

Title: Distinct roles of Hes1 in normal stem cells and tumor stem-like cells of the intestine

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Abstract

Cancer stem cells (CSC) have attracted attention as therapeutic targets, however, CSC-targeting therapy may disrupt normal tissue homeostasis because many CSC molecules are also expressed by normal stem cells (NSC). Here we demonstrate that NSC-specific and CSC-specific roles of the stem cell transcription factor Hes1 in the intestine enable the feasibility of a specific cancer therapy. Hes1 expression was upregulated in NSC and intestinal tumors. Lineage tracing experiments in adult mouse intestine revealed that Hes1 deletion in Lgr5⁺ or Bmi1⁺ NSC resulted in loss of self-renewal but did not perturb homeostasis. Further, in Lgr5⁺ NSC deletion of Hes1 stabilized β -catenin, limited tumor formation and prolonged host survival. Notably, in Lgr5⁺ or Dclk1⁺ tumor stem cells derived from established intestinal tumors, Hes1 deletion triggered immediate apoptosis, reducing tumor burden. Our results show how Hes1 plays different roles in NSC and CSC, in which Hes1 disruption leads to tumor regression without perturbing normal stem cell homeostasis, preclinically validating Hes1 as a cancer therapeutic target.

Introduction

Tumor stem cells (TSCs), capable of self-renewing and giving rise to progeny cells in the tumor, have attracted attention as promising targets for anti-tumor therapeutics (1-3). However, TSC-targeting therapy may disrupt normal tissue homeostasis because most of the TSC markers are also expressed by normal stem cells (NSCs) (4-8). To address this concern, two strategies can be employed: the identification of TSC-specific markers (9), and the identification of functional genes that act differently in NSCs and TSCs. Testing the feasibility of the latter approach, by targeting both NSCs and TSCs, requires proper in vivo models.

Hes1 is a basic helix-loop-helix transcription factor, and represses transcription by active and passive repression mechanisms (10). Because Hes1 protein negatively regulates its expression by binding to its own promoter, Hes1 expression oscillates in many cells, which plays an important role in the self-renewal of stem cells (11). Although Notch signaling is important in the maintenance of intestinal stem cells (ISCs) (12,13), the downstream target

required for ISC self-renewal remains elusive. Previously, we showed that the maintenance of ISCs is not affected after the prenatal triple deletion of Hes1, Hes3, and Hes5 (14). However, this result is not directly applicable to adult ISCs. Because early fetal and adult ISCs are regulated by different mechanisms (15,16), an unknown compensation may exist in the developmental phase. The role of Hes1 in adult ISCs needs clarification in order to explore the possibility of Hes1-targeting tumor therapy and to provide further insight into stem cell biology.

To address these issues, we examined the role of Hes1 in the NSCs of small intestine in adult mice. Hes1 deletion in NSCs led to the loss of self-renewal capacity but did not disrupt homeostasis. We investigated the role of Hes1 in intestinal tumors. Notably, we found that Hes1 deletion in the TSCs of established intestinal tumors led to tumor regression by inducing immediate apoptosis. We propose that Hes1 may be a novel tumor-specific therapeutic target that does not perturb intestinal homeostasis.

Materials and Methods

Animal models

Lgr5-EGFP-IRES-CreERT2 mice (JAX strain 008875), *Bmi1-CreER* mice (JAX strain 010531), *Apc^{Min}* mice (JAX strain 002020), and *Rosa26-LacZ* mice (JAX strain 003309) were obtained from the Jackson Laboratory (Bar Harbor, ME). *Dclk1-CreERT2-IRES-EGFP* mice and *Ctnnb1^{lox(ex3)}* mice were generated as previously described (9,17). *Hes1^{fllox}* mice were kindly gifted by Dr. Ryoichiro Kageyama (18). Mice were maintained on a C57BL/6 background. For induction of Cre-mediated recombination, 200 μ l of 20 mg/ml tamoxifen (Sigma-Aldrich, St. Louis, MO) in corn oil (for a single injection), or 100 μ l of 20 mg/ml tamoxifen either twice a day over two consecutive days or once a day over four consecutive days (for intensive induction) was intraperitoneally injected. For experiments using normal intestinal tissue, 8-week-old mice were used. For experiments using established intestinal tumors, 6-month-old mice were used. All experiments involving mice were approved by the animal research committee of Kyoto University and performed in accordance with Japanese government

regulations.

Human subjects

Surgically resected specimens were obtained from 20 colorectal cancer patients at Kyoto University Hospital. Analyses for human subjects were approved by the ethical committee of Kyoto University Hospital and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects.

Histological analyses and immunostaining

For histological analyses, mouse organs were isolated and fixed overnight in 4% paraformaldehyde, embedded in paraffin and sectioned at a thickness of 5 μ m. Sections were then deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). For immunohistochemical analyses, sections were incubated with primary antibody overnight at 4°C and washed with phosphate-buffered saline (PBS). Washed sections were incubated with

biotinylated secondary antibody for 1 h at room temperature. Sections were then incubated with avidin biotin-peroxidase complex (Vector Labs, Burlingame, CA), labeled with peroxidase, and colored with diaminobenzidine substrate (Dako, Glostrup, Denmark). For immunofluorescence, sections were incubated with primary antibody overnight at 4°C and washed with PBS. Washed sections were incubated with fluorescence-conjugated secondary antibody (Invitrogen, Carlsbad, CA) for 1 h at room temperature. Primary antibodies are listed in Supplementary Table 1.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissues or cells using the RNeasy Micro kit (Qiagen, Valencia, CA). Single-strand complementary DNA (cDNA) was synthesized using a Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Basel, Switzerland). qRT-PCR was performed using SYBR Green I Master (Roche Applied Science) and Light Cycler 480 (Roche Applied Science).

Values are expressed as arbitrary units relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers were listed in Supplementary Table 1.

β -galactosidase (LacZ) staining

Freshly isolated small intestine was incubated in ice-cold fixative solution (PBS containing 4% paraformaldehyde, 5 mM EGTA, 2 mM MgCl₂, 0.2% glutaraldehyde, and 0.02% NP-40) for 1 h at 4°C. After washing twice in PBS for 20 min, tissues were incubated with LacZ substrate (PBS containing 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂, 0.02% NP₄O, 0.1% sodium deoxycholate, and 1 mg/ml X-galactosidase) overnight at room temperature. After washing twice in PBS for 20 min, tissues were fixed overnight in 4% paraformaldehyde in PBS at 4°C. Paraformaldehyde was removed, and the stained tissues were transferred to tissue cassettes.

Spheroid Culture

Conditioned medium of L-cell line secreting Wnt3a, R-spondin 3 and Noggin (L-WRN CM) was created as previously described (19). L-WRN (ATCC; CRL-3276) was generated by Hiroyuki Miyoshi (2013) as previously described (14) and passaged for fewer than 6 months. Spheroid cultures were established as previously described (19,20). In short, crypts/glands units or tumor cell aggregates were isolated and embedded in Matrigel (BD Biosciences). For spheroid culture of normal epithelium, 50% L-WRN CM supplemented with 10 μ M Y-27632 (Tocris Bioscience, Bristol, UK) and 10 μ M SB431542 (Tocris Bioscience) was added to each well. CHIR99021 (Focus biomolecules, Plymouth Meeting, PA) and valproic acid (Tocris Bioscience) were added to the medium for *Lgr5*-GFP^{high} cell culture. For spheroid culture of intestinal tumors, advanced DMEM/F-12 (Invitrogen) supplemented with 50 ng/ml mouse EGF, 100 ng/ml Noggin (All from PeproTech, Rocky Hill, NJ), penicillin/streptomycin, 10 mmol/L HEPES, Glutamax, and 1xB27 (all from Invitrogen) was added to each well. For *in vitro* induction of Cre-mediated recombination, 1 μ M 4-hydroxytamoxifen (4-OHT) (Sigma) was added to the cultures.

Lgr5⁺ cell sorting

For sorting experiments, isolated crypts/glands units or tumor cell aggregates were additionally dissociated with TrypLE Express (Invitrogen) for 20 min at 37°C. Dissociated cells were passed through a 30-µm cell strainer and sorted by FACS Aria II (BD Biosciences). Single cells were gated by forward scatter pulse width and side scatter pulse width. Dead cells were excluded by labeling with 7-AAD. The data were analyzed using FlowJo software (version 10, Tree Star, Ashland, OR).

Statistics

All values are presented as mean ± SEM unless otherwise stated. The two-tailed Student's *t*-test was used for statistical analysis of continuous data. The log-rank test was used for statistical analysis of Kaplan-Meier survival curves. *P* values of < 0.05 were considered to be significant.

Results

Hes1 expression is upregulated in intestinal stem cells.

To confirm the pattern of Hes1 expression, we performed immunohistochemical analysis of Hes1 using samples of *Apc^{Min}* mice and human tissues with colon cancer. Consistent with previous reports (14,21-23), in the normal intestine, Hes1 was expressed in the crypt base columnar cells and transit-amplifying (TA) cells but not in Paneth cells or cells of the villi (Supplementary Fig. S1A and S1B). Hes1 was ubiquitously expressed in murine intestinal tumors and human colon cancers (Supplementary Fig. S1A). We sorted GFP-positive cells from intestinal epithelial cells of *Lgr5-EGFP-IRES-CreERT2* mice and performed qRT-PCR. Fluorescence-activated cell sorting (FACS) distinguished a population with high expression of *Lgr5*-GFP (*Lgr5*-GFP-high) and another with low expression of *Lgr5*-GFP (*Lgr5*-GFP-low), which have been reported to correspond to stem cells and their immediate TA daughters, respectively (20) (Supplementary Fig. S1C). The expression of *Axin2* mRNA, which reflects Wnt/ β -catenin activity, was

significantly higher in the *Lgr5*-GFP-high population; *Hes1* expression was also significantly higher in the *Lgr5*-GFP-high population (Supplementary Fig. S1D). The expression of *Notch1* and *Notch2* was significantly higher in the *Lgr5*-GFP-high population; however, the expression of other Notch effectors, *Hes3* and *Hes5*, was not upregulated in the *Lgr5*-GFP-high population (Supplementary Fig. S1E). Wnt/ β -catenin and Notch signaling has been reported to regulate *Hes1* expression respectively (10,21,24); these data imply that both Notch and Wnt/ β -catenin signaling may be important for the upregulation of *Hes1* in NSCs. The high expression of *Hes1* in NSCs prompted us to examine the role of *Hes1* in NSCs.

Hes1 deletion in *Lgr5*⁺ NSCs results in loss of self-renewal but does not perturb homeostasis.

To investigate the role of *Hes1* in NSCs, we deleted *Hes1* in *Lgr5*⁺ NSCs and performed lineage tracing using *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice. After administering 4 mg tamoxifen, LacZ-labeled blue cells

appeared in crypt base columnar cells at day 1, proliferated in the crypts at day 3, and gave rise to progeny cells that appeared as blue stripes from the crypts to the villi at day 5 (Fig. 1A). At day 7, these blue stripes started to disappear in the crypts, and only a few blue cells were detected on the tip of the villi (Fig. 1A). Provided that Lgr5⁺ NSCs divide every 24 h, and epithelial cells are renewed every 4 to 5 days (25), this indicates that Hes1-deleted Lgr5⁺ NSCs can give rise to progeny cells but cannot self-renew.

