

TSP50-mediated T β RII proteolysis maintains intestinal developmental and limits age-dependent susceptibility to colitis

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Abstract

Testis-specific protease 50 (TSP50) is essential for maintaining intestinal homeostasis, yet its biological role in orchestrating intestinal development remains to be elucidated. This study demonstrates that TSP50 deficiency significantly exacerbates age-dependent susceptibility to colitis in mice. Phenotypic analysis shows that TSP50-deficient mice exhibit abnormal intestinal development, characterized by premature differentiation of specific epithelial cell lineages during embryonic stages and premature crypt formation during the neonatal period, while the organoid-forming capacity of intestinal stem cells (ISCs) is markedly reduced in adulthood. Mechanistically, TSP50 exerts serine protease activity via its catalytic triad to target the juxtamembrane (JM) region of TGF- β receptor II (T β RII) and mediate proteolysis of its intracellular domain (ICD), thereby restraining TGF- β signaling intensity. Persistent TGF- β hyperactivation likely serves as a major contributor to the disrupted developmental timing and deteriorated organoid-forming capacity observed in TSP50-deficient mice. Importantly, Adeno-associated virus (AAV)-mediated restoration of TSP50 expression effectively suppresses aberrant TGF- β signaling and mitigates the exacerbated age-dependent susceptibility to colitis. Furthermore, clinical analysis reveals that TSP50 expression in inflammatory bowel disease (IBD) tissues is significantly and negatively correlated with both patient age and TGF- β pathway activity. Collectively, our findings suggest that TSP50 contributes to intestinal developmental progression, at least in part through modulation of TGF- β signaling activity, providing a promising molecular target for the prevention and treatment of age-related intestinal diseases.

Keywords: IBD; TSP50; TGF- β ; Organoid

INTRODUCTION

The intestinal epithelium is among the most rapidly self-renewing tissues in mammals, with its homeostasis maintained by a dynamic equilibrium between intestinal stem cell (ISC) self-renewal and differentiation. Recent advances in single-cell sequencing and spatial transcriptomics have provided systemic insights into these regulatory mechanisms at the cellular and microenvironmental levels, elucidating the precise spatiotemporal distribution and synergistic regulation of canonical Wnt, Notch, BMP, and TGF- β signaling pathways along the crypt-villus axis (Beumer and Clevers, 2021). During intestinal development, the transition from fetal to adult epithelial structures is not a continuous progression but rather follows a strictly programmed temporal sequence. The precise spatiotemporal establishment of distinct cell lineages and their microenvironments is decisive for adult barrier function and regenerative capacity. Spatial multi-omics studies further indicate that heterochrony or dysregulation of developmental timing is closely linked to impaired adult barrier function and increased susceptibility to various intestinal diseases (Elmentaite et al., 2021; Fawcner-Corbett et al., 2021). With aging, the intestine exhibits hallmarks of "inflammaging," including attenuated ISC regenerative capacity, compromised epithelial barrier integrity, and chronically elevated inflammatory levels (Brunet et al., 2023; Kolesnichenko, 2026). Thus, clarifying the molecular mechanisms governing intestinal development is essential for understanding the age-related pathogenesis of inflammatory bowel disease (IBD). The TGF- β signaling pathway exerts dichotomous roles in intestinal physiological homeostasis. Under homeostatic conditions, TGF- β acts as a potent inhibitor of cell proliferation, maintaining mucosal immune tolerance and suppressing tumorigenesis (Massague and Sheppard, 2023; Stolfi et al., 2020; Wu et al., 2024). Nevertheless, persistent or aberrant TGF- β activation is a critical molecular driver of intestinal fibrosis, epithelial senescence, and barrier dysfunction, contributing to age-related pathological processes (Jo et al., 2023; Ling et al., 2025; Ren et al., 2023). Although the type II receptor T β RII is recognized as the core receptor required for TGF- β signaling initiation, its proteostatic regulation in intestinal homeostasis remains to be systematically elucidated. Given the critical role of T β RII in TGF- β signaling, elucidating the molecular mechanisms governing T β RII stability in the intestinal epithelium is of great importance for understanding the establishment and maintenance of intestinal homeostasis, as well

as the mechanisms underlying barrier dysfunction.

Testis-specific protease 50 (TSP50) was initially identified as a potential tumor-associated antigen with conserved serine protease motifs. However, accumulating evidence has demonstrated that TSP50 is also widely expressed in intestinal tissues beyond the reproductive system and is closely associated with chemoresistance and clinical prognosis in colorectal cancer (Hilal et al., 2017; Li et al., 2025a; Li et al., 2025b; Lu et al., 2018; Youssef et al., 2021). Our previous studies revealed that TSP50 suppresses TGF- β signaling through interaction with T β RII, thereby regulating goblet cell differentiation, maintaining intestinal mucosal structural integrity, and reducing susceptibility to colitis (Li et al., 2024). However, the precise molecular mechanism by which TSP50 regulates TGF- β signaling through T β RII in the intestine remains unclear. In the present study, we found that TSP50 deficiency leads to abnormal intestinal development, characterized by premature crypt formation and premature differentiation of partial epithelial cell lineages. In adulthood, TSP50-deficient mice exhibit impaired organoid-forming capacity of intestinal stem cells (ISCs), elevated expression of senescence-associated markers, and increased susceptibility to age-related colitis, accompanied by aberrant activation of the TGF- β signaling pathway. Based on these observations, we hypothesized that TSP50 may regulate TGF- β signaling intensity by targeting T β RII protein stability in a protease activity-dependent manner, thereby influencing intestinal developmental progression and susceptibility to age-related colitis.

MATERIALS AND METHODS

Human IBD sample analysis

IBD patient samples (n=21) were obtained from the authors' previously established cohort(Li et al., 2024; Li et al., 2025b). Detailed patient information is provided in Supplementary Table S1. The study was approved by the institutional review board, and all diagnoses were histopathologically confirmed by pathologists following surgery. All procedures were conducted in accordance with the Declaration of Helsinki.

Antibodies and reagents

The antibodies and reagents used in this study are listed in Supplementary Table S2.

Induction of DSS-induced IBD

Acute colitis was induced in adult male mice by administering 3% DSS (molecular weight: 35–50 kDa) in their drinking water for a duration of 7 days. Subsequently, the drinking water was switched back to regular water. Throughout the process, the mortality rate of the mice and their body weight were monitored and recorded.

Animals

All experimental procedures were reviewed and approved by the Animal Advisory Committee at Northeast Normal University, China. The standard of the laboratory condition was specific pathogen-free (SPF). All of the mice were maintained under a 12-hour light/12-hour dark cycle (lights on at 6 a.m. and lights off at 6 p.m.). The mice were mated, bred, and genotyped in the Animal Experimental Center of National Engineering Laboratory for Druggable Gene and Protein Screening. All of the mice used in this study were maintained on a C57BL/6N background.

TSP50 heterozygous Knockout (KO) (*TSP50*^{+/-}) mice were generated by the GemPharmatech

Co., Ltd (Nanjing, China). All mice used in this study were male. The generation strategy and breeding plan for gene knockout mice were as previously described(Li et al., 2024).

Histologic scoring

Histologic scoring was performed on HE stained colonic tissue as follows (modified from(Johansson et al., 2014)). The scoring system included the assessment of various parameters: inflammatory cell infiltration (score range: 0–4), goblet cell depletion or decreased mucus accumulation (score range: 0–4), mucosa thickening (score range: 0–4), and loss of crypts (score range: 0–4). The individual scores for each parameter were summed to obtain a total score (maximum score: 16).

Disease activity index

The Disease Activity Index (DAI, 0–4 points) assesses colitis severity in mice via body weight loss, stool consistency, and bloody stool: 0 = no weight loss, normal stool, no occult blood; 1 = 1–5% weight loss, normal→soft stool, occult blood (–)→(+); 2 = 6–10% weight loss, soft stool, occult blood (+); 3 = 11–15% weight loss, soft→diarrhea, occult blood→gross blood; 4 = >15% weight loss, diarrhea, gross blood, with higher scores indicating more severe colitis.

Plasmid construction

pcMV–myc–TβR II (Li et al., 2024), pcDNA3.1–TSP50(Zhang et al., 2021), and pcMV–Flag–H153A/D206A/T310A(Gao et al., 2024a) were previously generated. Among these, pcMV–Flag–TβR II (ECD/JM/ICD) was prepared by Miaoling Bio.

Cell culture and plasmid transfection

HEK 293T and NCM 460 cell lines were obtained from the cell library of the Chinese Academy of Sciences. All cell lines were cultured in DMEM medium supplemented with 10% FBS,

100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO₂ incubator.

Upon reaching 80% confluence, a transfection procedure was conducted. Specifically, 200 µL of RPMI 1640 medium was mixed with 5 µL of X-tremeGENE HP (Roche) and 2.5 µg of plasmid DNA. The transfection mixture was then added to each well containing the cultured cells in medium after incubation at room temperature for 30 minutes.

Luciferase activity assay

HEK 293T cells were plated in 6-well plates. Upon reaching 70–80% confluence, cells were co-transfected with CAGA-Luc, PGL3 control, or pcMV-TSP50/H153A/D206A/T310A using Lipofectamine 3000. After 24 h, 10 ng/mL TGF-β1 was added, and cells were cultured for an additional 48 h. Cells were harvested for dual-luciferase assay, with the activity ratio indicating CAGA activity.

RNA extraction and PCR

The mice were anesthetized using isoflurane and subsequently euthanized for sampling purposes. The intestines were removed and rinsed with cold PBS. They were then cut to the same length and homogenized in Trizol (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted from the homogenized samples, followed by cDNA synthesis. Subsequently, PCR amplification was performed with the corresponding specific primers, using the same method as described earlier in this study (Song et al., 2014). The primer sequences used in this study are listed in Supplementary Table S3.

Western blotting analysis

The intestinal tissue was washed with ice-cold PBS, then cut and weighed before being lysed in ice-cold lysis buffer. Protein extracts were subjected to analysis by SDS-PAGE and Western

blotting. For SDS–PAGE, 20 µg of total protein per sample was resolved, and the separated proteins were transferred onto PVDF membranes (Bio–Rad Laboratories Inc). Immunoblotting was conducted using primary antibodies at dilutions recommended by the manufacturer, with GAPDH serving as a loading control. The immunoblots were detected using an ECL advanced Western blotting detection kit (Invitrogen). The quantification of the Western blotting results from independent repeated experiments was performed using ImageJ software.

Immunofluorescence (IF) analysis

The mice were anesthetized using isoflurane and subsequently euthanized for sampling purposes. The intestines were removed and intraluminally flushed once with cold PBS. They were then fixed in 4% paraformaldehyde for 12 hours at 4°C. Following fixation, the tissues were soaked in PBS containing 30% sucrose overnight at 4°C. Subsequently, the tissues were embedded in optimal cutting temperature compound (OCT), frozen, and cut into 5 µm sections. The frozen sections were washed with PBS, and antigen retrieval was performed by immersing the sections in 0.01 mol/L sodium citrate buffer (pH 6.0) at 98°C for 5 minutes. After antigen retrieval, the sections were blocked with 2% BSA in 0.2% Triton X–100/PBS for 1 hour and then incubated with primary antibodies overnight at 4°C. Following 5 washes with PBS, the sections were incubated with appropriate fluorochrome–conjugated secondary antibodies for 1 hour, followed by another 5 washes. Coverslips were mounted onto glass slides using 75% glycerol as a mounting medium. Images were acquired using a laser scanning confocal microscope (Zeiss, Germany).

