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**Extracellular SPON2 Protein Inhibits Metabolism by Blocking Glutamine Uptake to
Coordinate Mammary Morphogenesis and Cancer Suppression**

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ABSTRACT

Tissue morphogenesis and tumor progression are governed by reciprocal interactions between epithelial cells and their microenvironment, yet how epithelial niches regulate metabolic fitness during development and cancer remains poorly understood. Here, we identify SPON2 as a basal-derived extracellular protein that functions as a metabolic checkpoint during mammary gland morphogenesis and breast cancer suppression. *Spon2* was highly enriched in basal epithelial cells during pubertal branching, and its genetic deletion reaccelerated ductal elongation, epithelial proliferation, and branch formation in vivo. Mechanistically, SPON2 inhibits mitochondrial metabolism, ATP production, and mTORC1 activation independently of canonical TGF- β or FGF signaling. SPON2 directly binds the glutamine transporter SLC38A1 through its F-Spondin domain, reducing glutamine uptake and cell-surface transporter abundance. SPON2-mediated glutamine restriction impaired leucine-dependent mTOR Activation, resulting in mitochondrial dysfunction and suppression of epithelial expansion. Importantly, SPON2 expression was broadly reduced across major breast cancer subtypes, whereas restoration of SPON2 suppressed tumor growth in luminal, triple-negative, and patient-derived breast cancer models. Together, these findings establish an epithelial metabolic niche mechanism in which a basal-derived extracellular matrix protein directly gates nutrient accessibility to coordinate developmental homeostasis and suppress oncogenesis.

INTRODUCTION

It has been increasingly recognized that interactions between epithelial cells and their microenvironment are essential for organ development, tissue homeostasis, and disease progression¹⁻⁴. The therapeutic importance of tissue microenvironments is exemplified by the clinical success of anti-angiogenic agents and immune checkpoint inhibitors, which target vascular and immune components of the tumor microenvironment, respectively^{5,6}. Traditionally, studies of tissue microenvironments have focused primarily on stromal compartments, including fibroblasts, immune cells, and extracellular matrix (ECM) networks⁷⁻⁹. However, emerging evidence suggests that epithelial subtypes within tissues can themselves function as dynamic microenvironments or niches for one another^{10,11}. Such epithelial niche interactions may be particularly important in cancer, where reciprocal support between heterogeneous tumor cell states could contribute to tumor stability, progression, and therapeutic resistance^{12,13}. Accordingly, elucidating the signaling mechanisms that coordinate communication between epithelial cells and their niche compartments is essential not only for understanding organogenesis and tissue homeostasis, but also for identifying new therapeutic vulnerabilities in cancer.

The developing mouse mammary gland provides an exceptional model for dissecting functional interactions between epithelial cells and their stromal and epithelial microenvironments¹⁴. During branching morphogenesis, an intricate epithelial ductal tree is generated within the stromal fat pad through highly coordinated interactions among basal and luminal epithelial cells and surrounding stromal compartments^{15,16}. Stromal-derived signals play central roles in regulating this process¹⁷. For example, FGF10, produced by fibroblasts, promotes epithelial proliferation, migration, and bud bifurcation through epithelial FGFR2 signaling¹⁸⁻²⁰, whereas TGF- β acts as a key negative regulator^{21,22} that constrains excessive outgrowth, spaces ducts, and modulates extracellular matrix remodeling. Negative feedback regulators such as Sprouty (Spry) proteins further fine-tune FGF signaling to ensure balanced epithelial expansion and morphogenesis²³.

In addition to stromal regulation, basal and luminal epithelial cells engage in reciprocal signaling networks that coordinate mutual expansion and tissue architecture²⁴. For example, BMP7–INHBA-mediated signaling between epithelial compartments sustains branching morphogenesis through positive-feedback interactions¹². Increasing evidence further suggests that epithelial subtypes exhibit metabolic specialization and that niche-derived signals can regulate

nutrient utilization, energy production, and biosynthetic capacity. For instance, basal-derived EFNA3 modulates luminal cell oxidative phosphorylation and branching morphogenesis²⁵. Together, these findings indicate that epithelial niche interactions extend beyond canonical developmental signaling pathways to include metabolic coordination between distinct epithelial compartments. However, the molecular mechanisms through which epithelial niche factors regulate metabolic coupling and coordinated epithelial growth during mammary morphogenesis remain poorly understood.

Among the candidate epithelial niche factors identified in our previous studies, SPON2 (Spondin-2, also known as Mindin) emerged as a particularly intriguing regulator (**Fig. 1A**). SPON2 is a secreted extracellular matrix protein belonging to the F-Spondin family and has been implicated in innate immunity, integrin signaling, extracellular matrix organization, and tumor progression across multiple tissue contexts²⁶⁻²⁹. In cancer, SPON2 has been reported both as a diagnostic biomarker and as a pro-tumorigenic factor in prostate and several other malignancies³⁰⁻³⁴. Nevertheless, its biological function and mechanistic mode of action in epithelial development and tumor progression remain poorly characterized. In this study, we hypothesized that SPON2 is essential for mammary gland formation and its dysregulation promotes breast cancer development. To test this, we generated *Spon2* knockout mice, performed gain/loss-of-function transplants and organoids, conducted scRNA-seq and metabolic assays, which uncovered SPON2's mode of action and therapeutic relevance.

RESULTS

Candidate Breast Tumor Suppressor SPON2 Inhibits Mammary Epithelial Branching Morphogenesis

To investigate how SPON2 expression correlates with breast cancer, we examined its expression profile based on the Genotype-Tissue Expression (GTEx) and The Cancer Genome Atlas (TCGA) databases, which focuses on normal and cancer tissues, respectively. We found that SPON2 mRNA levels were significantly downregulated in breast tumors compared to adjacent normal tissues (**Fig. 1B**). Kaplan-Meier survival analysis showed that higher SPON2 expression was associated with better overall prognosis in breast cancer patients (**Fig. 1C**). Thus, contrary to studies reporting SPON2 as a poor prognostic marker in other cancer types, these findings suggest that SPON2 is a candidate breast tumor suppressor.

We predicted that, if SPON2 is a tumor suppressor, then its loss would likely cause accelerated mammary gland development in the mouse. To test this prediction, we determined SPON2's expression pattern during mammary gland development. Based on published single-cell RNA-sequencing (scRNA-seq) datasets³⁵ we found that *Spon2* mRNA was highly enriched in basal epithelial cells but absent in luminal cells at key postnatal stages (2.5 weeks, 5 weeks, and 10 weeks) when epithelial branching morphogenesis is active (**Fig. 1D**). Notably, expression peaked at 5 weeks, coinciding with the period of maximal postnatal branching activity (**Fig. 1D**). Using cDNA templates generated from basal and luminal cells from 5-week-old mammary glands, we performed qPCR and confirmed that *Spon2* mRNA was predominantly expressed in basal cells (**Fig. 1E**). Moreover, based on the above scRNA-seq datasets, we found that *Spon2* mRNA was more highly enriched in basal cells of the ductal epithelium, where side-branches form, than in basal cells of the TEBs (**Supplementary Fig. 1A, B**), where directional migration and branch-bifurcations are active³⁶.

To determine *Spon2* function in the mammary gland, we generated a global *Spon2* knockout (KO) mouse line by deleting all coding exons using CRISPR/Cas9 (**Supplementary Fig. 1C**). PCR genotyping and qPCR confirmed successful gene disruption and absence of *Spon2* mRNA in KO tissues (**Supplementary Fig. 1D, E**). As previously reported, female mice homozygous for the *Spon2* null allele appeared normal and were fertile²⁷. Pups born to *Spon2* null female mice survived and gained weight similarly as those born to control mice (**Supplementary Fig. 1F, G**).

Because we observed no differences in epithelial morphogenesis between wild-type (*Spon2*^{+/+}) and heterozygous (*Spon2*^{+/-}) mammary glands (**Supplementary Fig. 1H-J**), heterozygous littermates were used as controls in the subsequent analyses. Consistent with its normal organ physiology, *Spon2* null mammary glands showed no defects in cell differentiation, as indicated by the presence of K8⁺ luminal cells, K14⁺ basal cells, and their overall normal arrangement and tissue architecture when compared to the control gland (**Supplementary Fig. 1K**).

To determine whether epithelial morphogenesis was normal in the mutant mice, we harvested the inguinal mammary glands at several key branching stages, stained them with the Carmine alum dye, and photographed them whole-mount using a stereoscope. We found that both ductal elongation and branch point density were comparable between control and KO glands at the

3-week stage (**Fig. 1F, G, L, M**). However, at both the 6-week and 9-week stages, KO glands exhibited significantly greater ductal elongation and increased branch points per mm² compared to controls (**Fig. 1H–M**). Thus, consistent with our prediction, loss of *Spon2* caused an accelerated branching phenotype during the period of active pubertal development.

It is possible that the observed phenotypes resulted from systemic defects in the mutant mice, given *Spon2* expression in multiple tissues ²⁷. Therefore, we decided to test whether removal of *Spon2* function directly from the mammary epithelium and, specifically, from basal cells would produce a similar phenotype as observed in the global KO mice. To test the basal-intrinsic requirement for *Spon2*, we performed both loss-of-function (LOF) and gain-of-function (GOF) assays based on transplantation, where mammary epithelial aggregates containing wildtype luminal cells and manipulated basal cells were injected into the cleared fat pads of 3-week-old nude mice (**Fig. 1N**). For LOF, we screened four shRNA constructs and selected sh*Spon2*-3, which achieved ~70% knockdown efficiency by qPCR (**Supplementary Fig. 1L**). Transplanting sh*Spon2*-transfected epithelial cells resulted in enhanced regenerative outgrowth and increased branch-point formation at 5 weeks post-transplantation compared to shScr controls (**Fig. 1O–R**). By contrast, transplantation of mammary epithelium with basal cells overexpressing *Spon2*, where its mRNA expression increased by ~30 fold (**Supplementary Fig. 1M**), led to the opposite phenotype. Specifically, the regeneration outgrowth and branch-point formation were severely reduced in the GOF experimental glands when compared with control glands even by 7-weeks post-transplantation (**Fig. 1S–V**).

Together, the results from global KO and basal-specific LOF and GOF transplantation experiments are consistent with the prediction that *Spon2* is a candidate breast tumor suppressor. They demonstrate that SPON2 inhibits mammary epithelial branching morphogenesis in a basal-autonomous manner.

***Spon2* Suppresses Epithelial Expansion by Blocking Cell Proliferation and Promoting Cell Death**

Mammary branching morphogenesis is a multistep process involving branch-point formation, primarily driven by cell proliferation but also involving collective migration, and subsequent ductal elongation, dependent mainly on cell proliferation and rearrangement ^{36,37}. To determine if SPON2 affects migration, we used a 3D in vitro assay, where organoids directionally migrate to

point-source FGF10 delivered via soaked beads^{20,38,39}. Aggregates from shScr or sh*Spon2*-infected epithelial cells migrated toward the FGF10 bead over 72 hours with similar trajectories (**Supplementary Fig. 2A, B**), and quantitative measurements of displacement and organoid size showed no significant differences (**Supplementary Fig. 2C, D**).

Next, we employed an FGF2-supplemented 3D organoid culture system in which uniform growth factor exposure promotes isotropic branching driven primarily by cell number increase and cell rearrangement^{16,40,41}. We transfected basal cells with shScr or sh*Spon2*, sorted GFP⁺ transfected cells, mixed them 1:1 with wild-type luminal cells, and cultured aggregates for 7 days (**Fig. 2A, B**). Consistent with the *in vivo* results, we found that sh*Spon2* organoids exhibited ~30% more branches than controls (**Fig. 2C**). Immunofluorescence of Ki67, a marker of proliferating cells, showed that there were ~38% more Ki67⁺ cells in sh*Spon2* organoids than in control organoids (**Fig. 2D, Supplementary Fig. 2E, F**). By contrast, immunofluorescence of Caspase-3, an apoptotic marker, showed that there was a significant decrease in apoptotic cells in sh*Spon2* organoids compared to controls (**Fig. 2E, Supplementary Fig. 2G, H**).

We also sought to complement LOF with GOF by overexpressing *Spon2* in basal cells, mixed them with luminal cells, and cultured them in FGF2 medium. *Spon2* GOF reduced branch numbers by ~25% (**Fig. 2F–H**), with ~20% fewer Ki67⁺ cells (**Fig. 2I; Supplementary Fig. 2I, J**) and a significant increase in cleaved caspase-3⁺ (CC3⁺) cells (**Fig. 2J; Supplementary Fig. 2K–L**). Interestingly, addition of recombinant SPON2 protein (rSPON2) to the medium recapitulated the GOF phenotype by reducing branches in mixed organoids compared to BSA controls (**Fig. 2K–M**), indicating that the protein is bioactive. Therefore, for some of the following GOF experiments, we used this alternative method for its ease of use.

Previous studies show that luminal cells are the main drivers of epithelial branching, while basal cells are essential facilitators²⁵. Thus, we next focused on the effect of SPON2 on luminal cells. Unlike basal cells, which do not efficiently branch in the FGF2-based *in vitro* assay when cultured alone¹², luminal aggregates form branches in this condition but rSPON2 greatly inhibited branching of luminal aggregates by around half (**Fig. 2N–P**). Consistent with decreased branching, cell proliferation was reduced in treated luminal cells, as indicated by fewer Ki67⁺ cells (**Supplementary Fig. 2M–O**), and an increase in CC3⁺ cells in the rSPON2-treated organoids

(**Supplementary Fig. 2P–R**). The results thus indicate that SPON2 inhibits mammary epithelial branching by, at least in part, suppressing cell proliferation.

Does SPON2 limit cell proliferation only in luminal cells or also in basal cells? If the inhibition was only restricted to luminal cells, then we would predict that the luminal and basal cell composition would be affected in *Spon2* null epithelium because they normally remain constant during development and homeostasis^{10,42}. To determine the luminal and basal composition in the mammary epithelium, we stained their respective cell markers, followed by an FACS analysis. We found that luminal percentage was similar, at ~63% in both *Spon2* null and control epithelia (**Supplementary Fig. 2S, T**). Likewise, quantification of K8⁺ luminal cells in the null and control epithelia showed a similar result (**Supplementary Fig. 1K; Supplementary Fig. 2U**). Furthermore, rSPON2 addition to the culture medium reduced the number of colonies formed similarly by both luminal cells and basal cells (**Fig. 2Q–T**). Together, these results confirm that SPON2 exerts a similar inhibition on both luminal and basal cell numbers, thus explaining why the luminal/basal composition remained normal in the null mammary epithelium.

Finally, we sought to validate these in vitro data by in vivo mouse genetics using the 5-week branching mammary glands. Because there is little cell death at this stage¹⁹, we focused on the analysis of cell proliferation. We found that cell proliferation, as measured by the number of Ki67⁺ cells, increased by ~100% in KO versus heterozygous glands (**Fig. 2U, V**).

Taken together, these LOF, GOF, protein addition, and in vivo data indicate that SPON2 suppresses epithelial branching by limiting cell proliferation under physiological conditions and inducing cell death when overexpressed, with effects on both basal and luminal subtypes.

***Spon2* Inhibits Mitochondrial Energy Production Independently Of TGF- β Signaling**

TGF- β is well known for its inhibitory role in mammary gland branching^{21,43}. Therefore, we asked whether TGF- β exerts its inhibitory role by transcriptionally activating *Spon2*. We found that addition of TGF- β to the culture medium of mammary gland organoids or basal cells at different concentrations did not elicit significant changes in *Spon2* mRNA expression (**Supplementary Fig. 3A, B**). Moreover, unlike *Spon2*, which is specifically expressed in basal cells, especially in ductal basal cells, *Tgfbr1* and *Tgfbr2* (**Supplementary Fig. 3C, D**), encoding the receptors of TGF- β , and *Spry1* and *Spry2* (**Supplementary Fig. 3E, F**), which inhibit branching as target genes and negative-feedback regulators of FGF signaling^{20,44}, were more broadly distributed across the

different cell subtypes. The data suggest that *Spon2* is unlikely to function downstream of TGF- β or FGF signaling to inhibit epithelial branching.