To further validate these results, we maximized the induction efficiency by administering 2 mg tamoxifen twice a day over 2 consecutive days. Using this intensive induction, we also evaluated the effect of Hes1 deletion in NSCs on intestinal homeostasis. First, we sorted Lgr5⁺ NSCs 2 days after the last injection, and confirmed the downregulation of *Hes1* expression in Hes1-deleted Lgr5⁺ NSCs (Supplementary Fig. S2A). Surprisingly, *Hes3* and *Hes5* expression was not upregulated (Supplementary Fig. S2A), suggesting that, unlike the prenatal deletion of Hes1, Hes3 and Hes5 do not compensate for Hes1 deficiency in adult NSCs. Three days after the last injection, immunohistochemistry revealed that

the expression of Hes1 was almost lost in the LacZ-labeled crypts of *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice (Fig. 1B and C), confirming that recombination of the floxed Hes1 allele occurred efficiently. Cleaved caspase-3 was not expressed in LacZ-labeled crypts of *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice (Supplementary Fig. S2B), thereby excluding the possibility that Hes1-deleted *Lgr5*⁺ NSCs or their daughter TA cells were lost due to immediate apoptosis. Five days after the last injection, the proportion of LacZ⁺ crypt-villi axes in the proximal intestine did not differ between *Lgr5*^{CreERT2/+}; *Hes1*^{+/+}; *Rosa26*^{LacZ/+} and *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice (Supplementary Fig. S2C and D). Fourteen days after the last injection, most of the epithelial cells in proximal small intestine, where *Lgr5*⁺ NSCs are abundantly detected, were labeled blue in *Lgr5*^{CreERT2/+}; *Hes1*^{+/+}; *Rosa26*^{LacZ/+} control mice (Fig. 1D, E, and Supplementary Fig. S2D). This indicates that, with this intensive induction, recombination occurred in most of the *Lgr5*⁺ cells in proximal small intestine. Conversely, in *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice, most of the LacZ-labeled blue cells were depleted and repopulated with LacZ-negative cells

(Fig. 1D, E, and Supplementary Fig. S2D). These results further indicate that Hes1-deleted $Lgr5^{+}$ cells cannot self-renew. The repopulated LacZ-negative intestinal epithelium of $Lgr5^{CreERT2/+}; Hes1^{flox/flox}; Rosa26^{LacZ/+}$ mice did not display obvious morphological abnormalities (Fig. 1E). Immunohistochemical analysis revealed newly generated LacZ-negative $Lgr5^{+}$ cells at the base of the crypts and LacZ-negative $Hes1^{+}$ cells in the crypts of $Lgr5^{CreERT2/+}; Hes1^{flox/flox}; Rosa26^{LacZ/+}$ mice (Fig. 1E). The number of $Lgr5^{+}$ cells and $Hes1^{+}$ cells did not differ between $Lgr5^{CreERT2/+}; Hes1^{+/+}; Rosa26^{LacZ/+}$ control mice and $Lgr5^{CreERT2/+}; Hes1^{flox/flox}; Rosa26^{LacZ/+}$ mice at day 14 (Fig. 1C). Although $Lgr5^{+}$ NSCs cannot self-renew after the deletion of Hes1, $Lgr5^{+}$ NSCs were newly generated, likely from some of the $Lgr5^{-}$ reserve stem cells (e.g., $Bmi1^{+}$ cells), as previously reported (26). Furthermore, we also evaluated the effect of Hes1 deletion on the proliferative capacity and secretory cell differentiation before (at day 5) and after (at day 14) replacement with LacZ-negative cells; however, no significant difference was found (Supplementary Fig. S3A-C).

Lgr5-EGFP-IRES-CreERT2 mice have mosaic expression of the reporter (27); there remains some unlabeled *Lgr5*⁺ cells which escape the induction. To address this issue, we sorted single *Lgr5*-GFP^{high} cells from the normal intestinal epithelia of *Lgr5*^{CreERT2/+}; *Hes1*^{+/+} and *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox} mice; this approach circumvents mosaic reporter expression. Then, we cultured *Lgr5*-GFP^{high} cells in Matrigel, in 50% L-WRN CM, CHIR99021, and valproic acid, which facilitates homogenous *Lgr5*-GFP^{high} stem cell culture, with 4-OHT induction for 48 h from the start of the culture (Fig. 2A). Compared with that of the control, colony formation was almost completely abrogated in *Hes1*-deleted *Lgr5*-GFP^{high} single cells (Fig. 2B and C), showing that *Hes1* is required for the self-renewal of *Lgr5*-GFP^{high} stem cells. We also assessed the effect of *Hes1* deletion in *Lgr5*⁺ cells on established spheroids (Fig. 2D). Spheroids grown in 50% L-WRN CM were enriched for stem and/or progenitor cells (19). The *Lgr5* expression level in the spheroids decreased when cultured without CHIR99021 and valproic acid (Fig. 2E), suggesting the presence of *Lgr5*⁺ TA cells in these cultures. Three days after induction, the growth of established spheroids,

cultured in 50% L-WRN CM, was not affected, and Lgr5-positive cells were detected (Fig. 2F and G); these Lgr5-positive cells were likely generated from the Lgr5⁺ TA cells. These data demonstrate that deletion of Hes1 in NSCs does not affect intestinal homeostasis.

Hes1 deletion in Bmi1⁺ NSCs results in loss of self-renewal but does not perturb homeostasis.

Bmi1 is expressed in NSCs of the intestine (6); Bmi1⁺ cells serve as reserve stem cells in cases of injury or Lgr5⁺ cell ablation (26). We assessed whether Hes1 is important in the maintenance of these NSCs that can serve as reserve stem cells. We deleted Hes1 in Bmi1⁺ NSCs and performed lineage tracing using *Bmi1*^{CreER/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice. After injection with 4 mg tamoxifen, LacZ-labeled blue cells appeared four or five cells above the base of the crypt at day 1, and gave rise to progeny cells that arranged into blue stripes, reaching from the crypts to the villi, at day 3 (Fig. 3A). From days 5 to 7, blue

stripes started to disappear in some crypts, and only a few blue cells were detected on the tip of the villi (Fig. 3A).

We maximized the induction efficiency by administering 2 mg tamoxifen over 4 consecutive days. Immunohistochemistry was used to confirm that recombination of the floxed Hes1 allele occurred efficiently (Fig. 3B and C). Ten days after the last injection, most of the LacZ-labeled blue cells were depleted and repopulated with LacZ-negative cells in *Bmi1*^{CreER/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice compared with those in *Bmi1*^{CreER/+}; *Hes1*^{flox/+}; *Rosa26*^{LacZ/+} control mice (Fig. 3D and E). The number of Hes1⁺ cells did not differ between *Bmi1*^{CreER/+}; *Hes1*^{flox/+}; *Rosa26*^{LacZ/+} mice and *Bmi1*^{CreER/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice at day 10 (Fig. 3C and E). These data demonstrate that, regardless of the stem cell markers tested, Hes1 is necessary for the self-renewal of NSCs; however, Hes1 deletion in NSCs does not perturb intestinal homeostasis.

Concomitant Hes1 deletion in β -catenin-stabilized NSCs abrogates tumor formation.

To investigate the role of Hes1 in tumorigenesis, we stabilized β -catenin for intestinal tumor formation and deleted Hes1 in Lgr5⁺ cells using *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{flox/flox} mice. Twenty-eight days after injection with 2 mg tamoxifen over 4 consecutive days, we found that only microadenomas were formed in *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{flox/flox} mice, whereas large tumors were formed throughout the intestine in *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{+/+} control mice (Fig. 4A). Immunohistochemistry revealed the expression of Hes1 and nuclear accumulation of β -catenin in the large tumors of *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{+/+} mice and microadenomas of *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{flox/flox} mice (Fig. 4A). These results suggest that microadenomas in *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{flox/flox} mice were formed only from Lgr5⁺ cells that escaped deletion of Hes1 but had not escaped β -catenin stabilization. Because of the strongly suppressed tumor formation, *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{flox/flox} mice survived significantly longer than the control mice (median survival time: 96 days vs. 27 days, respectively; Fig. 4B). We analyzed the number and size of adenomas in *Lgr5*^{CreERT2/+};

Ctnnb1^{lox(ex3)/+}; *Hes1*^{fllox/fllox} mice at different times after the administration of tamoxifen. We found that the total number of adenomas did not increase, but the size of a few nascent adenomas gradually increased over time, resulting in the formation of some microadenomas and a few macroadenomas at 15 weeks (Fig. 4C). Immunohistochemistry revealed the expression of Hes1 in microadenomas, macroadenomas, and the normal intestinal epithelia of *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{fllox/fllox} mice (Fig. 4D), reaffirming that tumors were formed only from *Lgr5*⁺ cells that had escaped Hes1 deletion. We performed lineage tracing using *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{+/+}; *Rosa26*^{LacZ/+} and *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{fllox/fllox}; *Rosa26*^{LacZ/+} mice. Twenty-eight days after induction, most of the LacZ-labeled blue cells were depleted and repopulated by LacZ-negative epithelium in *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{fllox/fllox}; *Rosa26*^{LacZ/+} mice, whereas LacZ-labeled blue intestinal tumors formed in *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{+/+}; *Rosa26*^{LacZ/+} control mice (Fig. 4E and F). These results confirm that β -catenin stabilization cannot offset the inability of Hes1-deleted *Lgr5*⁺ NSCs to self-renew, which leads to substantial

suppression of intestinal tumor formation.

Hes1 deletion in *Lgr5*⁺ TSCs of established intestinal tumors results in tumor regression by inducing immediate apoptosis.

We have shown that concomitant Hes1 deletion in β -catenin-stabilized NSCs abrogates tumor formation; however, to recapitulate Hes1-targeting tumor therapy, it is necessary to examine the effect of Hes1 deletion in established intestinal tumors. It has been shown that *Lgr5* marks TSCs in the intestinal tumors of *Apc*-deficient mice (5). To investigate the role of Hes1 in the TSCs of established intestinal tumors, we deleted Hes1 in *Lgr5*⁺ TSCs of the intestinal tumors and performed lineage tracing using *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+}; *Rosa26*^{LacZ/+} mice. Five days after 4 mg tamoxifen administration, normal intestinal epithelia were intact and labeled blue; however, the intestinal tumors were injured and devoid of blue cells in *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+}; *Rosa26*^{LacZ/+} mice (Fig. 5A). In the *Lgr5*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+}; *Rosa26*^{LacZ/+} control mice, the normal intestinal epithelia and the intestinal tumors were

labeled blue (Supplementary Fig. S4A and B). We maximized induction efficiency by administering 2 mg tamoxifen over 4 consecutive days. The intestinal tumors were more severely injured at day 5 (Fig. 5A); the blue cells of the normal intestinal epithelia and the injured intestinal tumors were depleted at day 10 in *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+}; *Rosa26*^{LacZ/+} mice (Fig. 5A).

To further evaluate the effect of Hes1 deletion in *Lgr5*⁺ TSCs, we followed the time course of LacZ-labeled blue cells in the intestinal tumors after a single 4 mg injection of tamoxifen in *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+}; *Rosa26*^{LacZ/+} mice. Blue cells in the tumors were present at day 1 and depleted by day 3 (Fig. 5B). At day 1, immunohistochemistry showed cleaved caspase-3 expression in Hes1-deleted *Lgr5*⁺ TSCs (Fig. 5B). The proportion of cleaved caspase-3-positive cells in *Lgr5*⁺ cells was significantly higher in the intestinal tumors of *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice than in those of *Lgr5*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+} mice (15 % vs. 2.7 %, respectively), whereas cleaved caspase-3 expression was not detected in *Lgr5*⁺ cells of the normal intestinal epithelia of *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice at day 1 (Fig. 5C). To further

assess the behaviors of Lgr5⁺ NSCs and Lgr5⁺ TSCs after the deletion of Hes1, we sorted Lgr5⁺ NSCs and Lgr5⁺ TSCs from *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice at day 1 after a single 4 mg injection of tamoxifen, and used qRT-PCR to compare the levels of mRNA expression with those in the control mice. Hes1 expression was downregulated in Lgr5⁺ NSCs and Lgr5⁺ TSCs at day 1 after induction (Fig. 5D and Supplementary Fig. S4C). Of note, the expression of anti-apoptotic gene *Bcl2* was significantly downregulated, and the expression of pro-apoptotic gene *Bax* had a tendency to be upregulated in Hes1-deleted TSCs, whereas the expression levels of *Bax* and *Bcl2* were not altered in Hes1-deleted NSCs (Fig. 5D and Supplementary Fig. S4C). Previous studies showed that Hes1 directly suppresses Pten (28,29), which upregulates p53 activity (30) and downregulates the PI3K-Akt pathway (31) to induce apoptosis. Interestingly, *Pten* was upregulated in the Hes1-deleted TSCs, but not in the Hes1-deleted NSCs at day 1 (Fig. 5D and Supplementary Fig. S4C). These results demonstrate that Hes1 deletion results in immediate apoptosis of Lgr5⁺ TSCs, but not Lgr5⁺ NSCs.

