Phosphorylation of the cytoskeletal protein CAP1 controls its association with coflin and actin

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Abstract

Cell signaling can control the dynamic balance between filamentous and monomeric actin by modulating actin-regulatory proteins. One conserved actin-regulatory protein that controls actin dynamics is CAP (Cyclase-associated Protein). However, cell signals that regulate CAP remained unknown. We mapped phosphorylation sites on mouse CAP1 and found Ser307/Ser309 is a regulatory site. We further identified GSK3 (Glycogen Synthase Kinase 3) as a kinase for Ser309. The phosphomimetic mutant S307D/S309D lost binding to its partner cofilin, when expressed in cells, caused an accumulation of actin stress fibers similar to those in cells with reduced CAP expression. In contrast, the unphosphorylatable S307A/S309A mutant had drastically increased cofilin binding, but lost actin binding. These results suggest that the phosphorylation may serve to facilitate release of cofilin for a subsequent cycle of actin filament severing. Moreover, our results suggest that Ser307 and Ser309 function as tandem sites; neither the alterations in binding cofilin/actin, nor the defects in rescuing the phenotype in CAP1 knockdown cells was observed in single point mutants of either Ser307 or Ser309. In summary, we identify a novel regulatory mechanism of CAP1 through phosphorylation.
Introduction

The actin cytoskeleton is essential for many fundamental cell functions such as morphogenesis, cytokinesis, endocytosis, and cell polarization and migration. The cytoskeleton undergoes dynamic changes by regulating the balance of monomeric actin (G-actin) and filamentous actin (F-actin), a process called actin dynamics. One of the types of actin dynamics is “treadmilling” (Paavilainen et al., 2004; Pollard and Cooper, 2009), whereby actin filaments maintain a constant length but are in a state of dynamic equilibrium. During treadmilling, monomeric actin depolymerizes from the pointed end (or minus end) because it has a lower affinity, but polymerizes onto the barbed end (or plus end), which has a higher affinity. Energy to maintain this state comes from the hydrolysis of ATP. Treadmilling can be exploited by cells to cause rapid changes in dynamics. ADF (Actin Depolymerizing Factor) proteins, such as cofilin sever “aged” actin filaments to create shorter fragments, and hence increase the amount of filament pointed ends and consequent depolymerization of monomeric actin from these ends (Andrianantoandro and Pollard, 2006; Michelot et al., 2007). The monomers then undergo nucleotide exchange (replacing the bound ADP with ATP), and are recycled to the barbed end of the filament for next round actin polymerization (Pollard and Cooper, 2009). Repeated cycles of actin filament turnover drives actin cytoskeletal rearrangement. Cofilin plays a central role in driving actin filament turnover (DesMarais et al., 2005; Lappalainen and Drubin, 1997). The activity of cofilin is controlled by reversible phosphorylation at serine 3 (Yang et al., 1998). Thus by modulating phosphorylation at serine 3, cell signaling pathways can control the actin cytoskeleton to regulate cell motility and chemotaxis. However, cofilin regulates actin dynamics by itself, but in cells it is assisted by other proteins.

CAP (Cyclase-associated Protein) is a cytoskeletal protein that interacts with cofilin and actin to regulate actin dynamics (Hubberstey and Mottillo, 2002; Ono, 2013). Mutation or knockdown of CAP leads to problems with the actin cytoskeleton, including aberrant cell morphology, polarity and motility (Bertling et al., 2004; Freeman and Field, 2000; Kawamukai et al., 1992; Vojtek et al., 1991; Zhang et al., 2013). CAP regulation of the actin cytoskeleton is widely conserved and defects in CAP-deleted S. cerevisiae are rescued by expression of CAP homologues from even distant species (Kawamukai et al., 1992; Matviw et al., 1992; Zelicof et al., 1993; Zhou et al., 1998). CAP regulates actin dynamics at multiple levels. It binds and sequesters actin monomers to prevent them from polymerizing (Gerst et al., 1991; Gieselmann and Mann, 1992; Zelicof et al., 1996), which helps to maintain a sufficiently large and readily accessible G-actin pool essential for rapid actin cytoskeletal reorganization (Zhou et al., 2013). Studies over the past decade have revealed that multiple domains of CAP bind to actin and that CAP also
facilitates nucleotide exchange of ATP onto monomers (Hubberstey and Mottillo, 2002; Ono, 2013). Mammals have two CAP isoforms in CAP1 and CAP2. CAP1, which is more extensively studied, has now been solidly established as a regulator of the actin cytoskeleton and cell migration (Bertling et al., 2004; Moriyama and Yahara, 2002; Zhang et al., 2013; Zhou et al., 2013); this work focuses on CAP1.

CAP homologues have three conserved structural domains, the N-terminus domain (NT), the C-terminus domain (CT) and a proline-rich middle domain (Hubberstey and Mottillo, 2002; Ono, 2013). All three domains contribute to actin filament turnover through interactions with coflin, and both G- and F-actin (Ono, 2013). The CT binds and sequesters G-actin, and also catalyzes nucleotide exchange of ATP onto ADP-bound G-actin, while in yeast this function is further enhanced by a WH2 (Wasp Homology 2) domain, located towards the C-terminus of the middle domain (Chaudhry et al., 2010; Makkonen et al., 2013). Nucleotide recharging on ADP-G-actin in complex with coflin is a key rate-limiting step (Nishida, 1985) and CAP relieves the inhibitory effect of coflin on recharging. The NT of CAP binds the coflin/ADP-G-actin complex first for subsequent nucleotide exchange, and CAP can also directly bind F-actin to promote its severing (Chaudhry et al., 2013; Normoyle and Brieher, 2012). Moreover, the proline-rich middle domain interacts with profilin (Bertling et al., 2007; Makkonen et al., 2013). Finally, CAP was recently shown to assemble into a hexameric oligomer (Chaudhry et al., 2013). Regions within both the NT (Quintero-Monzon et al., 2009; Yusof et al., 2005; Yusof et al., 2006) and CT (Dodatko et al., 2004; Zelicof et al., 1996) can mediate CAP assembly into oligomers. The regulatory signals that modulate CAP, if any, are unknown.

We report here our findings that mouse CAP1 is a phospho-protein, with at least two phospho-regulatory sites. Phosphorylation at Ser307/Ser309 prevents coflin association, and expression of mutants that fail to undergo this phospho-regulation disrupts the actin cytoskeleton. We identify GSK3 (Glycogen Synthase Kinase 3) as a kinase that phosphorylates the Ser309 residue, and our results suggest that Ser309 functions along with Ser307 as tandem phospho-regulatory sites to control association with coflin and actin. Thus phosphorylation of Ser309 by GSK3 (and potentially additional kinases) is part of a phospho-regulatory mechanism that may facilitate association and dissociation of CAP1 with partner proteins coflin and actin, which are essential interactions for CAP1 to promote actin filament turnover.