To uncover the mechanism underlying SPON2's inhibitory effects, we sought to profile transcriptional alterations in *Spon2* KO mammary glands. We dissociated cells from 5-week heterozygous and KO glands, performed scRNA-seq with approximately 10,000 total cells captured per genotype, including basal, luminal, fibroblast, and immune cell populations, and visualized clusters via tSNE (**Fig. 3A**). Genotype-split tSNE showed similar cell type distributions (**Fig. 3B**), consistent with the normal differentiation observed earlier.

To identify SPON2-regulated genes in luminal cells, we generated a volcano plot of differentially expressed genes (DEGs) (**Fig. 3C**). We found that TGF- β signaling overall was not significantly changed in *Spon2* null basal cells, as indicated by the mRNA expression of the individual ligands, receptors, and downstream target readouts, as well as the Gene Set Enrichment Analysis (GSEA) of the pathway (**Supplementary Fig. 3G–J**). Likewise, TGF- β signaling was not significantly changed in *Spon2* null luminal cells, as shown by the mRNA expression of the individual components and GSEA of the pathway (**Supplementary Fig. 3K–N**). Similarly, we found that *Spry1/2* and the FGF signaling pathway were not significantly changed when *Spon2* was lost (**Supplementary Fig. 3O–R**).

Therefore, we took an unbiased approach and focused on the top 40 most differentially expressed genes (DEGs) (**Supplementary Fig. 4A**). Notably, Gene Ontology Biological Process (GOBP) analysis of these DEGs showed that, consistent with the results showing that *Spon2* limits epithelial proliferation, the process of “Regulation of epithelial cell proliferation” was enriched in the assay. Moreover, energy metabolism processes, including “Glycolysis,” “OXPHOS,” “ATP metabolism,” and “Gln-family amino acid metabolism,” were also enriched (**Fig. 3D**). This was further confirmed by a Gene Set Enrichment Analysis (GSEA), which showed that they were significantly increased in *Spon2* null luminal cells compared to control luminal cells (**Fig. 3E**).

Based on these LOF results, we predicted that SPON2 GOF would cause reduced energy metabolism. Therefore, we treated organoids with recombinant SPON2 and repeated the scRNA-seq experiment. Again, rSPON2 treatment did not change cellular composition (**Fig. 3F**). As predicted, GOBP analysis of the top DEGs showed that mitochondrial respiration and energy production, including “Energy metabolism,” “Mitochondrial ATP synthesis,” and “Electron

transport,” were enriched (**Fig. 3G**). GSEA analysis further confirmed that core metabolic pathways (glycolysis, fatty acid degradation, TCA cycle, and oxidative phosphorylation) were reduced in rSPON2-treated organoids (**Fig. 3H**). qPCR validated downregulation of representative genes in these pathways (**Supplementary Fig. 4B-E**).

To test if SPON2 inhibits metabolic disruption, as suggested by transcriptomics, we quantified ATP levels in organoids. *Spon2* knockdown significantly increased intracellular ATP, whereas SPON2 overexpression decreased it (**Fig. 3I, J**). To explore the mitochondrial basis of this ATP deficit, we used MitoTracker Red CMXRos staining, which accumulates based on mitochondrial membrane potential ($\Delta\Psi_m$). High-throughput flow cytometry across LOF (**Supplementary Fig. 4F, G**) and GOF models using OE-*Spon2* (**Supplementary Fig. 4H, I**) or rSPON2-treatment (**Supplementary Fig. 4J, K**) confirmed that SPON2 levels inversely correlated with MitoTracker intensity. Likewise, imaging of luminal cells also showed markedly reduced MitoTracker signal after rSPON2 treatment compared to controls (**Fig. 3K-M**).

To measure functional consequences on bioenergetic pathways, we conducted Seahorse XF Real-Time ATP Rate Assays. SPON2 treatment lowered both oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) (**Fig. 3N**). Detailed OCR dissection showed reduced basal respiration, ATP-linked OCR with near-abolished proton leak (**Supplementary Fig. 4L, M**), consistent with impaired mitochondrial function. Deconvolution quantified suppressed mitochondrial ATP production (**Supplementary Fig. 4N**), with no compensatory increase in glycolytic ATP—rather, it too was reduced (**Supplementary Fig. 4O**).

Together, these data demonstrate that SPON2 inhibits luminal cell function by suppressing mitochondrial metabolism. *Spon2* loss leads to increased metabolism, while its overexpression induces a metabolic crisis in luminal cells by collapsing $\Delta\Psi_m$ and failing oxidative phosphorylation, without glycolytic adaptation, leading to ATP depletion.

***Slc38a1* Is a *Spon2* Downstream Target Essential for Epithelial Branching**

As a secreted protein, SPON2 likely regulates luminal metabolism through a membrane target protein to initiate downstream events. To identify its protein-binding partner, we tagged SPON2 with a three-repeat FLAG peptide and used the fusion protein as bait in a pull-down assay using luminal cell lysates before the precipitates were analyzed by mass spectrometry (**Fig. 4A**). We then performed a volcano plot to identify enriched proteins (**Fig. 4B**) and focused on the 20 most

enriched candidates (**Fig. 4C**). Among these protein candidates, we were especially intrigued by SLC1A4 and SLC38A1 because they are annotated as neutral amino acid transporters responsible for glutamine uptake and metabolism, which is enriched in *Spon2* null luminal cells (**Fig. 3D**).

Next, we tested the candidacy of SLC1A4 and SLC38A1 as target proteins through which SPON2 regulates luminal metabolism. Mining of published scRNA-seq databases showed that *Slc1a4* (**Fig. 4D**) and *Slc38a1* (**Fig. 4E**) are expressed in both basal and luminal cells during the branching stages of the mammary gland, although the latter is more highly expressed than the former. Importantly, however, protein-binding assays based on co-immunoprecipitation (co-IP) showed that SLC38A1, but not SLC1A4, bound to SPON2 (**Fig. 4F, G**). Therefore, we focused on functional analysis of SLC38A1 in subsequent experiments.

Using shRNA technology, we reduced *Slc38a1* mRNA expression by ~60% compared to control (**Fig. 4H**). shRNA-mediated *Slc38a1* knockdown caused a 40% reduction in epithelial branching of mammary organoids (**Fig. 4I, J**). The results thus indicated that *Slc38a1* promotes epithelial branching, opposite to *Spon2* function in the mammary gland as expected. To confirm that *Slc38a1* indeed functions downstream of *Spon2*, we tested how *Slc38a1* LOF in luminal cells affected *Spon2* LOF and GOF in basal cells in organoids composed of both basal and luminal cells. Again, *Spon2* LOF in basal cells alone increased organoid branching; however, this increase in branching was reversed when *Slc38a1* was simultaneously downregulated in the luminal cells of the organoid (**Fig. 4K, L**). Conversely, although *Spon2* overexpression suppressed branching, this suppression was non-additive when *Slc38a1* was already knocked down, consistent with both acting within the same pathway (**Fig. 4M, N**). These data place SLC38A1 downstream of SPON2 in the regulation of branching morphogenesis.

Together, these data show that *Slc38a1* is a downstream target of *Spon2* and that *Spon2* inhibition of *Slc38a1* is essential for normal epithelial branching in the mammary gland. Loss of *Slc38a1* recapitulated the *Spon2*-GOF phenotype and suppressed epithelial branching. Moreover, considering that *Slc38a1* is also expressed in basal cells, *Spon2* may suppress cell proliferation via a similar mechanism in both basal and luminal cells.

SPON2 Suppresses Glutamine Import via Competition and SLC38A1 Endocytosis

Based on the above data, we predicted that *Spon2* blocks SLC38A1-mediated glutamine transport. To test this prediction, we visualized cytoplasmic glutamine, which was tagged by the fluorophore

Cy5, and examined how its levels, as indicated by Cy5 fluorescent intensity, were affected by changes in *Slc38a1* or *Spon2* expression. As expected, we found that *Slc38a1* LOF mediated by shRNA greatly diminished cytoplasmic glutamine (**Supplementary Fig. 5A, B**). Reduction in cytoplasmic glutamine levels, measured by Cy5 intensity on confocal microscopy (**Fig. 5A, B**) and flow cytometry (**Fig. 5C, D**), was also observed in SPON2-GOF, as mediated by rSPON2 addition to the medium, compared to control. Together, these data confirmed that SPON2 blocks glutamine import via targeting SLC38A1.

One possible mechanism by which SPON2 blocks glutamine intake is by competing with it for the transporter. If so, one would predict that glutamine addition into the medium would ameliorate SPON2 suppression of epithelial branching. Therefore, we overexpressed *Spon2* in basal cells of the organoid aggregates and compared the *Spon2* GOF effect in the absence or presence of glutamine addition. As expected, *Spon2* GOF in basal cells reduced organoid branching; however, this was partially reversed when glutamine was added to the medium (**Fig. 5E, F**). Together, the data suggest that SPON2 suppresses glutamine intake by, at least in part, blocking its access to the transporter SLC38A1.

Next, we sought to examine how physical binding by SPON2 may affect SLC38A1 cellular localization and, thus, its function. To this end, we created overexpression constructs where SPON2 and SLC38A1 were tagged by mCherry and GFP, respectively, at the C-terminus of the proteins. We then transduced the luminal HC11 line of cells with lentivirus carrying SLC38A1-GFP or SPON2-mCherry (**Fig. 5G**). As expected, SLC38A1-GFP was membranous, consistent with its being a transporter, in HC11 cells transduced with the virus (**Fig. 5H, H', J, K**). Interestingly, when SLC38A1-GFP⁺ cells were grown adjacent to SPON2-mCherry⁺ cells, there was significant cytoplasmic buildup of SLC38A1 in addition to its membranous presence (**Fig. 5I, I', J, K**).

The cytoplasmic SLC38A1 signal seemed punctate, presumably indicating the protein was en route to lysosome-mediated degradation because of SPON2-mediated endocytosis. To quantify SPON2-mediated loss of SLC38A1 protein expression, we isolated it from both the total lysate and cell-surface fraction of the expressing luminal cells. We found that rSPON2-mediated GOF did not significantly affect SLC38A1 expression level in the total lysate (**Fig. 5L, M**); interestingly, however, SLC38A1 surface localization was reduced by around 25% by rSPON2 (**Fig. 5N, O**).

Taken together, the above results show that *Spon2* suppresses glutamine intake by blocking its access to the transporter SLC38A1. Moreover, binding of SPON2 to SLC38A1 also reduces its cell-surface abundance, thus further blocking glutamine import.

The F-Spondin Domain Blocks mTOR Activation by Attenuating Gln-Leu Exchange

Can a part of the SPON2 full-length protein exert its inhibitory role on glutamine import? To answer this question, we generated Flag-tagged full-length SPON2 and domain-truncated constructs, including the F-Spondin domain and TSR domain fragments (**Fig. 6A**). Indeed, co-IP experiments confirmed that the Spondin domain, but not the TSR domain, bound to SLC38A1 protein (**Fig. 6B, C**). The data thus support that SPON2 suppresses glutamine intake via its F-Spondin domain-mediated SLC38A1 binding and functional inhibition. Consistent with this notion, we found that addition of the SPON2 F-Spondin domain fragment (SPON2-Fsp) alone into the culture medium recapitulated rSPON2's inhibition of glutamine import, as measured by Gln-Cy5 cytoplasmic fluorescent intensity measured by confocal microscopy (**Fig. 6D, E**) and flow cytometry (**Supplementary Fig. 6A, B**).

Glutamine is an essential metabolite and signaling molecule, e.g., via leucine exchange and mTOR activation, to regulate mitochondrial respiration and energy production. Therefore, we predicted that SPON2, via its suppression of glutamine import, would inhibit mTOR signaling. To test this, we used Western blotting and examined how mTOR activation was affected by SPON2 loss- or gain-of-function. We found that *Spon2* GOF, whether via OE-*Spon2* (**Fig. 6F, G**) or rSPON2 (**Fig. 6H, I**), caused a significant reduction (~20–30%) in mTOR activation. By contrast, mTOR activation was greatly increased in *Spon2* null mammary glands compared to control glands (**Fig. 6J, K**). Together, these data confirmed that SPON2 suppresses mTOR signaling, known to be essential for mitochondrial metabolism^{45,46}.

Next, we assessed how SPON2 and glutamine affected leucine function. We found that glutamine addition potentiated leucine-mediated mTOR activation, but this was dampened by the addition of rSPON2 into the culture medium (**Fig. 6L, M**). Finally, we measured how glutamine and leucine regulated each other's cytoplasmic concentration. As expected, addition of glutamine into the medium, whether alone or in combination with Leu, raised cytoplasmic glutamine concentration, while rSPON2 addition reduced it; however, addition of leucine into the medium had no obvious effects. These data thus suggest that leucine does not overtly affect glutamine

concentrations (**Fig. 6N**). By contrast, we found that glutamine addition, whether alone or in combination with simultaneous leucine addition, raised the cytoplasmic leucine level; and this could be reduced by the presence of rSPON2 (**Fig. 6O**).

Together, these data support the notion that cytoplasmic glutamine promotes leucine import via exchange and indirectly promotes mTOR signaling. Taken together, they support the working model that SPON2 suppresses mitochondrial metabolism and cell proliferation by targeting SLC38A1-mediated glutamine import and downstream mTOR signaling.

SPON2 Suppresses Distinct Breast Cancer Subtypes

The data so far demonstrating that SPON2 suppresses epithelial cell proliferation and branching via blocking glutamine-mediated mTOR activation and mitochondrial metabolism support its role as a breast tumor suppressor. Considering the well-known phenomenon of “glutamine addiction” in cancer development ⁴⁶ and the current lack of inhibitors or effective therapeutic interventions against transporters such as SLC38A1 ^{47,48}, we reasoned that a gain of SPON2 function may offer a novel therapy against breast cancer. We first tried to test this prediction in the luminal subtype of breast cancer.

To this end, we examined how *Spon2* LOF or GOF affected mTOR activation, which is well known for its promoting role in breast cancer ^{49,50}. First, we used the VO-PyMT line of cells, which are derived from the luminal-type PyMT model, for the analysis ⁵¹. Using Western blotting, we found that phospho-mTOR was increased in the VO-PyMT cells transfected by sh*Spon2*, compared to control VO-PyMT cells (**Fig. 7A, B**). By contrast, *Spon2* GOF suppressed mTOR activation (**Fig. 7C, D**). Consistent with these data, overexpression of SPON2 in VO-PyMT cells significantly impaired their clonogenic capacity (**Fig. 7E, F**). This established SPON2 as a negative regulator of this central growth pathway in a luminal model. To examine the tumor-suppressive function of SPON2 in vivo, we transplanted VO-PyMT cells carrying control or *Spon2*-GOF constructs into the cleared fat pads of immunodeficient BALB/c nude mice. We found that *Spon2*-GOF greatly inhibited tumor growth, reducing tumor volume and weight (**Fig. 7G, H; Supplementary Fig. 7A**).

In addition to suppressing the luminal breast cancer subtype, we were inspired by the observation that SPON2 metabolically inhibits both basal and luminal cells, which suggests that it suppresses other subtypes of breast cancer as well. To test this prediction, we analyzed its

expression across breast cancer subtypes. Indeed, SPON2 mRNA levels were significantly lower in tumor tissues compared to normal breast tissue across all major molecular subtypes—Basal-like, HER2-enriched, Luminal A, and Luminal B (**Fig. 7I**). This pan-subtype downregulation suggests that SPON2 loss is a common event in breast oncogenesis, prompting us to test its function in models representing distinct disease contexts.

To determine if this tumor-suppressive mechanism extends to more aggressive cancer type, we conducted parallel experiments in the 4T1 cell line, a model of triple-negative breast cancer (TNBC) ⁵². Consistent with the findings in VO-PyMT cells, *Spon2* knockdown in 4T1 cells enhanced mTORC1 activation (p-mTOR/mTOR) (**Supplementary Fig. 7B, C**), whereas its overexpression suppressed it (**Supplementary Fig. 7D, E**). As predicted, SPON2 overexpression suppressed the clonogenic growth of 4T1 cells (**Supplementary Fig. 7F, G**). Thus, the ability of SPON2 to inhibit mTORC1 signaling and cell expansion is conserved across luminal and TNBC models. Likewise, xenograft studies in nude mice showed that SPON2 overexpression suppressed tumor growth of 4T1 cells, reducing tumor volume and weight (**Supplementary Fig. 7H-J**).

