

PAR3 and aPKC regulate Golgi organization through CLASP2
phosphorylation to generate cell polarity

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Abbreviations used:

aPKC, atypical PKC; CLASP2, cytoplasmic linker associated protein 2; MT,
microtubule; TGN, *trans*-Golgi network

Abstract

The organization of the Golgi apparatus is essential for cell polarization and its maintenance. The polarity regulator PAR complex (PAR3, PAR6 and aPKC) plays critical roles in several processes of cell polarization. However, how the PAR complex participates in regulating the organization of the Golgi remains largely unknown. Here, we demonstrate the functional crosstalk of the PAR complex with CLASP2, which is a microtubule plus-end tracking protein and is involved in organizing the Golgi ribbon. CLASP2 directly interacted with PAR3 and was phosphorylated by aPKC. In epithelial cells, knockdown of either PAR3 or aPKC induced the aberrant accumulation of CLASP2 at the *trans*-Golgi network (TGN) concomitantly with disruption of the Golgi ribbon organization. The expression of a CLASP2 mutant that inhibited the PAR3-CLASP2 interaction disrupted the organization of the Golgi ribbon. CLASP2 is known to localize to the TGN through its interaction with the TGN protein GCC185. This interaction was inhibited by the aPKC-mediated phosphorylation of CLASP2. Furthermore, the non-phosphorylatable mutant enhanced the colocalization of CLASP2 with GCC185, thereby perturbing the Golgi organization. Based on these observations, we propose that PAR3 and aPKC control the organization of the Golgi through CLASP2 phosphorylation.

Introduction

Cell polarity is a fundamental property of most cell types. Polarized cells distribute their cellular components asymmetrically. This asymmetric organization enables the cells to perform cell-type-specific functions (Drubin and Nelson, 1996; Mellman and Nelson, 2008). In the trafficking required for cell polarity, the Golgi apparatus acts as a sorting center for newly synthesized membrane and proteins, directing them to the functionally specialized domains in the plasma membrane and cytoplasm of polarized cells, such as the leading edge of migrating cells (Kupfer *et al.*, 1982; Bergmann *et al.*, 1983), the developing axon in neurons (Zmuda and Rivas, 1998; de Anda *et al.*, 2005) and the apical or basolateral membranes of epithelial cells (Rodriguez-Boulan *et al.*, 2005; De Matteis and Luini, 2008).

In interphase mammalian cells, the Golgi apparatus itself is a polarized organelle. The Golgi apparatus consists of stacks of flattened cisternae, the Golgi stack, which has *cis* and *trans* faces to allow functionally directional transport (Farquhar and Palade, 1998; Glick and Nakano, 2009). The stacks are assembled, highly ordered and typically linked, thereby forming a twisted, ribbon-like network, the Golgi ribbon, which retains *cis-trans* polarity for the entire Golgi apparatus (Yadav *et al.*, 2009; Hurtado *et al.*, 2011; Vinogradova *et al.*, 2012). The Golgi ribbon localizes next to the centrosome in the juxtannuclear region (Infante *et al.*, 1999; Takahashi *et al.*, 1999). For polarized post-Golgi trafficking, the Golgi apparatus is reoriented toward and directs its *trans* face at a specific functional region as described above (Li and Gundersen, 2008; Conde and Caceres, 2009; Petrie *et al.*, 2009). Thus, the regulation of the polarized organization and orientation of the Golgi apparatus is considered essential for cell polarization.

A PAR complex comprised of PAR3, PAR6 and atypical PKC (aPKC) plays a critical role in establishing polarity in various cell types, including front-rear polarity in migrating cells, neuronal polarity and apico-basal polarity in epithelial cells (Suzuki and Ohno, 2006; Goldstein and Macara, 2007; Etienne-Manneville, 2008; Funahashi *et al.*, 2014). Previous studies investigating the PAR complex in post-Golgi trafficking have focused on the microtubule (MT) network and the Golgi orientation in migrating cells, as

described below. In various cellular processes, the PAR complex functions cooperatively with Rho family GTPases, including Cdc42, Rac1 and RhoA (Iden and Collard, 2008). Activated Cdc42 binds to PAR6, which then associates with aPKC and PAR3, leading to the activation of aPKC (Joberty *et al.*, 2000; Lin *et al.*, 2000; Qiu *et al.*, 2000; Garrard *et al.*, 2003). At the leading edge of migrating cells, activated aPKC inactivates GSK-3 β , thereby allowing several MTs-associated proteins, such as adenomatous polyposis coli (APC), to accumulate at and stabilize MTs; this is followed by the formation of an asymmetric MT network. This MT network, with Dynein, induces the relocation of the Golgi toward the leading edge (Etienne-Manneville and Hall, 2001, 2003). During the formation of the PAR complex, PAR3 interacts with the Rac-specific GEF Tiam1, which is essential for MT stabilization for directional migration (Nishimura *et al.*, 2005; Pegtel *et al.*, 2007). The PAR complex formation is disrupted by the RhoA effector Rho-kinase/ROCK through PAR3 phosphorylation (Nakayama *et al.*, 2008). In the wound-healing assay, PAR3 at the cell-cell contacts also relocates the centrosome and the Golgi apparatus anterior to the nucleus, via Dynein activity (Schmoranzler *et al.*, 2009). Thus, the regulatory mechanism of the MT network and the Golgi orientation by the PAR complex has been clarified. In addition, accumulating evidence indicates that the PAR complex controls intracellular trafficking including endocytosis and exocytosis for cell polarity (Nishimura and Kaibuchi, 2007; Horikoshi *et al.*, 2009; Lalli, 2009; Rosse *et al.*, 2009; Bryant *et al.*, 2010; Harris and Tepass, 2010; Sato *et al.*, 2011; Yoshihama *et al.*, 2011). However, the involvement of the PAR complex in the Golgi apparatus, especially its organization, has not been fully elucidated, but aPKC has been reported to localize to the *trans*-Golgi network (TGN) (Westermann *et al.*, 1996).

The microtubule plus-end tracking protein (+TIP) cytoplasmic linker associated protein 2 (CLASP2) and another CLASP isoform, CLASP1, are key regulators of post-Golgi trafficking in cell polarity (Miller *et al.*, 2009). In migrating cells, CLASPs localize at the MT ends and the TGN, depending on the other +TIPs EBs and the TGN protein GCC185 (Akhmanova *et al.*, 2001; Mimori-Kiyosue *et al.*, 2005; Efimov *et al.*, 2007;

Honnappa *et al.*, 2009). CLASPs at MT ends, which are regionally regulated by GSK-3 β , stabilize MTs at the leading edge via linkages to the cell cortex and to actin filaments, thereby contributing to the asymmetry of the MT network (Wittmann and Waterman-Storer, 2005; Drabek *et al.*, 2006; Tsvetkov *et al.*, 2007; Kumar *et al.*, 2009; Watanabe *et al.*, 2009). Meanwhile, CLASPs at the TGN are required for the nucleation of Golgi-derived MTs, most of which are directed toward the leading edge (Efimov *et al.*, 2007). These CLASP-dependent MTs are also necessary for the formation of the Golgi ribbon (Miller *et al.*, 2009). These studies indicate that CLASPs regulate directional trafficking from the Golgi apparatus through the asymmetry of the MT network and the organization of the Golgi ribbon.

In this study, we explored the role of the PAR complex in the regulation of the Golgi apparatus focusing on CLASP2 as a novel interactor of PAR3. CLASP2 binds directly with PAR3 and is phosphorylated by aPKC. Through CLASP2 phosphorylation, aPKC, together with PAR3, regulates the interaction of CLASP2 with GCC185, and the localization of CLASP2 to the TGN, thereby controlling the organization of the Golgi. Based on these findings, we propose a novel molecular mechanism of the PAR complex with CLASP2 for regulating the organization of the Golgi apparatus in cell polarization.

