Krüpple-like Factor 5 is required for proper maintenance of adult intestinal crypt cellular proliferation

Kristin N. Bell[1,2] and **Noah F. Shroyer**[1,2,3]

[1]Division of Gastroenterology, Cincinnati Children's Hospital Research Foundation, Cincinnati, OH 45229

[2]Graduate Program in Molecular Developmental Biology, University of Cincinnati, Cincinnati, OH 45229

[3]Department of Medicine, Baylor College of Medicine, Houston, TX 77030

Abstract

Background—Krüpple-like factor 5 (KLF5) is a transcription factor that is highly expressed in the proliferative compartment of the intestinal crypt. There, it is thought to regulate epithelial turnover and homeostasis.

Aim—In this study, we sought to determine the role for *Klf5* in the maintenance of cellular proliferation, cytodifferentiation and morphology of the crypt-villus axis.

Methods—Tamoxifen-induced recombination directed by the epithelial-specific *Villin* promoter (in *Villin-CreERT2* transgenic mice) was used to delete *Klf5* (in *Klf5$^{loxP/loxP}$* mice) from the adult mouse intestine, and analyzed by immunostaining and RT-qPCR. Control mice were tamoxifen-treated *Klf5$^{loxP/loxP}$* mice lacking *Villin-CreERT2*.

Results—Three days after tamoxifen-induced recombination, the mitosis marker phospho-histone H3 was significantly reduced within the *Klf5*-mutant crypt epithelium, coincident with increased expression of the apoptosis marker cleaved-caspase 3 within the crypt where cell death rarely occurs normally. We also observed a reduction in Chromagranin A expressing enteroendocrine cells, though no significant change was seen in other secretory or absorptive cell types. To examine the long term repercussions of *Klf5* loss, we sacrificed mice 5, 14, and 28 days post recombination and found reemerging expression of KLF5. Furthermore, we observed restoration of cellular proliferation, though not to levels seen *wildtype* intestinal crypts. Reduction of apoptosis to levels comparable to the *wildtype* intestinal crypt was also observed at later time points. Analysis of cell cycle machinery indicated no significant perturbation upon deletion of *Klf5*; however a reduction of stem cell markers *Ascl2, Lgr5*, and *Olfm4* was observed at all time points following *Klf5* deletion.

Conclusions—These results indicate that Klf5 is necessary to maintain adult intestinal crypt proliferation and proper cellular differentiation. Rapid replacement of *Klf5*-mutant crypts with

Corresponding Author: Noah F. Shroyer, PhD, Department of Medicine, Section of Gastroenterology, Baylor College of Medicine, One Baylor Plaza, Alkek Building, N1317, Houston, TX 77030, noah.shroyer@bcm.edu.

wildtype cells and reduction of stem cell markers suggests further that *Klf5* is required for self renewal of intestinal stem cells.

Keywords

Krüpple-like factor 5; proliferation; apoptosis; cellular differentiation

Introduction

The intestinal epithelium is a rapidly renewing surface that possesses the ability to regulate nutrient absorption, balance digestive hormone production, and protect the host from noxious lumenal contents [1-4]. The dynamic function of this organ relies on specific coordination between proliferation and differentiation to maintain proper homeostatic regeneration. In the adult intestine, the crypts of Lieberkühn are the niche for epithelial stem cells and contain all proliferative stem and progenitor cells. Differentiating cells exit the cell cycle and migrate out of the crypts and onto the surface epithelium of the intestine, where they perform their physiological roles (e.g., nutrient absorption by enterocytes; mucous secretion by goblet cells) and are eventually shed into the lumen [5, 6]. Homeostatic maintenance of these stem and progenitor cell populations is essential to support the rapid turnover of the intestinal epithelium.

Krüpple-like factors are a family of DNA-binding zinc finger transcription factors that regulate a wide variety cellular and context dependent processes such as proliferation, differentiation, and migration [7-9]. Among these, Krüpple-like factor 5 (KLF5) is a pro-proliferative transcription factor found to be essential in embryogenesis and organogenesis [10-14]. KLF5 is highly expressed in the proliferative compartments of epithelial surfaces such as the skin and gastrointestinal tract [15-17]. Cultured fibroblast and intestinal epithelial cell assays were among the first to show that KLF5 is a positive regulator of cellular proliferation. Furthermore, *Klf5* mRNA was found in actively proliferating cells, while transient overexpression of this transcription factor significantly enhanced proliferation, cell growth, and anchorage independent growth [13, 18-20].

Deregulated cellular proliferation can influence life-threatening intestinal pathophysiology such as inflammation or cancer. Studies investigating the role of *Klf5* in DSS-induced colitis determined it was requisite for the proliferative and migratory responses required for epithelial repair [21]. Furthermore, it was found to increase the proliferative capacity of the colon after bacterial infection [22]. Interestingly, the *KLF5* gene is amplified in colorectal cancers and frequently overexpressed in *KRAS* mutated colorectal cancers, suggesting a contributory role in tumorigenesis [23, 24]. *In vitro* analysis showed that KLF5 mediates the hyperproliferative phenotype found in HRAS and KRAS transformed cells through induction of mitogen-activated protein kinase signaling and cell cycle related genes[20, 23, 25]. *In vivo* studies further concluded that *Klf5* mediated tumorigenesis in intestines harboring *KRAS* mutations, loss of the tumor suppressor *adenomatous polyposis coli* (*APC*), or both *KRAS* and *APC* mutation: heterozygosity for *Klf5* attenuated both number and size of intestinal adenomas in both *Apc^{min}/KRAS* and *Apc^{min}* induced tumors [26, 27]. These data

implicate KLF5 as a key mediator of intestinal health; however, its primary role in regulation of normal epithelial homeostasis remains unclear.

A recent study examined the role of *Klf5* in the intestine by generating a mouse model in which conditional deletion of *Klf5* was directed by the epithelial-specific *Villin* promoter. It was concluded that loss of *Klf5* in the adult results in the loss of crypt architecture, loss of barrier function, impaired differentiation, migration, and proliferation[28]. However, this study also reported a 66% mortality rate shortly after birth in mice lacking *Klf5* in the intestinal epithelium. Subsequently it was determined that *Klf5* is integral part of villus formation and cellular differentiation in the embryonic small intestine at the time of villus formation [29]. Based on this result, it was unclear whether the reported adult phenotype of *Klf5* mutant mice was due to dysfunction of *Klf5* in the adult intestine or a result of maldeveloped tissue thus prompting the need for a novel inducible knockout of this gene. Therefore, we set out to distinguish these possibilities by determining the effect of *Klf5* loss in the adult intestinal epithelium utilizing an inducible *Klf5* deletion mouse model. In this present study, we found that absence of *Klf5* acutely inhibited proliferation and concomitantly increased cell death in the crypts. Long term loss of *Klf5* was not sustainable, and strong selection for expansion of the remaining undeleted epithelium allowed repopulation of the crypt with *wildtype* epithelium along with abatement of cell death. However, loss of stem cell markers, *Lgr5, Ascl2,* and *Olf4* as well as decreased proliferation remained through 28 days after recombination.

Materials and Methods

Animals

Vil^{CreERT2} mice [30] were mated with *Klf5$^{loxP/loxP}$* mice [31] to produce an F2 generation of *Klf5$^{loxP/loxP}$*; *Vil^{CreERT2}* experimental mice and *Klf5$^{loxP/loxP}$*; *Vil^{WT}* controls. Eight week old mice were given an intraperitoneal injection of 80mg/kg tamoxifen (Sigma-Aldrich, St. Louis, MO) dissolved in sesame oil, for two consecutive days. Animals were sacrificed 3, 5, 14, and28 days after first injection. DNA was extracted from tail clippings and utilized for PCR to determine genotype.

Tissue Staining

All intestinal specimens were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, and cut into 5μm sections. For immunofluorescence, antigen retrieval was performed in 10mM sodium citrate solution (pH 6.0). Tissue was blocked in 4% normal donkey serum and then stained with antibodies raised against KLF5 (a gift from Dr. Jeffrey Whitsett, Cincinnati Children's Hospital, OH; 1:500), Ki67 (Leica Biosystems, New Castle, UK; 1:1000), Chromagranin A (CHGA) (Immuostar, Hudson, WI; 1:5000), Mucin2 (MUC2) (Santa Cruz, Dallas, TX; 1:1000), Lysozyme (LYZ) (Zymed Laboratories, San Francisco, CA; 1:5000), or phospho-Histone H3 (PHH3) (Cell Signaling, Boston, MA; 1:1000). The sections were then mounted with Vectashield containing DAPI stain. For immunohistochemistry, antigen retrieval was performed in 10mM Tris, 1mM EDTA, 0.05% Tween solution (pH 9.0). Tissues were blocked with 0.3% H_2O_2 in Methanol, then Avidin/ Biotin(Vector Laboratories, Burlingame, CA), and finally in 4% normal goat serum.

Staining was performed for cleaved-caspase 3 (CC3) (Cell Signaling, Boston, MA; 1:100) using the Vector ABC kit (Vector Laboratories, Burlingame, CA). Development was completed utilizing liquid DAB (Vector Laboratories, Burlingame, CA) and counterstained with hemotoxylin (Leica Biosystems,New Castle, UK) . Intestinal tissue was evaluated from at least three animals per time point.

