PX-RICS mediates ER-to-Golgi transport of the N-cadherin/β-catenin complex

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Cadherins mediate Ca^{2+}-dependent cell–cell adhesion. Efficient export of cadherins from the endoplasmic reticulum (ER) is known to require complex formation with β-catenin. However, the molecular mechanisms underlying this requirement remain elusive. Here we show that PX-RICS, a β-catenin-interacting GTPase-activating protein (GAP) for Cdc42, mediates ER-to-Golgi transport of the N-cadherin/β-catenin complex. Knockdown of PX-RICS expression induced the accumulation of the N-cadherin/β-catenin complex in the ER and ER exit site, resulting in a decrease in cell–cell adhesion. PX-RICS was also required for ER-to-Golgi transport of the fibroblast growth factor-receptor 4 (FGFR4) associated with N-cadherin. PX-RICS-mediated ER-to-Golgi transport was dependent on its interaction with β-catenin, phosphatidylinositol-4-phosphate (PI4P), Cdc42, and its novel binding partner γ-aminobutyric acid type A receptor-associated protein (GABARAP). These results suggest that PX-RICS ensures the efficient entry of the N-cadherin/β-catenin complex into the secretory pathway, and thereby regulates the amount of N-cadherin available for cell adhesion and FGFR4-mediated signaling.

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Classic cadherins (simply referred to cadherins hereafter) comprise a family of single-pass transmembrane proteins that mediate Ca^{2+}-dependent cell–cell adhesion (Takeichi and Abe 2005; Halbleib and Nelson 2006). The extracellular domains of the same type of cadherins on adjacent cells bind to one another, and thereby mediate homophilic cell–cell adhesion. On the other hand, the cytoplasmic domains of cadherins directly interact with β-catenin, which bridges between cadherins and α-catenin-bound actin cytoskeleton. The β-catenin-mediated link of cadherins to the cytoskeleton is essential for the robust and stable cadherin-mediated cell–cell adhesion. Cadherins and β-catenin are assembled at the endoplasmic reticulum (ER) immediately after cadherins are synthesized and transported from the ER to the Golgi in a stoichiometric complex (Chen et al. 1999; Kurth et al. 1999; Wahl et al. 2003). In general, ER-to-Golgi protein transport relies on ER export signals at or near the C termini of the cargo proteins (Barlowe 2003), but no functional ER export motifs have been found in the cytoplasmic regions of cadherins (Chen et al. 1999). Instead, binding of β-catenin to cadherins is known to be required for efficient transport of cadherins out of the ER (Chen et al. 1999). These facts imply that ER-to-Golgi transport of cadherins is mediated by an unknown β-catenin-facilitated mechanism that is independent of canonical ER export motifs. However, it remains elusive how the association of cadherins with β-catenin is coupled to efficient ER export of the complex.

We previously identified a β-catenin-interacting GTPase-activating protein (GAP) for Cdc42, termed RICS (Okabe et al. 2003) (also designated as Grit, p200RhoGAP, p250GAP, or GC-GAP by other groups) (Nakamura et al. 2002; Moon et al. 2003; Nakazawa et al. 2003; Zhao et al. 2003). RICS is expressed predominantly in neurons of the brain and involved in neurite extension at the growth cones and presumably *N*-methyl-D-aspartate (NMDA) signaling at the post-synaptic density (PSD), where it forms a complex with β-catenin, N-cadherin, NMDA receptors, and PSD-95 (Okabe et al. 2003; Nasu-Nishimura et al. 2006). Recently, we identified a splicing variant of RICS, termed PX-RICS, which is expressed in a wide variety of tissues and cell lines (Hayashi et al. 2007). PX-RICS has several characteristics suggesting its involvement in ER- and/or Golgi-derived membrane dynamics (Fig. 1A). PX-RICS possesses a Phox homology

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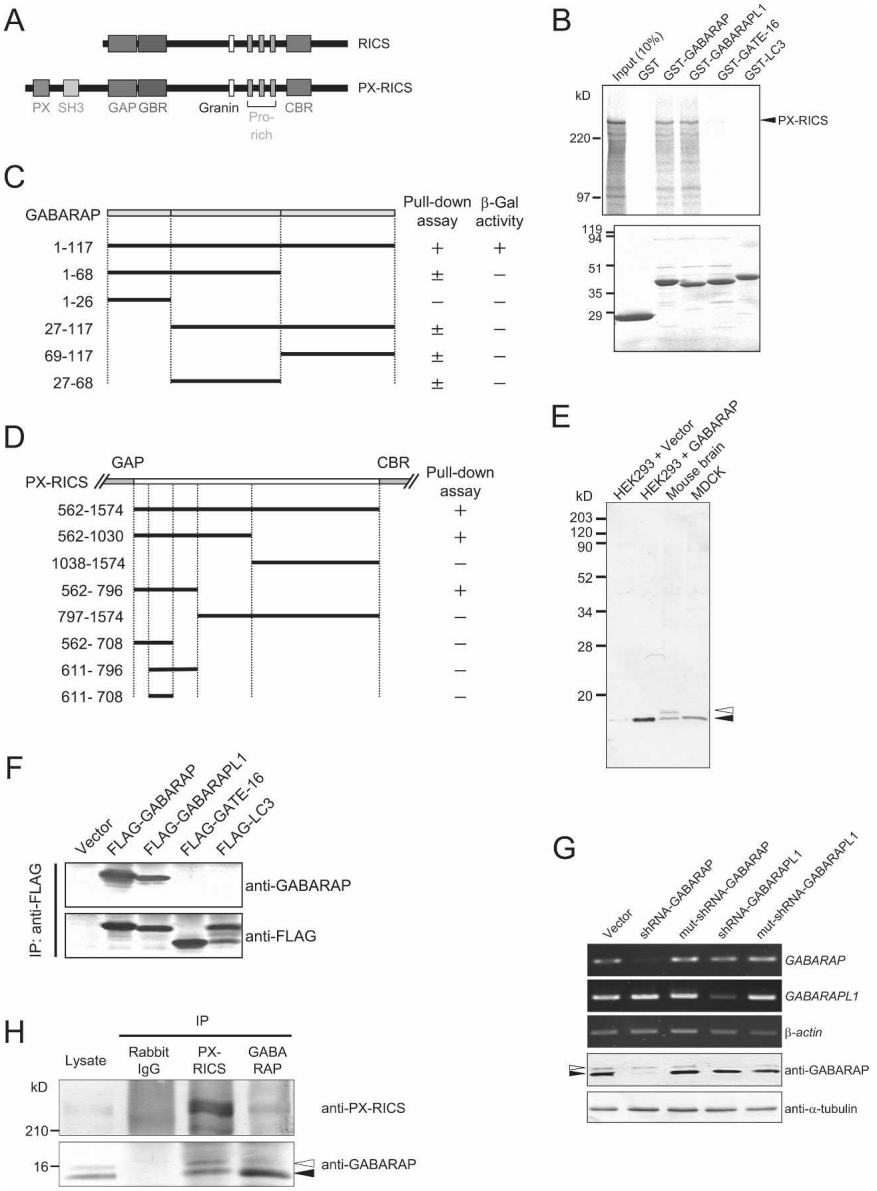

Figure 1. PX-RICS interacts with GABARAP/L1 in vitro and in vivo. (*A*) Schematic representation of RICS and PX-RICS. The domains and motifs contained in RICS and PX-RICS are shown. (PX) Phox homology domain; (SH3) Src homology 3 domain; (GAP) GTPase-activating protein domain; (GBR,) GABARAP-binding region; (Granin) granin motif; (Pro-rich) polyproline stretch; (CBR) β-catenin-binding region. (*B*) Association of GABARAP and GABARAPL1 with PX-RICS in vitro. In vitro translated ^{35}S-labeled PX-RICS was incubated with GST or GST fusion proteins as indicated and bound proteins were analyzed. The *bottom* panel shows Coomassie staining of GST and GST fusion proteins used in pull-down assays. (*C*) Mapping of regions in GABARAP required for binding to PX-RICS. GABARAP was divided into three regions based on the ability of each to interact with other known partners, including $GABA_AR\gamma2$, tubulin, gephyrin, NSF, and Unc-51-like kinase 1 (ULK1). GABARAP fragments fused to GST were analyzed for their ability to interact with in vitro translated ^{35}S-labeled PX-RICS. The results of yeast two-hybrid assays are also shown. (+) Detectable activity; (±) residual activity; (–) no detectable activity. (*D*) Mapping of regions in PX-RICS required for binding to GABARAP. In vitro translated ^{35}S-labeled fragments of PX-RICS were analyzed for their ability to interact with GST-GABARAP. (+) Detectable activity; (–) no detectable activity. (*E*) Detection of endogenous GABARAP/L1 with anti-GABARAP antibody. Lysates prepared from HEK293 cells transfected with empty vector or GABARAP, mouse brain, or MDCK cells were immunoblotted with anti-GABARAP antibody. The 15- and 16-kDa proteins are indicated by the solid and open arrowheads, respectively. (*F*) Specificity of anti-GABARAP antibody. HEK293 cells transfected with Flag-tagged GABARAP, GABARAPL1, GATE-16, orMAP1A/1B LC3 were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblotting with anti-GABARAP or anti-Flag antibody. (*G*) The 15- and 16-kDa proteins are down-regulated specifically by shRNAs against GABARAP and GABARAPL1, respectively. HeLa cells were transfected with empty vector, shRNA-GABARAP, shRNA-GABARAPL1, or their mutant forms, mut-shRNA-GABARAP or mut-shRNA-GABARAPL1, and subjected to semiquantitative RT–PCR. Cell lysates were subjected to immunoblotting analysis with anti-GABARAP antibody. GABARAP and GABARAPL1 are indicated by the solid and open arrowheads, respectively. Anti-α-tubulin antibody was used as a control. (*H*) Association of GABARAP/L1 with PX-RICS in vivo. HeLa cell lysates were subjected to immunoprecipitation, followed by immunoblotting with the indicated antibodies. GABARAP and GABARAPL1 are indicated by the solid and open arrowheads, respectively.

