The calcium pump PMCA4b promotes epithelial cell polarization and lumen formation

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Abstract

Loss of epithelial cell polarity is a hallmark of carcinogenesis, in which Ca^{2+} signaling plays a role. Here we demonstrate that the plasma membrane Ca^{2+} pump PMCA4 is downregulated in luminal breast cancer, and this is associated with shorter relapse-free survival of patients with luminal A and B1 sub-type tumors. We find that *in vitro* silencing of PMCA4 in MCF-7 breast cancer cells induces internalization of E-cadherin and Dlg1 leading to a severe loss of cell polarity while re-expression of the b variant of PMCA4 restores cell polarity and promotes lumen formation mediated by the Arf6-trafficking pathway. We also demonstrate via an *in vivo* model using *Drosophila melanogaster* that silencing of the single *pmca* gene destroys lumen morphology in the larval salivary gland suggesting a conserved role of PMCAs in lumen morphogenesis. Additionally, enhanced secretory vesicle accumulation in PMCA(4)-deficient cells indicates a profound secretion defect both *in vitro* and *in vivo*. Our findings point to a novel role of PMCA4 in restoring epithelial cell polarity, and maintaining normal glandular tissue architecture.

Introduction

Breast cancer is the most commonly occurring cancer in women representing about 10% of all cancers in this population worldwide (McGuire *et al*, 2015). The majority of breast cancers are hormone receptor-positive (HR^+), of which the luminal A (LUMA) sub-type is the least aggressive (low grade) while the more proliferative (Ki67-high) LUMB1 and the HER2-positive LUMB2 sub-types are of higher grades.

Intracellular Ca^{2+} homeostasis is frequently altered in cancer cells, and this can play a role in tumor initiation, progression, angiogenesis and metastasis (Cui *et al*, 2017; Monteith *et al*, 2017). Plasma membrane calcium ATPases (PMCAs) regulate

intracellular Ca^{2+} concentration by pumping Ca^{2+} out of the cytoplasm into the extracellular space in order to maintain the exceedingly low cytosolic Ca^{2+} concentration. In mammalian cells four genes (*ATP2B1-4*) encode PMCA1-4 proteins, and as a result of alternative splicing, more than 20 PMCA isoforms have been described. PMCA1 is the house-keeping form that together with PMCA4 is expressed in all cell types while PMCA2 and PMCA3 expressions are tissue specific (Krebs, 2015; Padanyi *et al*, 2016). Both PMCA2 and PMCA4 play a role in the adult mammary gland, but their functions are quite different (Reinhardt *et al*, 2000). PMCA2 is highly expressed during the lactating period and is responsible for setting Ca^{2+} concentration in the milk (Reinhardt *et al*, 2004). In contrast, PMCA4 is upregulated before lactation and during mammary gland involution (Reinhardt *et al.*, 2000; Reinhardt & Lippolis, 2009). It has been well established that serum estradiol levels gradually increase during pregnancy, and we showed that the “b” splice variant of PMCA4 (PMCA4b) expression was highly dependent on estradiol concentrations in ER-α positive MCF-7 breast cancer cells suggesting a role for this pump in mammary gland development (Varga *et al*, 2018).

Alterations in PMCA expressions have been reported in various cancer types (Islam, 2020; Monteith *et al.*, 2017). Among these, PMCA4b was shown to be down-regulated in estrogen receptor positive (ER^+) luminal-type breast cancer cells whereas its expression was relatively high in basal-type cells with a predominant localization to intracellular compartments (Varga *et al.*, 2018). Low expression of PMCA4b was detected in highly metastatic BRAF mutant melanoma cell lines, in which re-expression of PMCA4b was associated with decreased migration and metastatic activities suggesting a metastasis suppressor role for this PMCA variant (Hegedus *et al*, 2017a).

Luminal epithelial cells display apical-basal polarity that is regulated by specific protein modules under the control of the Par, Crumbs and Scribble polarity protein complexes (Martin *et al*, 2021). Lumen formation is an essential step in mammary gland development, and loss of lumens is a hallmark of tumorigenesis (Halaoui *et al*, 2017). It has been shown that primary mammary cells isolated from mice were able to form lumen-containing acini on reconstituted basement membrane matrix, however, after oncogene induction, the cells lost their epithelial polarity and lumen-forming capability (Jechlinger *et al*, 2009). In polarized cells directed vesicular transport is essential to

ensure proper protein and lipid composition of specialized membrane compartments (Jewett & Prekeris, 2018). The ADP-ribosylation factor 6 (Arf6) small GTPase protein regulates membrane trafficking pathways (Li *et al*, 2017), and it has also been implicated in cell polarization (Ikenouchi & Umeda, 2010; Osmani *et al*, 2010; Shteyn *et al*, 2011) and lumenogenesis (Clancy *et al*, 2018; Tushir *et al*, 2010). Although Arf6 is essential for cell polarization, Arf6 hyperactivity can promote invasiveness and metastatic activity of cancer cells (Li *et al.*, 2017; Nikolatou *et al*, 2023) by increasing their motility (Zobel *et al*, 2018).

In the present study we show that PMCA4(b) (assessed by a PMCA4-specific antibody) is downregulated in luminal-type breast carcinoma tissue samples, and that the expression level of the *ATP2B4* gene correlates with patient survival. We demonstrate that PMCA4b interacts with the polarity protein Dlg1 and promotes polarization and lumen formation of ER^+ MCF-7 breast cancer cells, and that this requires endosomal recycling by the Arf6-driven pathway and the previously identified di-leucine endocytic motif at the C-terminus (Antalffy *et al*, 2013). We also show that silencing of the Drosophila PMCA (dmPMCA) *in vivo* disrupts lumen morphology of larval salivary gland suggesting a conserved role of PMCAs in lumen formation.

Results

PMCA4 is downregulated in HR^+ breast cancer

Besides an analysis of publicly available gene expression datasets (Varga *et al.*, 2018), no clinically relevant data have been published on PMCA4 expression in breast cancer. Since a majority of all breast cancer cases (about 70-80%) are hormone receptor-positive (HR^+) (Harbeck *et al*, 2019), we investigated PMCA4 protein abundance in HR^+ breast carcinoma tissue samples (Suppl. Table 1) using the PMCA4-specific antibody JA9 (JA9 does not discriminate between PMCA4 “a” and “b” splice variants), and compared the results to those of the normal breast tissue samples obtained after breast reduction surgery. We found that PMCA4 was highly expressed in the ductal epithelium of normal breast tissue and in more differentiated regions of tumor samples when compared to fully de-differentiated regions of the tumor (Fig. 1A). In 76% of the breast cancer cases (83 out of 109 tissue samples) less than 5% of the tumor

cells showed membrane-associated PMCA4 staining. Analyzing separately the HR^+ sub-types no statistically significant differences were found between the LUMA and LUMB cases (Fig. 1B, Suppl. Table 1). Data from the Clinical Proteomic Tumor Analysis Consortium (Chandrashekar *et al*, 2022) (CPTAC) (https://ualcan.path.uab.edu) confirmed these findings and showed significantly reduced PMCA4b protein levels in luminal breast cancer types compared to normal breast tissue. In contrast to PMCA4b, PMCA1b expression was not different in the same comparison (Fig. 1C).

To assess the prognostic role of PMCA4 expression in the clinical outcome of HR^+ breast carcinoma sub-types, the publicly available KM Plotter Online Tool (Gyorffy, 2021) (www.kmplot.com) was used. According to this database, high PMCA4 gene expression was associated with greater probability of relapse-free survival (RFS) in grade 2 LUMA and in LUMB1 tumors, whereas an inverse correlation was observed in the HER2-expressing LUMB2 sub-type, in which poorer outcomes were associated with higher ATP2B4 expression. It is worth noting that in grade 1 and 3 LUMA tumors the effects were not significant (Suppl. Fig. 1). In contrast to PMCA4, no statistically significant association was detected between low and high PMCA1 gene expression and RFS in the LUMA and LUMB1 breast carcinoma sub-types, while in the LUMB2 sub-type high PMCA1 level was associated with a lower probability of RFS as also seen for high PMCA4 expression (Fig. 1D).

Changes in PMCA4b protein abundance alter E-cadherin localization in MCF-7 breast cancer cells

High PMCA4 protein levels in the normal mammary gland and its nearly complete loss in luminal subtype tumors suggest that PMCA4 might be involved in the development and/or maintenance of the normal mammary tissue. The PMCA4b splice variant is the ubiquitous form of PMCA4, which has been found in several luminal-type breast cancer cells including the estrogen-sensitive MCF-7 cells (Varga *et al.*, 2018; Varga *et al*, 2014). Although these cells express low levels of PMCA4b, they retain most of their epithelial features since they express the epithelial marker E-cadherin but not the mesenchymal markers N-cadherin and vimentin (Comsa *et al*, 2015). To understand the role of PMCA4(b) loss during tumor progression, GFP-tagged PMCA4b and its GFP-

tagged mutant form PMCA4b^{LA} with compromised endocytosis (Antalffy *et al.*, 2013) were stably over-expressed in MCF-7 cells, or the endogenous PMCA4b was silenced by a PMCA4-specific short hairpin RNA (sh-RNA). We found that neither silencing nor over-expression of PMCA4b affected the expression levels of the epithelial and mesenchymal markers E-cadherin, N-cadherin and vimentin (Fig. 2A, Suppl. Fig. 2A), respectively, or influenced cell growth (Suppl. Fig. 2B). However, silencing of PMCA4 induced internalization of E-cadherin, resulting in its reduced plasma membrane localization compared to the parental, GFP-PMCA4b- or the trafficking-mutant GFP-PMCA4b^{LA}-expressing MCF-7 cells, in which E-cadherin displayed a more prominent plasma membrane localization (Fig. 2B-E, Suppl. Fig. 2C). This suggests that loss of PMCA4 may induce partial epithelial-mesenchymal transition (EMT) through E-cadherin internalization without affecting the overall expression of EMT markers.

PMCA4b regulates epithelial cell polarity

E-cadherin is involved in cell-cell adhesion and cell polarization partly through its interaction with scribble (Scrib), a member of the apico-basal Scribble polarity complex that consists of lethal giant larvae (Lgl), discs large 1 (Dlg1) and Scrib proteins (Wang *et al*, 2018). It has been known for a long time that the C-terminal PDZ-binding sequence motif ETSV of PMCA4b binds to the PDZ-domains 1 and 2 of the scaffold protein Dlg1 (DeMarco & Strehler, 2001) (Fig. 3A). Here we show that Dlg1 is highly expressed in MCF-7 cells and its expression is not affected by either PMCA4b silencing or over-expression (Fig. 3B). Fluorescence immunostaining of PMCA4b expressing MCF-7 cells show co-localization between PMCA4b and Dlg1 at the plasma membrane, as expected (Fig. 3C), while PMCA4-silencing is associated with preferential cytoplasmic Dlg1-localization (Fig. 3D-G, Suppl. Fig. 3A-C), suggesting that PMCA4b is involved in targeting Dlg1 to the plasma membrane.

Ezrin is an F-actin-binding protein that is known to localize to the apical plasma membrane of polarized epithelial cells (Casaletto *et al*, 2011). The overall high level and/or atypical localization of ezrin in tumor cells were shown to correlate with poor prognosis in breast cancer (Jeong *et al*, 2019; Sarrio *et al*, 2006). While expression of sh-PMCA4, PMCA4b or the trafficking mutant PMCA4b^{LA} in MCF-7 cells did not alter

ezrin protein levels (Fig. 3H), variations in PMCA4b levels showed strikingly different ezrin localization patterns. In contrast to the PMCA4-silenced or trafficking mutant PMCA4b^{LA}-expressing MCF-7 cells where a large proportion of ezrin was localized to intracellular compartments, PMCA4b expression promoted asymmetric ezrin localization (Fig. 3I-K, Suppl. Fig. 3D, E). These data suggest that PMCA4b is involved in cell polarization through PDZ domain-mediated interaction with Dlg1 and show that not only PMCA4b expression but also its proper trafficking is required to accomplish apical-basal cell polarity.

PMCA4b enhances polarized vesicular trafficking

Epithelial cells require polarized vesicular trafficking for the proper distribution of proteins and lipids at specific plasma membrane domains to establish and maintain apico-basal cell polarity (Banushi *et al*, 2023). Wheat germ agglutinin (WGA) has been widely used to follow endosomal trafficking in live cells. Upon binding to N-acetylglucosamine and sialic acid moieties on the extracellular side of plasma membrane proteins WGA can be internalized and transported through the endosomal pathways (Liu *et al*, 2011). We performed a WGA uptake assay by MCF-7 cells and found that PMCA4b expressing cells collected WGA-positive vesicles to one side of the plasma membrane while these vesicles were located randomly in the cytoplasm of sh-PMCA4- and PMCA4b^{LA}-expressing cells (Fig. 4A-C, Suppl. Fig. 4). We found that PMCA4b strongly co-localized with WGA-positive vesicles in contrast to the PMCA4b^{LA} mutant that clearly separated from the WGA-positive puncta (Fig. 4D). These results show the importance of PMCA4b trafficking in cell polarization.

Arf6 regulates intracellular trafficking of PMCA4b

PMCAs require complex formation with neuroplastin or CD147/basigin for their proper plasma membrane localization and function (Korthals *et al*, 2017; Schmidt *et al*, 2017). In MCF-7 cells we found that PMCA4b and PMCA4b^{LA} over-expression induced elevated expression of CD147 (Fig. 5A) and increased its plasma membrane localization (Fig. 5B) where the two proteins showed strong co-localization (Fig. 5B, C). It has been demonstrated that internalization and recycling of CD147 is mediated by Arf6 (Qi *et al*,

2019). To study the role of Arf6 in PMCA4b trafficking we treated PMCA4b expressing MCF-7 cells by the Arf6 inhibitor NAV2729 (Yoo *et al*, 2016). After 24 hours of treatment, we observed an enrichment of PMCA4b-CD147 positive vesicles near the plasma membrane, and after 48 hours of treatment PMCA4b-CD147 vesicles accumulated in the cytoplasm (Fig. 5D) indicating that Arf6 was involved in the endocytic recycling of PMCA4b-CD147. This finding was confirmed by the strong co-localization between PMCA4b and Arf6 in intracellular compartments when wildtype Arf6 was transiently transfected in PMCA4b-expressing MCF-7 or HEK293 cells (Fig. 5E). Transient transfection with the constitutively active mutant form of Arf6 ($Arf6^{Q67L}$) caused accumulation of PMCA4b in Arf6-positive intracellular vesicles similarly to that described for other known Arf6 cargo proteins (Porat-Shliom *et al*, 2013; Sannerud *et al*, 2011) (Fig. 5F). These findings indicate that Arf6 is a novel regulator of PMCA4b-CD147 endosomal trafficking (Fig. 5G).

