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メタデータ	言語: eng 出版者: 公開日: 2019-04-25 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	https://doi.org/10.24517/00053860

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Stat3 is indispensable for damage-induced crypt regeneration but not for Wnt-driven intestinal tumorigenesis

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Running title: Stat3 in intestinal regeneration and tumorigenesis

Abbreviations

AFM-Wnt3a, afamin-associated Wnt3a; *Apc*, adenomatous polyposis coli; CAC, colitis-associated colon cancer; CRC, colorectal cancer; DSS, dextran sodium sulfate; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; EdU, 5-ethynyl-2'-deoxyuridine; FAK, focal adhesion kinase; GSK, glycogen synthase kinase; IL, interleukin; ROCK, Rho-associated protein kinase, RT-PCR, reverse transcription polymerase chain reaction; Stat3, signal transducer and activator of transcription 3; TGF- β ; transforming growth factor- β

ABSTRACT

Stat3 has been shown to play a role in intestinal regeneration and colitis-associated colon carcinogenesis. However, the role of Stat3 in the Wnt-driven sporadic intestinal tumorigenesis remains poorly understood. We examined the roles of Stat3 in intestinal regeneration and tumorigenesis by organoid culture experiments using *Stat3*^{ΔIEC} mouse-derived intestinal epithelial cells in which *Stat3* was disrupted. The regeneration of intestinal mucosa and organoid formation were significantly suppressed by *Stat3* disruption, which was compensated by Wnt activation. Furthermore, once organoids were recovered, Stat3 was no longer required for organoid growth. These results indicate that Stat3 and Wnt signaling cooperatively protect epithelial cells at the early phase of intestinal regeneration. In contrast, intestinal tumorigenesis was not suppressed by *Stat3* disruption in *Apc*^{Δ716} and *Apc*^{Δ716} *Tgfbr2*^{ΔIEC} mice, thus indicating that Stat3 is not required for Wnt activation-driven intestinal tumorigenesis. Mechanistically, *Itga5* and *Itga6* were downregulated by *Stat3* disruption, and FAK activation was also suppressed. Notably, FAK inhibitor suppressed the organoid formation of wild-type epithelial cells. These results indicate that Stat3 is indispensable for the survival of epithelial cells through the activation of integrin signaling and the downstream FAK pathway; however, it is not required for the Wnt signaling-activated normal or tumor epithelial cells.

KEY WORDS: colon cancer, organoids, anoikis, integrin, FAK,

INTRODUCTION

It has been established that inflammatory responses promote cancer development and malignant progression (1, 2). In the inflammatory tumor microenvironment, macrophages and fibroblasts express cytokines and chemokines, resulting in the activation of transcription factors, such as NF- κ B and signal transducer and activator of transcription 3 (Stat3). The aberrant activation of Stat3 was found in >70% of human cancers (3, 4), and Stat3 activation is correlated with the invasiveness, proliferation and stemness of human colon cancer cells (5-7). Furthermore, studies using a colitis-associated colon cancer (CAC) mouse model have shown that the disruption of *Stat3* in epithelial cells significantly suppresses CAC development through the suppression of proliferation and induction of apoptosis (8, 9). It has also been shown that IL-11 is a dominant IL-6 family cytokine in gastrointestinal tumors, and IL-11 receptor signaling promotes tumorigenesis through Stat3 activation (10). Based on these results, the Stat3 pathway is thought to be an important target in the development of anti-cancer drugs for various cancers, including CRC (11).

In contrast to these findings, several studies have demonstrated a tumor suppressor role of Stat3 in intestinal tumorigenesis (12, 13). In those reports, the disruption of *Stat3* in *Apc^{Min}* mice, a model of familial adenomatous polyposis, did not suppress tumorigenesis; instead, it induced the submucosal invasion of tumor cells, suggesting that Stat3 prevents the malignant progression of intestinal tumors. This discrepancy in the role of Stat3 in intestinal tumorigenesis may be due to the different mechanisms of cancer development in these models (i.e. inflammation and regeneration-associated tumorigenesis in the CAC model and Wnt activation-driven tumorigenesis in *Apc^{Min}* mice). A comprehensive genome analysis indicated that >90% of human CRCs carry genetic alterations in *APC* or *CTNNB1* resulting in Wnt

signaling activation (14); thus, it is important to investigate the precise role of Stat3 in Wnt activation-driven CRC development.

The Stat3 function has been shown to be absolutely required for the intestinal stem cell survival (15). Consistently, Stat3 plays a role in the regeneration of the damaged intestinal mucosa after irradiation or DSS treatment (16, 17). However, the mechanism through which Stat3 signaling is involved in the stem cell survival and proliferation during regeneration has not been fully elucidated. Wnt/ β -catenin signaling is a key driver of the tissue stem cell property, making the Wnt pathway important for both regeneration and cancer development (18, 19). Furthermore, pathways through focal adhesion kinase (FAK) or lysin methyltransferase SETD7 are responsible for both mucosal regeneration and tumorigenesis in the intestine through the activation of Wnt signaling (20, 21). However, the role of Stat3 in intestinal regeneration and tumorigenesis has not been studied in the context of Wnt-driven carcinogenesis.

In the present study, we used genetic mouse models and organoid culture systems to show that Stat3 is required for the survival and proliferation of the residual stem cells in damaged mucosa or isolated crypts, leading to crypt formation in the regenerating mucosa as well as organoid formation in Matrigel. However, Stat3 is not required for crypt development when Wnt signaling is activated by exogenous ligand stimulation or when the *Apc* gene is lost. Consistently, Stat3 is dispensable for Wnt activation-driven tumor development and malignant progression, including submucosal invasion and liver metastasis. These results suggest that the Wnt activation status and regeneration phenotype are important factors to consider with regard to the efficacy of Stat3 inhibitors in the prevention of CRC development.

MATERIALS AND METHODS

Mouse experiments

Apc^{Δ716}, *Stat3*^{flox/flox} and *villin-CreER* mice were previously described (22-24). *Tgfr2*^{flox/flox} mice were obtained from the Mouse Repository (NCI-Frederick, Frederick, MD, USA) (25). The genetic background of all strains used in this study is C57BL/6. For organoid culture experiments, *villin-CreER Stat3*^{flox/flox} mice were treated with Tamoxifen (Tam; 4 mg/mouse, intraperitoneal [i.p.]) for 3 continuous days at 4-8 weeks of age, resulting in *Stat3* disruption in the intestinal epithelial cells (hereafter, *Stat3*^{ΔIEC}). X-ray irradiation (9 Gy) was performed to induce intestinal mucosal regeneration (n=9 for wild-type mice, and n=12 for *Stat3*^{ΔIEC} mice). For the *in vivo* experiments, *Apc*^{Δ716 villin-CreER Stat3}^{flox/flox} mice and *Apc*^{Δ716 villin-CreER Tgfr2}^{flox/flox Stat3}^{flox/flox} mice were generated by crossing and treated with Tam (4 mg/mouse, i.p.) once per week from 6 to 19 weeks of age. The total number of intestinal polyps and the size distribution were scored under a dissection microscope (n=10 for *Apc*^{Δ716} mice, n=12 for *Apc*^{Δ716 Stat3}^{ΔIEC} mice, n=10 for *Apc*^{Δ716 Tgfr2}^{ΔIEC} mice, and n=8 for *Apc*^{Δ716 Tgfr2}^{ΔIEC Stat3}^{ΔIEC} mice). Invasion frequency was scored using hematoxylin and eosin (H&E) histology sections (n=4 each for *Apc*^{Δ716} and *Apc*^{Δ716 Stat3}^{ΔIEC} mice).

The protocols for the animal experiments were approved by the Committee on Animal Experimentation of Kanazawa University.

