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Supplemental Information

CRIPTO/GRP78 Signaling Maintains Fetal and Adult Mammary Stem Cells Ex Vivo

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Inventory of Supplemental Information:

4 Supplemental Figures:

Figure S1 is related to Figure 1. It shows additional details on the structure, purification and quality control of ALK4^{L75A}-Fc, the Cripto antagonist used throughout the manuscript.

Figure S2 is also related to Figure 1. This figure shows additional validation of the effects of Cripto and ALK4^{L75A}-Fc on the cellular behavior of the human mammary epithelial cell line (MCF10A) in diverse contexts.

Figure S3 is related to Figure 3. It shows representative fetal mammary stem cell derived colonies under the influence of Cripto pathway agonism or antagonism and a demonstration of these influences in fibroblast co-cultures.

Figure S4 is related to Figure 4 and provides representative flow cytometric and tissue histologic validation of transplant data scored in the main figure, composites images of additional representative colonies derived from GRP78^{high} and GRP78^{low} adult mammary epithelial populations, boundaries used to distinguish them during cell sorting, and alternative graphical representations of their differential transplant and *in vitro* serial passage capacity.

4 Supplemental Movies:

These movies are representative time lapse video microscopy of MCF10A-V (control) and MCF10A-Cr (CRIPTO-overexpressing) cells and illustrate a CRIPTO-dependent increase in cell motility that is blocked by ALK4^{L75A}-Fc.

Movie S1. MCF10A-V; IgG-treated

Movie S2. MCF10A-Cr; IgG-treated

Movie S3. MCF10A-V; ALK4^{L75A}-Fc-treated

Movie S4. MCF10A-Cr; ALK4^{L75A}-Fc-treated

Supplemental methods:

These provide detail on time-lapse microscopy, expression and purification of ALK4^{L75A}-Fc and production of GRP78 antibodies.

Supplemental references:

These references are comprised of those that relate specifically to the Detailed Experimental Procedures and Supplemental Figures.