Hematoxylin and eosin (HE) staining

The mice were anesthetized using isoflurane and subsequently euthanized for sampling purposes. The intestines were removed, intraluminally flushed once with cold PBS, and then fixed

in a formaldehyde solution for 48 hours. Following fixation, the tissues were dehydrated using a series of graded ethanol solutions, embedded in paraffin, and sectioned into 5 μ m slices. The paraffin sections were then dewaxed using xylene to remove the paraffin. Hematoxylin staining was performed for 6 minutes, followed by eosin staining for 2.5 minutes using a slide staining device. The stained sections were dehydrated, made transparent, and covered with coverslips using neutral balsam as a mounting medium. Light images of the stained sections were captured using a Nikon Eclipse microscope (Nikon, Japan).

Alcian Blue staining

The FFPE tissues or organoids were sectioned into 5 μ m slices, and the paraffin from the sections was removed using xylene. Subsequently, Alcian Blue staining were conducted using kits in accordance with the manufacturer's protocols.

Immunohistochemical (IHC) analysis

Formalin-fixed, paraffin-embedded (FFPE) tissue samples were cut into 5 μ m sections. The paraffin was subsequently removed from the sections using xylene. To retrieve the antigens, the deparaffinized and rehydrated sections were immersed in 0.01 mol/L sodium citrate buffer (pH 6.0) and subjected to high-pressure treatment for 3 minutes. After antigen retrieval, the slides were incubated with the primary antibody for 4 hours at room temperature, followed by washing with PBS and subsequent incubation with a secondary antibody. The antibodies were detected using a DAB staining kit. Hematoxylin was used as a counterstain, and the slides were mounted with neutral balsam and coverslips. Light images of the stained sections were captured using a Nikon Eclipse microscope.

Co-immunoprecipitation (Co-IP)

Co-immunoprecipitation (Co-IP) was performed using Protein A/G Magnetic Beads (HY-K0202, MedChemExpress, Monmouth Junction, NJ, USA) following the manufacturer's protocols to investigate protein-protein interactions. Firstly, 25 μ L of magnetic beads pretreated with 0.5% Triton X-100 in PBS (PBST) solution were mixed with PBST containing the antibodies at a final concentration of 5 μ g/mL and incubated at 4°C for 2 hours on a flip mixer. The proteins extracted from cells using IP lysis solution (87787, Thermo Fisher Scientific, Waltham, MA, USA) were then mixed with the antibody-magnetic-bead complexes and incubated overnight at 4°C on a flip mixer (88881002, Thermo Fisher Scientific, Waltham, MA, USA). After thorough washing, the antigen-antibody-magnetic-bead complexes were boiled in 25 μ L of 1 $\times$ SDS-PAGE loading buffer, and the supernatant was used for Western blotting analysis.

GST pull-down assay

The GST-TSP50 protein was prepared and purified as described previously (Gao et al., 2021). Total protein extraction from cells treated according to the experimental requirements was performed using RIPA lysate. Subsequently, the target proteins were incubated overnight at 4°C with GST pull-down protein binding buffer, along with an equilibrated 50% gel suspension and bait proteins. After centrifugation, the supernatant was removed, and the gel was resuspended using GST pull-down binding buffer to effectively wash away unbound proteins. Finally, GST pull-down elution buffer was added to the gel and incubated for 10 minutes. Following centrifugation at 1000 g for 2 minutes at 4°C, the supernatant was collected for Western blotting detection.

Crypt isolation and organoid culture

Mice were anesthetized with isoflurane prior to intestinal dissection, and isolated intestinal tissues were rinsed with pre-cooled DPBS. Tissues were cut into ~5 mm fragments and repeatedly

washed with cold DPBS until visible debris was completely removed. Tissue fragments were suspended in 20 mL DPBS supplemented with 20 mmol/L EDTA and incubated on a shaker for 30 min. After stationary sedimentation for 2 min, the supernatant was harvested and filtered through a 70 μ m cell strainer. The filtrate was centrifuged at 500 g for 5 min, the pellet was resuspended in 5 mL DMEM/F12 containing 5% FBS for washing, followed by another centrifugation at 500 g for 5 min to collect intact intestinal crypts. Crypts were counted under a microscope and serially diluted to attain an optimal seeding density, then mixed 1:1 with Matrigel to prepare crypt–Matrigel suspensions. Aliquots of the mixture were plated into a 24–well plate and allowed to solidify at 37°C. IntestiCult™ OGM Mouse Organoid Growth Medium was added to each well, and medium was refreshed every two days. All organoids applied in this study were generated from primary intestinal crypt culture without subsequent passaging.

AAV vectors delivery

Empty AAV9 vectors and PRSS50 (TSP50)–overexpressing AAV9 vectors (PRSS50: of human origin, Gene ID: 235631) were constructed by Vigene Biosciences (Shandong, China). These vectors (empty AAV9 vectors and PRSS50–overexpressing AAV9 vectors) were administered to mice via enema at a dose of $\sim 1 \times 10^{12}$ genome copies (GC) per mouse.

Bioinformatics analysis of public datasets

The transcriptome dataset GSE87473 was obtained from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE87473>). A total of 106 IBD samples with documented age metadata (range: 6.0–77.0 years) were included in this study for further analysis.

Gene Set Variation Analysis (GSVA)



To evaluate the activity of the TGF- β signaling pathway, Gene Set Variation Analysis (GSVA) was performed using the R package "GSVA." Enrichment scores for each sample were calculated based on the hallmark gene sets from the Molecular Signatures Database (MSigDB). These scores were subsequently utilized to analyze the correlations among pathway activity, TSP50 expression, and patient age.

Correlation and statistical analysis

Spearman's rank correlation was employed to assess associations between TSP50 expression, patient age, and TGF- β GSVA scores. For comparative analysis, patients were stratified by age into two cohorts: < 45 years (n=26) and $\geq$ 45 years (n=30). Additionally, samples were categorized into pathological/lesional (P) and non-pathological/non-lesional (NP) tissues to investigate tissue-specific regulation. Data visualization and statistical computations were conducted using R software and GraphPad Prism. A value of $P < 0.05$ was considered statistically significant.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software. The data are presented as means $\pm$ standard error of the mean (SEM). A two-tailed unpaired Student's t test was employed to calculate significant differences between two groups of samples or mice, with a significance threshold of $P < 0.05$. Asterisks were used to denote the level of significance, where ns indicates not significant, * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$. All data presented are representative of a minimum of three independent experiments.

RESULTS

TSP50 deficiency enhances age-dependent susceptibility to DSS-colitis in mice

Building upon previous findings that TSP50 deficiency increases susceptibility to DSS-induced colitis (Li et al., 2024), this study investigated whether this susceptibility is age-dependent (Chin et al., 2017; Kang et al., 2022; Kolev and Kaestner, 2023; Pann et al., 2020; Toth, 2018). In IBD patients, Spearman correlation analysis revealed a significant negative correlation between TSP50 expression and age ($R=-0.264$, $P=0.049$) (Figure 1A). Consistent with this clinical observation, we found that 7-month-old (7M) TSP50-deficient mice exhibited significantly higher mortality in the DSS model compared to 6-week-old (6W) and 3-month-old (3M) TSP50-deficient mice, whereas wild-type (WT) mice showed no significant age-related differences (Figure 1B, C). During the 10-day period following DSS induction, 7M *TSP50*^{-/-} mice displayed rapid weight loss and elevated Disease Activity Index (DAI) scores, accompanied by markedly shortened colon lengths, exacerbated intestinal epithelial damage, and a reduction in goblet cell numbers (Figure 1D–G). Collectively, these results demonstrate that the severity of colitis in TSP50-deficient mice exhibits an age-dependent pattern.

TSP50 deficiency induces premature intestinal development in mice

Normal timeline of intestinal development in wild type mice: intestinal tube closure completed by embryonic Day 9.5 (E9.5); intestinal villus structures formed between E14.5 and E15.5; the initial differentiation of goblet cells and enteroendocrine cells began at E16.5; and crypt structures and Paneth cell differentiation matured by postnatal Day 14 (P14) (Figure 2A) (Grosse et al., 2011; Guiu and Jensen, 2015; Sato and Clevers, 2013; Zorn and Wells, 2009). Our findings demonstrate that *TSP50*^{-/-} mice exhibited villous structure formation as early as E14.5, in contrast to WT mice. Furthermore, villus lengths at E15.5, E16.5, and E18.5 were significantly greater in *TSP50*^{-/-} mice compared to WT mice (Figure 2B). Crypt structures also formed prematurely on P7 (Figure 2C), accompanied by Paneth cell differentiation (Figure 2D); additionally, the number of Paneth cells in *TSP50*^{-/-} mice was significantly higher than that in WT mice on P14 (Supplementary Figure S1A). Regarding other intestinal cell populations, although the emergence of ISCs, enteroendocrine cells, and goblet cells was not advanced in *TSP50*^{-/-} mice, their

quantities were significantly greater than those in WT mice at E16.5, the time point of their initial differentiation (Figure 2E–G). Notably, only ISCs maintained this numerical advantage until P14 (Supplementary Figure S1B–C). In contrast, the numbers of enteroendocrine cells did not differ significantly between *TSP50*^{−/−} and WT mice at E18.5, P7, and P14 (Supplementary Figure S1D–F). For goblet cells, their counts were comparable to WT mice at E18.5 and P7 but significantly reduced in *TSP50*^{−/−} mice at P14 (Supplementary Figure S2A–C). These results suggest that *TSP50* deficiency disrupts the regulation of intestinal development, leading to precocious formation of intestinal villi and crypts, as well as premature differentiation of partial intestinal epithelial cell lineages.

***TSP50* deficiency increases senescence-associated marker expression in adult mouse intestine**

To further investigate phenotypes during adulthood, we examined the levels of intestinal barrier function and senescence-associated markers in adult mice aged 3 and 7 months. As oxidative stress is a key mediator of cellular aging (De Oliveira Belem et al., 2015), we quantified malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) levels in intestinal tissues. No significant differences in these oxidative stress markers were detected between *TSP50*^{−/−} and WT mice at 3 months of age. However, by 7 months, *TSP50*^{−/−} mice displayed markedly decreased SOD, CAT, and GSH activities (Figure 3A). Furthermore, 7M *TSP50*^{−/−} mice exhibited a significant upregulation of multiple senescence-associated markers, including P16, γ H2AX, SA- β -gal, as well as an increased number of apoptotic cells (Grosse et al., 2020; Ni et al., 2022; Sienkiewicz et al., 2023; Yang et al., 2025; Yang and Yuan, 2025), accompanied by reduced expression of intestinal tight junction proteins (Occludin) and elevated levels of inflammatory cytokines (TNF- α) and lipopolysaccharide (LPS) (Figure 3B–E, Supplementary Figure S3A–D). Collectively, these findings indicate that *TSP50* deficiency leads to elevated expression of senescence-associated markers and impaired intestinal barrier function in adult mice.

***TSP50* deficiency causes age-related changes in organoid forming capacity of ISCs**

Intestinal functional deterioration is associated with the progressive loss of ISC stemness and

functional decline (Gao et al., 2024b; Jasper, 2020; Wang et al., 2025). To investigate the role of TSP50 in this process, we first quantified ISC numbers. Compared to WT controls, *TSP50*^{-/-} mice exhibited a significant increase in ISC numbers during embryonic (E16.5, E18.5) and postnatal (P7, P14) stages (Figure 2E, Supplementary Figure S1B–C). However, no significant differences were observed between genotypes at 3 months or 7 months of age (Figure 4A). We established organoids at various developmental stages to assess the *in vitro* regenerative potential of ISCs (Jo et al., 2023; Nalapareddy et al., 2017). Morphological analysis revealed that during the postnatal period (P10, P12), TSP50 deficiency did not affect organoid formation efficiency but accelerated maturation, as evidenced by a significantly higher proportion of organoids with multiple buds (>5) in *TSP50*^{-/-} cultures on Day 7 (Figure 4B, Supplementary Figure S4A). In sharp contrast, the regenerative function of ISCs was significantly impaired in 3M and 7M *TSP50*^{-/-} mice. Specifically, organoids derived from 3M *TSP50*^{-/-} mice showed significantly reduced formation efficiency and budding rates compared to WT controls. By 7 months of age, the organoid-forming capacity of *TSP50*^{-/-} mice declined markedly (Figure 4C, D). This phenotype closely resembles the reduced organoid size and impaired growth typically observed in intestinal organoids derived from aged mice (Uchida et al., 2019). Collectively, these results indicate that TSP50 deficiency induces age-related changes in the organoid-forming capacity of mouse intestinal stem cells, manifested as organoids with increased budding numbers in the neonatal stage and markedly reduced organoid formation efficiency and budding numbers in adulthood (Figure 4E, Supplementary Figure S4B).

