METHODS AND MATERIALS

Milk production measurement

To measure milk production, we starved pups (7-9 pups per litter) for two hours before reuniting them with dams and measured the weight differences before and after 30 minutes of suckling. For direct milk production measurements, pups were removed for 3 hours on day 2 of lactation and dams were injected with 10 units of oxytocin to induce milk letdown. Milk was manually removed from the fourth mammary gland using a 20-µl-pipette tip (1).

Measurement of oxytocin and prolactin levels

Blood samples were collected from the orbital sinus and allowed to coagulate overnight at 4 °C. Plasma was collected by centrifugation and stored at -80 °C. Levels of oxytocin and prolactin were measured using the Oxytocin ELISA kit (Cat # JL12594-96T) and the PRL ELISA kit (Cat # JL12880-96T, Jianglai, Shanghai, China,) based on manufacturer-recommended protocols.

Oxytocin-induced milk expulsion assay

Mice were sacrificed on parturition day and pups were removed from the dam 3 hours prior to the assay. Abdominal skin was cut and peeled off in order to expose the mammary gland. PBS or 1 mg/ml oxytocin (Sangon Biotech, Shanghai, China) in PBS was applied directly onto the mammary glands for 1 min and then removed by PBS washes. Milk entry into the ducts was monitored using an Axio Zoom V16 stereoscope (Zeiss). Photographs were taken before and after PBS or oxytocin exposure. To visualize contraction in culture, primary organoids were seeded in Matrigel™. Organoids were culture in basal medium with or without 100ng/ml Oxytocin. Live imaging was done on a Zeiss Observer Z1 microscope. Images were processed using ImageJ.

Isolation of primary mammary organoids

Primary mammary organoids were isolated and cultured as previously described (Zhang et al., 2014). Briefly, mouse mammary glands were finely chopped and mince was digested in collagenase buffer [with collagenase 0.2%, (Sigma) and trypsin 0.2% (Life Technologies)]. Samples were centrifuged, washed several times, treated with DNase I. Then they were subjected to 5 rounds of differential centrifugation (a short-pulse centrifugation at 500x g), during which supernatant was collected and pellets were resuspended in DMEM/F12 (Invitrogen) for another

round of differential centrifugation. The pellet containing mammary organoids was further trypsinized to obtain a single-celled MEC solution.

Fluorescence activated cell sorting (FACS)

Single cells were dissociated and were fluorescently labeled by infection using Ad-Cre-GFP virus or by antibody staining. Antibodies against CD24 (FITC), CD49f (PE) and CD45 (PeCy7, eBioscience) were used together with 7-AAD (BD Biosciences) to sort for luminal and basal populations. Sorting was done using an Aria system and analysis using FACalibur system. Data were processed using FACS Diva software (BD Biosciences).

Quantitative RT-PCR

Total RNA was prepared from FACS-based luminal, basal, stromal cell partitions or whole mammary gland using Trizol reagents (Vazyme，R401-01). Equal amounts of RNA templates were then used in qPCR reactions using SYBR green with the BIO-RAD CFX Connect Real-Time Systems according to the manufacturer's protocol. Relative expression levels were calculated using the comparative CT method. Gene expression levels were normalized to *Actb*. The primers used are listed in Supplementary Table2.

Immunofluorescence analysis

For immunofluorescence analysis, mammary glands were harvested and fixed in 4% paraformaldehyde overnight at 4ºC. Then mammary glands were soaked in 15% sucrose and 30% sucrose prepared in PBS for 12 hours. 10μm frozen sections were cut using a Leica cryostat. Sections were blocked for 1hr at room temperature (RT) in PBS containing 10% goat serum and 0.2% Tween20, followed by incubation in primary antibodies for 1 hr. at RT or overnight at 4ºC. Primary antibodies used in this study were anti-Claudin 4 antibody (eBioscience, #364800, 1:100 dilution), anti-Claudin 7 antibody (eBioscience, #349100, 1:100 dilution), anti-Occludin antibody (Invitrogen, #406100, 1:200 dilution), anti-smooth-muscle-actin antibody (Sigma, #93777, 1:500 dilution), anti-Keratin 8 antibody (Proteintech , #10384-1-AP, 1:200 dilution), anti-ZO1 antibody (Proteintech, #21773-1-AP, 1:100), anti-E-Cadherin antibody (Invitrogen, #14-3249-82, 1:200), anti-GM 130-alexa-488 (BD, #560257, 1:100), anti-Ezrin antibody (Cell Signaling, #3145S, 1:200), while rabbit anti-mouse-β-Casein antibody was homemade. Phalloidin-633 was from

Invitrogen (#A22284, 1:200). Confocal microscopy was performed on a Leica SP8 STED confocal.

TJ permeability Assay

Primary MECs were incubated in freshly made 1 mg/ml EZ-Link™ Sulfo-NHS-LC-Biotin (Thermo Scientific, 21335#) in HBSS buffer (containing 1 mM CaCl2 and 1 mM MgCl2, Gibco) for 30 min. In some experiments, primary MECs were incubated with 1 mg/ml NHS-LC-biotin buffer containing 10 mM EGTA for 30 min at room temperature. After washing with HBSS three times, the surface-labeled primary MECs were fixed with 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 hr. at room temperature. Fixed samples were frozen in liquid nitrogen, and sectioned on a cryostat at a thickness of 12 μm. After rinsing in PBS containing 1% BSA for 1 hr., sections were incubated for 1 hr. with a mixture of fluorescein-phalloidin and XRITC-avidin (Pierce Chemical Co.), both of which were diluted 1:200 in PBS containing 1% BSA. The samples were embedded and examined by confocal fluorescence microscopy.