RNA Isolation and qRT-PCR

Jejunal segments of approximately 1cm were excised from the small intestine and immediately placed in TRIzol reagent (Life Technologies, Carlsbad, CA). RNA was isolated as described in the manufacturer's instructions and subsequently purified with the RNeasy kit (QIAGEN, Valencia, CA) with DNase treatment (QIAGEN, Valencia, CA). Five micrograms of RNA were used to synthesize of cDNA using Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Taqman assays were carried out utilizing ABsolute Blue QPCR Low ROX Mix (Thermo Scientific, Waltham, MA) with commercially available probe and primer sets were used for *Klf5* (Mm00456521_m1), *Ccnd1* (Catalog # Mm00432359_m1), *Ccnb1* (Catalog # Mm03053893_gH), and *Cdk1* (Catalog # Mm00772472_m1) (Life Technologies, Carlsbad, CA) with 3 to 6 animals per group. Relative RNA expression was found by absolute quantification with standard curves created from pooled cDNA, normalized to *Actb* (Mm00607939_s1), and reported expression relative to the control group. SYBR green assays were carried out utilizing Brilliant III Ultra Fast SYBR Green Master Mix (Agilent Technologies, Santa Clara, CA) with *ChgA* (F: 5′-CCCACTGCAGCATCCAGTT-3′ , R: 5′-AGTCCGACTGACCATCATCTTTC-3′), *Muc2* (F: 5′–ATGCCCACCTCCTCAAAGAC-3′, R: 5′–GTAGTTTCCGTTGGAACAGTGAA-3′), *Fabp1* (F: 5′–ATGAACTTCTCCGGCAAGTACC-3′, R: 5′–CTGACACCCCCTTGATGTCC-3′), *Gata4* (F: 5′–CCCTACCCAGCCTACATGG-3′, R: 5′–ACATATCGAGATTGGGGTGTCT-3′), *Lgr5* (F – ACATTCCCAAGGGAGCGTTC, R – ATGTGGTTGGCATCTAGGCG), *Ascl2* (F – AAGCACACCTTGACTGGTACG, R-AAGCACACCTTGACTGGTACG), and *Olfm4* (F – CAGCCACTTTCCAATTTCACTG, R – GCTGGACATACTCCTTCACCTTA) primers. Relative RNA expression was found by absolute quantification with standard curves created from pooled cDNA, normalized to *Gapdh* (F: 5′-AGGTCGGTGTGAACGGATTTG-3′, R: 5′-TGTAGACCATGTAGTTGAGGTCA-3′), and reported as expression relative to the control group.

Cell Quantification

Proliferation was calculated as an average of PHH3 positive cells per total crypt cell numbers for at least 20 crypts per region per mouse. Apoptosis was calculated as an average number of CC3 positive cells per crypt. The final numbers were averaged per mouse group (*wildtype* or *Klf5*-mutant at 3, 5, 14, or 28 days following tamoxifen treatment) to give the average proliferation or apoptosis rate for each segment of the intestine. These rates were then compared for each segment of the intestine, with significance assessed by Student's t-test. Data is reported as mean ± SD.

Results

KLF5 expression overlaps with proliferative cells in the small and large intestine

In the intestine, expression of KLF5 is predominantly localized in the proliferative crypt cells [32] extending up to the lower 1/3 of the villi in the small intestine [27, 28, 32]. This was confirmed by examining the co-expression of KLF5 with the proliferative marker Ki67. Along the epithelium, KLF5 expression is highest in the top two-thirds of the crypt with expression fading towards the bottom third. The expression of Ki67 is confined to the proliferative population of the crypts in both the large and small bowel. Immunoflourescent staining showed extensive overlap between Ki67 and KLF5 indicating that proliferative cells express this transcription factor (Figure 1A-C).

To determine the role of KLF5 in the adult intestine, we utilized conditional *Klf5* knockout mice that contain loxP sites flanking exons 2 and 3, as previously described [31]. Immunofluorescence was used to determine the efficiency of recombination in the adult mouse. Three days after the first intraperitoneal injection, histological staining showed efficient depletion of KLF5 throughout the proximal small intestine and a large decrease in KLF5 positive cells in the ileum and colon (Figure 1D-E). As *Klf5* loss was previously reported to be detrimental to barrier function and overall intestinal maintenance [28], we wanted to examine the long-term effects of *Klf5* absence. Interestingly, we found that *Klf5* loss was not maintained for 28 days for any experimental mice (Figure 1B). Intermediate time points were examined to determine when KLF5 reemerged. KLF5 remained low 5 days after tamoxifen treatment, whereas extensive reemergence of KLF5 could be seen by 14 days after recombination (Figure 1A, 1G-H). The results were confirmed by RT-qPCR on isolated jejunal segments which showed almost complete loss of *Klf5* mRNA at 3 and 5 days after recombination and restoration of *Klf5* expression at days 14 and 28 (Figure 1I).

Loss of *Klf5* affects cellular differentiation

It was previously reported that loss of *Klf5* in the adult intestine has a significant effect on villus formation and inhibits cytodifferentiation [28, 33]. To examine these effects, we utilized immunofluorescence analysis to visualize cellular differentiation within the intestine 3 and 5 days after tamoxifen treatment coincident with *Klf5* depletion. Furthermore, it is at these time points that turnover of the villus epithelium is nearly complete. The overall crypt-villus architecture of the small and large intestines was preserved in *Klf5*-mutant mice (Figure 2). Immunofluorescence indicated a decrease in the number of enteroendocrine cells (CHGA-positive cells) within the small intestine of the 3 and 5 day animals (Figure 2A-C). RNA analysis indicated a significant reduction in *ChgA* was maintained for 14 days, but returned to baseline levels by 28 days (Figure 2M). Normal numbers and placement of goblet cells (MUC2-positive) (Figure D-F) and enterocytes (DPP4-positive cells) (Figure G-I) were seen within the small and large intestines of *Klf5*-mutant mice (Figure 2D-F). RNA expression analysis confirmed normal expression of goblet cell marker, *Muc2*, and enterocyte markers, *Gata4* and *Fabp1*, 3 days post tamoxifen treatment (Figure M).

Paneth cell lifespans (approximately 60 days) are longer than cells migrating up the villi and thus deletion of Klf5 for 3-5 days is not expected to perturb the census of these long-lived

cells. However, it was previously reported that *Klf5* loss interfered with proper cell migration, specifically in Paneth cell placement [28]. Therefore we examined their localization in our knockout mice. Paneth cells (LYZ-positive) remained localized along the bottom of the small intestinal crypts of *Klf5*-mutant mice (Figure 2J-L). Together these data suggest that loss of *Klf5* results in enteroendocrine cell loss within the small intestine, but does not significantly affect the other cell types during the time periods examined.

Loss of *Klf5* reduces proliferation

KLF5 is a well-known pro-proliferative transcription factor, and its loss is associated with decreased proliferation in other epithelial cell types[11, 13]. As the expression of KLF5 overlaps with the proliferative transient amplifying population of cells, we hypothesized that loss of *Klf5* would have an effect on proliferation. Tissue analyzed three days after recombination showed a significant decrease in proliferating crypt cells as measured by the mitotic marker, PHH3. After 3 days, the number of cells in mitosis dropped significantly in all regions of the intestine. This drop was nearly three-fold in the duodenum ($9.13\pm0.97\%$ to $3.22\pm0.79\%$), jejunum ($8.86\pm0.83\%$ to $3.28\pm0.63\%$), and colon ($8.82\pm0.67\%$ to $3.68\pm0.12\%$). While magnitude of effect was slightly less, probably due to a less robust loss of *Klf5*, the loss in proliferation was also significant in the ileum ($7.05\pm0.35\%$ to $3.22\pm0.34\%$) (Figure 3A-B, 3F). Reduced proliferation was similar in the intestines after loss of *Klf5* for 5 days with proliferation rates also reduced nearly three-fold in ($3.75\pm1.26\%$ in the duodenum, $2.78\pm0.65\%$ in the jejunum, $2.64\pm0.29\%$ in the ileum, and $3.73\pm0.38\%$ in the colon) (Figure 3C, 3F).

Because KLF5 expression returned between 14 and 28 days after tamoxifen-induced recombination, we hypothesized that proliferation would increase accordingly. While there were increased numbers of PHH3+ mitotic figures found after 14 days ($4.92\pm0.45\%$ in the duodenum, $4.04\pm0.26\%$ in the jejunum, $3.62\pm0.94\%$ in the ileum, and $4.72\pm0.86\%$ in the colon) (Figure 3D, 3F), the rate of proliferation, as measured by this marker, was significantly improved only 28 days after *Klf5* deletion. The number of PHH3 positive cells rose in all regions of the epithelium: to $5.34\pm0.25\%$ in the duodenum, $5.82\pm0.66\%$ in the jejunum, 5.26 ± 0.28 in the ileum, and $5.31\pm0.28\%$ in the colon (Figure 3E-F). Although a restoration in the level of proliferation was seen 28 days after deletion of Klf5, this level of proliferation remained less than *wildtype* (Figure 3F). Altogether, these data indicate that disruption of *Klf5* has an immediate and perdurant effect on normal intestinal proliferation.

Loss of *Klf5* increases apoptosis in the crypt

In the uninjured intestine, cell death within the epithelium is rare and typically observed only at the villus tips. After cells migrate up the villi, they detach from the basement membrane and are shed into the luminal cavity, where they undergo detachment-induced apoptosis, called anoikis [34, 35]. Due to the decrease in proliferation and the lack of change in tissue morphology, we sought to determine the fate of cells within the crypt. Interestingly, the loss of *Klf5* was correlated with an acute increase in apoptosis in the crypts as visualized by CC3. On average, the number of apoptotic cells was about 1.5 cells per crypt three days after ablation of *Klf5*, with the number of dying cells per crypt ranging from 1-5 (Figure 4A-B, 4F). Apoptosis was still apparent in the crypt 5 days after loss of *Klf5*, but did was not

significantly increased as seen in 3 days (Figure 4A, 4C, and 4F). Intestinal tissues examined 14 and 28 days after deletion of *Klf5* had negligible amounts of crypt cell death comparable to the *wildtype* controls (Figure 4A, 4D-F). This may be explained by the reemergence of the KLF5 and restoration of normal homeostatic function. Collectively, these data indicate that acute loss of KLF5 expression correlates with increased apoptosis.

Loss of Klf5 does not globally affect cell cycle machinery *in vivo*, but does affect stem cell marker expression

As KLF5 has been shown to play a role in the regulation of cell cycle machinery such as CyclinD1 (*Ccnd1*), CyclinB1 (*Ccnb1*), and Cdc2 (*Cdk1*) [10, 20, 25, 36], we examined the RNA expression levels of these proteins from jejunal segments where deletion of *Klf5* was most robust (Figure 1). In contrast to the previous reports, no significant change was found in the expression of any of these genes (Figure 5A).