(PX) domain with the highest binding affinity to phosphatidylinositol-4-phosphate (PI4P) (Hayashi et al. 2007), a phospholipid that is known to be enriched in the ER and Golgi membranes (De Matteis et al. 2002; Di Paolo and De Camilli 2006) and implicated in vesicular budding and/or fusion events (Audhya et al. 2000; Bruns et al. 2002). Similarly, Cdc42, a target of the GAP domain of PX-RICS, is predominantly localized at the *cis*/middle cisternae of the Golgi, where it regulates Golgi-derived membrane trafficking (Erickson et al. 1996; Wu et al. 2000; Luna et al. 2002; Matas et al. 2004; Chen et al. 2005). Moreover, PX-RICS contains a putative granin motif, which is found specifically in proteins of the secretory pathway such as the granin family of proteins and cytosolic Golgi protein golgin-245/p230 (Huttner et al. 1991; Fritzler et al. 1995; Erlich et al. 1996). These facts prompted us to investigate whether PX-RICS is involved in protein transport between the ER and Golgi.

Here we report that PX-RICS interacts with γ-aminobutyric acid type A receptor-associated protein

(GABARAP) to mediate ER-to-Golgi transport of the N-cadherin/β-catenin complex, thereby regulating the amount of N-cadherin available for cell–cell adhesion. GABARAP was originally identified as a protein that interacts with the γ2 subunit of the $GABA_A$ receptor (Wang et al. 1999) and believed to mediate correct anchoring and clustering of the receptor (Chen et al. 2000). However, accumulating evidence indicates that GABARAP is involved in intracellular transport of proteins including the $GABA_A$ receptor (Kittler et al. 2001; Elazar et al. 2003; Leil et al. 2004), although it remains to be elucidated how GABARAP facilitates intracellular protein transport. We also demonstrate that PX-RICS regulates the amount of fibroblast growth factor-receptor 4 (FGFR4) associated with N-cadherin at the cell surface, thereby modulating FGF signaling. PX-RICS-mediated ER-to-Golgi transport was found to be dependent on multiple protein–protein (PX-RICS–GABARAP, PX-RICS–Cdc42, and PX-RICS–β-catenin) and protein–lipid (PX-RICS–PI4P) interactions. These results suggest the existence of a novel intracellular transport system that is independent of known ER export motifs. Furthermore, our findings suggest that ER-to-Golgi transport of cadherins could be a regulatory process critical for cadherin-mediated cell adhesion and FGF signaling.

Results

PX-RICS interacts with GABARAP and GABARAPL1

To gain a clue to understand the role of PX-RICS in intracellular transport, we performed a two-hybrid screen of a human brain cDNA library using PX-RICS as bait. The result suggested that PX-RICS could interact with GABARAP, which has been reported to be involved in intracellular protein transport (Elazar et al. 2003). The ability of GABARAP to interact with PX-RICS was confirmed by in vitro pull-down assays. We found that GABARAP fused to glutathione-S-transferase (GST), but not GST alone, associates with in vitro translated PX-RICS (Fig. 1B). Among GABARAP-related proteins, the most closely related protein GABARAP-like1 (GABARAPL1) was also found to interact with PX-RICS, whereas the Golgi-associated ATPase enhancer of 16 kDa (GATE-16) and light chain 3 (LC3) of microtubule-associated protein (MAP) 1A/1B exhibited markedly weak or no binding to PX-RICS (Fig. 1B). In vitro pull-down assays using a series of deletion fragments revealed that PX-RICS binds via its region downstream from the GAP domain to the entire region of GABARAP (Fig. 1C,D; data not shown).

We generated a polyclonal antibody against GABARAP and found that the antibody recognizes 15- and 16-kDa proteins in a mouse brain lysate (Fig. 1E). The antibody specifically recognized Flag-tagged GABARAP and GABARAPL1, but not Flag-GATE-16 and Flag-LC3 (Fig. 1F). This is probably because GABRAPL1 has a markedly high amino acid sequence similarity to GABARAP (94%), whereas GATE-16 and LC3 have relatively low similarities (76% and 57%, respectively). Knockdown experiments using shRNAs against GABARAP, shRNA-GABARAP, and GABARAPL1, shRNA-GABARAPL1, revealed that shRNA-GABARAP and shRNA-GABARAPL1 specifically suppress the expression of the 15- and 16-kDa proteins, respectively (Fig. 1G). On the other hand, either mutant shRNA-GABARAP or shRNA-GABARAPL1 did not show any effect on the expression of these proteins. These results clearly indicate that the 15- and 16-kDa proteins correspond to GABARAP and GABARAPL1, respectively. We then examined whether PX-RICS is associated with GABARAP and GABARAPL1 (referred to as GABARAP/L1 hereafter) in vivo. Immunoprecipitation experiments using HeLa cell lysates revealed that GABARAP/L1 coprecipitates with PX-RICS and, conversely, PX-RICS coprecipitates with GABARAP/L1 (Fig. 1H). These results suggest that PX-RICS is associated with GABARAP/L1 in living cells.

PX-RICS and GABARAP/L1 are colocalized at the ER and ER exit site

We next investigated the subcellular localization of PX-RICS and GABARAP/L1 by immunofluorescent staining. In HeLa cells, GABARAP/L1 was stained as a reticular or punctate pattern concentrated predominantly around the nucleus (Fig. 2A). GABARAP/L1 staining was not detected when the antibody was preabsorbed with an excess amount of the antigen used for immunization, suggesting that the staining signal is specifically derived from GABARAP/L1 (Fig. 2A). In double-immunostaining experiments, a significant fraction of PX-RICS was colocalized with GABARAP/L1, particularly in the perinuclear region (Fig. 2B). These staining patterns were very similar to those of calnexin, an ER-resident chaperone (Fig. 2C,D), and Sec23, a subunit of the coatmer complex (COP) II, which is located at the ER exit site (Fig. 2E,F). The *cis*-Golgi matrix protein GM130 exhibited a distribution pattern overlapping with a subset of PX-RICS, but not GABARAP/L1 (Fig. 2G,H). In contrast, the *trans*-Golgi network-specific protein p230 exhibited a staining pattern without any significant overlap with PX-RICS or GABARAP/L1 (data not shown). These results suggest that a major portion of PX-RICS and GABARAP/L1 is colocalized at the ER and ER exit site and that PX-RICS, but not GABARAP/L1, is also localized at the *cis*-Golgi.

