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The depalmitoylase APT1 directs the asymmetric partitioning of Notch and Wnt signaling during cell division

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Abstract

Asymmetric cell division results in two distinctly fated daughter cells. A molecular hallmark of asymmetric division is the unequal partitioning of cell fate determinants. We have previously established that growth factor signaling promotes protein depalmitoylation to foster polarized protein localization, which in turn drives migration and metastasis. Here, we report protein palmitoylation as a key mechanism for the asymmetric partitioning of the cell fate determinants Numb and β -catenin through the activity of the depalmitoylating enzyme APT1. Using point mutations, we show specific palmitoylated residues on Numb are required for asymmetric localization. By live-cell imaging, we show that reciprocal interactions between APT1 and the Rho-family GTPase CDC42 promote the asymmetric localization of Numb and β -catenin to the plasma membrane. This in turn restricts Notch- and Wnt-responsive transcriptional activity to one daughter cell. Moreover, we show altering APT1 expression changes the transcriptional signatures of MDA-MB-231 triple receptor-negative breast cancer cells similarly to changes in Notch and β -catenin-mediated Wnt signaling. We also show that loss of APT1 depletes a specific subpopulation of tumorigenic cells in colony formation assays. Together, our findings demonstrate that APT1-mediated depalmitoylation is a major mechanism of asymmetric cell division maintaining Notch and Wnt-associated protein dynamics, gene expression, and cellular functions.

Introduction

Asymmetric cell division yields two morphologically and functionally distinct daughter cells and serves as a major contributor to cellular heterogeneity during development and tissue homeostasis (1). In dividing stem and progenitor cells, cell fate determinant proteins are unequally segregated along the division axis and inherited by one cell, resulting in the differential activation of transcriptional networks that establish non-identical daughter cells (2–5). For example, *Drosophila melanogaster* neuroblasts divide asymmetrically to produce

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a self-renewing neuroblast and a differentiating cell (6, 7). Similarly, transformed cells can also exhibit cellular heterogeneity with variations in properties such as signaling activity, tumorigenicity, and drug resistance exhibited by distinct cell populations (8–10). The cause of tumor heterogeneity has generally been attributed to genomic instability, epigenetic alterations, or interactions with the tumor microenvironment (11–13), though it is possible that asymmetric cell division may also play a role.

The molecular mechanisms driving and maintaining asymmetric divisions are poorly understood, but developmental signaling pathways such as Notch and Wnt have been shown to be key factors. In *Drosophila* neuroblasts, polarized cell division results in asymmetric Numb (the Notch antagonist) localization at the plasma membrane, resulting in unequal inheritance by cells fated to differentiate into neurons (6, 7, 14–16). Numb is also partitioned asymmetrically in dividing mammalian cells such as mammary epithelial precursors, hematopoietic stem and progenitor cells, and T-lymphocyte precursors (2, 17, 18). Likewise, directionally applied Wnt signals restrict β -catenin (a critical intracellular mediator of canonical Wnt signaling) in a polarized manner to mouse embryonic stem cells and *C.elegans* seam cells that are fated to remain as progenitor cells rather than differentiate (4, 19). Differential spatial organization of proteins and the resulting cell polarity is established by the evolutionarily conserved Par-aPKC (atypical protein kinase C)-CDC42 complex (20–22). CDC42 is a small Rho-family GTPase that mediates polarized processes such as vesicle budding, trafficking, and directional cell migration by remodeling the actin and microtubule cytoskeleton networks (23). Although the CDC42 polarity complex is involved during asymmetric cell division, the mechanisms directing the asymmetric recruitment and retention of cell fate determinant proteins at the plasma membrane during cell division remain unclear.

Posttranslational lipid modification is a prevalent mechanism of targeting proteins to membranes that alters molecular conformations and increases protein affinity for hydrophobic environments (24). Lipid modifications are largely non-reversible and maintain protein enzymatic function and stability, protein-protein interactions, and intracellular trafficking (25). Palmitoylation, in contrast, is a reversible lipid modification that modulates subcellular polarity and signaling cascade activity, such as that of the epidermal growth factor receptor (EGFR) and the small GTPase Ras, by rapid protein shuttling between the cytosol and lipid rafts within the plasma membrane (26–32). Palmitoylation is modulated by two classes of enzymes: palmitoyltransferases that add palmitate to cysteine residues, and depalmitoyltransferases that remove palmitate at the membrane and promote protein localization to the cytosol (33). Thus, palmitoylation is an attractive, but untested, candidate mechanism for the dynamic asymmetric targeting of proteins to the plasma membrane during cell division to drive cellular heterogeneity in embryogenesis, development, and tumorigenesis.

In this study, we investigated the role of palmitoylation on the polarized partitioning of cell fate determinants during asymmetric cell division and clarified how palmitoylation contributes to cellular heterogeneity. Using human cancer cell lines derived from malignant tumors that exhibit high cellular heterogeneity, such as triple receptor-negative breast cancers and osteosarcomas (34–38), we found that the depalmitoylating enzyme acyl-protein

thioesterase 1 (APT1) (39–41), directs the asymmetric localization of Numb and β -catenin. Additionally, we found that APT1 activity during mitosis restricted Notch-, Wnt-, and Sox2-dependent transcription to one daughter cell. APT1 also maintained the gene expression signature and colony-forming potential of transformed cells. Moreover, our observations indicate that APT1 was required for the transcriptional output of different signaling pathways in cancer cells grown in serial colony formation assays, which correlates with cellular heterogeneity and tumorigenic potential. With these findings, we identify a palmitoylation-mediated mechanism of asymmetric cell division that promotes the generation of functionally heterogeneous cells in tumors.

Results

Activity of the depalmitoylating enzyme APT1 is required for asymmetric localization of Numb and β -catenin

We have previously shown that APT1 promotes the transient and asymmetric localization of cell adhesion molecules during interphase in response to extracellular signals (26). To test the hypothesis that APT1 directs the asymmetric localization of Notch and Wnt signaling-associated cell fate determinants during cell division, we examined the spatial organization of Numb and β -catenin in fixed cells. Using the MDA-MB-231 human triple receptor-negative breast cancer cell line, dividing cells were identified by immunostaining for acetylated tubulin, a marker of stabilized tubulin structures such as the mitotic spindle and cytokinetic midbody (42), and counterstained for Numb or β -catenin. In the absence of exogenous stimuli, we observed symmetric localization of both proteins (Fig. 1A, B), as assessed by measuring the percentage difference in the mean fluorescence pixel intensity of Numb or β -catenin across dividing cells (fig. S1A). The distribution of the percentage differences of all quantified cells was plotted, and cells with a percentage difference of 20 or greater were scored as asymmetric (Fig. 1C, D). Treating cells with Palmostatin B (PalmB), a pharmacological inhibitor of APT enzymes (43), reduced the asymmetric localization of Numb by 3.0-fold (29.4% vs. 9.9%) and β -catenin by 3.3-fold (26.1% vs. 8.0%) (Fig. 1E, F). These findings suggest that APT enzymes are required for establishing asymmetric localizations of Numb and β -catenin in dividing cells.

To address a specific requirement for APT1, we knocked down APT1 with a short hairpin RNA (shRNA) (fig. S1B). In APT1 knockdown cells, asymmetric localization of Numb was reduced by 6-fold (29.4% vs. 4.9%), and asymmetric localization of β -catenin was reduced by 4.1-fold (26.1% vs. 6.4%). Ectopic expression of wild-type human APT1 (APT1^{WT}) from a plasmid restored asymmetric localization of Numb and β -catenin to baseline control conditions (Fig. 1G, H). However, ectopic expression of a catalytically inactive mutant form of APT1, in Ser¹¹⁹ in the catalytic domain is mutated to Ala (APT1^{S119A}) (40, 41), failed to rescue asymmetric Numb and β -catenin localization (fig. S1B; Fig. 1G, H). This indicates that the catalytic activity of APT1 is critical for asymmetric Numb and β -catenin localization.

Of the 23 DHHC palmitoyltransferases found in mammalian cells, DHHC20 is one of three that is localized to the plasma membrane where we would expect it to be available to palmitoylate proteins like Numb and β -catenin (44). DHHC20 is also known to be expressed

in MDA-MB-231 cells, where it palmitoylates EGFR and attenuates EGFR signaling (28). We asked whether loss of DHHC20 would affect protein localization. Knockdown of DHHC20 reduced asymmetric localization of Numb and β -catenin in a manner similar to knockdown of APT1 (fig. S1C, D; Fig. 1I, J).

The dependence of Numb and β -catenin asymmetric localization on APT1 suggests that Numb and β -catenin may be palmitoylated. To evaluate protein palmitoylation, we used acyl-biotin exchange (ABE) assays (45). In the ABE assay, purified proteins are treated with N-ethylmaleimide to block free thiol groups, then the Cys-palmitoyl thioester linkages are cleaved with hydroxylamine (HAM), and the newly exposed thiol groups are coupled to biotin. The modified proteins are then purified with streptavidin. Results of ABE assays were consistent with Numb and β -catenin being palmitoylated under normal growth conditions (Fig. 1K-L). Treating cells with PalmB increased the total amounts of palmitoylated Numb and β -catenin (Fig. 1K, L), indicating that palmitoylation of these proteins was inhibited in part by APT enzymes. As a negative control, ABE assays did not detect palmitoylation of the extracellular signal-regulated kinase (ERK), which is not palmitoylated (Fig. 1M).