To evaluate whether *Hes1* deletion in *Lgr5*⁺ TSCs can be used as a tumor-specific treatment, we analyzed the number of intestinal tumors in *Lgr5*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+} control mice and *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice after administering 2 mg tamoxifen over 4 consecutive days. Compared with *Lgr5*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+} mice, the number of intestinal tumors was significantly reduced in *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice 5 days after intensive induction (Fig. 5E). Immunohistochemistry revealed that the number of *Lgr5*⁺ cells was unaltered in the normal intestinal epithelia, but significantly reduced in the remaining few intestinal tumors of *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice at day 5 (Fig. 5F). These results suggest that, in contrast with the normal intestinal epithelia, no new *Lgr5*⁺ cells were generated after *Hes1* deletion in the intestinal tumors.

To observe the time course of the same intestinal tumors after *Hes1* deletion, we generated spheroids (19) from the intestinal tumors of *Lgr5*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+} and *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice, using a medium containing EGF and Noggin; we, then, compared the spheroid growth after

4-OHT administration for 24 hours (Supplementary Fig. S5A). The spheroids from *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice ceased to expand, and *Lgr5*-GFP⁺ cells were depleted at day 3 (Supplementary Fig. S5B and C), in contrast with the unaltered growth of established spheroids generated from normal intestinal epithelia (Fig. 2D-G). These results demonstrate that *Hes1* deletion in *Lgr5*⁺ cells specifically suppresses tumor growth.

***Hes1* deletion in *Dclk1*⁺ TSCs of established intestinal tumors results in tumor regression by inducing immediate apoptosis.**

We have recently identified *Dclk1* as a TSC-specific marker in the intestine by murine lineage tracing experiments; we proposed that *Lgr5*⁺*Dclk1*⁺ cells are the bona fide TSCs in the intestinal tumors (9). To further confirm that *Hes1* deletion in TSCs leads to tumor regression, we deleted *Hes1* in *Dclk1*⁺ TSCs of established intestinal tumors and performed lineage tracing using *Dclk1*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+}; *Rosa26*^{LacZ/+} mice. Five days after administering 4 mg tamoxifen, the intestinal tumors of *Dclk1*^{CreERT2/+}; *Hes1*^{flox/flox};

Apc^{Min/+}; *Rosa26*^{LacZ/+} mice were injured and devoid of blue cells (Fig. 6A), which contrasted the blue intestinal tumors in *Dclk1*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+}; *Rosa26*^{LacZ/+} control mice (Fig. 6A). Next, we followed the time course of LacZ-labeled Hes1-deleted Dclk1⁺ cells after induction. Blue cells were present in the intestinal tumors at day 1 and depleted by day 3 (Fig. 6B). Immunohistochemistry revealed cleaved caspase-3 expression in Dclk1⁺ cells at day 1 (Fig. 6C), suggesting that deletion of Hes1 in Dclk1⁺ TSCs also induces immediate apoptosis.

We maximized induction efficiency by administering 2 mg tamoxifen over 4 consecutive days, and analyzed the number of intestinal tumors in *Dclk1*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+} and *Dclk1*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice. Compared with the number of intestinal tumors in the control mice, the number of intestinal tumors was significantly reduced in *Dclk1*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice 5 days after intensive induction (Fig. 6D). These results demonstrate that, irrespective of stem cell markers analyzed, Hes1 deletion in the TSCs leads to tumor regression by inducing immediate apoptosis in the

TSCs.

Discussion

In this study, we focused on the specific role of Hes1 in the NSCs and TSCs of the intestine, and showed that the different roles of Hes1 can enable stem cell-targeting tumor therapy. We first demonstrated that the deletion of Hes1 in the NSCs results in loss of self-renewal but does not perturb homeostasis. The Notch and Wnt/ β -catenin pathways play a crucial role in the maintenance of NSCs (12,13,32). Hes1 is a well-known Notch effector (10), and its expression is also directly regulated by the Wnt/ β -catenin pathway (21,24). Our study showed that while the expression of other Notch effectors, Hes3 and Hes5, was not upregulated, the expression of Hes1 was upregulated in the NSCs. Although the interaction between Notch and Wnt/ β -catenin pathways is complex and context-dependent (13,21,33,34), their role in the maintenance of NSCs may be dependent on their common downstream target, Hes1.

Previously, we employed the *Villin-Cre* mice to show that prenatal Hes1 deletion induces transient secretory differentiation, which is compensated by Hes3 and Hes5, and that the maintenance of NSCs is unaffected even after the

prenatal triple deletion of Hes1, Hes3, and Hes5 (14). We speculate that other Notch effectors (e.g., Hey1, Hey2, and HeyL) had compensated in the maintenance of NSCs. Recombination in *Villin-Cre* mice occurs early in the developmental stage (35) when the intestinal epithelium is generated from progenitors other than Lgr5-positive cells (15,16); these progenitors may transiently permit Hes1 deficiency and provide time for the compensation. In contrast, this study showed that Hes1 deficiency was not compensated in adult NSCs, indicating a novel role of Hes1 in the maintenance of adult NSCs, which was masked in our previous analysis of prenatal Hes1 deletion. However, *Lgr5-EGFP-IRES-CreERT2* mice have mosaic expression of the reporter (27); we cannot exclude the possibility that Hes1-expressing unlabeled Lgr5⁺ cells had replaced Hes1-deleted Lgr5⁺ cells by neutral drift dynamics (36) *in vivo*. To address this, we sorted *Lgr5-GFP^{high}* cells and showed that Hes1-deleted Lgr5⁺ stem cells cannot self-renew *in vitro*. Hes1 deletion using stem cell-specific reporter mice without the mosaic problem (27) could further corroborate the role of Hes1 *in vivo*. In contrast to prenatal Hes1 deletion, Hes1 deletion in adult

NSCs did not increase the number of secretory cells (e.g., goblet cells, enteroendocrine cells, and Paneth cells). This was probably because the depletion of Hes1-deleted NSCs did not provide sufficient time for the differentiation shift.

After Hes1 deletion in Lgr5⁺ cells, normal intestinal epithelia were replaced by wild-type epithelia with normal Hes1 expression in the crypts. The intact epithelium following Hes1 deletion in Lgr5⁺ NSCs could be explained by the previous report showing that ablation of Lgr5⁺ NSCs itself does not disrupt homeostasis because of compensation by reserve stem cells (26). To address the issue, we deleted Hes1 in Bmi1⁺ NSCs, which have the potential capacity of reserve stem cells (26). It has been shown that, in contrast to the ablation of Lgr5⁺ NSCs, the ablation of Bmi1⁺ NSCs leads to crypt loss, perturbing the intestinal homeostasis (6). We showed that Hes1 deletion in Bmi1⁺ cells does not perturb homeostasis either. It is possible that adjacent TA cells compensate for the loss of reserve stem cells, because Hes1 deletion, which results in loss of self-renewal, has milder influences on the adjacent TA cells than does ablation.

Further studies are required to corroborate this speculation.

Several reports have shown the potential of Hes1 as a target for anti-tumor therapeutics. *Hes1* knockdown suppresses cell proliferation in T-cell acute lymphoblastic leukemia and colon cancer cell lines (29,37,38), and Hes1 overexpression induces self-renewal properties in colon cancer cell lines (23). Our previous study showed that when Hes1 is deleted prenatally, the characteristics of *Apc*^{Min} tumors mimic those of normal intestinal epithelia, such as differentiation and low proliferative capacity (14). However, it is possible that compensation in the developmental phase permitted atypical tumor formation; additionally, we did not examine apoptosis or immediate changes after postnatal Hes1 deletion. Therefore, in this study, we performed TSC-specific Hes1 deletion, which indicated that the deletion of Hes1 in the TSCs of established tumors leads to tumor regression by inducing immediate apoptosis. These findings provide strong evidence for Hes1-targeting tumor therapy. The dramatic change in Hes1-deleted Lgr5⁺ TSCs can be attributed to the abrupt upregulation of Pten. Hes1 directly suppresses Pten (28,29), and Pten upregulation induces

apoptosis via direct upregulation of p53 activity (30) and downregulation of the PI3K-Akt pathways, such as the PI3K-Akt-mTOR and PI3K-Akt-GSK3 β pathways (29,31). Provided that Hes1 knockdown in the wild-type and mutant p53 colon cancer cell lines increases apoptosis (29), multiple pathways, such as Pten-p53 and Pten-Akt, may be involved in inducing apoptosis in Hes1-deleted TSCs.

In contrast to normal intestinal epithelia, Lgr5⁺ TSCs were not generated from the surrounding tumor cells after deletion of Hes1. The loss of Lgr5⁺ TSCs after Hes1 deletion can be attributed to the following two factors. First, the deletion of Hes1 in the TSCs results in changes that are more severe than those in NSCs (immediate apoptosis in the former compared to loss of self-renewal in the latter), and so the surrounding cells could not compensate for the loss of Lgr5⁺ TSCs due to apoptosis. Second, because the population of Lgr5⁺ cells expanded in the intestinal tumors (2.9% in the normal intestine compared to 13% in the intestinal tumors; Fig. 5F), it is possible that the Lgr5⁺ cells in the intestinal tumors consist of both stem cells and progenitor cells. The

first factor may be more plausible because Hes1 deletion in Dclk1⁺ TSCs also leads to tumor regression by inducing immediate apoptosis. Using murine lineage tracing experiments, we have recently shown that Dclk1 marks TSCs in the intestine, and proposed that Lgr5⁺Dclk1⁺ cells are the bona fide TSCs in intestinal tumors (9). In this study, we showed that Hes1 deletion in Dclk1⁺ cells as well as in Lgr5⁺ cells leads to tumor regression, implying that Hes1 deletion in Lgr5⁺Dclk1⁺ cells, which account for only a small subset of the tumor cells, may be sufficient for tumor regression.

Inhibition of Hes1 by small molecules, and in vivo delivery of Hes1 siRNA, are two approaches that can be employed to target Hes1 for therapeutic purposes. Several compounds can inhibit Hes1 activity (38-40). Agalloside, which directly binds to Hes1, has been recently identified as a naturally occurring Hes1 dimer inhibitor (39). Furthermore, siRNA delivery systems are being developed for cancer treatment and have already entered clinical trials (41). The advance of these technologies may realize the potential of Hes1-targeting tumor stem cell therapy in the future.

In summary, using several murine models with different NSC and TSC markers, we have shown the novel role of Hes1 in the maintenance of NSCs and TSCs in the adult intestine. Hes1 deletion in the NSCs leads to loss of self-renewal but does not disrupt homeostasis (Fig. 7A). Conversely, Hes1 deletion in the TSCs leads to tumor regression by inducing immediate apoptosis (Fig. 7B). Based on the different roles of Hes1 in the NSCs and TSCs, we propose that Hes1 can be used as a novel tumor-specific therapeutic target that does not perturb intestinal homeostasis.