PX-RICS and GABARAP facilitate ER-to-Golgi transport of the N-cadherin/β-catenin complex

The above results led us to examine whether PX-RICS and GABARAP/L1 are involved in protein transport between the ER and Golgi. We previously found that PX-RICS forms a tertiary complex with N- or E-cadherin and β-catenin (Okabe et al. 2003; Hayashi et al. 2007). Thus, we examined whether their localization at the sites of cell–cell contact is altered by knockdown of PX-RICS, GABARAP, or GABARAPL1. We generated shRNAs against human PX-RICS, shRNA-PX-RICS-1 and shRNA-PX-RICS-2, and confirmed their abilities to decrease the expression of PX-RICS in HeLa cells (Supplemental Fig. S1). When ei-

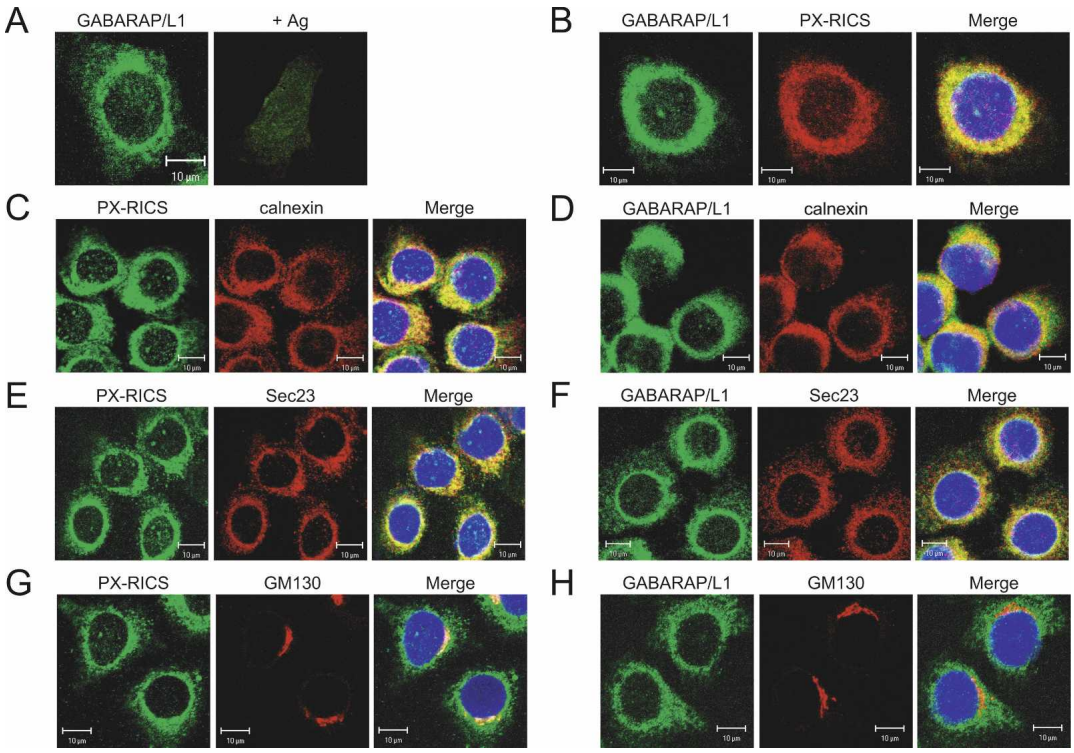

Figure 2. PX-RICS and GABARAP/L1 are colocalized at the ER and ER exit site. (*A*) HeLa cells were immunostained with anti-GABARAP antibody. (+Ag) Anti-GABARAP antibody was preabsorbed with immunizing antigen prior to use. (*B*) Colocalization of PX-RICS and GABARAP/L1 at the perinuclear region. HeLa cells were double-labeled with anti-PX-RICS and anti-GABARAP antibodies. (*C–H*) Subcellular localization of PX-RICS and GABARAP/L1. HeLa cells were double-labeled with the indicated antibodies. Bars, 10 µm.

ther shRNA-PX-RICS-1, shRNA-PX-RICS-2, or shRNA-GABARAP was expressed in HeLa cells, N-cadherin was no longer present at the cell–cell boundaries and, instead, was accumulated in the ER/Golgi-like compartment around the nucleus (Fig. 3A). The amount of β-catenin localized at the sites of cell–cell contact was also reduced, but intracellular accumulation of β-catenin appeared not to be significant. This may be because β-catenin is a multifunctional protein, and only its certain population is associated with cadherins and PX-RICS. On the other hand, HeLa cells transfected with shRNA-GABARAPL1 or mutant shRNAs did not show these changes (Fig. 3A). The perinuclear staining of accumulated N-cadherin clearly overlapped with that of calnexin and Sec23 (Supplemental Fig. S2), suggesting that the majority of N-cadherin is retained in the ER and ER exit sites. Similar changes in the subcellular localization of N-cadherin and β-catenin were observed when we used siRNA targeting the 3′-UTR of *GABARAP* (Fig. 3B; Supplemental Fig. S3). Furthermore, overexpression of *GABARAP* lacking its 3′-UTR restored the localization of N-cadherin and β-catenin in GABARAP-knockdown cells (Fig. 3B). By contrast, overexpression of *GABARAPL1* did not rescue the aberrant localization of N-cadherin and β-catenin. These results suggest that PX-RICS and GABARAP, but not GABARAPL1, are required for ER-to-Golgi transport of the N-cadherin/β-catenin complex. In addition, knockdown of PX-RICS or GABARAP had no effect on the subcellular distribution of the KDEL receptor (Supplemental Fig. S4), suggesting that shRNA-induced changes in the subcellular localization of N-cadherin and β-catenin are not due to a primary alteration in the retrograde (Golgi-to-ER) transport pathway.To confirm the above results, we performed subcellular fractionation analyses of HeLa cells that had been retrovirally transduced with shRNA-PX-RICS-1 or its control mutant. We found that the amounts of N-cadherin and β-catenin present in the plasma membrane are drastically reduced in cells expressing shRNA-PX-RICS-1 compared with those expressing control mutant shRNA (Fig. 3C). In contrast, the amounts of N-cadherin and β-catenin reside in the rough ER were significantly increased in shRNA-PX-RICS-1-expressing cells. It is notable that β-catenin is present in various fractions but N-cadherin localization is restricted to the plasma membrane and rough ER. This result is consistent with the fact that β-catenin is a multifunctional protein and only its certain population is associated with cadherins.

When we performed similar experiments using A431 cells, we also found that E-cadherin and β-catenin are barely detectable at the cell–cell contact sites (Supplemental Fig. S5A). However, in contrast to N-cadherin in HeLa cells, E-cadherin was found to be retained not only in the ER but also in the Golgi (Supplemental Fig. S5B).

