

CRB3 navigates Rab11 trafficking vesicles to promote γ TuRC assembly during ciliogenesis

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Running Title: CRB3 regulates ciliogenesis

Keywords: Primary cilium, Polarity protein, Breast cancer, CRB3, γ -TuRC assembly, Rab11-positive endosome

31 Abstract

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

50

51 Introduction

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

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

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

72 The primary cilium, an antenna-like microtubule-based organelle in most types of
73 mammalian cells, is a sensorial antenna that regulates cell differentiation, proliferation,
74 polarity, and tissue organization[9]. Dysfunction of the primary cilium can lead to
75 developmental and degenerative disorders called ciliopathies, such as polycystic
76 kidney disease, nephronophthisis, retinitis pigmentosa, and Meckel syndrome[10].

77 Remarkably, it has been reported that many cancers, including melanoma and breast,
78 renal, pancreatic, and prostate cancer, exhibit loss of the primary cilium, most likely
79 during the early stages of tumorigenesis[11-15]. The primary cilium is a significant
80 extension on the apical surfaces of epithelial cells and is maintained by polarized
81 vesicular traffic[16]. Some studies have shown that CRB3 localizes in the primary
82 cilium and that depletion of CRB3 leads to primary cilium loss in Madin-Darby
83 canine kidney (MDCK) cells[17, 18], indicating that CRB3 is necessary for the
84 initiation of ciliogenesis. However, how CRB3 promotes ciliogenesis on the apical
85 surfaces of epithelial cells remains unknown.

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

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

97 **Results**

98 ***Crb3* deletion mice exhibit smaller and anophthalmia.**

99 Since *Crb3* knockout mice die shortly after birth[19], we generated a novel transgenic
100 mouse model with conditional deletion of *Crb3* using the Cre-loxP system. The loxP
101 sites flanked either side of exon 3 in the *Crb3* gene (Supplementary Fig. 1A). The
102 positive embryonic stem (ES) cell clone was confirmed for the wild-type gene
103 (*Crb3*^{wt/wt}) and recombinant allele (*Crb3*^{wt/fl}) by Southern blotting (Supplementary Fig.
104 1B). The genotypes of the wild type, heterozygotes and homozygotes (*Crb3*^{fl/fl}) were
105 detected with specific primers (Supplementary Fig. 1C). We first intercrossed *Crb3*^{fl/fl}
106 with CMV enhancer/chicken β -actin promoter (CAG)-Cre mice and found that
107 *Crb3*^{fl/fl}, *Crb3*^{wt/fl}; CAG-Cre and *Crb3*^{fl/fl}; CAG-Cre pups were born normally and
108 looked well developed in size. However, most *Crb3*^{fl/fl}; CAG-Cre pups died within a
109 few days after birth. Few surviving *Crb3*^{fl/fl}; CAG-Cre mice were smaller and showed
110 anophthalmia than littermate *Crb3*^{fl/fl} mice at 4 weeks old (Fig. 1A-B). The eye is an
111 organ with perfect apical-basal cell polarity. However, *Crb3* knockout mice showed
112 ocular abnormalities. Together, these results indicated that *Crb3* is necessary for eye
113 development.

114 **Mammary epithelial-specific *Crb3* deletion causes ductal epithelial hyperplasia** 115 **and tumorigenesis.**

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

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

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

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

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

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

151 after 6 days (D6) and finally formed polarized acini with a hollow lumen at D14 (Fig.
152 2A). However, MCF10A cells, after CRB3 knockdown, formed more number, larger
153 diameter and aberrant acini after D6, and these acini had no lumen (Fig. 2A-D). Cell
154 proliferation assays of MCF10A cells showed that CRB3 knockdown increased the
155 proliferation rate and accelerated G1 to S phase progression (Supplementary Fig.
156 2C-E).

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

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

172 **CRB3 regulates primary cilium formation.**

173 The primary cilium, one of the apical antenna-like extensions in epithelial cells, has
174 been reported to play an important role in controlling lumen formation[23]. CRB3
175 knockdown leads to primary cilium loss in MDCK cells[17, 18], but the molecular
176 mechanism regulating ciliogenesis on apical surfaces is still unclear. Investigating the
177 altered effect of CRB3 on ciliogenesis *in vivo* and *in vitro*, we found that control
178 MCF10A cells formed primary cilia after becoming confluent, while CRB3
179 knockdown led to significant ciliogenesis defects (Fig. 3A). Compared with the
180 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.

200 **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 focus 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 focus 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

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

254 **CRB3 knockdown destabilizes γ TuRC assembly during ciliogenesis.**

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

whether CRB3 facilitate γ TuRC assembly, we fractionated MCF10A cytoplasmic proteins on sucrose gradients according to the method published[29]. We noticed that γ TuSC were regimented mainly in fractions 3 and γ TuRC sediment in fractions 6 (Fig. 5J). However, CRB3 downregulation caused most of GCP6 and GCP5 to be precipitated independently of γ TuRC in the low-density fractions (Fig. 5J). Together, these results have established that the depletion of CRB3 interferes with the molecular interactions between the γ TuRC proteins, failing microtubule formation in the primary cilium. However, it may not affect the expression of these molecules during γ TuRC assembly. According to these results, we hypothesized that CRB3 could mediate the polarized vesicle trafficking of some γ TuRC-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 γ TuRC-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 γ TuRC-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 γ TuRC-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 γ TuRC enrichment[29, 33]. Although CRB3 knockdown had little effect on the formation of GCP6/Rab11 trafficking vesicles, it could significantly inhibit the γ TuRC 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 Smoothed

(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

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

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

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

451 microtubule nucleation through longitudinal contacts with α -tubulin and β -tubulin[27].

452 The γ TuRC consists of multiple copies of γ TuSC, which is composed of two copies of

453 γ -tubulin and one each of GCP2 and GCP3. Then, GCP2 and GCP3 interact with

454 GCP4, GCP5 and GCP6 to form γ TuRC[46]. Our experiments showed that CRB3

455 knockdown does not affect the expression of the γ TuRC-specific proteins GCP3,

456 GCP6 and γ -tubulin but inhibits the interaction between GCP6 and GCP3, resulting in

457 destabilization of γ TuRC. Further analysis revealed significant colocalization between

458 GCP6- and Rab11-positive vesicles at low cell density, while at high cell density,

459 GCP6 accumulated at the centrosome together with GCP3. CRB3 knockdown results

460 in the disappearance of this accumulated GCP6. These results indicated that

461 GCP6/Rab11 trafficking vesicles must be transported to the basal body for

462 ciliogenesis. While it is still unclear how CRB3 interferes with γ TuRC assembly

463 during primary cilium formation, we hypothesize that Rab11-positive endosomes

464 mediate the intracellular trafficking of CRB3 and that CRB3, as a navigator

465 interacting with CEP290, can navigate GCP6/Rab11 trafficking vesicles to the

466 transition zone for γ TuRC assembly in primary cilium formation.

467 CEP290 is considered a centriolar or ciliary protein localized to the centrosomes in

468 dividing cells, the distal mother centriole in quiescent cells, and the transition zone in

469 the primary cilium[47]. *CEP290* mutations are associated with various diseases, such

470 as the devastating blinding disease Leber's Congenital Amaurosis (LCA),

471 nephronophthisis, Senior Løken syndrome (SLS), Joubert syndrome (JS),

472 Bardet-Biedl syndrome (BBS), and lethal Meckel-Gruber syndrome (MGS)[48]. In

473 particular, the phenotypes of CEP290-associated ciliopathies are very similar to those

474 in our *Crb3* knockout mouse model, with lethality and ocular and renal abnormalities.

475 CEP290 knockdown significantly decreased the number of cells with primary cilia,

476 and CEP290 knockout mice showed mislocalization of ciliary proteins in the

477 retinas[49, 50]. Additionally, the depletion of CEP290 disrupts protein trafficking to

478 centrosomes and affects the recruitment of the BBSome and vesicular trafficking in

479 ciliary assembly[51, 52]. Although CEP290 serves as a hub to recruit many ciliary

480 proteins, it has not been reported to be involved in polarized vesicle trafficking or

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

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

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

γ TuRC 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'-GTGGATTCCGACCAGTCTGA-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,

541 5'-TTACCACTCCCAGCAAGACAC-3', and amplification with one 247 bp band.
 542 The reaction conditions of *Crb3* and *Cre* PCR were as follows: 94°C for 5 min, 94°C
 543 for 20 s, 60°C for 30 s, and 72°C for 20 s for 35 cycles.