Cell proliferation and apoptosis assays

For cell proliferation analysis, mice were injected with EdU (1mg/100 g body weight) 3 hrs. prior to sacrifice. Mammary glands were harvested and fixed in 4% paraformaldehyde overnight at 4ºC. 10-μm frozen sections were obtained using a Leica cryostat. Assays were performed as recommended by the manufacturer (Ribobio, Guangzhou, China). Confocal microscopy was performed on a LSM710 Zeiss confocal. To assess apoptosis, TUNEL assay was done on frozen sections of mammary glands according to manufacturer's protocol (Vazyme, Cat# A111-01).

Western blotting and co-immuno-precipitation (Co-IP) assays

Organoid preparations were lysed in RIPA buffer (CWBIO,# CW2334S). Samples were cleared with centrifugation at 13,500 x *g* at 4 °C for 30 mins. Proteins were electrically transferred in a wet-tank to a PVDF membrane. After blocking with 5% BSA (sigma#WXBC3116V), target proteins were visualized using mouse anti-OCLN(proteintech， #66378-1, rabbit anti-stat5a (Santa Cruz Biotech, #sc-271542) and rabbit anti-p-stat5a antibodies (Thermo Fisher, #71-6900). For quantification of protein expression, band density was measured using an Amersham Imager 680.

For Co-IP assays, the 293T line of cells expressing FLAG-OCLN and HA-STX6/VAMP4/SNAP23 were harvested, washed twice with PBS, then lysed in buffer containing 20 mM HEPES, 150 mM NaCl, and 1% NP40. Samples were co-incubated with FLAG tagged magnetic beads (Bimake, #B26101) at 4 °C. Beads were then washed four times with washing buffer before co-incubated with 40 µl 2 X sample loading buffer to harvest FLAG-tagged proteins before they were subjected to Western Blotting analysis. The antibodies used were anti-FLAG (GNI, #GNI4110-FG-S), anti-HA (Cell Signaling Technology, #2999). Band density was measured using an Amersham Imager 680.

Ultrathin section and immuno-electron microscopy

Very small pieces (~1 mm3) of #4 mammary glands were fixed in 2% glutaraldehyde and 1% paraformaldehyde overnight. They were then fixed again in OsO4 for 2 hours before embedding using the Embed 812 Kit (Electron Microscopy Sciences, Cat# 14120). Ultrathin sections were cut using a diamond knife, double stained with uranyl acetate and lead citrate before examination.

For Immuno-Electron Microscopy, tissues were fixed in 0.2% glutaraldehyde and 4% paraformaldehyde before they were embedded in Epon 812. Ultra-thin sections were then cut and sequentially treated with sodium periodate, 0.1% glycine, and 1% BSA before they were incubated with OCLN antibody for 48 hours at 4 °C. After washing, samples were incubated with goat anti-rabbit IgG secondary antibody, which were conjugated with 10 nm gold particles (Sigma, #G7402) at a 1:40 dilution for 2 hours at the room temperature. Samples were re-fixed with 1% glutaraldehyde, double stained with uranyl acetate and lead citrate, then examined using a Joel JEM-1230 (Institute of Neurosciences, Academia Sinica) or a Talos L120C (ShanghaiTech University) transmission electron microscope at an accelerating voltage of 80 kV.

Bioinformatics analysis

For cell clustering, differentiation gene and GO analyses, we used the Cell Ranger R kit for quality control of the sequencing data, which were then rendered with Kmean Clustering of SC3. We chose Kmean = 2 and obtained two sub-groups with each having 10 marker genes. We then focused on the luminal group that had high Krt18 expression and further divided them into three subgroups using Kmean =3 and acquired their corresponding 10 marker genes. We defined the subgroup with high *Wap* expression as milk producing cells with the above 20 marker genes. For GO analysis,

we used Loupe Cell Browser to identify luminal cells and performed differential gene analysis with a P value cutoff at 0.05. They were then subjected to Cluster Profiler for GO analysis.

REFERENCE

1. R. Zhang *et al.*, Th-POK regulates mammary gland lactation through mTOR-SREBP pathway. *PLoS Genet* **14**, e1007211 (2018).

Supplementary Figure 1: Pups of female mice lacking *Ocln* died shortly after birth due to severe starvation because of defective mammary gland function.

(**A**) Relative expression of casein proteins as detected by mass spectrometry. (**B**) Diagram depicting the neurological and hormonal circuits governing feeding and milk production of lactating female mice.

Supplementary Figure 2: Tight junction integrity was not compromised by the absence of *Ocln* in mutant mammary glands.

(**A**) mRNA expression of members of the *Claudin* families as detected qPCR at the P17 and the L2 stages. Values were normalized against *actin* expression at P17. Multiple t-test was performed for statistical analysis and SEM was used.

(**B**) Permeability test of organoid epithelium from *Ocln* null and control mice using a biotin-labeled red fluorescent exclusion dye under the conditions indicated. Note EGTA treatments broke cell-cell junctions and made the epithelium more permeable, while PBS treatments did not. Organoids were counter stained using DAPI as a nuclear dye (blue). Scale bars: 20 μm.