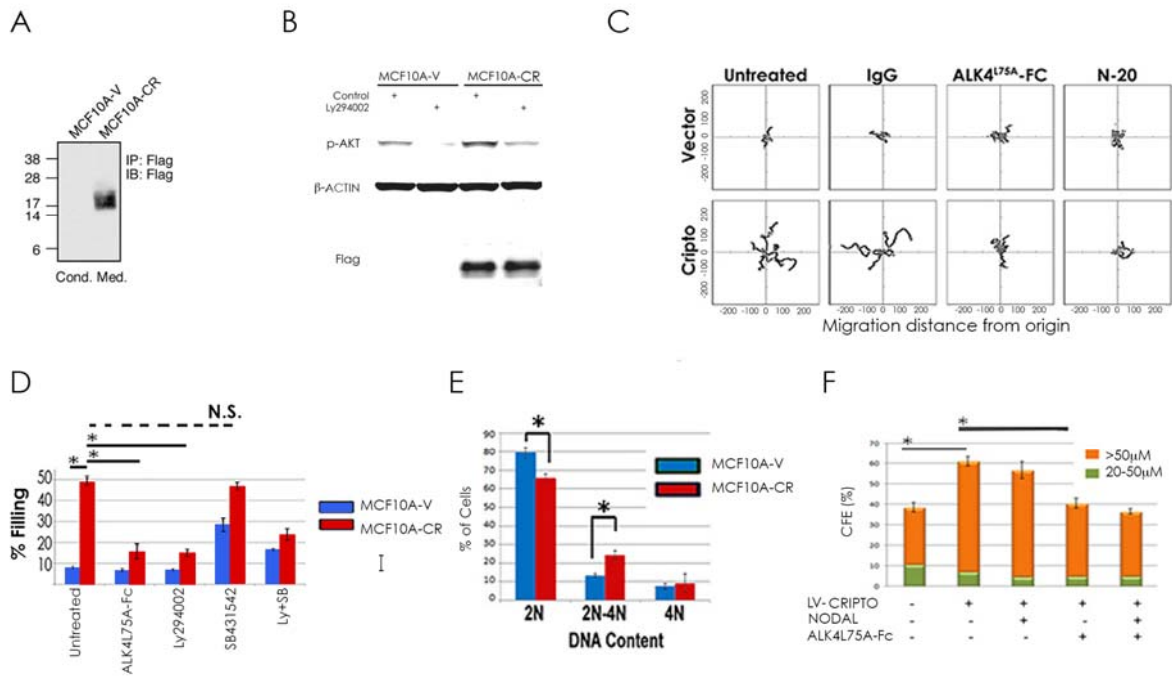


Figure S2, related to Figure 1. Effects of CRIPTO signaling and antagonism by ALK4^{L75A}-Fc in MCF10A Cells. (A) MCF10A-CR cells secrete CRIPTO-Flag. Conditioned media from MCF10A-V and MCF10A-CR cells was subjected to immunoprecipitation and western blotting with Flag antibody. (B) MCF10A-CR cells have higher phospho-AKT levels than MCF10A-V cells. Cell lysates were subjected to western blotting using antibodies directed toward phospho-AKT (p-AKT), actin or Flag as indicated. (C) Representative traces of migratory paths for MCF10A-CR and MCF10A-Vector control cells. MCF10A-CR cells exhibit elevated migration, a phenotype that is reversed by ALK4^{L75A}-Fc or the GRP78 neutralizing antibody N-20. See also Supplemental Movies S1-S4. (D) Suspension growth of MCF10A-V and MCF10A-CR cells was quantified by measuring the area filled by cells in images of individual wells. Cells were treated as indicated and data were normalized to areas measured for untreated MCF10A-V cells. * $p < 0.01$ Student's t-tests. (E) Suspension cultured MCF10A-CR cells have a significantly greater portion of cells with >2N DNA content indicative of greater S-phase fraction and mitotic index. (F) ALK4^{L75A}-Fc inhibited acinar growth of MCF10A-CR cells in Matrigel cultures while NODAL treatment did not affect the number of acini (>50μm) observed in the presence or absence of ALK4^{L75A}-Fc. * $p < 0.05$ t-tests. Results of two independent experiments with two replicates each are shown (D-F).

