

Centralspindlin assembly and 2 phosphorylations on MgcRacGAP by Polo-like kinase 1 initiate Ect2 binding in early cytokinesis

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Abbreviations: BRCT, BRCA1 C-terminal; Ect2, Epithelial cell transforming sequence 2; MgcRacGAP, Male germ cell RacGAP; MKLP1, Mitotic kinesin-like protein 1; Plk1, Polo-like kinase 1.

Cytokinesis is the final step of cell division which partitions genetic and cytosolic content into daughter cells. Failed cytokinesis causes polyploidy, genetic instability, and cancer. Kinases use phosphorylation to regulate the timing and location of the cytokinetic furrow. Polo-like kinase 1 (Plk1) is an essential mitotic kinase that triggers cytokinesis by phosphorylating MgcRacGAP to create a docking site for Ect2 at the central spindle. Ect2 binds to MgcRacGAP via its N-terminal BRCT domain (BRCA1 C-terminal), which docks at specific phosphorylated residues. Here we investigate the minimal Plk1-dependent phosphorylation sites required for cytokinesis onset. We demonstrate that phosphorylation of the major MgcRacGAP site, S157, is necessary but not sufficient to bind the Ect2 BRCT domain. Phosphorylation of an additional residue on MgcRacGAP at S164 is also required to elicit efficient binding. Surprisingly, BRCT binding additionally requires MKLP1 and its cognate interacting N-terminal domain of MgcRacGAP. Our findings indicate that central spindle assembly and 2 Plk1-dependent phosphorylations are required to establish efficient binding of the Ect2 BRCT in early cytokinesis. We propose that these requirements establish a high threshold to restrain premature or ectopic cytokinesis.

Introduction

Protein phosphorylation is a key post-translational modification for cellular signal transduction. It is involved in cell growth, differentiation, cell cycle progression, transcription, metabolism and cytoskeletal rearrangement.^{1,2} Phosphorylation can regulate functions such as enzyme catalytic activity and protein-protein binding. Up to 30% of proteins are phosphorylated on at least one residue,³ and a majority of these contain multiple phosphorylated sites.^{4,5} Multiple phosphorylations can establish a molecular barcode.⁶ For example, the retinoblastoma tumor suppressor protein (Rb) has 16 Cyclin-dependent kinase 1 (Cdk1) phosphorylation sites and each unique pattern specifies a particular set of interactions with other proteins.⁷ Conversely, multiple phosphorylations can elevate the threshold required to elicit a biologic effect, which may be important to limit critical biologic events to specific times or locales.⁸

Cytokinesis is a crucial step of cell division that is temporally-spatially regulated to maintain genomic integrity. The cleavage furrow is positioned between sister chromatids through a series

of signaling events that arise from the central spindle apparatus. At anaphase onset, the midzone microtubules are bundled by the central spindle complex (centralspindlin), a hetero-tetramer of 2 major components, MgcRacGAP (also known as RACGAP1 or HsCyk4) and Mitotic Kinesin-Like Protein 1 (MKLP1).^{9,10} The centralspindlin complex docks and bundles midzone microtubules in a process that is mediated by Aurora B phosphorylation on MKLP1.¹¹ Although the centralspindlin complex is most concentrated at the microtubule midzone, the MgcRacGAP/MKLP1 complex also operates at the equatorial cortex.¹² This finding explains how the activation of the small GTPase, RhoA occurs at the cell membrane to recruit an actomyosin ring and generate an equatorial furrow.

In order to trigger cytokinesis, centralspindlin recruits Epithelial cell transforming sequence 2 (Ect2).^{13,14} Ect2 was originally discovered as an oncogene that can regulate Rho and Rac small GTPases.¹⁵ Ect2 is a guanine nucleotide exchange factor (GEF) and is required for activation of RhoA and for cytokinesis.¹⁶ Phosphorylation regulates Ect2 localization and activity during mitosis.¹⁶ In particular, phosphorylation at T342 by Cdk1

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regulates its conformation to control its GEF activity¹⁷ and to restrain binding to centralspindlin prior to anaphase onset.¹⁸ Thus phosphorylation of Ect2 plays a crucial role in temporal-spatial recruitment and activation of Ect2.

Ect2 localization in anaphase additionally requires phosphorylation on MgcRacGAP.¹⁸ Ect2 recruitment to centralspindlin occurs via its N-terminal BRCT domain.¹⁹ BRCT domains are phosphopeptide-binding modules that dock upon phosphorylation of a direct binding partner.^{20,21} The BRCT domain of Ect2 is required for cytokinesis, and serves as a negative regulator of Ect2 GEF activity in early mitosis.¹⁹ At the molecular level, the Ect2 BRCT domain directly interacts with phosphorylated MgcRacGAP.¹⁸ The Ect2-MgcRacGAP interaction establishes local pool of active Ect2 at the spindle midzone, which can exchange with a MgcRacGAP-independent pool that acts at the adjacent equatorial membrane.²² Together, Ect2 and MgcRacGAP coordinate recruitment and activation of a tightly localized pool of RhoA to trigger cytokinesis.^{13,18,23} Their interaction is stimulated by Polo-like kinase 1 (Plk1).

Plk1 is a serine/threonine kinase with pleotropic mitotic functions.^{24–26} Plk1 co-localizes with centralspindlin to the microtubule midzone in anaphase,²⁷ but the function of this pool is concealed from genetic techniques by essential Plk1 functions in early mitosis.²⁸ Chemical inactivation of Plk1 activity at anaphase onset has demonstrated that Plk1 is required for cytokinesis and Ect2 localization.^{29–32} Plk1 first phosphorylates PRC1 at the spindle midzone, inducing its recruitment to the central spindle complex. Next, this pool of Plk1 phosphorylates MgcRacGAP on several residues, which elicits binding of the N-terminal BRCT domain of Ect2.^{33–35} Among the known MgcRacGAP phosphorylation sites, S157 is required to establish MgcRacGAP-Ect2 binding,³⁴ but other Plk1-dependent phosphorylations make contributions.³⁵ Although phosphorylation of MgcRacGAP by Plk1 is crucial to trigger cytokinesis, the specific requirements for BRCT binding are obscure.

Here, we establish the minimal requirements for the MgcRacGAP-BRCT binding interaction. Although BRCT domains have reported to interact with singly phosphorylated peptides,²¹ we find that phosphorylation of MgcRacGAP at S157 is not sufficient to bind to BRCT of Ect2, and that an additional Plk1-dependent phosphorylation is necessary for efficient binding. Moreover, we demonstrate that this binding interaction requires both MKLP1 and the cognate N-terminal binding domain of MgcRacGAP. We propose that centralspindlin assembly and 2 Plk1-dependent phosphorylations on MgcRacGAP establish a high threshold for Ect2 recruitment, thereby ensuring proper timing and location of the cytokinetic furrow.