KLF5 has also been shown to affect canonical Wnt signaling, a positive regulator of intestinal cellular proliferation, as reduction of *Klf5* reduced nuclear localization of β-catenin in the context of Apc[min] mice [26]. Therefore we examined RNA expression of well-known downstream targets of Wnt signaling in the intestine, *Ccnd1, Axin2, Lgr5 and Ascl2*. No significant decrease was seen in *Ccnd1* or *Axin2* levels; however loss of stem cell markers *Lgr5* and *Ascl2* were found to be significantly decreased (Figure 5B-C). Loss of *Lgr5* expression dropped to 55.2±15.0% of *wildtype* levels after 3 days, and continued to drop to 26.8±4.6% of *wildtype* levels 14 days after *Klf5* loss. Twenty-eight days after recombination, *Lgr5* expression remained below half of *wildtype* expression at 41.9±13.8%. *Ascl2* exhibited a more dramatic effect after recombination as expression dropped to 35.8±2.7% in 3 days, and continued to lower to 8.0±1.9% in 14 days. After 28 days, mRNA levels were still low at 16.0±5.1% of *wildtype*. This prompted us to examine another well-known stem cell marker *Olfm4*, which is thought to depend on Notch activity rather that beta-catenin transactivation [37] (Figure 5C). Expression of *Olfm4* plummeted to 17.5±4.4% of *wildtype* levels after 3 days, and continued to fall slightly to 15.8±4.8% after 14 days. As seen with the other two stem cell markers, there was some restoration of expression after 28 days (25%±5%), coincident with restored KLF5 protein expression, but this still remained significantly less than *wildtype* expression. Overall these data indicate that loss of *Klf5* does not globally disrupt cell cycle machinery nor canonical beta-catenin transactivation, but rather points to a role for this transcription factor in the maintenance of stem cells.

Discussion

The goal of this study was to determine the effects of *Klf5* loss in the adult intestine. Previously loss of *Klf5* was reported to have adverse effects on barrier function, cytodifferentiation, and migration. However, that prior study used the *Villin-Cre* driver to delete *Klf5* in the intestinal epithelium, resulting in perinatal death in two-thirds of the mutant offspring [28]. It was later discovered that this transcription factor plays an important role in intestinal morphogenesis wherein deletion of *Klf5* from the embryonic intestine disrupted villus emergence and cellular differentiation [29]. As these phenotypes emerged at the onset of villus outgrowth, we hypothesized that the effects of acute loss of *Klf5* in the

adult would be distinct from those phenotypes reported in the previous study using the continuously active *Villin-Cre* transgenic line, which we suggest may have developmental origins. To test this hypothesis, we used the tamoxifen-inducible *Villin-CreERT2* driver to delete *Klf5* in the adult intestine as a means to test its function in this context.

We found that loss of *Klf5* significantly decreased the number of proliferating cells per crypt along the entire region of the small and large intestines. Simultaneously, we observed an acute increase the number of apoptotic cells in *Klf5*-deficient crypts, and a decreased number of enteroendocrine cells in the small intestine. The diminished number of these cell types may indicate a role for *Klf5* specific to enteroendocrine cell differentiation. Additionally, we saw no change in intestinal morphology, suggesting that production of new epithelial cells continues in Klf5-mutant intestines. In contrast to the minimal changes in intestinal cellular differentiation, we observed decreased proliferation coincident with increased apoptosis in *Klf5* mutant intestines. We suggest that the lack of cell cycle progression potentiated the onset of cell death early after loss of *Klf5*. Caspase-3 is known to induce apoptosis after G1 phase cell cycle arrest [38]. As KLF5 is known to regulate expression of CyclinD1 [10, 36], which is required for G1/S phase transition, it is possible that loss of this transcription factor could result in a G1 phase arrest and activation of caspase activity and thus cellular apoptosis. However we did not see a significant change in *CyclinD1* mRNA expression. We note that RNA was isolated from whole jejunum, and therefore, these results measure the entire tissue including mesenchyme, and may have reduced the sensitivity of our assay to detect subtle changes in *CyclinD1* expression. As an alternative mechanism underlying the observed increased cell death, *Klf5* deletion may have caused a loss of a prosurvival gene such as *survivin*, which has previously been linked to KLF5 in leukemia [39], and thus may play a similar role in the intestine. Spontaneous crypt cell apoptosis is generally a sign of stress or toxicity in the epithelium [40] therefore we interpret our results to suggest that lack of KLF5 triggered a stress response within the intestinal epithelium.

Interesting, *Klf5*-deficient cells were rapidly replaced with *wildtype* cells, such that crypts had restored KLF5 expression within 14 to 28 days of induced deletion. While this is probably the result of the lack of 100% efficient knock out of *Klf5*, we suggest that, given the increased rate of apoptosis, there could be selection against *Klf5*-mutant cells that is required to reestablish the proliferative capacity of the crypt to maintain proper epithelial turnover. Our results are consistent with the previous studies of *Klf5*-deficient intestinal epithelium, in which surviving mice showed re-expression of KLF5 in tissues examined 8 weeks after birth [28] and, very recently, in inducible loss of *Klf5* in the colon [41]. Regardless of its origin, the reemergence of this transcription factor and incomplete loss of proliferation could explain the lack of disruption in cellular differentiation and intestinal morphology in *Klf5*-mutant mice.

The main conclusion of this study is that KLF5's primary function in the intestine is to maintain epithelial proliferation. Due to extensive turnover of the epithelium, three to five days is sufficient to see potential differentiation and morphological defects caused by acute KLF5 loss; however, loss of proliferation is the prominent phenotype observed in *Klf5*-mutant mice. Increased apoptosis is likely coupled to this effect early after recombination,

but the number of PHH3 positive cells remains significantly lower than *wildtype* 28 days post tamoxifen treatment suggesting a lasting effect from the mutation.

KLF5 has been linked to both the Wnt and MAPK pathways [17, 20, 23, 26], and has been reported to regulate cell cycle components such as *Cyclin D1, Cyclin B1*, and *Cdc2* [25, 36]. However, we did not see a significant decrease in the expression of any of these genes. As previously mentioned, analysis of whole jejunal tissue may have diminished the sensitivity of our assay to detect cell-specific changes in gene expression, for example specifically in transient amplifying or stem cells. It is interesting that in the developing intestine, *Klf5* deletion causes a profound loss of CyclinD1 expression, however loss of these genes had no effect on proliferation of the embryonic intestine [29]. Therefore the reduction in proliferation seen here may suggest that KLF5 regulates cell cycle in the adult intestine via mechanisms distinct from identified in tumors and cell lines..

Reemergence of *Klf5* coupled with the lasting effects of reduced proliferation indicates that the initial loss of *Klf5* may have an effect on another important element of homeostasis. . While it is thought that expression is rare or weak in adult stem cells [10], loss of Klf5 in Lgr5 positive stem cells has resulted in suppression of oncogenic transformation [42] indicating a prominent role for this transcription factor in this cell type. Indeed we saw a significant reduction in active intestinal stem cell markers, *Lgr5, Ascl2*, and *Olfm4* which can explain a decreased proliferative response in the intestine in the absence of an overt change in cell cycle machinery or canonical Wnt signaling. In contract to what we expected, there seems to be no lack of crypt cell numbers in these intestines which is possibly due to an incomplete loss of stem cells entirely. In any instance, it is clear that given its potential roles in the initiation of tumorigenesis and in stress induced epithelial repair, understanding the molecular mechanisms by which KLF5 controls homeostatic cellular proliferation and stem cell maintenance could provide valuable answers for future therapies of intestinal diseases.

