Supplementary Figures

**Fig. S1** Adipose tissue explants *ex vivo*. **a** Adipose tissue explants used for coculture assays or to generate conditioned medium. Culture medium incubated in parallel without adipose tissues was used as control for experiments with conditioned medium. **b** Bright field images of S1 cell acini cultured in the absence of adipose tissue (top) or cocultured with breast adipose tissue (bottom). The uneven hydrogel substrate revealed the footprint of adipocytes (right, image taken after removal of adipose tissue explants), indicating direct contacts between the tissue explants and the substrate and acini. Scale bar = 100 µm. **c** H&E staining of adipose tissues explants fixed directly after surgery (0h) or after two days in culture (48h). **d** Morphometric analysis of adipocytes. Size (adipocyte cross sections) and circularity were measured on H&E-stained tissues using ImageJ. n.s., not significant (*P* > 0.05, paired t-test, n = 4 patients; 40 adipocytes were quantified for each patient). Mean ± SEM are shown.
Fig. S2 Elevated leptin in DIO mice. a Immunohistochemical analysis of leptin levels in whole mount mammary glands from DIO mice and controls. DAB signals were isolated using spectral imaging. Scale bar, 20 µm. b Quantification of DAB signals in mice mammary glands. c Lep expression quantified by qPCR in mouse mammary glands. Results were normalized to HPRT expression. **, $P < 0.01$ (unpaired t-test). Means ± SEM are shown in the graphs.
Fig. S3 Leptin disrupts apical TJ in psHMEC and HMT-3522 S1 acini. a Detection of LEPR expression in psHMEC by western blot. b Confocal images of ZO-1 and claudin-1 immunostaining in psHMEC acini treated for 72h with vehicle or leptin. Scale bars = 20 μm. Quantification of the percentages of structures with apical ZO-1 and claudin-1 localization are displayed in the graphs. *, P < 0.05 (unpaired t-test, n = 3). Mean ± SEM are shown. c Validation of the approach for bioinformatics quantification of the radial distribution of ZO-1 staining in S1 acini. The curves represent normalized staining intensity (mean ± SEM) along the radii of the structures (see Supplementary Methods). Structures were visually selected as polarized (n = 72) or nonpolarized (n = 80), as illustrated by the images. d Concordance between the radial profile (RP) analysis and visual scoring of apical polarity (AP) in experimental dataset. Each symbol on the graph represents one experimental condition from one biological replicate, with AP quantified by visual scoring or using the RP algorithm. The Pearson correlation coefficient (r) and corresponding P-value are shown.
Fig. S4 Leptin alters the density and localization of zona adherens/desmosomes. 

a) Representative TEM images of S1 acini treated with vehicle or leptin for 72h. Epithelial junctions are highlighted with yellow circles and with arrows in the inset. Scale bars = 5 µm. 

b) Quantification of zona adherens/desmosomes density *, P < 0.05 (unpaired t-test; N = 16 acini; mean ± SEM shown).

c) Radial distribution of the junctions relative to the center of mass of the acini. Distances were normalized to the square root of the cross-section area and to the aspect ratio of the acini to compensate for size and elliptical effects. Statistical analysis using unpaired t-test. n = 285 (vehicle) and 290 (leptin).

d) Localization of AJ markers E-cadherin and β-catenin by immunostaining in S1 acini treated with vehicle or leptin. *, P < 0.05 and **, P < 0.01 (unpaired t-test; N = 3; mean ± SEM shown). Scale bars = 10 µm.
Fig. S5 Loss of apical polarity after leptin treatment is not mediated by STAT3 or MAPK signaling. **a** Confocal images showing localization of the tight junction marker ZO-1 in S1 acini treated for 72h with vehicle or leptin (100 ng/ml), alone or in combination with the STAT3 inhibitor static (10 µM) or the MEK inhibitor PD98059 (20 µM). **b** quantification of apical ZO-1 distribution in acini treated as in panel a. ***, P < 0.01, *, P < 0.05 (one-way ANOVA; n = 3; mean ± SEM shown). **c** Western blot for activated STAT3 (P-STAT3) or for P-MAPK in S1 cells treated with vehicle or leptin. Blots for total STAT3 and MAPK as well as Ponceau staining are shown as loading controls.
Fig. S6 Leptin does not alter proliferation, migration, and invasion of non-neoplastic breast epithelial cells but increases the proportion of cells with stem/progenitor characteristics. 

