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Supplemental Information

RANK Signaling Amplifies WNT-Responsive Mammary Progenitors through R-SPONDIN1

Purna A. Joshi, Paul D. Waterhouse, Nagarajan Kannan, Swami Narala, Hui Fang, Marco A. Di Grappa, Hartland W. Jackson, Josef M. Penninger, Connie Eaves, and Rama Khokha

Supplemental data

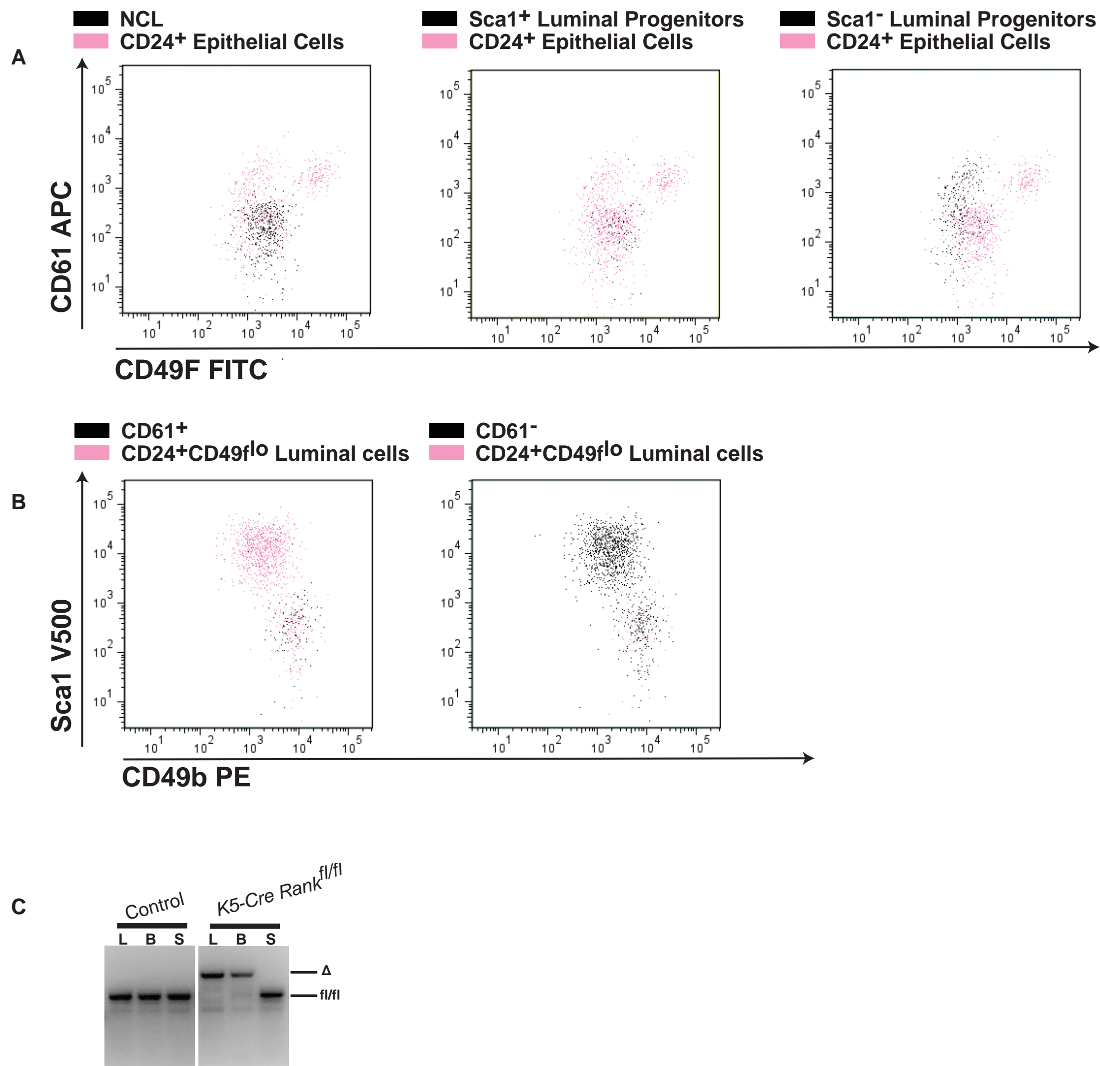

Figure S1, related to Figure 2. Co-staining of mammary epithelial populations for CD61 and Sca1, and *Rank* deletion in mammary cell compartments. FACS plots depicting the expression pattern of **(A)** NCL, Sca1^{+} and Sca1^{-} luminal cells within the CD24^{+}CD49f^{lo}CD61$^{+/-}$ cell compartments and **(B)** CD61^{+} and CD61^{-} luminal cells within the CD24^{+}CD49f^{lo}Sca1$^{+/-}$CD49b$^{+/-}$ fractions. (**C)** PCR on genomic DNA extracted from FACS-purified luminal (L:CD24^{+}CD49f^{lo}), basal (B:CD24^{+}CD49f^{hi}) and stromal (S:CD24^{-}CD49f^{-}) cells from *Rank*$^{fl/fl}$ (control) and K5-Cre *Rank*$^{fl/fl}$ mice to confirm *Rank* deletion; floxed (fl/fl) and deleted (Δ) *Rank* alleles.

