CRB3 navigates Rab11 trafficking vesicles to promote γTuRC assembly during ciliogenesis

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Abstract

The primary cilium plays important roles in regulating cell differentiation, signal transduction, and tissue organization. Dysfunction of the primary cilium can lead to ciliopathies and cancer. The formation and organization of the primary cilium are highly associated with cell polarity proteins, such as the apical polarity protein CRB3. However, the molecular mechanisms by which CRB3 regulates ciliogenesis and CRB3 location remain unknown. Here, we show that CRB3, as a navigator, regulates vesicle trafficking in γ-TuRC assembly during ciliogenesis and cilium-related Hh and Wnt signaling pathways in tumorigenesis. *Crb3* knockout mice display severe defects of the primary cilium in the mammary ductal lumen and renal tubule. CRB3 is essential for lumen formation and ciliary assembly in the mammary epithelium. We demonstrate that CRB3 localizes to the basal body and that CRB3 trafficking is mediated by Rab11-positive endosomes. Significantly, CRB3 directly interacts with Rab11 to navigate GCP6/Rab11 trafficking vesicles to CEP290, resulting in intact γ-TuRC assembly. In addition, CRB3-depleted cells cannot respond to the activation of the Hh signaling pathway, while CRB3 regulates the Wnt signaling pathway. Therefore, our studies reveal the molecular mechanisms by which CRB3 recognizes Rab11-positive endosomes to navigate apical vesicle trafficking in effective ciliogenesis, maintaining cellular homeostasis and tumorigenesis.

Introduction

Epithelial tissues are the most common, widely distributed, and perform several particular functions. The integrity of the specific cellular architecture composed of epithelial cells is necessary for these functions of epithelial tissues. Establishing apical-basal cell polarity within epithelial cells is a crucial biological process for epithelial homeostasis. Most human cancers originate from epithelial tissues, and loss of apical-basal cell polarity is one of the most significant hallmarks of advanced malignant tumors[1]. Several reports have shown that loss of some polarity proteins causes disordered cell polarity within epithelial cells, which cannot tightly control cellular growth and migration, ultimately leading to tumor formation, progression,

and metastasis[2, 3]. However, the relationship between polarity proteins and tumorigenesis remains incomplete.

The crumb complex, located apically, is vital in regulating and maintaining apical-basal cell polarity within epithelial cells. In mammals, three Crumbs orthologs are CRB1, CRB2, and CRB3, and only CRB3 is widely expressed in epithelial cells[4]. Our previous studies have shown that CRB3 is expressed at low levels in renal and breast cancers, and abnormal CRB3 expression leads to the disrupted organization of MCF10A cells in three-dimensional (3D) cultures[5-7]. Inhibition of CRB3 impairs contact inhibition and leads to migration, invasion and tumorigenesis of cancer cells[6, 8]. The contact inhibition results in growth arrest and causes epithelial cells to enter the quiescent phase, inducing primary cilium formation.

The primary cilium, an antenna-like microtubule-based organelle in most types of mammalian cells, is a sensorial antenna that regulates cell differentiation, proliferation, polarity, and tissue organization[9]. Dysfunction of the primary cilium can lead to developmental and degenerative disorders called ciliopathies, such as polycystic kidney disease, nephronophthisis, retinitis pigmentosa, and Meckel syndrome[10]. Remarkably, it has been reported that many cancers, including melanoma and breast, renal, pancreatic, and prostate cancer, exhibit loss of the primary cilium, most likely during the early stages of tumorigenesis[11-15]. The primary cilium is a significant extension on the apical surfaces of epithelial cells and is maintained by polarized vesicular traffic[16]. Some studies have shown that CRB3 localizes in the primary cilium and that depletion of CRB3 leads to primary cilium loss in Madin-Darby canine kidney (MDCK) cells[17, 18], indicating that CRB3 is necessary for the initiation of ciliogenesis. However, how CRB3 promotes ciliogenesis on the apical surfaces of epithelial cells remains unknown.

Here, we describe a novel conditional deletion of *Crb3* in mice and show that Crb3 is required to assemble the primary cilium in the gland, kidney and MEF cells from *Crb3* knockout mice and that *Crb3* deletion promotes breast cancer progression *in vivo*. We found that CRB3, which directly interacts with Rab11, navigates GCP6/Rab11 trafficking vesicles to the basal body of the primary cilium in mammary

epithelial cells. Additionally, we identified that CRB3 regulates the ciliary Hedgehog (Hh) and Wnt signaling pathways in breast cancer. Thus, we found unexpected diversity in the polarity protein CRB3 navigating polarized Rab11-dependent traffic, leading to a novel assembly mechanism of the γ-tubulin ring complex (γTuRC) in ciliogenesis.

Results

Crb3 deletion mice exhibit smaller and anophthalmia.

Since *Crb3* knockout mice die shortly after birth[19], we generated a novel transgenic mouse model with conditional deletion of *Crb3* using the Cre-loxP system. The loxP sites flanked either side of exon 3 in the *Crb3* gene (Supplementary Fig. 1A). The positive embryonic stem (ES) cell clone was confirmed for the wild-type gene ($Crb3^{wt/wt}$) and recombinant allele ($Crb3^{wt/fl}$) by Southern blotting (Supplementary Fig. 1B). The genotypes of the wild type, heterozygotes and homozygotes ($Crb3^{fl/fl}$) were detected with specific primers (Supplementary Fig. 1C). We first intercrossed $Crb3^{fl/fl}$ with CMV enhancer/chicken β-actin promoter (CAG)-Cre mice and found that $Crb3^{fl/fl}$, $Crb3^{wt/fl}$; CAG-Cre and $Crb3^{fl/fl}$; CAG-Cre pups were born normally and looked well developed in size. However, most $Crb3^{fl/fl}$; CAG-*Cre* pups died within a few days after birth. Few surviving $Crb3^{fl/fl}$; CAG-*Cre* mice were smaller and showed anophthalmia than littermate $Crb3^{fl/fl}$ mice at 4 weeks old (Fig. 1A-B). The eye is an organ with perfect apical-basal cell polarity. However, *Crb3* knockout mice showed ocular abnormalities. Together, these results indicated that Crb3 is necessary for eye development.

Mammary epithelial-specific *Crb3* deletion causes ductal epithelial hyperplasia and tumorigenesis.

Based on our earlier studies showing that inhibition of CRB3 impairs the organization of breast epithelium, we set out to directly explore the role of Crb3 in mammary gland development. We first developed epithelial cell-specific deletion of *Crb3* in the mammary gland by intercrossing $Crb3^{fl/fl}$ mice with mouse mammary tumor virus long terminal repeat promoter (MMTV)-*Cre* mice. We demonstrated the expression of

Crb3 in the mammary gland using immunoblotting, real-time quantitative PCR, and immunohistochemistry, and the level of Crb3 expression was deficient in mammary epithelial cells with Crb3 knockout (Supplementary Fig. 1D-F).

To assess the function of Crb3 in the development of mammary epithelial cells, we analyzed whole mammary mounts from 8-week-old virgin *Crb3*$^{fl/fl}$;MMTV-*Cre* and littermate *Crb3*$^{fl/fl}$ mice. *Crb3*$^{fl/fl}$;MMTV-*Cre* mice displayed significantly increased numbers of terminal end buds (TEBs) and bifurcated TEBs than *Crb3*$^{fl/fl}$ mice (Fig. 1 C-E). The histopathology results showed that normal ductal epithelial cells were arranged as a monolayer in *Crb3*$^{fl/fl}$ mice, while *Crb3* knockout led to ductal epithelial hyperplasia with increased ductal thickness in *Crb3*$^{fl/fl}$;MMTV-*Cre* mice (Fig. 1 F).

To observe the role of Crb3 in the tumorigenesis of breast cancer, we observed mammary glands from 9-week-old virgin polyomavirus middle T antigen (PyMT)-cKO-Crb3 and PyMT-WT mice whose mammary glands progressed to early carcinomas and lost their normal epithelial architecture[20]. The PyMT mouse model has been widely used in the study of tumor initiation and development with similar histological progression to human breast cancer. PyMT-WT mice exhibited loss of epithelial features and development of breast cancer, while PyMT-cKO-Crb3 mice grew larger tumors with faster tumor progression and a poorly differentiated phenotype (Fig. 1G). Thus, Crb3 knockout leads to ductal epithelial hyperplasia and promotes tumorigenesis, and it is essential for branching morphogenesis in mammary gland development and tumor progression.

CRB3 knockdown inhibits lumen formation in acini of mammary epithelial cells.

MCF10A cells are spontaneously immortalized human breast epithelial cells with the characteristics of normal breast epithelium[21]. MCF-10A cells could form acini similar to mammary epithelial acini under 3D basement membrane culture conditions. It has become a valuable system to study the morphogenesis and oncogenesis of the mammary epithelium[22]. To confirm the function of CRB3 in mammary epithelial acini formation, we first knocked down the expression of CRB3 by lentiviral shRNA in MCF10A cells (Supplementary Fig. 2A and B). Under the 3D culture system, we found that MCF10A cells infected with no target shRNA lentivirus could form acini

after 6 days (D6) and finally formed polarized acini with a hollow lumen at D14 (Fig. 2A). However, MCF10A cells, after CRB3 knockdown, formed more number, larger diameter and aberrant acini after D6, and these acini had no lumen (Fig. 2A-D). Cell proliferation assays of MCF10A cells showed that CRB3 knockdown increased the proliferation rate and accelerated G1 to S phase progression (Supplementary Fig. 2C-E).

Apoptosis and mitotic spindle orientation during lumen formation were further investigated in a 3D culture system. The immunofluorescence (IF) results showed that more internal cleaved-caspase 3-positive acini were observed in the control groups than in the CRB3 knockdown groups at D9 (Fig. 2E). CRB3 overexpression significantly promoted apoptosis in breast cancer cells (Supplementary Fig. 2F-H). Similarly, the staining of α-tubulin in mitotic cells exhibited more misorientation of the mitotic spindle in CRB3 knockdown groups (Fig. 2F-H).

In addition to verifying the effect of *Crb3* deletion on promoting proliferation and inhibiting apoptosis *in vivo*, these markers were detected in primary tumor tissue from PyMT-WT and PyMT-cKO-Crb3 mice. *Crb3* deletion increased the numbers of proliferative and mitotic cells and decreased the numbers of apoptotic cells in primary tumors (Fig 2I). These results demonstrate that CRB3 could promote the proliferation of mammary epithelial cells, disturb the mitotic spindle orientation, and protect the internal cells from apoptosis during acini formation, finally leading to irregular lumen formation and tumor progression.

CRB3 regulates primary cilium formation.

The primary cilium, one of the apical antenna-like extensions in epithelial cells, has been reported to play an important role in controlling lumen formation[23]. CRB3 knockdown leads to primary cilium loss in MDCK cells[17, 18], but the molecular mechanism regulating ciliogenesis on apical surfaces is still unclear. Investigating the altered effect of CRB3 on ciliogenesis *in vivo* and *in vitro*, we found that control MCF10A cells formed primary cilia after becoming confluent, while CRB3 knockdown led to significant ciliogenesis defects (Fig. 3A). Compared with the control, the number of cells with primary cilium was significantly increased, and the

length of the existing primary cilium was not different (Fig. 3B). Conversely, CRB3 conditional overexpression, adding doxycycline (+dox), resulted in restoring ciliary assembly in MCF7 cells (Fig. 3C). CRB3 overexpression increased the proportion of primary cilium formation in breast cancer cells, and the length of the restored primary cilium was increased (Fig. 3D). Importantly, the primary cilium could be directly visualized using scanning electron microscopy (SEM) (Fig. 3E). MCF10A cells with primary cilia were significantly decreased after CRB3 knockdown, while CRB3 conditional overexpression did not increase these populations (Fig. 3F).

To further corroborate these findings *in vivo*, we used breast and kidney tissue to assess ciliogenesis in the *Crb3* knockout mouse model. Renal epithelial cells can form prominent primary cilia, and the absence of primary cilia leads to ciliopathies. We used immunofluorescence to detect ciliary formation in the mammary ductal lumen and renal tubule. Compared with tissues of *Crb3*$^{fl/fl}$ mice, *Crb3* knockout led to the absence of primary cilium in the mammary ductal lumen and renal tubule from *Crb3*$^{fl/fl}$; CAG-*Cre* mice (Fig. 3G, H). In particular, the proportion of cells forming the primary cilium plummeted (Fig. 3G, H). This same phenomenon was also observed in mouse embryonic fibroblasts (MEFs) from *Crb3*$^{fl/fl}$; CAG-*Cre* mice (Supplementary Fig. 3A, B). We conclude that CRB3 plays a central role in ciliogenesis in various tissues and mammary cells.

CRB3 localizes to the basal body of the primary cilium.