Results

MgcRacGAP is phosphorylated by Plk1 to serve as a docking site for Ect2 at the spindle midzone, which is required to trigger localized RhoA activation and furrow formation. Plk1 can phosphorylate MgcRacGAP at multiple sites, as identified by either mass spectrometry or *in vitro* kinase assay (Fig. 1A).^{34,35}

Therefore, we reasoned that MgcRacGAP might exist in multiple phosphorylated forms owing to modest preference of sites as substrates for Plk1. To test this, we employed Phos-tag SDS-PAGE to detect the abundance of various forms of MgcRacGAP. The Phos-tag method enables detection several phosphorylated isoforms as multiple migrating bands, by selectively retarding migration of more highly-phosphorylated proteins.³⁶ When the MgcRacGAP N-terminal fragment (MgcRacGAP-N) is incubated with Plk1, it displays at least 3 major phospho-forms as a ladder, indicating that, indeed, Plk1 has varying substrate preferences for MgcRacGAP phosphorylation sites (Fig. 1B). The low phosphorylation level is the most abundant form. These observations suggest that Plk1 can generate various phosphorylated forms of MgcRacGAP.

Next, we examined the contribution of Plk1 phosphorylations on MgcRacGAP to binding of the Ect2 BRCT domain, using a biochemical assay with recombinant proteins. We anticipated that, although S157 phosphorylation is necessary to bind BRCT, the additional Plk1 phosphorylation sites may act to further stabilize the MgcRacGAP/Ect2 interaction.³⁴ To test this, MgcRacGAP-N (amino acids 1–177) was fused to Maltose Binding Protein (MBP) and used as a substrate for Plk1. To specify the site and number of phosphorylations on MgcRacGAP-N, 3 serine residues (S157, S164 and S170) were mutated to non-phosphorylatable alanine (Fig. 1C, left). These phosphorylation sites were found in common from 2 independent studies.^{34,35} All were assayed for Plk1-stimulated association with BRCT of Ect2. As shown in Fig. 1C, wild-type MgcRacGAP-N induced BRCT binding upon Plk1 phosphorylation. We found that S157 is the best substrate for Plk1-induced phosphorylation, and mutation of this site reduces phosphorylation to 20% of the wild-type fragment (Fig. S1). Moreover, mutation of S157 significantly decreased MgcRacGAP-BRCT binding, confirming that a serine residue at 157 is important for direct association with BRCT. However, mutation of all these sites did not eliminate binding altogether, suggesting that they are not strictly required for binding under the conditions of this assay, as observed previously in a direct binding assay.¹⁸ Importantly, alanine mutations of other residues, S164A and S170A, also reduced phosphorylation level (Fig. S1), and disrupted Plk1-stimulated MgcRacGAP/BRCT complex formation (92% of wild-type for S164A, and 81% for S164A/S170A) (Fig. 1C, right). These findings demonstrate that multiple Plk1 phosphorylations on MgcRacGAP contribute to its direct association with BRCT.

Our biochemical binding data not only support the previous finding that S157 is a critical Plk1 site but also raise the possibility that additional phosphorylations are required for the MgcRacGAP-Ect2 interaction in cells. To evaluate this, we performed co-immunoprecipitation assays between MgcRacGAP and the BRCT of Ect2. We generated Flag-tagged phosphomimetic mutants of MgcRacGAP whose serine residues were mutated to aspartic acid, either alone or in combination. Co-immunoprecipitation is performed with Myc-tagged BRCT of Ect2 after Plk1 is inactivated with BI-2536 (Fig. 2A). As described previously, when all 3 phosphorylation sites (S157, S164 and S170) are mutated to

