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Supplemental Information

RSK2 Maintains Adult Estrogen Homeostasis by Inhibiting ERK1/2-Mediated Degradation of Estrogen Receptor Alpha

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Supplemental Information

Ludwik&Sandusky_Supp_Figure_1

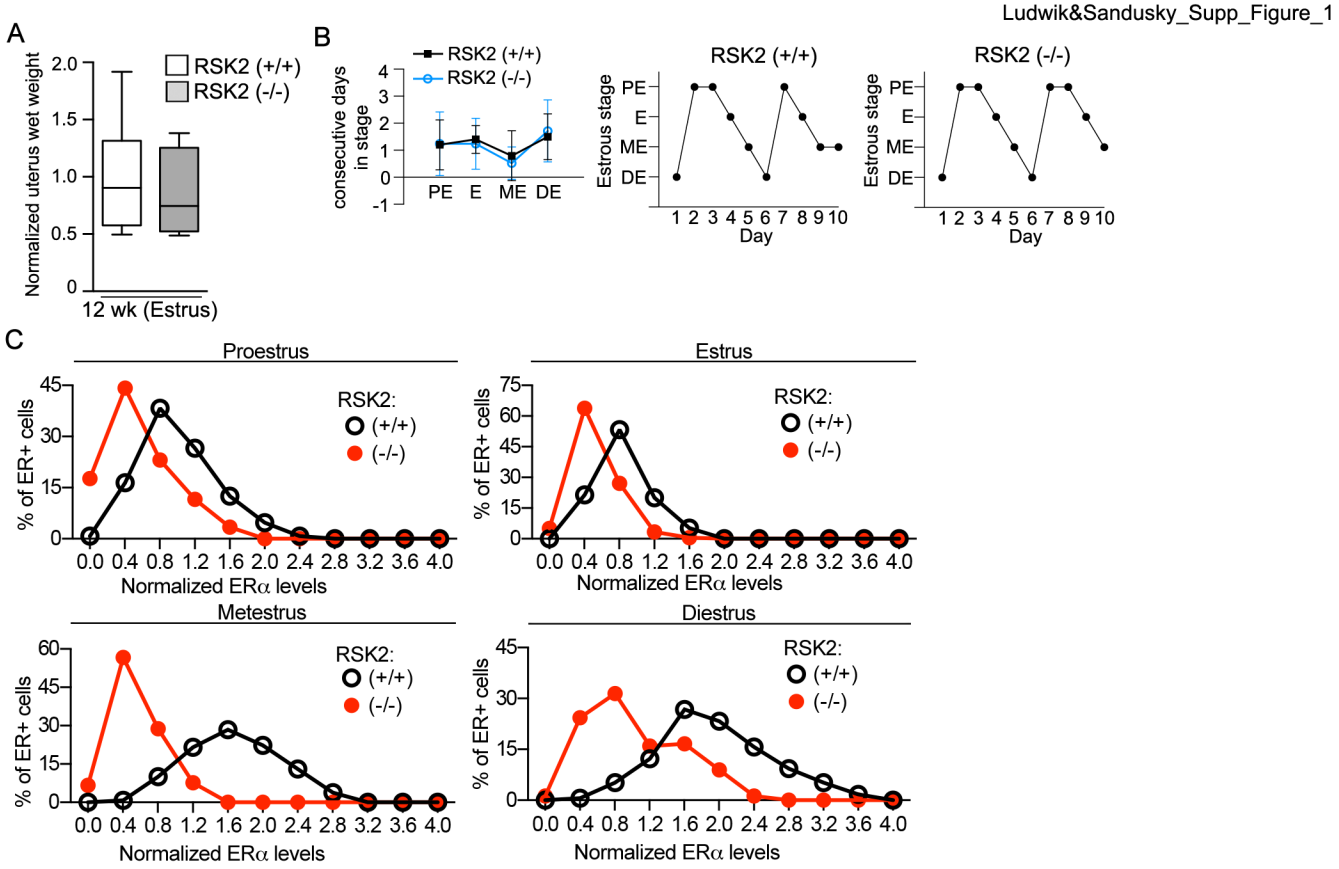
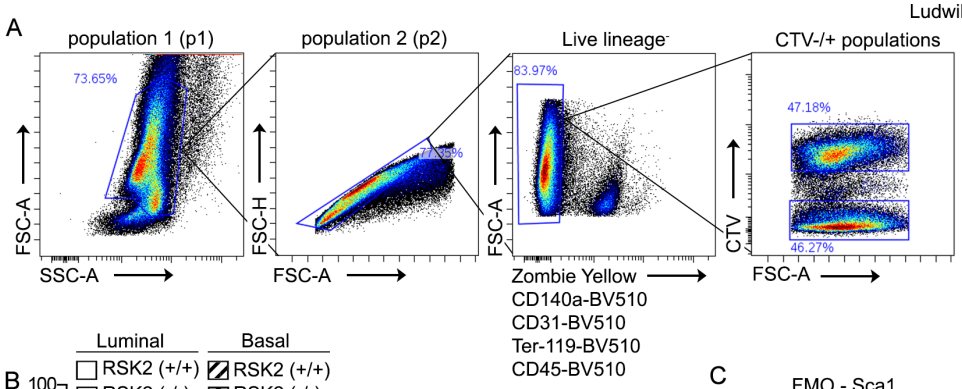
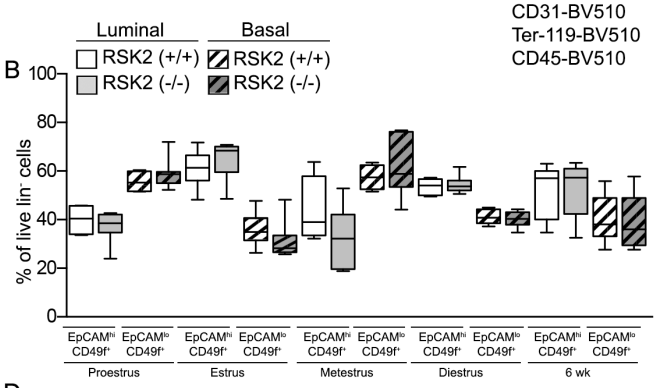