CRB3, a polarity protein, was mostly reported to be localized on the tight junctions of the apical epithelium membrane[24]. Additionally, we noted that CRB3 could be closely colocalized with centrosomes at prophase, metaphase, and anaphase[17]. To investigate the relationship between CRB3 and ciliary formation, we first examined CRB3 localization in MCF10A cells that expressed CRB3-GFP. In MCF10A cells, exogenous CRB3 was mainly localized at cell tight junctions and could colocalize with pericentrin (a centrosome marker) (Fig. 4A). And approximately 40% of cells had this co-localization of exogenous CRB3 and pericentrin (Fig. 4B). We observed the localization of endogenous CRB3 with another centrosome marker to verify this phenomenon. Similarly, we detected that CRB3 accumulated on one side of the

cytoplasm and had a CRB3 focous located at the basal body represented by γ-tubulin in MCF10A cells (Fig. 4C, D). Importantly, CRB3 knockdown disturbed the accumulation of γ-tubulin at the basal body in the confluent quiescence cells (Fig. 4C). To demonstrate the relationship of this colocalization to the primary cilium, we employed acetylated tubulin to mark the primary cilium. Double immunostaining showed that this CRB3 focous was the basal body of the primary cilium, and CRB3 knockdown inhibited ciliary assembly in MCF10A cells (Fig. 4E, F). These results suggest that CRB3 is located at the basal body of the primary cilium and that CRB3 knockdown disturbs the accumulation of γ-tubulin in quiescent cells.

CRB3 trafficking is mediated by Rab11-positive endosomes.

To better investigate the molecular mechanisms of CRB3 in regulating primary cilium formation, we detected a series of genes related to ciliogenesis, including markers of centriole or primary cilium, basal body (BB) components, intraflagellar transport (IFT)-A and IFT-B anterograde transport. However, CRB3 knockdown did not alter the mRNA expression of these genes (Supplementary Fig. 4A). Then, we carried out coimmunoprecipitation (co-IP) tandem mass spectrometry using tagged exogenous CRB3 as bait to identify its interacting proteins (Supplementary Fig. 4B). Pathway aggregation analysis of these proteins revealed that these interacting proteins of CRB3 were significantly involved in Golgi vesicle transport and vesicle organization (Fig. 5A). We focused on these pathways because smaller distal appendage vesicle (DAV) formation is critical for ciliogenesis initiation, and it requires the Rab GTPase Rab11-Rab8 cascade to function[25]. Interestingly, some Rab small GTPase family members identified as CRB3-binding proteins were aggregated in these pathways, such as Rab10, Rab11A, Rab11B, Rab14, Rab1A, Rab1B, Rab21, Rab2A, Rab32, Rab35, Rab38, Rab5B, Rab5C, and Rab6A. Several centriolar proteins were also identified, including tubulin gamma-1, tubulin gamma-2, CENP-E, CEP290, CEP192, CEP295, and CEP162 (Fig. 5B).

Rab11-positive vesicles bind to centrosomal Rabin8, leading to ciliary membrane formation[26]. This report suggested that the trafficking of Rab11-positive vesicles and polarized vesicles is an essential process for early ciliary assembly. Next, we

examined the polarized vesicle traffic of CRB3 in MCF10A cells. Our studies found that CRB3 could colocalize with EEA1-positive early endosomes, CD63-positive late endosomes and Rab11-positive recycling endosomes (Fig. 5C). In addition, after 2 hours of treatment with dynasore, an endocytosis inhibitor, CRB3 significantly accumulated at the cytomembrane, and the colocalization of CRB3 with EEA1-, CD63-, and Rab11-positive endosomes was significantly decreased (Fig. 5C, D). We have reported that CRB3 expression correlates with the contact-dependent change, which is CRB3 expression reduced considerably in confluent MCF10A cells[6]. Then, dynasore could also rescue the CRB3 downregulation induced by the increasing cell density of MCF10A cells (Fig. 5E). CRB3 knockdown did not affect Rab11 expression (Supplementary Fig. 4C). These results suggest that the intracellular trafficking of CRB3 is mediated by Rab11-positive endosomes at different cell densities.

CRB3 knockdown destabilizes γTuRC assembly during ciliogenesis.

According to our findings, we assumed that CRB3 might play an important role in polarized vesicle trafficking. The γ-Tubulin ring complex (γTuRC) is a central regulator of microtubule nucleation and is a nucleation site of α-Tubulin and β-Tubulin at the microtubule organizing center in mitotic cells[27]. The primary cilium is a microtubule-based structure attached to the basal body transformed from the mother centriole. As we found that CRB3 knockdown disturbed the accumulation of γ-tubulin, we analyzed γTuRC assembly in ciliogenesis. The γTuRC is composed of multiple copies of the γ tubulin small complex (γTuSC), GCP2 and GCP3, plus GCP4, GCP5, and GCP6[27, 28] (Fig. 5F). CRB3 downregulation did not affect the expression of γTuRC subunits (Fig. 5G). Then, we performed co-IPs directed against GCP6 in CRB3-depleted cells and assessed the relative levels of both γTuSC-specific proteins compared with the control shRNA-treated cells. Interestingly, CRB3 knockdown significantly reduced the interaction of GCP6 with GCP3 (Fig. 5H). To verify this phenomenon, we observed the localization of GCP3 with GCP6 foci in MCF10A cells. Correspondingly, CRB3 downregulation significantly disturbed the colocalization of GCP3 with GCP6 foci in quiescence cells (Fig. 5I). To investigate

whether CRB3 facilitate γTuRC assembly, we fractionated MCF10A cytoplasmic proteins on sucrose gradients according to the method published[29]. We noticed that γTuSC were regimented mainly in fractions 3 and γTuRC sediment in fractions 6 (Fig. 5J). However, CRB3 downregulation caused most of GCP6 and GCP5 to be precipitated independently of γTuRC in the low-density fractions (Fig. 5J). Together, these results have established that the depletion of CRB3 interferes with the molecular interactions between the γTuRC proteins, failing microtubule formation in the primary cilium. However, it may not affect the expression of these molecules during γTuRC assembly. According to these results, we hypothesized that CRB3 could mediate the polarized vesicle trafficking of some γTuRC-specific proteins during Rab11-positive endosomes.

CRB3 directly interacts with Rab11.

To fully prove this hypothesis, we first examined CRB3 interaction with Rab11-positive endosome and γTuRC-specific proteins in MCF10A cells. Using CRB3 as bait, we identified that CRB3 could interact with Rab11 instead of GCP6 and GCP3 (Fig. 6A). In contrast, Rab11 could interact with CRB3 and GCP6 instead of GCP3 by tagging Rab11 as bait (Fig. 6B). Thus, does CRB3 knockdown affect the trafficking of γTuRC-specific proteins during Rab11-positive vesicles? The co-IP results showed that CRB3 knockdown did not affect Rab11 interacting with GCP6, and the level of Rab11 binding to GCP6 had a tendency to decrease in MCF10A cells with CRB3 knockdown (Fig. 6C). The colocalization rate of Rab11 and GCP6 was significantly decreased in MCF10A cells with CRB3 knockdown (Supplementary Fig. 5A-C). Consistent with other reports[30], Rab11 knockdown significantly inhibited primary cilium formation in MCF10A cells (Supplementary Fig. 5D-F).

Based on these results, we further detected the region of CRB3 interacting with Rab11 by using coexpression and co-IP in HEK293 cells. CRB3 consists of a signal peptide (SP), extracellular domain, transmembrane (TM), FERM-binding domain (FBD), and carboxy-terminal PDZ-binding domain (PBD). Due to the specificity of the C- and N-terminal domains, we constructed CRB3-GFP fusion proteins where GFP tags were fused to the extracellular domain as previously reported[31] (Fig. 6D). According to

these domains, we also constructed the truncations of CRB3-GFP fusion proteins with serial C-terminal deletions (Fig. 6E) and another Flag-Rab11a fusion protein. The coexpression and co-IP results showed that Flag-Rab11a interacted with full-length CRB3 (1-120), CRB3 (1-116), CRB3 (1-83), and CRB3 (1-58) but not CRB3 (1-26) (Fig. 6F). Therefore, the region of CRB3 interacting with Rab11 is amino acids 27 to 58.

Rab11, a small GTP-binding protein, is an essential regulator of the dynamics in recycling endosomes, which are required to undergo GDP/GTP cycles. In particular, Rab11a[S25N] is a GDP-locked form, Rab11a[S20V], and Rab11a[Q70L] is a GTP-locked form[30]. To further investigate the GTP-bound form of Rab11a interacting with CRB3, we constructed these mutant variants of Rab11a. Next, we found that the weaker interaction of CRB3 and these mutant variants, Rab11a[Q70L], Rab11a[S20V] and Rab11a[S25N], compared with Rab11aWT, was observed using co-IP analysis (Fig. 6G). Together, these results indicate that CRB3 directly interacts with Rab11a, depending on the GTPase activity of Rab11a.

CRB3 navigates GCP6/Rab11 trafficking vesicles to CEP290 in the primary cilium.

Since CRB3 knockdown does not affect the interaction with Rab11-positive endosome and γTuRC-specific proteins GCP6, we wanted to know whether CRB3 affected the localization of GCP6/Rab11 trafficking vesicles to the basal body of the primary cilium. Reviewing the identification of CRB3 interacting proteins, there are some centriolar proteins. CEP290 is located in the transition zone of the primary cilium and is required for the formation of microtubule-membrane linkers[32]. Then, we verified that exogenous CRB3 could bind to CEP290, Rab11, GCP6 (Fig. 7A), and Rab11 also interacted with CEP290, CRB3-GFP, GCP6 (Fig. 7B). Similar to Fig. 6C, the amount of GCP6 bonded to Rab11 was reduced within CRB3 knockdown. Importantly, CRB3 knockdown showed that Rab11 hardly bonded to CEP290 (Fig. 7C). We checked the colocalization of the basal body foci of GCP6 and γ-tubulin, γ-tubulin and Rab11 in MCF10A and MEF cells to corroborate this result. In quiescence control cells, GCP6 foci and γ-tubulin formed a prominent focus at the

basal body of the primary cilium, as observed by immunofluorescence. However, CRB3 downregulation eliminated this focus and significantly disturbed the basal body foci of GCP6 and γ-tubulin in MCF10A cells (Fig. 7D), and γ-tubulin and Rab11 in MEF cells (Fig. 7E). At the beginning of this experiment, we also tried to show the interaction of CRB3, Rab11 and GCP6 with expressing fluorescence tagged GCP6 protein and FRET assay. However, the centrosomal foci of fluorescent GCP6 was difficult to capture because of too weak signal, and this may require re-overexpression of ninein-like protein (Nlp) to promote γTuRC enrichment[29, 33].

Although CRB3 knockdown had little effect on the formation of GCP6/Rab11 trafficking vesicles, it could significantly inhibit the γTuRC assembly during ciliogenesis, thus affecting the transport of GCP6/Rab11 trafficking vesicles to the basal body of the primary cilium. Since CRB3 affected the combination of Rab11 and CEP290, we speculate that CRB3 could be a navigator, navigating GCP6/Rab11 trafficking vesicles to the basal body of the primary cilium.

CRB3 regulates the Hh and Wnt signaling pathways in tumorigenesis

To examine the role of CRB3 in regulating ciliary assembly in breast cancer, we explored the relationship between CRB3 and the primary cilium in breast cancer tissues. We found that CRB3 was localized at the subapical surface of the mammary gland lumen in the paracarcinoma tissues. CRB3 had no obvious expression or localization in breast cancer tissues (Fig. 8A). The immunofluorescence analysis results showed that the intact primary cilium could be observed in the adjacent para-carcinoma tissues, whereas the number of cells with primary cilia was much lower in breast cancer tissues (Fig. 8B, C). Consistent with previous findings, CRB3 was localized at the basal body of the primary cilium in adjacent para-carcinoma tissues (Fig. 8D). These results again suggest that a defect in CRB3 expression inhibits ciliary assembly, which could be involved in tumorigenesis.

To detect some functional consequences of the absence of the primary cilium, we measured alterations in the Hedgehog (Hh) and Wnt signaling pathways in CRB3-depleted cells. We first detected the translocation of SMO into the primary cilium and the expression of the target gene GLI1. SAG is a potent Smoothened

(SMO) receptor agonist that activates the Hh signaling pathway. CRB3 knockdown did not promote the translocation of SMO into the primary cilium in MCF10A cells treated with SAG (Fig 8E). After SAG treatment, the mRNA expression of GLI1 was significantly decreased in CRB3-depleted cells (Fig 8F). Crb3 knockout did not alter the expression or cellular localization in primary tumors from PyMT-cKO-Crb3 mice (Fig 8H). We have reported that CRB3 expression was high in immortalized mammary epithelial cells while loss of CRB3 expression in breast cancer cells[6]. On the other hand, western blotting results showed that GSK3-β was significantly downregulated after CRB3 knockdown in MCF10A cells. In addition, β-catenin, the major effector of the canonical Wnt signaling pathway, was upregulated. CRB3 overexpression resulted in upregulation of GSK3-β and downregulation of β-catenin in breast cancer cells (Fig. 8G). Visibly, β-catenin was significantly upregulated and markedly localized in the nucleus from PyMT-cKO-Crb3 mice (Fig 8I). Together, these data suggest that CRB3 knockdown cannot activate the Hh signaling pathway but can activate the Wnt signaling pathway, which is ultimately involved in tumorigenesis.