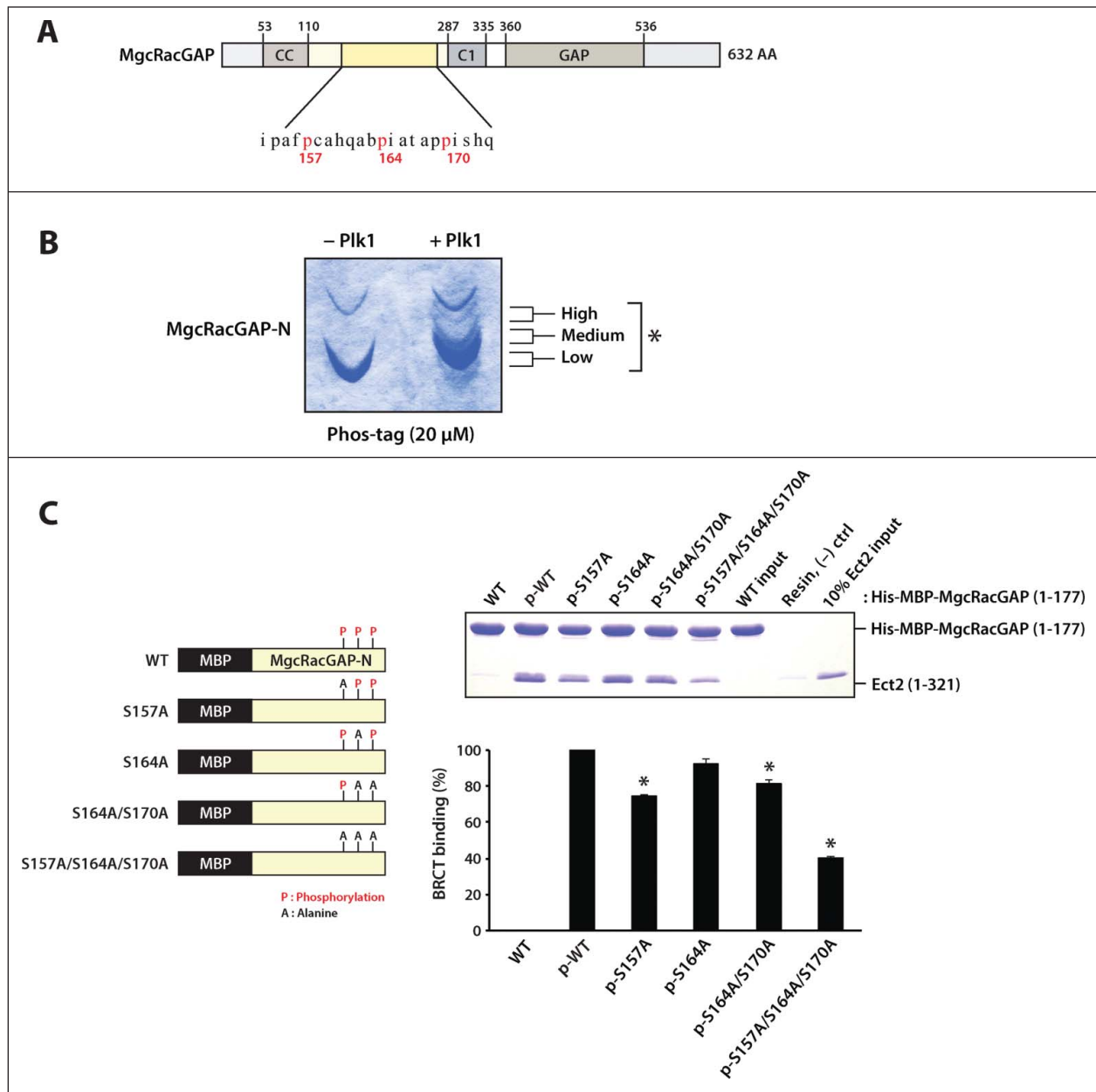


Figure 1. Multiple Plk1 phosphorylations on MgcRacGAP promote direct binding with BRCT of Ect2. **(A)** Domain organization of MgcRacGAP (CC, coiled-coil domain; C1, cysteine-rich domain; GAP, GTPase activating protein domain) and Plk1 phosphorylated sites in red (S157, S164, S170). AA, amino acid. **(B)** Mobility shift detection of phosphorylated forms of N-terminal MgcRacGAP. Recombinant MgcRacGAP (1–287aa) was incubated with ATP in the presence or absence of recombinant Plk1 kinase domain and analyzed by SDS-PAGE containing 20 μ M of acrylamide-coupled phosphate chelator (Phos-Tag), then detected by Coomassie. Phosphorylated forms are labeled as high, medium and low based on mobility. **(C)** *Left*, Schematic diagrams of MBP-tagged N-terminal MgcRacGAP phospho-forms (1–177aa). *Right top*, in vitro binding assay of N-terminal MgcRacGAP variants and BRCT of Ect2. MBP-MgcRacGAP or Plk1 phosphorylated MBP-MgcRacGAP was bound to amylose beads followed by incubation with Ect2-BRCT. Pulled down BRCT was separated on SDS-PAGE and visualized by Coomassie. *Right bottom*, Quantification of binding ability of MgcRacGAP derivatives to BRCTs. Relative band intensity of BRCT of Ect2 (1–321aa) is reported as a percentage of wild-type binding. Each was normalized to the band intensity of MgcRacGAP input. Error bars represent the mean \pm s.e.m. from 3 independent experiments. Asterisks indicate conditions with significant differences from p-WT, as determined by 2-tailed Student's t-test with $p < 0.05$.

aspartic acid, MgcRacGAP binds BRCT. However, single phosphomimetics at any site are insufficient to rescue the interaction with the Ect2 BRCT (Fig. 2B). To identify the minimal set of phosphorylations required for MgcRacGAP/

BRCT binding, we created a combination of double aspartic acid mutants (S157D/S164D, S157D/S170D and S164D/S170D). Consistent with the necessity of S157 phosphorylation site, the S157D/S164D restores efficient binding whereas

