The Par3-like polarity protein Par3L is essential for mammary stem cell maintenance

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The Par polarity proteins play key roles in asymmetric division of *Drosophila melanogaster* stem cells; however, whether the same mechanisms control stem cells in mammals is controversial. Although necessary for mammary gland morphogenesis, Par3 is not essential for mammary stem cell function. We discovered that, instead, a previously uncharacterized protein, Par3-like (Par3L), is vital for mammary gland stem cell maintenance. Par3L function has been mysterious because, unlike Par3, it does not interact with atypical protein kinase C or the Par6 polarity protein. We found that Par3L is expressed by multipotent stem cells in the terminal end buds of murine mammary glands. Ablation of Par3L resulted in rapid and profound stem cell loss. Unexpectedly, Par3L, but not Par3, binds to the tumour suppressor protein Lkb1 and inhibits its kinase activity. This interaction is key for the function of Par3L in mammary stem cell maintenance. Our data reveal insights into a link between cell polarity proteins and stem cell survival, and uncover a biological function for Par3L.

The Par3 protein sits at the apex of a signalling network that controls apical/basal polarity and spindle orientation, not only in differentiated epithelial cells but also in the *Drosophila* neuroblast, intestinal and sensory organ precursor stem cells. Loss of spatially organized Par signalling through atypical protein kinase C (aPKC) in these stem cells disrupts asymmetric cell division and perturbs self-renewal and the differentiation of daughter cells. Although the Par proteins are conserved throughout the metazoa, it remains unclear whether their functions have been retained in mammalian stem cells. For example, although the Par3 pathway does seem to play a role in the asymmetric divisions of radial glial progenitors, it is not essential for mammary gland regeneration from stem cells. Although the Par proteins are conserved throughout the metazoa, it remains unclear whether their functions have been retained in mammalian stem cells. For example, although the Par3 pathway does seem to play a role in the asymmetric divisions of radial glial progenitors, it is not essential for mammary gland regeneration from stem cells, and aPKC is completely dispensable for primitive and adult haematopoietic stem cell polarization, activity and blood formation.

Whereas in lower animals the Par proteins and aPKC are each encoded only by single genes, diversification has occurred in the vertebrate lineage, and the various isoforms can have distinct biological functions. For instance, isoforms of Par6 are not interchangeable in zebrafish. In 2002 we cloned a gene (*pard3b*) closely related to the Par3 gene (*pard3*), which expresses a protein we named Par3-like (Par3L; ref. 7). The function of Par3L has been enigmatic because, unlike Par3, it does not bind to either Par6 or aPKC, but exogenous Par3L can co-localize with these proteins and with Par3 at epithelial tight junctions. Nothing has been known about any biological roles for Par3L. It has not been shown to participate in cell polarization or in stem cell functions. Here we identify Par3L as an essential factor for mouse mammary stem cell maintenance, and show that it acts by suppressing the kinase activity of the Par4 or Lkb1 (also known as Stk11) polarity protein.

**RESULTS**

Par3L is expressed in cap cells and is required for mammary ductal outgrowth

To test for a function of Par3L in mammary gland development, we first developed Par3L-specific antibodies (Supplementary Fig. 1a,b) and stained sections from mature and developing murine mammary glands. In mature glands Par3L staining was restricted to the tight junctions of luminal epithelial cells, as predicted from previous studies. Strikingly, however, in the developing glands Par3L was detected at the cell cortex in the cap cells of terminal end buds (Fig. 1a). Cap cells express s-SHIP and, using a transgenic mouse model (Tg11.5kb–GFP) that expresses green fluorescent protein (GFP) from the s-SHIP promoter, they have been shown to be active, multipotent stem cells that can regenerate a functional mammary gland after transplantation into cleared fat pads of recipient mice. We refer to these cells as mammary repopulating units (MRUs). They are negative for cytokeratin 14 (CK14; known as Krt14 in mouse) and cytokeratin 8 (CK8; known as Krt8 in mouse), but are CD49f and CD24 positive.

