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The PDZ-binding motif of MCC is phosphorylated at position −1 and controls lamellipodia formation in colon epithelial cells

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ABSTRACT

In this study, we describe a new post-translational modification at position −1 of the PDZ-binding motif in the mutated in colorectal cancer (MCC) protein and its role in lamellipodia formation. Serine 828 at position −1 of this motif is phosphorylated, which is predicted to increase MCC binding affinity with the polarity protein Scrib. We show that endogenous MCC localizes at the active migratory edge of cells, where it interacts with Scrib and the non-muscle motor protein Myosin-IIB. Expression of MCC harboring a phosphomimetic mutation MCC-S828D strongly impaired lamellipodia formation and resulted in accumulation of Myosin-IIB in the membrane cortex fraction. We propose that MCC regulates lamellipodia formation by binding to Scrib and its downstream partner Myosin-IIB in a multiprotein complex. Importantly, we propose that the function of this complex is under the regulation of a newly described phosphorylation of the PDZ-binding motif at position −1.

1. Introduction

Mutated in colorectal cancer (MCC) is emerging as a multifunctional protein in epithelial cells. MCC is required for cell migration of epithelial cells [1] and also affects several other cellular processes and pathways, such as β-catenin (Wnt) signaling [2], NFκB signaling [3,4], the DNA damage response [5], and the cell cycle [6]. MCC contains a PDZ (PSD-95/Dlg/ZO-1) binding motif "ETSL" in its extreme carboxy terminus, which binds the polarity protein Scrib [1,7] and is predicted to interact with other cellular PDZ domain-containing substrates. The importance of this motif in the physiological role of MCC is underlined by its extreme evolutionary conservation. The consensus motif X-T/S-X-L/V/I is described as one of the most common protein–protein interaction motifs [8].

Scrib has four PDZ domains, two of which (PDZ-1 and PDZ-3) bind MCC. Scrib is involved in a variety of cellular functions including cell polarity and migration [9]. Scrib regulates cell migration by anchoring the PAK-interacting exchange factor (β-PIX), which in turn activates the p21 activating kinase (PAK) at the front of motile cells (lamellipodia). This Scrib–βPIX–PAK complex regulates a number of downstream substrates required for cell migration and cytoskeletal reorganization [10]. The molecular basis for MCC migratory activity is unknown, but unlike Scrib, it may not involve the activation of Rac1, Cdc42 or PAK [1].

The mechanism by which the Scrib–MCC complex mediates its signaling is still open to debate but is likely to involve precise spatial/temporal localization of key regulatory proteins [11]. Here we show that MCC interacts with both Scrib and the Myosin-IIB motor protein and regulates lamellipodia formation through phosphorylation of its PDZ-binding motif. Finally, we propose a mechanism by which Scrib plays a scaffolding role in bringing MCC/Myosin-IIB in close proximity with the βPIX/PAK complex. Importantly, this work presents the first functional report of a new post-translational modification at position −1 of the PDZ-binding motif.

2. Materials and methods

2.1. Cell culture

Caco2, MCF10A, HCT116 and HCT15 human cancer cell lines were obtained from the American Type Culture Collection and propagated in MEM, DMEM-F12 or RPMI 1640 media supplemented with fetal calf serum (10%), HEPES 10 mM, insulin 0.3 units/ml, 100 units/ml penicillin, and 100 μg/ml streptomycin. The HCT15 cells have a complete loss of endogenous MCC due to silencing of the MCC promoter [12] and Caco2 cells show greatly reduced MCC expression.

Abbreviations: MCC, mutated in colorectal cancer; PDZ, post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (DlgA), or Zonula occludens-1 protein (zo-1); PAK, p21 activated kinase

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2.2. Antibodies

The following antibodies were used: anti-MCC (610740, BD Transduction Laboratories, Franklin Lakes, NJ, USA), Scrib (C-20 sc-11049, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Myosin-IIB (M7939, Sigma, St Louis, MO, USA), E-cadherin (610181, BD Bioscience), beta-catenin (610153, BD Bioscience), total PAK (C-19 sc-881, Santa Cruz Biotechnology), Flag M2 Affinity Gel (2220, Sigma), pAKT (9271, Cell Signaling, Danvers, MA, USA), GAPDH (4300, Ambion, Austin, TX, USA), horseradish peroxidase-conjugated anti-mouse, or anti-rabbit IgG (Amersham, GE Healthcare, Rydalmere, Australia).

2.3. Transfection

The empty pQCXIP vector, pQCXIP containing full-length MCC cDNA (amplified from clone MGC:12731, Invitrogen) or MCC mutants were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Site directed mutagenesis and full sequencing was carried out as previously described [13].

2.4. In-gel protein tryptic digestion, LC–MS/MS

Endogenous MCC was immunoprecipitated from HCT116 cells using anti-MCC monoclonal antibody (BD Transduction Laboratories). Immunoprecipitated endogenous MCC was run on 10% polyacrylamide gel and the SYPRO-stained MCC band was excised and destained in 1 ml of 50% acetonitrile and 250 mM ammonium bicarbonate at room temperature for 45 min with shaking. The gel slice was dehydrated by incubation in 1 ml of 100% acetonitrile for 10 min at room temperature. All solutions were carefully removed prior to the addition of modified trypsin (12.5 ng/μl) in 100 mM NH_4HCO_3 and incubation overnight at 37 °C. Peptides were extracted by the addition of 0.1 ml of 5% formic acid and incubation at 37 °C for 1 h. Peptides were further extracted by the addition of 0.1 ml of 100% acetonitrile and incubation at 37 °C for 1 h. The gel slice was completely dehydrated by the addition of 0.5 ml of 100% acetonitrile and incubation at 37 °C for 10 min. The entire supernatant was then vacuum-dried. The peptides were redissolved in 20 μl of 5% formic acid for LC–MS/MS analysis. Data were processed, searched, and quantified using the MaxQuant software version 1.0.13.13 package and searches were performed using Mascot server version 2.2 against the entire International Protein Index (IPI) database. The settings used for the Mascot search were as follows: two missed cleavages were allowed; enzyme was trypsin cleaving after arginine and lysine; variable modifications were methionine oxidation, propionamide cysteine, and phosphorylation of serine, threonine, or tyrosine; no fixed modifications were used; a mass tolerance of 6 ppm was used for precursor ions; and a MS/MS tolerance of 0.5 Da was used for fragment ions. False recovery rate was less than 1% and localization score cut-off was greater than 75%.

2.5. Immunofluorescence microscopy

MCF10A and Caco2 cells were cultured as described above on glass coverslips. Cells were then fixed with fresh 4% paraformaldehyde in PBS for 15 min at room temperature (RT). The cells were then permeabilized in 0.5% triton for 5 min and blocked with 20% FCS. Labeling was performed in PBS containing 4% FCS for 45 min at RT using the appropriate primary antibody. Primary antibodies were detected with Alexa Fluor Cy2- or Cy3-conjugated secondary antibodies 1:500 (Invitrogen), DNA was stained with 4′,6-diamidino-2-phenylindole 1:200 (DAPI). Cells were mounted on slides using Dako anti-fading mounting media. Optical sections were analyzed by confocal microscopy or phase contrast microscopy on an inverted microscope (DM IRE2, Leica) using a 63×/1.4 NA HC Plan 10×/25 objective lens (Leica). The contrast was adjusted for all images with the same settings.

