Supplemental material

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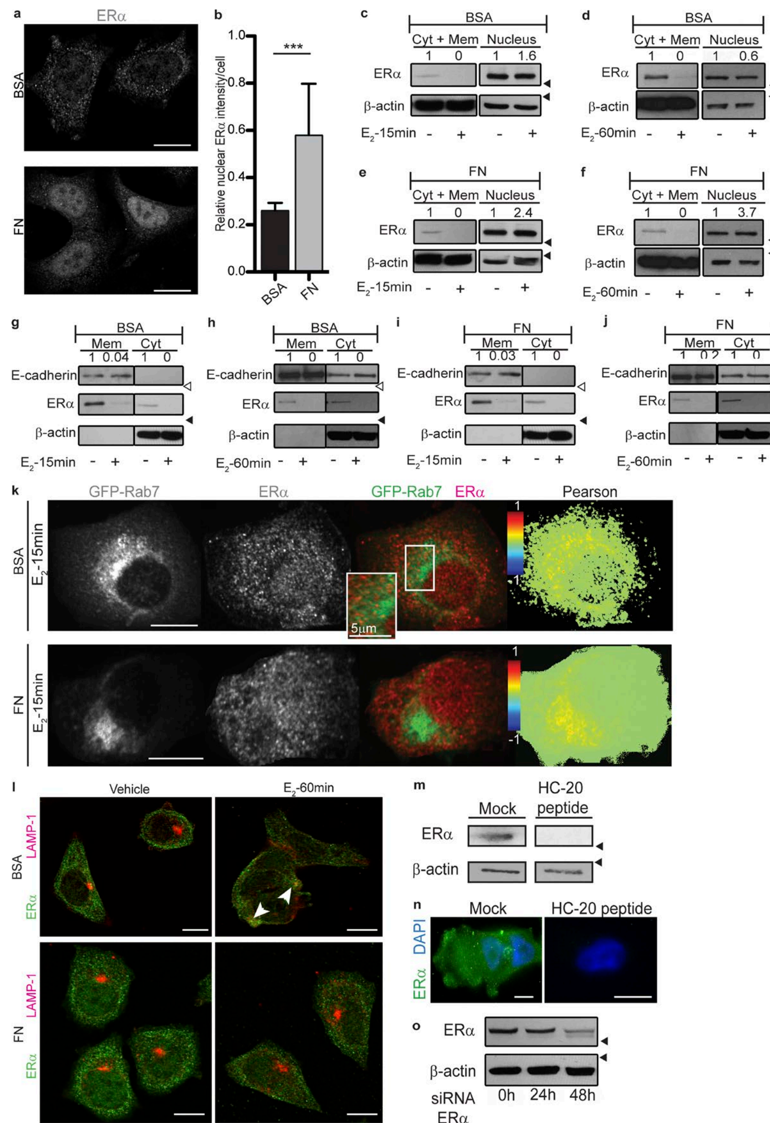


Figure S1. **FN stabilizes ERα. (a)** Confocal images of MCF7 cells seeded on BSA or FN treated with E_2 for 15 min and stained for ERα. **(b)** Quantification of a. For each experimental condition, shown is nuclear ERα intensity (mean gray value) per cell calculated using Fiji relative to the highest intensity recorded. Differences between groups were analyzed by one-tailed Student's *t* test (per replicate: BSA: n_{BSA} = 8 cells; n_{FN} = 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 LAMP-1 or ERα. Arrows indicate regions of colocalization between the two markers. **(m)** Western blots of MCF7 cells blotted with ERα antibody (clone HC-20) preincubated for 90 min at 4°C with HC-20 peptide or its control before using it in the blotting membranes following the standard protocol for Western blot. **(n)** Widefield images of T47D cells stained with ERα antibody preincubated with HC-20 peptide or its control. Counterstaining, DAPI. **(o)** 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 E_2. Bars, 10 µm (unless otherwise indicated).

