Fibronectin rescues estrogen receptor α from lysosomal degradation in breast cancer cells

Rocio G. Sampayo1–3, André M. Toscani4, Matthew G. Rubashkin5, Kate Thi6, Luciano A. Masullo7,8, Ianina L. Violi7, Jonathon N. Lakins5, Alfredo Cáceres5, William C. Hines9, Federico Coluccio Leskow4, Fernando D. Stefani10, Dante R. Chialvo10, Mina J. Bissell10, Valerie M. Weaver9, and Marina Simian1,3

Estrogen receptor α (ERα) is expressed in tissues as diverse as brains and mammary glands. In breast cancer, ERα is a key regulator of tumor progression. Therefore, understanding what activates ERα is critical for cancer treatment in particular and cell biology in general. Using biochemical approaches and superresolution microscopy, we show that estrogen drives membrane ERα into endosomes in breast cancer cells and that its fate is determined by the presence of fibronectin (FN) in the extracellular matrix; it is trafficked to lysosomes in the absence of FN and avoids the lysosomal compartment in its presence. In this context, FN prolongs ERα half-life and strengthens its transcriptional activity. We show that ERα is associated with β1-integrin at the membrane, and this integrin follows the same endocytosis and subcellular trafficking pathway triggered by estrogen. Moreover, ERα+ vesicles are present within human breast tissues, and colocalization with β1-integrin is detected primarily in tumors. Our work unravels a key, clinically relevant mechanism of microenvironmental regulation of ERα signaling.

Introduction

Estrogen receptor α (ERα) is a transcription factor present in different adult tissues such as mammary gland, ovaries, uterus, and brain (Couset al., 1997; Han et al., 2013). It regulates cell proliferation, migration, and survival. In the breast in particular, ERα controls mammary development and plays a key role in tumor growth. Therefore, understanding what regulates ERα activation and shutdown is fundamental for cell biology. ERα action can be blocked with tamoxifen (the most widely used selective ER modulator), although one third of breast cancer patients develop resistance, with ERα regaining activity (Nardone et al., 2015; Jeselsohn et al., 2017). The causes of this resistance are still unclear.

So far, the main proposed mechanism for ERα signaling shutdown is estrogen-induced ERα degradation. Estrogen binding to ERα induces its nuclear translocation. Once in the nucleus, ERα binds to its target promoters and is then ubiquitylated and subsequently degraded in cytosolic proteasomes. Therefore, ERα’s half-life decreases from 4 to 2 hours in the presence of estrogens. The pool of ERα attached to the plasma membrane by reversible S-palmitoylation on cysteine 447 (Acconcia et al., 2005; Marino et al., 2006; Adlanmerini et al., 2014) has been suggested to follow different degradation dynamics (La Rosa et al., 2012). Whether membrane-bound ERα has transcriptional activity is still a matter of debate (Levin, 2009). Understanding how membrane and cytoplasmic ERαs are regulated in breast cancer is crucial to develop strategies to overcome resistance to endocrine therapy.

The ECM plays a key role in cell fate, and evidence is accumulating that it modulates response to therapy in breast cancer as well (Ghajar and Bissell, 2008; Correia and Bissell, 2012). We previously described that ECM components affect the response of breast cancer cells to tamoxifen (Pontiggia et al., 2012). In particular, we found that fibronectin (FN), which correlates with lower survival when levels are increased (Yao et al., 2007; Hellemans et al., 2008), induces tamoxifen resistance in breast cancer.

1Universidad de Buenos Aires, Instituto de Oncología “Ángel H. Roffo”, Área Investigación, Buenos Aires, Argentina; 2Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Fisiología y Biología Molecular y Celular, Ciudad Universitaria, Buenos Aires, Argentina; 3Universidad Nacional de San Martín, Instituto de Nanosistemas, Campus Miguelete, San Martín, Argentina; 4Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Química Biológica, IQUIBICEN UBA-CONICET y Universidad Nacional de Luján, Departamento de Ciencias Básicas, Buenos Aires, Argentina; 5Division of Biological Systems and Engineering, Lawrence Berkeley National Laboratory, Berkeley, CA; 6Centro de Investigaciones en Bionanociencias, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina; 7Departamento de Física, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina; 8Instituto de Investigación Médica Mercedes y Martín Ferreyra, Córdoba, Argentina; 9Center for Complex Systems and Brain Sciences, Escuela de Ciencia y Tecnología, Universidad Nacional de San Martín and Consejo Nacional de Investigaciones Científicas y Tecnológicas, San Martín, Argentina.

Correspondence to Rocio G. Sampayo: ro.sampayo@gmail.com; Marina Simian: marsimian@gmail.com.

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cancer cells when bound to β1-integrin, its surface receptor. Therefore, we hypothesized that FN–β1-integrin pathway might have a direct effect on ERα signaling, modifying its response to hormone treatment.

We used two well-known cellular models of ERα-positive human breast adenocarcinoma: MCF7 and T47D. These cell lines have been widely used and validated for the study of ERα activity because primary culture of normal or tumor human breast tissues leads to the loss of ERα expression (Graham et al., 2009; Hines et al., 2016). We demonstrate that FN prolongs ERα half-life and strengthens its transcriptional activity. Mechanistically, we show that upon treatment with 17β-estradiol (E2), membrane ERα is endocytosed and travels in these vesicles through the cytoplasm and into the nucleus. In the absence of FN, it is degraded in lysosomes after 60 min of treatment. When FN is present, these endosomes escape lysosomal degradation, and ERα is localized in Rab11+ vesicles, typically involved in recycling. Using superresolution microscopy and coimmunoprecipitation assays, we found that ERα and β1-integrin colocalize at the plasma membrane and are endocytosed together after stimulation with E2. In these vesicles, β1-integrin is also degraded upon 60 min of treatment with E2, unless FN is present. We propose that FN-bound β1-integrin, following its recycling pathway, drags these ERα–β1-integrin vesicles back to the plasma membrane, thus bypassing the lysosomal compartment. We show that these endosomes are present in normal and tumor human breast tissues, although only tumor samples showed positive colocalization between ERα and β1-integrin. This indicates that the mechanism of ERα overactivation dependent on its association with FN–β1-integrin pathway would be particularly active within tumors. In light of these findings, we strongly suggest that a novel therapeutic strategy designed to interfere with the cross talk between FN and ERα signaling pathways would resensitize patients to endocrine therapy.

**Results**

### FN modulates ERα degradation and transcriptional activity

Given that we have previously shown that FN induces resistance to anti-estrogenic therapy (Pontiggia et al., 2012), we wondered whether FN has a direct effect on ERα activity. Research on ERα activity and dynamics in culture is challenging because primary culture of ERα-positive normal tissues and tumors leads to the loss of ERα expression (Hines et al., 2016). Therefore, we used two well-characterized human ERα-positive breast adenocarcinoma cell lines, MCF7 and T47D, that allowed us to modulate and study ERα regulation in culture. We first performed luciferase reporter assays with a construction that allowed us to measure ERα activity mediated by the estrogen response element (ERE). We found that, when cells are seeded on FN, this receptor has a stronger transcriptional activity in the presence of E2 compared with its activity on the control substrate (BSA; Fig. 1a). To study the mechanism through which FN regulates ERα activity, we analyzed the effect of FN on ERα degradation. We found that when cells are seeded on BSA, ERα completely localizes in the nucleus after 15 min of treatment with E2 (Fig. 1b). Knowing that E2 triggers ERα degradation, reducing ERα mean expression after 60 min (Reid et al., 2003), we increased the treatment time and found that as expected, total ERα levels drop after stimulation with E2 in cells seeded on BSA (Fig. 1c). When cells are seeded on FN, ERα is also completely localized in the nucleus after 15 min of treatment with E2 (Fig. 1d). However, we found that after a longer treatment with the hormone (>60 min), FN inhibits E2-induced ERα degradation (Fig. 1e). We confirmed these observations by immunofluorescence, showing a more intense signal of nuclear ERα after treatment with E2 in cells seeded on FN compared with BSA (Fig. S1, a and b). Similar results were obtained using T47D cells (Fig. S1, c–f). These data indicate that FN inhibits E2-stimulated ERα degradation. Of note, total ERα levels are increased when cells are seeded on FN even in the absence of E2, indicating that FN might also alter basal ERα degradation dynamics (Reid et al., 2003). Interestingly, when we performed ultracentrifugation to separate cytoplasmic and membrane fractions, we observed that membrane ERα follows a dynamics similar to cytoplasmic ERα (Fig. S1, g–j).

Moreover, we further tested the effect of FN on ERα shuttling kinetics. As shown in Fig. 1f, E2 stimulates ERα nuclear localization, reaching its maximum after 8 min of treatment. These kinetics are not affected by the presence of FN (Fig. 1g). However, upon 20 min of treatment, it can be already observed that degradation of both nuclear and cytoplasmic ERα is reduced when cells are seeded on FN. Altogether, these data indicate that FN modulates ERα degradation but does not alter ERα shuttling dynamics to the nucleus.

**ERα is degraded in lysosomes and can be rescued by FN**

We next tested whether FN was inhibiting E2-triggered proteasomal degradation of ERα, the best-characterized degradation pathway of this receptor. We found that upon E2 stimulation for 60 min, inhibition of the proteasomal pathway with bortezomib (BZ) increases ERα levels even in the presence of FN (Fig. 2a), suggesting that FN would inhibit a different mechanism of ERα degradation. Because FN has been found to modulate lysosomal degradation of membrane proteins (Caswell et al., 2009; Dozynkiewicz et al., 2012), we next asked whether ERα could be degraded in lysosomes upon E2 stimulation. Blocking the passage of late endosomes to lysosomes by inhibiting V-ATPase with bafilomycin-A1 (BAF; Li et al., 2013) impaired ERα degradation after 60 min of treatment with E2, in cells seeded on BSA (Fig. 2b). To further test that ERα is degraded in lysosomes after E2 treatment, we expressed GFP-tagged Rab7, a well-known small GTPase that determines the passage of late endosomes to lysosomes (Vanlandingham and Ceresa, 2009). As shown in Fig. 2c, E2 treatment triggers ERα colocalization with Rab7. Pearson’s and Manders’ correlation coefficients (PCCs and MCCs, respectively) were used to quantify the degree of colocalization observed between these proteins in each analyzed field as previously described (Dunn et al., 2011). The overall significance level of colocalization was calculated from these coefficients for each condition. A shorter treatment with E2 (15 min) revealed an increase in ERα localization closer to the Rab7 compartment, although practically no colocalization with Rab7+ endosomes was observed (Fig. S1k, top), indicating that a longer treatment is necessary for ERα+ vesicle localization to lysosomes.