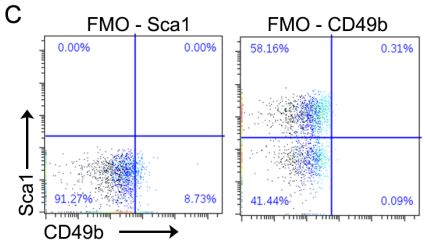
Figure 1 Estrogen responsiveness in WT and RSK2-KO mice.

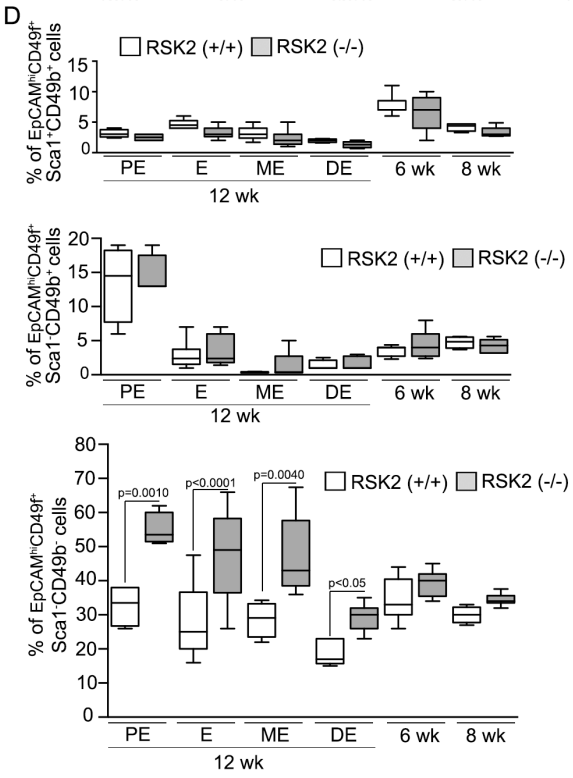
(A) Uterine wet weight is similar in WT and RSK2-KO. (median ± quartile, n≥8 mice/genotype, Student's t-test).
(B) Cycling through the estrous cycle is similar in WT and RSK2-KO mice. Left graph: (mean ± S.D., n≥10 mice/genotype); Right graph: Representative cycle.
(C) ERα protein expression levels are reduced in RSK2-KO at all stages of the estrous cycle in adult mammary glands. The graphs were generated from data shown in Figs. 1B and 1C.