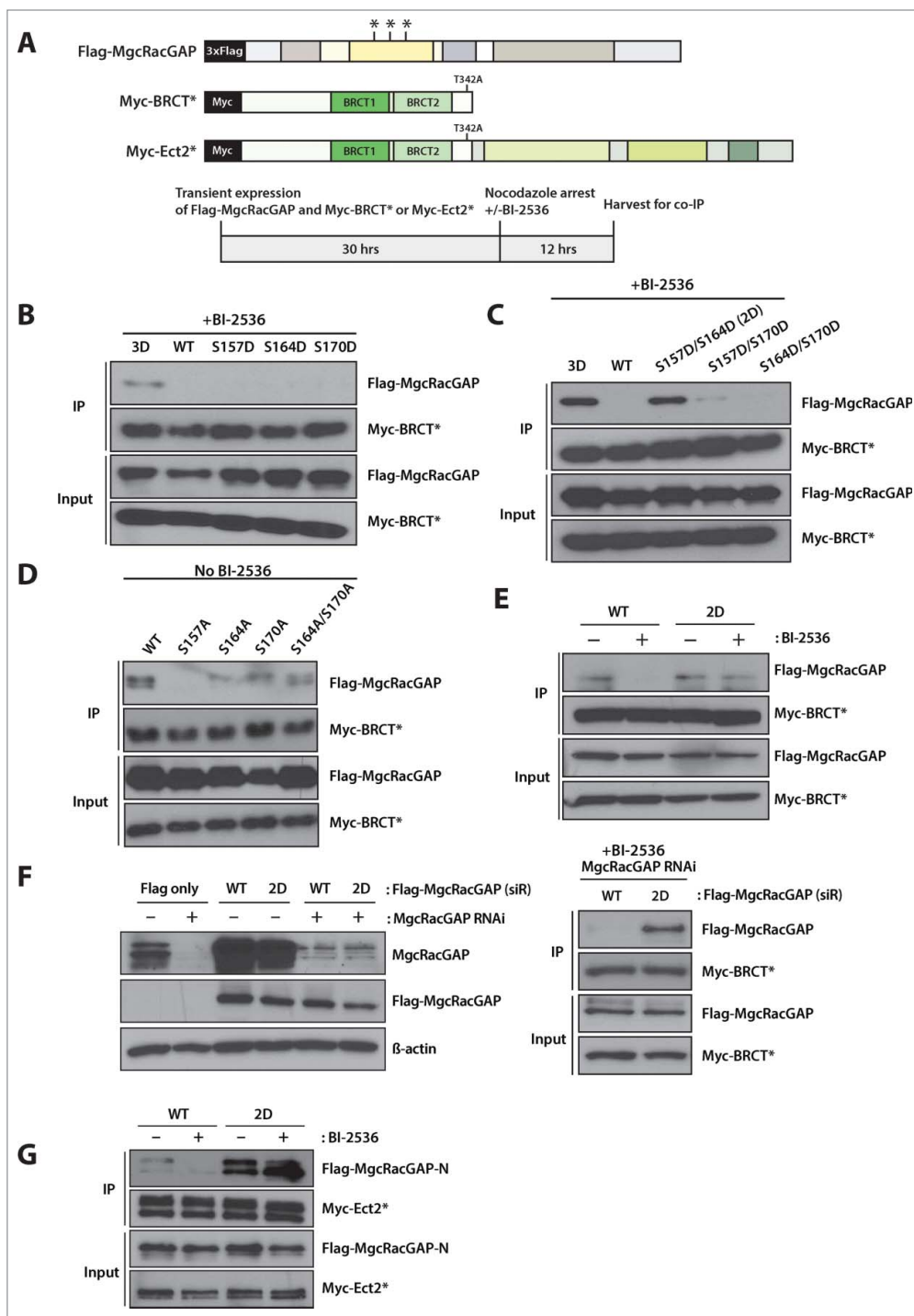


Figure 2. Diposphomimetic-MgcRacGAP at S157/S164 is sufficient for its binding to BRCT of Ect2. **(A)** Top, Recombinant human MgcRacGAP and Ect2 variants used for co-immunoprecipitation. Mutated Plk1 sites (either phosphomimetic or non-phosphorylatable) in MgcRacGAP are denoted by asterisks. Myc-BRCT and Myc-Ect2 lack an inhibitory Cdk1 phosphorylation site (T342A; permissive for MgcRacGAP binding in prometaphase, denoted here as Myc-BRCT^{*18} or Myc-Ect2^{*}). Bottom, Experimental scheme used for co-immunoprecipitation. HeLa cells were cotransfected with wild-type or derivatives of Flag-MgcRacGAP and Myc-BRCT^{*} or Myc-Ect2^{*}. At 30 h post-transfection, cells were incubated with 0.2 μ g/ml nocodazole \pm 100 nM BI-2536 for 12 h. Extracts were immunoprecipitated with Myc antibody, then analyzed by SDS-PAGE and Western blotting with Flag and Myc antibodies to assess binding. **(B–D)** Immunoblot analysis of protein extracts prepared and immunoprecipitated as described in **(A)**. **(E)** Mutation of S157A or S164A significantly reduces interaction of BRCT and MgcRacGAP. **(F and G)** Diposphomimetic-MgcRacGAP binding occurs at low expression level. **(F)** Immunoblot analysis indicating total MgcRacGAP and Flag-MgcRacGAP expression level. **(G)** Cell extracts cotransfected with MgcRacGAP siRNA and RNAi-resistant Flag-MgcRacGAP (siR) were immunoprecipitated by Myc antibody and analyzed for precipitation of Flag-MgcRacGAP as described in **(A)**. **(H)** Diposphomimetic-MgcRacGAP also rescues its binding to the full-length Ect2. HeLa cells were cotransfected with the N-terminal MgcRacGAP (Flag-MgcRacGAP-N; 1–287aa) and Myc-Ect2^{*}. Extracts were immunoprecipitated with Myc antibody and analyzed as described in **(A)**.

the other double phosphomimetic mutants (S157D/S170D and S164D/S170D) have minimal effects or fail to restore binding (Fig. 2C). Thus phosphomimetic mutations at S157 and S164 are sufficient to actuate binding of the Ect2 BRCT.

One limitation of the aforementioned experiments is that aspartic acid might not fully mimic phosphorylation, particularly since phosphate has a larger hydrated shell and greater negative charge compared with aspartic acid.³⁷ To overcome this limitation, we compared binding of phosphorylated wildtype

MgcRacGAP with the S157D/S164D (2D) phosphomimetic (Fig. 2D), and found that the diposphomimetic binds to BRCT to an extent similar to wild-type MgcRacGAP. Moreover, we tested whether blocking phosphorylation of S164 or S170 through non-phosphorylatable mutations would disrupt binding (Fig. 2E). Indeed, S164A mutation reduced Ect2 BRCT binding, confirming that phosphorylation on S164 is contributing to the binding of the BRCT domain of Ect2. To test if our results were due to overexpression of MgcRacGAP, we performed