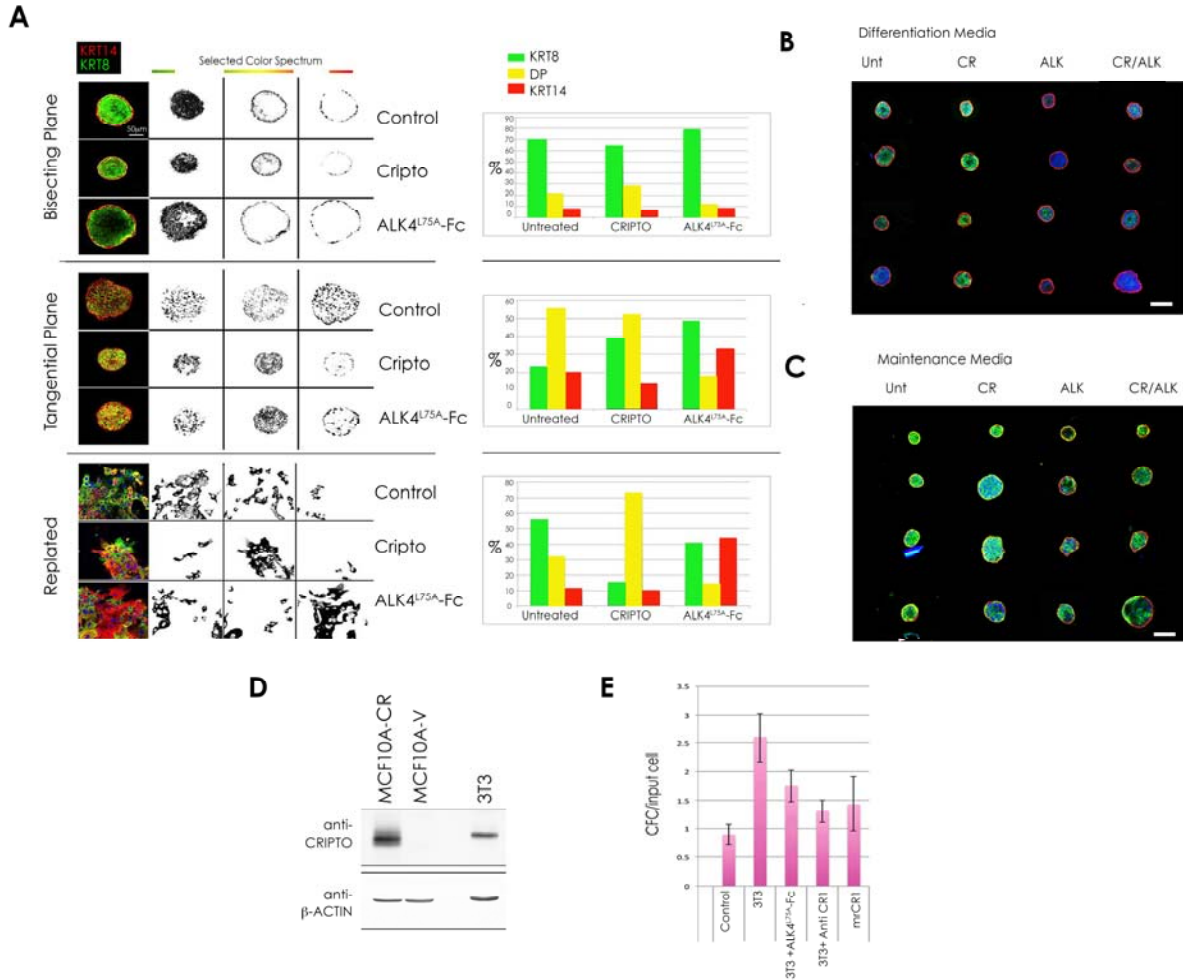


Figure S3, relates to Figure 3. Effects of CRIPTO stimulation or blockade on fMaSC derived organoids. (A) Representative organoids stained for Keratin 8 (green) and Keratin 14 (red) were quantified for distinct and overlapping keratin area (upper two panel sets) or the number of cells bearing distinct keratin expression patterns (bottom panels). Total pixels at the green, red and intermediate color ranges were selected and tallied in Adobe Photoshop for a central cross section (top panels) or peripheral section (middle panels) of day 7 organoids. In addition, nuclei contained within cells with predominantly KRT14, KRT8 or mixed staining cells were tallied following adherence and spread of individual colonies (bottom panels). In each case, ALK4^{L75A}-Fc treated colonies had more keratin single positive content relative to double positive content. Scale Bar = 50 μ m. (B, C) Composite images of additional representative organoids stained for Keratin 8 and Keratin 14 following growth under CRIPTO treatment or ALK4^{L75A}-Fc treatment in differentiation media (B) or maintenance media (C). Scale Bars = 100 μ m. (D) Anti-CRIPTO western blot of 3T3 fibroblast lysates in comparison with control MCF10A-V and MCF10A-CR cells. (E) Quantification of colony forming cell (CFC) numbers relative to epithelial input cell number following co-culture of fMaSC derived organoids with 3T3 fibroblasts. 3T3 co-culture leads to increased clonogenic growth upon subsequent plating in 2 dimensions and this effect is partially reversed by ALK4^{L75A}-Fc or anti-CRIPTO antibodies. Statistical test = pairwise Student's t-test. A representative experiment using technical duplicates is shown.