Next, we sought to evaluate *Spon2* as a therapeutic target in treating human breast cancer. To this end, we grew patient-derived organoids (PDOs) and treated them with either BSA placebo, rSPON2, or the rSPON2-Fsp. We found that treatment with both rSPON2 (**Fig. 7J-K; Supplementary Fig. 7K**) and rSPON2-Fsp (**Fig. 7L; Supplementary Fig. 7L**) significantly inhibited PDO growth, as measured by both the number of colonies and their sizes (**Supplementary Fig. 7K, L**). Finally, we wanted to test the efficacy of the Fsp domain fragment in breast cancer treatment using the PDO xenograft (PDOX) assays. We found that PDOs expressing the OE-SPON2-Fsp construct formed significantly smaller tumors than control PDOs in the NOD-SCID mice (**Fig. 7M, N; Supplementary Fig. 7M**).

Taken together, these data show that, consistent with SPON2 inhibition of glutamine intake, mTOR activation, and mitochondrial energy metabolism in development, all of which are essential for cancer development; its mRNA expression is greatly downregulated in different breast cancer subtypes. Forceful expression of SPON2 blocks cancer development of distinct breast cancer subtypes, highlighting its tumor-suppressing role.

DISCUSSION

Our study identifies SPON2 as a basal-derived extracellular matrix protein that functions as a metabolic checkpoint during mammary gland development and breast cancer suppression. Using genetic mouse models, transplantation assays, organoids, single-cell transcriptomics, metabolic analyses, and tumor models, we demonstrate that SPON2 restrains epithelial branching morphogenesis by suppressing glutamine-dependent mitochondrial metabolism and mTORC1 activation. Mechanistically, SPON2 directly binds the glutamine transporter SLC38A1 through its F-Spondin domain, thereby limiting glutamine uptake, reducing cell-surface transporter abundance, impairing leucine-dependent mTOR activation, and suppressing oxidative phosphorylation and ATP production. Beyond development, SPON2 expression was broadly reduced across major breast cancer subtypes, whereas restoration of SPON2 or its F-Spondin domain suppressed tumor growth in luminal, triple-negative, and patient-derived breast cancer models (Fig. 7O). Together, these findings establish a previously unrecognized epithelial metabolic niche mechanism in which a basal-derived extracellular matrix protein directly regulates nutrient accessibility to coordinate tissue homeostasis and suppress oncogenesis.

Epithelial Niche Signaling and Metabolic Coupling in Mammary Morphogenesis

Our results identify SPON2 as a basal-derived inhibitory niche factor that restrains mammary epithelial expansion during pubertal branching morphogenesis. *Spon2* was highly enriched in basal epithelial cells during active branching stages, and genetic deletion of *Spon2* accelerated ductal elongation, branch formation, and epithelial proliferation in vivo. Conversely, epithelial-specific gain of SPON2 function or recombinant SPON2 treatment suppressed branching and induced epithelial cell death in organoid systems. Mechanistically, these effects occurred independently of canonical TGF- β or FGF pathway regulation and instead reflected suppression of epithelial metabolic fitness and mitochondrial activity.

The mammary gland has long served as a model for understanding how local niche-derived signals coordinate epithelial growth and branching architecture. Classical studies established that stromal and epithelial growth factors, including FGF, WNT, BMP, and TGF- β family members, regulate branching through balanced stimulatory and inhibitory signaling networks^{14,36}. More recently, single-cell and lineage-tracing studies have revealed that basal and luminal epithelial populations actively communicate through reciprocal paracrine interactions that control progenitor activity and tissue organization^{10,24}. Our findings extend this framework by demonstrating that

epithelial niche regulation is not limited to transcriptional or morphogenetic signaling but also includes direct control of epithelial metabolism.

These findings further support and expand the concept of the “epithelial microenvironment” or “epithelial niche,” in which epithelial subtypes function as reciprocal regulatory environments for one another. In previous work, we showed that basal and luminal cells cooperate through an FGF–BMP7–INHBA signaling loop to promote mammary regeneration¹². More recently, EFNA3-mediated regulation of oxidative phosphorylation suggested that basal cells can positively regulate luminal metabolic activity²⁵. The present study identifies a complementary inhibitory arm in which basal-derived SPON2 restricts glutamine-dependent mitochondrial metabolism and mTORC1 activation. Together, these studies suggest that mammary epithelial homeostasis is maintained through balanced metabolic “accelerator” and “brake” signals that coordinately regulate nutrient sensing, energy production, and epithelial expansion during development.

Extracellular Matrix Modulation of Nutrient Accessibility

A central mechanistic advance of this study is the identification of a previously unrecognized mode of extracellular metabolic regulation in which an extracellular matrix-associated protein directly controls nutrient transporter function. We demonstrate that SPON2 binds the glutamine transporter SLC38A1 through its F-Spondin domain, suppresses glutamine uptake, and reduces cell-surface transporter abundance through endocytosis-associated internalization. This restriction of intracellular glutamine attenuated leucine-dependent mTORC1 signaling, resulting in impaired mitochondrial respiration, ATP depletion, and suppression of epithelial proliferation.

Current models of extracellular matrix biology primarily emphasize regulation of tissue mechanics, integrin signaling, growth factor availability, and cell polarity⁷⁻⁹. In parallel, nutrient transporters such as SLC38A1 and LAT1 have emerged as critical regulators of anabolic metabolism, mTOR activation, and proliferative capacity in both development and cancer^{47,53}. However, extracellular regulation of nutrient accessibility has remained poorly understood⁹. Our findings suggest that extracellular matrix proteins can directly modulate transporter accessibility and thereby influence intracellular metabolic state. Rather than functioning solely as structural scaffolds or signaling cofactors, extracellular proteins may act as gatekeepers of nutrient acquisition.

The SPON2–SLC38A1 axis also provides mechanistic insight into how glutamine metabolism is integrated with mTOR signaling during epithelial growth. Glutamine is increasingly recognized not only as a carbon and nitrogen source, but also as a signaling metabolite that promotes leucine exchange and mTORC1 activation ^{46,54}. By limiting glutamine availability upstream of these pathways, SPON2 simultaneously suppresses mitochondrial bioenergetics and nutrient-sensing signaling. These findings substantially broaden current understanding of how tissue niches regulate metabolism and raise the possibility that extracellular control of nutrient transport may represent a more general mechanism operating in other developmental systems and organ contexts.

Developmental Metabolic Checkpoints and Breast Cancer Suppression

Our findings further demonstrate that this developmental metabolic checkpoint is broadly relevant to breast cancer. SPON2 expression was significantly reduced across major molecular subtypes of human breast cancer, including luminal, HER2-enriched, and basal-like tumors, and higher SPON2 expression correlated with improved patient prognosis. Functionally, restoration of SPON2 suppressed mTOR activation, clonogenic growth, and tumor expansion in luminal and triple-negative breast cancer models, while recombinant SPON2 or its isolated F-Spondin domain inhibited growth of patient-derived organoids. These data support a model in which loss of SPON2-mediated metabolic restraint contributes to oncogenic metabolic reprogramming and tumor progression (**Fig. 7O**).

Cancer metabolism studies have traditionally focused on intracellular metabolic enzymes, particularly glutaminase-dependent glutaminolysis and downstream anabolic pathways ⁴⁷. Although glutamine addiction is now recognized as a hallmark of many aggressive tumors, therapeutic targeting of glutamine metabolism has proven challenging because intracellular metabolic pathways are highly adaptable and systemic inhibition often causes toxicity ⁵⁵. Our findings suggest an alternative framework in which nutrient acquisition itself is regulated by extracellular niche-derived suppressive mechanisms. Rather than directly targeting intracellular metabolism, restoring extracellular metabolic restraint may represent a complementary strategy for limiting tumor growth.

Importantly, the broad activity of SPON2 across multiple breast cancer subtypes suggests that epithelial metabolic checkpoints may operate upstream of subtype-specific oncogenic

programs. This is particularly notable given the substantial intratumoral heterogeneity and therapeutic resistance that characterize breast cancer³⁵. In this context, targeting shared metabolic dependencies imposed by the epithelial niche may provide advantages over approaches focused on genetically diverse tumor subclones. More broadly, our work suggests that tumors may evolve, at least in part, through escape from endogenous tissue-derived metabolic restraints that normally limit epithelial expansion during development and homeostasis.

Limitations and Future Directions

Collectively, our findings support a broader conceptual shift from viewing tissue homeostasis primarily through the lens of growth-factor signaling toward a model in which metabolic homeostasis is itself actively regulated by epithelial niches. We propose that basal epithelial cells do not merely provide instructive developmental signals but also regulate nutrient accessibility and bioenergetic capacity within epithelial tissues. This framework may be particularly relevant to other bilayered glandular organs, including the prostate, pancreas, and salivary gland, where basal-luminal interactions similarly coordinate tissue organization and regeneration.

Several important questions remain unresolved. The precise structural basis and *in vivo* dynamics of the SPON2–SLC38A1 interaction require further investigation, as does the potential involvement of additional nutrient transporters or stromal components. Moreover, while recombinant SPON2 and its F-Spondin domain showed therapeutic efficacy in organoid and tumor models, their pharmacologic optimization and long-term safety remain to be established. Future studies addressing these questions may help determine whether extracellular metabolic checkpoint restoration can be therapeutically exploited to overcome metabolic adaptation and treatment resistance in breast cancer and other malignancies.

MATERIALS AND METHODS

Mouse Strains and Animal Husbandry

Spon2 knockout mice were generated by CRISPR/Cas9-mediated deletion of all coding exons and were obtained from GemPharmatech (strain No. T017725; strain name C57BL/6JGpt-*Spon2*^{em15Cd13368/Gpt}). The engineered allele corresponds to a 13,368-bp genomic deletion removing exons 1–6 (based on the *Spon2*-201 transcript), resulting in functional disruption of *Spon2*. C57BL/6J and BALB/c-Nude mice were also obtained from GemPharmatech. All mice were housed in a specific pathogen-free vivarium with controlled temperature (18 - 23°C),

humidity (40–60%), and a 12-hour light, 12-hour dark cycle, with ad libitum access to food and water. Genotyping was performed by PCR on tail DNA according to the supplier's protocol, with primer information listed in **Supplementary Table 1**. All animal procedures were approved by the South China University Institutional Animal Care and Use Committee (IACUC# 2024#67) and conformed to ethical guidelines for animal research.

Public expression, survival and developmental dataset analyses

SPON2 expression in breast cancer was analyzed using the GEPIA web server, which integrates RNA-seq data from TCGA and GTEx. SPON2 mRNA levels were compared between breast cancer and normal breast tissues, and survival associations were evaluated with the Kaplan-Meier Plotter platform using platform-defined high- and low-expression groups, hazard ratios and log-rank P values. Published mouse mammary gland scRNA-seq datasets from Bach et al. and Pal et al. were re-analyzed in Seurat v5 to assess *Spon2*, *Slc38a1* and *Slc1a4* expression across epithelial subpopulations and developmental stages.

Mammary epithelial cell isolation, sorting and qPCR

Mammary glands from 8-10-week-old female mice were minced and digested in DMEM/F12 containing 2 mg/mL collagenase, 2 mg/mL trypsin, 5 µg/mL insulin, 5% FBS and 50 µg/mL gentamicin for 30-45 min at 37°C. Organoids were enriched by differential centrifugation. For sorting, dissociated primary mammary cells or cultured epithelial cells were incubated with antibodies listed in Supplementary Table 4 on ice for 40 min, washed, resuspended in PBS containing 2% FBS and 1% penicillin/streptomycin, filtered through a 40-µm mesh and sorted on a CytoFLEX SRT (Beckman Coulter). Flow cytometry data were analyzed using FlowJo 10.

Total RNA was extracted using the ABclonal RNA extraction kit (Cat# RK30120), reverse transcribed with ABScript Neo RT Master Mix for qPCR with gDNA Remover (ABclonal, Cat# RK20433) and quantified with 2× Universal SYBR Green Fast qPCR Mix (ABclonal, Cat# RK21203) on an Agilent AriaMx system. Gene expression was normalized to *Hprt* unless otherwise specified, and primer sequences are provided in **Supplementary Table 2**.

Cell Lines and Culture

HEK293T, VO, and 4T1 cells were cultured in DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin. HC11 cells were cultured in RPMI 1640 with 10% FBS, 10 ng/mL EGF, and 5 µg/mL insulin. All cell lines were routinely tested for mycoplasma contamination.

Mammary gland whole-mounts and transplantation

Inguinal (#4) mammary glands were dissected for whole-mount or transplantation analyses. For whole-mount imaging, glands were spread on glass slides, fixed in Carnoy's fixative, stained with carmine alum, dehydrated, cleared in xylene, mounted and imaged using a Zeiss Axio Zoom.V16 stereomicroscope. Ductal length and branch points per mm² were quantified with ImageJ. For mammary gland regeneration assays, 40,000 mammary epithelial cells were injected into cleared fat pads of 3-week-old female nude mice. Reconstituted glands were harvested at the indicated time points and analyzed by fluorescence imaging or carmine-stained whole-mount preparation.

Plasmids, shRNAs and lentivirus

shRNAs were designed using the BLOCK-iT RNAi Designer platform. Annealed shRNA fragments were ligated into AgeI/EcoRI-digested pLKO.1 (Addgene, Cat# 8453), confirmed by Sanger sequencing and screened for knockdown efficiency in basal cells. shRNA sequences are listed in **Supplementary Table 3**. For overexpression, the full-length Spon2 coding sequence was cloned into pLeGO-SFFV-P2A-mCherry using the ClonExpress Ultra One Step Cloning Kit V3 (Vazyme, Cat# C117). Lentivirus was produced by co-transfecting HEK293T cells with transfer plasmids, pMD2.G (Addgene, Cat# 12259) and psPAX2 (Addgene, Cat# 12260) using Eztrans reagent (Shanghai Life-iLab, Cat# AC04L099). Viral supernatant was collected at 60 and 88 h, concentrated by ultracentrifugation at 27,000 rpm for 2 h, and stored at -80°C.

Organoid migration, branching and colony assays

For migration assays, infected primary mammary epithelial cells were dissociated, resuspended in mammary stem cell medium at 20,000 cells/mL, seeded at 100 μ L per well in ultra-low attachment 96-well plates, and allowed to aggregate overnight. Heparin sulfate beads (100-150 μ m) were pre-soaked in 10 μ L FGF10 (100 μ g/mL; GenScript, Z03155). One epithelial aggregate and one FGF10 bead were positioned approximately 100 μ m apart in 80% growth factor-reduced Matrigel, overlaid with basal medium, and monitored by live-cell imaging for 72 h. Displacement from the origin and organoid size increase ratio were quantified from time-lapse images using ImageJ.

For 3D branching cultures, 500 flow-sorted basal cells and 500 flow-sorted luminal cells were aggregated overnight in ultra-low attachment plates, embedded in growth factor-reduced Matrigel, and maintained in mammary stem cell medium consisting of Advanced DMEM/F12, 5% FBS, 4 μ g/mL heparin, 10 ng/mL EGF, 20 ng/mL FGF2, 5 μ M Y-27632, 1% penicillin/streptomycin and 1% L-Ala-Gln. For branching assays, 10 ng/mL FGF2 was included, and medium was refreshed every 2-3 days. Recombinant SPON2 protein (Novoprotein, Cat. No. CM63), BSA control,

glutamine, leucine or the indicated genetic manipulations were applied as described in the figure legends. Endpoint brightfield images were used to quantify branch number per organoid with ImageJ. For glutamine rescue experiments, basal and luminal cells carrying the indicated loss- or gain-of-function manipulations were recombined in the same 3D culture system, treated with glutamine or matched controls, and scored for branching at the endpoint.

For 3D colony formation, single-cell suspensions were embedded in growth factor-reduced Matrigel and cultured in organoid medium. Primary mammary basal or luminal cells were seeded at 500 cells per condition, and VO or 4T1 cells were seeded at 1,000 cells per condition. Cultures were maintained for 7 days with medium replacement every 2-3 days. Colonies containing more than 50 cells were counted as positive colonies and reported relative to input cell number.