TSP50 deficiency diminishes ISC organoid-forming capacity via aberrant TGF- β pathway activation

Given that 7-month-old *TSP50*^{-/-} mice exhibited markedly reduced ISC organoid formation capacity and elevated senescence-associated markers, and considering the well-documented roles of the Wnt, Notch and TGF- β signaling pathways in regulating the self-renewal and differentiation of ISCs (Jiang et al., 2024), we further investigated the alterations of these pathways in the intestinal tissues of 7M *TSP50*^{-/-} mice. Here, we observed that compared to 7M WT mice, the Wnt, Notch, and TGF- β signaling pathways exhibited signatures of aberrant activation in the intestines of age matched *TSP50*^{-/-} mice. The Wnt (β -catenin, Cyclin D1, and c-Myc), Notch

(*Notch1*, *Dll1*, *Dll4*, *Jag1*, *Jag2*, and *Hes1*), and TGF- β (p-Smad2/3) signaling pathways were significantly upregulated in the intestinal tissues of 7M *TSP50*^{-/-} mice compared to 7M WT mice (Figure 5A–E, Supplementary Figure S5). To dissect the key molecular mechanisms driving these phenotypes, we treated intestinal organoids with specific inhibitors for each pathway. In WT organoids, inhibition of Wnt, Notch, or TGF- β signaling suppressed growth, confirming that the basal activity of these pathways is essential for normal organoid maintenance. However, in *TSP50*^{-/-} organoids, only the TGF- β inhibitor improved growth morphology, formation efficiency, and budding rates (Figure 5F–H). To further explore the relationship between TGF- β signaling and intestinal developmental abnormalities induced by TSP50 deficiency, we examined its changes across various developmental stages. The results revealed that p-Smad2/3 remained continuously upregulated in *TSP50*^{-/-} mice intestines during embryonic (E13.5, E18.5), postnatal (P7, P14), and adult (3M) stages (Figure 5I). Collectively, these findings suggest that sustained activation of TGF- β signaling caused by TSP50 deficiency may be an important contributor to the age-dependent alterations in the organoid-forming capacity of ISCs, thereby providing a potential explanation for the age-dependent exacerbation of the colitis phenotype observed in *TSP50*^{-/-} mice (Figure 1).

TSP50 binds the T β RII juxtamembrane region and promotes proteolytic destabilization of the intracellular kinase domain

Our previous work identified TSP50 as a protease structurally similar to serine proteases but characterized by a unique catalytic triad comprising His153, Asp206, and Thr310 (Song et al., 2014). To investigate its regulatory mechanism, we utilized a TGF- β -responsive CAGA reporter vector and generated TSP50 catalytic triad mutants (H153A, D206A, and T310A). Upon TGF- β 1 stimulation, WT-TSP50 significantly suppressed reporter activity, whereas the three mutants lost this inhibitory capacity (Figure 6A), suggesting that the negative regulation of TGF- β signaling by TSP50 depends on its intact protease activity. HADDOCK protein-protein docking analysis indicated that WT-TSP50 possesses binding potential for T β RII. Although the binding energy was slightly attenuated in the mutants, all HADDOCK scores remained below -30, indicating a preserved potential for interaction (Supplementary Figure S6A). This prediction was confirmed by immunofluorescence co-localization (Figure 6B) and Co-IP assays (Figure 6C), which

demonstrated that all TSP50 mutants retained the ability to bind T β RII. However, Western blot and *in vitro* cleavage assays revealed that only WT-TSP50 reduced T β RII protein levels and phosphorylation, whereas the mutants lost this hydrolytic function (Figure 6D–F, Supplementary Figure S6B). These results indicate that TSP50 regulates T β RII via direct proteolytic cleavage. Structurally, T β RII comprises an Extracellular Domain (ECD), Transmembrane Domain (TM), Juxtamembrane Region (JM), and Intracellular Kinase Domain (ICD) (Figure 6G) (Boesen et al., 2002; Graham et al., 2013; Hart et al., 2002; Hata and Chen, 2016). Notably, the JM region acts as an intrinsically flexible linker between the TM and ICD of T β R II, serving as a conformational hub for receptor dimerization and kinase activation (Lin et al., 2006). To map the specific domains mediating binding and cleavage, we generated T β RII truncation vectors encompassing the ECD, JM, or ICD. Co-IP and enzyme activity assays confirmed that TSP50 anchors to the T β RII–JM region for binding and promotes proteolytic cleavage–associated destabilization of its Ser/Thr-rich intracellular kinase domain (ICD) (Figure 6H, I).

TSP50 restoration alleviates age-dependent colitis via T β RII/TGF- β with clinical relevance in IBD

We further investigated whether *in vivo* restoration of TSP50 could alleviate the elevated levels of intestinal senescence markers and colitis susceptibility resulting from its deficiency. We restored TSP50 expression in 7M *TSP50*^{−/−} mice via intrarectal administration of Adeno-Associated Virus (AAV) vectors encoding full-length TSP50. Fourteen days post administration, expression of TSP50 in the colonic epithelium of 7M *TSP50*^{−/−} mice was significantly recovered (Supplementary Figure S7A). Subsequently, we examined changes in TGF- β signaling and senescence-associated markers. The results indicated that compared to untreated 7M *TSP50*^{−/−} mice, the levels of T β RII, p-T β RII, and p-Smad2/3, as well as the expression of P16 and γ H2AX, were significantly reduced in the intestinal epithelium of AAV-restored mice (Supplementary Figure S7A–D). Notably, following TSP50 restoration, the age-dependent exacerbation of colitis observed in 7M *TSP50*^{−/−} mice was significantly ameliorated, as evidenced by attenuated body weight loss, improved survival rates, and reduced intestinal epithelial damage and goblet cell loss (Figure 7A–E). To evaluate the clinical relevance of these findings, we analyzed the correlation between the TSP50/TGF- β axis, age, and disease progression using the GSE87473 database. Gene Set

Variation Analysis (GSVA) revealed that patient age was positively correlated with the enrichment score of the TGF- β pathway (Figure 7F, $R=0.32$, $P=0.011$), while TSP50 expression showed a significant negative correlation with the enrichment score of the TGF- β pathway (Figure 7G, $R=-0.38$, $P=0.0022$). Scatterplot and correlation analyses further suggested that older patients were characterized by lower TSP50 expression and higher TGF- β pathway activity (Figure 7G, Figure 1A). Stratified analysis by disease progression revealed that TSP50 and TGF- β were significantly and negatively correlated in pathological (P) tissues featuring high-grade dysplasia or early malignancy (Figure 7H; $R=-0.92$, $P<2.2e-16$). Further analysis of IBD clinical samples showed that patients aged ≥ 60 years exhibited significantly more severe histological damage than those aged ≤ 40 years, with significantly decreased TSP50 expression and significantly increased expression of T β RII, p-T β RII, p-Smad2/3, and P16 (Figure 7I). Collectively, these findings support the notion that loss of TSP50 contributes to age-related colitis and may promote its malignant progression by derepressing the TGF- β signaling pathway.

DISCUSSION

This study uncovers the molecular mechanism whereby TSP50 binds and proteolytically degrades T β RII in a catalytic triad-dependent manner, thereby modulating TGF- β signaling activity to influence intestinal development and age-associated susceptibility to colitis. Analysis across embryonic, postnatal, and adult stages revealed that TSP50 deficiency not only disrupts intestinal homeostasis, but may also cause sustained aberrant activation of TGF- β signaling throughout development and adulthood by relieving the proteolytic restriction on T β RII. This leads to altered intestinal development, characterized by premature differentiation of specific intestinal epithelial cell lineages and premature crypt formation during early life, reduced organoid-forming capacity in adulthood, and increased age-dependent susceptibility to colitis. This phenomenon aligns with recent studies based on single-cell transcriptomics and spatial omics. For instance, Guo et al. reported in *Cell* that temporal perturbations in cell differentiation trajectories during intestinal epithelial development lead to long-term remodeling of the stem cell niche, resulting in persistent functional consequences in adulthood (Fawcner-Corbett et al., 2021). These findings are also consistent with the antagonistic pleiotropy theory in aging biology, which posits that molecular events promoting growth or development in early life, if lacking precise restriction,

often transform into intrinsic drivers of aging and functional decline in later life(Long and Zhang, 2023; Wu et al., 2022).

Mechanistically, these developmental alterations are closely associated with persistent aberrant activation of TGF- β signaling. Research by Jo et al. indicates that the reduced organoid-forming capacity of intestines from aged mice depends on cell cycle arrest mediated by the TGF- β -Smad3-p16INK4a axis, and pharmacological inhibition of TGF- β signaling significantly improves regenerative capacity (Jo et al., 2023). Interestingly, TSP50 deficiency was accompanied by concurrent activation of the Wnt, Notch and TGF- β pathways, all of which are known regulators of intestinal development and epithelial lineage specification(Beumer and Clevers, 2021). During initial differentiation in development, intestinal stem cells, goblet cells and enteroendocrine cells increase synchronously, suggesting that TSP50 deletion may disturb epithelial lineage allocation rather than independently regulating a single specific cell population. Notably, despite concurrent alterations in multiple signaling pathways, only inhibition of TGF- β signaling significantly improved the abnormal organoid phenotype. Therefore, we speculate that aberrant activation of TGF- β signaling may be a key contributor to intestinal developmental abnormalities, whereas the changes in Wnt and Notch signaling are more likely to reflect signaling crosstalk associated with epithelial maturation and lineage differentiation.

In this study, we demonstrated that TSP50 functions as a serine protease that directly targets the juxtamembrane region of T β RII and mediates proteolysis of its intracellular kinase domain, thereby impairing the receptor's functional integrity and signaling capacity. Moreover, AAV-mediated restoration of TSP50 significantly suppressed aberrant TGF- β signaling and alleviated the associated phenotypes. Nevertheless, developmental stage-specific interventions are still needed in follow-up studies to further clarify the temporal role of TGF- β signaling in shaping long-term intestinal phenotypes. This finding serves as a mechanistic extension of our previous work, which showed that TSP50 deficiency leads to aberrant activation of TGF- β signaling in the intestinal epithelium and disrupts mucosal barrier integrity, thereby exacerbating the colitis phenotype (Li et al., 2024). Furthermore, abnormal intestinal developmental progression was observed only in systemic TSP50 knockout mice, whereas intestinal stem cell-specific TSP50 knockout mice did not exhibit evident developmental abnormalities. This suggests that the critical window for TSP50 activity likely occurs during early embryonic development. Existing

developmental biology studies indicate that mouse intestinal organogenesis and villus establishment mainly occur after embryonic Day E14.5, and strict regulation of signaling pathways during this stage is decisive for adult intestinal function (Ameku et al., 2025; Chin et al., 2017; Kwon et al., 2020). Therefore, under conditions of systemic TSP50 deficiency, aberrant activation of TGF- β signaling may lead to premature crypt formation and early differentiation of specific cell lineages during intestinal development, exert long lasting effects on intestinal homeostasis in adulthood, and ultimately increase age associated susceptibility to colitis. In contrast, our previous studies using neural-specific TSP50 knockout mice demonstrated a phenotype primarily characterized by impaired intestinal homeostasis during adulthood through altered neurotransmitter secretion and intestinal microbiota homeostasis (Li et al., 2025a; Li et al., 2025b). Although potential interactions between these mechanisms cannot be excluded, the current findings suggest that the developmental abnormalities described in the present study represent a distinct phenotype observed in systemic TSP50 knockout mice during embryonic intestinal development. A limitation of the present study is that all mechanistic analyses were performed using systemic TSP50 knockout mice, which precludes definitive discrimination between intestinal epithelial cell-autonomous mechanisms and systemic or microenvironmental regulation during intestinal development. Thus, the observed developmental phenotypes may reflect epithelial cell-autonomous effects, systemic or microenvironmental influences, or a combination of these mechanisms, and the current data are insufficient to disentangle their relative contributions. The current data also do not establish a direct causal relationship between early embryonic intestinal stem cell expansion and the subsequent decline in adult intestinal stem cell organoid-forming capacity. Future studies using tissue-specific conditional knockout models together with lineage-tracing approaches will be required to distinguish epithelial cell-autonomous, systemic, and microenvironmental mechanisms underlying these developmental phenotypes. Another limitation of the present study is the relatively small clinical cohort and the exclusive use of male mice. Future studies with larger cohorts will be needed to further evaluate potential sex-dependent differences. In summary, the present study demonstrates that TSP50 modulates the intensity of TGF- β signaling by restricting the stability of T β RII in a manner dependent on its intact catalytic triad, and suggests that this regulatory mechanism may contribute to the maintenance of intestinal developmental homeostasis and age-associated susceptibility to



508 colitis in mice. These findings suggest that regulatory disturbances occurring during intestinal
509 development may serve as an early trigger for age-related intestinal barrier dysfunction, which
510 helps to further understand the pathogenesis of age-associated intestinal disorders.

