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Tumor and Stem Cell Biology

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KLF5 Regulates the Integrity and Oncogenicity of Intestinal Stem Cells

Takeo Nakaya[1,2,3], Seishi Ogawa[2], Ichiro Manabe[4], Masami Tanaka[1,6], Masashi Sanada[2], Toshiro Sato[7], Makoto M. Taketo[8], Kazuki Nakao[5,9], Hans Clevers[11], Masashi Fukayama[3], Masahiko Kuroda[1], and Ryozo Nagai[4,10]

Abstract

The intestinal epithelium maintains homeostasis by a self-renewal process involving resident stem cells, including Lgr5$^+$ crypt-base columnar cells, but core mechanisms and their contributions to intestinal cancer are not fully defined. In this study, we examined a hypothesized role for KLF5, a zinc-finger transcription factor that is critical to maintain the integrity of embryonic and induced pluripotent stem cells, in intestinal stem-cell integrity and cancer in the mouse. Klf5 was indispensable for the integrity and oncogenic transformation of intestinal stem cells. In mice, inducible deletion of Klf5 in Lgr5$^+$ stem cells suppressed their proliferation and survival in a manner associated with nuclear localization of β-catenin (Catnb), generating abnormal apoptotic cells in intestinal crypts. Moreover, production of lethal adenomas and carcinomas by specific expression of an oncogenic mutant of β-catenin in Lgr5$^+$ stem cells was suppressed completely by Klf5 deletion in the same cells. Given that activation of the Wnt/β-catenin pathway is the most frequently altered pathway in human colorectal cancer, our results argue that KLF5 acts as a fundamental core regulator of intestinal oncogenesis at the stem-cell level, and they suggest KLF5 targeting as a rational strategy to eradicate stem-like cells in colorectal cancer. *Cancer Res; 74(10); 2882–91. ©2014 AACR.*

Introduction

The intestinal epithelium is maintained by a self-renewal in the crypts. In this process, the resident stem cells located in the crypt base produce proliferating progenitors, transit-amplifying cells, which move upward as coherent columns (1, 2). Intestinal epithelia include short-lived progenitors, long-lived progenitors, columnar cell progenitors, and pluripotential stem cells (3). The stem-cell pools include Lgr5$^+$ crypt-base columnar (CBC) cells and Lrig1$^+$ cells at the bases of crypts, and Hopx$^+$ or Bmi1$^+$ cells located at the +4 position from the bottom of the crypts (1, 4–8). Lgr5$^+$ cells constitute intestinal stem cells under normal condition (4, 9). When Lgr5$^+$ cells are specifically deleted in small intestine, Bmi-1$^+$ cells compensate for Lgr5$^+$ stem cells and crypts can be maintained (10). These "+4 cells" may be the quiescent Paneth cell precursors that can revert back into Lgr5$^+$ stem cells following crypt damage (11). These indicate plasticity of intestinal stem cells. Intestinal stem cells also show differential stem-cell activation with damage (12–14). The stem cells are maintained by the descendants of dominantly surviving stem cells in a crypt, under "neutral competition" between equipotent stem cells. This maintenance pattern is repeated to sustain the stem cells. The stem-cell survival is supported by stem-cell niche, including Paneth cells (2, 15).

Intestinal stem cells are also the focus on attention in the context of oncogenesis. Inducible deletion of the tumor-suppressor gene *adenomatous polyposis coli* (*Apc*) in the Lgr5$^+$ CBC stem cells of the mice gives rise to more tumors than deletion of *Apc* in more differentiated, nonstem cells (16). It has also been shown that in mice Lgr5$^+$ CBC cells constitute a subpopulation of adenoma cells that fuel the growth of established adenomas and function as cancer stem cells (CSC; ref. 17). We have recently established cancer cell lines from the samples of human colorectal cancers that express Lgr5 and possess CSC properties (18). These Lgr5$^+$ colonic CSCs, interconvert with drug-resistant Lgr5$^-$ cells after drug treatment and are capable of tumor reconstitution.

Thus, these observations lend support to the notion that oncogenic changes at the stem-cell stage are more critical than those in the differentiated nonstem stages during oncogenesis (19). A comprehensive genome-sequencing analysis has elucidated several altered genes and pathways in colorectal

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cancer, including the Wnt/β-catenin (Catnb), Ras–MAPK, PI3K, TGF-β, p53, and DNA mismatch-repair pathways (20). However, despite much attention to intestinal stem cells in the context of homeostasis and oncogenesis in the intestine, the mechanisms underlying the genetic pathways of cellular differentiation as well as those responsible for colorectal cancer development remain poorly understood.

In this study, we focused on the transcription factor, Krüppel-like factor 5 (KLF5), a zinc-finger transcription factor of the KLF family, which carries versatile functions in the homeostasis and pathogenesis of multiple systems, including the cardiovascular, metabolic, and renal (21). Intriguingly, KLF5 controls the stemness of embryonic stem (ES) cells (22) and has the potential to generate induced pluripotent stem (iPS) cells, *albeit* more weakly than KLF4 (23). Moreover, KLF2, KLF4, and KLF5 cooperatively constitute transcriptional autofeedback loops along with other iPS cell–inducing factors to sustain the stemness in ES cells (24). In contrast, the functions of KLF5 in adult somatic stem-cell regulations have remained to be elucidated. In the normal intestines, KLF5 is expressed in a polarized pattern: at higher levels in crypts in which stem cells reside, and lower levels in villi in which differentiated cells reside (21, 25). In contrast, KLF4 is expressed in a pattern complementary to that of KLF5 (26). Of further note, $Klf5^{+/-}$ mice have shorter and less mature villi than $Klf5^{+/+}$ mice (27), and noninducible and congenital deletion of *KLF5* in all intestinal epithelia through all developmental stages (*Villin Cre* $Klf5^{flox/flox}$ mice) resulted in the severe morphologic changes of intestine (28). On the basis of these observations, we hypothesized that KLF5, rather than KLF4, governed homeostasis and oncogenesis in stem and transit-amplifying cells of the intestinal epithelia. The functions of KLF5 in adult somatic stem-cell regulations, including oncogenesis, have remained to be clarified. The previous congenital deletions of *Klf5* showed the functions of KLF5 in the intestinal development, but did not demonstrate those in the homeostatic turnover of the fully developed intestinal epithelia from the stem cells. As such, we constructed a stem-cell–specific and tamoxifen-inducible deletion system for *Klf5* in mice. Furthermore, we used a novel system in which oncogenic activation of Wnt/Catnb signaling and deletion of *Klf5* could be induced simultaneously specifically in the intestinal stem cells.

Materials and Methods

Additional Materials and Methods are available as Supplementary Materials and Methods.

Mice

The mouse transgenics used were as follows: *Lgr5–EGFP–ires–CreERT2* (4), $Klf5^{flox/flox}$ (29), and $Catnb^{lox(\Delta ex3)}$ (30). All strains were backcrossed with C57BL/J background mice. Genotyping was performed by PCR, using genomic DNA purified from the tail, as described previously (4, 29, 30).