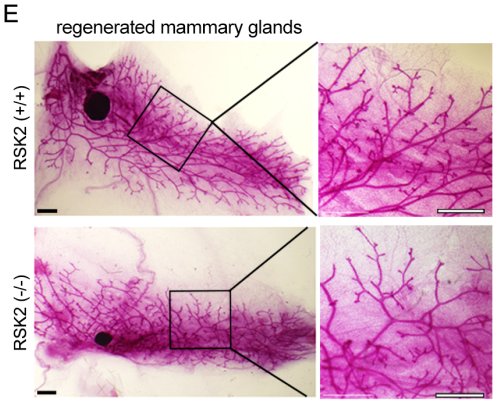
Related to Figures 1, 2 and 5


A
population 1 (p1)
population 2 (p2)
Live lineage-
CTV-/+ populations
73.65%
83.97%
47.18%
46.27%
FSC-A
SSC-A
FSC-H
FSC-A
FSC-A
Zombie Yellow
CD140a-BV510
CD31-BV510
Ter-119-BV510
CD45-BV510
CTV
FSC-A


B
Luminal
Basal
RSK2 (+/+)
RSK2 (-/-)
RSK2 (+/+)
RSK2 (-/-)
% of live lin- cells
EpCAMhi CD49f+
EpCAMlo CD49f+
EpCAMhi CD49f+
EpCAMlo CD49f+
EpCAMhi CD49f+
EpCAMlo CD49f+
EpCAMhi CD49f+
EpCAMlo CD49f+
EpCAMhi CD49f+
EpCAMlo CD49f-
Proestrus
Estrus
Metestrus
Diestrus
6 wk


C
FMO - Sca1
FMO - CD49b
0.00%
0.00%
58.16%
0.31%
91.27%
8.73%
41.44%
0.09%
Sca1
CD49b


D
RSK2 (+/+)
RSK2 (-/-)
% of EpCAMhiCD49f+ Sca1+CD49b- cells
PE
E
ME
DE
6 wk
8 wk
12 wk
% of EpCAMhiCD49f+ Sca1-CD49b+ cells
% of EpCAMhiCD49f+ Sca1-CD49b- cells
p=0.0010
p<0.0001
p=0.0040
p<0.05


E
regenerated mammary glands
RSK2 (+/+)
RSK2 (-/-)

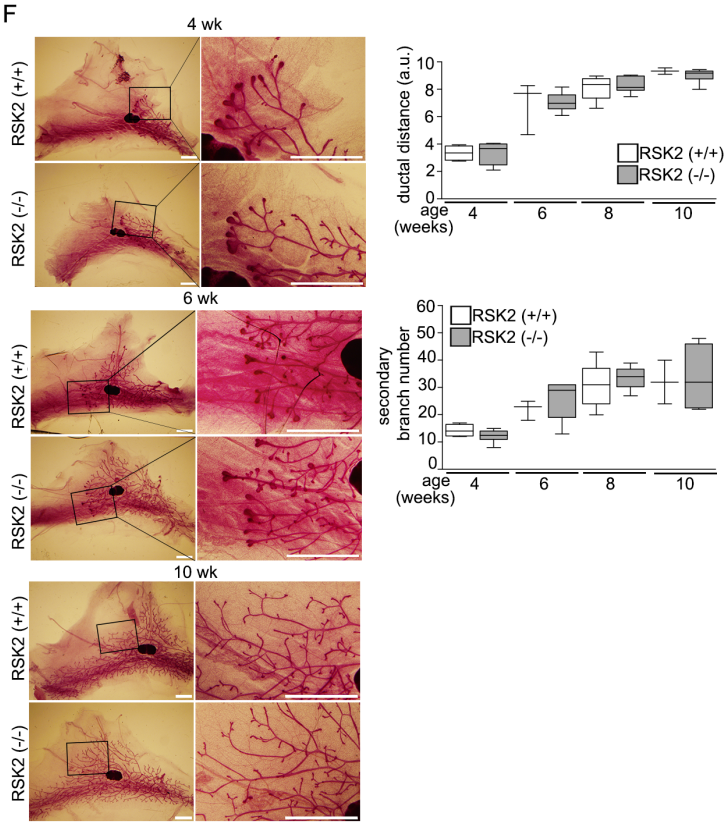

F
4 wk
6 wk
10 wk
RSK2 (+/+)
RSK2 (-/-)
ductal distance (a.u.)
secondary branch number
age (weeks)
4
6
8
10

Figure 2 Analysis of WT and RSK2-KO mammary glands.

(A) Gating strategy for flow cytometry analysis and sorting of mouse mammary epithelium. Cells were gated for forward (FCS-A) and side (SSC-A) scatter to remove debris. Single cells (p2) gated by FSC-H/A were then gated for live cells (ZombieYellow negative). Lineage^{+} (Cd140a^{+}; CD31^{+}; Ter-119^{+}; and CD45^{+}) cells were gated out. CellTraceViolet (CTV) positive and negative populations were separated.
(B) FACS analysis of luminal and basal epithelial populations in the mammary gland (median ± quartile, n≥4 mice/genotype and stage, one-way ANOVA with Holm-Sidak's correction for multiple comparisons) (Table S1). solid=luminal, hatched = basal
(C) Fluorescence minus one strategy for determining the gates for Sca1 and CD49b.
(D) FACS analysis of luminal progenitor and undefined epithelial populations (median ± quartile, n ≥ 3 mice/genotype and stage, one-way ANOVA with Holm-Sidak's correction for multiple comparisons) (Table S1).
(E) Representative whole mount image of the regenerated 4th mammary gland from WT or RSK2-KO ~ 20 wk after transplantation at 3 wk. Scale bar = 1 mm.
(F) Mammary gland development is similar in WT and RSK2-KO. (median ± quartile, n≥2 mice/genotype, one-way ANOVA with Holm-Sidak's correction for multiple comparisons) (Table S1). Scale bar = 2 mm.