To determine whether palmitoylation-dependent asymmetric localization was specific to Numb and β -catenin, dividing cells were immunostained for CD44 and RhoB, both of which are palmitoylated and unrelated to Wnt or Notch signaling (46–49), as well as for GFP in cells expressing a control GFP plasmid. The percentage of cells with asymmetrically localized GFP, CD44, or RhoB were not higher than the background percentages observed for β -catenin and Numb (approximately 8%), and were unaffected by PalmB treatment (fig. S1E-J). Together, these findings uncover a direct role for APT1 in the spatial distribution of the palmitoylated cell fate determinants Numb and β -catenin.

Asymmetric localization of Numb requires palmitoylation of the phosphotyrosine binding domain

Although we found β -catenin to be palmitoylated, β -catenin cortical localization is mediated through association with cadherins at tight junctions (50, 51). Thus, the necessity of palmitoylation for the membrane localization of β -catenin is unclear. However, the conserved phosphotyrosine binding (PTB) domain of Numb is required for association of Numb with the plasma membrane and for asymmetric localization of Numb in *Drosophila* through mechanisms that are still unknown (52). We sought to directly test whether palmitoylation of Numb is required for its asymmetric localization. Using the palmitoylation prediction algorithm CSS-Palm (53) and through identification of solvent-exposed cysteine residues within the PTB domain crystal structure (54), three conserved and potentially palmitoylated cysteine residues (Cys³⁷, Cys¹⁶⁰, and Cys¹⁶⁵) were identified and mutated to Ala (Fig. 2A). The Numb triple Cys-to-Ala mutant (Numb^{AAA}) showed reduced palmitoylation as measured by metabolic labeling of cells with palmitic acid azide (Fig. 2B). Endogenous β -catenin was also metabolically labeled with palmitic acid azide, confirming the efficiency of labeling in all reactions (Fig. 2B). These results demonstrate that Numb and β -catenin are continuously palmitoylated in MDA-MB-231 cells.

Next, we visualized the dynamic localization and segregation of fluorescently labeled Numb and APT1 during cell division by live-cell imaging. Because MDA-MB-231 cells shifted from a flat, spread morphology to a raised, rounded morphology out of the imaging plane during cell division, we instead utilized U2 OS human osteosarcoma cells, which maintained a consistent rounded morphology within the imaging plane during division. U2 OS cells were transduced to stably express an mCherry-Histone B (H2B) plasmid, allowing for the unambiguous identification of cells undergoing division in real time. Over the course of cell division, cyan fluorescent protein (CFP)-tagged APT1^{WT} (APT1^{WT}-CFP) and yellow fluorescent protein (YFP)-tagged Numb^{WT} (Numb^{WT}-YFP) exhibited highly dynamic asymmetric localization (Fig. 2C). At the beginning of the cell division cycle, Numb^{WT} was concentrated at one end of the cell at the plasma membrane, but as daughter cells formed, the localization of Numb shifted to membrane regions at and near the cleavage furrow. Finally, as daughter cells separated, Numb was partitioned to the plasma membrane of the cell that emerged from the same side of the mother cell to which Numb was initially concentrated. APT1^{WT} co-segregated with Numb to membrane regions and was retained in daughter cells with high Numb signal as indicated by line-scan analysis of YFP and CFP pixel intensity along the division axis (Fig. 2C, Movie S1–3, red arrows). This suggests that APT1 either responds to the same spatial cues as Numb. Alternatively, APT1 may direct Numb localization or vice versa. YFP-tagged Numb^{AAA} (Numb^{AAA}-YFP) was live-imaged to determine the contribution of Numb palmitoylation on its localization. Numb^{AAA} showed a 1.5-fold reduced asymmetric localization in dividing cells as compared to Numb^{WT} (Fig. 2D). Finally, knocking down APT1 reduced asymmetric Numb^{WT} localization but did not further reduce the asymmetry of Numb^{AAA} (Fig. 2D). Our results show that the asymmetric partitioning of Numb is actively maintained by a mechanism that requires both APT1-mediated depalmitoylation and palmitoylation of Cys³⁷, Cys¹⁶⁰, and/or Cys¹⁶⁵ within the PTB domain.

Asymmetric localization of APT1 during cell division requires APT1 catalytic activity

Having determined that APT1 activity is essential for asymmetric localization of Numb and β -catenin, we next examined whether the catalytic activity of APT1 is required for its own asymmetric localization. Immunostaining fixed cells showed that endogenous APT1 was asymmetrically localized in 22.3% of control cells. Expressing the catalytically inactive form APT1^{S119A} reduced this asymmetric localization by 2.4-fold, to 9.4%, whereas expressing APT1^{WT} had no significant effect (fig. S2A–C). Knocking down DHHC20 also reduced asymmetric APT1 partitioning, suggesting that DHHC20 promotes asymmetric localization of APT1 (fig. S2B, D).

To gain detailed insights into how APT1 activity promotes and maintains the dynamics of its own asymmetric localization, we compared the spatiotemporal distribution of ectopically expressed APT1^{WT}-CFP versus APT1^{S119A}-CFP by live-cell imaging (Fig. 2E–G; Movies S4–7). In 52.6% of dividing cells, APT1^{WT}-CFP asymmetry was maintained at the plasma membrane through cytokinesis (Fig. 2E,G; Movies S4–5). The asymmetric localization of APT1^{S119A}-CFP was significantly reduced to 20.6% of cells, similar to the asymmetric localization of GFP-vector (16.9%) (Fig. 2F,G; Movies S6–7). Additionally, the catalytically inactive mutant APT1^{S119A}-CFP was not discretely localized to the plasma membrane at the

start of division and appeared to be stuck at the cytokinetic midbody during cytokinesis (Fig. 2F, Movie S6–7). The data up to this point demonstrate that asymmetric localization of APT1 requires its catalytic activity and suggest a role for protein depalmitoylation activity at the site of asymmetric protein localization.

Palmitoylating and depalmitoylating enzymes localize asymmetrically with palmitoylated proteins during cell division

To clarify the purpose of APT1 accumulation at sites of asymmetrically localized proteins, we hypothesized that APT1 localizes to regions of high protein palmitoylation. Fixed MDA-MB-231 cells were immunostained for DHHC20, which was asymmetrically partitioned to the same region of a dividing cell as APT1 (Fig. 3A). Knocking down DHHC20 reduced asymmetric APT1 partitioning (fig. S2D). Additionally, the distribution of APT1 and DHHC20 overlapped with that of caveolin, a palmitoylated protein (55), at the plasma membrane (Fig. 3B, C). This would suggest DHHC20-mediated palmitoylation of substrates could recruit APT1 to the membrane.

The presence of both the depalmitoylating enzyme APT1 and palmitoylating enzyme DHHC20 in plasma membrane-associated domains led us to test whether these regions were also enriched for palmitoylated proteins. Because there are currently no palmitoylation-specific antibodies to visualize the localization of palmitoylated proteins, we modified the ABE assay for immunofluorescence and detected asymmetric palmitoylated protein localization at the cortex of dividing cells (Fig. 3D). This asymmetric localization depended on the activity of APT1, because PalmB treatment decreased the enrichment of palmitoylated proteins at the cortex and reduced the percentage of cells showing asymmetric localization of palmitoylated proteins by 4.1-fold (31.4% vs. 7.6%). The immunofluorescence signal generated by the modified ABE assay was specific to palmitoylated proteins; negative control staining of samples in which HAM was omitted from the ABE reaction showed greatly reduced signal and symmetric localization (Fig. 3D–F). To determine the localization of palmitoylated proteins relative to APT1 and DHHC20 and whether this asymmetric localization was only observed during mitosis, we performed the modified ABE immunofluorescence assay on non-dividing cells. Both APT1 and DHHC20 puncta were localized to regions enriched in palmitoylated proteins at membrane ruffles as assessed by confocal microscopy (Fig. 3G). The data thus far indicate that both APT1 and DHHC20 localize to regions enriched in palmitoylated proteins.

A constitutively active CDC42 mutant promotes asymmetric localization of APT1, Numb, and β -catenin during cell division

We next examined whether APT1-mediated asymmetric protein partitioning functions independently of known polarity-establishing mechanisms. The Par-aPKC-CDC42 polarity complex promotes the asymmetric subcellular distribution of cell fate determinants (15, 56). We knocked down CDC42 and PARD3 (the mammalian homolog of Par3) to assess the requirement of these factors for APT1, Numb, and β -catenin localization (fig. S3A, B). Asymmetric localization of endogenous APT1 was reduced by 2.6-fold (22.3% vs. 8.5%) in CDC42 knockdown cells, and by 4.3-fold (22.3% vs. 5.2%) in PARD3 knockdown cells (Fig. 4A, B; fig. S4C). PARD3 knockdown reduced the asymmetric localization of β -

catenin, but not that of Numb (fig. S3D-G). We next asked if CDC42 and APT1 double knockdown would completely abolish asymmetric Numb and β -catenin partitioning. The percentage of cells showing asymmetric partitioning of Numb and β -catenin in the double knockdown condition did not further decrease as compared to APT1 or CDC42 single knockdown (fig. S3D, E, H, I). This suggests there might be a basal level of asymmetric distribution for both of these proteins that is independent of both APT1 and CDC42.