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Figure 1 Par3L is required for mammary gland regeneration. (a,b) Immunostaining shows that Par3L is expressed specifically at the apical junctions of luminal epithelial cells (marked with CK8, red) in mature mammary ducts and in cap cells of the terminal end buds of developing mammary glands. (c) Par3L depletion results in abnormal terminal end bud morphology. (d) Carmine alum staining of whole mammary glands shows that Par3L depletion impairs mammary gland regeneration. (e) Quantification of regeneration, as percentage of fat pads showing any ductal outgrowth. (f) Depletion of Par3L significantly reduces the extent of outgrowth into the fat pads. This phenotype could be reversed by overexpression of human Par3L (PARD3B), which is resistant to the Par3L shRNAs. (g) Partial depletion of Par3L by Par3L shRNA 1 significantly reduces mammary ductal tree side branching. This phenotype could be rescued by overexpression of human Par3L. The graph shows the mean ± s.d. n represents the number of mammary glands examined in each experiment. Statistical significance was calculated for each experimental group, compared to the Luc shRNA control group; P values were calculated by the Student t-test.
Par3L is essential for mammary gland stem cell maintenance.

(a) Lentivirus-transduced (GFP+) colonies grown from single cells in Matrigel, first passage. (b) Serial colony-forming assay shows that Par3L depletion decreases the number of CFCs. The graph shows the mean ± s.d. for three experimental replicates (independent transductions). (c) Competition assay demonstrates that Par3L depletion impairs mammary repopulating unit (MRU) maintenance in a cell-autonomous manner. Equal numbers of red (control) and green (Par3 shRNA, top panel, or control, bottom panel) cells were mixed, injected into cleared fat pads and left to regenerate for 6 weeks.

Table 1. Limiting dilution mammary regeneration assay.

| Experiment       | Cells per injection into fat pad | Outgrowths (number/total) | Stem cell frequency (upper and lower limits) |
|------------------|---------------------------------|---------------------------|---------------------------------------------|
| Luc shRNA control| 1,000                           | 7/16                      |                                             |
|                  | 2,000                           | 4/6                       |                                             |
|                  | 4,000                           | 6/6                       | 1/2,062                                     |
|                  | 8,000                           | 5/6                       | (1/1,205–1/3,400)                           |
|                  | 16,000                          | 6/6                       |                                             |
| Par3L shRNA 2    | 16,000                          | 1/6                       | 1/214,960                                   |
|                  | 32,000                          | 2/6                       |                                             |
|                  | 64,000                          | 1/6                       |                                             |
|                  | 128,000                         | 2/6                       | (1/112,340–1/411,330)                       |
|                  | 256,000                         | 4/6                       |                                             |

Figure 2 Par3L is essential for mammary gland stem cell maintenance. (a) Par3L shRNA significantly reduced fat pad filling for those glands that did regenerate (Fig. 1d–f). Ductal branching was measured in the fat pads transplanted by mammary cells with Par3L shRNA 1, which was permissive for mammary gland outgrowth. The number of ductal branching points per unit area was significantly reduced (Fig. 1d–g). Importantly, these phenotypes were reversed by the expression of human Par3L, which is resistant to shRNA-mediated silencing, demonstrating that the defect is specific to Par3L knockdown rather than to off-target effects of the shRNAs. Because Par3L is expressed at either the cortex of cap cells in developing mammary glands or the tight junction of luminal epithelial cells in mature mammary glands, the phenotype caused by Par3L depletion could be due to defects in either type of cell. To further address this issue, we stained mammary gland sections to examine the structure of mammary glands. Silencing of Par3L substantially reduced anti-Par3L antibody staining of mammary glands (Fig. 1c). Moreover, the normal structure of terminal end buds was severely disrupted in glands that grew out from mammary stem cells transduced with Par3L shRNA 2. All the terminal end bud cells were CK8 positive and there was no distinct cap cell layer (Fig. 1c). This experiment demonstrates that Par3L silencing causes defects in ductal outgrowth and terminal end bud structures, probably through effects on the cap cell MRUs.