2.6. Cell fractionation

The cells were harvested using hypotonic buffer and collected by scraping. Lysates were incubated on ice for 10 min and then homogenized 5 times using a 19 G syringe. A sample was collected at this point to represent the total lysate. Lysates were centrifuged at 800 *g* at 4 °C for 15 min and the supernatant was collected and centrifuged again at maximum speed for 15 min. The supernatant collected was used as the cytoplasmic fraction. The pellet, corresponding to the membrane cortex, was washed twice in hypotonic buffer followed by centrifugation for 15 min at 2500 *g* at 4 °C. The washed pellet was resuspended in RIPA buffer, and corresponds to the membrane cortical fraction (referred to as the membrane cortex fraction in text).

2.7. Co-immunoprecipitation and western blotting

Immunoprecipitation was performed on whole cell lysates resuspended in cold TNE buffer using the appropriate antibody at 4 °C O/N. Immunoprecipitates were resolved on 10% or 8% SDS-PAGE. Proteins were transferred to nitrocellulose (Millipore, Bedford, MA), immunoprobed, and detected by enhanced chemiluminescence (Pierce Chemical, Rockford, IL). Western blotting was performed with the same antibodies used for the immunoprecipitation to control for loading. Phosphatase treatment was performed as previously described [5]. Lysate was treated with lambda phosphatase for 30 min and the reaction was stopped using a phosphatase inhibitor (phosphoSTOP, Roche) and 50 mM NaF. The wild type Flag-Scrib-PDZ3 sequence and the K/A mutant were cloned in pcDNA3.1 (GeneArt, Life Technologies, Mulgrave, VIC, Australia). The Scrib-PDZ3 sequence was derived from the Scrib transcript variant 1 (NM_182706.4), amino acids 1003–1092, and the K/A mutant harbored a lysine to alanine substitution at position 1040. The Flag-Scrib-PDZ3 protein was immunoprecipitated using anti-Flag M2 Affinity Gel (Sigma, #A2220).

3. Results

3.1. MCC PDZ-binding motif is phosphorylated at position −1, Serine 828

As phosphorylation at −2 regulates the binding activity of many PDZ binding motifs, we performed a LC–MS/MS experiment to determine whether the MCC PDZ-binding motif is also phosphorylated at this site. No phosphorylation was detected at the −2 position but the Serine 828, at position −1, was phosphorylated in HCT116 (Fig. 1A) and SW480 colon cancer cells (data not shown). Despite the large number of post-translational modifications in the PDZ-binding motif, to our knowledge, no functional studies have been reported on phosphorylation of the PDZ-binding motif residue −1 [14]. It is possible that this modification has not been previously described due to its close proximity with the threonine/serine residue at position −2, thus making its identification and validation by MS difficult. In our case, the presence of y2 ion in our MS/MS spectra (Fig. 1A, orange arrow) allows unambiguous assignment of MCC phosphorylation at Serine 828.

Remarkably, the importance of MCC PDZ-binding motif, and its phosphorylation at position −1, is highlighted by the strong evolutionary conservation. Hence, the only amino acid substitution within this PDZ-binding motif is found in *Drosophila* at position −1 where serine "ETSL" is substituted to another phosphorylable amino-acid threonine "ETTL" (Fig. 1B). MCC has recently been shown to bind to the polarity protein Scrib through its PDZ-binding motif [1]. The MCC/Scrib interaction is also conserved between mammals and flies, since *Drosophila* MCC efficiently co-immunoprecipitates with *Drosophila* Scribble [1].

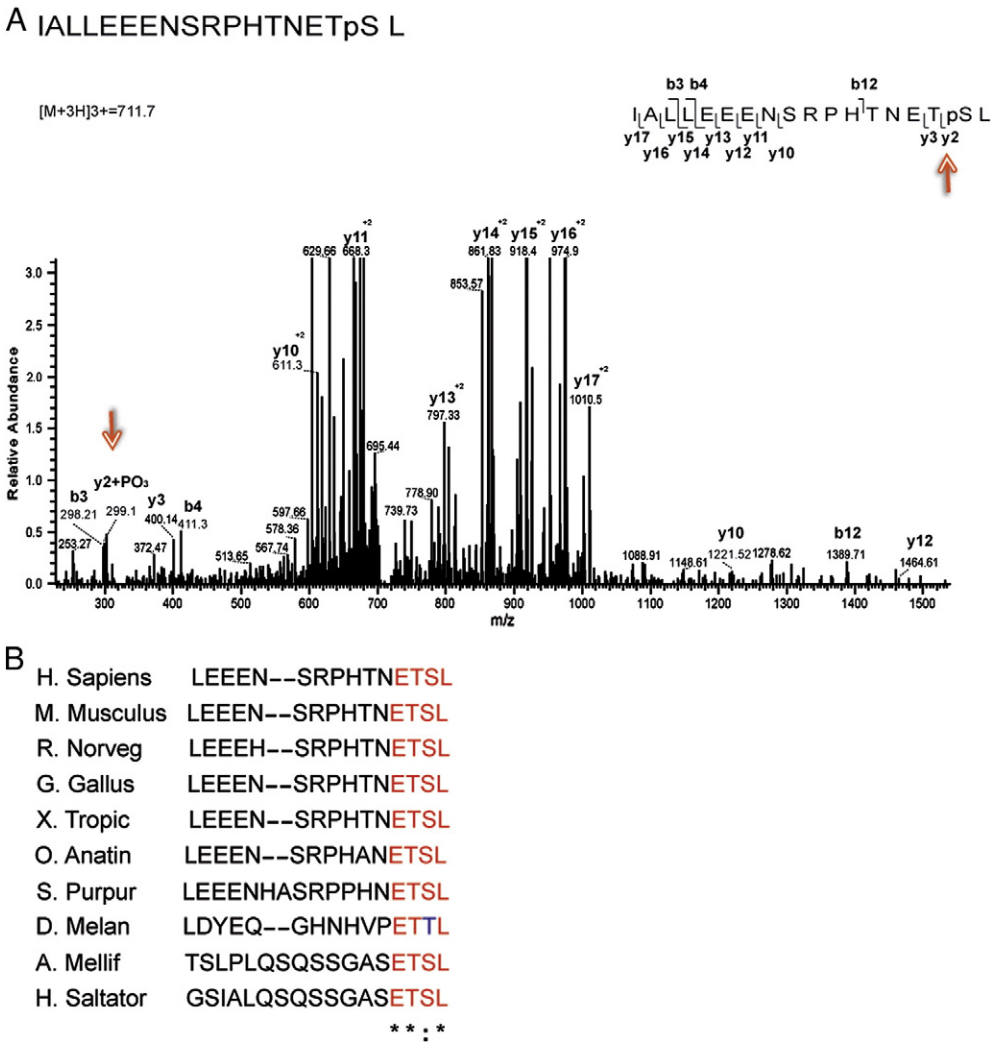

Fig. 1. MCC PDZ-binding domain is phosphorylated at the position −1, Ser828. A. LC–MS/MS experiment identifies phosphorylation of endogenous MCC at Serine 828. Endogenous MCC was immunoprecipitated from HCT116 cells using N-terminal mouse monoclonal antibody. MCC was excised from the SDS-PAGE gel, digested with trypsin and peptides were analyzed by LC/MS/MS. Search was performed using Mascot software. MS/MS spectra for the phospho-peptide IALLEEENSRPHTNET(pS)L, with annotated b and y ions, is shown. Phosphorylation at serine −1 is indicated by the presence of y2 ion (+80 Da for the mass of the phosphate group) at m/z = 299.1 (orange arrows). B. MCC PDZ-binding motif is strongly conserved among species. Sequence alignment shows a high degree of conservation of MCC-PDZ-binding motifs between species, including a conserved phosphorylable amino-acid at position −1.