**Results**

*Mapping of phosphorylation sites on CAP1*

We first tested potential phosphorylation of CAP1 *in vivo* through metabolic labeling of cells with orthophosphate followed by immunoprecipitation of CAP1 from cell lysate using an anti-CAP1 monoclonal antibody (Freeman and Field, 2000). Radioactivity was detected in CAP1 suggesting that CAP1 is a phosphoprotein (data not shown). We next mapped phosphorylation sites on mouse CAP1 by mass-spectrometry assays. To do this, we transiently expressed 6xHis-CAP1 in HEK293T cells (Zhang et al., 2013), the lysate was precipitated with Ni-NTA and the 6xHis-CAP1 band was excised from a Coomassie Blue-stained protein gel and subjected to mass-spectrometry. As shown in Table I, a total of nine phosphorylation sites were identified in three independent assays. Some of these sites, such as Ser36 (S36), Ser307 (S307), Ser309 (S309) and Thr314 (T314), were well conserved among the mammalian CAP1 homologues. The NCBI ID numbers of the CAP homologues compared are as following: (1) *S. pombe* CAP: NP_587817.1; (2) *M. musculus* CAP1: NP_031624.2; (3) *H. sapiens* CAP1: NP_001099000.1; (4) *S. cerevisiae* CAP: NP_014281.1; (5) *R. norvegicus* CAP1: NP_071778.2; (6) *C. elegans* CAS-2: NP_491324.1; (7) *D. melanogaster* Capt: AAF51408.2; (8) *A. thaliana* CAP1: NP_195175.1; (9) *M. musculus* CAP2: NP_080332.1.

*S36 and S307/S309 are phospho-regulatory sites*

To assess the significance of each phosphorylation site, we generated point mutants, expressed the mutants in cells and then evaluated their effects on the actin cytoskeleton. These sites were mutated to either an alanine (A), which mimics an unphosphorylatable residue or an aspartic acid (D), which is phosphomimetic. Where two phosphorylation sites are adjacent to each other (T24/S25), or are separated by a single residue (S307/S309), we mutated both sites together. As a result, we generated a total of fourteen constructs that express CAP1 mutants fused to a GFP (Green Fluorescence Protein), as listed in the Supplemental Table I. These mutants were: (1) T24A/S25A, (2) T24D/S25D, (3) S36A, (4) S36D, (5) S217A, (6) S217D, (7) S227A, (8) S227D, (9) S247A, (10) S247D, (11) S307A/S309A (AA), (12) S307D/S309D (DD), (13) T314A and (14) T314D.

We next stably expressed the mutants and compared them with wild-type CAP1 (WTCAP1) in NIH3T3 fibroblasts. Comparable expression levels of the exogenous proteins were verified by Western blotting with a GFP antibody (Figure 1A, shown only for WTCAP1 and the four mutants characterized further). In phase contrast microscopy we found that expression of WTCAP1 caused increased peripheral
ruffles (phase images not shown). In contrast, mutants of two phospho sites, S36 and S307/S309 caused significant yet distinct morphological phenotypes. Many cells expressing the S36D mutant were smaller with multiple lamellipodia that are randomly positioned. The cells expressing the DD mutant were larger and had reduced peripheral ruffling. In contrast, the AA expressing cells while also larger still had well developed lamellipodia. We also found that cells expressing T314A had a phenotype similar to the AA expressing cells. To quantify the changes, we measured the areas of 50 cells from three fields, and results from three independent experiments were analyzed using Student’s t-test as shown in Figure 1B. Consistently, the S36D expressing cells had significantly reduced cell size, while the AA, DD and T314A expressing cells had significantly increased cell size as compared to the WTCAP1 expressing cells. We also scored cells with prominent peripheral ruffling (lamellipodia) and found that the DD and S36A cells had the fewest cells with lamellipodia (Figure 1C). These data suggest that CAP1 is regulated by phosphorylation in two regions, the first is near serine 36 and the second is from amino acids 307-314. The rest of the mutants did not cause observable cell morphological phenotypes compared to WTCAP1, suggesting that their phosphorylation are less significant in regulating CAP1 function.

**Actin cytoskeletal phenotypes caused by the S36 and S307/S309 mutants**

We next stained cells with fluorescent Phalloidin to visualize F-actin. When we observed cells cultured on Nunc-chamber-slides under wide-field fluorescence imaging, we found that the control GFP vector expressing cells rarely developed lamellipodia, but had prominent stress fibers (Figure 2A, upper left panel, cells indicated with arrows). Expression of WTCAP1 stimulated lamellipodia (Figure 2A, upper right panel, cells indicated with arrows), which is a phenotype characteristic of motile cells because these structures help establish cell polarity and are required for cell migration. Interestingly, many S36D expressing cells had a disrupted actin cytoskeleton structure that is “contracted”, and some of them had lamellipodium-like protrusions on the “sides” of otherwise spindle shaped cells (Figure 2A, middle right panel, arrows indicate cells with a contracted actin cytoskeleton and arrowheads indicate randomly-positioned protrusions). Both the DD and AA expressing cells had enhanced stress fibers (Figure 2A, bottom panels, cells indicated with arrows), and looked similar to the vector expressing control cells. The stress fiber phenotype in DD and AA expressing cells is also similar to that observed in CAP1 knockdown fibroblasts and HeLa cells (Bertling et al., 2004; Zhang et al., 2013), presumably from reduced depolymerization of actin filaments due to impaired CAP1 activity. However, the AA expressing cells had more lamellipodia than the DD expressing cells. We calculated percentage of cells with prominent lamellipodia, similar as we did in Figure 1D, and found that S36D and AA expressing cells had similarly
high percentages of cells with developed lamellipodia as the WTCAP1 expressing cells (Figure 2B). The cell morphology and actin cytoskeleton phenotypes caused by the mutants are briefly summarized in *Supplemental Table I*.

We further examined the cytoskeleton by confocal imaging. In addition to the enhanced lamellipodia, cells expressing WTCAP1 had remarkably reduced stress fibers (Figure 2C, right top panel indicated with arrowheads and arrows). The mutants also affected stress fibers in some of the S36D expressing cells where the stress fibers had a discontinuous structure (Figure 2C, right middle panel, indicated with arrows), in contrast to the straight stress fibers seen in the control cells as well as stress fibers typical seen in cells (not shown). We speculate this abnormal stress fiber structure probably contributed to the contracted actin cytoskeleton in these cells. Also, we confirmed significantly enhanced stress fibers in the DD and AA expressing cells, which were also somewhat thicker than those in the vector cells (Figure 2C, bottom panels, indicated with arrows). A phenotype with thick stress fibers is known to hinder cell migration (Tojkander et al., 2012). The AA expressing cells had similar stress fiber phenotype to that of the DD expressing cells; however, unlike the DD expressing cells, the AA expressing cells still developed lamellipodia, as indicated with arrowheads.