Par3L is essential for mammary stem cell maintenance

To further investigate whether Par3L affects MRUs, we quantified the in vivo regeneration capacity of isolated mammary cells, after implantation into the cleared fat pads of syngeneic recipient mice, using limiting dilution assays. On the basis of positive outgrowths scored for each dilution, the MRU frequency was 1/2,100 for wild-type cells, in line with previous results, but only 1/215,000 for cells expressing Par3L shRNA 2 (Table 1). These data indicate that silencing of Par3L causes a highly significant 100-fold decrease in functional MRUs. By contrast, no significant decrease in MRUs was observed on depletion of the closely related Par3 gene.

As an in vitro assay for stem cell survival we used a serial colony-forming assay, a schematic representation of which is shown in Supplementary Fig. 2a. Single mammary cell suspensions were prepared from mammary cells transduced with lentivirus shRNAs against Par3L or Luc shRNA control, and seeded into Matrigel. Colones formed (Fig. 2a) after one week and were scored, then trypanosized and replated.
as single cells into fresh Matrigel. The number of CFCs increases in the second and third passages of the wild-type control (Fig. 2b). Importantly, this serial passage procedure is strongly enriching for MRUs, as shown by the very high efficiency with which the control cells from tertiary colonies could repopulate fat pads in vivo (Supplementary Fig. 2b). When Par3L was depleted, however, the number of CFCs was significantly lower, even in the primary culture, and dropped substantially in subsequent passages, for two independent shRNAs (Fig. 2a,b). These results are consistent with a rapid depletion of functional stem cells in the absence of Par3L expression.

We reasoned that Par3L might be required cell autonomously for MRU survival, or might alternatively support the stem cell micro-environment in a cell non-autonomous fashion. To distinguish these two possibilities, we used a competition assay to compare the efficiencies of ductal outgrowth by Luc shRNA control MRUs versus Par3L-depleted cells. Cells expressing the Par3L shRNA 2 were marked green with GFP, and those expressing the Luc shRNA were marked red with mApple. Equal numbers of cells were then mixed and implanted into cleared fat pads, as represented in Supplementary Fig. 2c. If Par3L were necessary for niche maintenance, or for secretion of factors that support stem cells, then it would be predicted that green, Par3L-depleted cells would be unable to form a niche and negatively impact the outgrowth of neighbouring control red cells. Conversely, the wild-type cells should provide a functional niche for green Par3L-depleted cells expressing Par3L shRNA 1 or Luc shRNA control. The ducts have normal myoepithelial and luminal epithelial organization. Partial depletion of Par3L has no effects on cleaved Caspase-3 or Ki67 staining in the ducts. (f) Quantification of Ki67 and cleaved Caspase-3 staining in the mammary gland ducts. The graph shows the mean of the number of mammary glands that were scored. Statistical significance was calculated for each experimental group, compared to the Luc shRNA control group. P values were calculated with a Student t-test.

Figure 3 Loss of Par3L causes mammary stem cell loss through apoptosis and/or failure of self-renewal. (a) Representative images of the staining of tertiary colonies. Par3L depletion decreases the percentage of CK14+CK8+ double-positive cells and increases cleaved Caspase-3+ cells, but does not change the Ki67+ cell ratio. (b–d) Quantification of Ki67+ cells (b), cleaved Caspase-3+ cells (c) and CK14+CK8+ cells (d). n represents the number of colonies scored. Original source data for c are provided in Supplementary Table 1. (e) Staining of mature mammary gland ducts regenerated from mammary cells expressing Par3L shRNA 1 or Luc shRNA control. The ducts have normal myoepithelial and luminal epithelial organization. Partial depletion of Par3L has no effects on cleaved Caspase-3 or Ki67 staining in the ducts. (f) Quantification of Ki67 and cleaved Caspase-3 staining in the mammary gland ducts. The graph shows the mean ± s.e.m. n represents the number of mammary glands that were scored. Statistical significance was calculated for each experimental group, compared to the Luc shRNA control group. P values were calculated with a Student t-test.
Par3L is necessary for colony-forming activity by s-SHIP GFP+ cells. GFP+ cells from six-week-old s-SHIP GFP transgenic mice were FACS sorted to homogeneity and infected by mApple–Luc shRNA control or mApple–Par3L shRNA 2 virus. (a, d) Serial colony-forming assay shows that Par3L depletion decreases the number of CFCs. All mApple-positive colonies were counted and presented in the bar graph. The graph represents the mean ± s.d. for three technical replicates (independent transductions of GFP+ cells from a single mouse). Original source data are provided in Supplementary Table 1. (b) Representative images of the staining of tertiary colonies for CK8 and CK14. Proliferation, as marked by Ki67 (also known as Mki67) or cleaved Caspase-3, and for CK8 as a luminal epithelial marker and CK14 as a myoepithelial marker. Proliferation, as marked by Ki67+

