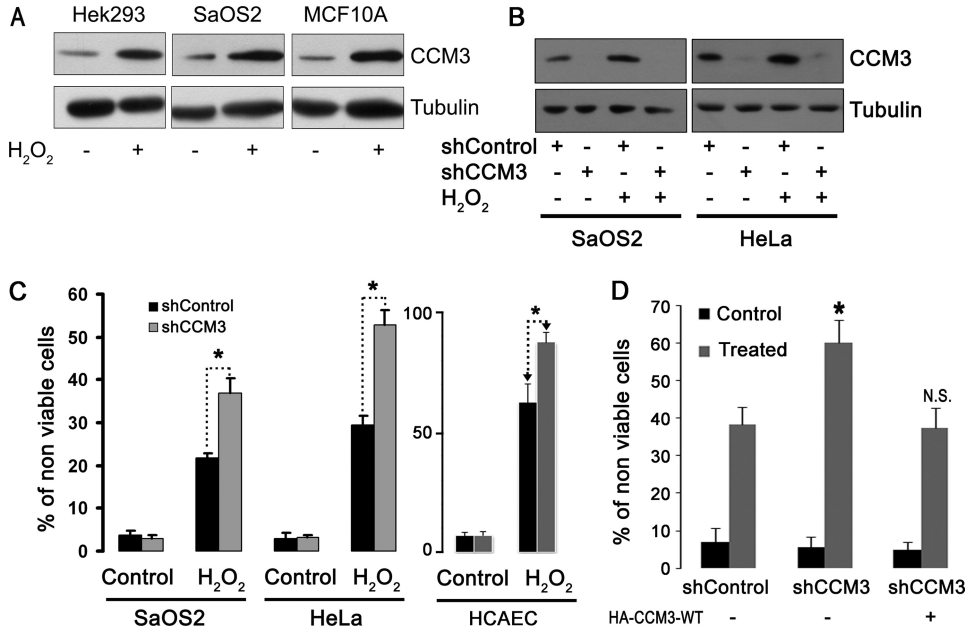

FIGURE 1. **CCM3 is up-regulated by reactive oxygen species and protects cells from death.** *A*, Western blot analysis of CCM3 in renal cells (Hek293), osteosarcoma cells (SaOS2), and mammary cells (MCF10A) treated with 500 μM hydrogen peroxide for 40 min. Tubulin is shown as a loading control. *B*, Western blot analysis of CCM3 in control and hydrogen peroxide-treated SaOS2 and HeLa cells (*left panel*), and HCAEC primary endothelial cells (*right panel*) after down-regulation by lentivirally encoded shRNA. Tubulin is shown as a loading control. *C*, effect of CCM3 shRNA on cell death after oxidative stress as assessed by trypan blue exclusion. Shown is mean ± S.D. of three different experiments. *, $p < 0.01$. *D*, effect of CCM3 expression on cell death after oxidative stress in CCM3 down-regulated cells as assessed by trypan blue exclusion. SaOS2 cells stably transduced with CCM3 or control shRNA were transfected with empty plasmid (HA) or HA-tagged CCM3 and subjected to the same treatment and analysis as in *D*. *, $p < 0.01$. *N.S.*, non-significant *versus* treated shControl cells.

overnight. The absorbance of each well was measured at 550 nm in a microplate reader.

For the staining of viable cells with propidium iodide (PI), ~40.000 SaOs2 cells were seeded on coverslips. After the indicated treatment, cells were washed with binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$) and then stained with 0.5 μg/ml PI and 5 μM Hoechst for 15 min at room temperature. Cells were washed again with binding buffer, and images were collected using a fluorescence microscope.

Statistical Analysis—The statistical significance of all data obtained was assessed by Mann-Whitney tests. All data were analyzed using SPSS software, version 12.0.0.

RESULTS

CCM3 Protects Cells from Accidental Death Induced by Oxidative Stress—CCM3 is up-regulated in several forms of cellular stress, but its regulation after oxidative stress has not been described. Thus, we measured CCM3 levels in HEK293 renal cells, SaOS2 cells derived from osteosarcoma, and MCF10A mammary cells both before and after oxidative stress treatment. Incubation with H_2O_2 resulted in a clear induction of CCM3 in all cells tested (Fig. 1*A*). This induction was dependent on reactive oxygen species (ROS) generated during the treatment because it could be inhibited by the ROS scavenger NAC (supplemental Fig. S1*A*) and was accompanied by an elevation of CCM3 mRNA levels (supplemental Fig. S1*B*). Thus, both CCM3 protein and mRNA are induced by oxidative stress.

