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Supplemental Information

Genome Toxicity and Impaired Stem Cell Function after Conditional Activation of CreER^{T2} in the Intestine

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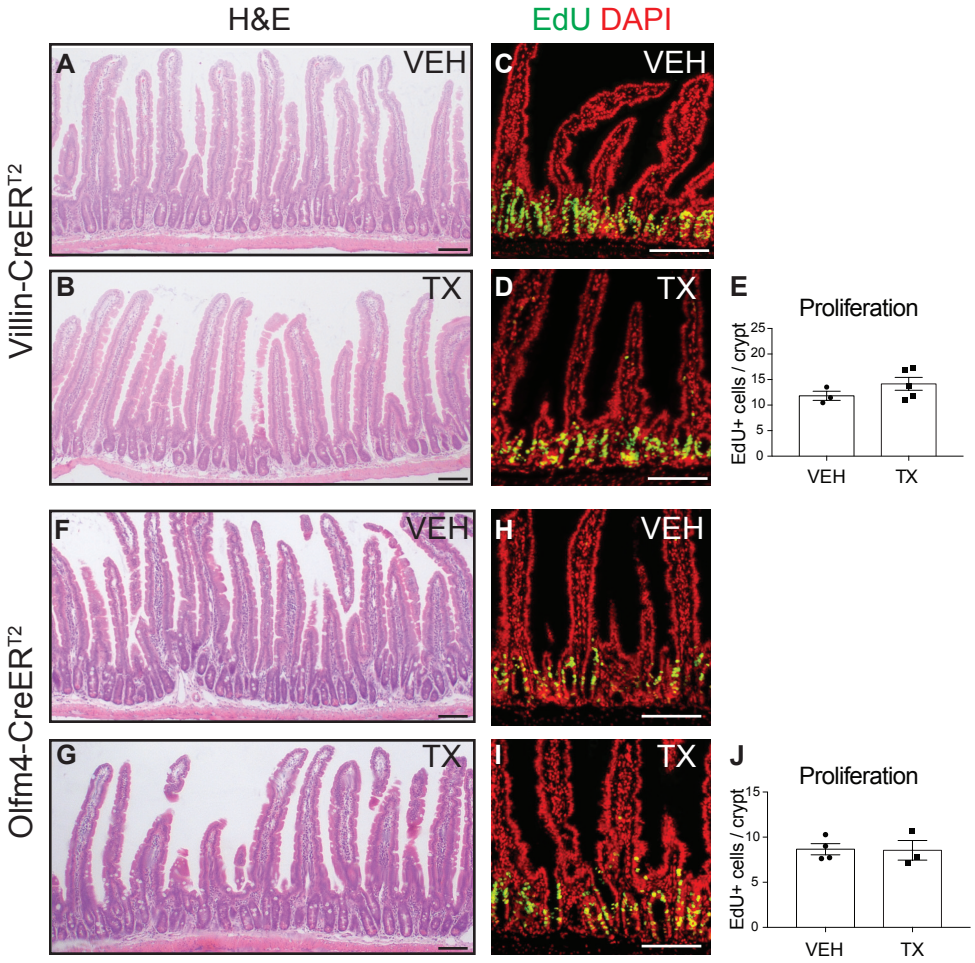

Figure S1. Normal intestinal histology in tamoxifen-treated *Villin-CreER^{T2}* and *Olfm4-CreER^{T2}* mice. Related to Figures 1 and 2.

Villin-CreER^{T2} and *Olfm4-CreER^{T2}* mice were treated with tamoxifen (TX; 100 mg/kg) or vehicle (VEH) daily for 5 days, and intestinal tissue was collected 1 day following the final injection. Duodenal histology was assessed by (A-B, F-G) H&E staining. (C-E, H-J) Cellular proliferation was assessed by EdU incorporation. Proliferating cells are presented as the number of EdU-positive cells per crypt (mean +/- SEM, n=3-5 mice/group).

Scale bars = 100µm.