We next investigated whether APT1 asymmetric localization depended on CDC42 by altering CDC42 activity. Work from other groups has shown that expression of either GTP-locked or GDP-locked mutant forms of CDC42 can interfere with CDC42-dependent cellular processes (20, 23, 57–60). We used live-cell imaging of APT1^{WT}-CFP and YFP-tagged CDC42 mutants to test the function of CDC42 activity on APT1 localization. Cells expressing a GTP-locked, constitutively active CDC42 mutant (CDC42^{V12}) or a GDP-locked, inactive CDC42 mutant (CDC42^{N17}) showed reduced asymmetry of APT1^{WT} during cell division as compared to cells expressing a control GFP vector (Fig. 4C). In fixed cells, knocking down CDC42 and expressing shRNA-resistant CDC42^{V12} or CDC42^{N17} also significantly reduced asymmetric APT1 and β -catenin localization. Compared to CDC42 knockdown, which had minimal effect, expressing CDC42^{V12} or CDC42^{N17} resulted in a strong reduction of asymmetric Numb localization (fig. S4A-G).

To demonstrate the requirement of CDC42 cycling activity on APT1 localization in cells, we expressed a YFP-tagged constitutively active CDC42 mutant that retains GTP-GDP cycling (CDC42^{F28L}). CDC42^{F28L} was previously shown by other groups to mimic CDC42-mediated effector binding and subcellular localization (57). Expressing shRNA-resistant CDC42^{F28L} in CDC42 knockdown cells was sufficient to rescue β -catenin and APT1 asymmetric localization, but only partially rescued asymmetric Numb localization in CDC42 knockdown cells (fig S4A-G). Additionally, expression of CDC42^{V12}, CDC42^{N17}, or CDC42^{F28L} did not significantly alter the low percentage of asymmetrically localized catalytically inactive APT1^{S119A} as compared to APT1^{WT} by live-cell imaging (Fig. 4C). These results demonstrate a requirement for the polarity complex and, specifically, CDC42 activity in promoting the asymmetric partitioning of APT1 and β -catenin to the membrane. However, although Numb asymmetric localization required CDC42 activity, overall it appears to be less dependent than APT1 and β -catenin on the canonical Par-aPKC-CDC42 polarity complex.

The reciprocal interaction between APT1 and palmitoylated CDC42 is sufficient to promote asymmetric protein partitioning during cell division

In addition to cycling between GTP and GDP, CDC42 function is also maintained in part by membrane association through a polybasic region and lipid modification of the C-terminal tail (23, 59). There are two known naturally occurring exon splice variants of CDC42 with distinct lipid modifications— a solely prenylated splice variant and a dually palmitoylated and prenylated splice variant (61). We asked whether either CDC42 splice variant could rescue asymmetric protein localization in CDC42 knockdown cells during division by expressing shRNA-resistant constructs of the dually lipid modified isoform (CDC42^{Palm}) or the solely prenylated CDC42 (CDC42^{Pren}) splice variants (fig. S4A) and examining the

localization of Numb, β -catenin, and APT1 in fixed cells. In CDC42 knockdown cells, expressing CDC42^{Palm} was sufficient to fully rescue APT1 asymmetry, whereas expression of CDC42^{Pren} appeared to inhibit asymmetric APT1 localization. CDC42^{Palm} and CDC42^{Pren} had little effect on asymmetric Numb localization in CDC42 knockdown cells. CDC42^{Palm} rescued asymmetric β -catenin localization to baseline control conditions, but CDC42^{Pren} had no observable effect (fig. S4B-D, H-J). These results suggest that palmitoylated CDC42 promotes asymmetric localization of specific downstream proteins, and APT1 may reciprocally promote asymmetric localization of palmitoylated CDC42 during cell division.

To spatiotemporally visualize CDC42 and APT1 localization during cell division, we measured the distribution of APT1^{WT}-CFP and CDC42^{Pren}-YFP or CDC42^{Palm}-YFP by live-cell imaging. CDC42^{Palm} expression increased the percentage of asymmetric APT1^{WT}-CFP in dividing cells by 1.5-fold (52.6% vs. 82.7%) whereas CDC42^{Pren} suppressed asymmetric partitioning of APT1^{WT}-CFP by 1.9-fold (52.6% vs. 27.2%) (Fig. 4D). In contrast, neither isoform sufficiently altered asymmetric partitioning of catalytically inactive APT1^{S119A} (Fig. 4D). This led us to ask whether APT1 was sufficient to promote asymmetric CDC42^{Palm} localization. Both APT1^{WT}-CFP and CDC42^{Palm}-YFP were asymmetric at the plasma membrane early during division, and asymmetrically redistributed to the membrane of one daughter cell upon cytokinesis (Fig. 4E, Movies S8–10). As expected, expression of APT1^{S119A} inhibited asymmetric redistribution of CDC42^{Palm}-YFP and retained CDC42^{Palm}-YFP at the cytokinetic midbody (Fig. 4F). We next asked whether CDC42^{Palm} was endogenously expressed in MDA-MB-231 cells using exon-spanning primers. We detected the endogenous transcript for this palmitoylated CDC42 splice variant by PCR (fig. S4K, L). Furthermore, we detected CDC42 palmitoylation in U2 OS cells by the ABE assay and demonstrated that treatment with PalmB increased the amount of palmitoylated CDC42, whereas treatment with the palmitoyltransferase inhibitor 2-bromopalmitate (2BP) decreased palmitoylation (fig. S4M). These results suggest that palmitoylated CDC42 could function with APT1 in these cells either at a basal level or in a subset of cells to promote asymmetric protein localization.

APT1 restricts Wnt and Notch transcriptional activity to one daughter cell

One downstream effect of asymmetrically partitioning cell fate determinants during cell division is the activation of different transcriptional networks in the two daughter cells, resulting in cells with unique transcriptional profiles (3, 4, 6). We hypothesized that APT1 could also mediate the partitioning of asymmetric transcriptional activity of the Notch and Wnt- β -catenin signaling pathways to one daughter cell. We expressed GFP reporter transgenes containing Notch-activated RBPJ (recombination signal binding protein for immunoglobulin kappa J region) binding sites (pGF1-Notch) (62, 63) or Wnt-activated TCF and Lef1 binding sites (pGF1-TCF/Lef1) in MDA-MB-231 cells, which were then immunostained for GFP and acetylated tubulin in daughter cells (Fig. 5A, B). After plotting the distribution of asymmetric divisions as described in fig. S1, we observed asymmetric Notch and TCF/Lef1 reporter signal in 22.7% and 31.7% of daughter cells, respectively. A control GFP reporter containing a minimal CMV promoter (mCMV) lacking pathway-specific promoter elements showed symmetric signal in most cells and confirmed that the

observed asymmetries were dependent on the TCF/Lef1 and Notch enhancer elements (Fig. 5C-H).

Pharmacologically inhibiting APT1 with PalmB treatment reduced asymmetric Notch reporter signal by 3.1-fold (31.7% vs. 10.2%), and Wnt reporter signal by 2.6-fold (22.7% vs. 8.7%), respectively (Fig. 5E, F). Knocking down APT1 or DHHC20 also reduced asymmetric Notch and Wnt reporter signal, indicating that palmitoylation can restrict Notch- and Wnt-dependent transcription to one daughter cell. Furthermore, knocking down CDC42 significantly disrupted asymmetric Wnt reporter signal, whereas the Notch reporter signal was unchanged, consistent with observations of asymmetric Numb localization (fig. S4). Additionally, CDC42 and APT1 may function in the same pathway that stimulates signal-activated transcription because double knockdown of APT1 and DHHC20 did not appreciably decrease asymmetric Wnt or Notch reporter signal over APT1 or CDC42 single knockdown (Fig. 5G, H). Together these findings suggest palmitoylation directs the localization of a Wnt activating factor and a Notch inhibitory factor to restrict transcription to one daughter cell.

APT1 induces Wnt, Notch, and mammary stem cell transcriptional signatures in MDA-MB-231 triple receptor–negative breast cancer cells

To date, APT1 has not been shown to drive transcriptional changes in the contexts of development or disease. To further investigate the observed influence of APT1 on asymmetric Notch and Wnt reporter activity, we asked whether APT1 influences a Notch or Wnt gene signature in cells. We performed RNA-seq and gene set enrichment analysis (GSEA) on wild-type control, APT1 knockdown, or APT1^{WT}-expressing MDA-MB-231 cells (table S1). We performed GSEA using a ranked list of differentially expressed genes and compared APT1^{WT}-expressing cells vs. control cells, APT1 knockdown cells vs. control cells, and APT1^{WT}-expressing cells vs. APT1 knockdown cells. In APT1 knockdown cells compared to control cells, we identified a high scoring signature [NES (normalized enrichment score) of 1.46 and a false discovery rate (FDR) q-value of 0.073] that positively correlated with genes reported to increase in cells overexpressing active β -catenin (BCAT_UP.V1_UP), suggesting that APT1 depletion promotes β -catenin signaling (Fig. 6A; fig. S5A). When comparing APT1^{WT}-expressing cells to control cells, we identified a negative correlation (NES 1.25 and FDR q-value 0.302), with a gene signature that includes genes reported to decrease in cells treated with a Notch inhibitor (NOTCH_DN.V1_DN), suggesting APT1 suppresses Notch signaling (Fig. 6B, fig. S5A). When APT1^{WT}-expressing cells were compared to APT1 knockdown cells, we identified a high scoring signature (NES 2.34, FDR q-value=0.0001) for genes reported to increase in mammary stem cells (PECE_MAMMARY_STEM_CELL_UP) (Fig. 6C). Visualization of the leading-edge genes from the mammary stem cell signature also indicated a strong decrease in expression with APT1 knockdown when normalized to control wild-type cells, (Fig. 6D).