Acknowledgments

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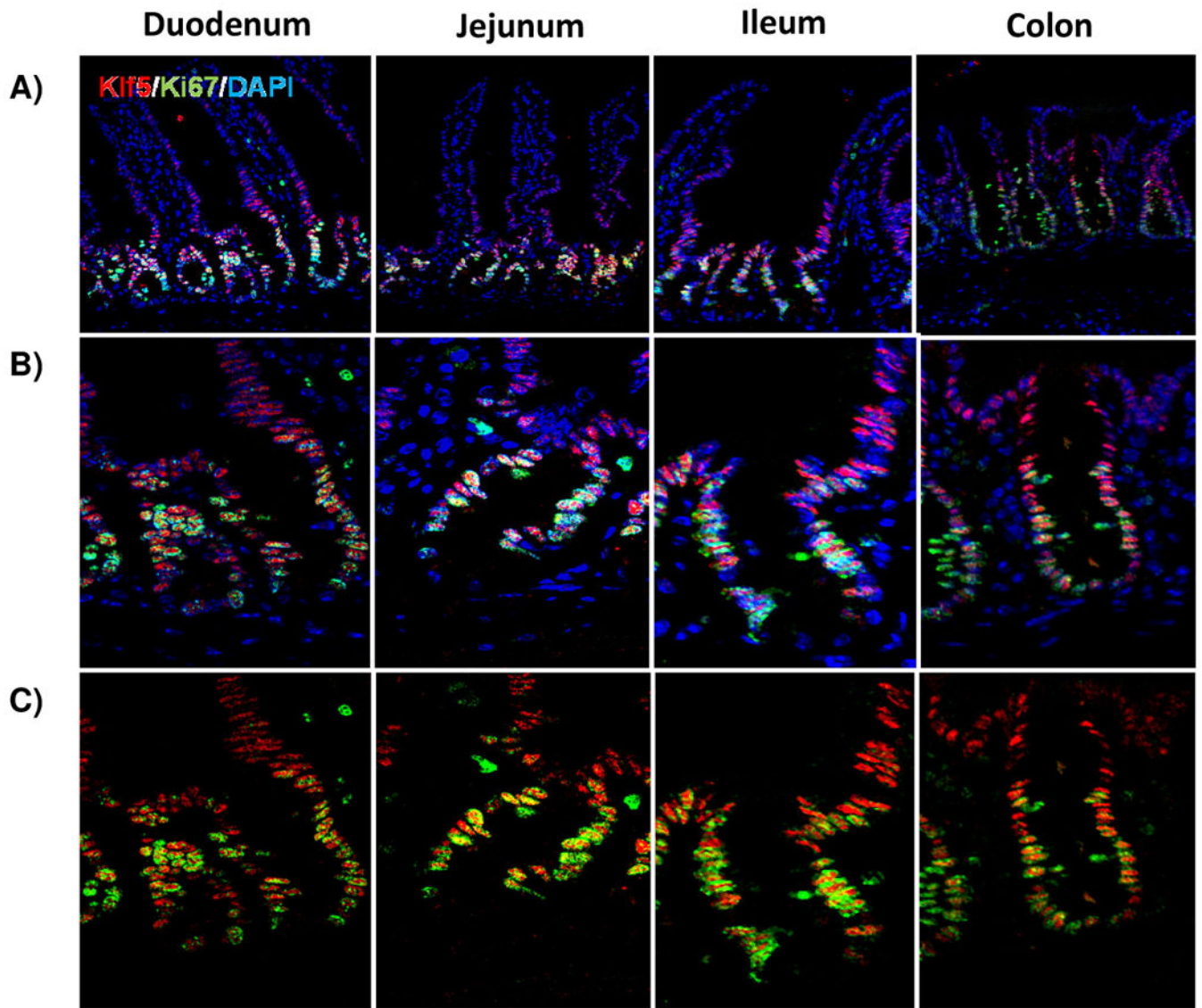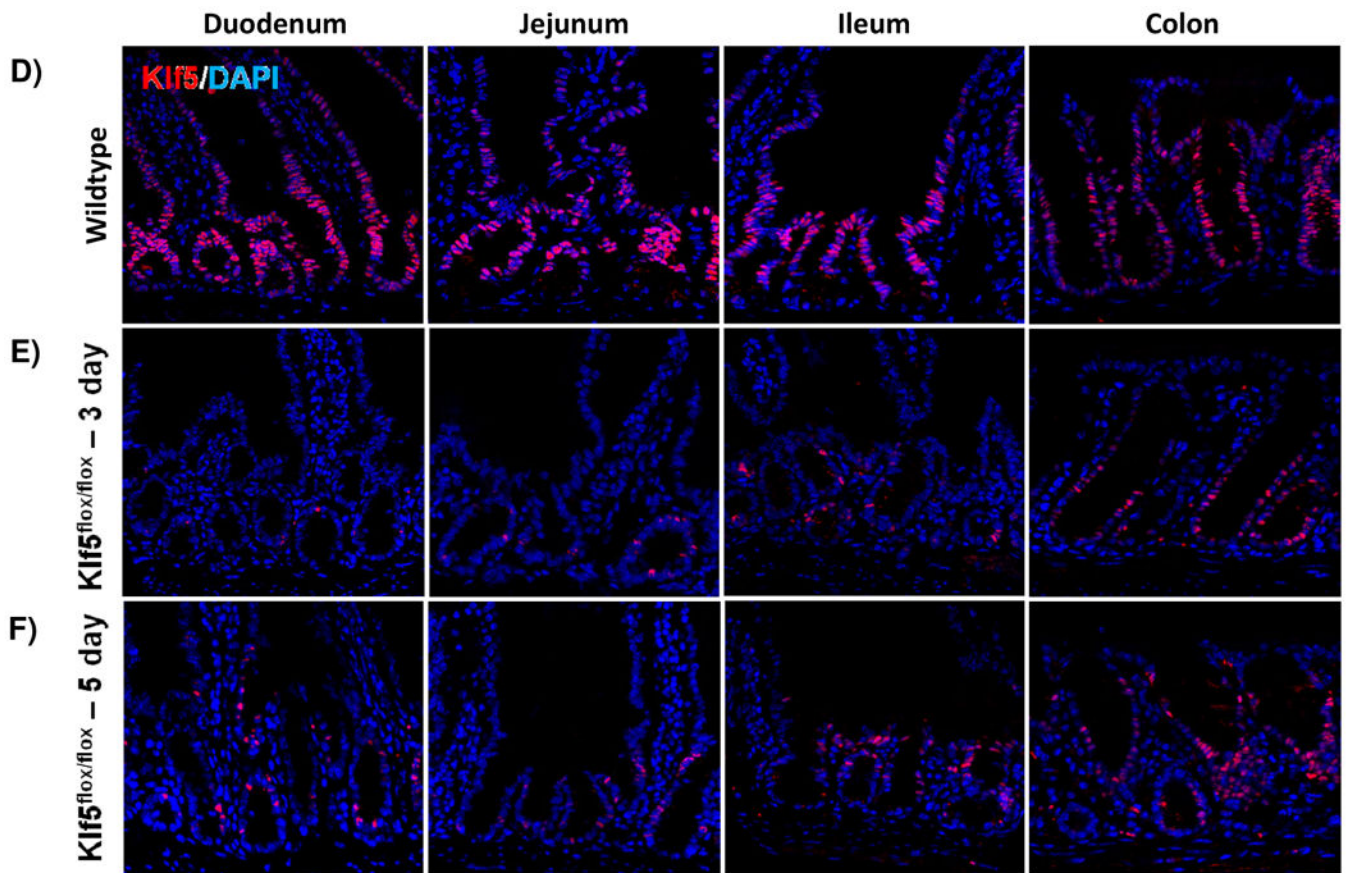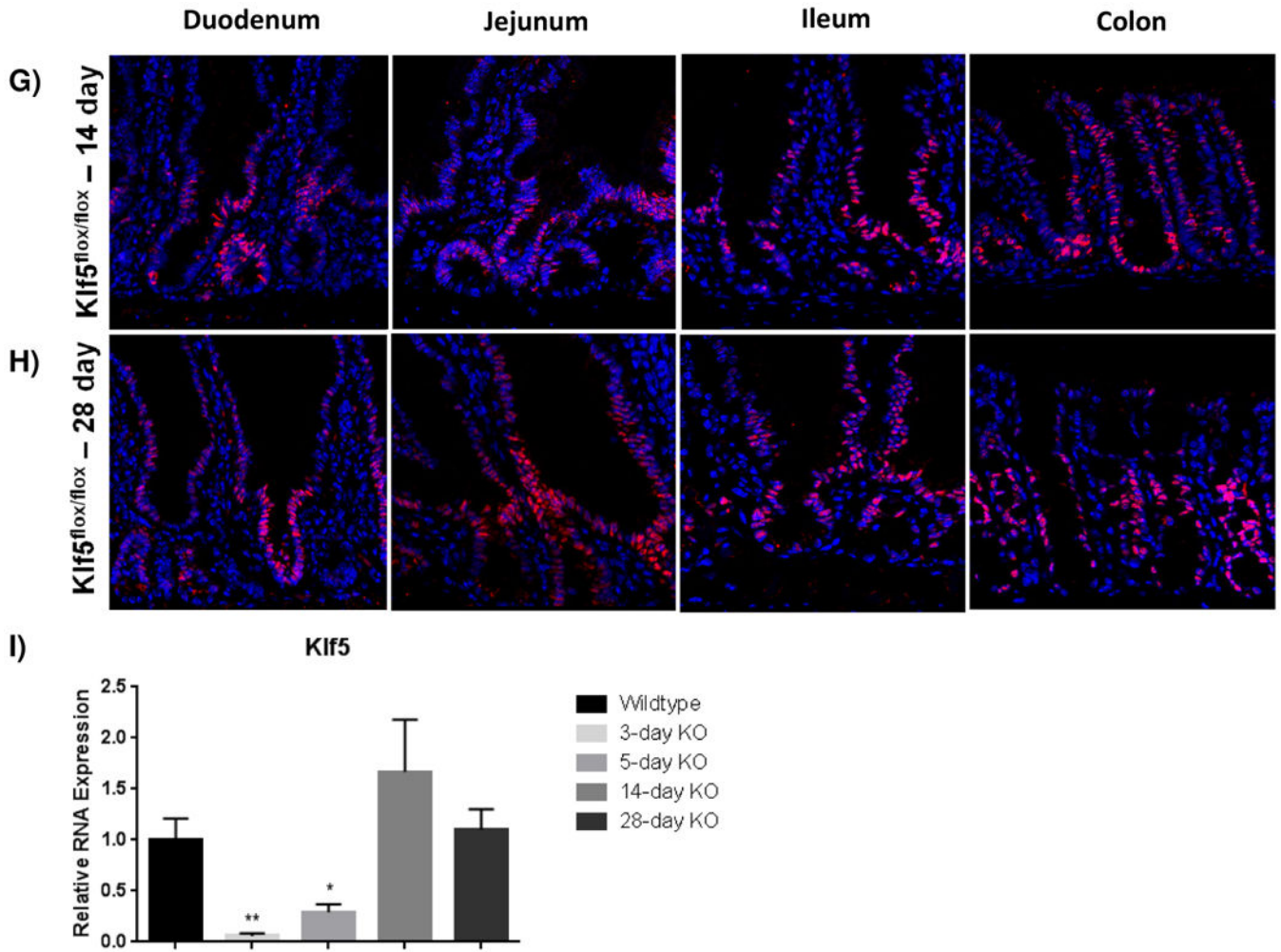
Figure 1. KLF5 is expressed in proliferative crypts of the small and large intestine
(A) Immunofluorescence staining for coexpression of KLF5 (red) and Ki67 (green). DAPI (blue) was utilized as a nuclear stain.(B) Immunofluorescence staining of the crypt with DAPI (B) and without DAPI (C) show regions of Klf5 and Ki67 expression overlap. Colocalization between these two proteins is seen in yellow. (D-H) Regional histological analysis of