Organoid culture experiments

The organoid cultures were prepared from the intestinal mucosa and tumors of the small intestine according to a previously described method (26). In brief, dissected tissues were soaked in 2 mM EDTA for 30 min, and crypts were isolated by shaking. Isolated crypts were

then collected and suspended in Matrigel with Advanced DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 50 ng/ml EGF (Invitrogen), 100 ng/ml Noggin (Peprotech, Rocky Hill, NJ, USA) and 10% R-Spondin1 conditioned medium prepared from human R-Spondin1-expressing 293T cells (kindly provided by Nick Barker's lab; A-STAR, Singapore). For mechanical passage, 15 organoids ($200\ \mu\text{m} < \phi < 400\ \mu\text{m}$) were collected using Cell Recovery Solution (CORNING, Corning, NY, USA), and crypt structures roughly dissociated into fragments by pipetting were mixed with Matrigel and transferred to 3 wells of a 48-well plate. For enzymatic passage, the collected organoids were dissociated by treatment with 25 mM trypsin for 5 min, suspended in Matrigel, and seeded at 10^5 cells/well (for wild-type and *Stat3^{ΔIEC}* mice) or 10^6 cells/well (for *Apc^{Δ716}* and *Apc^{Δ716} Stat3^{ΔIEC}* mice) to each well of 48-well plate. The respective passage experiments were performed three times, and the mean organoid numbers were counted. For the rescue experiments, 10 μM ROCK inhibitor (Y-27632; Wako, Osaka, Japan) and 5 μM GSK3 inhibitor (CHIR-99021; Tocris Bioscience, Bristol, UK) were added, and the total numbers of organoids derived from about 300 crypts were counted under a dissection microscope. Rescue experiments for each inhibitor combination were performed three times, and the mean organoid numbers were counted. For the activation of Wnt signaling, conditioned medium including afamin-associated Wnt3a (AFM-Wnt3a) was obtained from Dr. Toshiro Sato (Keio University, Japan) using a cell line established by Dr. Junichi Takagi (Osaka University, Japan) and added to the medium (27). The organoid cell proliferation was examined using the Click-iT EdU Imaging System (Invitrogen), and the EdU labeling index was calculated by counting the number of positive cells in at least five organoids. Organoids were treated with FAK inhibitor 14 (Merck, Darmstadt, Germany) at 15 μM .

Histology and immunohistochemistry

The tissue specimens were fixed in 4% paraformaldehyde, paraffin-embedded, and cut into 4- μ m-thick sections. Crypts were isolated by ethylenediaminetetraacetic acid (EDTA), and organoids were fixed in 4% paraformaldehyde and embedded in iPGell (Genostaff, Tokyo, Japan). The sections were stained with H&E. For immunohistochemistry, antibodies against Ki67 (Life Technologies, Grand Island, NY, USA), CD44s (Merck, Darmstadt, Germany), phosphorylated Stat3 at Tyr705 (Cell Signaling, Danvers, MA, USA) and phosphorylated FAK at Tyr397 (Cell Signaling) were used as the primary antibodies. Staining signals were visualized using a Vectastain Elite Kit (Vector Laboratories, Burlingame, CA, USA). Apoptosis was examined using an Apoptag Peroxidase *In Situ* Apoptosis Detection Kit (Merck).

Cell culture and transplantation experiments

A mouse metastatic intestinal cancer cell line (AKTP) was established as recently described (28). AKTP cells carry *Apc* ^{Δ 716} *Kras*^{G12D} *Tgfbr2*^{-/-} *Trp53*^{R270H} mutations. To disrupt the *Stat3* gene in AKTP cells, *Stat3*-specific sgRNA was cloned into the px330 expression vector (Addgene), which bicistronically expresses sgRNA and Cas9 nuclease, and transfected to AKTP cells. The sgRNA sequence was determined by the CRSPR Design Tool (<http://crispr.mit.edu/>). Disruption of the *Stat3* gene was confirmed by Western blotting using a Stat3 antibody (Cell Signaling). Cell proliferation was examined using a Cell Titer-Glo 2.0 Assay (Promega Corporation, Madison, WI, USA).

For the metastasis analysis, 1×10^6 cells were transplanted into the spleen of immunodeficient NOD/Shi-*scid* *Il2rg*^{-/-} mice (NSG mice; CIEA, Kanagawa, Japan) (n=3 for

each cell line), and metastatic liver lesions were examined histologically at 4 weeks after transplantation. The efficiency of metastasis was scored by the measurement of the areas of metastasized foci per total liver on H&E-stained sections.

Expression analyses

Total RNA was extracted from isolated crypts using an RNeasy Plus Micro extraction kit (Qiagen GmbH, Hilden, Germany). An expression analysis was performed using the RT² Profiler PCR Array (Mouse Cell Junction Pathway Finder).

Real-time reverse transcription (RT)-polymerase chain reaction (PCR)

For the real-time RT-PCR, total RNA was extracted from isolated crypts or organoids using an RNeasy Plus Micro extraction kit (Qiagen GmbH, Hilden, Germany), reverse-transcribed using a PrimeScript RT reagent kit (Takara, Tokyo, Japan), and amplified using ExTaqII SYBR Premix (Takara) on a Stratagene Mx3000P real-time thermocycler (Agilent Technologies, Santa Clara, CA, USA). The purchased primer sequences were as follows: *Itga5*-F:GAAGCTCTGAAGATGCCCTACCA, *Itga5*-R: TGATGATCCACAACGGGACAC; and *Itga6*-F:GTCACCGCTGCTGCTCAGAATA, *Itga6*-R:AGCATCAGAATCCCGGCAAG (TaKaRa, Shiga, Japan).

Statistical analyses

The data were analyzed using an unpaired *t*-test and are presented as the mean \pm standard deviation (*s.d.*). *P* values of < 0.05 were considered to indicate statistical significance.

RESULTS

Stat3 is required for the regeneration of the intestinal mucosa

The epithelial Stat3 expression has been shown to be required for the regeneration of the damaged intestinal mucosa (17, 29, 30). We confirmed that phosphorylated Stat3 (P-Stat3) was detected in the cryptic cells of the X-ray-irradiated wild-type mouse intestine, while P-Stat3 in the normal intestinal epithelia was below the detection limit (Fig. 1A). These results indicate that the Stat3 activation is significantly increased in epithelial cells of regenerating mucosa. We then constructed *Stat3^{ΔIEC}* mice, in which Stat3 was disrupted in the intestinal epithelial cells in a cell-specific manner by the treatment of *villin-CreER Stat3^{fllox/fllox}* mice with tamoxifen. We confirmed that P-Stat3 was not detected in the X-ray-irradiated *Stat3^{ΔIEC}* mouse intestine (Fig. 1A). X-ray irradiation caused a more severe phenotype in *Stat3^{ΔIEC}* mice than in wild-type mice; the 7-day survival rates of wild-type and *Stat3^{ΔIEC}* mice were 67% and 17%, respectively (Fig. 1B). Histologically, mucosal regeneration was significantly suppressed in *Stat3^{ΔIEC}* mice, and the number of remaining crypts at three days after irradiation in *Stat3^{ΔIEC}* mice was significantly decreased in comparison to wild-type mice (Fig. 1C, D). The numbers of Ki-67-positive proliferating cells and CD44-positive undifferentiated cells in the residual crypts were also significantly decreased in the *Stat3^{ΔIEC}* intestinal mucosa. These results indicate that the activation of epithelial Stat3 is required for crypt repopulation via the survival and proliferation of the residual stem cells in the damaged mucosa, which leads to regeneration.

Stat3 is required for intestinal organoid formation

To understand the biological mechanism of Stat3 in the regeneration of the intestinal mucosa, we next examined the *in vitro* organoid formation of intestinal epithelial cells, which mimics

crypt regeneration *in vivo*. Intestinal cryptic cells isolated from wild-type mice formed organoids through the construction of mini-crypt structures in Matrigel (Fig. 2A). Importantly, however, intestinal cryptic cells derived from *Stat3^{ΔIEC}* mice failed to generate organoids in Matrigel. Consistently, previous reports indicated that exogenous IL-22 signaling promotes organoid formation and mucosal regeneration through the induction of Stat3 activation (30, 31). Taken together, these results indicate that Stat3 activation is required for intestinal crypt reconstruction.

Next, we treated isolated cryptic cells from *Stat3^{ΔIEC}* mice with Rho-associated protein kinase (ROCK) inhibitor (Y-27632) and glycogen synthase kinase 3 (GSK3) inhibitor (CHIR-99021), as these inhibitors increase the efficiency of organoid formation through the suppression of anoikis and the activation of Wnt signaling, respectively (32). Notably, the addition of both inhibitors significantly increased the number of *Stat3^{ΔIEC}* mouse-derived organoids, while treatment with Y-27632 or CHIR-99021 alone was not sufficient for organoid recovery (Fig. 2A, B). We confirmed the disruption of *Stat3* in the rescued organoids by Western blotting, which ruled out the possibility of contamination of *Stat3* wild-type cells (Supplementary Fig. 1).