Supplementary Figure 3: *Ocln* was not required for alveolar differentiation.

(**A**) Levels of mRNA expression as detected by qPCR of several key regulators of alveologenesis at 10-weeks of age as virgins, during pregnancy (P) on day 5, 12, and 17. Values were normalized against *actin* expression and gene expression of the control gland at 10-weeks of age was set as the base value against which other stages were compared. No statistically significant differences were detected at the times indicated (multiple t-test).

(**B**, **C**) Protein expression of phosphorylated Stat5a (pStat5a) and total Stat5a as detected by Western-blotting. Internal control was Actin (**B**). Relative levels of active Stat5a as measured by the ratio of phosphorylated Stat5a and total Stat5a (**C**).

(**D**) Levels of mRNA expression as detected by qPCR of several key differentiation markers of alveologenesis during P5, 12, and 17. Values were normalized against *actin* expression and gene expression at P5 was set as the base value against which other stages were compared. No statistically significant differences were detected at the times indicated (multiple t-test). Abbreviations: P, pregnancy; L, lactation.

Supplementary Figure 4: *Ocln* deficient mammary glands had a lower than normal

percentage of milk-producing cells (MPCs) due in part to an increase in apoptosis.
(**A**, **B**) Cell proliferation as detected by EdU incorporation (red) on day 5, 10, and 17 during pregnancy (P), and on day 2 of lactation (L) (**C**) and was quantified (**D**). No statistically significant differences among the different regions at the times indicated (unpaired, two-tailed Student's t-test). Scale bars: 20 μm.

Supplementary Figure 5: Gene Ontology (GO) analyses based on single-cell transcriptomics.
(**A**, **B**) GO analysis of the differentially (P value <0.05) expressed genes (**A**) in single luminal cells of *Ocln* control and null mice to determine the biological processes these genes might be involved with (**B**). Data were from scRNA transcriptomics.

Supplementary Figure 6: Alveolar cells lacking *Ocln* show heightened ER stress and Unfolded Protein Response.
(**A**) Examples of some of the highly expressed ER stress genes from *Ocln* control and null single luminal cells. Each dot indicates the expression level based on log2 of the genes in a single cell. T-test was performed for statistical analysis。

Supplementary Figure 7: Epithelial cells lacking *Ocln* showed defective secretion.
(**A-D**) A range of anomalies of secretory vesicles in *Ocln* null mammary glands on day 2 of lactation as revealed by TEM. Note that SVs varied not only greatly in size but also by the numbers and irregular sizes of casein micelles they contained. Also note the deal cells in D' and D''. SV, secretory vesicle; Lu, lumen; Nu, nucleus. Scale bars are as indicated.

Supplementary Figure 8: OCLN localized on secretory vesicles and bound to SNARE proteins.
(**A, B**) Protein binding between OCLN and VAMP4 (A) or SNAP23 (B) as detected by co-immunoprecipitation assays. OCLN was tagged by Flag protein, whereas VAMP4 (**A**) and SNAP23 (**B**) were tagged by HA. Antibody against Flag was used for immunoprecipitation and antibody against HA was used for subsequent Western-Blotting analysis. Numbers indicate molecular weights of the markers (kilodalton).

(**C, D**) Time course of localization of OCLN and VAMP4 (**C**) or SNAP23 (**D**) as detected by fluorescent microscopy. GFP was fused in-frame with OCLN at the N-terminus, whereas mCherry was fused in-frame with VAMP4 (**C**) and SNAP23 (**D**). White arrowheads mark OCLN and VAMP4 (**C**) and SNAP23 (**D**) particles over the time course of observation. Note that 41% and 19% of OCLN particles co-localized with VAMP4 (**C**) and SNAP23 (**D**) particles, respectively.

Supplementary Movies:

Supplementary Movies 1: *Ocln*$^{-/+}$ control organoids responses to oxytocin treatment ex vivo cultures .

Supplementary Movies 2: *Ocln*$^{-/-}$ null organoids responses to oxytocin treatment ex vivo cultures .

Supplementary Movies 3: Live imaging of mammary epithelial cells co-transfected with GFP-Ocln and mCherry-Stx6.

Supplementary Movies 4: Live imaging of mammary epithelial cells co-transfected with GFP-Ocln and mCherry-VAMP4.

Supplementary Movies 5: Live imaging of mammary epithelial cells co-transfected with GFP-Ocln and mCherry-SNAP23.

SUPPLEMENTARY TABLE1. Colocalization coefficients of OCLN and SNARE proteins.