To evaluate the actions of CCM3 during stress, we inhibited its expression by RNA interference using a small hairpin RNA that down-regulated CCM3 efficiently (Fig. 1*B*). SaOS2, HeLa, and primary endothelial cells with inhibited CCM3 were more susceptible to death after ROS exposure (Fig. 1*C*). The effect of the shRNA was dependent on CCM3 down-regulation because it could be rescued by exogenous CCM3 (Fig. 1*D*), and cells transiently transfected with an unrelated synthetic CCM3 siRNA were also more susceptible to ROS exposure (supplemental Fig. S2*B*; see supplemental Fig. S2*A* for the efficiency of siRNA-mediated silencing). Moreover, the effect of CCM3 seemed to be to protect from cell death rather than simply delaying it, as the viability of cells without CCM3 was impaired even 24 h after hydrogen peroxide treatment (supplemental Fig. S3). We concluded that cells with down-regulated CCM3 are more susceptible to oxidative stress and that this is not dependent on the specific cell type or the strategy used to attain the down-regulation of CCM3.

CCM3 has been reported to be important in apoptotic cell death, although the exact nature of its involvement is the subject of controversy. On the other hand, ROS can induce several mechanisms of cell death depending, at least in part, on their levels. In our experimental conditions, hydrogen peroxide did not induce cytoplasmic release of cytochrome *c* to any significant degree, as opposed to a well known apoptosis inducer such as staurosporine (Fig. 2*A*). Although CCM3 seemed to prevent cytochrome *c* release after staurosporine treatment (Fig. 2*A*), as published previously (26), we did not detect significant differences when we compared cell death after this stimulus both by trypan blue exclusion and by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide viability assay (supplemental Fig. S4). Hydrogen peroxide did induce an important increase in permeability to the vital dye propidium iodide, and lack of CCM3 significantly increased this permeability (Fig. 2*B*), which

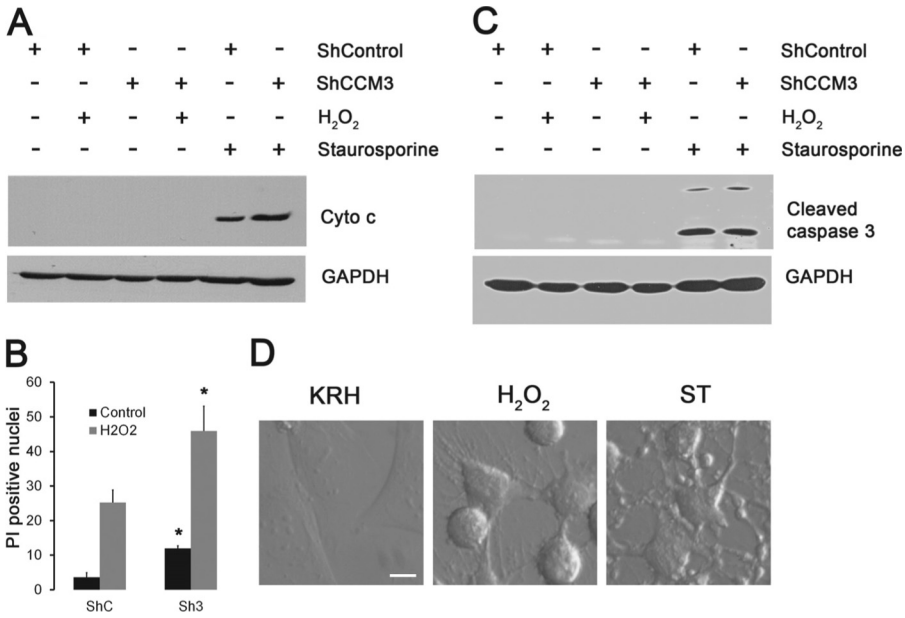

FIGURE 2. **CCM3 inhibition of cell death induced by reactive oxygen species.** *A*, SaOS2 cells transduced with control or CCM3 shRNA were incubated in KRH medium for 60 min and then left untreated, treated for 90 min with 500 μM H_2O_2, or treated for 5 h with 500 nM staurosporine as indicated. Cytoplasmic extracts were obtained, and Western blot analyses for cytochrome *c* and GAPDH were performed. *B*, percentage of nuclei positive for PI after incubation with PI and Hoechst 5 μM in SaOS2 cells stably transduced with CCM3 or control shRNA and treated for 90 min with 500 μM H_2O_2 or not. Mean ± S.D. in three independent experiments is shown. *, $p < 0.01$ *versus* ShC. NS, not significant, $p = 0{,}053$ *versus* ShC. *C*, SaOS2 cells transduced with control or CCM3 shRNA were incubated in KRH medium for 60 min and then left untreated or treated for 90 min with 500 μM H_2O_2 or 500 nM staurosporine as indicated. Cells were then allowed to recover for 3.5 h. Extracts were prepared, and Western blot analyses for activated caspase 3 and GAPDH were performed. *D*, SaOS2 control cells were treated as in *B*, and photographed by Hoffman contrast at the end of treatment.

is consistent with CCM3 protecting cells from accidental cell death (which is characterized by the loss of membrane integrity (29)) after oxidative stress. The differences between hydrogen peroxide and staurosporine effects on cell death were not due to different times of exposure to each death inducer because activated caspase 3 was readily detectable after 90 min of staurosporine treatment followed by 3.5 h of recovery in growth medium but not after the same regime of exposure to hydrogen peroxide (Fig. 2*C*). Besides, under these conditions staurosporine induced distinct morphological changes consistent with the generation of apoptotic bodies, whereas hydrogen peroxide did not (Fig. 2*D*). On the other hand, staurosporine did not induce ROS accumulation in treated cells to any significant degree, contrary to hydrogen peroxide (supplemental Fig. S5*A*).