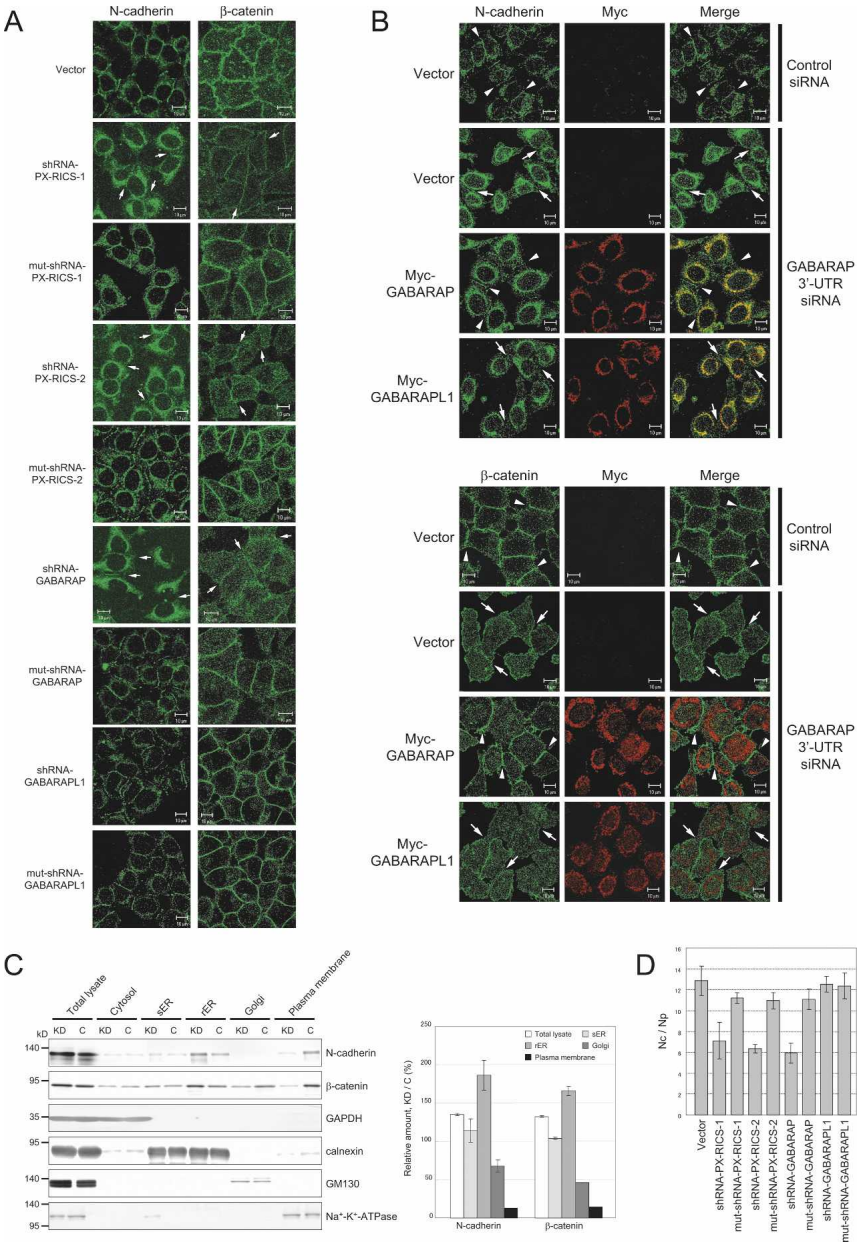

Figure 3. PX-RICS and GABARAP are critical for ER-to-Golgi transport of the N-cadherin/β-catenin complex. (*A*) Knockdown of PX-RICS or GABARAP results in the disappearance of N-cadherin and β-catenin at the cell–cell boundaries and intracellular accumulation of N-cadherin. HeLa cells were transfected with the indicated shRNAs and subjected to immunofluorescent staining with anti-N-cadherin or anti-β-catenin antibody. Arrows indicate the absence of N-cadherin and β-catenin at the borders between two neighboring cells. Bars, 10 µm. (*B*) Exogenous expression of GABARAP, but not GABARAPL1, restores the subcellular distribution of N-cadherin and β-catenin in GABARAP-knockdown cells. HeLa cells were cotransfected with siRNA for *GABARAP* 3′-UTR and Myc-tagged GABARAP or GABARAPL1, and subjected to immunostaining with the indicated antibodies. Arrowheads indicate N-cadherin or β-catenin enriched at the sites of cell–cell contact. The cell–cell boundaries lacking N-cadherin or β-catenin are indicated by arrows. Bars, 10 µm. (*C*) Subcellular distribution of N-cadherin and β-catenin in *PX-RICS*-knockdown (KD) and control (C) HeLa cells. (*Left* panel) After subcellular fractionation, the amounts of N-cadherin and β-catenin in each fraction were evaluated by immunoblotting. (*Right* panel) The results were analyzed by densitometry and the relative amounts in knockout versus control cells were quantified (mean ± SD [n = 3]). Each fraction was analyzed for the distribution of marker proteins specific for various organelles: GAPDH (cytosol), calnexin (ER), GM130 (Golgi), and Na^+/K^+-ATPase α1 (plasma membrane). (*D*) Ca^{2+}-dependent cell adhesion is abrogated by knockdown of PX-RICS or GABARAP. HeLa cells expressing the indicated shRNA were dissociated by pipetting in the presence of Ca^{2+}. Cell adhesion activity was quantified by counting the total number of cells (N_c) and the number of particles (cell clumps) (N_p). Error bars represent the mean ± SD (n = 5).

On the other hand, knockdown of either PX-RICS or GABARAP had no apparent effect on the intracellular distribution of the EGF receptor, IGF-1 receptor, HGF receptor (c-Met), transferrin receptor, potassium channel KCNK9, Na^+/K^+-ATPase α1, or integrin (data not shown).

Because the half-life of cadherins is quite short, retention of newly synthesized cadherins in the ER would immediately result in reduced cell surface expression and, consequently, reduced cell–cell adhesion. Thus, we examined whether knockdown of either PX-RICS or GABARAP/L1 changes adhesion of HeLa and A431 cells. After mechanical dispersal in the presence of Ca^{2+}, control cells remained as aggregates, whereas cells expressing shRNA-PX-RICS-1, shRNA-PX-RICS-2, or shRNA-GABARAP dissociated efficiently (Fig. 3D; Supplemental Fig. S5C). By contrast, knockdown of GABARAPL1 did not reduced cell–cell adhesion. These results suggest that knockdown of either PX-RICS or GABARAP results in a decrease in the amount of cadherins at the cell surface and a consequent decrease in cell–cell adhesion.

Taken together, our data suggest that PX-RICS and GABARAP facilitate ER-to-Golgi transport of the N- or E-cadherin/β-catenin complex, and consequently, regulate cell–cell adhesion. On the other hand, GABARAPL1

appears not to play a critical role in this transport system. We focused our further analysis on ER-to-Golgi transport of the N-cadherin/β-catenin complex.

ER-to-Golgi transport of the N-cadherin/β-catenin complex is dependent on the interaction of PX-RICS with GABARAP, PI4P, Cdc42, and β-catenin

PX-RICS contains several domains implicated in interactions with other molecules (Fig. 4A). We therefore assessed the contribution of these interactions to ER-to-Golgi transport of the N-cadherin/β-catenin complex. For this purpose, we expressed in HeLa cells a series of dominant-negative mutants of PX-RICS that block the interaction of PX-RICS with its associated molecules (Fig. 4A,B) and examined their effects on the subcellular localization of N-cadherins and β-catenin. We found that the amounts of N-cadherin and β-catenin at the cell–cell contact sites were markedly reduced, compared with surrounding untransfected cells, when we expressed the GABARAP-binding region (GBR), the PX domain (PX-WT), the β-catenin-binding region (CBR), or a mutant form of the GAP domain (GAP-RM) that is able to interact with Cdc42 but lacks GAP activity (Fig. 4C). By contrast, such changes were not induced by expression of a mutant form of the PX domain (PX-YA) that is unable to interact with phosphoinositides. These results suggest that PX-RICS-mediated ER-to-Golgi transport of the N-cadherin/β-catenin complex depends on its interaction with multiple binding molecules: GABARAP, PI4P, Cdc42, and β-catenin.

PX-RICS-mediated ER-to-Golgi transport of the N-cadherin/β-catenin complex affects FGFR4 signaling

There is accumulating evidence for the physical and functional interplay between N-cadherin and FGFRs (Halbleib and Nelson 2006). Interestingly, N-cadherin directly interacts with FGFR4 to form a multiprotein signaling complex (Cavallaro et al. 2001). Thus, we examined whether ER accumulation of N-cadherin would affect the subcellular distribution of FGFR4 in PX-RICS- or GABARAP-knockdown cells. We found that FGFR4 is accumulated in the perinuclear ER-like compartment, similar to N-cadherin (Fig. 5A). In contrast, knockdown of GABARAPL1 had not effect on the subcellular localization of FGFR4. In addition, FGFR4 was also found to be accumulated in the ER-like compartment when cells were transfected with PX-WT, GAP-RM, GBR, or CBR, but not PX-YA (Fig. 5B). These results suggest that PX-RICS mediates ER-to-Golgi transport of FGFR4 associated with the N-cadherin/β-catenin complex through its interaction with GABARAP, PI4P, Cdc42, and β-catenin. We were unable to specify the compartment where FGFR4 accumulates, because double-immunostaining experiments with anti-calnexin or anti-Sec23 antibody were not possible due to the differences in fixation conditions required for each antibody.

We next asked whether the retention of N-cadherin and FGFR4 in the ER-like compartment impairs FGFR4-mediated signaling activity. FGF stimulation rapidly induced phosphorylation of p42/44 MAPK in control cells (Fig. 5C). In contrast, FGF stimulation failed to promote phosphorylation of p42/44 in PX-RICS- or GABARAP-knockdown cells. These results suggest that FGF signaling is abrogated due to insufficient surface expression of FGFR4 caused by its retention in the ER-like compartment.

Subcellular localization of N-cadherin and β-catenin is altered in PX-RICS$^{-/-}$ MEFs

To confirm the results obtained with shRNA and dominant-negative mutants, we utilized mouse embryonic fibroblasts (MEFs) from wild-type and *PX-RICS*$^{-/-}$ mice (Nasu-Nishimura et al. 2006). We found that the total amounts of N-cadherin and β-catenin are almost the same in wild-type and *PX-RICS*$^{-/-}$ MEFs (Fig. 6A). However, subcellular fractionation analyses revealed that the amounts of N-cadherin and β-catenin present in the plasma membrane are drastically reduced in *PX-RICS*$^{-/-}$ MEFs compared with wild-type MEFs (Fig. 6A). In contrast, the amounts of N-cadherin and β-catenin reside in the ER, especially in the rough ER, are significantly increased in *PX-RICS*$^{-/-}$ MEFs (Fig. 6A). Immunostaining also revealed that, in *PX-RICS*$^{-/-}$ MEFs, N-cadherin is trapped in the perinuclear compartment or diffusely distributed throughout the cytoplasm (Fig. 6B), where it colocalized with the ER-resident protein calnexin (Fig. 6C). We also found that β-catenin is not present in the sites of cell–cell contact in *PX-RICS*$^{-/-}$ MEFs (Fig. 6B).

