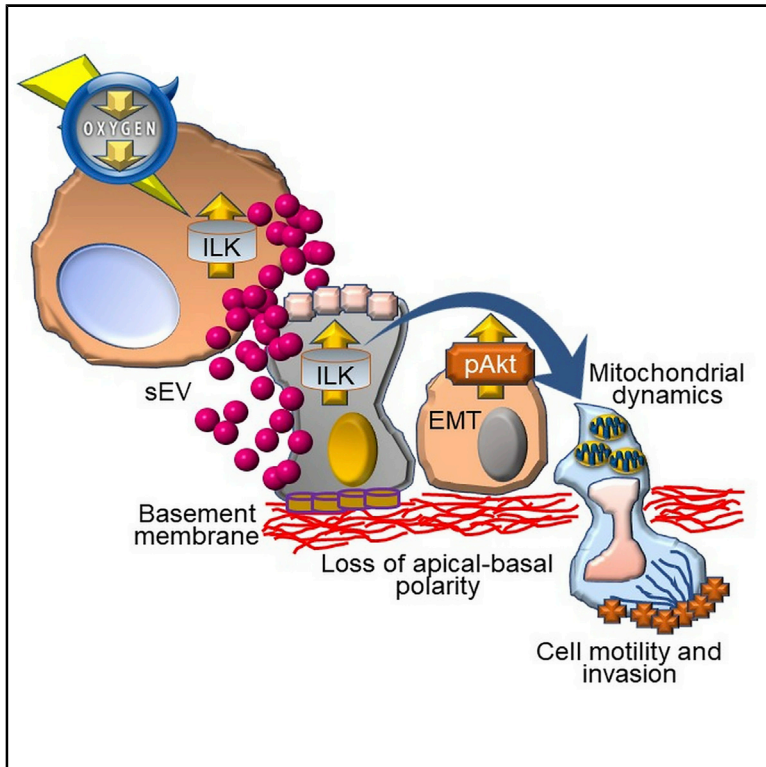


Developmental Cell

Small Extracellular Vesicle Regulation of Mitochondrial Dynamics Reprograms a Hypoxic Tumor Microenvironment

Graphical Abstract



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In Brief

The communication between tumor cells and their neighbors is important for disease progression. Here, Bertolini et al. show that hypoxic breast cancer cells release small extracellular vesicles that introduce multiple malignant changes in the normal mammary epithelium via reprogramming of mitochondrial functions. This pathway may promote local breast cancer recurrences.

Highlights

- Hypoxic breast cancer cells release signaling sEVs
- sEVs activate mitochondrial dynamics in recipient normal mammary epithelium
- Mitochondrial reprogramming stimulates ILK-Akt-dependent epithelial cell migration
- sEVs disrupt normal mammary gland morphogenesis with hallmarks of malignancy



Article

Small Extracellular Vesicle Regulation of Mitochondrial Dynamics Reprograms a Hypoxic Tumor Microenvironment

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SUMMARY

The crosstalk between tumor cells and the adjacent normal epithelium contributes to cancer progression, but its regulators have remained elusive. Here, we show that breast cancer cells maintained in hypoxia release small extracellular vesicles (sEVs) that activate mitochondrial dynamics, stimulate mitochondrial movements, and promote organelle accumulation at the cortical cytoskeleton in normal mammary epithelial cells. This results in AKT serine/threonine kinase (Akt) activation, membrane focal adhesion turnover, and increased epithelial cell migration. RNA sequencing profiling identified integrin-linked kinase (ILK) as the most upregulated pathway in sEV-treated epithelial cells, and genetic or pharmacologic targeting of ILK reversed mitochondrial reprogramming and suppressed sEV-induced cell movements. In a three-dimensional (3D) model of mammary gland morphogenesis, sEV treatment induced hallmarks of malignant transformation, with deregulated cell death and/or cell proliferation, loss of apical-basal polarity, and appearance of epithelial-to-mesenchymal transition (EMT) markers. Therefore, sEVs released by hypoxic breast cancer cells reprogram mitochondrial dynamics and induce oncogenic changes in a normal mammary epithelium.

INTRODUCTION

Epithelial tumorigenesis involves an intricate interplay between clones of transformed cells and a complex microenvironment (Gatenby and Gillies, 2008) composed of normal epithelium, immune-inflammatory cells, and disparate mesenchymal structures, such as stromal fibroblasts, blood vessels, and the extracellular matrix (Quail and Joyce, 2013). How this crosstalk is regulated is largely unknown. However, mechanisms of adaptive evolution (Greaves, 2015), intercellular communication (Tabasum and Polyak, 2015), and metabolic plasticity (Lehuédé et al., 2016) select clones with growth advantage and metastatic potential. These pathways may also contribute to the “field effect” of cancerization (Curtius et al., 2018), a poorly understood process that introduces genetic and phenotypic changes in histologically “normal” epithelia, potentially contributing to local and distant disease recurrence (Martincorena et al., 2015).

In this context, extracellular vesicles (EVs), including small (40–150 nm) extracellular vesicles (sEVs) from either the plasma

membrane (ectosomes) or the late endosomal trafficking machinery (exosomes) (Jaiswal and Sedger, 2019) have emerged as pleiotropic mediators of intercellular communication during tumorigenesis (Kalluri and LeBleu, 2020). Modulated by conditions of the microenvironment, such as hypoxia (King et al., 2012; Kucharzewski et al., 2013), and capable of horizontally transferring disparate bioactive molecules and nucleic acids to neighboring cells (Pathan et al., 2019), tumor-derived sEVs contribute to multiple oncogenic traits, such as metabolic reprogramming (Fong et al., 2015), drug resistance (Cornell et al., 2019), and various mechanisms of cell migration, invasion, and metastatic dissemination (Rodrigues et al., 2019; Wang et al., 2014).

Mechanistically, reprogramming of mitochondrial functions has been recently implicated in multiple aspects of tumorigenesis (Vyas et al., 2016). Fresh evidence has shown that rewiring of oxidative bioenergetics (Anderson et al., 2018); modulation of reactive oxygen species (ROS) signaling (Weinberg et al., 2010); and changes in mitochondrial dynamics, a process that



controls the size, shape, and subcellular position of mitochondria (Eisner et al., 2018), are key drivers of tumor growth *in vivo* (Sellers et al., 2015); cancer stemness (Park et al., 2019); and tumor cell invasion and metastasis (Altieri, 2019). What has remained unclear, however, is whether similar mechanisms affect “normal” epithelia in a tumor microenvironment, potentially facilitating the acquisition of transforming traits that enable disease progression (Quail and Joyce, 2013).

In this study, we examined mechanisms of intercellular communication between hypoxic breast cancer cells and the normal mammary epithelium.

RESULTS

Regulation of Mammary Epithelial Cell Motility by Breast-Cancer-Derived Hypoxic sEVs

We began this study by isolating sEVs from two HER2[−]/ER⁺/PR⁺ human breast cancer cell lines, MCF7 and T47D, under normoxic (5% O₂) or hypoxic (1% O₂) conditions. sEVs produced in hypoxia (sEV^{HYP}) or normoxia (sEV^{NORM}) had comparable shape by cryoelectron microscopy (Figure S1A) and expressed EV-associated markers, Tsg101, CD63, and flotillin 1, by western blotting (Figures S1B and S1C). In contrast, cellular markers, calnexin, voltage-dependent anion-selective channel (VDAC), catalase, and periaxin 1 (PRX1) were undetectable (Figures S1B and S1C). Size exclusion chromatography (SEC) demonstrated that both sEV^{NORM} or sEV^{HYP} produced by MCF7 cells mostly segregated within SEC fractions 7–9 with comparable particle size mean distribution of ~100 nm (Figures S1D and S1E) (Théry et al., 2018). Only SEC fractions 10–12 contained minor amounts of sEV^{NORM} or sEV^{HYP} particles, whereas other SEC fractions were negative (Figure S1D). Finally, fluorescence (PKH26)-labeled sEV^{HYP} or sEV^{NORM} were efficiently internalized by recipient MCF10A normal mammary epithelial cells starting at 2 h after addition, with comparable uptake by 6 h (Figures S1E and S1F). Unless otherwise noted, sEV^{HYP} or sEV^{NORM} were used at 3×10^8 particles per 10^4 recipient cells for all subsequent experiments. sEVs isolated from non-conditioned media were used as controls (Ctrl).

Using these conditions, breast-cancer-derived sEV^{HYP} increased directional migration of recipient MCF10A cells in a wound closure assay (Figures 1A and 1B), as well as polyethylene terephthalate (PET) inserts (Figures 1C and 1D). Treatment with sEV^{NORM} minimally increased cell migration compared to controls (Figures 1A–1D). In addition, sEV^{HYP} produced by MCF7 (Figure 1E) or T47D (Figure S2A) cells stimulated two-dimensional (2D) motility of recipient MCF10A cells, resulting in greater speed of cell movements and longer distance traveled by individual cells (Figures 1F and S2B). Consistent with these findings, sEV^{HYP} increased chemotaxis of MCF10A cells along a gradient of NIH3T3-cell-derived conditioned medium (Figure 1G), also resulting in increased speed of cell movements and longer distance traveled by individual cells, compared with controls (Figure S2C). In contrast, sEV^{NORM} did not significantly affect 2D (Figures 1E, 1F, S2A, and S2B) or chemotactic (Figures 1G and S2C) motility of recipient MCF10A cells. A prerequisite of cell movements is heightened focal adhesion (FA) turnover (Roussos et al., 2011). Consistent with this, sEV^{HYP} stimulated FA turnover in recipient MCF10A cells (Figure S2F), character-

ized by increased formation of new FA and reduction in stable FA, compared with that in controls (Figure 1H). sEV^{NORM} had no effect on FA turnover (Figures 1H and S2F).

We next carried out two sets of experiments to validate the specificity of these findings. First, we found that only SEC fractions 7–9, which contain the majority of breast-cancer-derived sEV^{HYP} (Figure S1D), stimulated MCF10A cell motility, increasing the speed of cell movements and the distance traveled by individual cells (Figure S2D). Other SEC fractions from normoxic or hypoxic samples had no effect on 2D cell motility (Figure S2D). Second, we observed that concentrations of sEV^{HYP} as low as 3×10^5 per mL were sufficient to stimulate 2D motility of MCF10A cells, quantitatively indistinguishable from higher concentrations of 3×10^6 – 3×10^8 particles/mL (Figure S2E). Therefore, breast-cancer-derived sEVs released under conditions of hypoxia potentially stimulate membrane dynamics of cell motility in a recipient normal mammary epithelium.

sEV^{HYP} Stimulate Mitochondrial Dynamics in Recipient Cells

Consistent with a role of mitochondrial dynamics in cell motility (Caino et al., 2016), sEV^{HYP} increased the subcellular trafficking of mitochondria in recipient MCF10A cells (Figure 1I), resulting in faster speed of organelle movements and greater distance traveled by individual mitochondria (Figure 1J). This was associated with increased accumulation of mitochondria at the cortical cytoskeleton of MCF10A cells in response to T47D-cell-derived sEV^{HYP} (Figures 2A and 2B) or MCF7-cell-derived sEV^{HYP} (Figures S3A and S3B). sEV^{NORM} had no effect on mitochondrial movements (Figures 1I and 1J) and did not promote mitochondrial accumulation at the cortical cytoskeleton on MCF10A cells (Figures 2A, 2B, S3A, and S3B). In the control experiments, breast-cancer-derived sEV^{HYP} or sEV^{NORM} had no effect on mitochondrial inner membrane potential of MCF10A cells (Figure 2C).