We next investigated the effect of FN on E2-induced ERα lysosomal degradation and found that ERα does not colocalize with...
Rab7 after 60 min (Fig. 2, e and f) or 15 min (Fig. S1 k, bottom) treatment in the presence of FN. These data indicate that FN is rescuing ERα from being degraded in the lysosomal compartment. We confirmed these results with LAMP-1, a lysosomal marker, which shows that after 60 min of treatment with E2, ERα colocalizes with LAMP-1 when cells are seeded on BSA, and this is reverted when cells are seeded on FN (Fig. S1 l).

To ensure that ERα signals observed in these assays correspond in fact with ERα, we checked the specificity of this antibody. We used the epitope this antibody was raised against as a blocking peptide and obtained no ERα signal in Western blot or immunofluorescence assays (Fig. S1, m and n). Moreover, knockdown of ERα significantly reduces the signal obtained with this antibody proving that it specifically recognizes this protein (Fig. S1 o).

ERα is rapidly endocytosed after estrogen treatment

We next asked whether ERα was present in endosomes that could end up in lysosomes upon E2 stimulation and whether this was...
Figure 2. **ERα is degraded in lysosomes and rescued by FN.** (a) Top: Western blot of T47D cells seeded on BSA or FN pretreated with BZ 8nM or its vehicle (saline) for 4 h and treated as indicated. Bottom: Densitometry. For each subcellular fraction, the mean ERα/β-actin density ratio is shown normalized to the mean control group. (b) Top: Western blot of a subcellular fractionation of T47D cells pretreated for 90 min with 25 nM BAF or its vehicle (DMSO) and then treated for 60 min with 10⁻⁸ M E2 or its vehicle (ethanol). Blotting antibodies are shown on the left. Bottom: Densitometry. For each experimental condition, the ERα/β-actin density ratio is shown, normalized to the mean control group. Each symbol represents a different experiment. Differences between groups were analyzed by one-tailed paired Student’s t test (n = 3 replicates). (c) Confocal images of T47D cells expressing GFP-Rab7 seeded on BSA treated for 60 min with vehicle or E2, and stained for ERα. In the inset, arrows indicate points of colocalization. (d) Quantification of c. For each experimental condition, Pearson’s correlation index and Manders’ coefficients (M1 and M2) were calculated within the areas of colocalization using Fiji. Data are represented as mean ± SD. Differences between groups were analyzed by one-tailed Student’s t test (per replicate: Pearson’s: n_vehicle = 11 fields, n_E2 = 12 fields; Manders’: n_vehicle = 8 fields, n_E2 = 9 fields). (e) Confocal images of T47D cells expressing GFP-Rab7 seeded on FN treated for 60 min with vehicle or E2, and stained for ERα. (f) Quantification of e. For each experimental condition, Pearson’s correlation index and Manders’ coefficients (M1 and M2) were calculated within the areas of colocalization using Fiji. Data are represented as mean ± SD. Differences between groups were analyzed by one-tailed Student’s t test (per replicate: n_vehicle = 9 fields, n_E2 = 7 fields). Treatments: ethanol (vehicle) or 10⁻⁸ M E2, 8 nM BZ. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Shown data are representative of at least three independent experiments. Black arrowheads indicate positions of 50-kD markers. White arrowheads indicate positions of 37-kD markers. Bars, 10 µm (unless otherwise indicated).
subcellular shuttling. This treatment should immediately block both membrane events: endocytosis and ATP-dependent vesicle trafficking (Letoha et al., 2003). We found that chilling cells to 0°C completely blocks subcellular shuttling of ERα (Fig. 3g), which is consistent with a static plasma membrane. We confirmed that the effect of low temperatures was reversible because prechilling the cells does not affect ERα shuttling (Fig. S2d).

There are two main mechanisms of integrin endocytosis: clathrin dependent and clathrin independent (Mayor and Pagano, 2007). Among clathrin-independent mechanisms, the
best described is the caveolin-dependent pathway. Given that ERα is known to interact with caveolin 1 and 3 (Schlegel et al., 1999; Chung et al., 2009), we hypothesized that ERα would be endocytosed through a caveolin-dependent mechanism in the presence of E2. To test this, we used filipin, a specific inhibitor of caveolin-mediated endocytosis/membrane recycling, and found that it inhibits E2-stimulated ERα nuclear translocation (Fig. 3b). The inhibition of clathrin-mediated endocytosis with phenylarsine oxide (PAO) does not affect ERα shuttling dynamics (Fig. 3b). We next assessed the effect of filipin treatment on ERα transcriptional activity, performing luciferase reporter assays with a construction that allowed us to measure ERα activity mediated by the ERE. As expected, we found that E2 is not able to induce ERα transcriptional activity in the presence of filipin, supporting the finding that ERα endocytosis has a major effect in gene transcription (Fig. 3i). We verified the specific action of filipin and PAO through the inhibition of their canonical endocytosis substrates (Fig. 4a and b). Together with these results, we found that caveolin 1 colocalizes with EEA1 in the cytoplasm upon 15 min of treatment with E2 (Fig. 4c). We further tested the effect of caveolin 1 knockdown on ERα action and found that it inhibits ERα transcriptional activity, similarly to what we found with its pharmacological inhibitor (Fig. 4d and e). Interestingly, we found that clathrin knockdown also impairs ERα transcriptional activity (Fig. 4d and e). This suggests that clathrin might also play at least a partial role in ERα dynamics. Altogether, these data indicate that E2 induces caveolin-mediated ERα endocytosis in cells seeded on BSA or FN.

Figure 4. ERα is endocytosed through a caveolin 1–dependent pathway. (a) Top: Confocal images of MCF7 cells treated for 15 min as indicated and stained for caveolin 1. Bottom: Merge between caveolin 1 signal and differential interference contrast (DIC) images. Arrows indicate internal or peripheral localization of caveolin 1. (b) Top: Confocal images of MCF7 cells, treated for 15 min as indicated, and stained for clathrin. Bottom: Merge between caveolin 1 signal and DIC images. Arrows indicate internal or peripheral localization of clathrin. (c) Confocal images of MCF7 cells treated for 15 min as indicated and stained for caveolin 1 or EEA1. Arrows indicate regions of colocalization between the two markers. (d) Western blots of MCF7 cells transfected with siRNAs against caveolin 1, clathrin HC, or scrambled for 48 h. Blotting antibodies are shown on the right. Fold change relative to scrambled siRNA is shown on the bottom. (e) Luciferase assay in MCF7 cells transiently transfected with pTK-ERE-Luc and pTK-Renilla and the respective siRNAs and treated for 14 h as indicated. Differences between groups were analyzed by two-way ANOVA followed by Bonferroni contrasts adjusted for multiple comparisons (n = 3 replicates). Data are represented as mean ± SD. **, P < 0.01. Shown data are representative of at least three independent experiments performed. Treatments: ethanol (vehicle) or 10⁻⁸ M E₂, 2.5 µg/ml filipin, 5 µM PAO. Bars, 10 µm.

ERα colocalizes with Rab11 in the presence of FN
Internalized endosomes typically avoid lysosomal degradation if recycled to the plasma membrane (Gould and Lippincott-Schwartz, 2009). Therefore, we explored whether FN promoted the recycling of ERα+ endosomes, therefore inhibiting...
its lysosomal degradation induced by E₂. We found that, when cells are seeded on FN and treated with E₂ for 15 min, there is a larger proportion of ERα⁺ vesicles closer to the basal plane (ventral membrane) than when cells are seeded on BSA as shown in Figs. 5 (a and b) and S2 e. Consistent with these findings, after a longer treatment with E₂, ERα distribution in the cytoplasm is peripheral when cells are seeded on FN compared with a more centered distribution on BSA (Fig. 5 c). These data suggest that in the presence of FN, ERα⁺ vesicles are more likely to be found closer to the plasma membrane than to the lysosomal–perinuclear compartment. 

To further explore whether ERα⁺ vesicles are more likely to be redirected to the plasma membrane in the presence of FN, we costained the cells with the recycling marker Rab11 (Grant and Donaldson, 2009). Rab11 is mostly localized in the perinuclear region and is further transported to the cell periphery to participate in membrane fusion when recycling is active (Cox et al., 2000; Takahashi et al., 2012). We found that the degree of colocalization of ERα with Rab11 is highest when cells are seeded on FN, particularly at the membrane tips, indicating that ERα is more likely to be localized in Rab11⁺ vesicles under these conditions (Fig. 5, d and e). Together with this, overall intensity of
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Rab11 is higher in the presence of FN, suggesting that this pathway is enhanced either by the presence of more Rab11+ vesicles or by an increased size of these vesicles (Fig. 5, d and f).

We further tested the effect of E2 on endocytosis using dextran (10 kDa) conjugated with a red fluorophore. We found that a 15-min treatment with E2 induces strong dextran endocytosis that also co-localizes with EEA1 (Fig. S2, f–h). In addition, we found that dextran co-localizes with Rab11 when cells are seeded on FN, suggesting that it is more frequently localized in Rab11+ vesicles in this condition (Fig. 5 g). Moreover, we measured the amount of dextran present in the supernatant after E2 treatment and found that it is significantly higher when cells are seeded on FN, further suggesting that dextran would be more likely to be recycled in the presence of FN (Fig. S2 i).