**a** Cell cycle distribution in S1 acini treated for 72h with vehicle or leptin (100 ng/ml) determined by flow cytometry. n.s., \( P > 0.05 \) (unpaired t-test; \( n = 3 \); mean ± SEM shown). 

**b** Migration of polarized S1 cell monolayers treated as in a, as measured by wound healing assays. n.s., \( P > 0.05 \) (unpaired t-test; \( n = 4 \); mean ± SEM shown). 

**c** Migration of individual S1 and psHMEC cells measured with time-lapse microscopy. Cell nuclei were stained with Hoechst and cells imaged for 4h in the presence or absence of leptin (100 ng/ml). Cells were pretreated with leptin or vehicle for 24h prior to the assay. The graphs show track lengths for individual cells (gray), and averages for each biological replicate (blue and red dots; \( n > 150 \) cells/replicate). n.s., \( P > 0.05 \) (unpaired t-test; \( n = 5 \)). Representative images of cell nuclei overlapped with migration tracks (red) are shown. Scale bar, 100 \( \mu \)m. 

**d** Morphometric analysis of S1 acini treated as in a. Bright field images illustrate the lack of invasive cells or cell protrusions in leptin-treated acini. Scale bar, 50 \( \mu \)m. 

**e** Representative cytometry plots of CD44/CD24 staining of S1 cells dissociated from acini treated as in a. Percentages of CD44+/CD44− populations are indicated. *, \( P < 0.05 \) (unpaired t-test; \( n = 3 \)). 

**f** ALDH-bright (ALDHbr) S1 cells in acini treated as in a. Representative cytometry plots are shown. Percentages of ALDHbr cells are displayed in the bar graph. *, \( P < 0.05 \) (unpaired t-test; \( n = 3 \)).
Fig. S7 Analysis of human breast tissue. a Confocal microscopy images of filamentous actin stained with fluorescent phalloidin in frozen sections from normal breast tissue. Cell nuclei were counterstained with DAPI and β4-integrin was detected by immunostaining to label the basal sides of the mammary acini and ducts. Scale bars, 50 µm. b Polarity scores for ZO-1, Par3 and actin in normal breast tissue from reduction mammoplasties (RM) and from mastectomies performed on breast cancer patients (BrC). c Tissue levels of leptin and adiponectin (ng/g of tissue), as well as adiponectin/leptin ratio, according to BMI categories in patients from the High Risk cohort. d Relative LEPI/ADIPOQ expression determined by qPCR in RM and BrC tissue punches. Statistical analyses using unpaired t-test (b) and Kolmogorov-Smirnov test (d).
Fig. S8 Polarity marker expression in S1 cells. Western blot analysis of ZO-1, claudin-1 and Par3 expression in S1 acini exposed for 72h to vehicle or leptin (100 ng/ml). Densitometry is shown in the graphs as mean ± SD. n.s., $P > 0.05$ (one sample t-test; n = 3).
Supplementary Materials and Methods

Procuration and analyses of human breast tissue

Written consent was obtained from all patients who participated in this study, which was conducted in accordance with recognized ethical guidelines (including the Belmont Report and U.S. Common Rule). Breast adipose tissue from reduction mammoplasties were obtained following the Purdue Institutional Review Board protocol # 1206012467, resected, minced to approx. 0.5 cm fragments, and placed in RPMI within 30 min after surgical excision. To generate conditioned medium, adipose tissue explants were transferred into H14 culture medium supplemented with penicillin (50 U/ml), streptomycin (50 µg/ml), and amphotericin B (0.5 µg/ml). Adipose tissue weight was 2.1 ± 0.2 g/ml of medium. After 48h incubation at 37°C in a humidified cell culture incubator, conditioned medium was collected, centrifuged to remove the buoyant lipids and pelleted cell debris, sterile-filtered, and snap-frozen in liquid nitrogen. For coculture experiments, tissue explants were added to acini in 3D culture (day 10 of culture).

