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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

RESEARCH LETTER

Limitations to Understanding Intestinal Stem Cell Activity via Cre-Lox–Based Lineage Tracing

The intestinal epithelium is a highly proliferative tissue with robust regenerative potential, governed by the intestinal stem cell (ISC) compartment. Our understanding of the cellular hierarchy within this compartment is drawn from Cre-lox–based lineage tracing studies, initially via the identification of crypt base columnar stem cells (CBCs) using an $Lgr5^{eGFP\text{-}IRES\text{-}CreER}$ allele and the $R26^{LSL\text{-}LacZ}$ reporter,[1] showing that CBCs reconstitute all epithelial cell types during homeostasis.[2] Despite their proliferative capacity, CBCs are exquisitely sensitive to injury, requiring facultative stem cells for postinjury regeneration.[3,4] Many groups have shown facultative ISC activity within populations marked by a Cre-estrogen receptor fusion (CreER) targeted to *Hopx*, *Bmi1*, *Lrig1*, and additional loci.[5–7] These Cre drivers mark heterogeneous yet overlapping populations that give rise to all epithelial cell types in response to injury.[8] More recently, plasticity and facultative ISC activity have been described in populations marked by CreER reporters in loci purportedly specific to terminally differentiated cells, particularly in the secretory lineages.[9–12]

Facultative ISCs are lineage-committed cells that reacquire stem cell function in response to injury to facilitate tissue regeneration and are defined by in vivo lineage tracing, usually using tamoxifen (Tam)-inducible CreER recombinase, expressed via a putative cell type–specific promoter, to excise a locus of X-over P1 (loxP)-flanked stop cassette (lox-stop-lox, or LSL) proceeding a ubiquitous promoter and preceding a reporter protein (usually a fluorophore or β-galactosidase). Tam treatment results in irreversible reporter activation, enabling lineage tracing, and thus functionally defining a stem cell. Lox-stop-lox reporters typically are inserted into the *ROSA26* locus, and different reporter alleles often are used with the same CreER driver interchangeably under the assumption that they mark analogous populations. However, clear differences in recombination efficiencies exist across lox-stop-lox reporters.

We aimed to address these differences by directly comparing recombination efficiencies between two commonly used reporter alleles within the same cell. We generated mice harboring a $Hopx^{CreER}$ allele (among the most broadly used reporters of facultative ISC activity[3]), and heterozygous for each of two *ROSA26* reporters: $R26^{LSL\text{-}tdTomato}$[13] and $R26^{LSL\text{-}eYFP}$[14] (Figure 1*A* and *B*). This allowed us to directly compare recombination efficiency within the same cell, eliminating potential discrepancies from differences in Cre activity or expression levels. We induced recombination in $Hopx^{CreER}::R26^{LSL\text{-}tdTomato/LSL\text{-}eYFP}$ mice and probed via flow cytometry and immunofluorescence for the relative number of tandem-dimer Tomato (tdTomato)+, enhanced yellow fluorescent protein (eYFP)+, and double-positive cells (Figure 1*C*–*E*).

The shortest Tam regimen (1 × 24 hours) often is used to characterize putative parental stem cells before cell division and tracing into progeny. In response to this Tam regimen, approximately 1.5% of cells were tdTomato+, while eYFP+ (and double-positive) cells were nearly undetectable. Histologically, tdTomato+ cells were observed around the +4 position near the crypt base, a location and frequency consistent with previous studies[5,8] (Figure 1*C*). Mice receiving a 5 × 24-hour Tam regimen had frequent tdTomato+ cells in the crypt base and scattered throughout the villi. We found only rare instances of eYFP+/tdTomato+ cells (Figure 1*C*). Consistent with the histology, approximately 15% of epithelial cells were tdTomato+ at this time point, and <0.2% were eYFP+ or eYFP+/tdTomato+ (Figure 1*D* and *E*). After the longest Tam chase period, we found frequent ribbons of tdTomato+ cells, but only rare eYFP+/tdTomato+ ribbons. Here, tdTomato+ cells represented approximately 13% of the epithelium, while eYFP+ and eYFP+/tdTomato+ cells made up <0.1% (Figure 1*C*–*E*). Thus, the recombination efficiency of the $R26^{LSL\text{-}eYFP}$ allele is markedly less efficient than that of the $R26^{LSL\text{-}tdTomato}$ allele.