The changes in lamellipodia and stress fibers of the S36D and DD expressing cells suggest that expressing these mutants may affect cell migration, such as what observed in Akt1 Knockout MEF (Mouse Embryonic Fibroblast) cells (Zhou et al., 2006). We tested motility of the S36D and DD cells, which had the strongest phenotypes, in Transwell migration assays. As shown in Figure 2D, expression of WTCAP1 stimulated cell migration compared to the vector cells. In contrast, expression of either S36D or the DD significantly reduced migration. These results suggest that the randomly-oriented protrusions in S36D cells probably caused difficulty in establishing and maintaining cell polarity during directional cell migration. In addition, it is also possible that the discontinuous stress fibers hindered cell migration, since stress fibers generate and transduce tension force to pull up the trailing edge of the cell during migration. As for the DD expressing cells, they had the lowest migration rate, which is probably due to a collective effect from both the stress fiber and cell polarity phenotypes in these cells.

*Effects of S307/S309 phosphorylation on association of CAP1 with cofilin and actin, and evidence supporting the two residues function as a tandem site*

The actin cytoskeletal phenotypes caused by S307/S309 mutants suggest impaired CAP1 activity, which led us to reason that phosphorylation on these sites may affect the association of CAP1 with cofilin
or actin, two binding partners of CAP1. To avoid the complications of interpreting results in cells caused by the presence of endogenous CAP1, we used CAP1 knockdown HeLa cells to re-express WTCAP1 and the mutants (Zhang et al., 2013). The plasmids expressing WTCAP1 and the mutants were modified by introducing mismatches to the shRNA target sequence so that they were not recognized by the snRNA used to stably knock down CAP1, but without changing the amino acid sequence. Stable re-expression of the WTCAP1, S36A, S36D, AA or DD mutants with a 6xHis-Xpress tag was confirmed by Western blotting and immunofluorescence (Figures 3 & 5A below). We tested cofilin binding in a GST-cofilin pull-down assay we developed previously (Zhang et al., 2013). Interestingly, we found striking differences between the DD and AA mutants in their capabilities to bind cofilin; the DD mutant virtually lost cofilin binding, while the AA mutant had drastically increased cofilin binding that is much higher than WTCAP1 (Figures 3A, quantified results shown in Figure 3B). We also found that the S36A mutant, similar to the DD mutant, had reduced cofilin binding. Noteworthy, NIH3T3 cells expressing the S36A and DD mutants were the cells that had the fewest lamellipodia (Figure 1D); these results suggest that cofilin binding is critical for CAP1 activity in facilitating the formation of these F-actin rich structures, and that cofilin binding is regulated by phosphorylation at S36 and S307/S309.

We next examined actin binding by precipitating 6xHis-CAP1 with Ni-NTA beads, followed by detection of co-precipitated actin in Western blotting (Zhang et al., 2013). We found that while the DD mutation did not affect binding but the AA mutation had significantly reduced actin binding (Figure 3C, quantified results shown in Figure 3D). Thus phosphorylation can modulate actin binding as well.

Since the AA/DD mutants harbor mutations at both S307 and S309, we constructed point mutations at either S307 or S309 to determine if either alone affected cofilin or actin binding. As shown in Figure 3E, and quantified in Figure 3F, neither S307A nor S309A showed increased binding to cofilin observed in the AA mutants. Similar results were observed when we tested actin binding. As shown in Figure 3G and quantified in Figure 3H, while the AA mutant lost actin binding, single mutants (S307A and S309A) both had normal actin binding. These results suggest that S307 and S309 function as tandem sites, and phospho-regulation at both residues collaboratively controls the interaction of CAP1 with cofilin and actin.

CAP1 binds both G- and F-actin, with a preference for G-actin (Ono, 2013). We next tested if the S307/S309 mutants had any altered preference in binding G- and F-actin. We treated lysates from HeLa cells re-expressing WTCAP1 and these mutants with Latrunculin A to depolymerize the F-actin. As
shown in *Supplemental Figure 1*, treatment with Latrunculin A increased actin binding for both WTCAP1 and the AA and DD mutants in a similar fashion, and the AA mutant consistently had the least actin binding with or without the treatment. These results suggest that the S307/S309 mutation does not alter the preference of CAP1 in binding which type of actin.

*GSK3 phosphorylates Ser309 on CAP1*

To aid in identifying kinase(s) for the S307/S309 site, we developed a phospho-specific antibody designed to detect phospho signals on both S307 and S309, as described in Materials and Methods. Western blotting of transiently expressed GFP-fusion WTCAP1 and the AA and DD mutants show that the phospho-specific antibody reacted specifically to GFP-WTCAP1 and endogenous CAP1, but not to the GFP-fusion AA or DD mutants (*Supplemental Figure 2A*). We further characterized the antibody in peptide blocking assays to see if the antibody only recognizes dual-phosphorylated antigen. As shown in *Supplemental Figure 2B*, the dual-phosphorylated peptide (S307p/S309p) completely blocked detection of the phospho-signal on CAP1 in cells, while the S309-phosphorylated peptide (S309p) modestly blocked the signal, suggesting that the antibody also recognizes CAP1 phosphorylated at S309 alone, although substantially less effective in blocking the signal. We detected phospho signals on CAP1 from all cells that we tested, including HEK293T, HeLa, NIH3T3 and a pancreatic cell line hTERT-HPNE (Lee et al., 2003) (Figures 4 & 6, not shown for NIH3T3). These results support S307/S309 as a phospho-regulatory site across different cell (and tissue) types, although the mass-spectrometry mapping was conducted with the protein expressed in HEK293T cells.

In predicting potential kinases that may phosphorylate CAP1 using the Net-Phos2.0 program ([http://www.cbs.dtu.dk/services/NetPhos/](http://www.cbs.dtu.dk/services/NetPhos/)), the serine/threonine kinase GSK3 (Kim and Kimmel, 2000; Woodgett, 2001) was predicted as one of the kinases for both the S307 and S309 sites (Table I). Since GSK3 is a regulator of cell polarity and migration, we further tested this candidate (Kim and Kimmel, 2006; Sun et al., 2009; Wu et al.). We used three common GSK3 inhibitors LiCl (Klein and Melton, 1996), 6-BIO (6-bromoindirubin-3’-oxime)(Meijer et al., 2003) and SB216763 (Coghlan et al., 2000) in Western blots with the phospho CAP1 antibody. Treatment of HEK293T cells with all three inhibitors reduced S307/S309 phosphorylation (Figure 4A, quantified in Figure 4B). LY294002 (PI3 kinase inhibitor) caused a modest stimulation of the phosphorylation, consistent with a negative regulation of GSK3 by PI3K/Akt signaling (Clodfelder-Miller et al., 2005). We also found that Forskolin, an activator of protein kinase A (PKA), reduced the phosphorylation, which may result from indirect inhibition of
GSK3 by activated PKA (Fang et al., 2000). We also tested several additional inhibitors, including ERK inhibitor U0126, JNK inhibitor XVI and ROCK inhibitor 1447, and none of them reduced the S307/S309 phosphorylation (Supplemental Figure 3A, quantified in Supplemental Figure 3B). Thus, the inhibitor data suggest that GSK3 is a kinase for the S307/S309 site. We also directly tested several kinases that regulate the actin cytoskeleton and/or cell migration, including Akt, LIM kinase and PAK1 (p21-activated kinase 1) in kinase assays with purified porcine CAP1 as a substrate, and none of these kinases phosphorylated CAP1 (not shown). We next silenced GSK3β in HeLa cells using two previously validated lentiviral shRNA constructs (Yoeli-Lerner et al., 2009). As expected, the constructs efficiently knocked down GSK3β, and also reduced the S307/S309 phosphorylation (Figure 4C). We further found that GSK3 co-precipitates with CAP1 in HeLa cells (Figure 4D) or purified from bacteria (not shown), suggesting a physical interaction between CAP1 and GSK3. Taken together, these results strongly support that GSK3 phosphorylates CAP1 at the S307/S309 site.