Discussion

Apical-basal cell polarity is essential for cellular architecture, development and homeostasis in epithelial tissues. Most malignant cancers lack cell polarity because of the unconstrained cell cycle and cell migration with decreased adhesion. CRB3, an important apical protein, is significantly reduced in various tumor cells and tissues and promotes metastasis and tumor formation in nude mice[5, 6, 8, 34-36]. Here, we generate a novel transgenic mouse model with conditional deletion of Crb3 to study the role of polarity proteins in tumorigenesis. *Crb3* knockout mice were reported to have cystic kidneys, improper airway clearance in the lung, villus fusion, apical membrane blebs, and disrupted microvilli in intestine airway clearances, which demonstrated that CRB3 is crucial for the development of the apical membrane and epithelial morphogenesis[19, 37]. Importantly, our studies in the *Crb3* knockout mouse model suggest that in addition to death after birth, it is characterized by smaller

size, ocular abnormality, bronchial smooth muscle layer defect, reduced pancreatic islets and hyperplasia mammary ductal and renal tubular epithelium (some data not shown). These important phenotypic changes are due to disruption of epithelial polarity homeostasis, which may ultimately lead to tumorigenesis.

In addition, we also found the absence of primary cilia in the mammary ductal lumen and renal tubule in the *Crb3* knockout mouse model. Consistent with other reports, CRB3 knockdown regulated ciliary assembly in MDCK cells[17, 18]. Furthermore, CRB3 was localized in the inner segments of photoreceptor cells and concentrated with the connecting cilium during the whole development of the mouse retina[38]. These ocular defect phenotypes remarkably resemble EHD1 knockout mice, which regulate ciliary vesicle formation in primary cilium assembly[25, 39, 40]. Hence, CRB3 can affect primary cilium formation in various epithelial tissues, such as the breast, kidney, and retina.

Previous literature has reported that CRB3 localizes in the primary cilium in differentiated MDCK cells[17, 18], but we mainly found that it is not located on the basal body of the primary cilium in mammary epithelial cells. The immunofluorescence results in these literatures showed that CRB3 was scattered on the primary cilia, and had a strong foci at the basal body. Especially in rat kidney collecting ducts, the localization of CRB3 on primary cilia was significantly reduced, while the obvious localization was basal body[18]. Another literature also reported the co-localization of CRB3 and γ-tubulin in MDCK cells[17]. This result is consistent with our conclusion, and we also verified its co-localization with centrosome by overexpressing CRB3 in mammary epithelial cells, indicating that CRB3 mainly localized to the basal body of the primary cilium. Given that CRB3 also alters primary cilium formation in the mammary epithelium, it is important to investigate the molecular mechanisms by which CRB3 regulates primary cilium formation.

Polarized vesicular traffic plays an important role in extending the primary cilium on apical epithelial surfaces, and the molecular mechanism and relationship to tumorigenesis remain unclear. Here, we reveal that Rab11-positive endosomes mediate the intracellular trafficking of CRB3 and that CRB3 can navigate

GCP6/Rab11 trafficking vesicles to CEP290 for correct γTuRC assembly during primary cilium formation. Moreover, in the presence of CRB3, the epithelium can form contact inhibition and an intact primary cilium in quiescence to achieve cellular homeostasis. In contrast, loss of contact inhibition and primary cilium formation with CRB3 deletion can activate the Wnt signaling pathway, affect the Hh signaling pathway, and disrupt the imbalance of this cellular homeostasis, resulting in significant cell proliferation during tumorigenesis (Fig. 9).

The formation of the primary cilium has been divided into several distinct phases. It begins with the maturation of the mother centriole, also known as a centriole-to-basal-body transition[9, 41]. DAVs, which are small cytoplasmic vesicles originating from the Golgi and the recycling endosome, accumulate in the vicinity of distal appendages of the mother centriole[25, 42, 43]. Rab8a is localized to cytoplasmic vesicles and the Golgi/trans-Golgi network in growing RPE1 cells, while Rab8-positive vesicles are rapidly redistributed to the mother centriole with binding to Rab11-positive recycling and are endosomes under serum starvation[26, 44]. The EHD1 protein converts these DAVs to form larger ciliary vesicles, which elongate through continuous fusion with Rab8-positive vesicles to produce the primary cilium membrane[25, 30, 40]. Thus, Rab11-positive recycling endosomes, as important transporters, are necessary for primary cilium formation and a centriole-basal-body transition in the early phase. Our study found that CRB3 could bind to some Rab small GTPase family members to significantly participate in Golgi vesicle transport and vesicle organization pathways through mass spectrometry identification. And coexpression and co-IP results showed that CRB3 directly interacted with Rab11 via extracellular domain, which mediated the intracellular trafficking of CRB3 within Rab11-positive endosomes at different cell densities.

Unlike motile cilia, primary cilia have only nine outer doublet microtubules without a central pair of microtubules (9+0 structure). Similar to microtubule assembly at microtubule organizing centers (MTOCs) during mitosis, γ-tubulin, which is a part of γTuRC, is also concentrated in the basal body of the cilium in interphase[45]. γ Tubulin complexes have been shown to form microtubule templates and regulate

microtubule nucleation through longitudinal contacts with α-tubulin and β-tubulin[27]. The γTuRC consists of multiple copies of γTuSC, which is composed of two copies of γ-tubulin and one each of GCP2 and GCP3. Then, GCP2 and GCP3 interact with GCP4, GCP5 and GCP6 to form γTuRC[46]. Our experiments showed that CRB3 knockdown does not affect the expression of the γTuRC-specific proteins GCP3, GCP6 and γ-tubulin but inhibits the interaction between GCP6 and GCP3, resulting in destabilization of γTuRC. Further analysis revealed significant colocalization between GCP6- and Rab11-positive vesicles at low cell density, while at high cell density, GCP6 accumulated at the centrosome together with GCP3. CRB3 knockdown results in the disappearance of this accumulated GCP6. These results indicated that GCP6/Rab11 trafficking vesicles must be transported to the basal body for ciliogenesis. While it is still unclear how CRB3 interferes with γTuRC assembly during primary cilium formation, we hypothesize that Rab11-positive endosomes mediate the intracellular trafficking of CRB3 and that CRB3, as a navigator interacting with CEP290, can navigate GCP6/Rab11 trafficking vesicles to the transition zone for γTuRC assembly in primary cilium formation.

CEP290 is considered a centriolar or ciliary protein localized to the centrosomes in dividing cells, the distal mother centriole in quiescent cells, and the transition zone in the primary cilium[47]. *CEP290* mutations are associated with various diseases, such as the devastating blinding disease Leber's Congenital Amaurosis (LCA), nephronophthisis, Senior Løken syndrome (SLS), Joubert syndrome (JS), Bardet-Biedl syndrome (BBS), and lethal Meckel-Gruber syndrome (MGS)[48]. In particular, the phenotypes of CEP290-associated ciliopathies are very similar to those in our *Crb3* knockout mouse model, with lethality and ocular and renal abnormalities. CEP290 knockdown significantly decreased the number of cells with primary cilia, and CEP290 knockout mice showed mislocalization of ciliary proteins in the retinas[49, 50]. Additionally, the depletion of CEP290 disrupts protein trafficking to centrosomes and affects the recruitment of the BBSome and vesicular trafficking in ciliary assembly[51, 52]. Although CEP290 serves as a hub to recruit many ciliary proteins, it has not been reported to be involved in polarized vesicle trafficking or

localization. Our study verified that CEP290 could interact with the polarity protein CRB3. CRB3 knockdown disrupts the interaction between CEP290 and Rab11-positive endosomes. Thus, these results validate our hypothesis. Although CRB3 navigates GCP6/Rab11 trafficking vesicles to CEP290, we further need to detect the region of CRB3 interacting with CEP290 and what other cargos are transported by CRB3/Rab11 trafficking vesicles in ciliary assembly.

In addition, our results showed that CRB3 knockdown could not activate the Hh signaling pathway with SAG in MCF10A cells. Under untreated conditions, PTCH1 located at the ciliary membrane inhibits SMO, and Suppressor of Fused (SuFu) sequesters Gli transcription factors, leading to its degradation. SAG can activate the Hh signaling pathway, resulting in dissociation into the nucleus to regulate the expression of downstream target genes[53]. The Hh signaling pathway mainly regulates cellular growth and differentiation and is abnormal in different types of tumors. Some studies indicate that cilia are double-sided, promoting and preventing cancer development through the Hh signaling pathway *in vivo*. It is thought that cilia deletion could activate SMO to inhibit tumor growth, while promoting carcinogenesis was induced by activated Gli2[54]. Although SAG cannot activate the Hh signaling pathway in CRB3-depleted cells, CRB3 regulation of the carcinogenic process through the ciliary Hh signaling pathway still needs further research. In addition, another canonical Wnt signaling pathway was altered after CRB3 knockdown. The Wnt signaling pathway is an important cascade regulating development and stemness, and aberrant Wnt signaling has been reported in many more cancer entities, especially colorectal cancer[55]. CRB3 downregulated the expression of β-catenin in our study. Thus, CRB3 can affect cilium-related signaling pathways in tumorigenesis.

In summary, our study provides novel evidence for the apical polarity protein CRB3 in the regulation of viability, mammary and ocular development, and primary cilium formation in germline and conditional knockout mice. CRB3 deletion causes irregular lumen formation and ductal epithelial hyperplasia in mammary epithelial-specific knockout mice. Further study uncovered a new function of CRB3 in directly interacting with Rab11, navigating GCP6/Rab11 trafficking vesicles to CEP290 for

γTuRC assembly during ciliogenesis. In addition, CRB3 also participates in regulating cilium-related Hh and Wnt signaling pathways in tumorigenesis. Understanding these polarity protein-mediated vesicle trafficking mechanisms will shed new light on luminal development and tumorigenesis.

Methods

Generation of floxed *Crb3* mice. All animal experiments were verified and approved by the Committee of Institutional Animal Care and Use of Xi'an Jiaotong University. Heterozygous *Crb3*$^{wt/fl}$ mice (C57BL/6J) were generated by Cas9/CRISPR-mediated genome editing (Cyagen Biosciences, Guangzhou, China). The gRNA to *Crb3* and Cas9 mRNA was coinjected into fertilized mouse eggs to generate targeted conditional knockout offspring. The sequences of the *Crb3* gRNA were gRNA1, 5’-GGCTGGGTCCACACCTACGGAGG-3’; gRNA2, 5’-ACCCACAAAGCCACGCCA GTGGG-3’. Mouse genotyping was screened by PCR with the primers F1, 5’-ACATAAGGCCTTCCGTTAAGCTG-3’; R1, 5’-GTGGATTCGGACCAGTCTGA-3’. *Crb3*$^{wt/fl}$ mice were intercrossed with MMTV-*Cre* mice or CAG-*Cre* mice to generate tissue-specific *Crb3* knockout mice. MMTV-*Cre* mice (FVB), CAG-*Cre* mice (C57BL/6N) and MMTV-PyMT mice (FVB) were purchased from Cyagen Biosciences. All mice were bred in the specific pathogen-free (SPF) animal houses of the Laboratory Animal Center of Xi’an Jiaotong University.

Mice were genotyped by using PCR and DNA gel electrophoresis. Genomic DNA from the mouse tail was extracted by using the Mouse Direct PCR Kit (Bimake, TX, USA, #B40015) according to the manufacturer’s instructions. The genotyping primers were F3, 5’-TTGAGAGTCTTAAGCAGTCAGGG-3’; R5, 5’-AACCTTTCCCAGGAGTA TGTGAC-3’. PCR results showed that *Crb3*$^{wt/wt}$ was one band with 163 bp, *Crb3*$^{wt/fl}$ was two bands with 228 bp and 163 bp, and *Crb3*$^{fl/fl}$ was one band with 228 bp. The primers for identifying *Cre* alleles were F, 5’-TTGAGAGTCTTAAGCAGTCAGGG-3’; R,

5’-TTACCACTCCCAGCAAGACAC-3’, and amplification with one 247 bp band. The reaction conditions of *Crb3* and *Cre* PCR were as follows: 94°C for 5 min, 94°C for 20 s, 60°C for 30 s, and 72°C for 20 s for 35 cycles.

Mouse mammary gland whole mount analysis. After mice were euthanized, the inguinal mammary gland tissues were removed intact and fully spread onto slides. Tissues were rapidly fixed in Carnoy’s fixative for 2 h at room temperature. Then, the tissues were washed in 70% ethanol solution for 15 min at room temperature and in 50 and 30% ethanol and distilled water for 5 min each. Staining was carried out in carmine alum solution at 4°C overnight. After washing with 70% ethanol solution, tissues were washed again in 70, 95 and 100% ethanol for 15 min each. Tissues were cleared in xylene overnight at room temperature and then mounted with neutral balsam. Images were obtained by using a microscope (Leica DMi8, Wetzlar, Germany).