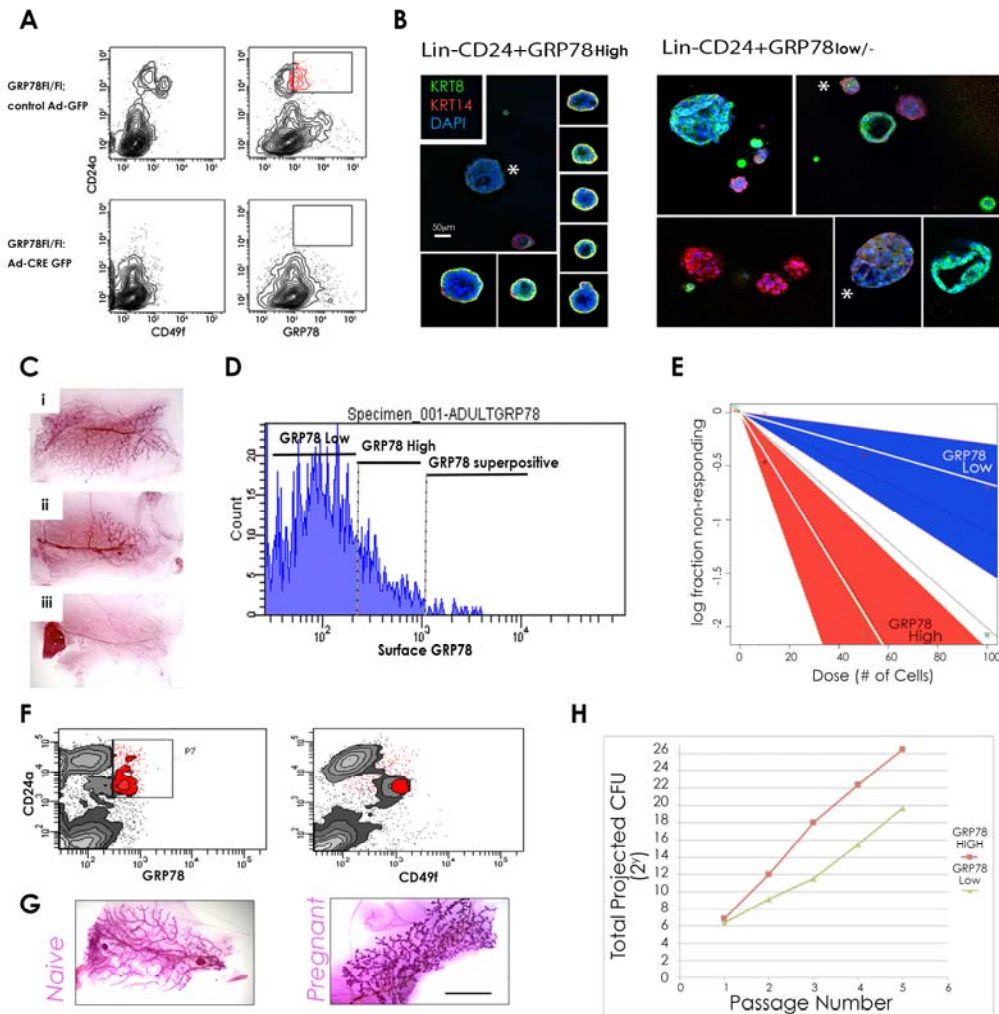


Figure S4, relating to Figure 4. Characterization of adult mammary epithelial cells with differing expression of cell surface GRP78. (A) Flow cytometric analysis of transplanted, conditionally GRP78-ablated mammary epithelial cells (GRP78^{FI/FI}; Ad-CRE, lower panels) and control mammary epithelial cells (GRP78^{FI/FI}; control Ad-GFP; upper panels) following 12 weeks outgrowth. (B) GRP78^{high}-derived colonies and GRP78^{low}-derived colonies collected from 2 independent experiments. KRT 8/14 co-labeling colonies were rare in the GRP78^{low} population while, conversely, colonies lacking KRT 8/14 co-labeling were more rare in the GRP78^{high} population than in the GRP78^{low} population (*). Scale Bar = 50 µm, applies to all panels. (C, i-iii) Carmine stained whole mounts of transplanted mammary glands used to visualize the extent of fat pad filling by mammary ductal epithelia. Glands with 50-100% fatpad filling were scored as outgrowths. (D) Histogram representation of GRP78 positivity in the Lineage+ CD24^{med} fraction of the normal mammary epithelium indicating approximate gating used to isolate GRP78^{Low-Neg.}, GRP78^{high} and GRP78^{high+} super-positive populations. (E) Limiting dilution analysis data for GRP78^{low} cells (white line in blue region), GRP78^{high} cells (white line in red region) and GRP78 super positive cells (black line; GRP78^{high+}). (F) Flow cytometric analysis of secondary transplants resulting from transplanted GRP78^{high} cells. The lineage negative profiles are indistinguishable from normal endogenous mammary gland profiles and include luminal and myoepithelial lineages and a GRP78^{high} fraction. (G) Tertiary outgrowths from serially transplanted GRP78^{high} cells. (H) Absolute cumulative colony forming units (CFU) calculated over serial passages in maintenance media (colonies produced/fraction of original cells plated at each round). Results of one experiment are shown. Subsequent experiments followed the same trend. Scale Bar = 1cm.