Related to Figure 1 and 2

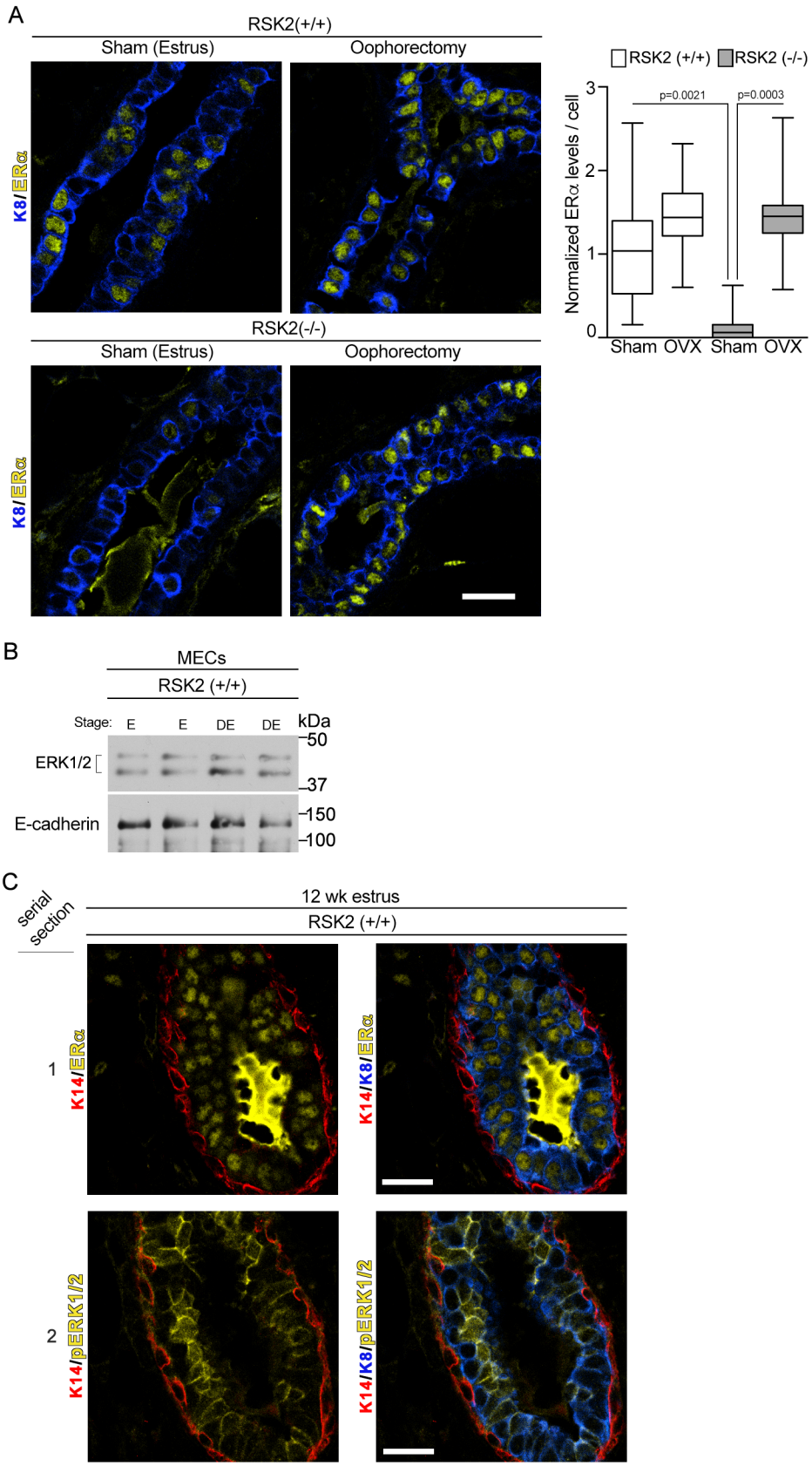

A
RSK2(+/+)
Sham (Estrus)
Oophorectomy
K8/ERα
RSK2(-/-)
Sham (Estrus)
Oophorectomy
K8/ERα
RSK2 (+/+)
RSK2 (-/-)
p=0.0021
p=0.0003
Normalized ERα levels / cell
0
1
2
3
Sham
OVX
Sham
OVX
B
MECs
RSK2 (+/+)
Stage: E E DE DE
kDa
ERK1/2
50
37
E-cadherin
150
100
C
serial section
12 wk estrus
RSK2 (+/+)
1
K14/ERα
K14/K8/ERα
2
K14/pERK1/2
K14/K8/pERK1/2

Figure 3 ERK1/2 is active in ER+ cells.

