

Supplementary information/materials

The epithelial Na⁺ channel (ENaC) in ovarian granulosa cells modulates Ca²⁺ mobilization and gonadotrophin signaling for estrogen homeostasis and female fertility

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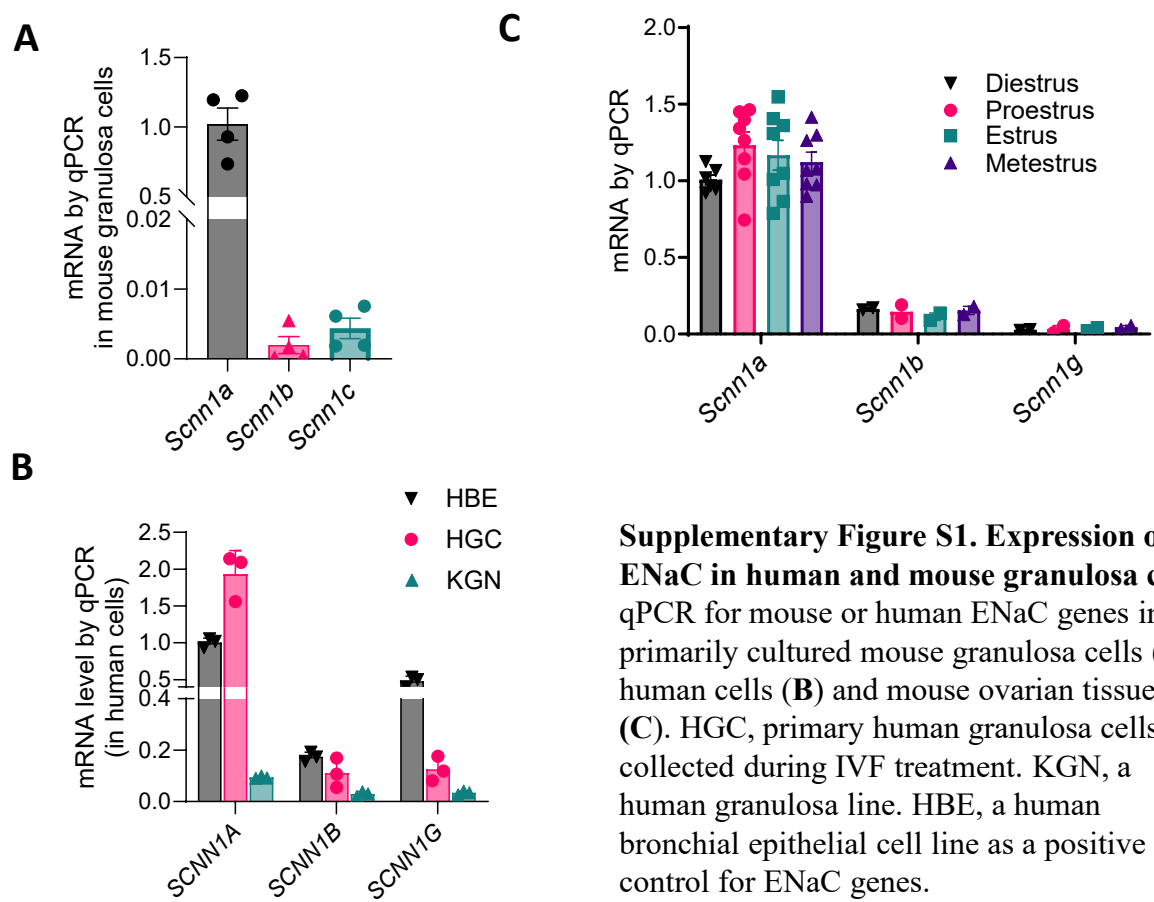
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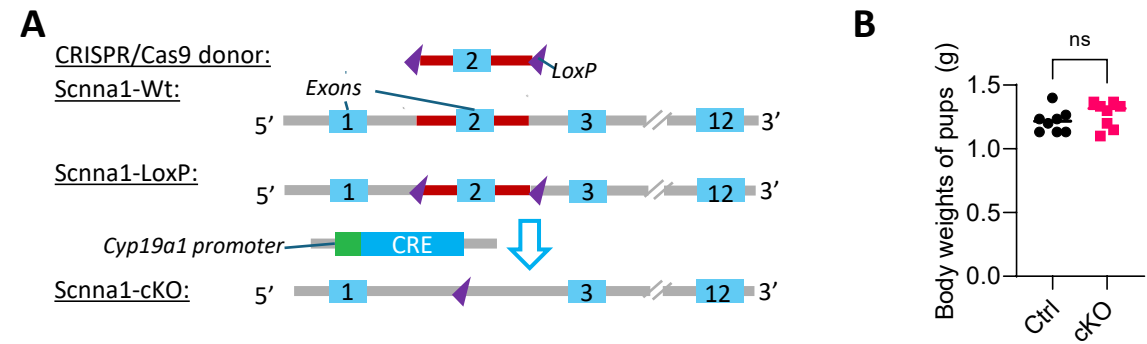
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Supplementary Figure S1.