511

uncorrected proof



DATA AVAILABILITY

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

AUTHORS' CONTRIBUTIONS

J.L. designed, performed experiments, analyzed data, and wrote the paper. J.R., X.Z., S.X., X.M., S.W., and F.G. performed experiments. Y.S., L.Z., and G.W. provided technical support. X.Y., X.L. conceived ideas, wrote the paper, and oversaw the research programme. All authors read and approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

ETHICAL APPROVAL

All animal studies were conducted with approval from the Animal Research Ethics Committee of Northeast Normal University (NENU/IACUC, AC-SMP-13-F-02) of China and performed in accordance with established guidelines.



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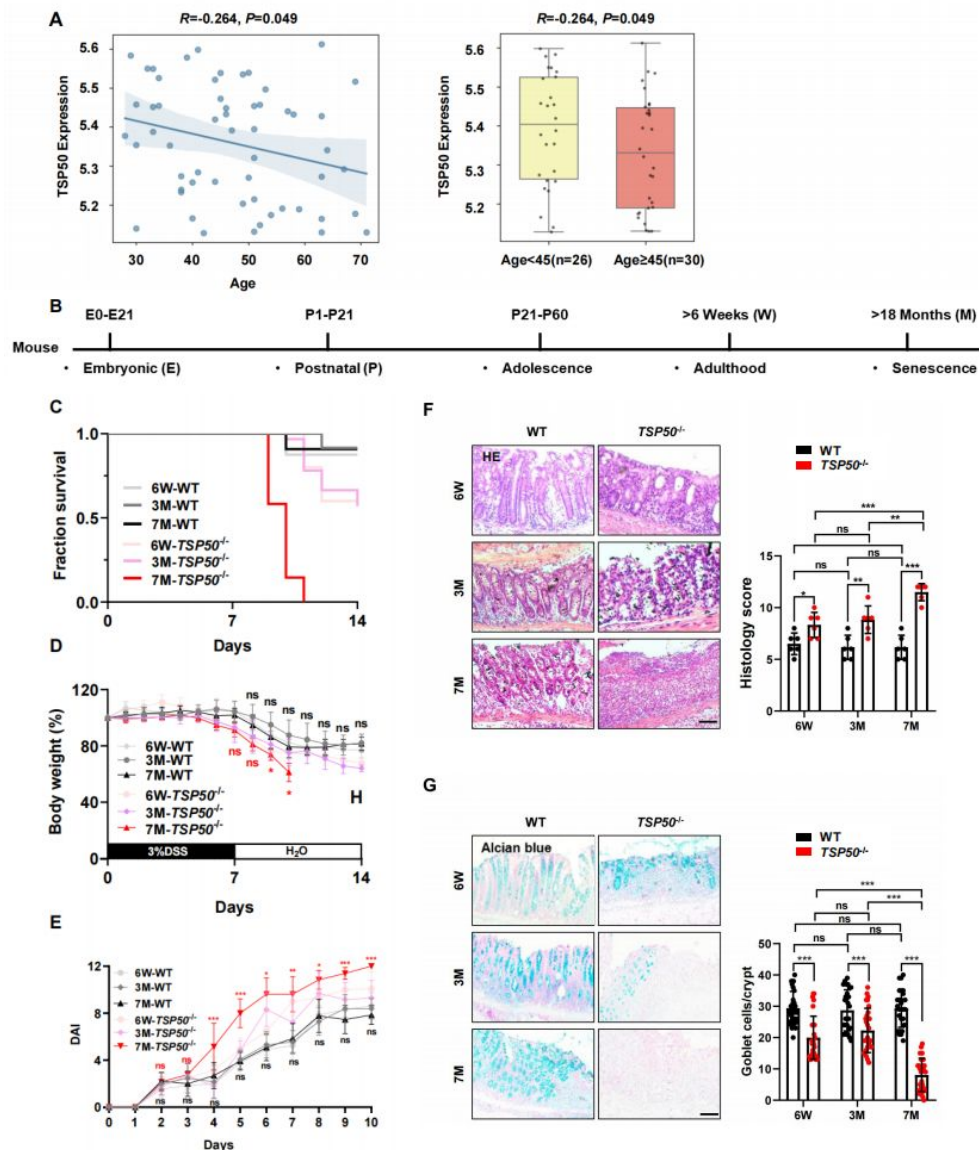


Figure 1. TSP50 deficiency exacerbates DSS-induced colitis in an age-dependent manner

A: TSP50 expression negatively correlates with age in IBD patients. Analysis of the GSE87473 dataset reveals a significant negative correlation between TSP50 expression and age ($R = -0.264$, $P = 0.049$), as shown by Spearman correlation analysis (left) and group comparison (right). For the box plot, patients were stratified into two groups: Age < 45 years (n=26) and Age $\geq$ 45 years (n=30). B: Schematic timeline of mouse developmental stages. C–E: Survival rates (C), percentage of body weight loss (D), and Disease Activity Index (DAI) scores (E) of 6-week-old (6W), 3-month-old (3M) and 7-month-old (7M), WT and *TSP50*^{-/-} mice following treatment with 3% DSS (n=10). Asterisks indicate significant differences between 3M and 7M groups

(Black*: WT; Red*: *TSP50^{-/-}*). F: Representative Hematoxylin and Eosin (HE) staining images of colonic tissues harvested on Day 10, along with histopathological scoring (n=6). Scale bar: 50 μ m. G: Representative Alcian Blue staining images of colonic tissues and quantification of positive goblet cells per crypt on Day 10 (n=6). Scale bar: 50 μ m. Data are presented as mean $\pm$ SEM. Two-way ANOVA was used to analyze (C–D), unpaired two-tailed Student's *t* test was used to analyze (E–G). ns: Not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

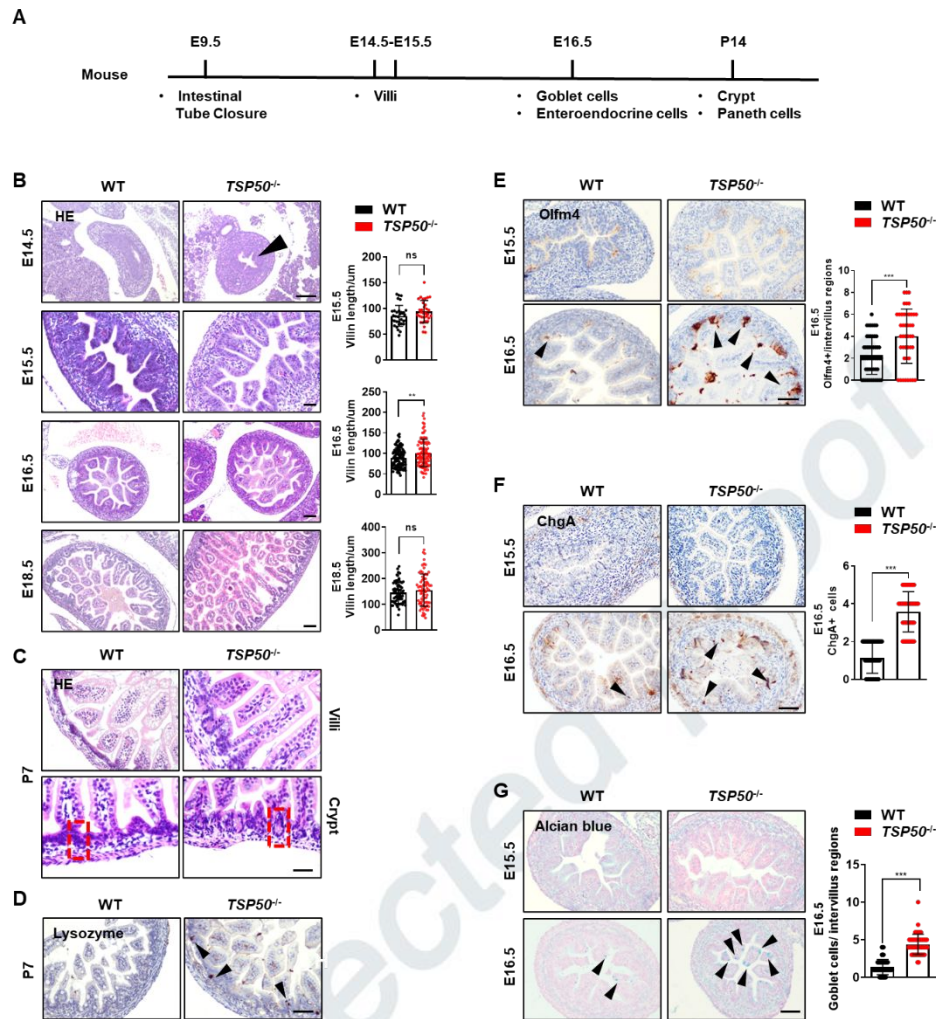


Figure 2. TSP50 deficiency induces premature intestinal epithelial cell differentiation and premature crypt formation

A: Timeline of key events in the intestinal development of normal wild-type (WT) mice. B: Representative Hematoxylin and Eosin (HE) staining images of intestinal tissues from WT and *TSP50*^{-/-} mice at embryonic days (E) 14.5, 15.5, 16.5, and 18.5. Quantification of villus length is shown on the right (n=6). Scale bar: 50 μm. C: Representative HE staining images of intestinal tissues at postnatal Day 7 (P7). Red dashed lines demarcate the intestinal crypt regions. Scale bar: 50 μm. D: Representative immunohistochemistry (IHC) images of Lysozyme (a marker for Paneth cells) in intestinal tissues at P7. Scale bar: 50 μm. E: Representative IHC images of Olfm4 (an intestinal stem cell marker) at E15.5 and E16.5. Quantification of Olfm4-positive cells per intervillous region is shown on the right (n=6). Scale bar: 50 μm. F: Representative IHC images of Chromogranin A (ChgA, an enteroendocrine cell marker) at E15.5 and E16.5. Quantification of