Par3L depletion significantly increases cleaved Caspase-3+ cells. The graph shows the mean ± s.e.m. for each experimental group, compared to the luc shRNA control group. P values were calculated with a Student t-test.

Depletion of Par3L promotes apoptosis and differentiation into CK8+ cells

Some MRUs in young mice are actively cycling. Therefore, a decrease in relative MRU number could arise from either reduced proliferation or increased cell death. To distinguish these possibilities we stained the colonies grown in Matrigel for Ki67 (also known as Mki67) or cleaved Caspase-3, and for CK8 as a luminal epithelial marker and CK14 as a myoepithelial marker. Proliferation, as marked by Ki67+
Figure 5 Par3L maintains mammary stem cells through interaction with Lkb1. (a) Par3L interacts with Lkb1 but not aPKC, whereas Par3 interacts with aPKC but not Lkb1. About 2% of Lkb1 was captured by Par3L, whereas only ~0.02% Lkb1 precipitated with Par3. (b) The endogenous interaction between Par3L and Lkb1 was confirmed by co-immunoprecipitation (co-IP) using mouse kidney lysates, which express relatively high levels of Par3L. WB, western blot. (c) The region on Par3L required for Lkb1 binding was mapped between PDZ2 and PDZ3 domains. Replacement of this region of Par3L with the corresponding region of Par3 disrupts the interaction between Lkb1 and Par3L. All full scan images are presented in Supplementary Fig. 6. (d,e) Mammary gland regeneration assay. Wild-type human Par3L can rescue mammary gland regeneration from Par3L-depleted mammary cells, whereas the Lkb1-binding-deficient Par3L mutant is unable to do so. (f) Representative images of the staining of tertiary colonies for CK8, CK14 and cleaved Caspase-3. (g,h) Quantification of CK14+CK8+ dual-positive (g) and cleaved Caspase-3+ (h) cells in in vitro serial colony-forming assay. Wild-type human Par3L, but not the mutant Par3L, was able to reverse the Par3L depletion effects. The graph shows the mean ± s.e.m. n represents the number of colonies that were scored. Statistical significance was calculated for each experimental group, compared to the Luc shRNA control group. P values were calculated with a Student t-test.
**Figure 6** Par3L maintains mammary stem cells by suppressing Lkb1 activity. (a) Par3L inhibits Lkb1 activity in HEK293T cells. Wild-type or mutant Par3L was overexpressed in HEK293T cells and phospho-AMPK was blotted as an indicator for Lkb1 activity. The full scan images are presented in Supplementary Fig. 6. (b) Wild-type Par3L reduced the phospho-AMPK level to about 40% of the control level, whereas mutant Par3L reduced the phospho-AMPK level to about 70% of the control level. The graph shows the means and ± s.e.m. for three independent experiments. *P* values were calculated with a paired Student’s t-test. (c) Mammary cells from female C3H mice were infected by vector control or Lkb1–2A–Stradα polycistronic expression virus and subjected to the serial colony-forming assay. (d) Lkb1 overexpression decreases the number of CFCs. The graph shows the mean ± s.d. for three experimental replicates (independent transductions). Original source data provided in Supplementary Table 1. (e) Representative images of the staining of tertiary colonies for CK8, CK14 and cleaved Caspase-3. (f) Quantification of cleaved Caspase-3+ cells in the *in vitro* serial colony-forming assay. Lkb1 overexpression significantly increases cleaved Caspase-3+ cells when compared to the vector control group. Graphs show mean ± s.e.m. *n* represents the number of colonies that were scored. Statistical significance was calculated for each experimental group, compared to the vector control group. *P* values were calculated with an unpaired Student’s t-test.

efficiently reversed by hPar3L expression (Fig. 3a,d). In contrast, we could detect no difference in either Ki67 or Caspase-3 staining for mature ducts arising from cells expressing Par3L shRNA 1 as compared to Luc shRNA controls, and there were no detectable CK8+/CK14+ cells (Fig. 3e,f). These data suggest that loss of Par3L specifically depletes MRUs by promoting apoptosis and/or preventing their self-renewal, but has no effect on the survival of differentiated mammary cells.