3.2. Effect of the Ser828 phosphorylation on the interaction with Scrib PDZ domains

Despite the general view of the −1 amino acid of the PDZ-binding motif being randomly assigned, Scrib PDZ-1 and PDZ-3 domains preferentially bind to motifs that have aspartic acid at the −1 position [15], as summarized in Fig. 2A. Aspartic acid is an amino acid commonly used for its phosphomimetic properties, suggesting that phosphorylation of MCC at Serine 828 might increase its binding affinity to Scrib. In addition, the consensus prediction shows that in addition to the last four amino acids forming a class 1 PDZ binding motif, amino acids at positions −4 and −5 also influence the binding affinity (Fig. 2A). This analysis reveals a strong preference for aromatic amino-acids (W, F, Y) at positions −4 and −5 giving a rational explanation for the reported higher affinity of β-PIX (WDETNL) for Scrib (PDZ-1 and 3) compared to MCC (TNETSL) [1].

We first confirmed the interaction between MCC and Scrib by co-immunoprecipitation (co-IP). HCT15 cells were transfected with either a wild type (WT) or a MCC construct deleted of its PDZ-binding motif (Δ). MCC was only detected in Scrib immunoprecipitates from cells expressing the WT construct, confirming that this interaction requires the PDZ-binding motif of MCC (last 4 C-terminal amino-acids). We also confirmed the interaction of MCC with β-catenin [2], which is independent of the PDZ-binding motif (Fig. 2B). As previously shown, MCC is a phosphoprotein that runs as two closely migrating bands on low percentage polyacrylamide gel representing MCC hyper- and hypo-phosphorylated forms [5]. We next ran the Scrib and β-catenin immunoprecipitates on a low percentage polyacrylamide gel and blotted for MCC. This showed that only the hyperphosphorylated form of MCC was found in complex with Scrib (upper band, upper red arrow) (Fig. 2C). We next treated the pre-IP lysate with lambda phosphatase and tested the ability of Scrib to immunoprecipitate MCC. Our data show that pre-treatment with lambda phosphatase strongly reduces the amount of MCC bound to Scrib compared to control (Fig. 2D). We next assessed the effect of MCC Ser828 phosphomutants on MCC/Scrib interaction by co-IP. MCC Ser828 was mutated by site directed mutagenesis to non-phosphorylable alanine (SA) or phosphomimetic aspartic acid (SD). As expected, mutation of Ser828 to alanine resulted in partial loss of interaction with Scrib, compared to wild type MCC, whereas substitution to aspartic acid had no detectable effect (Fig. 2E).

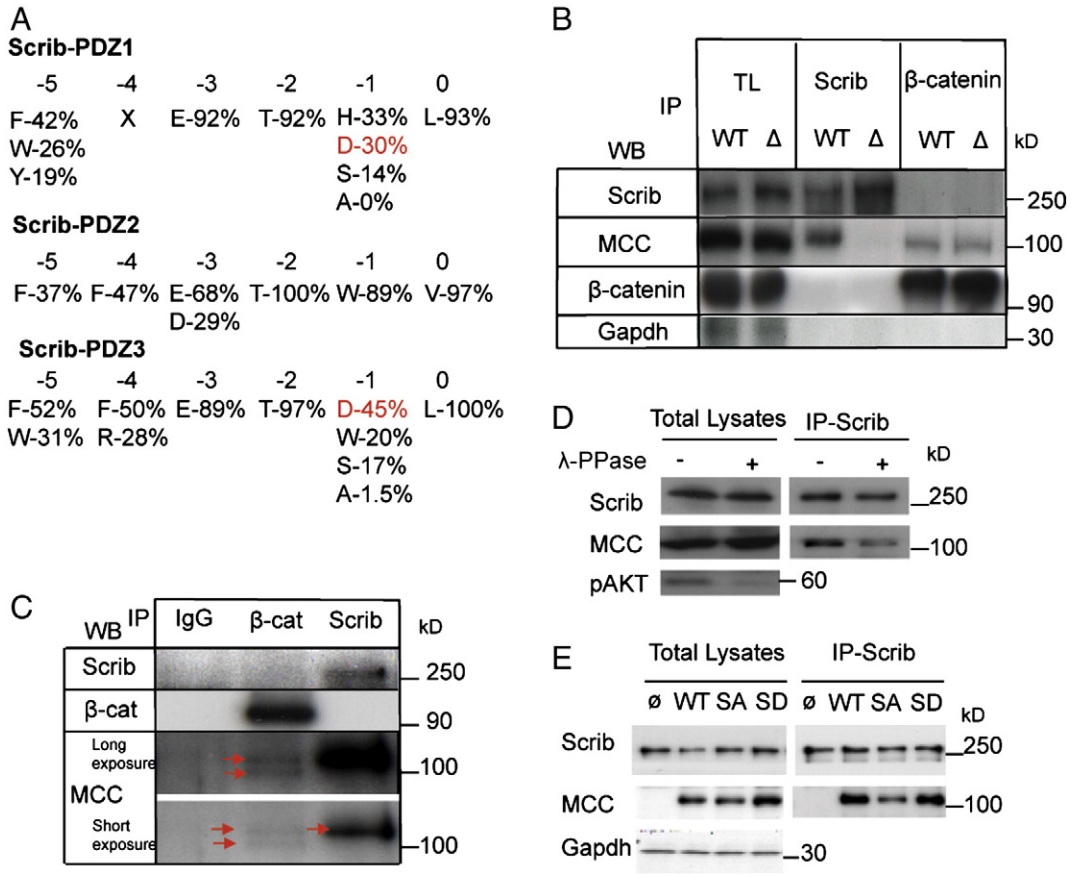

Fig. 2. Phosphorylation of the PDZ-binding motif regulates its interaction with Scrib PDZ domains. A. A randomly generated peptide binding assay [15] shows that a high proportion of PDZ-binding motifs interacting with Scrib PDZ domains 1 and 3, harbor an aspartic acid at position −1 (30% PDZ1 and 45% PDZ3). In addition to the last four amino acids forming the PDZ class 1 consensus, amino acids at positions −4 and −5 also influence the binding affinity. This consensus reveals a strong preference for aromatic amino acids (W, F, Y) at positions −4 and −5 giving a rational explanation for the reported higher affinity of β-PIX (WDETNL) for Scrib (PDZ1 and 3) compared to MCC (TNETSL) [1]. B. MCC–Scrib interaction is dependent on the PDZ-binding motif of MCC. HCT15 cells transfected with MCC-WT (WT) or MCC-ΔETSL (Δ) was lysed and subjected to immunoprecipitation using Scrib or β-catenin antibody. 10% of the whole lysate used for the co-IP was blotted for MCC to insure that equal amount of MCC-WT or MCC-ΔETSL was expressed. Scrib immunoprecipitates were blotted for Scrib to ensure that equal amounts of Scrib were immunoprecipitated in the samples, and blotted for MCC. C. The same IPs were run on an 8% polyacrylamide gel and blotted for β-catenin, Scrib and MCC. This assay shows that a hyper-phosphorylated form of MCC binds to Scrib (top red arrow) whereas β-catenin coimmunoprecipitates both hyper- and hypo-phosphorylated forms of MCC (lower and upper bands respectively, indicated by two red arrows). D. Lambda phosphatase treatment reduces the interaction between Scrib and MCC. MCC-WT was transfected into HCT15 cells. Prior to immunoprecipitation the lysates were treated with lambda phosphatase or mock-treated. 10% of the total lysates were blotted for MCC and pAKT to confirm that dephosphorylation occurs and that equal amounts of MCC-WT were expressed. Immunoprecipitates were blotted for Scrib, to confirm that an equal amount of Scrib was immunoprecipitated from each sample, as well as for MCC. E. Mutation of Ser828 to non-phosphorylable alanine resulted in partial loss of interaction with Scrib whereas substitution to pseudo-phosphorylated aspartic acid appeared to have no effect. Vector-only (Ø), MCC-WT (WT), MCC-S828A (SA) or MCC-S828D (SD) was transfected into HCT15 cells and immunoprecipitation was performed using Scrib antibody. 10% of the total lysates used for co-IP were blotted for MCC to confirm that equal amounts of MCC-WT and phosphomutants were expressed. Immunoprecipitates were run on a 10% polyacrylamide gel and blotted for Scrib, to confirm that an equal amount of Scrib was immunoprecipitated from each sample, as well as for MCC.