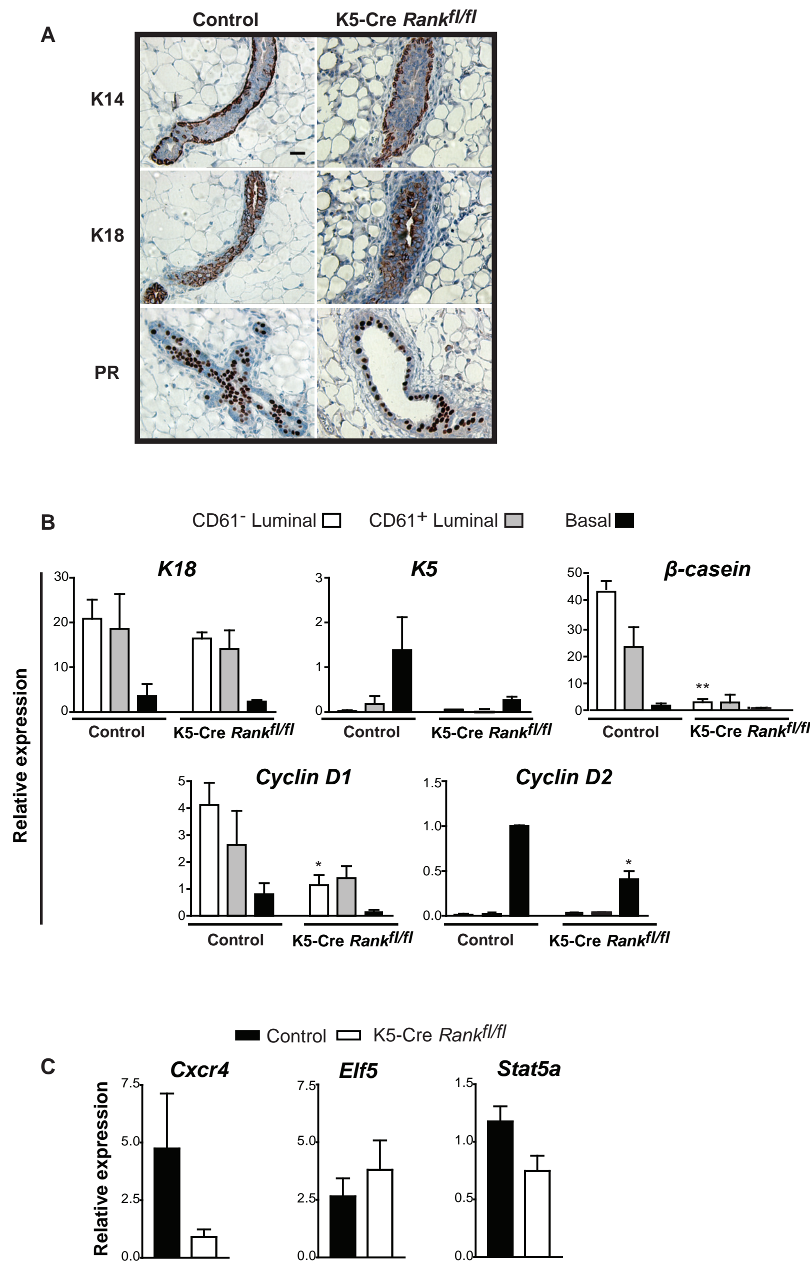

Figure S2, related to Figure 3. Expression of epithelial markers in the *Rank*-null mammary gland. (A) Immunostaining for Keratin 14, 18 and the progesterone receptor isoform A (PR) in paraffin-embedded K5-Cre *Rank*$^{fl/fl}$ and control mammary tissue sections; scale bar=20µm. **(B)** Expression of lineage markers and cyclins in FACS-sorted basal and CD61^{+}, CD61^{-} luminal mammary epithelial subsets from EP-stimulated control and *Rank*-null mice. (**C)** Expression of the indicated genes in purified Sca1^{-} luminal alveolar progenitors from control and *Rank*-null EP-treated mice. β-actin-normalized transcript levels are shown plotted relative to control basal cells in B and Sca1^{-} cells in C. Data represent mean± s.e.m of n=3/cell fraction/group for B and C.