Par3L expression is not entirely specific to cap cells, because it was also detectable at the tight junctions of luminal cells in mature ducts (Fig. 1a). Therefore, to test more definitively for a stem-cell-specific function of Par3L, we used fluorescence-activated cell sorting (FACS) to purify GFP+ MRUs to homogeneity from the Tg11.5kb–GFP mice (Supplementary Fig. 4a,b). The GFP+ cells were transduced with lentivectors expressing mApple plus Luc shRNA (control) or Par3L shRNA 2. The cells were then grown into colonies in Matrigel, and passaged as described above (Supplementary Fig. 2). Most of the control colonies continued to express GFP, and were positive for the lentiviral marker (Fig. 4a,d). However, very few cells transduced with Par3L shRNA 2 formed colonies (Fig. 4a,d). Moreover, those
colonies that grew out were generally smaller, and less positive for mApple (Fig. 4a,b), suggesting that there was selection against cells containing multiple copies of the shRNA–mApple vector. The effect of Par3L depletion is even more striking when GFP+ colonies are scored. After the third passage more than 50% of the colonies in the Lac shRNA control group were GFP+, whereas only 17% of surviving colonies in the Par3L shRNA 2 group were GFP+ (Fig. 4e). This result demonstrates that Par3L depletion causes rapid loss of GFP+ mammary stem cells. We next focused on the GFP+ mApple+ colonies to understand what effects Par3L depletion might have on these GFP+ MRUs. In surviving colonies from cells transduced with Par3L shRNA 2, over 60% of cells were CK8+, which is not different from the Lac shRNA control. However, only 16% of cells were CK14+ in the Par3L shRNA 2 group, a significant decrease when compared with 79% CK14+ cells for the Lac shRNA control (Fig. 4b,f). This result is consistent with our earlier observation that Par3L depletion causes loss of basal CK14+ or CK8+ CK14+ dual-positive cells (Fig. 3a,d). Moreover, the surviving Par3L-depleted colonies had a substantially higher apoptotic index (Fig. 4c,f). We conclude that Par3L possesses an MRU-specific function that is essential for stem cell maintenance.

**Par3L, but not Par3, associates with Lkb1**

How might Par3L act to maintain MRU survival? The Par3L protein does not bind to aPKC or Par6, so we tested other polarity proteins, and identified an unanticipated interaction with Lkb1, the mammalian homologue of the *Caenorhabditis elegans* PAR4 polarity protein (Fig. 5a). The endogenous association of the two proteins was confirmed using lysates from mouse kidneys, which express Par3L at relatively high levels (Fig. 5b). This interaction is specific, because Lkb1 does not bind the Par3 protein (which was able to bind aPKC, Fig. 5a). We next identified the region of Par3L required for Lkb1 binding. As shown in Supplementary Fig. 5 and Fig. 5c, the interaction involves the PDZ2–PDZ3 region of Par3L, and specifically requires a small region lying between the two PDZ domains, of no known function, and which shows no sequence similarity to the same region in Par3 (or to any other sequence in the GenBank database). Replacement of the Par3L sequence in this region with that from Par3 abolished Lkb1 binding by full-length hPar3L (Fig. 5c). Therefore, this short Par3L sequence is required for Lkb1 binding. To determine if the interaction has biological relevance, we asked if the mutant could rescue mammary outgrowth in the fat pad repopulation assay (Fig. 5a). We propose that Par3L maintains mammary stem cells through suppression of Lkb1 kinase activity, and that loss of this restraint results in a failure of self-renewal and increased apoptosis. This model is in accord with known functions of Lkb1, which is capable of driving p53-dependent apoptosis when overexpressed13,14, and which has been recently identified as an essential factor in a caspase-independent cell death and extrusion pathway in *C. elegans*15. Lkb1 is also required for the maintenance of haematopoietic stem cell dormancy16–18. Consistent with our model, the conditional deletion of Lkb1 in mammary glands promotes ductal hyperbranching, which is the opposite of the phenotype caused by loss of Par3L, and promotes tumorgenesis19. It will be of interest to determine if Par3L functions to restrain Lkb1 activity in other mammalian stem cell populations and perhaps acts as a tumour promoter through suppression of Lkb1 activity. Lkb1 is a master kinase that regulates 14 downstream protein kinases, and an important question for the future concerns which of these kinases mediates the effects of Lkb1 on mammary stem cell maintenance. We note that SIK1 couples Lkb1 to p53-dependent apoptosis triggered by cell detachment15. Finally, we speculate that in *Drosophila*—which does not contain a Par3L gene—the negative regulation of Lkb1 is occupied by Bazooka, the fly homologue of Par3.
ARTICLES