CCM3 Is Important for Mst4-dependent Phosphorylation of ERM Proteins after Oxidative Stress—Lack of CCM3 did not affect the levels of reactive oxygen species before or after oxidative stress (supplemental Fig. S5*B*). The induction of AKT, the MAP kinases JNK, p38, and ERK after oxidative stress was also intact in cells with silenced CCM3 (supplemental Fig. S6). Thus, we searched for other possible effectors of CCM3.

The GCKIII family of protein kinases, which bind to CCM3 and are stabilized by it (11), can phosphorylate and activate the ERM family of cytoskeletal regulators, which can bridge actin to membrane proteins. We first wanted to know if the phosphorylation status of the ERM proteins is altered after cellular stress. We found that treatment of control cells with H_2O_2 induced the phosphorylation of ERM proteins at their activating residue (Thr-567 ezrin/Thr-564 radixin/Thr-558 moesin). This enhanced phosphorylation was detectable after moderate levels of H_2O_2 and was still evident and consistent at 500 μM H_2O_2 (Fig. 3*A*). ERM phosphorylation depended on ROS, as it could be inhibited by two unrelated ROS scavengers, NAC and PBN (Fig. 3*B*), and it peaked at 40 min after treatment (*C*). Further, ERM was also phosphorylated after treatment with another unrelated oxidative stress inducer, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (30) (Fig. 3*D*).

We next assessed whether the phosphorylation of ERM proteins after oxidative stress was affected by CCM3 status. Both SaOS2 cells (Fig. 4*A*) and primary endothelial cells (*B*) with silenced CCM3 were partially defective in ERM phosphorylation after oxidative stress. This was dependent on CCM3 down-regulation, as expression of CCM3 rescued the effect (Fig. 5*E*). To study whether lack of ERM phosphorylation could account for the excessive cell death after oxidative stress in the absence of CCM3, we transfected an ezrin phosphomimetic mutant (T567D) (31) in CCM3 knockdown cells, in which endogenous ERM proteins are not effectively phosphorylated after oxidative stress. We found that it made them less susceptible to cell death after oxidative stress, something that did not happen when the T567A non-phosphorylatable mutant or the wild type version of ezrin was transfected (Fig. 4*C*). We concluded that the phosphomimetic ezrin mutant protects cells from cell death induced by oxidative stress when CCM3 is not present, which suggests that activated ERM proteins are important effectors of CCM3 upon oxidative stress.

As a first approach to understanding how CCM3 affects ERM phosphorylation, we measured the kinase activity of the three GCKIII kinases and found that all of them were activated by oxidative stress. This was already known for SOK1 and Mst3 (18, 20, 28), but this is the first time it is reported for Mst4. This activation correlated with CCM3 up-regulation (Fig. 1), cell