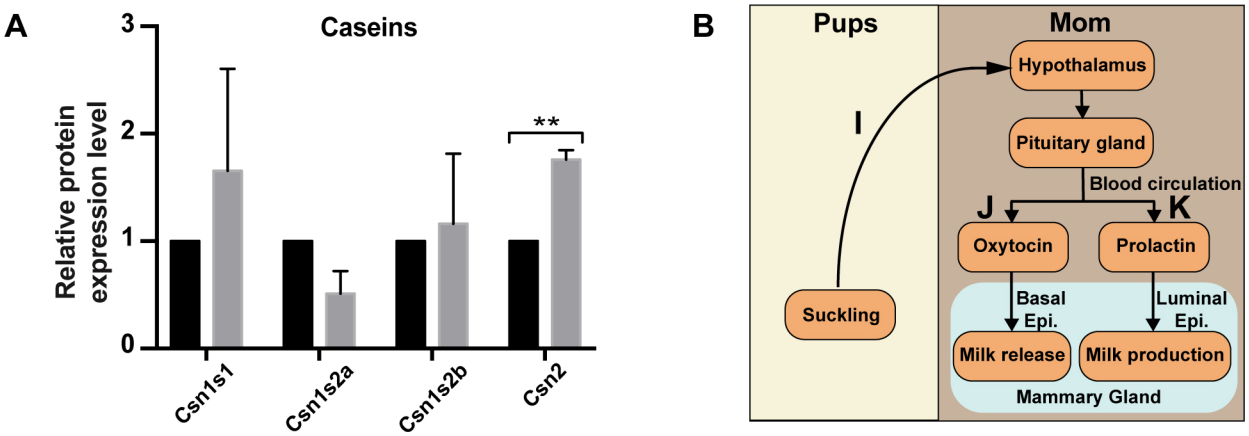
	Pearson's Coefficient	Overlap Coefficient	Manders' Coefficients M1	M2
OCLN and Stx6 colocalization	0.30±0.11	0.83±0.07	0.21±0.13	0.38±0.14
OCLN and VAMP4 colocalization	0.45±0.20	0.90±0.04	0.41±0.16	0.51±0.23
OCLN and SNAP23 colocalization	0.74±0.13	0.96±0.02	0.19±0.09	0.26±0.09

Note based on these coefficients, if n=1, it means two proteins have perfect colocalization; whereas if n=0, it means they do not colocalize.

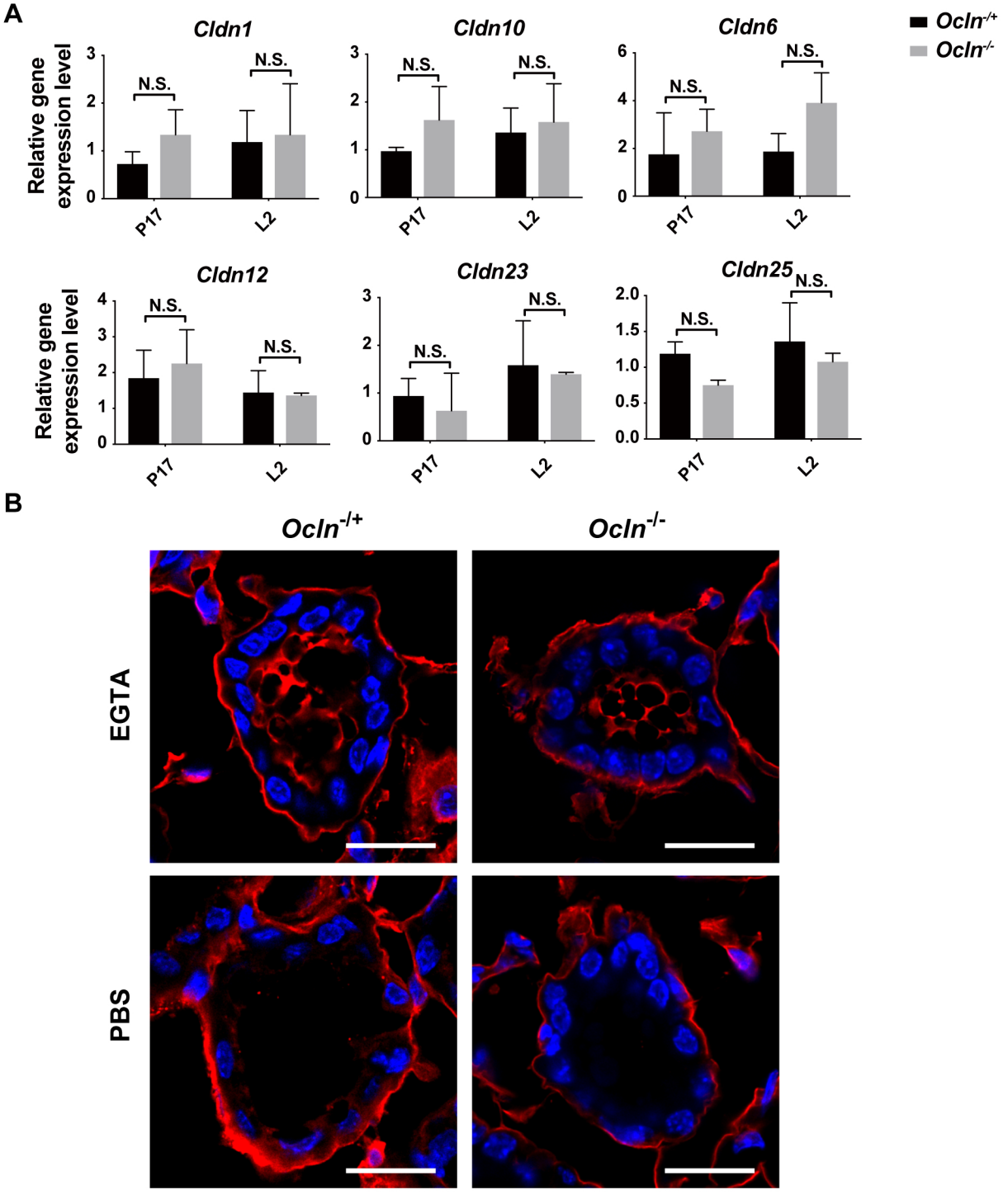
SUPPLEMENTARY TABLE2. Primers used in qPCR.