To pinpoint which of S307 or S309 is the actual GSK3 site, we next performed in vitro kinase assays using synthesized CAP1 peptides as substrates. GSK3 only phosphorylates substrates that are primed by prior-phosphorylation at a Ser or Thr 4 or 5 residues to the C-terminal from the actual GSK3 site (i.e. motifs S/TXXXS\textsuperscript{P}/T\textsuperscript{P} or S/TXXXXS\textsuperscript{P}/T\textsuperscript{P}, where X can be any residue) (Cole et al., 2004; Frame and Cohen, 2001). Since we also mapped T314 as a phosphorylation site (Table I), the combination of S309/T314, but not S307/T314, fits the motif for a GSK3 site (Cole et al., 2004). We thus predicted S309 as the GSK3 phosphorylation site, with T314 priming this site. To test this prediction, peptides were synthesized based on the mouse CAP1 sequence (QTSPSPKPATKK, a.a. 305-316) harboring an alanine (A) mutation at either S307 or S309 along with a phosphorylated or dephosphorylated T314. As shown in Figure 4E, GSK3β phosphorylated the S307/S309/T314\textsuperscript{P} peptide, but a single alanine mutation at S309 (S307/A309/T314\textsuperscript{P}) was sufficient to abolish the phosphorylation. In contrast, an alanine mutation at S307 (A307/S309/T314\textsuperscript{P}) did not have any effect on its phosphorylation by GSK3β. A known GSK3 substrate GSM (RRRPASVPPSPLSRH\textsuperscript{H}HQRR) was used as a positive control (Ryves et al., 2002). Thus we mapped S309 as the actual GSK3 site. We further demonstrated that T314 is the priming site for S309; whereas S307/S309/T314\textsuperscript{P} was phosphorylated by GSK3β, the same peptide harboring a dephosphorylated T314 (S307/S309/T314) abolished this phosphorylation (Figure 4F). Finally, we also demonstrate that GSK3α, the other GSK3 isoform, phosphorylated S309 as well (Figure 4F). While we found that GSK3 can regulate S307/S309, we note that other kinases may recognize this site because we found that treating cells with PMA (Phorbol 12-
Myristate 13-Acetate) reduced phosphorylation at S307/S309. Interestingly, when we tested the association between CAP1 and coflin, we found that the PMA treatment increased coflin binding (Supplemental Figure 4). These results further support our result from using the AA mutant that dephosphorylated CAP1 has increased binding with coflin. Moreover, it suggests that in addition to GSK3, PKC likely also regulate phosphorylation of CAP1 and its association with coflin.

Roles of Ser307 and Ser309 in CAP1 function and localization

Our biochemical assay results support that S307 and S309, which is a GSK3 site, function as tandem sites that regulate CAP1 function. To further assess the roles for each residue in CAP1 function in cells, we re-expressed the AA, DD as well as S309A and S309D mutants in the CAP1 knockdown HeLa cells, where at least 90% of the endogenous CAP1 is depleted (Zhang et al., 2013). As shown in Figure 5A and quantified results in Figure 5B, expression of the WTCAP1 rescued the phenotype of the enlarged cell size of the CAP1-depleted cells as we previously observed (Zhang et al., 2013). In contrast, the AA and DD mutants failed to rescue the phenotype (Figure 5A & 5B), consistent with the phenotypes caused in NIH3T3 cells by these mutants that suggest impaired CAP1 activities. Interestingly, the S309A and S309D mutants were both able to rescue the phenotype (Figure 5A & 5B), consistent with the normal actin and coflin binding for these S309 single point mutants (Figure 3E-H). In fact, the S309 mutants had even more pronounced effects on cell size compared to the WTCAP1, as literally no large cells were observed in cells rescued by these mutants, while in WTCAP1-rescued cells some large cells were still observed (data not shown). We also stained the transfected CAP1 with an Xpress antibody and found that the AA mutant localized predominantly to the cell periphery (indicated with arrows in Figure 5A) much more strongly than WTCAP1, while the DD mutant showed a diffuse localization across the cytosol (Figure 5A). The S309A mutant also localized to the cell periphery (indicated with arrows), although not as strongly as the AA mutant. The S309D mutant had a diffused localization similar to the DD mutant (Figure 5A). These results suggest that phosphorylation at S309 affects CAP1 localization, although dual phosphorylation at both S307 and S309 affects both localization and activity. We also tested the rescue by the S36A and S36D mutants, and found that both mutants were able to rescue the enlarged size of the CAP1-depleted cells (Supplemental Figure 5).

Inhibition of GSK3 leads to actin cytoskeletal changes and loss of peripheral CAP1 localization

We next tested effects of GSK3 inhibition on the actin cytoskeleton and CAP1 in hTERT-HPNE pancreatic cells, which have very well established cell polarity. We first confirmed that GSK3 inhibition
also reduced CAP1 phosphorylation in this cell line (Figure 6A). In a wound healing assay, cells treated with vehicle control (Figure 6B, upper panels) had a well established cell polarity (leading edge indicated with arrows). In contrast, cells treated with 6-BIO lost cell polarity (Figure 6B, lower panels, indicated with arrows). LiCl had a similar effect (not shown). These cells also had significantly enhanced and well organized stress fibers compared to the control cells. Moreover, treatment with 6-BIO also abolished CAP1 enrichment at the leading edge of the cell (Figure 6B). Instead, CAP1 showed a diffuse localization across the cytosol, including the cytosolic area immediately behind the leading edge, where virtually no CAP1 staining was observed in the control cells (Figure 6B, upper panels, indicated with arrowheads). These results are consistent with the phenotypes observed in NIH3T3 cells expressing the DD and AA mutants, both unable to undergo reversible phospho-regulation. However, the localization of CAP1 in GSK3-suppressed cells, which is expected to be dephosphorylated at S309 is not consistent with the cell peripheral localization of the S309A and AA mutants (Figure 5A). Thus an unknown localization mechanism may be involved in these cells, which is independent of phospho-regulation of CAP1 by GSK3. We tested this by inhibiting GSK3 with 6-BIO in CAP1 knockdown HeLa cells re-expressing WTCAP1 and the AA and DD mutants, and found that treatment abolished the peripheral localization of both WTCAP1 and the AA mutant (Figure 6C), supporting this is indeed the case. We next looked into potential effects of GSK3 suppression on cofilin, a binding partner of CAP1, and found that the inhibitor treatment reduced cofilin expression and also caused elevated cofilin phosphorylation at Ser3, suggesting loss of cofilin activity (Figure 6D). The effects of GSK3 inhibition on cofilin expression and phosphorylation are consistent with the phenotypes in stress fiber and loss of cell polarity. Since CAP1 activity depends on cofilin, the effects of GSK3 on cofilin may be epistatic to its effects on CAP (Moriyama and Yahara, 2002).