544 **Mouse mammary gland whole mount analysis.** After mice were euthanized, the
 545 inguinal mammary gland tissues were removed intact and fully spread onto slides.
 546 Tissues were rapidly fixed in Carnoy's fixative for 2 h at room temperature. Then, the
 547 tissues were washed in 70% ethanol solution for 15 min at room temperature and in
 548 50 and 30% ethanol and distilled water for 5 min each. Staining was carried out in
 549 carmine alum solution at 4°C overnight. After washing with 70% ethanol solution,
 550 tissues were washed again in 70, 95 and 100% ethanol for 15 min each. Tissues were
 551 cleared in xylene overnight at room temperature and then mounted with neutral
 552 balsam. Images were obtained by using a microscope (Leica DMI8, Wetzlar,
 553 Germany).

554 **Cell culture and transfection.** MCF10A, MCF7, T47D, MDA-MB-231, HCC1806,
 555 T47D, MDA-MB-453 cell lines were purchased from Shanghai Institute of
 556 Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China).
 557 MCF10A cells were routinely grown in DMEM/F12 (1:1) media (HyClone, UT, USA)
 558 supplemented with 5% horse serum, 20 ng/ml human EGF, 10 µg/ml insulin, and 0.5
 559 µg/ml hydrocortisone. MCF7, MDA-MB-231 and MDA-MB-453 cells were cultured
 560 in high glucose DMEM media (HyClone, UT, USA) supplemented with 10% FBS
 561 (HyClone, UT, USA, #SH30084.03). T47D and HCC1806 cells were grown in
 562 RPMI-1640 media (HyClone, UT, USA) supplemented with 10% FBS. All cell lines
 563 were incubated in 5% CO₂ at 37°C.

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

569 **DNA constructs and stable cell lines.** CRB3 was PCR amplified from RNA
 570 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 I and Xho I 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). DAPI (5 μ g/ml) was used for DNA staining, and images were taken by using a confocal microscope (Leica SP5 II, 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 μ g 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

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

636 **Immunoprecipitation and immunoblotting.** Cultured cells were lysed in RIPA
637 buffer supplemented with protease inhibitors (Roche, NJ, USA). Then, the cell lysates
638 were subjected to SDS–PAGE separation and transferred to PVDF membranes. The
639 membranes were subjected to immunoblot assays using antibodies against CRB3
640 (Santa Cruz, USA, sc-292449), GCP2 (Santa Cruz, USA, sc-377117), GCP3 (Santa
641 Cruz, USA, sc-373758), GCP4 (Santa Cruz, USA, sc-271876), GCP5 (Santa Cruz,
642 USA, sc-365837), GCP6 (Abcam, UK, ab95172), γ -tubulin (Proteintech, USA,
643 #66320-1-Ig), Rab11 (Abcam, UK, ab128913), GFP (Roche, USA, #11814460001),
644 Flag (Sigma, USA, F7425), CEP290 (Santa Cruz, USA, sc-390637), GSK3- β (CST,
645 USA, #12456), β -catenin (CST, USA, #8480), GAPDH (Proteintech, USA,
646 #HRP-6004), and β -actin (Proteintech, USA, #HRP-60008). HRP-conjugated
647 secondary antibodies (CST, USA, #7074, #7076) were incubated at room temperature
648 for 1 h in the dark. Final detection was detected by ECL Plus (Millipore, Germany,
649 WBULS0500). Proteins for the immunoprecipitation assay were lysed with NP40
650 buffer. The cell lysates were detected by a Dynabeads protein G immunoprecipitation
651 kit (Invitrogen, USA, #10007D) according to the manufacturer’s instructions. Elution
652 protein complexes were subjected to immunoblot assay.

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

659 **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 \pm 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.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 81872272 and 82173023), Innovation Capability Support Program of Shaanxi (No. 2020TD-046) and Clinical Research Award of the First Affiliated Hospital of Xi'an Jiaotong University (No. XJTU1AF-CRF-2017-007). We are grateful to Prof. Ceshi Chen (Kunming Institute of Zoology, China) and Prof. Yongping Shao (Xi'an Jiaotong University, China) for help in guiding the research. We thank Qi Tian, Lizhe Zhu and Yan Zhou for help with the experiments.

Conflict of interest

690 The authors declare no conflicts of interest.

691

692 Author contributions

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

697

698 Data availability

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

701

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915

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 μ m) 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 μ m) 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 μ m, right 50 μ m) Magnified areas of boxed sections are shown in the right panels. Bars represent the means \pm 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 μ m, bars represent means \pm 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

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

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

961

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

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

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

977

978 **Figure 5.** CRB3 trafficking is mediated by Rab11-positive endosomes, and CRB3
979 knockdown destabilizes γ TuRC assembly during ciliogenesis.

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

998

999 **Figure 6.** CRB3 directly interacts with Rab11.

1000 A. Coimmunoprecipitation of CRB3 with Rab11, GCP6 and GCP3 in MCF10A cells.
1001 B. Coimmunoprecipitation of Rab11 with CRB3, GCP6 and GCP3 in MCF10A cells.
1002 C. Coimmunoprecipitation of Rab11 with GCP6 in control and CRB3 knockdown
1003 MCF10A cells. D. Schematic diagram of CRB3 domains. E. Diagram truncations of
1004 CRB3-GFP fusion proteins with serial C-terminal deletions. F. Domain mapping of
1005 CRB3-GFP for Flag-Rab11a binding. Flag antibody co-IP of the full-length

1006 CRB3-GFP and truncations of CRB3-GFP with Flag-Rab11a were cotransfected into
1007 HEK293 cells for 48 h. Immunoblot analysis was performed using GFP and Flag
1008 antibodies. G. Coimmunoprecipitation of Rab11 mutant variants with full-length
1009 CRB3-GFP. Flag antibody co-IP of the full-length CRB3-GFP with Flag-Rab11aWT,
1010 Flag-Rab11a[Q70L], Flag-Rab11a[S20V] and Flag-Rab11a[S25N] were cotransfected
1011 into HEK293 cells for 48 h. Immunoblot analysis was performed using GFP and Flag
1012 antibodies. Bars represent the means \pm SD; Unpaired Student's *t* test, **P*<0.05.

1013

1014 **Figure 7.** CRB3 navigates GCP6/Rab11 trafficking vesicles to the basal body of the
1015 primary cilium.

1016 A. Coimmunoprecipitation of exogenous CRB3 with Rab11, GCP6, GCP3 and
1017 CEP290 in MCF10A cells. B. Coimmunoprecipitation of Rab11 with exogenous
1018 CRB3, GCP6, GCP3 and CEP290 in MCF10A cells. C. Coimmunoprecipitation of
1019 Rab11 with GCP6 and CEP290 in control and CRB3 knockdown MCF10A cells. D.
1020 Representative immunofluorescent images of GCP6 and γ -tubulin colocalization of
1021 the basal body foci in MCF10A cells with CRB3 knockdown. γ -Tubulin (green),
1022 GCP6 (red), and DNA (blue). (Colocalization marked by arrows; scale bars, 25 μ m) E.
1023 Representative immunofluorescent images of Rab11 and γ -tubulin colocalization of
1024 the basal body foci in MEF cells from *Crb3*^{fl/fl} and *Crb3*^{fl/fl};CAG-*Cre* mice. γ -tubulin
1025 (green), Rab11 (red), and DNA (blue). Foci marked by arrows; scale bars, 25 μ m.
1026 Bars represent the means \pm SD; Unpaired Student's *t* test, ****P*<0.001.

1027

1028 **Figure 8.** Defects in CRB3 expression inhibit ciliary assembly in breast cancer tissues,
1029 and activate the Wnt signaling pathway in mammary cells and PyMT mouse model.