Two other high scoring signatures found in APT1^{WT}-overexpressing cells compared to APT1 knockdown cells were signatures for Myc and Cyclin D1 overexpression, both of which are maintained by Wnt and Notch signaling (NES 2.08, FDR q-value 0.0001; NES 1.69, FDR q-value 0.014) (fig. S5B, C). Target genes with high log₂FC (fold change) values

were validated by qRT-PCR. Notable APT1-driven genes included *DKK1*, *BMP4*, *GATA6*, and *KLF5* (64–66) (fig. S5D, E; table S1).

Based on the high-scoring gene expression signature for mammary stem cells observed in APT^{WT}-overexpressing cells, it is possible APT1 asymmetrically restricts signaling pathways that are activated in stem cells. The transcription factor Sox2 has been implicated in mammary stem cell function (67). Expression of a Sox2-responsive reporter (pGF1-SRR2) in MDA-MB-231 cells revealed that its asymmetric signal was reduced with APT1 knockdown or PalmB treatment. DHHC20, CDC42, or DHHC20 and CDC42 double knockdown reduced the SRR2 asymmetric signal (Fig. 6E-H). We also observed asymmetric APT1 localization in dividing mouse embryonic stem cells expressing an APT1^{WT} plasmid (fig. S6). These results implicate a role for palmitoylation in promoting cell fate-related transcriptional signatures and maintaining asymmetric cell division in progenitor cells.

APT1 and CDC42 maintain unique cell populations in MDA-MB-231 colonies

Tumors are heterogeneous populations of cells that have various degrees of proliferative and self-renewal potential, and these properties contribute to tumorigenicity (35). Triple receptor-negative breast tumors, like that from which MDA-MB-231 cells were derived, contain subpopulations of cells that vary in proliferative and self-renewing, tumor-initiating potential (17, 34, 36). Whether asymmetric cell division contributes to the generation of functionally heterogeneous cells in tumors is unclear. We utilized the colony formation assay, which measures the growth of transformed cells in anchorage-independent conditions and correlates with tumorigenicity. Because only a subset of transformed cells can form colonies, this assay may also indicate the degree of heterogeneity within a cell population (17, 68). Knocking down APT1 reduced colony-forming potential of MDA-MB-231 cells by 2.3-fold (average colonies counted 39 vs. 90), whereas expressing APT1^{WT} increased colony numbers by 1.7-fold (average colonies counted 152 vs. 90), suggesting that APT1 maintains anchorage-independent growth and tumorigenic potential of transformed cells (fig. S7A).

Self-renewing cells within the colony are expected to form new colonies upon serial dissociation and replating, indefinitely. To test whether APT1-mediated asymmetry maintains a self-renewing population within colonies, we examined the self-renewal potential of APT1 knockdown or CDC42 knockdown MDA-MB-231 cells in colonies over 3 rounds of dissociation and replating. APT1 knockdown significantly reduced the number of colonies on the second and third plating, suggesting depletion of colony-initiating cells (Fig. 7A). Unexpectedly, CDC42 knockdown increased the number of colonies formed with each plating compared to control cells, suggesting an expansion of a colony-initiating cell population (Fig. 7B). Under normal two-dimensional culture conditions, we observed a slight lag in proliferation for APT1 knockdown, CDC42 knockdown, and double knockdown cells. Because we did not see a significant impairment of proliferation, this result would suggest that the reduced colony-forming potential is not due decreased proliferation under normal growth conditions (fig. S7B-D). Colonies from APT1 and CDC42 double knockdown cells showed reduced replating ability (fig. S7E), again demonstrating that the expansion of this colony-forming population is dependent on APT1.

Because the increase in colony-forming potential of CDC42 knock down cells was unexpected, we hypothesized this could be caused by increased symmetric divisions of colony-initiating cells. A subset of highly tumorigenic cells in basal breast cancers have been shown to reactivate developmental signaling pathways such as those dependent on Notch, Wnt, or Sox2 (34, 69–71). Pharmacologic inhibition of Wnt (with the Porcupine inhibitor IWP2) or Notch (with the γ -secretase inhibitor compound E) reduced the self-renewal potential of colonies similarly to APT1 knockdown or APT1 and CDC42 double knockdown (fig. S7E). Furthermore, APT1 knockdown increased Wnt reporter signal and decreased Notch and Sox2 reporter signals in colonies (fig. S7F). This is consistent with our GSEA analysis that indicated APT1 knockdown suppresses Notch signaling and promotes β -catenin signaling and pathways active in mammary stem cells.

Knocking down CDC42 reduced the Notch reporter signal, but increased the Sox2 reporter signal in most of the cells within the colony (fig. S7F). The Sox2 reporter has been previously shown to correlate with increased tumorigenicity (71); these results therefore suggest that the increase in colony formation caused by CDC42 knockdown is due to an increase in Sox2 transcriptionally-active cells.

To address the possibility that APT1 maintains a specific subpopulation of tumorigenic cells, we dissociated colonies and sorted the cells by flow cytometry to test for the cell surface marker profile that is associated with highly tumorigenic cells in breast cancers: CD44 high, CD24 low, and Aldehyde dehydrogenase high (CD44⁺/CD24^{lo}/ALDH⁺) (72). We first examined the size of the ALDH⁺ population, which was higher in APT1 knockdown cells (16.7%) as compared to control (7.3%) or CDC42 knockdown (7.8%) cells on the first replating (fig. S8). Further gating the ALDH⁺ population for CD44⁺/CD24^{lo} cells revealed that knocking down APT1 reduced CD44⁺/CD24^{lo} cells (2.6%), whereas knocking down CDC42 increased this population (14.4%), as compared to control cells (5.3%) (Fig. 7C). The trend of the number of CD44⁺/CD24^{lo}/ALDH⁺ cells being reduced in the APT1 knockdown condition and increased in the CDC42 knockdown condition was sustained on second and third platings (Fig. 7C). This pattern was observed to a lesser extent in adherent cells (fig. 7C; fig. S8). These results are consistent with the observed colony phenotypes and may explain why CDC42 knockdown cells form many colonies, whereas APT1 knockdown cells are unable to do so. Taken together, these findings demonstrate that in an anchorage-independent setting, an APT1-CDC42 axis maintains the expansion of a self-renewing, tumorigenic cell population and activation of transcriptional profiles required to maintain this population.

Discussion

Our results uncover a molecular mechanism through which palmitoylation restricts the localization of the cell fate determinants β -catenin and Numb and the downstream transcriptional responses to Wnt and Notch signaling to one daughter cell during cell division. Furthermore, palmitoylation directly promotes asymmetric Numb localization, because point mutations in conserved cysteine residues in Numb inhibit not only its palmitoylation but also its asymmetric localization. Inhibition of APT1 reduces the percentage of cells with asymmetrically localized cell fate determinants indicating it

facilitates a controlled, rather than a stochastic, process. The observation that both decreased palmitoylation of the Numb^{AAA} mutant or increased palmitoylation of Numb upon APT1 knockdown inhibits asymmetric localization would suggest it is the dynamic association with the plasma membrane that promotes asymmetry. The enrichment of both the palmitoyltransferase DHHC20 and the depalmitoylating enzyme APT1 to a discrete membrane domain could reinforce this process. APT1 inhibition did not completely block the asymmetric localization of β -catenin and Numb, but resulted in a residual asymmetry in about 8% of cells. This is similar to the percentage of cells with asymmetrically localized GFP that does not respond to APT1 inhibition, and is most likely independent of palmitoylation. This palmitoylation-independent asymmetric localization could be the effect of previously reported asymmetric partitioning of machinery that maintains protein abundance such as the proteasome (3, 73) or protein translation machinery (74, 75), which could result in localized differences in degradation or synthesis of proteins during cell division.

Our data show an interdependence of APT1 and CDC42 activity for their own asymmetric localization. This interdependence could occur through association of two distinct compartments of the plasma membrane: the lipid bilayer and the membrane-associated, cortical actin cytoskeleton. CDC42 establishes polarity in cells by spatially reorganizing actin to direct vesicle traffic to and fusion with the plasma membrane (23, 76, 77). Because active CDC42 is membrane-associated, APT1 could promote the recruitment of palmitoylated proteins that also stimulate CDC42 function, such as the CDC42 activator Intersectin, which is also activated by Numb (78), to the plasma membrane. This is consistent with current models of diffusible, membrane-bound molecules establishing distinct polarized domains at the plasma membrane in a feedforward manner (79, 80). It has also been hypothesized that the formation of polarized domains can be inhibited if the association rate outpaces the dissociation rate or vice versa (79, 81). This could explain why polarized membrane domains are sensitive to changes in CDC42 protein abundance, activity, and lipid-modification, as demonstrated by our findings.

Whereas the asymmetric distributions of CDC42 and APT1 appear to be interdependent, the depletion of either protein individually has distinct effects on the Wnt and Notch pathways. Knocking down CDC42 significantly reduces both APT1 and β -catenin asymmetric localization, without affecting the localization of Numb. This would suggest that although asymmetric β -catenin and Numb localization require APT1 activity, only the asymmetric localization of β -catenin requires asymmetric localization of APT1. CDC42 is known to promote canonical Wnt signaling activity by sequestering adenomatosis polyposis coli (APC), a component of the β -catenin destruction complex, to the plasma membrane in an actin-dependent manner (82, 83). Thus, maintenance of the actin cytoskeleton by CDC42 may be required for asymmetric β -catenin localization, but not for Numb. As such, the requirement for CDC42 may be necessitated by unique mechanisms of Wnt signal activation distinct from a requirement for APT1 activity.