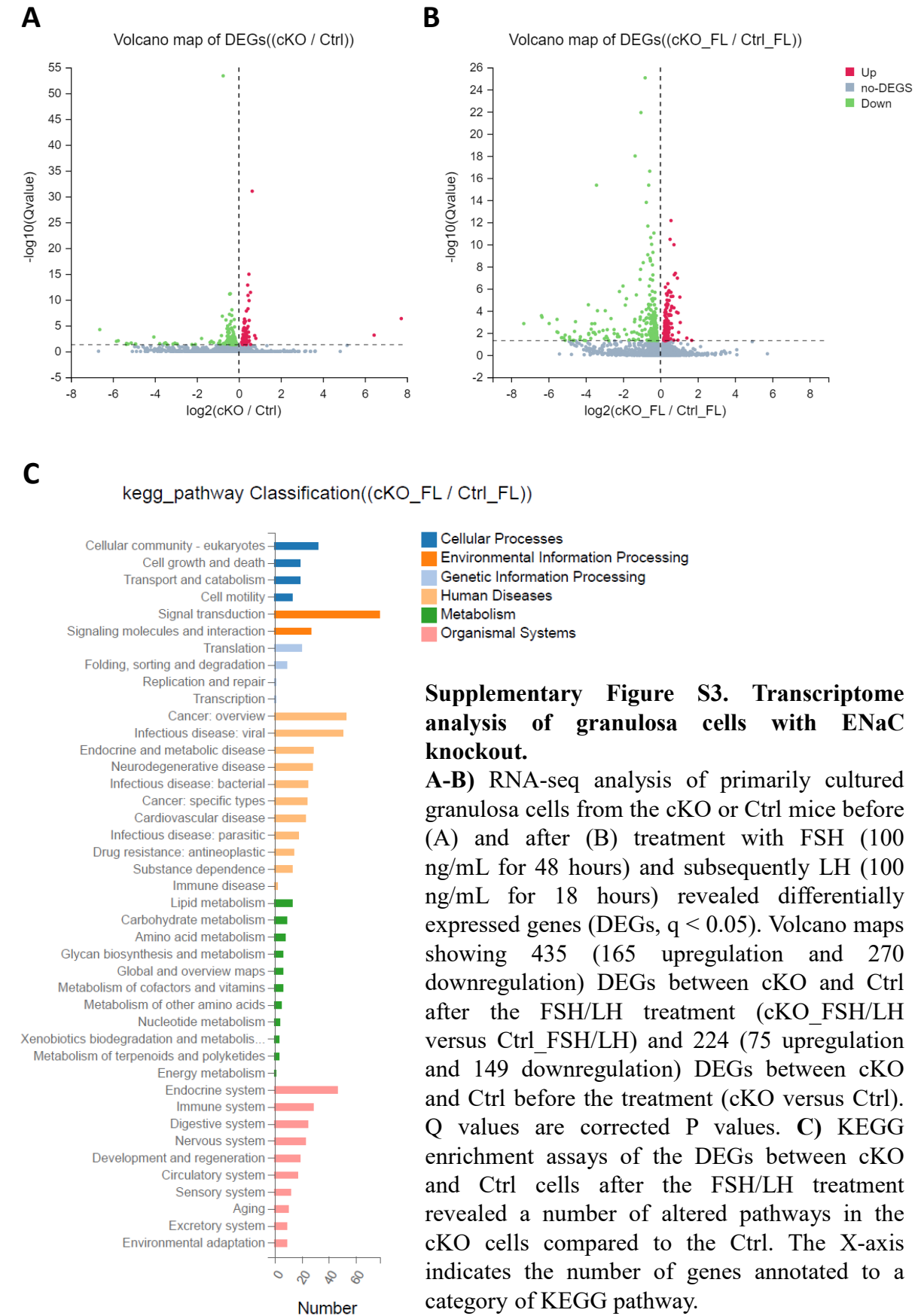
Supplementary Figure S1. Expression of ENaC in human and mouse granulosa cells. qPCR for mouse or human ENaC genes in primarily cultured mouse granulosa cells (A), human cells (B) and mouse ovarian tissues (C). HGC, primary human granulosa cells collected during IVF treatment. KGN, a human granulosa line. HBE, a human bronchial epithelial cell line as a positive control for ENaC genes.