Intriguingly, once the organoids were generated from *Stat3^{ΔIEC}* intestinal crypts in the presence of inhibitors, the rescued *Stat3^{ΔIEC}* organoids no longer required inhibitor treatment for further growth (Fig. 2C). The organoid-forming efficiency of *Stat3^{ΔIEC}* epithelial cells was unaltered compared with wild-type after mechanical passage. Furthermore, the EdU labeling efficiency of the rescued and mechanically passaged *Stat3^{ΔIEC}* organoids was similar to that of wild-type mouse-derived organoids (Fig. 2D). In contrast, the development of *Stat3^{ΔIEC}* organoids was significantly suppressed when the rescued organoids were passaged after

enzymatic dissociation by trypsin (Fig. 2E). These results suggest that Stat3 is required for crypt formation from the remnant stem cells in the damaged mucosa due to protection from anoikis, whereas Stat3 is no longer required for the maintenance and growth of the established crypts. It is noteworthy that the conditional *Stat3* disruption in the intestinal epithelial cells of adult mice did not change the mucosal morphology, proliferation rate or differentiation into lysozymes or Muc2-expressing cells (Supplementary Fig. 2). Furthermore, the numbers of SOX7-positive undifferentiated cells in the *Stat3^{ΔIEC}* mice were similar to those in wild-type mice. These results support the idea that Stat3 is only necessary to generate new crypts from the remnant stem cells following radiation injury.

Wnt activation compensates for *Stat3* disruption to support organoid formation

It has been shown that GSK3 inhibitor increases the colony formation efficiency of intestinal stem cells through Wnt signaling activation (32,33). Since treatment with CHIR99021 is required for the recovery of *Stat3^{ΔIEC}* organoids, it is possible that Wnt signaling activation is involved in the recovery of *Stat3^{ΔIEC}* organoid formation. To assess this possibility, we cultured *Stat3^{ΔIEC}* mouse intestinal crypts in medium supplemented with the active water-soluble form of lipidated Wnt3a, which is associated with bovine serum protein afamin (AFM-Wnt3a) (27). As expected, AFM-Wnt3a treatment increased the number and size of the organoids derived from wild-type mouse crypts in comparison to organoid cultures with control medium, which is consistent with the findings of a previous report in human intestinal organoids (27) (Fig. 3A). Importantly, AFM-Wnt3a treatment rescued the organoid formation of *Stat3^{ΔIEC}* mouse intestinal crypts, even in the absence of an inhibitor (Fig. 3B). These results indicate that Wnt signaling activation can compensate for *Stat3* disruption to support the survival and

proliferation of isolated intestinal cryptic cells.

We further examined whether or not Stat3 was required for organoid formation by Wnt signaling-activated epithelial cells using a different mouse model. Adenoma cells were isolated from the intestinal polyps of *Apc*^{Δ716} mice and *Apc*^{Δ716} *Stat3*^{ΔIEC} compound mice and cultured in Matrigel. In these tumor cells, Wnt/β-catenin signaling is constitutively activated because of an *Apc* mutation (22). When tumor cell organoids were mechanically passaged by pipetting, the organoids developed at the same level in both genotypes, similar to normal intestine-derived organoids (Fig. 3C). Notably, after the enzymatic dissociation of organoids by trypsin, *Apc*^{Δ716} *Stat3*^{ΔIEC} tumor cells formed organoids that were similar in total number and size distribution to simple *Apc*^{Δ716} tumor-derived organoids. Taken together, these results indicate that Wnt signaling activation compensates for *Stat3* disruption to support the survival and proliferation of the dissociated normal and tumor epithelial cells through protection from anoikis.

Stat3 is dispensable for Wnt activation-driven intestinal tumorigenesis

These results prompted us to examine the role of Stat3 in the Wnt activation-driven intestinal tumorigenesis by *Apc* mutations. We examined the phenotypes of *Apc*^{Δ716} *Stat3*^{ΔIEC} mice and age-matched simple *Apc*^{Δ716} mutant mice. P-Stat3 was detected in the nuclei of the tumor cells of *Apc*^{Δ716} mouse polyps but not in *Apc*^{Δ716} *Stat3*^{ΔIEC} mice, indicating that Stat3 is activated in the Wnt-driven sporadic tumor cells (Fig. 4A). However, there was no significant difference in the total number of tumors between *Apc*^{Δ716} and *Apc*^{Δ716} *Stat3*^{ΔIEC} mice (Fig. 4B). The size distributions of the tumors and the number of Ki67-positive cells were also similar, and no significant difference was found in *Apc*^{Δ716} *Stat3*^{ΔIEC} mice. These results indicate that Stat3 is

dispensable for Wnt activation-driven intestinal tumorigenesis.

Submucosal invasion was found in approximately 36% of intestinal polyps of >1 mm in size in the *Apc*^{Δ716} *Stat3*^{ΔIEC} mice, while invasion was not found in simple *Apc*^{Δ716} mice (Supplementary Fig. 3A and B), which is consistent with the previous findings in *Apc*^{Min} mice (12,13). We confirmed that the invasion frequency was significantly different between *Apc*^{Δ716} mice and *Apc*^{Δ716} *Stat3*^{ΔIEC} mice (Supplementary Fig. 3C). These results suggest a tumor suppressor role of Stat3; however, further investigations will be needed to confirm this point.

Next, we introduced the *Stat3*^{ΔIEC} allele into *Apc*^{Δ716} *Tgfbr2*^{ΔIEC} compound mice, which develop invasive adenocarcinomas due to a combination of Wnt activation and TGF-β suppression (34). Notably, nuclear-accumulated P-Stat3 was detected in the tumor cells and stromal cells at the invasion front of *Apc*^{Δ716} *Tgfbr2*^{ΔIEC} mouse tumors, whereas it was detected only in the stroma cells of *Apc*^{Δ716} *Tgfbr2*^{ΔIEC} *Stat3*^{ΔIEC} tumors (Fig. 4C). However, the total number of intestinal tumors and submucosal invasion efficiency in *Apc*^{Δ716} *Tgfbr2*^{ΔIEC} and *Apc*^{Δ716} *Tgfbr2*^{ΔIEC} *Stat3*^{ΔIEC} mice did not differ to a statistically significant extent (Fig. 4D), indicating that Stat3 is not required for the submucosal invasion of intestinal tumor cells.

Stat3 is dispensable in Wnt activation-associated intestinal tumor metastasis

We recently established tumor-derived organoid cells (AKTP cells) from the intestinal tumors of *Apc*^{Δ716} *Kras*^{G12D} *Tgfbr2*^{-/-} *Trp53*^{R279H} quadruple compound mice (28). AKTP cells efficiently metastasized to the liver when transplanted into the mouse spleen. To examine whether or not Stat3 is involved in the metastatic process of Wnt-activated CRC cells, we disrupted the *Stat3* gene in AKTP cells using the CRISPR/Cas9 system (Fig. 5A). The disruption of *Stat3* did not significantly change the cell proliferation rate of AKTP cells (Fig.

5B). Furthermore, multiple metastatic foci were developed in the livers of mice after the injection of two independent lines of *Stat3*-disrupted AKTP cells into the spleen (Stat3 KO#1 and Stat3 KO#2), and the multiplicity did not differ from that of control AKTP cells to a statistically significant extent ($p=0.158$ and 0.201 for control vs KO#1 and KO#2, respectively) (Fig. 5C). Moreover, there was no significant difference in the size distributions of metastatic foci between control AKTP cells and Stat3 KO#1 or Stat3KO#2 cells. Histologically, we confirmed that Stat3 is phosphorylated in metastasized AKTP tumor cells but not in Stat3 KO cells; however, the Ki67 labeling efficiency of the *Stat3*-disrupted AKTP cells did not differ from that of control metastasized tumor cells (Fig. 5D). Furthermore, the expression level of total and active form β -catenin in Stat3-disrupted cells was similar to that of control AKTP cells, indicating that Wnt signaling activation is not affected by *Stat3* gene disruption (Supplementary Fig. 4). These results indicate that Stat3 is not required for metastasis of Wnt activation-associated CRC cells.

Stat3-induced integrin expression is associated with FAK activation in crypts

When intestinal crypts were isolated by EDTA treatment, we realized that *Stat3^{ΔIEC}* mouse-derived crypts showed a distinct morphology from the clear cylinder-like structure of wild-type crypts (Fig. 6A, top). We therefore examined the histology of isolated crypts that were collected from the intestine of wild-type and *Stat3^{ΔIEC}* mice by EDTA treatment for 30 min. Notably, epithelial cell alignment was impaired, and apoptosis was induced in the isolated *Stat3^{ΔIEC}* crypts, while the alignment of columnar epithelial cells with the lateral expression of E-cadherin was confirmed in the wild-type crypts (Fig. 6A). These results indicate that Stat3 is important for the survival of cryptic cells after isolation from intestine following detachment

from the extracellular matrix (ECM).