1030 A. Representative immunofluorescent images of CRB3 in breast cancer tissues (n=50).
1031 CRB3 (green) and DNA (blue). (scale bars, 25 μ m) B. Representative
1032 immunofluorescent images of the primary cilium in breast cancer tissues (n=50).
1033 Acetylated tubulin (red), γ -tubulin (green), and DNA (blue). (scale bars, 10 μ m) C.
1034 Quantification of the proportion of cells with primary cilium formation in breast
1035 cancer tissues (n=50). D. Representative immunofluorescent images of CRB3 and

1036 γ -tubulin colocalization in adjacent paracarcinoma tissues. CRB3 (green), γ -tubulin
1037 (red), and DNA (blue). (scale bars, 25 μ m) E. Quantification of the proportion of
1038 MFC10A cells with SMO translocation after CRB3 knockdown (n=6). F. Real-time
1039 quantitative PCR showing the relative mRNA expression of GLI1 upon SAG
1040 treatment in CRB3-depleted MFC10A cells (n=6). G. Immunoblot analyses of the
1041 effect of CRB3 on the molecules of the Wnt signaling pathway in mammary cells. H.
1042 I. Immunohistochemical analyses of GLI1 and β -catenin in primary tumors from
1043 PyMT-WT and PyMT-cKO-*Crb3* mice at 9 weeks old, respectively. (scale bars, 25
1044 μ m) Bars represent the means \pm SD; Unpaired Student's *t* test, ****P*<0.001.

1045

1046 **Figure 9.** Schematic model of CRB3 regulating ciliary assembly.

1047 Graphic summary of prominent phenotypes observed after CRB3 deletion. CRB3 is
1048 localized on apical epithelial surfaces and participates in tight junction formation to
1049 maintain contact inhibition and cell homeostasis in quiescence. Inside the cell,
1050 Rab11-positive endosomes mediate the intracellular trafficking of CRB3, and CRB3
1051 navigates GCP6/Rab11 trafficking vesicles to CEP290. Then, GCP6 is involved in
1052 normal γ TuRC assembly in ciliogenesis. In CRB3 deletion cells, the primary cilium
1053 does not assemble properly, and the Wnt signaling pathway is activated through
1054 β -catenin upregulation and nuclear localization, but the Hh signaling pathway fails to
1055 be activated. This cellular imbalance is disrupted, leading to tumorigenesis. γ TuRC,
1056 γ -tubulin ring complex; TJ, tight junction; EE, early endosome; LE, late endosome.

1057

1058 **Supplementary Figure 1.** Generation of *Crb3* knockout mice by using the Cre-loxP
1059 system.

1060 A. Schematic representation of the strategy for loxP sequence insertion and PCR
1061 genotyping. The loxP sites (gray arrow) flanked either side of exon 3 (orange) in the
1062 *Crb3* gene. The primer pair F3&R5 and F4&R6 were used to distinguish the alleles. B.
1063 Southern blot analysis of a positive ES cell clone confirmed homologous
1064 recombination. The genomic DNA from ES cell clones and wild-type cells was
1065 digested with Avr II or Sac I and hybridized with a 5' external probe or 3' internal
1066 probe. C. PCR identification of mouse genotyping showing *Crb3*^{wt/wt}, *Crb3*^{wt/fl} and
1067 *Crb3*^{fl/fl} alleles. D. *Crb3* expression was detected by immunoblotting in mammary
1068 gland tissues in *Crb3*^{fl/fl} and *Crb3*^{fl/fl};MMTV-*Cre* mice at 8 weeks old. E. Real-time
1069 quantitative PCR showing the relative mRNA expression of *Crb3* in mammary gland
1070 tissues (n=10). F. *Crb3* expression was assessed by using immunohistochemistry in
1071 mammary gland tissues. The mammary epithelial cells were marked by areas of white
1072 dotted lines. (scale bars, 50 μ m) Bars represent the means \pm SD; Unpaired Student's *t*
1073 test, ****P*<0.001.

1074

1075 **Supplementary Figure 2.** CRB3 knockdown promotes proliferation of mammary
1076 epithelial cells, and overexpression promotes apoptosis of breast cancer.

1077 A. B. Immunoblot and real-time quantitative PCR analysis of CRB3 knockout
1078 efficiency in MCF10A cells. C. MCF10A proliferation assay for six successive days.
1079 D. Cell cycle showing the distribution of MCF10A cells. E. Quantification of the cell
1080 cycle distribution. F.G.H. Apoptosis of MCF7, T47D, and MDA-MB-453 breast
1081 cancer cells with CRB3 overexpression and quantification of apoptotic cells. Bars
1082 represent the means \pm SD; Unpaired Student's *t* test, * *P*<0.05, ***P*<0.01.

1083

1084 **Supplementary Figure 3.** CRB3 alters primary cilium formation in MEFs from
1085 *Crb3*^{fl/fl};CAG-*Cre* mice.

1086 A. Representative immunofluorescent staining of primary cilium formation in MEFs
1087 from *Crb3*^{fl/fl} and *Crb3*^{fl/fl};CAG-*Cre* mice. Acetylated tubulin (red), γ -tubulin (green),

1088 and DNA (blue). (scale bars, 10 μ m) B. Quantification of the proportion of cells with
1089 primary cilium formation (n=10). Bars represent the means \pm SD; Unpaired Student's
1090 *t* test, ****P*<0.001.

1091

1092 **Supplementary Figure 4.** The effect of CRB3 on a series of ciliogenesis-related
1093 genes and Rab11 expression.

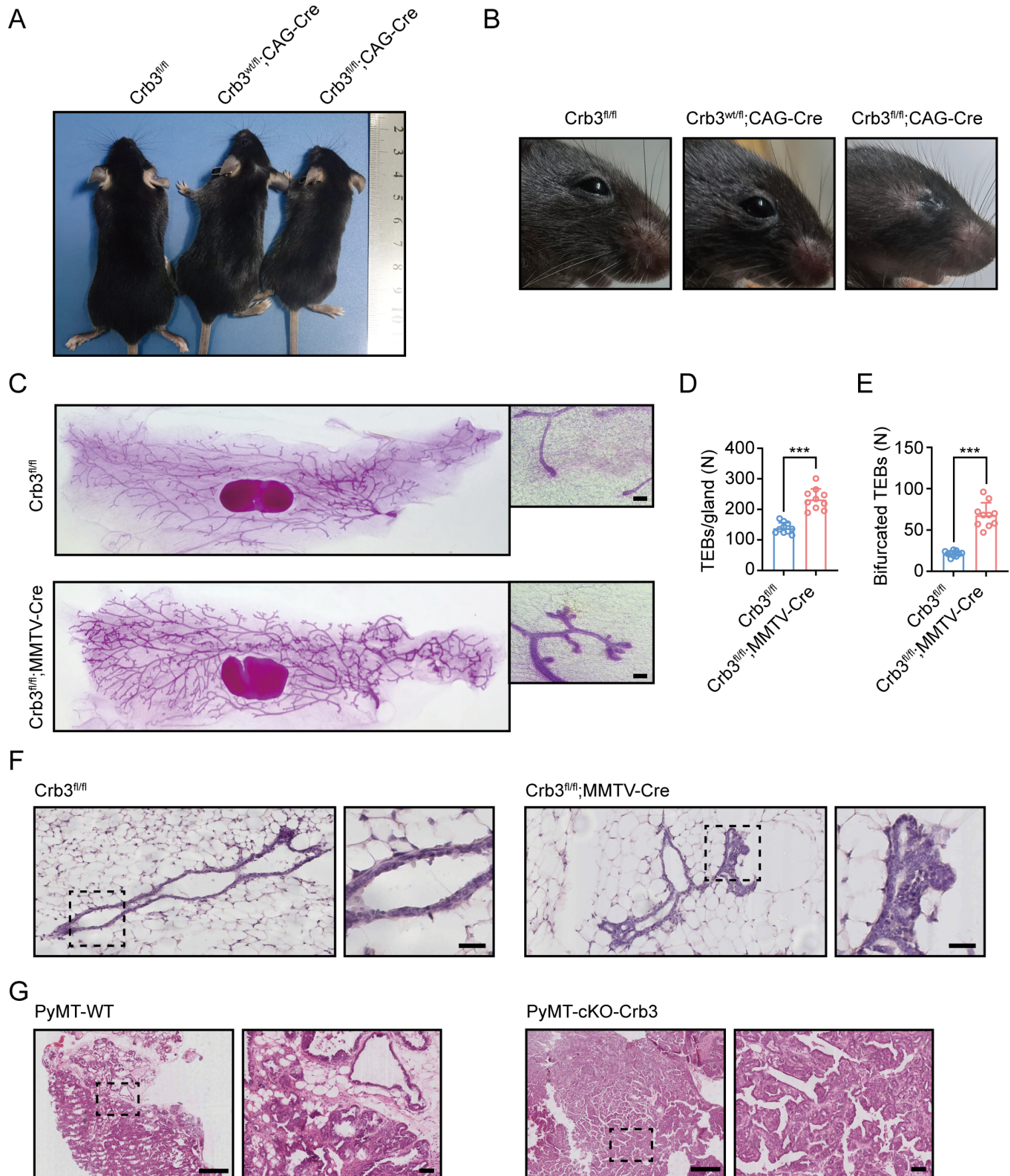
1094 A. Quantification of the mRNA expression of genes encoding markers of centriole or
1095 primary cilium, BB components, IFT-A and IFT-B anterograde transport (normalized
1096 to GAPDH). B. Coomassie blue staining showing the gel electrophoresis of
1097 exogenous CRB3 immunoprecipitated precipitates. C. Immunoblot analysis of the
1098 effect of CRB3 on Rab11 in MCF10A cells. Bars represent the means \pm SD; Unpaired
1099 Student's *t* test