In addition, sEV^{HYP} treatment of MCF10A cells was associated with activating Ser616 phosphorylation of dynamin-related protein 1 (Drp1) (Figure 2D), a key effector of mitochondrial fission (Eisner et al., 2018). This was accompanied by increased association of Ser616-phosphorylated Drp1 with mitochondria of recipient cells, whereas sEV^{NORM} had no effect (Figures 2E and 2F). Co-culture with sEV^{HYP}, but not sEV^{NORM}, also increased the expression of mitofusin-1 (MFN1) and mitofusin-2 (MFN2), which mediate mitochondrial fusion (Figure 2D). Taken together, real-time quantification of changes in mitochondrial volume by time-lapse videomicroscopy (Figure 2G) demonstrated that sEV^{HYP} increased the rates of both mitochondrial fusion (>1.5-fold mitochondrial volume) and fission (<1.5-fold mitochondrial volume) events in recipient MCF10A cells (Figure 2H). Conversely, sEV^{NORM} had no effect on mitochondrial volume, compared with controls (Figures 2G and 2H). Mechanistically, silencing of MFN1 or Drp1 by small interfering RNA (siRNA) in recipient MCF10A cells exposed to sEV^{NORM} or sEV^{HYP} (Figure 2I) abolished cell migration stimulated by sEV^{HYP} (Figure 2J). In contrast, MFN1 or Drp1 depletion had no effect on MCF10A cell migration in the presence of sEV^{NORM} (Figure 2J). Taken together, these findings demonstrate that sEV^{HYP} stimulate multiple aspects of mitochondrial dynamics in recipient cells,

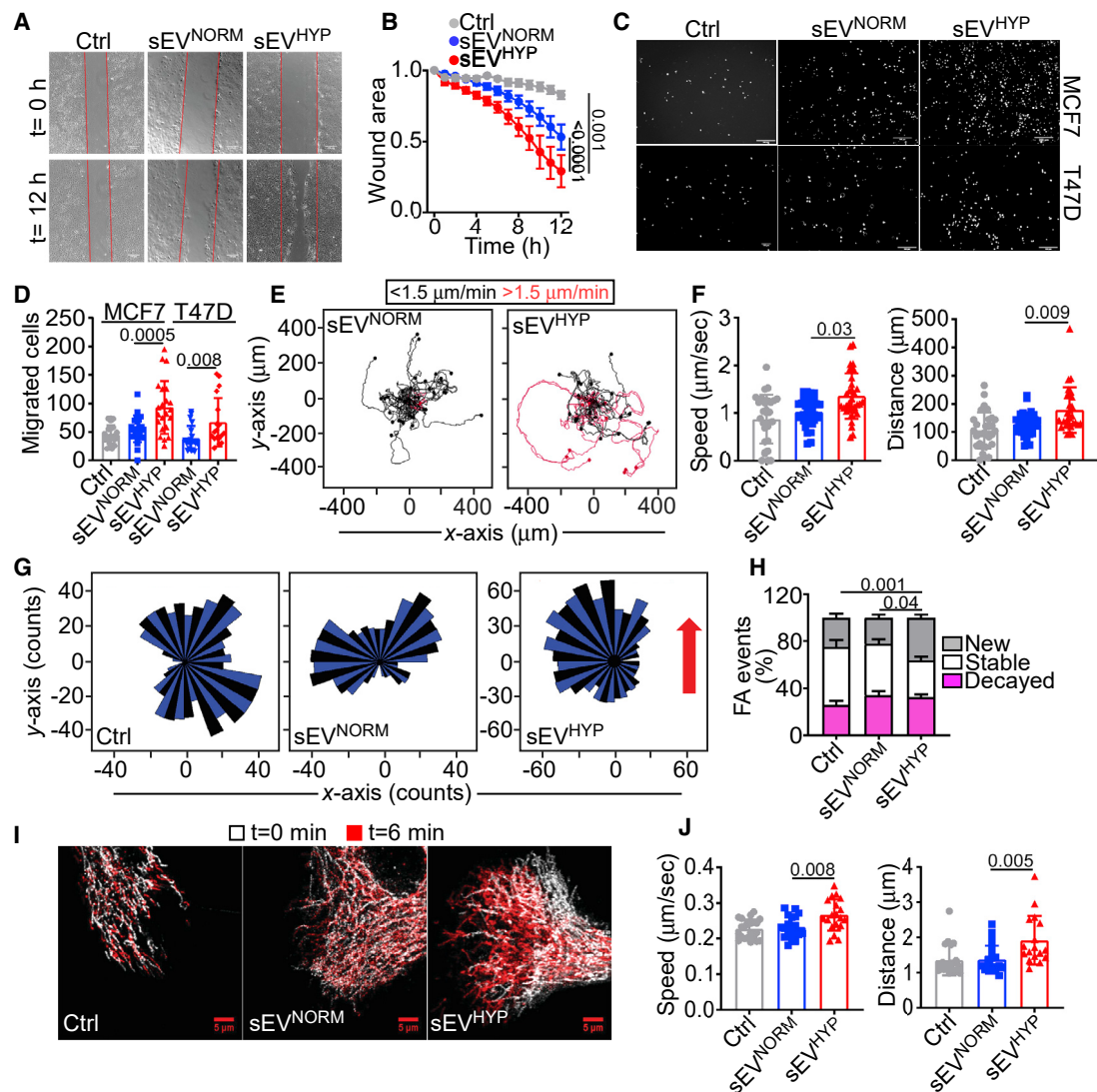


Figure 1. Breast-Cancer-Derived Hypoxic sEVs (sEV^{HYP}) Stimulate Motility of Normal Mammary Epithelial Cells

(A) Normal mammary epithelial MCF10A cells were treated with sEVs produced by MCF7 cells under normoxic (sEV^{NORM}) or hypoxic (sEV^{HYP}) conditions and analyzed for directional cell migration in a wound closure assay. Representative images at time (t) = 0 and 12 h. Scale bar, 150 μ m.

(B) The conditions are as in (A) and directional motility of sEV-treated MCF10A recipient cells was quantified. Mean $\pm$ SEM (n = 5). Numbers correspond to p values by two-way ANOVA with Tukey's multiple comparisons test.

(C and D) MCF10A cells treated with sEV^{NORM} or sEV^{HYP} produced by MCF7 (top) or T47D (bottom) cells were analyzed for migration on PET inserts (C, representative images of DAPI-stained nuclei of migrated cells) and quantified (D). Scale bar, 200 μ m. Mean $\pm$ SEM (n = 3). Numbers correspond to p values by one-way ANOVA with Tukey's posttest.

(E and F) MCF10A cultures treated with MCF7-cell-derived sEV^{NORM} or sEV^{HYP} were analyzed for cell motility in 2D contour plots (E), and the speed of cell movements (F, left) or total distance traveled (F, right) by individual sEV-treated MCF10A cells was quantified. Each tracing corresponds to an individual cell. The cutoff velocities for slow (black, <1.5 μ m/min) or fast (red, >1.5 μ m/min)-moving cells are indicated. Mean $\pm$ standard deviation (SD) (n = 3). Numbers correspond to p values by one-way ANOVA with Tukey's posttest.

(G) MCF10A cells treated with MCF7-cell-derived sEV^{NORM} or sEV^{HYP} were analyzed for chemotactic cell motility quantified by rose plots. Red arrow, direction of the chemotactic gradient. (n = 3).

(H) MCF10A cells treated with MCF7-cell-derived sEV^{NORM} or sEV^{HYP} were analyzed for FA turnover, and the percentage of new, stable, or decayed FA was quantified. Mean $\pm$ SEM (n = 3). Numbers correspond to p values by two-way ANOVA with Tukey's posttest.

(I and J) MCF10A cells as in (H) were analyzed for mitochondrial motility by time-lapse videomicroscopy (I, representative images) at t = 0 and 6 min, and the speed of mitochondrial movements (J, left) and total distance traveled by individual mitochondria (J, right) were quantified. Mean $\pm$ SD (n = 3). Scale bar, 5 μ m. Numbers correspond to p values by one-way ANOVA with Tukey's posttest.

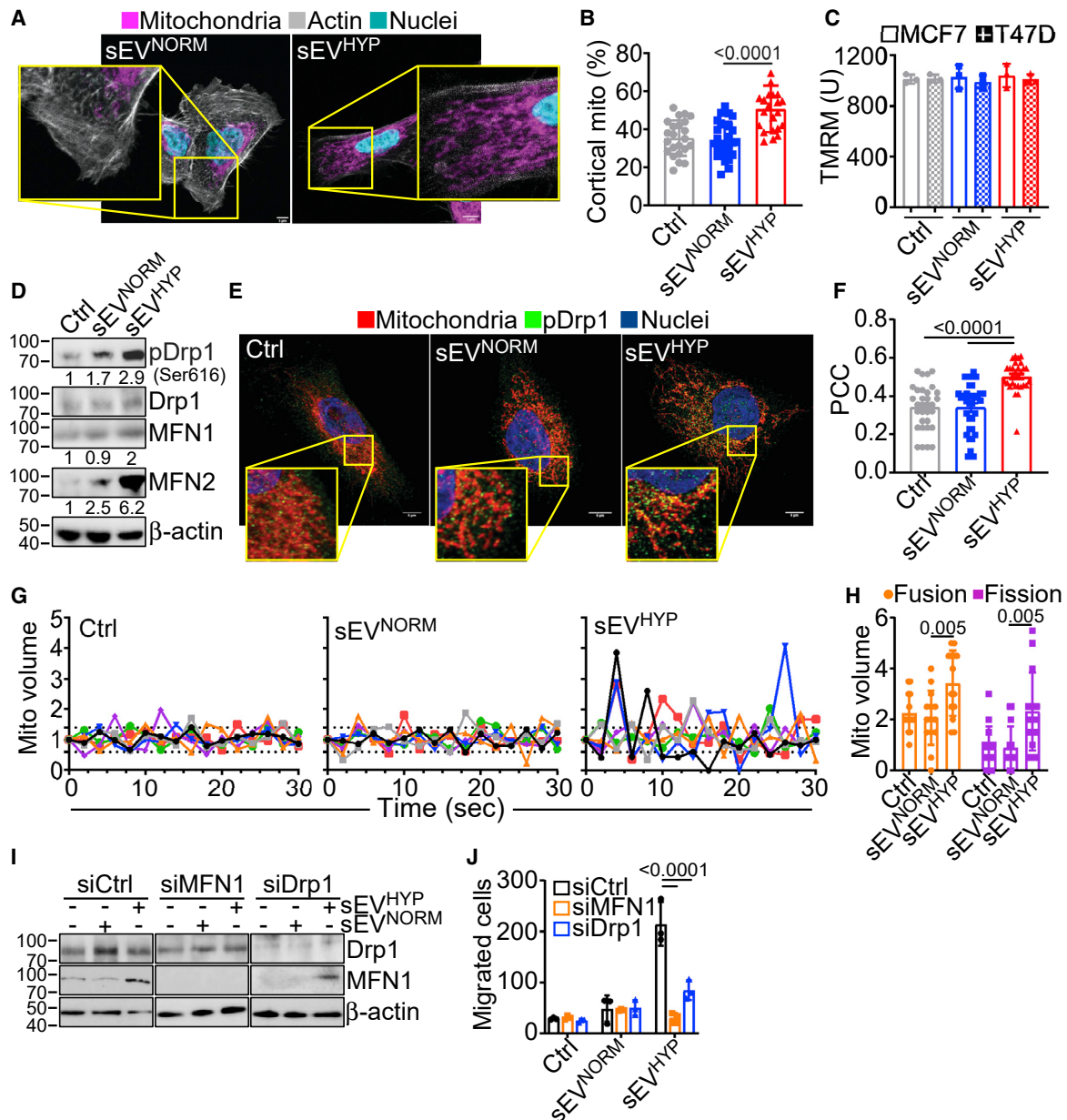


Figure 2. Breast-Cancer-Derived sEV^{HYP} Regulate Mitochondrial Dynamics

(A and B) MCF10A cells treated with T47D-cell-derived sEV^{NORM} or sEV^{HYP} were imaged after 24 h for subcellular mitochondrial distribution by confocal fluorescence microscopy (A, representative images), and the percentage of mitochondria localized to the cortical cytoskeleton was quantified (B). Insets, magnification of indicated areas. Scale bar, 5 μ m. Mean $\pm$ SD (n = 3). Numbers correspond to p values by one-way ANOVA with Tukey's posttest.

(C) MCF10A cells treated with MCF7- or T47D-cell-derived sEV^{NORM} or sEV^{HYP} were analyzed for changes in mitochondrial inner membrane potential by TMRM labeling and flow cytometry. Mean $\pm$ SD (n = 3).

(D) MCF10A cells treated with MCF7-cell-derived sEV^{NORM} or sEV^{HYP} for 8 h were analyzed by western blotting. p, phosphorylated. (n = 3).

(E and F) sEV^{NORM}- or sEV^{HYP}-treated MCF10A cells were analyzed for co-localization of Ser616-phosphorylated Drp1 (pDrp1) and mitochondria by confocal microscopy (E, representative images) and quantified (F). Insets, image magnification of indicated areas. Mean $\pm$ SD (n = 3). Scale bar, 5 μ m. Numbers correspond to p values by one-way ANOVA with Tukey's posttest. PCC, Pearson correlation coefficient.

(G and H) sEV-treated MCF10A cells were analyzed for changes in mitochondrial volume indicative of organelle fusion (>1.5-fold, positive y-scale) or fission (<1.5-fold, negative y-scale) over 30-s intervals (G), and the number of mitochondrial fusion and fission events was quantified (H), each line corresponds to changes in mitochondrial volume in a single cell. Mean $\pm$ SEM (n = 3). Numbers correspond to p values by two-way ANOVA with Sidak's multiple comparisons test. See also Figure S3.

(I and J) sEV-treated MCF10A cells transfected with control non-targeting siRNA (siCtrl) or siRNA directed to MFN1 (siMFN1) or Drp1 (siDrp1) were analyzed by western blotting (I) and cell migration on PET inserts (J). Mean $\pm$ SD (n = 3).

