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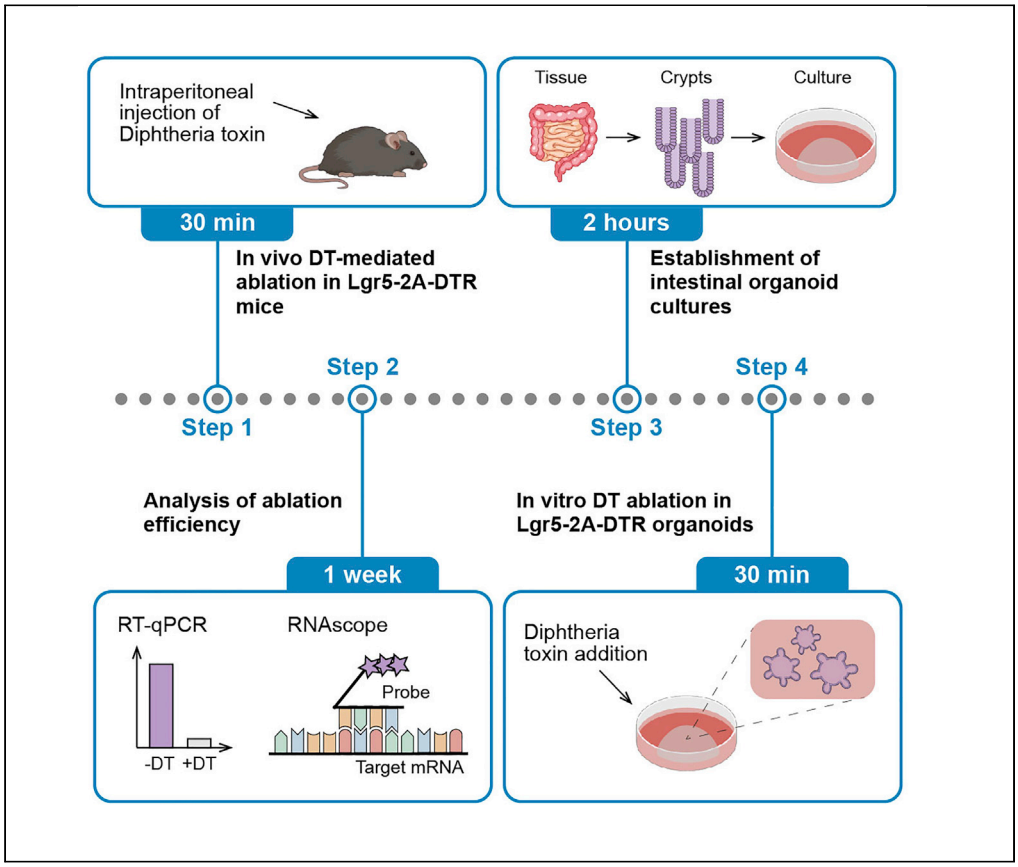
Targeted ablation of Lgr5-expressing intestinal stem cells in diphtheria toxin receptor-based mouse and organoid models



Intestinal cells marked by Lgr5 function as tissue-resident stem cells that sustain the homeostatic replenishment of the epithelium. By incorporating a diphtheria toxin receptor (DTR) cassette linked to the Lgr5 coding region, native Lgr5-expressing cells are susceptible to ablation upon DT administration *in vivo*. A similar strategy can be used for Lgr5-expressing cells within organoids established from DTR models. Together, these *in vivo* and *in vitro* approaches will facilitate dissection of the roles of Lgr5-expressing cells residing in different tissue compartments.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A diphtheria toxin (DT) system enables specific targeting of Lgr5+ cells in mouse models

DT administered *in vitro* also ablates Lgr5+ cells in mouse intestinal organoids

qPCR and histological analyses confirm ablation efficacy and phenotypes

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Targeted ablation of Lgr5-expressing intestinal stem cells in diphtheria toxin receptor-based mouse and organoid models

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SUMMARY

Intestinal cells marked by Lgr5 function as tissue-resident stem cells that sustain the homeostatic replenishment of the epithelium. By incorporating a diphtheria toxin receptor (DTR) cassette linked to the Lgr5 coding region, native Lgr5-expressing cells are susceptible to ablation upon DT administration *in vivo*. A similar strategy can be used for Lgr5-expressing cells within organoids established from DTR models. Together, these *in vivo* and *in vitro* approaches will facilitate dissection of the roles of Lgr5-expressing cells residing in different tissue compartments.