Next, we compared recombination efficiency using CreER alleles that mark mature cells within the secretory lineage, which recently has garnered attention as a source of facultative ISC activity.[9,10,11] First, to mark enteroendocrine cells, we used a $Chga^{CreER\text{-}2A\text{-}tdTomato}$ allele[15] coupled with either the $R26^{LSL\text{-}eYFP}$ or $R26^{LSL\text{-}LacZ}$ reporter.[1] Comparing the number of labeled cells in $Chga^{CreER}::R26^{LSL\text{-}eYFP}$ and $Chga^{CreER}::R26^{LSL\text{-}LacZ}$ mice, we observed significantly more LacZ-marked cells than eYFP-marked cells (Figure 2*A* and *B*). Next, to mark goblet cells, we used a novel $Muc2^{CreER}$ allele combined with either the $R26^{LSL\text{-}LacZ}$ or $R26^{LSL\text{-}tdTomato}$ reporter. We found significantly more tdTomato-marked cells than LacZ-marked cells (Figure 2*C* and *D*). Taken together, these results indicate that the $R26^{LSL\text{-}tdTomato}$ reporter is the most sensitive to Cre-mediated recombination, followed by the $R26^{LSL\text{-}LacZ}$, then $R26^{LSL\text{-}eYFP}$.

We postulate that discordance among reporters may result from differences in the size of the floxed stop cassette (because distance between loxP sites correlates inversely with recombination efficiency[16]), and/or variation in the sequences of the loxP sites. Indeed, the $R26^{LSL\text{-}tdTomato}$ reporter has a much smaller distance between the loxP sites (~900 bp) compared with the $R26^{LSL\text{-}LacZ}$ and $R26^{LSL\text{-}eYFP}$ alleles (~2.7 kb) (Figure 2*E*). However, neither distance nor loxP sequence can explain the difference in recombination efficiency between $R26^{LSL\text{-}LacZ}$ and $R26^{LSL\text{-}eYFP}$ alleles. It is possible that differences in detection methods