By manipulating APT1, we demonstrate that we can alter the asymmetric activation of Notch, Wnt, and Sox2 transcriptional reporters without directly altering the expression of transcription factors or upstream signaling factors. The fact that β -catenin and Notch gene

signatures were not as high-scoring as compared to those of mammary stem cells, Myc, and Cyclin D1 signatures may indicate that APT1 directs combinatorial signaling to determine cell identity. Myc and Cyclin D1 are canonical downstream transcriptional targets of both Wnt and Notch and are also involved in determining mammary stem cell identity (84–86). Within the mammary stem cell signature is a notable abundance of ribosomal proteins. A similar abundance in ribosomal proteins is also present in the Myc signature, consistent with its role in driving ribosomal biogenesis and protein translation in the mammary gland, among other tissues, and in cancer (87, 88). Because Myc is a critical factor for mammary stem cell identity and function (85), our results would suggest APT1 has a role in translation in mammary stem cells. What remains unclear is whether altering APT1 protein amounts induces the expansion of a population of cells expressing a mammary stem cell transcriptional signature or induces a de novo transcriptional signature in most cells. We also have evidence of asymmetric APT1 localization in dividing mouse embryonic stem cells expressing APT1^{WT}, suggesting that this mechanism may also have a broader and conserved role in development. Future experiments, including single-cell RNAseq, may address these questions.

Consistent with the concept of changing cell populations, our results suggest that APT1 is required to maintain a tumorigenic population of breast cancer cells whereas CDC42 appears to restrict the size of this population. Our findings indicate that APT1 may contribute to the activation of transcriptional programs that promote colony-forming potential, whereas CDC42 could restrict APT1 activity to one daughter during asymmetric division. This would be consistent with the increase in the CD44⁺/CD24^{lo}/ALDH⁺ population in CDC42 knockdown colonies. The implications of this study may be relevant to human disease because APT1 is amplified in various cancers, and this amplification is associated with poor patient prognosis, suggesting APT1 could function in the development and progression of human cancers (89, 90). Although the importance of self-renewing, tumor-initiating or stem-like cells in cancer is still unclear, the question of how tumors maintain and generate cells with diverse properties such as metastatic potential, dormancy, and drug resistance is still a critical one. Further exploration of the mechanisms that establish and direct asymmetric cell division and promote cellular heterogeneity may help us understand tumor development and progression.

Materials and Methods

Cell Culture

MDA-MB-231 and U2OS cells were cultured in DMEM + glutamax (Thermo Fisher; Cat. No. 10566-016) and 10% fetal bovine serum. For drug treatment, cells were treated with DMSO (Sigma; Cat. No. D2650), 10 μ M Palmostatin B (EMD Millipore; Cat. No. 178501) prepared in DMSO, or 5 μ M 2-bromopalmitate (Sigma; Cat. No. 21604-1G) prepared in DMSO for 16 hours before staining or harvesting for cell lysates. Cells were treated with 0.5 μ g/mL puromycin for selection. E14 ESCs were cultured in DMEM Knockout (ThermoFisher. Cat. 10829-018), 15% fetal bovine serum, 1% L-glutamine, 1% Pen Strep, 1% Non-essential amino acids, 0.1 mM 2 mercaptoethanol, 1000 units/mL Leukemia

inhibitory Factor (Sigma, Cat. L5158), 1 μ M MEK I/II Inhibitor (Millipore, Cat. 444966), 3 μ M GSK3 Inhibitor XVI (Millipore, Cat. 361559) on gelatin coated tissue culture plates.

Stable cell lines

HEK cells were transfected with 0.69 μ g/ μ L of a GAG, Rev, and Vsvg mix and 1.42 μ g/ μ L of the following plasmids: Scramble control, GFP-PRRL, APT1^{WT}-CFP-Flag-PRRL, APT1 (S119)-CFP-Flag-PRRL, FLAG- CDC 42 Pren -PRRL, FLAG-CDC42-V12-PRRL, FLAG-CDC42-N17-PRRL, FLAG-CDC42-F28-PRRL, FLAG-CDC42 Palm-PRRL, pGF-SRR2-mCMV-GFP-puro (System Biosciences; Cat. No. SR20071-PA-P), pGF-Notch-mCMV-GFP-puro (System Biosciences; Cat. No. TR020PA-P), pGF-TCF/Lef-mCMV-GFP-puro (System Biosciences; Cat. No. TR013PA-P), or pGF-mCMV-GFP (System Biosciences; Cat. No. TR011PA-1), for 24 hours with LT-1 transfection reagent (Mirus Bio. Cat. MIR2300). The aforementioned APT1 and CDC42 plasmids were designed with short hairpin resistant sequences for rescue studies. Virus was collected 72 hours after infection with 0.5-1mL virus used for stable cell line generation. U2OS cells were infected with APT1^{WT}-CFP-FLAG or APT1(S119)-CFP-FLAG lentivirus for 24 hours, then recovered in complete DMEM for 48 hours prior to cell culture. E14 ESCs were infected with APT1^{WT}-CFP-FLAG lentivirus for 24 hours, then recovered in complete DMEM-knockout for 48 hours prior to cell culture. E14 ESCs were infected with APT1^{WT}-CFP-FLAG lentivirus for 24 hours, and then recovered in complete DMEM for 48 hours prior to cell culture. MDA-MB-231 were infected with the aforementioned lentivirus for 24 hours and recovered in complete DMEM for 48 hours prior to cell culture.

Short hairpin design

shRNA for PARD3

F: 5'-

CCGGGCCATCGACAAATCTTATGATCTCGAGATCATAAGATTTGTTCGATGGC
TTTTTG-3'

R: 5'-

AATTCAAAAAGCCATCGACAAATCTTATGATCTCGAGATCATAAGATTTGTC
GATGGC-3'

F: 5'-

CCGGGCCATCGACAAATCTTATGATCTCGAGATCATAAGATTTGTTCGATGGC
TTTTTG-3'

R: 5'-

AATTCAAAAAGCCATCGACAAATCTTATGATCTCGAGATCATAAGATTTGTC
GATGGC-3'

F: 5'-

CCGGAGTCAATTGGATTTCGTTAACTCGAGTTTAACGAAATCCAATTGACT
TTTTTG-3'

R: 5' -

AATTCAAAAAAGTCAATTGGATTTTCGTAAACTCGAGTTTAACGAAATCCA
ATTGACT-3'

shRNA for CDC42

F: 5' -

CCGGCGGAATATGTACCGACTGTTTCTCGAGAAACAGTCGGTACATATTCCG
TTTTTG-3'

R: 5' -

AATTCAAAAACGGAATATGTACCGACTGTTTCTCGAGAAACAGTCGGTACA
TATTCCG-3'

F: 5' -

CCGGTGCTTGTTGGGACTCAAATTGCTCGAGCAATTTGAGTCCCAACAAGC
ATTTTTG-3'

R: 5' -

AATTCAAAAATGCTTGTTGGGACTCAAATTGCTCGAGCAATTTGAGTCCCA
ACAAGCA-3'

F: 5' -

CCGGAGATTACGACCGCTGAGTTATCTCGAGATAACTCAGCGGTCGTAATCT
TTTTTG-3'

R:

5' AATTCAAAAAAGATTACGACCGCTGAGTTATCTCGAGATAACTCAGCGGT
CGTAATCT-3'

shRNA for APT1

F 5' -

CCGGTAGGCCTGTTACATTAAATATCTCGAGATATTTAATGTAACAGGCCTAT
TTTTG-3'

R 5' -

AATTCAAAAATAGGCCTGTTACATTAAATATCTCGAGATATTTAATGTAACAG
GCCTA-3'

Alignment of Numb

Numb sequences from *D. melanogaster* (P16554), mouse (Q9QZS3), zebrafish (Q5FBC1), and human (P49757) were chosen from UniProt canonical sequences. Alignment was performed with Clustal Omega. Conserved domains were identified by the Conserved Domain Database (NCBI).

Mutagenesis

Site-directed mutagenesis of Numb C37, C160, and C165 to alanine, and APT1 (S119) to alanine were performed using QuikChange multisite-directed mutagenesis kit (Agilent; Cat. No. 210515). Potential palmitoylated cysteines on Numb were identified using CSS-Palm

3.0 developed by Zhou. *et al.* (53) and analysis of the PTB domain crystal structure. Mutants were sequence verified by the DNA Sequencing Facility at the Perelman School of Medicine, University of Pennsylvania.

Live-cell imaging

MDA-MB-231 cells were not conducive to studying the dynamics of asymmetric localization at the plasma membrane by live-imaging, as these cells frequently divided out of the focal imaging plane. Thus, U2 OS cells stably expressing mCherry-Histone H2B facilitated protein tracking over time and allowed us to study the dynamics of asymmetric localization at the plasma membrane over the course of cell division. U2 OS cells stably expressing APT1^{WT}-CFP-FLAG-PRRL or APT1(S119)-CFP-FLAG-PRRL were transfected with 2 µg of YFP-CDC42 Pren-PCDNA3.1, YFP-CDC42 Palm-PCDNA3.1, YFP-CDC42 V12-PCDNA3.1, YFP-CDC42 N17-PCDNA3.1, YFP-CDC42 F28-PCDNA3.1, or NUMB-YFP-FLAG-PRRL wild-type or mutant for 24 hours using LT-1 (Mirus Bio; Cat. No. MIR2300) according to manufacturer's protocol. Cells were imaged 48 hours after transfection in HBSS (Life Technologies; Cat. No. 14175079) containing 2% fetal bovine serum, 1 mg/mL glutamine, and 20mM HEPES pH 7.4 at 37°C. Cells were imaged using the Leica DMI6000 B inverted microscope.