PMCA4b promotes pre-lumen formation in 2D cell cultures through an Arf6-mediated endocytic recycling pathway

The establishment and maintenance of epithelial cell polarity are hallmarks of healthy mammary tissue organization featured by a complex ductal network. However, during neoplastic transformation the polarity of neoplastic mammary epithelial cells is lost and their capability to form mature mammary ducts with well-defined central lumens is also compromised (Chatterjee & McCaffrey, 2014). We found that in highly confluent 2D cell cultures PMCA4b over-expression led to the formation of significantly more pre-lumen-like structures resembling cellular intermediates of lumen formation than in cells expressing sh-PMCA4 or the trafficking mutant $PMCA4b^{LA}$ (Fig. 6A-C, E, Suppl. Fig. 5A). We observed a dynamic WGA-positive vesicular trafficking towards the pre-lumen area (Suppl. Fig. 5B) characteristic of *de novo* lumen formation (Datta *et al*, 2011; Levic & Bagnat, 2023).

Our experiments suggested that proper intracellular trafficking possibly mediated by Arf6 was required for the positive impact of PMCA4b on pre-lumen formation. Therefore, we treated PMCA4b-expressing MCF-7 cells with the Arf6 inhibitor NAV2729 and found significantly fewer F-actin labeled pre-lumen structures compared to the non-

treated PMCA4b-expressing cells, confirming the importance of Arf6-mediated trafficking in PMCA4b-induced pre-lumen formation (Fig. 6D, E, Suppl. Fig. 5C).

PMCA4b promotes central lumen formation in MCF-7 mammospheres

To confirm the role of PMCA4b in lumen formation, we cultured MCF-7 cells in Matrigel for 10 days to create mammospheres, and observed that the PMCA4b-expressing cells formed well-defined, F-actin-bordered central lumen-bearing spheroids with a single layer of cells around the lumen (Fig. 6F-H, Suppl. Fig. 6A-D) similar to that seen in cross-sections of healthy mammary duct (Hassiotou & Geddes, 2013). While the parental MCF-7 cells also formed some central lumen-bearing mammospheres, the lumen structures were not well-defined and their number was significantly lower compared to cell cultures with elevated PMCA4b expression (Fig. 6H, Suppl. Fig. 6B). Mammospheres generated by PMCA4 specific shRNA expressing MCF-7 cells did not form central lumens; rather, they displayed several small F-actin-rich areas (Fig. 6F-H, Suppl. Fig. 6A) resembling the microlumens commonly seen in non-invasive ductal carcinoma *in situ* (DCIS) (Winchester *et al*, 2000). The trafficking mutant PMCA4b^{LA}-expressing cells could not form central lumens either. Rather, they formed irregular actin-rich 3D structures (Fig. 6F-H, Suppl. Fig. 6D) indicating that not only PMCA4b abundance but also proper localization was required for appropriately positioned central lumen formation.

Imaging studies by electron microscopy indicated an enrichment of secretory vesicles in proximity of the plasma membrane. These secretory vesicles - especially the dense core-types - showed excessive accumulation in sh-PMCA4 expressing MCF-7 cells, suggesting altered secretion in the absence of PMCA4b (Fig. 6I, Suppl. Fig. 6E). Interestingly, in PMCA4b^{LA}-expresssing cells small empty endocytic vesicles accumulated near the plasma membrane (Fig. 6I) suggesting dysfunction in endosomal trafficking.

Drosophila PMCA is necessary for normal lumen morphology of the larval salivary gland

The *Drosophila melanogaster* (dm)PMCA (UniProt: Q9V4C7) shows 62.7% identity in amino acid sequence with the human PMCA4b protein (UniProt: P23634-6) (Suppl. Fig. 7A). For *in vivo* confirmation of the role of PMCA in lumen formation we examined whether silencing of dmPMCA affected lumen morphology of the larval salivary gland in Drosophila. We silenced dmPMCA using the UAS-GAL4 system with *daughterless* promoter-driven Gal4 and dmPMCA-specific UAS promoter-driven RNA interference construct that effectively reduced the expression of dmPMCA in 3rd stage larvae (Fig. 7A). For testing the expression of dmPMCA we used the pan-PMCA antibody 5F10 for the first time in Drosophila that was suitable for the detection of dmPMCA because of it recognizes a highly conserved PMCA-epitope (Fig. 7A, Suppl. Fig. 7A). We found that the endogenous dmPMCA localized to the basolateral and apical plasma membrane of the salivary gland cells in 3rd stage larvae (Fig. 7B) and showed strong co-localization with the *Drosophila melanogaster* Dlg protein (Fig. 7C) in good agreement with the potential PDZ-binding sequence present at the C-terminus of six out of the seven dmPMCA isoforms (Suppl. Fig. 7B).

Tissue-specific silencing of dmPMCA in salivary gland RNAi resulted in an aberrant F-actin pattern that normally borders the central lumen (Fig. 7D). Cross-sections of epoxy resin-embedded salivary gland samples showed a substantial reduction in central lumen size of the salivary gland as a result of dmPMCA silencing (Fig. 7E, F). Closer inspection of the dmPMCA deficient salivary gland cells by electron microscopy revealed a large secretory vesicle accumulation in the cytoplasm (Fig. 7G), suggesting a secretion defect similar to that seen in PMCA4-silenced MCF-7 mammospheres (Fig. 6I). These results highlight the role of PMCA proteins in the regulation of lumen morphology and suggest that secretion in glandular organs is conserved among species as distant as *Drosophila melanogaster* and human.

Discussion

Altered histological differentiation in malignancies (including breast carcinoma) characterized by the loss of tissue architecture and loss of cell polarity is associated with poor patient survival (Enane *et al*, 2018). Normal luminal epithelial cells in the mammary gland exhibit apico-basal polarity, and this is progressively lost during tumorigenesis

(Halaoui *et al.*, 2017). Recently, we demonstrated that PMCA4b over-expression induced cell polarization and actin cytoskeleton remodeling of BRAF mutant melanoma cells (Naffa *et al*, 2021). Moreover, a profound change in cell culture morphology and F-actin rearrangement was detected in MCF-7 cells in response to changes in PMCA4b protein abundance (Naffa *et al.*, 2021). In the current report, we show for the first time that PMCA4 is downregulated in tumor tissue samples derived from patients with luminal breast cancer when compared to healthy breast tissue, where PMCA4 expression is high, and that high PMCA4 expression is associated with longer relapse free survival in luminal A and B1 breast cancer sub-types (Fig. 1). To study whether the severe loss of PMCA4b in luminal breast cancer can be related to the loss of cell polarity we either silenced or increased the level of PMCA4b in MCF-7 cells. Silencing of PMCA4b caused cytoplasmic localization of E-cadherin and Dlg1 while it did not affect the expression of epithelial and mesenchymal markers or cell growth (Fig. 2), suggesting that PMCA4b-loss induced partial EMT. It is worth noting, however, that PMCA4 silencing induced full EMT and increased the migratory ability of gastric cancer cells (Wang *et al*, 2020). Low PMCA4 protein abundance is a characteristic of various tumor cells including MCF-7 that is associated with prolonged Ca^{2+} signaling (Hegedus *et al*, 2017b; Padanyi *et al.*, 2016; Varga *et al.*, 2014) and prolonged Ca^{2+} signaling has been implicated in partial EMT (Norgard *et al*, 2021). In the present study we show that re-expression of PMCA4b in MCF-7 cells induces recruitment of E-cadherin and Dlg1 to the plasma membrane, asymmetric localization of ezrin and polarized endosomal vesicular trafficking (Fig. 3, 4), suggesting that PMCA4b expression enhances cell polarization possibly through its interaction with the polarity protein Dlg1 (Fig. 8).

Loss of epithelial cell polarity is a hallmark of progression to DCIS and loss of ductal luminal structures during carcinogenesis (Chatterjee & McCaffrey, 2014). Here we show that PMCA4b over-expressing MCF-7 cells are able to create luminal structures with an F-actin-rich apical domain, whereas PMCA4-silenced MCF-7 cells rather formed microlumens typically found in DCIS (Figure 6) (Chatterjee & McCaffrey, 2014). A similar effect was observed in oncogenic Ras^{v12} expressing MCF10A cells, in which Scrib (the complex forming partner of Dlg1) overexpression was associated with the preservation of acinar organization (Dow *et al*, 2008). Higher capability of PMCA4b-expressing cells

to form a central lumen is also supported by the significantly elevated levels of pre-lumen-like structures in 2D cell cultures compared to the parental or PMCA4-silenced MCF-7 cells. Silencing the single *Drosophila melanogaster pmca* gene (encoding dmPMCA) resulted in larval salivary gland degeneration, suggesting an evolutionarily conserved role of PMCA proteins in lumen formation of glandular organs *in vivo*. It has been demonstrated that secretory activity requires cytoplasmic Ca^{2+} oscillations (Ahuja *et al*, 2020; Mikoshiba *et al*, 2008; Pedersen *et al*, 2019), and local elevation of Ca^{2+} concentration is needed during fusion of secretory granules with the plasma membrane (Xue *et al*, 2021). Our electron microscopy analysis of PMCA(4)-deficient MCF-7 mammospheres and Drosophila salivary glands revealed a pronounced secretion defect and abnormal accumulation of secretory vesicles both *in vivo* and *in vitro*. In the present study we show for the first time that dmPMCA can influence Drosophila salivary gland secretion, although revealing its exact role in merocrine secretion (characteristic of both the mammary epithelium and the Drosophila salivary gland) requires further investigation.

Our results highlight the importance of proper intracellular trafficking of PMCA4b in cell polarization and subsequent lumen formation and suggest that the small GTPase Arf6 may play a role in this process. Several studies showed that Arf6 regulates lumen formation in epithelial cells (Milanini *et al*, 2018; Monteleon *et al*, 2012; Tushir *et al.*, 2010; Zangari *et al*, 2014) and this is in good agreement with our current observations that inhibition of Arf6 prevented PMCA4b-induced pre-lumen formation in 2D cell cultures (Fig. 7D).

Loss of cell polarity and loss of cell-cell contacts are hallmarks of epithelial malignancy (Chatterjee & McCaffrey, 2014). Here we demonstrate that PMCA4b enhances cell polarity, and that the elevation of PMCA4b protein levels can induce re-polarization of MCF-7 cells and an increased ability to form lumens. Taken together with our observations on clinical outcome data (Fig. 1) this suggests that elevated levels of PMCA4b may be associated with a lower risk of cancer progression at early stages of luminal breast cancer. Tumor reversion could be a new strategy in cancer therapy that aims to transform cancer cells back to their original differentiated phenotype (Bizzarri *et al*, 2020; Lamartina *et al*, 2021). Some factors have been shown to promote cancer cell

differentiation and cancer regression including histone deacetylase inhibitors such as valproic acid (VPA) (Bizzarri *et al.*, 2020; Klepinina *et al*, 2021; Leiva *et al*, 2012) that can induce a significant upregulation of PMCA4b in breast cancer and melanoma cell lines (Hegedus *et al.*, 2017b; Varga *et al.*, 2018). Hence, VPA may be a potential candidate for supplementary breast cancer therapy against luminal breast cancer characterized by low PMCA4b expression at early stages (intraductal hyperplasia or DCIS) of the disease (Wawruszak *et al*, 2021). It is important to note, however, that certain cancer types may respond to such treatments differently. For instance, Sritangos and co-workers demonstrated high PMCA4 protein abundance in pancreatic ductal adenocarcinoma, and showed that knockdown of PMCA4 reduced the migratory activity of MIA PaCa-2 cells and increased their sensitivity to apoptosis (Sritangos *et al*, 2020). We also show here that whereas PMCA4 abundance is associated with longer relapse-free patient survival in luminal LUMA (grade 2) and LUMB1 breast cancer sub-types, the opposite is observed for patients with HER^+ positive LUMB2 breast cancer (Fig. 1D). Thus, PMCA4 may have cancer type-specific functions in various neoplastic processes, underlining the importance of precise histopathological and molecular characterization of individual tumor samples for optimal treatment choices.

In summary, our results suggest that PMCA4b is a novel polarity protein and point toward its potential evolutionarily conserved function in the formation of luminal structures in exocrine glandular organs. We show that elevated levels of PMCA4b preserve polarized phenotype of MCF-7 breast cancer cells derived from luminal epithelial cells. Our findings suggest that loss of PMCA4 is implicated in the early-onset luminal type breast carcinomas.

Material and Methods

Tissue samples

109 formalin fixed paraffin embedded HR^+ (LUMA n=39; LUMB1 n=50; LUMB2 n=20 study cohort) and 4 normal breast tissue samples obtained after breast reduction surgery were selected (Supplementary Table 1). The cases were diagnosed at the Department of Pathology, Forensic and Insurance Medicine, Semmelweis University, Hungary between 2000 and 2010. Clinicopathological data of the patients were obtained

from the files of the Semmelweis University, 2nd Dept. of Pathology and from the Semmelweis University Health Care Database with the permission of the Hungarian Medical Research Council (ETT-TUKEB 14383/2017). All patients were followed-up until death or censored at the date of January 30, 2020.

Immunohistochemistry to evaluate PMCA4 expression in breast tissue samples

Immunohistochemistry (IHC) staining on all human tissue samples was performed and evaluated at the Department of Pathology, Forensic and Insurance Medicine, Semmelweis University. Cut-off values for estrogen receptor (ER) and progesterone receptor (PgR) positivity were 1% of tumor cells with nuclear staining and defined as positive or negative. Human erb-b2 receptor tyrosine kinase 2 (HER2) status was determined either as protein overexpression or HER2 gene amplification detected by fluorescence in situ hybridization (FISH). Data were analyzed by Kruskal-Wallis and Mann-Whitney tests for comparison of quantitative variables by the statistical software SPSS V.25 (IBM Corp.).

PMCA4 IHC was performed with an automated immunostainer system (Ventana Benchmark Ultra, Roche Diagnostics) according to the manufacturer's instructions. Antigen retrieval was performed with ULTRA CC1 antigen retrieval solution for 90 min at 95°C. The primary anti-PMCA4 JA9 mouse monoclonal antibody (P1494, Merck) was used at a 1:200 dilution at 42°C for 32 min. For antibody visualization, the UltraView DAB Detection kit (Ventana) was applied. PMCA4 IHC reaction was semi-quantitatively evaluated on whole slides: 1) IHC score was defined as the percentage of positive epithelial cells counted on average in 3–5 high-power fields, 2) membrane positivity with or without cytoplasmic reaction was assessed. PMCA4 stained slides were scanned with slide-scanner (PANNORAMIC® 1000 DX, 3DHISTECH Ltd., Hungary). PMCA4 IHC scoring was performed on digitized slides by using the slide-viewing software CaseViewer (CaseViewer 2.3.0.99276, 3DHISTECH Ltd., Hungary).

Reagents and treatments

Phalloidin-TRITC (P1951, Sigma-Aldrich) and NAV2729 (SML2238, Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C at a

concentration of 80 µM and a 4.5 mM, respectively. Phalloidin-TRITC was used for F-actin labeling on confluent 2D cultures and 3D mammospheres of MCF-7 cells at a concentration of 80 nM. In the experiments studying the role of Arf6, MCF-7 cells were treated with 6.75 µM NAV2729 for 24 or 48 hours. To label nuclei 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) was used at a concentration of 0.5 µM. For *Drosophila melanogaster* salivary gland staining Rhodamine Phalloidin (ab235138, Abcam) was used at a 1000x dilution in PBS according to the manufacturer's instructions.