tamoxifen-induced recombination in $Klf5^{loxP/loxP}; Vil^{WT}$ control mice (D) and $Klf5^{loxP/loxP}; Vil^{CreERT2}$ experimental mice 3 (E), 5 (F), 14 (G), and 28 days (H). (I) Comparison of Klf5 mRNA expression analysis by RT-qPCR from jejunal segments of the small intestine. Student's t-test was performed to determine p-values; expression was normalized to Actb. (Mean ± SEM: P 0.05, **P 0.01, ***P 0.001, n = 3-6).
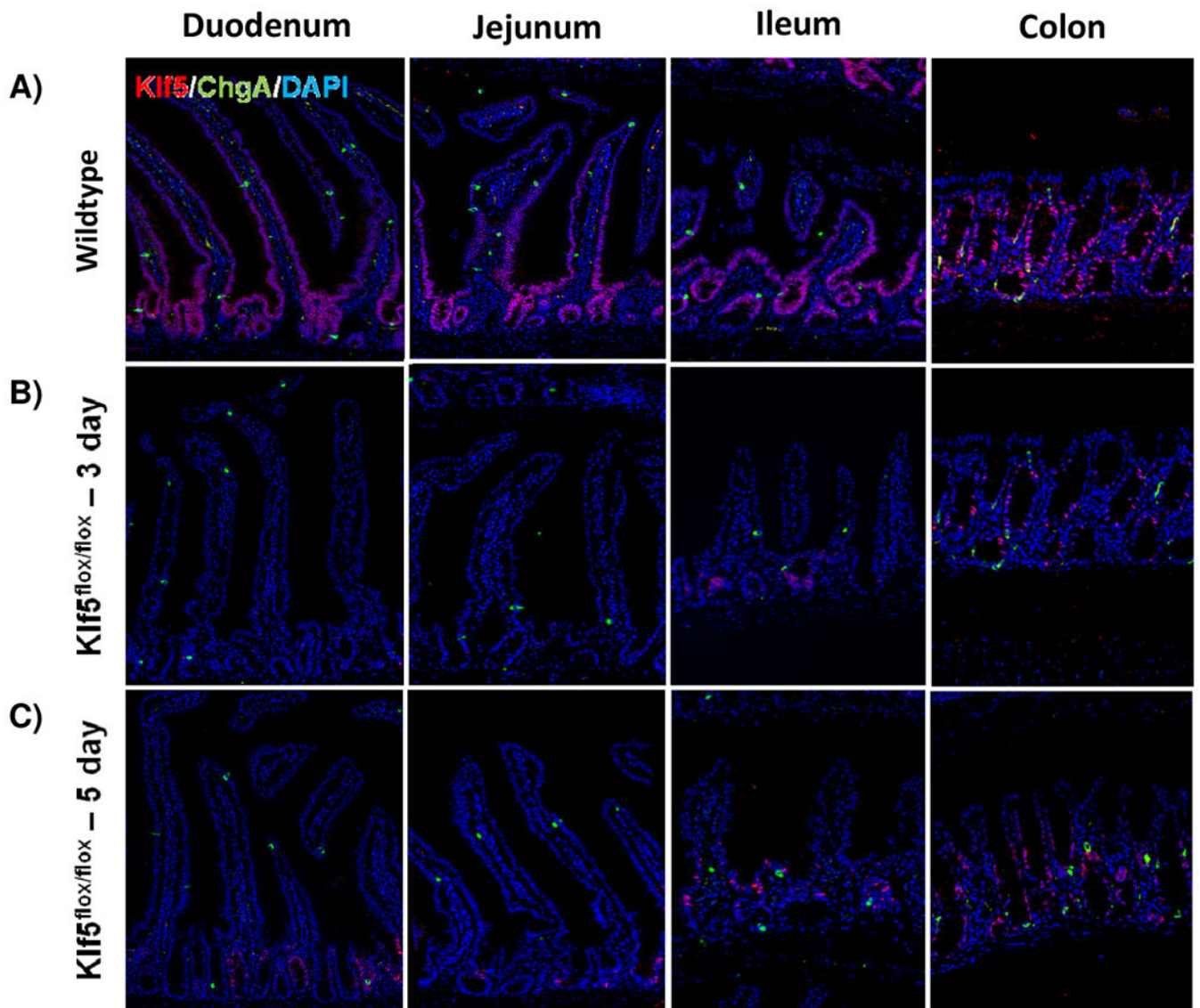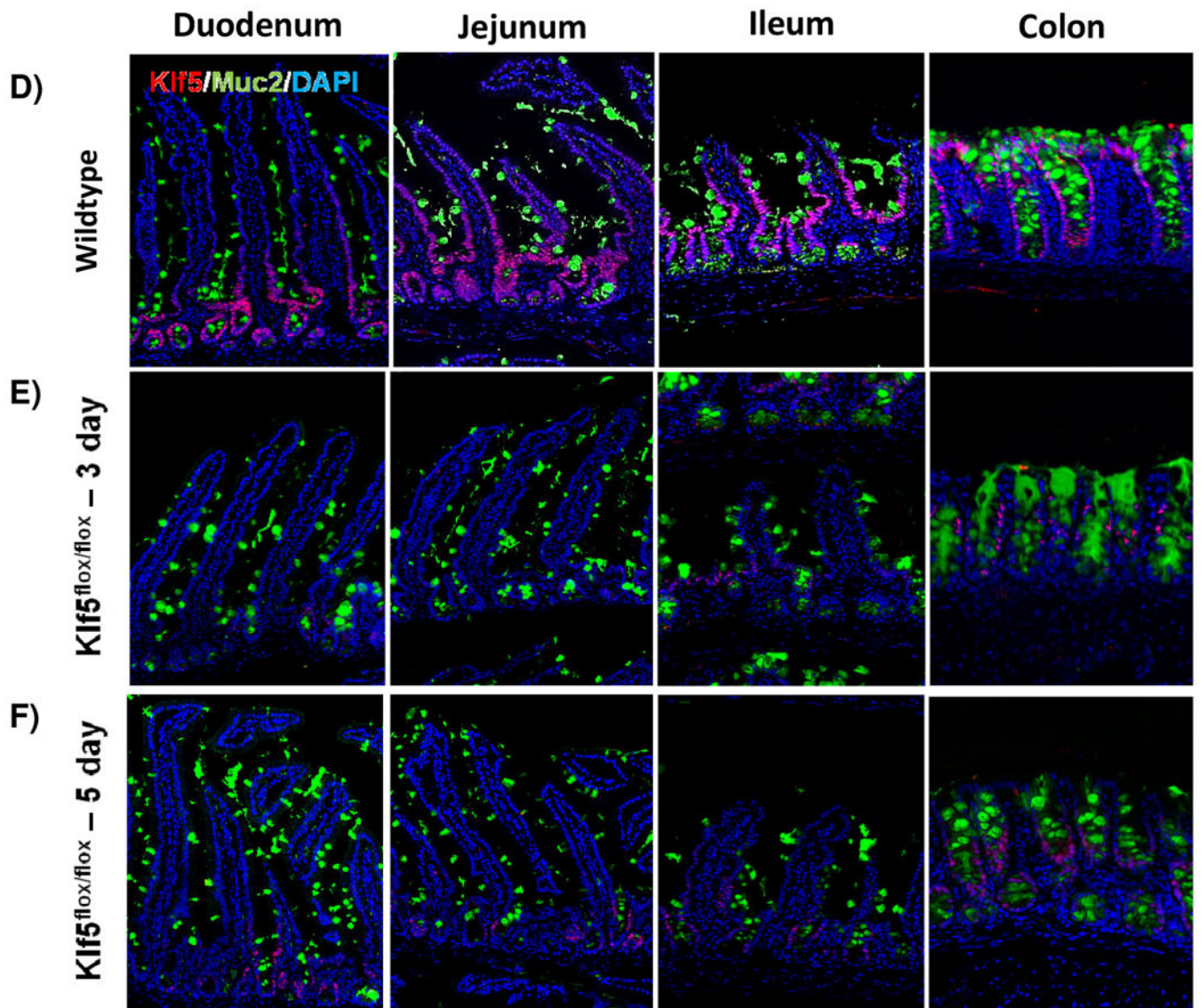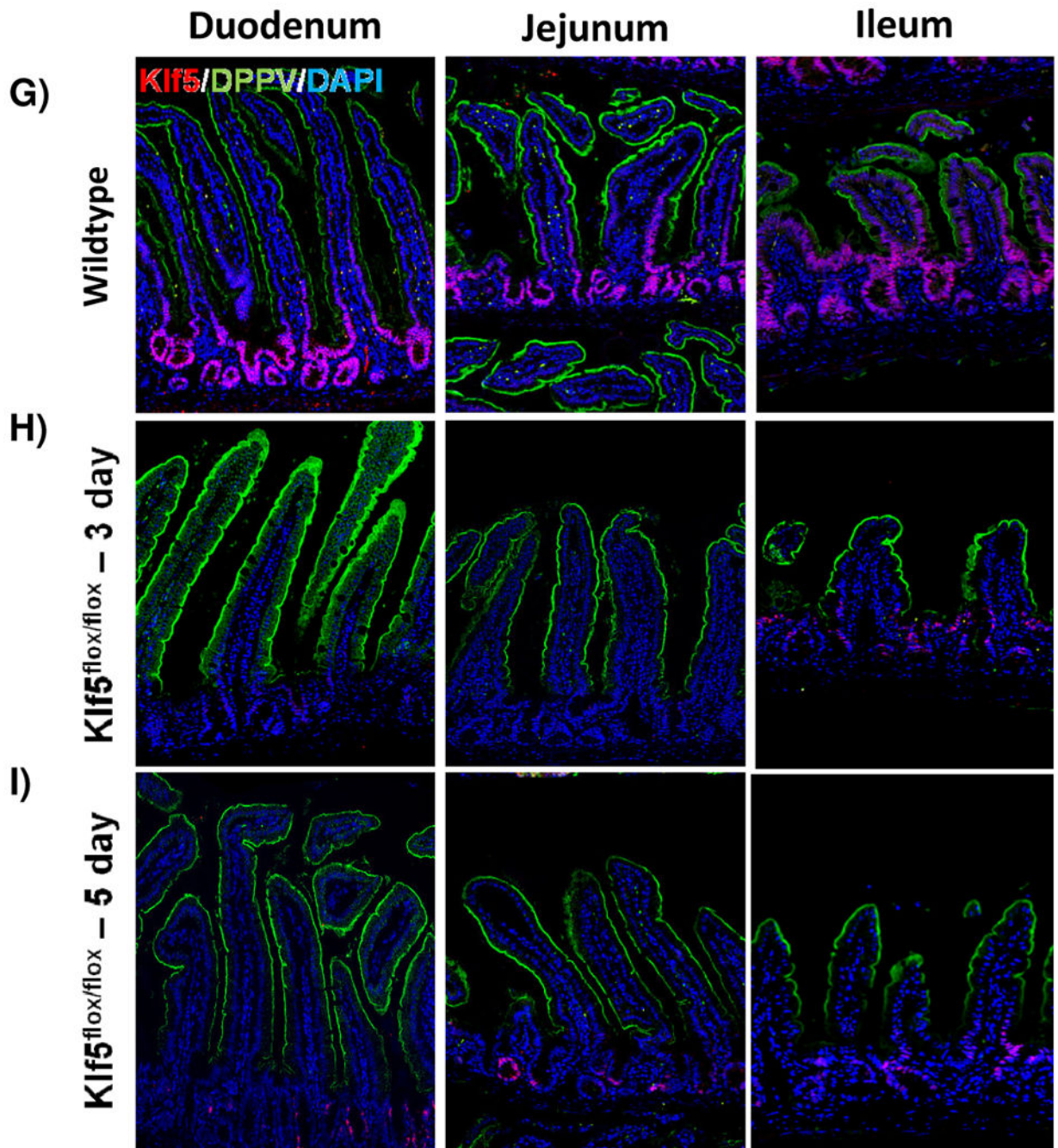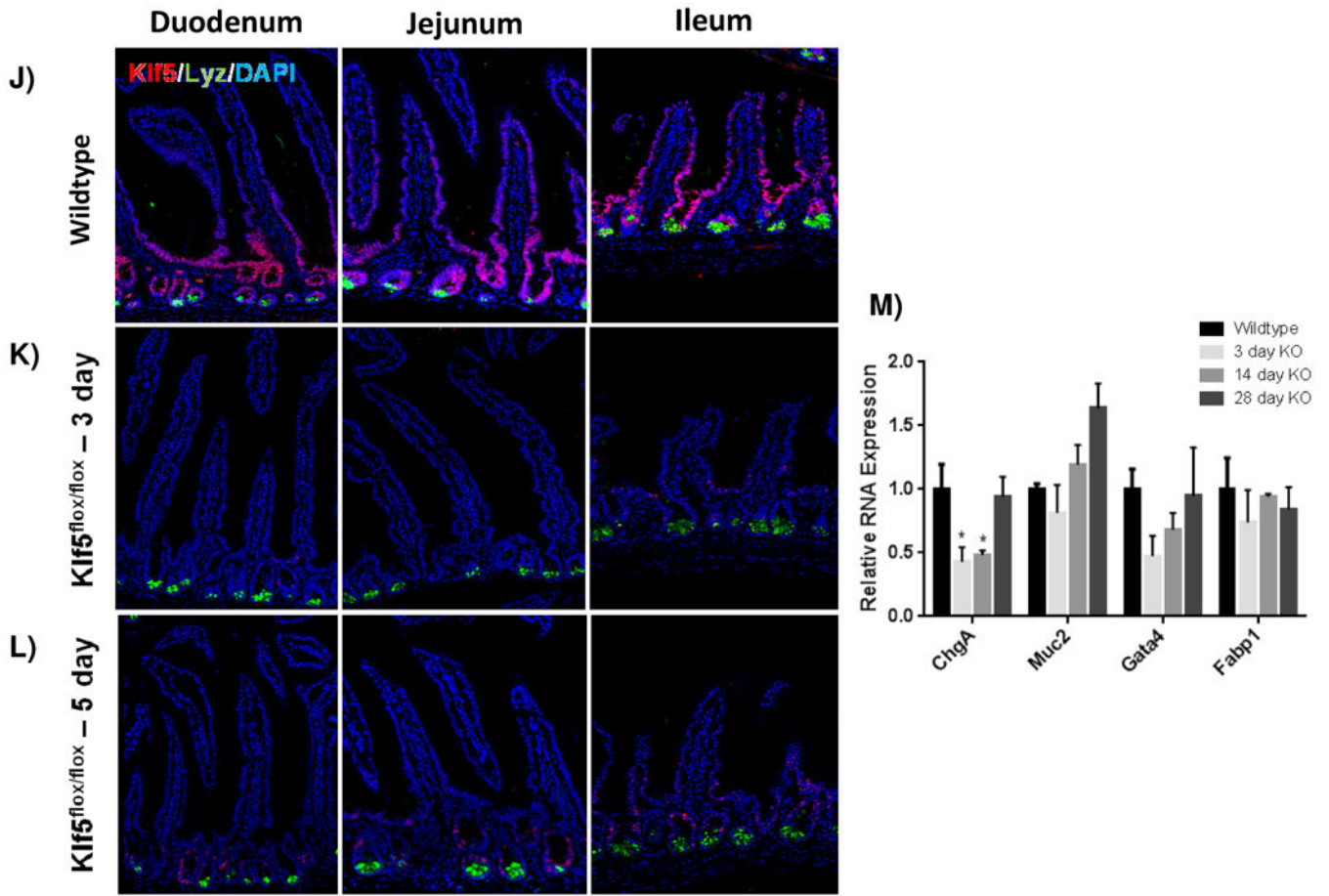
Figure 2. Loss of KLF5 affects cytodifferentiation