**Elevated S307/S309 phosphorylation in cells with a static actin cytoskeleton**

To assess role of the S307/S309 phosphorylation in CAP1 function in the actin cytoskeleton, we examined possible correlation of S307/S309 phosphorylation and actin dynamics, by comparing the phosphorylation in cells undergoing dynamic actin cytoskeletal reorganization with that in cells with a static actin cytoskeleton. HEK293T cells were cultured in suspension, in which cells have substantially reduced actin cytoskeletal reorganization, as well as on a fibronectin-coated plate, where cells undergo dynamic actin cytoskeletal rearrangement during the early and rapid spreading stage. Interestingly, we found remarkably elevated S307/S309 phosphorylation in suspension cells, as well as in cells cultured for extended time periods (e.g. 48 hrs; the left lane labeled as “0 min”) where cells have reached full
confluence (Figure 7A). In contrast, the phosphorylation is significantly reduced in cells cultured on fibronectin-coated plates during the spreading stage. We quantified both CAP1 and phospho-CAP1 signals and calculated the ratio of phospho-CAP1 to CAP1, as plotted in the graph in Figure 7B. The ratio was significantly reduced in cells during spreading stage. These results suggest that cells undergoing dynamic actin cytoskeletal reorganization have reduced phosphorylation. We propose that CAP1 phosphorylated at S307/S309 is an “inactive” form, consistent with the observation that the phosphor-mimetic DD mutant binds poorly to cofilin and localizes throughout the cytosol.
Discussion

We identify a phospho-regulatory mechanism for the first time on the versatile actin cytoskeletal protein CAP1. We show data supporting that dual phospho-regulation at S307 and S309 is important for CAP1 function through regulation of interactions with cofilin and actin. Single point mutants (S307 or S309), however, did not show alterations in binding cofilin or actin; thus phosphorylation at S307 (by another kinase(s), along with that at S309, collaboratively regulate CAP1 function. We found GSK3 can phosphorylate S309. GSK3 has primarily been studied for its role in development and Wnt signaling (Wu and Pan, 2010). However, it also regulates cell polarity and migration, and GSK3 targets that modulate tubulin can mediate some of the relevant cell signals. The role for GSK3 in actin regulation is less established, although p190RhoGAP was reported as a GSK3 target, and thus GSK3 may regulate the actin cytoskeleton through Rho (Jiang et al., 2008). One reason that CAP1 may not have been identified earlier is that the GSK3 site is not a canonical site. Nevertheless, we identify it as a GSK3 site using inhibitors, shRNA and in vitro kinase assays.

Mutations to aspartic acids at both S307 and S309 prevented cofilin binding, while the unphosphorylatable dual mutant had drastically increased cofilin binding, suggesting that phosphorylation may serve as a regulatory mechanism to control association/dissociation of cofilin with CAP1. Releasing cofilin (and actin) from association with CAP1 following completion of nucleotide exchange on G-actin would allow catalytic cycling of actin filament turnover by recycling cofilin. Although the DD mutant lost cofilin binding, it had normal actin binding. In contrast, while the AA mutant had dramatically increased cofilin binding, it had reduced actin binding. Actin binding is not necessary for CAP to bind to cofilin and assist depolymerization or filament severing (Chaudhry et al., 2013), consistent with our results that the S36A and the DD mutants, which lost cofilin association, had the fewest lamellipodia. Of note, there are two actin binding sites on CAP1, the cofilin/ADP-G-Actin binding site at the NT and the G-actin binding/sequestering site at the CT (Ono, 2013). The reduced actin binding in the AA mutant may reflect reduced binding of G-actin at the CT, since binding to cofilin (which is in complex with ADP-actin that initially binds to the NT) is drastically increased. The observation that the DD mutant, which lost cofilin binding, also caused lamellipodia phenotypes that are very similar to those in CAP1 knockdown cells, suggested that the phosphorylation causes inactivation of CAP1. Inactivation of CAP1 by phosphorylation is also in line with the literature that GSK3 typically phosphorylates and inactivates its substrates (Woodgett, 2001). Our results suggest that the DD mutant was dominant acting, which could be from (1) the inability to bind the cofilin/ADP-G-Actin to complete the nucleotide exchange or (2) interference with
CAP itself, since the functional form of CAP appears to be a hexameric oligomer. These results support a universal role for mammalian CAP1 in balancing the actin filaments vs. monomers, a property that cells can use to modulate cell polarity and migration (Bertling et al., 2004; Zhang et al., 2013). Moreover, given the important role for CAP1 function in controlling actin dynamics, it would not be surprising if multiple kinases phosphorylate both S307 and S309 residues on CAP1 to allow other cell signals to regulate CAP1.

Our findings that both the AA and DD mutants caused actin cytoskeletal phenotypes also suggest that phosphorylation may not merely inactivate CAP1, but is rather a step within a regulatory cycle where alternative phosphorylation and dephosphorylation may serve to control cycles of association/dissociation of cofilin and actin. The remarkable difference of the subcellular localization of the AA and DD mutant also support this notion. In this model, phosphorylation at S307/S309 on CAP1 may facilitate the release of cofilin so it can be recycled for the next round of severing. Also, kinase(s) and phosphatase(s) are expected to work in concert to achieve the reversible phosphorylation of CAP1 to drive the continuous cycles of actin filament turnover under physiological conditions, although we have yet to identify any specific phosphatase for these residues.

An unexpected finding of this study is that phosphorylation at S307/S309 regulates cofilin binding because this site is located near the CT of CAP1, which is distant from the minimal cofilin binding site closer to the NT, and it thus remains unclear how phosphorylation at S307/S309 can regulate the cofilin binding. However, we note that the cofilin binding site has not been clearly defined. Additionally, mutations at the extreme NT of yeast CAP homologue (Srv2) can prevent localization and SH3 binding at the P2 site, which is near the equivalent of S307/S309 (Yu et al., 1999). Thus there are other CAP mutations distant from direct binding sites that can affect binding to partners. We suggest that phosphorylation may indirectly prevent cofilin binding.