Cell culture and transfection. MCF10A, MCF7, T47D, MDA-MB-231, HCC1806, T47D, MDA-MB-453 cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). MCF10A cells were routinely grown in DMEM/F12 (1:1) media (HyClone, UT, USA) supplemented with 5% horse serum, 20 ng/ml human EGF, 10 μg/ml insulin, and 0.5 μg/ml hydrocortisone. MCF7, MDA-MB-231 and MDA-MB-453 cells were cultured in high glucose DMEM media (HyClone, UT, USA) supplemented with 10% FBS (HyClone, UT, USA, #SH30084.03). T47D and HCC1806 cells were grown in RPMI-1640 media (HyClone, UT, USA) supplemented with 10% FBS. All cell lines were incubated in 5% CO_2 at 37°C.

siRNAs were purchased from GenePharma company (Shanghai, China). Cultured cells were transfected with siRNAs or plasmids by using Lipofectamine 2000 (Invitrogen, CA, USA, #11668-019) following the manufacturer’s protocol. The negative control shRNA (NC) and shRNA against CRB3 were packaged into lentivirus as previously described[5, 6].

DNA constructs and stable cell lines. CRB3 was PCR amplified from RNA extracted from MCF10A cells and cloned into the pCMV-Blank vector (Beyotime,

Shanghai, China, D2602) using T4 ligase (NEB, MA, USA). Then, the GFP sequence was inserted between the extracellular and transmembrane CRB3 domains to generate the pCMV-CRB3-GFP vector as described previously[31]. The full-length CRB3 (1-120), CRB3 (1-116), CRB3 (1-83), CRB3 (1-58) and CRB3 (1-26) were amplified by PCR from the pCMV-CRB3-GFP vector using specific primers to create EcoR and Xho restriction sites and cloned into the pCMV-Blank vector. Full-length was cloned into the lentiviral vector pLVX-TetOne-Puro (Clontech, Takara, #631849). pECMV-3×FLAG-RAB11A was purchased from SinoBiological (Beijing, China). The plasmids of pECMV-3×FLAG-RAB11A[S20V]/[S25N]/[Q70 L] mutants were constructed by using the *Fast* Mutagenesis System (Transgen, Beijing, China, FM111-01).

Lentiviruses were produced in HEK293T cells. CRB3-GFP in MCF10A and MCF7 cells was generated by using a lentivirus expression system. Screening of stable cell lines used puromycin after lentiviral infection for 72 h.

3D morphogenesis. The growth factor-reduced Matrigel (Corning, USA, #354230) was added to the four-well chamber slide system (Corning, USA, #177437) and then incubated at 37°C for solidification. MCF10A cells were plated into this chamber slide and cultured in DMEM/F12 (1:1) media (HyClone, UT, USA) supplemented with 2% horse serum, 5 ng/ml human EGF, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 2.5% Matrigel. 3D morphogenesis was photographed using a microscope (Leica DMi8, Wetzlar, Germany) at different time points. 3D MCF10A cells were fixed and stained on day 14 after cell culture.

Immunohistochemistry. The paraffin-embedded sections of samples were baked, deparaffinized in xylene, and rehydrated sequentially in gradient concentrations of ethanol, sequentially. Then, antigen retrieval was performed in Tris-EDTA buffer (pH 9.0) heated to 95°C for 20 min. Endogenous peroxidase activity was reduced in 3% hydrogen peroxide for 10 min at room temperature. Then, 5% goat plasma was used for blocking at 37°C for 30 min, and the sections were incubated with specific primary antibodies overnight at 4°C. Ki67 (Abcam, UK, ab279653), phospho-histone H3 (Abcam, UK, ab267372), cleaved caspase 3 (CST, USA, #9661), GLI1 (CST,

USA, #3538), and β-catenin (CST, USA, #8480) were used as primary antibodies. The following steps used biotin-streptavidin HRP detection kits (ZSGB-BIO, China, SP-9001, SP-9002) according to the manufacturer's instructions. The sections were stained with DAB (ZSGB-BIO, China, ZLI-9017), counterstained with hematoxylin, dehydrated in gradient concentrations of ethanol, cleared in xylene, and mounted with neutral balsam, sequentially. Images were obtained by using a slide scanner (Leica SCN400, Wetzlar, Germany).

Immunofluorescence. Cells were plated on coverslips and fixed in 4% paraformaldehyde for 30 min. Then, the membranes were permeabilized by using 0.2% Triton X-100. The cells were blocked with 5% BSA solution for 1 h at room temperature. Cells were incubated with specific primary antibodies overnight at 4°C. Caspase 3 (CST, USA, #9662S), α-tubulin (CST, USA, #3873), acetylated tubulin (Proteintech, USA, #662001-IG), γ-tubulin (Proteintech, USA, #15176-1-AP), pericentrin (Abcam, UK, ab4448), CRB3 (Sigma, USA, HPA013835), EEA1 (BD, USA, #610456), Rab11 (BD, USA, #610823), and GCP6 (Abcam, UK, ab95172) were used as primary antibodies. The secondary antibodies were Alexa Fluor 488-labeled or 594-labeled (Invitrogen, USA, A32731, A32744). DPIA (5 μg/ml) was used for DNA staining, and images were taken by using a confocal microscope (Leica SP5 , Wetzlar, Germany).

MEFs isolation and maintenance. The embryos were harvested from *Crb3*$^{wt/fl}$;CAG-Cre mice crosses at E13.5. Head, tail and all innards were removed from these embryos. DNA could be extracted from the tails for genotyping. Then, the body was minced and digested with trypsin in a 37°C incubator for 30 min. Cells were resuspended in MEF media (DME, 10% FBS, 1% penicillin/streptomycin) and plated on 10 cm cell culture dishes with one embryo. They were passaged 1:3 with MEF media for further expansion.

Real-time PCR assay. Total RNA from cell lines was isolated by using TRIzol reagent (Invitrogen, USA, #15596026), and 5 μg RNA was converted to cDNA with the RevertAid first strand cDNA synthesis kit (Thermo, USA, K1622) according to the manufacturer's instructions. Real-time PCR was prepared with SYBR qPCR

Premix (Takara, Japan, RR420L) and then performed with a real-time PCR detection instrument (Bio–Rad CFX96, USA). The primers used for real-time PCR were purchased from Tsingke Biotech (Beijing, China), and the sequences are listed in Table 1. The mRNA expression was normalized to *GAPDH*, and fold changes were calculated by using the ΔΔCt method.

Immunoprecipitation and immunoblotting. Cultured cells were lysed in RIPA buffer supplemented with protease inhibitors (Roche, NJ, USA). Then, the cell lysates were subjected to SDS–PAGE separation and transferred to PVDF membranes. The membranes were subjected to immunoblot assays using antibodies against CRB3 (Santa Cruz, USA, sc-292449), GCP2 (Santa Cruz, USA, sc-377117), GCP3 (Santa Cruz, USA, sc-373758), GCP4 (Santa Cruz, USA, sc-271876), GCP5 (Santa Cruz, USA, sc-365837), GCP6 (Abcam, UK, ab95172), γ-tubulin (Proteintech, USA, #66320-1-Ig), Rab11 (Abcam, UK, ab128913), GFP (Roche, USA, #11814460001), Flag (Sigma, USA, F7425), CEP290 (Santa Cruz, USA, sc-390637), GSK3-β (CST, USA, #12456), β-catenin (CST, USA, #8480), GAPDH (Proteintech, USA, #HRP-6004), and β-actin (Proteintech, USA, #HRP-60008). HRP-conjugated secondary antibodies (CST, USA, #7074, #7076) were incubated at room temperature for 1 h in the dark. Final detection was detected by ECL Plus (Millipore, Germany, WBULS0500). Proteins for the immunoprecipitation assay were lysed with NP40 buffer. The cell lysates were detected by a Dynabeads protein G immunoprecipitation kit (Invitrogen, USA, #10007D) according to the manufacturer's instructions. Elution protein complexes were subjected to immunoblot assay.

Protein identification and bioinformatics analyses. LC−MS/MS analysis was conducted by PTM Bio (Zhejiang, China). Q Exactive™ Plus (Thermo, MA, USA) was used for tandem mass spectrometry data analysis. The Blast2GO program against the UniProt database was used to analyze the functional annotation and classification of the identified proteins. Then, pathway enrichment analysis was enriched by using DAVID tools.

Scanning electron microscope (SEM) observations. CRB3-GFP and CRB3

knockdown MCF10A cells were plated on culture slides, and doxycycline (Dox) was added to induce the expression of CRB3-GFP. After the cells were fully confluent, the slides were fixed with 4% paraformaldehyde at 4°C overnight and then with 1% osmium tetroxide at 4°C for 1 h. Dehydration was performed at room temperature, and the samples were dried using the critical point drying method. Images were obtained by using a scanning electron microscope (Hitachi TM1000, Japan).

Clinical data. Patient data, breast cancer tissues and adjacent para-cancerous tissues were collected from the First Affiliated Hospital of Xi'an Jiaotong University (Shaanxi, China). All patients signed informed consent forms before surgery. This research was authorized by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University and conducted in conformity with the Declaration of Helsinki.

Statistics. Statistical analysis was performed using SPSS statistics 23.0 for Windows (IBM, Armonk, USA). All experiments were repeated at least three times. The values are expressed as the mean ± standard deviation (SD). Unpaired Student's *t* test was used to compare the differences between two groups. One-way ANOVA followed by Dunnett's multiple comparisons test was used for multiple comparisons. The χ^2 test was used to assess the significance of the observed frequencies. $P<0.05$ was considered an indicator of statistical significance.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

P.L. designed the research; B.W., Z.L., T.T, Y.J., Y.S., R.W., H.C., J.L. and P.L. performed the experimental work, and M.Z., X.G., J.L. and S.S. analyzed the data; B.W., Z.L., Y.R. and P.L. wrote the original manuscript. P.L. conceived the study and critically revised the manuscript. All authors read and approved the final manuscript.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figure 1. *Crb3* knockout mice exhibit smaller sizes and ocular abnormalities, and mammary epithelial cell-specific *Crb3* knockout leads to ductal epithelial hyperplasia and promotes tumorigenesis.

A, B. Representative whole bodies (A) and eyes (B) from littermate *Crb3*$^{fl/fl}$, *Crb3*$^{wt/fl}$;CAG-*Cre* and *Crb3*$^{fl/fl}$;CAG-*Cre* mice at 4 weeks old. C. Representative mammary whole mounts from littermate *Crb3*$^{fl/fl}$ and *Crb3*$^{fl/fl}$;MMTV-*Cre* mice at 8 weeks old with Carmine-alum staining. (scale bars, 200 μm) D, E. Quantification of the average number of TEBs (n=10) and bifurcated TEBs (n=10) in littermate *Crb3*$^{fl/fl}$ and *Crb3*$^{fl/fl}$;MMTV-*Cre* mice at 8 weeks old. F. Representative images of mammary glands in littermate *Crb3*$^{fl/fl}$ and *Crb3*$^{fl/fl}$;MMTV-*Cre* mice stained with H&E. (scale bars, 50 μm) G. Representative images of primary tumors stained with H&E in PyMT-WT and PyMT-cKO-*Crb3* mice at 9 weeks old. (scale bars, left 500 μm, right 50 μm) Magnified areas of boxed sections are shown in the right panels. Bars represent the means ± SD; Unpaired Student's *t* test, ***$P<0.001$.

Figure 2. CRB3 knockdown inhibits acinar formation of mammary epithelial cells in a 3D culture system.

A. Representative effect of CRB3 on acinar formation in the 3D culture system at days 3, 6, 9 and 14. (Magnified areas of marked arrows are shown in the lower right corner) B-D. Quantification of the average number, diameter and aberration of acini (n=10). E. Immunofluorescence showing apoptosis during lumen formation. Caspase 3 (red), α-tubulin (green), and DNA (blue). F. Immunofluorescence showing the mitotic spindle orientation during lumen formation. α-Tubulin (green) and DNA (blue). G, H. Quantification of division angle. I. Immunohistochemical analyses of Ki67, phospho-histone H3 and cleaved caspase 3 in primary tumors from PyMT-WT and PyMT-cKO-*Crb3* mice at 9 weeks old. (positive cells marked by arrows) Scale bars, 25 μm, bars represent means ± SD; Unpaired Student's *t* test, * $P<0.5$, ** $P<0.01$, ***$P<0.001$.

Figure 3. CRB3 alters primary cilium formation in mammary cells, mammary ductal

lumen and renal tubule from *Crb3*$^{fl/fl}$;CAG-*Cre* mice.

A. C. Representative images of immunofluorescent staining of primary cilium formation with CRB3 knockdown in MCF10A cells and CRB3 conditional overexpression upon dox induction in MCF7 cells. Acetylated tubulin (red), γ-tubulin (green), and DNA (blue). (primary cilium marked by arrows; scale bars, 10 μm) B. D. Quantification of the proportion of cells with primary cilium formation and the length of the primary cilium (n=10). E. Representative scanning electron microscope images of primary cilium formation with CRB3 knockdown and conditional overexpression in MCF10A cells. (primary cilium marked by arrows; scale bars, 50 μm) F. Quantification of the proportion of cells with primary cilium formation (n=10). G. H. Representative immunofluorescent staining of primary cilium formation in the mammary ductal lumen and renal tubule from *Crb3*$^{fl/fl}$ and *Crb3*$^{fl/fl}$;CAG-*Cre* mice, respectively. Acetylated tubulin (red), γ-tubulin (green), and DNA (blue). (n=10; scale bars, 25 μm) Bars represent the means ± SD; Unpaired Student's *t* test, **P*<0.05, ****P*<0.001.