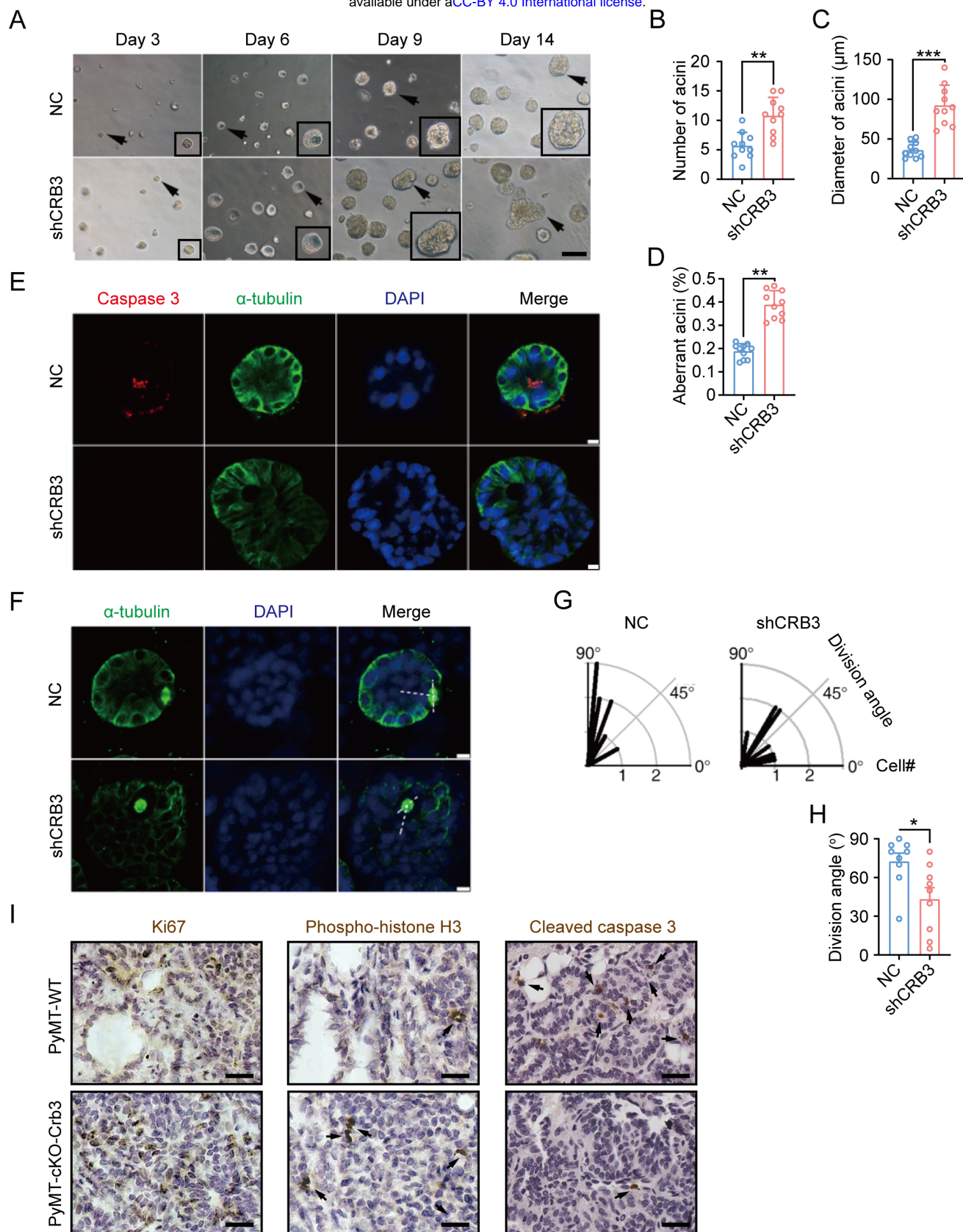
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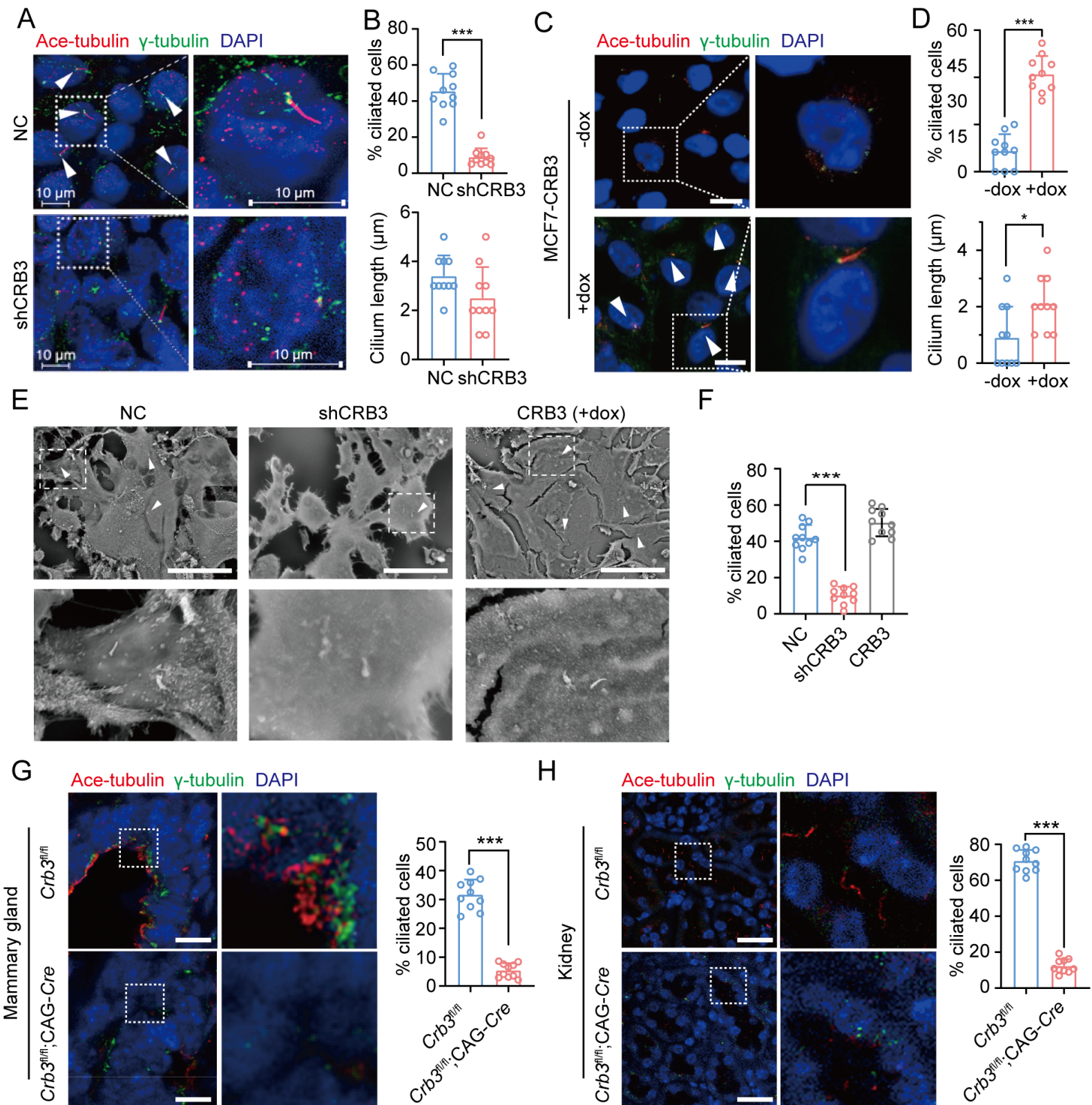
1101 **Supplementary Figure 5.** CRB3 knockdown disturbed the colocalization of GCP6
1102 and Rab11, and Rab11 knockdown caused primary cilium defects.