Results

PAR3 and CLASP2 form a complex through direct interaction

By comprehensive screening for interactors of PAR3, we previously identified CLASP2 as a candidate interactor (Itoh *et al.*, 2010). We first verified our finding by immunoprecipitating the complex of PAR3 and CLASP2. Endogenous CLASP2 was specifically precipitated with endogenous PAR3, and vice versa (Figure 1A). Furthermore, Myc-fused CLASP2 α and CLASP2 γ were precipitated with GFP-fused PAR3 (Figure 1B). CLASP1 α was also precipitated with PAR3 (Figure 1C). These results indicate that PAR3 physiologically associates with CLASPs irrespective of its isoforms.

We next characterized the interaction of PAR3 with CLASP2 through a series of biochemical analyses. To narrow down the CLASP2 binding region, we prepared four fragments covering the full length of PAR3 as GST-fusion proteins (Figure 1D) and performed pull-down assays. CLASP2 was detected in the eluates of GST-PAR3-2N (252-697 aa) and -4N (937-1337 aa), and more CLASP2 was precipitated with the former PAR3 fragment (Figure 1E). We also found that Myc-PAR3 bound to CLASP2-N2 (310-670 aa) in an immunoprecipitation assay (Figure 1F and G), indicating that PAR3-2N and CLASP2-N2 are primarily responsible for the interaction between PAR3 and CLASP2. To examine whether this interaction is direct, we then performed an *in vitro* binding assay using purified GST-PAR3-2N and MBP-tagged CLASP2-N2. MBP-CLASP2-N2 bound to GST-PAR3-2N in a dose-dependent and saturable manner. Scatchard plot analysis revealed that the K_d value of this interaction was approximately 6 nM (Figure 1H). These results indicate that PAR3 and CLASP2 form a complex through direct interaction.

aPKC phosphorylates CLASP2

PAR3 acts as an escort protein for aPKC substrates such as Numb and Tiam1, and it is required for their phosphorylation (Nishimura and Kaibuchi, 2007; Wang *et al.*, 2012). We therefore investigated whether aPKC phosphorylates CLASP2. In *in vitro* phosphorylation assays, aPKC ζ and

aPKC λ phosphorylated His-CLASP2 γ but not His-RhoGDI, which was used as a control (Figure 2A and data not shown). The phosphorylation sites were mapped to the serine/arginine (S/R)-rich and carboxyl (C)-terminal regions in CLASP2 (Supplemental Figure 1A and Figure 2B), which are responsible for its localization to MT ends and the TGN (Akhmanova *et al.*, 2001; Mimori-Kiyosue *et al.*, 2005; Efimov *et al.*, 2007). Because aPKC depletion did not affect its localization to MT ends but did influence its localization to the TGN (see below), we focused on the phosphorylation of the C-terminal region. aPKC ζ phosphorylated GST-CLASP2-C1 (872-1294 aa) but not GST-CLASP2-C (1017-1294 aa; Figure 2B), indicating that aPKC phosphorylation occurs in the region between amino acid 872 and amino acid 1016 in CLASP2. To identify the exact phosphorylation site, we prepared a series of CLASP2-C1 point mutants whose potential PKC phosphorylation sites ([S/T]-X-[R/K], [R/K]-X-[S/T] and [R/K]-X-X-[S/T]) in this region were mutated to Ala. Ala substitutions at Ser940 (S940A), Ser952 (S952A) and Ser967 (S967A) significantly reduced the phosphorylation by aPKC ζ (Figure 2C), suggesting that these three Ser residues are major sites of phosphorylation by aPKC. To verify the *in vivo* phosphorylation of CLASP2, we attempted to generate phospho-specific antibodies against these residues, and we successfully produced anti-S940-P antibody. In immunoblot analysis, anti-S940-P antibody detected the phosphorylation of CLASP2-C1 by aPKC ζ *in vitro* but not that of CLASP2-C1-S940A (Figure 2D), indicating that anti-S940-P antibody specifically detects the Ser940 phosphorylation of CLASP2. This antibody did not detect the phosphorylation of endogenous CLASP2 in RPE-1 cells under normal growth conditions. However, treatment of the cells with calyculin-A, a phosphatase inhibitor for PP1 and PP2A, increased the phosphorylation level of CLASP2 and that increase was suppressed by pretreatment with an aPKC pseudosubstrate inhibitor (Figure 2E) and by depletion of aPKC (Figure 2F, see below). Under the same condition, depletion of PAR3 also reduced the phosphorylation of Ser940 in CLASP2 (data not shown). Because the phosphorylation levels of the proteins are regulated by the balance between kinases and phosphatases, these results suggest that aPKC, together with

PAR3, phosphorylates CLASP2 *in vivo* and that the turnover of this phosphorylation is rapid.

PAR3 and aPKC cooperatively regulate the localization of CLASP2 to the TGN and the organization of the Golgi ribbon.

CLASPs accumulate at the Golgi apparatus and the plus ends of MTs, depending on GCC185 and EBs (Mimori-Kiyosue *et al.*, 2005; Efimov *et al.*, 2007). We utilized RNA interference to examine whether PAR3 and aPKC participate in the localization of CLASP2 in RPE-1 cells. Because RPE-1 cells are supposed to express CLASP2 α and its N-terminal truncated alternative isoform CLASP2 γ , we used siRNA targeting their common sequence to deplete both isoforms (Akhmanova *et al.*, 2001; Mimori-Kiyosue *et al.*, 2005; Miller *et al.*, 2009; Watanabe *et al.*, 2009). Depletion of PAR3, aPKC or CLASPs in RPE-1 cells with siRNA was confirmed by immunoblot analysis (Figure 3A and Supplementary Figure 2). Depletion of CLASPs also reduced the level of GCC185 to approximately 25.3% of that in control cells. CLASP2 accumulated at the Golgi apparatus and partially overlapped with GCC185 in scramble-transfected cells (Figure 3B), as previously described (Efimov *et al.*, 2007). Those cells displayed tightly packed and elongated intact Golgi morphology, Golgi ribbons, judging from the fluorescence of the *cis*-Golgi protein GM130. However, depletion of CLASPs diminished the signal of CLASP2 and changed the Golgi ribbon morphology into a circular appearance (Miller *et al.*, 2009). Under the same conditions, depletion of PAR3 and aPKC increased the accumulation of CLASP2 at the TGN and enhanced the colocalization of CLASP2 and GCC185 concomitantly with disruption of the Golgi ribbon (Figure 3B). Based on the method described in the previous report (Miller *et al.*, 2009), we quantified the disruption of the Golgi ribbon by measuring the circularity index of the Golgi. Depletion of PAR3 and aPKC increased the circularity of the Golgi ribbon (Figure 3D). Of note, depletion of PAR3 and aPKC did not noticeably affect the localization of CLASP2 at the plus ends of microtubules under this condition (data not shown).

To test whether PAR3 and aPKC cooperate to regulate the

localization of CLASP2 and the organization of the Golgi, we performed a rescue experiment in the PAR3 depleted cells. The expression of siRNA-resistant PAR3-GFP partially restored the localization of CLASP2 and the organization of the Golgi ribbon, whereas the expression of PAR3-S827/9A, which is defective in aPKC binding (Nagai-Tamai *et al.*, 2002; Horikoshi *et al.*, 2009), failed to rescue those phenotypes (Figure 3C and D). These results indicate that PAR3 and aPKC cooperatively regulate the localization of CLASP2 to the TGN and the organization of the Golgi ribbon.