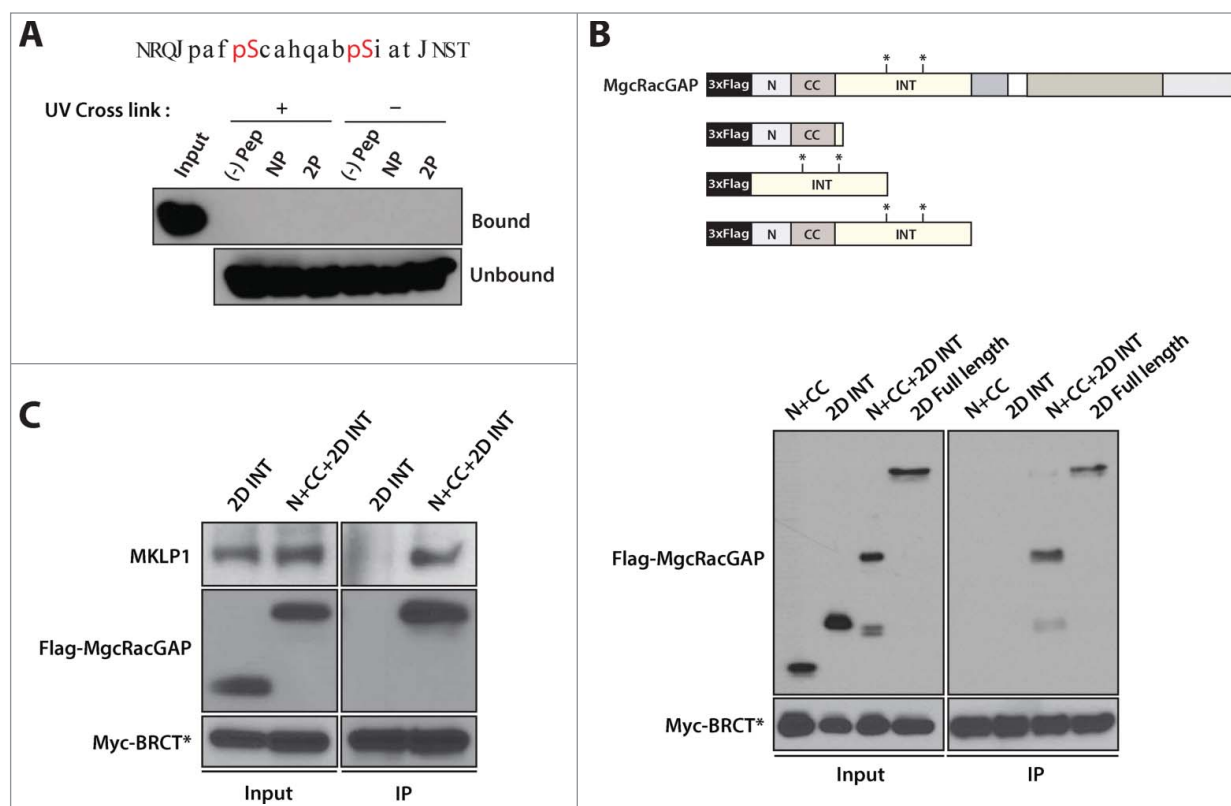


Figure 3. MgcRacGAP coiled-coil domain is necessary for BRCT binding. **(A)** *Top*, Peptide sequence (154–167aa). Phosphorylated S157 and S164 are highlighted in red. *Bottom*, Phosphopeptide binding assay. Streptavidin-coated beads were decorated with each biotinylated peptide (1 nmole) followed by incubation with 500 μ g of HeLa extracts expressing Myc-BRCT*. UV cross linking was performed on ice with 1200 J/m². Both Bound and Unbound fraction are detected by Myc antibody. **(B)** *Top*, Schematic representation of Flag-tagged truncation mutants of MgcRacGAP. Asterisks in the INT domain indicate the phosphomimetic mutations at S157 and S164. *Bottom*, Western Blotting analysis of coimmunoprecipitation of Flag-truncated MgcRacGAP with Myc-BRCT*. Protein extracts were prepared as described in Fig. 1A. **(C)** MKLP1 coimmunoprecipitates with Myc-BRCT*, but requires the N-terminal domains of MgcRacGAP. BI-2536-treated mitotic cells expressing the indicated Flag-MgcRacGAP truncation and Myc-BRCT* were immunoprecipitated with Myc antibody and were blotted with MKLP1 antibody.

knockdown of endogenous MgcRacGAP and replacement with expression of low levels of FLAG-MgcRacGAP-2D, which nevertheless interacted with the Ect2 BRCT (Fig. 2F and G). Finally, we verified that full-length Ect2 also interacts with 2D MgcRacGAP-N (Fig. 2H). Collectively, the data support the model that MgcRacGAP requires 2 phosphorylations for efficient Ect2 binding.

Previous work with BRCA1-derived BRCT domain showed that comparable domain could bind a short peptide sequence flanking the phosphorylated residue.³⁸ To test this, we synthesized biotinylated peptides 14-amino acids in length and they were either non-phosphorylated (NP) or doubly-phosphorylated at S157 and S164 (2P). We performed a streptavidin-affinity binding assay to test their ability to pull down Myc-tagged BRCT from transfected HeLa extracts. Surprisingly, neither peptide precipitated BRCT (Fig. 3A), suggesting that the doubly phosphorylated 14-amino acid peptide is insufficient for BRCT binding. One possible interpretation of this finding is that BRCT binding requires contacts provided by the context of additional MgcRacGAP domains. To address this and to identify the

required domains, we constructed truncated mutants of MgcRacGAP (Fig. 3B, top). We found that binding requires the N-terminus and coiled-coil domains (N+CC+2D INT) in addition to phosphomimetic mutations (Fig. 3B, bottom). Since the N-terminal domain of MgcRacGAP is known to interact with MKLP1 to form the centralspindlin complex,⁹ we speculated that MKLP1 may be important for Ect2 BRCT binding with MgcRacGAP. In support of this idea, BRCT pulls down MKLP1 as well as N+CC+2D INT MgcRacGAP (Fig. 3C). If so, the Ect2 BRCT may recognize both an assembled and highly phosphorylated centralspindlin complex.

If formation of the centralspindlin complex is a prerequisite, depletion of MKLP1 would impair MgcRacGAP-BRCT binding. Consistent with this, MKLP1 knock-down diminishes MgcRacGAP-BRCT binding (Fig. 4A) and similar results are obtained when cells were co-depleted for MgcRacGAP/MKLP1 and rescued with RNAi-resistant MgcRacGAP (Fig. 4B). These data suggest that the MKLP1-MgcRacGAP scaffold is necessary to dock BRCT of Ect2.