(A) ERα protein levels increase in response to oophorectomy (median ± quartile, n ≥2 mice/genotype and procedure, ≥ 3 fields/mouse, one-way ANOVA with Holm-Sidak's correction for multiple comparisons) (Table S1). Scale bar= 20 μm.
(B) ERK1/2 protein levels are similar in estrus and diestrus in mammary epithelial cells isolated from WT adult mammary glands.
(C) The image on the left is shown without K8 to facilitate the visualization of ERα and pERK1/2. Serial sections were necessary to avoid antibody interference. Scale bar = 20 μm.

Related to Figures 3 and 6

Ludwik&Sandusky_Supp_Figure_4

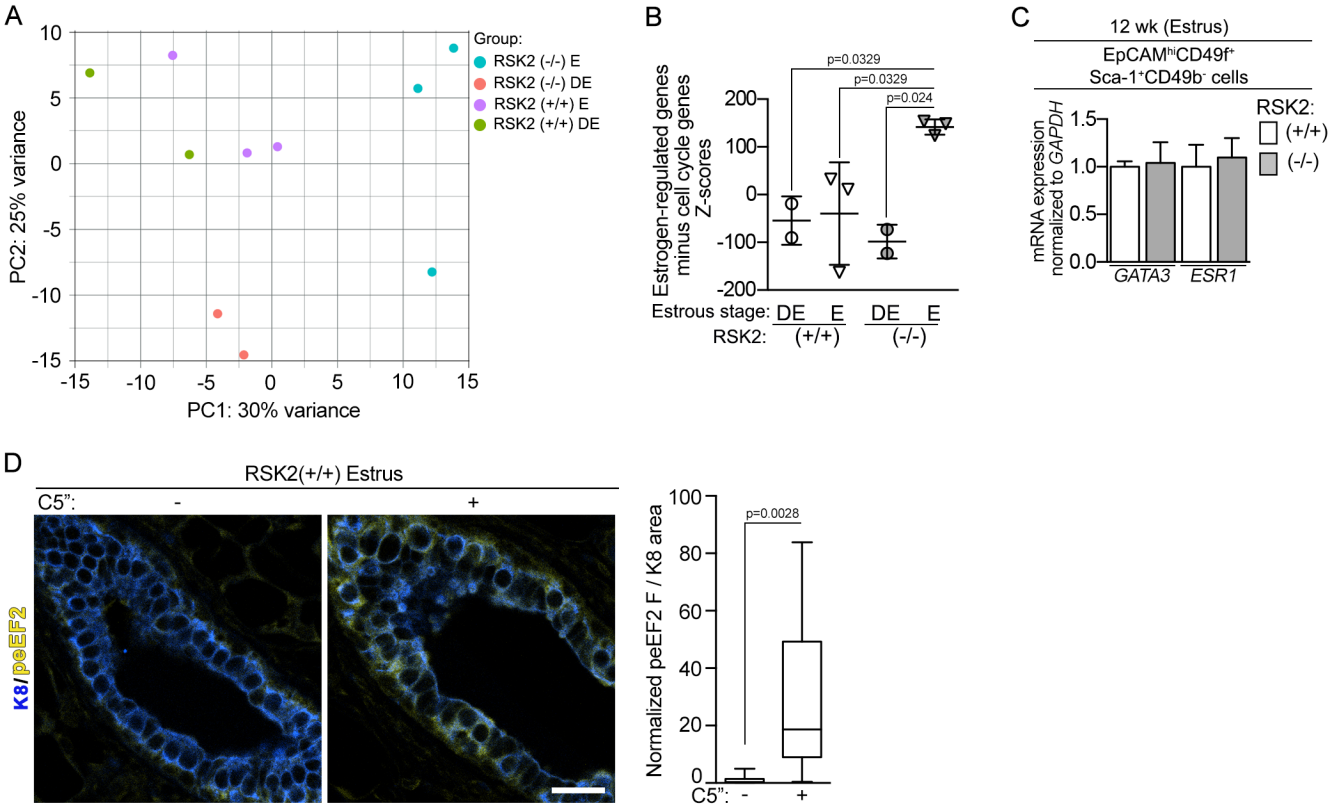


Figure 4 Transcriptomic analysis of the NCL population.