3.3. Mutation of a basic residue at position β3C5 of Scrib-PDZ3 reduces the binding with MCC

The effect of phosphorylation at position −2 of the PDZ-binding motif is well described and is predicted to prevent an interaction between the unphosphorylated serine/threonine at position −2 and a histidine residue in the PDZ domain (Fig. 3A, blue arrow), subsequently weakening the PDZ interaction [14]. The way that residues at position −1 interact with the PDZ domain is still open to debate but an interaction with the fifth residue of the third beta-sheet of the PDZ domain (β3C5) is likely to be involved [16].

In agreement with this model, both Scrib PDZ-1 and PDZ-3, which show poor overall identity (43%), harbor a basic residue at position β3C5 (Fig. 3A, red arrow). In addition, alpha-carbon backbone representation of Scrib-PDZ-1 shows the β3C5 arginine residue pointing towards the PDZ-binding motif, represented as a thick red arrow (Fig. 3B, left panel). The right panel shows Scrib PDZ1 hydrophobicity representation with the hydrophobic PDZ groove in red (Fig. 3B, right panel). Finally, multiple sequence alignment of Scrib PDZ-1 shows conservation of a positively charged residue at position β3C5 between species (Fig. 3C). Therefore, we tested the requirement of a basic residue at position β3C5 of Scrib-PDZ3 by substituting it with a non-charged alanine. WT and mutant versions of Scrib-PDZ3 were co-expressed with MCC and tested for their ability to interact with MCC by co-IP. Our data clearly show that lysine β3C5 to alanine substitution strongly reduces the ability of Scrib-PDZ3 to bind to MCC (Fig. 3D).

3.4. MCC localizes at the leading edge of polarized cells

In order to address the functional role of MCC phosphorylation at the PDZ-binding motif position −1, we first examined endogenous MCC intra-cellular localization by confocal microscopy. We detected endogenous MCC at the leading edge of polarized MCF10A cells where it co-localized with actin. MCC localization was confined to the outer margins of the lamellipodia structure as shown by phase contrast cell imaging (Fig. 4A, white arrow), and relative clearing within the inner margins (Fig. 4A, white brackets). This staining was