Normal breast epithelium was collected from reduction mammoplasties, as well as from mastectomies performed on breast cancer patients (Purdue IRB protocol # 1206012467). For the mastectomy cases, tissue was taken distal from the tumor site to minimize field effects. Tissue explants were minced (2-4 mm cubes), cryoprotected with two sucrose steps (18 and 30%), and frozen in optimal cutting temperature compound to prepare thin (10 µm) sections.

Cell culture and treatments

HMT-3522 S1 cells were obtained from Dr. Mina Bissell (Lawrence Berkeley Laboratory) and propagated between passages 54 and 60 in H14 medium [1]. Mycoplasma tests were negative (last testing February 2018). Acinar differentiation was achieved by culturing the cells in 3D for ten days on top of a layer of Matrigel matrix (Corning, Corning NY, USA) in chambered slides (MilliporeSigma, Burlington MA, USA), as described [2].

For quantification of mitotic spindle alignment, polarized monolayers of S1 cells were cultured on glass coverslips coated with laminin (Corning; 2.8 µg/cm²). For transepithelial permeability assays, cells were seeded as monolayers at a density of 90 000 cells/cm² on culture inserts (Fisher, Asheville NC, USA; 0.4 µm pore sizes) coated with laminin. Evans blue-labeled albumin (EBA) was added to the transwell insert. The EBA concentration in the abluminal chamber was measured after 1h incubation by spectrophotometry at OD 650 nm. Each measurement was done in triplicates.

The XtremeGene HP (Roche, Basel, Switzerland) reagent was used for Akt plasmid transfection in HMT-3522 S1 cells. Cells were transfected as monolayers during their exponential growth phase (3-4 days after seeding), seeded in 3D culture 24h after transfection, and analyzed 7 days after transfection. For LEPR silencing, small interfering RNAs (siRNA) and short hairpin RNA (shRNA) constructs were delivered in S1 cells by nucleofection (Amaxa Solution L and program A-20; Lonza). Nucleofected cells were directly seeded in 3D culture and analyzed 5 days later. shRNA targeting LEPR (TR311760A) and a scramble control (TR30012) were from Origene (Rockville MD, USA). siRNA (ON-TARGET plus, Dharmacon, Lafayette CO, USA) were used at 50 nM final concentration.

Cells were treated with human recombinant leptin (Protein Laboratories Rehovot, Rehovot, Israel); human leptin receptor antagonist (SLAN-2, PLR); leptin-neutralizing antibodies (R&D Systems, AF398; 150 µg/ml); human adiponectin (Abcam, Cambridge, UK); LY294002
Antibodies used for immunofluorescence

The following antibodies were used for immunostaining cell cultures and human tissue: afadin (R&D Systems, MAB78291, 5 µg/ml); P-Akt (Ser 473, Cell Signaling, Danvers MA, USA, #9271, 1:50); E-cadherin (Cell Signaling, #3195, 1:200); β-catenin (Santa Cruz, Dallas TX, USA, SC-7199, 1 µg/ml); claudin-1 (Abcam, Ab15098, 1 µg/ml); β4-integrin (MilliporeSigma, clone 3E1, 1:300); Ki67 (Thermo Fisher Scientific, Waltham MA, USA, PA5-19462, 1 µg/ml); LEPR (Abcam, Ab104403, 1 µg/ml); NuMA (Bethyl Laboratories, Montgomery TX, USA, A301-509A, 1 µg/ml); Par3 (MilliporeSigma, #07-330, 13 µg/ml); ZO-1 (ThermoFisher, 33-9100, 2.5 µg/ml). Secondary antibodies conjugated with fluorescent dyes (AF488, AF568, or AF647) were from ThermoFisher and were used at 1:500 dilutions.