For complete details on the use and execution of this protocol, please refer to Tan et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the steps taken to establish a DT-mediated ablation strategy for targeted elimination of Lgr5-expressing cells within both an Lgr5-2A-DTR mouse model as well as murine intestinal organoids derived from the same mouse line. As Lgr5 is known to label stem cells in a number of adult epithelial tissues, this protocol can be further expanded to accommodate the study of stem cell behavior during homeostasis and under injury or disease contexts within other tissues of interest. We include in this protocol suggested steps to modify the DT dosing regimen when applying this to other tissues of interest, and how to evaluate ablation efficiency and resulting experimental outcomes. Moreover, this workflow can also be adapted for other ablation models, including our previously published Aqp5-2A-DTR model targeting Aqp5-expressing pyloric stem cells (Tan et al., 2020).

Institutional permissions

All animal procedures described in this protocol were approved by the A*STAR Institutional Animal Care and Use Committee (IACUC) and conducted in strict compliance with ethical guidelines and regulations. Approval must be sought from the relevant institutional review boards prior to the commencement of any animal work.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-activated Caspase 3 (1:200 final dilution)	Cell Signaling Technology	9661; RRID: AB_2341188

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rabbit monoclonal anti-Ki-67 (1:200 final dilution)	Thermo Scientific	MA5-14520; RRID: AB_10979488
Rabbit polyclonal anti-Lysozyme (1:200 final dilution)	Dako	A0099; RRID: AB_2341230
Mouse polyclonal anti-ChgA (1:200 final dilution)	Abcam	15160; RRID: AB_301704
Rabbit EnVision+	Dako	K4003; RRID: AB_2630375
Mouse EnVision+	Dako	K4001; RRID: AB_2827819
Chemicals, peptides, and recombinant proteins		
Diphtheria toxin	Sigma-Aldrich	D0564
Critical commercial assays		
Intesticult Organoid Growth Medium (Mouse)	STEMCELL Technologies	06005
Matrigel Growth Factor Reduced Basement Membrane Matrix	Corning	356231
RNAscope 2.5 HD Detection Reagents-BROWN	ACD	322310
RNeasy Universal Plus Mini Kit	QIAGEN	73404
Superscript III Reverse Transcriptase	Thermo Fisher Scientific	18080093
GoTaq qPCR Master Mix	Promega	A6002
Experimental models: Organisms/strains		
Mouse: Lgr5-2A-DTR line (adults with minimum age of 7–8 weeks, not selected for gender)	Seishima et al. (2019)	N/A
Mouse: C57BL/6J line (adults with minimum age of 7–8 weeks, not selected for gender)	InVivos	C57BL/6JInv
Oligonucleotides		
Lgr5 qPCR forward primer: AGAGCCTGATACCATCTGCAAAC	Tan et al. (2021)	N/A
Lgr5 qPCR reverse primer: TGAAGGTCGTCCACACTGTTGC	Tan et al. (2021)	N/A
Other		
1 mL syringe	BD	302100
25G needles	BD	305127
70 μm cell strainer	Falcon	352350
Nunc Cell-Culture Treated 48-well plate	Thermo Fisher Scientific	150687
1.5 mL Protein LoBind tube	Eppendorf	0030108442
C-Chip disposable hemocytometer	iNCYTO	DHC-N01
Dulbecco's phosphate-buffered saline (DPBS) without Calcium and Magnesium, 1×	Gibco	14190144
0.1 M EDTA Solution, pH 7.4	1st BASE	BUF-1051-500mL-pH7.4
Paraformaldehyde 16% Solution	Electron Microscopy Sciences	15710
Ethanol	Merck	100983
Xylene	Merck	108633
Target Retrieval Solution, pH 9 (10×)	Dako	S2367
CAS-Block	Life Technologies	008120
DAB+ Substrate Buffer	Dako	K3468
DAB+ Chromogen	Dako	K3468
Liquid Blocker Super Pap Pen	Agar Scientific	AGL4197S
Paraplast	Leica	39601006
Surgipath Micromount Mounting Medium	Leica	3801732
TRIzol Reagent	Invitrogen	15596026
RNaseZap	Invitrogen	AM9780
RNAscope Probe- Mm-Lgr5	ACD	312171
RNAscope Probe- Mm-Alpi	ACD	436781
RNAscope Positive Control Probe- Mm-Ppib	ACD	313911
RNAscope Negative Control Probe- DapB	ACD	310043

MATERIALS AND EQUIPMENT

Diphtheria toxin stock solution		
Reagent	Final concentration	Amount
Diphtheria toxin (lyophilized)	n/a	750 μg
Sterile distilled water	n/a	1 mL
Total	750 ng/μL	1 mL

Note: Diphtheria toxin stocks should be aliquoted as 20 μL solutions and stored at −20°C.