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References

1. Clevers H. The cancer stem cell: premises, promises and challenges. *Nat Med* **2011**;17:313-9
2. Beck B, Blanpain C. Unravelling cancer stem cell potential. *Nat Rev Cancer* **2013**;13:727-38
3. Medema JP. Cancer stem cells: the challenges ahead. *Nat Cell Biol* **2013**;15:338-44
4. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, *et al.* Identification of stem cells in small intestine and colon by marker gene *Lgr5*. *Nature* **2007**;449:1003-7
5. Schepers AG, Snippert HJ, Stange DE, van den Born M, van Es JH, van de Wetering M, *et al.* Lineage tracing reveals *Lgr5*⁺ stem cell activity in mouse intestinal adenomas. *Science* **2012**;337:730-5
6. Sangiorgi E, Capecchi MR. *Bmi1* is expressed in vivo in intestinal stem cells. *Nat Genet* **2008**;40:915-20
7. Snippert HJ, van Es JH, van den Born M, Begthel H, Stange DE, Barker N, *et al.* Prominin-1/CD133 marks stem cells and early progenitors in mouse small intestine. *Gastroenterology* **2009**;136:2187-94 e1
8. Zhu L, Gibson P, Currle DS, Tong Y, Richardson RJ, Bayazitov IT, *et al.* Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. *Nature* **2009**;457:603-7
9. Nakanishi Y, Seno H, Fukuoka A, Ueo T, Yamaga Y, Maruno T, *et al.* *Dclk1* distinguishes between tumor and normal stem cells in the intestine. *Nat Genet* **2013**;45:98-103
10. Kageyama R, Ohtsuka T, Kobayashi T. The *Hes* gene family: repressors and oscillators that orchestrate embryogenesis. *Development* **2007**;134:1243-51
11. Imayoshi I, Isomura A, Harima Y, Kawaguchi K, Kori H, Miyachi H, *et al.* Oscillatory control of factors determining multipotency and fate in mouse neural progenitors. *Science* **2013**;342:1203-8
12. VanDussen KL, Carulli AJ, Keeley TM, Patel SR, Puthoff BJ, Magness ST, *et al.* Notch signaling modulates proliferation and differentiation of

- intestinal crypt base columnar stem cells. *Development* **2012**;139:488-97
13. Tian H, Biehs B, Chiu C, Siebel CW, Wu Y, Costa M, *et al.* Opposing activities of Notch and Wnt signaling regulate intestinal stem cells and gut homeostasis. *Cell Rep* **2015**;11:33-42
14. Ueo T, Imayoshi I, Kobayashi T, Ohtsuka T, Seno H, Nakase H, *et al.* The role of Hes genes in intestinal development, homeostasis and tumor formation. *Development* **2012**;139:1071-82
15. Fordham RP, Yui S, Hannan NR, Soendergaard C, Madgwick A, Schweiger PJ, *et al.* Transplantation of expanded fetal intestinal progenitors contributes to colon regeneration after injury. *Cell Stem Cell* **2013**;13:734-44
16. Mustata RC, Vasile G, Fernandez-Vallone V, Strollo S, Lefort A, Libert F, *et al.* Identification of Lgr5-independent spheroid-generating progenitors of the mouse fetal intestinal epithelium. *Cell Rep* **2013**;5:421-32
17. Harada N, Tamai Y, Ishikawa T, Sauer B, Takaku K, Oshima M, *et al.* Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J* **1999**;18:5931-42
18. Imayoshi I, Shimogori T, Ohtsuka T, Kageyama R. Hes genes and neurogenin regulate non-neural versus neural fate specification in the dorsal telencephalic midline. *Development* **2008**;135:2531-41
19. Miyoshi H, Stappenbeck TS. In vitro expansion and genetic modification of gastrointestinal stem cells in spheroid culture. *Nature protocols* **2013**;8:2471-82
20. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **2009**;459:262-5
21. Peignon G, Durand A, Cacheux W, Ayrault O, Terris B, Laurent-Puig P, *et al.* Complex interplay between beta-catenin signalling and Notch effectors in intestinal tumorigenesis. *Gut* **2011**;60:166-76
22. Kayahara T, Sawada M, Takaishi S, Fukui H, Seno H, Fukuzawa H, *et al.* Candidate markers for stem and early progenitor cells, Musashi-1 and Hes1, are expressed in crypt base columnar cells of mouse small intestine. *FEBS Letters* **2003**;535:131-5

23. Gao F, Zhang Y, Wang S, Liu Y, Zheng L, Yang J, *et al.* Hes1 is involved in the self-renewal and tumourigenicity of stem-like cancer cells in colon cancer. *Sci Rep* **2014**;4:3963
24. Hatzis P, van der Flier LG, van Driel MA, Guryev V, Nielsen F, Denissov S, *et al.* Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. *Mol Cell Biol* **2008**;28:2732-44
25. Clevers H. The intestinal crypt, a prototype stem cell compartment. *Cell* **2013**;154:274-84
26. Tian H, Biehs B, Warming S, Leong KG, Rangell L, Klein OD, *et al.* A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. *Nature* **2011**;478:255-9
27. Schuijers J, van der Flier LG, van Es J, Clevers H. Robust cre-mediated recombination in small intestinal stem cells utilizing the *olm4* locus. *Stem Cell Reports* **2014**;3:234-41
28. Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M, *et al.* Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nat Med* **2007**;13:1203-10
29. Gao F, Huang W, Zhang Y, Tang S, Zheng L, Ma F, *et al.* Hes1 promotes cell proliferation and migration by activating Bmi-1 and PTEN/Akt/GSK3beta pathway in human colon cancer. *Oncotarget* **2015**;6:38667-80
30. Freeman DJ, Li AG, Wei G, Li HH, Kertesz N, Lesche R, *et al.* PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. *Cancer Cell* **2003**;3:117-30
31. Franke TF, Hornik CP, Segev L, Shostak GA, Sugimoto C. PI3K/Akt and apoptosis: size matters. *Oncogene* **2003**;22:8983-98
32. Korinek V, Barker N, Moerer P, van Donselaar E, Huls G, Peters PJ, *et al.* Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat Genet* **1998**;19:379-83
33. Rodilla V, Villanueva A, Obrador-Hevia A, Robert-Moreno A, Fernandez-Majada V, Grilli A, *et al.* Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer. *Proc Natl Acad*

- Sci U S A **2009**;106:6315-20
34. Fre S, Pallavi SK, Huyghe M, Lae M, Janssen KP, Robine S, *et al.* Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. *Proc Natl Acad Sci U S A* **2009**;106:6309-14
 35. el Marjou F, Janssen KP, Chang BH, Li M, Hindie V, Chan L, *et al.* Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genesis* **2004**;39:186-93
 36. Snippert HJ, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, *et al.* Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* **2010**;143:134-44
 37. Wendorff AA, Koch U, Wunderlich FT, Wirth S, Dubey C, Bruning JC, *et al.* Hes1 is a critical but context-dependent mediator of canonical Notch signaling in lymphocyte development and transformation. *Immunity* **2010**;33:671-84
 38. Schnell SA, Ambesi-Impiombato A, Sanchez-Martin M, Belver L, Xu L, Qin Y, *et al.* Therapeutic targeting of HES1 transcriptional programs in T-ALL. *Blood* **2015**;125:2806-14
 39. Arai MA, Ishikawa N, Tanaka M, Uemura K, Sugimitsu N, Suganami A, *et al.* Hes1 inhibitor isolated by target protein oriented natural products isolation (TPO-NAPI) of differentiation activators of neural stem cells. *Chem Sci* **2016**;7:1514-20
 40. Sail V, Hadden MK. Identification of small molecule Hes1 modulators as potential anticancer chemotherapeutics. *Chem Biol Drug Des* **2013**;81:334-42
 41. Kanasty R, Dorkin JR, Vegas A, Anderson D. Delivery materials for siRNA therapeutics. *Nat Mater* **2013**;12:967-77

Figure Legends

Figure 1.

Hes1 deletion in $Lgr5^+$ NSCs results in loss of self-renewal, but does not perturb intestinal homeostasis. (A) Macroscopic images and H&E staining of LacZ staining in the small intestine in $Lgr5^{CreERT2/+}; Hes1^{flox/flox}; Rosa26^{LacZ/+}$ mice at day 1, 3, 5, and 7 after tamoxifen induction. (B) Immunostaining of Hes1 in $Lgr5^{CreERT2/+}; Hes1^{+/+}; Rosa26^{LacZ/+}$ and $Lgr5^{CreERT2/+}; Hes1^{flox/flox}; Rosa26^{LacZ/+}$ mice at day 3 after intensive induction. (C) Quantification of Hes1⁺ cells per crypt at day 3 after intensive induction and $Lgr5$ -GFP⁺ cells and Hes1⁺ cells per crypt at day 14 after intensive induction. n = 20-30, each. * $P < 0.05$. (D) Macroscopic images of LacZ staining in the small intestine in $Lgr5^{CreERT2/+}; Hes1^{+/+}; Rosa26^{LacZ/+}$ and $Lgr5^{CreERT2/+}; Hes1^{flox/flox}; Rosa26^{LacZ/+}$ mice at day 14 after intensive induction. (E) H&E staining and immunostaining of $Lgr5$ -GFP and Hes1 in $Lgr5^{CreERT2/+}; Hes1^{+/+}; Rosa26^{LacZ/+}$ and $Lgr5^{CreERT2/+}; Hes1^{flox/flox}; Rosa26^{LacZ/+}$ mice at day 14 after intensive induction. Scale bars: 20 μ m (A,B,E).

Figure 2.

Spheroid formation was abrogated in Hes1-deleted *Lgr5*-GFP^{high} single cells. (A) Scheme of Hes1 deletion in *Lgr5*-GFP^{high} single cells. (B-C) The number of spheroids formed from 1000 *Lgr5*-GFP^{high} single cells, sorted from *Lgr5*^{CreERT2/+}; *Hes1*^{+/+} and *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox} mice, at day 6 (B); images were acquired at days 0 and 6 (C). n = 11-13, each. (D) Scheme of Hes1 deletion in *Lgr5*⁺ NSCs of established spheroids. (E) qRT-PCR analysis of *Lgr5* in spheroids cultured in 50% L-WRN CM, CHIR99021, and Valproic acid and in spheroids cultured in 50% L-WRN CM. n = 4, each. (F-G) Time course images (F) and diameter (G) of spheroids generated from *Lgr5*^{CreERT2/+}; *Hes1*^{+/+} and *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox} mice after induction. n = 20, each. Scale bars: 400 μm (C,F); 200 μm (black boxes in panel F).

Figure 3.

Hes1 deletion in *Bmi1*⁺ NSCs results in loss of self-renewal, but does not perturb intestinal homeostasis. (A) Macroscopic images and H&E staining of LacZ

staining of the small intestine in *Bmi1*^{CreERT/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice at day 1, 3, 5, and 7 after tamoxifen induction. (B) Immunostaining of Hes1 in *Bmi1*^{CreERT/+}; *Hes1*^{flox/+}; *Rosa26*^{LacZ/+} and *Bmi1*^{CreERT2/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice 3 days after intensive induction. (C) Quantification of Hes1⁺ cells per crypt at day 3 and day 10 after intensive induction. n = 20, each. **P* < 0.05. (D) Macroscopic images of LacZ staining in the small intestine in *Bmi1*^{CreERT/+}; *Hes1*^{flox/+}; *Rosa26*^{LacZ/+} and *Bmi1*^{CreERT/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice 10 days after intensive induction. (E) H&E staining and immunostaining of Hes1 in *Bmi1*^{CreERT/+}; *Hes1*^{flox/+}; *Rosa26*^{LacZ/+} and *Bmi1*^{CreERT/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice 10 days after intensive induction. Scale bars: 20 μm (A,B,E).

Figure 4.

Concomitant Hes1 deletion in β-catenin-stabilized NSCs abrogates tumor formation. (A) Macroscopic images of the duodenum, H&E staining, and immunostaining of β-catenin and Hes1 in *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{+/+} and *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{flox/flox} mice at day 28 after tamoxifen

induction. Arrowheads indicate microadenoma. (B) The log-rank test revealed a significantly longer survival of *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{flox/flox} mice compared with *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{+/+} control mice after tamoxifen induction ($P < 0.0001$). MST: median survival time. $n = 15$, each. (C) Time course of change in size and number of intestinal tumors of *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{flox/flox} mice after tamoxifen induction. (D) Immunostaining of Hes1 in macroadenoma, microadenoma, and normal mucosa of *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{flox/flox} mice at week 15 after tamoxifen induction. (E-F) Macroscopic images (E) and H&E staining (F) of LacZ staining in the small intestine in *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{+/+}; *Rosa26*^{LacZ/+} and *Lgr5*^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+}; *Hes1*^{flox/flox}; *Rosa26*^{LacZ/+} mice at day 28 after tamoxifen induction. Scale bars: 50 μ m (A,D,F).