**ERα is associated to β1-integrin in estrogen-triggered endosomes**

To gain insight into the possible mechanism responsible for triggering ERα localization in Rab11+ vesicles on cells seeded on FN after E2 treatment, we explored the possibility that upon endocytosis, ERα+ endosomes might contain integrins that, if engaged with FN, would trigger membrane recycling, therefore making the whole complex avoid lysosomal degradation (Caswell et al., 2009; Sung and Weaver, 2011; Dozynkiewicz et al., 2012; De Franceschi et al., 2015). Because we have previously found that FN-induced endocrine resistance is mediated by β1-integrin (Pontiggia et al., 2012), we asked whether this could be the bona fide integrin associated with ERα at the plasma membrane and, therefore, present in E2-induced endosomes. We performed total internal reflection fluorescence microscopy (TIRFM), which allows the detection only of those fluorophores localized on the ventral plasma membrane, at the cell-substrate interface. This assay showed that β1-integrin and ERα co-localize at the ventral membrane in MCF7 cells (Fig. 6, a and b; and Video 1). For β1-integrin detection, live-staining technique was used to intensely detect integrin present in the periphery of the cell, although it does not stain cytoplasmic integrin. Therefore, most of the colocalization structures were found at the periphery of the cell, where further colocalization analysis was run as described previously (Dunn et al., 2011). We also found colocalization structures in T47D cells (Fig. S3 a). As a positive control, colocalization between β1-integrin and its well-known partner FAK was assayed with TIRFM, and a similar colocalization pattern was found (Fig. S3 b).

Consistent with these results, co-immunoprecipitation experiments showed that ERα and β1-integrin immunoprecipitate together (Figs. 6 c and S3, c and d). Moreover, we found that β1-integrin has a sequence of five amino acids (LXXLL) within the cytoplasmic-proximal region of its transmembrane domain that is present among all steroid hormone receptor coactivators such as steroid receptor coactivator 1 (SRC1; Fig. 6 d; Mak et al., 1999). Indeed, this conserved motif called NR-box is known to be sufficient to mediate the interaction of coactivators with nuclear receptors such as ERα. For ERα in particular, this interaction is established within its helix 12 in the AF-2 domain (Heery et al., 1997; Savkur and Burris, 2004). In addition, we found that only β1- and β3-integrins contain this sequence (Fig. 6 d), and remarkably, these two integrins share several extracellular ligands and moreover are known to have transmembrane and cytoplasmic domains that are functionally interchangeable (Solowska et al., 1991). Fig. 6 e shows our proposed model for ERα–β1-integrin interaction. Further analyses needed to confirm ERα–β1-integrin physical association are being conducted at our laboratory.

We further investigated whether β1-integrin followed the same endocytosis/degradation pathway as ERα. We found that as with ERα, 60-min treatment with E2 generates a strong reduction in β1-integrin levels (Figs. 6 f and S3 e). As shown in Fig. 5 f, E-cadherin levels remain unchanged after this treatment, indicating that E2-induced endocytosis and posterior degradation is specific for certain plasma membrane proteins spatially associated with ERα. As expected, when cells were seeded on FN, this ECM protein rescued β1 integrin from E2-induced degradation (Figs. 6 g and S3 f).

Using the antibody feeding technique, we followed β1-integrin internalization dynamics and found that 15-min treatment with E2 stimulates the internalization of β1-integrin (Fig. S3, g and h). This technique allows the detection of β1-integrin+ endosomes in a cleaner manner, making it possible to see a small fraction of them without the background signal from cytoplasmic β1-integrin. Along with this, β1-integrin and ERα colocalize in a proportion of E2-induced endosomes (Fig. 6, h and i). Moreover, β1-integrin shows a strong colocalization with Rab11 after stimulation with E2 in cells plated on FN, indicating that as with ERα, β1-integrin is largely localized in Rab11+ vesicles under these conditions (Fig. 6, j and k). As another control, cells negative for ERα (such as MDA-MB-231) do not exhibit alterations in β1-integrin levels after prolonged treatment with E2 (Fig. S3, i and j), suggesting that E2-induced β1-integrin degradation is in fact mediated by ERα.

**Estrogen treatment stimulates ERα–β1-integrin clustering**

To investigate the interaction between ERα and β1-integrin in higher detail, we performed two-color superresolution microscopy using stochastic optical reconstruction microscopy (STORM; Rust et al., 2006; Bates et al., 2007). Fig. 7 a shows representative STORM images taken in regions of the filopodia of MCF7 cells.

Two-color STORM is a fairly new technique, and therefore there is no consensus yet on the optimal method to quantify correlations between biomolecules. PCC or MCC indices have been used to measure the degree of cooccurrence of the two colors in the same pixel within very small areas of the image where physical colocalization happens (He et al., 2015; Johnson et al., 2016). In principle, the changes in the association between any pair of biomolecules can be estimated by three pairwise quantities: mutual distances between their domains, relative densities, and spatial heterogeneity. We started by analyzing the images using a recently published method (Bermudez-Hernandez et al., 2017) that accounts for two of these quantities: mutual distances and other with higher correlation (>0.4). In fact,
Figure 6. ERα is spatially associated with β1-integrin and they are endocytosed together. (a) Widefield (top) and TIRFM (bottom) images of a coimmunofluorescence in MCF7 cells, using antibodies against β1-integrin (live-stained) and ERα. In the inset, white arrowheads indicate points of colocalization. Pearson’s correlation maps corresponding with the white box shown on the right. White arrowheads indicate points of positive Pearson’s correlation. (b) Quantification of a. Top left: Polar transformation of TIRFM images was performed using Fiji to align areas of the cell periphery where colocalization is found. For each experimental condition, Pearson’s correlation index and Manders’ coefficients (M1 and M2) were calculated within the areas of colocalization (ROI) and compared with random areas without colocalization (Null), using Fiji. For Pearson’s correlation, datasets are plotted and mean ± SD are shown on the graph. For Manders’ coefficients, the table shows mean and SD for each dataset. Differences between groups were analyzed by one-tailed Student’s t test (per replicate: Pearson’s: nnull = 9 fields, nROI = 9 fields; Manders’: nnull = 14 fields, nROI = 9 fields). (c) Western blot of a coimmunoprecipitation in MCF7 cells, using antibodies against β1-integrin or ERα. Blotting antibodies are shown on the right. Input, whole lysate. IP, immunoprecipitated fraction. (d) ClustalW alignment of the eight β-integrins present in humans. The sequence of SRC1 is shown on top. NR-box motif is indicated in red. On the sequences of β1-integrin and β3-integrin, underlined in black is the region corresponding with their transmembrane domain, and in green is their cytoplasmic domain. The topology was predicted using the algorithm TMpred from the website ExPASy and the algorithm from the website TOPCONS. (e) Cartoon showing β1-integrin structure and putative interaction site with ERα. Black box indicates the localization of NR-box motif (LXXLL) within β1-integrin transmembrane domain, and the arrow indicates where its helix 12 would be localized within the AF-2 domain. (f and g) Top: Western blot of total lysates of MCF7 cells, seeded on BSA (f) or FN (g) and treated for 60 min as indicated. Blotting antibodies are shown on the left. Bottom: Densitometry. For each experimental condition, shown is the β1-integrin/β-actin density ratio normalized to the mean control group. Each symbol represents a different experiment. Differences between groups were analyzed by one-tailed paired Student’s t test (n = 3 replicates). (h) Confocal images of MCF7 cells treated for 15 min as indicated and stained for β1-integrin (live stained) and ERα. Arrows indicate points of colocalization. Corresponding Pearson’s correlation maps are shown on the right, respectively. White arrows indicate points of positive Pearson’s correlation. (i) Quantification of h. For each experimental condition, Pearson’s correlation index and Manders’ coefficients (M1 and M2) were calculated within the areas of colocalization (ROI) and compared with random areas without colocalization (Null) using Fiji. For Pearson’s correlation, datasets are plotted and mean ± SD are shown on the graph. For Manders’ coefficients, the table shows mean and SD for each dataset. Differences between groups were analyzed by one-tailed Student’s t test (per replicate: Pearson’s: nnull = 14 fields, nROI = 15 fields; Manders’: nnull = 10 fields, nROI = 11 fields). (j) Confocal images of MCF7 cells seeded on BSA or FN treated with E2 for 15 min and stained for β1-integrin and Rab11. Full images are shown in the insets. (k) Quantification of j. For each experimental condition, Pearson’s correlation index was calculated within the areas of colocalization using Fiji. Data are represented as mean ± SD. Differences between groups were analyzed by one-tailed Student’s t test (per replicate: nBSA = 4 fields, nFN = 4 fields). *, P < 0.05; **, P < 0.01; ***, P < 0.001. Shown data are representative of at least three independent experiments. Black arrowheads indicate positions of 100-kD markers. White arrowheads indicate positions of 50-kD markers. Treatments: ethanol (vehicle) or 10−8 M E2. Bars, 10 µm (unless otherwise indicated).
Figure 7. Effect of E2 treatment on the conditional distribution of ERα versus β1-integrin. (a) Images from STORM of filopodia of MCF7 cells treated as indicated for 15 min and stained for ERα or β1-integrin. Insets in the top left corners show the same images taken with widefield microscopy. Inside the zoomed areas, arrows show regions of superposition of the two markers (yellow pixels). Blue squares outline representative areas of 500 × 500 pixels used for subsequent analyses. In the treated cell, arrow inside the blue square shows a region of dense clustering between ERα and β1-integrin. (b) Tables showing the IF calculated as described previously (Bermudez-Hernandez et al., 2017) for 10 representative frames of filopodia of MCF7 cells under control (top) or treated (bottom) conditions. R-G, red–green correlation; G-R, green–red correlation. Red, β1-integrin; green, ERα. (c) IF calculated for one treated cell (frame 7) and for two sub-ROIs of this frame, showing how this index changes between areas of different β1-integrin/ERα densities. Bars 2 µm. (d) Histogram for normalized frequencies of MD between β1-integrin and ERα in filopodia of MCF7 cells among all the analyzed frames for each condition. For each domain detected, centroids were identified, and MDs were calculated from each β1-integrin to its nearest ERα domain throughout each 500 × 500-pixel frame. Frequencies were normalized to the highest value. The graph shows a slight shift toward smaller MDs for treated cells. (e) Mean density covariance between ERα and β1-integrin.
this method shows a high variability depending on where the region of interest (ROI) is chosen as shown in Fig. 7 c. Therefore, based on this IF, one conclusion would be that there is no difference between control and treated cells, ignoring any change in β1-integrin/ERα clustering that could have occurred in specific domains of the filopodia of these cells. Indeed, this index, similarly to FCCs and MCCs, is biased toward studying the cooccurrence of the two colors in the same pixel, which makes sense for diffraction-limited microscopy but is not enough for the resolution that STORM offers (20–30 nm).