Cell culture

MCF-7 (ATCC HTB-22) and HEK-293 cell lines were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (DMEM-HXA, Capricorn Scientific) supplemented with 10% fetal bovine serum (FBS; ECS0180L, Euroclone), 2mM L-glutamine (STA-B, Capricorn Scientific), 100 U/ml Penicillin and 100 µg/ml streptomycin (PS-B, Capricorn Scientific) in a humidified 5% CO_2 incubator at 37 °C. In order to maintain the stable expression of PMCA4b transgenes and of the shRNA construct, 100 ng/ml puromycin dihydrochloride (sc-108071, Santa Cruz Biotechnology) was added to the culture media.

Generation of stable cell lines

PMCA4-specific shRNA MCF-7 cells were generated by using the PMCA4-specific shRNA plasmid (sc-42602-SH, Santa Cruz Biotechnology, Santa Cruz, CA, USA), as described previously (Naffa *et al.*, 2021). GFP-PMCA4b and GFP-PMCA4b^{LA}-expressing MCF-7 and GFP-PMCA4b-expressing HEK-293 cell lines were generated by stable transfection of the cells with *SB-CAG-GFP-PMCA4b-CAG-puromycin* or *SB-CAG-GFP-PMCA4b-LA-CAG-puromycin* constructs that contain a Sleeping Beauty transposon system, using the protocol described previously (Hegedus *et al.*, 2017a; Naffa *et al.*, 2021).

Transgenic *Drosophila melanogaster* lines

Flies were raised at 25°C on standard cornmeal, yeast and agar containing medium. The w^{1118} strain (FlyBase ID: FBst0003605) was used as control and *da-Gal4* fly line (exact genotype: *w*;P(UAS-da.G)52.2*, FlyBase ID: FBst0051669) were obtained from the Bloomington Drosophila Stock Center (BDSC), the $PMCA^{2165R-3}$ *RNAi* line (stock ID: 2165R-3) was obtained from the Fly Stocks of National Institute of Genetics (Nig-Fly) and the PMCA-GFP line was obtained from Kyoto Drosophila Stock Center (exact genotype: *w1118; PBac(681.P.FSVS-1)PMCACPTI001995*, DGRC number: 115256). The *fkh-Gal4* fly line was provided by Eric H. Baehrecke (Berry & Baehrecke, 2007) (University of Massachusetts Medical School, Worcester, MA). Genotypes of Drosophila larvae used in generating data for Figure 7 are displayed in Supplementary Table 2.

Immunofluorescence

1. Immunocytochemistry of MCF-7 cells

Cells were seeded in removable 8-well chambers (80841, Ibidi) and 96 hours later rinsed with phosphate buffered saline (PBS) at 37°C, and fixed with 4% PFA diluted in PBS for 10 min. After rinsing twice and washing twice with PBS for 5 min, cells were permeabilized for 10 min. with 0.2% Triton X-100 diluted in PBS. After rinsing twice and washing twice with PBS, the cells were treated with Image-iT™ FX Signal Enhancer (I36933, Invitrogen) for 30 min to reduce non-specific binding of fluorophores. After rinsing twice with PBS, the samples were incubated in blocking solution containing 2 mg/ml bovine serum albumin (A7030, Sigma-Aldrich), 1% gelatin from cold water fish skin (G7765, Sigma-Aldrich), 0.1% Triton X-100 (X100PC, Sigma-Aldrich) and 5% goat serum (G9023, Sigma-Aldrich) diluted in PBS, for 1 hour at room temperature (RT). Samples were then incubated in the following primary antibodies diluted in blocking solution: anti-E-cadherin (24E10, CST 3195, Cell Signaling, dilution 1:200), anti-ezrin (E1281, Sigma-Aldrich, dilution 1:200), anti-Dlg1/SAP97 (sc-25661, Santa Cruz Biotechnology, dilution 1:100), anti-CD147/basigin (10186-R125, Sino Biological, dilution 1:200), or anti-HA (H3663, Sigma-Aldrich, dilution 1:500). After rinsing twice and washing twice for 5 min with PBS, the Alexa Fluor 594-conjugated goat anti-rabbit and anti-mouse IgG secondary antibodies (A-11012 and A-11005, Invitrogen, dilution 1:500)

were added. Samples were rinsed twice and washed twice with PBS for 5 min and the nucleus was labeled with DAPI (details in section *Reagents and treatments*). The silicon part of the chamber was then removed, and the samples were mounted with Ibidi Mounting Medium (50001, Ibidi).

2. Phalloidin staining of MCF-7 cells

Cells were seeded, grown, fixed, permeabilized and mounted as described in the previous section. Phalloidin-TRITC (P1951, Sigma-Aldrich) was added to the cells after permeabilization and incubated for 1 hour.

3. IHC staining of Drosophila larval salivary gland

Salivary glands were dissected from late L3 stage Drosophila larvae in PBS and pre-permeabilized for 20 sec in 0.05% Triton X-100 diluted in PBS. Salivary glands were then fixed for 40 min at room temperature in 4% PFA diluted in PBS, rinsed twice and washed twice with 0.1% Triton X-100 diluted in PBS (PBST) for 15 min and incubated for 40 min at room temperature (RT) in blocking solution that contained 10% fetal bovine serum and 0.1% Triton X-100 diluted in PBS; then salivary glands were incubated for 24 hours at 4°C with the following primary antibodies: anti-*pan*-PMCA (5F10, MABN1802, Sigma-Aldrich, dilution 1:250), anti-Dlg (4F3, Developmental Studies Hybridoma Bank, dilution 1:100), anti-GFP (A10262, Invitrogen, dilution 1:1000) diluted in blocking solution. After rinsing twice and washing twice the samples with PBST for 15 min, they were incubated with the secondary antibodies diluted in blocking solution: Alexa Fluor 488-conjugated goat anti-chicken IgY (A-11039, Invitrogen, dilution 1:1000) and Alexa Fluor 594-conjugated goat anti-mouse IgG (A-11020, Invitrogen, dilution 1:1000). After rinsing twice and washing twice the samples with PBST for 15 min, nuclei were labeled with DAPI (as in section *Reagents and treatments*) and samples were mounted with Vectashield (H-1000-10, Vector Laboratories).

4. Phalloidin staining of Drosophila larval salivary glands

Salivary glands were dissected, permeabilized and fixed the same way as described in the previous section. For F-actin labeling, the samples were incubated with

Rhodamine Phalloidin (details in section *Reagents and treatments*) for 1 hour, then washed, nuclei labeled with DAPI and mounted the same way as described for IHC.

Imaging

Fluorescent images were obtained with an Axio Imager M2 microscope (ZEISS) with an ApoTome2 grid confocal unit (ZEISS) using a Plan-Apochromat 63×/1.4 Oil (ZEISS) objective for human cells, a Plan-Apochromat 40×/0.95 Air (ZEISS) objective for semi-thin sections of Drosophila salivary glands, a Plan-Neofluar 20x/0.5 Air objective for mammospheres and Drosophila salivary glands with an Orca Flash 4.0 LT sCMOS camera (Hamamatsu Photonics) using the Efficient Navigation 2 software (ZEN 2) (ZEISS). Images in Figure 6A were obtained with LSM 710, AxioObserver confocal laser-scanning microscope (ZEISS) using a Plan-Apochromat 63×/1.4 Oil DIC M27 objective (ZEISS). Images in Figure 6D and Supplementary Figure 5C were obtained with a Nikon Eclipse Ti2 confocal microscope with a Plan-Apochromat 60×/1.4 Oil objective.

Growing and staining mammospheres

5000 cells from the parental, GFP-PMCA4b, GFP-PMCA4b^{LA} and PMCA4-specific shRNA expressing MCF-7 lines were mixed with cold Matrigel (Corning Matrigel, DLW356231, Sigma-Aldrich) and 15 µl of cell-Matrigel mixtures were added to removable 8-well chambers (80841, Ibidi) and incubated at 37 °C for Matrigel polymerization. After 15 min, FBS supplemented DMEM was added to the wells, and cells were grown for 10 days until they formed mature mammospheres. For F-actin staining the samples were washed with PBS at 37 °C, fixed for 40 min with 4% PFA diluted in PBS, permeabilized for 20 min with 0,2% Triton X-100 in PBS, and after washing TRITC-Phalloidin was added for F-actin staining (see further details in section *Reagents and treatments*). Samples were mounted with Ibidi Mounting Medium.

Transfection

GFP-PMCA4b-expressing MCF-7 ($5x10^4$) and HEK293 ($7x10^4$) cells were seeded in removable 8-well chambers (80841, Ibidi). Next day cells were transiently transfected

with the following plasmids: *pcDNA3/hArf6(WT)-mCherry* (Addgene plasmid #79422) and *pcDNA3/hArf6(Q67L)-HA* (Addgene plasmid # 79425) (the plasmids were a gift from Kazuhisa Nakayama (Makyio *et al*, 2012)) using the FuGENE HD Transfection Reagent (E2311, Promega) according to the manufacturer's instructions. 24 hours after transfection, cells were fixed, permeabilized, stained with DAPI and mounted. In the case of $Arf6^{Q67L}$ the cells were fixed and labeled with anti-HA antibody on the next day (details are the same as described in the section *Immunofluorescence*).

WGA uptake assay

$5x10^4$ cells from the parental, GFP-PMCA4b, GFP-$PMCA4b^{LA}$ and PMCA4 specific shRNA expressing MCF-7 lines were seeded in removable 8-well chambers (80841, Ibidi) and were grown for 5 days in FBS-supplemented DMEM. Cells were rinsed with cold (4°C) HBSS (Hanks′ Balanced Salt solution; H15-009, PAA Laboratories) twice and incubated with wheat germ agglutinin (WGA; W21404, Life Technologies) at a concentration of 5 µg/ml in HBSS for 10 min at room temperature. Unbound WGA was removed by rinsing the cells twice with PBS, the cells were then fixed, permeabilized and stained with DAPI, as described in the section *Immunofluorescence*.

Sulforhodamine B (SRB) Assay

MCF-7 cells at densities indicated in Supplementary Figure 2B were seeded in a 96-well plate and cultured for 96 hours. Then cells were fixed with 10% trichloroacetic acid at 4 °C for 1 hour and washed with distilled water. After air dry overnight, 0.4% SRB was added to the wells and incubated for 15 min at room temperature. Excess stain was removed by washing with 1% acetic acid solution, and the bound SRB dye was solubilized in 10 mM Tris base solution for 10 min at RT with agitation. Optical density was measured at 570 nm using a microplate reader (EL800, BioTek Instruments, Winooski, VT, USA).

Western blot analysis

The total proteins of MCF-7 and A375 cells were extracted by using the protocol described previously (Hegedus *et al.*, 2017b). *Drosophila melanogaster* third-instar larvae were collected, homogenized in Laemmli buffer and centrifuged to separate the protein containing supernatant. Equal amounts of proteins were loaded on 10% polyacrylamide gel, electrophoresed and electroblotted onto polyvinylidene difluoride (PVDF) membrane. Primary antibodies used for immunostaining were: anti-PMCA4 (JA9, P1494, Sigma-Aldrich, dilution 1:1000), anti-*pan*-PMCA (5F10, MABN1802, Sigma-Aldrich, dilution 1:2500), anti-E-cadherin (CST 3195, Cell Signaling, dilution 1:1000), anti-N-cadherin (sc-8424, Santa Cruz Biotechnology, dilution 1:500), anti-vimentin (M7020, Dako Products, dilution 1:1000), anti-β-actin (A1978, Sigma-Aldrich, dilution 1:2000), anti-Dlg1/SAP97 (sc-25661, Santa Cruz Biotechnology, dilution 1:1000), anti-CD147/basigin (10186-R125, Sino Biological, dilution 1:1000), anti-β-tubulin (ab6046, Abcam, dilution 1:1000). Horseradish peroxidase-conjugated secondary antibodies were used (715-035-151 and 711-035-152, Jackson ImmunoResearch, dilution 1:10,000) for detection with Pierce ECL Western Blotting Substrate (Thermo Scientific) and luminography on CL-XPosure Film (Thermo Scientific) or Amersham Hyperfilm ECL (GE Healthcare) films.

Transmission electron microscopy (TEM)

MCF-7 cells were grown in Matrigel as described above for 14 days to create mammospheres. After the medium was removed, mammospheres were washed with FBS-free DMEM and fixed in a 3.2% methanol free, ultrapure formaldehyde, 0.25% glutaraldehyde, 0.029 M sucrose, 0.002 M $CaCl_2$ and 0.1 M sodium cacodylate containing fixing solution for 24 hours at 4 °C. Samples were then washed for 2x5 min with 0.1 M sodium cacodylate, post-fixed in 1% ferrocyanide-reduced OsO_4 diluted in 0.1 M sodium cacodylate, pH 7.4, at 4 °C for 1 hour and washed again for 2x5 min with 0.1 M sodium cacodylate. For contrasting, samples were washed for 2x5 min with ultrapure distilled water and incubated in 1% uranyl-acetate diluted in distilled water for 30 min. Then samples were dehydrated by using a graded series of ethanol and were embedded in Spurr low viscosity epoxy resin (EM0300, Sigma Aldrich). 70-nm ultrathin sections were made with an Om U3 microtome (Reichert Austria), collected on copper

grids (AGG214, Agar Scientific) and stained in Reynold's lead citrate. For examination we used a transmission electron microscope (JEM-1011; JEOL, Tokyo, Japan) equipped with a digital camera (Morada; Olympus) using iTEM software (Olympus).

Production of semi-thin cross-sections of *Drosophila melanogaster* larvae

Salivary glands were dissected from late L3 stage Drosophila larvae then fixed in a 3.2% methanol free, ultrapure formaldehyde, 0.25% glutaraldehyde, 0.029 M sucrose, 0.002 M $CaCl_2$ and 0.1 M sodium cacodylate containing fixing solution for 24 hours at 4 °C. Samples were than immobilized in 1.5% agar dissolved in distilled water (this helps to keep orientation and integrity of the samples), and embedded in Spurr resin the same way as described in section *Transmission electron microscopy*. 0.5-1 µm semi-thin sections were made with an Om U3 microtome (Reichert Austria) and stained with a 1% toluidine blue, 1% Azure II, 1% borax and 25% sucrose containing solution.

Image analysis and statistics

IHC data were analyzed by Kruskal-Wallis and Mann-Whitney tests for comparison of quantitative variables by statistical software SPSS V.25 (IBM Corp.).To compare the prognostic impact of *ATP2B4* (PMCA4) and *ATP2B1* (PMCA1) expression in different breast carcinoma sub-types the publicly available database KM Plotter Online Tool (http://www.kmplot.com) was used. Only JetSet best probe set and best cutoff values were selected and Relapse Free Survival (RFS) plots were visualized by the Kaplan–Meier plotter using microarray data analysis (Lanczky & Gyorffy, 2021). Data for PMCA4 (NP_001675.3:S328) and PMCA1 (NP_001673.2:S1182) protein expression in normal and luminal breast cancer sub-types were derived from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) and analyzed by using the UALCAN web portal (Chandrashekar *et al.*, 2022) (https://ualcan.path.uab.edu/analysis-prot.html). Student's t test p-values were considered significant below 0.05.