Table S1, related to Figure 2.
Effect of *Rank* deletion on mammary repopulation by alveolar outgrowths.

No. of cells injected/ fat pad	No. of fat pads positive for outgrowths/ total no. of fat pads injected	
	K5-Cre *Rank*$^{fl/fl}$	**Control**
10,000	1/3	3/3
5,000	2/7	7/7
1,000	2/7	7/7
500	1/8	5/8
MRU frequency	**1/10,594**	**1/385**
(95% CI)	**(1/4,517 – 1/24,848)**	**(1/199 – 1/743)**

Single cells isolated from mammary glands of ovariectomized 11-14 week old K5-Cre *Rank*$^{fl/fl}$ and control mice stimulated with 17β-estradiol+progesterone were injected at limiting dilution into cleared inguinal contralateral fat pads of 3 week-old syngeneic recipients which were mated 3 weeks following transplantation. Outgrowths were scored following isolation of the fat pads from mid-pregnant hosts. Outgrowth numbers reported are pooled from at least 2 independent experiments for each group.

Supplemental Experimental Procedures

Deletion analysis

To determine Cre-mediated *Rank* deletion in K5-cre *Rank*$^{fl/fl}$ mice, genomic DNA was extracted from FACS-sorted populations of epithelial and stromal cells. *Rank*^{flox} allele was detected by PCR using primers P88: AGTGTGCCTGGCATGTGCAGACCTT and P105: CTGGTGGTTG TTCTCCTGGTGTCAT, and *Rank*$^{\Delta}$ allele using primers P87:GGCAGAACTCGGATGCAC AGATTGG and P88: AGTGTGCCTGGCATGTGCAGACCTT.

Whole-mount analysis and immunostaining

Murine thoracic mammary glands were analyzed for morphology by carmine-alum whole-mount staining, as described previously (Fata et al., 2001). For immunohistochemistry, 4% paraformaldehyde-fixed paraffin-embedded tissue sections were de-paraffinized in xylene, gradually rehydrated in descending concentrations of ethanol, and subsequently treated in Borg Decloaker antigen retrieval solution (pH 9) for 30 minutes at 121°C and 10 seconds at 90°C using a Decloaking chamber (Biocare Medical). For RANKL, TCF1 and AXIN2 immunostaining, sodium citrate antigen retrieval solution (pH 6) was used. Tissue sections were stained using HRP-AEC tissue staining kits according to manufacturer's instructions (R&D Systems). Antibodies used were anti-mouse against K14 (Covance Cat.#PRB-155P or #SIG-3476, Santa Cruz Cat.#sc-53253), K18 (Cedarlane Cat.#10R-C161A), Ki67 (Thermo scientific Cat.#RM-9106-S0), PR (Thermo Scientific Cat.#MA5-12658 or Santa Cruz Cat.# sc-7208), SMA (Sigma Cat.#A2547), RANKL (R&D Cat.#AF462), TCF1 (Cell Signaling Technology Cat.#2203S), AXIN2 (Abcam Cat.#ab32197) and beta Galactosidase (Abcam Cat.#ab9361). Ki67 positive cells were quantified by counting ~500 cells per mouse mammary tissue section. For immunofluorescence, paraffin-embedded tissue sections were stained with primary antibodies followed by anti-mouse, anti-rabbit or anti-chicken secondary antibodies conjugated to Alexafluor 488, Alexafluor 647 (Life Technologies Cat.#A11008, A21235) or Cy3-Affinipure (Jackson Immunoresearch Cat.#103-165-155), and mounted using ProLong Gold Anti-fade reagent with DAPI. Human paraffin-embedded breast tissue sections were processed similarly although treated with the antigen retrieval solution (pH 9) for only 30 seconds at 125°C, and subsequently stained with antibodies against Ki67 and RANKL (kindly provided by Amgen). RANKL, TCF1 in mouse mammary gland, and RANKL, Ki67 in human breast were quantified by measuring the ratio of positive pixels to total positive+negative pixels within glandular regions using Aperio Imagescope software (Aperio technologies Inc.).