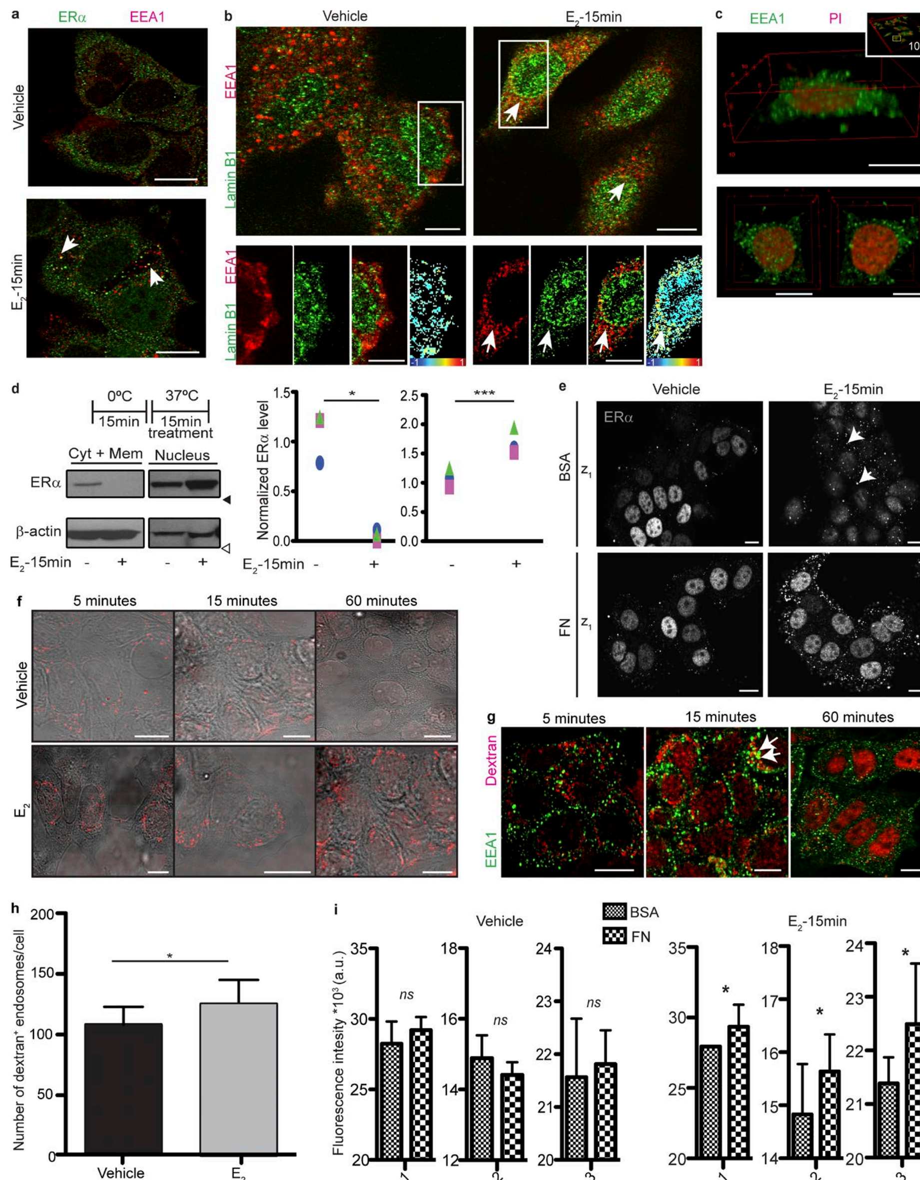


Figure S2. **ERα is endocytosed in the presence of E_2. (a)** Confocal images of MCF7 cells treated for 15 min as indicated and stained for EEA1 or ERα. Arrows indicate regions of colocalization between the two markers. **(b)** Confocal images of MCF7 cells treated for 15 min as indicated and stained for Lamin B1 or EEA1. Arrow indicates 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 E_2 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 E_2 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 E_2 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 E_2 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 E_2, 10 µg/ml dextran-CF543. Bars, 10 µm.