(A) Principal component (PC) analysis of the transcriptomic data.
(B) Proliferation genes do not drive the enrichment for estrogen -regulated signature in RSK2 KO estrus mice. Cumulative Z-scores were generated for each mouse by summing individual Z-scores of genes up regulated in estrogen-regulated signature in which the cell cycle genes were removed and subtracting individual Z-scores of genes down regulated. (median ± quartile, one-way ANOVA with Holm-Sidak's correction for multiple comparisons) (Table S1).
(C) ESR1 and GATA3 mRNA levels are similar in NCL cells isolated from RSK2-KO and WT mice during the estrus stage (mean ± S.D., n=3 mice/genotype in triplicate, Student's t-test).
(D) On target increase in peEF2 *in vivo* by *C5"-n*-propyl cyclitol SL0101 (C5"). Adult mice staged at estrus were treated with vehicle or *C5"* (40 mg/kg) IP twice every 7 h before euthanasia and isolation of the mammary gland (median ± quartile, n ≥ 2 mice/genotype in triplicate, Student's t-test).

Related to Figure 4

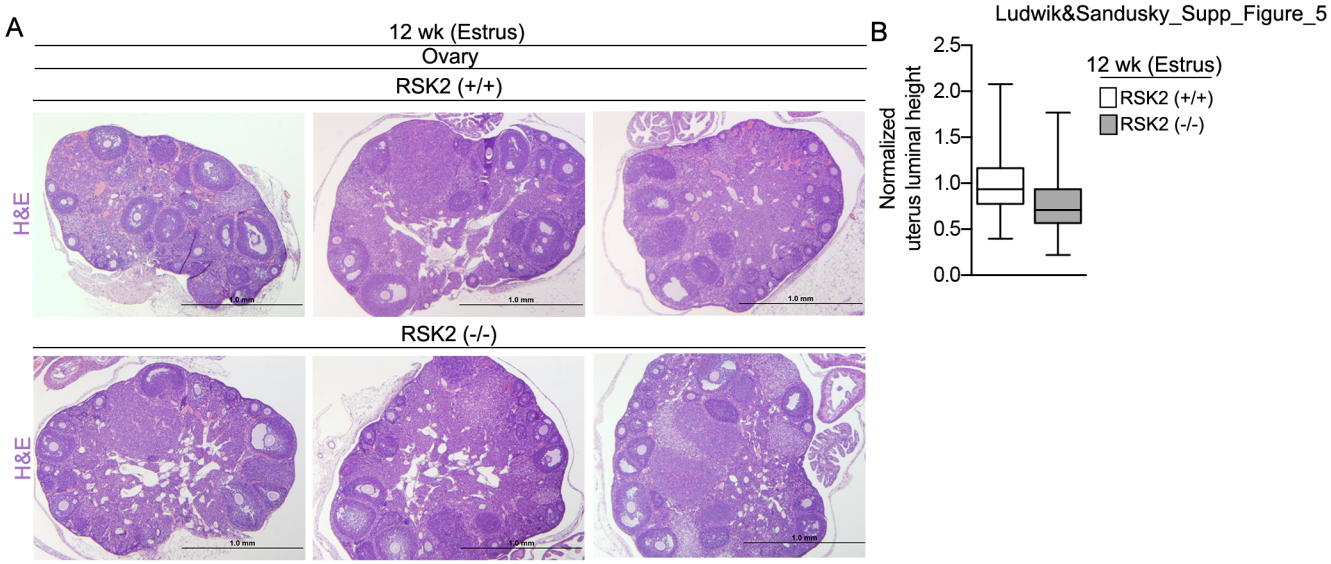


Figure 5 The hypothalamic-pituitary-ovarian axis is not impaired in RSK2-KO mice.

(A) Representative H&E images of ovaries. Scale bar = 1 mm.
(B) Luminal height in the uterus in the WT and RSK2-KO are similar. Measurements from ≥30 randomly selected regions from each animal (median ± quartile, n≥3 mice/genotype, ≥ 3 fields/mouse, Student's t-test).