Supplementary Figure S2.



Supplementary Figure S2. Granulosa cell-specific knockout of ENaCa in mice. A) Construction of the granulosa cell-specific ENaCa (*Scnn1a*) knockout mouse model. Two loxP sequences were inserted to flank the exon 2 of *Scnn1a* gene by CRISPR/Cas9 techniques. *Scnn1a* conditional knockout mice (*Scnn1a^{fl/fl} Cyp19a1^{Cre+}*) were generated by crossing the *Scnn1a* floxed transgenic mouse line (*Scnn1a^{fl/fl}*) with another transgenic line, where Cre-recombinase under the promoter of *Cyp19a1* (a granulosa-specific gene) was inserted (*Cyp19a1^{Cre+}*). B) Body weight of newborn (1-day-old) pups of Ctrl and cKO mice. ns: not significant, by Unpaired Student's t-test. n = 21.

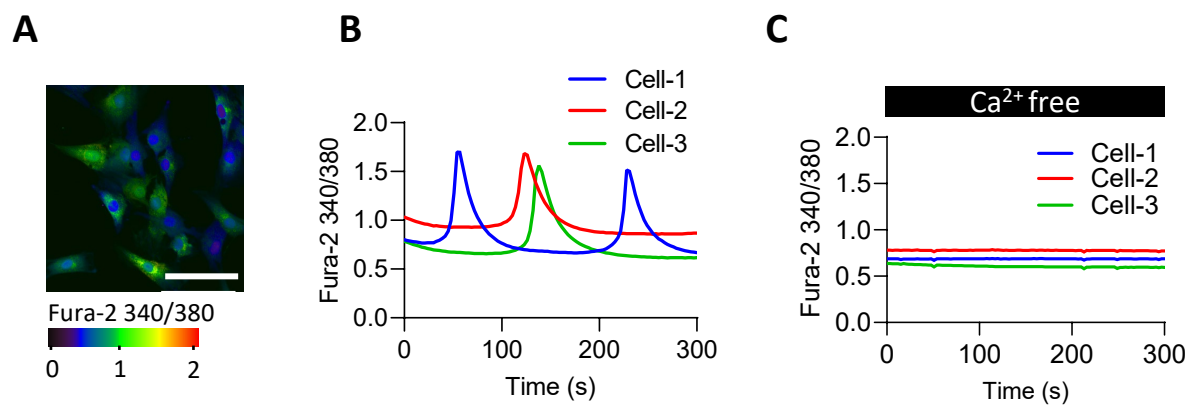
Supplementary Figure S3.



Supplementary Figure S3. Transcriptome analysis of granulosa cells with ENaC knockout.