Figure 4. CRB3 localizes to the basal body of the primary cilium.

A. Immunofluorescence showing the colocalization of exogenous CRB3 with centrosomes in MCF10A cells. Pericentrin, a marker of centrosome (red), CRB3-GFP (green), and DNA (blue). (Colocalization marked by arrows; scale bars, 10 μm) B. Quantification of the proportion of cells with pericentrin and exogenous CRB3 colocalization (n=10). (bars represent the means ± SD) C. Another colocalization of endogenous CRB3 with the basal body in MCF10A cells. γ-Tubulin is a marker of the centrosome and basal body of the primary cilium (green), CRB3 (red), and DNA (blue). (Colocalization marked by arrows; scale bars, 10 μm) D. Corresponding fluorescence intensity profile across a section of the array, as indicated by the dashed white line in (C). E. Double immunostaining displaying the colocalization of CRB3 with the primary cilium in MCF10A cells. Acetylated tubulin (green), CRB3 (red), and DNA (blue). (Colocalization marked by arrows; scale bars, 10 μm) F. Fluorescence 3D reconstruction of CRB3 and primary cilium colocalization.

Acetylated tubulin (red), CRB3 (green), and DNA (blue).

Figure 5. CRB3 trafficking is mediated by Rab11-positive endosomes, and CRB3 knockdown destabilizes γTuRC assembly during ciliogenesis.

A. Pathway aggregation analysis of CRB3 binding proteins identified by mass spectrometry in MCF10A cells. B. Table of some Rab small GTPase family members and centriolar proteins identified as CRB3 binding proteins. C. Immunofluorescence showing the colocalization of CRB3 with EEA1-, CD63-, and Rab11-positive endosomes in MCF10A cells. EEA1, CD63, Rab11 (green), CRB3 (red), and DNA (blue). (scale bars, 10 μm) D. Quantification of the proportion of cells with these markers and CRB3 colocalization (n=10). E. Western blots showing the levels of CRB3 in MCF10A cells treated with dynasore at different cell densities. F. Structure diagram of γTuRC (γ-tubulin ring complex). G. Immunoblot analysis of the effect of CRB3 on γTuRC molecules in MCF10A cells. H. Coimmunoprecipitation showing the interacting proteins with GCP6 in MCF10A cells with CRB3 knockdown. I. Representative images of immunofluorescent staining of GCP3 and GCP6 colocalization in MCF10A cells with the corresponding fluorescence intensity profile. GCP3 (red), GCP6 (green), and DNA (blue). (scale bars, 10 μm) J. Comparison of cytoplasmic extracts from MCF10A cells and cells with CRB3 knockdown after fractionation in sucrose gradients. The γTuSC sedimentation was mainly in fractions 3, and γTuRC sedimentation was mainly in fractions 6. Bars represent the means ± SD; Unpaired Student's *t* test, ***$P<0.001$.

Figure 6. CRB3 directly interacts with Rab11.

A. Coimmunoprecipitation of CRB3 with Rab11, GCP6 and GCP3 in MCF10A cells. B. Coimmunoprecipitation of Rab11 with CRB3, GCP6 and GCP3 in MCF10A cells. C. Coimmunoprecipitation of Rab11 with GCP6 in control and CRB3 knockdown MCF10A cells. D. Schematic diagram of CRB3 domains. E. Diagram truncations of CRB3-GFP fusion proteins with serial C-terminal deletions. F. Domain mapping of CRB3-GFP for Flag-Rab11a binding. Flag antibody co-IP of the full-length

CRB3-GFP and truncations of CRB3-GFP with Flag-Rab11a were cotransfected into HEK293 cells for 48 h. Immunoblot analysis was performed using GFP and Flag antibodies. G. Coimmunoprecipitation of Rab11 mutant variants with full-length CRB3-GFP. Flag antibody co-IP of the full-length CRB3-GFP with Flag-Rab11aWT, Flag-Rab11a[Q70L], Flag-Rab11a[S20V] and Flag-Rab11a[S25N] were cotransfected into HEK293 cells for 48 h. Immunoblot analysis was performed using GFP and Flag antibodies. Bars represent the means ± SD; Unpaired Student's *t* test, $^{*}P<0.05$.

Figure 7. CRB3 navigates GCP6/Rab11 trafficking vesicles to the basal body of the primary cilium.

A. Coimmunoprecipitation of exogenous CRB3 with Rab11, GCP6, GCP3 and CEP290 in MCF10A cells. B. Coimmunoprecipitation of Rab11 with exogenous CRB3, GCP6, GCP3 and CEP290 in MCF10A cells. C. Coimmunoprecipitation of Rab11 with GCP6 and CEP290 in control and CRB3 knockdown MCF10A cells. D. Representative immunofluorescent images of GCP6 and γ-tubulin colocalization of the basal body foci in MCF10A cells with CRB3 knockdown. γ-Tubulin (green), GCP6 (red), and DNA (blue). (Colocalization marked by arrows; scale bars, 25 μm) E. Representative immunofluorescent images of Rab11 and γ-tubulin colocalization of the basal body foci in MEF cells from *Crb3*$^{fl/fl}$ and *Crb3*$^{fl/fl}$;CAG-*Cre* mice. γ-tubulin (green), Rab11 (red), and DNA (blue). Foci marked by arrows; scale bars, 25 μm. Bars represent the means ± SD; Unpaired Student's *t* test, $^{***}P<0.001$.

Figure 8. Defects in CRB3 expression inhibit ciliary assembly in breast cancer tissues, and activate the Wnt signaling pathway in mammary cells and PyMT mouse model.

A. Representative immunofluorescent images of CRB3 in breast cancer tissues (n=50). CRB3 (green) and DNA (blue). (scale bars, 25 μm) B. Representative immunofluorescent images of the primary cilium in breast cancer tissues (n=50). Acetylated tubulin (red), γ-tubulin (green), and DNA (blue). (scale bars, 10 μm) C. Quantification of the proportion of cells with primary cilium formation in breast cancer tissues (n=50). D. Representative immunofluorescent images of CRB3 and

γ-tubulin colocalization in adjacent paracarcinoma tissues. CRB3 (green), γ-tubulin (red), and DNA (blue). (scale bars, 25 μm) E. Quantification of the proportion of MFC10A cells with SMO translocation after CRB3 knockdown (n=6). F. Real-time quantitative PCR showing the relative mRNA expression of GLI1 upon SAG treatment in CRB3-depleted MFC10A cells (n=6). G. Immunoblot analyses of the effect of CRB3 on the molecules of the Wnt signaling pathway in mammary cells. H. I. Immunohistochemical analyses of GLI1 and β-catenin in primary tumors from PyMT-WT and PyMT-cKO-*Crb3* mice at 9 weeks old, respectively. (scale bars, 25 μm) Bars represent the means ± SD; Unpaired Student's *t* test, ***$P<0.001$.

Figure 9. Schematic model of CRB3 regulating ciliary assembly.

Graphic summary of prominent phenotypes observed after CRB3 deletion. CRB3 is localized on apical epithelial surfaces and participates in tight junction formation to maintain contact inhibition and cell homeostasis in quiescence. Inside the cell, Rab11-positive endosomes mediate the intracellular trafficking of CRB3, and CRB3 navigates GCP6/Rab11 trafficking vesicles to CEP290. Then, GCP6 is involved in normal γTuRC assembly in ciliogenesis. In CRB3 deletion cells, the primary cilium does not assemble properly, and the Wnt signaling pathway is activated through β-catenin upregulation and nuclear localization, but the Hh signaling pathway fails to be activated. This cellular imbalance is disrupted, leading to tumorigenesis. γTuRC, γ-tubulin ring complex; TJ, tight junction; EE, early endosome; LE, late endosome.

Supplementary Figure 1. Generation of *Crb3* knockout mice by using the Cre-loxP system.

A. Schematic representation of the strategy for loxP sequence insertion and PCR genotyping. The loxP sites (gray arrow) flanked either side of exon 3 (orange) in the Crb3 gene. The primer pair F3&R5 and F4&R6 were used to distinguish the alleles. B. Southern blot analysis of a positive ES cell clone confirmed homologous recombination. The genomic DNA from ES cell clones and wild-type cells was digested with Avr □ or Sac □ and hybridized with a 5' external probe or 3' internal probe. C. PCR identification of mouse genotyping showing *Crb3*$^{wt/wt}$, *Crb3*$^{wt/fl}$ and *Crb3*$^{fl/fl}$ alleles. D. Crb3 expression was detected by immunoblotting in mammary gland tissues in *Crb3*$^{fl/fl}$ and *Crb3*$^{fl/fl}$;MMTV-*Cre* mice at 8 weeks old. E. Real-time quantitative PCR showing the relative mRNA expression of *Crb3* in mammary gland tissues (n=10). F. Crb3 expression was assessed by using immunohistochemistry in mammary gland tissues. The mammary epithelial cells were marked by areas of white dotted lines. (scale bars, 50 μm) Bars represent the means ± SD; Unpaired Student's *t* test, ***P<0.001.

Supplementary Figure 2. CRB3 knockdown promotes proliferation of mammary epithelial cells, and overexpression promotes apoptosis of breast cancer.

A. B. Immunoblot and real-time quantitative PCR analysis of CRB3 knockout efficiency in MCF10A cells. C. MCF10A proliferation assay for six successive days. D. Cell cycle showing the distribution of MCF10A cells. E. Quantification of the cell cycle distribution. F.G.H. Apoptosis of MCF7, T47D, and MDA-MB-453 breast cancer cells with CRB3 overexpression and quantification of apoptotic cells. Bars represent the means ± SD; Unpaired Student's *t* test, * P<0.05, **P<0.01.

Supplementary Figure 3. CRB3 alters primary cilium formation in MEFs from *Crb3*$^{fl/fl}$;CAG-*Cre* mice.

A. Representative immunofluorescent staining of primary cilium formation in MEFs from *Crb3*$^{fl/fl}$ and *Crb3*$^{fl/fl}$;CAG-*Cre* mice. Acetylated tubulin (red), γ-tubulin (green),

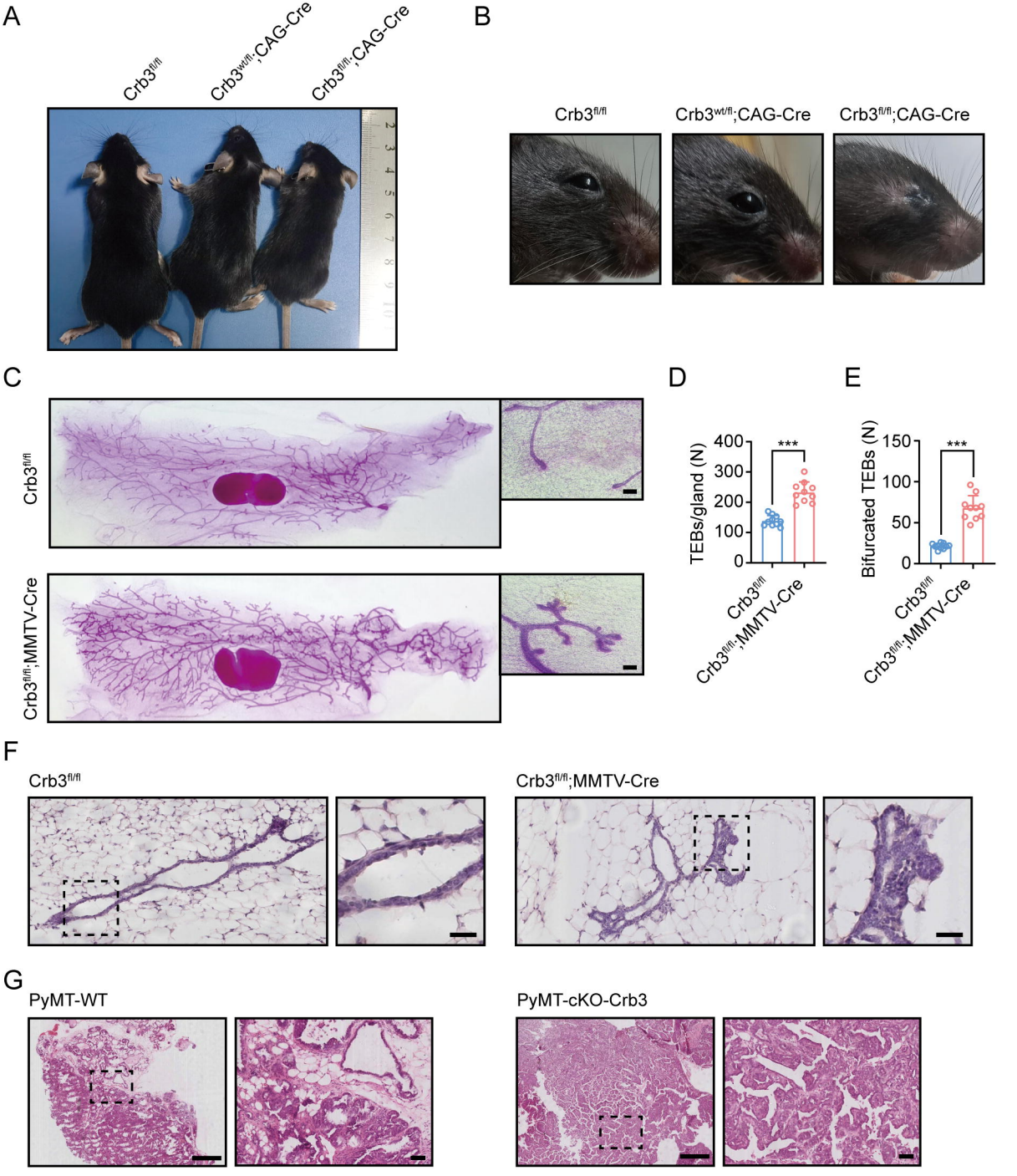
and DNA (blue). (scale bars, 10 μm) B. Quantification of the proportion of cells with primary cilium formation (n=10). Bars represent the means ± SD; Unpaired Student's *t* test, ***P<0.001.