We therefore examined the changes in the expression of cell adhesion molecules using a PCR array. Notably, the expression of *Gjb1*, *Gjb2* and *Gjb3*, which encode the gap junction proteins connexin 32, 26 and 31, respectively, and *Itga5* and *Itga6*, which encode integrins $\alpha 5$ and $\alpha 6$, respectively, was significantly downregulated in the isolated *Stat3^{ΔIEC}* crypts (Fig. 6B). We confirmed that the expression of *Itga5* and *Itga6* was significantly decreased in the isolated *Stat3^{ΔIEC}* mouse intestinal crypts but not in the inhibitor-rescued *Stat3^{ΔIEC}* organoids by RT-PCR (Fig. 6C). Integrin $\alpha 5$ and $\alpha 6$ bind integrin $\beta 1$ to form receptor complexes for fibronectin and laminin, respectively. It has been shown that the signaling of these ECM components through integrin complexes is important for the survival of epithelial cells through their activation of FAK, which protects cells from anoikis after detachment from the ECM (35). Importantly, phosphorylated FAK (P-FAK) was detected by immunostaining in epithelial cells of wild-type mouse-derived crypts; however, the staining intensity for P-FAK was significantly decreased in the *Stat3^{ΔIEC}* mouse-derived cryptic cells (Fig. 6D). To examine whether or not FAK signaling is indeed required for the organoid formation of the isolated crypts, we treated cultured organoids derived from wild-type crypts and inhibitor-rescued *Stat3^{ΔIEC}* organoids with FAK inhibitor. Of note, FAK inhibition significantly suppressed organoid development in both genotypes (Fig. 6E, F). Taken together, these results indicate that Stat3 prevents anoikis of the isolated crypts that are detached from the ECM by inducing the expression of integrin, which is associated with the activation of the FAK pathway.

DISCUSSION

It has been shown that Stat3 is required for regeneration of the damaged intestinal mucosa (17,23). We confirmed the role of Stat3 in intestinal regeneration as well as organoid formation in Matrigel, which mimics crypt formation *in vitro*. Importantly, however, we showed that Stat3 is not required for the growth of established organoids and that organoids can be maintained indefinitely by mechanical dissociation in the absence of Stat3. In contrast, *Stat3*-disrupted organoid cells were unable to survive when organoids were enzymatically dissociated by trypsin. These results indicate that while Stat3 is important in the protection of intestinal epithelial cells from acute injury and the subsequent induction of anoikis, it is not required for the maintenance and proliferation of steady-state cryptic cells (Fig. 7).

One possible mechanism underlying the impaired survival of *Stat3*^{AIEC} epithelial cells after tissue damage or crypt isolation is the downregulation of integrin $\alpha 5$ and $\alpha 6$, which complex with integrin $\beta 1$ to form the receptors for fibronectin and laminin, respectively. It has been shown that integrin signaling from ECM activates FAK, which is important for the survival of epithelial cells through the further activation of the PI3K/Akt and Ras/MEK signaling pathways (35). It has also been shown that signaling through $\alpha 5\beta 1$ integrin protects the intestinal epithelial cells from apoptosis (36). Thus, Stat3-dependent integrin signaling may contribute to the survival of epithelial cells through the activation of FAK signaling.

Importantly, we showed that Wnt signaling activation by an exogenous ligand compensated for *Stat3* disruption to support organoid development. It has been shown that c-Myc-induced FAK activation is required for mucosal regeneration downstream of the Wnt signaling pathway (20). It has also been shown that treatment of *Apc*^{Min} mice with FAK inhibitor significantly suppressed intestinal polyposis (37). Accordingly, it is possible that the

Stat3 pathway and Wnt signaling cooperatively regulate the survival of the epithelial cells in the damaged mucosa and isolated crypts through the activation of integrin/FAK signaling. It was recently shown that subepithelial telocytes are a source of Wnt ligands for intestinal epithelial cells (38). It is therefore possible that such a stromal network lining the intestinal crypts in lamina propria is disrupted in the damaged intestinal mucosa, resulting in a decrease in Wnt signaling activity in regenerating epithelial cells, which may impair the stem cell properties of the residual stem cells. Under such Wnt-suppressed circumstances, Stat3 is important for the survival of epithelial cells through the activation of FAK signaling. In contrast, when Wnt signaling is sufficiently activated, Stat3 is not required for the survival of epithelial cells (Fig. 7). This hypothesis may explain why Wnt activation-driven tumor cells do not require Stat3 activation.

It has been shown that Paneth cells generate a stem cell niche through Wnt ligand secretion (39), and IL-6-induced Stat3 activation in Paneth cells increases organoid cell proliferation through Wnt activation (40). Accordingly, it is also possible that suppression of Paneth cell-dependent Wnt signaling causes impaired regeneration and crypt formation in *Stat3^{AIEC}* mice.

With regard to the role of Stat3 in tumorigenesis, it has been shown that the blocking of IL-6 and IL-11 cytokine signaling or downstream transcription factor Stat3 causes the significant suppression of CAC development in mouse models, indicating that Stat3 is indispensable for inflammation-associated colonic tumorigenesis (8-10). However, we showed in the present study that Stat3 was not required for intestinal tumor development in *Apc^{A716}* mice and that submucosal invasion was not suppressed in *Apc^{A716} Tgfr2^{AIEC}* mice by Stat3 disruption. Furthermore, Stat3 is also dispensable for liver metastasis of *Apc^{A716} Kras^{G12D}*

Tgfr2^{-/-} *Trp53*^{R270H} tumor cells. These genetic results indicate that Stat3 does not play an important role in Wnt activation-driven colonic tumorigenesis or the malignant progression of CRC (Fig. 7); thus, its role differs from that in CAC development. Accordingly, in CAC development, it is possible that Stat3 is required for the survival and proliferation of tumor cells initiated in the ulcerative colitis mucosa, similarly to normal epithelial cells. In contrast, in sporadic CRC development, Wnt signaling is constitutively activated by the genetic alteration of *APC* or *CTNBI*, which is sufficient to support the survival of tumor cells independently of the Stat3 pathway. In the present study, we showed that the epithelial Stat3 pathway is not required for Wnt-driven tumorigenesis; however, Stat3 in stromal cells is also activated in the inflammatory microenvironment of intestinal tumors. Thus, it is still possible that stromal Stat3 plays a role in sporadic tumorigenesis.

We also found that Stat3 disruption induced the submucosal invasion of intestinal tumors of *Apc*^{A716} mice, which is consistent with the findings of previous reports using *Apc*^{Min} *Stat3*^{-/-} compound mice (12,13). It has also been shown that Stat3 suppresses KRAS-induced lung tumorigenesis and p19^{ARF} expression-associated prostate cancer metastasis (41, 42). Thus, Stat3 may play a tumor suppressor role in the malignant progression of CRC. However, a further analysis to compare the results of mouse experiments and human CRC findings is needed to draw hard conclusions in this regard.

In conclusion, Stat3 is required for the survival and proliferation of the remaining epithelial cells in the damaged mucosa; thus, Stat3 is indispensable for mucosal regeneration and CAC development. In contrast, Stat3 is not required for Wnt activation-driven tumor development or the malignant progression of CRC (Fig. 7). Mechanistically, Wnt signaling and Stat3 cooperate to support the survival of cryptic epithelial cells through the activation of the

integrin/FAK signaling pathway, which protects epithelial cells from anoikis. When Wnt signaling is sufficiently activated, Stat3 is no longer required for the survival or proliferation of normal and tumor epithelial cells. Thus, the degree of Wnt activation in cancer cells has an important impact on the efficacy of Stat3 inhibitor treatment in CRC patients.

ACKNOWLEDGEMENTS

We thank Manami Watanabe, Ayako Tsuda and Yoshie Jomen for their technical assistance. This study was supported by AMED-CREST (JP17gm0410014) and AMED (JP17ck0106259), Japan Agency for Medical Research and Development, Japan; and Grants-in-Aid for Scientific Research (A) (JP18H04030) and (C) (JP16K07111) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Takeda Science Foundation; and Mitsubishi Foundation. The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

H. Oshima, D.C. Voon, and M. Oshima designed the study; H. Oshima, S.Y. Kok performed the study; M. Nakayama and K. Murakami provided resources and analytic tools; M. Oshima wrote the manuscript; all authors discussed the results and edited the final manuscript.