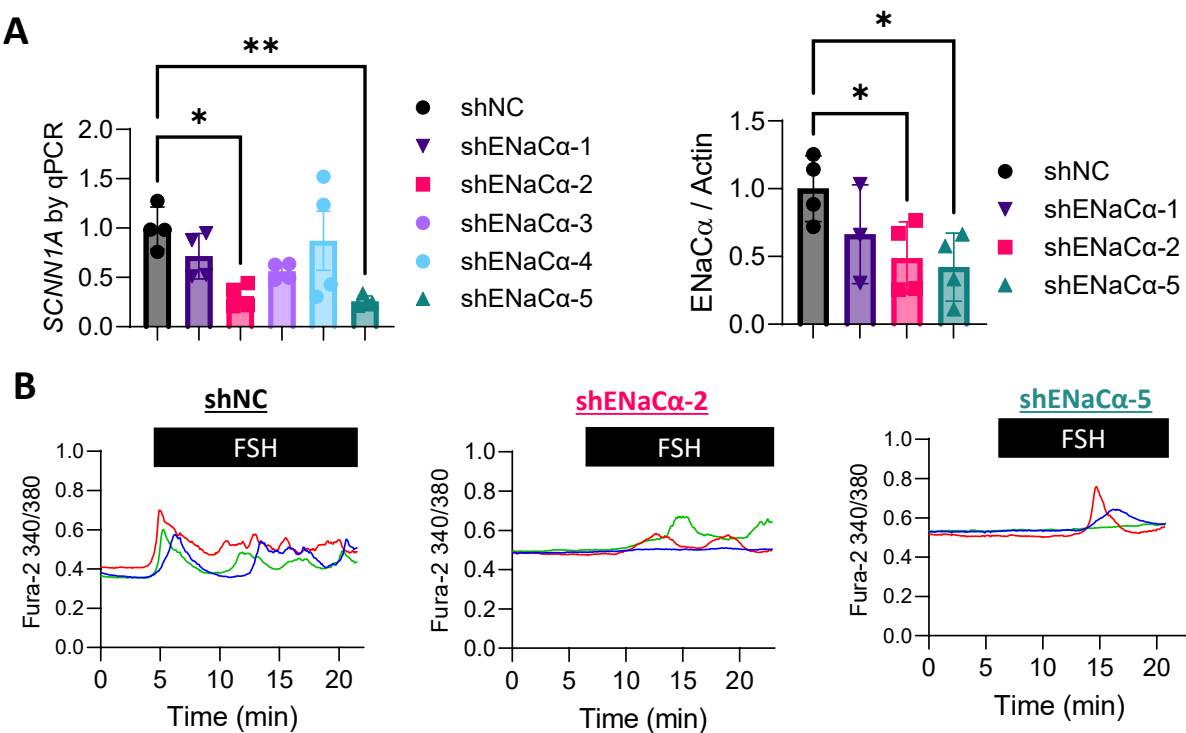
A-B) RNA-seq analysis of primarily cultured granulosa cells from the cKO or Ctrl mice before (A) and after (B) treatment with FSH (100 ng/mL for 48 hours) and subsequently LH (100 ng/mL for 18 hours) revealed differentially expressed genes (DEGs, $q < 0.05$). Volcano maps showing 435 (165 upregulation and 270 downregulation) DEGs between cKO and Ctrl after the FSH/LH treatment (cKO_FSH/LH versus Ctrl_FSH/LH) and 224 (75 upregulation and 149 downregulation) DEGs between cKO and Ctrl before the treatment (cKO versus Ctrl). Q values are corrected P values. **C)** KEGG enrichment assays of the DEGs between cKO and Ctrl cells after the FSH/LH treatment revealed a number of altered pathways in the cKO cells compared to the Ctrl. The X-axis indicates the number of genes annotated to a category of KEGG pathway.

Supplementary Figure S4.



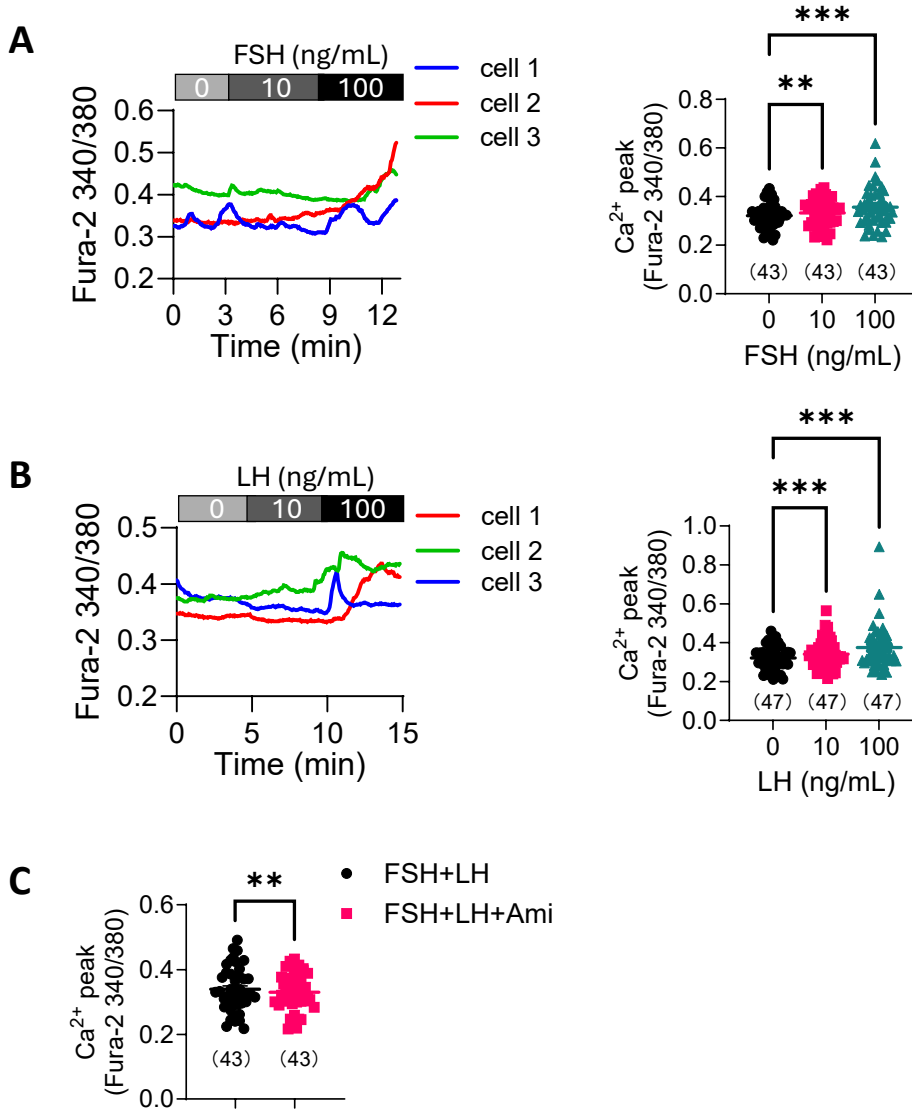
Supplementary Figure S4. Spontaneous Ca^{2+} oscillations in KGN cells. Fura-2 imaging (**A**) and time-course traces (**B-C**) of intracellular Ca^{2+} levels in representative KGN cells bathed in normal (containing 2.5 mM Ca^{2+} , **B**) or Ca^{2+} -free (**C**) Margo-Ringer solution. Pseudo colors from purple to red indicate Fura-2 340/380 from low to high. Scale bar, 100 μm .