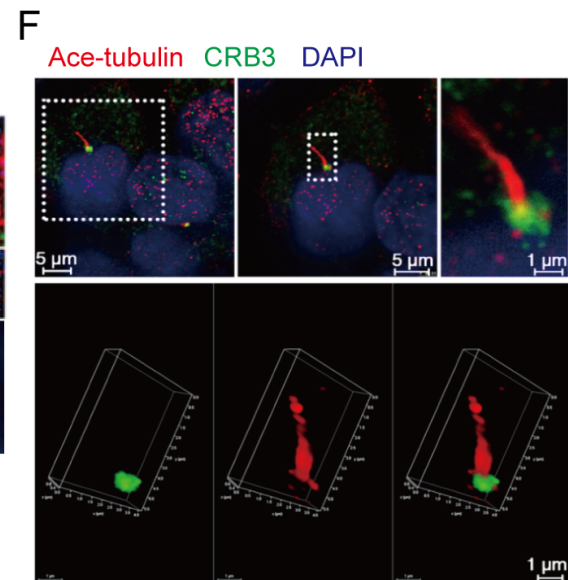
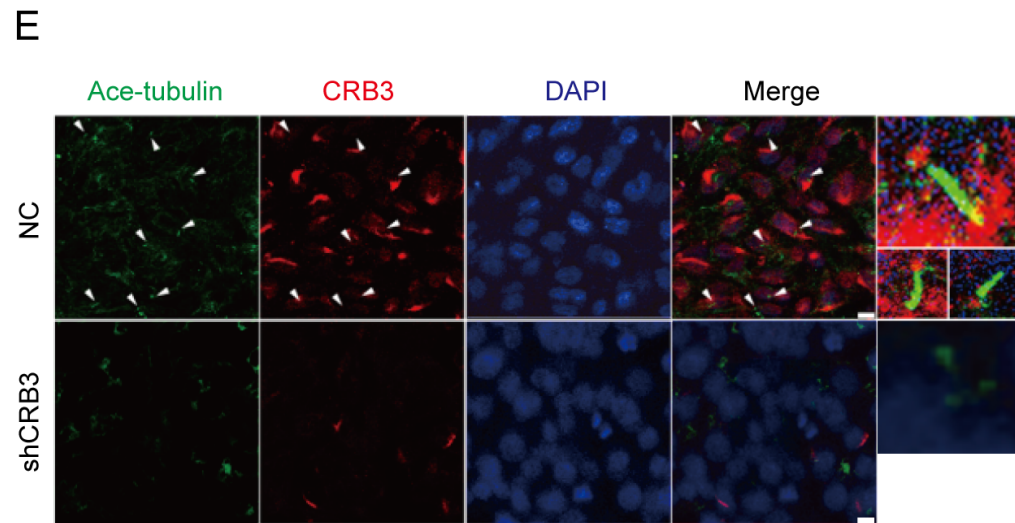
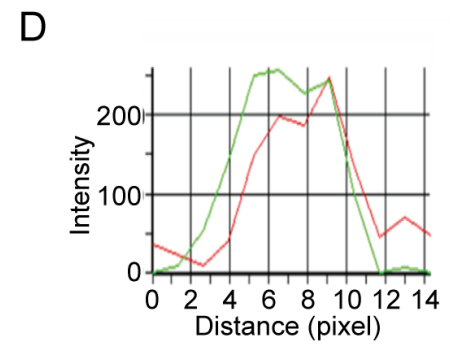
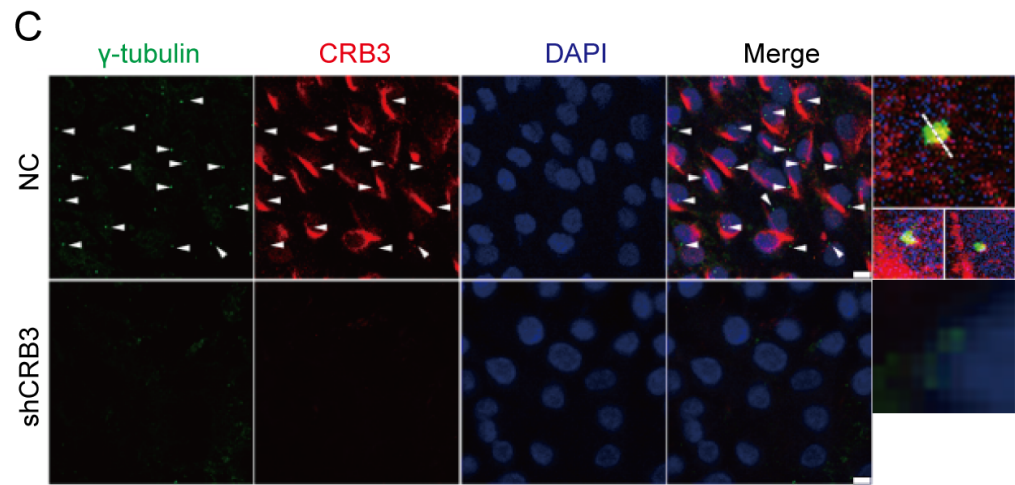
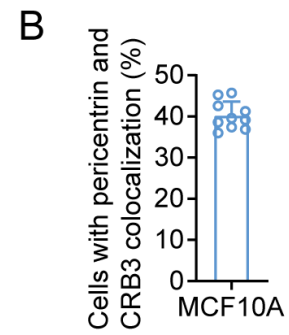
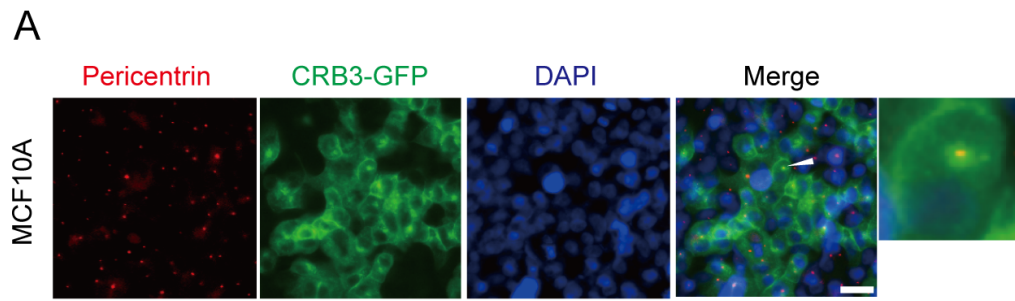
1103 A. Representative immunofluorescent staining of the colocalization between Rab11
1104 and GCP6 in MCF10A cells. GCP6 (red), Rab11 (green), and DNA (blue). (scale bars,
1105 20 μ m) B. Fluorescence colocalization analysis in (A). C. Quantification of the
1106 proportion of cells with Rab11 and GCP6 colocalization. D. Representative images of
1107 immunofluorescent staining of primary cilium formation with Rab11 knockdown in
1108 MCF10A cells. Acetylated tubulin (red), γ -tubulin (green), and DNA (blue). (primary
1109 cilium marked by arrows; scale bars, 20 μ m) E. Quantification of the proportion of
1110 cells with primary cilium formation. F. Immunoblot analysis of Rab11 knockout
1111 efficiency in MCF10A cells. Bars represent the means \pm SD; Unpaired Student's *t* test,
1112 ***P*<0.01, ****P*<0.001.