Immunofluorescence

MDA-MB-231 cells were plated on glass coverslips and treated as described. Cells were fixed in 10% formalin, blocked in 5% BSA in TBS containing 0.1% Triton-X (Roche), incubated in 1:500 primary antibody (β-catenin, Cell Signaling Technologies; Cat. No. 9581S), (PARD3 (ab64646)/ Numb (ab14140)/ APT1 (ab91606)/ GFP (ab290)/ Caveolin (ab17052), Abcam), (DHHC20, Sigma; Cat. No. HPA014483), (Acetylated tubulin, Santa Cruz; Cat. No. sc23950), 1:1000), for 1-2 hours at room temperature, incubated in secondary antibody (Alexafluor 488 goat anti-mouse (A11001)/ Alexafluor 594 goat anti-rabbit (A11012), Life Technologies; 1:1000) for 1 hour at room temperature, and mounted in DAPI-mount (Southern Biotech; Cat. No. 0100-20). ESCs were cultured without LIF, MEK or GSK inhibitors for 24 hours before staining with 1:1000 anti-GFP (ab290) as described. Cells were imaged using the Leica DMI6000 B inverted microscope on 40X magnification and colonies were imaged on 20X magnification.

Western Blotting

200,000 MDA-MB-231 cells were plated on 60mm tissue culture dishes and were lysed in Tris lysis buffer (50mM Tris pH 7.4 buffer, 150mM NaCl, 2% Triton-X, 1µg/ml leupeptin, 1µg/ml aprotinin, 2µg/ml pepstatin A). Proteins were run out on 10% acrylamide gel and probed with PARD3 1:1000, CDC42 1:500 (Cytoskeleton; Cat. No. ACD03), Flag 1:500 (Sigma; Cat. No. F3165), APT1 1:500, and DHHC20 1:1000, at 4°C, overnight, then incubated in secondary anti-rabbit HRP (Jackson ImmunoResearch; Cat. No. 211-032-171) or anti-mouse HRP (Jackson ImmunoResearch; Cat. No. 115-035-003) for 1 hour at room temperature. Blots were developed in Pierce ECL Chemiluminescence solution (Thermo Fisher; Cat. No. 32106).

Acyl-Biotin Exchange (ABE) Assay

The protocol is adapted from Wan et al., 2007. : 200,000 MDA-MB-231 cells were plated on 60mm tissue culture dishes and were harvested by scraping in ABE lysis buffer (50mM HEPES pH 7.4, 1% Triton X-100, 150mM NaCl, 5mM EDTA, 50mM N-ethyl-maleimide (NEM), 1µg/ml leupeptin, 1µg/ml aprotinin, 2µg/ml pepstatin A). Lysates were clarified by centrifugation at 15,000 RPM for 10 minutes, and incubated with NEM overnight at 4°C. The samples were m/c precipitated twice then resuspended in 80µL 4%SDS buffer. The samples were split in half and 160µL of hydroxylamine buffer (0.7M hydroxylamine pH 7.4, 50mM HEPES pH 7.4, 0.2% Triton X-100, 150mM NaCl, 5M EDTA) was added to one half of the sample and control 0.2% Triton X-100 buffer (50mM HEPES pH 7.4, 0.2% Triton X-100, 150mM NaCl, 5mM EDTA) was added to the remaining sample and incubated at room temperature for 1 hour. The samples were m/c precipitated and resuspended in 40µL 4%SDS buffer containing 10µM Biotin-HPDP (Thermo Fisher; Cat. No. 21341). 160µL of 0.2% Triton X-100 buffer + 10µM Biotin-HPDP was added and incubated at RT for 1 hour. The samples were m/c precipitated and resuspended in 20µL of 4%SDS buffer followed by addition of 800µL of 1% Triton X-100 buffer (50µL removed for analysis as “input”). 30µL of streptavidin agarose buffer (50mM HEPES pH 7.4, 0.2% Triton X-100, 150mM NaCl, 5mM EDTA) was added to the remaining sample and incubated at room temperature for 1 hour. The samples were m/c precipitated and resuspended in 40µL 4%SDS buffer containing 10µM Biotin-HPDP (Pierce). 160µL of 0.2% Triton X-100 buffer + 10µM Biotin-HPDP was added and incubated at RT for 1 hour. The samples were m/c precipitated and resuspended in 20µL of 4%SDS buffer followed by addition of 800µL of 1% Triton X-100 buffer (50µL removed for analysis as “input”). 30µL of streptavidin agarose beads (Thermo Scientific; Cat. No. 20349) were added to the samples and incubated 20 overnight at 4°C rotating. The samples were washed in 1% Triton-X100 buffer and analyzed by SDS PAGE.

ABE Assay for Immunofluorescence

Washes were performed in 1X ABE buffer + 0.2% Triton-X + 0.1% SDS. Cells were seeded onto coverslips. Cells were fixed in 10% formalin + 50mM NEM for 10 minutes at room temperature and washed once before being incubated overnight in 1X ABE buffer + 0.2% Triton-X + 0.1% SDS* + 50mM NEM at 4°C. The following day, 3 × 15 minute washes were performed. Cells were incubated in HA+ or HA- buffer for 2 hours at room temperature, then washed 3 × 15 minutes. Cells were incubated in Biotin-HPDP buffer for 1 hour at room temperature and washed for 3 × 20 minutes. Cells were Incubated in primary antibody (Biotin, 1:500; Abcam; Cat. No. ab53494), at 4°C overnight, and immunofluorescence was performed as described above.

Click Chemistry Assay for Palmitoylation

200,000 U2 OS cells were plated on 60mm tissue culture dishes and were transfected with 2µg Numb wt or Numb^{AAA} YFP-Flag for 24 hours, labeled with 100µM palmitic acid azide (Life Technologies; Cat. No. C10265). Cells were prepared using the Click-IT protein reaction buffer (Life Technologies; Cat. No. C10276) according to manufacturer’s protocol and analyzed with Western blot as described above.

Quantification and linescan analysis

Cells were quantified by drawing around the mitotic spindle poles, or around each daughter cell in cytokinesis using the Leica LAS AF software as shown in Fig. S1A. Percent difference was calculated from the Mean Gray Values generated in Leica LAS AF and calculated as described in Fig. S1A. The distribution of acquired percentage differences for each experimental condition were plotted as dot plots. Cells with a percentage difference of 20 or greater were counted as asymmetric and plotted in a bar graph. All graphs were generated with Prism software. Linescan analysis of pixel intensity was performed on still frames from live-cell movies using ImageJ software.

Colony Assays

MDA-MB-231 cells were plated on 6-well, low adhesion plates (Corning; Cat. No. 3471) in 2mL of WIT-P plus serum-free supplement growth media (Cellaria; Cat. No. 00-0045-500) containing 20ng/mL FGF (Life Technologies; Cat. No. PHG0024), 20ng/mL EGF (Life Technologies; Cat. No. PHG0311), and 10 µg /mL heparin (StemCell Technologies; Cat. No. 07980). Each well contained 4,000 cells. Cells were treated with 1 µM GSI (Compound E; Millipore; Cat. No. 565790), 5 µM IWP2 (StemCell Technologies; Cat. No. 72124), or DMSO (Sigma; Cat. No. D2650) and grown for 7 days before counting. For replating, cells were grown into colonies as described. On Day 7, colonies were spun down at 1000rpm × 5min, resuspended in 0.05% Trypsin-EDTA, reconstituted in growth media, and plated as described above. Process was repeated for three replatings in duplicate.

Proliferation Assays

15,000 MDA-MB-231 cells were plated on a 12-well dish. At 24, 48, and 72 hours, cells were washed with 1X PBS, trypsinized, and spun at 1000rpm × 5 minutes. Pellets were resuspended in DMEM and counted by a hemacytometer.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen; Cat. No. 74104) and cDNA was synthesized from 2,000ng total RNA using SuperScript III First-Strand Synthesis SuperMix (Life Technologies; Cat. No. 18080400). qRT-PCRs were performed in triplicate using standard SYBR green reagents and protocols on a StepOnePlus Real-Time PCR system (Applied Biosystems). Target genes with high log₂FC values were chosen for validation. The target mRNA expression was quantified using Ct method and normalized to GAPDH expression. The following primers were used for validation:

hDKK1

F: 5' - CAGGCGTGCAAATCTGTCT - 3'

R: 5' - AATGATTTTGATCAGAAGACACACATA - 3'

hFGF5

F: 5' - CCCAGAATCAGCCCTACAAG - 3'

R: 5' - GAGGAGGAAGGACAAGCTCA - 3'

hGATA6

F: 5' - GCAAAAATACTTCCCCCACA - 3'

R: 5' - TCTCCCGCACCCAGTCATC - 3'

hKLF5

F: 5' - CTGCCTCCAGAGGACCTG - 3'

R: 5' - TCGTCTATACTTTTTATGCTCTGGAAT - 3'

hITGB4

F: 5' - TCAGCCTCTCTGGGACCTT - 3'