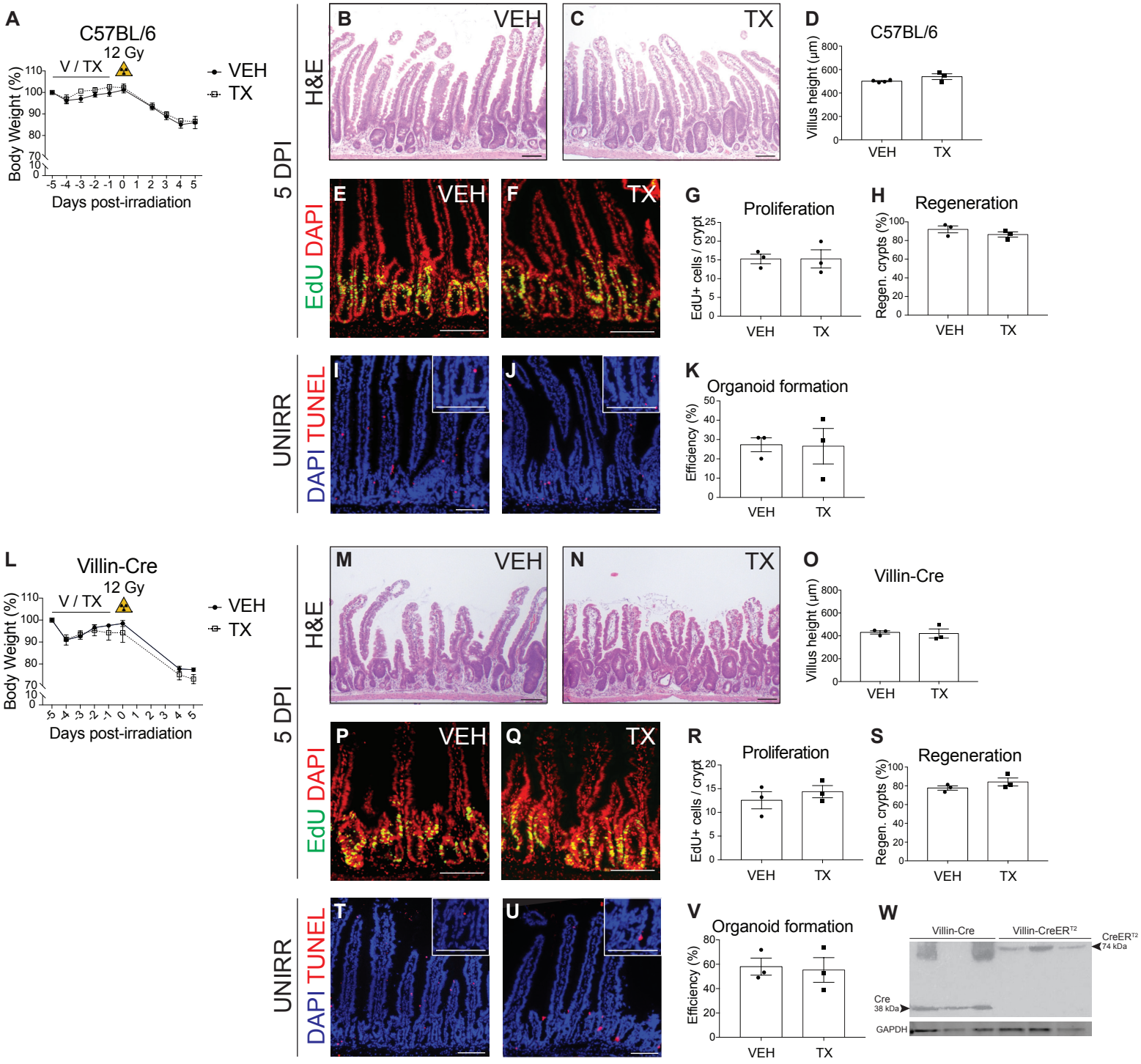

Figure S2. Normal intestinal regeneration and organoid formation in tamoxifen-treated C57BL/6 and *Villin-Cre* mice. Related to Figure 1.

(A-H) C57BL/6 and (L-S) *Villin-Cre* mice were treated with TX or VEH daily for 5 days, irradiated (12Gy) 1 day later, and intestinal tissue was collected at 5 DPI.

(A, L) Mouse body weight relative to weight at the initiation of treatment is presented as mean +/- SEM (n=3-6 mice/group). Duodenal crypt regeneration post-irradiation was assessed by (B-C, M-N) H&E staining and (E-F, P-Q) EdU incorporation.

(D, O) Villus height measurements presented as mean +/- SEM (n=3-4 mice/group).

(G, R) Proliferating cells are presented as the number of EdU-positive cells per crypt.

(H, S) Regenerating crypts were defined as intact crypts with 4 or more EdU-positive cells and presented as percent of the total crypts. Quantitative data are presented as mean +/- SEM (n=3 mice/group).

(I-J, T-U) TUNEL staining of unirradiated (UNIRR) TX- and VEH-treated C57BL/6 and *Villin-Cre* mice 24h following the last day of injection.