⚠ CRITICAL: Diphtheria toxin is highly toxic if inhaled or ingested. Proper laboratory PPE is required when handling this reagent.

STEP-BY-STEP METHOD DETAILS

Part 1: *In vivo* DT ablations

Intraperitoneal injection of DT into Lgr5-2A-DTR mice

Timing: 15 min

This step delivers DT into the peritoneal cavity of the mouse, enabling *in vivo* ablation of Lgr5-positive intestinal cells co-expressing the DT receptor. As the Lgr5-2A-DTR mouse model also allows for ablation of Lgr5-expressing cells in other tissues, we provide a suggested framework for testing and evaluating DT dosing regimens that can be applied to other tissue contexts.

1. Prepare fresh DT diluted to the desired concentration in 300 μL sterile PBS per 30 g of mouse weight. Previously published DT dosages and administration regimens are listed in the following table as a starting guide:

Tissue of interest	Mouse model	DT dosage (per 30 g mouse weight)	Administration regimen	References
Small intestine	Lgr5-2A-DTR	0.6 μg	4 consecutive doses daily	Tan et al. (2021)
Small intestine	Lgr5-DTR-GFP	1.5 μg	4 consecutive doses, once every 48 h	Tian et al. (2011)
Stomach	Aqp5-2A-DTR	0.5 μg	Once	Tan et al. (2020)
Stomach	Lgr5-DTR-GFP	0.6 μg	Once	Sakitani et al. (2017)
Skeletal muscle	Lgr5-2A-DTR	0.3 μg	Once	Leung et al. (2020)
Neonatal uterus	Lgr5-DTR-GFP	0.5 μg	Once	Seishima et al. (2019)

Note: If evaluating DT ablation in a new tissue of interest for the first time, perform a series of DT titrations in the range of 0.1–2.0 μg per 30 g mouse weight to determine the optimal dose that maximizes ablation efficacy while minimizing toxicity or off-target lethality effects.

Note: DT solution is kept on ice until use.

2. Aspirate the DT solution into a 1.0 mL syringe with a 26G needle attached.
3. Weigh each mouse prior to injection and adjust the volume of DT injected accordingly based on the mouse weight.

4. Carefully restrain the mouse by holding the loose skin on its back (scruffing). Prior to injection, tilt the mouse slightly downwards head-first.
5. Insert the needle into the lower right quadrant of the mouse abdomen at a 30°–45° angle.

   Note: The plunger should be drawn up slightly to check for negative pressure within the peritoneal cavity. If urine, blood, or other material is aspirated at this step, the needle may have entered the bladder, blood vessels or abdominal organs and the injection location should be adjusted.

6. Depress the plunger slowly until the full volume of DT is injected.
7. Return the mouse to its cage and monitor it for the next 3 days [troubleshooting 1].

Lgr5 expression analysis on ablated tissue

Timing: 1 day

This section of the protocol evaluates the efficiency of DT ablation by comparing Lgr5 transcript levels within ablated and non-ablated intestinal tissues. Intestinal tissue is harvested from treated and untreated Lgr5-2A-DTR mice 6 h after the last round of DT administration, and subsequently processed for RNA extraction, cDNA synthesis, and RT-qPCR to assess endogenous Lgr5 levels. The changes in Lgr5 expression levels following DT ablation compared to wild-type levels provide a quantitative readout for the efficiency of DT ablation. This expression analysis can be adapted for other tissues of interest with appropriate tissue isolation and washing steps prior to RNA extraction.