To evaluate the number of E-cadherin- and Dlg1-positive vesicles, the fluorescent images from original, unmodified single focal planes were analyzed with the Image J software (Rueden *et al*, 2017). The fluorescence signal was set by auto threshold, transformed into binary format and particles were counted with the Analyze Particles

command, in which particle circularity was set between 0.5 and 1, and edges were excluded. Data were normalized to the total cell number determined by the nuclear dye DAPI image. Number of pre-lumens in 2D cell cultures and lumens in 3D cultures were quantified manually. To quantify the size of the lumens in Drosophila salivary glands, we measured the total area of the gland, as well as the lumen area in the cross-section images using ZEN 2 software, and their ratio was determined by dividing the lumen area with the total area of the gland. Data were imported into the GraphPad Prism 8.0 (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com) software and tested for normality of data distribution using D'Agostino & Pearson and Shapiro-Wilk tests. When data showed normal distribution with both tests, we used one-way ANOVA and Tukey's multiple comparisons tests; when data showed non-normal distribution we used Kruskal-Wallis and Dunn's multiple comparisons tests.

To define the ratio of polarized MCF-7 cells in a 2D cell cultures, the number of cells with either asymmetric or symmetric distribution of the apical marker ezrin was quantified manually in 16 fields of view for all cell lines. The total number of cells of each type was collected into contingency tables and data was analyzed by the Chi-square test.

To analyze planar distribution of WGA positive vesicles in MCF-7 cells we defined the center of nucleus with the Centroid command of the Image J software. The center of nucleus was then connected to the WGA-positive vesicles and the angle of the lines was defined by Image J. The obtained data were imported into an Excel table. Because Image J defines angles in a range of -180° to +180°, the data were converted into the range of 0° to 360°, and the angles were normalized to 180°. Then we calculated the mean values for each cell and determined their deviation from 180°. This deviation was added to the angles of each corresponding cell to get the final dataset. If the obtained value was higher than 360°, we subtracted 360 from it, if it was lower than 0° it was corrected by the addition of 360. Then we calculated the percentage of data in each bin for the whole datasets. We evaluated at least 15 cells of each cell type, and the data were copied to the Minitab (v.21) (https://www.minitab.com) followed by distribution analysis and histogram generation. Data were analyzed by the Levene’s probe using the

“Two sample variance test” for each combination of the groups as illustrated by a box plot diagram.

For *Homo sapiens* PMCA4b and *Drosophila melanogaster* PMCA amino acid sequence comparison the P23634-6 and Q9V4C7 sequences were used from UniProt (UniProt, 2023). Alignment was depicted and analyzed by the Jalview 2.11.3.1 software (Waterhouse *et al*, 2009) (Waterhouse 2009) using the “Percentage Identity” dynamic color scheme. Conservation values were automatically calculated in Jalview as described in the paper of Livingstone and Barton (Livingstone & Barton, 1993).

The number of evaluated images (n) and experiments are indicated in figure legends.

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Conflict of interests

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. PMCA4 is downregulated in HR^+ breast cancer sub-types. **A** PMCA4-specific antibody staining of normal and neoplastic LUMA, LUMB1 and LUMB2 sub-type mammary gland tissue samples. GR1 or 2 indicates grade 1 or grade 2. **B** Fraction of cancer cells showing positive membranous PMCA4 staining; $n_{(normal)}=4$ (obtained after breast reduction surgery), $n_{(LUMA)}=39$, $n_{(LUMB1)}=50$, $n_{(LUMB2)}=20$. **C** Protein expression of PMCA4b (NP_001675.3:S328) and PMCA1b (NP_001673.2:S1182) in normal breast tissue and luminal breast cancer sub-types. Data derived from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) (http://ualcan.path.uab.edu) that are expressed as z-values and analyzed by Student's t test; $n_{(PMCA4b/normal)}=18$, $n_{(PMCA4b/luminal)}=64$, $n_{(PMCA1b/normal)}=18$, $n_{(PMCA4b/luminal)}=64$; ***$p<0.001$, $^{ns}p>0.05$; ns indicates non-significant difference. **D** Kaplan–Meier relapse free survival analysis in breast cancer patients with LUMA, LUMB1 or LUMB2 sub-type tumors with low and high *ATP2B4*/PMCA4 and *ATP2B1*/PMCA1 expression levels. GR 2 indicates grade 2. Data were collected by the online survival analysis tool (http://www.kmplot.com) using microarray data analysis; $n_{(ATP2B4/LUMA\text{-}GR2/low)}=97$, $n_{(ATP2B4/LUMA\text{-}GR2/high)}=297$, $n_{(ATP2B4/LUMB1/low)}=99$, $n_{(ATP2B4/LUMB1/high)}=301$, $n_{(ATP2B4/LUMB2/low)}=150$, $n_{(ATP2B4/LUMB2/high)}=108$, $n_{(ATP2B1/LUMA\text{-}GR2/low)}=257$, $n_{(ATP2B1/LUMA\ GR2/high)}=254$, $n_{(ATP2B1/LUMB1/low)}=239$, $n_{(ATP2B1/LUMB1/high)}=161$, $n_{(ATP2B1/LUMB2/low)}=89$, $n_{(ATP2B1/LUMB2/high)}=169$.

Figure 2. Over-expression of PMCA4b promotes plasma membrane localization of E-cadherin. **A** Western blot analysis of epithelial (E-cadherin) and mesenchymal (N-cadherin, vimentin) marker proteins in PMCA4 specific shRNA (sh-PMCA4) expressing,

parental, GFP-PMCA4b and GFP-PMCA4b^{LA} expressing MCF-7 cell lines; exp. denotes time of exposure is the abbreviation of exposure. **B** E-cadherin immunostaining of PMCA4 specific shRNA (sh-PMCA4), GFP-PMCA4b and GFP-PMCA4b^{LA} expressing MCF-7 cell lines. DAPI (4′, 6-diamidino-2-phenylindole, blue) labels nuclei. **C** Outline of the E-cadherin positive cell compartments generated by the Image J software. **D** Fluorescence intensity profiles of E-cadherin and GFP-PMCA4b corresponding to the white line shown in B. **E** Statistical analysis of the number of E-cadherin positive particles in PMCA4 specific shRNA (sh-PMCA4) expressing, parental, GFP-PMCA4b and GFP-PMCA4b^{LA} expressing MCF-7 cell lines. Graph displays means with 95% confidence intervals (CI); data were collected from 2 independent experiments, $n_{(sh-PMCA4)}$=17, $n_{(MCF-7)}$=13, $n_{(PMCA4b)}$=13, $n_{(PMCA4b^{LA})}$=16. Data were analyzed with Kruskal-Wallis and Dunn's multiple comparisons tests, adjusted p values: **p<0.01, ***p<0.001. Non-significant differences are not indicated.

Figure 3. PMCA4b promotes plasma membrane localization of Dlg1 and cell polarization. **A** Model of the interaction between PMCA4b and the Scrib-Dlg1-Lgl polarity complex. PDZb denotes the PDZ-binding sequence motif of PMCA4b. **B** Western blot analysis of Dlg1 in PMCA4-specific shRNA (sh-PMCA4) expressing, parental, GFP-PMCA4b- and GFP-PMCA4b^{LA}-expressing MCF-7 cell lines. **C** Immunostaining of Dlg1 in GFP-PMCA4b-expressing MCF-7 cell line. Insets show magnification of the area framed by the dotted line. DAPI labels nuclei. **D** Immunostaining of Dlg1 in PMCA4 specific shRNA (sh-PMCA4) and GFP-PMCA4b expressing MCF-7 cell lines. DAPI labels nuclei. **E** Outline of the Dlg1 positive cell compartments generated by the ImageJ software. **F** Fluorescence intensity profiles of Dlg1 and GFP-PMCA4b corresponding to the white line shown on panel D. **G** Statistical analysis of the number of Dlg1 positive particles in PMCA4-specific shRNA (sh-PMCA4) expressing, parental and GFP-PMCA4b-expressing MCF-7 cell lines. Graph displays means with 95% CI; data were collected from 2 independent experiments, $n_{(sh-PMCA4)}$=20 $n_{(MCF-7)}$=15, $n_{(PMCA4b)}$=17; data were analyzed with Kruskal-Wallis and Dunn's multiple comparisons tests; adjusted p values: ^{ns}p>0.05, ***p<0.001; ns indicates non-significant difference. **H** Western blot analysis of ezrin in PMCA4 specific shRNA (sh-PMCA4) expressing, parental, GFP-

PMCA4b- and GFP-PMCA4b^{LA}-expressing MCF-7 cell lines. **I** Ezrin immunostaining on PMCA4-specific shRNA (sh-PMCA4), GFP-PMCA4b- and GFP-PMCA4b^{LA}-expressing MCF-7 cell lines. DAPI labels nuclei. **J** Fluorescence intensity profiles of ezrin and GFP-PMCA4b corresponding to the white lines shown on panel I. **K** Statistical analysis of cell polarization based on ezrin distribution in PMCA4-specific shRNA (sh-PMCA4) expressing, parental, GFP-PMCA4b- and GFP-PMCA4b^{LA}-expressing MCF-7 cell lines. Data were collected from 16 fields of view from 2 independent experiments; $n_{(sh-PMCA4)}$=1699, $n_{(MCF-7)}$=1589, $n_{(PMCA4b)}$=1462, $n_{(PMCA4b^{LA})}$=1511. Data were analyzed with Chi-square test, p values: **p<0.01, ***p<0.001.

Figure 4. PMCA4b promotes polarized vesicular trafficking. **A** WGA uptake assay was performed on PMCA4-specific shRNA (sh-PMCA4), GFP-PMCA4b- and GFP-PMCA4b^{LA}-expressing MCF-7 cell lines. After 10 minutes incubation with WGA, cells were washed and transferred to a 37 °C incubator for 20 minutes. During this time frame WGA positive endocytic vesicles were collected in the cytosol. Dashed white rectangles indicate areas for magnification on D. DAPI labels nuclei. **B** The yellow lines connect the center of the cell nucleus with WGA positive endocytic vesicles accumulated in the cytosol. **C** Intracellular distribution analysis of WGA positive vesicles. Red line indicates the distribution fit curve. Data were collected from 2 independent experiments, $n_{(sh-PMCA4)}$=15, $n_{(PMCA4b)}$=28, $n_{(PMCA4b^{LA})}$=21, and analyzed as described in the Methods section. Statistical analysis is shown in *Supplementary Fig. 3D*. **D** Magnification of the areas indicated by the dashed white rectangles panel A. Arrows indicate colocalized WGA and PMCA4b positive vesicles.

Figure 5. Arf6 regulates endocytic recycling of PMCA4b. **A** Western blot analysis of PMCA4 specific shRNA (sh-PMCA4) expressing, parental, GFP-PMCA4b and GFP-PMCA4b^{LA} expressing MCF-7 cell lines. **B** CD147 immunostaining of PMCA4 specific shRNA (sh-PMCA4) and GFP-PMCA4b expressing MCF-7 cell lines. DAPI labels nuclei. **C** Fluorescence intensity profiles of CD147 and PMCA4b corresponding to the white lines shown in panel B. **D** CD147 immunostaining of control and NAV2729-treated GFP-PMCA4b-expressing MCF-7 cell lines. Insets show magnified areas indicated by the

dashed white rectangles. **E-F** Transfection of GFP-PMCA4b-expressing MCF-7 and HEK293 cell lines with wild-type (WT) and Q67L mutant form of Arf6. **G** A scheme depicting the role of Arf6 in PMCA4b trafficking. RE: recycling endosome.

Figure 6. PMCA4b promotes lumen formation in 2D and 3D cultures of MCF-7 cells. **A** Phalloidin staining of PMCA4 specific shRNA (sh-PMCA4), GFP-PMCA4b and GFP-PMCA4b^{LA} expressing MCF-7 cell lines. White and yellow arrowheads point to pre-lumens. **B** A schematic figure of a typical pre-lumen structure in a 2D cell culture. **C** Magnification of the area indicated by the dashed white rectangle on panel A. **D** Phalloidin staining of control and NAV2729 treated PMCA4 specific shRNA (sh-PMCA4) and GFP-PMCA4b-expressing MCF-7 cell lines. Scaled-down insets show the GFP-PMCA4b signal. The yellow arrowhead points to a pre-lumen. **E** Statistical analysis of pre-lumen formation in control and NAV2729 treated PMCA4 specific shRNA (sh-PMCA4) expressing, parental, GFP-PMCA4b and GFP-PMCA4b^{LA} expressing MCF-7 cell lines. Graph displays means with 95% CI corrected with the total area of the images; data were collected from 2 independent experiments, $n_{(sh\text{-}PMCA4)}$=23, $n_{(sh\text{-}PMCA4+NAV2729)}$=21, $n_{(MCF\text{-}7)}$=26, $n_{(MCF\text{-}7+NAV2729)}$=22, $n_{(PMCA4b)}$=25, $n_{(PMCA4b+NAV2729)}$=23, $n_{(PMCA4b^{LA})}$=23, $n_{(PMCA4b^{LA}+NAV2729)}$=17. Data were analyzed with Kruskal-Wallis and Dunn's multiple comparisons tests, adjusted p values: $^{ns}p\geq0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$; ns indicates non-significant difference. **F** Phalloidin staining of mammospheres formed by PMCA4-specific shRNA (sh-PMCA4), GFPPMCA4b and GFP-PMCA4b^{LA} expressing MCF-7 cell lines cultured in Matrigel for 10 days. **G** Schematic figures demonstrating structure of mammospheres created by PMCA4-specific shRNA (sh-PMCA4), GFP-PMCA4b and GFP-PMCA4b^{LA} expressing MCF-7 cell lines. **H** Statistical comparisons of the ratios of mammospheres with central lumen in 3D cultures between PMCA4-specific shRNA (sh-PMCA4) expressing, parental, GFP-PMCA4b- and GFP-PMCA4b^{LA}-expressing MCF-7 cell lines cultured in Matrigel. Graph displays means with 95% CI, *n*=8 images with 10-15 mammospheres per image for each cell type. Data were analyzed with ordinary one-way ANOVA and Tukey's multiple comparisons tests, adjusted p values: $^{ns}p\geq0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, non-significant differences are not labeled. **I** Transmission electron microscope images of 14 days old

mammospheres formed by PMCA4-specific shRNA (sh-PMCA4), GFP-PMCA4b and GFP-PMCA4b^{LA} expressing MCF-7 cell lines cultured in Matrigel. Insets show magnified areas indicated by the dashed black rectangles.