(K, V) C57BL/6 and *Villin-Cre* duodenal crypts were isolated from UNIRR TX- or VEH-treated mice and plated (200 crypts/well) to form organoids. Organoid formation efficiency is presented as mean +/- SEM (n=3 mice/group with 3 technical replicates per mouse).

(W) Western blot probing for Cre, and loading control GAPDH in duodenal crypt lysates of *Villin-Cre* and *Villin-CreER^{T2}* mice.

Duodenum images scale bars = 100µm.

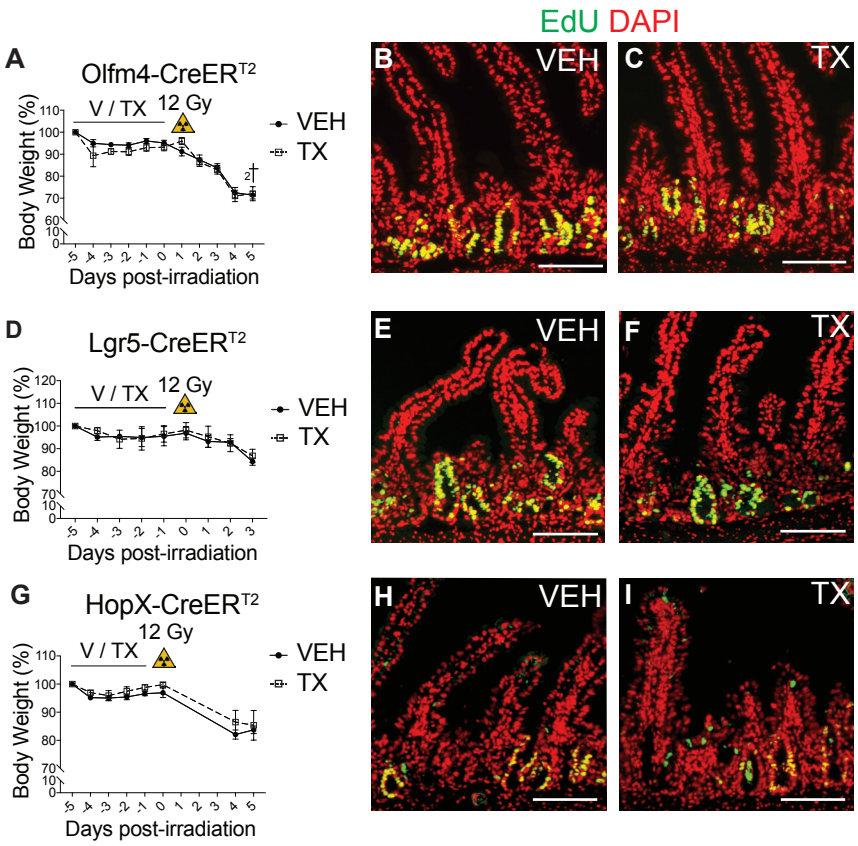

Figure S3. Normal post-irradiation regenerative responses after CreER^{T2} activation in intestinal stem cells. Related to Figure 2.
Mouse strains with TX-inducible CreER^{T2} drivers specific for active (*Olfm4-CreER^{T2}* and *Lgr5-CreER^{T2}*) or facultative (*HopX-CreER^{T2}*) intestinal stem cells were treated with TX or VEH daily for 5 days, irradiated (12Gy) 1 day later and tissue was collected at 3DPI.
(A, D, G) Mouse body weight relative to weight at the initiation of treatment is presented as mean +/- SEM (n=3-14 mice/group).
(B-C, E-F, H-I) Cellular proliferation was assessed by EdU incorporation (n=3-8). Scale bars = 100μm.