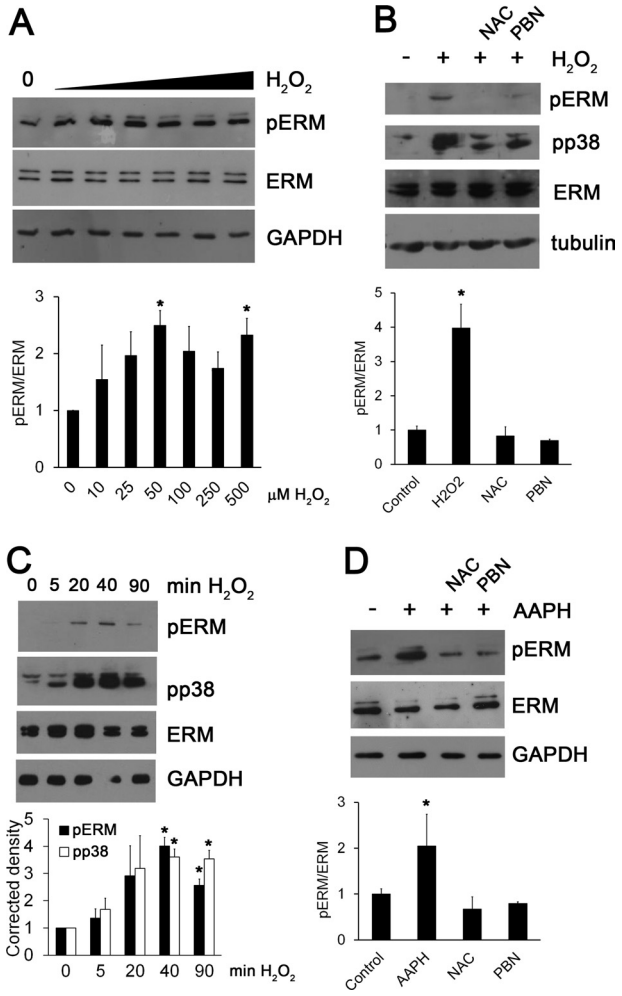

FIGURE 3. **Oxidative stress induces ERM phosphorylation.** *A*, Western blot analyses for Thr-567 ezrin/Thr-564 radixin/Thr-558 moesin (pERM), total ERM, and GAPDH of SaOS2 cells treated for 40 min with 0, 10, 25, 50, 100, 250, and 500 μM H_2O_2. B, Western blot analyses of pERM, total ERM proteins (*pp38*), and GAPDH of SaOS2 cells treated with 500 μM H_2O_2 in the absence or the presence of 25 mM *N*-acetyl-cysteine and 20 mM PBN. *C*, Western blot analyses of pERM, total ERM proteins, phosphorylated p38 (*pp38*), and GAPDH of SaOS2 cells treated for the indicated amounts of time minutes with 500 μM H_2O_2. *D*, Western blot analyses for pERM, total ERM, and GAPDH of SaOS2 cells treated with 500 μM H_2O_2 in the absence or the presence of 25 mM *N*-acetyl-cysteine and 20 mM PBN. Densitometric scans are shown below the Western blot analyses. *, $p < 0.05$ with respect to control.

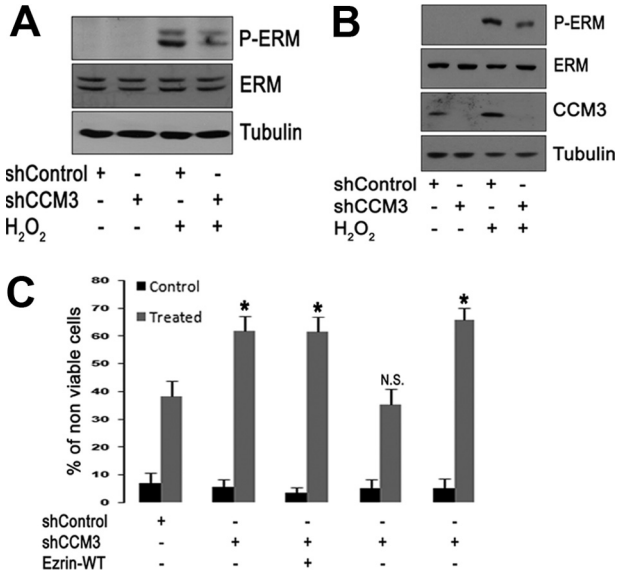

FIGURE 4. **ERM phosphorylation after oxidative stress depends on CCM3 and has a prosurvival action.** *A*, Western blot analyses for pERM, total ERM, and GAPDH in SaOS2 cells transduced with control or CCM3 shRNA and either left untreated or treated with 500 μM hydrogen peroxide for 40 min. *B*, Western blot analyses for pERM, total ERM, and GAPDH in HCAEC primary endothelial cells transduced with control or CCM3 shRNA and either left untreated or treated with 500 μM hydrogen peroxide for 40 min. *C*, cell death was assessed by trypan blue exclusion in SaOS2 cells transduced with control shRNA or shRNA against CCM3; transfected with empty plasmid or with the wild type, T567D, or T567A ezrin; and either left untreated or treated with 500 μM H_2O_2 for 90 min. Shown is the mean ± S.D. of three different experiments. *, $p < 0.01$. *N.S.*, non-significant *versus* treated shControl cells.

death, and ERM phosphorylation in three different cell types (supplemental Fig. S7). Lack of CCM3 did not affect the activation of Mst3 or SOK1 but significantly blunted the activation of Mst4 (Fig. 5*A*). The lack of effect of CCM3 on activation of SOK1 is consistent with its lack of effect on the phosphorylation of the SOK1 substrate 14-3-3ζ after oxidative stress (supplemental Fig. S6*B*). We found the lack of effect of CCM3 inhibition on oxidative stress-induced activity of SOK1 and Mst3 surprising. Lack of CCM3 results in a shorter half-life and lower levels of these two kinases (11), and its lack of effect after oxidative stress suggests the existence of different cellular pools of these kinases, which is consistent with results published recently (24). We are pursuing this interesting observation now, but for this study, it implies that if a GCKIII kinase is downstream of CCM3 in its effects on ERM phosphorylation under oxidative stress, this is likely to be Mst4.