The interaction between PAR3 and CLASP2 is essential for Golgi ribbon organization

To examine whether the PAR3-CLASP2 interaction regulates the localization of CLASP2 to the TGN and the organization of the Golgi ribbon, we attempted to generate a CLASP2 mutant that inhibits the PAR3-CLASP2 interaction in a dominant negative manner. Because the CLASP2-N2 region is also responsible for interacting with EBs, IQGAP1 and MTs (Mimori-Kiyosue *et al.*, 2005; Watanabe *et al.*, 2009), we sought to identify the CLASP2 region that specifically interacted with PAR3. CLASP2-N2-1 (481-550 aa) bound to PAR3-2N and -4N but not to IQGAP1 (Figure 4A and Supplemental Figure 3). CLASP2-N2-1 has two EB-binding motifs (Ser-X-Ile-Pro)(Honnappa *et al.*, 2009), and replacement of the Ile-Pro with Ser-Ser impaired the interaction with EB1 but had no effect on the interactions with PAR3-2N and -4N (Figure 4A and Supplemental Figure 3). These results indicate that the CLASP2-N2-1 mutant that is defective in EB-binding, called CLASP2-DN, specifically interacts with PAR3. MBP-CLASP2-DN inhibited the interaction of CLASP2 with GST-PAR3-2N or -4N in a dose-dependent fashion (Figure 4B). Thus, CLASP2-DN appears to function as a dominant-negative mutant by competing with the PAR3-CLASP2 binding. The expression of HA-CLASP2-DN in RPE-1 cells increased the accumulation of CLASP2 at the TGN concomitantly with disruption of the Golgi ribbon, whereas that of HA-CLASP2-N1, used as a control, had little effect (Figure 4C and D). The analysis of the circularity

index of the Golgi confirmed that the CLASP2-DN expression significantly disrupted the Golgi ribbon morphology (Figure 4D). These results suggest that the PAR3-CLASP2 interaction regulates the localization of CLASP2 to the TGN and the organization of the Golgi ribbon.

PAR3 and aPKC regulate the interaction of CLASP2 with GCC185

To explore the mechanism regulating the localization of CLASP2 to the TGN dependent on PAR3 and aPKC, we examined the involvement of PAR3 and aPKC in the interaction between CLASP2 and GCC185. Based on a previous report (Lin *et al.*, 2011), we employed dithiobis succinimidyl propionate (DSP; Pierce) as a cross-linker for the immunoprecipitation of endogenous CLASP2-GCC185 complex. Under the condition where CLASP2 was coprecipitated with GCC185 from control cells, PAR3 depletion increased the amount of CLASP2 coprecipitated with GCC185 (Figure 5A), suggesting that PAR3 negatively regulates the interaction between CLASP2 and GCC185. The effects of aPKC depletion were inconclusive because the treatment of the cross-linker with aPKC-depleted cells drastically decreased the GCC185 solubility for the immunoprecipitation.

To examine whether aPKC regulates the interaction between CLASP2 and GCC185, we employed a pulldown assay with GST-fused GCC185-1N, which was proved to be the CLASP2 binding region in the immunoprecipitation assay (Figure 5B and C). Myc-CLASP2 γ was pulled down with GST-GCC185-1N, whereas the coexpression of HA-tagged aPKC ζ catalytic kinase domain (226-592 aa, aPKC ζ -cat) inhibited this interaction (Figure 5E), indicating that aPKC inhibits the CLASP2-GCC185 interaction. We further examined whether this inhibition by aPKC depends on the phosphorylation of CLASP2. Because of two regions phosphorylated by aPKC, the CLASP2-N2 and -C regions, GCC185 bound to the latter (Figure 5D), we used the C-terminal region for further experiments. In pulldown assays with the CLASP2-3A mutant in which three aPKC phosphorylation sites (Ser940, Ser952, and Ser967) in the C-terminal region were simultaneously mutated into Ala, the mutant was precipitated with GCC-185-1N to a similar extent to wild type, irrespective of the presence of

aPKC ζ -cat (Figure 5E). These results indicate that aPKC inhibits the interaction of CLASP2 with GCC185 through the phosphorylation of the CLASP2 C-terminal region.

CLASP2 phosphorylation by aPKC is essential for the organization of the Golgi ribbon.

Finally, we performed rescue experiments in CLASP-depleted cells with GFP-CLASP2 γ -3A. The expression of the siRNA-resistant wild type CLASP2 γ restored the organization of the Golgi ribbon to that observed in control cells. However, GFP-CLASP2 γ -3A failed to restore the organization of the Golgi ribbon (Figure 6A and B). In addition, compared with the wild type, GFP-CLASP2 γ -3A strongly colocalized with GCC185, which is similar to the behavior of endogenous CLASP2 in PAR3⁻ and aPKC-depleted cells (Figure 3B and 6A). These results suggest that PAR3 and aPKC regulate the organization of the Golgi ribbon through the phosphorylation of CLASP2.

Discussion

The PAR complex regulates the organization of the Golgi through CLASP2 for directional trafficking

Accumulating evidence suggests the involvement of the PAR complex in directional trafficking in polarized cells. In migrating epithelial cells, the Golgi apparatus is a center of directional trafficking, and its regulation leads to the formation of the polarity axis. Previous studies have revealed that the PAR complex orients the Golgi apparatus toward the leading edge through the asymmetric MT network for directional trafficking. Here, we found that depletion of PAR3 and the inhibition of aPKC induced aberrant accumulation of CLASP2 at the TGN, resulting in the disruption of the Golgi ribbon organization (Figure 3 and 6). Regulation of the Golgi organization is also essential for directional trafficking from the Golgi apparatus. The disruption of the organization of the Golgi by depolymerizing MTs or depletion of Golgi proteins does not affect the intra-Golgi transport or overall secretion to the plasma membrane, but it impairs the directional trafficking (Miller *et al.*, 2009; Yadav *et al.*, 2009; Hurtado *et al.*, 2011), which is most likely caused by the disorganization in the overall *cis-trans* polarity of the Golgi ribbon but each individual Golgi stack retains its *cis-trans* polarity (Cole *et al.*, 1996; Lowe, 2011; Vinogradova *et al.*, 2012). Consistently, depletion of PAR3 and the inhibition of aPKC appeared to disrupt the overall *cis-trans* polarity of the Golgi ribbon (Figure 3 and 6). Furthermore, cisternal unstacking and remodeling are required for the reorientation of the Golgi apparatus and centrosome (Bisel *et al.*, 2008). Our studies, together with these findings, suggest that the PAR complex orchestrates the reorientation and organization of the Golgi apparatus for directional trafficking. In concert with the reorientation of the Golgi, the PAR complex may accelerate the Golgi dynamics by regulating CLASP2 accumulation at the TGN downstream of Cdc42 or other factors (see below).

Regulation of CLASP2 localization to the TGN by the PAR complex

CLASPs are key regulators of directional trafficking from the Golgi apparatus, thereby establishing and maintaining cell polarity. CLASPs

localize to the TGN and regulate the organization of the Golgi ribbon. At the TGN, CLASPs stabilize MTs and anchor these MTs to the TGN membrane through GCC185, contributing to Golgi-derived MT nucleation, which is required for Golgi ribbon formation (Efimov *et al.*, 2007; Miller *et al.*, 2009). Both the formation and the maintenance of the Golgi depend on MTs (Cole *et al.*, 1996; Chabin-Brion *et al.*, 2001; Shorter and Warren, 2002). The Golgi structure, especially the TGN, is dynamically regulated for both post-Golgi trafficking and Golgi reorientation (Bisel *et al.*, 2008; De Matteis and Luini, 2008; Sengupta and Linstedt, 2011). However, the regulation of CLASPs at the TGN remains unclear. In this study, we reveal novel roles for the PAR complex in localizing CLASP2 to the TGN in the Golgi apparatus. aPKC and PAR3 inhibit the interaction of CLASP2 with GCC185 by phosphorylating CLASP2 (Figure 5), thereby regulating CLASP2 localization to the TGN. This regulation of CLASP2 localization to the TGN is required for the correct organization of the Golgi ribbon (Figure 3 and 6). Based on these findings, we speculate that CLASP2 phosphorylation by aPKC promotes the turnover of CLASP2 at the TGN, thereby facilitating the dynamicity of the MT-TGN linkage and ensuring that the Golgi structure remains dynamic. This Golgi dynamicity might contribute to the remodeling for the Golgi reorientation, as well as to the maintenance of the overall *cis-trans* polarity of the Golgi ribbon, for directional trafficking from the Golgi apparatus.