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper

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AUTHOR CONTRIBUTIONS
Y.H. carried out the experiments; Y.H. and I.G.M. analysed the data and prepared the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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METHODS

Mice. All C3H female mice were purchased from Charles River. s-SHIP–GFP transgenic (Tg11.5kb–GFP) mice were provided by Larry Rohrschneider. To isolate mammary gland cells, the third to fifth pairs of mammary glands were removed from eight-week-old C3H female mice, minced with scissors and digested in digestion medium (DMEM/F12, 2 mg ml\(^{-1}\) collagenase I (Roche), 5 mg ml\(^{-1}\) insulin (Sigma), 200 U ml\(^{-1}\) Nystatin (Sigma), 100 U ml\(^{-1}\) penicillin/streptomycin) for 45 min at 37 °C. Epithelial organoids were collected by centrifugation at 1,000 rpm for 5 min. The cell pellet was washed in 5 ml of DMEM/F12 containing DNase I and centrifuged at 1,000 rpm for 5 min. The pellet was resuspended in 5 ml DMEM/F12 10% fetal bovine serum followed by 15 s of centrifugation at 1,500 rpm five times. The cells were resuspended in 1 ml of fresh trypsin/EDTA (Invitrogen) and incubated at 37 °C for 12 min, dissociated into a single-cell suspension and passed through a cell strainer (BD) to obtain a single-cell suspension of mammary gland cells. Freshly isolated mammary gland cells were transduced by lentivirus in a final volume of 200 μl for 1 h. After transduction, cells were seeded into six-well low attachment dishes (Corning) and grown as mammosphere cultures for 2–5 days before transplantation. Transplantation of the mammary cells into the cleared fat pads of three-week-old recipients were as described in ref. 20. Briefly, incisions were made around each nipple of the fourth pair and parts of the fat pads were pulled through the openings. The endogenous mammary gland was removed. Cells were suspended in DMEM/F12 supplemented with 2% B27 and 10% growth-factor-reduced Matrigel and injected into the cleared fat pad. Skin was closed by tissue adhesive. The number of mice used in each experiment was predetermined by power analysis. Transplanted mice were killed after 6–8 weeks to examine the mammary gland outgrowth. Experiments were not randomized or blinded to allocation during analysis. Transplanted mice were killed after 6–8 weeks to examine the mammary gland outgrowth. Experiments were not randomized or blinded to allocation during analysis. All experiments were carried out in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval.

Plasmids. pLVTHM, pPAX2 and pMD2.G were provided by Didier Trono\(^{21}\). pLVTHM was modified to change the enhanced green fluorescent protein tag to mApple and a BamHI–NotI–EcoRI linker was incorporated after the tag. pLVTHM was modified to change the enhanced green fluorescent protein tag to mApple and a BamHI–NotI–EcoRI linker was incorporated after the tag. ShRNAs were cloned into the ClaI–MluI sites of pLVTHM or pLVTHM–mApple. Plasmids. pLVTHM–Venus, Lkb1 and Strad\(^{\alpha}\)Merrifield and subcloned into pLVTHM (ref. 22). Par3L was amplified by PCR using the two-tailed Student’s t-test. All other analyses for significance were carried out using the two-tailed Student’s t-test.