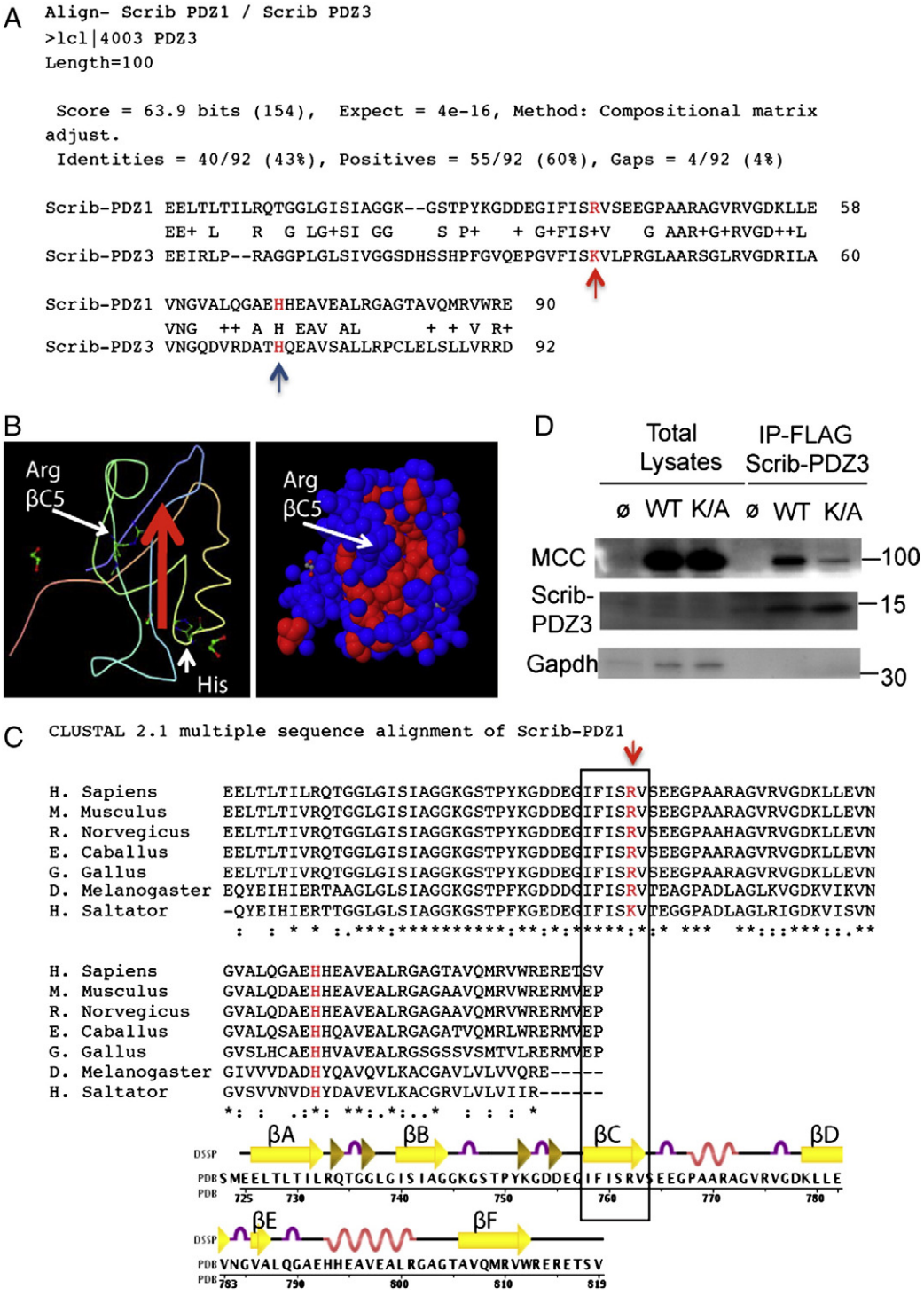

Fig. 3. Mutation of a basic residue at position of β3C5 of Scrib-PDZ3 reduces the binding with MCC. A. Sequence alignment of Scrib-PDZ1 and 3 shows a basic residue at position β3C5 (red arrow) and a histidine residue in the alpha helix2 (blue arrow) for both Scrib PDZ 1 and 3. Protein Scribble homolog isoform a [*Homo sapiens*] ref: NP_874365.2. B. The conserved β3C5_Arg residue points toward the hydrophobic PDZ binding pocket. PDZ binding motif is represented by a red arrow (N- to C-terminal). Representation of Scrib-s (PDB 2W4F) alpha carbon backbone (left) and hydrophobicity CPK (right). The figure was made using Jmol. C. Multiple sequence alignment of Scrib PDZ1 between species shows conservation of a positively charged residue at position β3C5. Multiple alignments were performed using the software ClustalW2.1. The conserved positively charged residue β3C5 (red arrow) and the histidine residue in alpha2 that hydrogen bonds with Ser/Thr at position (−2) of the peptide, are shown in red. Secondary structure elements are indicated at the bottom of the alignment as yellow arrow (beta strand) and red sinusoidal (alpha helix). D. The lysine to alanine mutation of residue β3C5 strongly reduces the binding of Scrib-PDZ3 with MCC. HCT15 cells were transfected with Flag-Scrib-PDZ3-WT (WT)/vector-alone (Ø), Flag-Scrib-PDZ3-WT (WT) /MCC-WT, or Flag-Scrib-PDZ3 mutated at position β3C5 (K/A) /MCC-WT. Flag-Scrib-PDZ3 WT or the K/A mutant were immunoprecipitated using Flag M2 Affinity Gel and the immunoprecipitates were blotted for MCC and Flag.

reminiscent of another protein involved in lamellipodia formation, cortactin [17].

Similar to endogenous MCC, exogenous wild type MCC co-localized with actin at lamellipodia (white arrow) and along spike-like filopodia (blue arrow) in polarized Caco2 cells. Endogenous MCC expression in Caco2 cells is almost absent and is not detectable by immunofluorescence (Fig. 4B). Heat map intensity gradient also clearly indicated exogenous MCC accumulating along the filopodia extensions (Fig. 4B,

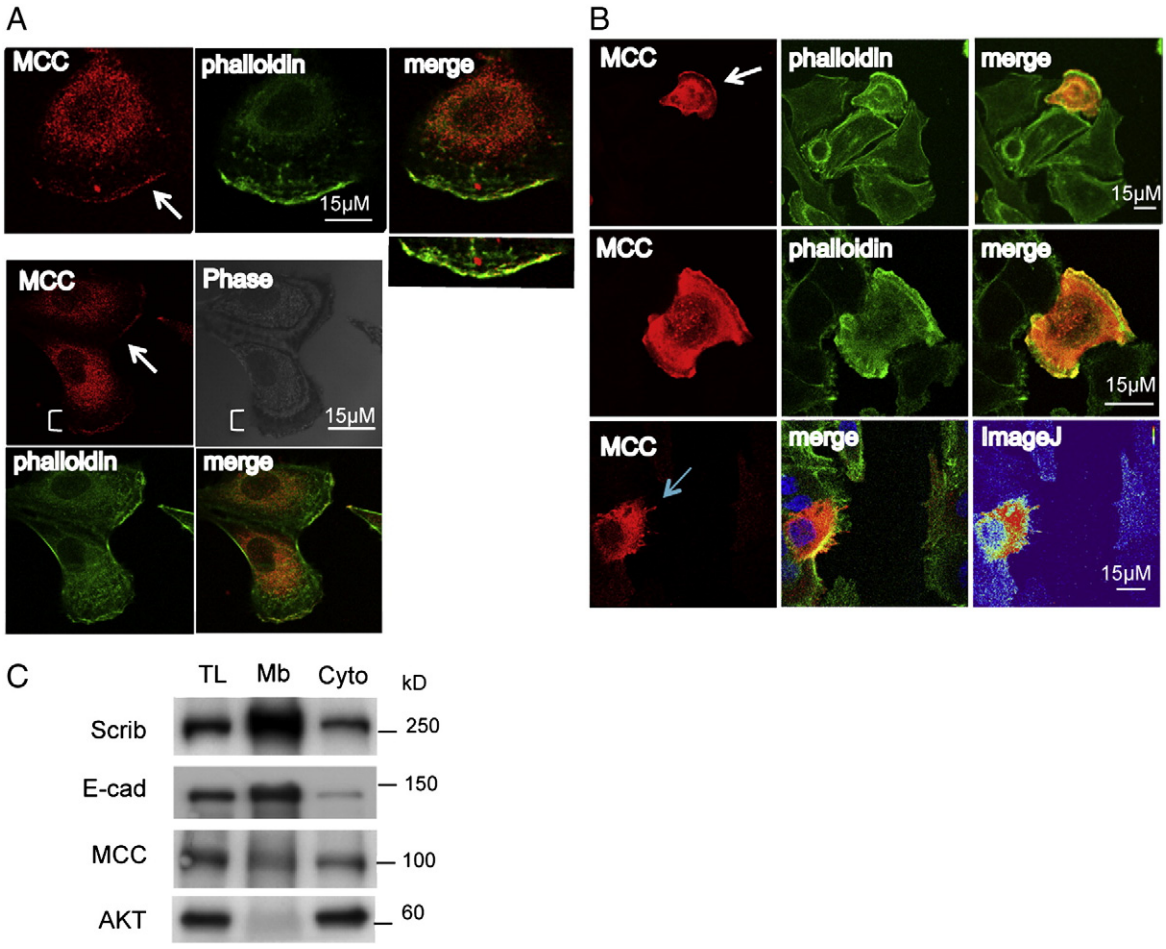

Fig. 4. MCC localizes at the leading edge of polarized cells. MCC sub-cellular localization was assessed by immunofluorescence and confocal acquisition. A. MCF10A cells were grown O/N on coverslips, permeabilized, fixed and stained using MCC antibody. FITC-conjugated phalloidin was used to stain actin. White arrow indicates MCC localizing at lamellipodia. White bracket indicates the inner margins. Higher magnification: MCC/phalloidin co-localization in lamellipodia. B. Exogenous MCC localizes at the leading edge of polarized Caco2 cells. MCC was over-expressed for 36 h in Caco2 cells and was stained using MCC antibody. FITC-conjugated phalloidin was used to stain actin. White arrow indicates lamellipodia and blue arrow indicates filopodia. C. MCC is present in the membrane cortex fraction of MCF10A cells. Total lysate (TL), membrane (Mb) and cytoplasmic (Cyto) fractions from MCF10A cells were run on a 10% polyacrylamide gel and blotted for MCC and Scrib. E-cadherin (membrane marker) and AKT (cytoplasmic marker) were used to assess the purity of the fractionation.

ImageJ). Finally, cell fractionation assay in MCF10A cells identified the presence of endogenous MCC in the membrane cortical fraction (Fig. 4C).

3.5. Expression of MCC bearing the substitution of Serine 828 to aspartic acid but not to alanine reduces the formation of lamellipodia

We next assessed the impact of MCC Ser828 phosphomutants on lamellipodia formation. MCC-WT or MCC phosphomutants (S828A or S828D) were expressed in Caco2 cells for 36 h and the cells were assessed for their ability to form lamellipodia. Cells expressing the phospho-deficient mutant (S828A) were able to maintain lamellipodia formation at levels indistinguishable from cells expressing wild type MCC, whereas cells expressing the phospho-mimetic mutant (S828D) showed reduced lamellipodia formation (Fig. 5A). Representative confocal images of Caco2 cells expressing MCC-WT or the MCC-S828 phosphomutants are shown in Fig. 5B.

We next assessed the ability of MCC phosphomutants to localize at lamellipodia by assessing MCC staining by confocal microscopy. We found that localization of MCC at the lamellipodia is strongly affected by mutation of Ser828. Hence, although MCC-S828D expressing cells had fewer lamellipodia, the intensity of MCC staining at the membrane was stronger (Fig. 5C). Hence, more than 50% of the cells expressing MCC-S828D show high fluorescence MCC intensity at the lamellipodia, compared to less than 30% for MCC-WT and MCC-S828A. These results suggest that excess MCC-S828D protein at the membrane cortex could be detrimental to lamellipodia formation. Representative confocal images of MCC staining intensity and scoring are shown (Fig. 5D).