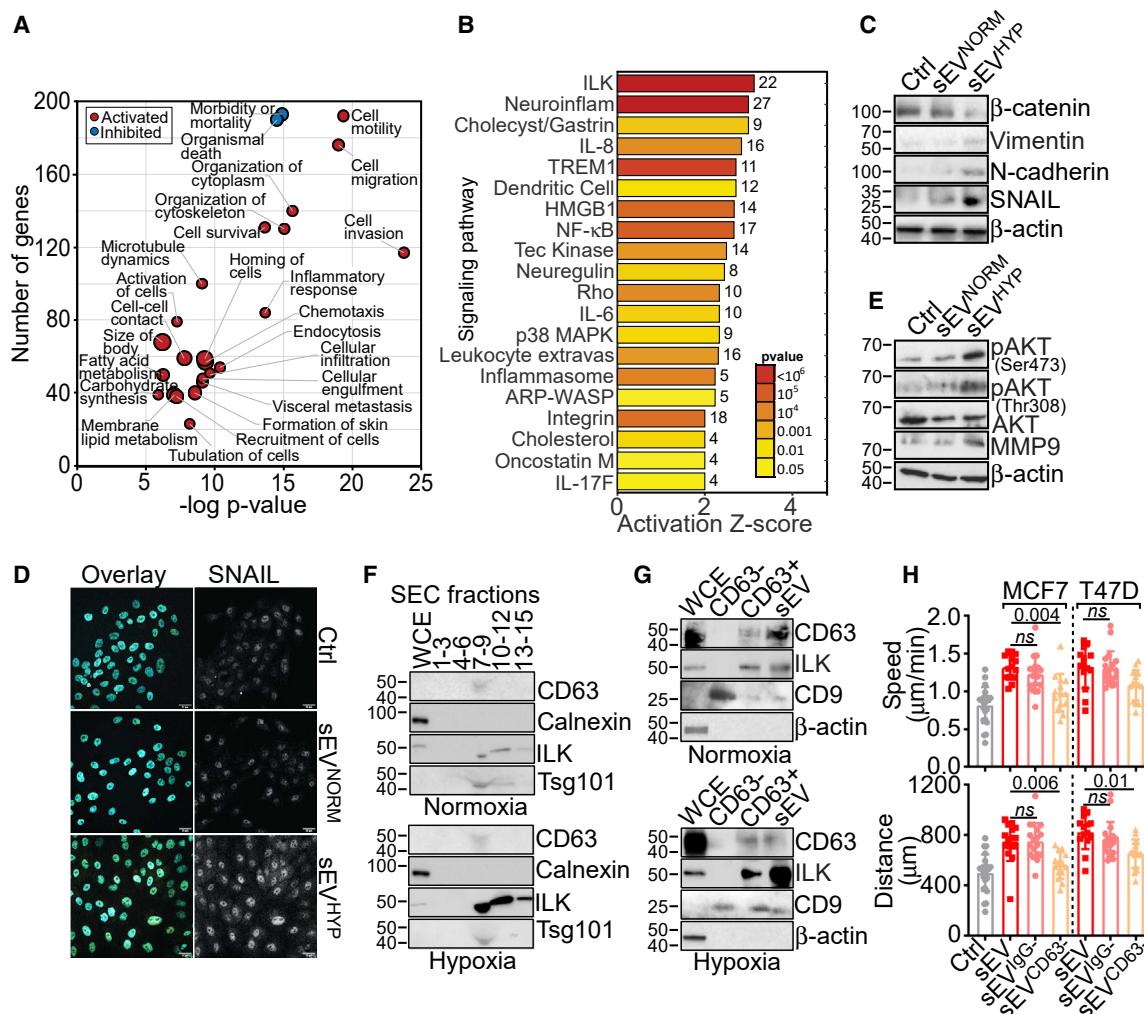


Figure 3. sEV^{HYP}-Induced Reprogramming of Normal Mammary Epithelial Cells

(A) Ingenuity pathway analysis of genes significantly modulated (red, activated; blue, inhibited) in MCF10A cells treated with sEV^{HYP} by RNA-seq ($p < 0.05$). (B) Bioinformatics analysis of gene pathways regulated by sEV^{HYP} in recipient MCF10A cells by RNA-seq. The activation Z score and p values are indicated. (C) sEV^{NORM}- or sEV^{HYP}-treated MCF10A cells were analyzed by western blotting ($n = 3$). (D) The conditions are as in (C), and MCF10A cells were analyzed for nuclear accumulation of SNAIL by fluorescence microscopy (representative images, $n = 3$). Scale bar, 25 μ m. (E) sEV^{NORM}- or sEV^{HYP}-treated MCF10A cells were analyzed by western blotting ($n = 3$). (F) WCE or the indicated sEV-containing SEC fractions isolated from normoxic (top) or hypoxic (bottom) MCF7 cells were analyzed by western blotting ($n = 2$). (G) WCE, unfractionated sEVs or CD63-positive (CD63⁺) or CD63-negative (CD63⁻) sEVs isolated from normoxic (top) or hypoxic (bottom) MCF7 cells were analyzed by western blotting. An antibody to CD9 was used as a control ($n = 3$). (H) MCF10A cells treated with total sEV, IgG-negative sEVs (sEV^{IgG}), or CD63-negative sEVs (sEV^{CD63}) isolated from MCF7 or T47D cells were analyzed for 2D motility with quantification of speed of cell movements (top) and total distance traveled by individual cells (bottom). Non-binding-sEVs to IgG-coated beads were used as control. Mean $\pm$ SD ($n = 3$). Numbers correspond to p values by one-way ANOVA with Tukey's posttest.

including organelle motility, subcellular distribution, and fusion and fission events.

sEV^{HYP}-ILK Reprogramming of Normal Mammary Epithelial Cells

To understand the mechanisms of sEV^{HYP} signaling, we next profiled recipient MCF10A cells by RNA sequencing (RNA-seq). Mirroring the observed increase in cell migration, sEV^{HYP} treatment was associated with a global transcriptional response in recipient cells, with activation of multiple pathways of cell

movements, chemotaxis, cytoskeletal organization, and cell-to-cell contact (Figure 3A). In addition, sEV^{HYP}-treated cells exhibited reduced cell death and increased pro-inflammatory responses (Figure 3A), including NF- κ B-, IL6-, and IL8-associated pathways (Figure 3B). Consistent with these data, sEV^{HYP} treatment induced markers of pro-migratory epithelial-to-mesenchymal transition (EMT) in recipient cells, with increased expression of the EMT transcriptional activator, Zinc finger protein SNAIL (SNAIL), as well as mesenchymal markers, N-cadherin and vimentin, and lower levels of β -catenin (Figure 3C).

Functionally, this was associated with increased nuclear accumulation of SNAIL compared with controls, whereas sEV^{NORM} had no effect (Figures 3D and S3C). In addition, sEV^{HYP}, but not sEV^{NORM}, increased the phosphorylation of Akt (Ser473 and Thr308) in recipient MCF10A cells, as well as upregulation of MMP9 (Figure 3E), two critical mediators of cell motility and invasion.

Bioinformatics analysis of RNA-seq data identified integrin-linked kinase (ILK) as the pathway most activated in sEV^{HYP}-treated MCF10A cells (Figure 3B). Consistent with this, exposure to hypoxia increased the accumulation of ILK in breast-cancer-derived sEVs (Figure S3D). This effect was specific, because ILK levels were not affected in whole-cell extracts (WCEs) from hypoxic or normoxic samples (Figure S3D). Next, we mapped the association of ILK with sEV-containing SEC fractions from normoxic or hypoxic MCF7 cells. We found that ILK was prominently enriched in SEC fractions 7–9 from sEV^{HYP} (Figure 3F), containing sEV markers, CD63, and Tsg101, but not ER marker, Calnexin (Figure 3F), and directly mediating MCF10A cell motility (Figure S2D). A pool of ILK was also present in SEC fractions 10–12 from sEV^{HYP} (Figure 3F). In contrast, SEC fractions 7–9 or 10–12 isolated from normoxic MCF7 cells showed little to no ILK (Figure 3F). Using an independent approach, ILK co-localized with CD63-expressing sEVs in hypoxic and, to a lesser extent, normoxic breast cancer cells, as observed by confocal microscopy (Figure S3E). As a control, siRNA silencing of ILK abolished the co-localization of ILK with CD63-positive sEVs in hypoxic or normoxic MCF7 or T47D cells, whereas a control, non-targeting siRNA had no effect (Figure S3E).

To establish the relevance of CD63-ILK association, we next immunodepleted CD63-reactive material from normoxic or hypoxic sEVs. In these experiments, CD63 depletion removed ILK expression from sEV^{NORM} or sEV^{HYP} (Figure 3G). Conversely, ILK was abundantly present in CD63-expressing sEVs or unfractionated sEVs from hypoxic and, to a lesser extent, normoxic MCF7 cells (Figure 3G). Similar results were obtained by flow cytometric staining of CD63-positive sEVs, where ILK was highly expressed in hypoxic, compared with normoxic samples (Figure S3F). Under these conditions, immunodepletion of CD63 abolished sEV^{HYP}-induced 2D motility of recipient MCF10A cells, normalizing the speed of cell movements and the total distance traveled by individual cells to levels of control cultures (Figure 3H). Consistent with the data above, depletion of CD63-sEVs with CD63-coated beads abolished the effect of sEVs on cell motility, whereas incubation of sEVs with immunoglobulin G (IgG)-coated beads had no effect on sEV-stimulated cell motility (Figure 3H). Finally, co-culture with sEV^{HYP} increased ILK protein levels in recipient MCF10A cells, whereas sEV^{NORM} had no effect on ILK levels in recipient cells (Figure S3G). Consistent with these data, only sEV-containing SEC fractions 7–9 transferred ILK to recipient MCF10A cells, whereas SEC fractions 10–12 had no effect (Figure S3H). Therefore, sEV^{HYP} globally reprogram the transcriptome of recipient mammary epithelial cells accompanied by delivery of ILK in these settings.

Role of ILK in sEV^{HYP} Regulation of Mitochondrial Dynamics

Based on these data, we next asked if sEV-vehiculated ILK participated in mitochondrial reprogramming and cell motility in

recipient cells. We found that sEVs isolated from hypoxic MCF7 cells transfected with ILK-directed siRNA failed to stimulate mitochondrial trafficking to the cortical cytoskeleton in recipient MF10A cells (Figures 4A and 4B). Instead, sEV^{HYP} from MCF7 cells transfected with non-targeting siRNA potently stimulated mitochondrial repositioning to the cortical cytoskeleton (Figures 4A and 4B). In addition, siRNA silencing of HIF1 α in sEV-producing cells also suppressed sEV^{HYP}-mediated mitochondrial trafficking (Figures 4A and 4B). Consistent with these data, treatment with a small molecule ILK inhibitor, Cpd22, which was identified by a cell-based assay to inhibit ILK-mediated signaling (Lee et al., 2011), suppressed sEV^{HYP}-induced mitochondrial trafficking in recipient MCF10A cells (Figures S4A and S4B). Cpd22 also inhibited Ser616-phosphorylated Drp1 recruitment to mitochondria mediated by sEV^{HYP} (Figures S4C and S4D). In contrast, Cpd22 did not affect mitochondrial trafficking to the cortical cytoskeleton (Figures S4A and S4B) or Drp1 association with mitochondria (Figures S4C and S4D) in the presence of sEV^{NORM}. Consistent with a potential direct effect on mitochondrial dynamics (Onodera et al., 2018), a pool of ILK associated with mitochondria in transfected cells, as observed by confocal microscopy (Figures S4E and S4F).

Using an independent experimental approach, siRNA silencing of ILK, but not control siRNA, suppressed mitochondrial dynamics induced by sEV^{HYP}, reducing both fusion and fission events to levels of control cultures (Figure S4G). In contrast, knockdown of ILK had no effect on mitochondrial dynamics in the presence of sEV^{NORM} (Figure S4G). Under these conditions, siRNA knockdown of ILK or treatment with Cpd22 prevented the increased turnover of FA mediated by sEV^{HYP}, lowering the percentage of new FA while increasing the number of stable FA (Figure 4C). In contrast, ILK targeting by siRNA or Cpd22 treatment did not affect FA turnover in sEV^{NORM}-co-cultured cells (Figure 4C). Finally, treatment with Cpd22 (Figure S4H) or HIF1 α or ILK silencing (Figure 4D) suppressed the increase in 2D motility stimulated by sEV^{HYP} in MF10A cells, normalizing the speed of cell movements and the total distance traveled by individual cells to levels of control cultures (Figures 4D and S4H). Together, these data demonstrate that sEV^{HYP} stimulation of ILK signaling mediates reprogramming of mitochondrial functions in recipient mammary epithelial cells.