Regulation of the PAR complex at the Golgi apparatus

The PAR complex is activated downstream of migratory stimuli such as integrin engagement to ECM through Cdc42, which is required for the reorientation of the Golgi apparatus, as described above (Etienne-Manneville and Hall, 2001; Iden and Collard, 2008). In other cellular processes including endocytosis and exocytosis for cell polarity, the PAR complex acts as a Cdc42 effector (Martin-Belmonte *et al.*, 2007; Harris and Tepass, 2010), suggesting that Cdc42 plays a central role in cell polarization in conjunction with the PAR complex. Cdc42 localizes to the Golgi apparatus (Erickson *et al.*, 1996; Fukata *et al.*, 2003). The GTP/GDP cycling of Cdc42 at the Golgi apparatus appears to be essential for the

organization of the Golgi ribbon (Chen *et al.*, 2005; Dubois *et al.*, 2005). At the *cis* side of the Golgi, Cdc42 and its effectors regulate the Golgi-to-ER transport (Wu *et al.*, 2000; Luna *et al.*, 2002; Matas *et al.*, 2004). At the *trans* side, Cdc42 is activated (Nalbant *et al.*, 2004; Egorov *et al.*, 2009) and is involved in the sorting of vesicles from the TGN to the plasma membrane in the apico-basal polarity of epithelial cells (Kroschewski *et al.*, 1999; Cohen *et al.*, 2001; Musch *et al.*, 2001). However, the mode of action of Cdc42 at the *trans* side is not fully elucidated. It is tempting to speculate that Cdc42 is involved in the post-Golgi trafficking for cell polarity through the regulation of the PAR-CLASP complex.

Materials and methods

Reagents and chemicals

The anti-CLASP2 antibody and the anti-PAR3 antibody were described previously (Nishimura *et al.*, 2005; Watanabe *et al.*, 2009). Anti-S940-P-CLASP2 polyclonal antibody was raised against the phosphopeptide (EDIYSS(-P)LRGVT; Sigma-Aldrich, St. Louis, MO) and was affinity purified against the phosphopeptide. The anti-GCC185 antibody was kindly provided by Suzanne R. Pfeffer (Stanford University). The following commercial antibodies were used: anti-PAR3 (Millipore, Billerica, MA), anti-aPKC λ (BD Biosciences, San Jose, CA), anti-aPKC ζ (Santa Cruz Biotechnology, Santa Cruz, CA), anti-CLASP2, anti-CLASP1, anti-GCC2 (Abcam, Cambridge, MA), anti-GM130 (BD Biosciences), anti-GFP (Roche Diagnostics, Basel, Switzerland; MBL, Nagoya, Japan; Nacalai Tesque, Kyoto, Japan), anti-GST (Sigma-Aldrich), anti-Myc (9E10), anti-HA (12CA5 and 3F10). The following siRNAs from Sigma-Aldrich were used: Control Scramble, 5'-CAGUCGCGUUUGCGACUGG-3'; siPAR3-#1, 5'-GGCAUGGAGACCUUGGAAG-3'; siPAR3-#2, 5'-GAAUGUAUUUAGUACGACU-3'; siCLASP1-a, 5'-GCCAUUAUGCCAACUAUCU-3'; siCLASP2-a, 5'-GACAUACAUGGGUCUUAGA-3'; siCLASP1-b, 5'-GGAUGAUUUACAAGACUGG-3'; siCLASP2-b, 5'-GUUCAGAAAGCCCUUGAUG-3'; siaPKC λ -#1, 5'-CGAACAGCUCUUCACCAUG-3'; siaPKC ζ -#1, 5'-AGAGGAUCGACCAGUCAGA-3'; siaPKC λ -#2, 5'-CUUCCAAGCCAAGCGUUU-3'; siaPKC ζ -#2, 5'-GAUUGACCCUUUAACUGUA-3'. For depletion of CLASP and aPKC, we used a mixture of appropriate siRNAs. GST-aPKC ζ used for phosphorylation assay was obtained from CARNA Bioscience (Kobe, Japan). The aPKC pseudosubstrate inhibitor was provided by CALBIOCHEM (San Diego, CA). Other chemicals were obtained from commercial sources.

Plasmid constructions

The cDNAs encoding PAR3, CLASP1 and CLASP2 were obtained as

described previously (Nishimura *et al.*, 2005; Watanabe *et al.*, 2009). GCC185 cDNA was kindly provided by Paul A. Gleeson (The University of Melbourne). aPKC ζ cDNA was obtained from Kazusa DNA Research Institute (Chiba, Japan) and its catalytic kinase domain (aPKC ζ -cat, 226-592 aa) was used. Fragments of cDNA were amplified using PCR and subcloned into the pGEX (GE Healthcare Bio-Sciences, Uppsala, Sweden), pEGFP (Takara Bio Inc., Otsu, Japan), pCAGGS-Myc and pCAGGS-HA vectors. For the rescue experiments, siRNA-resistant CLASP2 and PAR3 harboring mutations in the siRNA target sequences were described previously (Nishimura *et al.*, 2005; Watanabe *et al.*, 2009). PAR3 or CLASP2 alanine mutations were generated with a site-directed mutagenesis kit (Stratagene, Santa Clara, CA).

Cell culture and transfection

COS-7 cells were cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum, and hTERT RPE-1 cells (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium/F12 with 10% fetal bovine serum. COS-7 cells were transfected with Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and RPE-1 cells were transfected with X-tremeGENE HP (Roche Diagnostics) according to the manufacturer's protocol. For siRNA transfection, Lipofectamine RNAiMAX (Invitrogen) was used.

Protein purification and biochemistry

Recombinant proteins were produced in *Escherichia coli* (XL-1 Blue, BL21DE3, or RosettaDE3) and purified as described previously (Kakeno *et al.*, 2014). Briefly, the collected bacteria were suspended and sonicated. After the ultracentrifugation for 1 h at 100,000 g, the supernatants were applied to the columns containing glutathione sepharose for GST-fusions (GE Healthcare Bio-Sciences). After washing the columns, the proteins were eluted with the buffer containing reduced glutathione, and they were then dialyzed against the appropriate buffer. All protein-purification procedures were performed at 4 °C.

Pulldown and immunoprecipitation assays

For the pulldown assay, transfected COS-7 cells were washed with PBS and lysed with Buffer A (20 mM Tris/HCl [pH 7.5], 1% NP-40, 50 mM NaCl, protease inhibitors). After removing debris by centrifugation, the lysates were incubated with 20 pmol GST-fusion proteins and glutathione beads for 1 h at 4 °C. The beads were washed with Buffer A and dissolved in SDS sample buffer. In the immunoprecipitation assay, appropriate antibodies and Protein A sepharose (GE Healthcare Bio-Sciences) were used instead of GST-fusion proteins and glutathione beads. The immunoprecipitation assay for endogenous GCC185-CLASP2 complex was described elsewhere (Lin *et al.*, 2011) and was performed with some modifications. Briefly, RPE-1 cells were cross-linked with 1 mM dithiobis succinimidyl propionate (DSP, Pierce Biotechnology, Rockford, IL) for 15 min before lysis.