PX-RICS restores the subcellular distribution of N-cadherin and β-catenin in PX-RICS$^{-/-}$ MEFs

Finally, we examined whether exogenous expression of PX-RICS or a series of its mutants (Fig. 7A,B) restores the localization of N-cadherin and β-catenin in *PX-RICS*$^{-/-}$ MEFs. When wild-type PX-RICS (PX-RICS-WT) was expressed in *PX-RICS*$^{-/-}$ MEFs, subcellular localization of N-cadherin and β-catenin was restored to nearly the same location as in wild-type MEFs (Fig. 7C,D). In contrast, PX-RICS-RM, a GAP activity-deficient mutant, failed to restore the localization of N-cadherin and β-catenin. This result suggests that the GAP activity of PX-RICS is required for PX-RICS-mediated transport of the N-cadherin/β-catenin complex. We also found that PX-RICS-YA, a point mutant lacking PI4P-binding activity but retaining intact GAP activity, cannot rescue the aberrant localization of N-cadherin and β-catenin. Interestingly, expression of wild-type RICS (RICS-WT), which has higher GAP activity than PX-RICS but lacks the PX domain, also could not restore the localization of N-cadherin and β-catenin. Similarly, the aberrant localization of N-cadherin and β-catenin was not rescued by exogenous expression of PX-RICS-ΔGBR or PX-RICS-ΔCBR, mutant PX-RICS lacking the GBR or CBR, respectively.

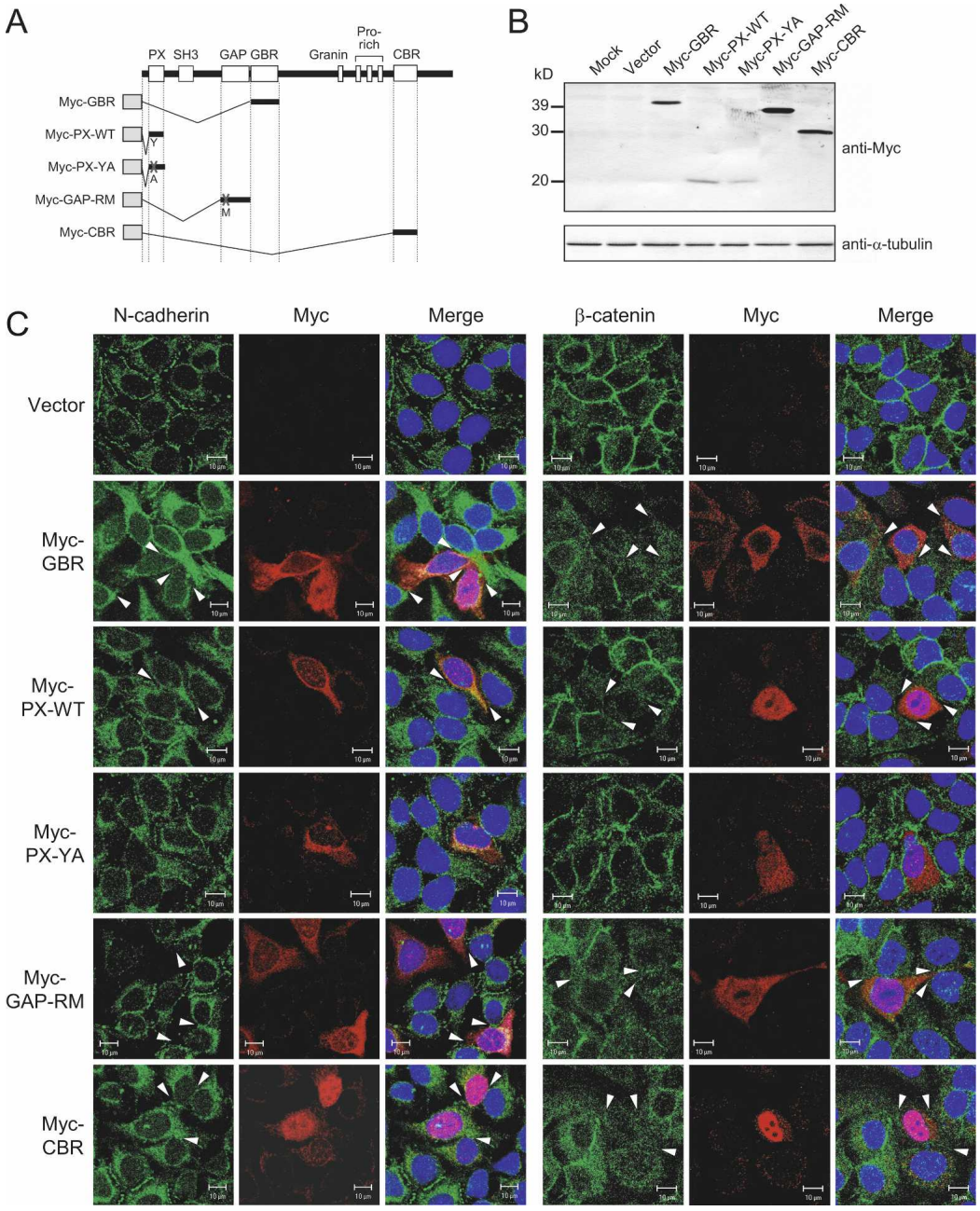

Figure 4. ER-to-Golgi transport of the N-cadherin/β-catenin complex is dependent on the interaction of PX-RICS with its associated molecules. (*A*) Schematic representation of the dominant-negative mutants of PX-RICS that block the interaction of PX-RICS with its associated molecules. (PX) Phox homology domain; (SH3) Src homology 3 domain; (GAP) GTPase-activating protein domain; (GBR) GABARAP-binding region; (Granin) granin motif; (Pro-rich) polyproline stretch; (CBR) β-catenin-binding region. Shaded rectangles indicate Myc tag. (*B*) Protein expression of dominant-negative mutants of PX-RICS in HeLa cells. Lysates prepared from HeLa cells expressing the indicated Myc-tagged fragments of PX-RICS were immunoblotted with anti-Myc tag antibody. Anti-α-tubulin antibody was used as a control. (*C*) Block of the interaction between PX-RICS and its associated molecules results in the disappearance of N-cadherin and β-catenin from the cell–cell boundaries. HeLa cells were transfected with the indicated Myc-tagged fragments of PX-RICS and processed for immunostaining with anti-Myc tag antibody plus anti-N-cadherin or anti-β-catenin antibody. N-cadherin and β-catenin disappeared from the cell–cell boundaries of HeLa cells expressing dominant-negative mutants of PX-RICS (arrowheads). Bars, 10 μm.

Collectively, these results suggest that ER-to-Golgi transport of the N-cadherin/β-catenin complex is dependent on both the GAP activity of PX-RICS and its interaction with PI4P, GABARAP, and β-catenin.

Discussion

In the present study, we have shown that PX-RICS and its novel binding partner GABARAP play a critical role