Related to Figure 5

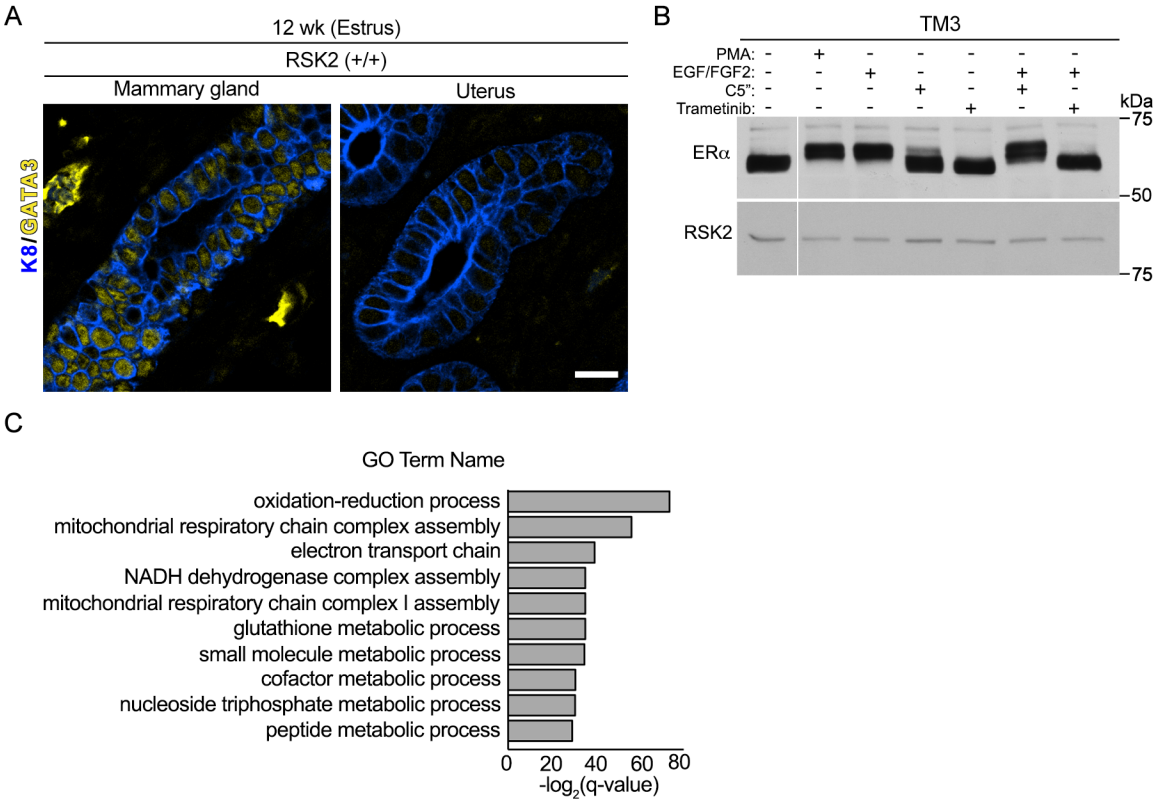


Figure 6 Phosphorylation of Ser-118 ERα correlates with degradation of ERα.

(A) GATA3 is expressed at very low levels in the uterus compared to the mammary gland. Scale bar = 20 μm. (B) Ser118- ERα phosphorylation occurs in response to agents that stimulate ERα degradation. Serum starved TM3 were treated with PMA (0.5 μM, 20 min) or an EGF/FGF7 cocktail (12.5 nM each, 5 min) with or without *C5"* (20 μm, 2h) or trametinib (1 μm, 1 h as a pretreatment). The white vertical line indicates that conditions not relevant to the manuscript were removed. (C) GO enrichment analysis for NCL population in RSK2-KO glands at estrus.