In vitro binding assay

The *in vitro* binding assay was performed as described previously (Watanabe *et al.*, 2009). Briefly, purified MBP-tagged CLASP2-N2 was mixed with affinity beads coated with GST-PAR3-2N in buffer A. The beads were then washed with buffer A, and the bound proteins were eluted with buffer A containing 10 mM reduced glutathione. The eluates were subjected to SDS-PAGE, followed by immunoblot analysis using an anti-MBP antibody. The amount of CLASP2-N2 bound to GST-PAR3-2N was detected in a linear range using serial dilutions of standards by chemiluminescent detection and estimated with a Densitograph (ATTO, Tokyo, Japan) or with the ImageQuant LAS 4010 (GE Healthcare Bio-Sciences). Purified CLASP2-N2 was used as the standard for quantification.

In vitro phosphorylation assay

The kinase reaction was conducted in 50 μ l kinase buffer (50 mM Tris-HCl at pH 7.5, 5 mM MgCl₂, 0.3 mM dithiothreitol) containing 100 μ M [γ -³²P]ATP, 40 ng/ μ l recombinant kinases (GST-aPKC ζ or GST-aPKC λ -cat), and 1 μ M substrate (purified His or GST fusion proteins). After incubation for 2 h at

30°C, the reaction mixtures were boiled in SDS sample buffer and subjected to SDS-PAGE and silver staining. The radiolabeled bands were visualized with an image analyzer (Typhoon FLA 9000; GE Healthcare Bio-Sciences).

Immunohistochemical analysis

The cells were fixed with methanol at -30°C for 20 min, or with 2% PFA for 20 min, followed by permeabilization with 0.2% Triton X-100. After incubation with blocking buffer (0.2% Tween20, 1 mg/ml BSA, PBS) for 60 min at room temperature, the cells were incubated overnight at 4°C with the indicated primary antibodies and for 1 h at room temperature with the indicated secondary antibodies. The cells were observed using a 1.4 NA CFI Plan-Apo VC 60X or Plan-Apo 63X oil immersion objective lens under a confocal laser microscope (LSM780, Carl Zeiss, Jena, Germany).

To quantify the disruption of the Golgi ribbon, the circularity index of the Golgi morphology was measured, as described elsewhere (Miller *et al.*, 2009). Briefly, a freehand selection option in ImageJ software was used to outline the Golgi based on GM130 staining. Circularity index values were assigned to Golgi outlines by the ImageJ circularity plugin (<http://rsb.info.nih.gov/ij/plugins/circularity.html>), where circularity = 4π (area/perimeter²). A circularity value of 1 corresponds to a perfect circle.

Statistical analysis

Statistical analyses were performed with GraphPad Prism, version 5.0 (GraphPad Software, La Jolla, CA). $P < 0.05$ was considered to indicate statistical significance.

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Figure Legends

Figure 1. PAR3 and CLASP2 form a complex through direct interaction.

(A) RPE-1 cell lysates were precipitated with the indicated antibodies. Endogenous PAR3 was specifically coprecipitated with CLASP2, and vice versa. (B, C) Lysates of COS-7 cells expressing PAR3-GFP and Myc-CLASP isoforms were precipitated with anti-GFP antibody. PAR3 formed a complex with each CLASP. (D) The domain structures of PAR3 and its fragments are represented. Numbers refer to amino acids. CR, conserved region; PDZ, PSD95/Discs-Large/ZO-1 domain; aPKC BR, aPKC binding region. (E) Indicated GST-fusion proteins were incubated with lysates from COS-7 cells and precipitated with glutathione beads. CLASP2 was detected in the eluates of GST-PAR3-2N and -4N. (F) Schematic of CLASP2. The domain structures of CLASP2 and its various fragments are represented. CR, conserved region; S/R-rich, serine/arginine-rich. (G) Lysates of COS-7 cells expressing Myc-PAR3 and the indicated GFP-CLASP2 fragments were immunoprecipitated with anti-GFP antibody. Myc-PAR3 was coprecipitated with GFP-CLASP2-N2. (H) Saturable interaction of purified GST-PAR3-2N with MBP-CLASP2-N2. The indicated concentrations of MBP-CLASP2-N2 were incubated with beads coated with GST-PAR3-2N (20 pmol), and the K_d value was calculated by Scatchard analysis. The K_d value of the interaction between PAR3-2N and CLASP2-N2 was approximately 6 nM. The data represent the means ± SD of more than three independent experiments.

Figure 2. aPKC phosphorylates CLASP2 both *in vitro* and *in vivo*.

(A-C) Purified full-length CLASP2 γ or its mutants were incubated with recombinant aPKC ζ in the presence of [³²P]ATP, followed by silver staining and autoradiography. (A) aPKC phosphorylated His-CLASP2 γ *in vitro*. (B) Schematic of CLASP2 C-terminal fragments (top). aPKC phosphorylated GST-CLASP2-C1 but not -C (bottom). (C) Ala substitutions at Ser940 (S940A), Ser952 (S952A) and Ser967 (S967A) in CLASP2-C1 reduced the phosphorylation by aPKC ζ . (D) GST, GST-CLASP2-C1 wild type (WT) and S940A were incubated with aPKC ζ in the presence or absence of ATP, followed by immunoblotting with anti-GST and anti-S940-P antibody.

Anti-S940-P antibody detected GST-CLASP2-C1 phosphorylated by aPKC ζ but did not detect phosphorylation of the other constructs. (E) RPE-1 cells were incubated with or without 10 μ M aPKC pseudosubstrate inhibitor (aPKC-PS) for 20 min, followed by treatment with 100 nM calyculin-A for 10 min. Incubation with aPKC-PS diminished the phosphorylation of Ser940 in CLASP2. (F) RPE-1 cells transfected with the indicated siRNA were treated with 100 nM calyculin-A for 10 min. aPKC depletion reduced the phosphorylation of Ser940 in CLASP2. All results are representative of three independent experiments.

Figure 3. PAR3 and CASP2 cooperatively regulate the localization of CLASP2 to the TGN and the organization of the Golgi ribbon.

(A) RPE-1 cells were transfected with indicated siRNA, followed by immunoblotting. The transfection of siRNAs reduced the expression of their respective target proteins to undetectable levels. (B) RPE-1 cells transfected with the indicated siRNAs were fixed with methanol at -30°C for 20 min, followed by immunostaining with CLASP2 (gray and green), GCC185 (magenta) and GM130 (cyan). Depletion of PAR3 and aPKC increased CLASP2 at the TGN, enhanced the colocalization of CLASP2 and GCC185, and disrupted the organization of the Golgi ribbon. Insets in the left panel are magnified in the right panel. Bars, 10 μ m. (C) Rescue experiments for CLASP2 localization and the Golgi ribbon organization. RPE-1 cells were transfected with siRNA for PAR3 along with the indicated plasmids. These cells were fixed with methanol at -30°C for 20 min, followed by immunostaining with anti-GFP, anti-CLASP2 and anti-GM130 antibodies. The expression of siRNA-resistant PAR3-GFP partially restored CLASP2 localization and the organization of the Golgi ribbon, but expression of PAR3-S827/9A, which is defective in aPKC binding, failed to do so. Insets in the left panel are magnified in the right panel. Bars, 10 μ m. (D) The circularity index of the Golgi morphology was calculated (see Materials and methods). $n>30$. * $p<0.05$, *** $p<0.001$. All results are representative of three independent experiments.

Figure 4. The interaction of CLASP2 with PAR3 is essential for the organization of the Golgi ribbon.