Immunofluorescence, imaging and flow cytometry readouts

Tissues were fixed in 4% paraformaldehyde overnight at 4°C, cryoprotected in 30% sucrose, embedded in OCT and sectioned at 10 µm. Sections were permeabilized with 0.5% Triton X-100, blocked with goat serum-containing buffer, incubated with primary antibodies overnight at 4°C and secondary antibodies for 2 h at room temperature, and counterstained with DAPI. Antibodies are listed in **Supplementary Table 4**. Whole-mount organoid immunofluorescence was performed using a suspension staining and glycerol-fructose clearing protocol¹; organoids were fixed in 4% paraformaldehyde for 45 min before staining. Cleaved Caspase-3-positive and Ki67-positive cells were quantified from confocal images with ImageJ. Confocal microscopy was performed using Zeiss LSM 900 or Nikon CSU-W1 Sora systems.

TGF-β Treatment Assays

To test whether SPON2 acts downstream of TGF-β signaling, published transcriptomic data from TGF-β-treated basal mammary epithelial cells were re-analyzed, and primary basal cells were treated with recombinant TGF-β protein at 0, 5, 10 or 30 ng/mL. Spon2 mRNA was measured by qPCR, normalized to Gapdh and calculated by the 2-ΔΔCt method.

Single-Cell RNA Sequencing and Bioinformatics Analysis

For in vivo single-cell RNA sequencing, mammary epithelial cells were isolated from mouse mammary glands, enriched by fluorescence-activated cell sorting, and submitted to Shanghai Majorbio Bio-pharm Technology Co., Ltd. for single-cell library preparation and sequencing. For in vitro single-cell RNA sequencing, cultured organoids were dissociated into single cells, and viable cells were sorted by fluorescence-activated cell sorting and submitted to Guangzhou IGE

Biotechnology Co., Ltd. for single-cell library preparation and sequencing. For single-cell RNA sequencing, the Cell Ranger tool (v7.1.0) was used for quality control and quantification of the raw sequencing data from 10x Genomics. The Seurat R package (v5) was utilized for data input, quality control, clustering, and differential gene expression analysis ⁵⁶. Basal and luminal cell marker genes, 1814 and 2139 genes, respectively, in transplanted samples were identified by differential analysis based on a previously published adult mammary gland single-cell transcriptome dataset ³⁵. For embryonic mammary gland development analysis, the GSE111113 dataset was used, and the Canonical Correlation Analysis (CCA) method in Seurat was applied to integrate different samples.

Downstream analyses were performed using several R packages: Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and GSEA analyses were performed using the clusterProfiler (v4.6.2) ⁵⁷. Cell-cell communication analysis was conducted using the CellChat (v2.1.2) R package ⁵⁸.

Mitochondrial and metabolic assays

Cellular ATP was measured with CellTiter-Glo (Promega, Cat# G7572). Mitochondrial membrane potential was assessed by staining cells with 100 nM MitoTracker Red CMXRos (Invitrogen, Cat# M7512) for 30 min at 37°C, followed by flow cytometry or confocal microscopy. ATP production rate was measured with the Seahorse XF Real-Time ATP Rate Assay on an Agilent XFe96 analyzer according to the manufacturer's protocol.

SPON2 pull-down, co-immunoprecipitation and Western blotting

To identify extracellular SPON2-interacting proteins, luminal epithelial cells expressing SPON2-3×FLAG or GFP control were lysed under non-denaturing conditions with protease inhibitors, and clarified lysates were incubated with anti-FLAG affinity beads at 4°C. Bound proteins were eluted after stringent washing, digested with trypsin, analyzed by LC-MS/MS, and compared between SPON2-3×FLAG and GFP controls. Quantitative proteomic results are provided in **Supplementary Table 5**; enriched candidates were visualized by volcano plot, and membrane-associated candidates were prioritized for validation.

For Co-IP, HEK293T cells were co-transfected with the indicated plasmids, harvested 36-48 h later, and lysed in non-denaturing buffer. Equal protein amounts were incubated with anti-FLAG affinity beads, washed, eluted in SDS sample buffer, and immunoblotted with anti-HA and anti-FLAG antibodies. For domain-mapping experiments, HEK293T cells were co-transfected with

HA-SLC38A1 and the indicated FLAG-tagged SPON2 truncation constructs, including TSR-containing and F-Spondin domain fragments. For Western blotting, cells were lysed in RIPA buffer with protease and phosphatase inhibitors, quantified by BCA assay, resolved by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies listed in **Supplementary Table 4**. Signals were detected by enhanced chemiluminescence, quantified with ImageJ, and phosphoprotein levels were normalized to the corresponding total proteins.

Glutamine uptake and SLC38A1 localization assays

For glutamine uptake, cells were incubated with 100 μ M L-Glutamine-Cy5 (Ruixi Bío, Cat# R-M1-8469) in uptake buffer for 1h at 37°C. Uptake was stopped with ice-cold PBS and analyzed by flow cytometry or confocal microscopy. To test the isolated SPON2 F-Spondin domain, cells were treated with control or SPON2 F-Spondin domain protein before Glutamine-Cy5 labeling, and mean fluorescence intensity was quantified with ImageJ or FlowJo.

For SLC38A1 localization, HC11 cells expressing SLC38A1-GFP alone or together with SPON2-mCherry were analyzed by confocal microscopy. Membrane-to-cytoplasmic fluorescence profiles and co-localization were quantified from line-scan analyses in ImageJ. Cell-surface SLC38A1 abundance was measured by membrane-impermeable biotinylation of intact luminal cells treated with control or rSPON2. Biotinylated surface proteins were captured by affinity purification and analyzed together with total lysates by Western blotting. ATP1A1 was used as a plasma membrane marker, and β -tubulin was used to assess intracellular protein contamination.

Amino acid supplementation and mTOR signaling assays

For amino acid stimulation experiments, luminal cells were treated with leucine, glutamine or both, with or without recombinant SPON2 protein, and collected for amino acid measurements or mTOR signaling analysis. Intracellular and extracellular glutamine were measured using a Glutamine Content Assay Kit (Biosharp, Cat# BL1411A; 450 nm), and leucine was measured using a Human Leucine ELISA kit (Keaibo Biotechnology, Cat# CB12470-Hu; 450 nm). Cell pellets were extracted in kit buffer, culture supernatants were clarified before measurement, and ELISA samples were diluted when required to fall within the standard curve. Concentrations were calculated from standard curves and normalized to cell number, total protein or dilution factor as appropriate.

Breast cancer colony, xenograft and PDO assays

For breast cancer colony assays, VO and 4T1 single-cell suspensions were embedded in growth factor-reduced Matrigel and cultured as described above at 1,000 cells per condition. For orthotopic xenograft assays, VO or 4T1 cells were implanted into the #4 inguinal mammary fat pad of female immunodeficient BALB/c nude mice. For PDO-derived xenografts, PDOs were transduced with control or SPON2-Fsp-overexpressing lentivirus, resuspended in sterile DMEM/F12 mixed 1:1 with growth factor-reduced Matrigel, and implanted into female NOD-SCID mammary fat pads. Unless otherwise stated, 50,000 cells in 20 μ L were injected per mouse. Tumor volume was calculated as $V = (\text{length} \times \text{width}^2) / 2$. Mice were euthanized at study endpoints or humane endpoints, and tumors were excised, photographed, weighed, and processed for downstream analysis when indicated.

PDOs were maintained in growth factor-reduced Matrigel under organoid culture conditions and treated with recombinant SPON2 protein, isolated SPON2 F-Spondin domain protein or matched control protein as indicated. PDO growth was assessed from endpoint brightfield images by quantifying organoid number and maximum diameter with ImageJ. Treatment duration, dose and replicate information are provided in the corresponding figure legends.

Quantification and Statistical Analysis

Sample size for each figure is denoted in the figure legends. Statistical significance between conditions was assessed by two-tailed Student's *t*-tests. For comparisons among three or more groups, one-way ANOVA followed by Tukey's multiple-comparisons test was used. All error bars represent SD, and significance is denoted as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. n.s. denotes not significant.

Data Availability

The scRNA-seq data generated in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number **GSE326296**. During peer review, the data are available through GEO reviewer access.

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AUTHOR CONTRIBUTIONS

Z.D. performed most of the experiments, acquired and analyzed the data, and drafted the manuscript.

Y.H. performed the mass spectrometry experiments and contributed to proteomic data acquisition and analysis.

R.M. assisted with bioinformatics analysis and interpretation of the sequencing and proteomic data.

Yiting Li performed experiments and contributed to data acquisition.

Yuting Li assisted with figure assembly and data presentation.

J.C. assisted with experimental work, sample collection, and data acquisition.

J.F. provided clinical samples and contributed to sample collection.

L.W. prepared schematic illustrations and contributed to figure presentation.

J.L. provided intellectual input and participated in manuscript revision.

P.L. conceived the study, supervised the project, acquired funding, curated the data, and revised the manuscript.

All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict interests.

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FIGURE LEGENDS**Figure 1: Candidate Breast Tumor Suppressor SPON2 Inhibits Mammary Epithelial Branching Morphogenesis.**

(A) Strategy used to prioritize *Spon2* as a basal-enriched epithelial niche factor that may regulate luminal epithelial behavior during mammary development and breast cancer. Basal epithelial cells (Ba) and luminal epithelial cells (Lu) were compared to identify Ba-enriched ligand-encoding genes, which were further prioritized based on the expression of their cognate receptors in Lu cells. The heatmap shows the top 25 Ba-enriched candidate ligands, with *Spon2* highlighted in red.

(B) *SPON2* mRNA expression in normal breast tissue and breast tumors from the GTEx and TCGA datasets. Data are shown as $\log_2(\text{FPKM}+1)$. Box plots indicate median and quartiles; whiskers indicate minimum and maximum values. Tumor, $n = 1085$; normal, $n = 291$. *** $P < 0.001$.

(C) Kaplan-Meier survival curve of breast cancer patients stratified by high (light blue, $n = 2773$) or low (dark blue, $n = 2156$) *SPON2* expression. P value from log-rank test. HR and log-rank P are indicated in the panel.

(D) Bar plots showing average *Spon2* expression (log-normalized counts) in basal and luminal cells from scRNA-seq of 2.5-, 5-, and 10-week mouse mammary glands.

(E) qPCR analysis of *Spon2* expression in sorted basal and luminal cells from 5-week mammary glands. Data were normalized to *Hprt* and are shown relative to basal cells. Mean $\pm$ SD, $n = 3$ biological replicates. * $P < 0.05$ (unpaired t-test).

(F-K) Representative whole-mount Carmine alum staining of control (*Spon2*^{+/-}) and *Spon2*-null mammary glands at 3 weeks (F, G), 6 weeks (H, I), and 9 weeks (J, K) of age. Scale bars, 2mm.

(L, M) Quantification of branch points per mm² (L) and ductal elongation (M) from (F-K). Each point represents one gland. Mean $\pm$ SD, $n = 5$ mice per genotype per time point. * $P < 0.05$, ** $P < 0.01$ (two-way ANOVA with Tukey's post-hoc test).

(N) Schematic of the mammary gland transplantation assay.

(O, P) Representative whole-mount images of glands reconstituted with control shRNA (shScr) or *Spon2* knockdown (sh*Spon2*) epithelial cells. Scale bars, 2 mm.

(Q, R) Quantification of ductal elongation (Q) and branch points (R) from (O, P). Mean $\pm$ SD, $n = 12$ transplants per group. * $P < 0.05$, ** $P < 0.01$ (unpaired t-test).

(S, T) Representative whole-mount images of glands reconstituted with control vector (OE-Ctrl) or *Spon2*-overexpressing (OE-*Spon2*) epithelial cells. Scale bars, 2 mm.

(U, V) Quantification of ductal elongation (U) and branch points (V) from (S, T). Mean $\pm$ SD, n = 12 transplants per group. **P < 0.01 (unpaired t-test).

Figure 2: *Spon2* Suppresses Epithelial Expansion by Blocking Cell Proliferation and Promoting Cell Death.

(A, B) Representative brightfield images of 7-day organoids derived from basal cells infected with shScr (A) or sh*Spon2* (B), co-cultured with wild-type luminal cells in FGF2-containing medium. Scale bars, 100 μ m.

(C) Quantification of branch number per organoid from (A, B). Mean $\pm$ SD, n $\geq$ 17 organoids per group from 3 independent experiments. *P < 0.05 (unpaired t-test).

(D, E) Quantification of the percentage of Ki67⁺ proliferating cells (D) and CC3⁺ apoptotic cells (E) in organoids from (A–C). Mean $\pm$ SD, n $\geq$ 10 organoids per group. *P < 0.05, **P < 0.01 (unpaired t-test).

(F–H) Representative brightfield images and quantification of branch number per organoid derived from basal cells infected with control (OE-Ctrl) or *Spon2*-overexpression construct (OE-*Spon2*). Scale bars, 100 μ m. Mean $\pm$ SD, n $\geq$ 18 organoids. ***P < 0.001 (unpaired t-test).

(I, J) Quantification of Ki67⁺ cells (I) and CC3⁺ cells (J) in organoids from (F–H). Mean $\pm$ SD, n $\geq$ 10 organoids. **P < 0.01 (unpaired t-test).

(K–M) Representative brightfield images and quantification of branch number per organoid in mixed epithelial organoids treated with control protein or recombinant SPON2 (rSPON2). Scale bars, 100 μ m. Mean $\pm$ SD, n $\geq$ 48 organoids. **P < 0.01 (unpaired t-test).

(N–P) Representative brightfield images and quantification of branch number in luminal-only organoids treated with control protein or rSPON2. Scale bars, 100 μ m. Mean $\pm$ SD, n $\geq$ 12 organoids. ***P < 0.001 (unpaired t-test).

(Q, R) Representative images of colonies formed by luminal cells treated with control protein (Q) or rSPON2 (R). Scale bars, 50 μ m.

(S, T) Quantification of colony number formed by luminal (S) and basal (T) cells treated with rSPON2 or control protein. Colonies (>50 cells) were counted after 7 days and are shown as colony

number per 500 input cells. Mean $\pm$ SD, n = 10 technical replicates. **, P < 0.001, *, P < 0.05 (unpaired t-test).

(U, V) Representative immunofluorescence images and quantification of Ki67⁺ epithelial cells in 5-week *Spon2*^{+/-} and *Spon2*^{-/-} mammary glands. Scale bars, 20 μ m. Mean $\pm$ SD. ~100% increase in KO samples (*P < 0.05, unpaired t-test).

Figure 3: *Spon2* Inhibits Mitochondrial Energy Production Independently Of TGF- β Signaling.

(A) t-SNE plot of scRNA-seq data from *Spon2*^{+/-} and *Spon2*^{-/-} mammary glands, colored by major cell types.

(B) Genotype-split t-SNE showing similar cell type distributions.

(C-E) Volcano plot of DEGs (C), GOBP enrichment and GSEA of metabolic pathways (D, E) in luminal cells from *Spon2*^{-/-} versus control glands.

(F-H) t-SNE plot, GOBP enrichment, and GSEA of metabolic pathways in luminal cells from organoids treated with vehicle (Ctrl) or rSPON2.

(I, J) Relative ATP levels in organoids with *Spon2* knockdown (I) or *Spon2* overexpression (J). Mean $\pm$ SD, n = 4 biological replicates. *P < 0.05 (unpaired t-test).

(K-M) Representative confocal images of MitoTracker Red CMXRos staining in control and rSPON2-treated luminal cells.

(N) Seahorse XF Real-Time ATP Rate Assay traces showing ECAR and OCR in control and rSPON2-treated luminal cells. Mean $\pm$ SD, n = 6 biological replicates from two independent experiments.

Figure 4: *Slc38a1* Is a *Spon2* Downstream Target Essential for Epithelial Branching.

(A) Workflow of the SPON2-3 $\times$ FLAG pull-down and mass spectrometry assay using luminal cell lysates.

(B) Volcano plot of proteins enriched in SPON2 pull-down versus control.

(C) Top 20 differentially abundant membrane protein candidates.

(D, E) Bar plots showing *Slc1a4* (D) and *Slc38a1* (E) expression in basal and luminal cells from scRNA-seq of 2.5-, 5-, and 10-week mouse mammary glands.