Figure 7. *Drosophila melanogaster* (dm)PMCA regulates lumen size and morphology in larval salivary gland. **A** Western blot analysis of dmPMCA expression in control and ubiquitous *daughterless* promoter-driven dmPMCA-specific RNAi expressing 3rd stage *Drosophila melanogaster* larvae. 5F10 anti-human pan-PMCA antibody was used to stain dmPMCA. **B** Immunostaining of dmPMCA in different planes of salivary gland of 3rd stage larvae. **C** Immunostaining of *Drosophila melanogaster* Dlg and dmPMCA in salivary gland of 3rd stage larvae. **D** Phalloidin staining of control and salivary gland specific *forkhead* promoter-driven dmPMCA RNAi expressing 3rd stage larvae. Images in the lower row show distal parts of the lumen in the same salivary gland as above using higher magnification. **E** Toluidine blue-stained semi-thin cross-sections of salivary glands from 3rd stage control and *forkhead* promoter-driven dmPMCA specific RNAi expressing larvae. The lumen is outlined by red lines. **F** Statistical comparisons of the lumen area ratios on semi-thin cross-sections shown on panel E. Graph displays means with 95% CI, n=10. Data were analyzed with Kruskal-Wallis and Dunn's multiple comparisons tests, adjusted p values: $^{ns}p \geq 0.05$, $^{**}p < 0.01$; ns indicates non-significant difference. **G** Transmission electron microscope images from cross-sections of salivary gland of control and salivary gland specific *forkhead* promoter-driven dmPMCA RNAi expressing 3rd stage larvae. Magnified areas indicated by the dashed black rectangles are shown on the right. The lumen is outlined by red line. L: lumen.

Figure 8. A model on the role of PMCA4b in luminal epithelial cell polarization. Polarized PMCA4b expressing cell lines maintain stable cell-cell contacts through E-cadherin at adherent junctions. PMCA4b interacts with Dlg1 at the lateral plasma membrane suggesting a potential role in the recruitment of the Scribble polarity complex. In PMCA4b expressing cell lines ezrin is localized to the apical plasma membrane where it interacts with cortical F-actin. Furthermore, PMCA4b displays intensive intracellular trafficking involved in maintaining epithelial cell polarity, lumen morphology and

secretion activity of glandular tissues. PMCA4b loss during cancer progression can result in the loss of polarized epithelial cell phenotype associated with enhanced E-cadherin, Dlg1 and ezrin mislocalization and thus the loss of luminal structures.

Supplementary figure legends

Supplementary Figure 1. Kaplan–Meier relapse free survival analysis in breast cancer patients with LUMA sub-type tumors with low and high *ATP2B4*/PMCA4 and *ATP2B1*/PMCA1 expression levels. GR1 and GR3 indicate grade 2 and 3. Data were collected by the online survival analysis tool (http://www.kmplot.com) using microarray data analysis; p values and the sample sizes are indicated in the figure.

Supplementary Figure 2. MCF-7 cells display epithelial characteristics. **A** Western blot analysis of epithelial (E-cadherin) and mesenchymal marker (N-cadherin, vimentin) proteins in mesenchymal-type A375 cells, and in epithelial-type PMCA4 specific shRNA (sh-PMCA4) expressing, parental, GFP-PMCA4b and GFP-PMCA4b^{LA} expressing MCF-7 cell lines. A375 cells were used for N-cadherin and vimentin validation. **B** Cell density analysis of PMCA4 specific shRNA (sh-PMCA4) expressing, parental, GFP-PMCA4b and GFP-PMCA4b^{LA} expressing MCF-7 cell lines by using sulforhodamine B assay. **C** E-cadherin immunostaining of parental MCF-7 cells. **D** Outline of the E-cadherin-positive cell compartments generated by the Image J software. **E** Fluorescence intensity profiles of E-cadherin and GFP-PMCA4b across the line shown in C.

Supplementary Figure 3. Dlg1 and ezrin localization in MCF-7 cells. **A** Immunostaining of Dlg1 in parental MCF-7 cell line. DAPI (4′, 6-diamidino-2-phenylindole) labels nuclei. **B** Outline of the Dlg1-positive cell compartments generated by the Image J software. **C** Fluorescence intensity profiles of Dlg1 and GFP-PMCA4b across the line shown in A. **D** Ezrin immunostaining of parental MCF-7 cells. **E** Fluorescence intensity profiles of ezrin and GFP-PMCA4b across the lines shown in D.

Supplementary Figure 4. Endosomal vesicle trafficking is only slightly polarized in parental MCF-7 cells. **A** WGA uptake assay was performed on MCF-7 cells. After 10

minutes incubation in WGA, cells were washed and transferred to a 37 °C incubator for 20 minutes. During this time frame WGA positive endocytic vesicles were collected in the cytosol. DAPI labels nuclei. **B** The yellow lines connect the center of the cell nucleus with WGA positive endocytic vesicles accumulated in the cytosol. **C** Cellular distribution analysis of WGA positive vesicles. Red line indicates the distribution fit curve. Data were collected from 2 independent experiments, and analyzed as described in the Methods section. **D** Statistical analysis of the intracellular distribution of WGA vesicles as illustrated by a box plot diagram. Data were collected from 2 independent experiments ($n_{(sh\text{-}PMCA4)}$=15, $n_{(MCF\text{-}7)}$=15, $n_{(PMCA4b)}$=28, $n_{(PMCA4b^{LA})}$=21) and analyzed with Levene's test. P values: **P<0.01, ***P <0.001. No significant difference was found between shPMCA4 and PMCA4b^{LA}.

Supplementary Figure 5. Pre-lumen formation in 2D culture of MCF-7 cells. **A** Phalloidin staining of parental MCF-7 cells. Arrow points to a pre-lumen. **B** WGA uptake assay on GFP-PMCA4b-expressing MCF-7 cells with 20 minutes incubation after WGA removal. Pre-lumen creating cells are encircled with yellow line. **C** Phalloidin staining of control and NAV2729-treated parental and GFP-PMCA4b^{LA}-expressing MCF7-cells. Scaled-down insets show GFP-PMCA4b^{LA} signal. Arrow points to a pre-lumen.

Supplementary Figure 6. Lumen formation in MCF-7 mammospheres. **A-D** Z-stack images of PMCA4 specific shRNA (sh-PMCA4) expressing, parental MCF-7, GFP-PMCA4b and GFP-PMCA4b^{LA} expressing MCF-7 cells that were grew in Matrigel for 10 days and stained with phalloidin. The range is 1.25 µm between slices. **E** Transmission electron microscope image of a 14 days old mammosphere formed by parental MCF-7. Inset shows magnified area framed by dotted line.

Supplementary Figure 7. Amino acid sequence comparison of human PMCA4b and *Drosophila melanogaster* PMCA sequences. **A** Alignment of human PMCA4b and *Drosophila melanogaster* (dm)PMCA amino acid sequences using the Jalview software with the region recognized by the 5F10 antibody (located 719-738 in the human amino acid sequence) marked with red line. Purple colors indicate the rate of similarities

between amino acids in the same position (ranges are indicated at right) with Percentage Identity Coloring in Jalview. Conservation row in yellow and brown shows conservation rate based on physico-chemical properties. Conserved columns are indicated by asterisks and columns with conserved changes are indicated by "+". **B** Multiple sequence alignment of the C termini of dmPMCA isoforms (black) and human PMCA4b (yellow) by CLUSTAL O (1.2.4). Red color labels the PDZ-binding sequence of human PMCA4b, blue color labels Drosophila sequences with only 1 and green color labels 2 amino acid difference from human sequence.

Supplementary Table 1. Clinicopathological characteristics of the breast cancer patients' samples. ER (estrogen receptor), PgR (progesterone receptor), HER2 (human erb-b2 receptor tyrosine kinase 2) status, and Ki67 (marker of proliferation Ki67; low means below 5%) index were evaluated by immunohistochemistry. The following data were taken into consideration: age at diagnosis, histological grade, pathologic tumor size (pT), nodal involvement (pN), surrogate breast carcinoma sub-types. T1: the tumor in the breast is 20 millimeters (mm) or smaller in size at its widest area; T2: the tumor is larger than 20 mm but not larger than 50 mm. N0: no cancer was found in the lymph nodes; N1: metastatic lymph nodes. Non-parametric statistical analysis was performed using Kruskal-Wallis and Mann-Whitney tests for comparison of quantitative variables. Statistical significance was defined at $p<0.05$.

Supplementary Table 2. Accurate genotypes of *Drosophila melanogaster* larvae on Figure 7.

Tables

Supplementary Table 1: Clinicopathological characteristics of the breast cancer patients' samples

		PMCA4			
		Low	High	Total	P values
ER	Positive	83	26	109	-
PR	Negative	22	6	28	0.727
	Positive	61	20	81	
	Total	83	26	109	
Ki67	Low	56	13	69	0.107
	High	27	13	40	
	Total	83	26	109	
HER2+	Negative	71	17	88	0.048
	Positive	12	8	20	
	Total	83	25	108	
Sub-types	LUMA	30	9	39	0.142
	LUMB1	41	9	50	
	LUMB2	12	8	20	
	Total	83	26	109	
Age	Mean	57.54	61.27		0.186
	SD	12.59	12		

	Range	27-80	44-83		
	Total	83	26	109	
Grade	1	23	6	29	0.519
	2	41	10	51	
	3	18	8	26	
	Total	82	24	106	
pT	1	49	13	62	0.492
	2	33	12	45	
	Total	82	25	107	
pN	0	49	14	63	0.923
	1	33	9	42	
	Total	82	23	105	
Metastatic	Yes	13	3	16	0.621
	No	68	22	90	
	Total	81	25	106	

Supplementary Table 2: Accurate genotypes of *Drosophila melanogaster* larvae on Figure 7

Panel	Genotype
A	*da-Gal4 / w^{1118}*
	da-Gal4 / $PMCA^{2165R-3}$
B	*w^{1118}* (homozygous)
C	*w^{1118}; PBac(681.P.FSVS-1)PMCACPTI001995 (PMCA-GFP)* (homozygous)
D-G	*fkh-Gal4 / w^{1118}*
	fkh-Gal4 / $PMCA^{2165R-3}$

Figure 1: PMCA4 is downregulated in HR^{+} breast cancer sub-types

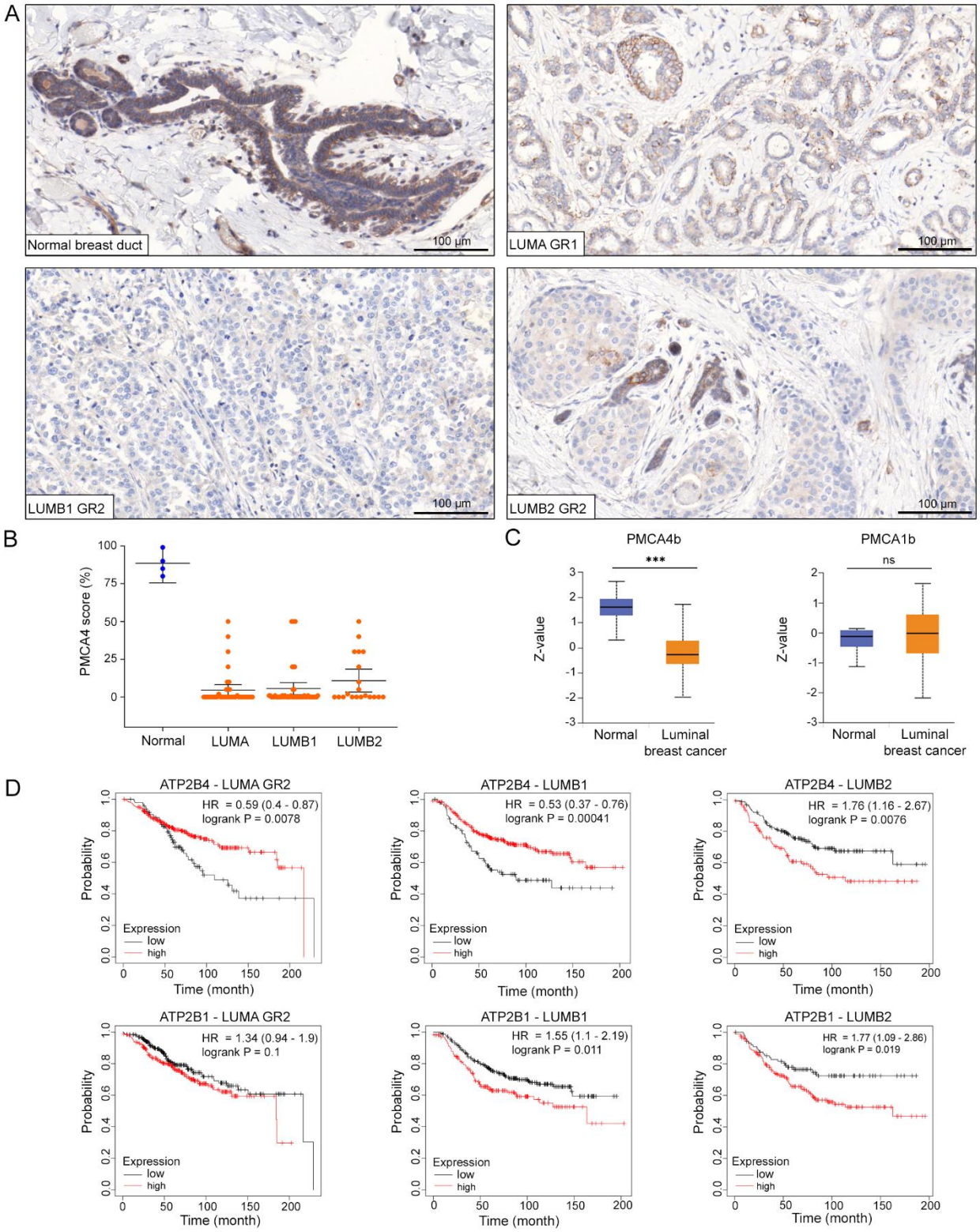


Figure 2: Over-expression of PMCA4b promotes plasma membrane localization of E-cadherin

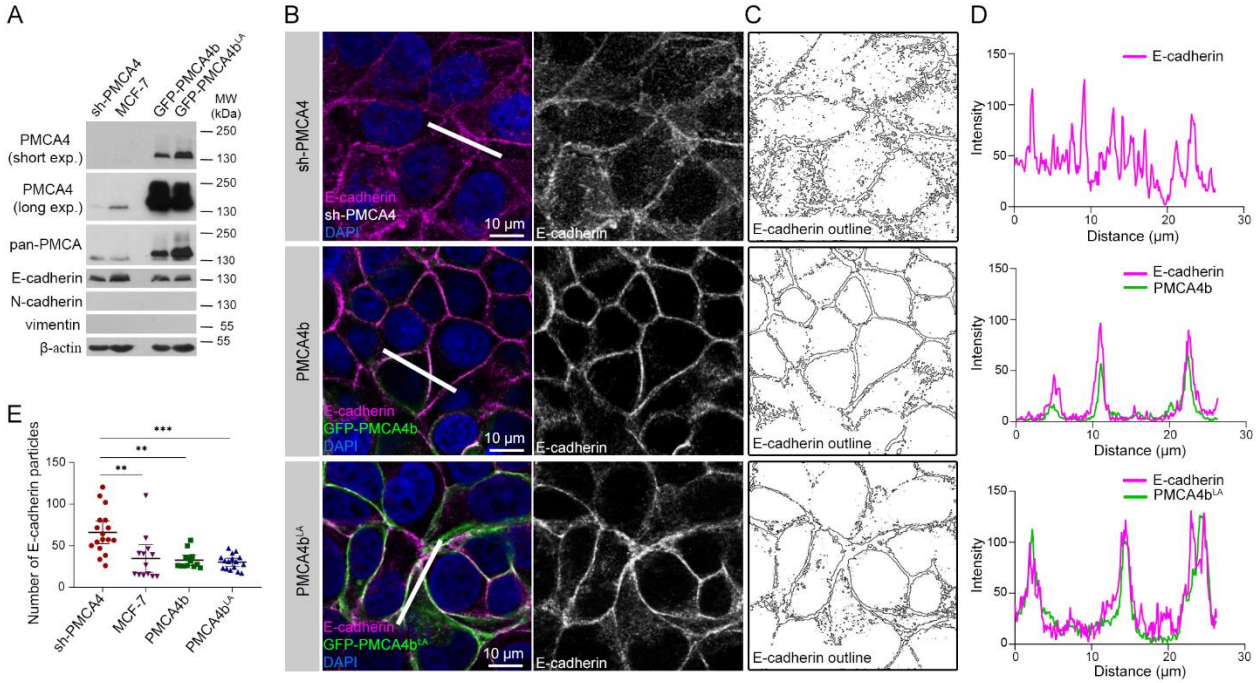


Figure 3: PMCA4b promotes plasma membrane localization of Dlg1 and cell polarization