Figure 5.

Hes1 deletion in *Lgr5*⁺ TSCs of established intestinal tumors results in tumor regression by inducing immediate apoptosis. (A) Macroscopic image of LacZ

staining in the small intestine in *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+}; *Rosa26*^{LacZ/+} mice at day 5 after tamoxifen induction revealed injured intestinal tumors devoid of blue cells (Red circles). H&E staining at day 5 after intensive induction revealed severely injured intestinal tumor (black circle), and macroscopic image of LacZ staining at day 10 after intensive induction revealed tumor regression.

(B) H&E staining of *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+}; *Rosa26*^{LacZ/+} mice at day 1 and day 3 after tamoxifen induction. Immunofluorescent staining of cleaved caspase-3 and *Lgr5*-GFP in *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice at day 1 after tamoxifen induction. (C) Proportion of cleaved caspase-3⁺ cells in *Lgr5*-GFP⁺ cells in the normal intestine and intestinal tumors of *Lgr5*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+} and *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice at day 1 after tamoxifen induction. **P* < 0.05. (D) qRT-PCR of *Hes1*, *Bcl2*, *Bax*, and *Pten* in sorted *Lgr5*⁺ cells from intestinal tumors of *Lgr5*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+} and *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice at day 1 after tamoxifen induction. n = 3, each. **P* < 0.05. (E) Macroscopic images and the number of intestinal tumors in *Lgr5*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+} and *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice at day

5 after intensive tamoxifen induction. $n = 7-11$, each. $*P < 0.05$. (F)

Immunostaining of *Lgr5*-GFP in *Lgr5*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+} and *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice at day 5 after intensive induction. Proportion of *Lgr5*-GFP⁺ cells in normal intestinal epithelia and intestinal tumors in *Lgr5*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+} and *Lgr5*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+} mice at day 5 after intensive induction. $*P < 0.05$. Scale bars: 100 μm (A); 20 μm (B); 200 μm (F).

Figure 6.

Hes1 deletion in *Dclk1*⁺ TSCs of established intestinal tumors results in tumor regression by inducing immediate apoptosis. (A) Macroscopic images and H&E staining of LacZ staining in the small intestine in *Dclk1*^{CreERT2/+}; *Hes1*^{+/+}; *Apc*^{Min/+}; *Rosa26*^{LacZ/+} and *Dclk1*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+}; *Rosa26*^{LacZ/+} mice at day 5 after tamoxifen induction. Red circles indicate intestinal tumors. Black circle indicates the injured tumor. (B) H&E staining of LacZ staining in *Dclk1*^{CreERT2/+}; *Hes1*^{flox/flox}; *Apc*^{Min/+}; *Rosa26*^{LacZ/+} mice at day 1 and day 3 after tamoxifen

induction. (C) Immunofluorescent staining of cleaved caspase-3 and Dclk1 in *Dclk1^{CreERT2/+}; Hes1^{flox/flox}; Apc^{Min/+}* mice at day 1 after tamoxifen induction. (D) Macroscopic images and the number of intestinal tumors of *Dclk1^{CreERT2/+}; Hes1^{+/+}; Apc^{Min/+}* and *Dclk1^{CreERT2/+}; Hes1^{flox/flox}; Apc^{Min/+}* mice at day 5 after intensive tamoxifen induction. n= 7-8, each. **P* < 0.05. Scale bars: 50 μ m (A-C).

Figure 7.

Hes1 can be a tumor-specific therapeutic target. (A) Scheme of Hes1 deletion in NSCs. Hes1-deleted NSCs cannot self-renew, but Hes1⁺ NSCs are newly generated; mucosa remains intact. (B) Scheme of Hes1 deletion in TSCs. Hes1-deleted TSCs undergo immediate apoptosis, which leads to tumor regression.