Supplementary Figure S5.



Supplementary Figure S5. FSH-induced Ca^{2+} responses in KGN cells. **A)** qPCR (left) and western blot (right) for ENaCa in shNC- or shENaCa-1 to -5-treated KGN cells. $n = 4$. $*P < 0.05$, $**P < 0.01$ by one-way ANOVA with post-hoc Tukey's test. **B)** Representative time-course measurement of intracellular Ca^{2+} activities in shNC- or shENaC-2/-5-treated KGN cells before and after the addition of FSH (100 ng/mL).

Supplementary Figure S6.



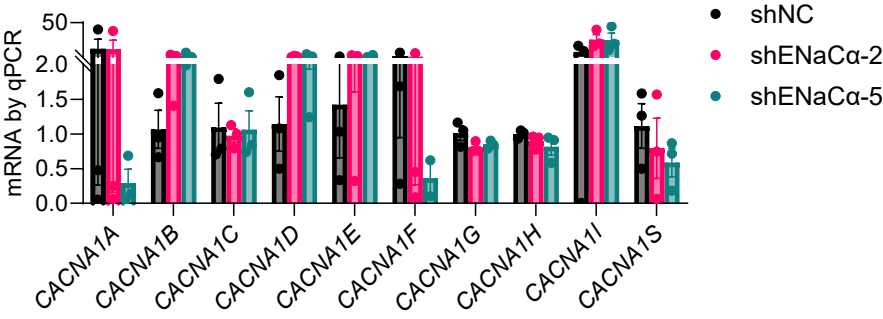
Supplementary Figure S6. FSH/LH induced Ca²⁺ response in primarily cultured mouse granulosa cells.

A-B) Time-course changes in intracellular Ca²⁺ level (indicated by Fura-2 ratio at 340/380 nm) in 3 representative primarily cultured granulosa cells from wild-type mice before and after the addition of FSH (10 to 100 ng/mL, **A**) or LH (10 to 100 ng/mL, **B**) with corresponding quantification of maximal responses (Ca²⁺ peak) under different conditions. n is shown for each group. ** $P < 0.01$, *** $P < 0.001$ by one-way ANOVA with post-hoc Tukey's test.

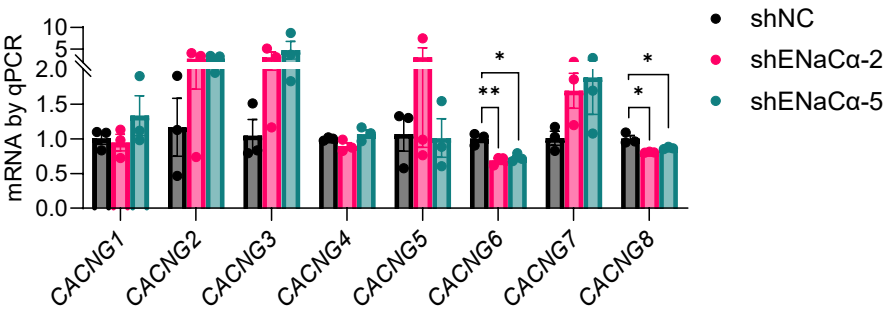
C) FSH/LH-induced Ca²⁺ peak in mouse granulosa cells in the presence or absence of amiloride (Ami, 1 μ M). n = 43 cells. ** $P < 0.01$ by Paired Student's t-test.