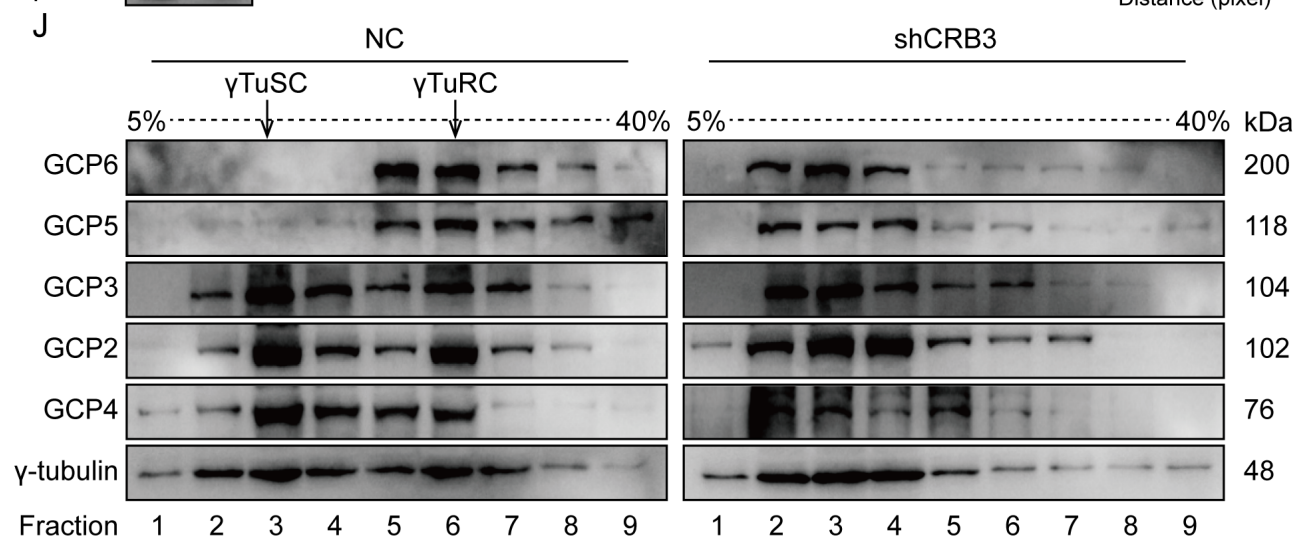
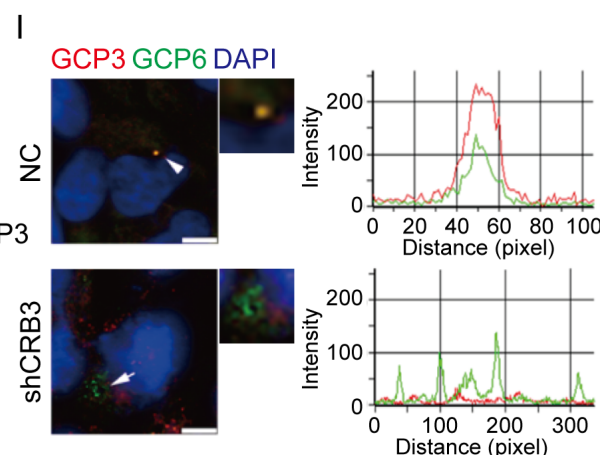
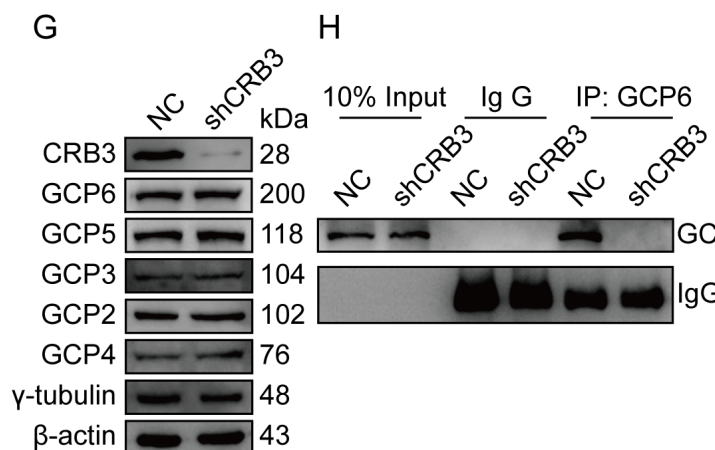
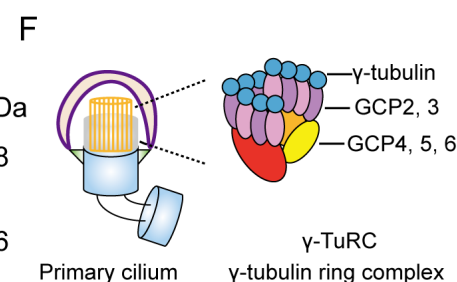
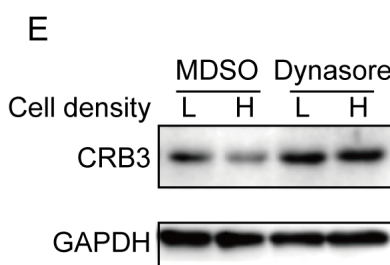