Induction of activated Cre recombinase

To induce the activation of Cre recombinase and genomic recombination at flox sites, mice were given daily intraperitoneal injections of 200 μL of tamoxifen (Sigma-Aldrich; 10 mg/mL) dissolved in sunflower oil, for 3 to 10 days.

Tissue isolation and histopathology

Small intestines were removed immediately from sacrificed mice, opened, washed with 1× PBS, fixed overnight in 10% neutral-buffered formalin, and embedded in

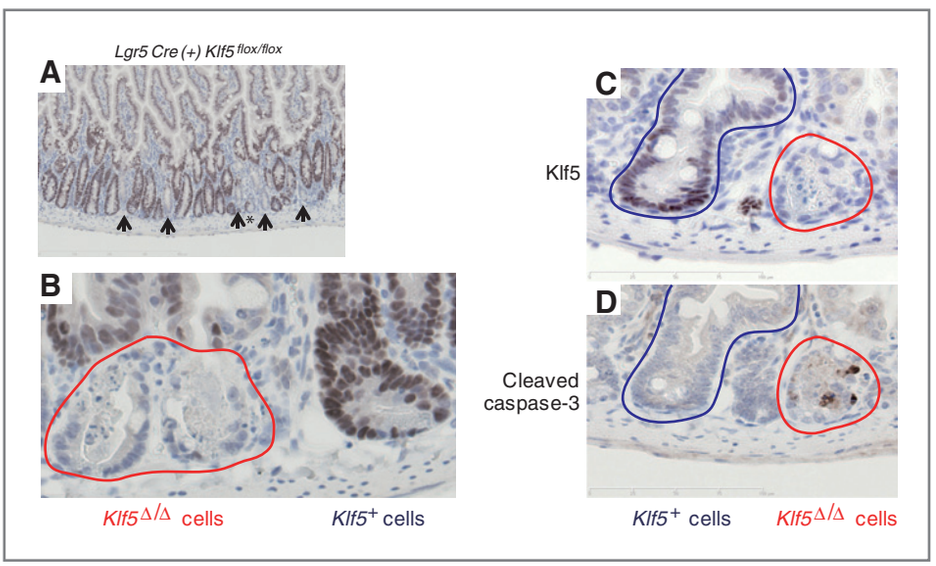

Figure 1. An inducible deletion of *Klf5* in $Lgr5^+$ stem cells generated abnormal apoptotic cells in the crypts. A, IHC of Klf5 in *Lgr5–EGFP–ires–CreERT2* $Klf5^{flox/flox}$ mouse crypts 7 days after Cre induction (arrows, $Klf5^{\Delta/\Delta}$ crypts). Some crypts included Klf5-deleted epithelium (arrows), and the crypt that was chimeric in Klf5 expression (*) was included. B, inducible deletion of *Klf5* in $Lgr5^+$ stem cells produced some dead or abnormal $Klf5^{\Delta/\Delta}$ crypt cells (inside red line) in *Lgr5–EGFP–ires–CreERT2* $Klf5^{flox/flox}$ mouse, whereas there were no such cells in Klf5-positive epithelia (7 days after Cre induction). C and D, IHC of Klf5 (C) and cleaved caspase-3 (D) in the serial sections of abnormal $Klf5^{\Delta/\Delta}$ crypt cells (inside red outline) and *Klf5*-positive ($Klf5^+$) cells in the same mouse (inside blue outline).

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paraffin. Immunohistochemical/immunofluorescence staining (detailed methods are described in Supplementary Materials and Methods) and hematoxylin–eosin (H&E) staining were performed on the paraffin slides of the mice intestines or the surgical specimens of human colorectal cancers, fixed in neutral-buffered formalin.

Isolation of intestinal stem cells and transit-amplifying cells from Lgr5–EGFP–ires–CreERT2 mice

Intestinal crypts were isolated from the duodenum and jejunum (3–4 cm in length from the pylorus) from *Lgr5–EGFP–ires–CreERT2* mice. The GFP^{++} stem and GFP^{+} transit-amplifying cells were sorted from the trypsinized epithelial cells by FACSAria (Becton Dickinson). The total RNAs from these cells were extracted and subjected to the microarrays (Agilent) or cDNA preparations for real-time PCR (RT-PCR) analysis. The detailed methods are described in Supplementary Materials and Methods.

Results

KLF5 in homeostatic maintenance and proliferation of intestinal epithelial stem cells

We first examined the function of Klf5 in normal homeostasis and turnover of stem-cell–derived intestinal epithelia in mice with a stem-cell–specific deletion of *Klf5* that were generated by crossing *Lgr5–EGFP–ires–CreERT2* and $Klf5^{flox/flox}$ mice (4, 29). Because Cre–ERT2 fusion gene activity is inducible following tamoxifen administration, this experimental system has the advantage of being able to analyze the specific function of Klf5 in the intestinal epithelial stem cells, thereby avoiding artifacts that might arise from deficiency of *Klf5* throughout development (4, 28).

As shown in Fig. 1, intraperitoneal injection of tamoxifen successfully deleted both *Klf5* alleles in $Lgr5^{+}$ stem cells in a subset of crypts, yielding $Klf5^{\Delta/\Delta}$ stem cells that produced $Klf5^{\Delta/\Delta}$ epithelia (Fig. 1A). The efficiency of *Klf5* deletion was highest in the duodenum and oral jejunum, which might reflect the expression pattern of Cre recombinase in *Lgr5–EGFP–ires–CreERT2/*$Klf5^{flox/flox}$ mice (4). Some crypts were chimeric in Klf5 expression (Fig. 1A).

Interestingly, we found the inducible deletion of *Klf5* in $Lgr5^{+}$ stem cells resulted in the appearance of some dead or abnormal-looking $Klf5^{\Delta/\Delta}$ cells, whereas no such cells were observed in Klf5-positive crypt epithelia in the same mice (Fig. 1B). Consistent with this, some abnormal $Klf5^{\Delta/\Delta}$ crypt epithelia were found to be positive for cleaved caspase-3, a hallmark of apoptosis, whereas no such cells were present among $Klf5^{+/+}$ crypt cells (Fig. 1C and D). Nonetheless, these apoptotic cells did not predominate among the $Klf5^{\Delta/\Delta}$ cells in the crypts, and most of the Klf5-deficient cells seemed histologically normal (Fig. 1A).