R: 5' - TCCTTATCCACACGGACACA - 3'

hBMP4

F: 5' - TCCACAGCACTGGTCTTGAG - 3'

R: 5' - TGGGATGTTCTCCAGATGTTCT - 3'

hPTK7

F: 5' - CAGAGGACTCACGGTTCGAG - 3'

R: 5' - TACCAGGGTCTCTGCCACTC - 3'

hGAPDH

F: 5' - ACA CCA TGG GGA AGG TGA AG-3'

R: 5' -AAG GGG TCA TTG ATG GCA AC -3'

hCDC42 palm

F: 5' -TGGAGTGTCTGCACTTACA-3;

R: 5' -GAATATACAGCACTTCCTTTTGGG-3'

hCDC42 prenyl

F: 5' -AGGCTGTCAAGTATGTGG-3'

R: 5' -TAGCAGCACACACCTGCG-3'

RNA Isolation and analysis for RNA-seq

Total RNA was harvested from MDA-MB-231 control, APT1^{WT}-overexpressing or shAPT1 cells using the RNeasy RNA isolation kit (Qiagen; Cat. No. 74104). 100ng of RNA was used to generate cDNA libraries with the Illumina TruSeq mRNA Library Prep Kit for NeoPrep library preparation system (Illumina; Cat. No. NP-202-1001) and analyzed on a NextSeq500 sequencer. Analysis was prepared by the DNA Sequencing Facility at the Perelman School of Medicine, University of Pennsylvania as follows: estimated transcript levels were ranked with Salmon, TX Import was used to condense transcript levels to gene intensity, and Deseq2 was used to calculate statistical levels for each condition. The scaled values (determined by DeSeq2) were input to Gene Set Enrichment Analysis software (Broad

Institute) and analyzed against the C2 Chemical and Genetic perturbations, C6 Oncogenic, and Hallmark signatures gene matrix applying classic enrichment statistic. Heatmaps were generated by taking the $\log_2$ values with an offset of 1 for all conditions and targets were chosen by taking the leading-edge targets from GSEA BCAT, NOTCH, and mammary stem cell sets. The average of the 3 control wild-type values were subtracted from each individual value. Values were clustered for samples and genes using Euclidian similarity measure with average linkage.

PCR

To confirm the expression of FLAG-CDC42 plasmids in shCDC42 MDA-MB-231 cells, we isolated RNA and prepared cDNA from FLAG-prenylated CDC42, FLAG-CDC42-V12, FLAG-CDC42-N17, FLAG-CDC42-F28, FLAG-palmitoylated CDC42 cell lines. PCR reaction was performed using 2ug of cDNA, 10X PCR buffer (Sigma; Cat. No. P2192), 10mM dNTPs (Life Technologies; Cat. no. 18252015), Taq polymerase (Sigma; Cat. No D1806) and 10 μ M of hCDC42-palm and hCDC42-prenyl primers mentioned above, which are designed to span the alternatively spliced exon. The reaction was run out on a 1.4% agarose gel.

Flow cytometry

Adherent non-confluent MDA-MB-231 cells or colony-dissociated cells were treated to detect ALDH activity using the ALDEFLUOR assay (Stem Cell Technologies; Cat. No. 01702) according to manufacturer's protocol. Diethylaminobenzaldehyde (DEAB) was used as a negative control to set ALDH + gates. Cells were stained for surface markers with CD44-APC (BD Biosciences; Cat. No. 560890), CD24-PE (BD Biosciences; Cat. No. 560991), and Live/Dead Violet (Thermo Fisher; Cat. No. L34963) for viability. Compensation was performed using Ultra Comp beads (Thermo Fisher; Cat. No. 01-2222-41). Experiments were run on the Attune NxT flow cytometer system (Life Technologies) and analysed with FlowJo software. ALDH+ cells (Fig. S7) were gated for CD44⁺/CD24^{lo} (Fig. 7C) for comparison.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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One-Sentence Summary

Palmitoylation-dependent asymmetric partitioning of cell fate determinants correlates with tumor cell heterogeneity.

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Editor's Summary

Lipid modification promotes cancer cell heterogeneity

Asymmetric cell division generates daughter cells that have distinct fates and is accomplished through the unequal distribution of cell fate determinants or signaling pathway components. Stypulkowski *et al.* found that the depalmitoylase APT1 was asymmetrically localized in dividing breast cancer and osteosarcoma cells and required for the asymmetric localization of the Notch antagonist Numb and the Wnt signaling mediator β -catenin, both of which are palmitoylated. The catalytic activity of APT1 was required for asymmetric localization of APT1, Numb, and β -catenin, and the polarity complex component CDC42 was required for the asymmetric localization of both APT1 and β -catenin. APT1-mediated asymmetric partitioning of Numb and β -catenin restricted Notch and Wnt signaling to one daughter cell or the other and induced a mammary stem cell transcriptional signature in breast cancer cells. The absence of APT1 or CDC42 reduced heterogeneity and self-renewal capacity in breast cancer cell populations. These results identify palmitoylation-dependent asymmetric partitioning of cell fate determinants as a potential driver of tumor cell heterogeneity, which has been associated with tumor progression and metastatic potential.

Fig. 1. Activity of the depalmitoylating enzyme APT1 is required for asymmetric localization of Numb and β -catenin

(**A** and **B**) Images of dividing MDA-MB-231 cells stained to show endogenous Numb (**A**) and β -catenin (**B**) in red, acetylated tubulin in green, and nuclei in blue. Arrowheads indicate asymmetric localization of Numb and β -catenin. Scale bars, 15 μ m. (**C** and **D**) Distribution dot plots showing the difference in mean fluorescence pixel intensity of endogenous Numb (**C**) and β -catenin (**D**) across dividing MDA-MB-231 cells. The distribution of the percentage differences of all quantified cells was plotted, and cells with a difference of >20% (black dotted line) were scored as asymmetric. $n = 508$ –582 cells scored for each experimental group. Each dot represents a single cell. Asterisks indicate statistically significant differences between the indicated groups. (**E** and **F**) Quantification of dividing MDA-MB-231 cells showing asymmetric Numb (**E**) and β -catenin (**F**) localization after treatment with PalmB or DMSO. (**G** and **H**) Quantification of the number of dividing MDA-MB-231 cells showing asymmetric Numb (**G**) and β -catenin (**H**) localization when APT1 was knocked down with shAPT1, and when wild-type APT1 (APT1^{WT}) or the catalytically inactive APT1^{S119A} mutant was coexpressed with shAPT1. Cells expressing a scrambled (Scr) shRNA sequence were used as a negative control for APT1 knockdown, and cells expressing the empty vector were used as the negative control for the APT1 rescue experiments. (**I** and **J**) Quantification of the number of dividing MDA-MB-231 cells

showing asymmetric Numb (I) and β -catenin (J) localization when DHHC20 was knocked down with shDHHC20. (**K to M**) Immunoblots showing biotin-labeled Numb (K), β -catenin (L), and Erk (M) in MDA-MB-231 cell lysates following acyl-biotin exchange (ABE) assays and pulldown on streptavidin beads (PD). Cells were grown in either in the presence either PalmB or vehicle control (DMSO). Input lanes show cell lysates prior to pulldown. Samples without hydroxylamine (-HAM) were negative controls for the ABE reactions. * $P < 0.05$; ** $P < 0.01$ T-test (E and F) or ANOVA (G to J). Error bars indicate standard deviation (SD).

Fig. 2. Palmitoylation and APT1 activity drive Numb localization
(A) Sequence comparison of the N-terminus of Numb from fruit fly (*D. melanogaster*), zebrafish (*D. rerio*), mouse (*M. musculus*), and human (*H. sapiens*). The phosphotyrosine domain (PTB) is highlighted in green, and the putative palmitoylated cysteines are highlighted in yellow. Conserved residues indicated by asterisk (*). **(B)** Immunoblot showing transgenically-expressed wild-type Numb (Numb^{WT}) or the Numb^{AAA} mutant and endogenous β -catenin in U2 OS cell lysates following purification of palmitoylated proteins. Cells were metabolically labelled with palmitic acid azide or treated with DMSO (vehicle

control), then lysates were subjected to click chemistry to convert the palmitic acid moiety to biotin, pulled down on streptavidin beads, and used for immunoblotting. Input was taken from cell lysates prior to pulldown. **(C)** Time-lapse images of dividing U2 OS cells co-expressing Numb^{WT}-YFP (yellow), APT1^{WT}-CFP (blue), and mCherry-Histone H2B (red). Fluorescence pixel intensity was measured along the division axis (dashed line) and the corresponding pixel values of Numb (yellow line) and APT1 (blue line) along the division axis were plotted on graphs. Red arrowheads on images and graphs indicate the peak Numb and APT1 pixel intensity at the membrane or cytokinetic midbody. Time shown in minutes, m. Arbitrary units, a.u. Scale bar, 15 μ m. **(D)** Quantification of the number of dividing U2 OS cells showing asymmetric localization of Numb^{WT}-YFP (black bar) and Numb^{AAA}-YFP (grey bar) when each was coexpressed with shAPT1. Cells expressing a scrambled (Scr) shRNA sequence were used as a negative control for APT1 knockdown. **(E and F)** Time-lapse images of dividing U2 OS cells coexpressing either APT1^{WT}-CFP (E) or APT1^{S119A}-CFP (F) with mCherry-Histone H2B (red). Fluorescence pixel intensity was quantified as in (C). Time shown in minutes, m. Arbitrary units, a.u. Scale bars, 15 μ m. **(G)** Quantification of the number of dividing U2 OS cells showing asymmetric APT1^{WT}-CFP or APT1^{S119A}-CFP localization. Cells expressing an empty GFP plasmid (Vector) were used as a negative control. n= 102-143 cells scored for each group from three independent experiments. * P < 0.05; ** P < 0.01 T-test and ANOVA. Error bars indicate standard deviation (SD).