688 ChgA-positive cells per intestinal cross-section is shown on the right (n=6). Scale bar: 50 μ m. G:
689 Representative Alcian Blue (AB) staining identifying goblet cells at E15.5 and E16.5.
690 Quantification of AB-positive cells per intervillous region is shown on the right (n=6). Scale bar:
691 50 μ m. Data are presented as mean $\pm$ SEM. Unpaired two-tailed Student's *t* test was used to
692 analyze. ns: Not significant; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.
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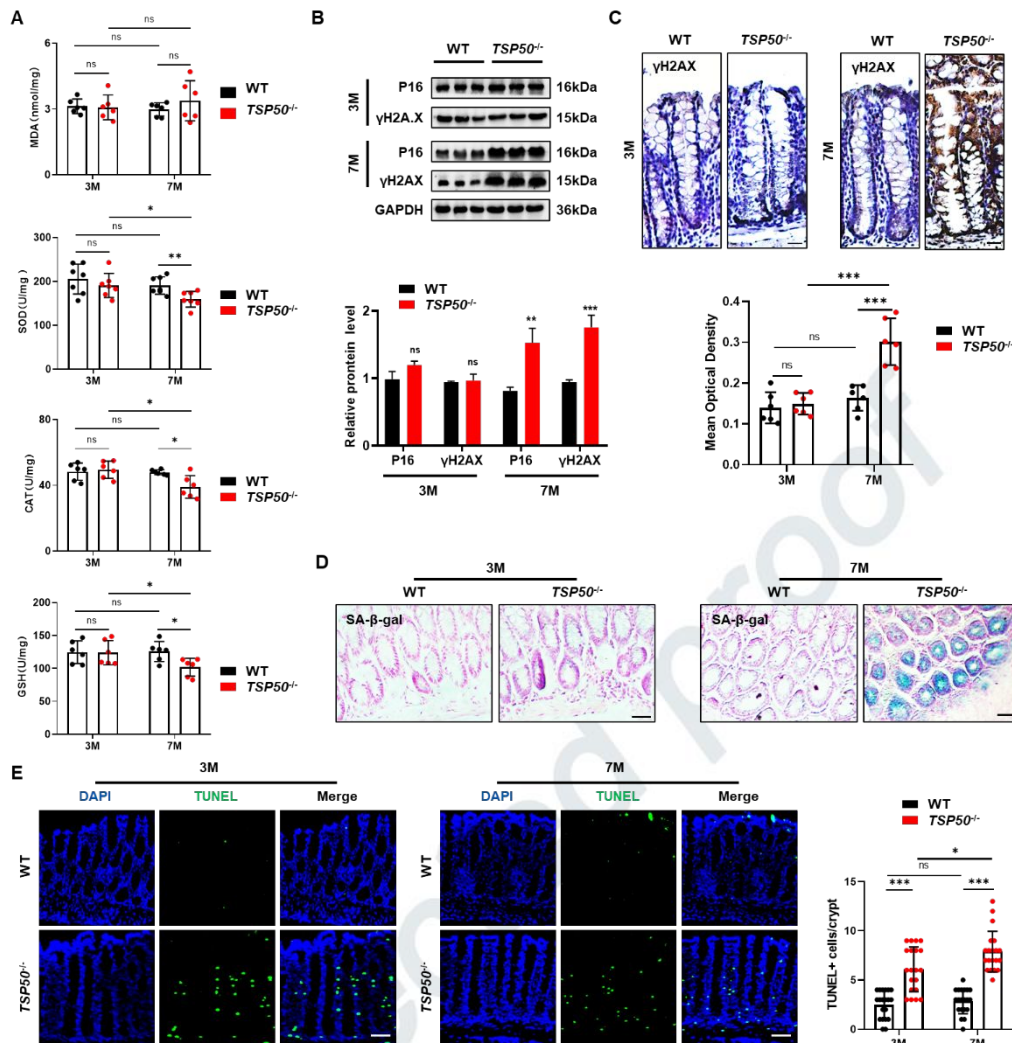


Figure 3. TSP50 deficiency increases senescence-associated marker expression in adult mouse intestine

Experiments were performed using 3-month-old (3M) and 7-month-old (7M) wild-type (WT) and *TSP50*^{-/-} mice. A: ELISA analysis of MDA, SOD, CAT, and GSH levels in intestinal tissues (n=6). B: Western blot analysis of P16 and γH2AX protein expression. Quantification is shown below (n=6). C: Representative IHC staining of γH2AX. Quantification is shown below (n=6). Scale bar: 10 μm. D: Representative images of Senescence-Associated β-galactosidase (SA-β-gal) staining. Scale bar: 20 μm. E: Representative TUNEL immunofluorescence staining. Quantification of TUNEL-positive cells per crypt is shown on the right (n=6). Scale bar: 50 μm. Data are presented as mean ± SEM. Unpaired two-tailed Student's *t* test was used to analyze. ns: Not significant; *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.

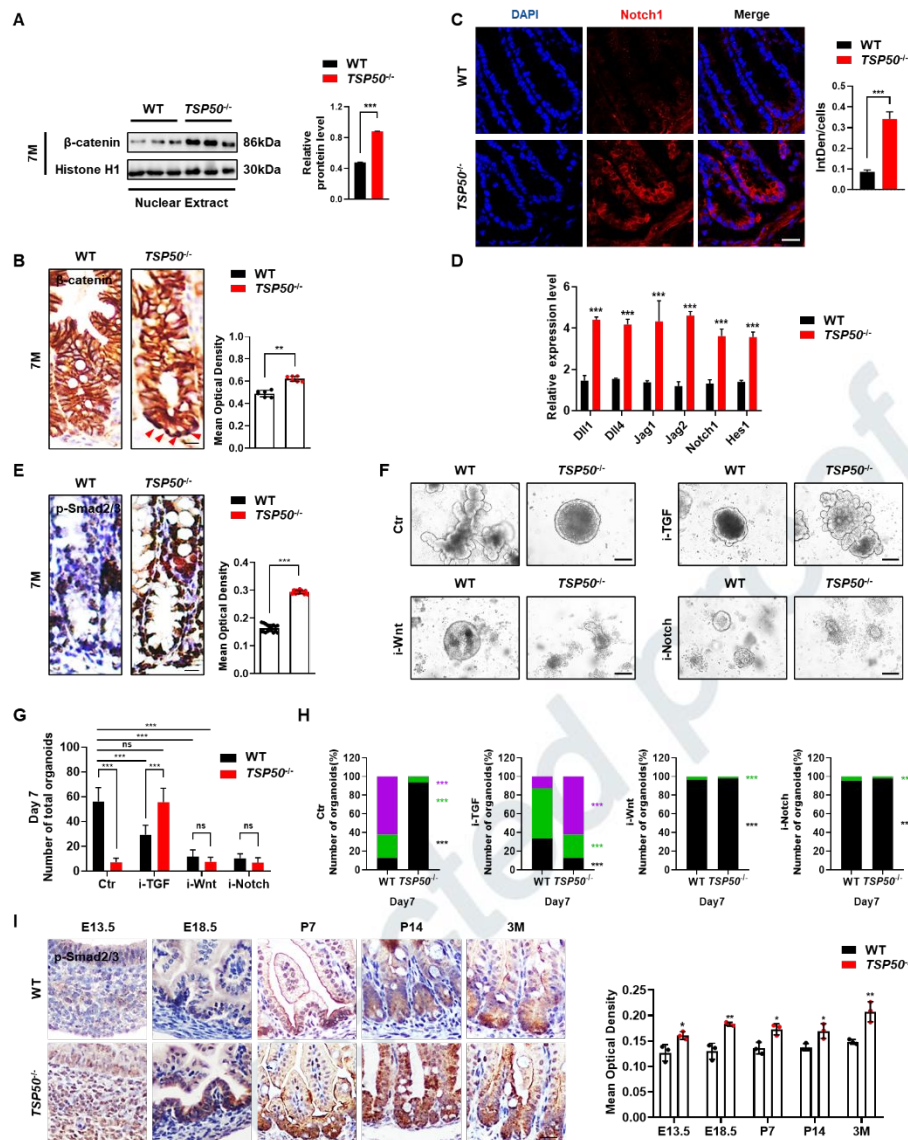


Figure 5. TSP50 deficiency leads to aberrant activation of Wnt, Notch, and TGF- β signaling, and TGF- β inhibition rescues the defective organoid phenotype

A: Western blot analysis of nuclear β -catenin levels in intestinal tissues from 7M WT and *TSP50*^{-/-} mice. Quantification is shown on the right (n=6). B: Representative IHC staining of β -catenin in intestinal tissues from 7M WT and *TSP50*^{-/-} mice. Black arrows indicate nuclear accumulation of β -catenin. Quantification is shown on the right (n=6). Scale bar: 10 μ m. C: Representative IF staining of Notch1 in intestinal tissues from 7M WT and *TSP50*^{-/-} mice. Quantification is shown on the right (n=6). Scale bar: 20 μ m. D: RT-qPCR analysis of Notch signaling components (Dl11, Dl4, Jag1, Jag2, Notch1, and Hes1) in intestinal tissues from 7M WT and *TSP50*^{-/-} mice (n=3). E: Representative IHC staining of p-Smad2/3 in intestinal tissues from 7M WT and *TSP50*^{-/-} mice. Quantification is shown on the right (n=6). Scale bar: 10 μ m. F: Representative images of

intestinal organoids derived from 7M WT and *TSP50*^{-/-} mice treated with solvent control (Ctr) or
 specific signaling pathway inhibitors (5 μ M) starting from Day 0 and cultured for 7 days. i-TGF:
 TGF- β inhibitor (GW788388); i-Wnt: Wnt inhibitor (Salinomycin sodium salt); i-Notch: Notch
 inhibitor (Dibenzazepine). Scale bar: 10 μ m. G-H: Quantification of organoid formation
 efficiency (seeded with 200 crypts per well) (G) and the percentage distribution of budding
 numbers (H) in organoids derived from 7M mice (n=3). (Black, <2 buds; Green, 3–5 buds;
 Purple, >5 buds). I: Representative IHC staining of p-Smad2/3 in intestinal tissues from WT and
TSP50^{-/-} mice at E13.5, E18.5, P7, P14, and 3M. Quantification is shown on the right (n=6). Scale
 bar: 20 μ m. Data are presented as mean $\pm$ SEM. Unpaired two-tailed Student's *t* test was used to
 analyze. ns: Not significant; *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.

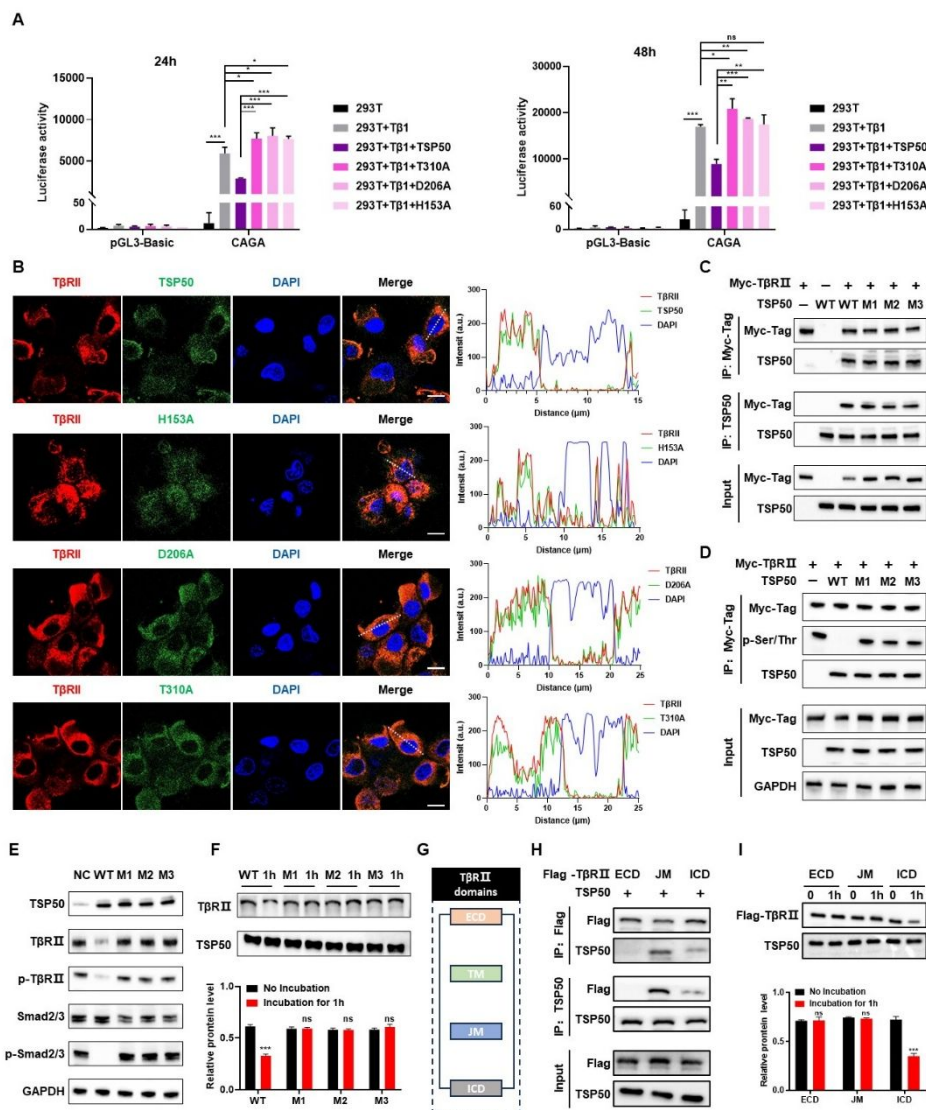


Figure 6. TSP50 binds the TβRII juxtamembrane region and promotes proteolytic destabilization of the intracellular kinase domain