Gene name	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
Cldn1	AGCACCGGGCAGATACAGT	GCCAATTACCATCAAGGCTCG
Cldn10	GGTCAGCCTGGGATTTTTC	GAACAGCCTGTCATGGAACA
Cldn12	CTTCCTGTGTGGTATTGCCTC	AAATCGTCAGGTTCTTCTCGTTT
Cldn23	CCCGACGAGTGGAACTACTTC	GGCCAGCGACGAAAAACAC
Cldn3	ACCAACTGCGTACAAGACGAG	CAGAGCCGCCAACAGGAAA
Cldn4	GTCCTGGGAATCTCCTTGGC	TCTGTGCCGTGACGATGTTG
Cldn5	GCAAGGTGTATGAATCTGTGCT	GTCAAGGTAACAAAGAGTGCCA
Cldn6	ATGGCCTCTACTGGTCTGCAA	GCCAACAGTGAGTCATACACCTT
Cldn7	GGCCTGATAGCGAGCACTG	TGGCGACAAACATGGCTAAGA
Cldn8	GCAACCTACGCTCTTCAAATGG	TTCCCAGCGGTTCTCAAACAC
Cldnd1(Cldn25)	ATGGATAACCGTTTTGCTACTGC	CATACCAGAAGTCCGTGCCTA
ZO-1	GCACAGCAATGGAGGAAACA	TCAGCTGGCCCTCCTTTTAA
ZO-2	ATGGGAGCAGTACACCGTGA	GCTGAACGGCAAACGAATGG
ZO-3	TCGGCATAGCTGTCTCTGGA	GTTGGCTGTTTTGGTGCAGG
C/ebpβ(Cebpb)	CAAGAAGACGGTGGACAAGC	AGCTGCTCCACCTTCTTCTG
Nf1	GTGGCCCCTACAAATCATTCTC	TGCTTTCCGTAAACTGTCCAG
Etv4(Pea3)	TCCCGGAAATGGGAGCTT	GAGCCACGTCTCTTGGAAGT
Elf5	ACCGATCTGTTCAGCAATGAAG	CGCTTGGTCCAGTATTCAGG
Prlr	TGCTTACATGCTGCTTGTCC	TGAATAATTGGTGGGGAGTCC
Pgr	AGGTCTACCCGCCATACCTT	CAGCTCCCACAGGTAAGCAC
RankL(OPGL/Tnfsf11)	GTCTGCAGCATCGCTCTGT	CAGGTAGTGTGTCTTCACTCTCCA
CSF1	CTGCCCTTCTTCGACATGG	TTCAGGTGTCCATTCCCAAT
Igf2	CGTGGCATCGTGGAAGAGT	ACGTCCCTCTCGGACTTGG
Esr1	CCTCCCGCCTTCTACAGGT	CACACGGCACAGTAGCGAG
Lao1	CCAGAATGGGCTCCATACCTC	TCCAAGATGGTTACCTCGTGA
Glycam1	CCACCAGCTACACCAGTGAG	CCTGGGCCTCTTGATTCTCTG
Wap	CGCTCAGAACCTAGAGGAACA	GAGTTTTGCGGGTCCTACCAC
α-lactalbumin	TTCAGAAGGTGAATCTCATGGGA	ATGTTTTGTGGGACGGGATTG