(A) Schematic presentation of CLASP2 mutants and their binding abilities to PAR3-2N, -4N, EB1 and IQGAP1. CLASP2-DN bound to PAR3-2N and -4N but neither EB1 nor IQGAP1 (see Supplemental Figure 3). Asterisks indicate the locations of EB-binding motif. The red “X” indicates the mutation site in EB-binding motif. (B) GST-, GST-PAR3-2N- or -4N-immobilized beads were incubated with COS-7 cell lysates expressing GFP-CLASP2 γ in the presence or absence of MBP-fusion proteins. The bound proteins were analyzed by immunoblotting. Increasing amounts of MBP-CLASP2-DN inhibited the interaction of CLASP2 with PAR3-2N and -4N. (C) To examine the effect of the expression of CLASP2-DN on CLASP2 localization, RPE-1 cells expressing HA-CLASP2-N1 or -DN were fixed with methanol at -30°C for 20 min, followed by immunostaining with anti-HA, anti-CLASP2, and anti-GM130 antibodies. The ectopic expression of CLASP2-DN increased the accumulation of CLASP2 at the Golgi apparatus. Bars, 10 μ m. (D) To monitor the expression level of CLASP2-DN and to examine its effect on the Golgi organization, the indicated RPE-1 cells were fixed with 2% PFA and permeabilized with 0.2% Triton X-100, followed by immunostaining with anti-HA (gray), anti-GCC185 (magenta), and anti-GM130 (cyan). Bars, 10 μ m. The right graph shows circularity index of the Golgi morphology in indicated cells. The ectopic expression of CLASP2-DN induced the circular appearance of the Golgi. n>30. ***p<0.001. All results are representative of three independent experiments.

Figure 5. PAR3 and aPKC regulate the interaction of CLASP2 with GCC185.

(A) Control and PAR3-depleted RPE-1 cells were cross-linked with 1 mM dithiobis succinimidyl propionate (DSP), followed by precipitation with anti-GCC185 antibody. Depletion of PAR3 increased the association between CLASP2 and GCC185. (B) The domain structures of GCC185 and its fragments are represented. CC, Coiled-coil domain; GRIP, GRIP domain. (C,

D) Lysates of COS-7 cells expressing indicated Myc-fusion proteins and GFP-fusion proteins were immunoprecipitated with anti-GFP antibody. Myc-CLASP2 γ was coprecipitated with GFP-GCC185-1N (C), and Myc-GCC185-1N was coprecipitated with GFP-CLASP2-C1 (D). (E) COS-7 cells were transfected with the indicated constructs, and their lysates were pulled down with GST-GCC185-1N. The coexpression of aPKC ζ catalytic kinase domain (226-592 aa, aPKC ζ -cat) impaired the interaction of GCC185-1N with CLASP2 γ -WT but not with -3A. All results are representative of three independent experiments.

Figure 6. CLASP2 phosphorylation by aPKC is essential for Golgi ribbon organization.

(A) Rescue experiments for Golgi ribbon organization. RPE-1 cells were transfected with siRNA for CLASPs along with the indicated plasmids. These cells were fixed with methanol at -30°C for 20 min, followed by immunostaining with anti-GFP (gray and green), anti-GCC185 (magenta) and anti-GM130 (cyan) antibodies. The expression of siRNA-resistant CLASP2 γ -WT restored Golgi ribbon organization, but CLASP2-3A failed to do so. Insets in the left panel are magnified in the right panel. Bars, 10 μ m. (B) The circularity index of the Golgi morphology in indicated cells was calculated. $n > 40$. *** $p < 0.001$. All results are representative of three independent experiments.

Supplemental Figure 1. aPKC phosphorylates the S/R-rich region of CLASP2.

Purified GST-CLASP2-N2 was incubated with recombinant GST tagged aPKC λ catalytic kinase domain (210-586 aa, aPKC λ -cat) in the presence of [³²P]ATP, followed by silver staining and autoradiography. aPKC phosphorylated CLASP2-N2 *in vitro*.

Supplemental Figure 2. Depletion of PAR3, aPKC and CLASPs in RPE-1 cells

(A-B) RPE-1 cells were transfected with the indicated siRNAs, followed by

immunoblotting. (A) The transfection of siRNAs reduced the expression of the target proteins to undetectable levels. (B) CLASP depletion reduced the expression of GCC185.

Supplemental Figure 3. CLASP2-DN specifically interacts with PAR3-2N and -4N but not with either EB1 or IQGAP1.

(A-C) Lysates of COS-7 cells expressing the indicated CLASP2 mutants were pulled down by GST-fusions, and bound proteins were analyzed by immunoblotting. GFP-CLASP2-DN interacted with GST-PAR3-2N and -4N but not with either GST-EB1 or GST-IQGAP1-CT, which is the CLASP2-binding region.

Figure 1.

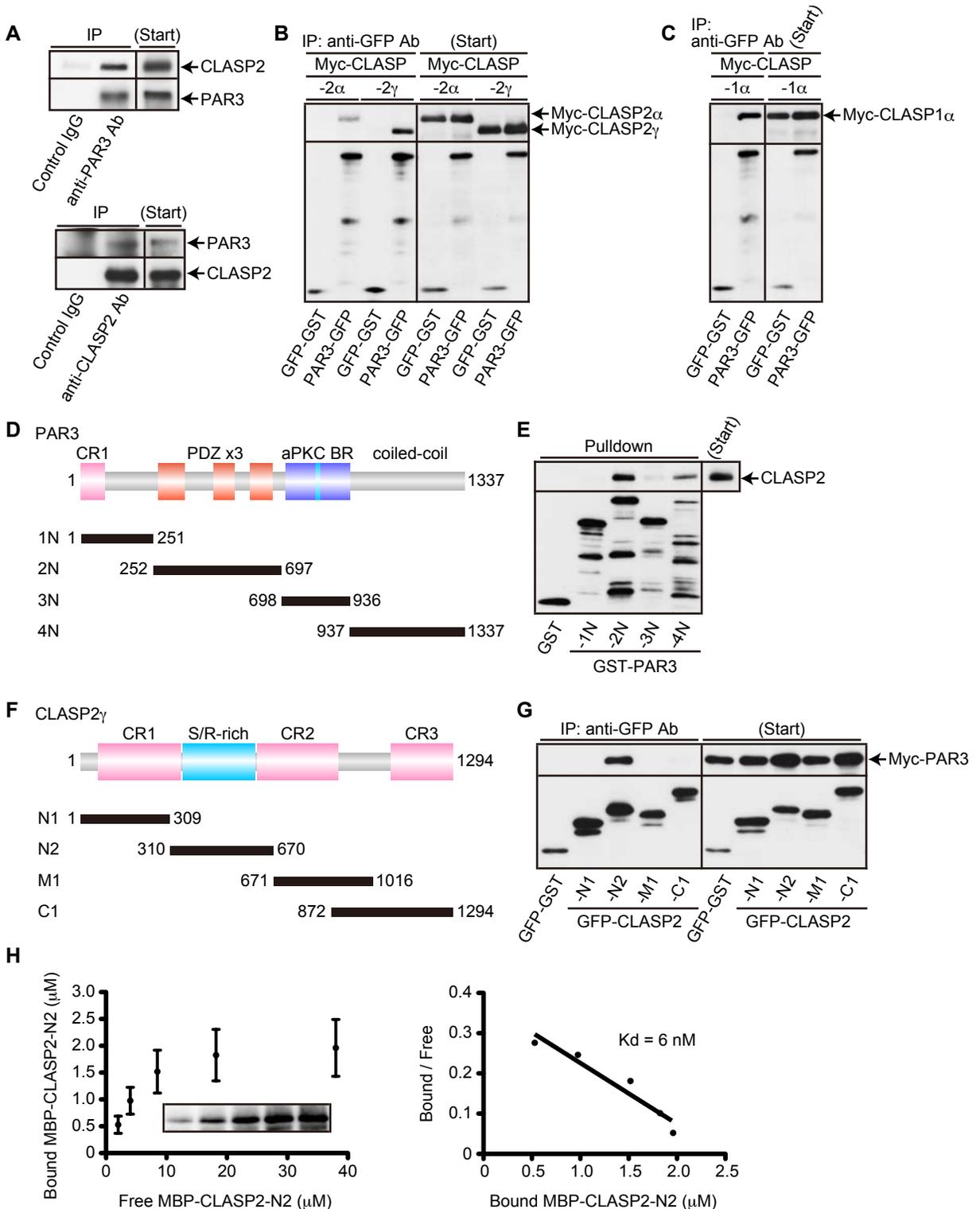


Figure 2.