Related to Figure 6

Ludwik&Sandusky_Supp_Figure_7

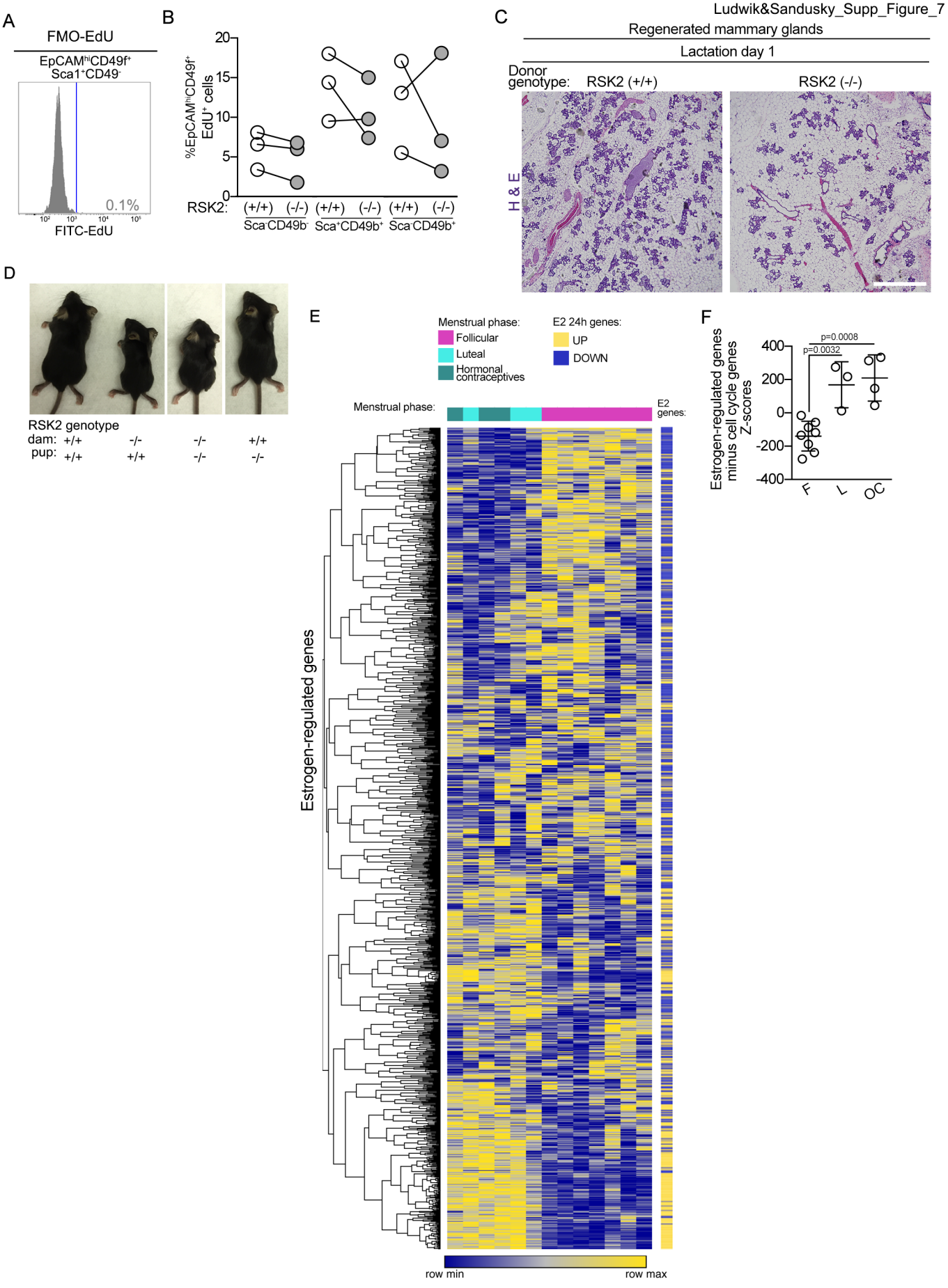


Figure 7 RSK2-KO dams fail to provide adequate nutrition for their pups.

(A) Fluorescence minus one strategy for determining the gate for FITC-EdU.
(B) FACS analysis of proliferation of mammary glands using RSK2-KO or WT MECs regenerated in a WT mouse. (n=3 glands/genotype; paired Student's t-test).
(C) Alveolar expansion is reduced in mammary glands regenerated from RSK2-KO mammary epithelial cells as shown by the H&E stains of mammary glands isolated from the same WT dam 1 d after birth. Scale bar = 1 mm.
(D) Representative images of WT and RSK2-KO pups at 21 d nursed by either WT or RSK2-KO dams.
(E) Heat map illustrating that estrogen-regulated signature is enriched in the luteal phase and by oral contraceptive use.
(F) Proliferation genes do not drive the enrichment for the estrogen-regulated signature in individuals in the luteal phase or those taking oral contraceptives. Cumulative Z-scores were generated for each individual by summing individual Z-scores of genes up regulated in estrogen-regulated signature and subtracting individual Z-scores of genes down regulated. (mean ± S.D., one-way ANOVA with Holm-Sidak's correction for multiple comparisons) (Table S1).

Related to Figure 7

Supplemental Table 3. Statistical analysis of gene set overlaps from the NCL populations.
Related to Figure 4

Gene sets in overlap		Fisher's exact test for overlap
Genes UP in R2KO-E vs R2KO-DE	E2_24h_UP	0.00001
Genes DOWN in R2KO-E vs R2KO-DE	E2_24h_DOWN	0.00001
Genes UP in R2KO-E vs R2KO-DE	Cell cycle genes	0.0007
Genes UP in WT-E vs WT-DE	E2_24h_UP	no overlap
Genes DOWN in WT-E vs WT-DE	E2_24h_DOWN	no overlap
Genes UP in WT-E vs WT-DE	Cell cycle genes	0.5785