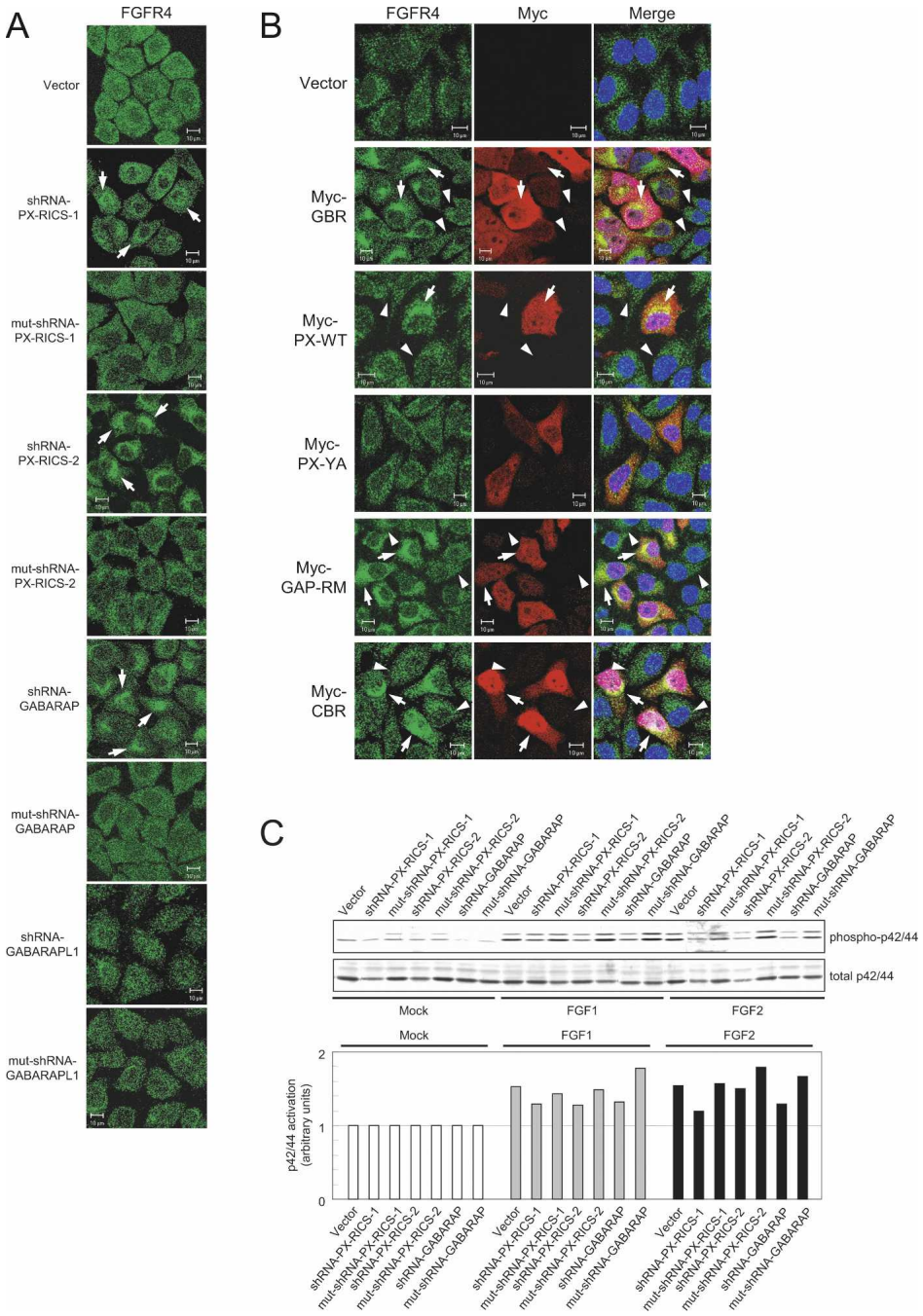

Figure 5. The N-cadherin/β-catenin complex is accompanied by FGFR4 in PX-RICS/GABARAP-mediated transport. (*A*) Knockdown of PX-RICS or GABARAP induces the accumulation of FGFR4 in the perinuclear ER-like compartments. HeLa cells were transfected with shRNAs or mutant shRNAs as indicated and probed with anti-FGFR4 antibody. Perinuclear accumulation of FGFR4 is indicated by arrows. Bars, 10 µm. (*B*) Accumulation of FGFR4 in HeLa cells expressing dominant-negative mutants of PX-RICS. HeLa cells were transfected with Myc-tagged fragments of PX-RICS and processed for immunostaining with anti-FGFR4 and anti-Myc tag antibodies. Note that FGFR4 is accumulated in Myc-positive cells (arrows), including those with low expression levels. Arrowheads indicate the surrounding untransfected cells. Bars, 10 µm. (*C*) FGF signaling is suppressed by knockdown of PX-RICS or GABARAP. HeLa cells expressing the indicated shRNAs were assessed for FGF1- or FGF2-induced activation of p42/44 MAPK, downstream effecters of FGF signaling, by immunoblotting with antibodies specific for the phosphorylated (activated) form of p42/44 (phospho-p42/44) and with antibodies against all forms of p42/44 (total p42/44) as indicated. The results were quantified by densitometry and the ratio of phosphorylated to total p42/44 in each sample is shown.

in the ER-to-Golgi transport of the N-cadherin/β-catenin complex, and thereby modulate the amount of N-cadherin and N-cadherin-associated FGFR4 available for cell-cell adhesion and FGF signaling. We have also shown that the

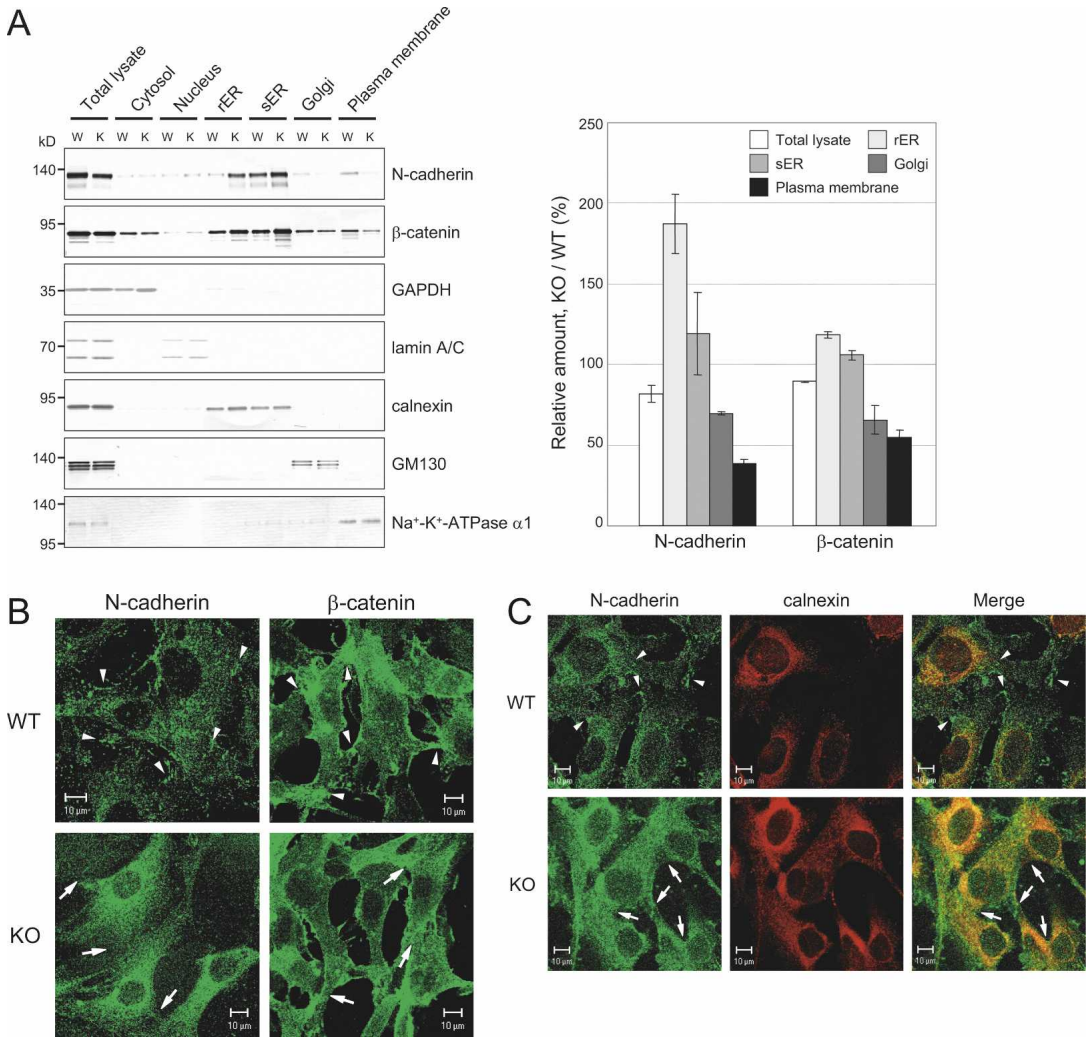

Figure 6. PX-RICS regulates ER-to-Golgi transport of N-cadherin and β-catenin in MEFs. (*A*) Subcellular distribution of N-cadherin and β-catenin in MEFs. (*Left* panel) Subcellular fractionation was performed for wild-type (W) and *PX-RICS*-knockout (K) MEFs, and the amounts of N-cadherin and β-catenin in each fraction were evaluated by immunoblotting. (*Right* panel) The results were analyzed by densitometry and the relative amounts in knockout versus wild-type cells were quantified (mean ± SD [$n = 3$]). Each fraction was analyzed for the distribution of marker proteins specific for various organelles: GAPDH (cytosol), lamin A/C (nuclei), calnexin (ER), GM130 (Golgi), and Na^+/K^+-ATPase α1 (plasma membrane). (*B*) Immunocytochemical localization of N-cadherin and β-catenin in *PX-RICS*$^{-/-}$ MEFs. Primary MEFs prepared from wild-type (WT) or *PX-RICS*$^{-/-}$ (KO) mice were probed with antibodies to N-cadherin or β-catenin. Arrowheads indicate N-cadherin or β-catenin enriched at the sites of cell–cell contact. The cell–cell contact sites lacking N-cadherin or β-catenin are indicated by arrows. Bars, 10 μm. (*C*) ER accumulation of N-cadherin in *PX-RICS*$^{-/-}$ MEFs. Wild-type (WT) or *PX-RICS*$^{-/-}$ (KO) MEFs were processed for double-staining with anti-N-cadherin and anti-calnexin antibodies. Arrowheads indicate N-cadherin enriched at the sites of cell–cell contact. The cell–cell contact sites lacking N-cadherin are indicated by arrows. Bars, 10 μm.

other PX-RICS-associated molecules, PI4P and Cdc42, participate in the PX-RICS/GABARAP-mediated transport of the N-cadherin/β-catenin complex.