Molecular Requirements of ILK Regulation of Mitochondrial Functions in Recipient Cells

To test the specificity of the findings above, we next carried out three sets of complementary experiments. First, we asked if reconstitution with sEV^{HYP} could restore ILK activity in ILK-silenced breast cancer cells. Co-culture with sEV^{HYP} restored ILK protein expression in siILK-knockdown cells (Figure 4E). Functionally, reconstitution with sEV^{HYP} rescued the recruitment of Ser616-phosphorylated Drp1 to mitochondria (Figures 4F and 4G) and promoted mitochondrial accumulation at the cortical cytoskeleton, compared with controls (Figure 4H). As a second approach, we reconstituted ILK-silenced MCF7 cells with wild-type (WT) ILK or ILK L207W dominant-negative mutant that interferes with the pseudocatalytic site and disrupts downstream signaling leading to stress fiber formation, cell spreading, and migration (Vaynberg et al., 2018). Reconstitution of ILK knockdown cells with WT ILK (Figure S5A) restored mitochondrial

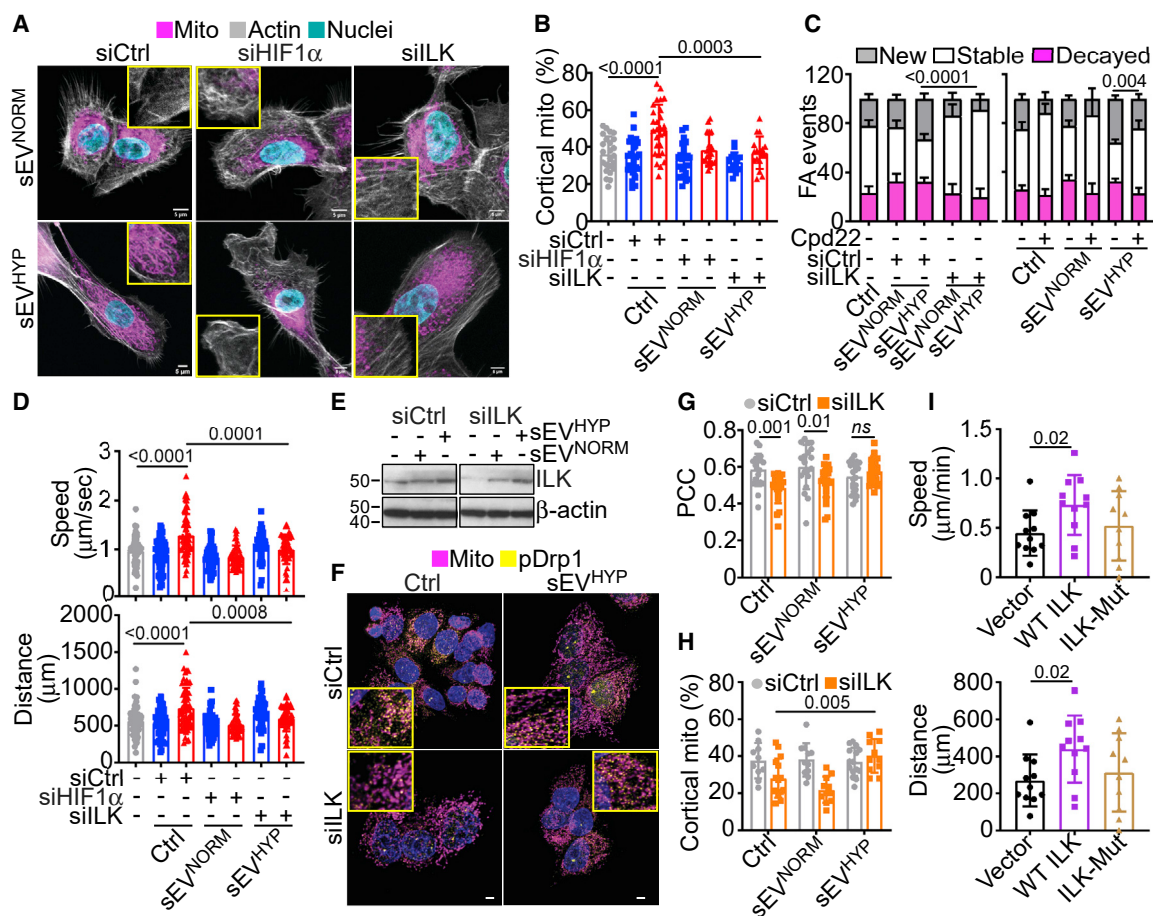


Figure 4. ILK Regulation of Exo^{HYP}-Induced Mitochondrial Dynamics

(A and B) MCF10A cells treated with MCF7-cell-derived sEV^{NORM} or sEV^{HYP} after transfection with siCtrl, siHIF1 α , or siILK were analyzed for subcellular mitochondrial localization by confocal fluorescence microscopy (A, representative images), and the percentage of mitochondria accumulated at the cortical cytoskeleton was quantified (B). Insets, image magnification of indicated areas. Scale bar, 5 μ m. Mean $\pm$ SD (n = 3). Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test.

(C) MCF10A cells treated with MCF7-cell-derived sEV^{NORM} or sEV^{HYP} after transfection with siCtrl or siILK (left) or treated with small molecule ILK inhibitor, Cpd22 (right) were analyzed for FA turnover by time-lapse videomicroscopy, and the percentage of new, stable, and decayed FA events under the various conditions tested was quantified. Mean $\pm$ SD (n = 3). Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test.

(D) MCF10A cells co-cultured with MCF7-cell-derived sEV^{NORM} or sEV^{HYP} after transfection with siCtrl, siHIF1 α , or siILK were analyzed for 2D motility, and the speed of cell movements (top) or total distance traveled by individual cells (bottom) was quantified. Mean $\pm$ SD (n = 3). Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test.

(E) MCF7 cells transfected with siCtrl or siILK were reconstituted with sEV^{NORM} or sEV^{HYP} and analyzed after 8 h by western blotting (n = 3).

(F and G) MCF7 cells transfected with siCtrl or siILK were treated with sEV^{NORM} or sEV^{HYP} and analyzed for co-localization of Ser616-phosphorylated Drp1 (pDrp1) with mitochondria by confocal microscopy (F, representative images) and quantified (G, n = 3). Insets, image magnification of indicated areas. Scale bar, 5 μ m. Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test. PCC, Pearson correlation coefficient. ns, not significant.

(H) The reconstitution conditions are as in (E), and MCF7 cells were analyzed for subcellular accumulation of mitochondria at the cortical cytoskeleton. Mean $\pm$ SD (n = 3). Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test.

(I) Normal primary mammary epithelial cells transfected with ILK or L207W ILK mutant (ILK-Mut) were analyzed for 2D motility, and the speed of cell movements (top) and total distance traveled by individual cells (bottom) were quantified. Mean $\pm$ SD (n = 3). Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test.

trafficking to the cortical cytoskeleton (Figures S5B and S5C) and normalized mitochondrial dynamics, re-establishing cycles of organelle fusion and fission events comparably to control transfectants (Figures S5D and S5E). In contrast, reconstitution with L207W ILK mutant had no effect on mitochondrial trafficking to the cortical cytoskeleton (Figures S5B and S5C) and did not restore mitochondrial dynamics in transfected cells (Figures S5D and S5E).

Third, to rule out that this response was selective for MCF10A cells, we transfected primary, normal mammary epithelial cells with WT or L207W ILK mutants. Similar to the data above, WT ILK, but not ILK L207W mutant, transfected in primary mammary epithelial cells (Figure S5F) associated with mitochondria, as observed by confocal microscopy (Figures S5G and S5H). Functionally, WT ILK stimulated subcellular mitochondrial trafficking to the cortical cytoskeleton (Figures S5I and S5J) and promoted

2D motility of normal mammary epithelial cells, increasing the speed of cell movements and the total distance traveled by individual cells (Figure 4I). Conversely, transfection of L207W ILK mutant did not promote increased mitochondrial trafficking (Figures S5I and S5J) or 2D motility (Figure 4I) in primary mammary epithelial cells. Therefore, mechanisms of pseudocatalytic ILK signaling regulate mitochondrial dynamics and subcellular organelle trafficking.

Requirement of Akt for sEV^{HYP} Signaling in Normal Mammary Epithelial Cells

The signaling requirements for how sEV-ILK regulates mammary epithelial cell responses were next investigated. In these experiments, siRNA silencing of ILK or HIF1 α in sEV-producing breast cancer cells abolished Akt phosphorylation (Ser473) induced by sEV^{HYP} in recipient MCF10A cells (Figure 5A). Similar results were obtained with a small molecule Akt inhibitor, MK2206, which also suppressed Akt phosphorylation (Ser473) mediated by sEV^{HYP} in MCF10A cells (Figure S6A). MK2206 treatment also moderately reduced total Akt levels in these cells (Figure S6B). In addition, MK2206 inhibited the association of Ser616-phosphorylated Drp1 with mitochondria (Figures 5B and 5C) and suppressed mitochondrial trafficking to the cortical cytoskeleton in sEV^{HYP}-treated cultures (Figures S6B and S6C). Consistent with these data, MK2206 blocked mitochondrial motility (Figure 5D) and suppressed mitochondrial dynamics mediated by sEV^{HYP}, decreasing the rate of fusion and fission events to levels of sEV^{NORM} treatment (Figure 5E). Finally, MK2206 blocked the increase in 2D motility mediated by sEV^{HYP} in recipient cells, lowering the speed of cell movements and the total distance traveled by individual cells to levels of sEV^{NORM}-treated cultures (Figure S6D).

As an independent approach, we next silenced by siRNA individual Akt isoforms, Akt1 or Akt2, in recipient cells (Figure S6F). This inhibited Akt phosphorylation in MCF10A cells (Figure S6E) and shut off the accumulation of mitochondria at the cortical cytoskeleton mediated by sEV^{HYP} treatment (Figures 5F and 5G). In contrast, depletion of Akt1 or Akt2 had no effect on mitochondrial trafficking in the presence of sEV^{NORM} (Figures 5F and 5G). Finally, we reconstituted MCF10A cells transfected with siCtrl or siAkt with WT Akt or dominant-negative (DN) Akt mutant (Figure 5H). In these experiments, reconstitution with WT Akt restored the increase in mitochondrial trafficking to the cortical cytoskeleton mediated by sEV^{HYP}, indistinguishably from control transfectants (Figure 5I). In contrast, transfection of DN Akt did not rescue the defect of mitochondrial motility induced by Akt knockdown, whereas reconstitution with WT or DN Akt had no effect on mitochondrial trafficking in the presence of sEV^{NORM} (Figure 5I). Altogether, these findings demonstrate that an ILK-Akt signaling axis is required for mitochondrial dynamics and downstream regulation of cell motility in mammary epithelial cells.

sEV^{HYP} Regulation of Mammary Gland Morphogenesis

Based on these data, we next examined the effect of sEV^{HYP} in a three-dimensional (3D) model of mammary gland development. In these experiments, addition of sEV^{HYP} increased the surface area of MCF10A 3D acini, *in vitro*, compared with controls (Figures 6A and 6B). This was associated with extensive morphological changes in 3D acini, with decrease in multiacinar sphericity

(Figures 6A and 6B) and more extensive filling of the acinar lumen (Figure 6C). Co-culture with sEV^{NORM} had no effect on MCF10A 3D acini in culture, compared with controls (Figures 6A–6C). In addition, MCF10A 3D acini treated with a single addition of sEV^{HYP} (Figures 6D and 6E) or sequentially with sEV^{HYP} additions every 4 days (Figure 6F) exhibited comparable increase in cell proliferation, by Ki67 staining and decreased apoptosis (cleaved caspase-3⁺ cells). Instead, treatment with sEV^{NORM} did not induce changes in cell proliferation or apoptosis in MCF10A 3D acini after single (Figures 6D and 6E) or sequential (Figure 6F) additions.

Consistent with the data above, sEV^{HYP}-treated 3D acini exhibited sustained phosphorylation of Akt (Ser473), persisting up to 21 days after seeding (Figures 6G and 6H), and appearance of EMT markers, including nuclear accumulation of SNAIL and increased expression of vimentin, compared with controls or sEV^{NORM}-treated acini (Figures 6G and 6H). Sequential treatment with sEV^{HYP} every 4 days gave similar results, with sustained phosphorylation of Akt (Ser473), nuclear accumulation of SNAIL, and increased levels of vimentin, compared with controls (Figures S7A and S7B).