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Figure Legends

Figure 1. The histological analysis of the X-ray-irradiated mouse small intestine. *A)* Representative sections immunohistochemically stained for phosphorylated Stat3 (P-Stat3) in the small intestine of control wild-type and irradiated wild-type and irradiated *Stat3^{AIEC}* mice. Bars, 100 μ m. Insets indicate enlarged images of the boxed areas. *B)* The survival rate of wild-type and *Stat3^{AIEC}* mice after X-ray-irradiation (n=9 for wild-type and n=12 for *Stat3^{AIEC}* mice). *C)* Representative histology sections from wild-type (*left*) and *Stat3^{AIEC}* (*right*) mouse intestines (H&E) (*top*) and immunohistochemical staining for Ki67 (*middle*) and CD44 (*bottom*). Insets show enlarged image views of the boxed areas. Bars, 100 μ m. *D)* The mean number of crypts in the microscopy fields (*left*) and Ki67-positive cells per crypt (*right*) are shown (mean \pm *s.d.*). Asterisk, $p < 0.05$.

Figure 2. The role of Stat3 in organoid formation from intestinal crypts. *A)* A schematic illustration of tamoxifen (Tam) treatment and organoid formation (*top*). Representative microscopic photographs of organoid cultures of intestinal crypts of wild-type and *Stat3^{AIEC}* mice on day 2 (*left*) and day 4 (*right*). Arrowheads indicate organoids rescued by inhibitors (Y-27632 and CHIR-99021). Bars, 250 μ m. *B)* The mean numbers of organoids per well developed from the intestinal crypts of wild-type (*top*) and *Stat3^{AIEC}* mice (*bottom*) in the indicated inhibitor conditions are shown (mean \pm *s.d.*). Asterisk, $p < 0.05$; and N.S., not significant. Inhibitor treatment experiments were performed three times for each mouse genotype. *C)* A schematic illustration of organoid passaging by mechanical pipetting (*top*). Representative microscopic photographs of organoids passaged from wild-type and *Stat3^{AIEC}* rescued organoids (*bottom*). Insets show enlarged views. Bars, 250 μ m. The mean numbers of organoids

> 150 μm are shown in the bar graph (mean \pm *s.d.*). The mechanical passage experiments were performed three times. *D*) Immunohistochemical staining of wild-type (*top*) and *Stat3^{AIEC}* (*bottom*) rescued organoids for EdU (*green*) and E-cadherin (*red*). EdU labeling efficiency is shown in the bar graph (mean \pm *s.d.*). N.S., not significant. *E*) A schematic illustration of organoid passaging by trypsin treatment (*top*). Representative microscopic photographs of organoids passaged from wild-type (*top*) and *Stat3^{AIEC}*-rescued organoids (*bottom*). Bars, 200 μm (day 0) and 1mm (day 10). The mean numbers of organoids are shown in the bar graph (mean \pm *s.d.*). Asterisk, $p < 0.05$. The enzymatic passage experiments were performed three times.

Figure 3. Wnt activation compensates for *Stat3* disruption to support organoid formation. *A*) A schematic illustration of Tam treatment and organoid formation (*top*). Representative microscopic photographs of organoids of the intestinal crypts from wild-type mice cultured with control medium (*top*) and AFM-Wnt3a (*bottom*) on day 2 (*left*) and day 5 (*right*). Bars, 250 μm . *B*) Representative microscopic photographs of organoid cultures of the intestinal crypts from *Stat3^{AIEC}* mice cultured with control medium (*top*), AFM-Wnt3a without inhibitor (*middle*) and with inhibitor (*bottom*) on days 3 (*left*) and 5 (*right*). Bars, 250 μm . *C*) A schematic illustration of Tam treatment, tumor-derived organoid formation and treatment with pipetting or trypsin to passage (*top*). Representative microscopic photographs of the primary organoids (*left*; Bars, 250 μm) and organoids passaged by pipetting (*middle*; Bars, 250 μm) and trypsin treatment (*right*; Bars, 500 μm) derived from intestinal tumors of *Apc^{A716}* simple mutant mice (*top*) and *Apc^{A716} Stat3^{AIEC}* compound mice (*bottom*). The mean numbers of organoids (*top*) and size distributions (*bottom*) after passaging with trypsin treatment in the respective

genotype are shown in the bar graphs (mean \pm *s.d.*). N.S., not significant. The experiments were performed three times.

Figure 4. The dispensable role of Stat3 in Wnt-driven intestinal tumorigenesis. *A*) A schematic illustration of Tam treatment of *Apc*^{A716} mice (n=10) and *Apc*^{A716} *villin-CreER Stat3*^{flx/flx} mice (n=12) (*top*). Representative histology sections of small intestinal tumors of *Apc*^{A716} mice (*left*) and *Apc*^{A716} *Stat3*^{AIEC} mice (*right*) (H&E) (*top*) and immunohistochemical staining for Ki67 (*middle*) and phosphorylated Stat3 (P-Stat3) (*bottom*). Insets show enlarged views. Bars, 200 μ m. *B*) The total numbers of polyps in each mouse are shown with dots with the mean values (*top*). The size distribution is shown in the bar graph (mean \pm *s.d.*). *C*) A schematic illustration of the Tam treatment of *Apc*^{A716} *villin-CreER Tgfbr2*^{flx/flx} mice (n=10) and *Apc*^{A716} *villin-CreER Tgfbr2*^{flx/flx} *Stat3*^{flx/flx} mice (n=8) (*top*). Representative histology sections of small intestinal tumor of *Apc*^{A716} *Tgfbr2*^{AIEC} mice (*left*) and *Apc*^{A716} *Tgfbr2*^{AIEC} *Stat3*^{AIEC} mice (*right*) for H&E (*top*) and immunohistochemical staining for P-Stat3 (*bottom*). Insets show enlarged views of boxed areas. Bars, 400 μ m. *D*) The total numbers of polyps (*top*) and invasive polyps (*bottom*) per section are shown with the mean number. N.S., not significant.

Figure 5. The dispensable role of Stat3 in Wnt activation-associated cancer cell metastasis. *A*) Western blotting results for Stat3 in control AKTP cells (Cont) and Stat3-targeted AKTP cell lines, Stat3 KO #1 and #2, by CRISPR/Cas9. β -catenin was used for internal controls. *B*) The results of the cell proliferation analysis of control and Stat3 KO#1 and #2 AKTP cells (mean \pm *s.d.*). *C*) Control AKTP cells (Cont) and Stat3-targeted AKTP cell lines (Stat3 KO#1 and #2) were transplanted to the spleen of NSG mice (n=3 for each cell line). Representative

macroscopic photographs of mouse livers at four weeks after spleen transplantation are shown (*top*). Arrowheads indicate metastatic foci. The mean metastasis tumor areas (*bottom left*) and size distribution of metastatic foci (*bottom right*) on the histological sections are shown in the bar graphs (mean \pm *s.d.*). N.S., not significant. *D*) Representative low-powered images of liver metastasis (H&E) (*top*) and high-powered images of serial sections of metastatic foci (H&E) and immunohistochemical staining for P-Stat3 and Ki67 (from *top* to *bottom*). Dotted lines indicate metastatic foci for each genotype. Insets show enlarged views inside of metastatic foci. Bars, 200 μ m.

Figure 6. The Stat3-dependent integrin expression and FAK activation. *A*) Representative photographs of isolated crypts under a dissection microscope, a histological section of isolated crypts (H&E), and immunohistochemical staining for E-cadherin and ApopTag (from *top* to *bottom*) of wild-type (*left*) and *Stat3^{ΔIEC}* isolated intestinal crypts (*right*). Insets show enlarged views. Bars, 100 μ m. *B*) Results of the filter array expression analysis are shown in the dot graph, which displays the upregulated (> 4 fold) and downregulated (< 0.25 fold) genes in the *Stat3^{ΔIEC}* crypts in different colors. *C*) The relative mRNA levels in the isolated crypts (*top*) and rescued organoids (*bottom*) (mean \pm *s.d.*) were examined by RT-PCR for *Itega5* and *Itega6*. Asterisk, < 0.05 . *D*) Isolated crypts from wild-type (*left*) and *Stat3^{ΔIEC}* mice (*right*) that were subjected to immunohistochemical staining for phosphorylated FAK (P-FAK). Bars, 50 μ m. *E*) Representative microscopic photographs of organoids derived from wild-type (*top*) and *Stat3^{ΔIEC}* mouse (*bottom*) crypts in the absence (*left*) or presence (*right*) of FAK inhibitor in the Matrigel. Insets show enlarged views. Note that crypt growth was suppressed by FAK inhibitor regardless of genotypes. Bars, 250 μ m. *F*) The mean numbers of developed organoids (budding

number > 5) in the Matrigel with or without FAK inhibitor (*FAKi* and *NT*, respectively) are shown (mean \pm *s.d.*). Asterisk, $p < 0.05$.