Supplementary Figure S7.

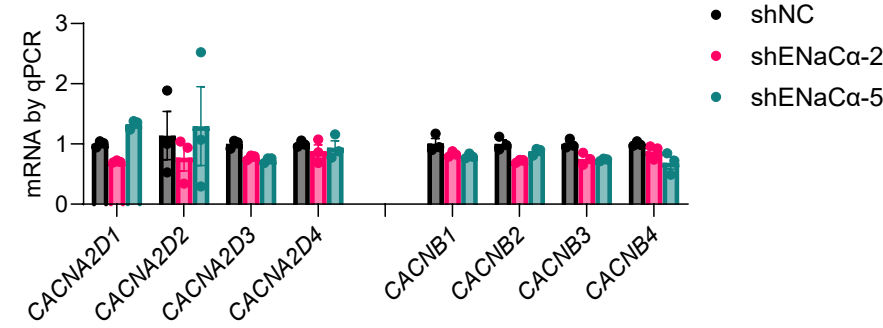
A



B



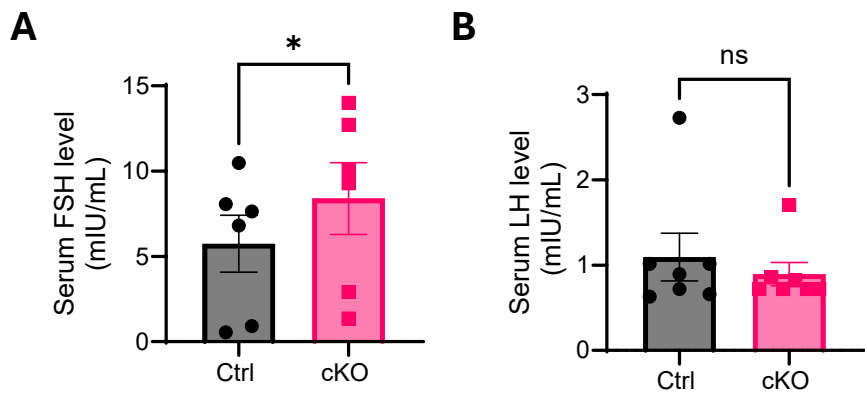
C



Supplementary Figure S7. Effect of ENaC knockdown on the expression of voltage-gated Ca²⁺ channels subunits genes in KGN cells.

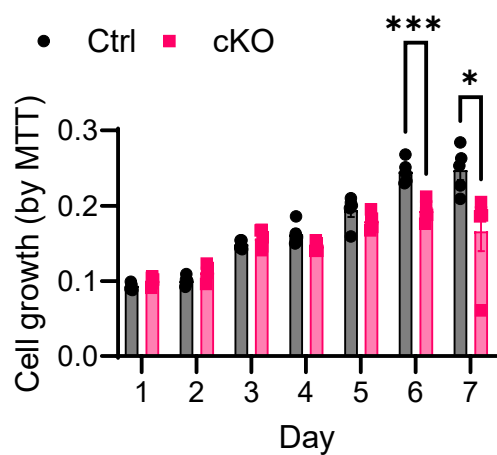
qPCR for voltage-gated Ca²⁺ channels subunits genes *CACNA* (calcium voltage-gated channel alpha1 subunits, **A**), *CACNG* (calcium channel auxiliary gamma subunits, **B**), *CACNA2D* (calcium voltage-gated channel auxiliary alpha2 delta subunits, **C**) and *CACNB* (calcium voltage-gated channel auxiliary beta subunits, **C**) in shNC or shENaCα-2/-5-treated KGN cells. n = 3. **P* < 0.05, ***P* < 0.01, by one-way ANOVA with post-hoc Tukey's test.

Supplementary Figure S8.



Supplementary Figure S8. Serum FSH and LH levels in the Ctrl or cKO mice. ELISA measurement of serum FSH (A) and LH (B) levels in the Ctrl or cKO mice. n = 6-7. * $P < 0.05$ by Paired Student's t-test.

Supplementary Figure S9.



Supplementary Figure S9. Knockout of ENaC impairs granulosa cell growth in vitro.

MTT assessment of cell growth in 7-day cultures of granulosa cells isolated from the Ctrl or cKO mice. n = 5. * $P < 0.05$, *** $P < 0.001$ by two-way ANOVA with post-hoc Tukey's test.

Supplementary Table 1. Information of woman subjects.