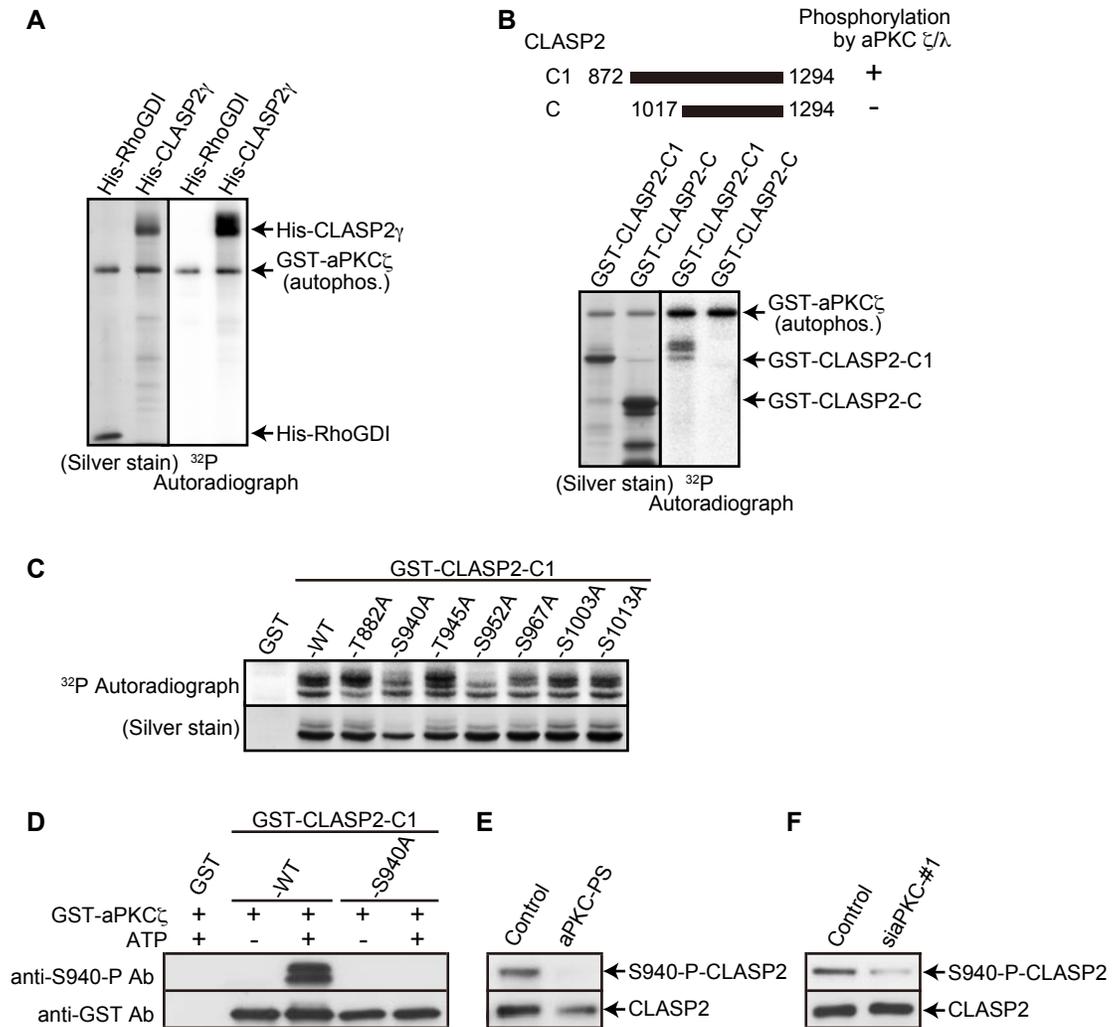


Figure 3.