This calls for alternative ways to incorporate the heterogeneities of the distribution of the proteins in this analysis. Therefore, we implemented a numerical estimation of the changes in the three quantities to describe the interaction between ERα and β1-integrin. To facilitate the analysis, these calculations were done using only the centroids of each domain, a simplification further justified by the fact that the sizes of the domains of both proteins were shown to be invariant between the different conditions analyzed (see Fig. S4 e).

Of the three pairwise quantities, the simplest one is the estimation of the minimum distance (MD) calculated over all pairs of ERα and β1-integrin molecules. The first analyses from these datasets reveal that the overall mean MD between ERα and β1-integrin is ~100 nm (Fig. S4 a), clearly below the diffraction limit. This indicates that these molecules are probably part of the same subcellular nanodomain and that their localization is not well resolved by conventional diffraction-limited microscopy. Using this approach, we also investigated whether distribution of ERα and β1-integrin was affected by 15-min treatment with E2. Overall, of all frames, we found a small shift toward smaller MD for treated cells (Fig. 7 d). However, when comparing individual frames, we observed that this shift was in the other direction in three of the 10 treated frames analyzed, suggesting that this overall difference is not significant and is highly dependent on the analyzed frame.

The second pairwise quantity estimates the covariation of densities, which is computed as the number of domains per unit area (for each ERα and β1-integrin). The functional association of these two molecules was investigated by computing their covariation as a function of increasing areas. The densities of the two proteins in fact covary, denoted by a positive linear regression coefficient between their densities as shown on the graph of Fig. S4 b. This covariation is shown to be statistically significant when tested against a null model constructed by randomizing the spatial positions of the centroids. This indicates that ERα and β1-integrin exhibit some degree of spatial cooccurrence and that such localization cannot be simply attributed to a random process (as indicated by the values of the z scores in the inset in Fig. S4 b). With this tool, we further sought to explore whether the density covariation was influenced by E2 treatment. In this case, we found no significant difference between control and treated cells (Fig. 7 e).

Finally, the third analysis considers the spatial spread of both molecules, which seems highly heterogeneous. Specifically, we computed the ratio between the two densities (number of ERα centroids over number of β1-integrin centroids) inside a square ROI of a given size. An example of that heterogeneity is presented (for ROI side length = 50 nm) in Fig. S4 c. The revealed heterogeneity calls for caution when reporting overall means because they might not be representative of changes that are very important in one cell but negligible in others. For that reason, we decided to explore a novel measure with the potential to avoid the limitations. The idea is very simple and uses a Voronoi transformation of the ERα receptor centroid positions (Nicovich et al., 2017). This mathematical transformation identifies a “shell” containing all the points in space that are closer to a given ERα protein than to any other ERα protein. After the transformation, the analysis estimates the size of the shells as well as their β1-integrin contents, i.e., the number, distance, and distribution of β1-integrin centroids inside each shell.

Two examples of the Voronoi transformation (control and treated cells) are presented in Fig. 7 f, where the size of the shells are labeled with colors: centroids of each ERα domain with empty circles and locations of the β1-integrin centroids with full black circles. For each frame, we computed in each shell the average distance (AD) of all β1-integrins to the ERα centroid (notice that this is different from the previous computation that only accounted
ingly, there are a couple of E2-treated cells that showed the oppositely higher bunching index than the control ones. Interestingly, our data showing that ERα and β1-integrin are internalized in human tissues, we analyzed normal human tissues from.

To further explore whether ERα endocytosis takes place within the plasma membrane region of breast tumor cells. We present now the membranes of the treated cells would be the first step preceding caveolin-dependent internalization as has been extensively shown previously (Mayor et al., 1994; Upla et al., 2004; Bacia et al., 2005). Conversely, faster-responding cells might have already internalized most of the endosomes containing ERα–β1-integrin clusters that would therefore not be present anymore in the membrane region analyzed, explaining why some treated cells showed fewer ERα–β1-integrin clusters than control ones.

Moreover, we studied whether size or number of ERα or β1-integrin domains were affected by the treatment and found that there is no significant effect on these variables (Fig. S4, d–f). Interestingly, sizes of these domains are scale free, i.e., they are well represented by a power law distribution, as often happens in many biological systems (Fig. S4 e; Honerkamp-Smith et al., 2009). We also verified that STORM clearly reveals nuclear accumulation of ERα upon E2 treatment as shown in Fig. S5 (a and b).

ERα–β1-integrin complexes are present in tumor and normal human samples

To further explore whether ERα endocytosis takes place within human tissues, we analyzed normal human tissues from reduction mammoplasties and tumor samples from patients with mammary adenocarcinoma. We found that ERα is present in endosome-like bodies in both normal and tumor samples (Fig. 8, a and b). Sizes of the vesicles observed are compatible with early endosomal vesicles (<500 nm), late endosomes (>600 nm), or multivesicular bodies (>1 µm; Luzio et al., 2007; Su et al., 2016). We confirmed these observations using the ERα antibody typically used for clinical analysis (clone SPI) to stain different sections of the same samples used in Fig. 8 a. This antibody reveals ERα endosomes in both normal and tumor tissues (Fig. S5, c and d).

Interestingly, ERα colocalizes with β1-integrin in several areas of the analyzed tumor samples, showing a higher degree of colocalization compared with normal tissues (Fig. 8, a and c). Remarkably, membrane localization of ERα in the tumor samples is higher than in normal tissues; this might account for the increased degree of colocalization with β1-integrin within these samples.

To further explore the clinical relevance of ERα–β1-integrin association, we analyzed TCGA data through cBioPortal (Cerami et al., 2012; Gao et al., 2013) and found that alterations in ERα (ESR1) and β1-integrin (ITGB1) genes present a tendency to be mutually exclusive in breast cancer (Fig. 8 d). As has been intensely studied, alterations that affect the same pathway tend to not co-occur in the same patient (Cancer Genome Atlas Network, 2012; Ciriello et al., 2012). Therefore, mutual exclusivity would be further evidence indicating that ERα and β1-integrin signaling have a close relationship. Moreover, breast cancer patients with genetic alterations in ESR1 or ITGB1 have decreased survival (Fig. 8 e).

These preliminary clinical findings reveal that even though ERα+ vesicles are present in both normal and tumor tissues, ERα and β1-integrin might only be co-endocytosed within tumors. Therefore, FN-induced strengthening of ERα signaling would be a tumor-specific phenomenon, which further suggests this pathway as a target for new antitumor therapies.

Discussion

In this study, we demonstrate by biochemical approaches and high-resolution microscopy that E2 induces endocytosis of ERα by a mechanism involving caveolin 1. In the presence of FN, ERα avoids lysosomal degradation and is localized in Rab11+ recycling endosomes. We found that ERα is functionally associated with β1-integrin at the plasma membrane of breast tumor cells. We show that β1-integrin follows the same endocytosis/degradation dynamics in the presence of E2 and would be responsible for dragging ERα to Rab11+ vesicles in the presence of FN, avoiding lysosomal degradation. In this context, FN has a direct, positive impact on ERα’s transcriptional activity. ERα+ vesicles are present within human breast tissues, and colocalization with β1-integrin is detected primarily in tumors. The mechanism we describe in this study unravels a new level of regulation of cancer cell signaling by the ECM and provides a putative target for new treatments directed to resensitize patients to endocrine therapy.

We found that upon E2 treatment, activated membrane ERα is endocytosed in a caveolin-dependent manner and travels in...
Figure 8. Endosomes containing ERα are present in normal and tumor human breast tissues. (a) Top: Confocal images of a normal human breast tissue (reduction mammoplasty; sample N211) stained for ERα (HC-20 clone), β1-integrin, and DAPI. In the inset, arrows indicate the presence of ERα+ endosomes. Similar results were obtained in the four different specimens analyzed. Bottom: Confocal images of a human breast tumor (Luminal A subtype adenocarcinoma; sample T171) stained for ERα (HC-20 clone), β1-integrin, and DAPI. Yellow arrows indicate ERα+ endosomes. Similar results were obtained in the three different specimens analyzed. (b) Magnification from the inset shown in panel a (top). Diameters of ERα+ vesicles are shown on the right. (c) Table showing mean and SD of Pearson's correlation index calculated for the overall colocalization between ERα and β1-integrin. Differences between groups were analyzed by two-tailed Student's t test (per replicate: nnormal = six fields; ntumor = seven fields). (d) OncoPrint from http://www.cbioportal.org (Cerami et al., 2012; Gao et al., 2013) showing the alterations found in ERα (ESR1) and β1-integrin (ITGB1) genes in different patients obtained from the search in four different datasets: British Columbia, Nature 2014 (Eirew et al., 2015); TCGA, Nature 2012 (Cancer Genome Atlas Network, 2012).
endosomes through the cytoplasm and into the nucleus. In addition, inhibition of clathrin also impairs ERα transcriptional activity, suggesting that the clathrin-dependent pathway might be also, at least partially, involved in ERα endocytosis. The shuttling of plasma membrane proteins into the nucleus through endosomes has been described for other endosomal proteins (Chaumet et al., 2015) and transmembrane receptors such as FGFR (Malecki et al., 2004). A mechanism for E2-induced ERα endocytosis has been proposed for ERα-positive neurons (Kisler et al., 2013). We propose that binding of E2 to ERα would occur at the plasma membrane so that at least a proportion of hormones could exert their action without actually crossing through the membrane. We show in this study that E2-induced endosomes containing ERα can also be targeted to lysosomes, where ERα is degraded. Therefore, we propose that E2-induced lysosomal degradation of ERα is a relevant mechanism of desensitization to E2. However, this mechanism is lost when cells are in a FN-rich matrix, where ERα escapes lysosomal degradation and its transcriptional activity is enhanced. We present evidence indicating that FN promotes ERα localization in Rab11+ vesicles that would therefore inhibit its lysosomal degradation.