Figure 7. A schematic illustration of the role of Stat3 in regeneration and tumorigenesis of intestinal crypts. Wild-type (*top*) and *Stat3^{AIIEC}* crypts (*bottom*) are shown. Stat3 is required for regeneration from damaged mucosa, which may be compensated by Wnt activation (*left*). In contrast, Stat3 is dispensable for Wnt activation-driven tumorigenesis (*right*).

Figure 1

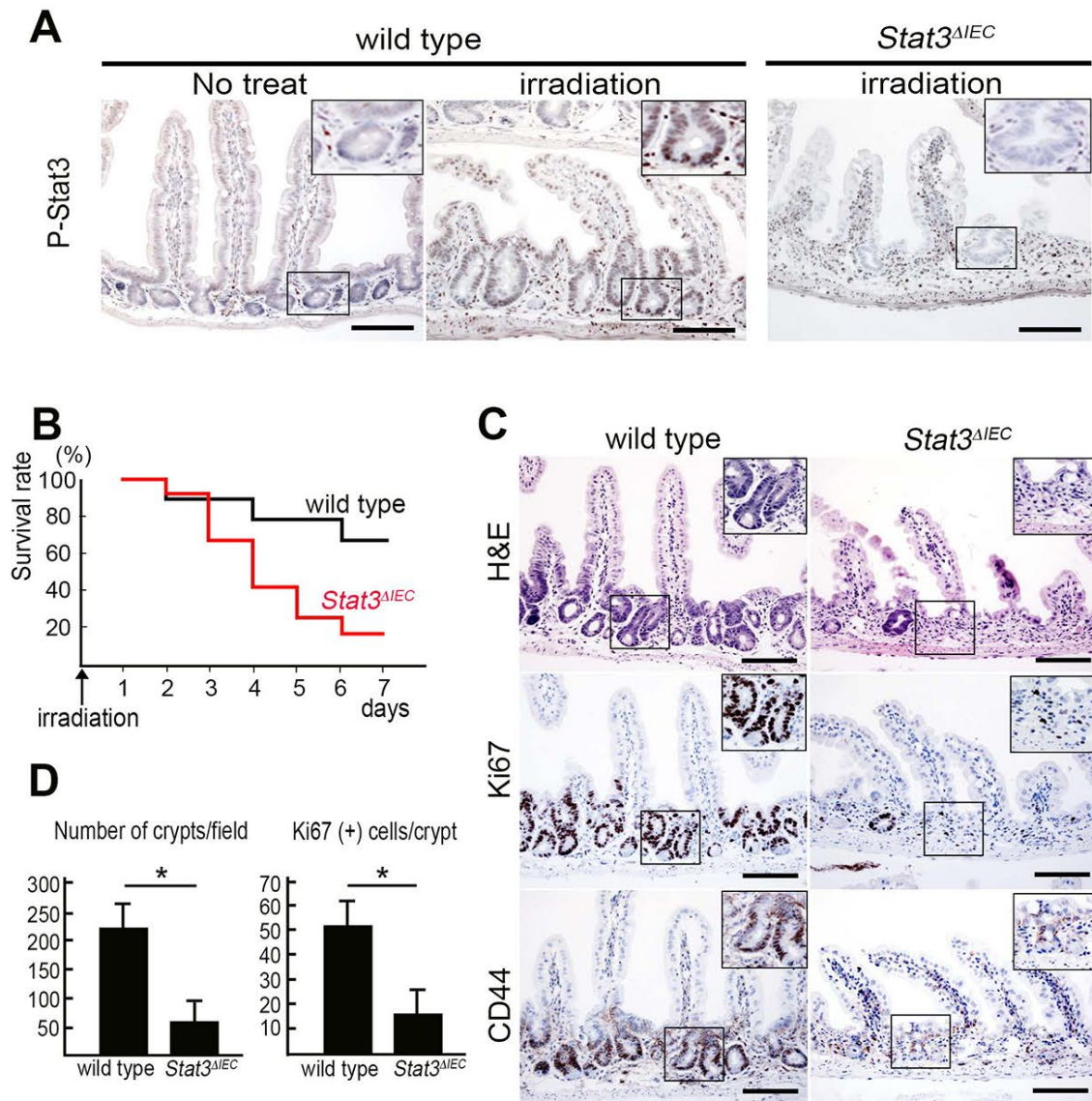


Figure 2

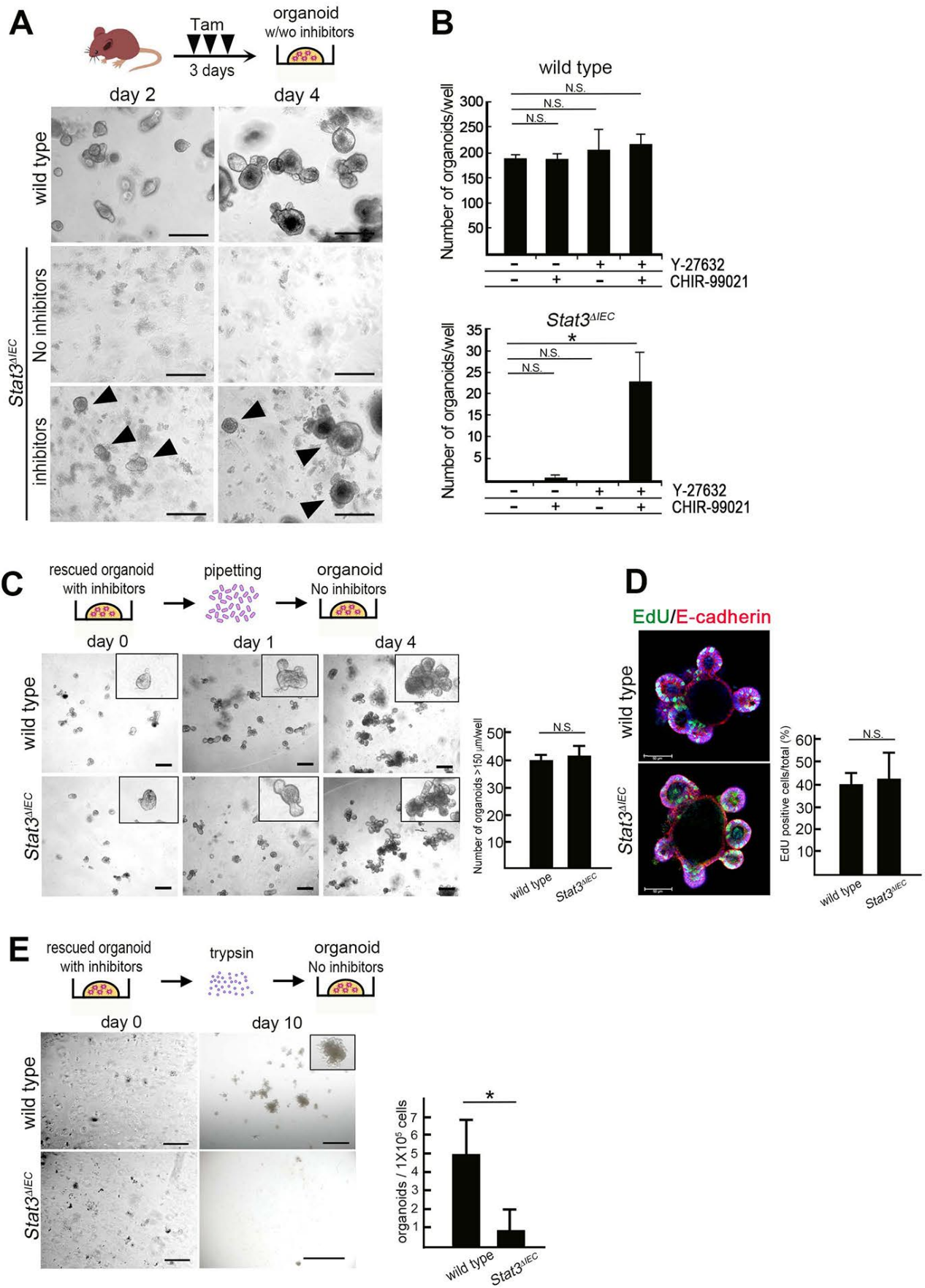


Figure 3

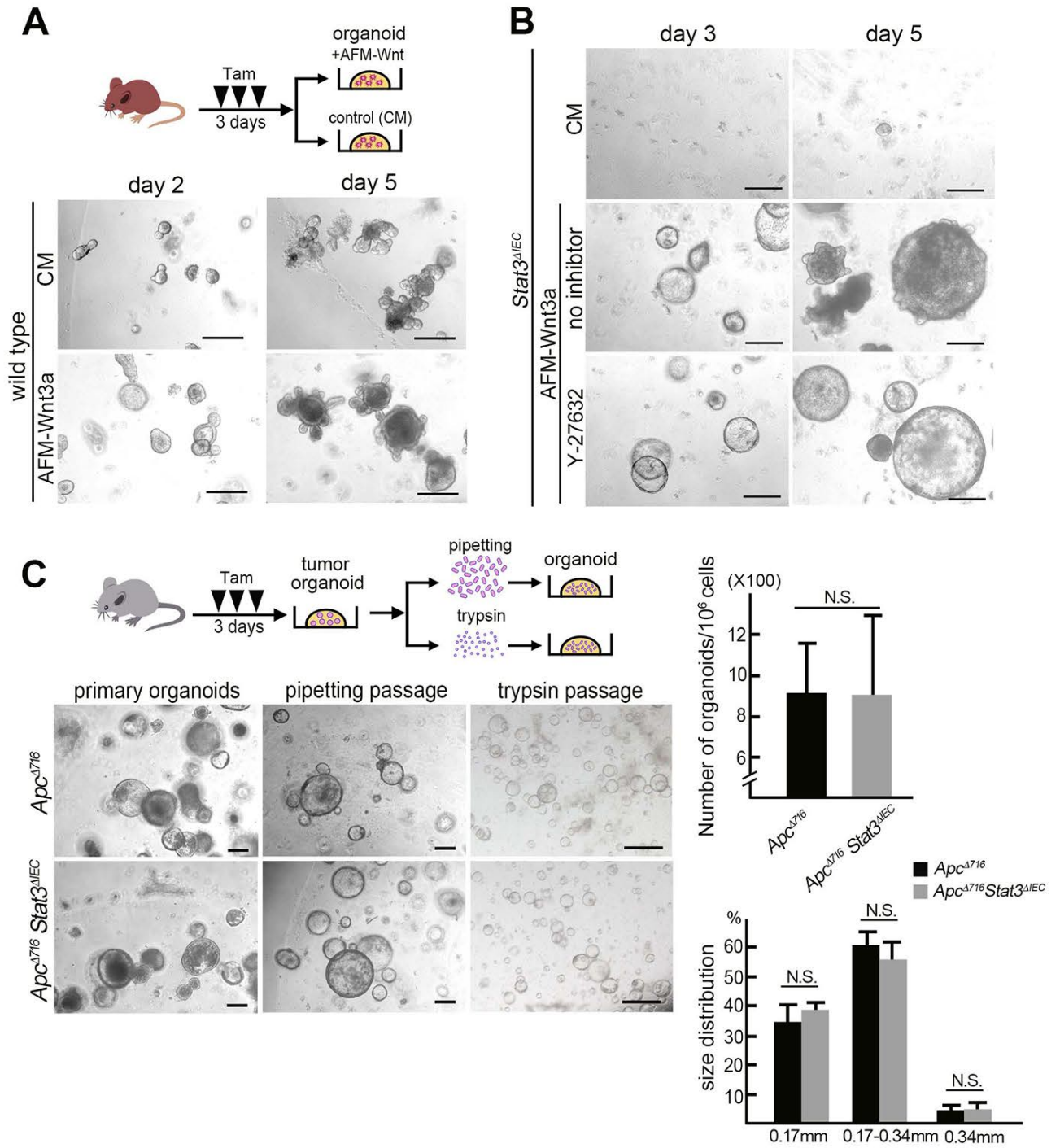


Figure 4