In addition, sEV^{HYP} treatment disrupted the apical-basal polarity of 3D acini assessed 21 days after seeding, as reflected by deregulated topography of markers of apical polarity (GM130), basolateral polarity (hDlg), cell cortex-cytoskeletal linkages (phosphorylated ezrin, radixin, and moesin complex: pERM), as well as disorganized actin filament arrangement (phalloidin) (Figure 7A). 3D acini in control cultures or acini treated with sEV^{NORM} maintained apical-basal polarity and a well-defined arrangement of cell cortex-cytoskeleton and actin filaments (Figure 7A). Sequential addition of sEV^{HYP} every 4 days during a 21-day culture comparably disrupted apical-basal polarity and actin filament organization in 3D acini, whereas sequential treatment with sEV^{NORM} had no effect, compared with controls (Figures 7A and S7C). The above changes in mammary gland morphogenesis, together with deregulated cell proliferation, reduced apoptosis, and appearance of EMT markers are all hallmarks of malignant transformation, and a potential correlation between ILK expression and breast cancer progression was next investigated. In an analysis of publicly available databases, we found that high levels of ILK strongly correlated with reduced overall survival in patients with triple-negative breast cancer (Figure 7B).

DISCUSSION

In this study, we have shown that small EVs released by hypoxic breast cancer cells, sEV^{HYP}, target mitochondrial dynamics in recipient normal mammary epithelium, activating cycles of fusion and fission, promoting organelle motility and repositioning mitochondria at the cortical cytoskeleton (Figure 7C). In turn, this fuels heightened FA turnover, chemotaxis, and mammary cell migration (Figure 7C). Mechanistically, sEV^{HYP} signaling involved transfer of ILK to recipient cells, with downstream activation of Akt and transcriptional upregulation of various cell motility pathways. As a result, co-culture with sEV^{HYP} introduced traits typical of oncogenic transformation in 3D models of mammary gland morphogenesis, with deregulated cell proliferation, reduced apoptosis, appearance of EMT markers, and disruption of apical-basal polarity (Figure 7C).

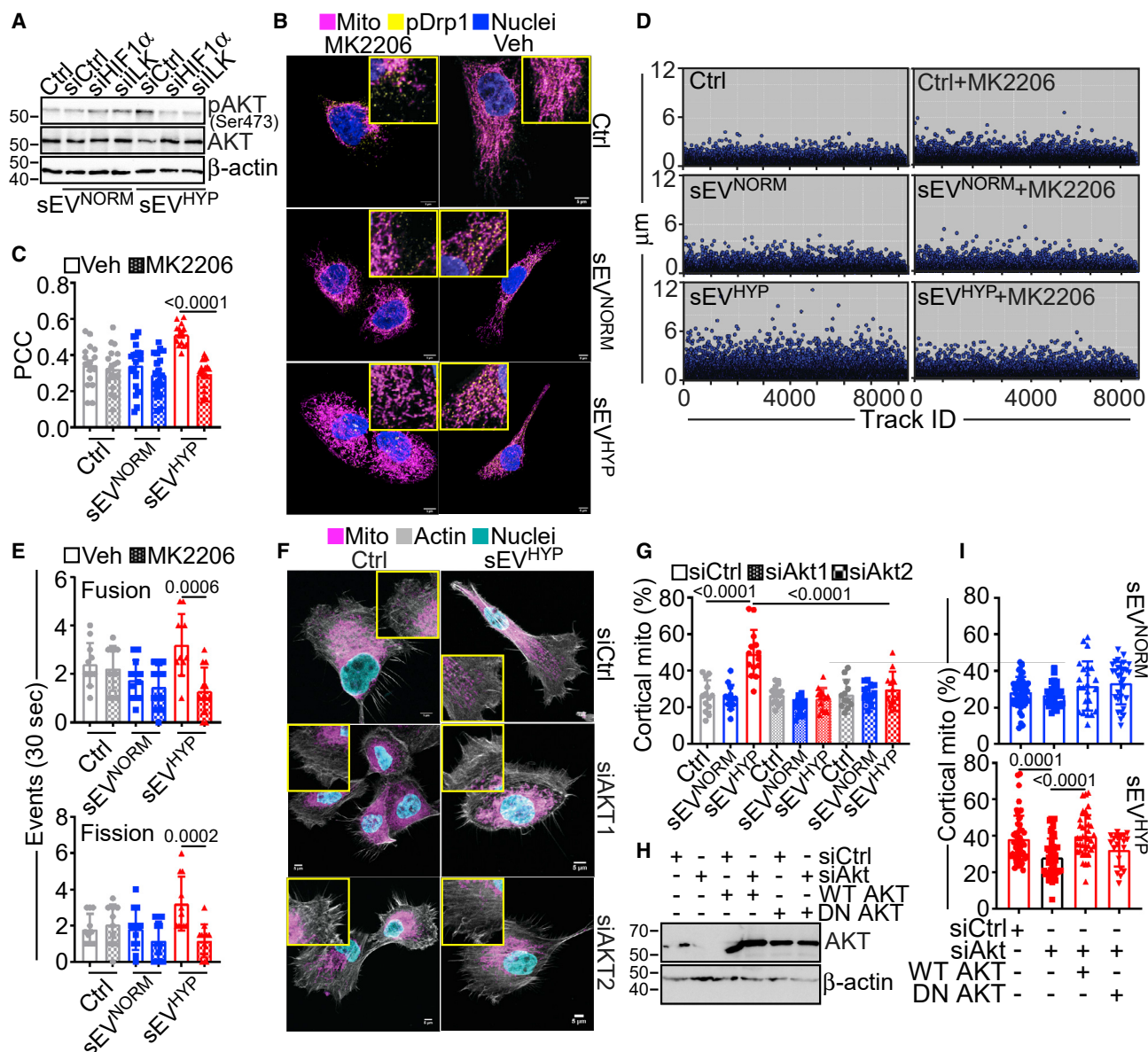


Figure 5. Akt Regulation of sEV^{HYP}-Dependent Mitochondrial Dynamics

(A) MCF10A cells treated with sEV^{NORM} or sEV^{HYP} isolated from MCF7 cells transfected with siCtrl, siHIF1α, or siLK were analyzed by western blotting. p, phosphorylated (n = 3).

(B and C) MCF10A cells treated with MCF7-cell-derived sEV^{NORM} or sEV^{HYP} were incubated with vehicle (Veh) or small molecule Akt inhibitor, MK2206 and co-localization of Ser616-phosphorylated Drp1 (pDrp1) with mitochondria was analyzed by confocal microscopy (B, representative images) and quantified (C). Scale bar, 5 μm. PCC, Pearson correlation coefficient. Mean ± SEM (n = 3). Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test.

(D) MCF10A cells treated with MCF7-cell-derived sEV^{NORM} or sEV^{HYP} were treated with vehicle (Veh) or MK2206 and analyzed for mitochondrial motility by time-lapse videomicroscopy. Each symbol corresponds to an individual mitochondrion. Representative experiment (n = 3).

(E) MCF10A cells co-cultured with MCF7-cell-derived sEV^{NORM} or sEV^{HYP} were treated with vehicle (Veh) or MK2206 and analyzed for changes in mitochondrial dynamics by time-lapse videomicroscopy, and fusion and fission events were quantified over 30-s intervals. Mean ± SD (n = 3). Numbers correspond to p values by two-way ANOVA with Tukey's posttest.

(F and G) MCF10A cells treated with sEV^{NORM} or sEV^{HYP} isolated from MCF7 cells transfected with siCtrl, siAkt1, or siAkt2 were analyzed for subcellular mitochondrial localization by confocal microscopy (F, representative images), and the percentage of mitochondria accumulated at the cortical cytoskeleton was quantified (G). Insets, image magnification of indicated areas. Scale bar, 5 μm. Mean ± SD (n = 3). Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test.

(H) MCF10A cells transfected with siCtrl or siAkt were reconstituted with WT Akt or DN Akt cDNA and analyzed by western blotting. (n = 3).

(I) MCF10A cells reconstituted as in (H) and treated with MCF7-cell-derived sEV^{NORM} (top) or sEV^{HYP} (bottom) were quantified for mitochondrial accumulation at the cortical cytoskeleton by confocal microscopy. Mean ± SD (n = 3). Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test. See also Figures S5 and S6.

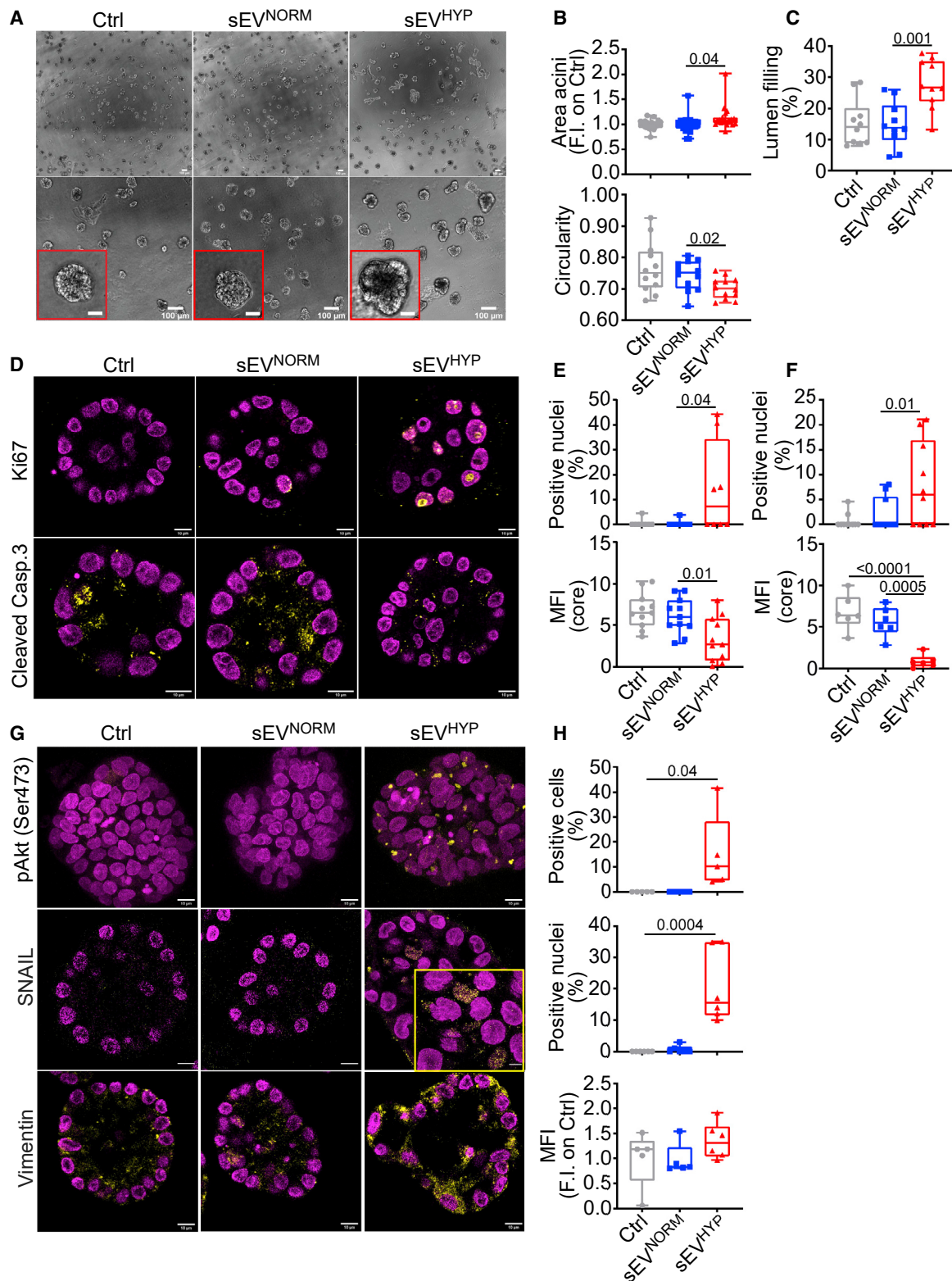


Figure 6. sEV^{HYP} Regulation of 3D Mammary Acini Development

(A) MCF10A cells were seeded in 3D acini culture in the presence of Ctrl, sEV^{NORM}, or sEV^{HYP} for 21 days and imaged by bright-field microscopy. Insets, image magnification of indicated areas. Scale bar, 100 μ m. (n = 3).

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EVs are recognized mediators of autocrine signaling for tumor cells, but less is known about their effect(s) on neighboring “normal” cells in the microenvironment (Kalluri and LeBleu, 2020). There is evidence that breast-cancer-derived EVs, or exosomes, reprogram normal epithelial cells and fibroblasts to activate metabolic changes (Fong et al., 2015), mitogenic responses (Antonyak et al., 2011), and oncogenic transformation (Melo et al., 2014), but a paracrine role of sEV^{HYP} on normal mammary epithelial cell motility has not been previously reported. Here, this pathway was unexpectedly centered on sEV^{HYP} activation of mitochondrial dynamics in recipient cells (Eisner et al., 2018), a mechanism not previously associated with EV signaling, involving upregulation of MFN1 and MFN2, recruitment of Ser616-phosphorylated Drp1 to mitochondria, and increased cycles of organelle fusion and fission.