Figure 1

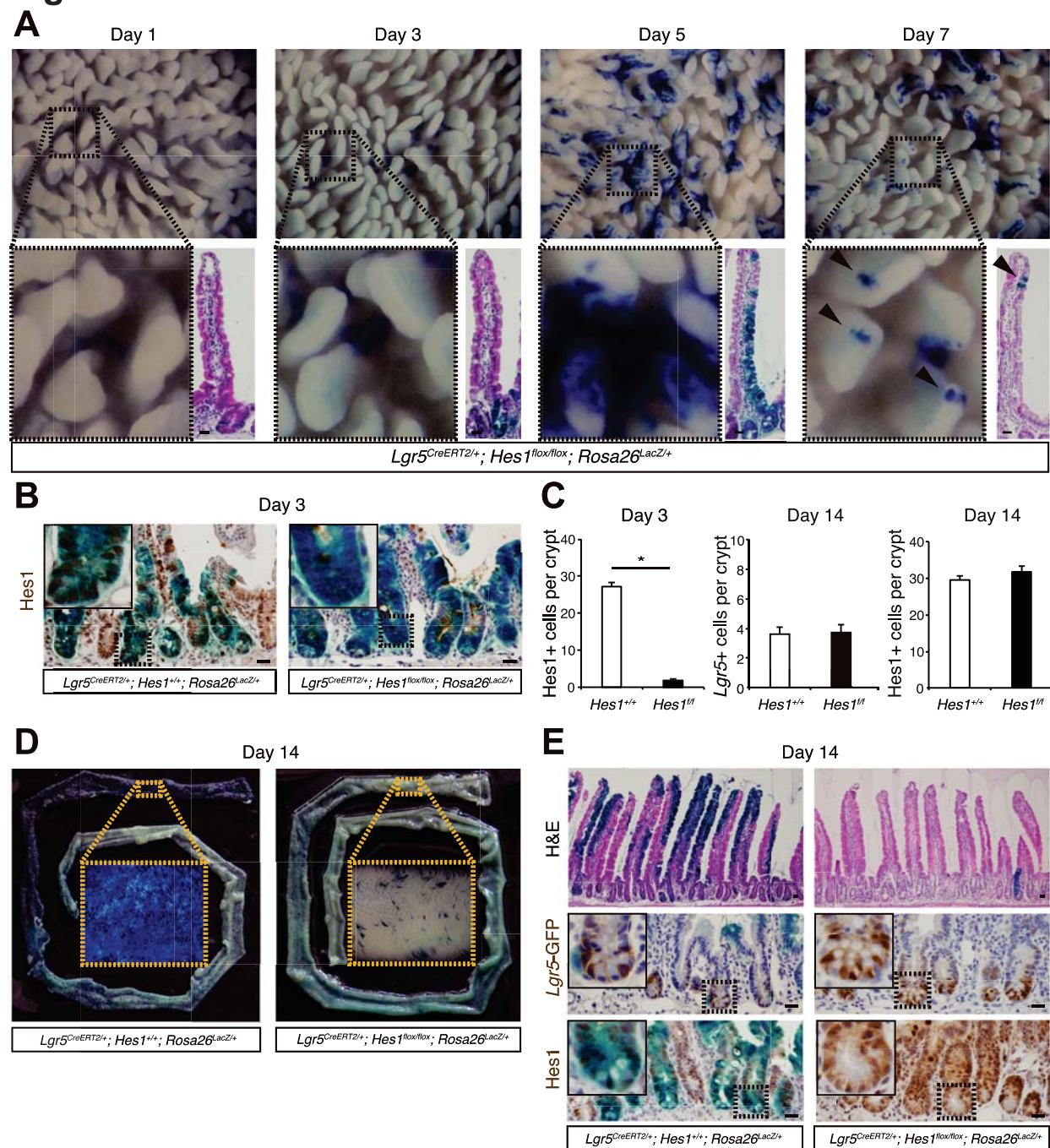


Figure 2

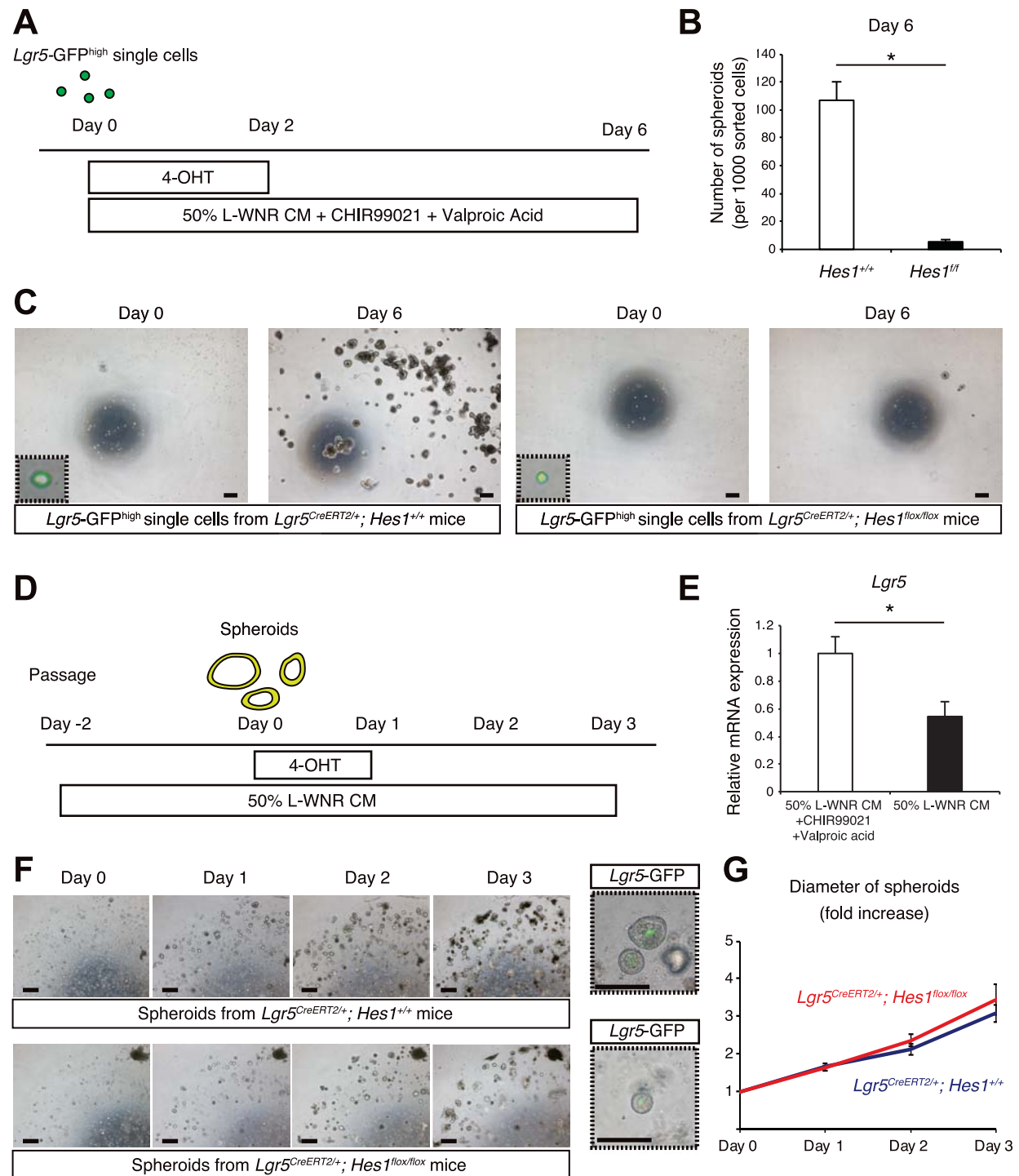


Figure 3

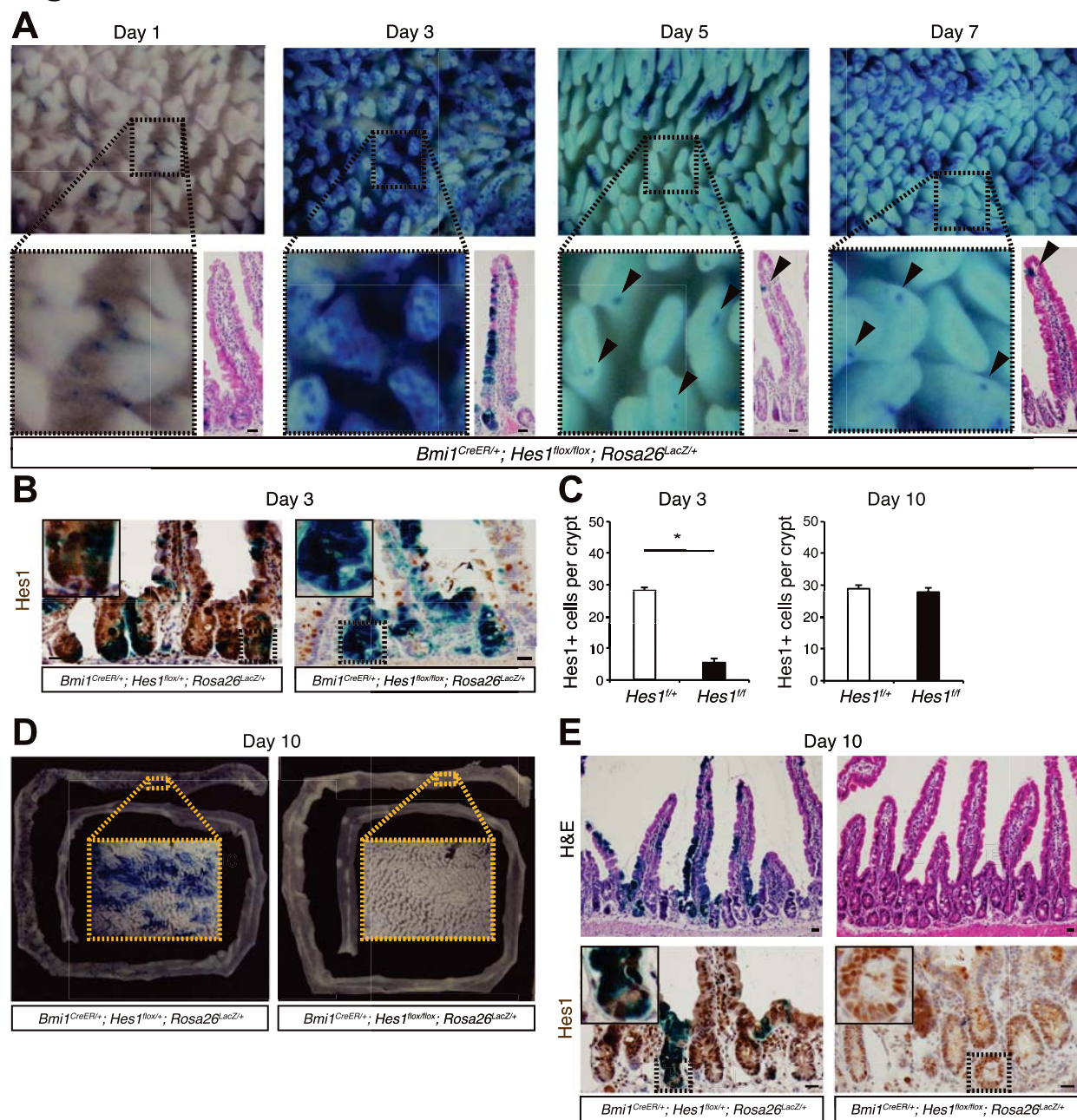


Figure 4

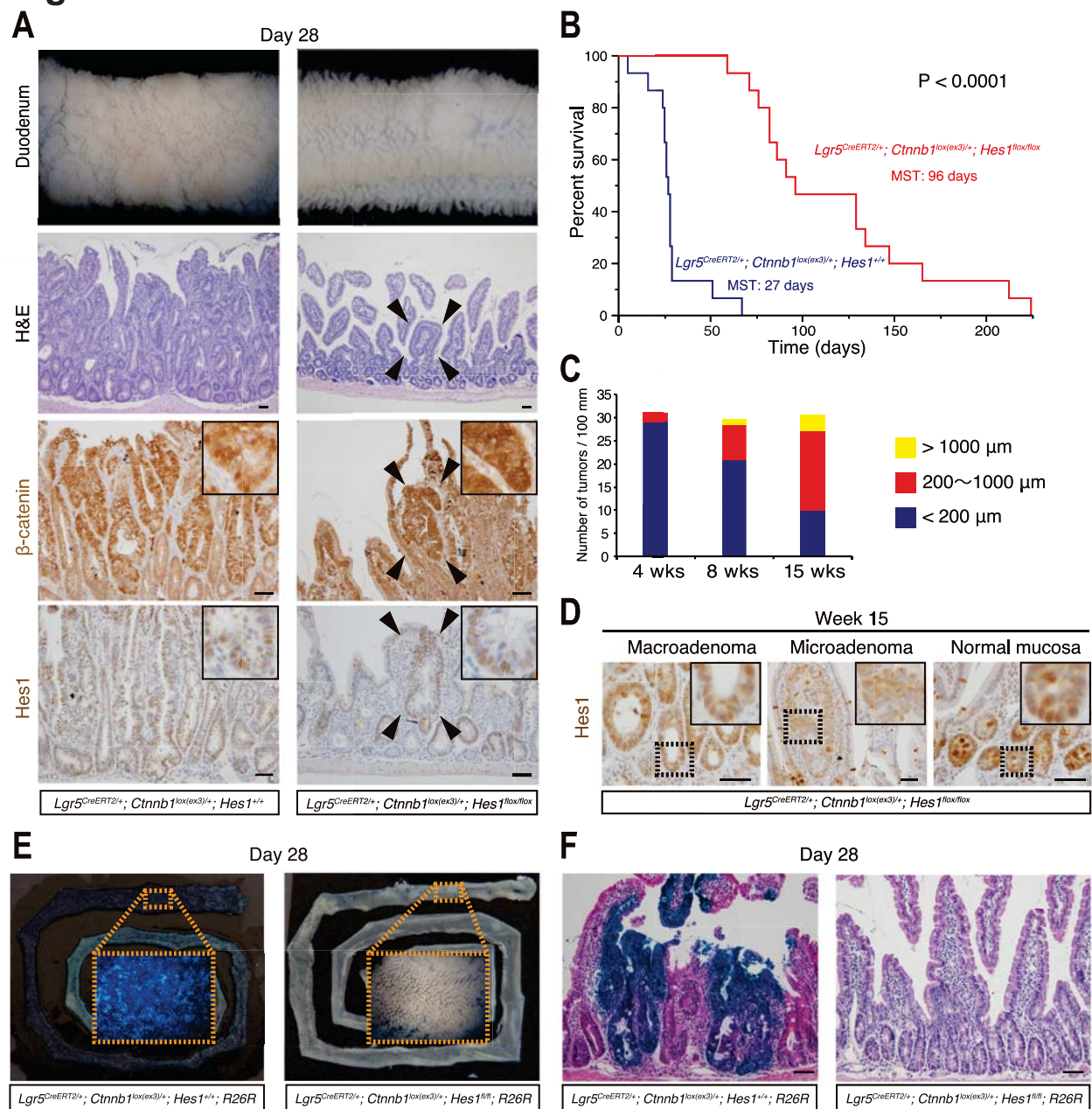


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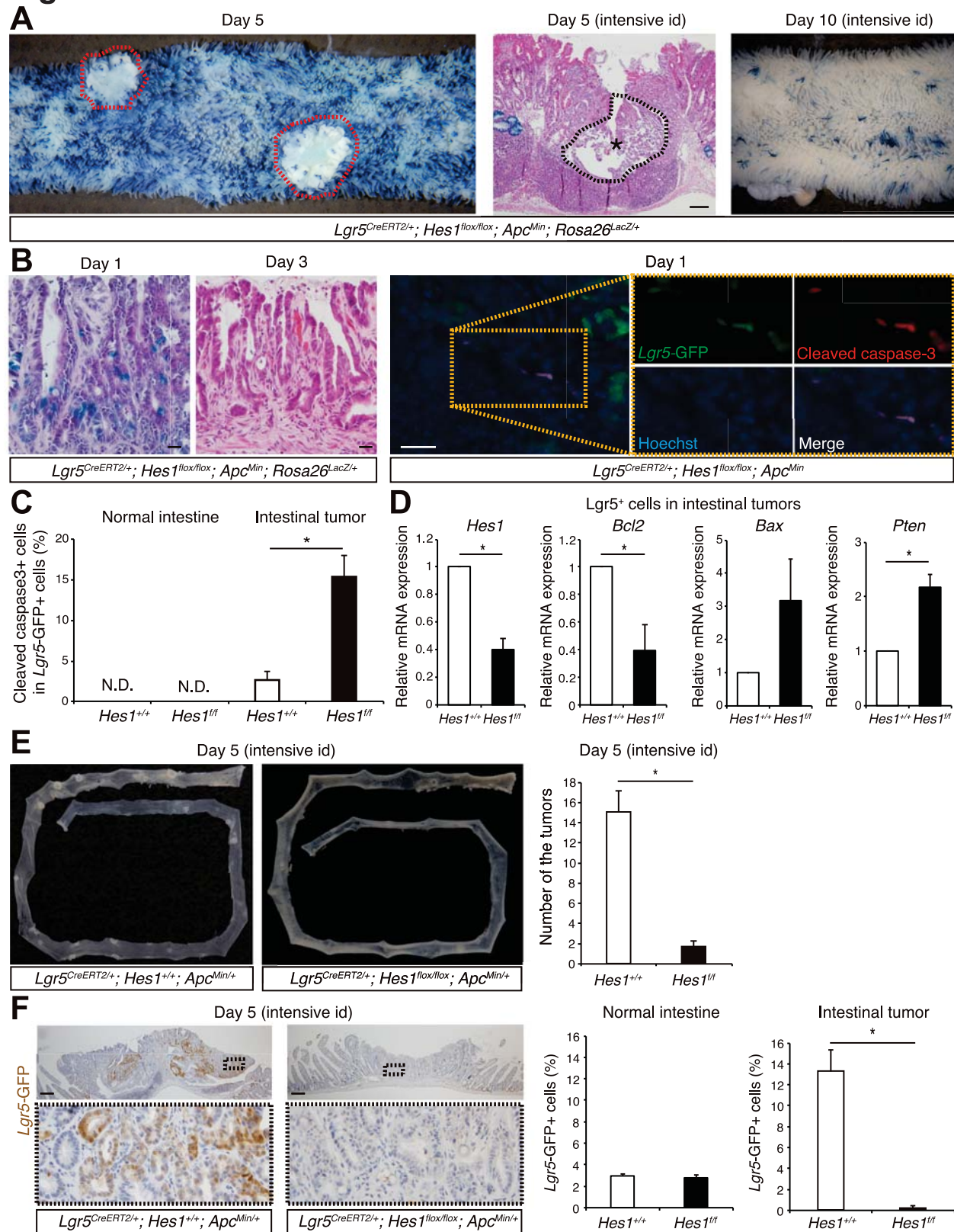