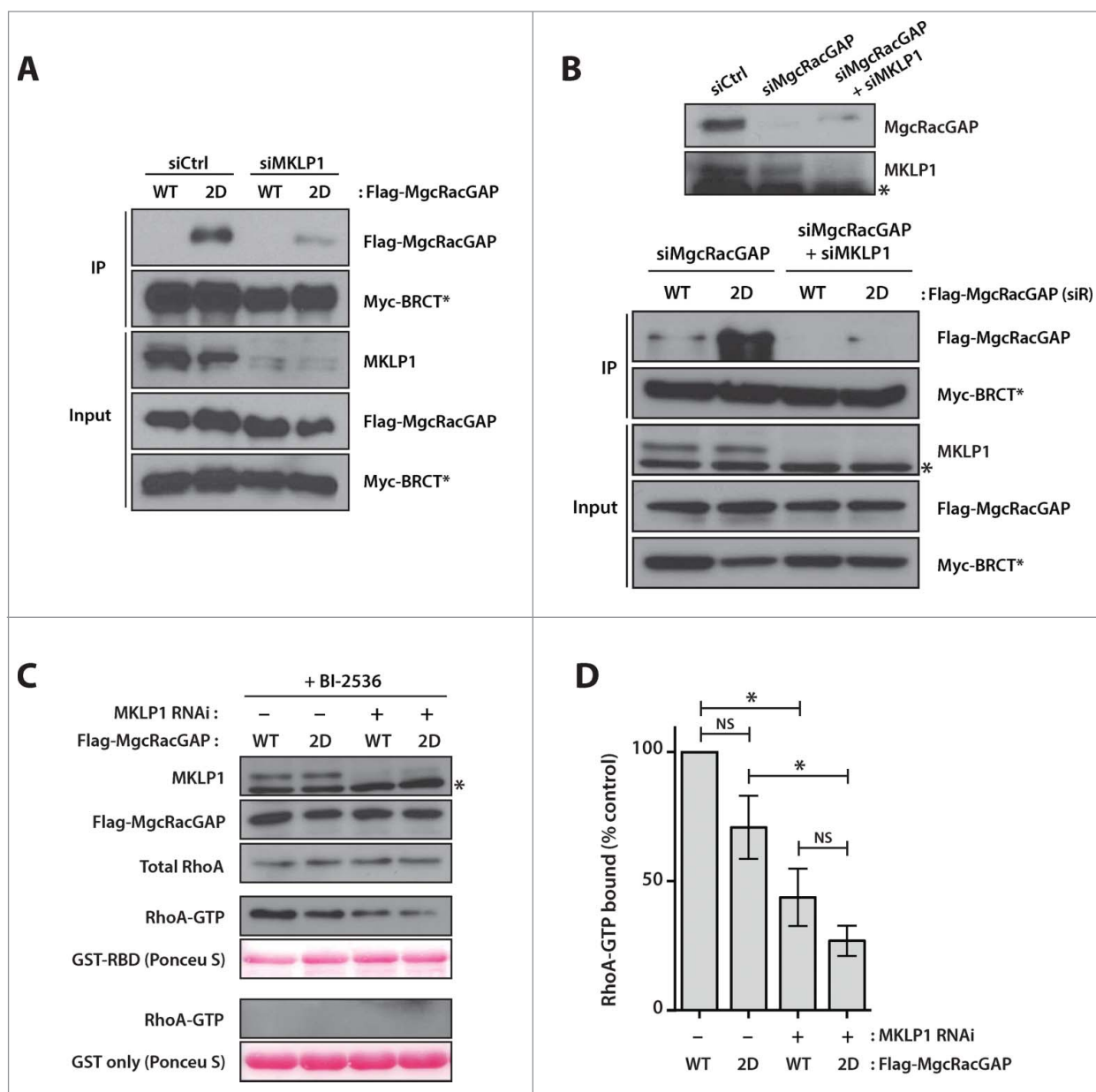


Figure 4. MKLP1 depletion attenuates binding of MgcRacGAP-Ect2 BRCT and activity of RhoA. (**A** and **B**) HeLa cells were transfected with either MKLP1 siRNA alone in **A** or in combination with MgcRacGAP siRNA in **B**. At 12 h after siRNA transfection, cells were subjected to a sequential transfection with the indicated Flag-MgcRacGAP and Myc-BRCT*. Cells were then arrested in mitosis by BI-2536 treatment for 12 h and harvested 24 h later. Cell lysates were immunoprecipitated by Myc antibody, resolved by SDS-PAGE and detected by immunoblotting with Flag antibody. The asterisk marks a non-specific band detected by this batch of anti-MKLP1 polyclonal antibody. (**B, top**) Protein levels of MgcRacGAP and MKLP1 are shown for each RNAi condition. (**C**) Representative western blot from a pull-down experiment to detect GTP-bound active RhoA. HeLa cells were sequentially transfected with MKLP1 siRNA and the indicated Flag-MgcRacGAP. After 24 h cells were arrested in mitosis with 0.2 μ g/ml nocodazole and 200 nM BI-2536 for 15 h and then treated with 10 μ M Cdk1-specific inhibitor RO-3306 for 30 min to induce mitotic exit. The asterisk marks a non-specific band. (**D**) Histogram shows quantification from 3 independent pull-down experiments in (**C**). Error bars represent mean \pm s.d. Comparisons were made with a 2-tailed Student's t-test (* p 0.05; NS, not significant).

Localized Ect2 docking at the spindle midzone and adjacent equatorial membrane plays a key role in activating RhoA.¹³ However, in the absence of MKLP1, RhoA can nevertheless be activated to some extent, albeit without focus at the equatorial membrane.¹⁸ However, it is possible that total RhoA activity is reduced under these conditions. We therefore pulled down active

RhoA in the presence or absence of MKLP1. To do this, we forced Ect2 targeting to centralspindlin with 2D MgcRacGAP in the presence of Plk1 inhibition, then measured the RhoA activity by guanosine triphosphate (GTP) binding. Interestingly, there is a small but statistically insignificant difference in the GTP loading on RhoA between wide-type *versus* 2D MgcRacGAP

transfected cells. However, total RhoA activity in extracts is significantly decreased when MKLP1 is depleted (Figs. 4C and D). These findings demonstrate that MKLP1 is required to highly activate RhoA, but that this might not be mediated through docked Ect2.

Taken together, our data demonstrate that the Ect2 BRCT requires at least 2 phosphorylation events on MgcRacGAP for its binding. Moreover, the N-terminus of MgcRacGAP interacts with MKLP1 to enhance BRCT binding.

Discussion

Over 700,000 unique protein phosphorylation sites have been detected in eukaryotic cells and thousands are specific to the mitotic state.^{5,39–41} The majority of cell-cycle specific phosphorylation occurs in mitotic cells⁴¹ and the mitotic substrates sites are often highly occupied by phosphate.⁴² For example, 44 phosphorylation sites have been identified on human MgcRacGAP,⁴³ comprising 7% of its amino acids; of these, 73% are present in mitosis.^{5,41,44–46} Thus, it will be important to identify the sites that are physiologically functional and understand how these sites control its functions.

In this study, we carefully characterized the complex formation between centralspindlin and Ect2-BRCT. Previous data supported conflicting models—one identified a strict requirement for S157 whereas another suggested a partial binding requirement distributed across the Plk1 phosphorylation sites.^{34,35} The data here resolve this discrepancy and support a model wherein both S157 and S164 sites contribute to this early event in cytokinesis onset. The S157 is preferentially phosphorylated by Plk1 and its phosphorylation contributes significantly to BRCT binding (Fig. 1; Fig. S1). Phosphorylation of both S157 and S164 are the necessary and sufficient Plk1-dependent sites to dock BRCT of Ect2. This conclusion is based on the importance of S164 phosphorylation: (1) aspartic acid mutation at other sites do not fully restore BRCT binding in the absence of Plk1 activity, and (2) it is not due to nonspecific negatively charged amino acids as 3 aspartic acids excluding S164, fail to enhance BRCT binding (Fig. 2C; Fig. S2). Second, our data identify additional structural elements of MgcRacGAP required for Ect2 binding. The MgcRacGAP N-terminal domain and coiled-coil domain are also required for BRCT binding. The N-terminal domain of MgcRacGAP binds to MKLP1 and the coiled-coil domain mediates its homodimerization (Fig. 3B).^{9,10} Importantly, MKLP1 depletion resulted in diminished MgcRacGAP-Ect2 BRCT binding, suggesting that intact centralspindlin enhances phospho-dependent MgcRacGAP-Ect2 BRCT interaction (Figs. 4A and B).