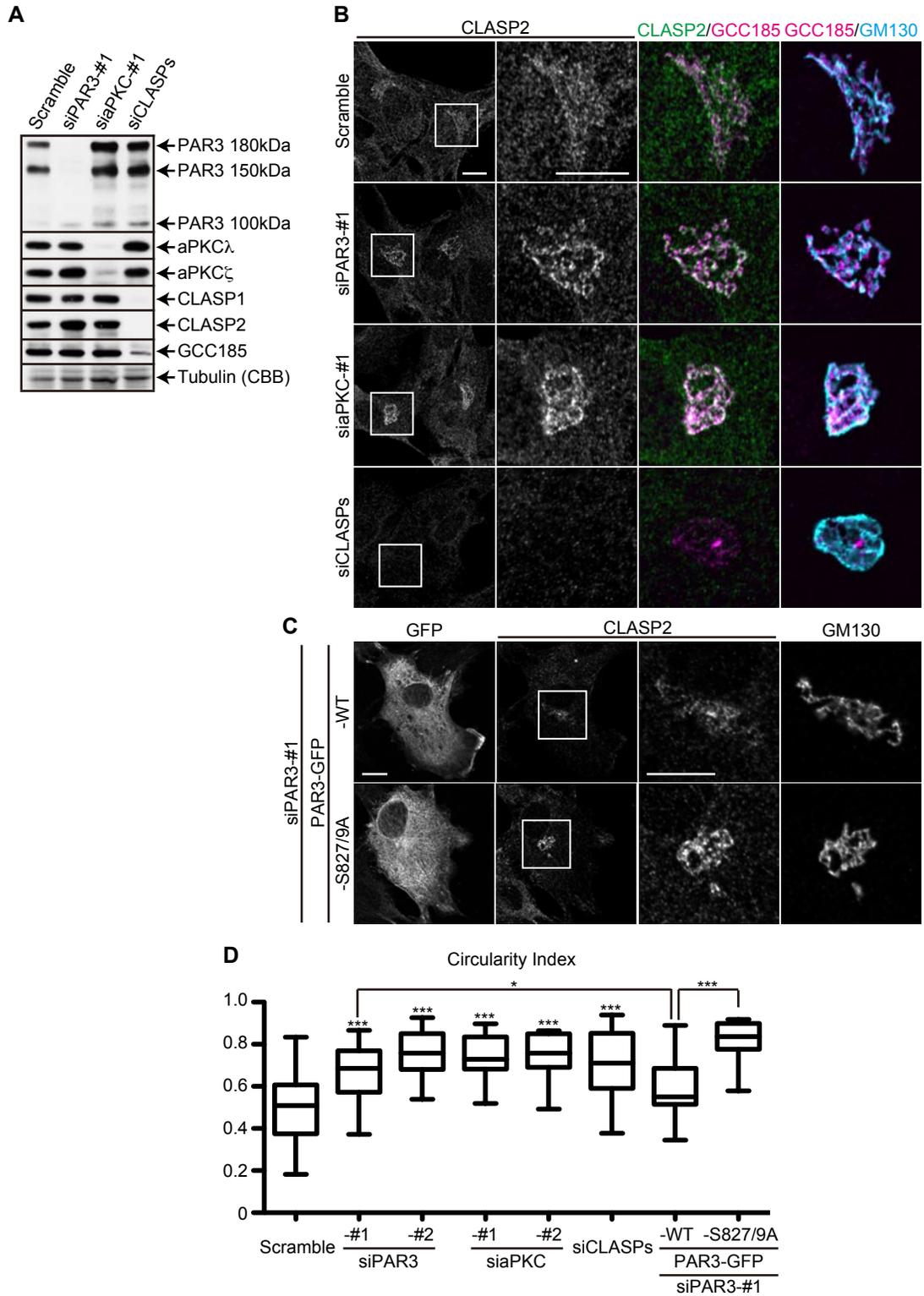


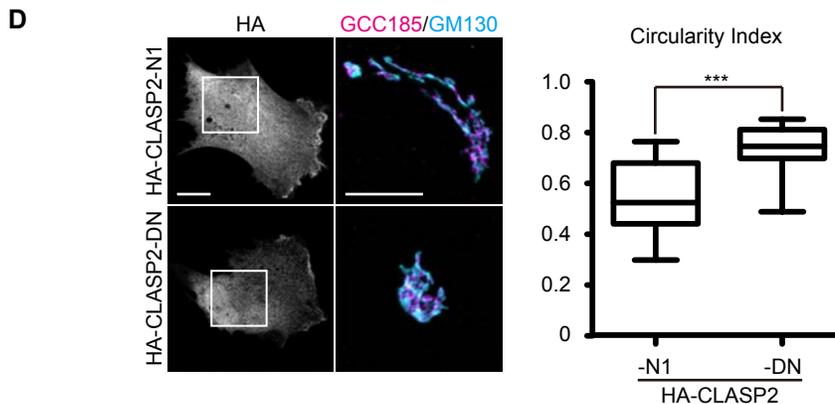
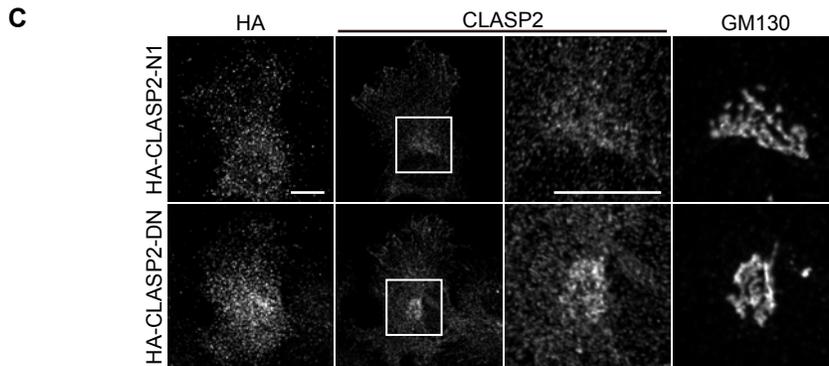
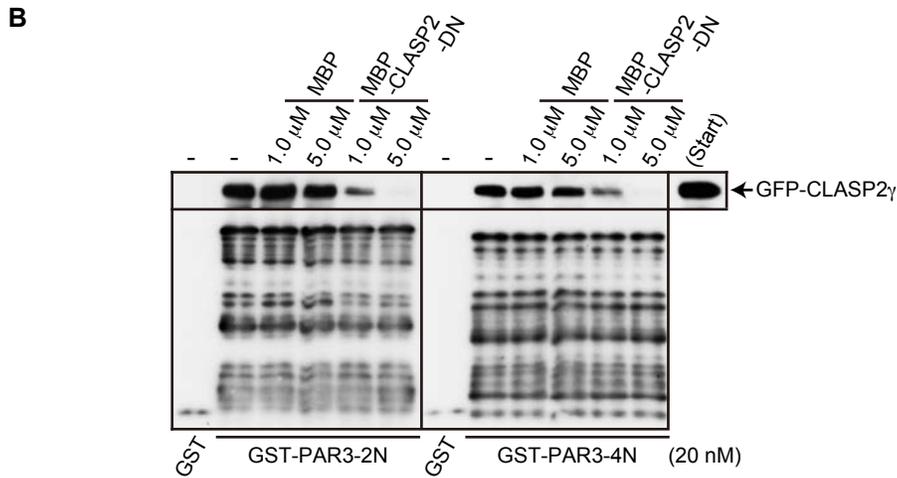
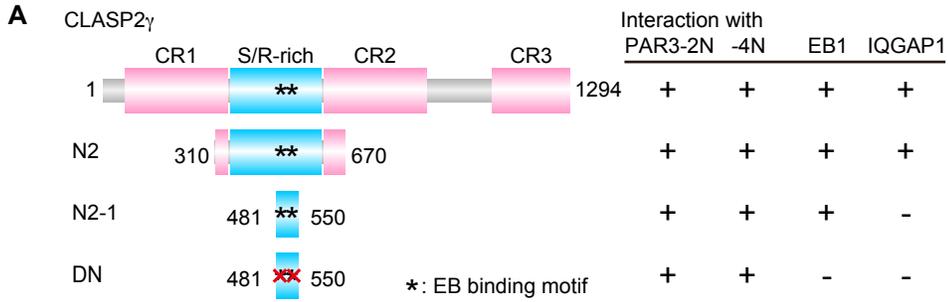
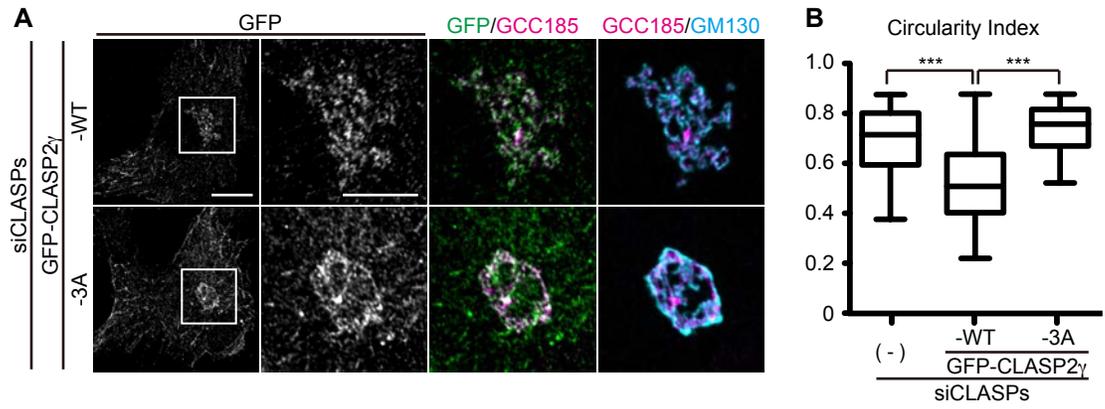
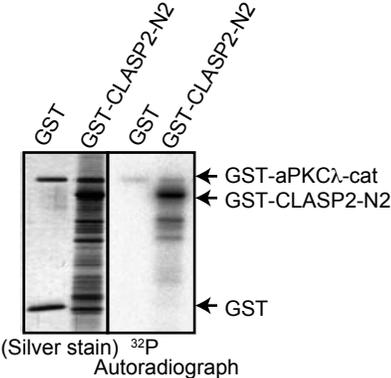
Figure 4.

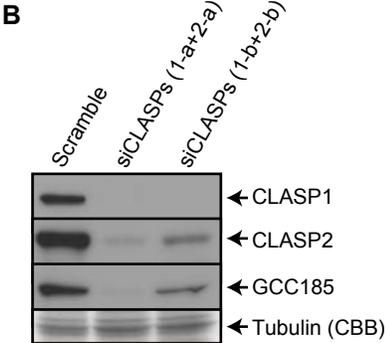
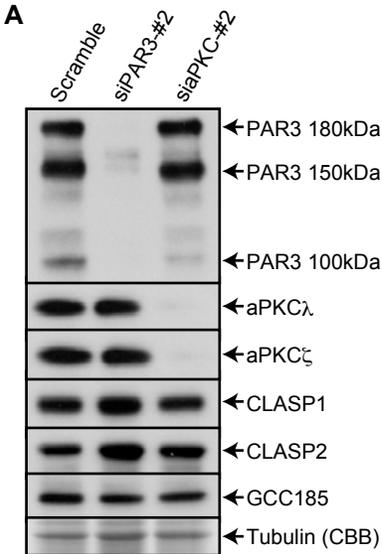
Figure 6.



Supplemental Figure 1.



Supplemental Figure 2.



Supplemental Figure 3.