Role of PI4P and GABARAP in PX-RICS-mediated ER-to-Golgi transport

PI4P is the predominant phosphoinositide in the ER and Golgi membranes (De Matteis et al. 2002; Di Paolo and De Camilli 2006) and plays important roles in vesicular budding and/or fusion (Audhya et al. 2000; Bruns et al. 2002). Our results suggest that the interaction of PX-RICS with PI4P via its PX domain is critical for ER-to-Golgi transport of the N-cadherin/β-catenin complex. In general, the interaction between phosphoinositides and cytosolic proteins is thought to be of low specificity and affinity. As a cellular mechanism to overcome this, the concept of "coincidence detection" has been proposed, in which an additional binding cue within the membrane acts in concert with phosphoinositides to achieve membrane–protein interactions with higher specificity and affinity (Di Paolo and De Camilli 2006). GABARAP

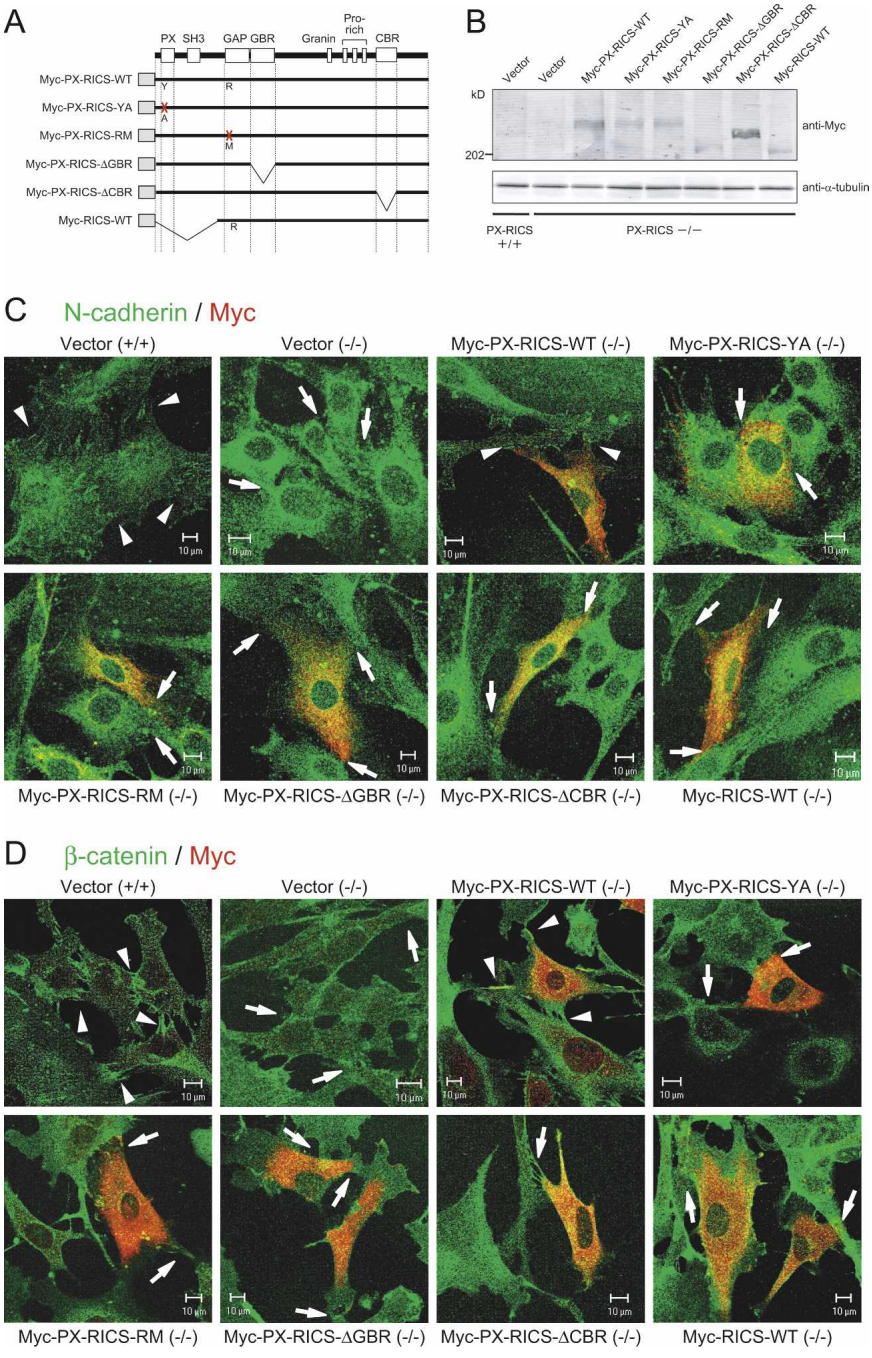

Figure 7. Exogenous expression of PX-RICS, but not its mutant forms, restores the subcellular distribution of N-cadherin and β-catenin. (*A*) Schematic representation of the PX-RICS mutants. (PX) Phox homology domain; (SH3) Src homology 3 domain; (GAP) GTPase-activating protein domain; (GBR) GABARAP-binding region; (Granin) granin motif; (Pro-rich) polyproline stretch; (CBR) β-catenin-binding region. Shaded rectangles indicate Myc tag. (*B*) Protein expression of wild-type and mutant forms of PX-RICS and RICS in MEFs. Wild-type and *PX-RICS*$^{-/-}$ MEFs were transfected with indicated Myc-tagged expression plasmids, and the cell lysates were immunoblotted with anti-Myc tag antibody. Anti-α-tubulin antibody was used as a control. (*C*,*D*) Exogenous expression of PX-RICS, but not its mutant forms, restores the subcellular distribution of N-cadherin (*C*) and β-catenin (*D*) in *PX-RICS*$^{-/-}$ MEFs. Primary *PX-RICS*$^{-/-}$ MEFs were transfected with Myc-tagged wild-type or mutant PX-RICS, and were subjected to immunostaining with the indicated antibodies. Arrowheads indicate N-cadherin or β-catenin enriched at the sites of cell–cell contact. The cell–cell contact sites lacking N-cadherin or β-catenin are indicated by arrows. Bars, 10 μm.

is known to be associated with the ER membrane presumably through its hydrophobic phosphatidylethanolamine-conjugated moiety (Kittler et al. 2001; Kabeya et al. 2004). Thus, GABARAP may act as a binding cue to stabilize the interaction between PI4P and PX-RICS and facilitate the selective recruitment of PX-RICS to the ER membrane.

We found that PX-RICS also interacts with GABARAPL1. Because GABARAP and GABARAPL1 are very closely related small molecules, our antibody recognized both proteins. Thus, we could not discriminate between GABARAP and GABARAPL1 in our immunofluorescent staining. However, we clearly showed that knockdown of GABARAPL1 does not affect ER-to-Golgi transport of the N-cadherin/β-catenin complex. Furthermore, unlike GABARAP overexpression, GABARAPL1 overexpression did not rescue the aberrant subcellular distribution of N-cadherin and β-catenin in GABARAP-knockdown cells. Thus, in spite of the high amino acid sequence similarity to GABARAP, GABARAPL1 appears not to play a critical role in ER-to-Golgi transport of the N-cadherin/β-catenin complex.

Role of Cdc42 in PX-RICS-mediated ER-to-Golgi transport

We have shown that the GAP activity of PX-RICS for Cdc42 is required for transport of the N-cadherin/β-catenin complex. It has been reported that the reciprocal conversion of coatmer-bound Cdc42 between the GTP- and GDP-bound states is important for the regulation of dynein recruitment to COPI vesicle (Wu et al. 2000; Chen et al. 2005). Thus, it is interesting to speculate that PX-RICS also regulates the interaction among Cdc42, coatmer proteins, motor proteins, and microtubules through its GAP activity for Cdc42.

The C terminus of Cdc42 is known to be geranylgeranylated (Wennerberg and Der 2004), which enables a significant fraction of Cdc42 to localize at the Golgi membrane, particularly at the *cis*/middle cisternae (Erickson et al. 1996; Luna et al. 2002; Matas et al. 2004). Interestingly, our immunostaining experiments revealed that a subset of PX-RICS is colocalized with the *cis*-Golgi marker protein GM130, but not with the *trans*-Golgi marker p230. Therefore, we speculate that Cdc42 associated with the Golgi membrane could serve as a coreceptor together with PI4P to facilitate the recruitment of cytosolic PX-RICS to the *cis*-face of the Golgi stacks like GABARAP in the ER membrane (Di Paolo and De Camilli 2006).

Relationship between ER-to-Golgi transport of the N-cadherin/β-catenin complex and FGF signaling

It has been shown that signaling from cell adhesion molecules including N-cadherin is mediated by FGFR (Halbleib and Nelson 2006). N-cadherin is directly associated with FGFR4 via their extracellular domains, and assembles a number of transmembrane and cytosolic proteins into a large signaling complex (Cavallaro et al. 2001). Consistent with these observations, we found that shRNA-mediated knockdown of PX-RICS or GABARAP induced the simultaneous accumulation of N-cadherin and FGFR4 in the ER-like compartment and caused inhibition of signaling cascades downstream from FGFR4. These results suggest that formation of the N-cadherin/FGFR4 complex occurs at the ER before their export to the Golgi. Therefore, exit from the ER seems to be a potential regulatory point for cadherin functions such as intercellular adhesion and FGFR-mediated signaling.