(F, G) Co-immunoprecipitation assay showing an absence of binding to SLC1A4 (F), but binding to SLC38A1 (G) by SPON2 protein.

(H) qPCR analysis of *Slc38a1* knockdown efficiency. Mean $\pm$ SD, n = 3. ***P < 0.001 (unpaired t-test).

(I, J) Representative brightfield images and quantification of branch number in organoids with *Slc38a1* knockdown. Scale bars, 100 μ m. Mean $\pm$ SD, n $\geq$ 20 organoids. ~40% reduction (*P < 0.01, unpaired t-test).

(K-N) Representative brightfield images and quantification of organoid branching in epistasis experiments combining basal *Spon2* LOF or GOF with luminal *Slc38a1* knockdown. Scale bars, 100 μ m. Mean $\pm$ SD, n $\geq$ 20 organoids per condition. Statistics as indicated (one-way ANOVA with Tukey's post-hoc test).

Figure 5: SPON2 Suppresses Glutamine Import via Competition and SLC38A1 Endocytosis.

(A-D) Representative confocal images (A, B) and flow cytometry histogram and quantification (C, D) of cytoplasmic Gln-Cy5 intensity in control versus rSPON2-treated cells. Scale bars, 20 μ m. Mean $\pm$ SD, n = 3-4 independent experiments. **P < 0.01 (unpaired t-test).

(E, F) Representative brightfield images and quantification of organoid branching under glutamine rescue in *Spon2* GOF conditions. Scale bars, 100 μ m. Mean $\pm$ SD. Statistics as indicated (one-way ANOVA).

(G-K) Effect of SPON2 expression on SLC38A1 cellular localization. (G) Diagram depicting the experimental flow. (H, I) Confocal micrographs of SLC38A1-GFP cultured alone (H), or together with SPON2-mCherry cells (I). Squares with white dashed lines indicate insets that are zoom-in on the right (H', I'). Note that (I) showed a magnified view of a SLC38A1-GFP depicted in the inset with red frame of dashed line; it is adjacent to a SPON2-mCherry cell at the upper-right corner of an area with a white frame. Yellow dashed lines in (H) and (I) indicate the cross-sections scanned by the laser with fluorescent intensity shown in (J). White arrowhead in (I') indicate punctate cytoplasmic signals, presumably because of endocytosis. (K) Quantification of the membrane-to-cytoplasm ratio of SLC38A1-GFP.

(L-O) Western blot analysis and quantification of total (L, M) versus cell-surface (N, O) SLC38A1 in control and rSPON2-treated luminal cells. Mean $\pm$ SD, $n = 3$. * $P < 0.05$ for surface fraction (~25% reduction, unpaired t-test).

Figure 6: The F-Spondin Domain Blocks mTOR Activation by Attenuating Gln-Leu Exchange.

(A) Diagram depicting SPON2 protein domain structure and co-IP construct design.

(B) Co-IP experiment showing protein-protein binding between the F-Spondin domain of SPON2 and SLC38A1.

(C) Co-IP experiment showing that the TSR domain of SPON2 does not bind to SLC38A1.

(D, E) Gln-Cy5 uptake after treatment with SPON2 F-Spondin domain fragment (SPON2-Fsp). Mean $\pm$ SD. * $P < 0.05$ (unpaired t-test).

(F-I) Western blot analysis and quantification of phospho-mTOR (Ser2448) and total mTOR in *Spon2* GOF (F, G) and rSPON2 (H, I) conditions. Mean $\pm$ SD, $n = 3$. * $P < 0.05$ (unpaired t-test; ~20–30% change).

(J, K) Western blot analysis and quantification of phospho-mTOR (Ser2448) and total mTOR in control and *Spon2*-null mammary glands. Mean $\pm$ SD, $n = 3$. * $P < 0.05$ (unpaired t-test; ~30–40% change).

(L-M) Western blot analysis of mTOR activation under Leu stimulation, and Gln/Leu/rSPON2 treatment. Mean $\pm$ SD, $n = 3$. Statistics as indicated.

(N, O) Cytoplasmic glutamine (M) and leucine (N) levels under indicated treatments. Mean $\pm$ SD, $n = 3$.

Figure 7: SPON2 Suppresses Distinct Breast Cancer Subtypes.

(A-D) Western blot analysis and quantification of phospho-mTOR and total mTOR in VO luminal breast cancer cells with *Spon2* knockdown (A, B) or overexpression (C, D). Mean $\pm$ SD, $n = 3$. * $P < 0.05$, ** $P < 0.01$ (unpaired t-test).

(E, F) Representative images (E) and quantification (F) of colony formation in VO-PyMT cells with *Spon2* overexpression. Scale bars, 200 μm . Mean $\pm$ SD, $n = 8$ replicates. $P < 0.01$ (unpaired t-test).

(G, H) Representative images of excised VO xenograft tumors **(G)** and quantification of tumor volume **(H)** in nude mice. Mean $\pm$ SD, n = 4 per group. P < 0.01 (unpaired t-test).

(I) SPON2 mRNA expression across breast cancer molecular subtypes from TCGA.

(J-L) Representative images of patient-derived organoids (PDOs) treated with control protein or rSPON2 **(J, J')**, and quantification of colony number after treatment with rSPON2 **(K)** or SPON2-Fsp **(L)**. Scale bars, 100 μ m. Mean $\pm$ SD; P < 0.01 (unpaired t-test).

(M, N) Representative images of PDOX tumors **(M)** and quantification of tumor volume **(N)** after orthotopic mammary fat pad implantation of control or OE-SPON2-Fsp PDOs into NOD-SCID mice. Mean $\pm$ SD; P < 0.05 (unpaired t-test).

(O) Schematic diagrams depicting SPON2 function in the mammary gland. Basal cells produce SPON2 to suppress epithelial ductal elongation and branching by inhibiting luminal cell proliferation and survival. This is achieved by binding to the glutamine transporter SLC38A1 and blocking Gln import, leading to reduced Gln-Leu exchange, and downstream events including mTOR activation, mitochondrial respiration and ATP production. Basal-derived SPON2 suppression and negative regulation of the metabolic coupling between epithelial cells and their niche are essential for normal branching. SPON2 loss upregulates the signaling and metabolic events, leading to excessive branching and possibly cancer development; while gain of SPON2 function leads to reduced branching and is a powerful inhibitor of breast cancer progression.