Subject No.	Age	Reason for IVF
1	39	Tubal problem (unilateral), adenomyosis
2	40	Male factor
3	38	Male factor
4	41	Male factor
5	34	Male factor
6	33	Male factor
7	33	Male factor
8	44	Hydrosalpinx

Supplementary Table 2. Primers for qPCR.

Gene	Host	Forward (5'-3')	Reverse (5'-3')
<i>Housekeeping genes</i>			
<i>GAPDH</i>	human	AGGGTCATCATCTCTGCC	CCATCACGCCACAGTTTC
<i>Gapdh</i>	mouse	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC
<i>ENaC genes</i>			
<i>SCNN1A</i>	human	CCCTCTGTCACGATGGTCAG	TCCGGAACCTGTGCAGTAAC
<i>Scnn1a</i>	mouse	ACTCCAGGCATGGAAGACAT	CCTGGGCAGCTTCATCTTTA
<i>SCNN1B</i>	human	GCCCTTCAGCAGGTACTTCTT	GTGTCCCAGTGTCAACCAACA
<i>Scnn1b</i>	mouse	CCGTGGGTGTTGGTGTATTT	CACACTGGAGCAGCTTCCTA
<i>SCNN1G</i>	human	CCTCCGTGCACTGACTGTAG	GCAGGATGAGTATCCCTTCG
<i>Scnn1g</i>	mouse	CCAGGAAGGGGTTGTACTCA	ACAGAGAAAACGCCACCATC
<i>Ovarian steroidogenesis related genes</i>			
<i>CYP19A1</i>	human	AGCTCCTCCAGCTCCTCTTT	CAGCCTCAACATCAACCTCA
<i>Cyp19a1</i>	mouse	TCGTCGCAGAGTATCCAG	CGATGTACTTCCCAGCAC
<i>Fshr</i>	mouse	TGTCGCAGCTGTATGTTATG	GAGAAAGTCCGTGAAGATGA G
<i>Lhcgr</i>	mouse	CCGACTATCTCTCACCTATCT C	GCACCGGGTTCAATGTATAG
<i>Hsd3b2</i>	mouse	CTCAATTGTGCCTGCCTTCAG	AGAAAGGCTGGTTCTGGTCTG
<i>Cyp11a1</i>	mouse	GCTGGCTAAAGGACTTTCCCT	GGTTTAGCCAACCATTGTGCG
<i>Star</i>	mouse	TAAGGCAGCGCACTTGATCT	ACACAGCTTGAACGTAGCGA
<i>Ca²⁺ channel related genes</i>			
<i>CACNA1A</i>	human	AGACCCCCGAGAATAGCCTT	TCACTTGTACGACGGTGTGG
<i>CACNA1B</i>	human	CAGTGGTTCGGTGAATGGGA	GTTGGCCGTCTTGTAGGTGA
<i>CACNA1C</i>	human	ACATGCTCTTCACTGGCCTC	TGCTGCCAATTACGATGAGGA
<i>CACNA1D</i>	human	GAGGACCTGGCGGATGAAAT	TATGAGGCACTTTTCCCCTGG