Supplementary Figure 4. The effect of CRB3 on a series of ciliogenesis-related genes and Rab11 expression.

A. Quantification of the mRNA expression of genes encoding markers of centriole or primary cilium, BB components, IFT-A and IFT-B anterograde transport (normalized to GAPDH). B. Coomassie blue staining showing the gel electrophoresis of exogenous CRB3 immunoprecipitated precipitates. C. Immunoblot analysis of the effect of CRB3 on Rab11 in MCF10A cells. Bars represent the means ± SD; Unpaired Student's *t* test

Supplementary Figure 5. CRB3 knockdown disturbed the colocalization of GCP6 and Rab11, and Rab11 knockdown caused primary cilium defects.

A. Representative immunofluorescent staining of the colocalization between Rab11 and GCP6 in MCF10A cells. GCP6 (red), Rab11 (green), and DNA (blue). (scale bars, 20 μm) B. Fluorescence colocalization analysis in (A). C. Quantification of the proportion of cells with Rab11 and GCP6 colocalization. D. Representative images of immunofluorescent staining of primary cilium formation with Rab11 knockdown in MCF10A cells. Acetylated tubulin (red), γ-tubulin (green), and DNA (blue). (primary cilium marked by arrows; scale bars, 20 μm) E. Quantification of the proportion of cells with primary cilium formation. F. Immunoblot analysis of Rab11 knockout efficiency in MCF10A cells. Bars represent the means ± SD; Unpaired Student's *t* test, **P<0.01, ***P<0.001.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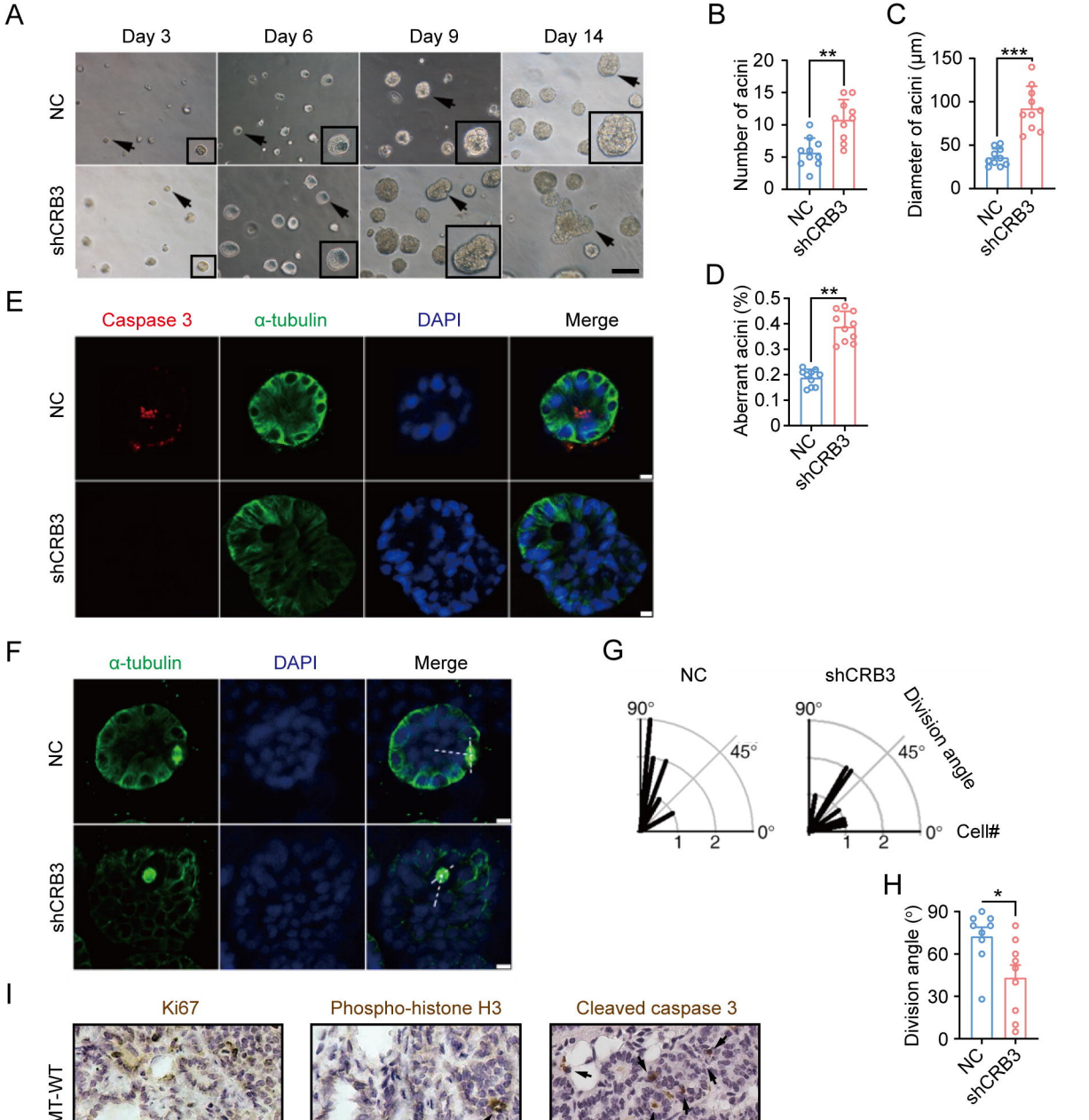
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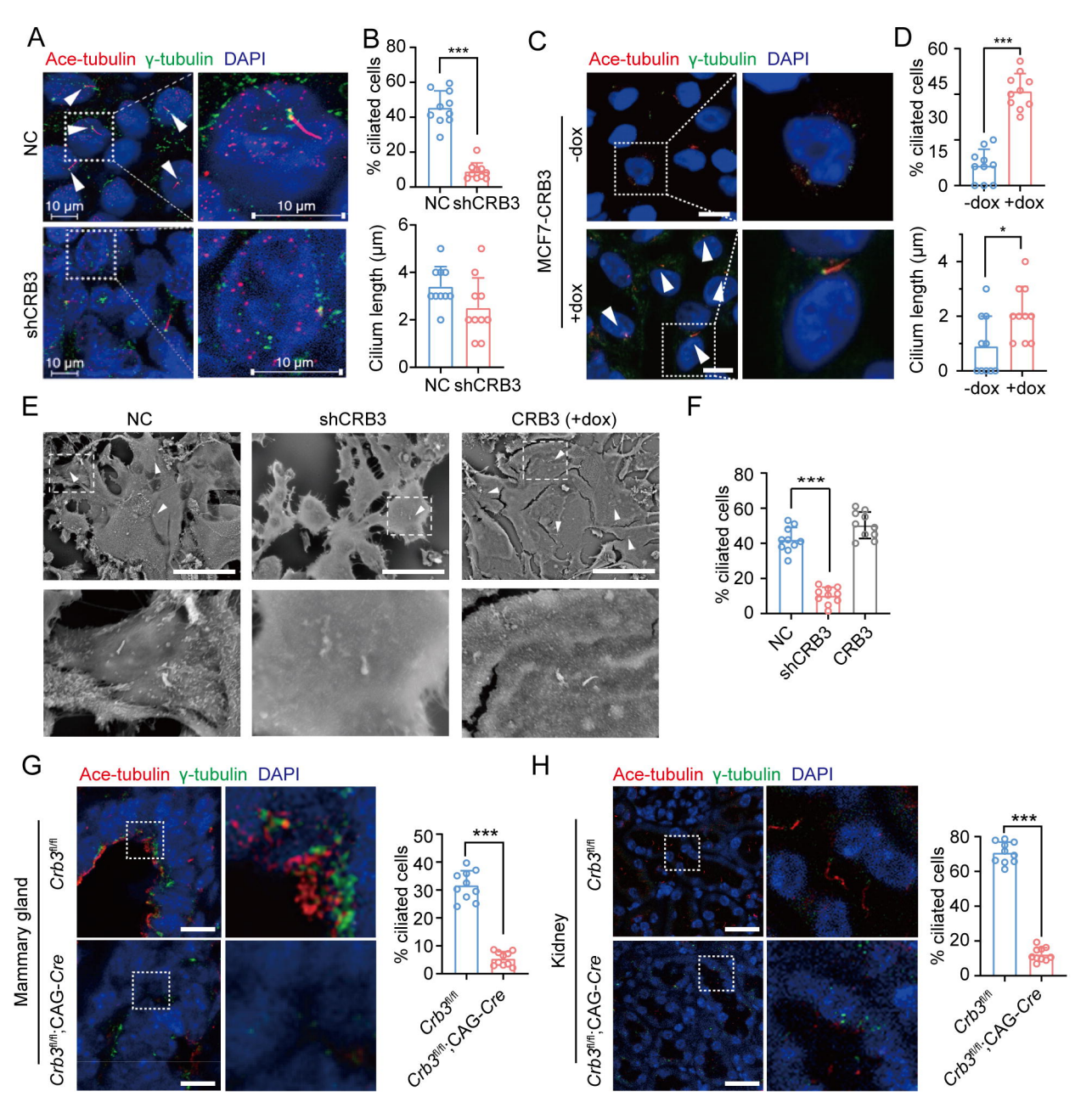
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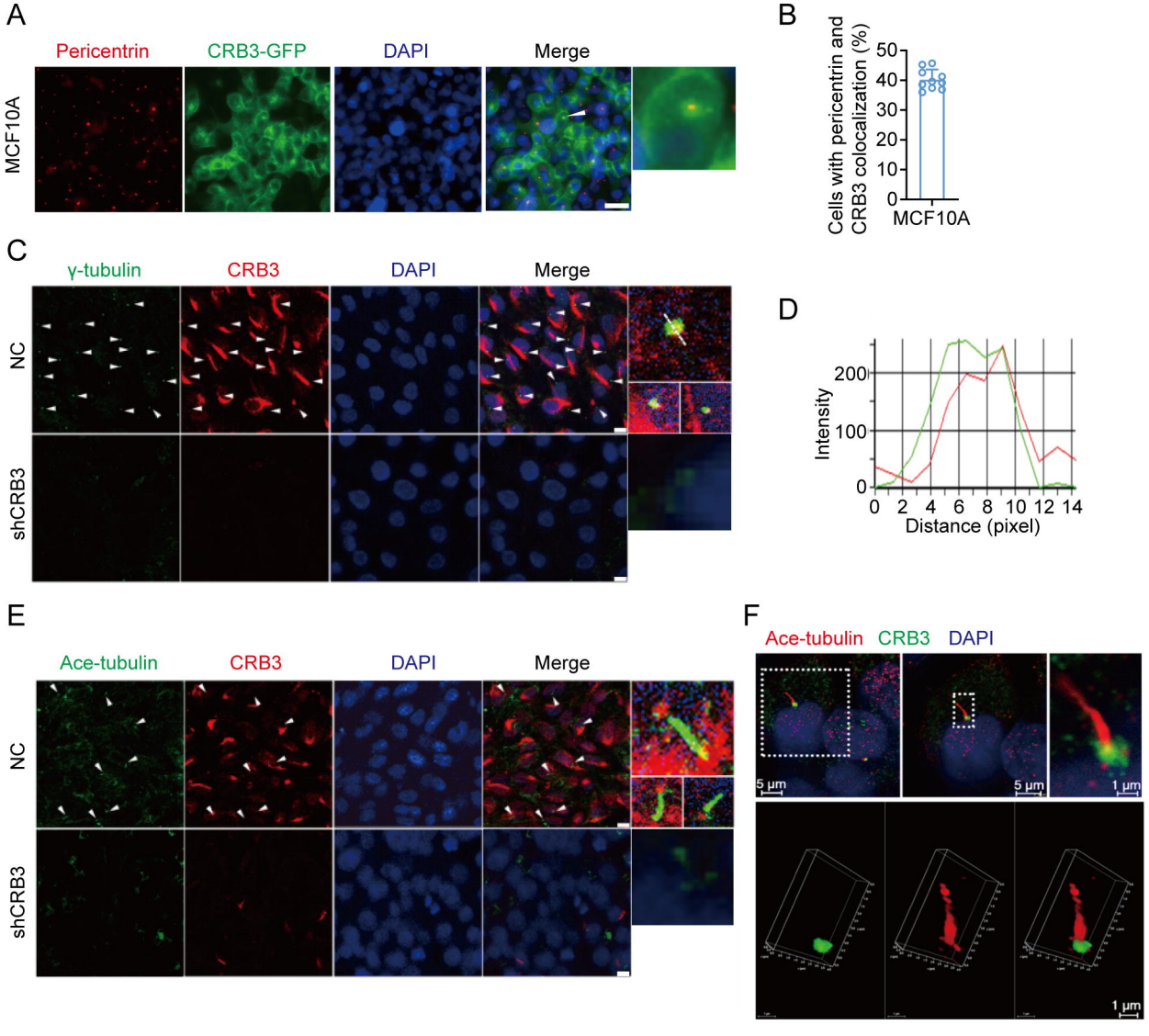
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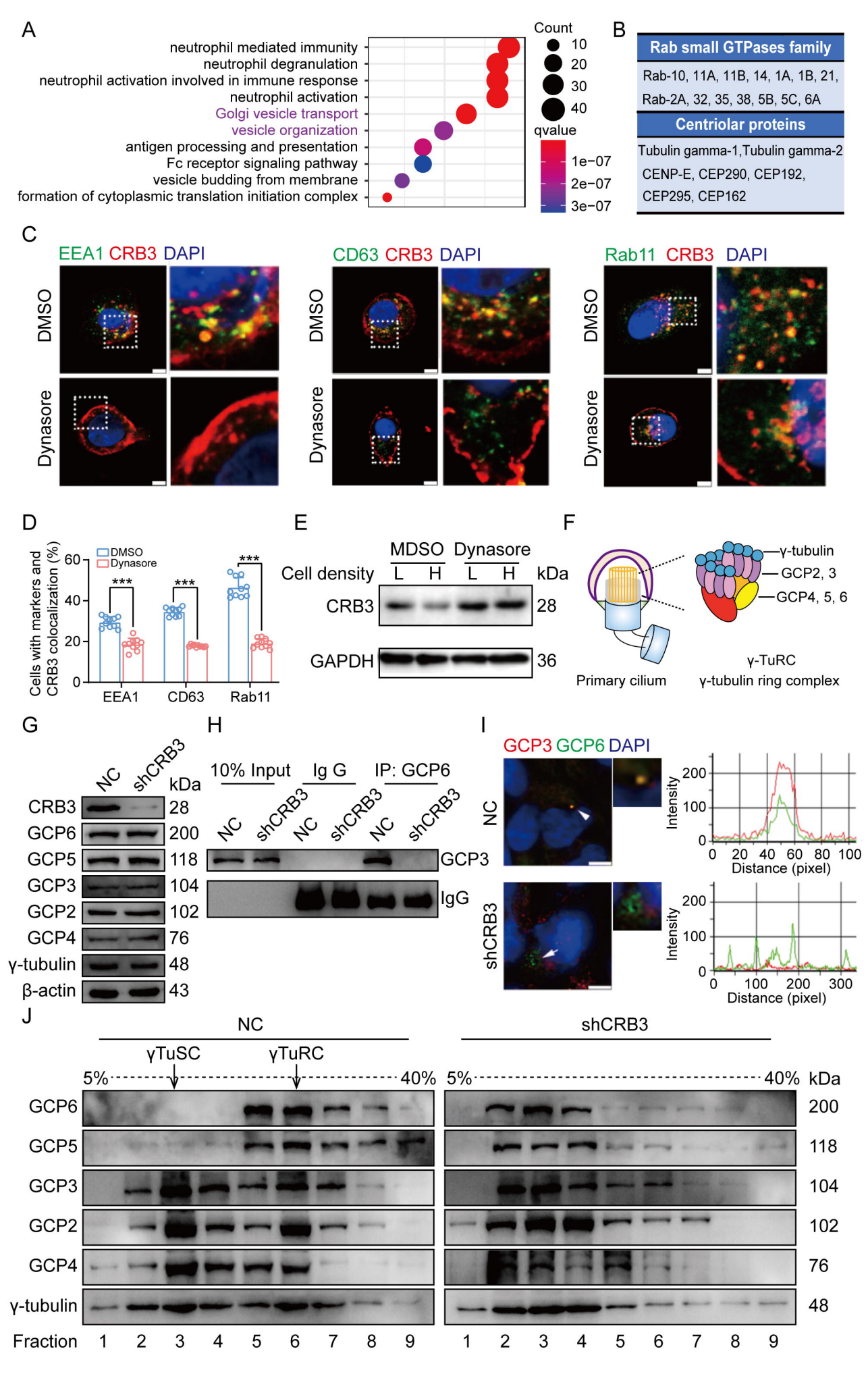
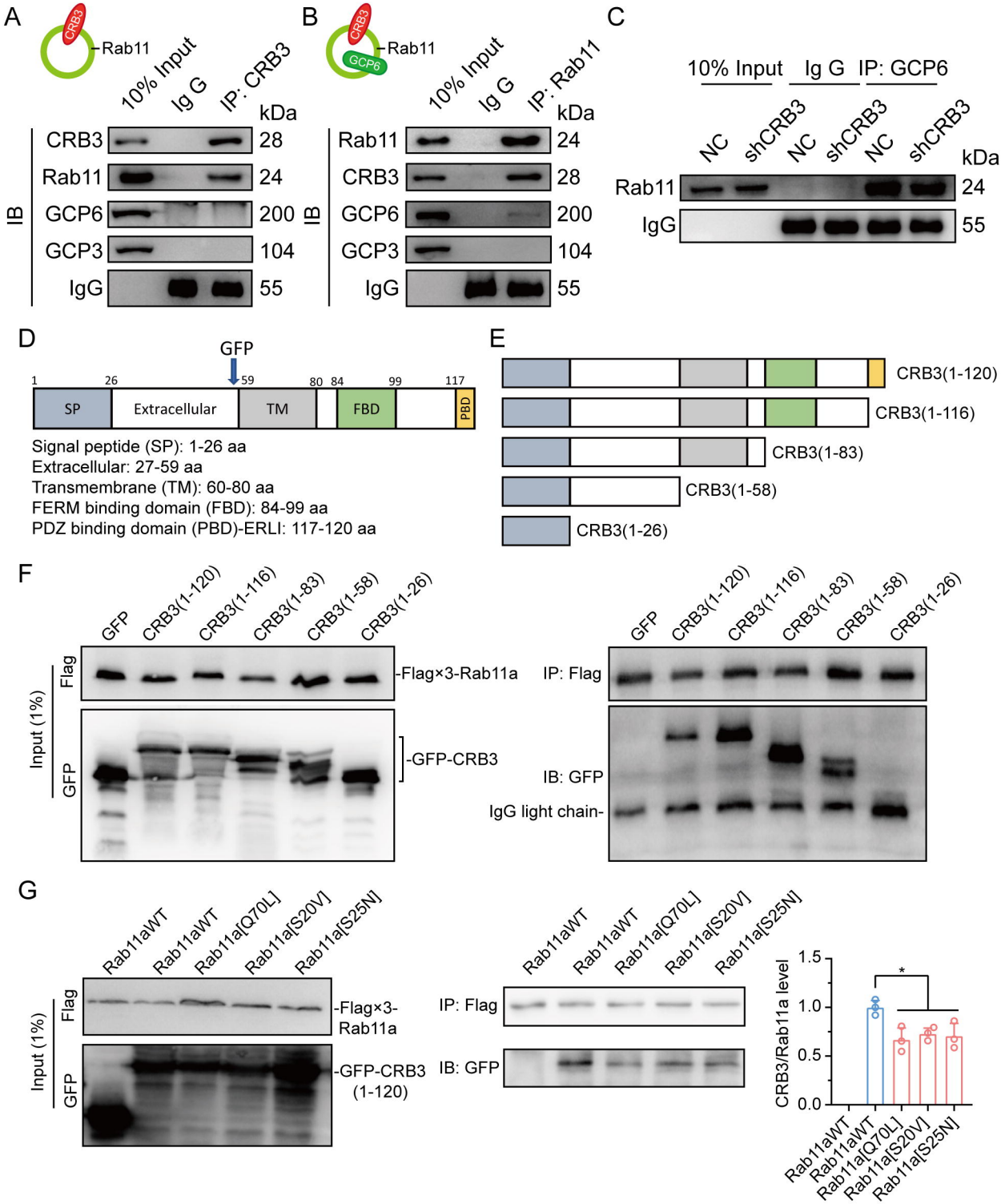
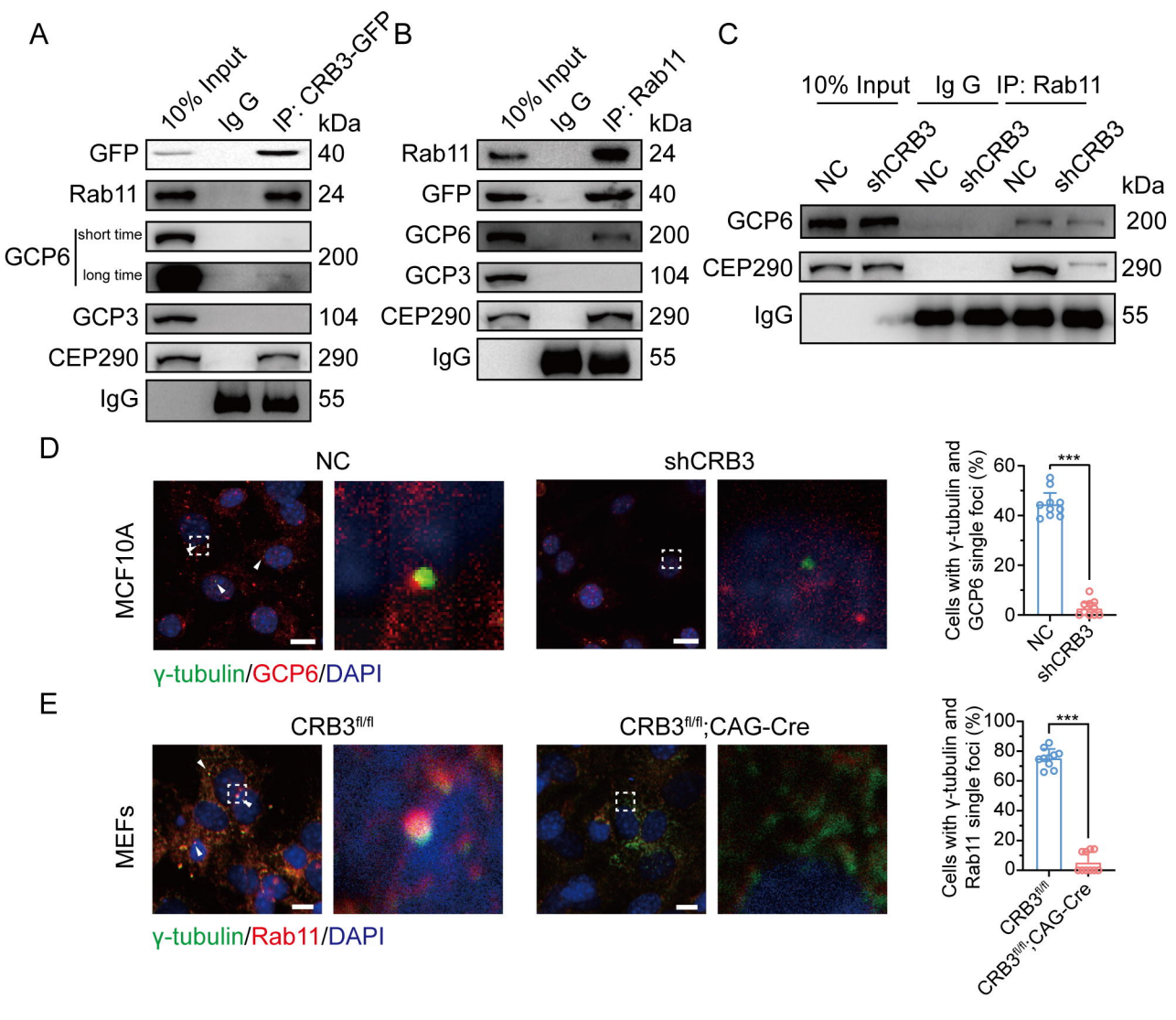
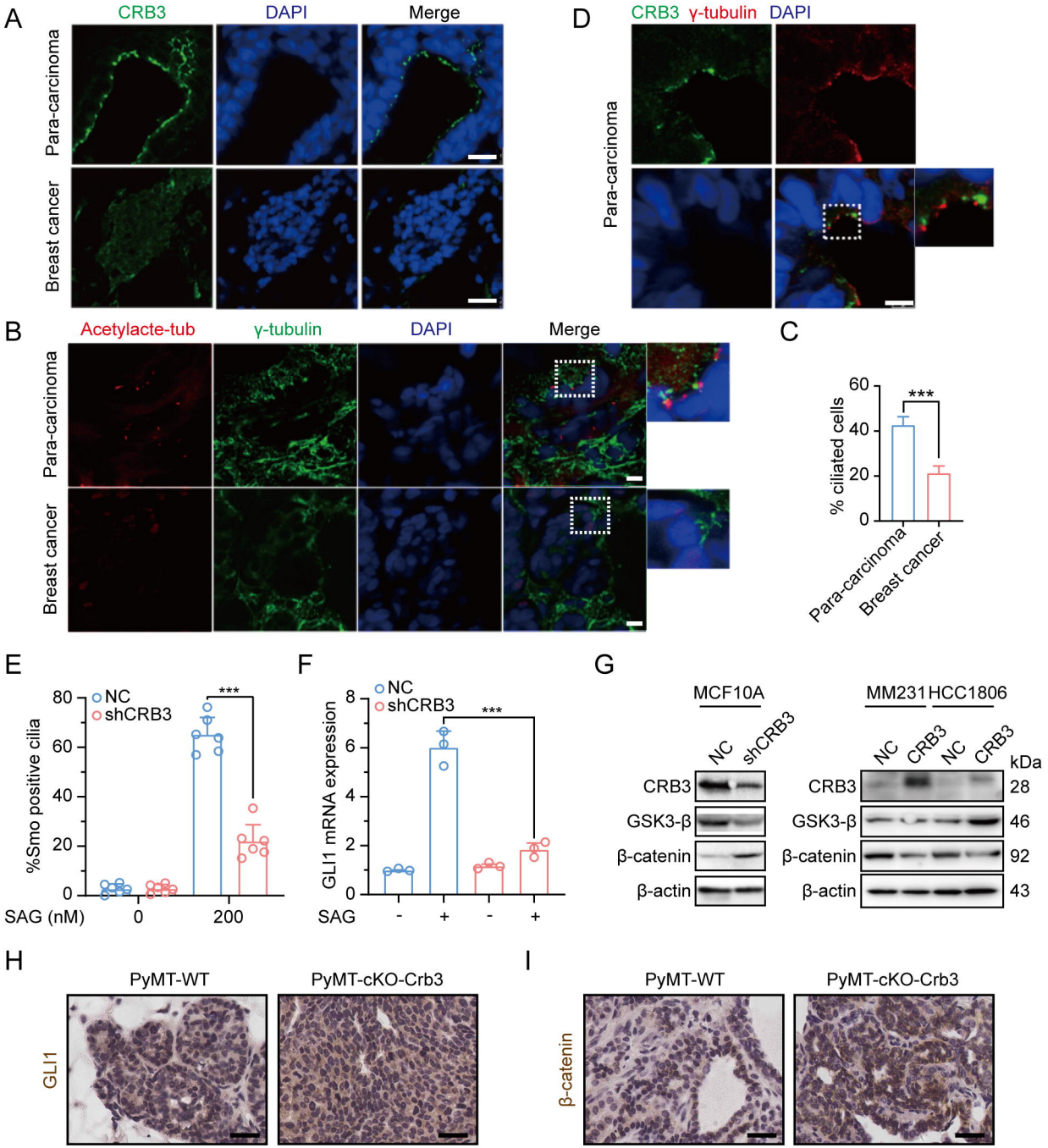
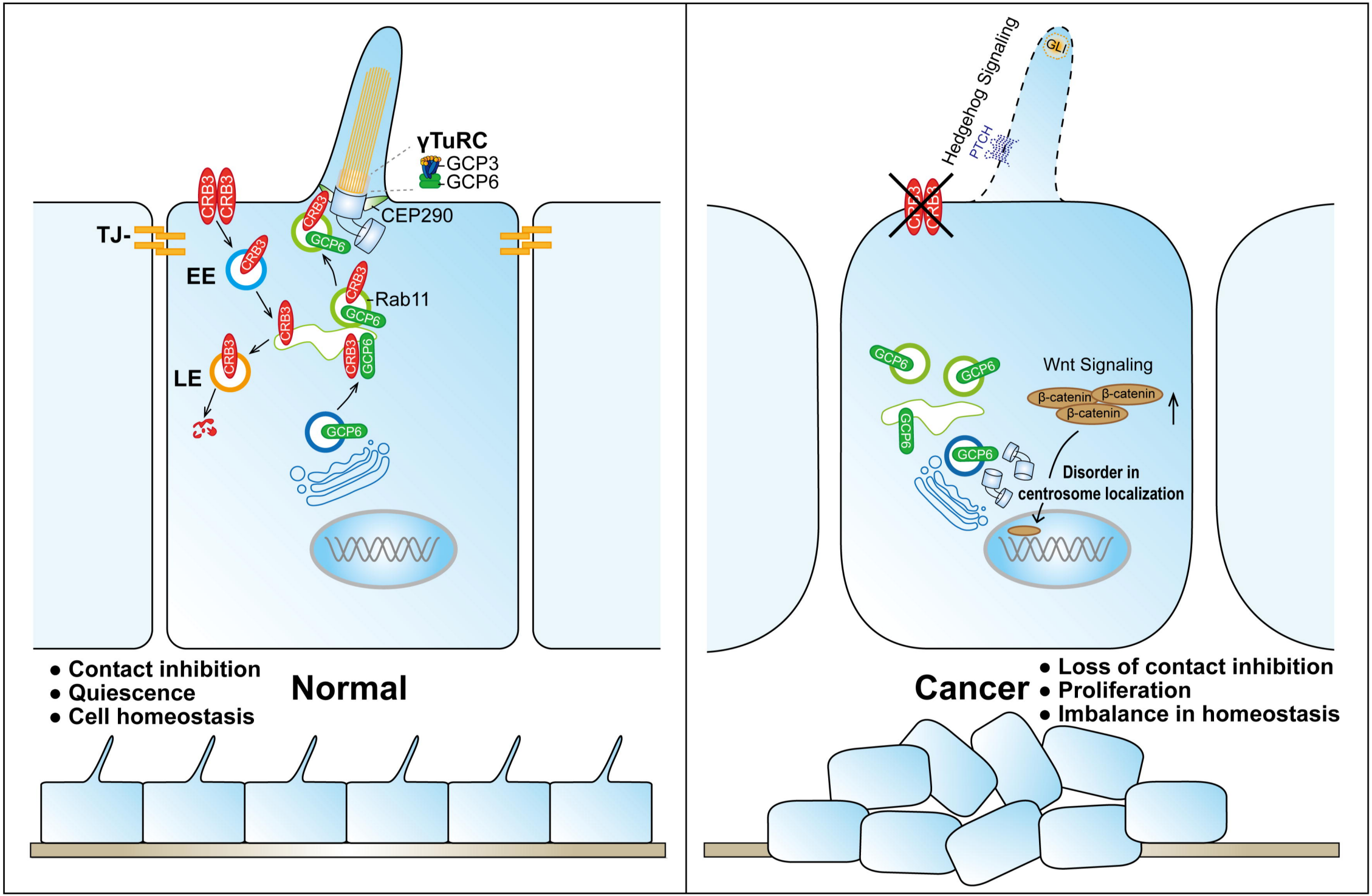
MCF10A
B
Cells with pericentrin and CRB3 colocalization (%)
50
40
30
20
10
0
MCF10A
C
γ-tubulin
CRB3
DAPI
Merge
NC
shCRB3
D
Intensity
200
100
0
0 2 4 6 8 10 12 14
Distance (pixel)
E
Ace-tubulin
CRB3
DAPI
Merge
NC
shCRB3
F
Ace-tubulin CRB3 DAPI
5 μm
5 μm
1 μm
1 μm