Next, we compared the ratios of the crypts including $Klf5^{\Delta/\Delta}$ cells among the total crypts in the duodenums/jejunums approximately 3 cm from the pylori of mice. Deletions of *Klf5* in *Lgr5–EGFP–ires–CreERT2/*$Klf5^{flox/flox}$ mice were restricted in

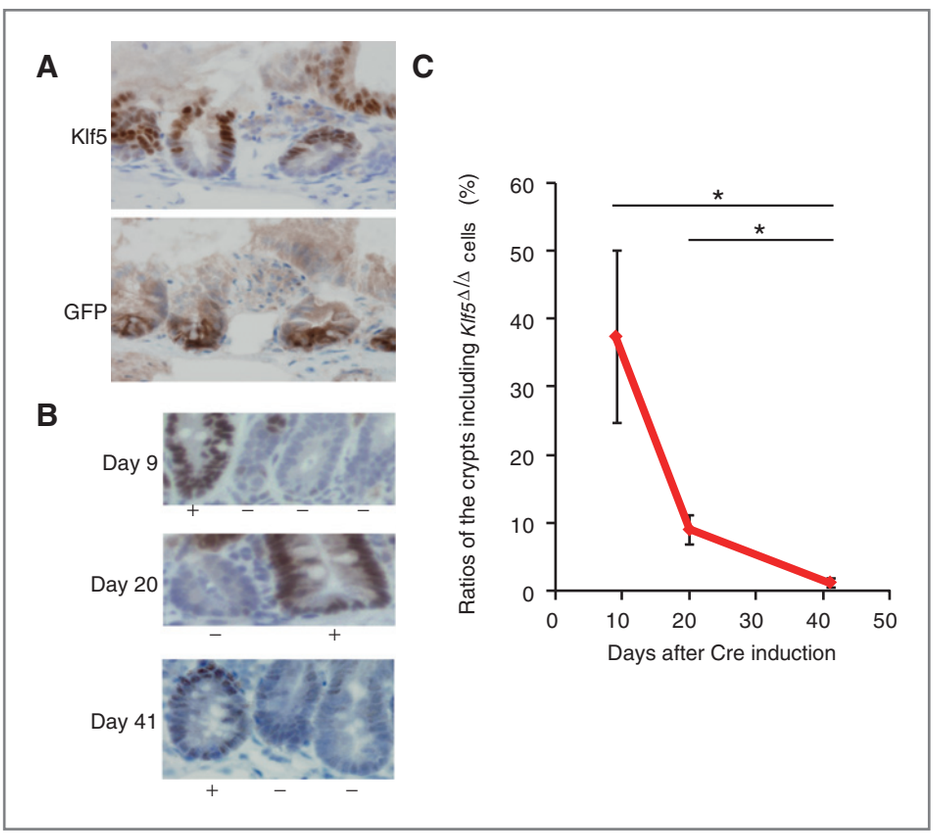

Figure 2. *Klf5*-deleted crypt epithelia at $Lgr5^{+}$ stem-cell levels had disadvantages for the survival under the neutral competitions of the stem cells. A, representative IHC of GFP and Klf5 in the mutually consecutive mirror sections of *Lgr5–EGFP–ires–CreERT2* $Klf5^{flox/flox}$ mice crypts 1 day after Cre induction. B, representative IHC of Klf5-positive crypts without $Klf5^{\Delta/\Delta}$ cells (+) and the crypts, including $Klf5^{\Delta/\Delta}$ epithelia (–), 9, 20, and 41 days after Cre induction (performed at day 0, 1, 2, and 3) in the same aged *Lgr5--EGFP–ires–CreERT2* $Klf5^{flox/flox}$ mice. C, the average ratios with SDs of the crypts, including $Klf5^{\Delta/\Delta}$ cells in the duodenums/jejunums, are indicated (B) *, $P_{score} < 0.05$.

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the GFP-expressing Lgr5^{+} stem cells, 1 day after Cre induction (Fig. 2A). The ratios of the crypts, including *Klf5*$^{\Delta/\Delta}$ cells in the duodenums/jejunums, declined after Cre induction (Fig. 2B and C). This indicated that *Klf5* deletions in the stem cells were disadvantageous for the survival of the stem cells and the descendant epithelia under the neutral competitions, defeating other stem cells (2, 15).

Double-immunofluorescence staining of Klf5 and Ki-67, a marker of proliferating cells, revealed fewer proliferating *Klf5*$^{\Delta/\Delta}$ cells as compared with *Klf5*$^{+/+}$ cells in the crypts (Fig. 3A and B), supporting the notion that Klf5 critically controls the proliferation of stem/transit-amplifying cells. Of further note, the inducible ablation of *Klf5* resulted in a drastic reduction of nuclear localization of Catnb at the bottoms of crypts (Fig. 3C and D), an observation that corroborates with the presumed role of Klf5 in regulating stem-cell proliferation via the enhancement of Wnt/Catnb signaling.

Inducible ablation of *Klf5* in the stem cells did not significantly influence the localization of Paneth cells, which constitute the Lgr5^{+} stem-cell niche at the bases of crypts (Fig. 4A and B; ref. 31).

Gene expression analysis demonstrated that deleting *Klf5* in Lgr5^{+} stem cells decreased the expression of *Ascl2*, which is a Wnt signal target gene and controls intestinal stem-cell fate (32), both in the stem and transit-amplifying cells (Fig. 4C–E; Supplementary Figs. S1 and S2; Supplementary Tables S7–S14). This may lend support to the notion that the Klf5 and Wnt/Catnb pathways are interconnected in the context of the regulation of the intestinal stem cells.

Expression status of KLF5 and genome amplifications at *KLF5* locus in human colorectal cancers

In addition to the role of KLF5 in lineage reprogramming and stemness acquisition, there has been much attention focused on its role in oncogenesis (21). Noncancerous tissue from patients with colorectal cancer exhibited polarized expression patterns of KLF5 in which expression was found to be stronger in the bottoms of crypts where the stem and transit-amplifying cells reside, and weaker as epithelial cells differentiated (Fig. 5A, left). On the other hand, KLF5 was expressed more strongly in cancer epithelia than in normal epithelia, both in colorectal mucosa and in liver metastases. Indeed, we found these colorectal cancer cells lost their polarity of KLF5 expression (Fig. 5A and D–G, right). Gastric cancers also had stronger expressions of KLF5 than the noncancerous epithelia (Fig. 5H–J).