Finally, consistent with a loss of cell migration following MCC knockdown [1], expression of MCC deleted of its PDZ-binding domain resulted in a severe reduction of lamellipodia formation and membrane localization (Fig. 5A–D).

3.6. MCC interacts with Myosin-IIB in a PDZ-independent manner and expression of MCC-S828D, but not MCC-S828A, resulted in Myosin-IIB accumulation at the membrane cortex

Scrib/β-PIX has been shown to participate in lamellipodia formation through the activation of PAK1 at the leading edge of migratory cells [10]. However, loss of migration caused by MCC knockdown was not associated with altered PAK1 activation in a previous study [1]. We have further shown that expression of the MCC-S828D mutant reduced lamellipodia formation while still binding to Scrib, suggesting that additional proteins, other than Scrib, are likely to be affected by re-expression of the MCC-S828D mutant.

We focused our attention on Myosin-IIB, a non-muscle motor protein implicated in lamellipodia formation [18] and identified as a

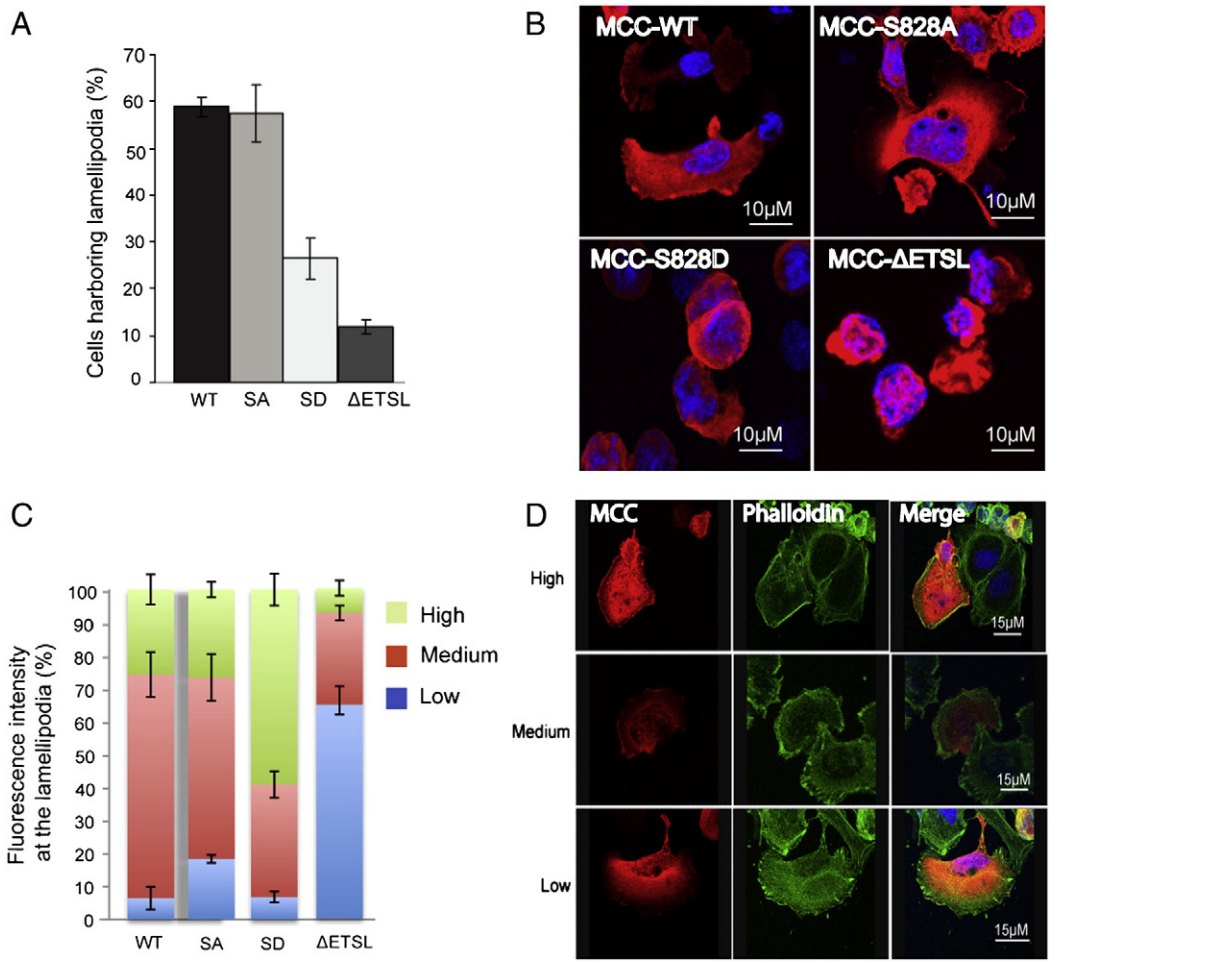

Fig. 5. Expression of MCC bearing the substitution Serine 828 to aspartic acid but not to alanine reduces the formation of lamellipodia. A. Percentage of Caco2 cells expressing MCC-WT, MCC-ΔETSL or phosphomutants forming lamellipodia. Graph values represent the mean percentage and standard deviation of MCC expressing cells which display lamellipodia formation. B. Representative field of Caco2 cells expressing MCC-WT (WT), MCC-ΔETSL, MCC-S828A (SA) or MCC-S828D (SD) using confocal microscopy. Caco2 cells were transfected with MCC-WT, MCC-ΔETSL, MCC-S828A or MCC-S828D for 36 h before being stained for MCC. C. Scoring of MCC intensity at the lamellipodia. Caco2 lamellipodia positive cells expressing MCC wild type or mutant were scored for MCC intensity at the lamellipodia. Graph values represent the mean score of MCC intensity at lamellipodia and the deviation from the mean. D. Representative confocal images of the different MCC intensity staining of MCC at the lamellipodia.

MCC-interacting protein in a large-scale mass spectrometry analysis [19] and recently confirmed by us [4]. Myosin-IIB is a critical generator of contractile force during cell migration by re-organizing the cytoskeleton including lamellipodia formation [18]. We confirmed that the binding of MCC with Myosin-IIB is independent of the PDZ-binding motif of MCC (Fig. 6A). To confirm the relevance of MCC/Myosin-IIB interaction in lamellipodia formation, the subcellular localization of both endogenous proteins was determined. MCC and Myosin-IIB clearly co-localized at the leading edge of migrating MCF10A cells (Fig. 6B).

Among the many kinases regulating Myosin-IIB activity is PAK1, which reduces myosin light chain (MLC) phosphorylation through MLCK inhibition, and increases Myosin-IIB heavy chain phosphorylation by activating aPKC-zeta. These modifications are part of complex signaling pathways which vary in different cellular conditions, and result in a dynamic assembly/disassembly of myosin filaments at the membrane cortex which is required for lamellipodia formation [20]. However, the detailed mechanism of the full process is poorly understood and is likely to involve modification of several amino acids in the Myosin-IIB heavy chain [21,22].