<i>CACNA1E</i>	human	GCTTCGCAGAGGTTGCATTT	GCAGAAAGACACACGGAGAC T
<i>CACNA1F</i>	human	CAGACCCTTGGAGCAAGCAT	TTGGAGTCGTCCTCAGGGAA
<i>CACNA1G</i>	human	TTCGGCAACTACGTGCTCTT	TGGGTGAGAAGAAATCGGGC
<i>CACNA1H</i>	human	CATCTTCAGCATCCTGGGCA	CACAGCAGGGAGTCGAAGTT
<i>CACNA1I</i>	human	GACTTTTTGCAAGTCCGCGT	ATGACCTGAGGCTGCTTGAC
<i>CACNA1S</i>	human	TTTGGAGACCCCTGGAATGT G	TCCACCCAGGCAATACAGTC
<i>CACNG1</i>	human	CCAAAATGCTGAAGGTCCGC	CTCGCAGGTAGTGTTGTGGT
<i>CACNG2</i>	human	TCCGTGAGCTTTCTTACCTCC	TCTGGTTCTCGGGAGAGTGT
<i>CACNG3</i>	human	CCAAAGTCCCTGTGGATGCT	TCATGGTACTCTGGACGGCT
<i>CACNG4</i>	human	AATCAACGCCAGGAGAGCAA	TCCACTCCGTCCTGTTAGGT
<i>CACNG5</i>	human	CGGACGATACTGGCCTTTGT	AACAAGAGTGTGGCGAGGTT
<i>CACNG6</i>	human	AGACTTCTCTAAGCAACCAC CG	CAGTTTCTCTAGGAGATGGGG C
<i>CACNG7</i>	human	TCCCTGGAAGGTCCCATTCT	CAAGAGCCCCCTAAAAGGCA
<i>CACNG8</i>	human	TGCCATTTTTGGCCATCTGC	TGGATGGCGGTGTTTGATGA
<i>CACNA2D 1</i>	human	TCTTCGATCGCGAAGATGGC	TTGATAGTGACGGCCGAAGG
<i>CACNA2D 2</i>	human	AACGTTGGTCAAGTCCCTGG	CTGATTGGCAACTTGACCTGC
<i>CACNA2D 3</i>	human	ACCAAGCCATGTGTAGAGCC	GCTGTCATATCGGAGTGCCA
<i>CACNA2D 4</i>	human	CTGACCTGTGTTTTGGCAAG G	CCAGGATTGAGAGGAGCGTT
<i>CACNB1</i>	human	AGCCTATTGGAAGGCCACAC	AGGTTTCTCCTGAGCGAGGT
<i>CACNB2</i>	human	GCCAATGGTCCAAAGGGACA	TCACAAAGGAAGGAGAAGGC G
<i>CACNB3</i>	human	TCTGACTCCAGTGCAACCTTC	AATGCCACAGGTTTGTGCTTG
<i>CACNB4</i>	human	TTGAAAAGATCCGATGGCAG C	TCTCCTGTCTGAATTGCTTCCC
<i>ITPR1</i>	human	CGAGGCTGGAAATGAAGGGT	CCTCATTGCAGCCTGGGTTA
<i>ITPR2</i>	human	AGCAATGAAGGCGACAGTGA	TGGGGTGTGTTTGATCCGAG
<i>ITPR3</i>	human	GCCATGAGTGAAATGTCCAG C	GTGCTGATGAAGCCATTGACG
<i>MICU1</i>	human	GCATCATTCGCTCCCAAACC	CAAGCCAGACTTGAGGGTGT
<i>MICU2</i>	human	GCGGGATGGCAGTTTTACAG	TGAAGCGCTGCTTACGAAGA
<i>CALHM5</i>	human	TTCCCTCTGTGCATCTCCAAC	GGAAAACCAGCCCATAGGTCA
<i>S100A10</i>	human	CCCTCTGGCTGTGGACAAAA T	ATGGTGAGGCCCCGCAATTAG
<i>STIM2</i>	human	GCAGTGCACGAACCTTCATTT	CGTATAAGCAAACCAGCAGCC

Supplementary Table 3. Primary antibodies.

Poly/monoclonal	Host specie	Against	Catalog	Company	Application
Polyclonal	Rabbit	ENaC α	SC21012	Santa Cruz	Mouse ovarian tissues, IF (1:50)
Polyclonal	Rabbit	ENaC α	Ab272878	Abcam	Mouse ovarian tissues, IHC (1:500)
Polyclonal	Rabbit	ENaC α	PA1-920A	Thermo Fisher Scientific	Primary mouse and human granulosa cells, KGN (a human granulosa line) cells and mouse ovarian tissues, WB (1:500) and IF (1:100)
Monoclonal	Rabbit	pCREB	9198	Cell signaling technology	Primary mouse granulosa cells and mouse ovarian tissues, WB (1:500) and IF (1:100)
Monoclonal	Rabbit	CREB	9197s	Cell signaling technology	Primary mouse granulosa cells and mouse ovarian tissues, WB (1:1000)
Monoclonal	Mouse	GAPDH	Sc47724	Santa Cruz	Primary mouse granulosa cells and mouse ovarian tissues, WB (1:2000)
Monoclonal	Mouse	Actin	mAB1501R	Sigma	Primary mouse and human granulosa cells, KGN cells and mouse ovarian tissues, WB (1:2000)

IF: Immunofluorescence, IHC: Immunohistochemistry, WB: Western blot.

Supplementary Table 4. Secondary antibodies.

Host specie	Against	Conjugated to	Catalog	Company	Application
Goat	Rabbit IgG	HRP	170-6515	Bio-Rad	WB (1:10000) IHC (1:1000)
Goat	Mouse IgG	HRP	170-6516	Bio-Rad	WB (1:10000)
Goat	Rabbit IgG	FITC	111-095-003	Jackson ImmunoResearch (West Grove, PA, USA)	IF (1:200)
Goat	Rabbit IgG	CY3	111-165-003	Jackson ImmunoResearch (West Grove, PA, USA)	IF (1:200)

IF: Immunofluorescence, IHC: Immunohistochemistry, WB: Western blot.