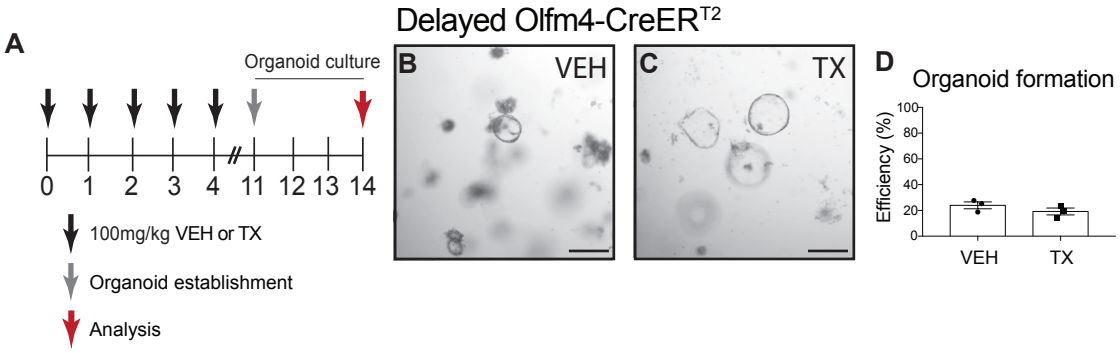

Figure S4. Tamoxifen-induced *Olfm4-CreER^{T2}* toxicity is abated by delay. Related to Figure 4.
(A) Schematic of organoid formation efficiency assay. Organoids were established from duodenal crypts isolated from *Olfm4-CreER^{T2}* mice 7 days after treatment with VEH or TX.
(B,C) Brightfield images of organoids 3 days post-establishment.
(D) Organoid forming efficiency was determined by counting the number of organoids in each well at 3 days post-establishment (n=3 mice/group with 3 technical replicates per mouse).
Scale bars = 250μm.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice

Mice were housed in ventilated and automated watering cages with a 12-hour light /dark cycle under specific pathogen-free conditions. The following mouse strains were employed: *Villin-CreER^{T2}* (el Marjou et al., 2004), *Villin-Cre* (Madison et al., 2002), *Olfm4-ires-EGFP-CreER^{T2}* (gift from Dr. Hans Clevers) (Schuijers et al., 2014), *Lgr5-EGFP-ires-CreER^{T2}* (JAX strain 008875) (Barker et al., 2007), *HopX-CreER^{T2}* (JAX strain 017606) (Takeda et al., 2011). Mice were maintained on a C57BL/6 strain background. Mice of both sexes aged 1.5-4 months were used.

Tissue collection

Intestinal tissue was harvested following *ad libitum* feeding and fixed in 4% paraformaldehyde in 1X PBS overnight before paraffin processing, as previously described (VanDussen et al., 2012). Intestinal crypts were harvested from duodenum, as previously described (Carulli et al., 2015).

Organoid culture

Mouse intestinal organoid cultures were established from duodenal crypts and maintained as described (Demitrack et al., 2015), with modifications. Longitudinally opened 6 cm of proximal intestinal tissue was washed in ice cold DPBS (Gibco), with antibiotics penicillin-streptomycin (1X) and gentamycin (1X; Gibco) for 20 min, cut into 1 cm pieces, and incubated in 15 mM EDTA in DPBS with antibiotics for 35 min at 4°C on a rocking platform. Tissue was vortexed for 2 min., and the solution was passed through a 70-µm filter. Crypts were gravity settled for 10min, the supernatant was decanted, the remaining pellet was resuspended in 1X DPBS with antibiotics, and centrifuged at 150x*g* for 10 min. The resulting crypt pellet was resuspended in complete culture media [50% L-WRN-conditioned media (Miyoshi and Stappenbeck, 2013), 20% fetal bovine serum (Atlas Biologicals), antibiotics, 2 mM L-glutamine (Gibco), 1X Fungizone (Gibco) and Y- 27632 (10 µM; Tocris) in advanced DMEM/F12 (Gibco)]. To test organoid formation efficiency, 600 crypts (extrapolated by determining crypt number per µL by counting crypts from a 5 µL droplet of crypt suspension) were mixed with 120 µL Matrigel (BD Biosciences), and 40 µL aliquots were plated in pre-warmed 24-well plates. After 30 min at 37°C, 500 µL complete culture media was overlaid. Culture media without Y-27632 was replaced every other day. The efficiency of organoid formation was determined by counting organoids at 3 days following plating, and normalizing to the number of plated crypts.

Immunohistochemistry

Immunostaining with rabbit α-Ki67 (1:200, Thermo Scientific RM-9106), and rabbit α-γ-H2AX (1:50, Cell Signaling 9718) was performed as described (Lopez-Diaz et al., 2006). A goat anti-rabbit IgG Alexa Fluor 488 polyclonal secondary antibody was used (1:400, Invitrogen A27034). EdU-Click-it kit (Life Technologies) was used to identify proliferating cells. Images were captured on a Nikon E800 microscope with Olympus DP controller software, except for γ-H2AX-immunostained images, which were captured on a Leica SP5 inverted confocal microscope with Leica software.