A: Luciferase reporter assays. Cells were co-transfected with the TGF-β-responsive CAGA-luciferase reporter (or pGL3-Basic as a control) together with wild-type (WT) TSP50 or its catalytic triad mutants (H153A, D206A, T310A). At 24 h and 48 h post-transfection, cells were stimulated with 10 ng/mL TGF-β1 for 30 min. Luciferase activity was subsequently measured. **B:** IF co-localization analysis of TβRII with WT-TSP50 or its mutants (H153A, D206A, T310A) in NCM460 cells. Scale bar: 10 μm. **C:** Co-immunoprecipitation (Co-IP) analysis of the interaction between TβRII and TSP50 (or mutants) in HEK 293T cells. Cells were co-transfected with pcMV-Myc-TβRII and pcDNA3.1-TSP50 (or mutants). M1: H153A; M2: D206A; M3: T310A. **D:** Assessment of TβRII phosphorylation. HEK 293T cells were co-transfected with pcMV-Myc-TβRII and pcDNA3.1-TSP50 (or mutants). IP was performed using anti-Myc antibody,

followed by Western blot analysis using anti-TSP50 and anti-phospho-Ser/Thr antibodies. M1: H153A; M2: D206A; M3: T310A. E: Western blot analysis of TβRII, p-TβRII, Smad2/3, and p-Smad2/3 expression in NCM460 cells transfected with pcDNA3.1-TSP50 or its mutants. M1: H153A; M2: D206A; M3: T310A. F: *In vitro* cleavage assay. Purified GST-TSP50 or Flag-tagged mutant proteins (H153A, D206A, T310A) were incubated with TβRII protein in reaction buffer (20 mmol/L Tris-HCl, pH 7.6) at 37°C for 1 h. TβRII protein levels were assessed by Western blot. Quantification is shown below. M1: H153A; M2: D206A; M3: T310A. G: Schematic diagram of TβRII functional domains. ECD: Extracellular Domain; TM: Transmembrane Domain; JM: Juxtamembrane Region; ICD: Intracellular Domain. H: Co-IP analysis of the interaction between TSP50 and TβRII domains. HEK 293T cells were co-transfected with pcMV-Flag-TβRII-ECD/JM/ICD constructs and pcDNA3.1-TSP50. I: *In vitro* cleavage assay of TβRII domains. Purified Flag-tagged TβRII-ECD/JM/ICD proteins were incubated with GST-TSP50 in reaction buffer (20 mmol/L Tris-HCl, pH 7.6) at 37°C for 1 h. Protein levels were analyzed by Western blot. Data are presented as mean ± SEM. Unpaired two-tailed Student's *t* test was used to analyze. ns: Not significant; *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.

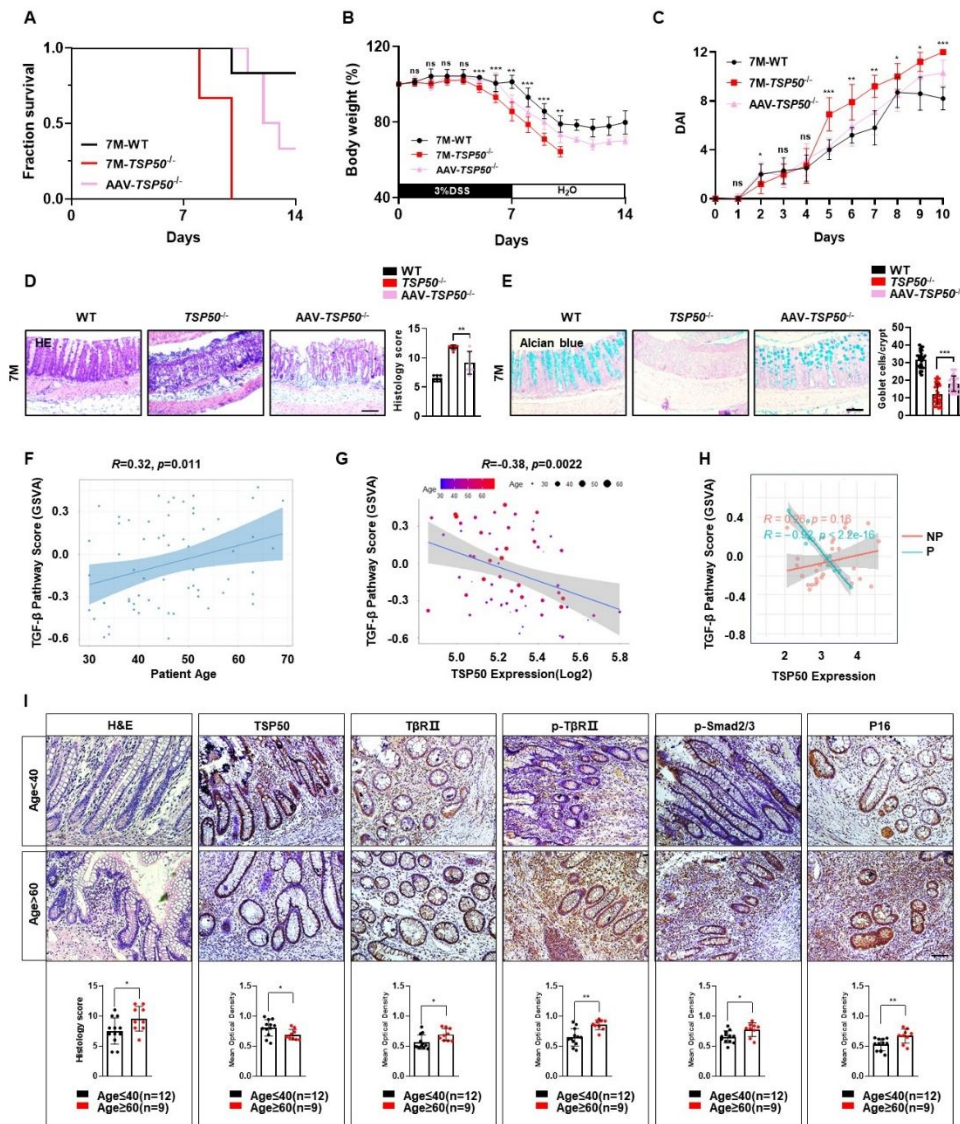
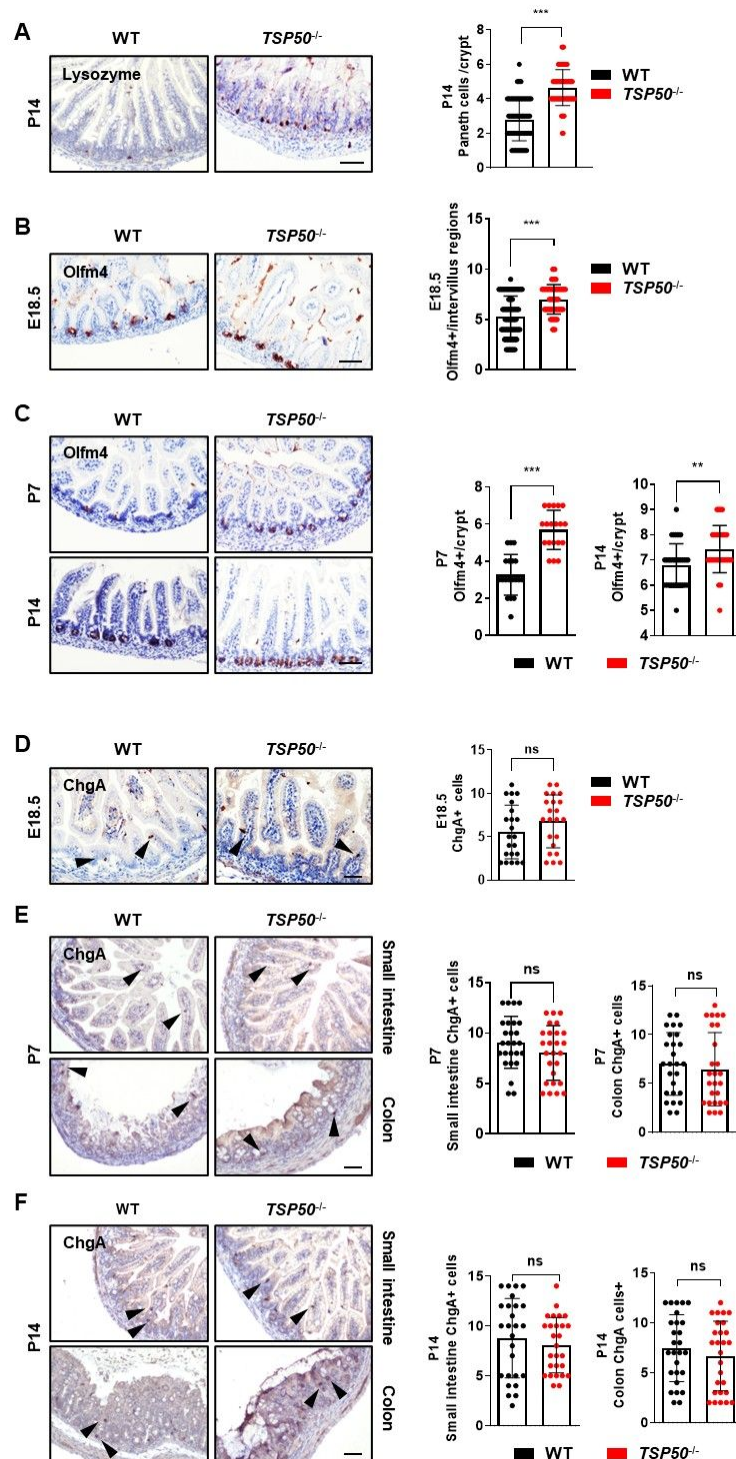


Figure 7. Restoration of TSP50 suppresses aberrant TGF-β signaling and alleviates age-dependent colitis severity