Histological comparison of *Klf5^{loxP/loxP}; Vil^{WT}* (WT) and *Klf5^{loxP/loxP}; Vil^{CreERT2}* (Klf5-mutant) intestinal tissues for cellular differentiation utilizing immunofluorescent staining for chromagranin A (green; enteroendocrine cells) (A-C),mucin2 (green; goblet cells) (D-F), lysozyme (green; Paneth cells) (G-I), and dipeptidyl peptidase-4 (green; enterocytes) (J-L). Nuclear stain is DAPI (blue). (M) Comparison of ChgA, Muc2, Gata4, and Fabp1 mRNA expression analysis by RT-qPCR from jejunal segments of the small intestine. Student's t-test was performed to determine p-values; expression was normalized to Gapdh. (Mean ± SEM: P 0.05, **P 0.01, ***P 0.001, n = 3-6).
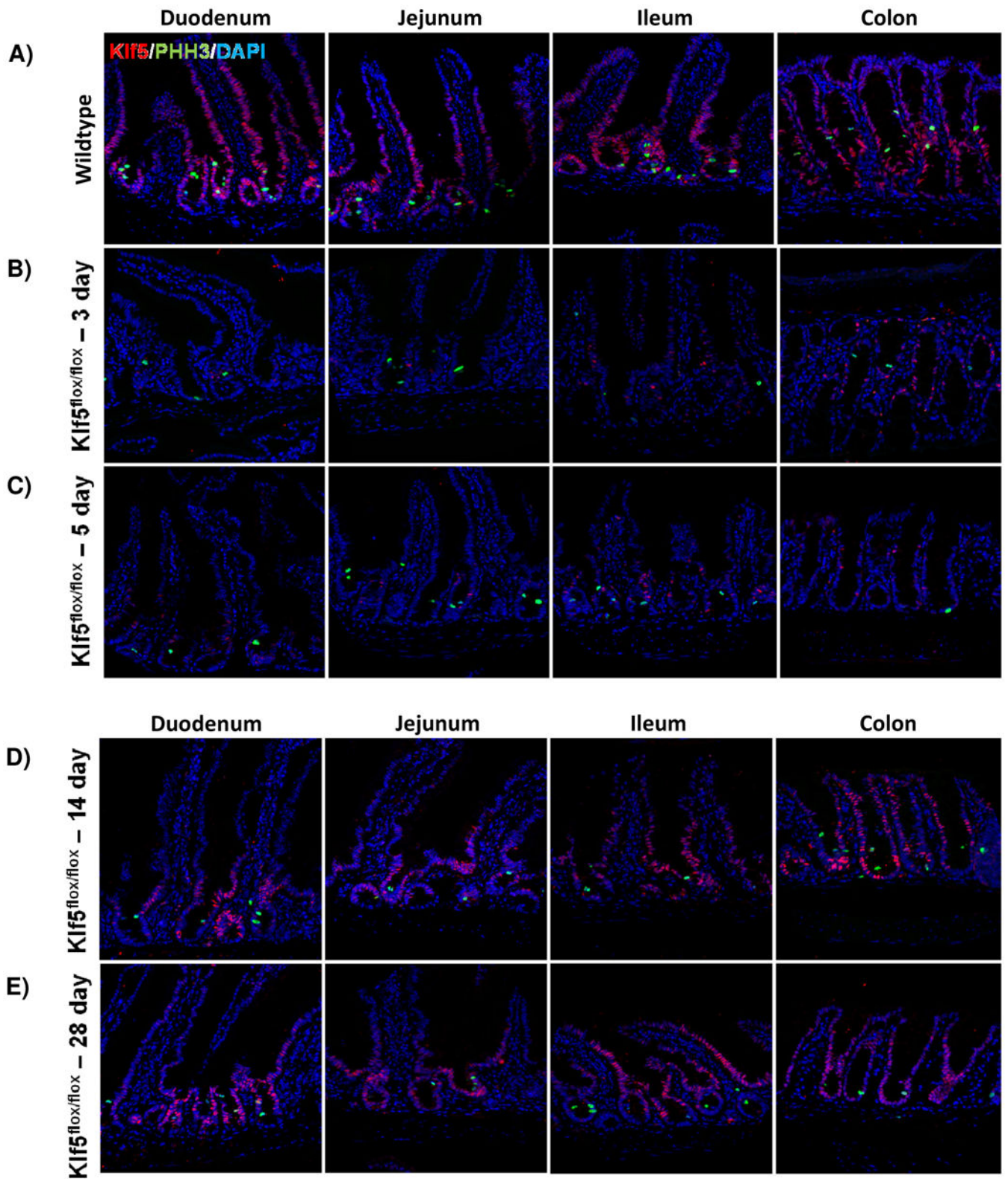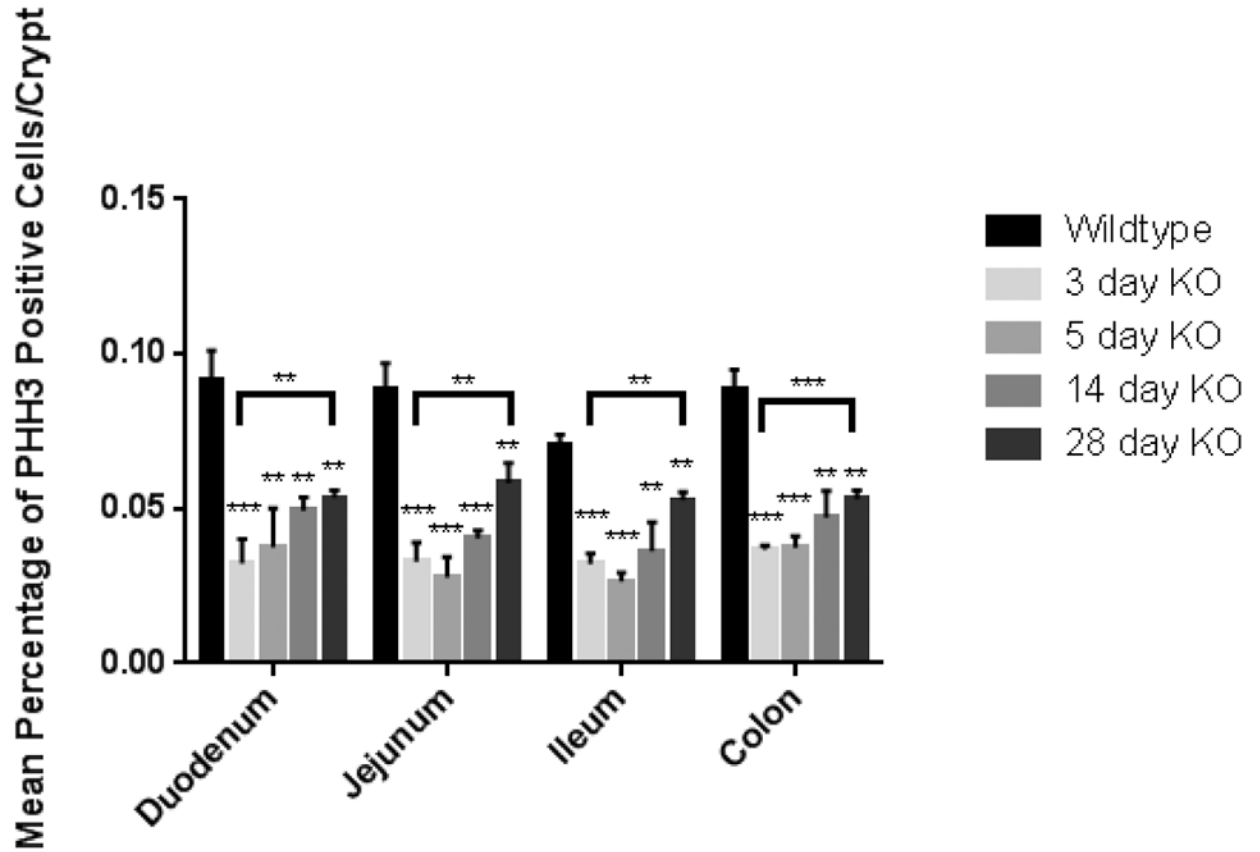
Figure 3. Loss of KLF5 attenuates cellular proliferation in the adult intestine