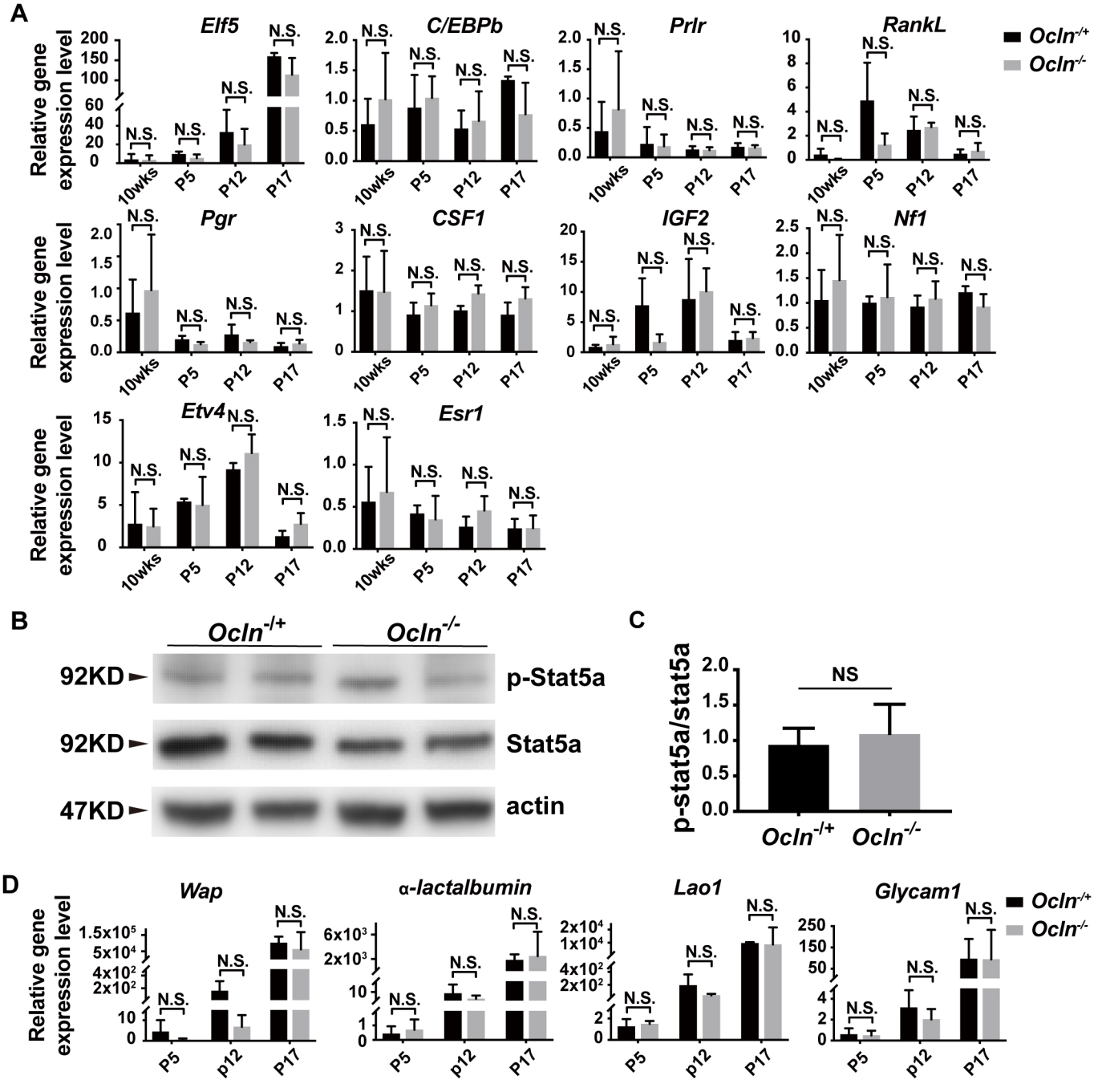
Supplementary Figure 1. Pups of female mice lacking *Ocln* died shortly after birth due to severe starvation because of defective mammary gland function.



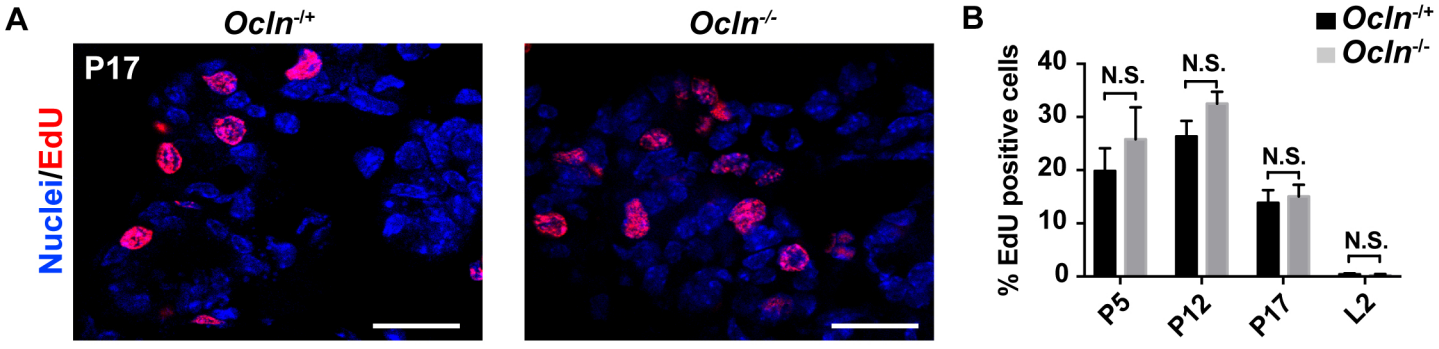
Supplementary Figure 2. Tight junction integrity was not compromised by the absence of *Ocln* in mutant mammary glands.



Supplementary Figure 3. ***Ocln*** **was not required for alveolar cell differentiation.**



Supplementary Figure 4. ***Ocln*** **deficient mammary glands had a lower than normal percentage of milk-producing cells due in part to an increase in apoptosis.**