Here, we performed an analysis of genome copy-number changes in human cancer tissues by array comparative genomic hybridization (CGH; ref. 33). The genes mapped in frequently genome-amplified regions in cancers often possess oncogenic and/or tumor-promoting functions. We revealed that focal genome amplifications, including the *KLF5* locus, were

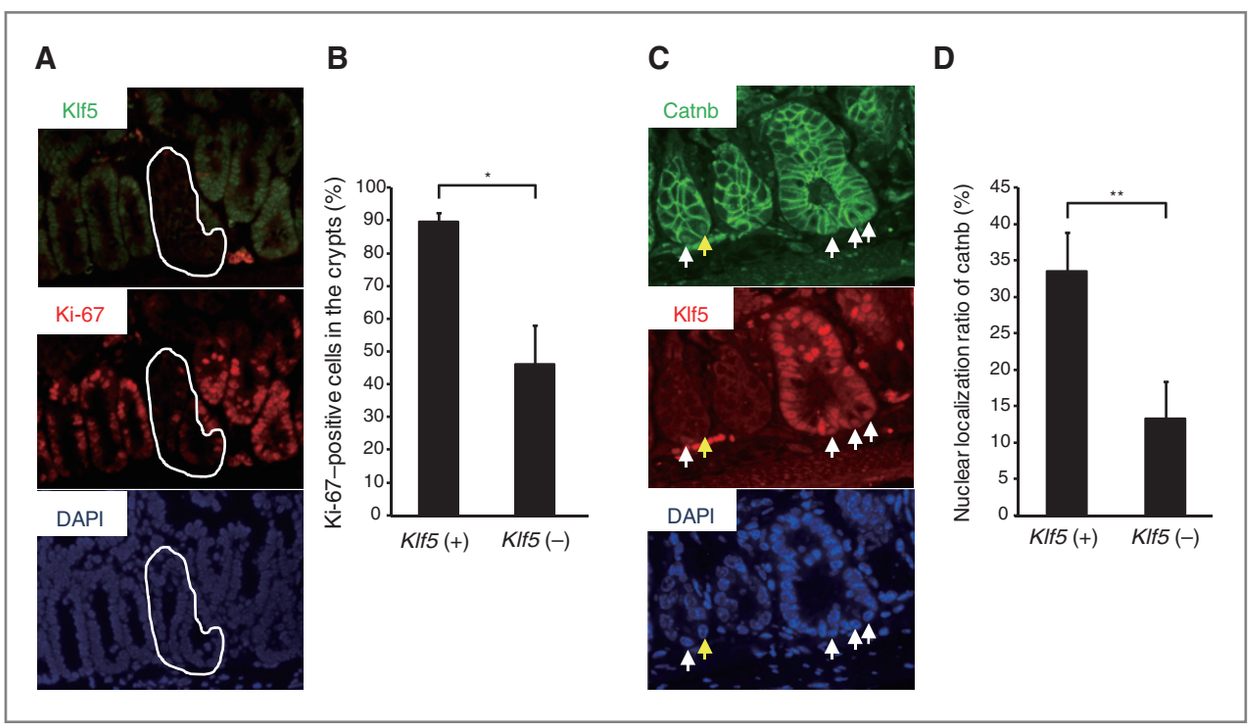

Figure 3. Inducible deletion of *Klf5* in Lgr5^{+} stem cells suppressed proliferation and nuclear localization of Catnb in the crypts. A, double immunofluorescence staining of Klf5 and Ki-67 of duodenal crypts from *Lgr5–EGFP–ires–CreERT2 Klf5*$^{flox/flox}$ mice 7 days after Cre induction. The Klf5-negative region is inside the white outline. B, the average ratios with SDs of Ki-67–positive cells among *Klf5*$^{\Delta/\Delta}$ [Klf5 (–)] and Klf5-positive [Klf5 (+)] epithelia of crypts from the duodenum and oral jejunum of *Lgr5–EGFP–ires–CreERT2 Klf5*$^{flox/flox}$ mice (*, $P_{score} < 0.05$). C, representative picture of double immunofluorescence staining of Catnb and Klf5 of crypts from *Lgr5–EGFP–ires–CreERT2 Klf5*$^{flox/flox}$ mice 6 days after Cre induction (white arrows, Klf5-positive cells with nuclear localization of Catnb; yellow arrow, *Klf5*$^{\Delta/\Delta}$ cells with nuclear localization of Catnb). D, ratios of cells with nuclear localization of Catnb in the bottom of crypts in the duodenum/jejunum in *Klf5*$^{\Delta/\Delta}$ [Klf5 (–)] and Klf5-positive [Klf5 (+)] epithelia of *Lgr5–EGFP–ires–CreERT2 Klf5*$^{flox/flox}$ mice (**, $P_{score} < 0.01$).

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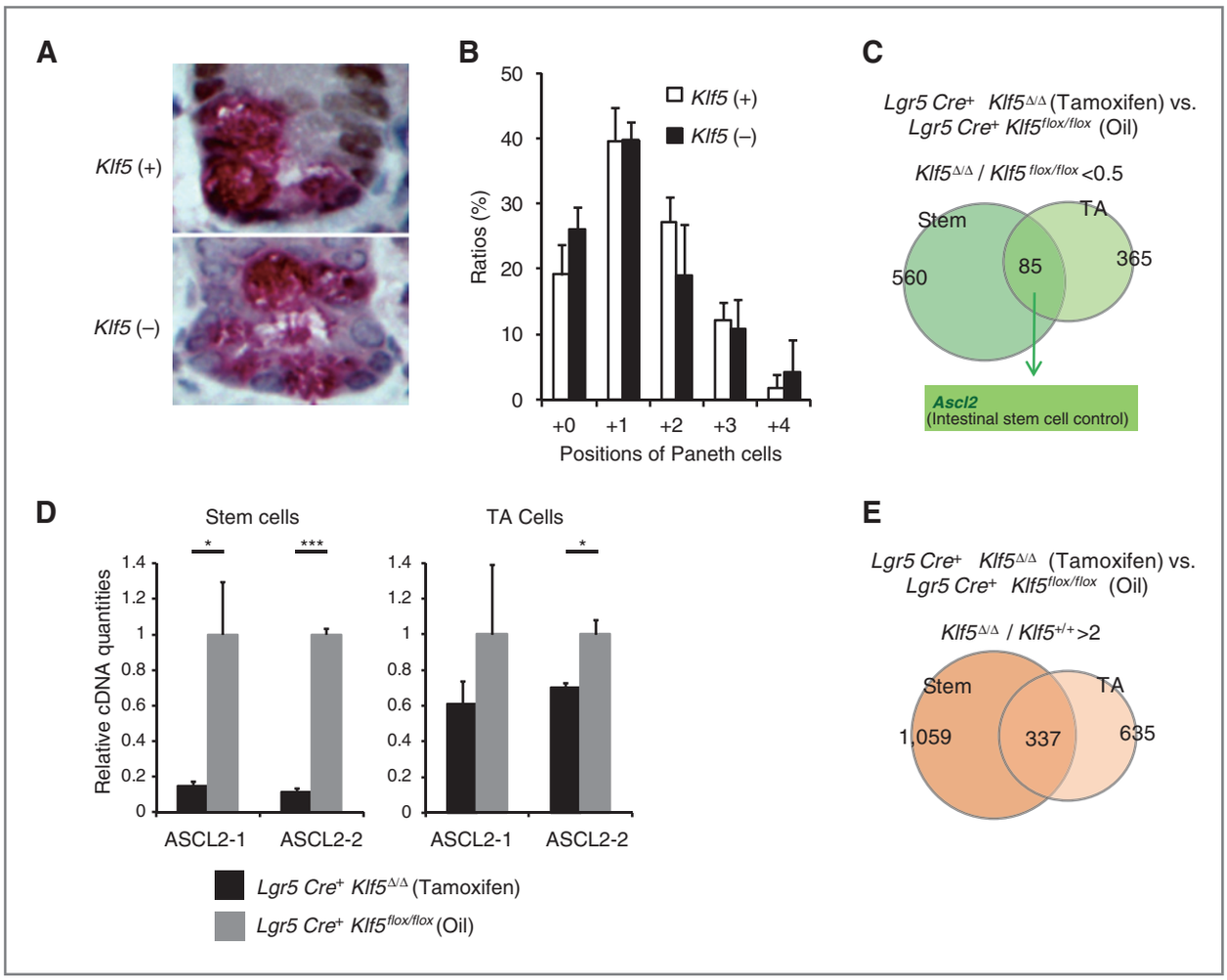