Inhibition of Mst4 by RNA interference, but not inhibition of Mst3 or SOK1, rendered cells more susceptible to oxidative stress (Fig. 5, *B* and *C*), consistent with Mst4 being in the same pathway as CCM3, and overexpression of Mst4 restored the susceptibility of cells lacking CCM3 to normal levels (*D*). Conversely, overexpression of SOK1 or Mst3 in the same conditions induced massive cell death (data not shown), which is consistent with published results (18, 19). Taking these results together with the kinase activity measurements, we concluded that Mst4, but not SOK1 or Mst3, was downstream of CCM3 in its cell protective function. Expression of Mst4 also rescued the lack of ERM phosphorylation in CCM3-depleted cells (Fig. 5*E*), which strongly suggests that Mst4 is downstream of CCM3 in this effect. On the other hand, lack of Mst3 or Mst4, but not of SOK1, inhibited phosphorylation of ERM proteins after oxidative stress (Fig. 5*F*). Given the ability of Mst4 to phosphorylate Ezrin *in vitro* (32) and to rescue both the enhanced cell death and the lack of ERM phosphorylation seen in the absence of CCM3, we focused our attention in this kinase. It is important to note that we could not perform rescue experiments with Mst3 because of the massive cell death induced by its overexpression (see above).

Mst4 Colocalizes with pERM Proteins at the Cell Periphery upon Oxidative Stress—To analyze the phosphorylation of ERM proteins further, we stained cells with a phosphospecific ERM antibody by immunofluorescence. Cells treated with H_2O_2 accumulated phospho-ERM proteins at the periphery of the cell, and this was inhibited when CCM3 was down-regulated (figure 6*A*). As expected for the kinase that