A–C: Assessment of colitis severity in 7M mice treated with 3% DSS following TSP50 restoration. Survival rates (A), percentage of body weight loss (B), and Disease Activity Index (DAI) scores (C) are shown (n=10). D: Representative Hematoxylin and Eosin (HE) staining and histopathological scoring of colonic tissues harvested on Day 10 from the 3% DSS-induced colitis model following TSP50 restoration (n=6). Scale bar: 50 μm. E: Representative Alcian Blue (AB) staining of colonic tissues harvested on Day 10 from the 3% DSS-induced colitis model following TSP50 restoration. Quantification of AB-positive goblet cells per crypt is shown on the right (n=6). Scale bar: 50 μm. F–H: GSEA and correlation analysis of TSP50 and TGF-β signaling in IBD patients (GSE87473). (F) Scatter plot illustrating a significant positive correlation between

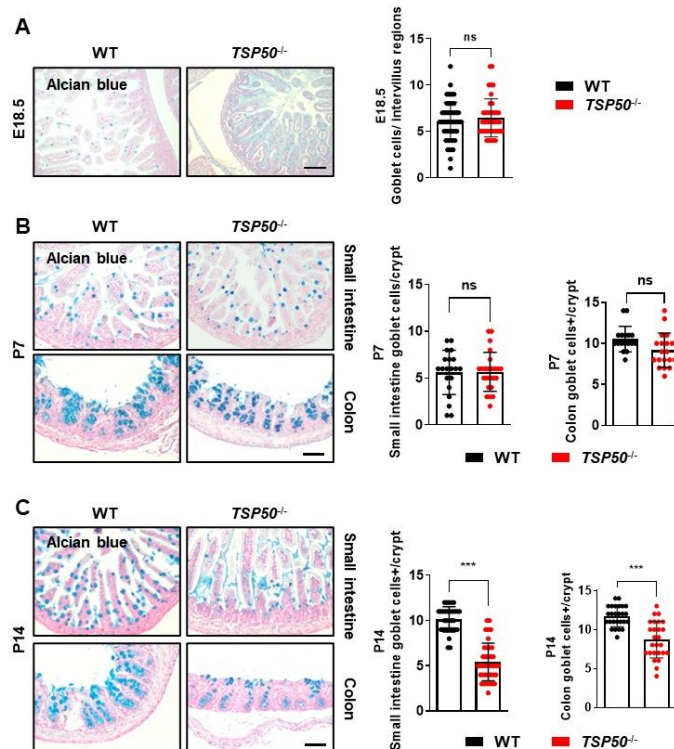
patient age and TGF- β pathway score calculated by Gene Set Variation Analysis (GSVA) ($R=0.32$, $P=0.011$). (G) Negative correlation between TSP50 expression and TGF- β pathway score ($R=-0.38$, $P=0.0022$); dots are color-coded by patient age. (H) Correlation analysis between TSP50 and TGF- β score stratified by disease progression. A remarkably strong negative correlation was observed in pathological (P) tissues featuring high-grade dysplasia or early malignancy (blue line, $R=-0.92$, $P<2.2e-16$), whereas no significant correlation was found in stable, non-dysplastic (NP) tissues (red line, $R=0.26$, $P=0.16$). I: Representative images of H&E and immunohistochemistry (IHC) staining for TSP50, T β RII, p-T β RII, p-Smad2/3, and P16 in colonic tissues from Age ≤ 40 ($n=12$) and Age ≥ 60 ($n=9$) IBD patients. Scale bar: 50 μ m. Bottom panels show the quantitative comparison of histology scores and Mean Optical Density (MOD) for each marker between the two age groups. Data are presented as mean $\pm$ SEM. Two-way ANOVA was used to analyze (B–C), unpaired two-tailed Student's t test was used to analyze (D–E, I). ns: Not significant; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.



Supplementary Figure S1. Analysis of intestinal cell differentiation at late embryonic and postnatal stages

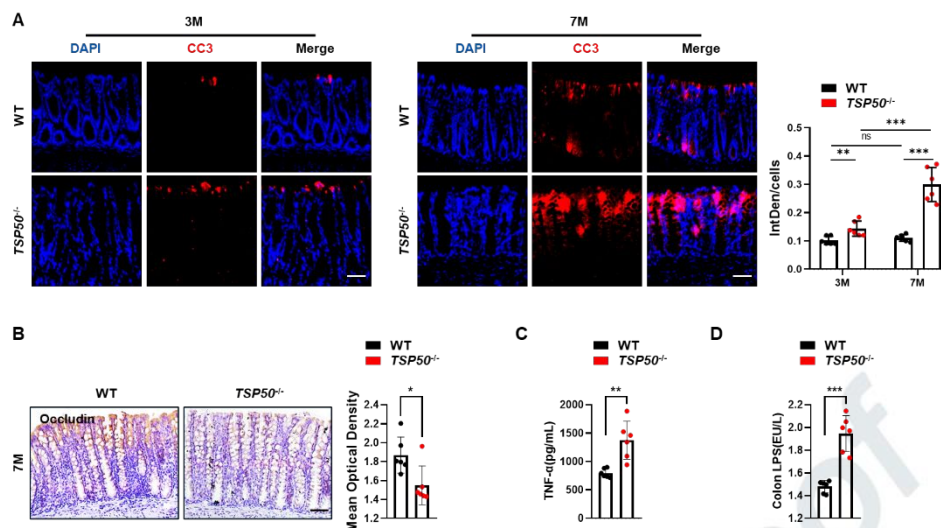
A: Representative immunohistochemistry (IHC) images of Lysozyme (a Paneth cell marker) in intestinal tissues at postnatal Day 14 (P14). Quantification of Lysozyme-positive cells per crypt is shown on the right (n=6). B, C: Representative IHC images of Olfm4 (an intestinal stem cell

marker) at embryonic Day 18.5 (E18.5) and postnatal days 7 and 14 (P7, P14). Quantification of Olfm4-positive cells per intervillous region (for E18.5) or per crypt (for P7 and P14) is shown (n=6). D–F: Representative IHC images of Chromogranin A (ChgA, an enteroendocrine cell marker) at E18.5, P7, and P14. Quantification of ChgA-positive cells per intestinal cross-section is shown (n=6). Scale bars: 50 μ m. Data are presented as mean $\pm$ SEM. Unpaired two-tailed Student's *t* test was used to analyze. ns: Not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.



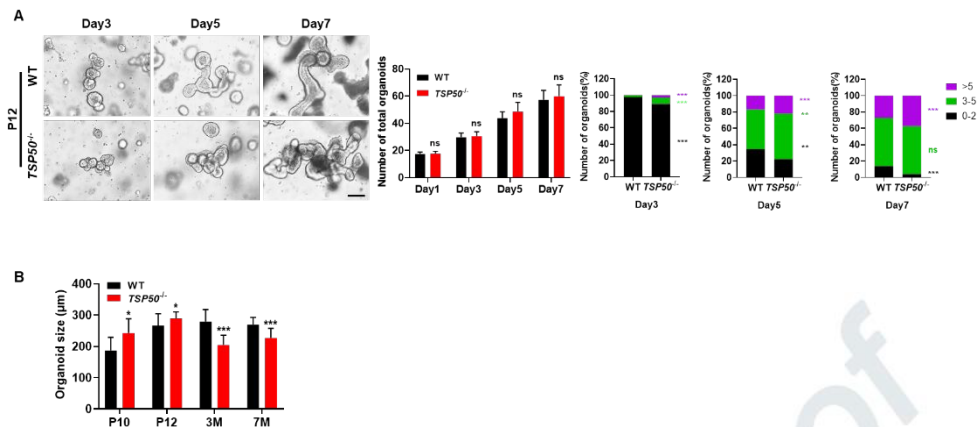
Supplementary Figure S2. Goblet cell differentiation at late embryonic and postnatal stages

A–C: Representative Alcian Blue (AB) staining identifying goblet cells at E18.5, P7, and P14. Quantification of AB-positive cells per intervillous region (for E18.5) or per crypt (for P7 and P14) is shown (n=6). Scale bar: 50 μ m. Data are presented as mean $\pm$ SEM. Unpaired two-tailed Student's *t* test was used to analyze. ns: Not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.



Supplementary Figure S3. Detection of Cleaved Caspase-3, Occludin, TNF- α and LPS levels in mouse intestinal tissues

A: Representative immunofluorescence staining of Cleaved Caspase-3 (CC3) in intestinal tissues from 3-month-old (3M) and 7-month-old (7M) WT and *TSP50*^{-/-} mice. Quantification is shown on the right (n=6). Scale bar: 50 μ m. B: Representative IHC staining images of the tight junction protein Occludin in intestinal tissues from 7M WT and *TSP50*^{-/-} mice. Quantification results are presented on the right (n=6). Scale bar: 50 μ m. C, D: Levels of TNF- α (C) and lipopolysaccharide (LPS) (D) in intestinal tissues from 7-month-old WT and *TSP50*^{-/-} mice measured by ELISA (n=6). Data are presented as mean $\pm$ SEM. Unpaired two-tailed Student's *t* test was used to analyze. ns: Not significant; *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.

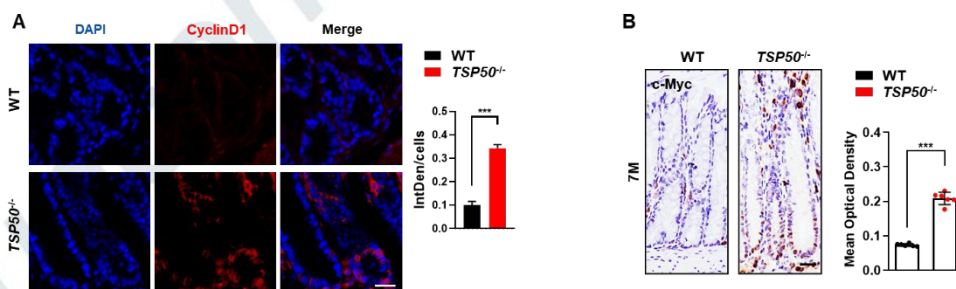


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835 Supplementary Figure S4. Organoid culture characteristics of P12 mice

836 A: Analysis of intestinal organoids derived from P12 mice. Representative images at days 3, 5,
 837 and 7 of culture are shown. Quantification includes the number of organoids formed (seeded with
 838 200 crypts per well) and the percentage distribution of budding numbers (n=3) (Black, <2 buds;
 839 Green, 3–5 buds; Purple, >5 buds). Scale bar: 50 µm. B: Maximum size of individual organoids on
 840 Day 7 of P10, P12, 3M, and 7M mouse intestinal organoid culture (n=3). Data are presented as
 841 mean ± SEM. Unpaired two-tailed Student's *t* test was used to analyze. ns: Not significant; *: *P*
 842 *P*<0.05; **: *P*<0.01; ***: *P*<0.001.