(A) Histological analysis for the comparison of cellular proliferation utilizing immunofluorescent staining for mitotic marker, phosphohistoneH3 (green) in *wildtype* and *Klf5* loss-of-function mice (3, 5, 14, and 28 days post tamoxifen-induced recombination). Nuclear stain is DAPI (blue). (B) Quantification of average percentage of phosphohistoneH3 positive cells per crypt. Significance determined between groups of two (wildtype vs. loss of Klf5 for 3 days, wildtype vs. loss of Klf5 for 5 days, wildtype vs. loss of Klf5 for 14 days, wildtype vs. loss of Klf5 for 28 days, and loss of Klf5 for 3 vs. 28 days). (Mean ± SD, T-test: * P   0.05, **P   0.01, ***P   0.001, n = 3)
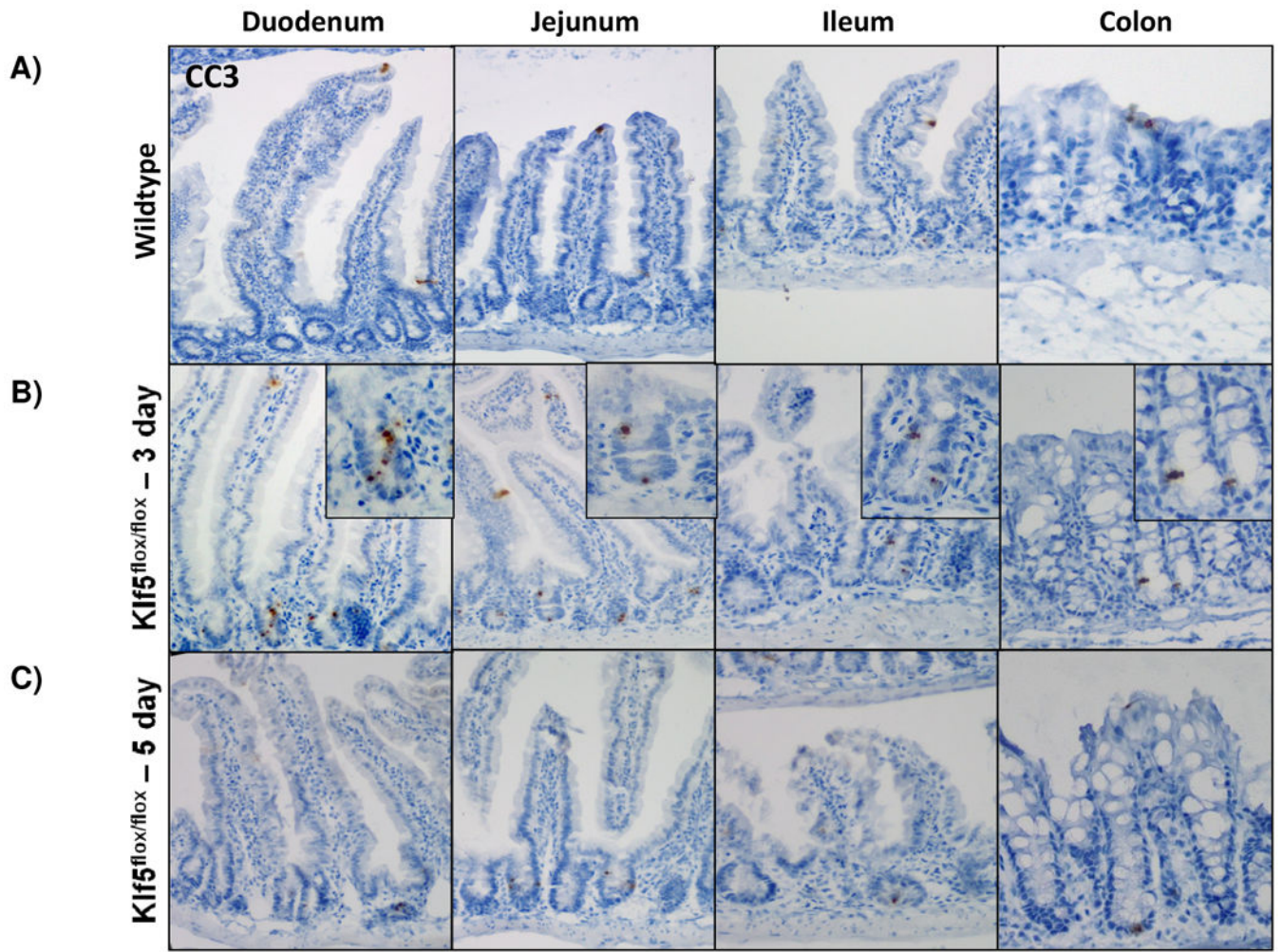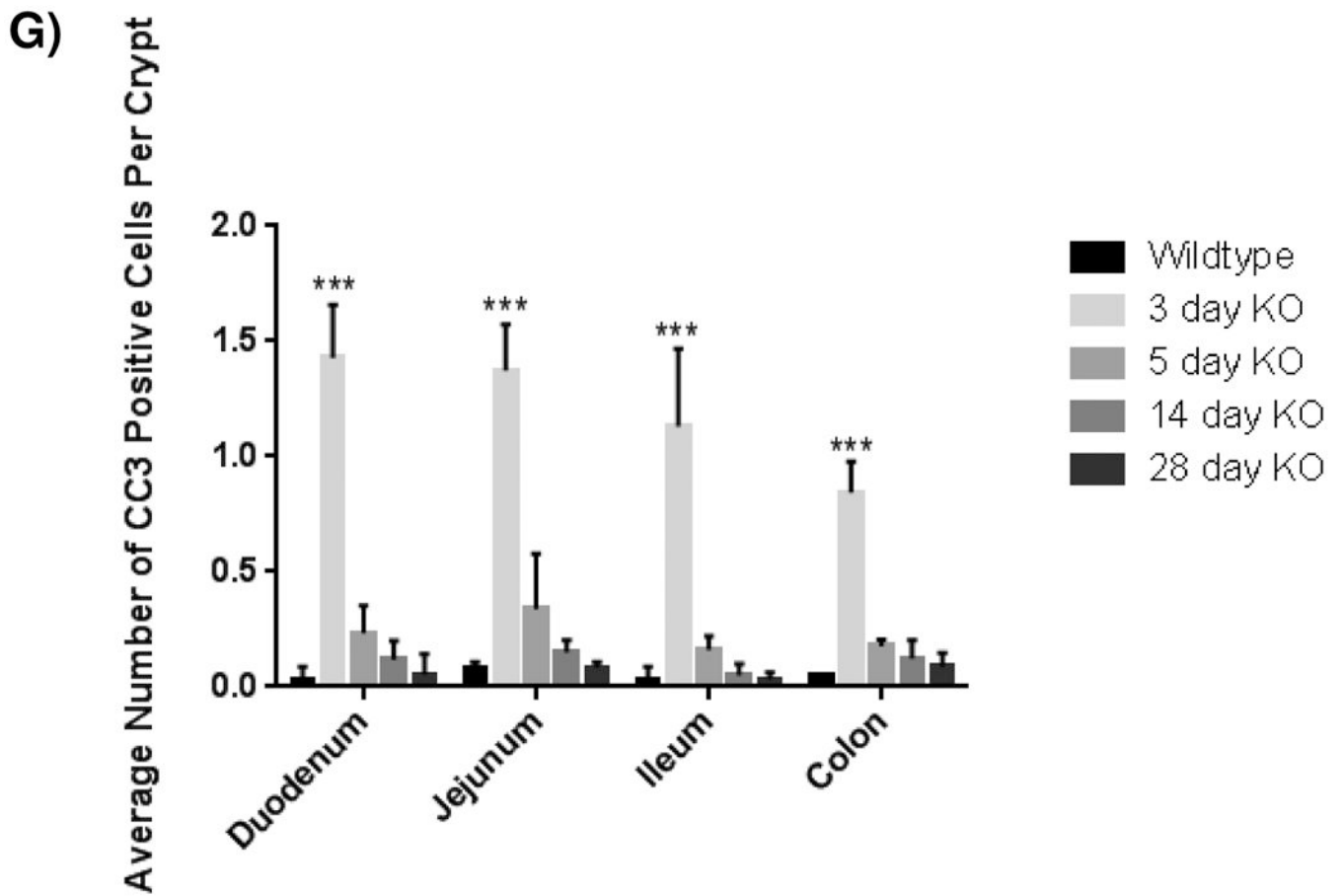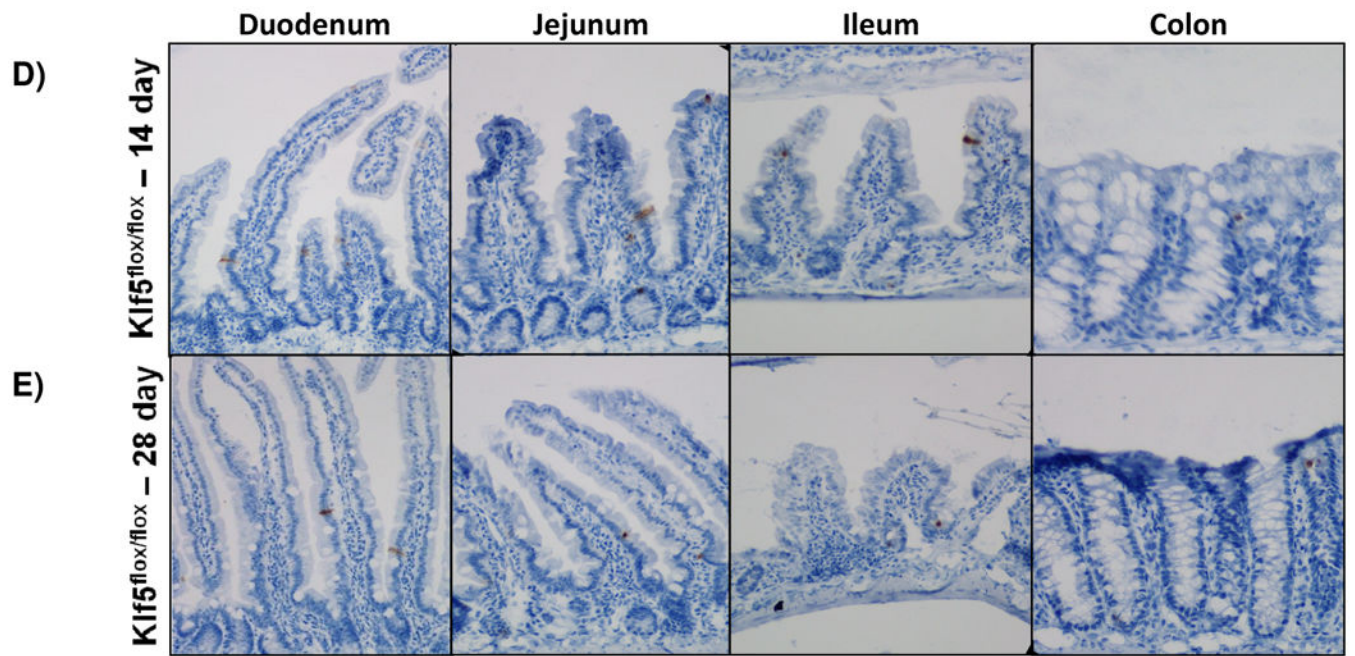
Figure 4. Intestinal crypt cell death is increased early after loss of KLF5, but disappears over time