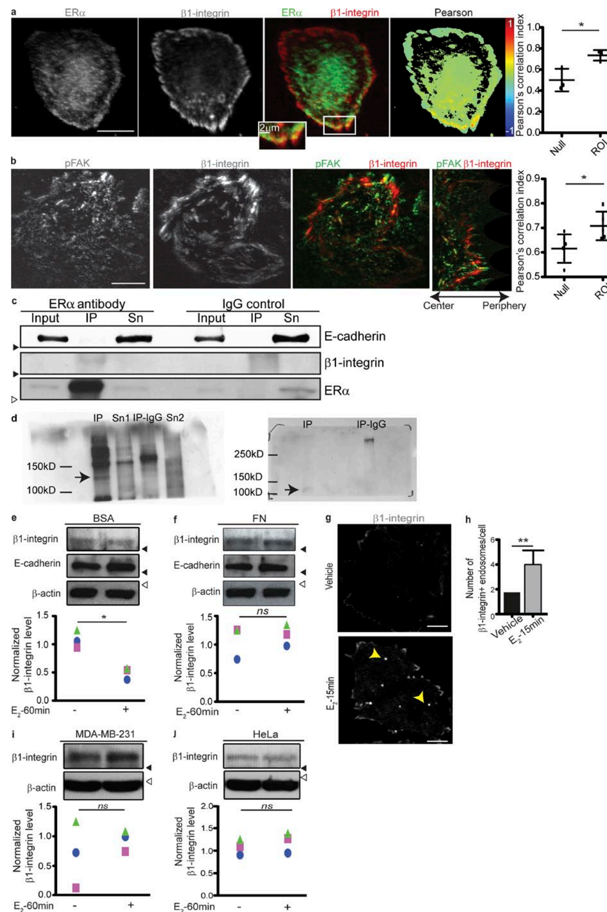


Figure S3. **ERα and β1-integrin colocalize at the plasma membrane and are internalized after treatment with E_2. (a)** Confocal images of T47D cells stained for ERα and β1-integrin. Pearson's correlation map is shown on the right. Right: Quantification. For each experimental condition, Pearson's correlation 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 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). **(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 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). *, $P < 0.05$. 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 E_2. Bars, 10 µm (unless otherwise indicated).