Supplemental Movies:

Supplemental Movie S1. MCF10A-V untreated

Supplemental Movie S2. MCF10A-V ALK4^{L75A}-Fc

Supplemental Movie S3. MCF10A-CR untreated

Supplemental Movie S4. MCF10A-CR ALK4^{L75A}-Fc

Supplemental Methods:

Expression and Purification of ALK4^{L75A}-Fc

ALK4^{L75A}-Fc was purified according to previously described methods (Harrison et al., 2004). 293T cells were plated in DMEM + 10% FBS + Ciproflaxin at 4×10^6 cells/15-cm plate coated with poly-D-lysine (up to 50+ plates). ~16 h following plating, cells were transfected with ALK4^{L75A}-Fc DNA in phenol red free DMEM containing 2.5% FBS using 24 μ g DNA/36 μ g PEI transfection reagent per plate. After transfection, cells were incubated ~48-72 hr at 37 degrees to allow protein expression and accumulation. Conditioned media was then collected, filtered through a 0.2 μ m filter and neutralized by addition of 1/10th volume 1 M Tris pH 8. An additional 20 ml fresh phenol red free DMEM + 2.5% FBS was added to each plate and following an additional 48 hr conditioned media was again collected, filtered and neutralized.

ALK4^{L75A}-Fc proteins were purified by sequential protein A and M2-Flag agarose affinity chromatography. 10 cm x 1 cm columns were used with a large volume adaptor at the top for the protein A affinity column and ~3-5 ml affinity resin was used for each column. The protein A agarose bed was equilibrated with at least 10 column volumes protein A IgG binding buffer (21001 Pierce). Filtered, neutralized conditioned media was then gently added to column and allowed to flow through by gravity. The column was then washed extensively with protein A IgG binding buffer (> 10 column volumes) and the protein concentration of the last wash fraction was checked to make sure all unbound protein had been removed from the column. 10 mL elution buffer (0.1 M glycine, pH 2.8) was added to the column and collected into a tube containing 1 ml 1M Tris pH 8.8 to neutralize the eluate. Following elution, the protein A column was washed with an additional 2 column volumes of elution buffer and then the column was re-equilibrated with at least 10 column volumes of protein A IgG binding buffer. The M2-Flag column was equilibrated by washing with 10 column volumes of PBS. The sample to be purified (Protein A eluate) was loaded onto the Flag agarose column and allowed to flow through by

gravity. The flow through material was then loaded onto the column once more and allowed to flow through. The Flag agarose column was washed with 10 column volumes of Flag Wash Buffer (TBS) and then eluted with 10 ml of elution buffer (0.1 M Glycine pH 2.8) into a tube containing 1 ml Tris HCl pH 8.0. Samples were concentrated and buffer exchanged with PBS using an Amicon Ultra centricon with a 30 kDa cutoff (Millipore). Samples were centrifuged 3 times at 2750 rpm for 20 min at 4 degrees C using a Beckman J-6M swinging bucket centrifuge with adaptors for 50 ml tubes. Concentrated samples were typically between 1-5 mg/ml and 2-5 mg of pure protein was generally obtained for each set of 50 plates. After use, columns were stored in binding buffer containing 0.05% azide at 4 degrees.