(A-E) Immunohistochemical analysis of apoposis is shown using cleaved-caspase 3 (brown) counterstained with hemotoxylin (blue) in wildtype and Klf5 loss-of-function mice (3, 5, 14, 28 days post tamoxifen-induced recombination). (F) Quantification of the number of cleaved-caspase 3 positive cells per crypt. Significance determined between groups of two (wildtype vs. loss of Klf5 for 3 days, wildtype vs. loss of Klf5 for 5 days, wildtype vs. loss of Klf5 for 14 days, wildtype vs. loss of Klf5 for 28 days, and loss of Klf5 for 3 vs. 28 days). (Mean ± SD, T-test: * P   0.05, **P   0.01, ***P   0.001, n = 3)
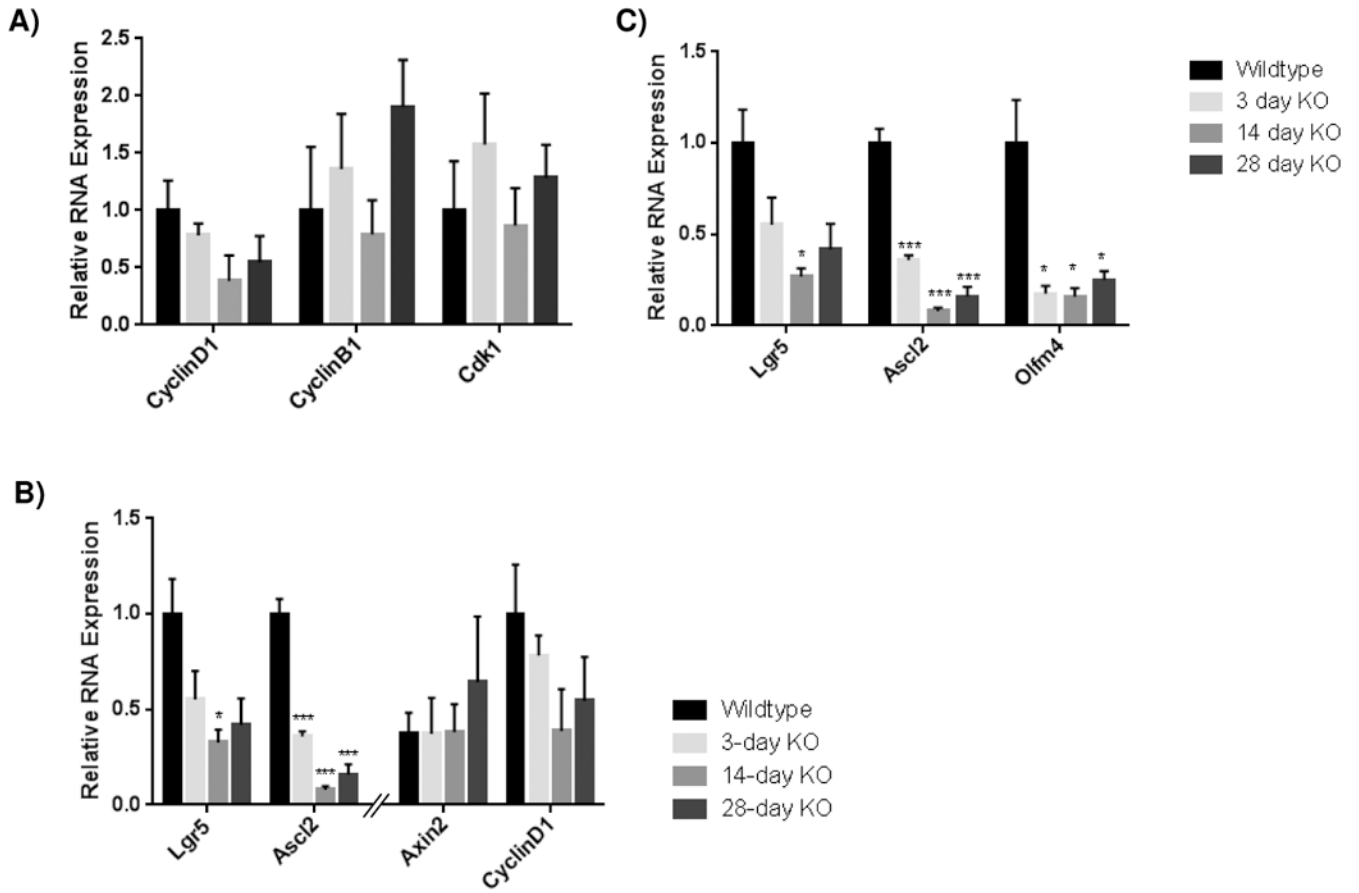
Figure 5. Loss of Klf5 results in loss of active stem cell marker expression in the small intestine
Results from of mRNA of expression for (A) cell cycle proteins (CyclinD1, CyclinB1, and Cdk1) , (B) downstream targets of canonical Wnt signaling (Lgr5, Ascl2, Axin2, and CyclinD1), and (C) active intestinal stem cell markers (Lgr5, Ascl2, Axin2, CyclinD1) by RT-qPCR from jejunal segments of the small intestine. Student's t-test was performed to determine p-values; expression was normalized to Gapdh (SYBR) or Actb (Taqman). (Mean ± SEM: P 0.05, **P 0.01, ***P 0.001, n = 3-6). Note that some qRT-PCR results for relative RNA expression appear on two graphs for comparison purposes.