Figure 4. The mRNA expression changes in Lgr5$^+$ stem cells and transit-amplifying (TA) cells induced by *Klf5* deletion in Lgr5$^+$ stem cells. *Klf5* deletion in Lgr5$^+$ stem cells does not influence the position of Paneth cells, which constitute stem-cell niche. A, double staining of Klf5 (dark brown) and lysozyme (red), a Paneth cell marker, in the duodenum/jejunum of *Lgr5–EGFP–ires–CreERT2 Klf5$^{flox/flox}$* mice 9 days after Cre induction (day 0, 1, 2, and 3). B, the ratios of positions (from the bottoms of crypts) of Paneth cells of *Klf5*-deleted (−) or Klf5-positive (+) Paneth cells in duodenum/oral jejunum (E). C, the Venn diagram of the genes with the microarray fold change in expression (*Lgr5 Cre$^+$ Klf5$^{\Delta/\Delta}$/Lgr5 Cre$^+$ Klf5$^{flox/flox}$*) <0.5 in stem cells and transit-amplifying cells. Of note, 560 genes (Supplementary Table S7) met this criterion in the stem cells and 385 genes (Supplementary Table S9) in the transit-amplifying cells; 85 genes (Supplementary Table S11) met the criterion in both the stem and transit-amplifying cells. D, the relative expressions of *Ascl2* mRNA in the FACS-sorted stem cells and transit-amplifying cells of duodenum/jejunum of *Lgr5–EGFP–ires–CreERT2 Klf5$^{\Delta/\Delta}$* mice (tamoxifen, *Klf5*-deleted) or *Lgr5–EGFP–ires–CreERT2 Klf5$^{flox/flox}$* mice (oil, control, and *Klf5*-positive), quantified by quantitative RT-PCR. The cells were collected 5 days after Cre induction (day 0, 1, 2; tamoxifen) or control oil injection into abdominal cavities (oil). The expressions of *Ascl2* in the stem or transit-amplifying cells in *Lgr5 Cre$^+$ Klf5$^{flox/flox}$* (oil) mice are indicated as one in the bar graphs. (*, $P_{score} < 0.05$; ***, $P_{score} < 0.001$) E, the genes with the fold change (*Lgr5 Cre$^+$ Klf5$^{\Delta/\Delta}$/Lgr5 Cre$^+$ Klf5$^{flox/flox}$*) >2 in stem cells and transit-amplifying cells. Of note, 1,059 genes (Supplementary Table S8) met this criterion in the stem cells and 635 genes (Supplementary Table S10) in the transit-amplifying cells; 337 genes (Supplementary Table S12) met the criterion in both the stem and transit-amplifying cells.

accumulated in human colorectal cancer and gastric cancers (Fig. 5B). Furthermore, we observed frequent chromosomal amplification of the 13q region, the location where the *KLF5* gene is mapped, in colorectal cancer (Fig. 5C). Thus, these data suggested that *KLF5* is a candidate oncogene for intestinal tumors, acting at the level of the stem-cell homeostasis (20, 34).

Klf5 in the oncogenesis of intestinal epithelial stem cells

The above observations prompted us to investigate the function of Klf5 in the intestinal stem cells during intestinal oncogenesis in our mouse model. For this purpose, we generated two additional mouse crosses: *Lgr5–EGFP–ires–CreERT2/Catnb$^{lox(\Delta ex3)}$/Klf5$^{flox/flox}$* and *Lgr5–EGFP–ires–CreERT2/Catnb$^{lox(\Delta ex3)}$/Klf5$^{+/+}$* (Supplementary Fig. S3A; ref. 30). Briefly, we observed that the *Lgr5–EGFP–ires–CreERT2/Catnb$^{lox(\Delta ex3)}$/Klf5$^{+/+}$* strain allowed Lgr5$^+$ stem-cell–specific induction of an active *Catnb* mutant that is resistant to proteolysis, which caused the formation of severe adenomas and carcinomas (Fig. 6A, B and D and Supplementary Fig. S3B). Indeed, more than 70% of these mice died within 49 days due to the enlargement of lethal adenomas or carcinomas (Fig. 6F). Interestingly, this phenotype was found to be completely

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suppressed in the *Lgr5–EGFP–ires–CreERT2 Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{flox/flox}$ strain, wherein the *Klf5* gene is ablated simultaneously in *Lgr5*-positive cells (Fig. 6A, and C). Prognoses and survival rates of the *Lgr5–EGFP–ires–CreERT2 Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{flox/flox}$ mice were drastically improved relative to the *Lgr5–EGFP–ires–CreERT2 Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{+/+}$ mice (Fig. 6F). Some microadenomas were detected in the *Lgr5–EGFP–ires–CreERT2 Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{flox/flox}$ mice after Cre recombinase induction (Fig. 6C and E). Because the microadenoma cells in the *Lgr5–EGFP–ires–CreERT2 Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{flox/flox}$ mice expressed Klf5 (Fig. 6E), we speculate that these microadenomas were caused by insufficient deletion of *Klf5* by Cre recombinase in cells expressing the active mutant of *Catnb*. In view of the fact that alterations in the Wnt/Catnb pathway are the most frequently mutated in tumors, 92% to 97% of tumors affected in colorectal cancer according to one report (20), these results indicate that the Klf5–Wnt/Catnb axis in the stem cells is essential for the oncogenesis of most intestinal tumors.

Functional relationship between Klf5 and the Wnt/Catnb pathway

Finally, to reveal the molecular mechanisms underlying the above phenotype and given the interaction between the Klf5 and Wnt/Catnb pathways in stem/transit-amplifying cells, we compared RNA expression profiles of FACS (fluorescence-activated cell sorting)-sorted stem cells and transit-amplifying cells from *Lgr5–EGFP–ires–CreERT2/Catnb*$^{lox(\Delta ex3)}$*/Klf5*$^{+/+}$ and *Lgr5–EGFP–ires–CreERT2/Catnb*$^{lox(\Delta ex3)}$*/Klf5*$^{\Delta/\Delta}$ mice (Fig. 7A and B; Supplementary Fig. S4; Supplementary Tables S1–S6, S13–S16; ref. 32). We found that the simultaneous deletion of *Klf5* under the induction of the active mutant of *Catnb* suppressed the transcription of *Hdac1*, which promotes colorectal cancer, in both stem and transit-amplifying cells (Fig. 7A; Supplementary Table S5; ref. 35). The tumor-suppressive microRNAs (miRNA) *miR-451*, *miR-193b*, *miR–125a-5p*, *let-7d*, and *let-7e* were upregulated upon deletion of *Klf5*; in contrast, *miR-18a*, which is expressed at higher levels in colorectal cancer, was downregulated (Supplementary Tables S15–S16). These transcriptional changes related to oncogenesis may explain the almost complete suppression of oncogenesis when *Klf5* is deleted simultaneously to the induction of the active mutant of *Catnb*.