Inhibition of GSK3 can cause loss of cell polarity as well as accumulation of stress fibers. We propose that GSK3 regulates CAP1 through at least two mechanisms. Firstly, GSK3 (and potentially other kinases) phosphorylate CAP1 at S309 and promote CAP1 localization to the cytosol. Secondly, phosphorylation at 309 affects protein-protein interactions with actin and cofilin. Our data suggest the phosphorylation is not exclusively at S309, but likely requires phosphorylation at T314 as a primer, and requires at least another kinase for S307. The relative contribution of GSK3 in regulating CAP1 may
vary with different cells and signals compared to the other kinases. The loss of this phospho-regulation by GSK3 inhibition is expected to disrupt CAP1 function and actin dynamics. Additionally GSK3 can also affect actin dynamics through other mechanisms, such as cofilin phosphorylation and down-regulation as we observed (Figure 6). We show that reduced cofilin activity (including reduced expression) could be responsible for the unexpected CAP1 localization in GSK3-suppressed cells, overriding the direct effects of GSK3 on CAP1.

The results comparing the single point mutants of S309 and S307 with the AA and DD mutants in biochemical and cell biological analyses support that the two residues function as tandem sites. However, it remains to be determined if the differences in binding cofilin and actin, as well subcellular localization, are solely responsible for the phenotypes caused by these mutants. The cellular effects may involve additional mechanisms such as effects on nucleotide exchange of G-actin and CAP1 oligomerization, and studies are underway to unravel these scenarios. In addition to S307/S309, we also identified S36 as the other phospho-regulatory site, although we have yet to identify the responsible kinase. Phospho-regulation may also be employed in fungal CAP, as we previously found that the NT of CAP homologues from *L.edodes* and *S.pombe* both interact with 14-3-3 homologues (Zhou et al., 2000), a family of chaperon proteins that bind phosphoproteins. We did not observe significant alterations in the morphology of cells expressing CAP1 harboring mutations at the other phosphorylation sites that we found; however, we cannot exclude the possibility that they may impact other aspects of CAP1 such as subcellular localization. Some of these sites, such as S227 and S247 are within the middle domain, which is involved in CAP localization in budding yeast (Freeman et al., 1996).

In summary, our work reveals a phospho-regulatory mechanism of CAP1, by identifying a direct phosphorylation of CAP1 by GSK3 at S309, which, along with phospho-regulation at S307, collaboratively regulates CAP1 function through association with cofilin and actin to control the actin cytoskeleton. These findings contribute mechanistic insights into how cell signaling controls the actin cytoskeleton through molecules that regulate actin dynamics.
Materials and Methods

Miscellaneous reagents, cell culture, transfection and Western blotting

Monoclonal antibodies against human CAP1 has been previously described (Freeman and Field, 2000). Mouse anti-GAPDH, goat anti-actin and rabbit anti-GSKβ were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). GSK3α and phosphor-cofilin antibodies were from Cell Signaling Technology Inc. (Danvers, MA). Anti-His antibody, Alexa-Flour 488 Phalloidin, Alexa-Flour 594 Phalloidin, Alexa-Flour 488 goat anti-mouse IgG (H+L), fibronectin and PDGF (Platelet-Derived Growth Factor) were all from Life Technologies Inc. (Carlsbad, CA). Cofilin antibody was from the Cytoskeleton Inc. (Denver, CO). The phspo-specific polyclonal antibody against S307/S309 was developed by synthesizing and injection of the peptides APKPQT(SP)(SP)PKPA (a.a. 301-313) into rabbits followed by affinity-purification of the antibody (Genscript Inc., Piscataway, NJ). Lentiviral packaging mix was from Sigma Aldrich (St. Louis, MO). PH797804, LY294002 and MEK inhibitor U0126 were from Sellekchem (Houston, TX). Forskolin and H-89 were from LC Laboratories (Woburn, MA). 6-BIO was from Biovision Inc. (Milpitas, CA) and SB216763 was from Cayman Chemical Co. (Ann Arbor, MI). JNK inhibitor XVI and ROCK inhibitor RKI-1447 were from EMD Millipore (Billerica, MA). Peptide substrates were synthesized by Neo-Peptide™ (Cambridge, MA). FBS (Fetal Bovine Serum) was from Hyclone Laboratories Inc. (Logan, UT). Cells were cultured in the DMEM except for the hTERT-HPNE cells, which were maintained in a medium of 75% DMEM supplemented with 25% medium M3 base. Cell transfection was conducted using FuGene 6 from Roche Diagnostics. Recombinant active GSK3α and GSK3β, as well as the control GSK3 substrate peptide GSM [RRRPASVPPSPSLSRSRHSSHQRR] were from Millipore (Billerica, MA). Tissue culture dishes for immunofluorescence were from MatTek Corp. (Ashland, MA) and Transwell plates were from Corning Inc. (Corning, NY). Western blotting was conducted following standard procedures, and cell culture, transfection and preparation of cell lysates were conducted following procedures previously described (Zhou et al., 2003).

Mapping of phosphorylation sites on CAP1 by Mass-Spectrometry

A plasmid expressing mouse CAP1 with a 6xHis-tag to the N-terminus was transfected into HEK293T cells, and the expressed 6xHis-tagged CAP1 was precipitated from the cell lysate with Ni-NTA beads as described (Zhang et al., 2013). The samples were resolved on SDS-PAGE followed by Coomassie Blue staining of the gel. The tagged CAP1 band was isolated, reduced with Dithiothreitol (DTT), and trypsin-digested. Part of the recovered sample was used for protein identification, and the remaining sample was loaded onto a TiO2 column for purification of the phosphorylated peptides (Cantin
et al., 2007). The samples were subjected to a nanospray/Qstar-XL analysis and the data obtained was searched against the Swiss protein database. The mass-spectrometry and data analysis were performed by the Proteomics Core Facility at the University of the Pennsylvania Perelman School of Medicine.

Construction of plasmids and establishment of NIH3T3 stable cells expressing the mutants

The plasmid that expresses a 6xHis-tagged mouse CAP1 has been described (Zhang et al., 2013). To express the S36 and S307/S309 mutants in CAP1 knockdown HeLa cells, DNA fragments of mouse CAP1 mutants were amplified using high-fidelity DNA polymerase and sub-cloning them into the pcDNA4 vector, following similar procedures for the WT CAP1 gene (Zhang et al., 2013). Point mutants of CAP1 that harbor unphosphorylatable (A) or phosphomimetic (D) mutation(s) at the phosphorylation site(s) were generated based on a pEGFP-N1 vector using the primers listed in Supplemental Table II. NIH3T3 cells stably expressing WTCAP1 and the point mutants were generated by transfection of cells followed by selection with 1,000 \( \mu \text{g/ml} \) Neomycin for two weeks and pooling the cells that had survived the selection.