We hypothesized that expression of MCC-S828D mutant could impair lamellipodia formation by interfering with the normal subcellular localization of Myosin-IIB and its regulation by PAK1. To test this hypothesis, we exogenously expressed MCC-WT, MCC-S828A or MCC-S828D in Caco2 cells for 36 h. Our cell fractionation data clearly showed that the amount of Myosin-IIB was increased in the membrane cortex fraction of MCC-S828D expressing cells compared to MCC-WT, MCC-S828A or mock transfected cells (Fig. 6C). Similarly, accumulation of Myosin-IIB at the membrane cortex was evident in MCC-S828D but not in S828A expressing cells by immunofluorescence microscopy (Fig. 6D and E). This resembles the phenotype observed after transfection of a dominant negative PAK1 mutant (devoid of kinase activity) in a prostate cancer cell line [20]. This PAK1 mutant was associated with defective Myosin-IIB filament disassembly at the membrane cortex and a lamellipodia defect. This increase in Myosin-IIB at the membrane cortex is consistent with the observed "contracted" morphology of MCC-S828D expressing cells (Fig. 5B) and reduced lamellipodia formation (Fig. 5A).

These data suggest that the phosphomimetic mutant MCC-S828D, "locks in" a phenotype, where Myosin-IIB is trapped in the membrane cortex and lamellipodia formation is reduced.

4. Discussion

Following our previous work on the role of MCC phosphorylation in the DNA damage response [5] we now report a new phosphorylation of MCC within its PDZ-binding domain at position −1 and its role in lamellipodia formation. This work presents the first functional characterization of phosphorylation of the PDZ-binding motif at position −1.

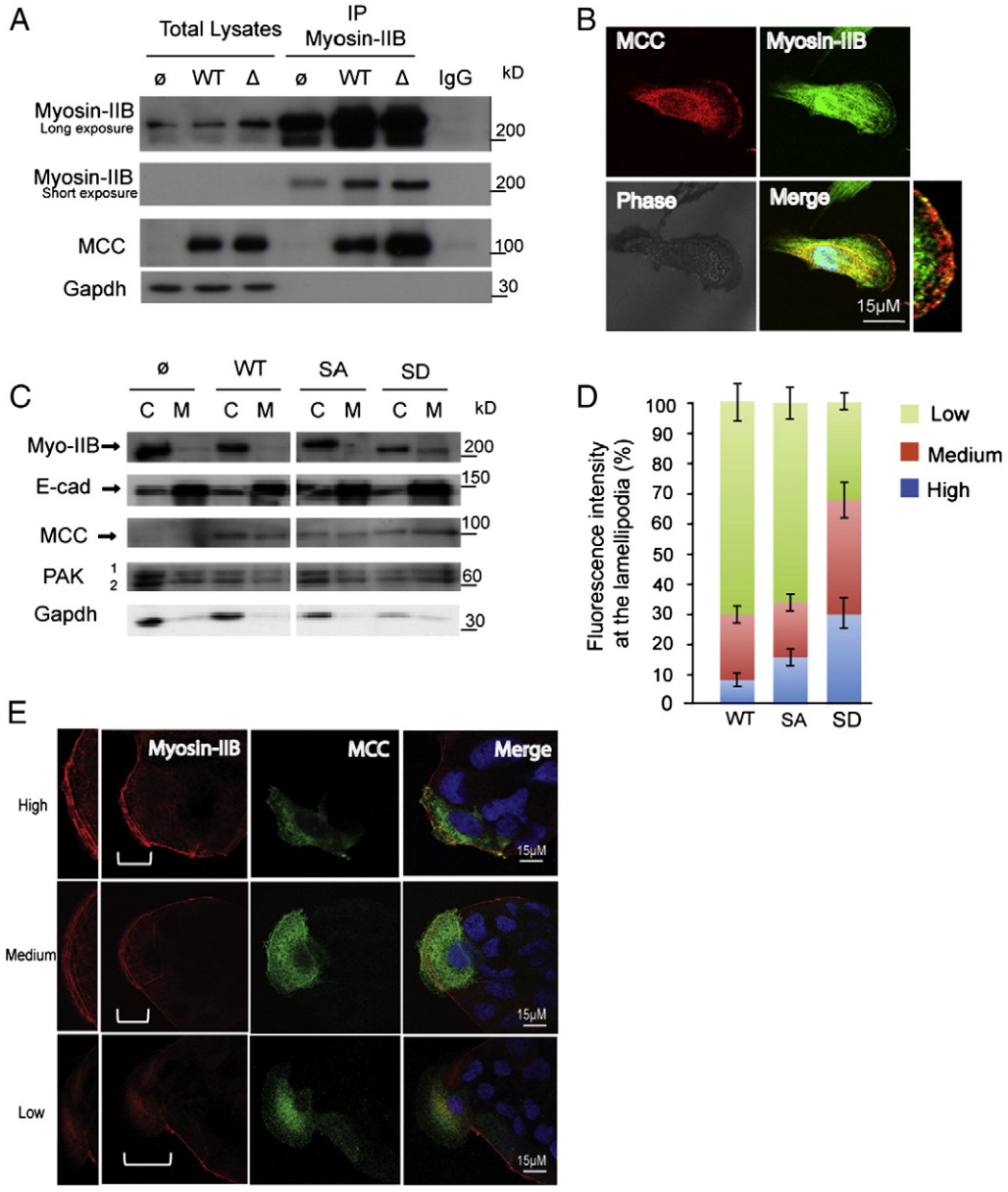

Fig. 6. MCC interacts with Myosin-IIB in a PDZ-independent manner and expression of MCC-S828D but not MCC-S828A increases the amount of Myosin-IIB at the membrane. A. MCC interacts with Myosin-IIB independently of its PDZ-binding motif. HCT15 cells were transfected with vector only (Ø), MCC-WT (WT) or MCC-ΔETSL (Δ) for 36 h. Immunoprecipitation was performed using Myosin-IIB antibody or IgG control and run on a 10% polyacrylamide gel. 10% of the total lysate used for co-IP was run to show that a similar amount of MCC-WT and MCC-ΔETSL was expressed. Gel was blotted for Myosin-IIB, MCC and Gapdh. B. Endogenous MCC co-localizes with Myosin-IIB at the lamellipodia. Endogenous MCC (red) co-localized with endogenous Myosin-IIB (green) in polarized MCF10A cells. Higher magnification: MCC/Myosin-IIB co-localization in the lamellipodia. C. Caco2 cells were transfected with vector only (Ø), MCC-WT (WT), MCC-S828A (SA) or MCC-S828D (SD) for 36 h. 5 μg of membrane (M) or cytoplasmic (C) fraction was loaded and gel was blotted for Myosin-IIB, MCC and total PAK. E-cadherin and Gapdh were used as membrane and cytoplasmic markers respectively. D. Caco2 cells were transfected with MCC-WT, MCC-S828A or MCC-S828D for 36 h before being stained for MCC and Myosin-IIB. Graph values represent the mean score of Myosin-IIB intensity at lamellipodia in MCC expressing cells and the deviation from the mean. E. Representative confocal images of the different Myosin-IIB intensity staining at the lamellipodia of MCC expressing Caco2 cells.

The PDZ-binding motif of MCC belongs to the class I family, which is characterized by the sequence X-(S/T)-X-(V/L/I), where X at position −1 is any amino acid. The PDZ-binding motif is one of the most common protein–protein interaction motifs and phosphorylation at position −2 of the class I motif is widely reported as a way to modulate this type of interaction [14]. Despite the large number of proteins harboring PDZ-binding motifs (>250) in the human genome, phosphorylation has only been described at positions p(−2) to p(−10) but not at p(0) or p(−1) [14]. The residue at position −1 of the PDZ binding motif was originally described as unimportant for PDZ interaction [23]; however, a number of studies are now slowly changing this view [24,25].