As with other membrane proteins such as caveolin 1 and membrane-associated proteins such as integrin-linked kinase (Schlegel et al., 1999; Acconcia et al., 2006; Chung et al., 2009), ERα colocalizes with β1-integrin in plasma membrane structures. Through high-resolution microscopy and coimmunoprecipitation analyses, we show evidence of a close association between these proteins that would be mediated by the NR-box that we identified within the β1-integrin sequence. Ongoing experiments in our laboratory are aiming to confirm the physical association between these two proteins and the domains involved.

Through STORM superresolution microscopy, we showed that ERα and β1-integrin are present in the same nanodomains within the filopodia and, moreover, are organized in structures of size consistent with early endosomes. In addition, STORM revealed that ERα-β1-integrin clustering is incremented upon E2 treatment, further supporting the presented evidence that indicates that ERα-β1-integrin complexes are endocytosed together upon E2 treatment. Membrane β1-integrin could be in both its active (high-affinity) or inactive (low-affinity) conformations, each of which normally undergo different endocytosis/recycling pathways (De Franceschi et al., 2015). Inactive β1-integrins are rapidly recycled to the cell membrane to form protrusions such as lamellipodia and filopodia, following a fast recycling pathway. Active β1-integrins are less efficiently recycled and are targeted to the Rab7 compartment (Arjoner et al., 2012). However, in the presence of FN, ligand-occupied active β1-integrins are rapidly recycled from the lysosomal compartment to the rear of the cell, keeping their active conformation. Interestingly, this process occurs specifically in cancer cells and is a way by which cells remodel their ECM (Dzoyinkiewicz et al., 2012). Therefore, we propose that E2 induces internalization of at least active β1-integrins associated with ERα (Fig. 9). In the absence of FN, active β1-integrin and ERα are directed to the lysosomal compartment, where both proteins are degraded. However, when FN is present, ligand-occupied active β1-integrin would be recycled to the plasma membrane in Rab11+ vesicles, carrying ERα with it and inhibiting its lysosomal degradation (Fig. 9). The role of integrins as masters of endosomal trafficking has been also demonstrated for other receptors and cargos such as VEGFR2 and lipid rafts (Caswell et al., 2009). In this context, an FN-rich matrix represents a double advantage for breast tumor cell survival because it triggers proliferative signals transduced through β1-integrin (Han and Roman, 2006; Moreno-Layseca and Streuli, 2014) and also intensifies E2 signaling. Ongoing experiments in our laboratory are aiming to determine whether E2 affects active and inactive β1-integrins differently.

Several functions have been associated with membrane ERα and are mainly related to nonclassic (extranuclear) ERα signaling pathways (Levin, 2009). However, some authors have begun to suggest that there is a direct link between membrane ERα and its classic nuclear activity (Pedram et al., 2002; La Rosa et al., 2012). Our results provide a link between genomic and nonepigeneic effects of E2 through the activity of membrane-bound ERα. We show evidence suggesting that membrane ERα travels in endosomes into the nucleus, where it would also have transcriptional (genomic) activity. The evidence of the signaling pathway shown in this study is, to our knowledge, the first demonstration of the previously suggested hypothesis of an active mechanism responsible for E2 shuttling into the nucleus (Pietras and Szego, 1984; Razandi et al., 2002). Further analyses are needed to unravel whether membrane ERα dimerizes within these endosomes and at which stage of its subcellular shuttling.

Endocytosis and subsequent degradation of ERα in lysosomes described in this study is a novel pathway of subcellular signaling and negative feedback induced by E2. Totta et al. (2014, 2015) recently proposed that membrane-bound ERα could be degraded in lysosomes as well as in the proteasomal compartment. However, the authors did not demonstrate what membrane events take place in response to E2, leading to ERα internalization and further lysosomal degradation. In this study, we show robust evidence of this phenomenon, describing how this process is regulated and what is the response of the membrane proteins involved. However, it still remains to be elucidated how ERα enters the lysosomal lumen for its degradation. In this sense, it has been proposed that the molecular pump LAMP-2, which has been shown to interact with ERα, would be responsible for allowing its uptake into the lysosomal lumen (Totta et al., 2014; Wang and Robbins, 2014). The signal that triggers ERα lysosomal degradation after prolonged exposure to E2 and whether this mechanism requires ERα’s previous translocation to the nucleus are still unknown.
show that alterations in ERα and β1-integrin genes are mutually exclusive, suggesting that they might be implicated in the same signaling pathway. In addition, alterations in these genes correlate with decreased survival. The findings presented in this study have direct therapeutic implications for breast cancer as blocking FN-dependent activation of ERα, potentially by inhibiting the interaction between ERα and β1-integrin, arises as a novel target for new therapies. This would be a breakthrough approach to overcome endocrine resistance induced by the ECM in breast cancer.

Materials and methods

Cell culture

MCF7 and T47D cell lines were purchased from ATCC and regularly checked for mycoplasma. These cell lines were routinely maintained in DMEM/F12 cell culture medium (Sigma-Aldrich) supplemented with 10% FBS (Internegocios) and gentamicin, in a humidified 5% CO₂/air atmosphere. Serial passages were performed by treatment of 80% confluent monolayers with 0.25% trypsin (Invitrogen) and 0.02% EDTA in Ca²⁺-free and Mg²⁺-free PBS.

Reagents

E₂ was purchased from Sigma-Aldrich; BAF and BSA from Santa Cruz Biotechnology; and FN from EMD Millipore. Filipin and PAO, both from Sigma-Aldrich, were provided by C. Davio (University of Texas, Houston, TX). pTK-renilla was purchased from Thermo Fisher Scientific; LyoVec transfection reagent InvivoGen; phalloidin from Sigma-Aldrich, and DAPI from Research Organics.

DNA constructs and RNA interference sequences

GFP-Rab7 expression construct was a gift from C. Arregui (Universidad de Buenos Aires, Buenos Aires, Argentina). BZ was purchased from Velcade; Lipofectamine 2000 from Thermo Fisher Scientific; Stealth against ERα was purchased from Invitrogen as the following sequences: sense 5’-CAGAGGCCUCUAAACUAAGAAA-3’, and antisense 5’-UUUCUUUAAGUUGACCCUCUG-3’. siRNA against caveolin 1 (sc-29241), siRNA against clathrin–heavy chain (HC; sc-35067), and scrambled siRNA (sc-37007) were purchased from Santa Cruz Biotechnology.

Antibodies

The following antibodies were used in this study and were purchased from Santa Cruz Biotechnology unless otherwise noted (including dilutions/amounts used for immunofluorescence, Western blot [WB], and immunoprecipitation [IP]): ERα (HC-20 rabbit; 1:100 immunofluorescence, 1:200 WB; 3 µg IP), ERα (F-10 mouse; 3 µg IP), β1-integrin (LM534 mouse; 1:100 immunofluorescence; EMD Millipore), β1-integrin (M-106 rabbit; 1:300 WB; 3 µg IP), E-cadherin (H-108 rabbit; 1:1,000 WB), β-actin (C4 mouse; 1:10,000 WB), Rab11 (H-87 rabbit; 1:200 WB), Rab7 (sc-376362 mouse; 1:100 immunofluorescence), and caveolin 1 (sc-53564

The finding that FN strengthens ERα activity suggests that tumor-like stromas would enhance ERα’s activity. FN has been shown to be incremented within cancer cells (Nam et al., 2010; Bae et al., 2013) and is known to contact breast epithelial cells when the normal structure of the basement membrane is disrupted, which occurs during malignant transformation (Ghajar and Bissell, 2008; Lu et al., 2011, 2012). Within tumors, not only ECM composition changes but also its stiffness (Acerbi et al., 2015). Therefore, it is tempting to speculate that not only the presence of FN but also the distorted tissue architecture in stiff substrates (i.e., more cell-substrate contacts, loss of cell apical-basal polarity) might affect ERα degradation in tumors. The loss of normal cellular polarity and subcellular compartmentalization might lead to a different frequency of ERα–β1-integrin interactions in tumor cells. Indeed, we show that although ERα endosomes are present in normal as well as tumor human samples, ERα colocalizes with β1-integrin fundamentally in tumors. These data are consistent with studies showing that high levels of FN and β1-integrin in breast tumors are associated with lower survival (Yao et al., 2007; Helleman et al., 2008). Moreover, clinical data of breast cancer patients from TCGA databases
and 1 mM EGTA; Abcam). The Abcam subcellular fractionation bodies, all purchased from Thermo Fisher Scientific. goat anti–rabbit Alexa Fluor 488–, 555–, and 647–conjugated anti–rabbit Alexa Fluor 488–, 555–, and 647–conjugated antibodies, all purchased from Thermo Fisher Scientific.

Subcellular fractionation
1,000,000 cells were seeded in 60-mm cell culture dishes (Greiner-Bio-One) coated with FN (2 µg/cm² in PBS) in regular culture medium. Culture dishes coated with BSA (2 µg/cm² in PBS) were used as control. After 16 h, cells were washed three times with PBS, and culture medium was changed to phenol red–free DMEM/F12 plus 1% charcoal-stripped FBS and left in the incubator for another 24 h. Cells were then treated with 10⁻⁸ M E2 at 37°C for the indicated times and washed twice with PBS, and protein extracts were prepared by homogenizing fresh cells on ice in subcellular fractionation buffer (250 mM sucrose, 20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, and 1 mM EGTA; Abcam). The Abcam subcellular fractionation protocol was followed. Briefly, cells were centrifuged at 720 g to obtain the nuclear pellet, and the supernatant was recentrifuged at 3,000 g to obtain the cytoplasmic and membrane fraction. After two further ultracentrifugations, cytosolic and membrane fractions were obtained. These fractions were subsequently analyzed by SDS-PAGE followed by Western blotting with the indicated antibodies. Efficiency of nuclear/cytoplasmic separation was verified using nuclear-specific protein H2A.X (Fig. S5 e). E-cadherin was used to verify the efficiency of membrane purification (Fig. S1, g–j).