Figure 1

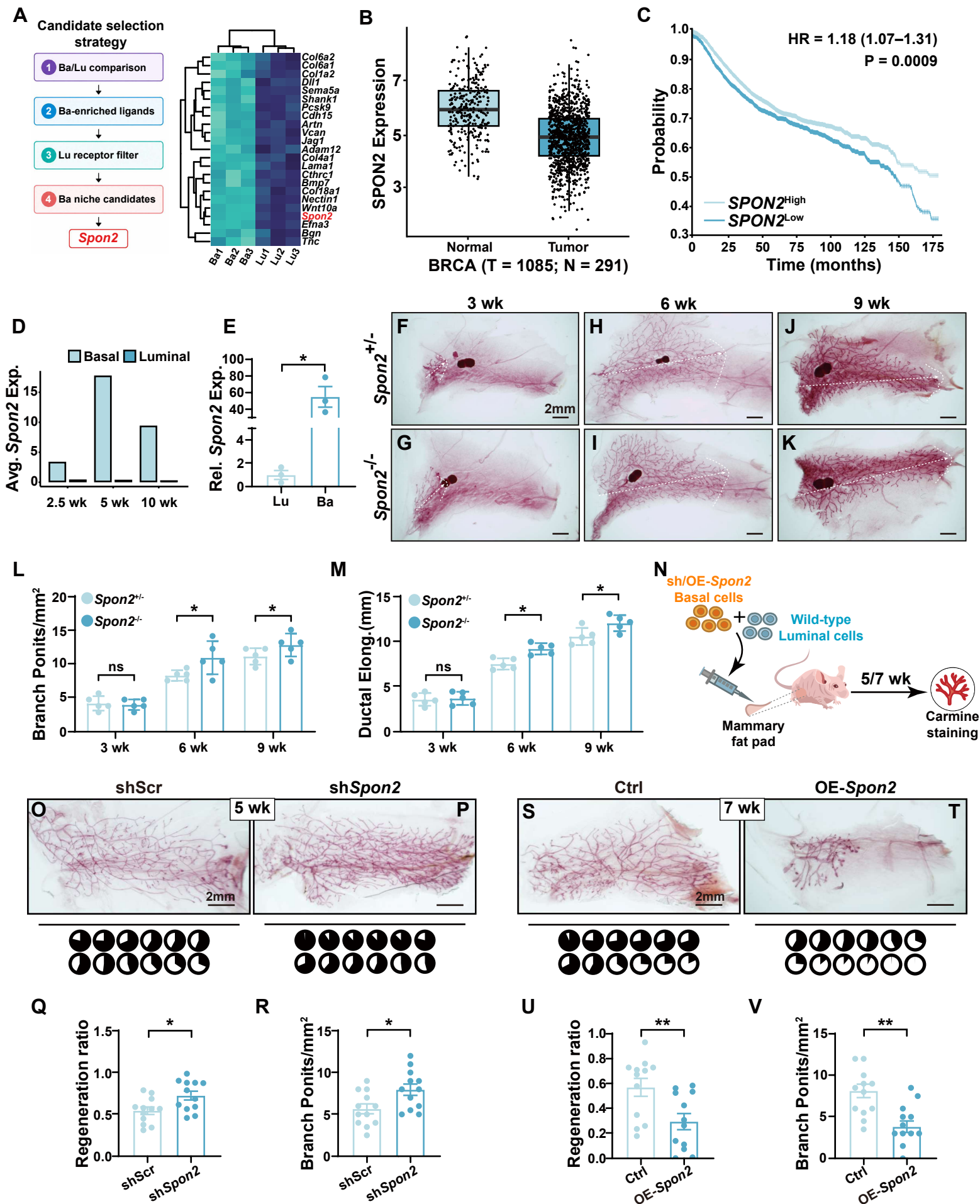


Figure 2

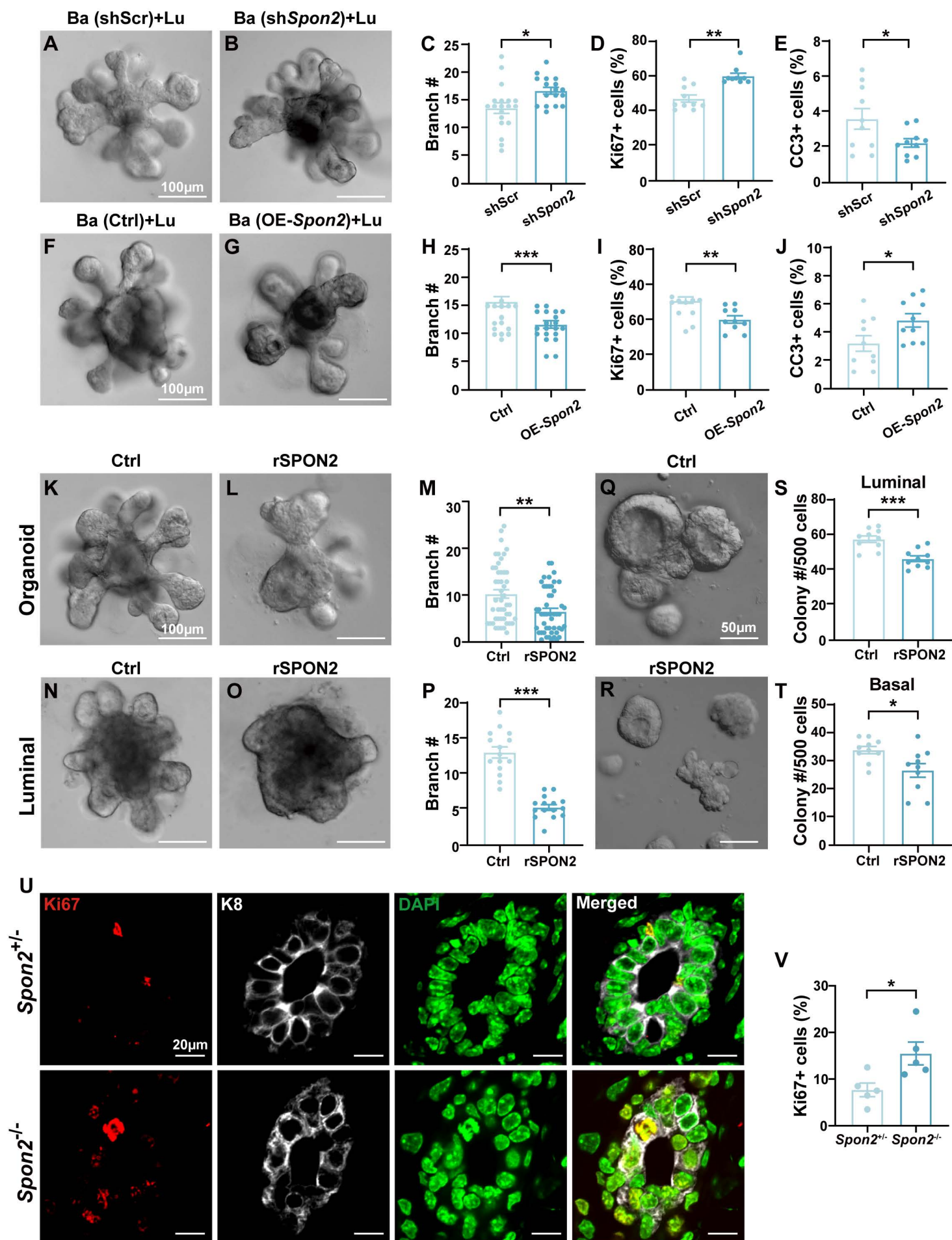


Figure 3

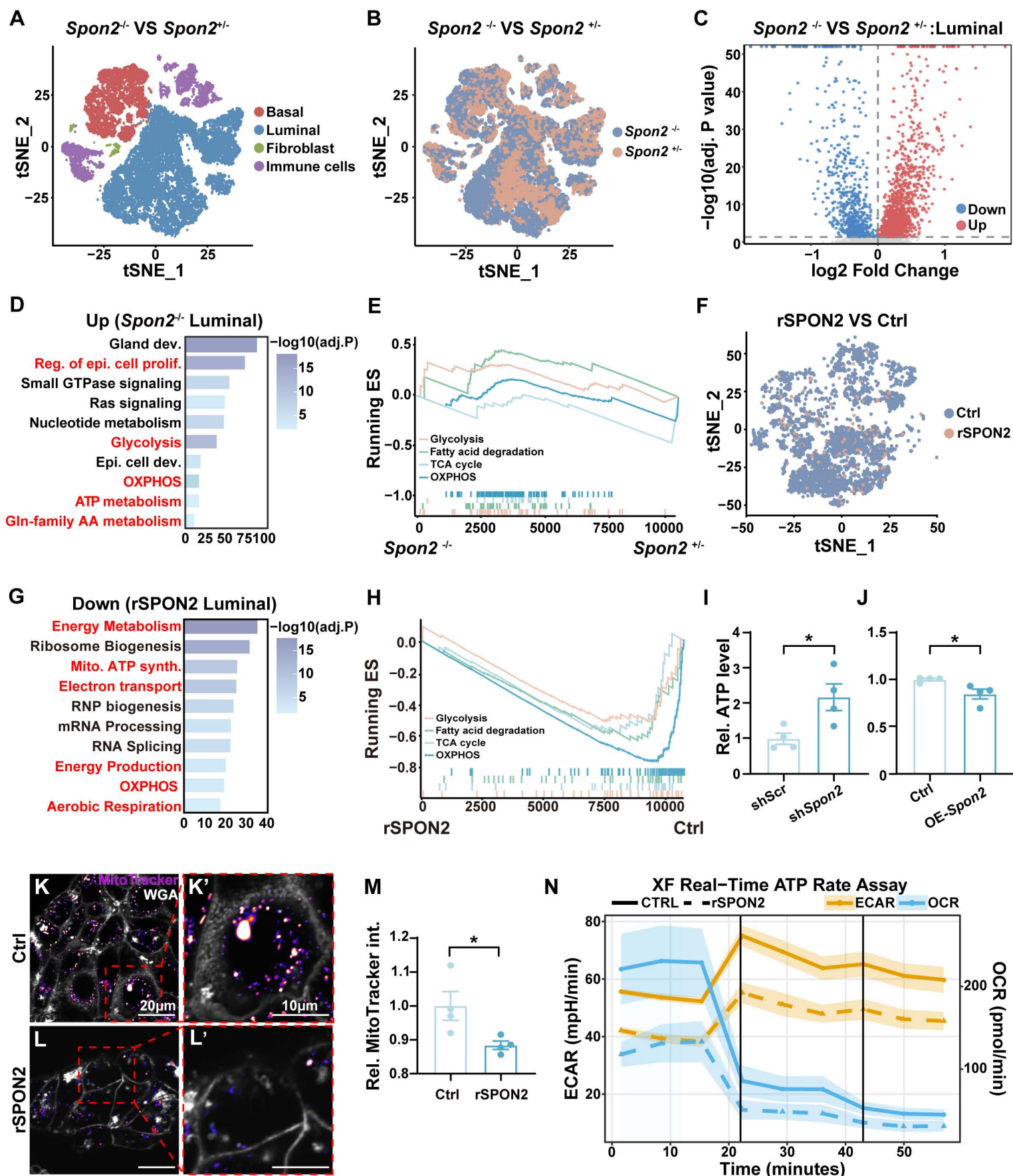


Figure 4

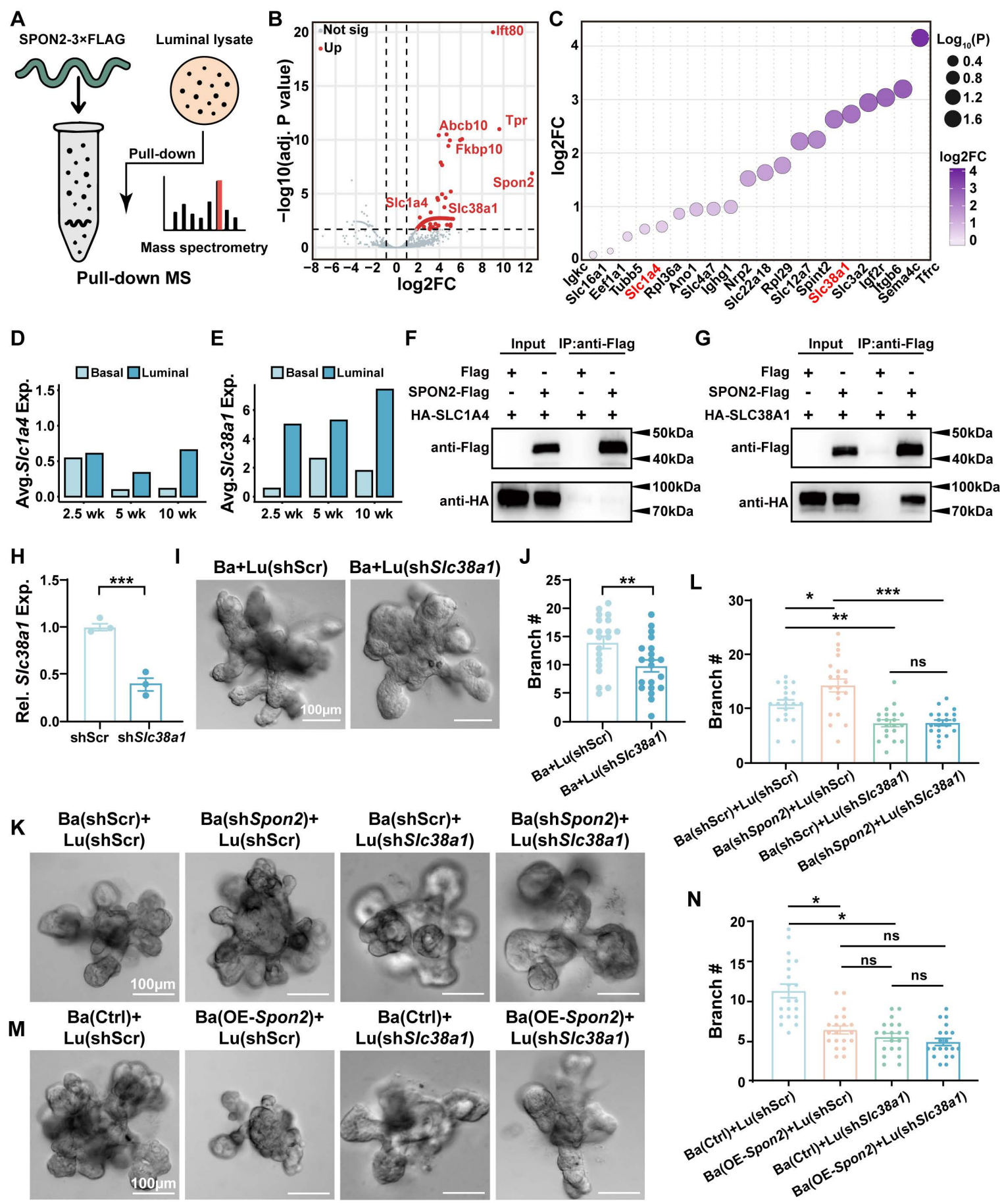


Figure 5

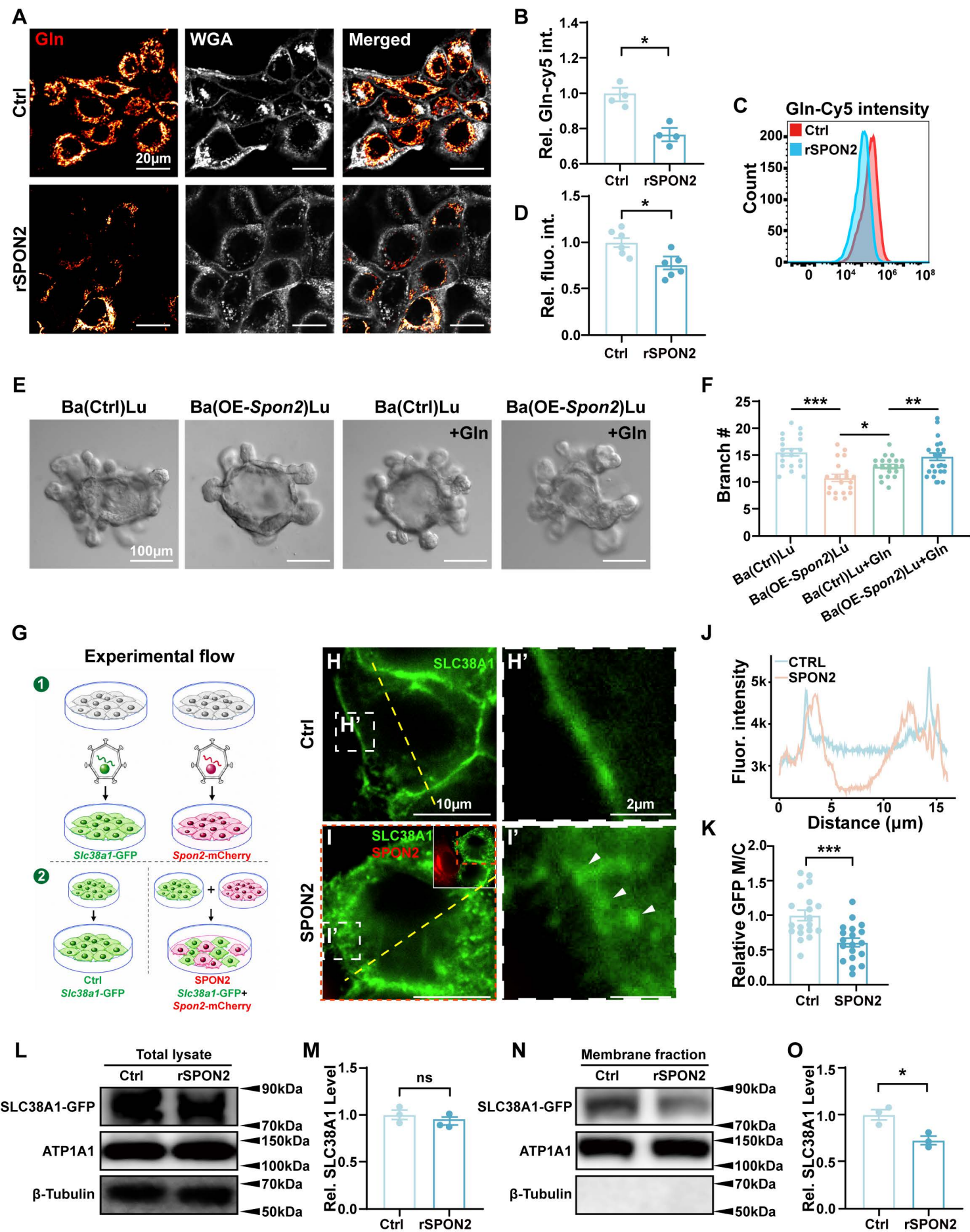


Figure 6

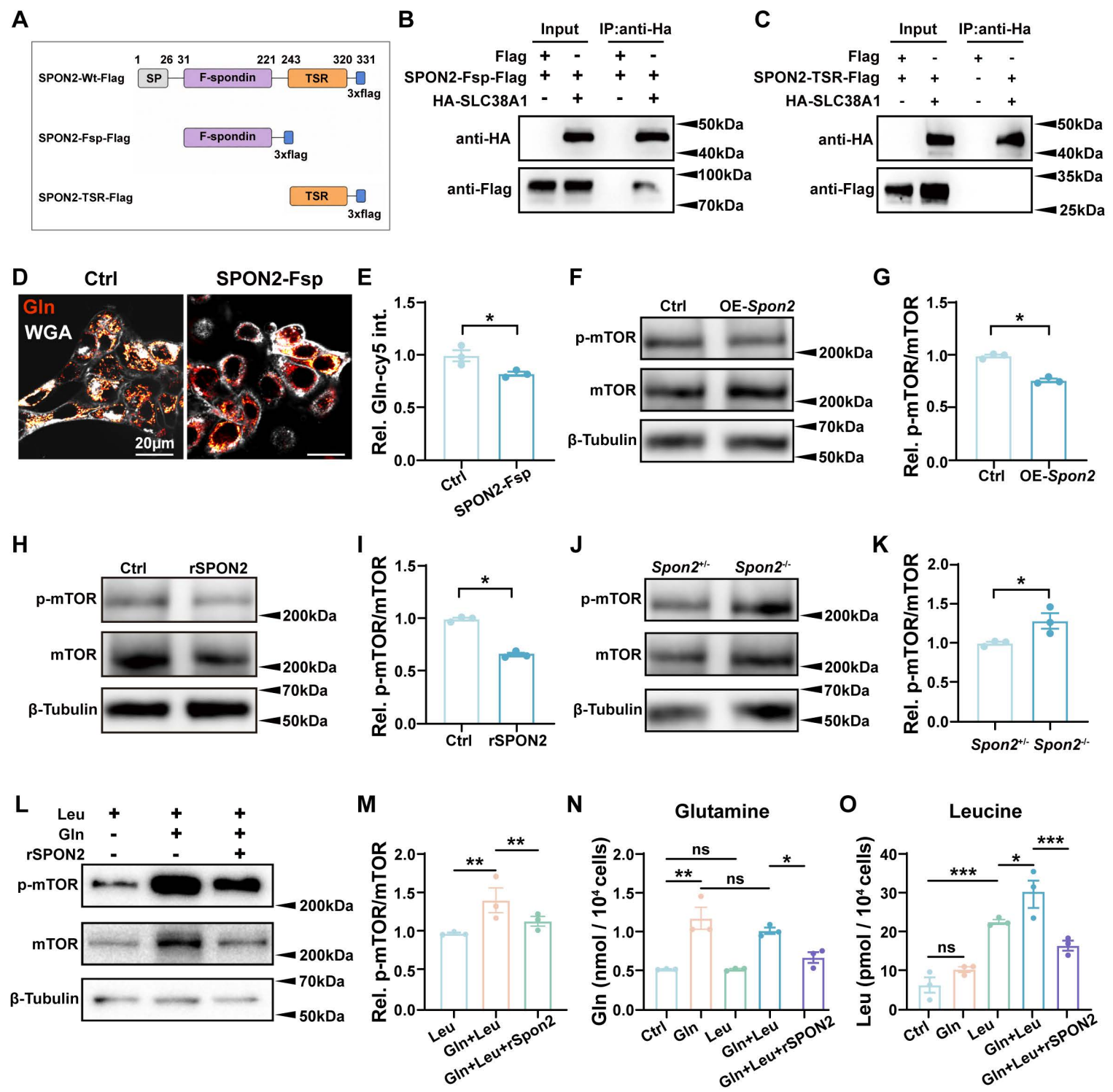
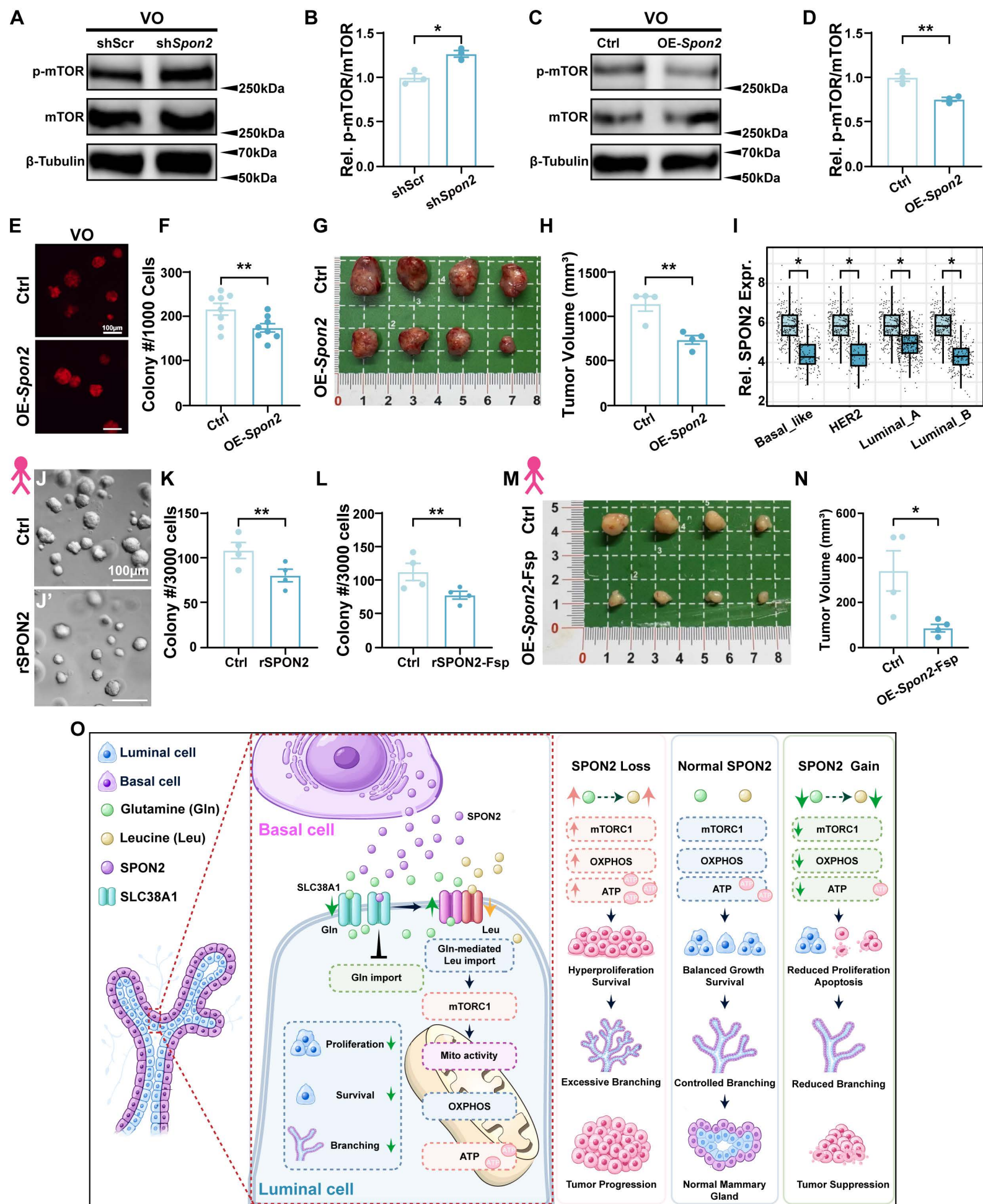


Figure 7



Supplementary Figure 1: Candidate Breast Tumor Suppressor SPON2 Inhibits Mammary Epithelial Branching Morphogenesis.