Discussion

In the present study, we investigated the role of KLF5 in the homeostatic regulation of intestinal stem cells and in oncogenesis induced by the Wnt/Catnb axis. KLF5 is strongly expressed in epithelial cells at the base of the crypts of the intestine, but not in the differentiated epithelial cells in the villi, suggesting the potential of KLF5 to control intestinal stem cells and immature epithelial cells, including transit-amplifying cells (21, 25). Our results using mice with a stem-cell–specific inducible deletion of *Klf5* indicate that Klf5 positively controls the proliferation and survival of the intestinal stem cells as well as transit-amplifying cells (Figs. 1–3).

It has been shown that in ES cells, KLF5 collaborates with KLF4, Sox2, Oct4, and other iPS cell–generating factors in an autofeedback loop that maintains stemness (24). In contrast, in intestinal epithelial cells, KLF5, rather than KLF4, is essential for stem-cell control as shown in our study.

It has been reported previously that an inducible deletion of *Oct4*, another iPS cell–generating factor, does not affect the homeostasis of intestinal epithelia, although the induction of *Oct4* causes dysplasia in intestinal epithelia (36, 37). It will be interesting to investigate gene regulatory networks aimed at revealing the similarities and differences between the stemness control mechanisms of somatic intestinal epithelial stem cells by KLF5 and these transcription factors.

Of note, we found the increase in the number of apoptotic cells upon the inducible deletion of *Klf5* in the Lgr5^{+} stem cells (Fig. 1). It has been reported that KLF5 has an antiapoptotic function by inducing the expression of survivin, a factor making cells resistant to apoptosis, and that KLF5 binds to the promoter of the survivin gene and interacts with p53 to interfere with its function (38). Thus, although further work will be required, it is possible that the KLF5–survivin pathway may be critical to the survival of the intestinal stem cells.

The stem cells and transit-amplifying cells have the deviations of original intracellular conditions among them. We speculate that's why inducible deletion of *Klf5* caused apoptosis only in the restricted population of stem and transit-amplifying cells, which were originally prone to apoptosis (Fig. 1). This speculation is consistent with "neutral competitions of equipotent stem cells," in which slightly strong stem cells for survival can leave their offspring as stem cells, defeating other stem cells (2, 15). The decrease of *Klf5*-deleted stem cells also supports this speculation (Fig. 2C).

There has been accumulating reports showing abnormal expression of KLF5 in many cancer types, though its function therein is still controversial. The expression of KLF5 mRNA is frequently reduced or absent in cancerous cells, including those of intestinal tumors and adenomatous polyposis adenomas (39). In contrast, the congenital haploinsufficiency of *Klf5* reduced the intestinal tumor of congenital *Apc*^{min} mice (40). The expression of KLF5 mRNA is also increased in breast cancer (41). Thus, KLF5 may have context-dependent functions in oncogenesis.

Our inducible and stem-cell–specific gene alteration system triggers Wnt/Catnb signal activation by the induction of active mutant of Catnb. Most of Wnt/Catnb signal activation in human colorectal cancer is caused by the loss or mutation of *Apc*, and *Catnb* is rarely mutated in human colorectal cancer (20). Apc has other functions besides regulating nuclear Catnb and different mutations in *Apc* have different strengths (42). Furthermore, *Lgr5–Cre* needs to remove both *Apc* alleles to cause adenomas, whereas other drivers like the *Lrig-1 Cre* only needs to remove one allele of *Apc* to induce tumor formation, because the second allele is lost by loss of heterozygosity in this situation (8, 16). These observations show potential differences in the ability of the cells to form tumors in different contexts.

In addition to these, human colorectal cancer are developed by the accumulation of genetic changes. Thus, our experimental system is not a perfect simulation of human colorectal cancer. However, our experimental system has the advantage to be much more similar to the sporadic genome changes in the stem cells, which actually occur in the oncogenesis of most

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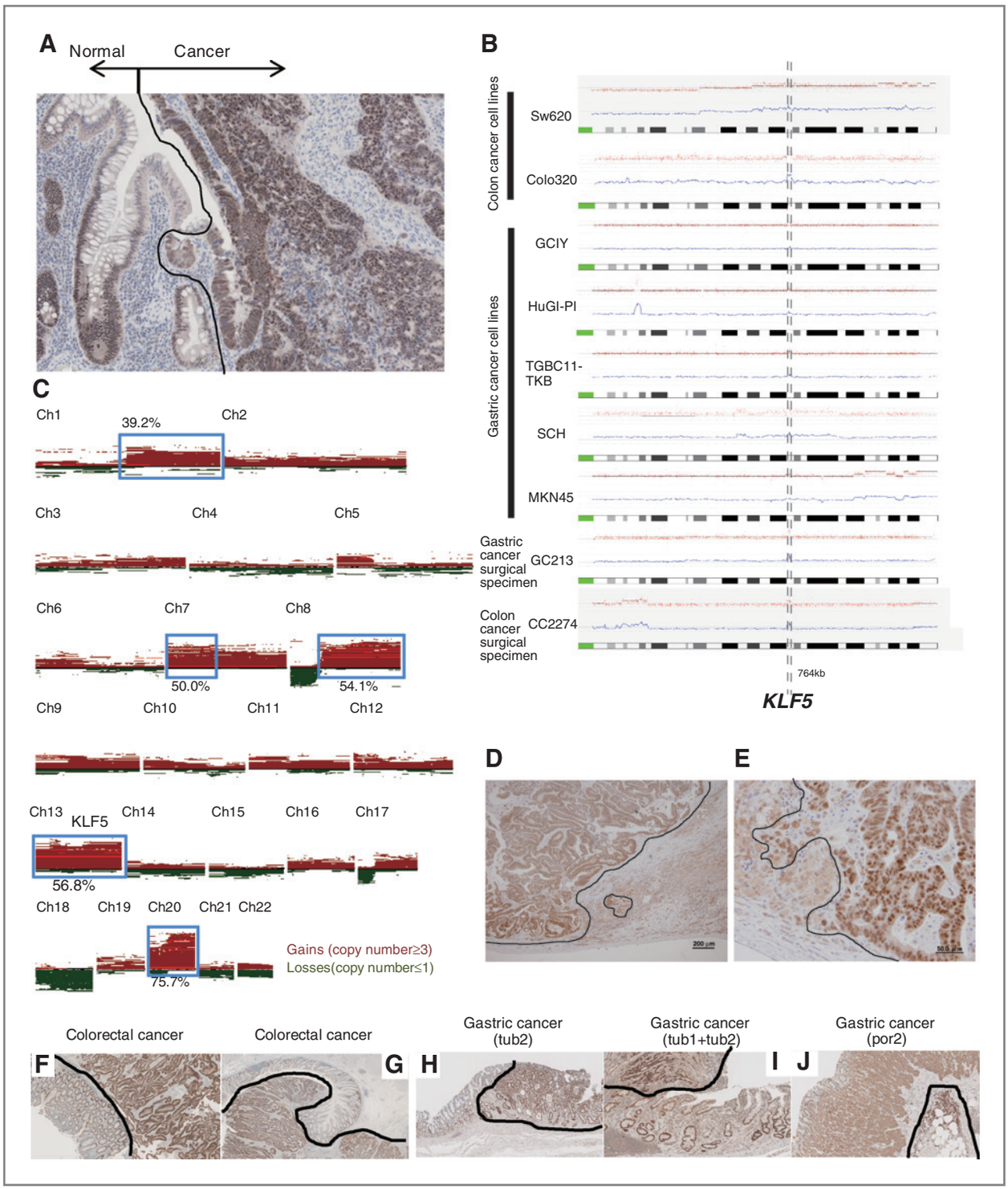