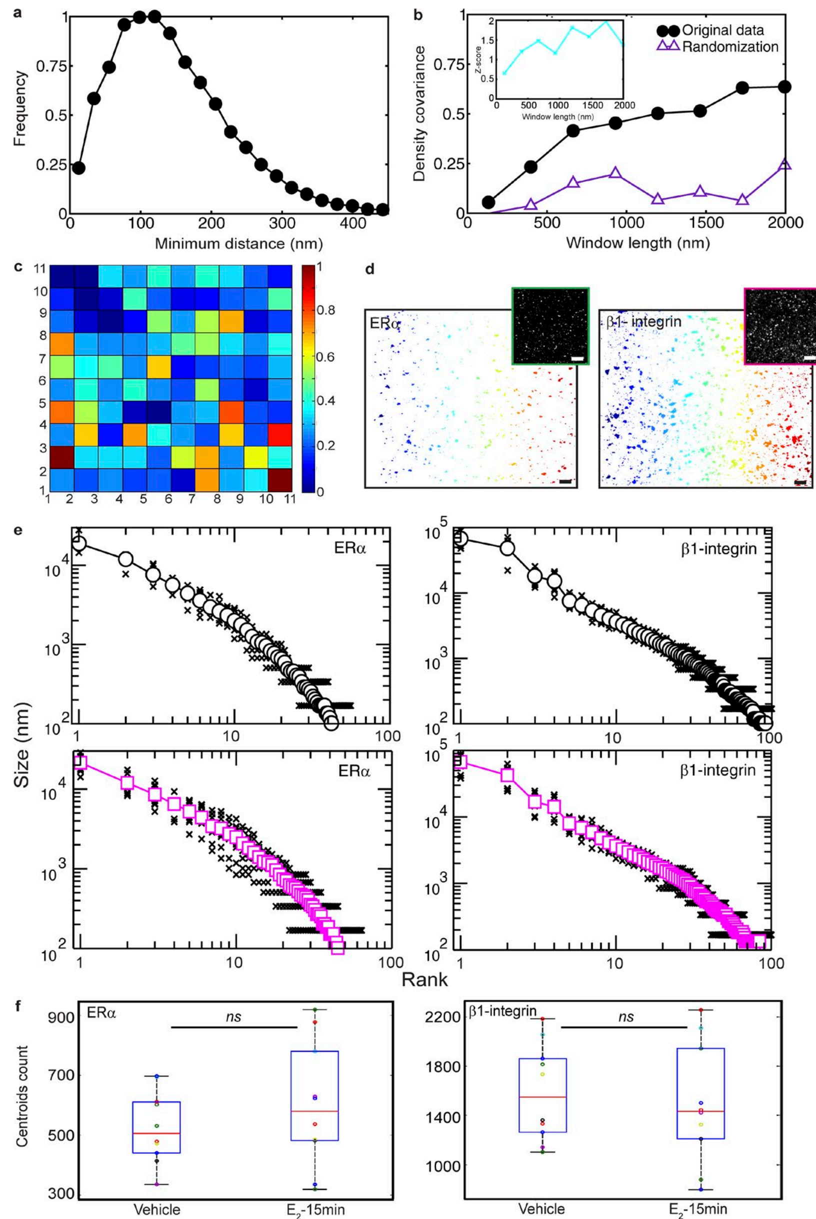
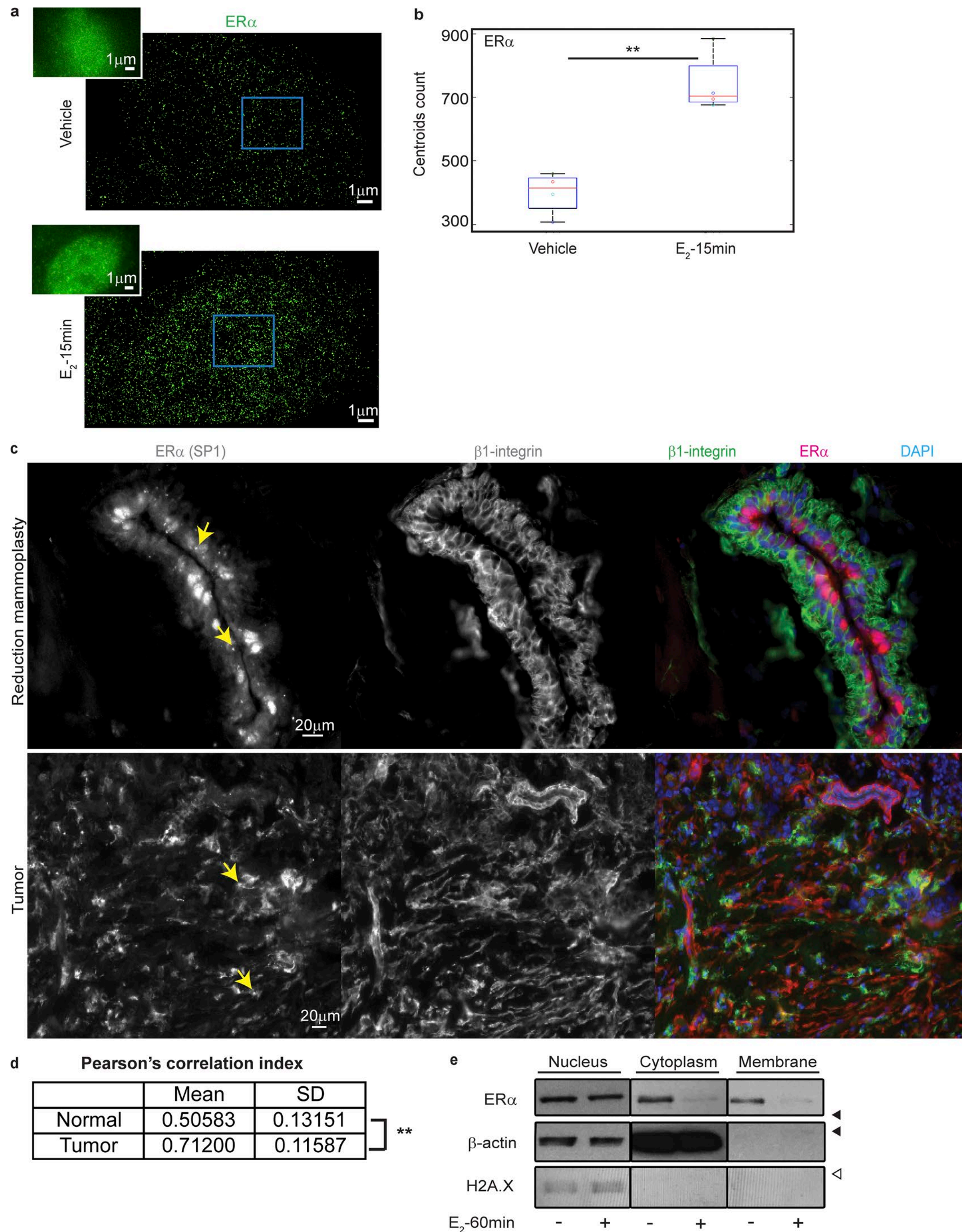
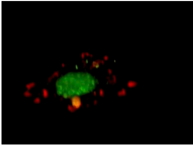


Figure S4. **Conditional distribution of ERα versus β1-integrin and its statistical properties. (a)** Shown is the histogram for normalized frequencies for the MDs between β1-integrin and ERα in filopodia of MCF7 cells among all the analyzed fields from STORM images. For each domain detected, centroids 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 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 E_2-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 E_2. Bars, 2 µm.



	Mean	SD
Normal	0.50583	0.13151
Tumor	0.71200	0.11587

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 E_2-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 E_2 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: n_{normal} = 6 fields; n_{tumor} = 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 50-kD markers. White arrowheads indicate positions of 20-kD marker. Treatments: ethanol (vehicle) or 10^{-8} M E_2.



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.