8. Sacrifice two Lgr5-2A-DTR mice, one untreated and one having received the DT dosing regimen, by cervical dislocation or CO_2 asphyxiation.
9. Dissect a 3–5 cm piece of duodenum from each mouse and place the tissue into a 10 cm petri dish filled with ice-cold PBS.
10. Flush out the contents of the intestine using an oral gavage needle with ice-cold PBS.
11. After washing, transfer the tissue into a 1.5 mL Eppendorf tube containing 1 mL TRIzol reagent. Pipette the solution up and down several times to thoroughly homogenize the sample.
12. Add 200 μL of chloroform to the TRIzol lysate and vortex the suspension for 10 s.
13. Centrifuge the suspension at 13,500 g for 10 min at 4°C to achieve a clear separation of the upper aqueous layer containing RNA and the lower organic layer containing DNA and proteins.
14. Transfer the clear aqueous upper layer into a new 1.5 mL Eppendorf tube, while discarding the lower organic layer.
15. To the aqueous solution, add an equal volume of 70% ethanol to precipitate the RNA, and mix the solution well by pipetting up and down for 10 s.
16. Perform RNA extraction according to manufacturer's instructions from the QIAGEN RNeasy Universal Plus Mini Kit (https://www.qiagen.com/us/resources/resourcedetail?id=a8f31442-95db-4cbd-9f9d-9e6a63b4af58&lang=en). Elute the RNA from the column using 30 μL RNase-free water.
17. Perform cDNA synthesis using random hexamer primers based on manufacturer's instructions from the Superscript III kit (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FsuperscriptIII_man.pdf). Add 3 μL of total RNA obtained from the previous step to each cDNA synthesis reaction.

cDNA synthesis cycling conditions

Steps	Temperature	Time
Combine RNA, random hexamer primers, dNTP, and water		
Denaturation	65°C	5 min
	4°C	>1 min

(*Continued on next page*)

Continued		
Steps	Temperature	Time
Addition of buffer, $MgCl_2$, DTT, RNase and Reverse Transcriptase to reaction mix		
Annealing	25°C	10 min
cDNA synthesis	50°C	50 min
Termination	85°C	5 min
Hold	4°C	Indefinitely

18. Run the Lgr5 RT-qPCR reactions in triplicate with the resulting cDNA preparation using Promega SYBR Green dye and validated Lgr5 primers from Tan et al. (2021), following manufacturer's instructions (https://www.promega.sg/-/media/files/resources/protocols/technical-manuals/101/gotaq-qpcr-master-mix-protocol.pdf?rev=b4a61a4c901142789c009b8d891a82c7&sc_lang=en) on a Quantstudio7 qPCR machine (or similar) [troubleshooting 2].

RT-qPCR cycling conditions			
Steps	Temperature	Time	Cycles
Activation	50°C	2 min	1
Pre-soak	95°C	10 min	
Denaturation	95°C	15 s	40
Annealing and extension	60°C	1 min	
Melt curve	95°C	15 s	1
	60°C	1 min	
	95°C	15 s	

Histological validation of ablation efficacy

Timing: 1 week

This step describes the procedures for performing standard histological analyses on formalin-fixed paraffin-embedded (FFPE) sections of isolated intestinal tissue. To complement the whole-tissue Lgr5 expression analysis described in the previous step, the effectiveness of the DT ablation strategy can be further verified by Lgr5 RNAscope staining performed on FFPE sections of ablated and non-ablated intestinal tissues isolated from Lgr5-2A-DTR mice, 6 h after administration of the final DT dose. This RNAscope protocol enables a direct evaluation of the prevalence of Lgr5 RNA transcripts within the intestinal stem cell compartment at the crypt base. At the same time, immunohistochemistry (IHC) performed against the apoptotic marker Caspase3 can reveal the presence and location of cell death occurring within the intestinal tissue following DT-mediated ablation of Lgr5-expressing cells. Due to morphological differences between the proximal and distal intestine, both qPCR and RNAscope/IHC analyses should be performed on a similar region of the tissue for a more reliable assessment of DT ablation effectiveness.

19. Sacrifice an Lgr5-2A-DTR mouse by cervical dislocation or CO_2 asphyxiation.
20. Dissect out the entire intestine and place it in a 10 cm ice-cold PBS-filled petri dish (Figure 1A).

Note: All tools should be cleaned with RNaseZap prior to handling the tissue to eliminate RNases from the environment that can compromise the RNA integrity in the isolated tissue. All reagents used should also be RNase-free.