Western blot
Protein extracts from whole cells were prepared by scraping the culture dishes on ice with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 8.0, containing 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, and 1% NP-40) containing protease inhibitors (40 µM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 50 µg/ml aprotinin, and 200 µM orthovanadate). Protein extracts form subcellular fractions were obtained as described above. Protein concentration was measured using the Bradford method (Bradford, 1976). After adding sample buffer containing β-mercaptoethanol, samples were heated at 95°C for 5 min. 50 µg of each sample was then run in SDS-PAGE mini-gels and transferred to polyvinylidene difluoride membranes (Amersham Biosciences). Membranes were blocked for 1 h at RT in 5% fat-free milk in Tris-buffered saline plus 0.1% Tween-20 (TBST). Primary antibodies were prepared in blocking medium and incubated at 4°C overnight. After washing with TBST, membranes were incubated with secondary antibodies for 1 h at RT. Signal was detected with an enhanced chemiluminescence kit (ECL; Amersham Biosciences). Densitometry was performed using the gel analyzer plugin of Fiji (ImageJ; National Institutes of Health; Schindelin et al., 2012) and the plugin Gels. The standard process with this software was to select the ROI in each lane individually and then plot the intensity measurements. The area under the curve was quantified, which represents the final intensity for each band. The intensities were further analyzed as has been extensively reported previously (Degasperi et al., 2014; McDonough et al., 2015). We then normalized each intensity value to the intensity measured for β-actin in the corresponding lane for each one of the three repetitions of the experiment. The three results for the density ratio of the control group were then averaged, and each ratio was normalized to the control mean, so that the control value will be 1 with its correspondent SD.

Membrane fluidity reduction, endocytosis/membrane recycling blockade, and lysosomal inhibition
For membrane fluidity reduction, a 15-min pretreatment of chilling cells at 0°C followed by a treatment with E2 at 0°C or 37°C was performed. For endocytosis/membrane recycling blockade, filipin (2.5 µg/ml), or PAO (5 µM) were administrated together with E2 for the indicated times. For lysosomal inhibition, a 90-min pretreatment with BAF (25 nM) at 37°C was done. After these treatments, subcellular fractionation and Western blot were performed as described above.

Dextran endocytosis assay
We followed the protocol described previously for substrate endocytosis/recycling (Gillespie et al., 2013). Briefly, cells were seeded at 80% confluence in 24-well plates. After 16 h, cells were washed three times with PBS and serum-starved for 24 h. Cells were then treated with 10⁻⁸ M E2 or its vehicle for 15 min at 37°C. Then 10 µg/ml dextran-CF543 (80111; Biotium) was added, and cells were left at 37°C for another 10 min. Subsequently, cells were washed once with cold serum-free medium and twice with 0.2 M sodium acetate, pH 4.5, and washed again once with cold serum-free medium. For the chase, cells were incubated another 15 min at 37°C with serum-free medium. After the chase, the medium was recovered before washing one more time with 0.2 M sodium acetate, pH 4.5. Fluorescence from acid washes and medium was measured on a FilterMax F3 Multi-Mode Microplate-Reader (Molecular Devices) at 535/595-nm absorption/emission.

IP
The protocol described by Bonifacino et al. (2001) was followed with slight modifications. Briefly, fresh cells were lysed with weak RIPA buffer. 3 µg antibody was preincubated with protein A/G agarose beads (Santa Cruz Biotechnology) with gentle mixing for 1 h at RT. Antibody–bead complexes were then mixed overnight at 4°C with 500 µg protein. After several washes with weak RIPA, samples were analyzed by SDS-PAGE followed by Western blotting with the indicated antibodies.

Luciferase assay
100,000 cells per well were seeded in 48-well culture dishes coated or not coated with FN (2 µg/cm²) in the presence of
DNA constructs pTK-Renilla and pTK-ERE-Luc were used in a 10:1 ratio. After 18 h, cells were washed three times with PBS, and culture medium was changed to phenol red–free DMEM/F12 plus 1% charcoal-stripped FBS and left in the incubator for another 24 h. Cells were then incubated in the presence of $10^{-8}$ M E$_2$ at 37°C for 14 h. Dual-luciferase reporter assay system kit (Promega) was used to reveal luciferase or renilla signals, following the instructions described by the manufacturer.

**Immunofluorescence and confocal microscopy**

50,000 cells were seeded on glass coverslips (Marienfeld) in 24-well plates coated with BSA or FN (2 μg/cm$^2$) in regular culture medium. After 16 h, cells were washed three times with PBS, and culture medium was changed to phenol red–free DMEM/F12 plus 1% charcoal-stripped FBS and left in the incubator for 24 h. Cells were then treated with $10^{-8}$ M E$_2$ for the indicated times. When cells were transfected before this treatment, $3 \times 10^4$ cells were seeded on glass coverslips in 24-well plates covered or uncovered with FN (2 μg/cm$^2$) in regular culture medium. After 16 h, cells were transfected using Lipofectamine 2000, following the protocol described by the manufacturer. Briefly, culture medium was replaced by OptiMEM (Thermo Fisher Scientific), and cells were incubated for 24 h with lipofectamine–DNA (5:1) complexes. Treatment was then administered as described above.

For immunofluorescence staining, the protocol described by Debnath et al. (2003) was followed with slight modifications. In brief, cells were fixed for 20 min at RT with 4% PFA in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min at 4°C, then blocked for 90 min at RT with immunofluorescence buffer (130 mM NaCl, 7 mM Na$_2$HPO$_4$, 3.5 mM NaH$_2$PO$_4$, 7.7 mM NaN$_3$, 0.1% BSA, 0.2% Triton X-100, and 0.05% Tween-20) plus 10% goat serum. They were subsequently stained with the indicated primary antibodies (prepared in blocking medium) overnight at 4°C, followed by incubation with the secondary antibodies for 1 h at RT. For β1-integrin staining, when indicated, cells were live-stained: antibody was used to detect fibronectin (2 μg/cm$^2$) in regular culture medium. After 16 h, cells were seeded on glass coverslips in 24-well plates covered or uncovered with FN (2 μg/cm$^2$) in regular culture medium. After 16 h, cells were transfected using Lipofectamine 2000, following the protocol described by the manufacturer.

**Western blot** to evaluate the loss of the ERα signal. For specific siRNA described above, followed by its detection through Western blot to evaluate the loss of the ERα signal.

**Verification of antibody specificity**

Every antibody used in this paper was chosen based on its wide usage in the literature. In particular, the antibody used to detect ERα has been used in >200 papers (Gao et al., 2015; Arnal et al., 2017). β-integrin antibodies used throughout this paper have also been widely used in the literature (Tiwari et al., 2011; Waxmonskey and Conner, 2013; Long et al., 2016). In the case of the antibody used to detect ERα, the most thoroughly used in the present paper, its specificity was tested for Western blot and immunofluorescence. For immunoneutralization assays, ERα antibody (clone HC-20; Santa Cruz Biotechnology) was preincubated for 90 min at 4°C with HC-20 peptide (Santa Cruz Biotechnology) or its control before using it in the blotting membranes from MCF7 cell lysates or for immunofluorescence of these cells. Considering that each antibody has two HC-20 epitopes and that an excess (at least 10-fold) of the peptide is needed to ensure efficient blocking (Skliiris et al., 2009), the amount of blocking peptide used was calculated as

$$\text{mol HC-20 peptide} = \left[2 \times (\text{mol ERα antibody})\right] \times 10.$$
TIRFM
This technique is based in the formation of an evanescent electromagnetic field generated when the incident light is totally internally reflected at the glass-water interface. The evanescent field decays exponentially from the interface and thus only illuminates fluorophores that are close to the glass. Therefore, it is used to selectively detect molecules localized in the ventral plasma membrane, within a radius of ~100 nm from the glass surface (Ambrose, 1956; Axelrod, 1981, 2001). 300,000 cells were seeded on BSA- or FN-coated (2 µg/cm²) 25-mm coverslips in six-well plates. After 16 h, cells were live-stained for β1-integrin as explained for confocal microscopy, or fixed with 4% PFA/4% sucrose and stained with the antibody against ERα. Coverslips were mounted in PBS on the stage of a fully motorized Nikon TE2000-E inverted microscope equipped for widefield and TIRFM. In all cases, cells were visualized through a TIRF 60×/1.45-NA water objective, at RT, and imaged using an ORCA II ER charge-coupled device (CCD) camera controlled by the MetaMorph software (Molecular Devices). Images were then processed with the software Fiji. For TIRFM, cells were illuminated using a 488-nm argon laser. Evanescent wave penetration depth was calculated to be ~210 nm using the following parameters: 488 nm as λ, 1.52 as n₁, 1.33 as n₂, and 62° as the incident light beam angle. For image processing, when indicated, polar transformation was performed using Fiji and the plugin Polar Transformer (https://imagej.nih.gov/ij/plugins/polar-transformer.html). This transformation takes an input image in a Cartesian space and transforms it into polar coordinates. This transformation is useful for “unwrapping” images with a generally round object. As a consequence of this transformation, if the image has a visible background, the four angles of the original can be often seen as four triangle-like shapes to the right of the transformed image (giving rise to five black arches, which is the color of the underlying background).

STORM Setup
The STORM microscope is custom-built on an Olympus IX-73 inverted microscope. Two continuous-wave lasers of wavelength 642 nm (2RU-VFL-P-1500-642; MPB Communications) and 532 nm (Ventus 532; Laser Quantum) and output power of 1.5 W are used for fluorescence excitation/deactivation (van de Linde et al., 2011), and a 405-nm continuous-wave, 50-mW laser (RGB Photonics Lambda Mini) is used for fluorescence reactivation. The lasers are combined with dichroic mirrors (LM01-552-25 and LM01-427-25; Semrock), magnified, and then focused to the back focal plane of the oil-immersion objective Olympus Plan Apochromat 60× NA 1.42. A multiband dichroic mirror (Di03-R 405/488/532/635-t1 25 × 36; Semrock) is used for decoupling of the fluorescence emission of the sample from the laser excitation. Further blocking of the illumination lasers is performed with a multi-edge notch filter (NF03-405/488/532/635E-25; Semrock). The emission light is further divided into two channels with a longpass dichroic (αt647rcd; Chroma) and expanded with a 2× telescope so that the pixel size of the electron-multiplying CCD camera (Andor iXon3 897) matches an optimal value for single-molecule localization, in this case a pixel size of 133 nm. Both channels are filtered with appropriate emission dichroics for Alexa Fluor 565 and 647 (Semrock 582/75 BrightLine HC and Chroma ET700/75m) and imaged side by side into the same electron-multiplying CCD camera (Andor iXon3 897) by using a D-shaped mirror.