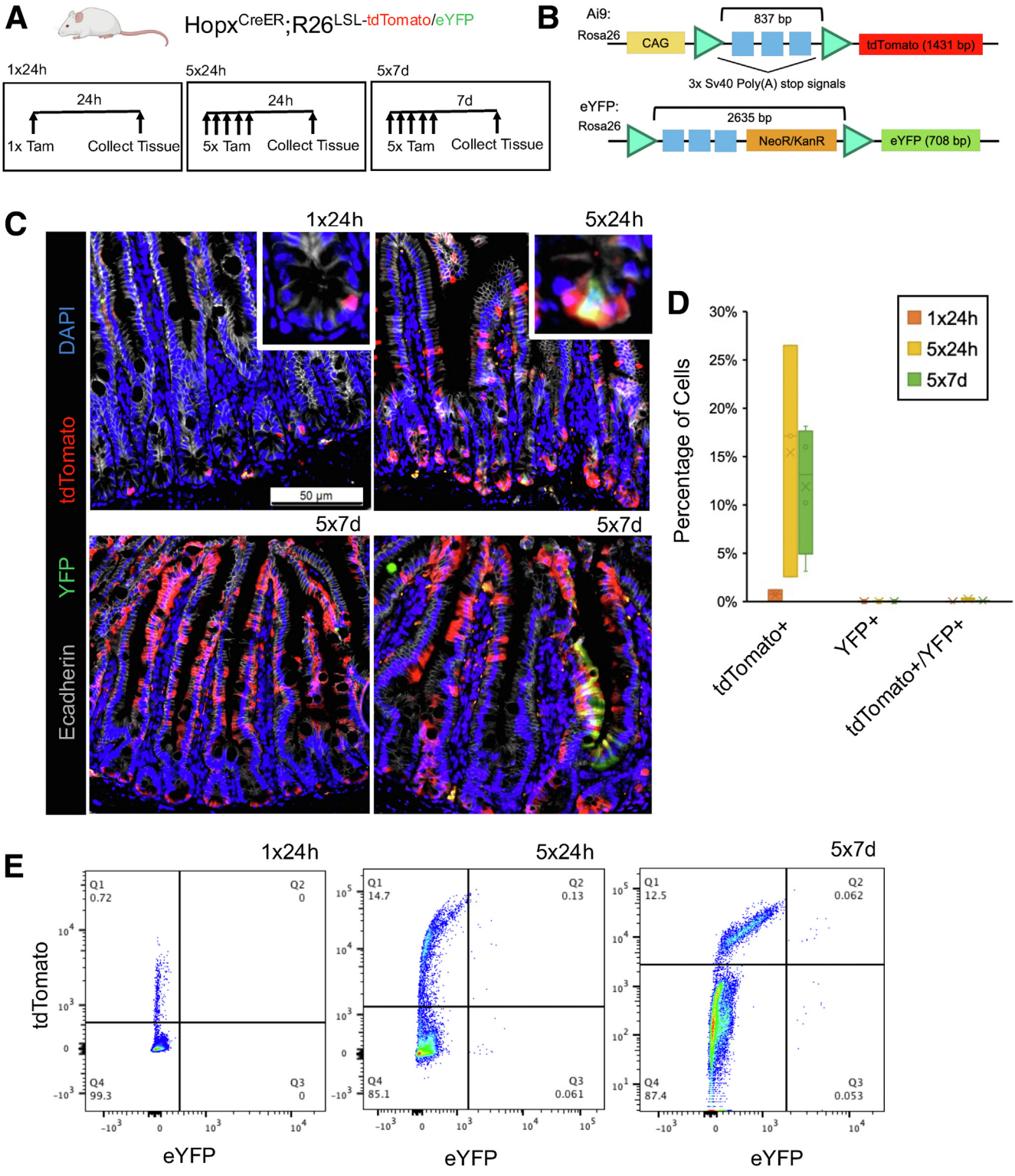

Figure 1. ***R26*$^{LSL\text{-}tdTomato}$ and *R26*$^{LSL\text{-}eYFP}$ recombination efficiencies within single *Hopx*$^{CreER+}$ cells.** (*A*) Tamoxifen schematic. (*B*) Schematic of *R26*$^{LSL\text{-}tdTomato}$ and *R26*$^{LSL\text{-}eYFP}$ reporter alleles. (*C*) Representative images of tdTomato and eYFP immunofluorescence staining of jejunum after different TAM regimens. *Scale bar*: 50 um. (*D*) Percentage of tdTomato+ and eYFP+ cells in small intestinal epithelium after different Tam regimens, measured by fluorescence-activated cell sorter. N = 3 mice/group. (*E*) Representative flow plots from mice quantified in panel *D*. CAG, (**C**) the cytomegalovirus (CMV) early enhancer element, (**A**) the promoter, the first exon and the first intron of chicken beta-actin gene, (**G**) the splice acceptor of the rabbit beta-globin gene; DAPI, 4′,6-diamidino-2-phenylindole.