Figure 5. Increased expression of KLF5, loss of polarity in KLF5 expression, and genomic amplifications of the *KLF5* locus in human colorectal cancer. A, IHC of KLF5 in a surgical specimen of human colorectal cancer. Noncancer part is the left of the line and the cancer part is the right of the line. B, focal genome amplifications of the *KLF5* locus in cell lines and surgical specimens of colorectal cancer [Sw620, the same origin as Colo320, human colorectal cancer cell lines (focal amplification of *KLF5* locus in one cell line among 36 cells); Cc2274, human colorectal cancer surgical specimen (1 among 79 specimens)], and gastric cancers [GCIY, TGBC-11TKB, HuGI-PI, MKN-45, and SCH: human gastric cancer cell lines (5 among 32 cells); GC213, human gastric cancer surgical specimen (1 among 12 specimens)], detected by array CGH analysis. Red dots show the original CGH array data, and blue lines and red lines show the genome copy numbers calculated by the data. (*Continued on the following page*).

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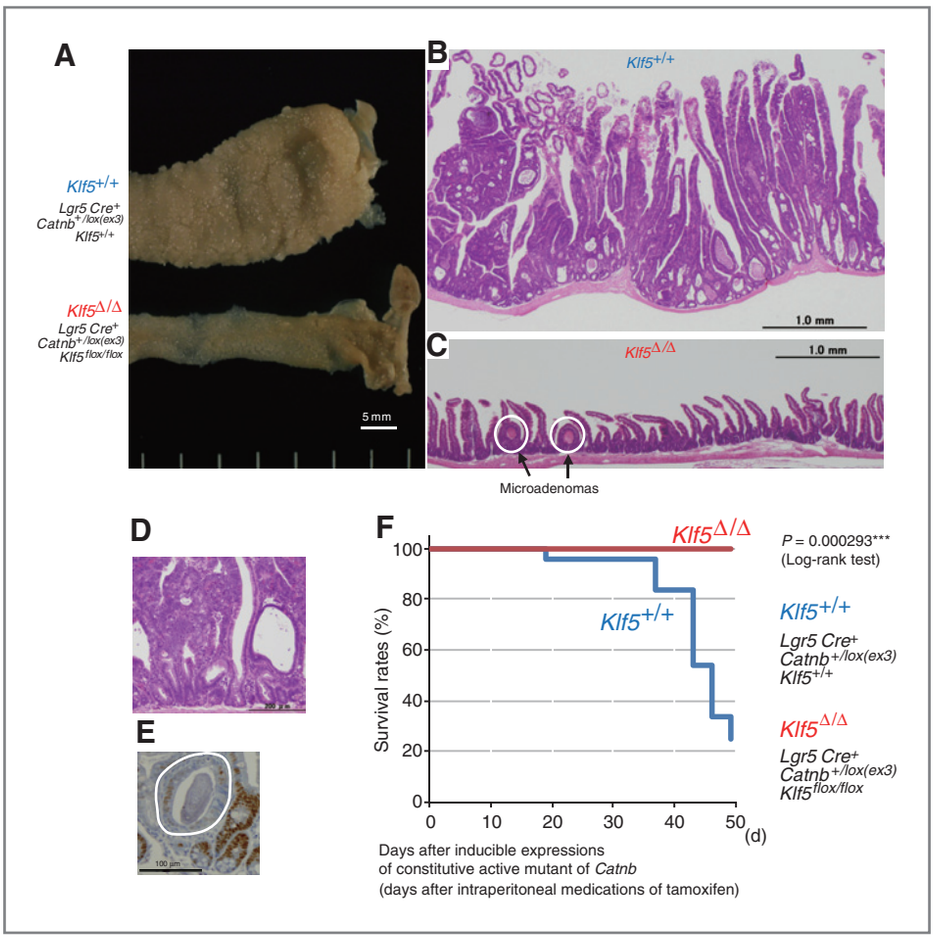

Figure 6. Klf5 is essential for oncogenesis of intestinal tumors. A, the duodenum of *Lgr5–EGFP–ires–CreERT2 Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{+/+}$ mice and *Lgr5–EGFP–ires–CreERT2 Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{flox/flox}$ mice 49 days after Cre induction. B and D, H&E–stained slides of the duodenum from a *Lgr5–EGFP–ires–CreERT2 Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{+/+}$ mouse 49 days after Cre induction, shown in A. Severe adenomas with some carcinomas (B and D). Tumors included carcinomas, including some cribriforms (D). C, H&E–stained slide of the duodenum from a *Lgr5–EGFP–ires–CreERT2 Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{flox/flox}$ mouse 49 days after Cre induction, shown in A. Oncogenesis was almost completely suppressed, except for a few microadenomas that arose from Klf5-positive epithelia (cf. E). E, IHC of Klf5 in *Lgr5–EGFP–ires–CreERT2 Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{flox/flox}$ mouse duodenum 54 days after Cre induction. A microadenoma (inside the white line) was Klf5-positive. F, survival rates of *Lgr5–EGFP–ires–CreERT2 Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{+/+}$ (24 mice) and *Lgr5–EGFP–ires–CreERT2 Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{flox/flox}$ mice (19 mice), following Cre inductions (***, $P_{score} < 0.001$).

human colorectal cancer, than the previous congenital, non-inducible, and nonstem-cell–specific gene alteration systems (16, 40). In our present study, we have given the clear answer to the previous controversial discussions (Fig. 6).