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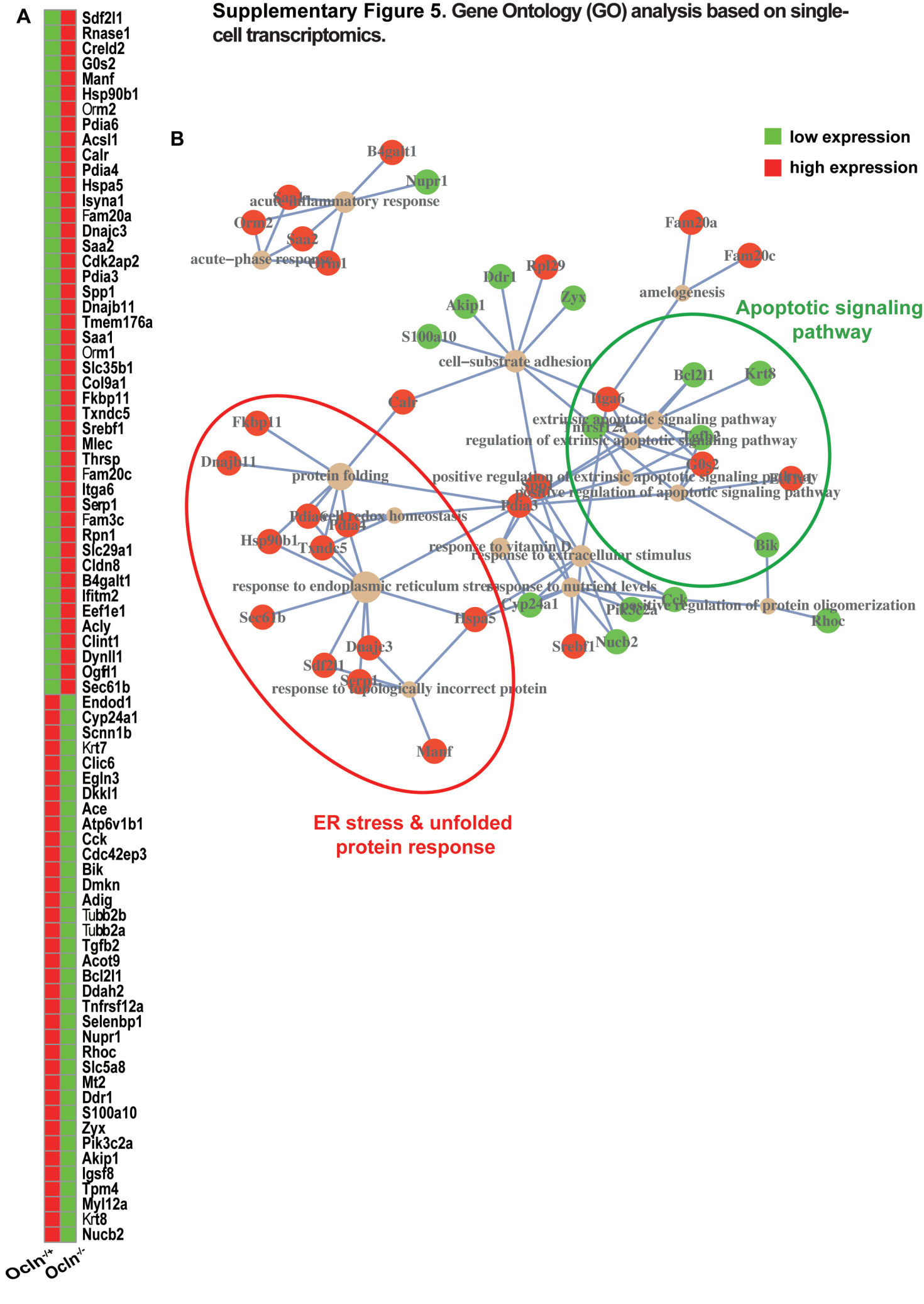