21. Flush out the contents of the intestine using an oral gavage needle with ice-cold PBS.

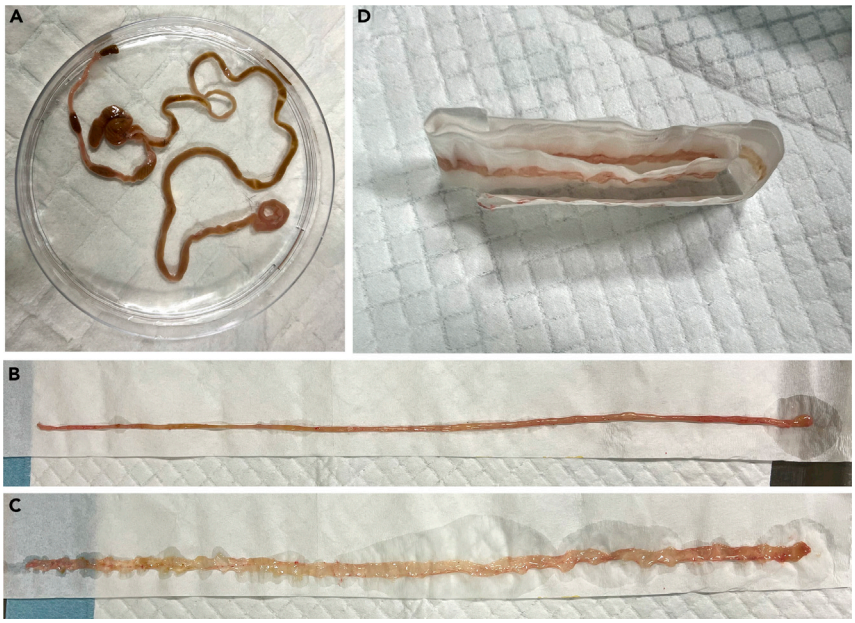

Figure 1. Images depicting small intestine dissection procedure

(A) Freshly dissected small intestine in ice-cold PBS in a petri dish.

(B) Small intestine laid out on a paper towel strip after being flushed of its contents.

(C) Small intestine spread open after being cut longitudinally.

(D) Final assembly and folding of paper towel over the small intestine tissue just prior to PFA fixation.

22. Lay out the flushed intestine in a straight line on a C-fold towel (Figure 1B).
23. Using a pair of dissecting scissors, cut open the intestine longitudinally and spread open the edges to flatten the tissue (Figure 1C).
24. Gently scrape the tissue with a cover slip once longitudinally to remove any excess mucus or remaining contents.
25. Carefully fold the C-fold towel over the tissue to flatten the tissue evenly (Figure 1D).
26. Place the C-fold cladded intestine in 4% paraformaldehyde (PFA) in PBS and incubate for 16–24 h on the bench at 20°C–25°C.
27. Following PFA fixation, wash the tissues three times in ice-cold PBS for 10 min each time on a shaker.
28. Carefully unfold and separate the C-fold towel from the tissue. Hold one end of the intestine strip in place using a pair of curved forceps, and slowly twist the strip around the forceps to create a "swiss roll" (Figures 2A–2C).
29. Transfer the rolled-up tissues into tissue cassettes and leave them submerged in 70% ethanol at 4°C for at least 16 h.

Pause point: Cassettes can be left in 70% ethanol for up to a week before dehydration.

30. The next day, dehydrate the tissues by placing the cassettes sequentially in a new 70% ethanol solution, two separate 95% ethanol solutions, and two separate 100% ethanol solutions. Leave the cassettes shaking for 1 h at 4°C in each solution.
31. Place the cassettes in two separate xylene solutions for 1 h at 20°C–25°C each time, before transferring them into paraffin at 65°C for at least 16 h.
32. The following day, transfer the cassettes into a new container of paraffin and keep them submerged for 1 h at 65°C.
33. Embed the tissues in paraffin within plastic molds and leave the paraffin blocks on a cool plate for at least 1 h to fully set.

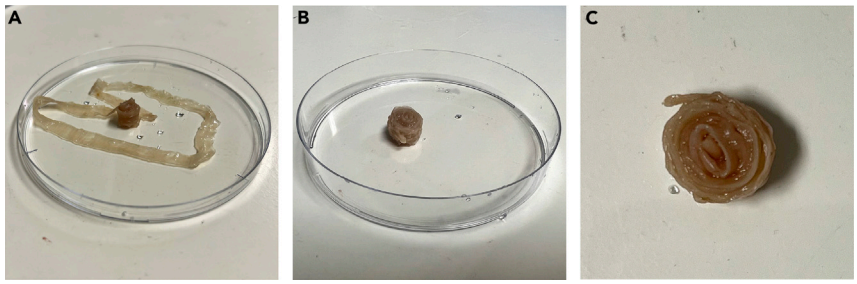

Figure 2. Images showing procedure for preparing the small intestine tissue for histological analysis
(A) Partially rolled tissue, highlighting the flattened strip of tissue following PFA fixation in C-fold towel.
(B) Fully-rolled tissue in a petri dish.
(C) Close-up view of the "swiss roll" from the fully-rolled tissue.