(A) t-SNE plot of scRNA-seq data of mammary glands with a focus on basal and luminal cells in the ducts and TEBs.

(B) Violin plots showing average *Spon2* expression (log-normalized counts) in ductal and TEB basal and luminal cells from scRNA-seq of the mouse mammary glands. Note the preferential *Spon2* expression in ductal basal cells relative to TEB basal cells.

(C) Schematic of the *Spon2* knockout strategy using CRISPR/Cas9.

(D) PCR genotyping of wild-type (+/+), heterozygous (+/-), and knockout (-/-) mice. WT allele, 305 bp; KO allele, 275 bp.

(E) qPCR of *Spon2* mRNA in control and knockout mammary glands. Mean $\pm$ SD, n = 3. ***P < 0.001 (unpaired t-test).

(F, G) Pup survival rate (F) and body weight at weaning (G) from control and KO mothers. Mean $\pm$ SD, n $\geq$ 3 litters.

(H-J) Representative whole-mount Carmine alum staining of *Spon2*^{+/+} and *Spon2*^{+/-} mammary glands (H) and quantification of epithelial branching based on branch points per mm² (I) and ductal elongation (J). Mean $\pm$ SD, n = 6 mice per genotype. n.s., not significant (unpaired t-test). Scale bars, 2 mm.

(K) Immunofluorescence of K14 (basal) and K8 (luminal) showing normal bilayer organization in KO glands. Scale bars, 10 μ m. Mean $\pm$ SD, n = 5 mice per group. n.s., not significant (unpaired t-test).

(L) qPCR screening of shRNA constructs for *Spon2* knockdown. sh-*Spon2*-3 achieves ~70% knockdown. Mean $\pm$ SD, n = 3. **P < 0.01 (one-way ANOVA).

(M) qPCR of *Spon2* after overexpression (OE-*Spon2*) showing ~25-fold increase. Mean $\pm$ SD, n = 3. ***P < 0.001 (unpaired t-test).

Supplementary Figure 2: *Spon2* Suppresses Epithelial Expansion by Blocking Cell Proliferation and Promoting Cell Death.

(A, B) Time-lapse images of shScr and sh*Spon2* organoids migrating toward an FGF10-soaked bead over 72 h. Circles with white dashed lines indicate the original location of the organoids at time 0. Scale bars, 100 μ m.

(C, D) Quantification of organoid displacement from origin (C) and size increase ratio (D). Mean $\pm$ SD, n = 20 organoids per group. n.s., not significant (unpaired t-test).

(E-H) Representative immunofluorescence of Ki67 (E, F) and CC3 (G, H) in shScr vs. sh-*Spon2* organoids. Scale bars, 100 μ m. Corresponding quantifications are in Fig. 2D, 2E.

(I-L) Representative immunofluorescence of Ki67 (I, J) and CC3 (K, L) in OE-Ctrl vs. OE-*Spon2* organoids. Scale bars, 100 μ m. Quantifications in Fig. 2I, 2J.

(M-O) Representative immunofluorescence (M, N) and quantification (O) of Ki67 in luminal organoids treated with BSA or rSPON2. Scale bars, 100 μ m. Mean $\pm$ SD, n = 10 organoids per group. ***P < 0.001 (unpaired t-test).

(P-R) Representative immunofluorescence (P, Q) and quantification (R) of CC3 in luminal organoids treated with BSA or rSPON2. Scale bars, 100 μ m. Mean $\pm$ SD, n = 10 organoids per group. **P < 0.01 (unpaired t-test).

(S, T) Flow cytometry of basal (CD49^{hi}CD24⁺) and luminal (CD49^{lo}CD24⁺) populations in control and KO glands (S) and quantification (T). Mean $\pm$ SD, n = 3 mice. n.s. not significant.

(U) Quantification of luminal cell percentage in the developing mammary epithelium based on immunofluorescence of luminal/basal markers shown in Supplementary Fig. 1K.

Supplementary Figure 3: *Spon2* Inhibits Mitochondrial Energy Production Independently of TGF- β Signaling.

(A, B) qPCR of *Spon2* expression in organoids (A) or primary basal cells (B) treated with TGF- β at indicated concentrations. No significant changes. Mean $\pm$ SD, n = 3. n.s., not significant (unpaired t-test).

(C, D) Violin plots showing normalized expression of *Tgfb1* and *Tgfb2* across ductal basal cells (DuBa), ductal luminal cells (DuLu), TEB basal cells (TEBa), and TEB luminal cells (TELu) in scRNA-seq data. Unlike *Spon2*, these genes are ubiquitously expressed.

(E, F) Violin plots showing normalized expression of *Spry1* and *Spry2* across the same epithelial subtypes in scRNA-seq data.

(G-I) Bar plots of mean log-normalized expression of TGF- β ligands (G), receptors (H), and downstream target genes (I) in basal cells from control and KO glands.

(J) GSEA plot showing that the TGF- β signaling pathway is not significantly changed in *Spon2*-null basal cells.

(K-M) Bar plots of mean log-normalized expression of TGF- β ligands (K), receptors (L), and target genes (M) in luminal cells.

(N) GSEA plot for TGF- β signaling in *Spon2*-null luminal cells.

(O, P) Bar plots of mean log-normalized expression of *Spry1* (O) and *Spry2* (P).

(Q, R) GSEA plot showing that the FGF signaling pathway is not significantly changed in *Spon2*-null basal or luminal cells.

Supplementary Figure 4: *Spon2* Inhibits Mitochondrial Energy Production Independently Of TGF- β Signaling.

(A) List of the top 40 differentially expressed genes (DEGs) in luminal cells from *Spon2*^{-/-} vs. *Spon2*^{+/-} glands.

(B-E) qPCR validation of representative metabolic genes downregulated by rSPON2: glycolysis (B), fatty acid degradation (C), TCA cycle (D), oxidative phosphorylation (E). Data normalized to Hprt and shown relative to Ctrl. Mean $\pm$ SD, n = 3 biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired t-test).

(F, G) MitoTracker Red MFI in control vs. sh-*Spon2* luminal cells. *Spon2* LOF increases $\Delta\Psi_m$. Mean $\pm$ SD, n = 3 independent experiments. P < 0.05 (unpaired t-test).

(H, I) MitoTracker Red in OE-Ctrl vs. OE-*Spon2* cells. SPON2 GOF decreases $\Delta\Psi_m$. Mean $\pm$ SD, n = 3. *P < 0.05 (unpaired t-test).

(J, K) MitoTracker Red in BSA- vs. rSPON2-treated cells. rSPON2 reduces $\Delta\Psi_m$. Mean $\pm$ SD, n = 3. *P < 0.05 (unpaired t-test).

(L, M) Quantification of basal respiration (L) and ATP-linked OCR (M) from Seahorse assays (Fig. 3N). rSPON2 reduces both. Mean $\pm$ SD, n = 6 biological replicates. **P < 0.01, ***P < 0.001 (unpaired t-test).

(N, O) Mitochondrial ATP production (N) and glycolytic ATP production (O) from Seahorse data. rSPON2 suppresses both. Mean $\pm$ SD, n = 6. **P < 0.01, ***P < 0.001 (unpaired t-test).

Supplementary Figure 5: SPON2 Suppresses Glutamine Import via Competition and SLC38A1 Endocytosis.

(A, B) Representative confocal images (A) and quantification (B) of Gln-Cy5 uptake in luminal cells infected with shScr or sh*Slc38a1*. *Slc38a1* LOF greatly diminishes cytoplasmic glutamine. Scale bars, 20 μ m. Mean $\pm$ SD, n = 4 biological replicates. ***P < 0.001 (unpaired t-test).

Supplementary Figure 6: The F-Spondin Domain Blocks mTOR Activation by Attenuating Gln-Leu Exchange.

(A, B) Flow cytometry histograms (A) and quantification of Gln-Cy5 MFI (B) in cells treated with control protein or the SPON2 F-Spondin domain fragment (SPON2-Fsp). Note the F-Spondin domain reduces glutamine uptake. Mean $\pm$ SD, n = 4 biological replicates. *P < 0.05 (unpaired t-test).

Supplementary Figure 7: SPON2 Suppresses Distinct Breast Cancer Subtypes.

(A) Tumor weight of VO-PyMT xenografts in nude mice (OE-Ctrl vs. OE-*Spon2*). Mean $\pm$ SD, n = 4 per group. **P < 0.01 (unpaired t-test).

(B, C) Western blot (B) and quantification (C) of p-mTOR/mTOR in 4T1 cells with shScr or sh*Spon2*. *Spon2* loss enhances mTOR activation. Mean $\pm$ SD, n = 3. **P < 0.01 (unpaired t-test).

(D, E) Western blot (D) and quantification (E) of p-mTOR/mTOR in 4T1 cells with OE-Ctrl or OE-*Spon2*. SPON2 GOF suppresses mTOR activation. Mean $\pm$ SD, n = 3. **P < 0.01 (unpaired t-test).

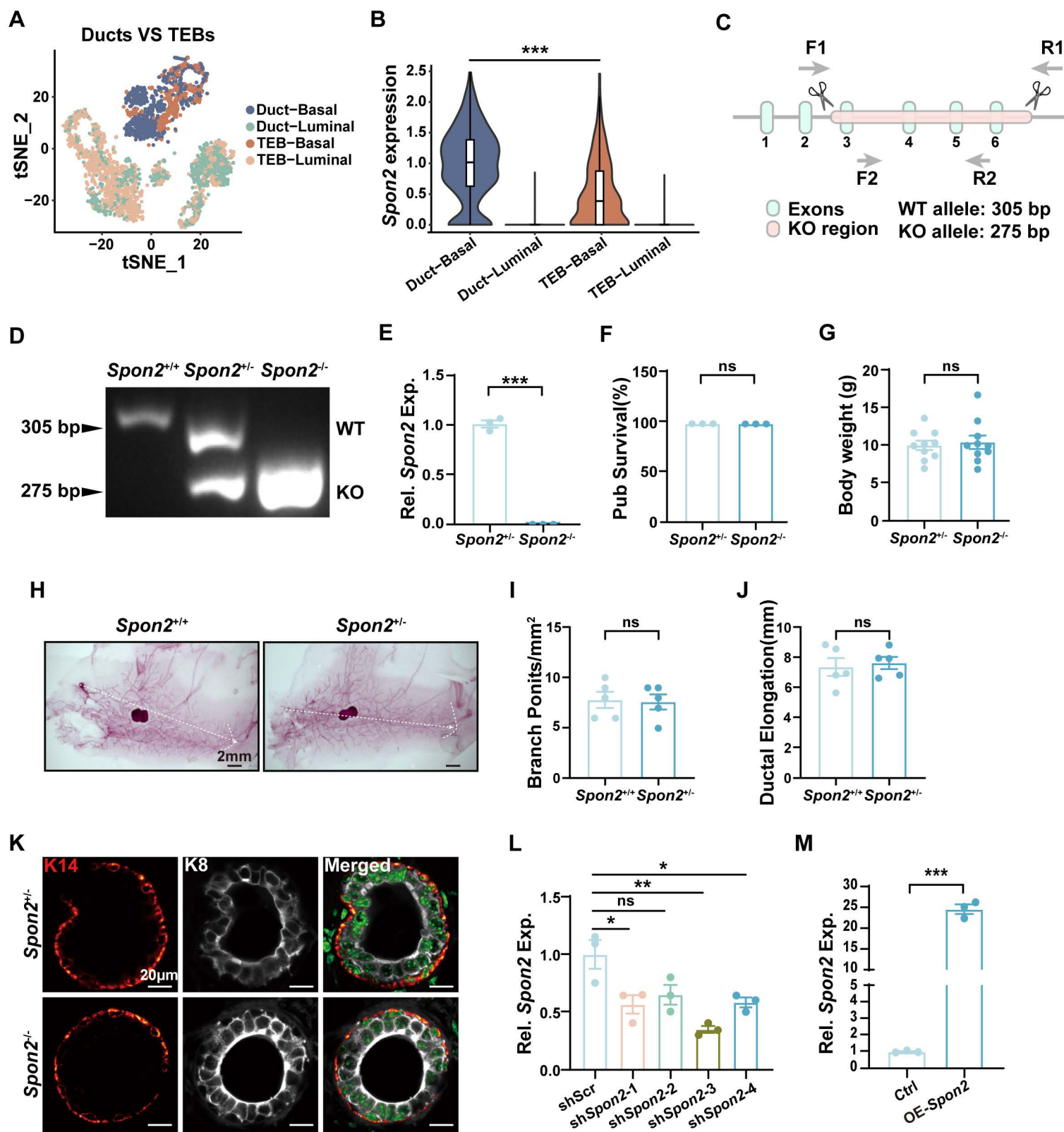
(F, G) Clonogenic growth (F) and quantification (G) of 4T1 cells with OE-Ctrl or OE-*Spon2*. Colonies (>50 cells) counted after 7 days. Mean $\pm$ SD, n = 8 replicates. **P < 0.01 (unpaired t-test).

(H–J) Representative images of excised 4T1 xenograft tumors **(H)**, tumor volume **(I)**, and tumor weight **(J)** in nude mice with OE-Ctrl or OE-Spon2. Mean $\pm$ SD, n = 5 per group. **P < 0.01 (unpaired t-test).

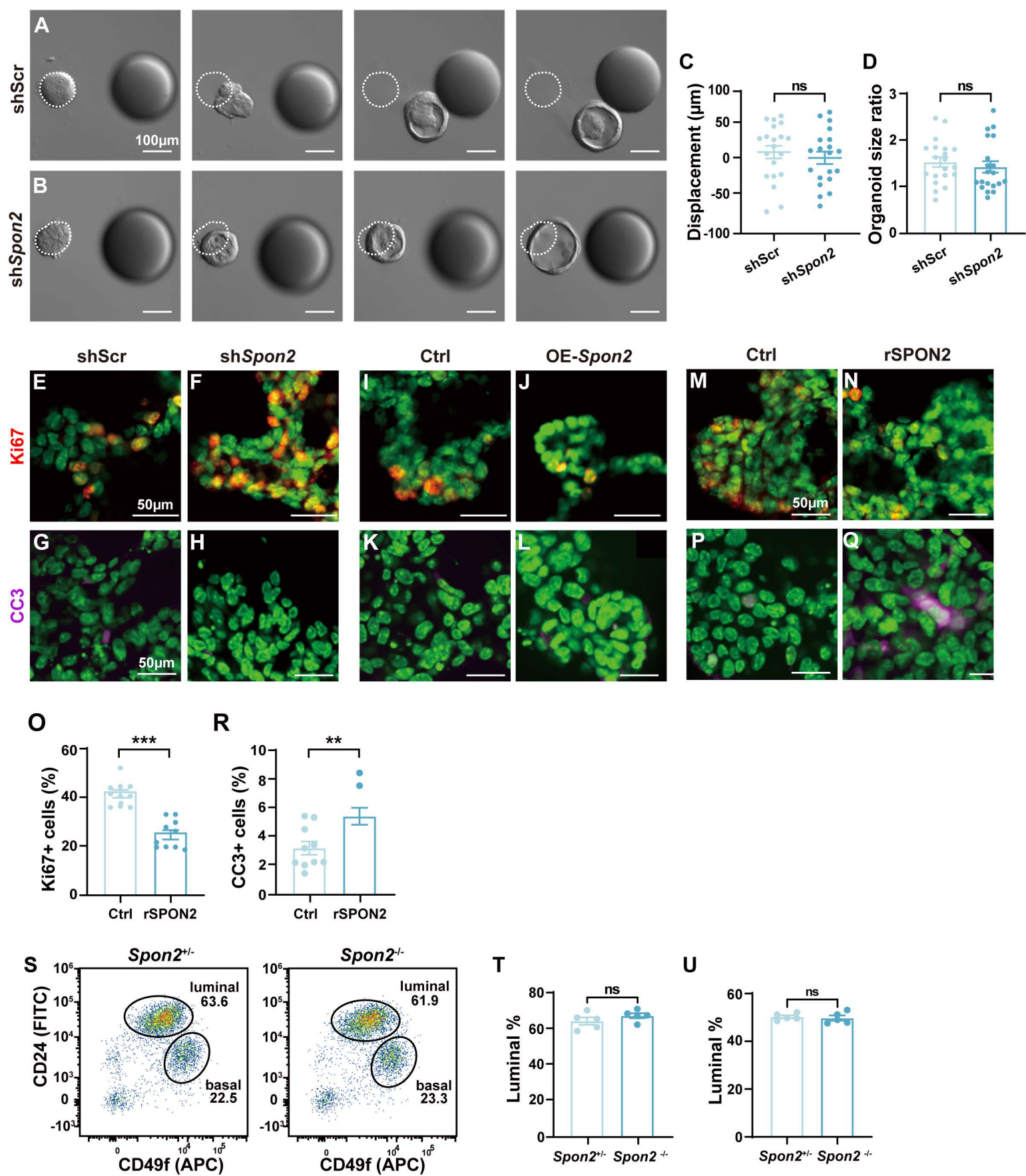
(K, L) Quantification of maximum diameter of patient-derived organoids (PDOs) treated with control protein, rSPON2 **(K)**, or rSPON2-Fsp **(L)**. Mean $\pm$ SD. **P < 0.01 (unpaired t-test).