Western Blot Analysis

Isolated duodenal crypts were lysed in RIPA buffer (Thermo, 89900) containing protease and phosphatase inhibitor cocktail (Thermo Scientific, 78440). Cell lysates (40 µg protein) were mixed with NuPAGE LDS Sample Buffer (Thermo, NP0007) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using NuPAGE MOPS SDS Running Buffer (Thermo, NP0001) and NuPAGE 4-12% Bis-Tris gels (Thermo, NP0335), following manufacturer recommendations. Protein transfer onto 0.45µm pore size nitrocellulose membrane (GE Healthcare) at 100V for 45 min preceded blocking in Odyssey Blocking Buffer (Li-COR, 927-40000) for 1 hour at room temperature. Immunoblotting with rabbit α-γ-H2AX (1:50, Cell Signaling 9718), rabbit α-cleaved caspase 3 (1:500, Cell Signaling 9664), and mouse α-GAPDH (1:10,000, Thermo Scientific MA5-15738) was performed on a rocking platform overnight at 4°C. IRDye 800CW Goat α-rabbit (1:10,000, LI-COR 925-32211) and IRDye 680RD Goat α-mouse (1:10,000, LI-COR 925-68070) secondary antibodies were used to visualize probed proteins. Membrane was scanned on an Odyssey Imager (LI-COR). Western blot analysis was performed using the free Image Studio Lite software (LI-COR).

Gene integrity analysis

Quantitative polymerase chain reaction was performed as previously described (Lopez-Diaz et al., 2006), using 40ng DNA and *clox*P primers with sequences: GGT CTG AGC TAT ACT TAC AAA GGT (forward) and GCT ATC

ACA ATG GTG GTC CG (reverse), which yielded a 300 bp amplified product size. Assays for each sample were run in triplicate and normalized to *Gapdh* as an internal control, with primer sequences: TCA AGA AGG TGG TGA AGC AGG (forward) and TAT TAT GGG GGT CTG GGA TGG (reverse), which yielded a 350 bp amplified product size.

Statistical analysis

All experiments were performed with at least 3 biological replicates per group. Quantitative data are presented as mean ± SEM. Comparisons between 2 groups were conducted with unpaired two-tailed Student *t* tests using the Prism software (Graphpad). Significance is reported as * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), and $^{\#}$ ($P<0.0001$).

SUPPLEMENTAL REFERENCES

Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., et al. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature *449*, 1003–1007.

Carulli, A.J., Keeley, T.M., Demitrack, E.S., Chung, J., Maillard, I., and Samuelson, L.C. (2015). Notch receptor regulation of intestinal stem cell homeostasis and crypt regeneration. Dev. Biol. *402*, 98–108.

Demitrack, E.S., Gifford, G.B., Keeley, T.M., Carulli, A.J., VanDussen, K.L., Thomas, D., Giordano, T.J., Liu, Z., Kopan, R., and Samuelson, L.C. (2015). Notch signaling regulates gastric antral LGR5 stem cell function. EMBO J. *34*, 2522–2536.

Lopez-Diaz, L., Hinkle, K.L., Jain, R.N., Zavros, Y., Brunkan, C.S., Keeley, T., Eaton, K.A., Merchant, J.L., Chew, C.S., and Samuelson, L.C. (2006). Parietal cell hyperstimulation and autoimmune gastritis in cholera toxin transgenic mice. Am. J. Physiol. Liver Physiol. *290*, G970–G979.

el Marjou, F., Janssen, K.-P., Chang, B.H.-J., Li, M., Hindie, V., Chan, L., Louvard, D., Chambon, P., Metzger, D., and Robine, S. (2004). Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis *39*, 186–193.

Miyoshi, H., and Stappenbeck, T.S. (2013). In vitro expansion and genetic modification of gastrointestinal stem cells in spheroid culture. Nat. Protoc. *8*, 2471–2482.

Schuijers, J., van der Flier, L.G., van Es, J., and Clevers, H. (2014). Robust cre-mediated recombination in small intestinal stem cells utilizing the olfm4 locus. Stem Cell Reports *3*, 234–241.

Takeda, N., Jain, R., LeBoeuf, M.R., Wang, Q., Lu, M.M., and Epstein, J.A. (2011). Interconversion between intestinal stem cell populations in distinct niches. Science *334*, 1420–1424.

VanDussen, K.L., Carulli, A.J., Keeley, T.M., Patel, S.R., Puthoff, B.J., Magness, S.T., Tran, I.T., Maillard, I., Siebel, C., Kolterud, Å., et al. (2012). Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. Development *139*, 488–497.