Mammary cell preparation and flow cytometry

Mouse mammary glands were digested for 2.5 hours at 37°C in DMEM: F12 (3:1) with 750Uml^{-1} collagenase and 250Uml^{-1} hyaluronidase as described previously (Joshi et al., 2010). Organoids obtained after vortexing were subjected to red blood cell lysis in NH_4Cl, further dissociation in 0.25% trypsin for 2 min, 5 mgml^{-1} dispase with 0.1 mgml^{-1} DNaseI for 2 min, and filtered through a 40 μm mesh to obtain single cells. All reagents were from STEMCELL Technologies. Cells were incubated with biotinylated mouse/human chimera cocktail containing anti-CD45^{+}/Ter119^{+} (STEMCELL Technologies) and anti-CD31 (BD Biosciences Cat.#553371) to label hematopoietic and endothelial cells respectively which were excluded by flow cytometry following secondary conjugation with streptavidin-PE-Cy7 (BD Biosciences Cat.#557598). Alternatively, CD45, Ter119, CD31 antibodies directly conjugated to PE-Cy7 (eBioscience Cat.#25-0451-82, 25-5921-81, 25-0311-81) were used. Dead cells were excluded with propidium iodide staining (PI, Sigma). To identify and isolate mammary epithelial cell populations, the following antibodies were used: anti-CD49f-FITC (clone GoH3; BD Biosciences Cat.#555735), anti-CD24-PE (clone M1/69; BD Biosciences Cat.#553262) or anti-CD24-APC-eFluor 780 (clone M1/69; eBioscience Cat.#47-0242-80), anti-CD61-APC (clone HMbeta3-1; Invitrogen Cat.#MCD6105), anti-Sca-1-APC-cy7 (clone D7; Biolegend Cat.#108126) or anti-Sca-1-V500 (clone D7; BD Biosciences Cat.#561228) and anti-CD49b-Alexafluor 647 (HMa2; Biolegend Cat.#103511) or anti-CD49b-PE (HMa2; Biolegend Cat.#103506).

***In vivo* limiting dilution assays**

Total mammary cells were generated as single cell suspensions from EP-stimulated K5-Cre *Rank*$^{fl/fl}$ and *Rank*$^{fl/fl}$ control mice. Cells were resuspended in a 10 μL volume containing 50% Matrigel and 50% Hanks' balanced salt solution plus 2% FBS and 0.02% Trypan blue (Sigma). Viable cells (10,000, 5,000, 1,000 and 500) were injected into cleared mammary fat pads of pre-pubescent, 21 day old syngeneic recipient females. Cells derived from K5-Cre *Rank*$^{fl/fl}$ mice and controls were transplanted into the two contra-lateral 4th inguinal glands of the same recipient mouse to allow cells from knockout and control animals to be exposed to identical hormonal milieu. After 3 weeks, mice were mated and mammary glands dissected at mid-pregnancy. Glands were then carmine alum-stained for whole mount analysis. Fat pads were scored as positive or negative for ductal and alveolar outgrowths and the frequency of mammary repopulating units (MRUs) in the cell suspensions thus assayed was calculated using Poisson statistics and the method of maximum likelihood using L-Calc software (Stem Cell Technologies). An estimate of the MRU frequency range was obtained using one-sided 95% Clopper Pearson Limits, and

goodness of fit to a single-hit Poisson model was tested using Chi-squared statistics as described (Stingl et al., 2006)..

CFC assays

FACS-sorted mammary epithelial cells from individual mice were plated with irradiated fibroblasts in DMEM:F12 (3:1) medium containing 10% FBS, insulin (Life Technologies), cholera toxin (Sigma), adenine (Sigma), hydrocortisone (STEMCELL Technologies), and Rock inhibitor (Reagents Direct)(Makarem et al., 2013). Basal and luminal cells were cultured in 5% oxygen conditions. Colonies were scored after 7-10 days. Human mammary CFC assays were performed in dishes precoated with 1.6% Matrigel (BD Biosciences) in SF7 medium with 5% fetal bovine serum (STEMCELL Technologies), 20 μM ROCK inhibitor (Reagents Direct), and irradiated feeders as previously documented (Kannan et al., 2013).

RNA isolation and real-time PCR analysis

Total RNA was prepared from FACS-sorted primary mammary cell subpopulations using the PicoPure RNA Isolation Kit (Arcturus). The quality and concentration of RNA was determined by visualizing purified RNA samples on SyBr Green II (Invitrogen) stained formaldehyde agarose gels and by analysis with a NanoDrop 2000 Spectrometer (260/280 ratio; Thermo Scientific). Isolated and purified total RNA was reverse transcribed into first strand cDNA and amplified using the SMARTer PCR cDNA Synthesis Kit and Advantage2 PCR Kit (Clontech) as previously reported(Joshi et al., 2010). Relative quantification Real-time PCR ($\Delta\Delta$Ct) was performed on 2 ng of cDNA generated from sorted primary mammary cells using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). TaqMan gene expression assay mix containing unlabelled PCR primers and FAM-labelled TaqMan MGB probes were used to detect expression of specific genes as listed by catalogue number in the table below. All raw data were analyzed using Sequence Detection System software Version 2.1 (Applied Biosystems). The threshold cycle (C_T) values were used to calculate relative RNA expression levels. Transcript levels were normalized to endogenous β-actin transcripts and compared to the control basal or luminal population (Relative Expression = 1).