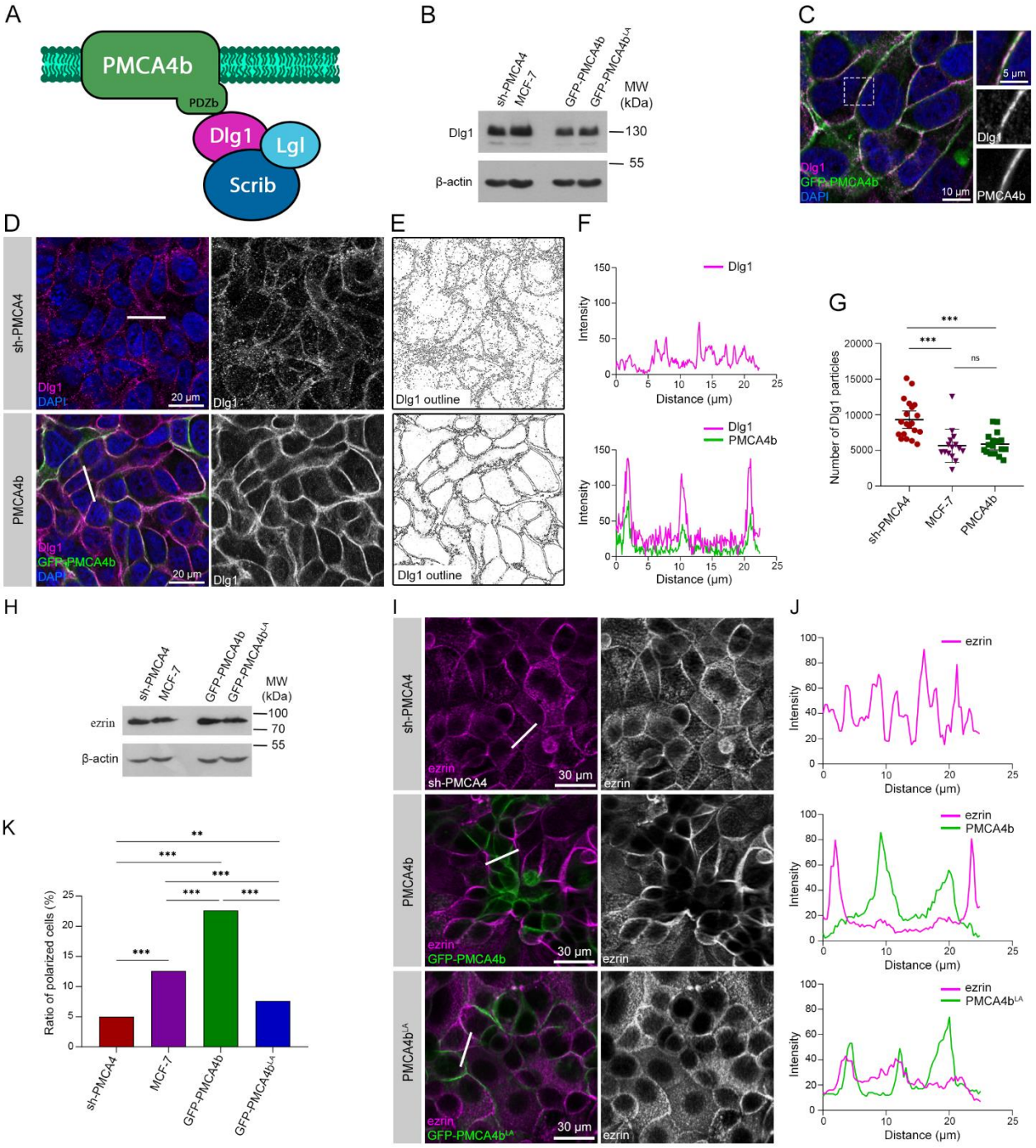


Figure 4: PMCA4b promotes polarized vesicular trafficking

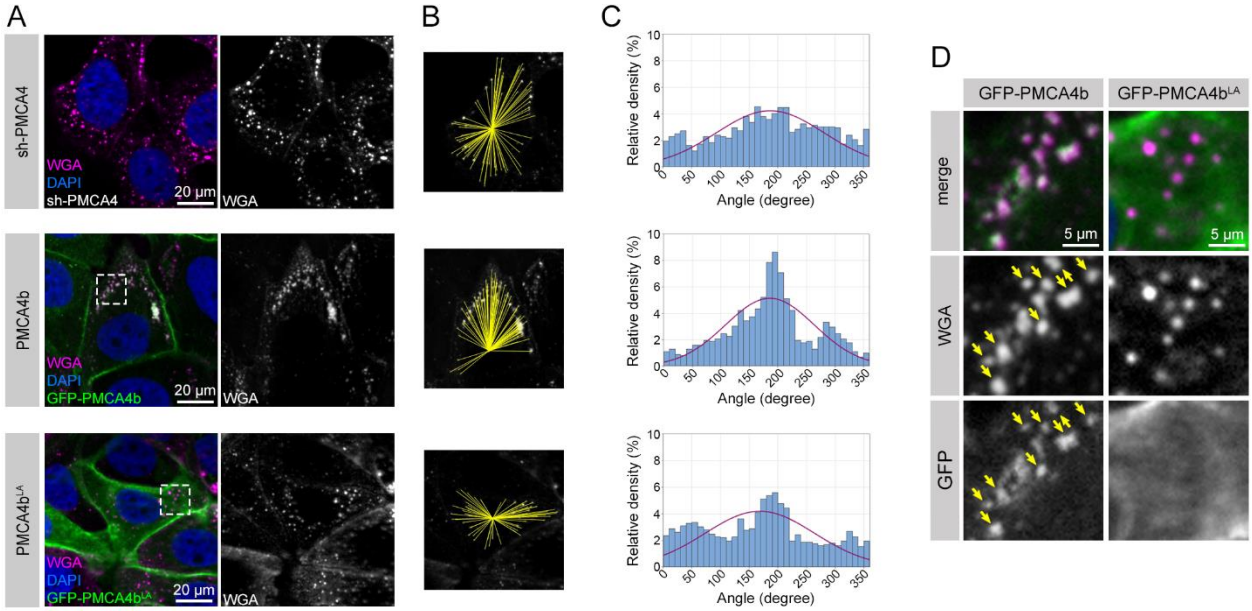


Figure 5: Arf6 regulates endocytic recycling of PMCA4b

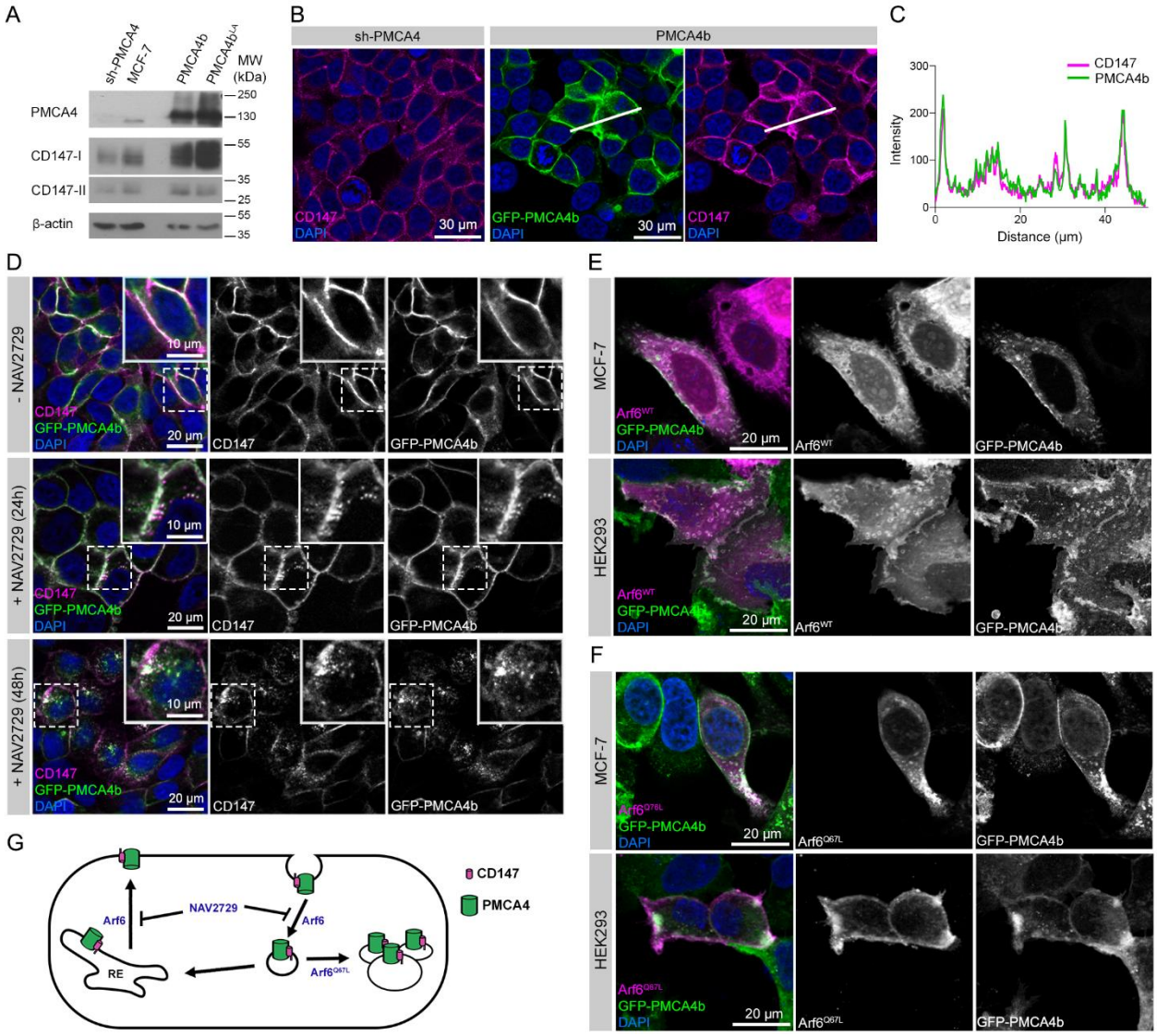


Figure 6: PMCA4b promotes lumen formation in 2D and 3D cultures of MCF-7 cells

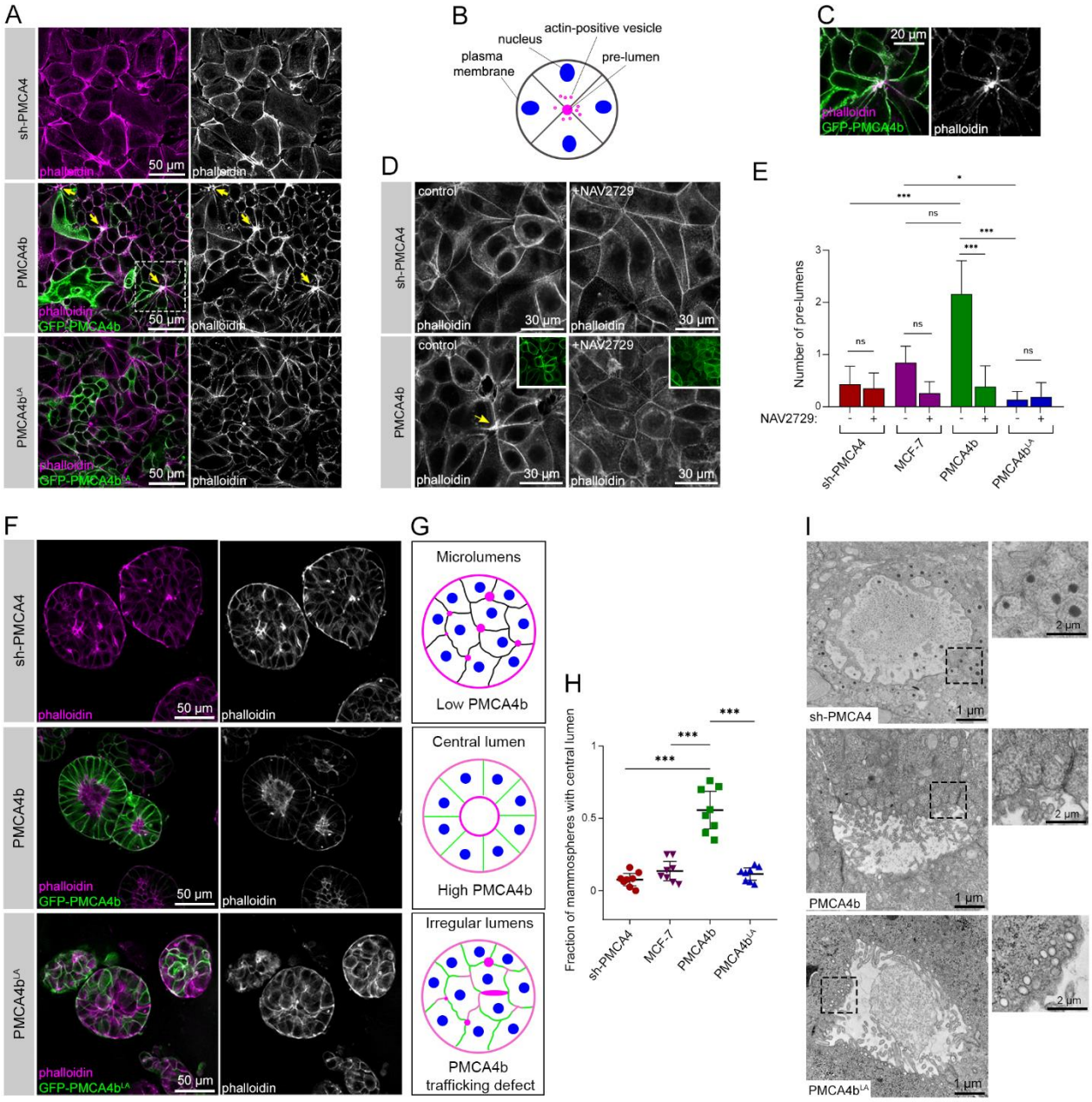


Figure 7: *Drosophila melanogaster* (dm)PMCA regulates lumen size and morphology in larval salivary gland