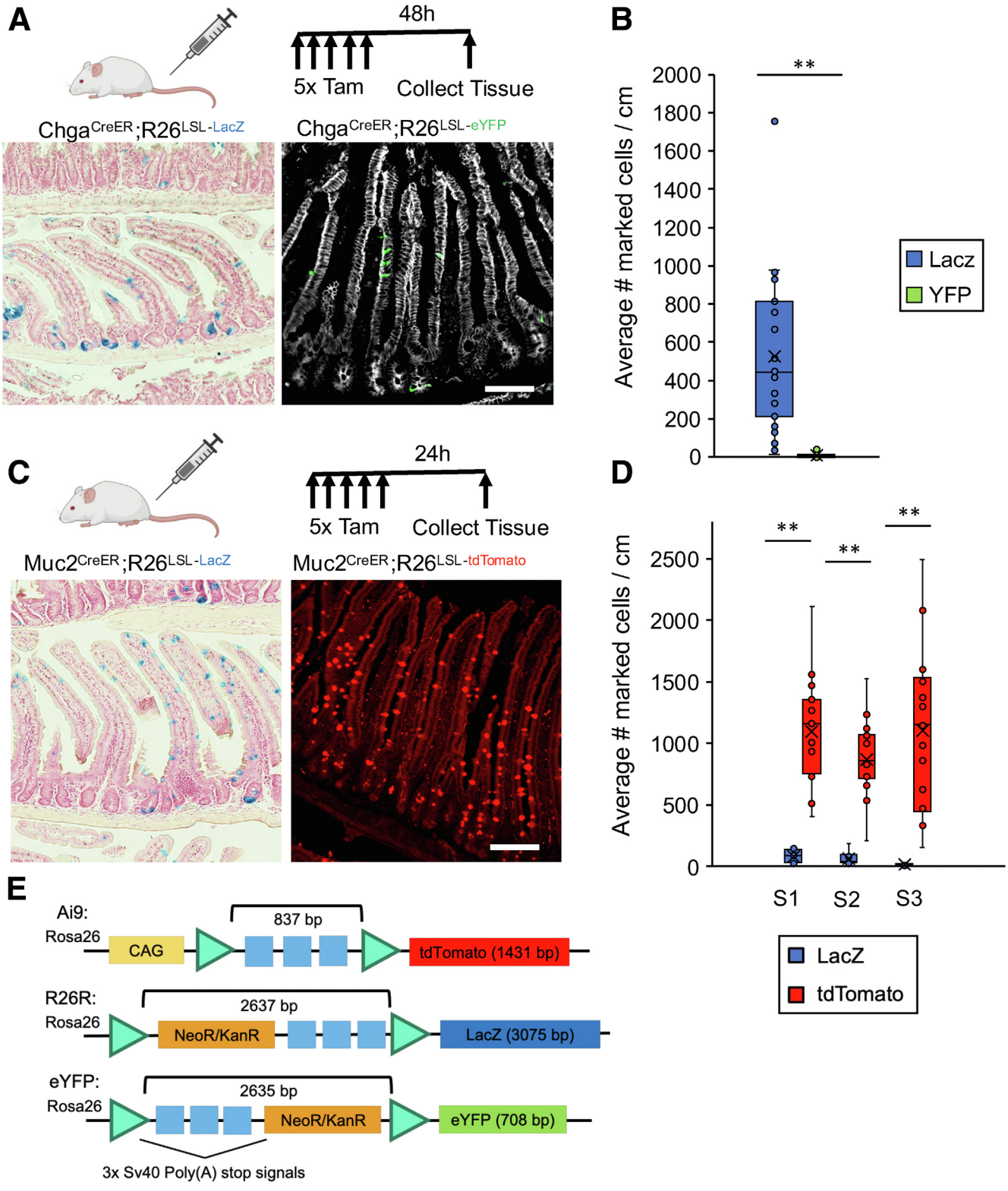

Figure 2. Relative efficiencies of *R26*$^{LSL\text{-}tdTomato}$, *R26*$^{LSL\text{-}LacZ}$, and *R26*$^{LSL\text{-}eYFP}$ reporters in goblet and enteroendocrine cells. (*A*) Histology and immunofluorescence of *Chga*^{CreER}*::R26*$^{LSL\text{-}LacZ}$ and *Chga*^{CreER}*::R26*$^{LSL\text{-}eYFP}$ small intestine 48 hours after 5 daily TAM doses. *Scale bar*: 100 um. (*B*) Quantification of marked cells from histology in panel *A*. N = 3 mice/group, n = 4 fields of view/mouse. $^{**}P < 10^{-6}$. (*C*) Histology and immunofluorescence of *Muc2*^{CreER}*::R26*$^{LSL\text{-}LacZ}$ and *Muc2*^{CreER}*::R26*$^{LSL\text{-}tdTomato}$ small intestine 24 hours after 5 daily TAM doses. *Scale bar*: 100 um. (*D*) Quantification of marked cells from histology in panel *C*. N = 3 mice/group, n = 4 fields of view/mouse. S1, S2, and S3 represent proximal, middle, and distal small intestine segments, respectively. S1, $^{**}P < 10^{-8}$; S2, $^{**}P < 10^{-9}$; and S3, $^{**}P < 10^{-6}$. (*E*) Schematic of *R26*$^{LSL\text{-}tdTomato}$, *R26*$^{LSL\text{-}LacZ}$, and *R26*$^{LSL\text{-}eYFP}$ alleles. CAG, (**C**) the cytomegalovirus (CMV) early enhancer element, (**A**) the promoter, the first exon and the first intron of chicken beta-actin gene, (**G**) the splice acceptor of the rabbit beta-globin gene.

could explain the greater proportion of LacZ+ cells (enzymatic detection, more sensitive) vs eYFP+ cells (immunofluorescence, less sensitive). We did not, however, directly compare all 3 reporter alleles with the same Cre driver, and thus these data should be interpreted with that limitation in mind.