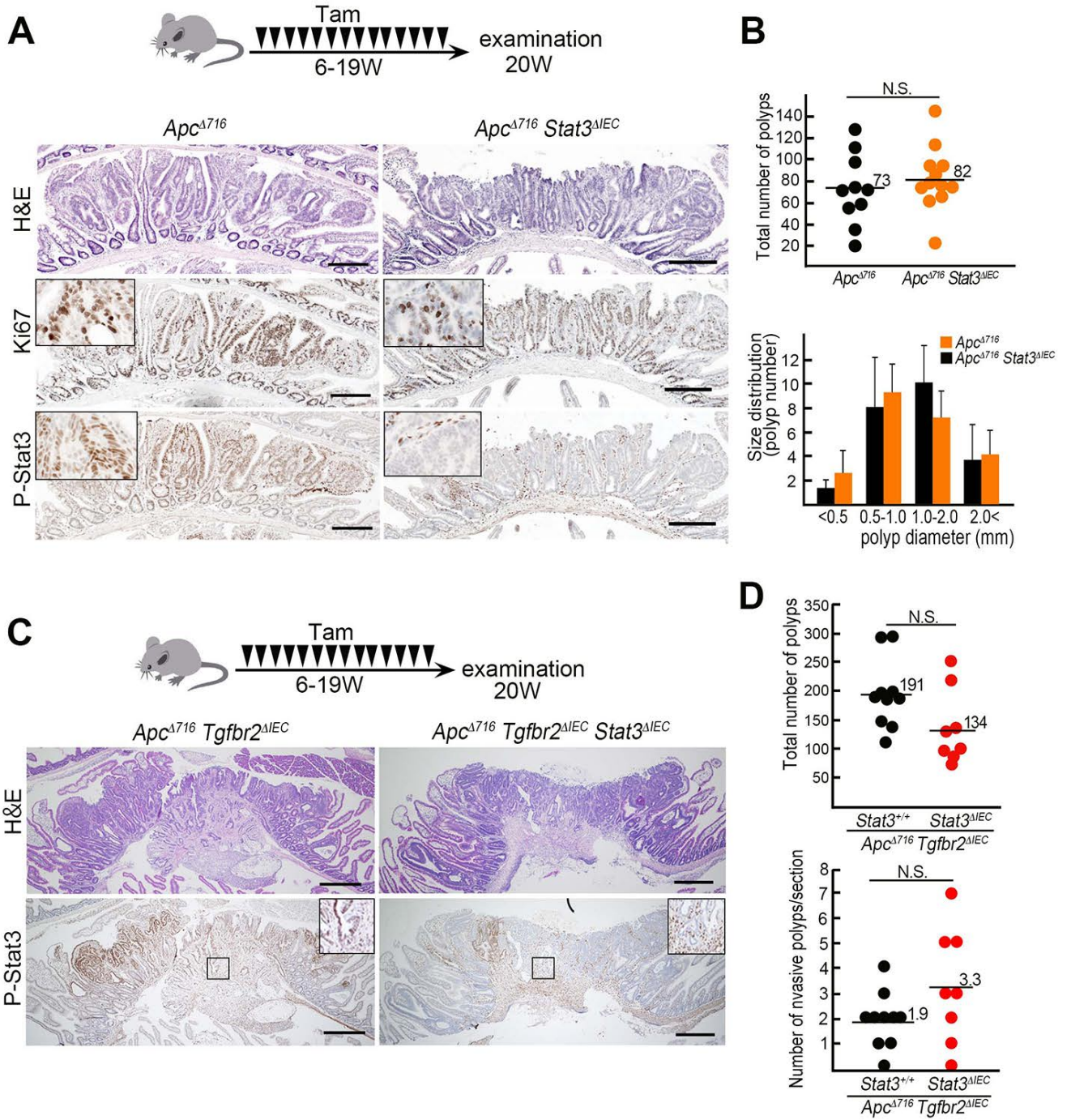


Figure 5

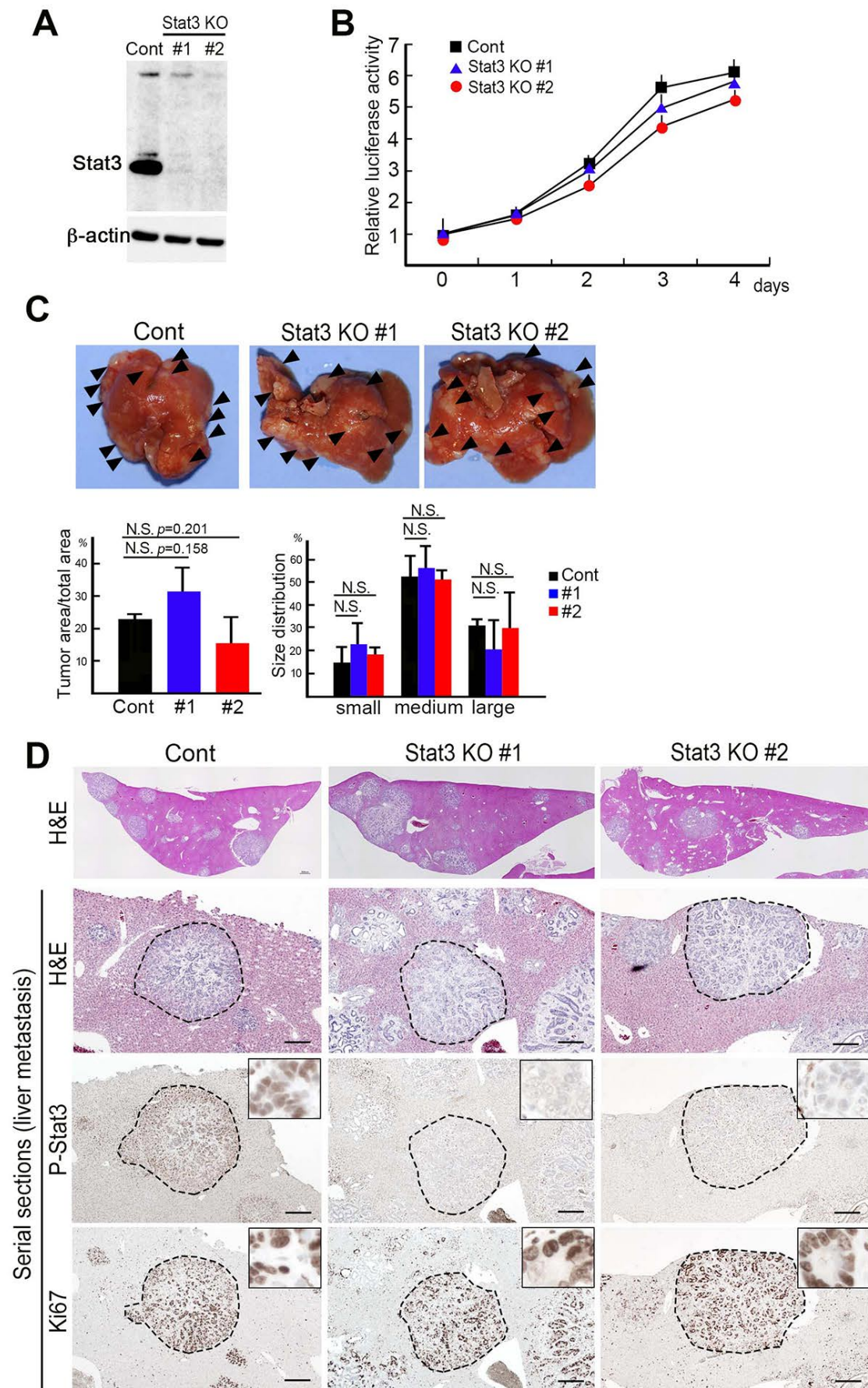


Figure 6

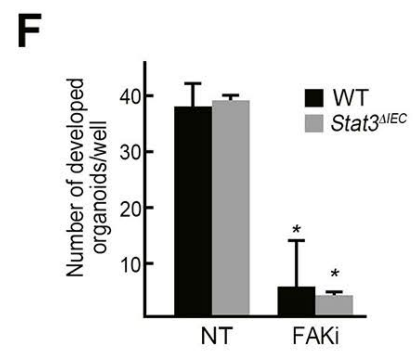
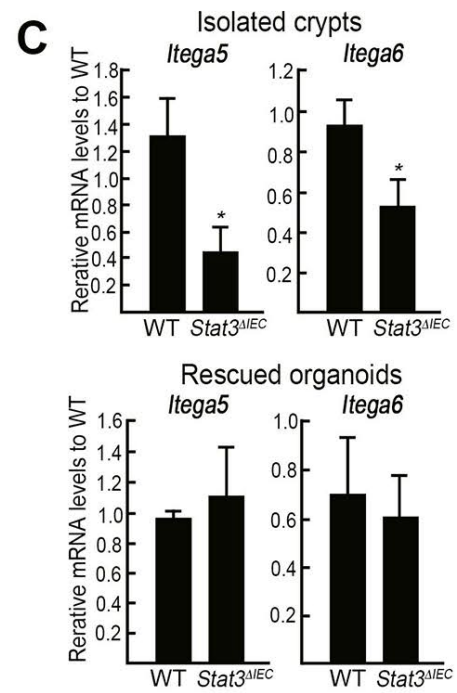
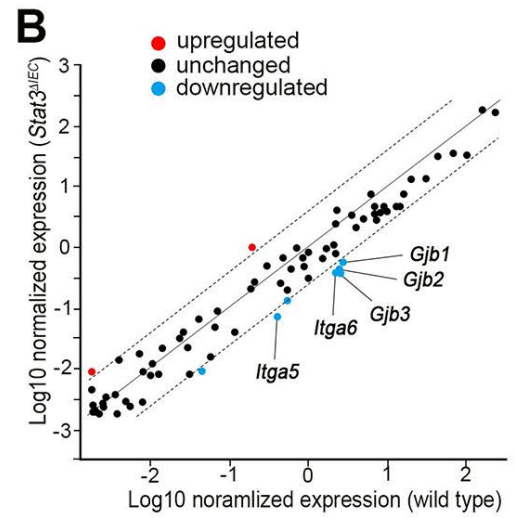
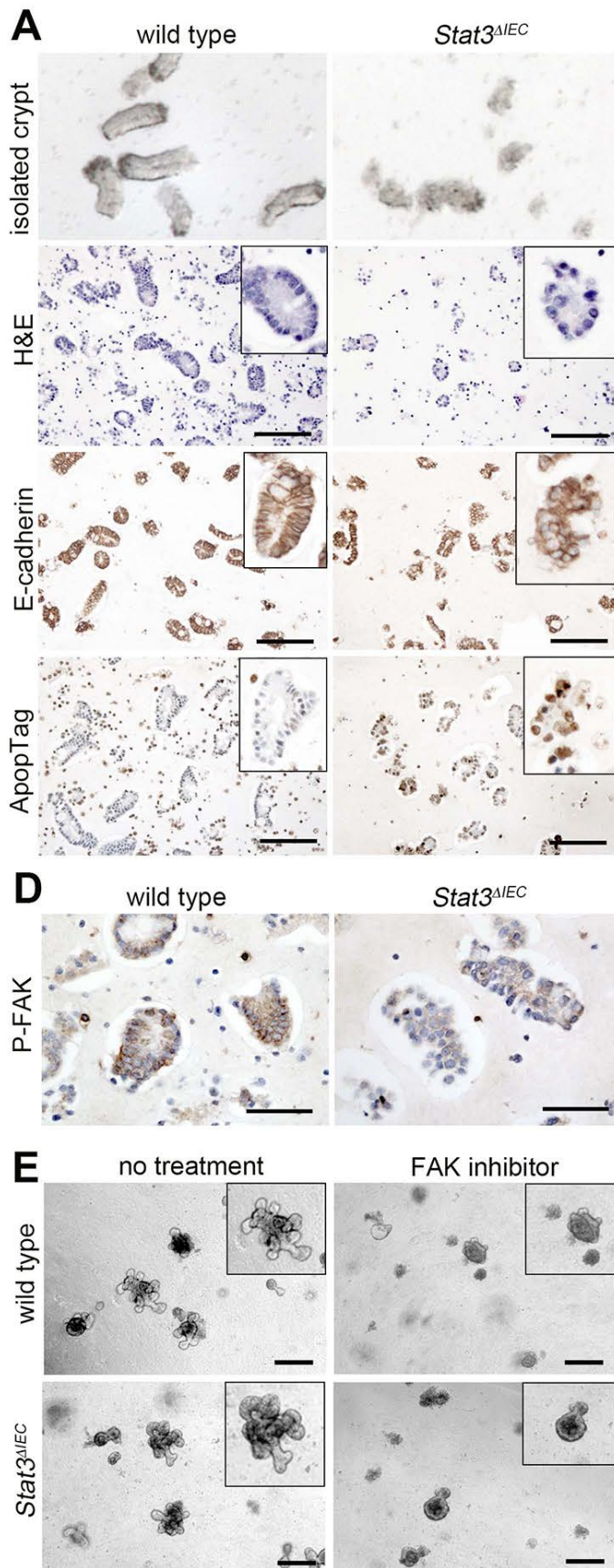


Figure 7