This finding is interesting because it has been reported that KLF5 is a target gene of Wnt-1 signaling linked to a protein kinase C pathway, but not to the Catnb-Lef/T-cell factor pathway (43, 44). Although further work will be required to clarify the complex network of the KLF5 and Wnt/Catnb pathways, these pathways may contribute to the enhancement of the proliferative and oncogenic pathways in the intestinal stem cells. In *Ah Cre* mice, *Myc* deletion was reported to rescue *Apc* deficiency in the intestine (45). In our system, *Klf5* deletion rescued the phenotype of abnormal *Catnb* activation, which is biologically similar to that of *Apc* deficiency. Regulatory network of stemness in ES cells, revealed by combining protein–proteins and protein–DNA interactions, is composed of three modules (core module, including KLF5/4, polycomb module, and Myc module). The Myc module, independent of the core module, is active in acute myeloid leukemias, bladder cancers, and breast cancers, and its activity predicts cancer outcome (46). Here, our study indicates that the Core module is fundamentally important for the oncogenesis of intestines.

We investigated the mRNA and miRNA expression changes of the FACS-sorted stem cells and transit-amplifying cells in *Lgr5–EGFP–ires–CreERT2/Catnb*$^{lox(\Delta ex3)}$*/Klf5*$^{+/+}$ and *Lgr5–EGFP–ires–CreERT2/Catnb*$^{lox(\Delta ex3)}$*/Klf5*$^{\Delta/\Delta}$ mice (Fig. 7A and B). The Cre induced materials obtained from *Lgr5–EGFP–ires–CreERT2* mice intestines are small in quantity, despite of the excellence of the mice as a stem-cell–specific/inducible gene

(*Continued*). C, genome copy-number changes in surgical specimens of colorectal cancer compared with noncancerous tissues of the same patients (74 specimens). *KLF5* is mapped to chromosome 13q, in which many colorectal cancer had genome amplifications [56.8%; brown lines, genome copy-number gain in the cancers compared with the noncancer genome of the same patients (copy number $\geq$ 3); green lines, genome copy-number losses in the cancers (copy number $\leq$ 1); no lines, no genome copy-number changes in the cancers (copy number = 2)]. D and E, KLF5 expressions (IHC) in liver metastases of human colorectal cancer. The top left and the bottom central region are separated by the line in D and the right side in E represents the metastases. F and G, KLF5 expressions in human colorectal cancer. The right side of the line in F and the bottom side in G are the colorectal cancer lesions. H–J, KLF5 expressions in human gastric cancers. The top right region of the line is a tub2 cancerous lesion in H, the top left region is a tub1+tub2 lesion in I, and the lower left region is a por2 lesion in J.

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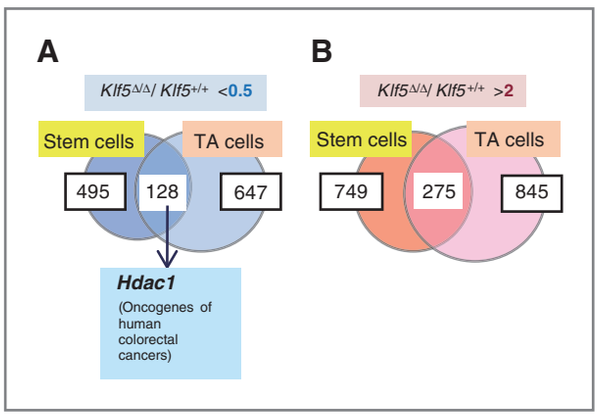

Figure 7. The mRNA expression changes in the stem cells and transit-amplifying (TA) cells, induced by *Klf5* deletion in Lgr5^{+} stem cells with the activations of Wnt/Catnb signaling. A, the Venn diagram of the genes with fold change in expression (*Lgr5 Cre*$^{+}$ *Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{\Delta/\Delta}$/*Lgr5 Cre*$^{+}$ *Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{+/+}$) <0.5 in stem cells and transit-amplifying cells. Of note, 495 (listed in Supplementary Table S1) met this criterion in stem cells and 647 genes (Supplementary Table S3) in transit-amplifying cells; 128 genes (Supplementary Table S5) met the criterion in both stem and transit-amplifying cells, including *Hdac1*. B, the Venn diagram of the genes with fold change in expression (*Lgr5 Cre*$^{+}$ *Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{\Delta/\Delta}$/*Lgr5 Cre*$^{+}$ *Catnb*$^{lox(\Delta ex3)}$ *Klf5*$^{+/+}$) >2 in stem cells and transit-amplifying cells. Of note, 749 (Supplementary Table S2) genes met this criterion in stem cells and 845 genes in transit-amplifying cells (Supplementary Table S4); 275 genes (Supplementary Table S6) met the criterion in both stem and transit-amplifying cells.

alteration system. For revealing more profound molecular mechanisms, innovations are desired to overcome the experimental limitation. Our results demonstrate for the first time that Klf5 is essential for the oncogenesis of intestinal tumors at the stem-cell level. Furthermore, colorectal cancer cells maintained their high levels of KLF5 expression, even in liver metastases (Fig. 5D and E), implying that KLF5 has essential functions not only in the oncogenesis of colorectal cancer, but also in the maintenance of the tumor even after metastasis. Knockdown of *KLF5* in human colorectal cancer cell lines suppresses cell growth (47), which is consistent with our results. These findings underscore the role of KLF5 as a fundamental regulator of intestinal stem cells in homeostatic and neoplastic proliferation.

Currently, monoclonal antibody against EGFR and VEGF are used for colorectal cancer therapy in conjunction with chemical drugs, fluorouracil, and oxaliplatin. However, most colorectal cancer depend on Wnt/Catnb signaling, whereas EGFR represents an alternative pathway (20). Therefore, blocking EGFR pathways may be insufficient to cure colorectal cancers. Intestinal tumors are generated mainly from stem cells, and are maintained by dedicated and slow-growing stem cells (16–18). Because anticancer drugs attack proliferating cells, they are not effective against slowly growing cells. Therefore, for colorectal cancer therapy to be optimally effective, interventions will be required to target the slowly growing CSCs. In line with this, it was shown that nanoparticle delivery of siRNA for suppressing *KLF5* in transplanted lung carcinomas reduced the tumor size in mice (48). Thus, our present findings on the critical role of KLF5 in the oncogenic transformation of intestinal stem cells may provide a novel target for colorectal cancer therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Nakaya, S. Ogawa, M. Tanaka, M. Sanada, M. Kuroda, R. Nagai
Writing, review, and/or revision of the manuscript: T. Nakaya, S. Ogawa, H. Clevers, M. Kuroda
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Nakaya, S. Ogawa, I. Manabe, T. Sato, M. Fukayama, M. Kuroda, R. Nagai
Study supervision: T. Nakaya, I. Manabe, M. Fukayama, M. Kuroda, R. Nagai

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