In an attempt to address the importance of this newly characterized PDZ-binding motif modification, we undertook an in silico search, which revealed that 146 proteins contain a class 1 PDZ-binding motif with a phosphorylable amino acid at position −1 [S/T][S/T][L/V/I]-COOH. Importantly, phosphorylation has been found for 31 of them in large-scale phosphomapping studies (Fig. 7 and Supplementary Table 1), highlighting the potential significance of this post-translational modification in a wide range of cellular processes.

The effect of phosphorylation at position −2 of the PDZ-binding motif is well described and is predicted to prevent an interaction between the unphosphorylated serine/threonine at position −2 and a histidine residue in the PDZ domain, subsequently weakening the

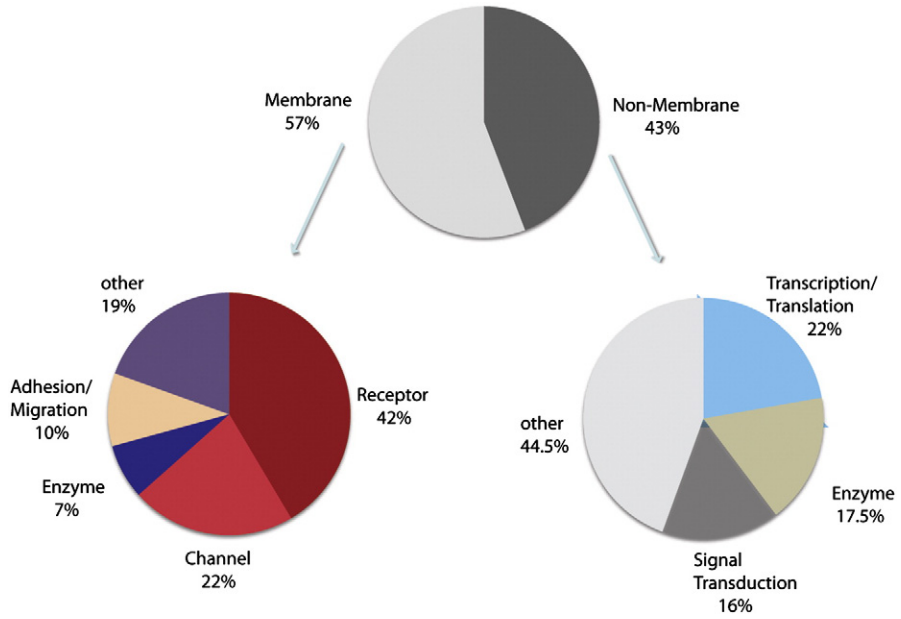

Fig. 7. Sub-cellular and functional classification of 146 proteins harboring a class 1 PDZ binding motif with a phosphorylable amino-acid at position −1. As expected, the majority of these proteins are found at the membrane. Within the membrane fraction (left pie chart), 64% are transmembrane proteins (42% receptor and 22% channel) supporting a role for this phosphorylation in membrane transport and membrane signal transduction. On the other hand, 22% of the proteins in the non-membrane fraction (right pie chart) are nuclear proteins having a role in transcription/translation, highlighting the role of this post-translational modification in a multitude of biological functions.

PDZ interaction [14]. The way that residues at position −1 interact with the PDZ domain is still open to debate but an interaction with the fifth residue of the third beta-sheet of the PDZ domain (β3C5) is likely to be involved [14,16]. Several studies also suggest a correlation between the presence of a charged β3C5 residue within the PDZ domain and an oppositely charged residue at position −1 of the PDZ-binding motif, supporting an electrostatic type of interaction or a salt bridge [26,27]. In agreement with this model, a randomly generated peptide binding assay identified aspartic acid (acidic residue) as the preferential −1 residue interacting with Scrib PDZ-1 and −3 (Fig. 2A), which harbor a basic residue at position β3C5 in close proximity to the interacting PDZ-binding motif in the crystal structure (Fig. 3A and B). Furthermore, as shown here, substitution of the lysine residue at Scrib PDZ3 β3C5 for alanine strongly reduced the MCC/PDZ3 interaction, providing further evidence for this binding mechanism.

Therefore, contrary to the effect of phosphorylation at residue −2, our data suggest that phosphorylation of MCC at serine −1 could increase its binding affinity with Scrib. As predicted, our co-immunoprecipitation assay shows a reduction in binding affinity between Scrib and MCC-S828A (non-phosphorylable amino acid) compared to MCC-WT and a reduction in MCC binding to Scrib following phosphatase treatment. However, despite the in vitro prediction, no detectable difference was observed between MCC-S828D (pseudo-phosphorylated) and MCC-WT. It is possible that this is because the majority of MCC-WT is already phosphorylated at Ser828 in these cells.

Finally, the importance of MCC/Scrib interaction, the MCC PDZ-binding motif, and its phosphorylation at position −1, is highlighted by the strong evolutionary conservation. Hence, in addition to a reported interaction between MCC and Scrib (scribble) in *Drosophila* [1], conservation analyses also predict that phosphorylation at position −1 is likely to be conserved. The only amino acid substitution within the PDZ-binding motif is found in *Drosophila* at position −1 where the serine "ETSL" is substituted to another phosphorylable amino acid threonine "ETTL" (Fig. 1B).

Based on the data reported here, we propose that phosphorylation of the MCC PDZ-binding motif in the −1 position regulates lamellipodia formation. We also propose that this function could be mediated through the formation of a transient complex that contains MCC, Scrib and Myosin-IIB. We found that MCC interacts with Myosin-IIB independently of its PDZ-binding domain and that MCC and Myosin-IIB co-localize at the migratory edge of the cells (Figs. 4A, 6A and B). We next found that expression of MCC-S828D, but not MCC-S828A, resulted in Myosin-IIB accumulation at the membrane cortex (Fig. 6C) and loss of lamellipodia. These results are similar to the phenotype observed following expression of a PAK1 dominant negative mutant, which lacks its kinase domain [20]. Hence, expression of $PAK1^{DN}$, a binding partner of the Scrib complex, resulted in excess Myosin-IIB accumulation at the membrane cortex and aberrant lamellipodia formation. This PAK1 mutant was associated with defective Myosin-IIB filament disassembly at the membrane cortex.

Further work is required to determine how the MCC-S828D/Scrib/Myosin-IIB interactions might cause reduced lamellipodia formation but this could involve dysregulation of myosin filament assembly/disassembly. Based on our data, we propose a model in which efficient lamellipodia formation requires cyclic association/dissociation of a MCC/Myosin-IIB complex with Scrib. While the majority of MCC in the cell is phosphorylated on S828, a small pool at the cell cortex undergoes regulated phosphorylation/dephosphorylation at this residue and this regulates the binding affinity of MCC towards Scrib. Dephosphorylation represents a critical element of this regulation mechanism and in its absence, as modeled by MCC S828D, the protein complex cannot dissociate, resulting in prolonged localization of MCC/Myosin-IIB at the cell cortex and impaired lamellipodia formation.

In conclusion, this study presents the first functional characterization of phosphorylation at position −1 of the PDZ-binding motif unveiling another level of regulation of PDZ–ligand interaction. Contrary to phosphorylation at position −2, we propose that phosphorylation at position −1 might increase the binding affinity possibly through the formation of an additional interaction with the residue β3C5 of the PDZ domain. Future work will now try to address the detailed mechanism by which phosphorylation of Ser828 affects MCC/Scrib binding, Myosin-IIB function and lamellipodia formation.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2012.03.011.

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