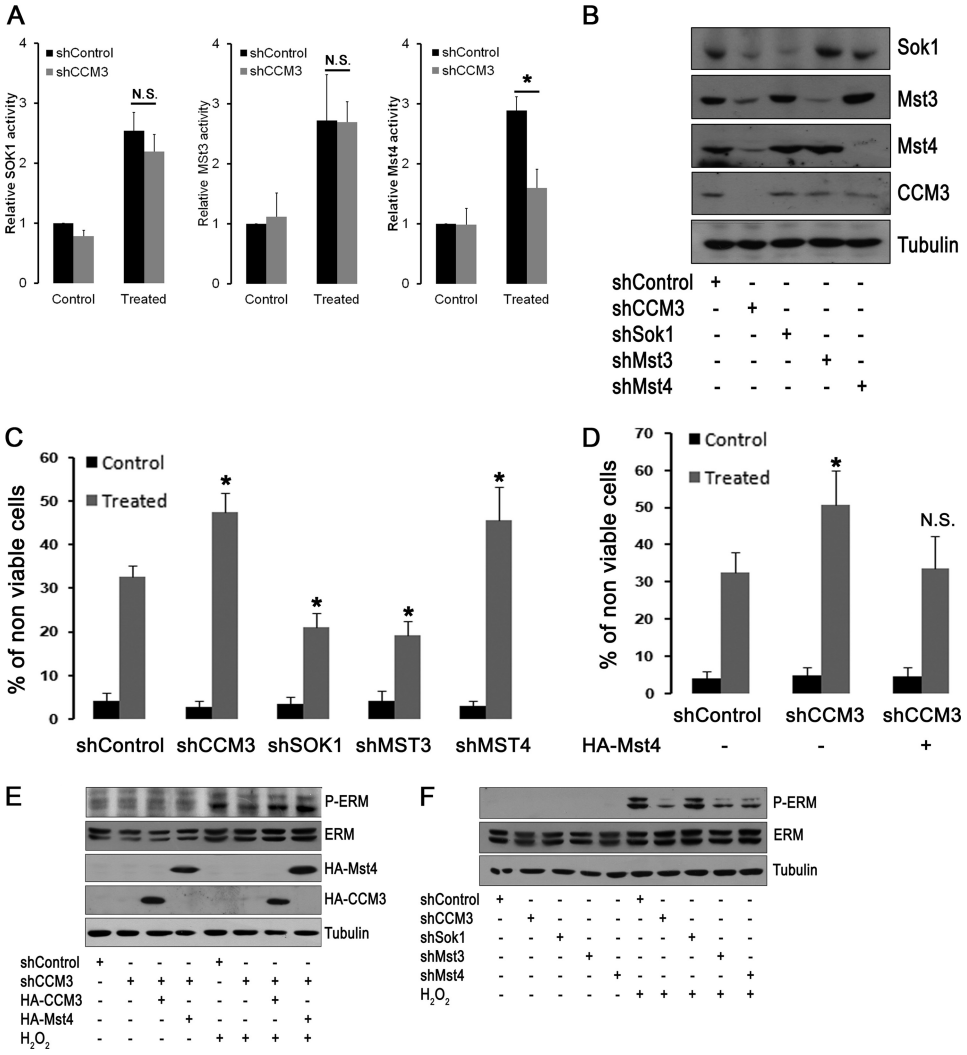

FIGURE 5. **The GCKIII kinase Mst4 mediates ERM phosphorylation and cell survival by CCM3.** *A*, kinase assays of SOK1, Mst3, and Mst4 in SaOS2 cells transduced with control or CCM3 shRNA, untreated or treated with 500 μM H_2O_2 for 40 min. Shown is the mean ± S.D. of three different experiments. *N.S.*, non-significant differences. *, $p < 0.01$. *B*, Western blot analyses of GCKIII proteins, CCM3, and tubulin after RNA interference of the indicated genes. *C*, effect of down-regulation of GCKIII proteins and CCM3 on cell death after treatment with 500 μM H_2O_2 for 90 min, as assessed by trypan blue exclusion. Shown is the mean ± S. D. of three different experiments. *, $p < 0.01$ *versus* treated shControl cells. *D*, effect of Mst4 expression on cell death after oxidative stress in CCM3 down-regulated cells as assessed by trypan blue exclusion. *, $p < 0.01$. *N.S.*, non-significant *versus* treated shControl cells. *E*, Western blot analyses for pERM, ERM, HA (shown in *two stripes* for convenience), and tubulin in SaOS2 cells transduced with control or CCM3 shRNA, transfected with empty plasmid, HA-CCM3, or HA-Mst4 and either left untreated or treated with hydrogen peroxide. *F*, Western blot analyses for p-ERM, ERM, and tubulin, in SaOS2 cells transduced with shRNAs against CCM3, SOK1, Mst3, Mst4, or control shRNA and either left untreated or treated with hydrogen peroxide.

phosphorylates ERM proteins, Mst4 colocalizes with them after oxidative stress. In untreated cells, endogenous Mst4 had a cytoplasmic distribution with some accumulation in the perinuclear region, which is consistent with Mst4 being localized partly in the Golgi apparatus but less than the other GCKIII kinases (11). After oxidative stress, Mst4 concentrated in the cell periphery, forming accumuli that were similar to those formed by pERM proteins. When CCM3 was down-regulated, Mst4 was even more clearly visible in the Golgi apparatus (Figure 6*B*), which suggests that the pool of Mst4 that is down-regulated in this situation is out of the Golgi. Upon oxidative stress, Mst4 formed significantly less accumuli in CCM3-depleted cells than in normal cells (15 ± 11.2 accumuli per cell in normal cells *versus* 5.2 ± 3.9 in CCM3-depleted cells, $p = 0.001$) (Fig. 6*B*), and a visible perinuclear signal remained in many cells. This partial location of Mst4 at the cell periphery may be due to incomplete silencing of CCM3 or due to the fact that CCM3 is not absolutely required for it. Consistent with Mst4 dissociating from the Golgi complex upon stress, when it was costained with the Golgi protein GM130 (Fig. 6*C*), we found colocalization of Mst4 with GM130 in untreated cells (Pearson's correlation 0.4875), whereas the loss of GM130 signal prevented colocalization quantitation in hydrogen-peroxide-treated cells.

We could not costain Mst4 with pERM proteins because of antibody incompatibility. To overcome this, we overexpressed GFP-Mst4 and stained cells with pERM antibody. GFP-Mst4, but not GFP, colocalized with pERM in treated cells (Pearson's correlation −0.055 for untreated *versus* −0.026 for treated GFP cells; −0.153 *versus* 0.742 in GFP-Mst4 cells) (Fig. 6*D*), suggesting that Mst4 colocates with pERM after oxidative stress.