Antibodies. The primary antibodies were for CK14 (Covance, PRB-155P, 1:1,000), CK8 (Troma-1, 1:250), cleaved Caspase-3 (Cell Signaling, 9664, 1:100), Ki67 (Invitrogen, 18-0191Z, 1:75), LKB1 (Cell Signaling, 3050, 1:1,000), αPKC (Santa Cruz, sc-216, 1:1,000), Myc (9E10), AMPK (Cell Signaling, 2532, 1:1,000), Phospho-AMPK (Cell Signaling, 2535, 1:1,000), β-catenin (BD, 610153, 1:5,000). Draq5 (Cell Signaling, 4084, 1:200) was used to detect DNA. The Par3L antibody was created by Cocalico in guinea pigs. The antigen was a keyhole limpet haemocyanin-conjugated peptide against the carboxy-terminal sequence of murine Par3L. The antibody was purified using peptide-coupled beads and used at 1:500 dilution in immunoblots and 1:150 dilution in immunostaining. A pressure cooker (Retriever 2100) was used for antigen retrieval. Alexa Fluorochrome-conjugated secondary antibodies were purchased from Invitrogen and used at 1:1,000 dilution for staining. Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Immunolab and used at 1:10,000 dilution for western blots. Confocal microscopy images were taken on an LSM510 confocal microscope (Zeiss) at the VUMC Cell Imaging Shared Resource.

FACS. Freshly isolated mammary gland cells from Tg11.1kbGFP mice were labelled for lineage markers PE-CD31 (MEC13.3, BD), PE-CD45 (30-F11, BD) and PE-Ter119 (TER-119, BD) for 30 min at 4 °C. 7AAD (1:200, BD) was added before sorting. Cells were sorted on a FACSDiva. GFP+ PE 7AAD− single cells were collected for the experiments.

In vitro assay. Mammary gland cells were cultured in suspension for 3 days after virus transduction to enable recovery. Cell clusters were dissociated into single cells by 0.05% trypsin–EDTA at 37 °C for 30–60 min. Cells were imbedded into growth-factor-reduced Matrigel (BD) and cultured in mammosphere medium\(^{7}\) for 1 week before imaging and scoring. To passage the colonies Matrigel was dissolved in Cell Recovery Solution (BD) at 4 °C for 30 min. Colonies were recovered and dissociated for the next round of culture in Matrigel. For immunostaining, colonies were fixed in 4% paraformaldehyde (Sigma) for 30 min and then permeabilized in 0.25% Triton X-100 for 30 min. Antibodies were applied in PBS supplement with 10% western blocking reagent (Roche). Images were taken on an LSM510 confocal microscope (Zeiss) at the VUMC Cell Imaging Shared Resource.

Statistical analysis. The analysis for limiting dilution assays was carried out using a webtool described previously\(^{21}\). All other analyses for significance were carried out using the two-tailed Student’s t-test.

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ERRATUM

The Par3-like polarity protein Par3L is essential for mammary stem cell maintenance