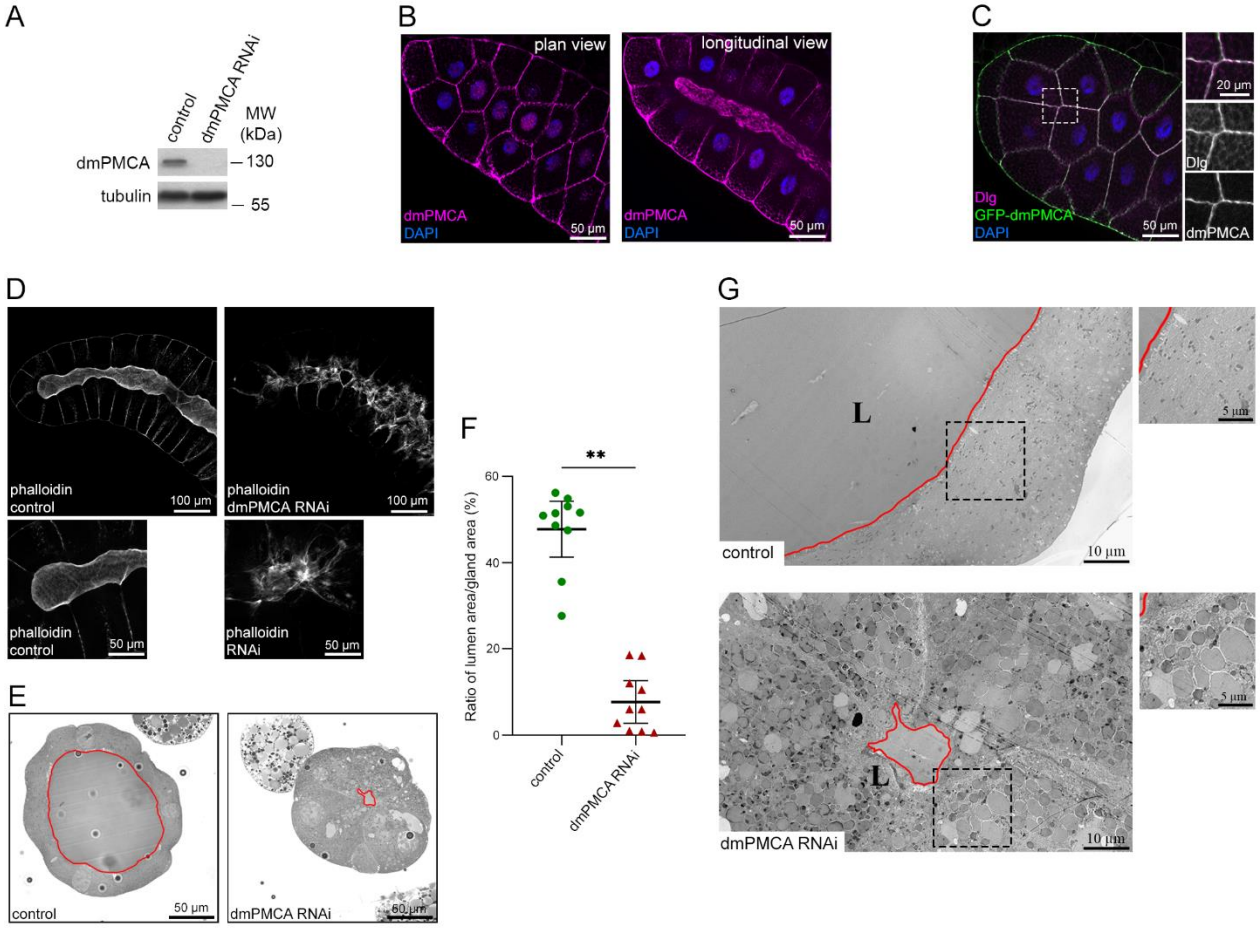
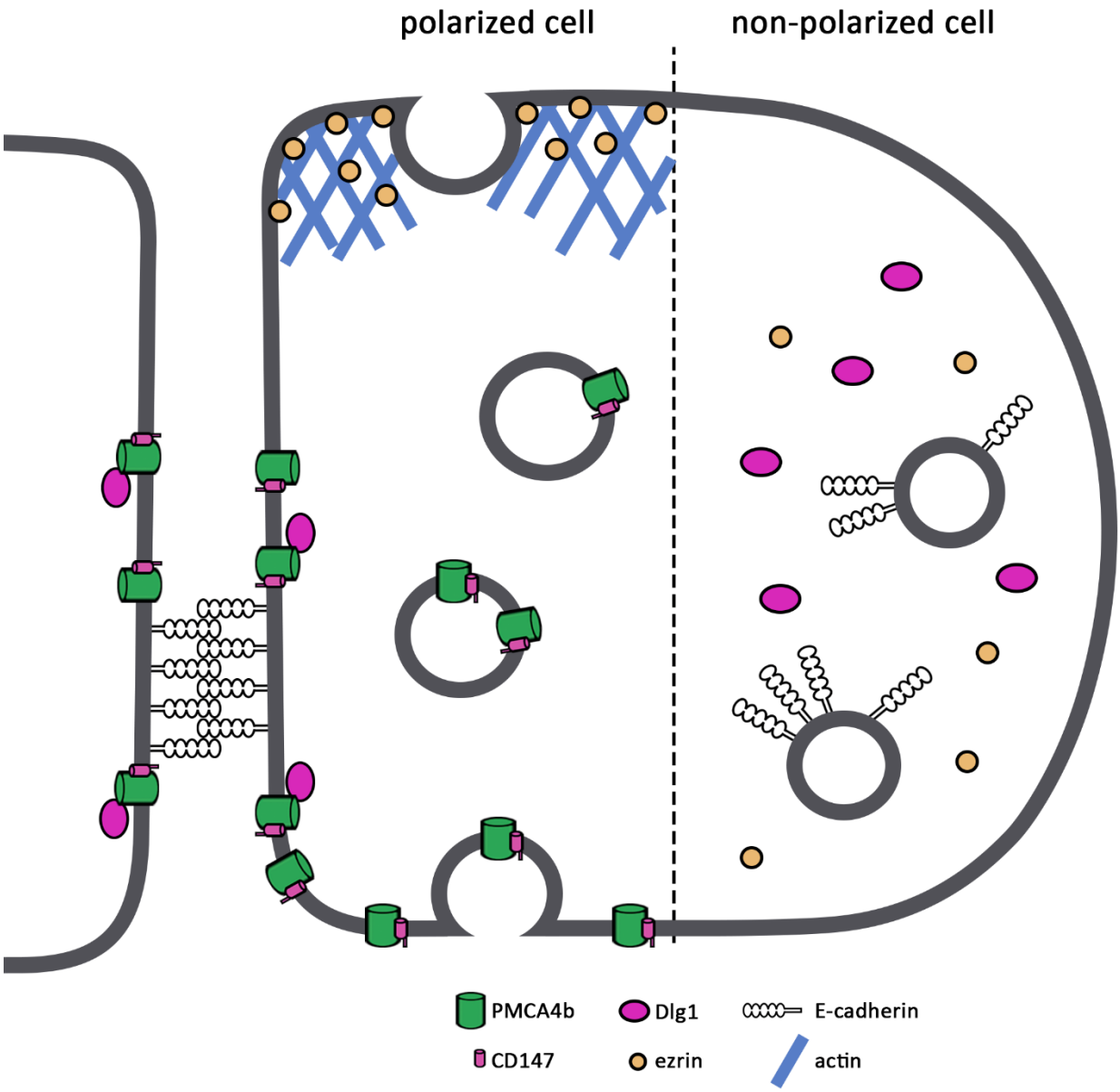
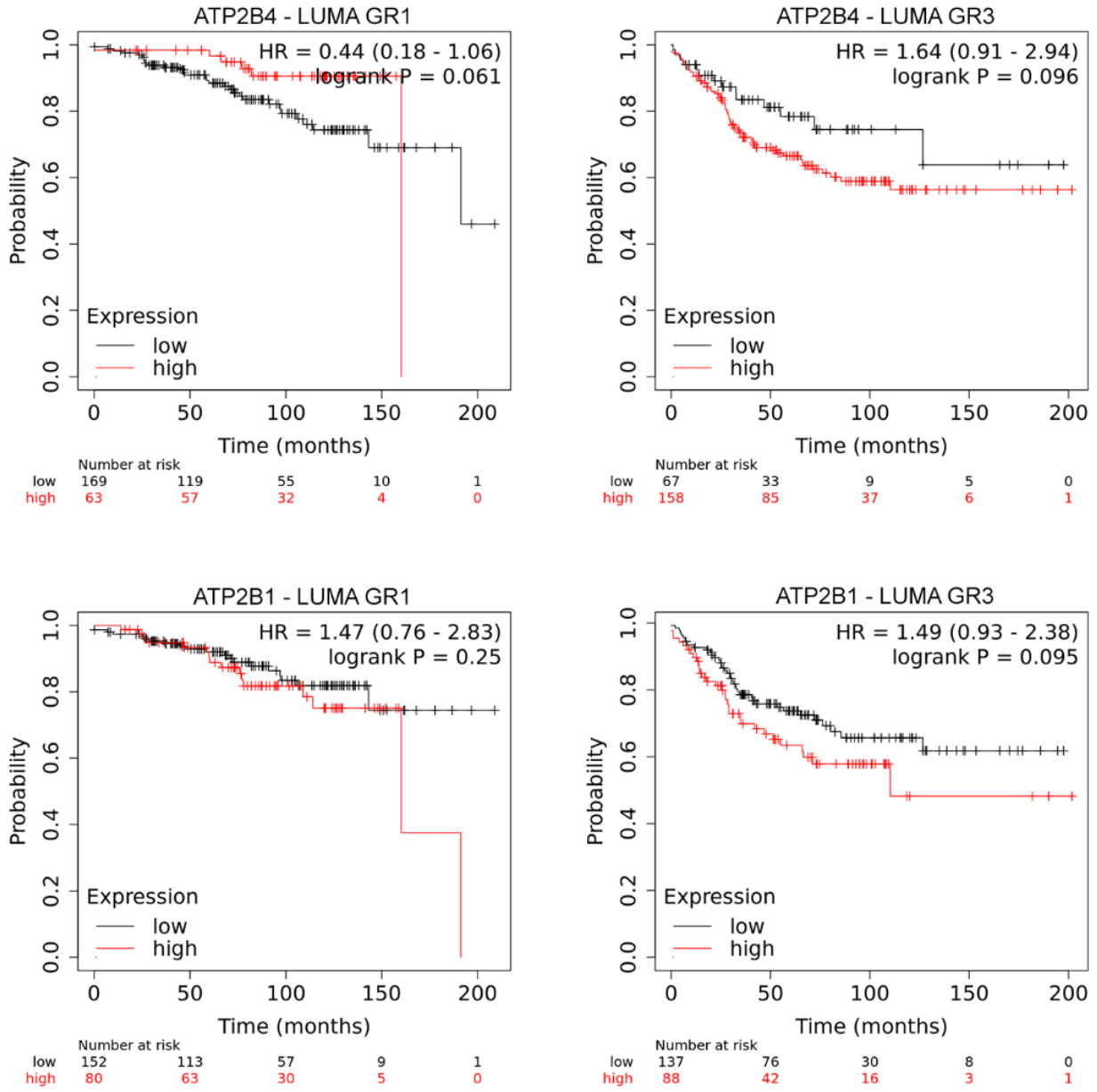


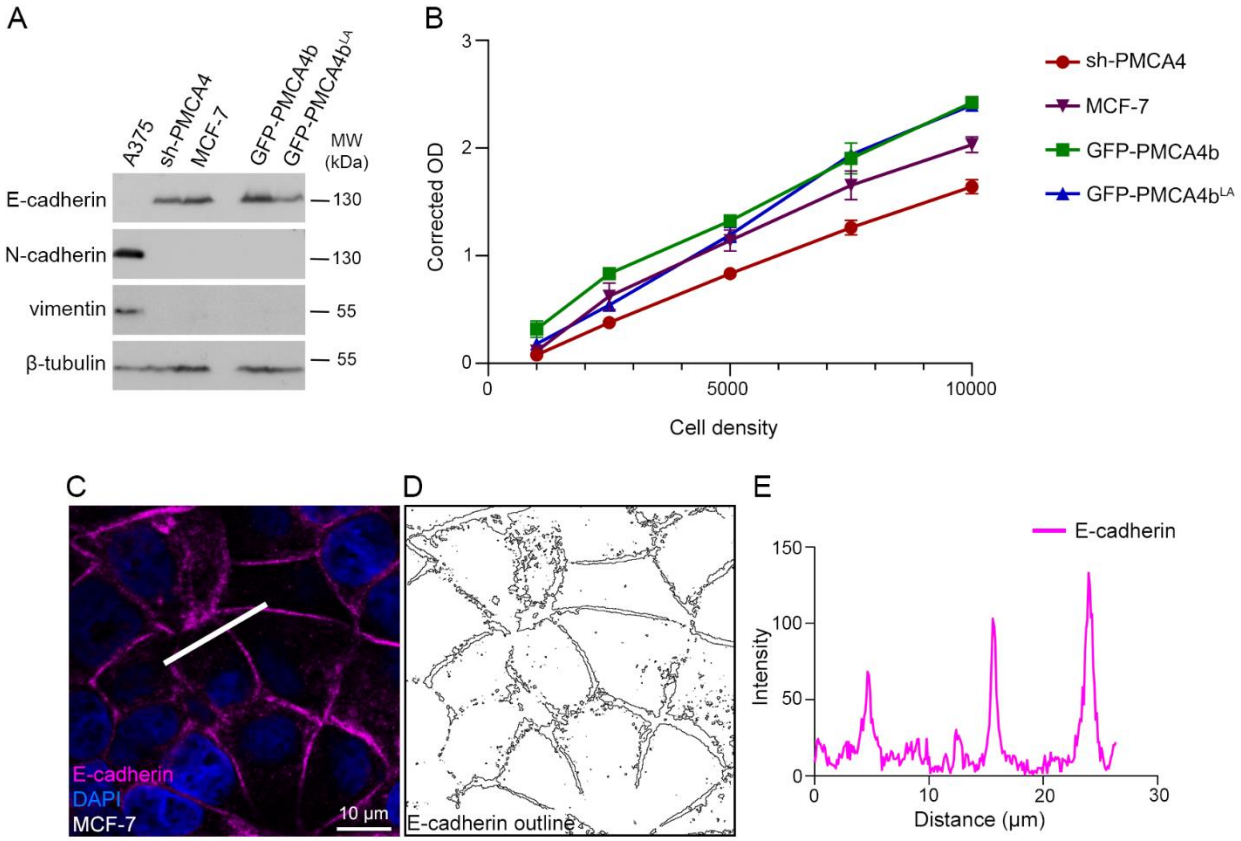
Figure 8: A model on the role of PMCA4b in luminal epithelial cell polarization