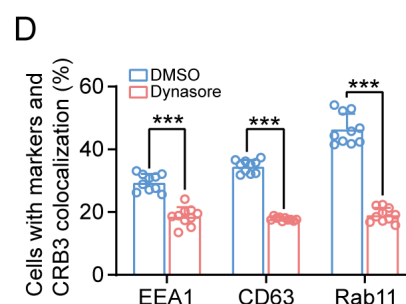
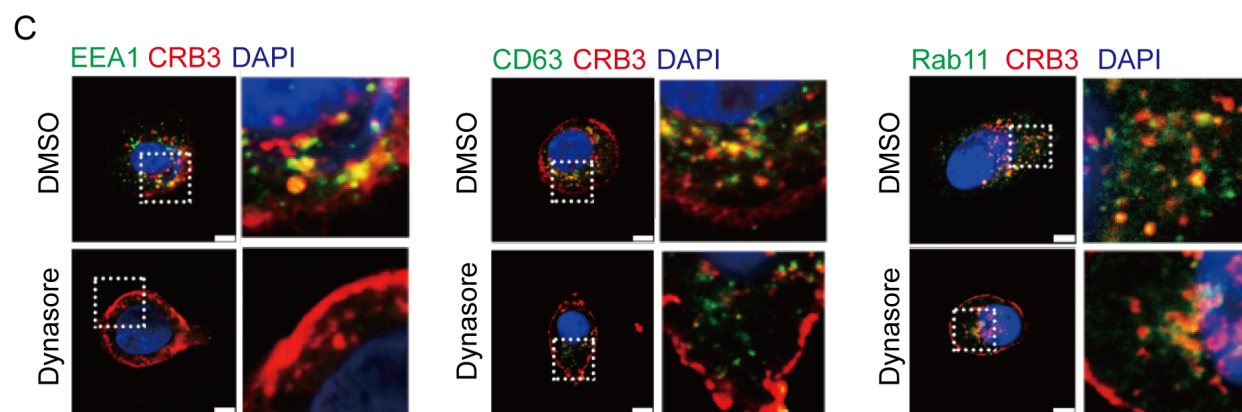
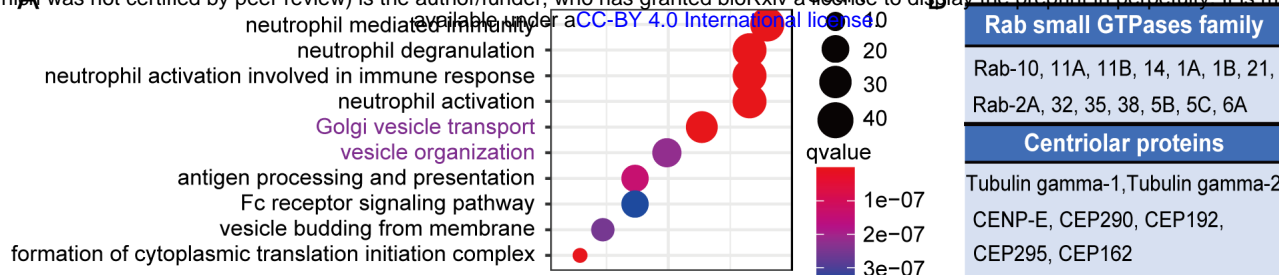
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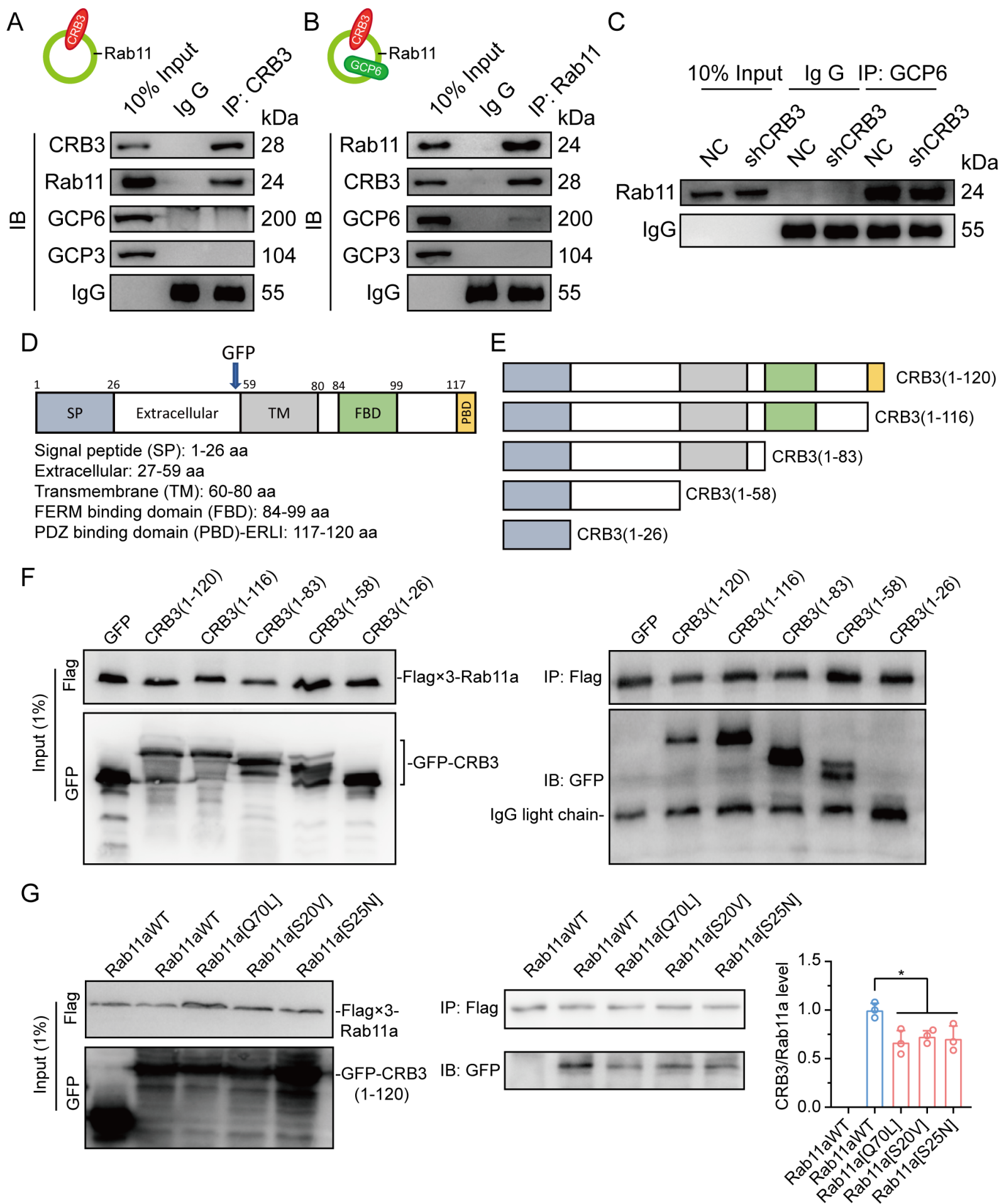