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In the version of this Article originally published, the scale bars in Fig. 2c should have been labelled ‘3 mm’. In addition, a small red arrow indicating the location of the mutation was missing from Fig. 5c (shown below). These errors have now been corrected in the online versions of the Article.
Supplementary Figure 1 (a, b) Specificity of the Par3L antibody. Par3L antibody recognizes over-expressed human YFP-tagged Par3L in HEK293T cells and endogenous murine Par3L in mouse kidney lysates. (c) Par3L staining co-localizes with GFP staining in the mammary glands of 6-week old s-Ship-GFP transgenic mice. (d, e) Immunoblot to test the efficiency of shPar3L hairpin RNAs. HEK293T cells over-expressing mouse Par3L gene were infected by Par3L hairpin virus. shPar3L#1 reduced Par3L expression by 70%, and shPar3L#2 completely depleted Par3L protein. Graph represents mean ± SEM for n= 3 independent experiments. All full scan images of western blots can be found in supplementary figure 6.
Supplementary Figure 2 (a) Scheme of serial colony-forming assay. Mammary gland cells were isolated from adult female C3H or s-SHIP-GFP transgenic mice. Cells were cultured for two days in suspension after virus infection to allow recovery. Before Matrigel culture cells were dissociated in fresh 0.05% trypsin for 45 min at 37°C to obtained single cells. Three hundred to 1,000 cells in 5μl of medium were mixed with 45μl growth factor reduced phenol-red free Matrigel. The mixture were laid on the bottom of ultra-low attachment 24-well cluster (Corning) as a drop and solidified at 37°C for 30 min. The solidified Matrigel drops were covered by 500μl of mammosphere medium. The cultures were fed every other day. Colonies were scored and imaged using an Olympus SZX16 microscope. For passage, colonies were recovered in 0.5 ml Cell Recovery Solution (BD) at 4°C for 30 min. Single cells were obtained by trypsinization and plated in Matrigel culture as described previously. After the third passage, colonies were analyzed by immuno-staining in vitro or by transplantation. (b) Summary of limiting dilution assay using control tertiary colonies and a representative image of a GFP+ mammary gland regenerated in the limiting dilution assay from these control cells. Mammary gland cells expressing ShLuc were used for the serial colony-forming assay. After the third passage, colonies of the indicated numbers were injected into cleared fat pad for the limiting dilution assay. Mammary gland regeneration was tested in 8 cleared mammmary fat pads. (c) Scheme of competition assay. Mammary gland cells were isolated from adult female C3H mice. The cells were infected with mApple-ShLuc virus, EGFP-ShLuc virus or EGFP-ShPar3L#2 virus. After 2 days recovery in suspension culture, 50,000 mApple-ShLuc virus infected cells were combined with an equal number of EGFP-ShLuc virus infected cells or EGFP-ShPar3L#2 virus infected cells and injected into cleared fat pads of 3 week old C3H mice. Mammary glands were scored for outgrowth after 6 weeks.
Supplementary Figure 3  Representative images of mature mammary ducts stained for CK8, CK14, or cleaved Caspase-3. Those mammary ducts that grew out from cells depleted of Par3L show a normal morphology with a single layer of luminal epithelial cells surrounded by a single layer of myoepithelial cells. The number of cleaved Caspase-3+ cells is very low and not significantly different from the ShLuc control.
Supplementary Figure 4 (a) Scheme of mammary stem cell colony-forming assay. Mammary gland cells were isolated from 6-week old female s-SHIP-GFP transgenic mice. GFP+ mammary gland stem cells were purified by FACS sorting as described in METHODS. The sorted cells were then infected with mApple-ShLuc virus or mApple-ShPar3L#2 virus. After recovery for 2 days in suspension culture the cells were plated in Matrigel. (b) Scheme of FACS for the GFP+ mammary gland stem cells. Mammary gland cells from s-SHIP-GFP transgenic mice were stained by the following lineage antibodies: PE-CD31 (MEC13.3, BD), PE-CD45 (30-F11, BD), and PE-Ter119 (TER-119, BD). 7AAD (BD) was added before sorting. GFP+PE-7AAD- single cells were collected for the experiments.
Supplementary Figure 5  The region between PDZ2 and PDZ3 on Par3L protein is necessary for the interaction between Par3L and LKB1. (a) schematic view of the Par3L truncation mutants used for co-immunoprecipitation with LKB1. (b) co-precipitation of LKB1 and Par3L truncation mutants group #1. LKB1 binding to Par3L protein required the region from PDZ2 to PDZ3 domain. (c) co-precipitation of LKB1 and Par3L truncation mutants group #2. LKB1 was unable to bind to any one of the 3 isolated PDZ domains. (d) co-precipitation of LKB1 and Par3L truncation mutants group #3. LKB1 binding to Par3L protein requires PDZ domains 2 and 3 plus the intervening region. All experiments were conducted 3x independently. All full scan images of western blots can be found in supplementary figure 6.
Supplementary Figure 6: Original data for the immunoblots presented in the other figures.
Supplementary Table 1 statistics source data