Applied Biosystems Gene Expression Assay catalogue numbers and sequences:

Gene	**Catalogue number**
Keratin 18	Mm01601706_g1
Keratin 5	Mm00503549_m1

Keratin 14	Mm00516876_m1
PR	
Probe	5’ - CACGCCATAGTGACAGCCAGATGCTT - 3’
Forward	5’ - CACAGTATGGCTTTGATTCCTTACCTC - 3’
Reverse	5’ - TGCCCTCTTAAAGAAGACCTTGC - 3’
Rankl	Mm01313944_g1
Rank	Mm01286484_m1
Wnt4	Mm01194003_m1
Lrp5	Mm01227476_m1
Lrp6	Mm00999795_m1
Tcf1	Mm01293215_m1
Axin2	Mm01265783_m1
Rspo1	Mm00507077_m1
β-casein	Mm04207885_m1
Cyclin D1	Rn00596851_g1
Cyclin D2	Mm03053712_s1
Cxcr4	Mm01996749_s1
Elf5	Mm00468732_m1
Stat5a	Mm03053818_s1
β-actin (ACTB)	Mm01205647_g1

For qRT-PCR measurements in human mammary epithelial cells, we isolated total RNA from FACS-purified cells using Trizol (Invitrogen) according to the manufacturer’s protocol. We then converted the RNA into cDNA using SuperScript III RT (Invitrogen) with random hexamer primers.

Sequence-specific primers designed using qPrimerDepot:

Gene	**Primer Sequence**
PR-F	CGATGCAGTCATTTCTTCCA
PR-R	AATCTGTGGGGATGAAGCAT
RANK-F	GATGATGTCGCCCTTGAAGT
RANK-R	GGAGGCTCCCAAGCTCAG

RANKL-F	ATGTGCTGTGATCCAACGAT
RANKL-R	TGAGACTCCATGAAAATGCAGA
Wnt4-F	AGTTTCTCGCACGTCTCCTC
Wnt4-R	CTCGTCTTCGCCGTCTTCT
TCF1-F	GACTTGACCATCTTCGCCAC
TCF1-R	CCTCAAAGAGCTGGAGAACCT
GAPDH-F	CCCATCACCATCTTCCAGGAG
GAPDH-R	CTTCTCCATGGTGGTGAAGACG

Supplemental References

De Gasperi, R., Rocher, A.B., Sosa, M.A., Wearne, S.L., Perez, G.M., Friedrich, V.L., Jr., Hof, P.R., and Elder, G.A. (2008). The IRG mouse: a two-color fluorescent reporter for assessing Cre-mediated recombination and imaging complex cellular relationships in situ. Genesis *46*, 308-317.

Fata, J.E., Chaudhary, V., and Khokha, R. (2001). Cellular turnover in the mammary gland is correlated with systemic levels of progesterone and not 17beta-estradiol during the estrous cycle. Biology of reproduction *65*, 680-688.

Joshi, P.A., Jackson, H.W., Beristain, A.G., Di Grappa, M.A., Mote, P.A., Clarke, C.L., Stingl, J., Waterhouse, P.D., and Khokha, R. (2010). Progesterone induces adult mammary stem cell expansion. Nature *465*, 803-807.

Kannan, N., Huda, N., Tu, L., Droumeva, R., Aubert, G., Chavez, E., Brinkman, R.R., Lansdorp, P., Emerman, J., Abe, S., *et al.* (2013). The luminal progenitor compartment of the normal human mammary gland constitutes a unique site of telomere dysfunction. Stem cell reports *1*, 28-37.

Makarem, M., Kannan, N., Nguyen, L.V., Knapp, D.J., Balani, S., Prater, M.D., Stingl, J., Raouf, A., Nemirovsky, O., Eirew, P., *et al.* (2013). Developmental changes in the in vitro activated regenerative activity of primitive mammary epithelial cells. PLoS biology *11*, e1001630.

Stingl, J., Eirew, P., Ricketson, I., Shackleton, M., Vaillant, F., Choi, D., Li, H.I., and Eaves, C.J. (2006). Purification and unique properties of mammary epithelial stem cells. Nature *439*, 993-997.