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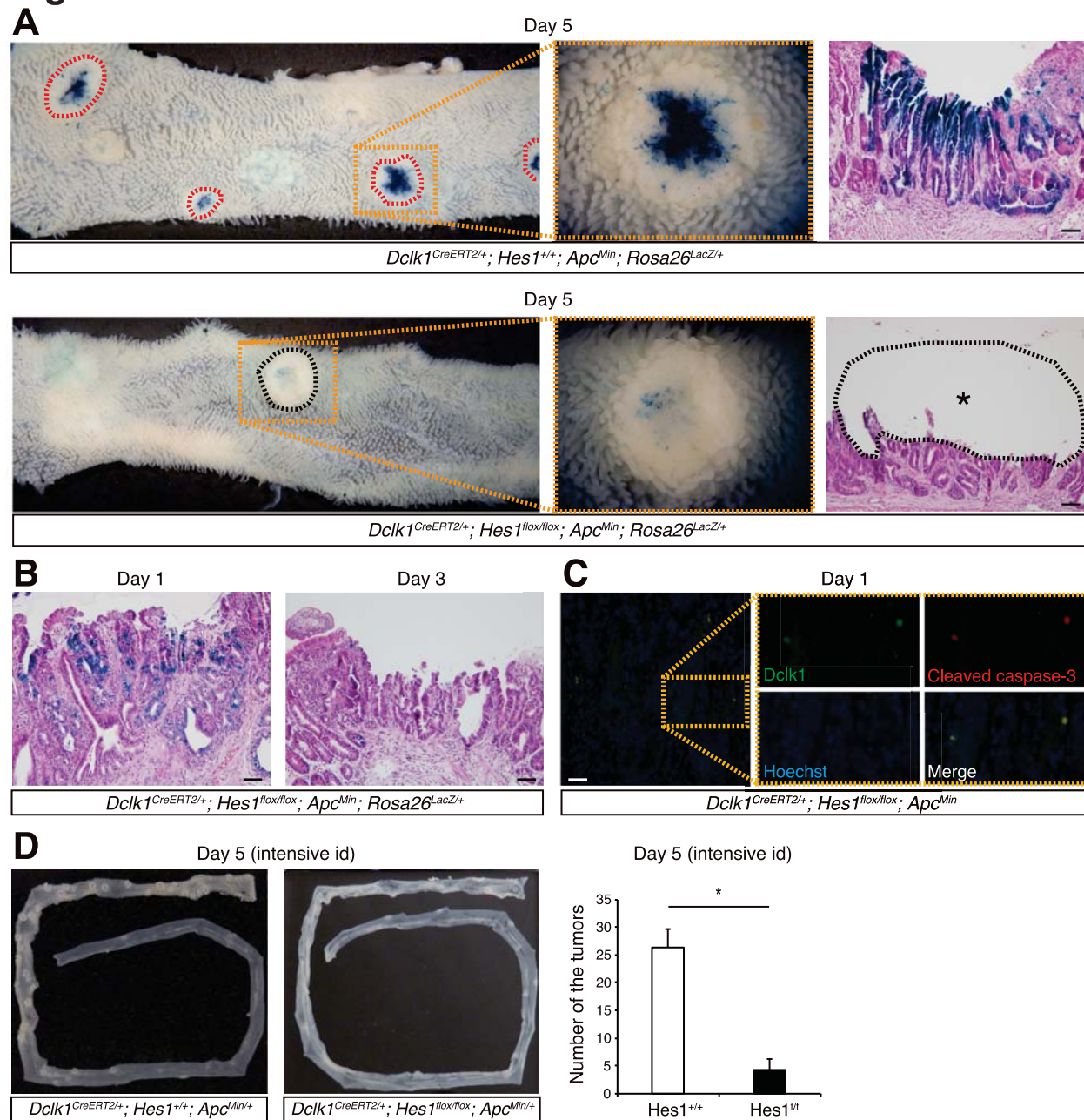
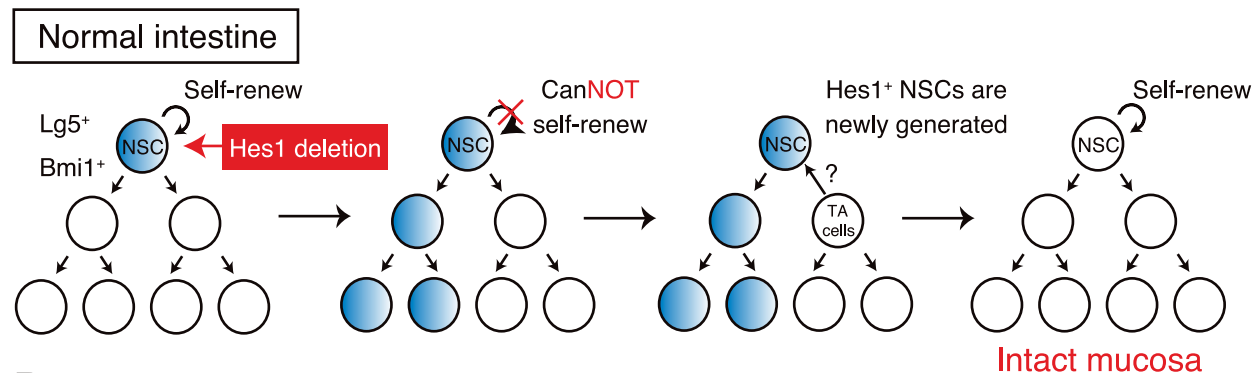
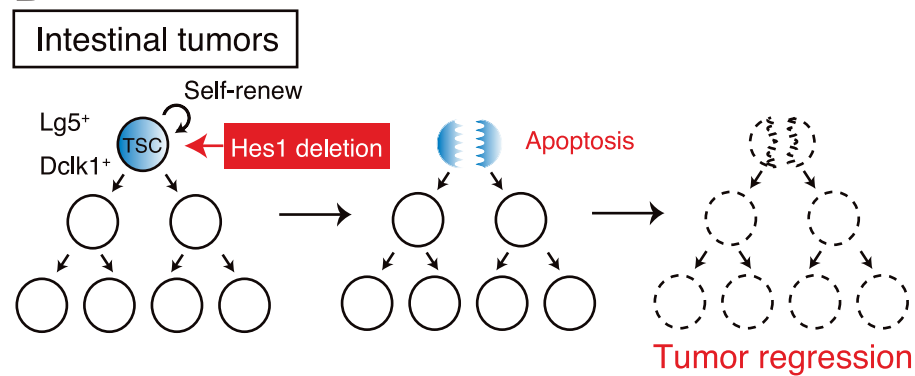


Figure 7

A



B



Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Distinct roles of Hes1 in normal stem cells and tumor stem-like cells of the intestine

Norihiro Goto, Taro Ueo, Akihisa Fukuda, et al.

Cancer Res Published OnlineFirst May 23, 2017.

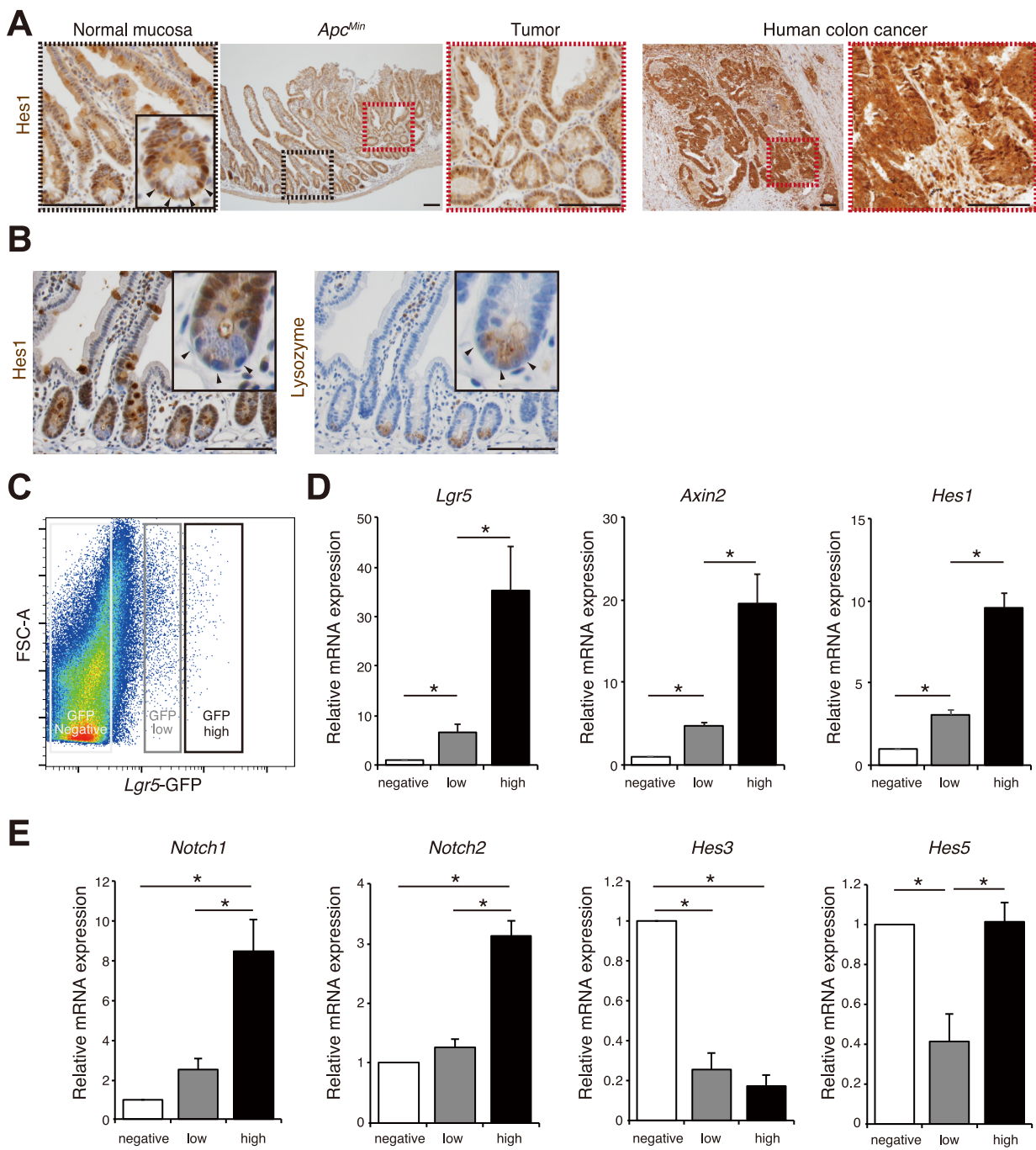
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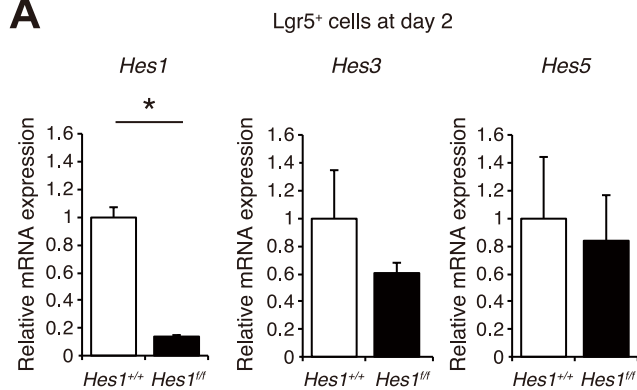
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Supplementary Figure 1