A motorized platform is used to laterally displace the illumination (two mirrors and lens), focusing at the back focal plane of the objective. This allows to switch among conventional widefield, HILO, and TIRF illumination. To prevent defocusing within the relatively long STORM imaging acquisition, the setup is equipped with a custom-built focus stabilization system that senses the total internal reflection of an IR diode laser at the interface between the coverslip and the sample and then actuates by mechanically correcting the objective’s axial position. The camera, lasers, motorized parts of the microscope, and focus stabilization system are controlled and integrated with Tormenta, open-source, free Python software for fluorescence microscopy control and measurements (Barabas et al., 2016).

Data acquisition
Cells cultured on 18-mm coverslips were placed in a holder, and imaging was performed in 50 mM Tris, pH 8, 10 mM NaCl buffer, at RT. The imaging buffer was supplemented with 10% wt/vol glucose, 100 mM 2-mercaptoethanolamine, 1 µg/ml glucose oxidase (Sigma-Aldrich), and 0.5 µg/ml catalase (Sigma-Aldrich) as oxygen scavenging system.

Before STORM imaging, conventional fluorescence images of the ROI were acquired by setting the excitation laser intensity to 1–5 W cm⁻². STORM data acquisition was then started by changing the excitation lasers’ intensity to 5–15 kW cm⁻², thus inducing on-off switching of the fluorescent marker in the tens of milliseconds time range. A camera rate of 20 Hz was found appropriate considering both Alexa Fluor 647 and 565 switching times. Throughout the whole acquisition, the activation 405-nm laser power (1–10 µW cm⁻²) was increased manually in steps whenever the density of single-molecule events decreased below ~1–2 molecules per frame. Typically, 15,000 frames were recorded to assure a high density of localizations.

To correct for chromatic aberrations, the two channels were calibrated using fluorescent nanoparticles emitting in both channels (TetraSpeck microspheres; Thermo Fisher Scientific). An affine matrix was computed as the best transformation that matches the location of the beads in both channels (Hartley and Zisserman, 2004). Only calibrations resulting in an error <10 nm were used. A preprocessing background subtraction step is performed to match the background level of both channels for subsequent analysis. The localization analysis and the rendering of the final superresolved image were performed with ThunderSTORM software (Ovesný et al., 2014).

Data analysis
MatLab software (release 2014a; MathWorks) was used to analyze all the images using codes developed by our laboratory to study distribution of distances, densities, and architecture of the proteins imaged by STORM as described for each corresponding figure.
Human breast tissues
Breast tissues from reduction mammoplasties and tumors were acquired from the Cooperative Human Tissue Network, a program funded by the National Cancer Institute. All specimens were collected with patient consent; reduction mammoplasties were reported negative for proliferative breast disease by board-certified pathologists. Use of anonymous samples was granted exemption status by the University of California, Berkeley, Institutional Review Board in accordance with the Code of Federal Regulations 45 CFR 46.101.

Statistical analysis
All statistical analyses were performed using SPSS (IBM SPSS Statistics for Mac OS X, v.23.0; IBM Corp.) or Prism 5 (v.5.0c for Mac OS X; GraphPad Software). To detect differences between media, we used Student’s t test when comparing between two media. For multiple contrasts, we used two-way ANOVA followed by Bonferroni contrast adjusted for multiple comparisons. Statistical tests and the corresponding contrasts used for each assay are indicated in the figure legends.

Online supplemental material
Fig. S1 shows that FN stabilizes ERα and verifies specificity of the antibodies used. Fig. S2 provides further evidence that ERα is endocytosed in the presence of E2. Fig. S3 confirms that ERα and β1-integrin colocalize at the plasma membrane and are internalized upon treatment with E2 in both MCF7 and T47D cells. Fig. S4 shows the conditional distribution of ERα and β1-integrin and its statistical properties from superresolution microscopy analyses. Fig. S5 shows the nuclear localization of ERα through STORM and provides evidence for ERα⁺ endosomes in human breast tissues using the antibody typically used for clinical analysis (clone SP1). Video 1 provides a 3D render to show that ERα and β1-integrin colocalize in membrane structures in breast tumor cells.

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Author contributions: R.G. Sampayo and M. Simian conceived the project; R.G. Sampayo and M. Simian designed experimental approaches; R.G. Sampayo performed most of the experiments and analyzed all the data; R.G. Sampayo and A.M. Toscani developed TIRFM and confocal microscopy analyses; A.M. Toscani also helped in the experimental design; A. Cáceres provided expertise in high-resolution microscopy and the tools to develop TIRFM experiments; M.G. Rubashkin and J.N. Lakins provided technical support for developing confocal and high-resolution microscopy; K. Thi and W.C. Hines performed immunofluorescence staining and imaging of human samples; W.C. Hines also provided key advice for manuscript preparation; F.C. Leskow provided experimental advice and insightful ideas on data interpretation and contributed to the confocal microscopy analyses; L.A. Masullo, I.L. Violi, and F.D. Stefani provided the necessary equipment to perform STORM, helped to carry out the measurements, and contributed in the elaboration of the superresolution section of this manuscript; D.R. Chialvo provided the numerical tools applied in this study to analyze superresolution microscopy images, performed the analysis with R.G. Sampayo, and contributed in the elaboration of the superresolution section of this manuscript; M.J. Bissell and V.M. Weaver. Weaver provided expertise in ECM biology, conceptual advice, and experimental support; V.M. Weaver provided expertise in biophysics of cell–matrix interaction; R.G. Sampayo and M. Simian wrote the paper. All authors read and extensively critiqued the manuscript.