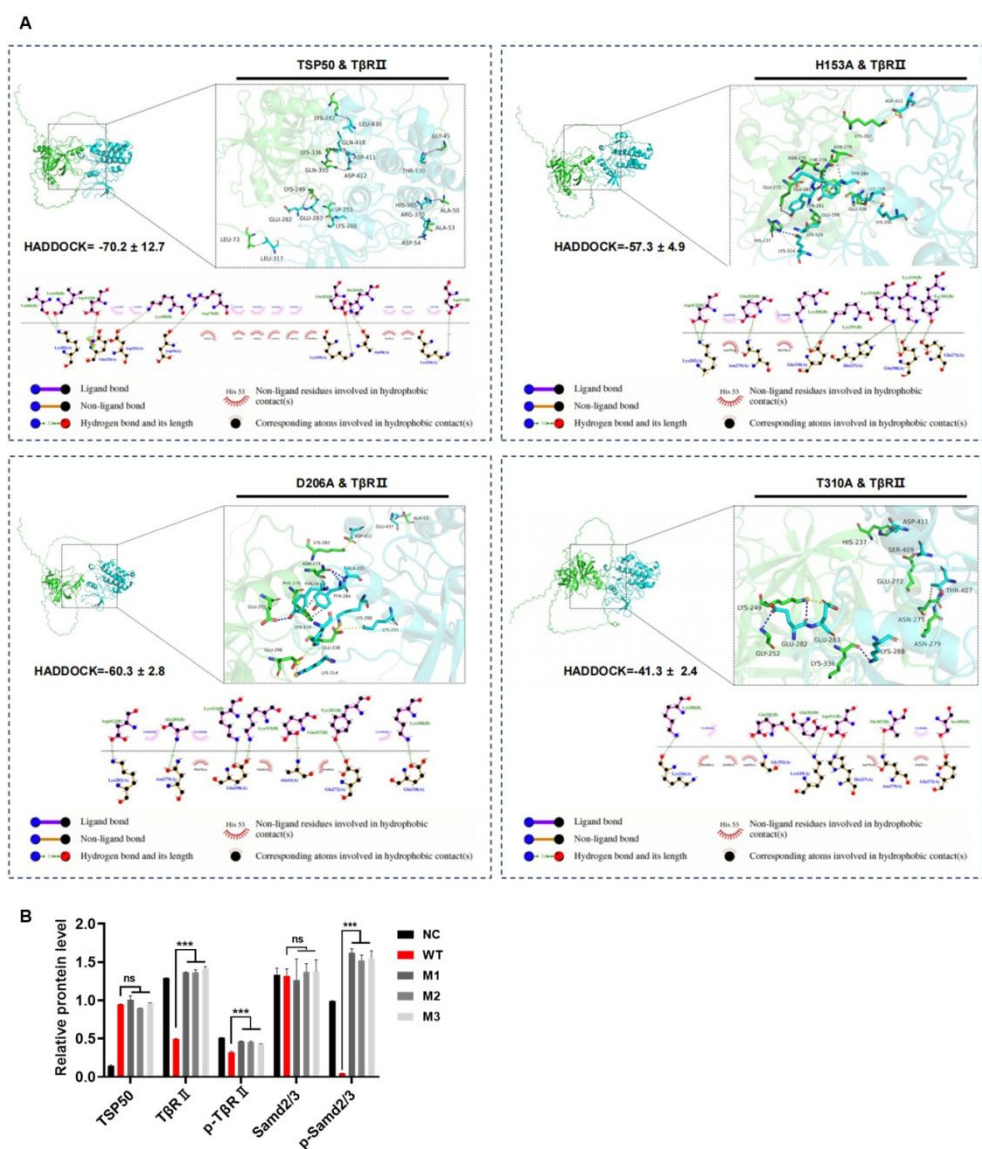
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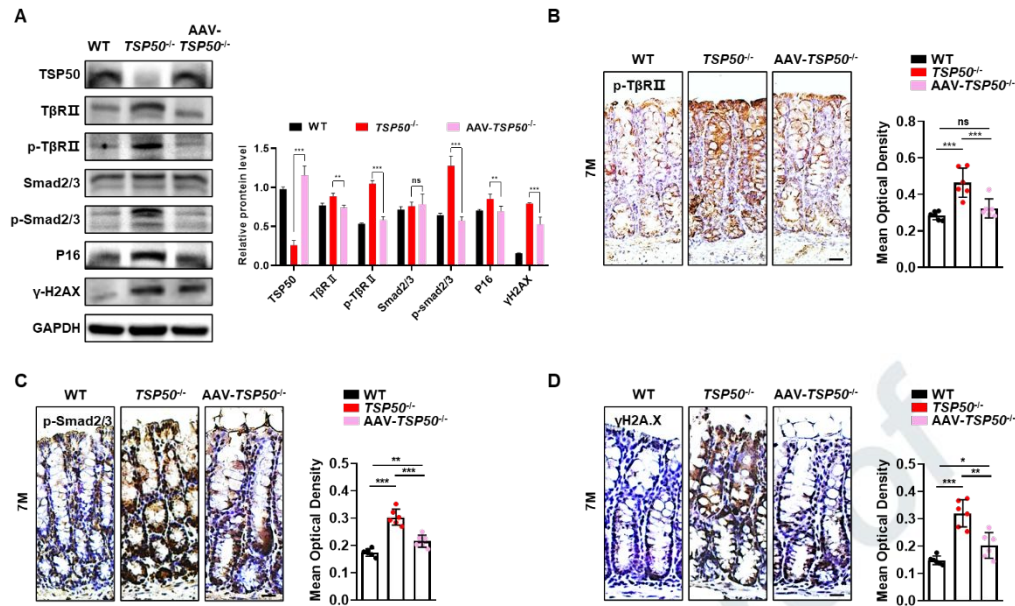
845 Supplementary Figure S5. Upregulation of Wnt target genes

846 A: Representative IF staining of Cyclin D1 in intestinal tissues from 7M WT and *TSP50*^{-/-} mice.
 847 Quantification is shown on the right (n=6). Scale bar: 20 µm. B: Representative IHC staining of c-
 848 Myc in intestinal tissues from 7M WT and *TSP50*^{-/-} mice. Quantification is shown on the right
 849 (n=6). Scale bar: 10 µm. Data are presented as mean ± SEM. Unpaired two-tailed Student's *t* test
 850 was used to analyze. ns: Not significant; *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.



Supplementary Figure S6. TSP50 suppresses TGF- β transcriptional activity in a catalytic activity-dependent manner

A: Protein-protein docking analysis of T β RII with WT-TSP50 or its catalytic triad mutants (H153A, D206A, T310A) performed using HADDOCK. Representative 3D and 2D interaction diagrams are shown along with HADDOCK scores, where lower values indicate higher predicted binding potential. B: Western blot analysis of T β RII, p-T β RII, Smad2/3, and p-Smad2/3 expression in NCM460 cells transfected with pcDNA3.1-TSP50 or its mutants. M1: H153A; M2: D206A; M3: T310A. Phosphorylation levels were calculated as the p/total ratio, and other protein levels were normalized to GAPDH (n=3). Data are presented as mean $\pm$ SEM. Unpaired two-tailed Student's *t* test was used to analyze. ns: Not significant; *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.



Supplementary Figure S7. Restoration of TSP50 inhibits the TGF-β pathway and reduces the expression of intestinal epithelial senescence markers

A: Western blot and quantitative analysis of TSP50, TβRII, p-TβRII, Smad2/3, p-Smad2/3, P16, and γH2AX in the intestinal epithelium of 7M TSP50^{-/-} mice following TSP50 restoration. Phosphorylation levels were calculated as the p/total ratio, and other protein levels were normalized to GAPDH (n=3). B–D: Representative IHC staining and quantification of p-TβRII (B), p-Smad2/3 (C), and γH2AX (D) in the colonic epithelium of 7M mice following TSP50 restoration (n=6). Scale bar: 10 μm. Data are presented as mean ± SEM. Unpaired two-tailed Student's *t* test was used to analyze. ns: Not significant; *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.

876 **Supplementary Table S1. Clinical data of patients with IBD**

IBD patients				
No.	Age	Sex	Organ	Pathological Diagnosis
1	21	Male	Colon	Crohn's disease (CD)
2	23	Male	Colon	CD
3	27	Male	Colon	Ulcerative Colitis (UC)
4	30	Male	Colon	UC
5	31	Male	Colon	UC
6	33	Male	Colon	UC
7	35	Female	Colon	UC
8	37	Male	Colon	UC
9	38	Male	Colon	UC
10	39	Male	Colon	UC
11	40	Male	Colon	UC
12	40	Female	Colon	UC
13	60	Male	Colon	UC
14	62	Male	Colon	UC
15	64	Male	Colon	UC
16	65	Male	Colon	UC
17	67	Male	Colon	UC
18	71	Male	Colon	UC
19	74	Male	Colon	UC
20	77	Male	Colon	UC
21	78	Male	Colon	UC

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879 **Supplementary Table S2. Antibodies and reagents used in this study**

Name	Manufacturer	Cat. No.
Lysozyme antibody	Abcam	ab108508
Olfm4 antibody	Proteintech	28432-1-AP
Chromogranin A antibody	Proteintech	23342-1-AP
γ H2AX antibody	Proteintech	83307-2-RR
P16 antibody	Proteintech	10883-1-AP
Cleaved Caspase 3 antibody	Proteintech	25128-1-AP
β -catenin antibody	Proteintech	51067-2-AP
Smad2/3 antibody	Abcam	ab305325
p-Smad2/3 antibody	Proteintech	80427-2-RR
T β R II antibody	Cell Signaling Technology	41896
p-T β R II antibody	YaJi	7420R
Notch1 antibody	Abcam	ab52627
Cyclin D1 antibody	Proteintech	26939-1-AP
c-Myc antibody	Abcam	ab32072
Myc-Tag antibody	Cell Signaling Technology	2278S
Flag antibody	Solarbio	K107723P
HE Kit	Solarbio	G1120
Alcian blue Kit	Solarbio	75881-23-1
MDA Kit	Solarbio	BC6415
SOD Kit	Solarbio	BC5160

CAT Kit	Solarbio	BC0205
GSH Kit	Solarbio	BC1175
TUNEL Kit	Beyotime	C1086
SA- β -gal Kit	Beyotime	C0602

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904 **Supplementary Table S3. Primer sequences**

Name		
<i>β-actin</i>	F-Primer	CGTGCGTGACATTAAGGAGAAG
	R-Primer	GGAAGGAAGGCTGGAAGAGTG
<i>Dll1</i>	F-Primer	CAGGACCTTCTTTCGCGTATG
	R-Primer	AAGGGGAATCGGATGGGGTT
<i>Dll4</i>	F-Primer	TTCCAGGCAACCTTCTCCGA
	R-Primer	ACTGCCGCTATTCTTGTCCC
<i>Jag1</i>	F-Primer	CCTCGGGTCAGTTTGAGCTG
	R-Primer	CCTTGAGGCACACTTTGAAGTA
<i>Jag2</i>	F-Primer	CAATGACACCACTCCAGATGAG
	R-Primer	GGCCAAAGAAGTCGTTGCG
<i>Notch1</i>	F-Primer	GATGGCCTCAATGGGTACAAG
	R-Primer	TCGTTGTTGTTGATGTCACAGT
<i>Hes1</i>	F-Primer	CCAGCCAGTGTC AACACGA
	R-Primer	AATGCCGGGAGCTATCTTTCT

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TSP50 介导的 T β RII 蛋白水解维持肠道发育并限制年龄相关性结肠炎易感性

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摘要

睾丸特异性蛋白酶 50 (TSP50) 对维持肠道稳态至关重要, 但其在调控肠道发育中的生物学作用尚不明确。本研究证实, TSP50 缺失会显著加剧小鼠年龄依赖性结肠炎易感性。表型分析结果显示, TSP50 缺失小鼠存在肠道发育异常, 具体表现为胚胎期特定上皮细胞谱系提前分化、新生儿期隐窝过早形成; 同时, 成年后肠道干细胞 (ISC) 的类器官形成能力显著下降。机制研究表明, TSP50 借助其催化三联体发挥丝氨酸蛋白酶活性, 靶向转化生长因子- β 受体 II (T β RII) 的近膜区 (JM), 介导该受体胞内结构域 (ICD) 的蛋白水解, 进而抑制 TGF- β 信号通路的激活强度。TGF- β 信号持续过度激活很可能是造成 TSP50 缺失小鼠发育时序紊乱以及类器官形成能力受损的主要原因。重要的是, 腺相关病毒 (AAV) 介导的 TSP50 表达回补, 可有效抑制异常激活的 TGF- β 信号, 并缓解加剧的年龄依赖性结肠炎易感性。此外, 临床分析结果表明, 炎症性肠病 (IBD) 组织中 TSP50 的表达水平与患者年龄及 TGF- β 通路活性均呈显著负相关。综上, 本研究结果提示, TSP50 参与肠道发育进程, 该作用至少部分通过调控 TGF- β 信号通路活性实现, 为年龄相关性肠道疾病的预防与治疗提供了具有潜力的分子靶点。

关键词: 炎症性肠病; TSP50; 转化生长因子- β ; 类器官