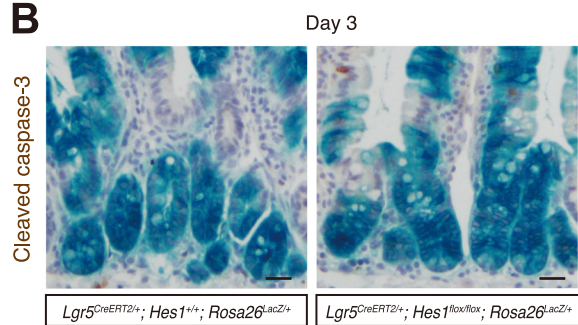


Supplementary Figure 2

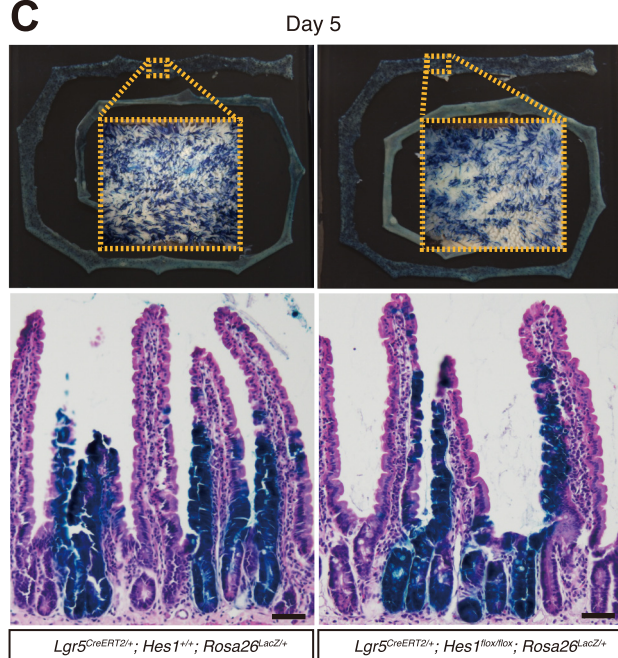
A



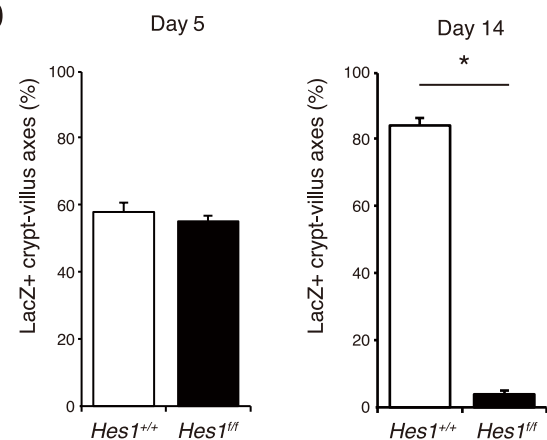
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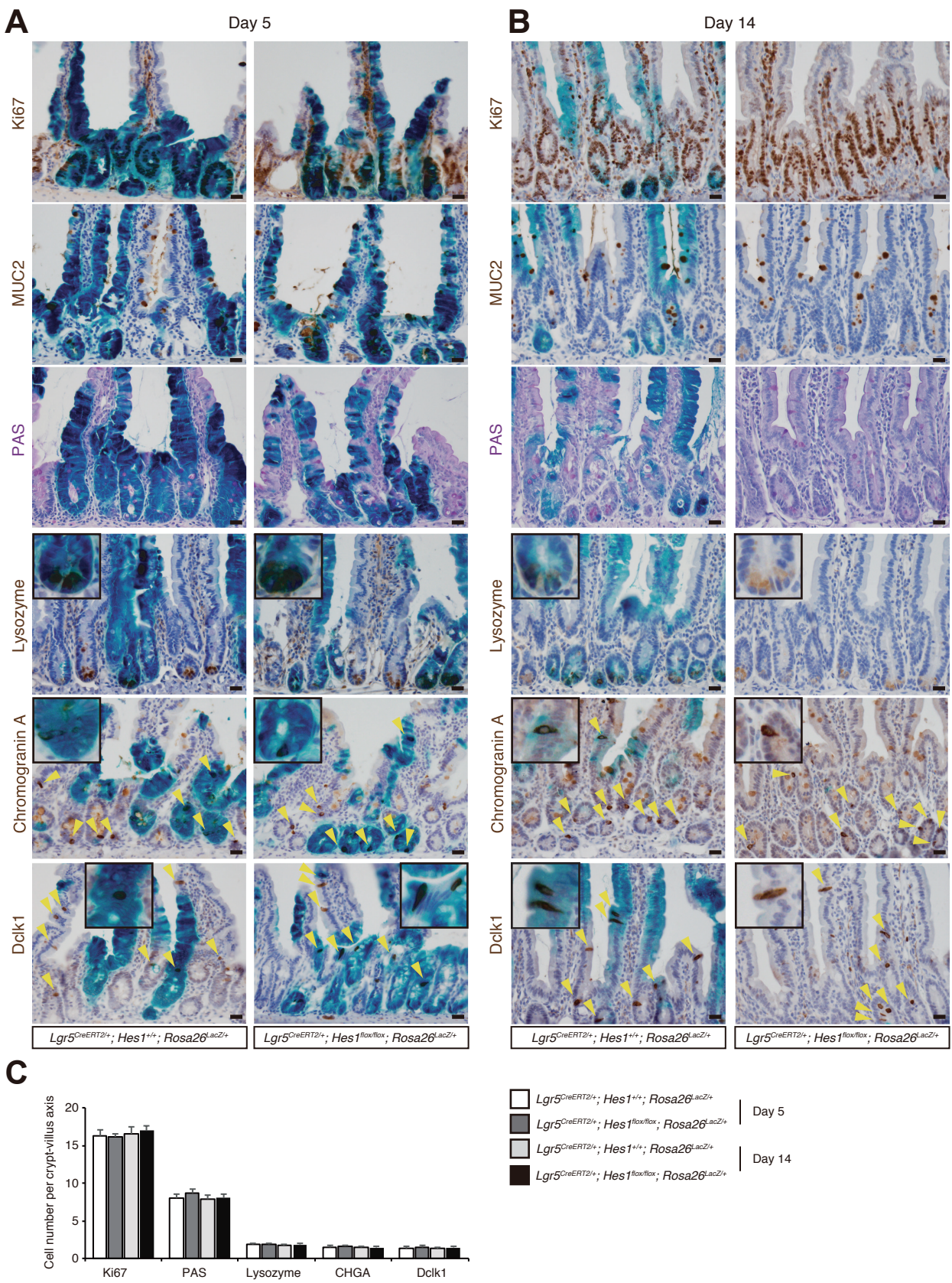
C



D

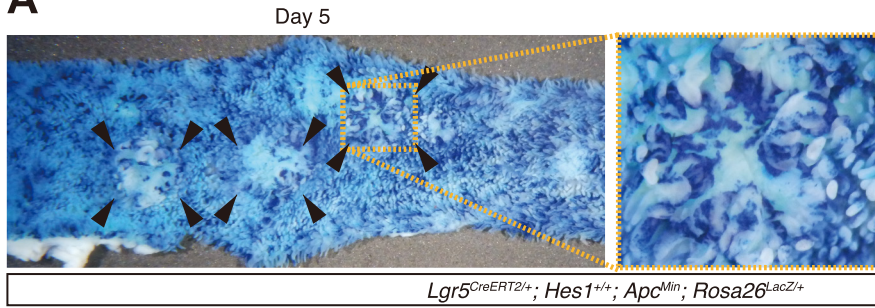


Supplementary Figure 3

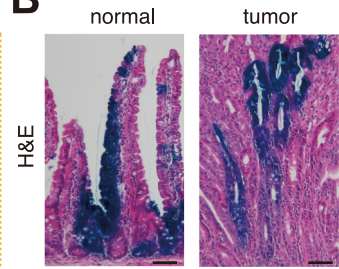


Supplementary Figure 4

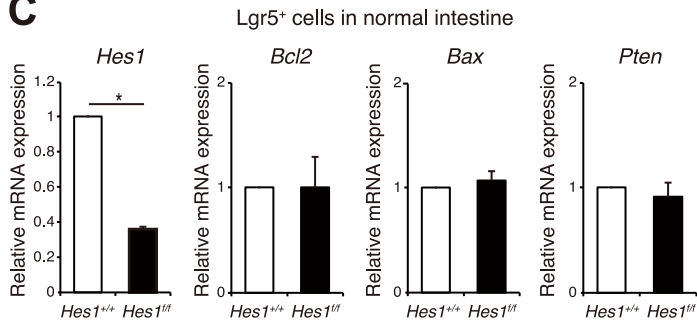
A



B

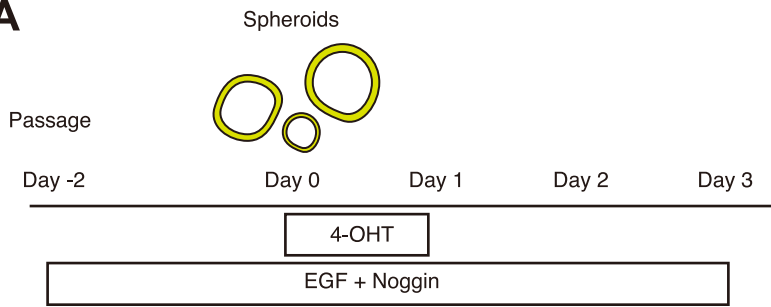


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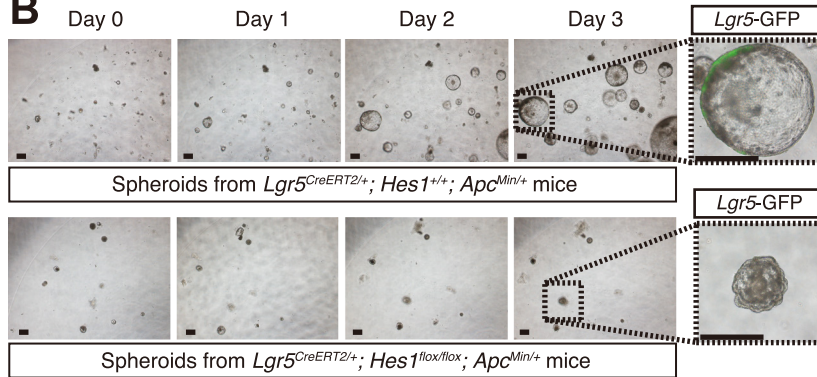


Supplementary Figure 5

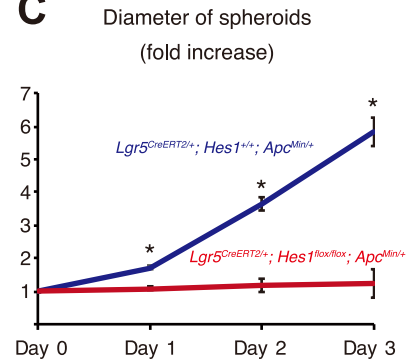
A



B



C



Supplementary Table 1: Antibodies and qRT–PCR primers used in this study

Immunohistochemystry Antibodies

Target	Host	Manufacturer	Catalog number	Dilution
Hes1	rabbit	gift from Dr. Sudo (41)		1:1000
β-catenin	mouse	BD Biosciences, San Jose, CA	610153	1:100
GFP	goat	Abcam , Cambridge, MA	ab6673	1:500
Cleaved caspase-3	rabbit	Cell Signaling Technology, Danvers, MA	9664	1:200
Ki67	mouse	BD biosciences, San Jose, CA	550609	1:500
MUC2	rabbit	Santa Cruz Biotechnology, Santa Cruz, CA	sc-15334	1:100
Lysozyme	rabbit	Abcam , Cambridge, MA	ab108508	1:200
Chromogranin A	rabbit	Abcam , Cambridge, MA	ab15160	1:200
Dclk1	rabbit	Abcam , Cambridge, MA	ab31704	1:200
Dclk1	goat	Santa Cruz Biotechnology, Santa Cruz, CA	sc-46312	1:200

qRT-PCR Primers

Target	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')
<i>Gapdh</i>	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
<i>Hes1</i>	ATAGCTCCCGGCATTCCAAG	GCGCGGTATTTCCCCAACA
<i>Hes3</i>	GCACGCATCAACGTGTCAC	TGAGTTCTGGAGGCTTCTCAT
<i>Hes5</i>	AGTCCCAAGGAGAAAAACCGA	GCTGTGTTTCAGGTAGCTGAC
<i>Notch1</i>	GATGGCCTCAATGGGTACAAG	TCGTTGTTGTTGATGTCACAGT
<i>Notch2</i>	ATGTGGACGAGTGTCTGTTGC	GGAAGCATAGGCACAGTCATC
<i>Lgr5</i>	TCCTAGAAGAGTTACGTCTTGCT	CCTTGGGAATGTGTGTCAAAGC
<i>Axin2</i>	TGACTCTCCTTCCAGATCCCA	TGCCCACACTAGGCTGACA
<i>Bcl2</i>	GGGAGAACAGGGTATGATAACCG	TAGCCCCTCTGTGACAGCTTA
<i>Bax</i>	TGAAGACAGGGGCCTTTTTG	AATTCGCCGGAGACACTCG
<i>Pten</i>	TGCACAGTATCCTTTTGAAGACC	GAATTGCTGCAACATGATTGTCA