Aberrant mitochondrial dynamics is commonly observed in tumors (Senft and Ronai, 2016) and mechanistically linked to drug resistance (Zhang et al., 2016), cancer stemness (Wu et al., 2019), and metastatic competence (Caino et al., 2015; Jung et al., 2016). As a component of this process, subcellular mitochondrial trafficking has attracted attention as a prerequisite of heightened cell motility and invasion (Altieri, 2019) for both normal and transformed cell types. In particular, oncogenes, such as Myc (Agarwal et al., 2019), have been recently shown to hijack a cellular machinery for anterograde and retrograde mitochondrial movements, originally characterized in neurons (Birsá et al., 2013), to reposition mitochondria at the leading edge of migration. In turn, this is thought to fuel FA turnover, membrane lamellipodia dynamics, and heightened cell motility and invasion, *in vivo* (Caino et al., 2016; Cunliffe et al., 2016). A similar paradigm emerged here for sEV^{HYP} signaling, where increased mitochondrial dynamics in recipient cells enabled the redistribution of mitochondria at the cortical cytoskeleton to sustain FA turnover, chemotaxis, and cell movements.

A key regulator of this response uncovered here was ILK (Qin and Wu, 2012), which was packaged in breast-cancer-derived sEV^{HYP}. ILK was originally proposed as a kinase regulating integrin-mediated cell-extracellular matrix polarization and cell motility (Hannigan et al., 1996). However, its kinase activity has remained controversial (Qin and Wu, 2012), and recent studies have suggested that ILK acts non-catalytically to regulate stress fiber formation, cell polarization, and cell movements (Vaynberg et al., 2018).

Here, a pool of ILK was localized to mitochondria, whereas targeting of ILK with genetic (siRNA silencing), molecular (L207W mutant) (Vaynberg et al., 2018), or pharmacologic (small molecule Cpd22 inhibitor) approaches suppressed mitochondrial trafficking, organelle dynamics, and cell motility. Accordingly, ILK has been recently implicated in blocking retrograde mitochondrial movements and regulates cell invasion via inhibition of RhoT1-TRAK2 complex(es) at the organelle outer membrane (Onodera et al., 2018). Whether a similar mechanism is involved here is currently not known. What is known is that ILK contributes to the activation of Akt (Qin and Wu, 2012), a critical mediator of mitochondrial trafficking and associated cell movements (Caino et al., 2015), potentially by targeting it to the plasma membrane via its binding partner Parvin (Fukuda et al., 2003) that directly binds to Akt (Kimura et al., 2010).

A key consequence of sEV^{HYP}-mediated signaling was a general disruption of normal mammary epithelial morphogenesis, with loss of apical-basal polarity in 3D cultures, persistent cell proliferation with concomitantly reduced apoptosis, and acquisition of EMT markers, including nuclear accumulation of SNAIL. Although a role of EVs in mammary epithelial morphogenesis has not been previously proposed, there is abundant evidence that the phenotype observed here is one of the earliest events in carcinoma initiation (Martin-Belmonte and Perez-Moreno, 2011). Mechanistically, this is thought to involve deregulated gene expression maintained by cell-cell contacts (Balda and Matter, 2000), disruption of a developmentally timed cell death-cell proliferation balance (Anderson et al., 2010), and impaired control of cell-cycle entry (Chandramouly et al., 2007). The molecular details of how sEV^{HYP} drive oncogenic transformation of the normal mammary epithelium remains to be elucidated. It is intriguing that exposure to sEV^{HYP} triggered extensive transcriptional changes in recipient cells, affecting not only intercellular communication, cell movements, and cell death pathways but also NF κ B signaling, which has been associated with mammary gland tumorigenesis (Liu et al., 2010). There is also evidence that sustained phosphorylation of Akt, which in our system persisted up to 21 days after co-culture with sEV^{HYP}, contributes to snail family transcriptional repressor 2 (Slug) upregulation and EMT induction (Carpenter et al., 2015).

In terms of biological context, the oncogenic changes induced by sEV^{HYP} in “normal” mammary epithelium may

(B) The conditions are as in (A), and the surface area (top) and circularity (bottom, 1 = perfect circle) of sEV-treated MCF10A acini in 3D culture were quantified. Each dot represents the mean of MCF10A acini in one 10 $\times$ microscopy field. An average of 12 independent microscopy fields were used for counting. Data are expressed as boxplots (min to max) of at least three independent experiments. Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test.

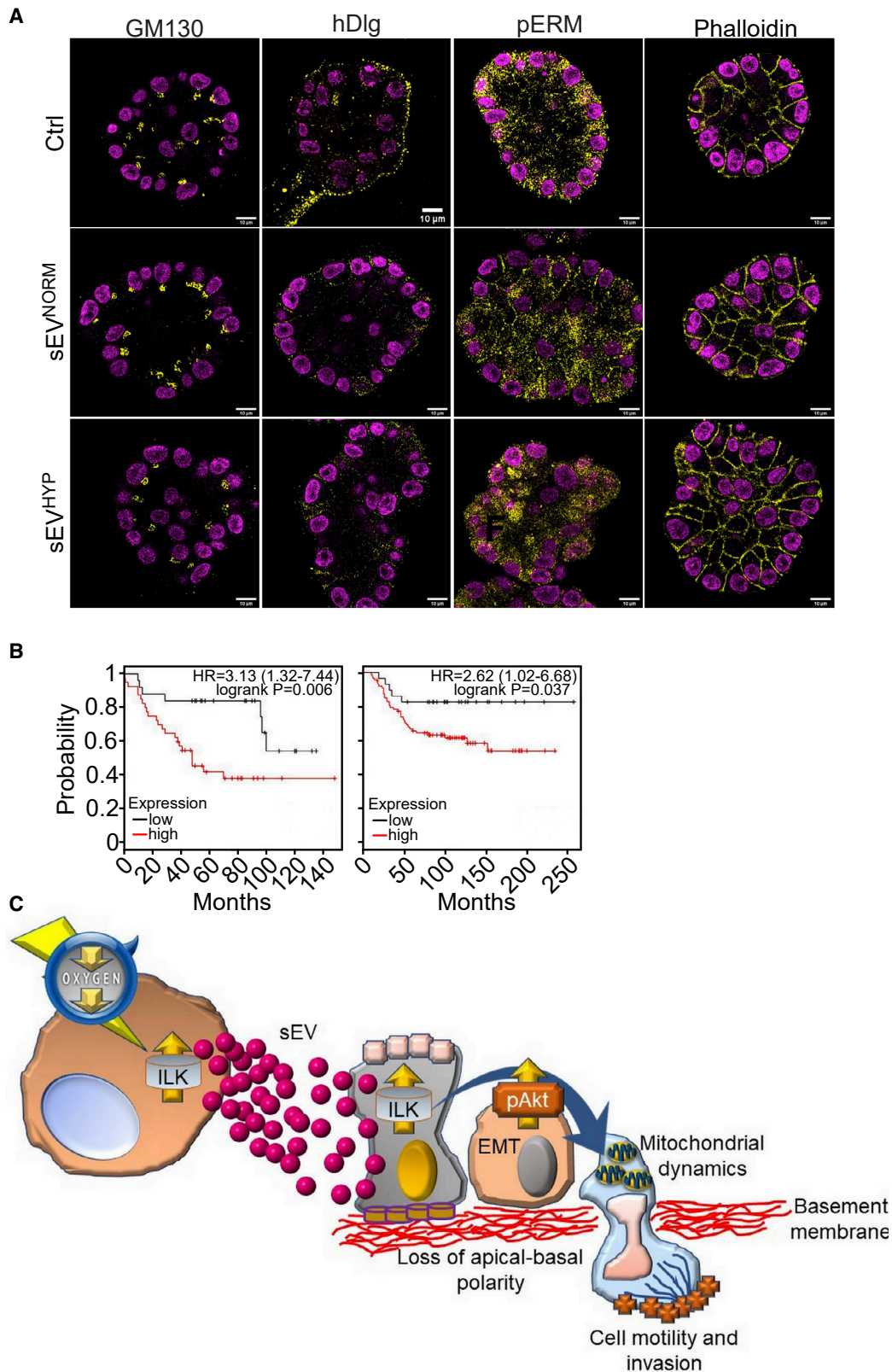
(C) The conditions are as in (A), and the lumen filling of sEV-treated MCF10A acini in 3D was quantified. Data are expressed as boxplots (min to max) of at least three independent experiments. Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test.

(D) sEV-treated MCF10A acini in 3D as in (A) were analyzed for Ki67 (top) or cleaved caspase 3 (bottom) reactivity after 8 days, by immunohistochemistry. Representative equatorial section images. Scale bar, 10 μ m.

(E and F) The conditions are as in (D), and the percentage of Ki67⁺ nuclei and cleaved caspase 3-expressing cells was quantified for sEVs added once at t = 0 (E) or sequentially every 4 days (F). MFI, mean fluorescence intensity. Data are expressed as boxplots (min to max) of at least three independent experiments. Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test.

(G) The conditions are as in (D), and sEV-treated MCF10A acini in 3D were analyzed for expression of pAkt (Ser473, top), nuclear accumulation of SNAIL (middle), or levels of vimentin (bottom) after 21 days by immunohistochemistry. Representative confocal microscopy images are shown; inset, magnification of selected field. Scale bar, 10 μ m.

(H) The conditions are as in (G), and the percentage of pAkt⁺ cells (max projection), SNAIL⁺ nuclei, and expression of vimentin was quantified. Data are expressed as boxplots (min to max) of at least three independent experiments (8 independent acini per condition). MFI, mean fluorescence intensity. Numbers correspond to p values by one-way ANOVA with Tukey's multiple comparisons test, n = 3. See also Figure S7.



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contribute to a “field effect” of cancerization (Curtius et al., 2018). Although linked to somatic mutations (Martincorena et al., 2015) and epigenetic changes (Ushijima and Hattori, 2012), it seems plausible that sEV^{HYP} reprogramming of mitochondrial dynamics, with disruption of developmental tissue morphogenesis, and induction of EMT (Leung and Brugge, 2012), may expand the footprint of mammary epithelial transformation to enable both local and distant disease recurrence(s) (Gatenby and Gillies, 2008). On the one hand, as a key effector of sEV signaling, this model is consistent with an important oncogenic role of ILK (Hannigan et al., 2005), including in mammary tumorigenesis (Hinton et al., 2008) and linked to abbreviated patient survival (this study). On the other hand, therapeutic targeting of mitochondrial reprogramming (Zhang et al., 2016), as well as ILK (Hannigan et al., 2005), may provide viable strategies to disrupt early pro-tumorigenic changes in the microenvironment (this study), as well as mechanisms of metastatic competence (Caino et al., 2016).

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.devcel.2020.07.014>.

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AUTHOR CONTRIBUTIONS

I.B. and D.C.A. conceived the study; I.B. performed experiments of exosome-mediated mitochondrial reprogramming, cell motility, developmental morphogenesis in 3D culture, and immunohistochemistry; J.C.G. examined exosome function in mitochondrial trafficking and oxidative stress; A.V.K. performed bioinformatics analysis of RNA-seq studies; S.M. and S.R.K. examined exosome characteristics and parameters; J.Q. provided wild-type and mutant ILK constructs, L.R.L., V.V., J.Q., E.F.P., and D.C.A. analyzed data; and I.B. and D.C.A. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Figure 7. Role of sEV^{HYP}-ILK Signaling in Mammary Gland Transformation

(A) MCF10A acini in 3D culture seeded in the presence of Ctrl, sEV^{NORM}, or sEV^{HYP} (added once at t = 0) were analyzed after 21 days for expression of apical polarity marker, GM130, basolateral polarity marker, hDlg, plasma membrane marker, pERM or actin cytoskeleton marker, phalloidin by immunohistochemistry. Representative confocal microscopy images (n = 3). Scale bar, 10 μ m.

(B) Correlation between ILK expression and overall survival in two independent series of triple-negative breast cancer patients. Kaplan-Meier curves for each dataset are indicated. HR, hazard ratio.