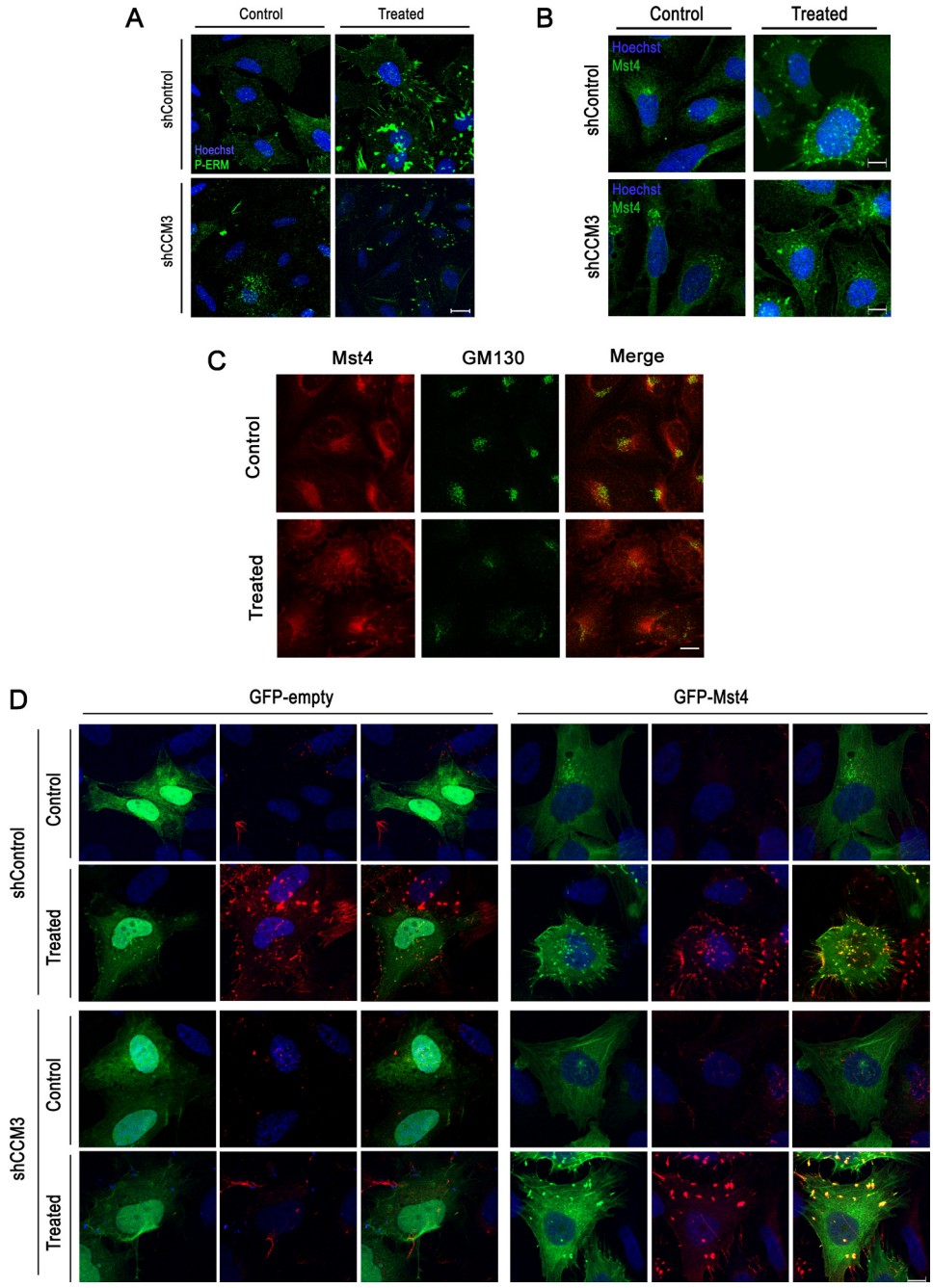

FIGURE 6. **Mst4 translocates from the Golgi complex to the cell periphery upon oxidative stress.** *A*, fluorescence image of Hoechst 33342 (*blue*) and pERM (*green*) in SaOS2 cells transduced with control shRNA or shRNA against CCM3, untreated or treated with 500 μM H_2O_2 for 40 min. *Scale bar* = 20 μm. *B*, fluorescence image of Hoechst 33342 (*blue*) and endogenous Mst4 (*green*) in cells transduced with control shRNA and treated as in *A*. *Scale bar* = 10 μm. *C*, immunofluorescence of endogenous Mst4 and GM130 in cells transduced with control shRNA and treated as in *A*. *D*, fluorescence of pERM (*red*), GFP fluorescence (*green*), Hoechst 33342 (*blue*), in SaOS2 cells transduced with control or CCM3 shRNA, transfected with GFP or GFP-Mst4, and either left untreated, or treated with 500 μM H_2O_2. *Scale bar* = 20 μm.

DISCUSSION

Here, we describe a novel pathway that is important in the life-or-death decision the cell has to make when subject to oxidative stress. It involves phosphorylation of the activating residue of ERM proteins by the GCKIII kinase Mst4, which is activated and redistributed, in turn, upon oxidative stress in a manner that is dependent on the adaptor protein CCM3 (see Fig. 7 for a schematic of the pathway).

Before this report, Mst4 had been shown to phosphorylate ezrin in enterocytes where polarization had been induced by Ste20-related kinase adapter alpha overexpression (32). In this work, we show for the first time that ERM phosphorylation by Mst4 plays a role in a pathophysiological relevant situation, the response to stress. Several different kinases can phosphorylate ERM proteins to activate them. Rho kinase has been shown to be essential for ERM phosphorylation in response to lysophosphatidic acid, in formation of microvilli, and in cell transformation induced by Rho activation (33–35), whereas PKCα has been proposed to phosphorylate these proteins in wound healing assays (36). Significantly, Mst4 is not the first Ste20 kinase to