Phase imaging and immunofluorescence

Phase contrast imaging and immunofluorescence were conducted as described (Zhang et al., 2013). The actin cytoskeleton was stained with fluorescent Phalloidin (Alexa-Fluor 488 or 594). For immunofluorescence of CAP1, cells were blocked with 3% BSA for 1 hr after fixation and permeabilization, followed by incubation with a CAP1 monoclonal antibody diluted at 1:200 (Freeman and Field, 2000) or an X-press antibody. Signals were visualized by incubation with an Alex-Fluor 594 goat anti-mouse secondary antibody. The samples were observed and wide-field images were taken using a Zeiss fluorescence microscope as previously described (Zhou et al., 2003), and the confocal imaging was conducted similarly as we did previously (Zhang et al., 2013).

Measurement of cell area, percentage of cells with lamellipodia and Transwell migration assays

Cell areas were measured using ImageJ software (http://rsb.info.nih.gov/ij/) and analyzed similarly as described (Zhang et al., 2013). To quantify and compare percentage of cells with peripheral ruffling, cells with prominent peripheral ruffling were scored and the percentage was calculated. 100 cells per field were counted from three fields for each experiment, and results from three independent experiments were analyzed using Student’s \( t \)-test and plotted in the graph where the error bars represent S.E.M. Transwell assays were conducted similarly as previously described (Zhou et al., 2006). Briefly, sub-confluent stable
cells were serum-starved overnight and ~3x10^4 cells were plated in triplicate into Transwell inserts with 8μm pores placed in 12-well plates containing DMEM supplemented with 10 μg/ml PDGF. Cells were incubated for ~16 hrs before staining and scoring the migrated cells.

**Kinase assays**

Kinase assays were conducted similarly as previously described (Zhou et al., 2006). 20μg peptide substrate was mixed with 0.2μg GSK3 (α or β) in a 25 μl reaction mixture containing 20μM cold ATP in 1x kinase assay buffer (10mM MgCl2, 40mM HEPES, pH7.4). The reactions were initiated by addition of 5μCi γ-32P ATP, the samples were incubated at 30°C for 30 min and the reactions were terminated by adding 25 μl of the 2xTris-Tricine sample buffer (Bio-Rad). The samples were boiled and resolved on 10-20% Tris-Tricine Gradient Gel (Bio-Rad), and signals were detected by autoradiography.
Acknowledgements

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Author contributions

G.L.Z conceived, designed and supervised the most of the project, and wrote the manuscript. G.L.Z., H.Z., H.W. and P.G. conducted the experiments and analyzed the data. J.F. conceived and supervised the project at the early stage, and H.Z. organized the figures and tables.
Funding

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Figure legends

Figure 1. Expression of phospho mutants of S36 and S307/S309 in NIH3T3 fibroblasts caused morphological phenotypes. (A) Expression of GFP-fusion WTCAP1 and mutants. Cell lysates from the NIH3T3 stable cells harboring an empty GFP vector, a plasmid expressing GFP-fusion WTCAP1 and the four mutants were resolved on 10% SDS-PAGE followed by Western blotting using a GFP antibody. GAPDH was used as a loading control. (B) Effects of phospho mutants on cell size. Areas of 50 cells per field were measured individually using ImageJ software from three fields for each experiment, and results from three independent experiments were analyzed using Student’s t-test, and plotted in the graph where the error bars represent S.E.M. (* indicates P<0.05, and ** indicates p<0.01). (C) S36A and S307D/S309D mutants reduced percentage of cells with lamellipodia (LP). Cells with lamellipodia were counted and the percentages were calculated. 100 cells per field were counted from three fields for each cell type, and results from three independent experiments were analyzed and shown similarly as in (B).

Figure 2. Actin cytoskeletal alterations caused by expression of CAP1 phospho mutants. (A) Wide-field fluorescence imaging showing alterations in lamellipodia and stress fibers in cells expressing the mutants. The actin cytoskeleton was stained with fluorescent Phalloidin (Alexa-Fluor 594), and cells indicated with arrows show typical actin cytoskeletal phenotypes discussed for S36D and S307/S309 cells. The arrowheads indicate randomly-positioned protrusions in the S36D cells. (B) Percentage of cells with prominent lamellipodia. 50 cells were counted for each cell type and the experiment was repeated for three times. The data was analyzed using Student’s t-test and shown as mean ± S.D. (** indicates P<0.01). (C) Confocal fluorescence microscopy showing further details of the actin cytoskeletal alterations. F-actin was stained with fluorescent Phalloidin (Alexa-Fluor 488) and images were taken under a 60x lenses with a BD Pathway 855 imaging system (the stably-expressed GFP is of low level and does not interfere with Alexa-Flour 488 in microscopy). The arrows show stress fiber phenotypes in each cell type as discussed. (D) Transwell migration assays revealed reduced motility in both S36D and DD cells. The data was analyzed and shown similarly as in (B). (* indicates P<0.05; ** indicates P<0.01). The experiment was repeated for three times with similar results.

Figure 3. Altered binding with cofilin and actin for the S307/S309 mutants, and evidence supporting S307 and S309 function as a tandem site. (A) GST-cofilin pull-down revealed loss of cofilin binding for the DD mutant, while the AA mutant had drastically increased cofilin binding. Equal amounts of GST-cofilin bound to glutathione beads (~10-20µg) were added to lysates containing ~400µg total protein
harvested from CAP1 knockdown HeLa cells re-expressing WTCAP1 or the mutants. Tagged CAP1 co-precipitated with coflin was detected with a 6xHis antibody and GST-cofilin was detected with a coflin antibody. (B) Quantification of results in (A). Three independent experiments of GST-cofilin pull-down were measured by densitometry, and the results were analyzed and shown in the graph where the error bars represent S.E.M. (* indicates P<0.05; ** indicates P<0.01). (C) Actin co-precipitation assays reveal reduced actin binding for the AA mutant. Cell lysates prepared from CAP1 knockdown HeLa cells re-expressing WTCAP1 or the mutants were incubated with Ni-NTA beads, and the beads were precipitated and washed with lysis buffer containing 40mM imidazole. Samples were resolved on SDS-PAGE, and detected with antibodies against actin and 6xHis in Western blotting. (D) Quantification of results in (C). Three independent experiments were measured by densitometry, and the results were analyzed and shown in the graph where the error bars represent S.E.M. (* indicates P<0.05; ** indicates P<0.01). (E) & (F) A single alanine mutation at S307 and S309 (S307A & S309A) did not mimic the drastic increase in coflin binding, the experiments and data analysis were done similarly as in (A) and (B). (G) & (H) A single alanine mutation at S307 and S309 (S307A & S309A) did not mimic the reduced actin binding, the experiments and data analysis were done similarly as in (C) and (D). (** indicates P<0.01).