¹²⁵I-CRIPTO binding

Recombinant mouse CRIPTO (R&D Systems) was iodinated and used for intact cell binding assays as previously described (Kelber et al., 2009).

Production of GRP78 antibody

Antisera were raised against synthetic amino acids 19-39 of human GRP78 or amino acids 81-97 of mouse CRIPTO conjugated to human α -globulins via bisdiazotized benzidine using a protocol previously described in detail for inhibin subunits (Vaughan et al., 1989). Antisera were produced in rabbit using complete Freund's adjuvant (initial injection) or incomplete Freund's adjuvant (booster injections) mixed with an equal volume of phosphate buffered saline containing hGRP(19-39)- or mCR(81-97) α -globulins conjugate. Host animals were immunized every 3 wk with 0.5 mg conjugate (initial injection) or 0.25 mg conjugate (booster injection) delivered in multiple intradermal sites. Rabbits were bled and sera were harvested 7 days after each booster injection. Rabbit #PBL 6197 (GRP78) and #PBL 6900 (CRIPTO) serum was used for these studies.

Time-Lapse Imaging and Migration Analysis

MCF10A-V or MCF10A-CR cells were plated on Matrigel (1:10 dilution) at 1×10^4 cells/well in duplicate in a 24-well plate and allowed to adhere in the presence of the following treatments (untreated, 2 $\mu\text{g}/\text{mL}$ Goat IgG, 10 $\mu\text{g}/\text{mL}$ ALK4^{L75A}-Fc, or 2 $\mu\text{g}/\text{mL}$ N-20) for four hours prior to imaging. Sequential images were captured at 10x magnification and 10-minute intervals for three different fields per well at 37 °C for ~14 hours. Cell migration dynamics were quantified for individual cells using Metamorph Imaging/Tracking software. Velocity (pixels/minute) and persistence (D-displacement/T-track length) values were calculated and averages were plotted and statistics were calculated using a 2-way ANOVA test and Graph Pad Prims. Representative rose plots were generated using cell position data from approximately 10 cells per condition relative to an x,y origin of 0,0 in Excel. Image stacks were exported from Metamorph and compiled into quick time movies.

Proliferation

Cells were plated in triplicate in 96 well plates and quantified at the indicated times on a Celigo microplate cytometer (Cytellect).

Cell Cycle Analysis

Cells were incubated for 15 min at room temperature with 4 $\mu\text{g}/\text{ml}$ Hoechst33342 DNA dye and with 1 $\mu\text{g}/\text{ml}$ propidium iodide (PI). Hoechst+PI- cells were registered on an LSRII flow cytometer (BD) in linear scale with the major population designated as 2N DNA content.

Primary Cell Preparation

Cells were prepared according to the Stem Cell Technologies protocol with modifications

described previously that shorten the dissociation time (90 min) and omit trypsinization for fetal derived material (Spike et al., 2012).

Primary Cell Flow Cytometry

Flow cytometry and cell sorting were carried out as described previously (Spike et al., 2012) incorporating the polyclonal rabbit antibodies targeting GRP78 described above.

In Vitro Colony Formation Assays

For suspension cultures of MCF10A cells, 2-3 replicates of equivalent cell numbers were seeded at a density of 2000-5000 cells/ml in complete CnT-27 media and grown for up to 2 weeks in low adherence tissue culture plates with feeding every 5 days by media addition. Individual wells were photographed and the percentage of the field occupied by cells was quantified. Alternatively, cells were suspended in MCF10A media containing 1% methylcellulose and followed for 4 weeks and spheres were quantified and measured under phase contrast microscopy. For Matrigel cultures, cells were seeded at 5000 cells/ml and grown in 2% growth factor reduced Matrigel (BD Biosciences) in MCF10A media in 96 well low adherence dishes (Corning). For primary mouse mammary epithelia, cells were seeded in 2% Matrigel at the single cell densities described above or were seeded at the time of sorting as single cells per well in 96 well plates. Cells were isolated for serial passage with cell recovery solution (BD Biosciences) and trypsinization according to the manufacturer's recommendations. For confocal microscopy, cells were grown in 75% Matrigel on 8 well glass chamber slides. AKT activation was determined after four days of organoid culture followed by three hours of serum starvation with the indicated treatments. 3T3 co-culture experiments were carried out as previously described (Makarem et al., 2013), with minor modifications. Briefly, 3T3 fibroblasts were arrested with Mitomycin-C (10 μ g/ml) and admixed with fMaSC cells. Cells were subsequently co-cultured in differentiation media with 2% Matrigel for 7 days before being plated at low