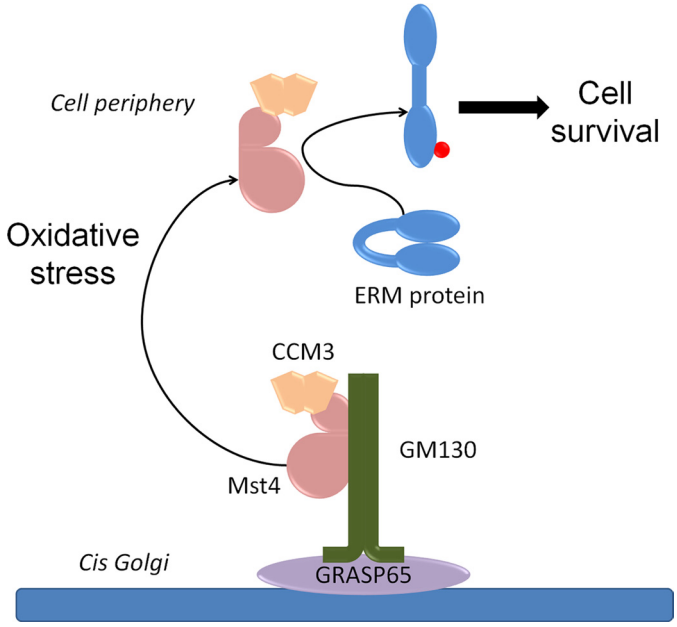

FIGURE 7. **Schematic of the CCM3-Mst4-ERM pathway.** Mst4 is at least partially located on the cis-Golgi, forming a complex with GM130 in unstressed cells. After oxidative stress, it is activated and relocated to the cell periphery in a CCM3-dependent manner. There, it phosphorylates (represented as a *red dot*) the activating residues of ERM proteins. As a result, the cell is protected from death.

be known as an ERM kinase. Nck-interacting kinase phosphorylates ERM proteins to promote the formation of lamellipodia (37), and Slik is the major kinase of moesin in *Drosophila melanogaster* (38). All these data suggest that ERM proteins may act as hubs, being targets of different pathways and kinases, depending on the stimuli the cells receive.

Mst3 is also necessary for normal phosphorylation of ERM proteins after oxidative stress. However, Mst3 is also cleaved by caspases in late stages of apoptosis. The resulting catalytic fragment enters the nucleus, where it has undefined pro-death actions (18). These pro-death functions are likely to overcome any Mst3 prosurvival action because of ERM phosphorylation. Moreover, apart from their function in apoptosis, all GCKIII kinases may have a role in necrosis. In fact, the three of them are activated during this process, as shown in this paper and as already shown for SOK1 (39).

The adaptor protein CCM3 is important for ERM phosphorylation after stress. CCM3 can bind to GCKIII kinases, forming a complex with the cis-Golgi protein GM130 and also as a part of the STRIPAK complex. We find that, in the absence of CCM3, Mst4 is more concentrated in the perinuclear region of the cell and that its relocation to the cell periphery upon stress is less efficient (although it still happens to a small degree, see figure 6*B*). This is consistent with the results reported by Kean *et al.* (24), who have shown that CCM3 is important for the distribution of Mst4 between GM130 and the STRIPAK complex. Thus, CCM3 seems to act as a switch for Mst4 between GM130 and STRIPAK. When CCM3 levels are down-regulated, Mst4 redistributes to the cell periphery less efficiently, and its levels are lower (Fig. 4*B*), probably because of enhanced degradation (11).

Mutations in the CCM3 gene give rise to cerebral cavernous malformations, a clinically important type of vascular malformations. The involvement of CCM3 in the response to oxidative stress may also be important for CCM development, as ROS have been shown to alter tight junctions in endothelial cells through alteration of the cytoskeleton, and defects in the blood-brain barrier are a hallmark of CCMs. Intriguingly, another CCM gene, CCM1, has also been shown to be important for cell response to oxidative stress. CCM1 affects the handling of ROS by modulating the expression and activity of the transcription factor FoxO1 (40), a manner that is different from the one described here. The fact that two different CCM genes affect the same cellular function independently, suggest that this function is important in the pathogenesis of CCMs. On the other hand, the involvement of CCM3 in the regulation of ERM proteins in situations other than oxidative stress can also play a role in the pathogenesis of CCMs.

Death of cells after their exposure to stress occurs in many pathophysiologically relevant scenarios. Depending on the kind of stress and its intensity, death can happen through several mechanisms, such as apoptosis, autophagy, and necrosis, among others (41). Rupture of the plasma membrane is considered the point of no return during necrotic cell death (29), and membrane anchoring to the cytoskeleton is important for its integrity. Thus, changes in the linkage of the cytoskeleton to the plasma membrane could, in principle, modulate cell resistance to necrosis. The phosphorylated ERM proteins link filamentous actin in the cell cortex to membrane proteins on the surface of cells (42, 33). Given our results, we hypothesize that phosphorylation of ERM proteins upon oxidative stress strengthens the linkage of the actin cytoskeleton to membrane proteins, providing structural support to the plasma membrane. In fact, ERM has been shown to be phosphorylated after other kinds of stress, such as osmotic stress, where the membrane integrity is also at risk (43).

In summary, we have unveiled a novel mechanism of protection against extreme forms of stress that inhibits accidental cell death or necrosis by activating a cytoskeletal regulator. These results shed new light on how cells protect themselves when subject to stress and open a new avenue of research on how cytoskeletal regulation may be related to necrosis.

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