Possible model for the action of PX-RICS and its interacting molecules during ER-to-Golgi transport of the N-cadherin/β-catenin complex

Based on our results, we propose a hypothesis for the action of PX-RICS and its interacting molecules (Supplemental Fig. S6). Prior to ER exit of the N-cadherin/β-catenin complex, PX-RICS may be selectively recruited to the ER membrane through its interaction with PI4P and GABARAP and link the N-cadherin/β-catenin complex with GABARAP via its ability to interact with β-catenin. The PX-RICS-binding region of β-catenin (armadillo repeats 10–12) is distinct from the critical region for cadherin binding (armadillo repeats 3–8) (von Kries et al. 2000; Huber and Weis 2001; Okabe et al. 2003). Thus, it is conceivable that PX-RICS can interact with cadherin-associated β-catenin. The concerted action of these molecules may ensure sorting of the complex into budding vesicles. The ability of GABARAP to bind to microtubules, actin filaments, and N-ethylmaleimide-sensitive factor (NSF) may contribute to prime ER exit and/or transport of the complex (Wang and Olsen 2000; Kittler et al. 2001). PI4P may also function at the *cis*-face of the Golgi stacks as a coreceptor with the membrane-bound, active form of Cdc42 to facilitate the recruitment of PX-RICS to the Golgi membrane. This complex may act to supplement the function of the major tethering complex during Golgi fusion of the N-cadherin/β-catenin complex-containing vesicles. In conclusion, our findings suggest that PX-RICS provides a molecular basis to explain why assembly of cadherins with β-catenin is essential for efficient ER exit of cadherins.

Materials and methods

PX-RICS mutants

Details of the PX-RICS mutants used in this study are as follows: PX-YA and PX-RICS-YA, Tyr-173indispensable for their interaction with phosphoinositides was replaced with Ala; GAP-RM and PX-RICS-RM, Arg-407 essential for GAP activity was replaced with Met; PX-RICS-ΔGBR, the GBR (amino acids 562–796) was deleted; PX-RICS-ΔCBR, the CBR (amino acids 1531–1720) was deleted. All mutant forms were generated by PCR-based mutagenesis.

Antibodies

Rabbit polyclonal antibody to PX-RICS was generated as described previously (Hayashi et al. 2007). Rabbit polyclonal antibody to GABARAP was generated by immunizing rabbits with recombinant GST-GABARAP. Antibodies were purified by affinity chromatography using columns to which the antigens used for immunization had been linked. The following antibodies were used: rabbit anti-c-Myc tag (MBL), rabbit anti-calnexin (Stressgen), rabbit anti-FGFR4 (Santa Cruz Biotechnology), goat anti-Sec23 (Santa Cruz Biotechnology), mouse anti-Flag tag (M2) (Sigma), mouse anti-c-Myc tag (9E10) (Santa Cruz Biotechnology), mouse anti-α-tubulin (Calbiochem), mouse anti-calnexin (BD Biosciences), mouse anti-GM130 (BD Biosciences), mouse anti-p230 (BD Biosciences), mouse anti-E-cadherin (BD Biosciences), mouse anti-β-catenin (BD Biosciences), mouse anti-lamin A/C (BD Biosciences), mouse anti-N-cadherin (13A9) (Millipore), mouse anti-GAPDH (Millipore), mouse anti-Na^+-K^+-ATPase α1 (Millipore), mouse anti-KDEL receptor (Stressgen), and rat anti-HA tag (3F10) (Roche).

Cell culture and transfection

HEK293, MDCK, HeLa, and A431 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Cansera International). HEK293, HeLa, and A431 cells were transfected with plasmids using FuGene 6 (Roche) according to the manufacturer's instruction.

RNAi experiments

Human *PX-RICS* and *GABARAP* sequences targeted by shRNAs are as follows: shRNA-PX-RICS-1 (human), 5′-GCTGCA CAGCATTCATTGA-3′; shRNA-PX-RICS-2 (human), 5′-GAC TACGCCATGAATTTGA-3′; shRNA-GABARAP (human), 5′-GAAATACCTGGTGCCTTCT-3′; shRNA-GABARAPL1 (human), 5′-GGCCTACAGTGATGAGAGT-3′.

For mutant shRNAs, the sequences synthesized in the reverse order were used. DNA oligonucleotides encoding shRNAs were subcloned into the H1 promoter-driven vector pSUPERretro. puro (OligoEngine). HeLa or A431 cells were transfected with the shRNA expression constructs, cultured for 24 h, and then treated with puromycin (4 µg/mL for HeLa cells; 2 µg/mL for A431 cells) for 48 h to remove untransfected cells. Surviving cells were then harvested and subjected to semiquantitative RT–PCR, immunoblotting, or immunofluorescence.

Stealth RNAi that targets the 3′-UTR of the human *GABARAP* gene (target sequence, 5′-CATCCTGCTGTAGA CTTCTTGATTG-3′) was obtained from Invitrogen. HeLa cells were transfected with Stealth RNAi and the expression plasmid carrying only the coding region of *GABARAP* using Lipofectamine 2000 according to the siRNA-plasmid cotransfection protocol.

Retrovirus-mediated shRNA introduction

PlatA packaging cells were plated on 10-cm tissue culture dish (4.2×10^6 cells per dish) and transfected with pSUPERretro.puro-shRNA constructs using FuGene 6. Forty-eight hours after transfection, culture medium was recovered and added to HeLa cell culture (~80% confluent in a 10-cm tissue culture dish) with polybrene (10 µg/mL). Infected cells were selected with puromycin (4 µg/mL) for 48 h, and surviving cells were subjected to subcellular fractionation.

Immunofluorescence

HeLa or A431 cells were plated on coverslips in six-well tissue culture plates (1×10^5 cells per well). After 48 h of incubation at 37°C, cells were fixed with cold methanol and permeabilized with 0.2% Triton X-100 in Tris-buffered saline. The cells were double-stained with the appropriate combination of primary antibodies for 60 min at room temperature. Staining patterns were visualized by incubating with Alexa Fluor 488- or Alexa Fluor 594-labeled donkey secondary antibody (Invitrogen) for 60 min at room temperature. The cell images were obtained with a LSM510META laser scanning confocal microscope (Zeiss). For double-staining experiments with anti-PX-RICS and anti-GABARAP antibodies, they were labeled directly with Alexa Fluor 488 and Alexa Fluor 594, respectively, using the Zenon Tricolor Rabbit IgG labeling Kit (Invitrogen). The nuclei were counterstained with TO-PRO-3 (Invitrogen). Blocking of antibodies was done by preincubating the antibodies for 2 h at 4°C with an excess of the antigens used for immunization.

Cell dissociation assays

Cells were scraped in phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and suspended by 10 times repeated pipetting. The extent of cell dissociation was quantified by counting the number of cell clumps (Np) and the total number of cells (Nc), and was represented by the ratio Nc/Np.

FGF signaling assays

HeLa cells expressing shRNA were starved of serum for 48 h and then stimulated with 20 ng/mL FGF1 (Sigma) plus 10 µg/mL heparin (Sigma) or 20 ng/mL FGF2 (Roche) for 20 min. Cells were lysed in buffer A and the cleared lysates were subjected to immunoblotting with anti-p42/44 or anti-phospho-p42/44 antibody (Cell Signaling Technology).

MEFs

Primary MEFs were obtained from day 13.5 embryos and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Transfection of expression plasmids was performed using Nucleofector electroporation (Amaxa).

Subcellular fractionation

Cytosolic and nuclear fractions were prepared according to the standard ultracentrifugation method. A plasma membrane-enriched fraction was prepared using the Qproteome plasma membrane protein kit (Qiagen). Preparation of rough ER-, smooth ER- and Golgi-enriched fractions was performed as described elsewhere (Bole et al. 1986). HeLa cells or MEFs (5×10^7) were resuspended in 2 mL of isotonic sucrose (0.25 M sucrose in 5 mM HEPES-KOH at pH 6.8) and disrupted with 30 gentle strokes in a tight-fitting glass homogenizer. After centrifugation at 800*g* for 10 min, the post-nuclear supernatant was layered over a discontinuous sucrose gradient containing 1 mL/2.0 M, 3.4 mL/1.3 M, 3.4 mL/1.0 M, 2.75 mL/0.6 M sucrose in 5 mM HEPES-KOH (pH 6.8). After 2 h of centrifugation at 285,000*g* in a Beckman SW40 rotor, 22 fractions containing 500 µL of each were collected from the bottom of the tube. Membranes enriched for rough ER, smooth ER and Golgi apparatus are known to be recovered at three interfaces of the sucrose layers.

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