There are some limitations of our findings. We do demonstrate that low-physiologic levels of MgcRacGAP is sufficient to sustain binding with BRCT, and that full-length Ect2 is capable of binding. However, we have not demonstrated interaction with endogenous Ect2. Moreover, we have not tested whether MKLP1 and phosphorylation of MgcRacGAP at S157 and S164 are sufficient to trigger cytokinesis. Based on previous data, we

anticipate that additional Plk1-dependent phosphorylations are required.^{34,35} It will be important to elucidate the other contributions of Plk1 to trigger cytokinesis.

Although Ect2 is known to interact with centralspindlin, it is unclear whether this interaction mediates GEF activation. To address this, we measured a biochemical RhoA activity to monitor the GDP/GTP exchange activity of Ect2, assuming Ect2 is a main GEF for RhoA activation during early cytokinesis.⁴⁷ However, forced docking of Ect2 to phosphomimetic MgcRacGAP in the absence of Plk1 activity did not increase total active RhoA. Thus, Ect2 may retain full GEF activity in the absence of interaction with MgcRacGAP, relying solely on its own conformational change at anaphase onset. Consistent with this, recent evidence suggests that Ect2 is active even prior to anaphase onset.⁴⁸ Alternatively, redundant GEF activity generated by other RhoA GEFs, such as GEF-H1,⁴⁹ may compensate for loss of Ect2s GEF activity toward RhoA activation. Yuce et al. demonstrated that MKLP1 knockdown disrupts a focused zone of active RhoA at the equatorial cortex, yet RhoA is active even at diffused cortical localization.¹⁸ However, our results demonstrate that loss of MKLP1 reduces total RhoA activity (Figs. 4C and D). These results could be explained by the residual active RhoA, which might nevertheless be cortically localized. This raises the intriguing possibility that centralspindlin assembly is required for high activation of RhoA, but that this effect might not be mediated through centralspindlin-docked Ect2.

It is interesting to speculate why multiple phosphorylations on MgcRacGAP are required to recruit Ect2, rather than an optimal single phosphorylation site. One possible reason is that multiple phosphorylations produce a sharper on-off switch or set a higher threshold for activation than single phosphorylations.⁸ For example, the yeast cyclin-dependent kinase inhibitor, Sic1, has a high threshold for binding the ubiquitin ligase SCF^{Cdc4} which requires 6 or more phosphorylations prior to its degradation commits cells to S-phase entry.⁵⁰ Likewise, 2 required phosphorylations on MgcRacGAP can create a higher threshold for commitment than a single phosphorylation. Considering the crucial importance of timing and localization of cytokinesis onset, multiple phosphorylations may restrain premature or ectopic recruitment of Ect2 to the midzone. In other words, multi-site phosphorylation may be a mechanism to delay cytokinesis onset until a high threshold of Plk1 activity is reached at the central spindle. Although MgcRacGAP could be occasionally phosphorylated at S157 by low Plk1 activity before anaphase, this will be insufficient to trigger Ect2 binding until midzone Plk1 activity has exceeded the threshold level for multiple phosphorylations. Once co-localized at the spindle midzone in anaphase, multiply phosphorylated MgcRacGAP can respond decisively to high local Plk1 activity for robust recruitment of Ect2 (model shown in Fig. 5A). Moreover, phosphorylations of MgcRacGAP may be maintained in a dynamic equilibrium between kinases and phosphatases at spindle midzone. MgcRacGAP is known to interact with B56ε, a PP2A regulatory subunit and is dephosphorylated by PP2A.⁵¹ Additionally, the PP2A regulatory subunits B56α and B56β are also present at the central spindle at anaphase and may provide additional phosphatase activity that is exceeded to trigger cytokinesis.⁵² These

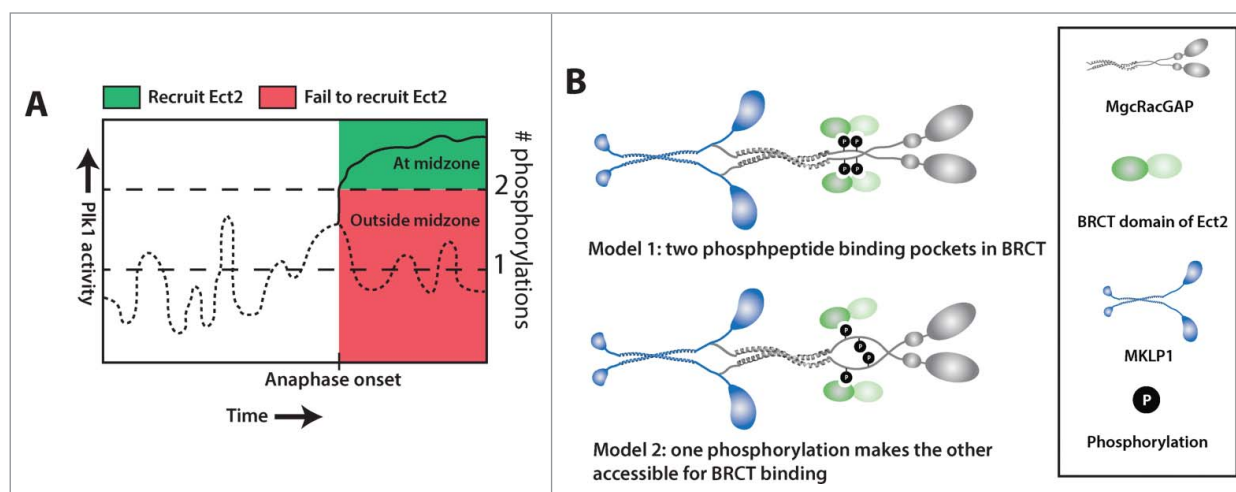


Figure 5. Model showing multisite phosphorylation mechanism. **(A)** Hypothetical model of the requirement for double phosphorylation for successful Ect2 recruitment. Dashed horizontal lines indicate threshold of Plk1 activity to phosphorylate either S157 (bottom) or both S157 and S164 (top), assuming that the more phosphorylation events requires the higher kinase activity. Subcellular Plk1 activity either at midzone (solid line) or outside midzone (dotted line) is plotted over time. Ect2 recruitment to midzone is illustrated in either green (above double phosphorylation threshold) or red (below double phosphorylation threshold). **(B)** Schematic models of how BRCT of Ect2 responds to phosphorylations on MgcRacGAP. *Top*, BRCT repeats of Ect2 have 2 phosphopeptide binding pockets. *Bottom*, One phosphorylation makes the other favorable for BRCT docking.

findings suggest that PP2A could function to balance phosphorylation level on MgcRacGAP at the midzone.