**Figure 4. GSK3 phosphorylates S309 on CAP1.** (A) Treatment of HEK293T cells with GSK3 inhibitors LiCl, 6-BIO and SB216763 all reduced CAP1 phosphorylation at S307/S309. Cells were treated with the inhibitors for 14 hrs, and cell lysates were prepared for Western blotting with both CAP1 and phospho-CAP1 antibodies. (B) Signals from three independent experiments were measured using densitometry and analyzed using Student’s t-test and plotted in the graph where the error bars represent S.E.M. (* indicates P<0.05; ** indicates P<0.01). (C) Knockdown of GSK3β with lentiviral shRNA in HeLa cells reduced S307/S309 phosphorylation. HEK293FT cells were transfected with Lentiviral Packaging Mix along with the shRNA constructs. The supernatant media were collected 48 hrs post transfection and used to infect HeLa cells. HeLa cells were harvested 48 hrs post infection and the lysates were prepared and used in Western Blotting. (D) CAP1 co-precipitates with GSK3β. Lysates from CAP1 knockdown HeLa cells re-expressing 6xHis-CAP1 or a control vector were incubated with Ni-NTA beads to precipitate 6xHis-CAP1. The precipitates were resolved on SDS-PAGE and blotted with both 6xHis and GSK3β antibodies. (E) Kinase assays mapping S309 as the GSK3 site. Peptides harboring an alanine mutation at either S307 or S309 with a phosphorylated or dephosphorylated T314 (a.a. 305-316, QTSPSPKPATKK) were used as substrates in the kinase assays to test their phosphorylation by GSK3β; the GSK3 substrate GSM peptide (RRRPASVPPSPLSRHSSHQR) was used as a positive control. The samples were resolved on 10-
20% Tris-Tricine gradient gel (Bio-Rad) and signals were detected by autoradiography. "*" indicates the quantified signals from the phosphorylated peptide and the arrowhead indicates partially degraded substrate. The numbers below the gels indicate the intensity of the signals compared to the positive control (folds). (F) Kinase assay results supporting T314 as the priming site for S309. A dephosphorylated T314 abolished the phosphorylation by GSK3; also both GSK3α and GSK3β phosphorylated S309.

**Figure 5. Effects of single and dual mutations on CAP1 subcellular localization and rescue of the enlarged size in the CAP1 knockdown HeLa cells.** (A) Confocal images showing cells re-expressing the mutants (S309A, S309D, AA, DD, S36A and S36D) and WTCAP1. Cells were fixed with 3.7% formaldehyde and stained with mouse anti-Xpress followed by Alexa-Flour 594 for CAP1 and Alexa-Flour 488 Phalloidin for F-actin. The arrows indicate peripheral localization of CAP1 mutants (AA and S309A). (B) Quantified results of cell area measured using ImageJ software similarly as we did in Figure 1C. Results from three independent experiments were analyzed using Student’s t-test and plotted in the graph where the error bars represent S.E.M. (**) indicates P<0.01.

**Figure 6. Effects of GSK3 inhibition on the actin cytoskeleton and CAP1 localization.** (A) Treatment with 6-BIO reduced S307/S309 phosphorylation of CAP1 in hTERT-HPNE cells. (B) GSK3 suppression caused accumulated stress fibers, reduced cell polarity as well as loss of CAP1 enrichment at the leading edge of the cell. Control cells (top panels) had well-established cell polarity and leading edges (indicated with arrows), cells treated with 6-BIO for 5 hrs lost this polarity (bottom panels; cells indicated with arrows). Cells were stained with CAP1 antibody and visualized with an Alex-Fluor 594 goat anti-mouse secondary antibody in addition to Phalliodin 488. CAP1 is enriched at the leading edge of the control cells, whereas the cytosolic areas immediately behind the leading edges had very weak CAP1 staining (indicated with arrowheads). In contrast, CAP1 had a diffuse localization across the cytosol in cells treated with 6-BIO, including the area mentioned above. (C) GSK3 suppression caused relocalization of both WTCAP1 and the AA mutant. CAP1 knockdown HeLa cells expressing both WTCAP1 and AA and DD mutants were treated with 6-BIO, and tagged CAP1 was stained with an X-press antibody followed by visualization using an Alex-Fluor 594 goat anti-mouse secondary antibody. The arrows indicate peripheral localization of WTCAP1 and the AA mutant, and both of which were abolished by suppression of GSK3. (D) GSK3 inhibition led to reduced cofilin activity, including down-regulation of cofilin as well as elevated cofilin phosphorylation at Ser3. HeLa cells were treated with 5μM 6-BIO for 5 hrs, and cofilin and Ser3-phosphorylated cofilin were detected in Western blotting.
Figure 7. Phosphorylation at S307/S309 is reduced in cells undergoing dynamic actin cytoskeletal reorganization. (A) Phosphorylation at S307/S309 was compared between HEK293T cells cultured in suspension and cells shortly after plating onto fibronectin-coated culture plates. Cells were harvested at the indicated time points of a suspension culture (SUS) and cultured on the fibronectin-coated plates (FN), and lysates were prepared for Western blotting with antibodies for CAP1 and the S307/S309-phosphorylated CAP1. GAPDH blotting was used as a loading control. (B) CAP1 and phospho-CAP1 signals from Western blots of three independent experiments were measured with densitometry, quantified and the ratios of phospho-CAP1 (pCAP1) versus CAP1 were calculated and plotted in the bar graph where the error bars represent S.E.M. (* indicates P<0.05; ** indicates P<0.01).
References


**Table I: Phosphorylation sites identified on mouse CAP1**

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<th>Sites</th>
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<td>T314</td>
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**A.thaliana** CAP1 36 SSAAGIDIASSDP 306 **--TKPAPS**KTGPP
*S.cerevisiae* Srv2 64 SAENAPEVEQDPK 358 **--PKPSTLKT**KRPP
*S.pombe* CAP1 92 SLTSTSAVEAVPA 384 **--P**KPAET**APV**KP
*C.elegans* CAS2 27 **--KE**TPVDATFQ 289 **--V**EKKPEKIHE
*D.melanogaster* capt 1  **---------**M 257 **--A**AAAP**SA**A**A**AKA
*M.musculus* CAP2 32 CGEVNGVIAGVAP 311 **--T**PSK**YPSQ**KA
*R.norvegicus* CAP1 32 ADS--P**SK**GAAAP 306 TP**SPKRAT**KEPA
*H.sapiens* CAP1 32 GDS--P**SK**GAVP 307 TS**SPKPA**KEPA
*M.musculus* CAP1 32 GDS--P**SK**GAVP 306 TS**SPKPA**KEPA
Figure 5

(A) Phalloidin Anti-Xpress Merge

Vec  
WT  
AA  
DD  
S309A  
S309D  

10μM
Figure 5 (B)

Cell Area (Pixels)

lec WT AA DD S309A S309D 36A 36D
Figure 7

(A)  

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pCAP1  
Fold  
1.00  
1.01  
1.04  
1.08  
0.11  
0.09  
0.39  

- 55 kDa

CAP1  
- 55 kDa

GAPDH  
- 37 kDa

(B)  

- 1.2

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pCAP1  
- 0.9

CAP1  
- 0.9

pCAP1/CAP1  
- 0.3

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