A
neutrophil mediated immunity
neutrophil degranulation
neutrophil activation involved in immune response
neutrophil activation
Golgi vesicle transport
vesicle organization
antigen processing and presentation
Fc receptor signaling pathway
vesicle budding from membrane
formation of cytoplasmic translation initiation complex
Count
10
20
30
40
qvalue
1e−07
2e−07
3e−07
B
Rab small GTPases family
Rab-10, 11A, 11B, 14, 1A, 1B, 21,
Rab-2A, 32, 35, 38, 5B, 5C, 6A
Centriolar proteins
Tubulin gamma-1,Tubulin gamma-2
CENP-E, CEP290, CEP192,
CEP295, CEP162
C
EEA1 CRB3 DAPI
CD63 CRB3 DAPI
Rab11 CRB3 DAPI
DMSO
Dynasore
D
DMSO
Dynasore
Cells with markers and CRB3 colocalization (%)
60
40
20
0
***
EEA1
CD63
Rab11
E
MDSO
Dynasore
Cell density
L
H
L
H
kDa
CRB3
28
GAPDH
36
F
γ-tubulin
GCP2, 3
GCP4, 5, 6
Primary cilium
γ-TuRC
γ-tubulin ring complex
G
NC
shCRB3
kDa
CRB3
28
GCP6
200
GCP5
118
GCP3
104
GCP2
102
GCP4
76
γ-tubulin
48
β-actin
43
H
10% Input
Ig G
IP: GCP6
NC
shCRB3
NC
shCRB3
NC
shCRB3
GCP3
IgG
I
GCP3 GCP6 DAPI
NC
shCRB3
Intensity
200
100
0
0
20
40
60
80
100
Distance (pixel)
Intensity
200
100
0
0
100
200
300
Distance (pixel)
J
NC
shCRB3
γTuSC
γTuRC
5%
40%
5%
40%
kDa
GCP6
200
GCP5
118
GCP3
104
GCP2
102
GCP4
76
γ-tubulin
48
Fraction
1
2
3
4
5
6
7
8
9
1
2
3
4
5
6
7
8
9


A
CRB3
Rab11
10% Input
Ig G
IP: CRB3
kDa
IB
CRB3 28
Rab11 24
GCP6 200
GCP3 104
IgG 55
B
CRB3
Rab11
GCP6
10% Input
Ig G
IP: Rab11
kDa
IB
Rab11 24
CRB3 28
GCP6 200
GCP3 104
IgG 55
C
10% Input
Ig G
IP: GCP6
NC
shCRB3
NC
shCRB3
NC
shCRB3
kDa
Rab11 24
IgG 55
D
GFP
1
26
59
80
84
99
117
SP
Extracellular
TM
FBD
PBD
Signal peptide (SP): 1-26 aa
Extracellular: 27-59 aa
Transmembrane (TM): 60-80 aa
FERM binding domain (FBD): 84-99 aa
PDZ binding domain (PBD)-ERLI: 117-120 aa
E
CRB3(1-120)
CRB3(1-116)
CRB3(1-83)
CRB3(1-58)
CRB3(1-26)
F
GFP
CRB3(1-120)
CRB3(1-116)
CRB3(1-83)
CRB3(1-58)
CRB3(1-26)
Input (1%)
Flag
GFP
-Flag×3-Rab11a
-GFP-CRB3
IP: Flag
IB: GFP
IgG light chain-
G
Rab11aWT
Rab11aWT
Rab11a[Q70L]
Rab11a[S20V]
Rab11a[S25N]
Input (1%)
Flag
GFP
-Flag×3-Rab11a
-GFP-CRB3 (1-120)
IP: Flag
IB: GFP
CRB3/Rab11a level
1.5
1.0
0.5
0.0
*


A
10% Input
Ig G
IP: CRB3-GFP
kDa
GFP
40
Rab11
24
GCP6
short time
long time
200
GCP3
104
CEP290
290
IgG
55
B
10% Input
Ig G
IP: Rab11
kDa
Rab11
24
GFP
40
GCP6
200
GCP3
104
CEP290
290
IgG
55
C
10% Input
Ig G
IP: Rab11
NC
shCRB3
NC
shCRB3
NC
shCRB3
kDa
GCP6
200
CEP290
290
IgG
55
D
NC
shCRB3
MCF10A
γ-tubulin/GCP6/DAPI
Cells with γ-tubulin and GCP6 single foci (%)
60
40
20
0
***
NC
shCRB3
E
CRB3fl/fl
CRB3fl/fl;CAG-Cre
MEFs
γ-tubulin/Rab11/DAPI
Cells with γ-tubulin and Rab11 single foci (%)
100
80
60
40
20
0
***
CRB3fl/fl
CRB3fl/fl;CAG-Cre


A
CRB3
DAPI
Merge
Para-carcinoma
Breast cancer
D
CRB3
γ-tubulin
DAPI
Para-carcinoma
B
Acetylacte-tub
γ-tubulin
DAPI
Merge
Para-carcinoma
Breast cancer
C
% ciliated cells
60
40
20
0
***
Para-carcinoma
Breast cancer
E
NC
shCRB3
%Smo positive cilia
80
60
40
20
0
***
SAG (nM)
0
200
F
NC
shCRB3
GLI1 mRNA expression
8
6
4
2
0
***
SAG
-
+
-
+
G
MCF10A
NC
shCRB3
MM231
HCC1806
NC
CRB3
NC
CRB3
kDa
CRB3
GSK3-β
β-catenin
β-actin
28
46
92
43
H
PyMT-WT
PyMT-cKO-Crb3
GLI1
I
PyMT-WT
PyMT-cKO-Crb3
β-catenin


γTuRC
GCP3
GCP6
CEP290
TJ-
CRB3
GCP6
EE
Rab11
LE
Normal
• Contact inhibition
• Quiescence
• Cell homeostasis
Hedgehog Signaling
GLI
PTCH
Wnt Signaling
β-catenin
Disorder in
centrosome localization
Cancer
• Loss of contact inhibition
• Proliferation
• Imbalance in homeostasis