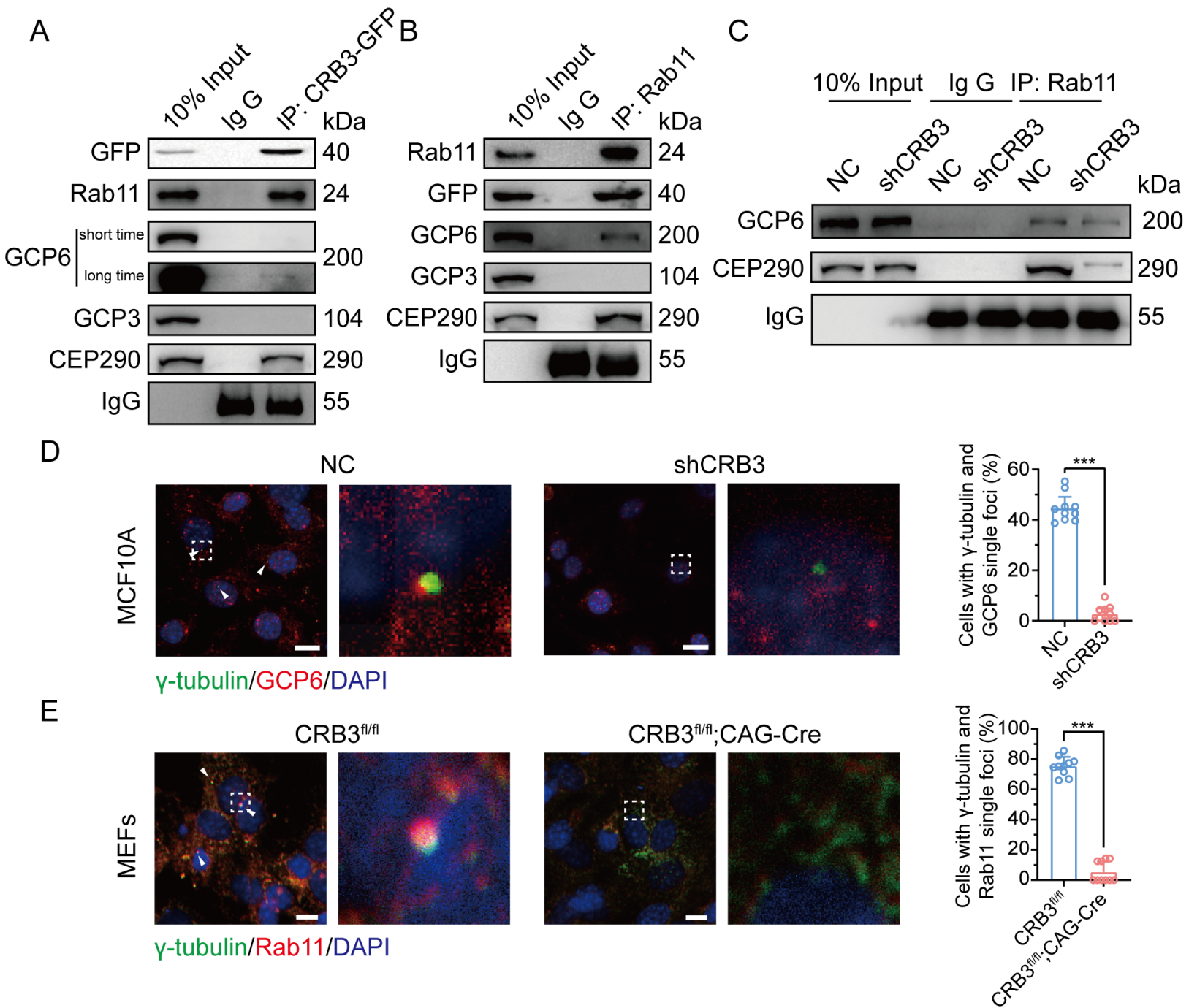


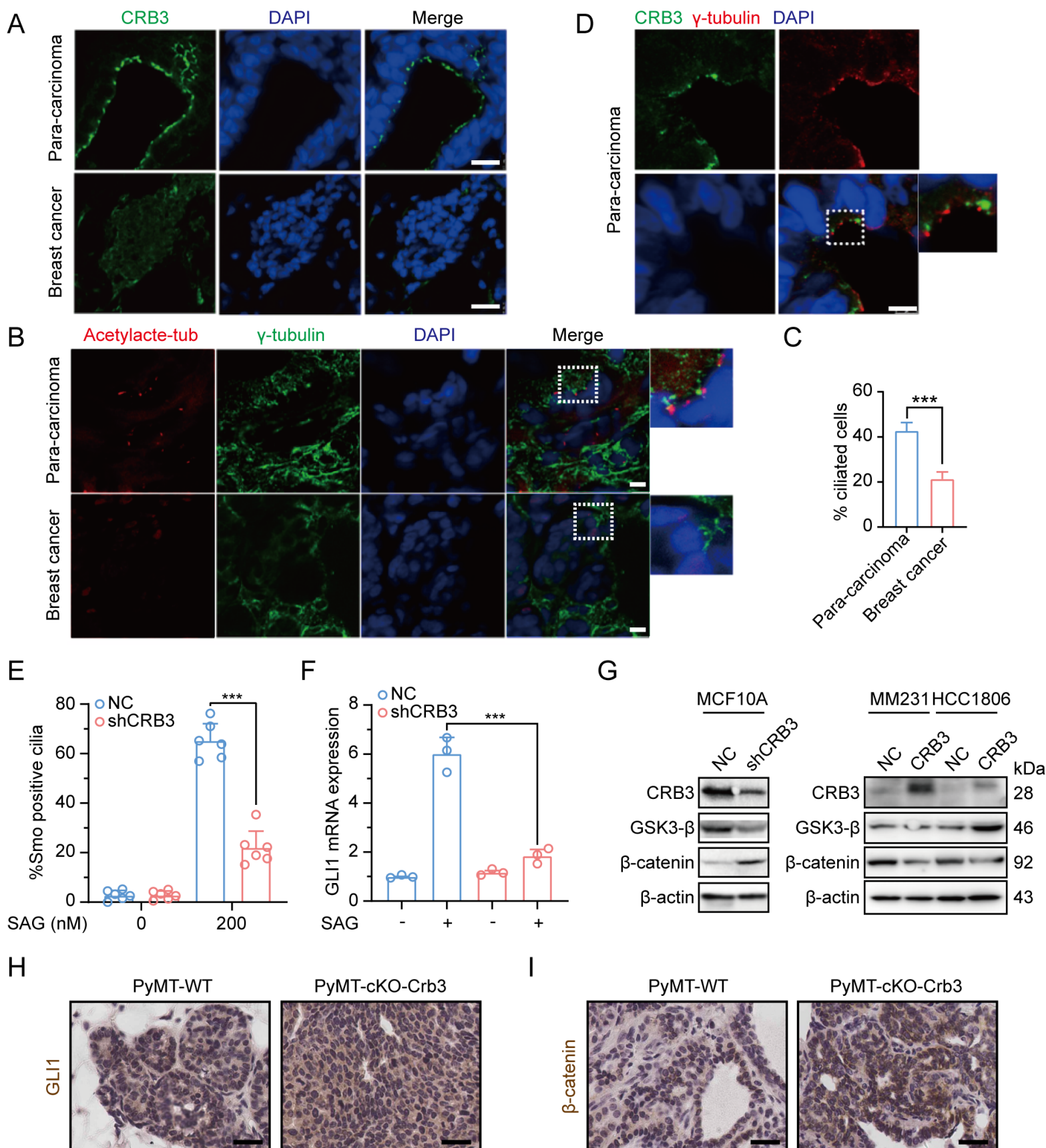


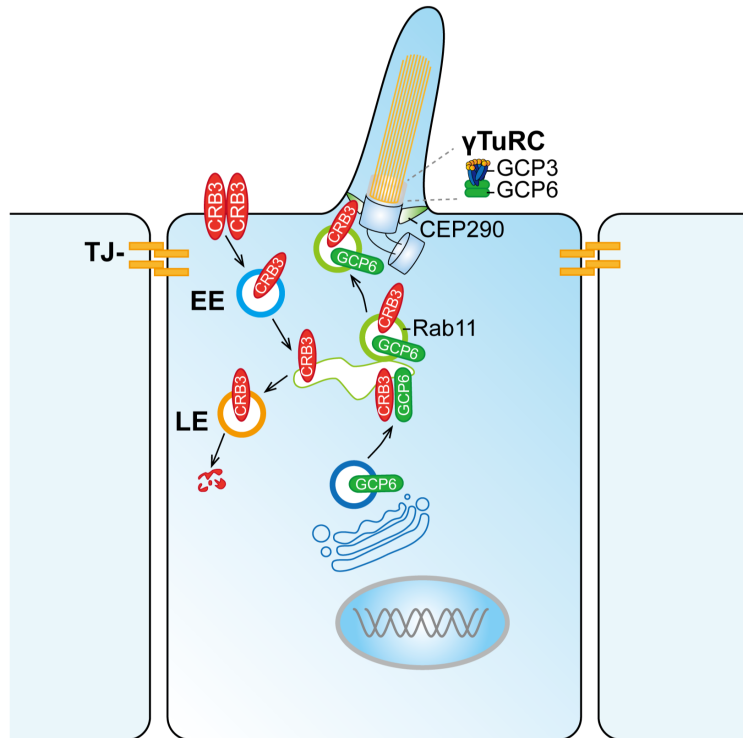






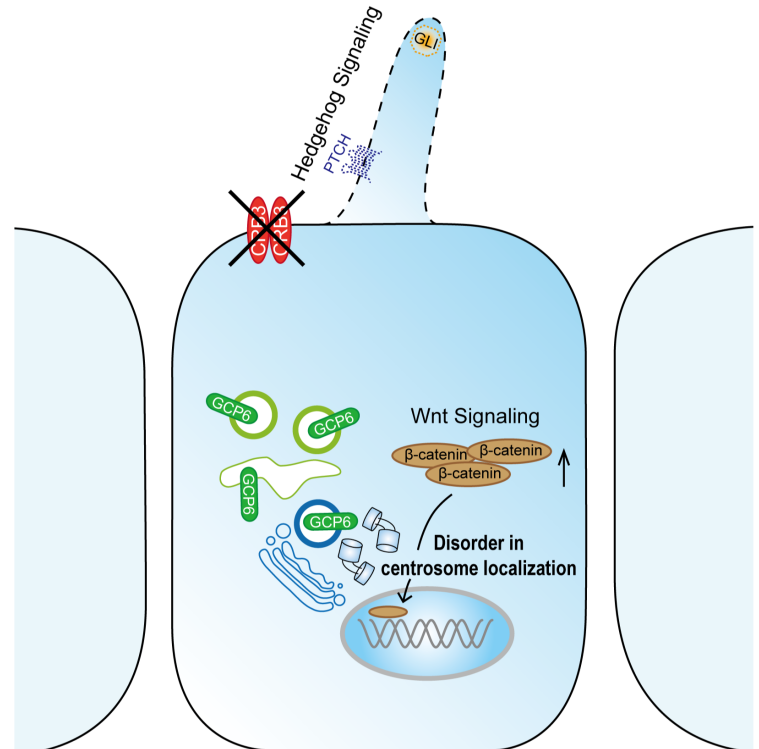
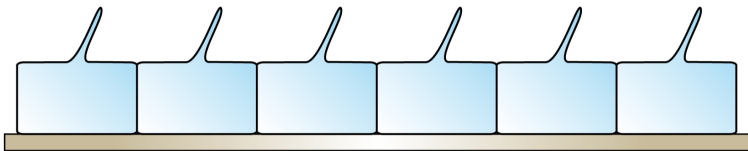






- Contact inhibition
- Quiescence
- Cell homeostasis

Normal



- Loss of contact inhibition
- Proliferation
- Imbalance in homeostasis

Cancer