Reporter choice is particularly important in the study of intestinal stem cell biology because quantifying the degree to which different cell populations contribute to regeneration is greatly influenced by reporter efficiency. To date, many Cre drivers have been reported to mark facultative ISCs (eg, *Dll1*, *Mex3a*, *Hopx*, *Bmi1*, *Lyz1*, *Clu*, *Atoh1*, *Krt19*, *Alpi*, *Lrig1*, *Sox9*, *mTert*, *Dclk1*, *Prox1*, and H2B-split-Cre), with postinjury lineage tracing events occurring at varying frequencies, from robust (>40% with $Hopx^{CreER}$), to exceedingly rare (<1% with $Dll1^{CreER}$).[17] Our findings highlight the importance of understanding recombination efficiencies when interpreting the literature describing these various proxy markers of facultative ISC activity. Our studies suggest that $R26^{LSL\text{-}YFP}$ is too inefficient to reliably gauge stem cell frequency, and although $R26^{LSL\text{-}tdTomato}$ readily recombines in target cell types, it may suffer from excess sensitivity because spurious recombination or recombination in progenitors upstream of the target cell type can be observed. The $R26^{LSL\text{-}LacZ}$ reporter is perhaps the best alternative, offering robust recombination with the added benefit of being amenable to whole-mount imaging, enabling the quantification of relatively rare events across large regions of tissue. Ultimately, interpretation of the literature describing facultative intestinal stem cell activity should be performed with caution and respect to reporter allele choice.

NICOLETTE M. JOHNSON
Medical Scientist Training Program
Perelman School of Medicine
Department of Biomedical Sciences
School of Veterinary Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

JEEYOON NA
KEARA E. MONAGHAN
Department of Biomedical Sciences
School of Veterinary Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

ALAN T. TANG
Department of Medicine
Perelman School of Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

YUHUA TIAN
NICOLAE A. LEU
NING LI
Department of Biomedical Sciences
School of Veterinary Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

MARK L. KAHN
Department of Medicine, Perelman School of Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

CHRISTOPHER J. LENGNER
Department of Biomedical Sciences
School of Veterinary Medicine
Department of Cell and Developmental Biology, Perelman School of Medicine
Institute for Regenerative Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

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Abbreviations used in this letter: **CBC, crypt base columnar stem cells; CreER, Cre-estrogen receptor fusion; eGFP, enhanced green fluorescent protein; eYFP, enhanced yellow fluorescent protein; IRES, internal ribosome entry site; ISC, intestinal stem cell; loxP, locus of X-over P1; LSL, lox-stop-lox; Tam, tamoxifen; tdTomato, tandem dimer tomato.**

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Correspondence
Corresponding author: Christopher Lengner, PhD, Biomedical Sciences, University of Pennsylvania, 3800 Spruce Street, Room 309EA, Philadelphia, Pennsylvania 19104. e-mail: lengner@vet.upenn.edu.

Conflicts of interest
The authors disclose no conflicts.

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Supplementary Materials and Methods

Animals

All mice used for these studies were between 20 and 30 weeks of age, fed ad libitum, and housed under standard University Laboratory Animal Resources conditions. The following mice were obtained from Jackson Laboratories (Bar Harbor, ME): Hopx-CreER (017606), R26-eYFP (006148), R26-tdTomato (007909), and R26-LacZ (003474); Chga-CreER mice were generated in house as previously described.[15] Muc2-CreER mice were generated as described later.

The Muc2-2A-CreERT2 targeted allele was generated by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-assisted homologous recombination in mouse embryonic stem cells. Briefly, a targeting construct was synthesized by Genscript (Piscataway, NJ) to insert a mouse codon optimized 2xV5 epitope tag-T2A-CreERT2 sequence in frame immediately before the Muc2 stop codon in the terminal exon. This open reading frame was followed by a flippase recognition target (FRT)-flanked Neomycin resistance cassette, which subsequently was removed by breeding to R26-FLPo germline deleter mice. A guide RNA sequence against the immediate downstream 3' untranslated region 5'-GACCTTCTCCACTCCTGGCT-3' was cloned into the eSpCas9(1.1) plasmid (71814; Addgene) and co-transfected with the targeting construct in V6.5 mouse embryonic stem (ES) cells with subsequent neomycin selection and propagation of appropriately targeted ES cells initially screened by polymerase chain reaction, and then verified by full-length sequencing of the insertion using primers flanking the arms of homology.