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Figure S1. FN stabilizes ERα. (a) Confocal images of MCF7 cells seeded on BSA or FN treated with E2 for 15 min and stained for ERα. (b) Quantification of a. For each experimental condition, shown is nuclear ERα intensity (mean gray value) per cell calculated using Fiji relative to the highest intensity recorded. Differences between groups were analyzed by one-tailed Student’s t test (per replicate: BSA: nBSA = 8 cells; nFN = 15 cells). (c and d) Western blots of a subcellular fractionation of T47D cells seeded on BSA and treated for 15 (c) or 60 min (d) as indicated. Blotting antibodies are shown on the left. For each subcellular fraction, shown is the ERα/β-actin density ratio normalized to the control group. (e and f) Western blots of a subcellular fractionation of T47D cells seeded on FN and treated for 15 (e) or 60 min (f) as indicated. Blotting antibodies are shown on the left. For each subcellular fraction, shown is the ERα/β-actin density ratio normalized to the control group. (g and h) Western blots of a subcellular fractionation of MCF7 cells seeded on BSA and treated for 15 (g) or 60 min (h) as indicated. Blotting antibodies are shown on the left. For the membrane fraction, the ERα/E-cadherin density ratio is shown normalized to the control group. For the cytoplasmic fraction, shown is the ERα/β-actin density ratio normalized to the control group. (i and j) Western blots of a subcellular fractionation of MCF7 cells seeded on FN and treated for 15 (i) or 60 min (j) as indicated. Blotting antibodies are shown on the left. For the membrane fraction, the ERα/E-cadherin density ratio is shown normalized to the control group. For the cytoplasmic fraction, shown is the ERα/β-actin density ratio normalized to the control group. (k) Confocal images of T47D cells expressing GFP-Rab7 seeded on BSA (top) or FN (bottom), treated for 15 min as indicated, and stained for ERα. Pearson’s correlation maps are shown on the right. (l) Confocal images of MCF7 cells seeded on BSA or FN, treated for 60 min as indicated, and stained for ERα. Pearson’s correlation maps are shown on the right. (m) Western blots of ERα knockdown by siRNA in MCF7 cells. Blotting antibodies are shown on the left. Data are represented as mean ± SD. ***, P < 0.001. Shown data are representative of at least three independent experiments. Black arrowheads indicate positions of 50-kD markers. White arrowheads indicate positions of 100-kD markers. Treatments: ethanol (vehicle) or 10−8 M E2. Bars, 10 µm (unless otherwise indicated).
**Figure S2.** ERα is endocytosed in the presence of E2. (a) Confocal images of MCF7 cells treated for 15 min as indicated and stained for EEA1 or ERα. Arrows indicate regions of colocalization between the two markers. (b) Confocal images of MCF7 cells treated for 15 min as indicated and stained for Lamin B1 or EEA1. Arrow shows regions of colocalization between the two markers. White rectangles outline the areas whose magnifications are presented in the bottom panels, showing each channel separately and their corresponding merge. Pearson’s correlation maps are shown on the bottom right. Arrows indicate regions of colocalization between the two markers. (c) 3D reconstruction from confocal z stacks of MCF7 cells treated for 15 min with E2 and stained for EEA1 or propidium iodide (PI). Top: Rendered image of a side view of the cell outlined with a yellow rectangle in the inset. Bottom left: Rendered image of the top view of the outlined cell. Bottom right: Rendered image of the bottom view of the outlined cell. Renderizations were done using the plugin 3D viewer of Fiji. The inset shows the full reconstructed field. (d) Left: Outline of the protocol followed and Western blot of a subcellular fractionation of MCF7 cells treated as indicated. Blotting antibodies are shown on the left. Right: Densitometry. For each subcellular fraction, the ERα/β-actin density ratio is shown, normalized to the mean control group. Each symbol represents a different experiment. Differences between groups were analyzed by one-tailed paired Student’s t test (n = 3). (e) Images of confocal microscopy of the nuclear/cytoplasmic (apical) focal plane (z1) of MCF7 cells seeded on BSA or FN treated for 15 min as indicated and stained for ERα. White arrows indicate ERα+ vesicles, determined as punctae of 10–15 pixels in diameter (~200 nm). (f) MCF7 cells were treated with E2 for the indicated times, in the presence of dextran-CF543. Differential interference contrast (DIC) images merged with the red channel (dextran) are shown. (g) Images of confocal microscopy of MCF7 cells treated with E2 for the indicated times in the presence of dextran-CF543 and stained for EEA1. Arrows indicate regions of colocalization between the two fluorophores. (h) Quantification of g. For each experimental condition, the number of dextran+ endosomes per cell is shown after a 15-min treatment. Differences between groups were analyzed by one-tailed Student’s t test (per replicate: n = 9 fields). (i) Dextran recycling assay. MCF7 cells seeded on BSA or FN were pretreated for 15 min with E2 or its vehicle, followed by a 10-min incubation with dextran-CF543, and then were chased for 15 min to measure the amount of dextran-CF543 in the conditioned medium. For each experimental condition, shown is the fluorescence intensity measured for three independent experiments. Data are represented as mean ± SD. Differences between groups were analyzed by one-tailed paired Student’s t test (n = 3 replicates). *, P < 0.05; ***, P < 0.001. Shown data are representative of at least three independent experiments. Black arrowheads indicate positions of 50-kD markers. White arrowheads indicate positions of 37-kD markers. Treatments: ethanol (vehicle) or 10−8 M E2, 10 µg/ml dextran-CF543. Bars, 10 µm.
Figure S3. ERα and β1-integrin colocalize at the plasma membrane and are internalized after treatment with E2. (a) Confocal images of T47D cells stained for ERα and β1-integrin. Pearson’s correlation map is shown on the right. Right: Quantification. For each experimental condition, Pearson’s correlation index was calculated within the areas of colocalization (ROI) and compared with random areas without colocalization (Null), using Fiji. Differences between groups were analyzed by one-tailed Student’s t test (per replicate: n_null = 3 fields, n_ROI = 3 fields). (b) TIRFM images of MCF7 cells stained for pFAK and β1-integrin. Polar transformation performed with Fiji is shown on the right. Right: Quantification. For each experimental condition, Pearson’s correlation index was calculated within the areas of colocalization (ROI) and compared with random areas without colocalization (Null), using Fiji. Datasets are plotted, and mean ± SD are shown on the graph. Differences between groups were analyzed by one-tailed Student’s t test (per replicate: n_null = 4 fields, n_ROI = 4 fields). (c) Western blot of a coimmunoprecipitation assay on MCF7 cells. IP antibodies are shown on the top. Blotting antibodies are shown on the right. Input, whole lysate; IP, immunoprecipitated fraction; Sn, supernatant fraction from the IP. (d) Left: IP experiment following the protocol described by Bonifacino et al. (2001) with slight modifications. Immunoblot: β1-integrin (1981-LM534). The specific band corresponding with β1-integrin upon IP with ERα (F10) antibody, and blotted with β1-integrin LM534 antibody can be seen. As expected, IP with control IgG does not show the specific β1-integrin band. Lanes: IP, ERα antibody; Sn1, supernatant from IP with ERα antibody; IP-IgG, control IgG; Sn2, supernatant from IP with control IgG. Right: Improved IP protocol adding more stringent washing conditions to remove the remaining IP primary antibodies, leading to cleaner IPs. Immunoblot: β1-integrin. This blot is one of the replicates of the original Western blot shown in c. The specific, albeit faint, β1-integrin band can be seen in the IP lane, whereas this band is absent in the lane from control IgG. Lanes: IP, ERα antibody; IP-IgG, control IgG. Arrows indicate the band corresponding with β1-integrin. (e) Top: Western blot of total lysates of T47D cells seeded on BSA and treated for 60 min as indicated. Blotting antibodies are shown on the left. Bottom: Densitometry. For each experimental condition, shown is the β1-integrin/β-actin density ratio normalized to the mean control group. Each symbol represents a different experiment. Differences between groups were analyzed by one-tailed paired Student’s t test (n = 3 replicates). (f) Top: Western blot of total lysates of T47D cells seeded on FN and treated for 60 min as indicated. Blotting antibodies are shown on the left. Bottom: Densitometry. For each experimental condition, shown is the β1-integrin/β-actin density ratio normalized to the mean control group. Each symbol represents a different experiment. Differences between groups were analyzed by one-tailed paired Student’s t test (n = 3 replicates). (g) Widefield images of MCF7 cells live-stained for β1-integrin and then treated for 15 min as indicated to chase β1-integrin internalization dynamics. Arrows indicate β1-integrin vesicles determined as punctae of ∼200-nm diameter (10–15 pixels). (h) Quantification of g. For each experimental condition, shown is the number of β1-integrin vesicles per cell, among cells with these endosomes. Shown data are mean ± SD. Differences between groups were analyzed by one-tailed Student’s t test (per replicate: n = 5 fields). (i) Top: Western blot of total lysates of MDA-MB-231 cells (human mammary adenocarcinoma) seeded on BSA and treated for 60 min as indicated. Blotting antibodies are shown on the left. Bottom: Densitometry. For each experimental condition, shown is the β1-integrin/β-actin density ratio normalized to the control group. Each symbol represents a different experiment. Differences between groups were analyzed by one-tailed paired Student’s t test (n = 3 replicates). (j) Top: Western blot of total lysates of HeLa cells (human cervical cancer) seeded on BSA and treated for 60 min as indicated. Blotting antibodies are shown on the left. Bottom: Densitometry. For each experimental condition, shown is the β1-integrin/β-actin density ratio normalized to the control group. Each symbol represents a different experiment. Differences between groups were analyzed by one-tailed paired Student’s t test (n = 3 replicates). *, P < 0.05. Shown data are representative of at least three independent experiments. Black arrowheads indicate positions of 100-kD markers. White arrowheads indicate positions of 50-kD markers. Treatments: ethanol (vehicle) or 10−8 M E2. Bars, 10 µm (unless otherwise indicated).
Figure S4. Conditional distribution of ERα versus β1-integrin and its statistical properties. (a) Shown is the histogram for normalized frequencies for the MDs between β1-integrin and ERα in filopodia of MCF7 cells among all the analyzed fields from STORM images. For each domain detected, centroids were identified, and MDs were calculated from each β1-integrin to its nearest ERα domain throughout each 500 × 500-pixel frame. Frequency of each distance bin was normalized to the highest frequency detected. The graph shows that the mean MD between ERα and β1-integrin is ~100 nm. (b) Shown is the mean density covariance between ERα and β1-integrin domains. Each frame was divided into square ROIs of different sizes as depicted in c. Within each window, the density of ERα or β1-integrin was computed, and the correlation coefficient (C) between these densities was calculated for each frame analyzed. The mean of C among all the analyzed cells (black full circles) or randomized data generated by mixing β1-integrin images with random ERα images (violet empty triangles) was plotted as a function of the window side length. The inset shows the z score. It was calculated as the difference between the mean of the original dataset for each window minus the mean of the randomized group divided by the square root of the sum of the SD of each group normalized by n. This result shows a significant difference in density covariance between both groups, indicating that ERα and β1-integrin have a positive spatial association and that this organization is not a consequence of a random process. (c) Each 500 × 500-pixel field was divided into squares or windows of different sizes (from 130 nm [10 pixels] to 2,000 nm [150 pixels] in side length) to evaluate ERα and β1-integrin intensities in each window. The figure shows one field divided into 50 × 50-pixel windows and colored as a function of the ratio between ERα and β1-integrin densities, going from 0 to 1 as shown in the color bar on the right. (d) Each color represents a different ERα or β1-integrin domain from the STORM fields of filopodia of MCF7 cells shown on the top right corners. (e) Rank size distribution plot of the domains depicted in d. From the largest sizes on the left and decreasing to the right of the plot, the empty symbols represent averages over 10 frames denoted by crosses. A simple visual inspection already reveals no significant differences between the datasets obtained under vehicle (upper) or treated (bottom) conditions. (f) Box plots representing the total number of domains identified among all the analyzed fields for ERα or β1-integrin in control or E2-treated cells. Central red marks represent the median, and the bottom and top edges of each box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the most extreme data points. This plot shows no significant differences in the number of domains between control and treated cells. Student’s t test (n = 3 replicates). Shown data are representative of at least three independent experiments. Treatments: ethanol (vehicle) or 10⁻⁸ M E2. Bars, 2 µm.
Figure S5. Nuclear distribution of ERα and colocalization with β1-integrin in human breast samples. (a) Images from STORM of the nuclear region of MCF7 cells treated as indicated for 15 min and stained for ERα or β1-integrin. Insets in the top left corners show the same images taken with widefield microscopy. Blue squares outline representative areas of 500 × 500 pixels used for subsequent analysis. (b) Box plot representing the total number of centroids (domains) identified among all the analyzed nuclear fields for ERα in control or E2-treated cells. Central red mark represents the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the most extreme data points. This plot shows that E2 treatment significantly increases the number of ERα nuclear domains compared with control. Student’s t test (n = 3 replicates). (c) Top: Confocal images of a normal human breast tissue (reduction mammoplasty; sample N211) stained for ERα (SP1 clone), β1-integrin, and DAPI. Arrows indicate the presence of ERα+ endosomes. Similar results were obtained in the four different specimens analyzed. Bottom: Confocal images of a human breast tumor (Luminal A subtype adenocarcinoma; sample T171) stained for ERα (SP1 clone), β1-integrin, and DAPI. Arrows indicate the presence of ERα+ endosomes. Similar results were obtained in the three different specimens analyzed. (d) Table showing mean and SD of Pearson’s correlation index calculated for the overall colocalization between ERα and β1-integrin. Differences between groups were analyzed by two-tailed Student’s t test (per replicate: nnormal = 6 fields; ntumor = 6 fields). (e) Western blot of a subcellular fractionation of MCF7 cells, seeded on BSA and treated for 60 min as indicated. Blotting antibodies are shown on the left. **, P < 0.01. Shown data are representative of at least three independent experiments. Black arrowheads indicate positions of 20-kD marker. White arrowheads indicate positions of 50-kD markers. Treatments: ethanol (vehicle) or 10−8 M E2.
Video 1. **ERα and β1-integrin colocalize in membrane structures.** Fiji 3D-reconstruction of confocal images of a cell (MCF7) stained for ERα and β1-integrin. Frame rate: seven frames per second.