(M) Tumor weight of PDOX tumors generated from control or OE-SPON2-Fsp PDOs after orthotopic mammary fat pad implantation into NOD-SCID mice. Mean $\pm$ SD. ** P < 0.01 (unpaired t-test).

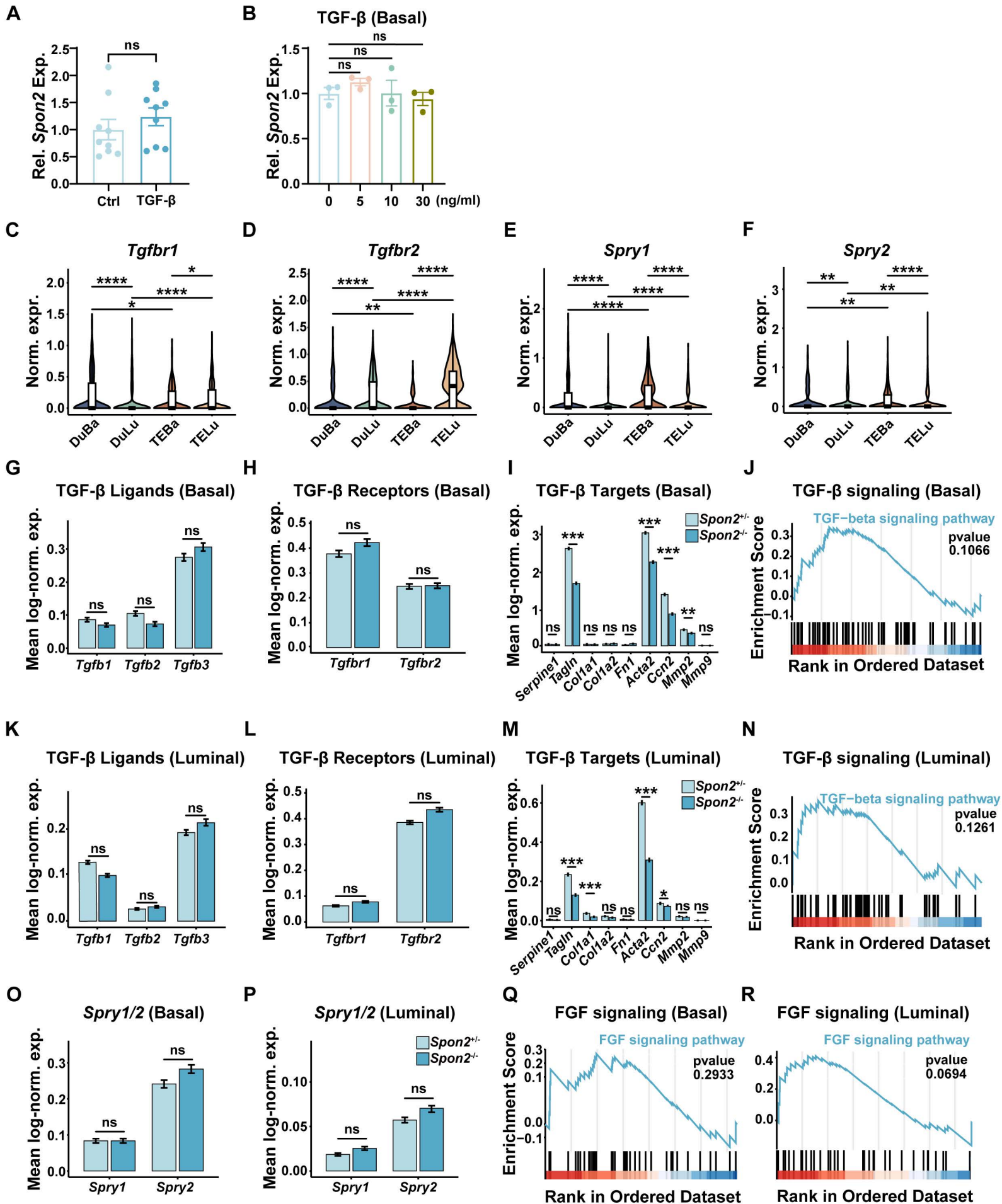
Supplementary Figure 1



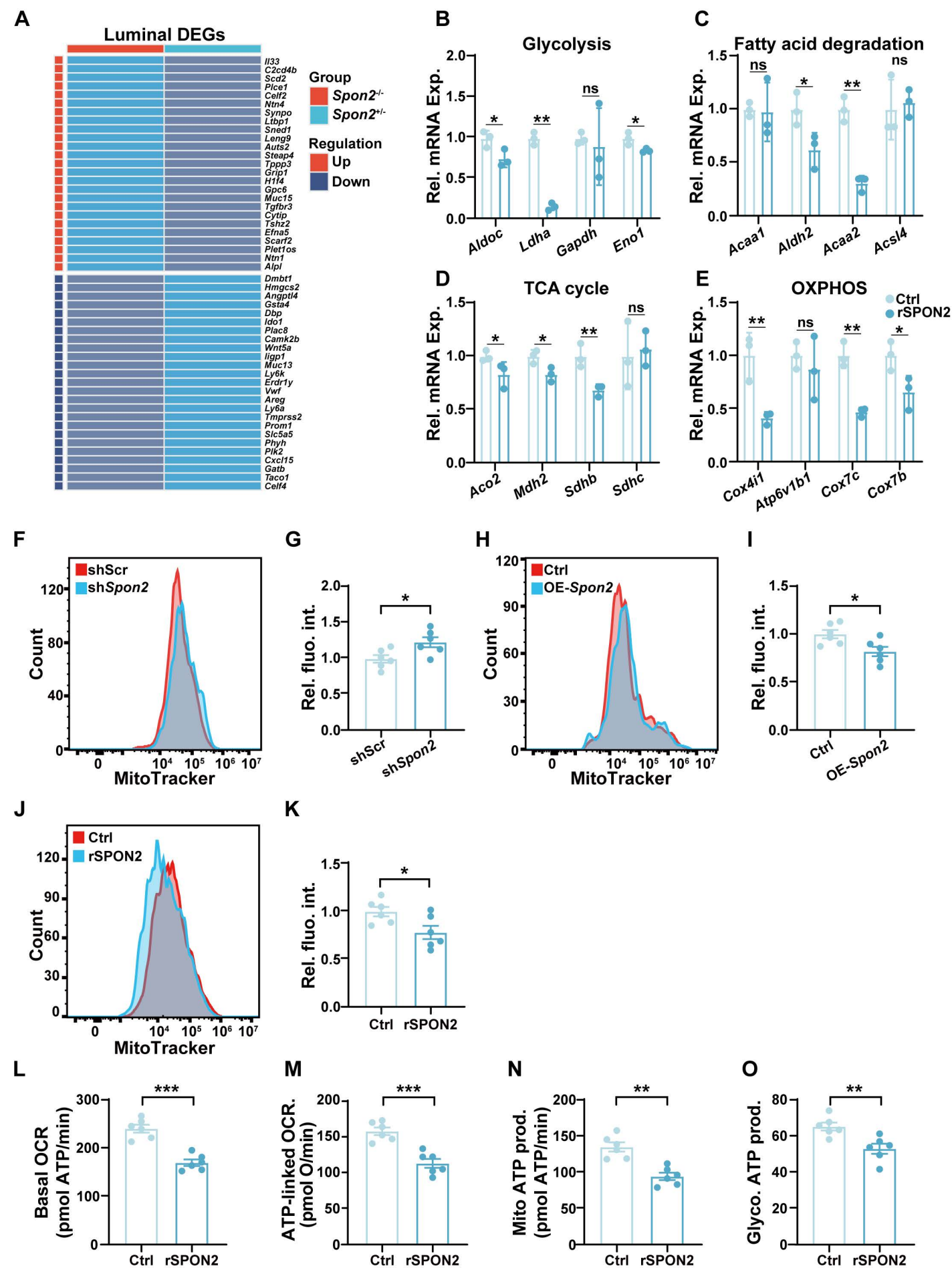
Supplementary Figure 2



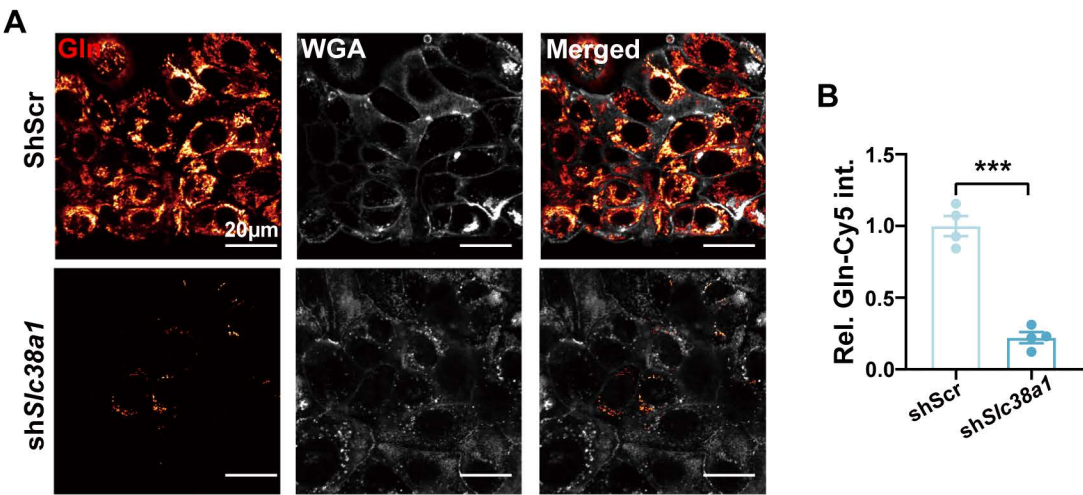
Supplementary Figure 3



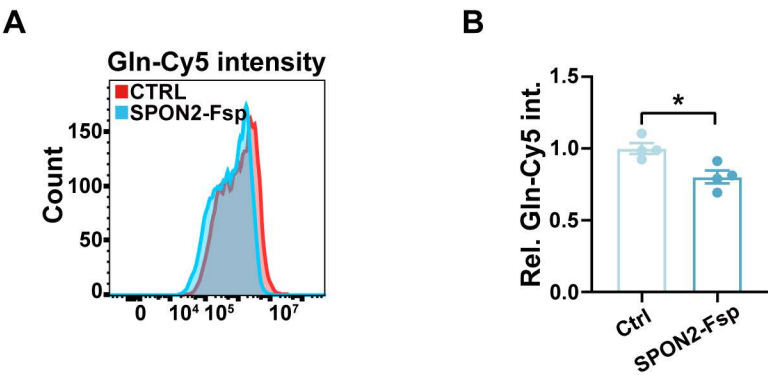
Supplementary Figure 4



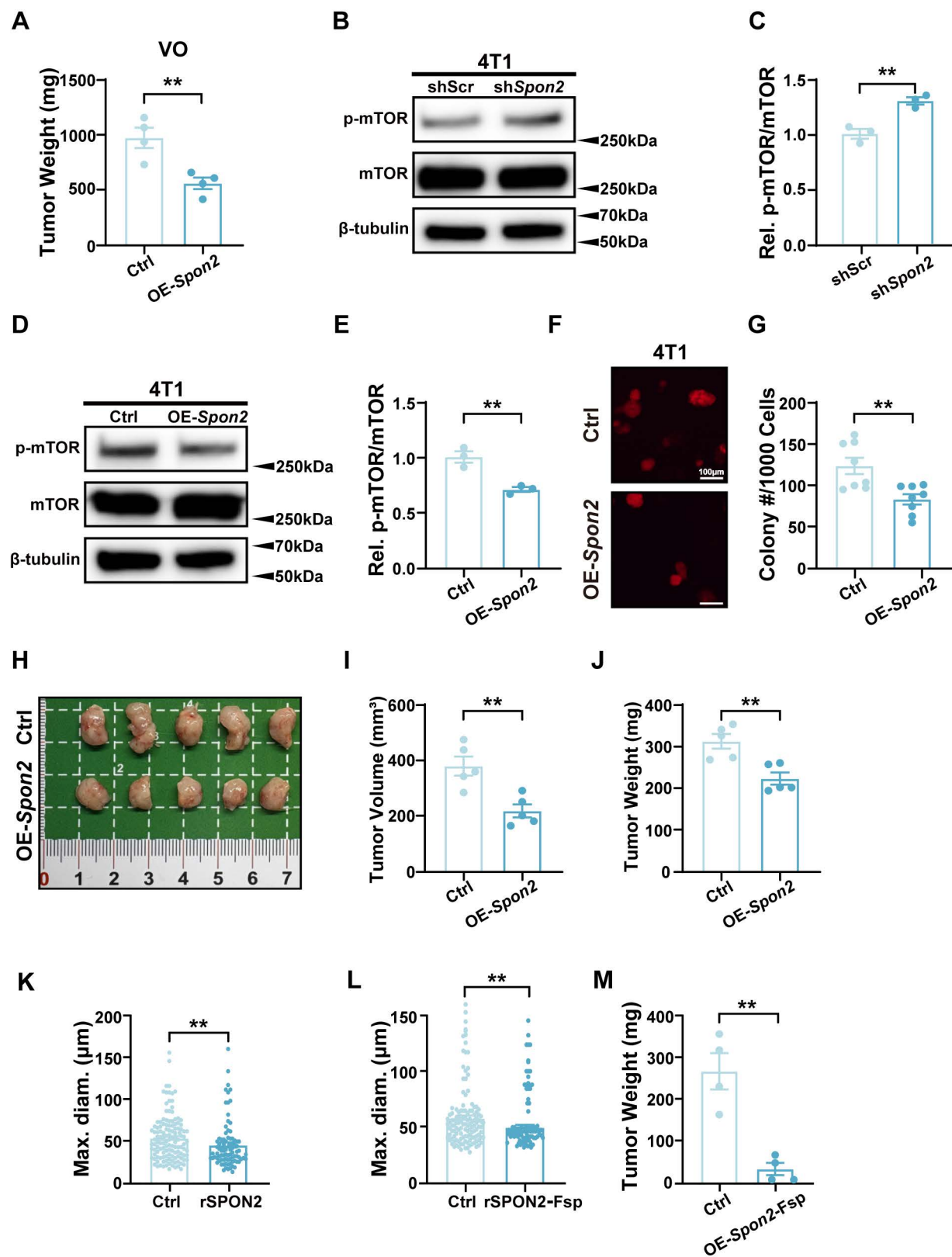
Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7



Supplementary Files

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- [SupplementaryTable3.xlsx](#)