Genotyping primers used that distinguish zygosity: 5'-3' wild-type forward: GGATCACAGGTGCTCTTGCT; wild-type reverse: ATGTGCACGGTACAACCCAT; and mutant reverse: ACTTCCCCTGCCCTCTCC; wild-type band: 219 bp; mutant band: 310 bp.

To activate CreERT2-based alleles, mice received 1 mg tamoxifen doses dissolved in corn oil via intraperitoneal injection. To activate the Hopx-CreER allele, mice received one of the following tamoxifen regimens: 1 dose followed by cell harvest 24 hours later; 5 consecutive daily doses with harvest 24 hours later; or 5 consecutive daily doses followed by harvest 7 days later. To activate Chga-CreER and Muc2-CreER alleles, mice received 5 consecutive daily doses of tamoxifen with harvest 48 hours later.

Isolation of Small Intestinal Crypts and Fluorescence-Activated Cell Sorter Analysis

After the mice were killed, the gastrointestinal tract of the mice was dissected and the first 2 cm of duodenum was removed, the next 5 cm of jejunum was taken for histology, and the following 10 cm was isolated in phosphate-buffered saline. The tissue was briefly washed in fresh phosphate-buffered saline and subsequently was splayed open and transferred to a tube containing 10 mL 1× Hank's balanced salt solution with 1 mmol/L N-acetyl cysteine. After collection, the tissue was vortexed for 15 seconds followed by a 15-second rest on ice; this was performed repeatedly during a 2-minute period. The tissue then was transferred to a tube containing 10 mL 1× Hank's balanced salt solution with 1 mmol/L NAC and 10 mmol/L EDTA and was placed on a rotator in 4ºC for 45 minutes. After the incubation period, the tissue was vortexed for 30 seconds followed by a 30-second rest period on ice; this was performed repeatedly during a 3-minute period. After vortexing, the tissue digestion was filtered through a 70-umol/L filter and the flow-through was centrifuged at 300 × *g* for 3 minutes. To generate a single-cell suspension, the cell pellet was resuspended in a single-cell suspension buffer containing DNAse (35 ug/mL) and Liberase (20 ug/mL) (Sigma-Alrich, St. Louis, MO) and was incubated at 37ºC for 20 minutes. After digestion, the cells were washed in phosphate-buffered saline and resuspended in fluorescence-activated cell sorter buffer (phosphate-buffered saline with 4% fetal bovine serum) before fluorescence-activated cell sorter analysis. The viability dye 4′,6-diamidino-2-phenylindole was used to exclude dead cells. Cells were analyzed on an LSRFortessa (BD Biosciences, Franklin Lakes, NJ) and data analysis was performed using FlowJo software (BD Biosciences, Franklin Lakes, NJ).

Immunofluorescence and LacZ Staining

For immunofluorescence staining, the first 2 cm of duodenum was removed and the subsequent proximal 5 cm of jejunum was cut open lengthwise, Swiss-rolled, and fixed overnight in zinc formalin and then processed for paraffin embedding. Sections (5 um) from paraffin blocks were used for immunofluorescence staining with the following primary antibodies: tdTomato (dsRed mouse, 632392; Takara Biosystems, Kusatsu, Shiga, Japan; rabbit, 632496; Takara Biosystems, Kusatsu, Shiga, Japan), GFP (6673; Abcam, Cambridge, United Kingdom), E-cadherin (mouse, 610182; BD Biosciences, Franklin Lakes, NJ). All secondary antibodies were used at a 1:600 dilution.

LacZ staining was performed as previously described.[18] The entire length of the small intestine was divided into 4 segments, labeled as S1, S2, S3, and S4, with S1 being the most proximal and S4 being the most distal. Each segment was flushed with fixative and stained with X-Gal (10703729001; Sigma-Aldrich), Swiss rolled, embedded in paraffin, sectioned, and stained with neutral red.

Statistical Methods

Data were analyzed using unpaired and paired 2-tailed Student *t* tests, and *P* values are indicated in individual Figures. Specific experimental replicates are described in each Figure legend.

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