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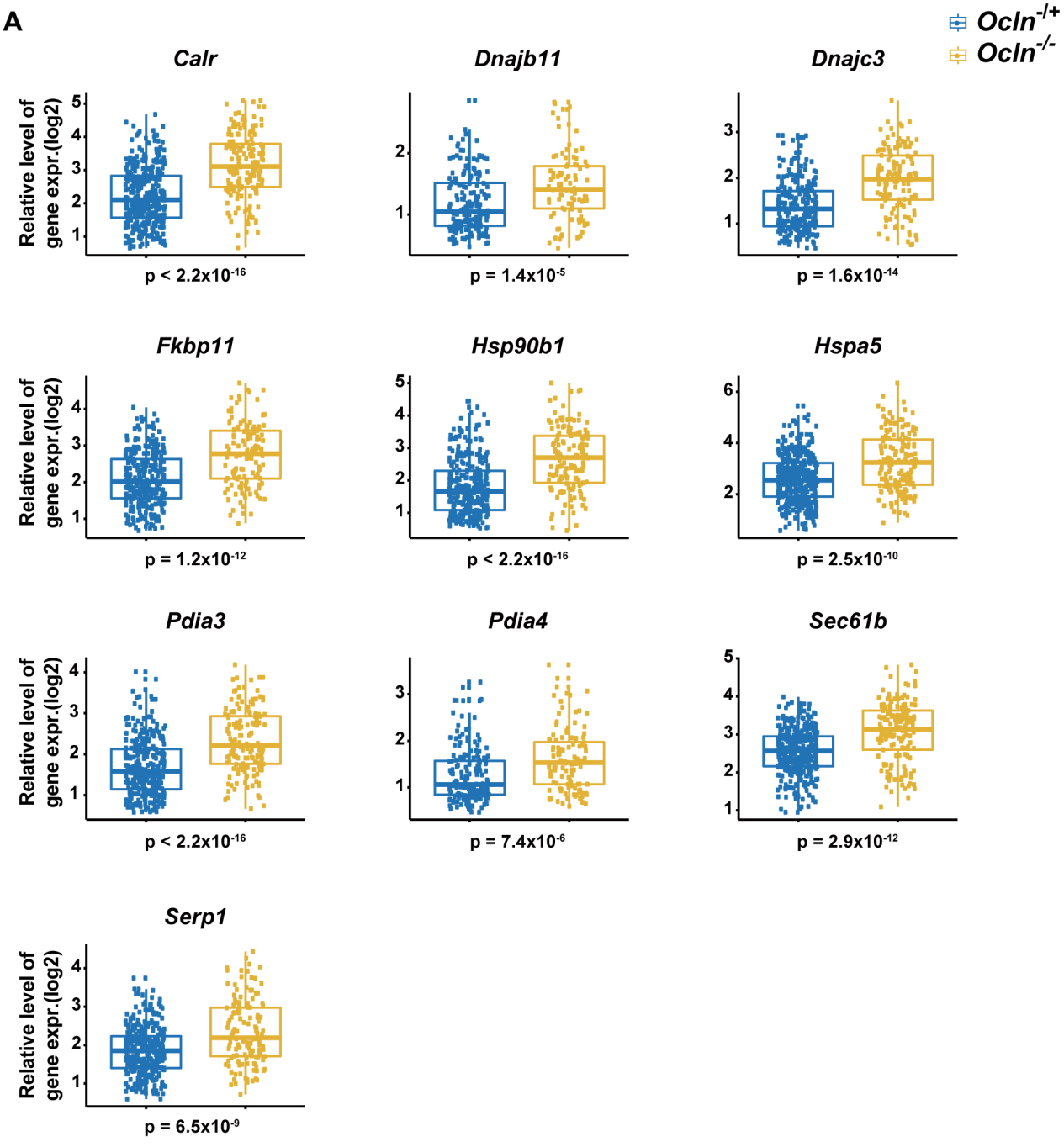
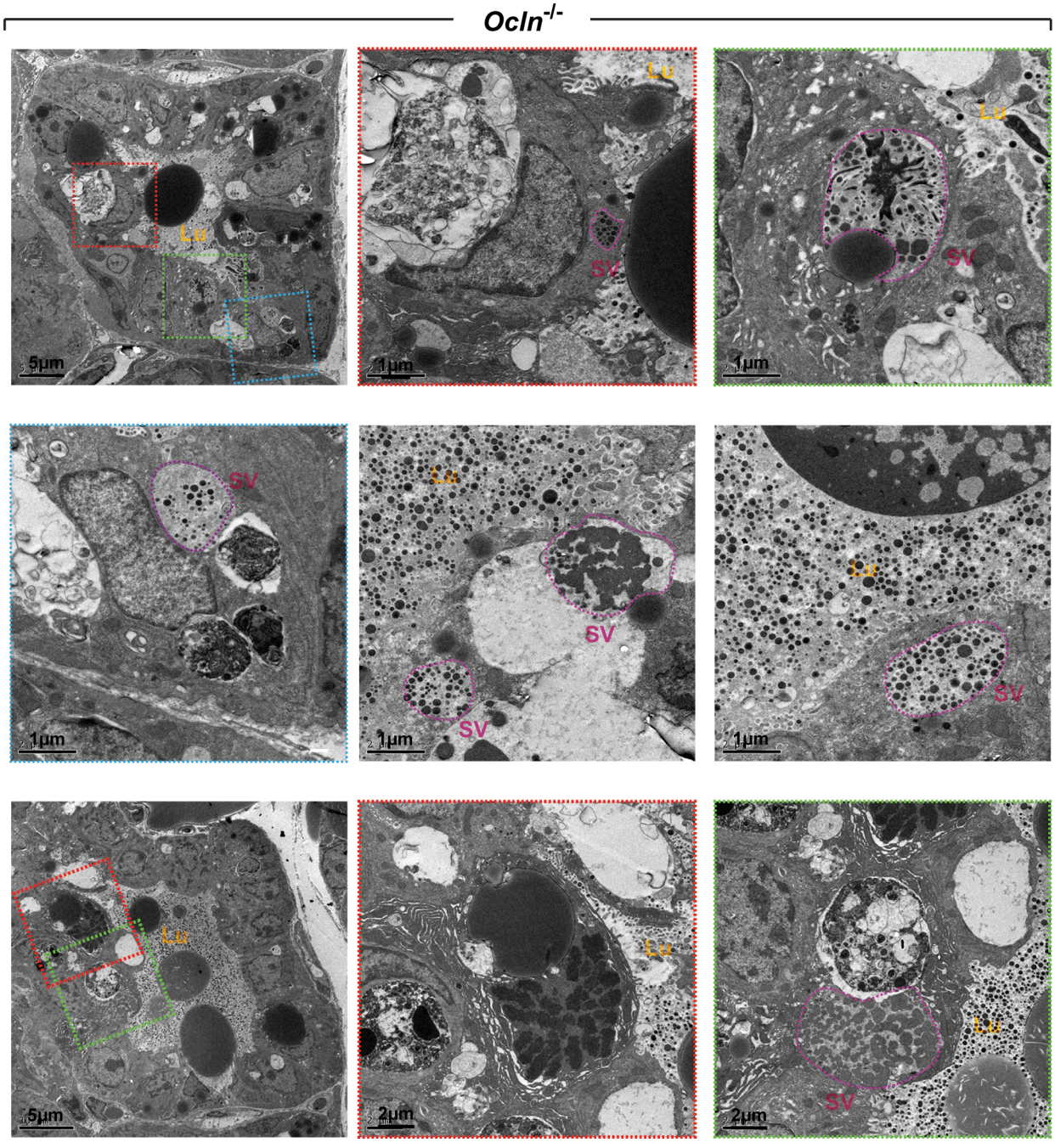


Supplementary Figure 7. Epithelial cells lacking *Ocln* showed defective secretory pathway.



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