(C) Proposed model for sEV^{HYP} regulation of mitochondrial functions and cell motility in transformation of the normal mammary epithelium via ILK and Akt signaling. See text for details. See also Figure S7.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-Ser616-phosphorylated Drp1	Cell Signaling	Cat #4494; RRID: AB_11178659
Mouse monoclonal anti-Drp1	Cell Signaling	Cat #14647; RRID: AB_2798554
Rabbit monoclonal anti-MFN1	Cell Signaling	Cat #14739; RRID: AB_2744531
Rabbit monoclonal anti-MFN2	Cell Signaling	Cat #9482; RRID: AB_2716838
Rabbit monoclonal anti-Ser473-phosphorylated AKT	Cell Signaling	Cat #9271; RRID: AB_329825
Rabbit monoclonal anti-Thr308-phosphorylated Akt	Cell Signaling	Cat #4056; RRID: AB_331163
Rabbit monoclonal anti-AKT	Cell Signaling	Cat #9272; RRID: AB_329827
Rabbit monoclonal anti-EGFR	Cell Signaling	Cat #4267; RRID: AB_2246311
Rabbit monoclonal anti-MMP9	Cell Signaling	Cat # 13667; RRID: AB_2798289
Rabbit monoclonal anti-ILK	Cell Signaling	Cat # 3862; RRID: AB_2127050
Rabbit monoclonal anti-HIF1 α	Cell Signaling	Cat # 36169; RRID: AB_2799095
Rabbit monoclonal anti-Calnexin	Cell Signaling	Cat # 2679; RRID: AB_2228381
Rabbit monoclonal anti-VDAC	Cell Signaling	Cat # 4661; RRID: AB_10557420
Rabbit monoclonal anti-Flotillin1	Cell Signaling	Cat # 18634; RRID: AB_2773040
Rabbit monoclonal anti-E-cadherin	Cell Signaling	Cat # 3195; RRID: AB_2291471
Rabbit monoclonal anti-N-cadherin	Cell Signaling	Cat # 13116; RID: AB_2687616
Rabbit monoclonal anti-SNAIL	Cell Signaling	Cat # 3879; RRID: AB_2255011
Rabbit monoclonal anti-Cleaved-Caspase 3	Cell Signaling	Cat # 9661; RRID: AB_2341188
Mouse monoclonal anti-Ki67	Cell Signaling	Cat # 9449; RRID: AB_2797703
Rabbit monoclonal anti-pERM	Cell Signaling	Cat # 3726; RRID: AB_10560513
Rabbit monoclonal anti-Vimentin	Cell Signaling	Cat # 5741; RRID: AB_10695459
Mouse monoclonal anti-hDlg (SAP97)	Santa Cruz	Cat # sc-9961; RRID: AB_2092015
Rabbit monoclonal anti-Tsg101	Protein Tech	Cat # 14497-1-AP; RRID: AB_2208090
Mouse monoclonal anti-CD63	Abcam	Cat # ab59479; RRID: AB_940915
Rabbit monoclonal anti-CD9	Abcam	Cat# ab92726; RRID: AB_10561589
Mouse monoclonal anti- β -actin	Sigma	Cat # A5441; RRID: AB_476744
Chemicals, Peptides, and Recombinant Proteins		
Human Epidermal growth factor	Peptotech	Cat # AF-100-15
Hydrocortisone	Sigma	Cat # H0888
Cholera toxin	Sigma	Cat # C8052
Growth Factor Reduced Matrigel	BD Biosciences	Cat # 354230
Talin-red fluorescent protein (RFP) BacMam	ThermoFisher	Cat # C10612
PKH67 Green Fluorescent Cell Linker	Sigma	Cat # PKH67GL-1KT
MitoTracker deep red	ThermoFisher	Cat # M22426
CellLight™ Mitochondria-RFP	ThermoFisher	Cat # C10601
CellROX Green Reagent	ThermoFisher	Cat # C10444
TMRM	ThermoFisher	Cat #M20036
RNAiMAX	ThermoFisher	Cat #13778100
X-tremeGENE HP	Sigma	Cat #366244001
MK-2206 AKT inhibitor	Selleck Chemicals	Cat S1078; CAS: 1032350-13-2
Cpd22 ILK inhibitor	Sigma	Cat #407331-5MG

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
qEV size exclusion column	IZON	qEVoriginal / 70nm
Deposited Data		
RNA-seq data	NCBI GEO	Accession number GSE154340
Experimental Models: Cell Lines		
MCF7	ATCC	ATCC® HTB-22
T47D	ATCC	ATCC® HTB-133™
MCF10A	ATCC	TCC® CRL-10317™
Oligonucleotides		
siRNA targeting HIF1 α	Dharmacon	Cat # L-004018
siRNA targeting ILK	Sigma	Cat # PDSIRNA2D
siRNA targeting AKT1	Santa Cruz Biotech	Cat # sc-29195
siRNA targeting AKT2	Santa Cruz Biotech	Cat # sc-29197
siRNA targeting MFN1	Santa Cruz Biotech	Cat # sc-270320
siRNA targeting DRP1	Santa Cruz Biotech	Cat # sc-43732
Recombinant DNA		
896 pcDNA3 T7 Akt1	Ramaswamy et al., 1999	Addgene Cat #9003
1014 pcDNA3 T7 Akt1 K179M T308A S473A	Ramaswamy et al., 1999	Addgene Cat #9031
ILK WT	Vaynberg et al., 2018	N/A
L207W ILK mutant	Vaynberg et al., 2018	N/A
Software and Algorithms		
ImageJ	Commercial	https://imagej.nih.gov/ij
Prism 8	Commercial	https://www.graphpad.com/scientificsoftware/prism
TrackMate plugin	Commercial	Tinevez et al., 2017
Chemotaxis and Migration Tool v. 2.0	Ibidi	https://ibidi.com/chemotaxis-analysis/171-chemotaxis-and-migration-tool.html
Bowtie 2	(Langmead and Salzberg, 2012)	N/A
RSEM v1.2.12 software	Li and Dewey, 2011	N/A
DESeq2 algorithm	Love et al., 2014	N/A
QIAGEN's Ingenuity® Pathway Analysis software	IPA®, QIAGEN	www.qiagen.com/ingenuity

RESOURCE AVAILABILITY

Lead Contact

Further information and request for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Dario Altieri (daltieri@wistar.org).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The RNA-seq data was submitted to NCBI GEO under accession number GSE154340.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Culture

Breast cancer epithelial MCF7 (female) and T47D (female) cells and normal breast epithelial MCF10A (female) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF7 and T47D cells were maintained in culture in RPMI 1640 medium with 10% fetal bovine serum (FBS) plus 1% streptomycin and penicillin. MCF10A cells were grown in DMEM F12

medium with 5% horse serum (HS), 20 ng/ml epidermal growth factor (EGF), 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μ g/ml insulin and 1% streptomycin and penicillin. Conditioned media used for cell invasion assays was prepared from exponentially growing cultures of NIH 3T3 cells (ADCC) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/liter d-glucose, sodium pyruvate, 10 mM HEPES, and 10% FBS for 48 h. All cell lines were maintained at 37°C.

METHOD DETAILS

Antibodies and Reagents

PKH67 was purchased from Sigma, MitoTracker deep red and CellLight™ Mitochondria-RFP, BacMam 2.0 were obtained from ThermoFisher. Growth Factor Reduced Matrigel was obtained from BD Biosciences (BD No. 354230). Antibodies to Ser616-phosphorylated Drp1, Drp1, MFN1, MFN2, Ser473-phosphorylated AKT, Thr308-phosphorylated Akt, Akt, EGFR, MMP9, ILK, HIF1 α , Calnexin, VDAC, Flotillin 1, E-cadherin, Vimentin, SNAIL, Ki67, cleaved-Caspase 3, phosphorylated Ezrin-Radixin-Moesin (pERM) complex were obtained from Cell Signaling. An antibody to hDlg was from Santa Cruz. An antibody to Tsg101 was from ProteinTech. Antibodies to CD63 and CD9 was from Abcam. An antibody to β -actin was from Sigma. The Akt inhibitor MK2206 (1 μ M) was purchased from Selleck Chemicals and ILK inhibitor Cpd22 (600 nM) was purchased from Sigma.

Small Extracellular Vesicles (sEV) Isolation

MCF7 or T47D cells at 70% of confluency were cultured in RPMI medium with EV-depleted FBS for 24 h in normoxic or hypoxic condition (5% or 1% O₂, respectively). At the collection of supernatants, cells were counted and cell death was evaluated by trypan blue staining (death cells < 5%). Cells and debris were removed by sequential centrifugation (350 g for 5 min and 1000 g for 10 min) and the supernatants were concentrated using Amicon Ultra centrifugal filter tubes (Merk Millipore). Larger vesicles were eliminated by centrifugation at 10,000 g for 30 minutes at 4°C. Finally, EV were isolated by qEV size exclusion column (SEC – iZON) as described (Lobb et al., 2015). Briefly, 500 μ l aliquots of concentrated supernatant were overlaid on qEV columns followed by elution with PBS, 500 μ l phases were harvested in Eppendorf tubes and EV were collected in phases 7-8-9 (Witwer et al., 2013). To confirm the presence of sEV in fraction 7-8-9, the first 15 fractions (500 μ l each) were collected and analyzed by NTA (nanoparticle tracking analysis). In particular, the fractions were pooled (1 to 3, 4 to 6, 7 to 9, 10 to 12 and 13 to 15) and analyzed using ZetaView NTA (Particle Metrix). Isolated EV were stored at -80°C before analysis or RNA/protein extraction. As control, fresh RPMI medium was processed as culture medium and stored at -80°C. The concentration and size distribution of particles were analyzed using Nanosight NS300 (Malvern, Instruments Ltd Worcestershire). Unless otherwise noted, 3x10⁸ EV were used per every 10⁴ recipient cells. For internalization experiments, sEV were precipitated by ultracentrifugation (100,000 g for 2 h at 4°C), suspended in 400 μ l of diluent C, stained with 5 μ l PKH67 for 5 min at 22°C and quenched by addition of 100 μ l of 20% BSA. Subsequent sEV isolation was carried out using qEV SEC as described above.

Immuno-Affinity Depletion of CD63

Five hundred μ l of isolated sEV were incubated overnight at 4°C with CD63-coated microbeads (10606D, Thermo Fisher) and/or Dynabeads protein A (10001D, Thermo Fisher) following the manufacturer's instructions. Briefly, 100 μ l of magnetic beads were washed with PBS – 0.1% BSA and mixed with sEV solution and incubated overnight. Unbound sEV were collected and transferred to a fresh tube, and concentration and size distribution of CD63-depleted sEV were analyzed using the ZetaView NTA. For protein isolation, CD63-depleted sEV were concentrated by ultracentrifugation at 100,000 g at 4°C for 2 h and then lysed with RIPA buffer. For CD63+ vesicles, RIPA buffer was directly added to the magnetic beads, incubated for 2 h at 4°C. For flow cytometry analyses, CD63+ vesicles coated to CD63-microbeads were stained according to the manufacturer's specifications. Briefly, sEV were permeabilized with Triton 0.5% for 15 min at 22°C and incubated overnight with a primary antibody to ILK and/or IgG isotype control at 4°C on a sample shaker. The bead-bound sEV were washed twice with PBS – 0.1% BSA and incubated with a fluorescent secondary antibody for 1 h at room temperature on a sample shaker. Data were acquired on a BD Biosciences (BD) FACSCelesta, and analysis after acquisition was performed using FlowJo version 9.9.6 (Tree Star Inc).

Cryo-EM Sample Preparation and Data Collection

A 3- μ l aliquot of each sample was applied onto 200-mesh copper grids (Quantifoil R1.2/1.3) that were glow discharged for 60 s. The excess solution was blotted with filter paper for 6 sec using Vitrobot Mark IV (FEI Netherlands) at 4°C and the grids were immediately flash-frozen by rapidly plunging the grid into liquid ethane at -165°C. CryoEM data for each sample were collected on a Tecnai F20 TEM microscope operated at 200 keV. Images were recorded on Falcon III direct electron detector at a magnification of 25,000x. Each micrograph was generated by averaging individual dose fractionated frames collected at a rate of 40 frames/s for 4 sec exposure. The frames were motion-corrected and summed into a single micrograph. The micrographs collected were in the range of 2.0–4.0 μ m under focus.

Plasmid and siRNA Transfection

Small interference RNA (siRNA) experiments were carried out using control, non-targeting small interfering RNA (siRNA) pool (Dharmacon, cat. no. D-001810) or individual siRNA targeting HIF1 α (L-004018, Dharmacon), ILK (PDSIRNA2D, Sigma), Akt1 (sc-29195, Santa Cruz Biotech) or Akt2 (sc-29197, Santa Cruz Biotech), MFN1 (sc-270320, Santa Cruz Biotech) and Drp1 (sc-43732, Santa Cruz

Biotech). For gene silencing experiments, pooled or individual siRNA oligonucleotide sequences were transfected at 10 nM concentrations in the presence of Lipofectamine RNAiMAX in a 1:1 ratio (Invitrogen). Cells were incubated for 48 h, confirmed for target protein knockdown by Western blotting, and processed for subsequent experiments. For rescue experiments, plasmid DNA transfections were performed 48 h after siRNA transfection in MCF10A cells using X-tremeGENE HP DNA transfection reagent (Roche). Plasmids encoding 896 pcDNA3 T7 Akt1 (wild type), 1014 pcDNA3 T7 Akt1 K179M T308A S473A (dominant negative) (Addgene) (Ramaswamy et al., 1999) were used. Plasmids encoding WT ILK or loss-of-function L207W ILK mutant were as described (Vaynberg et al., 2018).