Antibodies used for western blot analyses

The following antibodies were used for immunoblotting: afadin (R&D Systems, MAB78291, 0.5 µg/ml); P-afadin (Cell Signaling, #5485, 1:50); Akt (Cell Signaling, #9272, 1:1000); P-Akt (Ser473, Cell Signaling, #9271, 1:100); claudin-1 (Abcam, 8 µg/ml); HA tag (Cell Signaling, clone 6E2, 1:500); lamin B (Abcam, Ab16047, 0.2 µg/ml); LEPR (Abcam, Ab104403, 1 µg/ml); MAPK (ERK1/2, Cell Signaling, clone 197G2, 1:1000); P-MAPK (ERK1/2 Thr202/Tyr204, Cell Signaling, clone 137F5, 1:1000); NuMA (clone B1C11, 1:2, a gift from Dr. Jeffrey Nickerson, UMass, Worcester, USA); Par3 (MilliporeSigma, #07-330, 2 µg/ml); STAT3 (Cell Signaling, clone 79D7, 1:1000); P-STAT3 (Tyr705, Cell Signaling, clone D3A7, 1:1000); and ZO-1 (ThermoFisher, 1A12, 5 µg/ml). Secondary antibodies conjugated to horse radish peroxidase (GE Healthcare, Little Chalfont) were used at 1:10 000.

Sequences of primers used for qRT-PCR

Forward and reverse primers were 5’ CTATGTCCAAGCTGTGCCCA 3’ and 5’ GAGACTGACTGCGTGTGA 3’ (human leptin), 5’ ATGGCCCCTGCACTCTCA 3’ and 5’ CAGGGATGAGTTCGGCACTT 3’ (human adiponectin), and 5’ TCAAGACCATTGTCAACCA 3’ and 5’ TGAAGGCCAGGAATGAGTC 3’ (murine leptin).

Computational approach to quantify apical polarity

ImageJ (http://rsbweb.nih.gov/ij/) was used to quantify the radial distribution of apical polarity signals in acini, as validation for visual scoring. The approach was similar to the Radial Profile Plot, an ImageJ plugin written by Paul Baggeethun, which produces a plot of normalized integrated intensities of concentric circles as a function of the distance from a point in the image (https://imagej.nih.gov/ij/plugins/radial-profile.html). Based on the same concept, our program identifies acini from a DAPI image and computes for each acinus the normalized radial profile for the fluorescence channel corresponding to the polarity marker.

Each acinus within the image is first identified as a region of interest (ROI) by thresholding the DAPI image and filtering particle sizes. These circular ROIs are then applied to the
fluorescence channel used for the radial profile analysis. For each acinus, the program identifies the center of the ROI and loops through all of the pixels within the ROI to populate a two-dimensional array. This array contains the quantity of pixels on each concentric circle within the ROI and the average pixel intensity of each concentric circle. Before running the program, the user enters a value for the number of radial bins (or concentric areas) that will be used for analysis (ten in our case). After the average intensity of each concentric area is determined, the radius of the ROI is normalized according to the user-indicated number of bins (center = 0 and periphery = 1), and intensity values are normalized so that average intensity equals one. Normalizing signal intensities and structure diameters avoids influences from the staining procedure or structure sizes. For each acinus, the program outputs a table listing the average intensities for each radial area, which can be visualized as line profiles (Fig. S3c). Steep profiles represent apically polarized structures, whereas horizontal curves (y = 1) represent nonpolarized acini. Summary values of polarity are generated as the sum of the distances between the curve and the horizontal line at y = 1. Computer code will be made available upon request.