Fig. 3. Palmitoylating enzymes and depalmitoylating enzymes are asymmetrically partitioned during cell division

(A) Images of dividing MDA-MB-231 cells stained to show endogenous APT1 (red), DHHC20 (green), and nuclei (blue). (B and C) Images of dividing MDA-MB-231 cells stained to show endogenous APT1 (B) or DHHC20 (C), caveolin, and nuclei. Asymmetric localization is indicated by arrowheads (A to C). (D) Images of dividing MDA-MB-231 cells treated with PalmB or DMSO and stained to show biotin-labeled palmitoylated proteins (red), acetylated tubulin (green), and nuclei (blue) by ABE immunofluorescence. Samples

without hydroxylamine (-HAM) were negative controls for the ABE reaction. **(E)** Distribution dot plots showing the difference in mean fluorescence pixel intensity of biotin-labeled palmitoylated proteins. The distribution of the percentage differences of all quantified cells was plotted, and cells with a difference of >20% (dotted line) were scored as asymmetric. $n = 91-101$ cells scored for each experimental group. Each dot represents a single cell. Asterisks indicate statistically significant differences between the indicated groups. **(F)** Quantification of the number of dividing MDA-MB-231 cells showing asymmetric palmitoylated proteins after treatment with PalmB or DMSO control. **(G)** Confocal images of ABE immunofluorescence in non-dividing MDA-MB-231 cells showing all palmitoylated proteins (green), APT1 or DHHC20 (red), and nuclei (blue) by ABE immunofluorescence. White dotted boxes indicated magnified areas shown directly below (Zoom). Samples without hydroxylamine (-HAM) were negative controls for the ABE reaction. Scale bars (including zoom), $15\mu\text{m}$. $*P < 0.05$ T-test. Error bars indicate standard deviation (SD).

Fig. 4. The reciprocal interaction between APT1 and CDC42 establishes and maintains asymmetric protein partitioning during cell division

(A and B) Quantification of the number of dividing MDA-MB-231 cells showing asymmetric localization of APT1 when CDC42 was knocked down with shCDC42 (A) or PARD3 was knocked down with shPARD3 (B). Cells expressing a scrambled (Scr) shRNA sequence were used as a negative control for knockdown. (C and D) Quantification of the number of dividing U2 OS cells showing asymmetric APT1^{WT}-CFP or APT1^{S119A}-CFP in cells expressing constitutively active (CDC42^{V12} and CDC42^{F28L}) or dominant negative (CDC42^{N17}) forms of CDC42 (C), or expressing isoforms of CDC42 that are prenylated (CDC42^{Pren}) or both palmitoylated and prenylated (CDC42^{Palm}) (D). (E and F) Time-lapse images of dividing U2 OS cells co-expressing CDC42^{Palm}-YFP (yellow) and either APT1^{WT}-CFP (E) or APT1^{S119A}-CFP (F) (blue), and mCherry-Histone H2B (red). Overlapping CFP and YFP signal in the merge (green). Fluorescence pixel intensity was measured along the division axis (dashed line) and the corresponding pixel values of CDC42 (yellow line) and APT1 (blue line) along the division axis were plotted. Red arrowheads on images and corresponding graphs mark the peak asymmetric CDC42 and APT1 accumulation at the membrane or cytokinetic midbody. Time shown in minutes, m. Arbitrary units, a.u. Scale bars, 15 μ m. n = 152-288 cells scored for each group from 4 independent experiments. * P < 0.05; ** P < 0.01 T-test (A–D) and ANOVA (C, D). Error bars indicate standard deviation (SD).

Fig. 5. APT1 restricts Wnt and Notch transcriptional activity to one daughter cell
(A and B) Images of cytokinetic MDA-MB-231 cells stained to show expression of the pGF1-Notch GFP reporter (A) or pGF1-TCF/Lef1 GFP reporter (B) (red), acetylated tubulin (green), and nuclei (blue). Arrowhead indicate asymmetric localization. Scale bars, 15 μ m. **(C and D)** Distribution dot plots showing the difference in mean fluorescence pixel intensity of pGF1-Notch reporter (C) or pGF1-TCF/Lef1 reporter (D) across dividing cells. Cells expressing an empty pGF1-mCMV GFP reporter were used as a negative control for the reporters. The distribution of the percentage differences of all quantified cells was plotted, and cells with a difference of >20% (dotted line) were scored as asymmetric. $n = 784-822$ cells scored for each experimental group. Each dot represents a single cell. Asterisks indicate statistically significant differences between the indicated groups. **(E and F)** Quantification of dividing MDA-MB-231 cells showing asymmetric localization of pGF1-Notch GFP reporter (E) or pGF1-TCF/Lef1 GFP reporter (F) (black bars) following treatment with PalmB or DMSO vehicle control. Cells expressing an empty pGF1-mCMV reporter (grey bars) were used as a negative control for reporter expression. **(G and H)** Quantification of the number of dividing MDA-MB-231 cells showing asymmetric localization of the pGF1-Notch GFP reporter (G) or pGF1-TCF/Lef1 GFP reporter (H)

(black bars) when coexpressed with shAPT1, shCDC42, both shAPT1 and shCDC42 (DKD), and shDHHC20. Cells expressing an empty pGF1-mCMV GFP reporter (grey bars) were used as a negative control for reporter expression, and cells expressing a scrambled (Scr) shRNA sequence were used as a negative control for knockdown. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ T-test (between reporters and pGF1-mCMV) or ANOVA (C to H). Error bars indicate standard deviation (SD).

Fig. 6. Altering APT1 expression changes β -catenin and Notch gene signatures in MDA-MB-231 cells

Gene set enrichment analysis (GSEA) of (A) a top-scoring β -catenin overexpression transcriptional signature in MDA-MB-231 cells when APT1 was knocked down and compared against control cells (FDR q-value= 0.073), (B) a Notch inhibition signature when APT1^{WT} was overexpressed and compared against control cells (FDR q-value= 0.302) (B), and (C) a mammary stem cell signature when APT1^{WT} was overexpressed and compared against shAPT1 cells (FDR q-value= 0.0001). (D) Heat map of leading edge genes obtained

from the mammary stem cell signature shown in (C). Data is grouped by APT1 knockdown (kd) cells, APT1^{WT}-overexpressing (oe) cells, and control (wt) cells. (E) Images of cytokinetic MDA-MB-231 cells stained to show the pGF1-SRR2 GFP reporter (red), acetylated tubulin (green), and nuclei (blue). Arrowheads indicate asymmetric localization of pGF1-SRR2. Scale bar, 15µm. (F) Distribution dot plots showing the difference in mean fluorescence pixel intensity of the pGF1-SRR2 reporter across dividing MDA-MB-231 cells. Cells expressing an empty pGF1-mCMV GFP reporter were used as a negative control for the reporters. The distribution of the percentage differences of all quantified cells was plotted, and cells with a difference of >20% (dotted line) were scored as asymmetric. n = 959 cells scored for the experimental group. Each dot represents a single cell. Asterisks indicate statistically significant differences between the indicated groups. (G) Quantification of dividing MDA-MB-231 cells showing asymmetric localization of the pGF1-SRR2 GFP reporter after treatment with PalmB or DMSO control (black bars). Cells expressing an empty pGF1-mCMV reporter (grey bars) were used as a negative control for reporter expression. (H) Quantification of the number of dividing MDA-MB-231 cells showing asymmetric localization of pGF1-SRR2 GFP reporter (black bars) when coexpressed with shAPT1, shCDC42, both shAPT1 and shCDC42 (double knockdown, DKD), or shDHHC20. Cells expressing an empty pGF1-mCMV GFP reporter (grey bars) were used as a negative control for reporter expression, and cells expressing a scrambled (Scr) shRNA sequence were used as a negative control for knockdown conditions. ** $P < 0.01$; **** $P < 0.0001$ T-test (between reporters and pGF1-mCMV) or ANOVA (H). Error bars: SD.

Fig. 7. APT1 and CDC42 maintain tumorigenic cell populations in MDA-MB-231 colonies (A and B) Quantification of the average number of colonies formed from MDA-MB-231 shAPT1 cells (A) or shCDC42 cells (B) over 3 serial replatings. Cells expressing a scrambled (Scr) shRNA sequence were used as a negative control for knockdown. Each graph shows means taken from three independent experiments. * $P < 0.05$; *** $P < 0.0001$ as measured by T-test. Error bars indicate standard error of the mean (SEM). (C) Representative flow cytometry analysis showing gating strategy of CD44⁺/CD24^{lo} cells (red box) in cells dissociated from colonies or adherent. The population was gated off of ALDH⁺ cells shown in fig. S7. The percentages inside the red box indicate the relative proportion of the CD44⁺/CD24^{lo} cell population. Cells were stained with phycoerythrin (PE)-conjugated anti-CD24 (CD24-PE) (x-axis) and allophycocyanin (APC)-conjugated CD44-APC (y-axis). Flow cytometry plots are representative of results from six independent experiments.