The apparent requirement for multiple phosphorylations raises a fundamental question about the molecular basis of phospho-specific binding: how does BRCT of Ect2 recognize 2 phosphorylation sites of MgcRacGAP? Known BRCT domains recognize and bind a single phosphorylated site.^{20,21} However, if Ect2's BRCT has a second phospho-binding pocket, this could explain why it binds multiply phosphorylated MgcRacGAP more efficiently (Fig. 5B, top). Although mutations of equivalent phospho-coordinating residues in Ect2 disrupt MgcRacGAP binding,³⁵ a weak secondary site may contribute to stable binding with the necessity of primary site. Alternatively, multiple phosphorylations may simply position the phospho-S157 to favor BRCT binding (Fig. 5B, bottom).³⁵ If the intermediate domain of MgcRacGAP is highly disordered (as predicted by XtalPred), the N-terminal BRCT and the phosphorylation at S164 could lock this domain conformation to allow the BRCT to engage phosphorylated S157. In either scenario, dual phosphorylation is required to fully dock the BRCT.

In conclusion, binding with the Ect2 BRCT requires 2 phosphorylations on MgcRacGAP as well as its MKLP1-interacting domain. We propose that these requirements establish a high threshold to restrain ectopic or premature cytokinesis onset.

Materials and Methods

Plasmids for expression in human cells

MgcRacGAP was inserted into pCDNA-Flag and its variants were created using polymerase chain reaction (PCR) and site-directed mutagenesis. For RNAi rescue experiments, 6 silent mutations were introduced into the target site (nt 1,294–1,312).

Full length Ect2* (T342A) was cloned in pCMV-Myc. The pCMV-Myc BRCT* (Ect2 amino acids 1 to 352 with T342A) and pGEX1 λ T RBD (Rho binding domain; Rhotekin 1–89aa) were kindly provided by Michael Glotzer.^{18,53} For transient transfection, HeLa cells were transfected using FuGENE HD (Promega) and analyzed 24 h to 48 h post transfection.

siRNA transfection

The following siRNA duplexes were used as follows: control (Thermo Scientific siGENOME Non-Targeting siRNA #2 D-001206–14), MgcRacGAP (40 nM; Thermo Scientific, custom order; CCUCUUCUGACCUUUCGCCUU), MKLP1 (100 nM; Thermo Scientific, ON-TARGETplus SMARTpool, L-004956–00). Lipofectamine 2000 (Invitrogen) was used for siRNA/add back transfection. Cells were analyzed in 48 h after transfection.

Co-immunoprecipitation

Thirty hours after transfection, HeLa cells were arrested in 200 ng/ml nocodazole for 12 h. Cells were lysed in lysis buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail and phosphatase inhibitor cocktail). Whole cells extracts were incubated with Myc antibody (9E10) at a concentration of 1 μ g of antibody/1 mg of total protein in lysis buffer and followed by incubation with protein A- and G-Sepharose beads (GE Healthcare). Beads were washed 3 times with lysis buffer and immunoprecipitated protein complexes were analyzed by SDS-PAGE and immunoblotting.

Recombinant protein preparation

All constructs and point mutations for MBP pull-down assay were generated using a standard PCR-based cloning strategy.

GST-Ect2 (1–321aa) and His-MBP-MgcRacGAP (1–177aa) wild-type and mutants were cloned in pQlink vector (Addgene) harboring GST-tag and His-MBP-tag, respectively. The proteins were overexpressed at 23°C for 20 h in *E. coli* strain DH5 α . The soluble fraction of the *E. coli* cell lysate was purified over Glutathione sepharose 4 Fast Flow resin (GE Healthcare) or Ni-NTA resin (Qiagen). His-MBP-MgcRacGAP (1–177aa) proteins further fractionated by Gel Filtration Chromatography (Superdex 200, GE Healthcare) to separate the free His-MBP from target protein. GST-tag was removed from Ect2 (1–321aa) by TEV protease before further purified by anion exchange chromatography (Source 15Q, GE Healthcare).

Plk1 kinase assay

For MBP-mediated pull down assay, 100 μ g His-MBP-MgcRacGAP (1–177aa) wild-type or mutant proteins were mixed with 10 μ g of His-Plk1 (1–352aa) and 0.5 mM ATP in 200 μ l of Kinase assay buffer (50 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 1 mM DTT) before incubating at 30°C water bath for 40 min.

MBP-mediated pull-down assay

Approximately 5 μ g of His-MBP-MgcRacGAP (1–177aa) or phospho-His-MBP-MgcRacGAP (1–177aa) (wild-type and mutants) was bound to 10 μ l of 50% Amylose resin slurry via MBP tag. The resin was washed with 200 μ l of assay buffer (25 mM Tris-Cl, pH 8, 150 mM NaCl, 3 mM DTT) 3 times to remove the excess unbound protein. Eight μ g of pure Ect2 (1–321aa) was added to the resin resuspended in 100 μ l assay buffer. The mixture was washed with 200 μ l assay buffer twice before examination by SDS-PAGE, and visualization by Coomassie-blue staining. The molar binding ratio of Ect2/MgcRacGAP on SDS-PAGE was calculated by Image J based on the triplicate data.

GTP-RhoA pull-down

Cells were lysed in lysis buffer and lysates were incubated with ~10 μ g of GST-RBD fusion conjugated with glutathione beads at 4°C for 4 h. The beads were washed twice with lysis buffer and subjected to SDS-PAGE. Bound RhoA was detected by immunoblotting using a RhoA antibody.

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Antibodies and chemicals

The following antibodies were used: mouse monoclonal anti-Flag (1:2000; M2, Sigma-Aldrich), mouse monoclonal anti-Myc (1:2000; a gift from P. Jallepalli), mouse monoclonal anti-MgcRacGAP (1:1000; Abnova), rabbit anti-MKLP1 (1:1000; Santa Cruz Biotechnology), mouse monoclonal anti-RhoA (1:1000; Santa Cruz Biotechnology), mouse monoclonal anti- β actin (1:20,000; abcam). Chemicals used in this study are nocodazole (EMD Biosciences), BI-2536 (a gift from P. Jallepalli), and RO-3306 (R&D Systems).

Phos-tag SDS-PAGE

GST-MgcRacGAP (1–287aa) was expressed in *E. coli* (BL21) and purified using Glutathione Sepharose 4 Fast Flow (GE Healthcare). Various phosphorylated forms of GST-MgcRacGAP were separated in an SDS polyacrylamide gel containing 20 μ M Phos-tag (Wako chemicals USA, Phos-tagTM Acrylamide AAL-107) and 100 μ M MnCl₂.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental data for this article can be accessed on the publisher's website.

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