Electron microscopy

S1 acini cultured in 6-well plates were collected by gentle scraping. Pelleted acini were fixed with 2.5% glutaraldehyde in 0.1 M Millonig’s phosphate buffer pH 7.3, washed three times in buffer and post-fixed with 1% osmium tetroxide in phosphate buffer for one hour. After three washes in buffer, the pellets were dehydrated through a graded ethanol series. For preparation of resin infiltration, the pellets were incubated in propylene oxide for two changes of 15 minutes. Finally, the pellets were gradually infiltrated with Spurr’s resin which was allowed to cure overnight. Sections (90 nm) obtained with a Reichert-Jung (Vienna, Austria) Ultracut E ultramicrotome were stained with lead citrate and uranyl acetate and viewed with a FEI Tecnai (ThermoFisher) Spirit TEM operating at 80 kV. Images were obtained with an AMT (Woburn MA, USA) 2Vu CCD camera. Tiled images taken at 6800x were stitched and used for the analysis of cell-cell junctions.

Cell migration

Migration was assessed in 35 mm dishes with silicone inserts (ibidi, Martinsried, Germany). Cells were imaged with a 4x objective immediately after removal of the insert and 12h later. This time point was chosen to avoid any potential confounding effect from cell proliferation. The areas over which cells migrated were quantified using ImageJ. Time-lapse experiments were used to quantify migration of individual cells (or small groups of cells). Nuclei were labeled with Hoechst 33342 (ThermoFisher; 5 µg/ml; 30 min) and imaged for 4h (5 min intervals) with a 20x objective mounted on an IX83 microscope (Olympus) with environmental control (37°C, 5% CO₂; PeCon, Erbach, Germany). Cell tracks were generated and analyzed using the TrackMate plugin for ImageJ [3].

Cell cycle analysis

S1 acini cultured for 10 days in Matrigel were retrieved from Matrigel using dispase (Corning; cat# 354235; 30 min at 37°C) and dissociated into single cells with a short (8 min) treatment with 0.25% trypsin/1 mM EDTA. Trypsin was inactivated with soybean trypsin inhibitor (MilliporeSigma) and acini were washed in H14 medium. Cell suspensions were fixed in cold ethanol:acetone (1:1), stained with propidium iodide (PI, ThermoFisher; 0.1 mg/ml) in the
presence of 20 mg/ml RNase A, and analyzed by flow cytometry using an Accuri C6 Analyzer (BD Biosciences, Franklin Lakes NJ, USA). Cell cycle distribution was quantified with MultiCycle AV for FCS Express (DeNovo Software, Glendale, CA, USA), using a first order S phase polynomial (+S Order = 1) model, which consistently yielded the lowest $\chi^2$ values.

**Evaluation of stem cell populations**

Single cell suspensions ($10^6$ cells/ml) derived from S1 acini (as above) were stained with APC-conjugated CD44 antibodies (1:3,000) and FITC-conjugated CD24 antibodies (1:10), both from BioLegend (San Diego CA, USA). Fluorescence was quantified by flow cytometry (Accuri C6 Analyzer). For quantification of aldehyde dehydrogenase (ALDH) activity, the ALDEFLUOR kit (STEMCELL Technologies, Cambridge MA, USA) was used following the manufacturer’s protocol.

**REFERENCES**

1. Briand P, Petersen OW, Van Deurs B. A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. *In Vitro Cell Dev Biol*. 1987; 23: 181-8.
2. Vidi PA, Bissell MJ, Lelievre SA. Three-dimensional culture of human breast epithelial cells: the how and the why. *Methods in molecular biology*. 2013; 945: 193-219.
3. Tinevez JY, Perry N, Schindelin J, Hoopes GM, Reynolds GD, Laplantine E, et al. TrackMate: An open and extensible platform for single-particle tracking. *Methods*. 2017; 115: 80-90.