Immunofluorescence

Ten thousand cells were seeded on cover-glasses. MCF7 and T47D cells were transfected with ILK-directed siRNA for 48 h. MCF10A cells were treated with sEV for 24 h, with or without the small molecule Akt inhibitor, MK2206 or ILK inhibitor, Cpd22 and then stained with 300 nM MitoTracker deep red FM dye for 30 min at 37°C. Cells were fixed with 4% paraformaldehyde for 10 min, quenched with 20 mM glycine for green auto-fluorescence for 20 min and then permeabilized with Triton 0.5% for 15 min at 22°C. Samples were saturated with 10% BSA for 1 h at 22°C. Primary antibodies (ILK, SNAIL and Ser616-phosphorylated Drp1, 1:100; CD63, 1:50 in 10% PBS-BSA) were incubated for 18 h at 4 °C, whereas secondary antibodies (1:1000) were incubated for 1 h at 22°C. Nuclei were stained with Hoechst 3342 (Cell Signaling) for 5 min at 22°C and slides were mounted using the Prolong reagent (ThermoFisher Scientific). Confocal images were generated on a Leica TCS SP5 confocal microscope (Leica Microsystems) with a magnification of 40X or 63X. For colocalization experiments, images were acquired with a pixel size <80 nm in xy and a z-stack of 0.12 μm. Images were deconvolved using Hyugens software and co-localization was quantified as Pearson Correlation Index (PCI).

RNA Sequencing (RNA-Seq)

Total RNA was extracted from MCF10A cells after 24 h of co-culture with MCF7 cell-derived sEV using Direct-zol RNA MiniPrep kit (Zymo Research). RNA quantity was determined using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and the quality was validated using the TapeStation RNA ScreenTape (Agilent). One hundred ng of total RNA was used to prepare a library for Illumina Sequencing using the Quant-Seq 3'mRNA-Seq Library Preparation Kit (Lexogen). Library quantity was determined using qPCR (KAPA Biosystem), and overall library size was determined using the Agilent TapeStation and the DNA High Sensitivity D5000 ScreenTape (Agilent). Equimolar amounts of each sample library were pooled, denatured and Mid-Output, 75 bp paired-end Next Generation Sequencing was carried out on a NextSeq 500 (Illumina). RNA-seq data were aligned using bowtie 2 (Langmead and Salzberg, 2012) against hg19 version of human genome, and RSEM v1.2.12 software (Li and Dewey, 2011) was used to estimate gene-level read counts using Ensemble transcriptome information. Raw gene counts were used in DESeq2 (Love et al., 2014) algorithm to estimate significance of differential expression between group pairs. Expression heatmaps were plotted using mean-centered log₂-scaled normalized by DESeq2 expression values. Gene set enrichment analysis was done using QIAGEN's Ingenuity® Pathway Analysis software (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) on genes that passed nominal p<0.05 using "Disease and Functions" and "Canonical Pathways" options. General cellular functions that passed p<10⁻⁵ threshold and pathways that passed p<0.05 threshold were considered significant. Only results with significantly predicted activation state (|Z-score|>2) were reported.

Mitochondrial Time-Lapse Videomicroscopy

To study mitochondrial dynamics two different experimental approaches were utilized. In both cases, MCF10A and/or MCF7 cells (1 × 10⁴) were seeded on high-optical-quality glass-bottom 35-mm plates (MatTek Corporation) for 24 h. When using MitoTracker, MCF10A cells were co-cultured with sEV and/or treated with small molecule Akt inhibitor, MK2206. For MCF7 cells, cultures were seeded after 48 h of transfection with control non-targeting siRNA (siCtrl) or ILK-directed siRNA (siILK) and then co-cultured with sEV. After 24 h, cells were stained with 300 nM (MCF10A) or 100 nM (MCF7) MitoTracker deep red FM dye for 30 min at 37°C. Alternatively, cells were incubated with 2 μl of CellLight™ Mitochondria-RFP, BacMam 2.0 O.N. before co-culture with sEV and drug treatment.

Using both approaches, cells were imaged on a Leica TCS SP8 X inverted confocal laser scanning microscope using a 63X, 1.40-numerical-aperture (NA) oil objective. Live imaging time-lapse videomicroscopy was performed using a Tokai Hit incubation chamber equilibrated to bidirectional scanning at 8,000 Hz at 37°C with 5% CO₂. Images were acquired every 2 sec for a 6-min interval. Individual 12-bit images were acquired using a white-light supercontinuum laser (0.2% at 643 nm) and hybrid detectors at a 4 × digital zoom with a pixel size of 70 nm by 70 nm and a step size of 0.260 μm. At least 6 single cells under each condition were collected for analysis. Initial postprocessing of 3D sequences was carried out with Hyugens software to deconvolve the images. Images were imported into ImageJ Fiji and individual mitochondria were automatically tracked using TrackMate plugin (Tinevez et al., 2017) or into Leica LAS X software to study fission and fusion events as described (Wang et al., 2019).

Cortical Mitochondria Quantification

MCF10A cells were seeded on coverslip and co-cultured with sEV or transfected with siCtrl or siAkt for 48 h and then co-cultured with sEV or treated with Akt inhibitor, MK2206 or ILK inhibitor, Cpd22. After 24 h, cells were stained with 300 nM MitoTracker deep red FM dye for 30 min at 37°C, washed, and fixed with 4% formaldehyde for 10 min at 22°C. Actin filaments were stained with phalloidin-rhodamine (Molecular Probes) 488 for 1 h at 22°C and nuclei were stained with Hoechst (Molecular Probe) for 5 min at 22°C. Slides

were analyzed on a Leica TCS SP5 confocal laser microscope with a 63X oil objective and images were analyzed in ImageJ as described (Agarwal et al., 2019).

Time-Lapse Videomicroscopy Analysis of Focal Adhesion (FA) Dynamics

MCF10A cells were seeded on high-optical-quality 35 mm glass-bottom plates and transduced with talin-red fluorescent protein (RFP) BacMam virus for 18 h, followed by co-culture with sEV with or without vehicle or ILK inhibitor, Cpd22 for additional 24 h. Time-lapse videomicroscopy was carried out using a Leica TCS SP8 confocal laser scanning microscope system with an HCX PL APO CS 40X, 1.40-NA oil UV objective. Acquisition of live cells using integrated Leica LAS software was performed every 2 min per frame for a total time interval of 30 min. Sequences were imported into ImageJ for further analysis. The initial and final frames were duplicated and assembled as composite images. FA complexes were manually counted and classified (according to the presence in some or all the time frames) into three groups: decaying, newly formed, and stable (merged areas). The analysis was carried out with 10 cells per condition in 3 independent time-lapse experiments using the LASX software package.

ROS and Mitochondria Depolarization Analyses

To detect total ROS, MCF10A cells were co-cultured with sEV for 24 h, and incubated with 2.5 μ M of CellROX Green Reagent (Invitrogen) for 30 min at 37°C, according to the manufacturer's instructions. After three washes in PBS, pH 7.4, cells were harvested and analyzed on a BD Biosciences FACSCelesta flow cytometer. To evaluate mitochondrial inner membrane depolarization, MCF10A cells co-cultured with sEV for 24 h, were stained with Tetramethylrhodamine (TMRM, Invitrogen, 100 nM) for 30 min at 37°C. Cells were analyzed on a BD Biosciences FACSCelesta flow cytometer and intact cells were gated in the FSC/SSC plot to exclude small debris. The resulting data were plotted on a histogram.

Tumor Cell Motility and 2D Chemotaxis

For 2D motility studies, MCF10A cells (1×10^4) were seeded in 4-well Ph+ chambers (Ibidi) in complete medium. For 2D chemotaxis, MCF10A cells (2×10^4) were placed in 8- μ m chemotaxis slides (Ibidi). After 5 h from sEV addition, time-lapse videomicroscopy was performed over a 10-h interval, as described (Agarwal et al., 2019). Stacks were imported into ImageJ Fiji software for analysis, and 30 cells per each condition tested were tracked using the Manual Tracking plugin for ImageJ Fiji in 3 independent experiments. Tracking data were exported into Chemotaxis and Migration Tool v. 2.0 (Ibidi) for graphing and calculation of means of speed and accumulated distance of movement. For analysis of directional cell migration using a wound closure assay, MCF10A cells were seeded on 24 MW and co-cultured with sEV for 24 h. "Wounds" in the cell monolayer were made with a 20- μ l pipette tip and cell debris was washed. Time-lapse imaging of migrating cells was performed using a TE300 inverted microscope (Nikon) equipped with an incubator set at 37°C with 5% CO₂ and 95% relative humidity. Each image was acquired using a 10 $\times$ objective of the same fields at 1-h time intervals for a total of 12 h.

Cell Migration

Cell migration and invasion experiments were performed essentially as described (Agarwal et al., 2019), using growth factor-reduced Matrigel-coated 8- μ m PET (cell invasion) and PET (cell migration) Transwell chambers (Corning). MCF10A cells were seeded on Transwell filters (10^5 cells/well) in medium containing 0.1% bovine serum albumin (BSA) with or without sEV. Conditioned medium from NIH 3T3 fibroblasts was placed in the lower chamber as a chemoattractant. Cells were allowed to invade or migrate for 24 h, non-invading cells were removed whereas invaded cells were fixed in methanol. Membranes were mounted in medium containing DAPI (Vector Laboratories) and analyzed by fluorescence microscopy. Ten fields at 10X magnification were collected and the number of invading/migrating cells were counted using ImageJ.

3D Acini Formation and Immunofluorescence

Three-dimensional (3D) culture of MCF10A cells was performed as described (Debnath et al., 2003). Each well of a μ -Slide 8 Well chamber (Ibidi) was coated with 40 μ l of Growth Factor-Reduced Matrigel (BD Biosciences). MCF10A cells (5000/well) were suspended in Assay Medium (DMEM/F12, 2% horse serum, 0.5 μ g/ml Hydrocortisone, 100 ng/ml Cholera Toxin, 10 μ g/ml Insulin and Pen/Strep) plus 2% Matrigel and 5 ng/ml EGF with or without sEV and seeded on coated Matrigel. Cultures were supplemented every 4 d with assay medium containing 2% Matrigel and 5 ng/ml EGF. sEV were added once at t=0 or, alternatively, every 4 d. Bright-field images were collected at d 7, 14 or 21 using TE300 inverted microscope (Nikon) equipped with an incubator set at 37°C with 5% CO₂ and 95% relative humidity with a 10X objective.

At d 21, MCF10A 3D acini were fixed in 4% formalin for 20 min at 22°C and processed for immunofluorescence (Debnath et al., 2003). Briefly, cultures were permeabilized with 0.5% PBS-Triton for 20 min at 4°C, rinsed three times with PBS/glycine, incubated with 200 μ l of IF buffer (130 mM NaCl, 7 mM Na₂HPO₄, 3.5 mM NaH₂PO₄, 7.7 mM Na₂SO₄, 0.1% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween-20) plus 10% goat serum for 1 h at 22°C (primary block). For secondary block, 10% goat serum and 20 μ g/ml goat anti-mouse F(ab')₂ fragment (Jackson ImmunoResearch) were added to the IF buffer and cells were incubated for additional 40 min. Finally, primary antibodies were diluted in secondary block solution (GM130 and hDlg, 1:50; pERM, Ki67, cleaved caspase 3, pAKT (Ser473), vimentin and SNAIL, 1:100) and incubated for 18 h at 22°C. Isotype-matched secondary antibodies and phalloidin were diluted 1:200 in IF buffer plus 10% goat serum and further incubated for 1 h at 22°C. Nuclei were counterstained with Hoechst

1:500 in PBS for 15 min at 22°C. Images were captured using a Leica TCS SP8 confocal laser scanning microscopy system with an HCX PL APO CS 63 $\times$, 1.40-NA oil UV objective, zoom 2X and a pinhole of 0.7 μ m.

Bioinformatics Analysis of ILK Expression in Breast Cancer Patients

For these experiments, Kaplan Meier curves for ILK were generated (Györfy et al., 2010) using two different databases (Liu_2014 (n=126) and Tang_2018 (n=118)). Both cohorts contained patients with triple-negative breast cancer (HER2⁻, ER⁻, PGR⁻). For the survival analysis generated from the Liu_2014 database, the p value was 0.0367 and FDR > 50%. For the survival analysis generated from the Tang_2018 database, the p value was 0.0068 and FDR 50%.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 8 software (La Jolla, CA). All biological experiments were performed at least three times (all single experiments have a technical duplicate). To compare means for two groups, a Mann Whitney t-test with Tukey's pos-test was performed. For three or more group comparison, a one-way Anova test was used. To determine how a response is affected by two factors, a two-way Anova test was used.