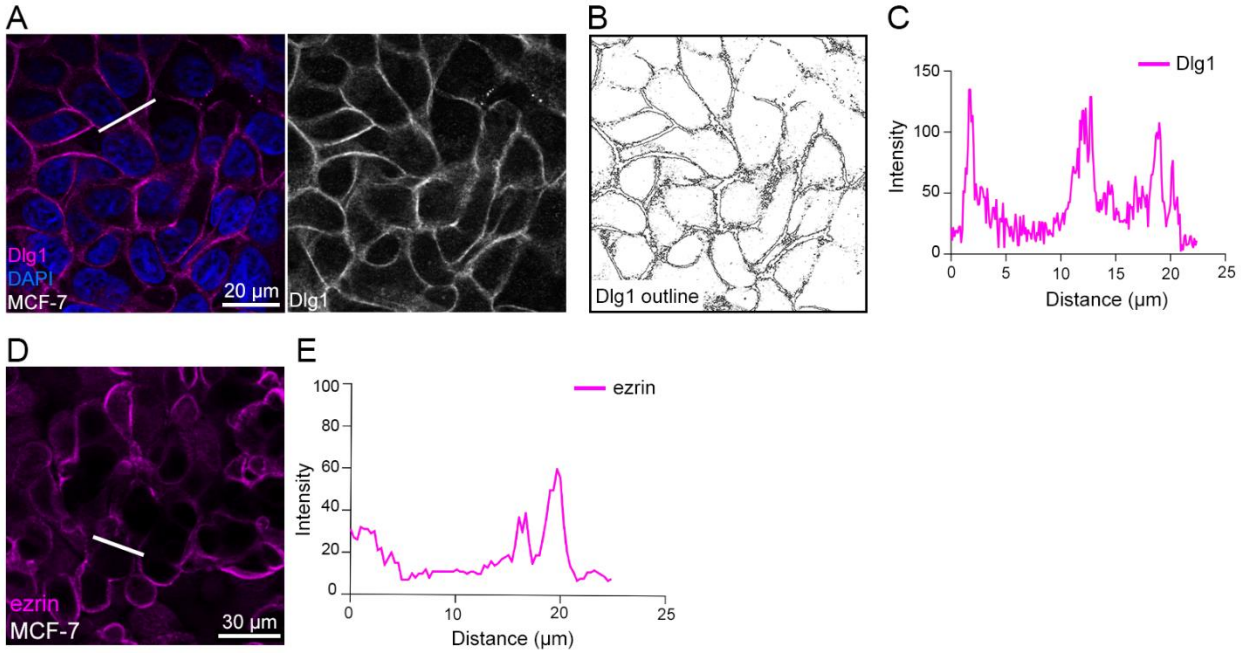
Supplementary Figure 1: Kaplan–Meier relapse free survival analysis in breast cancer patients with LUMA sub-type tumors with low and high *ATP2B4*/PMCA4 and *ATP2B1*/PMCA1 expression levels



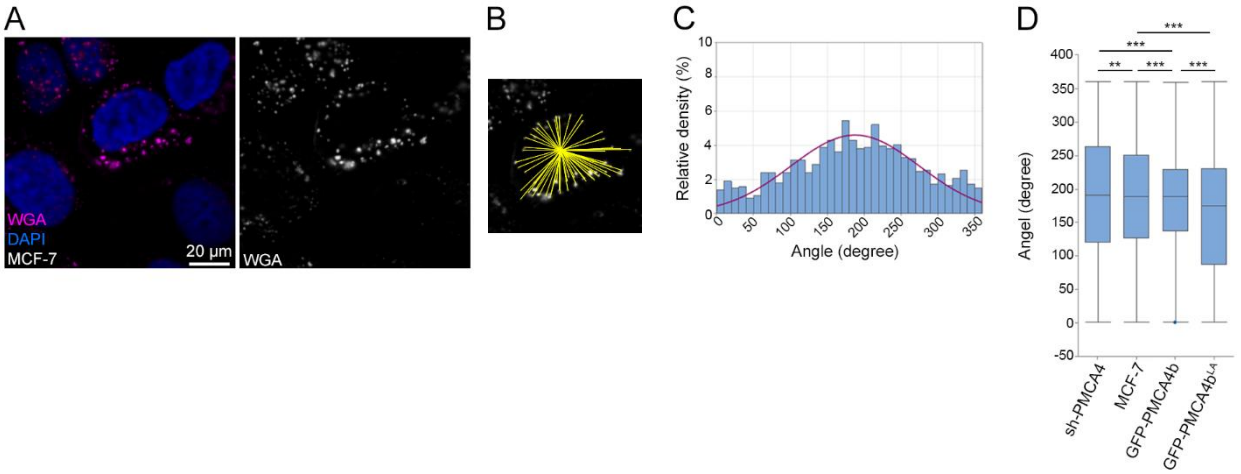
Supplementary Figure 2: MCF-7 cells display epithelial characteristics



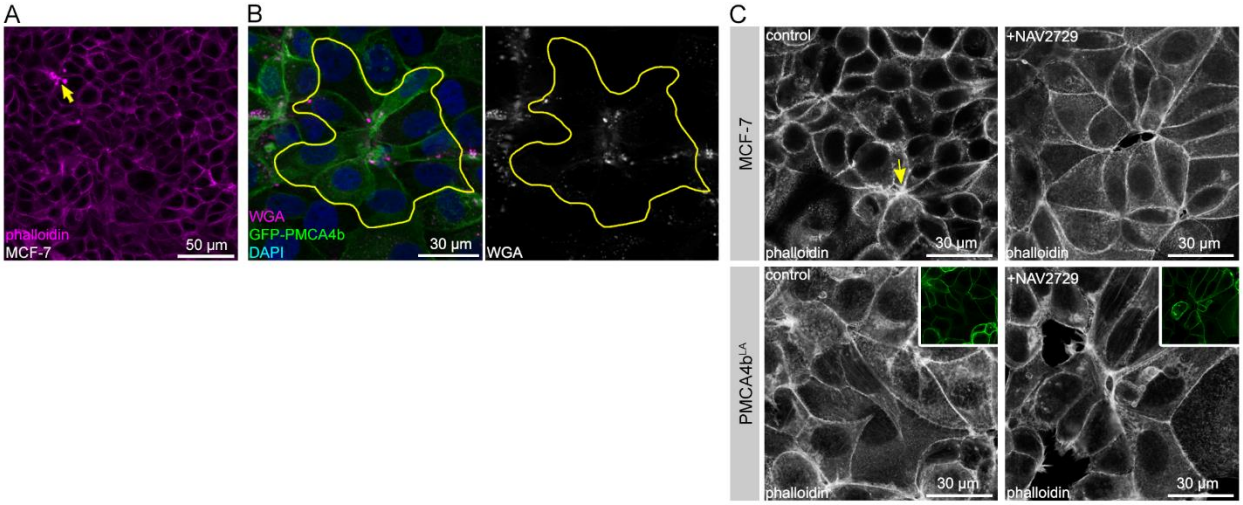
Supplementary Figure 3: Dlg1 and ezrin localization in MCF-7 cells



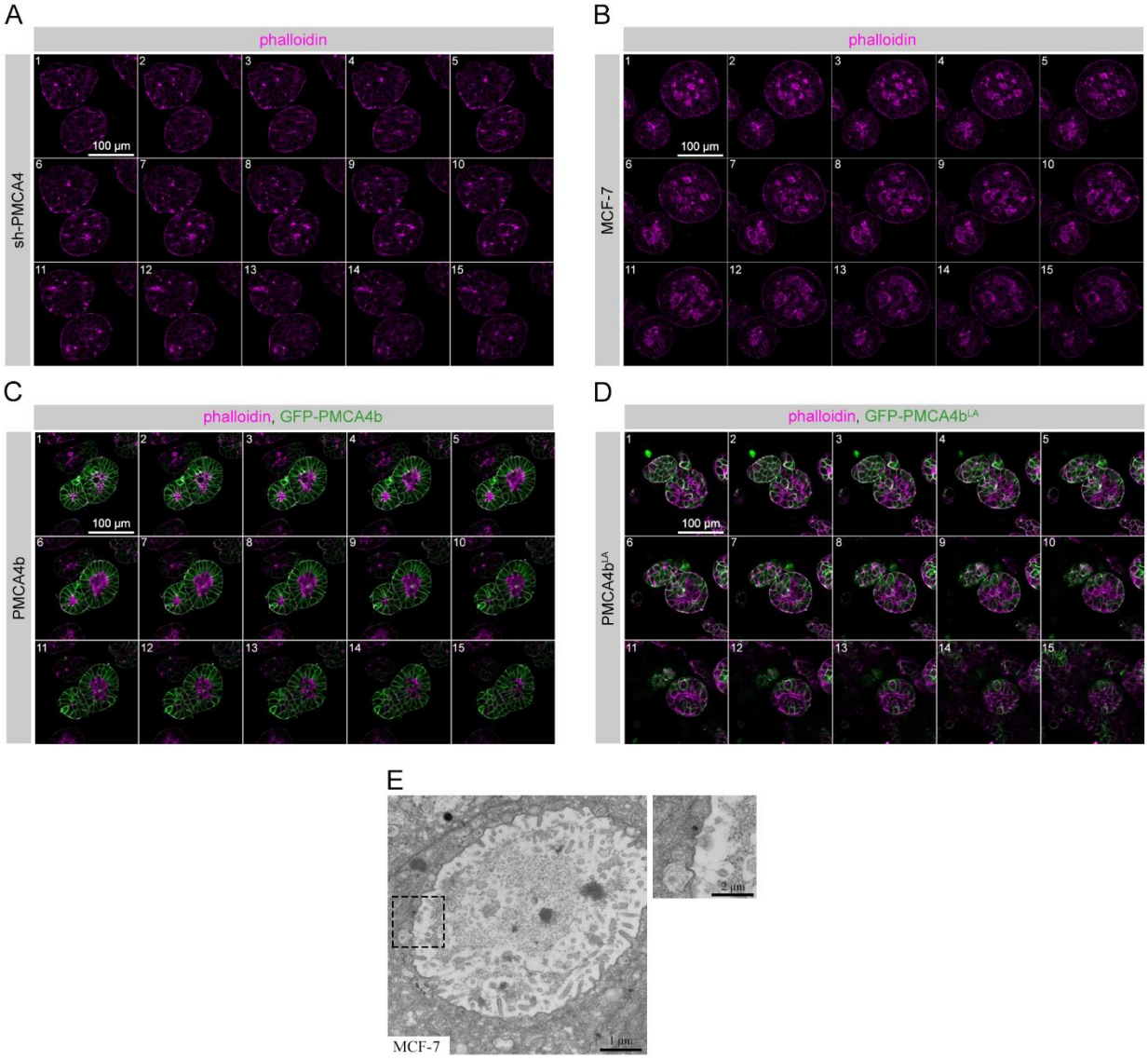
Supplementary Figure 4: Endosomal vesicle trafficking is only slightly polarized in parental MCF-7 cells



Supplementary Figure 5: Pre-lumen formation in 2D culture of MCF-7 cells

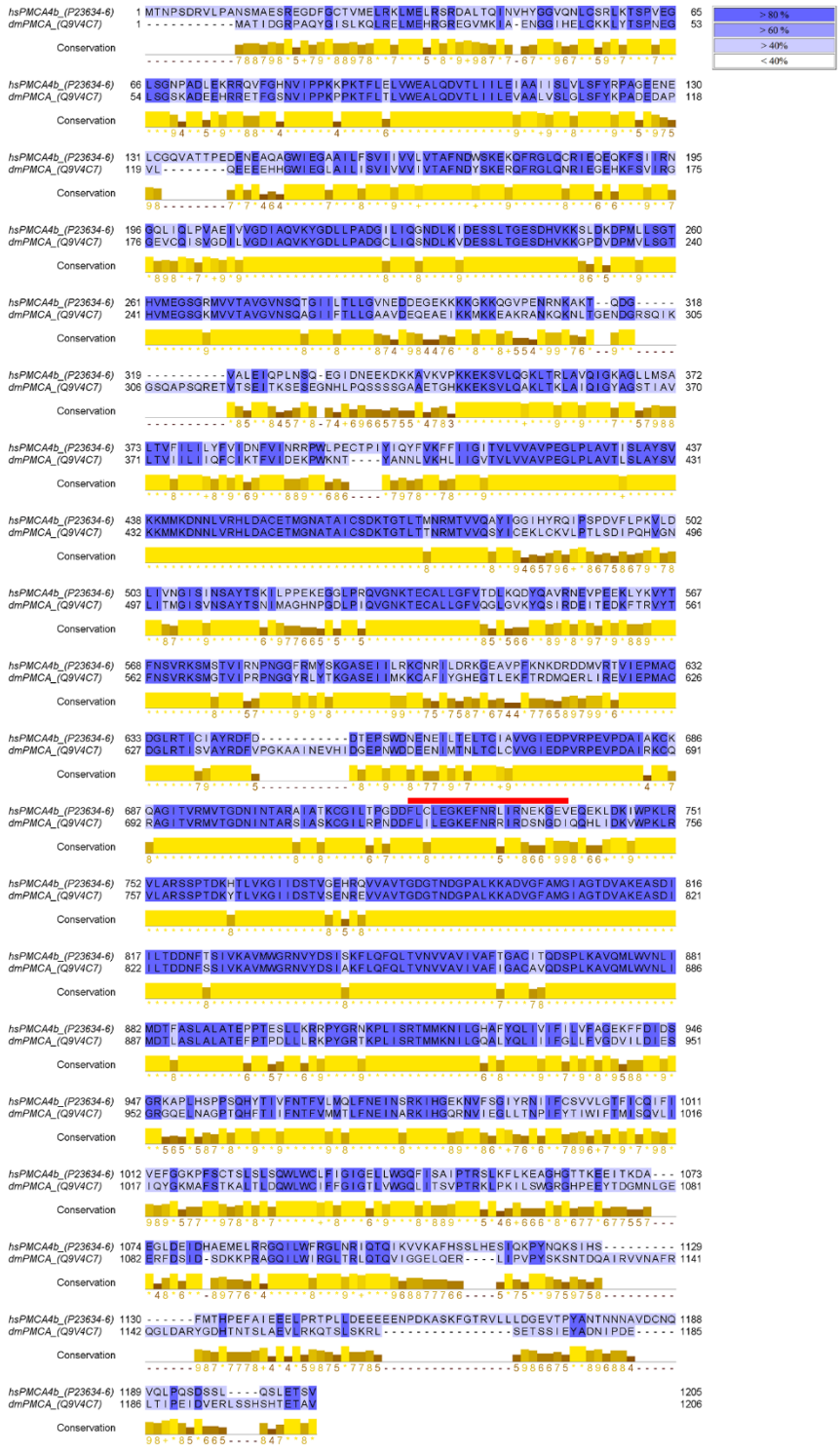


Supplementary Figure 6: Lumen formation in MCF-7 mammospheres



Supplementary Figure 7: Amino acid sequence comparison of human PMCA4b and *Drosophila melanogaster* PMCA sequences

A



B

Q59DP8_dm	- - - - - - - - - - - - - - -	
Q59DQ0_dm	SHSHTETAV	1183
E6EK17_dm	SHSHTETAV	1187
E6EK18_dm	SHSHTETAV	1210
Q59DP9_dm	SNGQNETRI	1255
Q9V4C7_dm	SHSHTETAV	1206
E6EK15_dm	SHSHTETAV	1194
A0A024R968_hs	SLQSLETSV	1205