density into 2-dimensional culture with maintenance media and grown for 4 days for colony counting. Polyclonal rabbit antibodies directed against amino acids 81-97 of mouse CRIPTO were added to sequester CRIPTO where indicated.

RT-PCR

Relative mRNA expression levels of *TDGF1* (*CRIPTO*) were obtained using the comparative CT method between *TDGF1* directed Taqman assays (Mm03024051_g1, Applied Biosciences) and *HPRT* (Mm01324427_m1, Applied Biosciences). Samples were amplified as technical triplicates and quantified on an ABI 7900HT Fast Real-Time PCR system.

Immunostaining

Cells were fixed with 10% neutral buffered formalin (NBF) and CRIPTO or GRP78 protein was detected with the standard avidin-biotin immunoperoxidase procedures, sodium citrate antigen retrieval methods, and the VECTASTAIN Elite ABC Kit (Vector Laboratories) using rabbit polyclonal anti-CRIPTO (AbCam) 1:1000; goat polyclonal anti-GRP78 (SC Biotechnology) 1:400. Cytokeratin staining was conducted as previously described (Spike et al., 2012) using antibodies against KRT14 (AF-64, Covance, 1:1000), KRT8 (Troma-1, DSHB, 1:100) and DAPI 500 ng/ml. Whole mount immunofluorescent staining was carried out on adult sections as above for IHC and on E18.5 fetal mammary rudiments in suspension using goat antibody N-20 (1:400) to detect GRP78, KRT8 antibodies and DAPI as described above and polyclonal rabbit antibodies directed against amino acids 81-97 of mouse CRIPTO. Macrophages were detected with APC-conjugated anti-F4/80 antibodies (BM8, eBioscience, 1:250). The rudiments were fixed for 1hr in 10% neutral buffered formalin at 4°C, blocked and stained in 4% horse serum in PBS/0.1% Triton X-100 with primary antibodies and subsequently with species specific Alexa Fluor 488, 568 and/or 660 conjugated secondary antibodies, before mounting in Prolong Gold (Life Technologies) and imaging on a Zeiss LSM780 Microscope. The 2-dimensional cultures of

fMaSCs used for Ki67 detection (B56, BD, 1:100) were treated as indicated on day 1 and day 6 and were then fixed on day 7 with 10% NBF and cold Methanol Acetone (3:1) prior to staining.

Mammary Fat Pad Transplantation and Analysis

Mammary transplantation analyses were conducted as previously described (Spike et al., 2012), using ELDA to estimate stem cell frequencies (<http://bioinf.wehi.edu.au/software/elda/>). Here, donor material comprised primary cells grown in 2% Matrigel as described above for 5 days under the indicated treatments. 20 colonies (by dilution) were injected with Matrigel into cleared fat pads of 21 day old SCID hosts (Charles River) and outgrowth was determined at 8-12 weeks by carmine staining as previously described (Figure S4Ci-Ciii) (Spike et al., 2012). Alternatively, donor material comprised primary adult mammary epithelial cells from wild type mice sorted as described previously (Stingl et al., 2006) or sorted on the basis of surface GRP78 expression detected with polyclonal GRP78 antibodies. Additionally, cells from GRP78 floxed mice (a kind gift of Amy Lee, University of Southern California) were sorted as above and transduced by 60 min spinoculation with Adeno-viral vectors expressing GFP and CRE recombinase or GFP alone, grown overnight in maintenance media, sorted for GFP expression and transplanted as described.

Supplemental References:

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