Pause point: Paraffin tissue blocks can be stored for months (except for RNAscope protocols).

34. Trim and section the paraffin blocks to obtain 7 μm tissue sections. Collect the tissue sections on glass slides and leave the slides at 20°C–25°C for at least 5 h to air dry.

Pause point: Slides can be stored for months before staining (except for RNAscope protocols).

After step 34, slides can be used for RNAscope:

35. Perform the RNAscope 2.5 High-Definition Brown Assay according to manufacturer's instructions (https://acdbio.com/system/files_force/322310-QKG%20RNAscope%202.5%20HD%20Brown.pdf?download=1), using the Mm-Lgr5 probe to detect Lgr5 transcripts, and the Mm-Ppib and DapB RNAscope probes as positive and negative controls respectively [troubleshooting 3].

After step 34, Caspase3 staining can also be performed in parallel on a separate set of tissue sections following standard immunohistochemistry protocols as described below:

36. Slot the slides into a slide rack and leave them baking in an oven set to 60°C for 10 min.
37. Deparaffinate the slides by dipping them sequentially in two separate xylene solutions, two separate 100% ethanol solutions, and two separate 70% ethanol solutions for 3 min each time.
38. Transfer the slides into a large container filled with running tap water and leave them submerged for 10 min.
39. After washing, transfer the slides into 80 mL of 1× pH9 Target Retrieval Solution diluted with distilled water at 20°C–25°C, then perform antigen retrieval in a pressure cooker heated to 121°C for 30 min.
40. Following antigen retrieval, leave the slides to cool to 20°C–25°C within the pressure cooker before placing on ice for 30 min.
41. Wash the slides by submerging them in running tap water for 10 min followed by PBS on a laboratory platform shaker for 5 min.
42. To block endogenous peroxidase activity, place the slides within a solution of 20 mL hydrogen peroxide diluted in 180 mL PBS and leave them shaking on a laboratory platform shaker for 20 min.
43. Wash the slides again by submerging them in running tap water for 10 min followed by PBS on a laboratory platform shaker for 5 min.
44. Remove each slide from the PBS and flick them strongly several times to remove excess water clinging to the tissue.

45. Using a liquid blocker wax pen, draw a hydrophobic barrier around the boundary of each tissue section, with care taken to avoid drawing across the tissue.
46. Immediately add 2–3 drops of CAS-block to the tissue.

Note: The entire tissue should be covered in CAS-block; if some areas are exposed, use a pipette tip to gently spread the solution across the exposed areas of the tissue.

47. Place the slides in a dark box for 1.5 h on the bench at 20°C–25°C.
48. During the incubation with CAS-block, prepare the Caspase3 primary antibody mix by diluting the antibody in 2% BSA at a concentration of 1:200.
49. After the CAS-block incubation, tap off the CAS-block from each slide and immediately add 2–3 drops of primary antibody mix to each tissue section. As before, all parts of the tissue should be covered with the antibody solution.
50. Place the slides in a dark box at 4°C for 16–24 h.
51. The next day, wash the slides three times in PBS on a laboratory platform shaker for 10 min each time.
52. Remove each slide from the PBS and flick them strongly several times to remove excess water clinging to the tissue.
53. Immediately add 2–3 drops of rabbit EnVision+ secondary antibody to the tissue. As before, all parts of the tissue should be covered with the antibody solution.
54. Place the slides in a dark box for 1 h on the bench at 20°C–25°C.
55. Following the secondary antibody incubation, wash the slides three times in PBS on a laboratory platform shaker for 10 min each time.
56. Add 1 drop of DAB+ chromogen to 2 mL of DAB+ substrate buffer and invert the solution several times to mix well.

Note: DAB is carcinogenic and should be handled with care following standard laboratory safety protocols.

57. Add 2–3 drops of DAB solution to the tissue for 3 min or until sufficient brown coloration has developed [troubleshooting 4].
58. Tap off the DAB solution from the slide to stop the reaction.
59. Perform counterstaining by placing the slides in Mayer's hematoxylin for 5 min, washing in running water for 5 min, then Methylene blue for 5 min, and washing again in running water for 5 min.
60. Dehydrate the slides by dipping them successively in two separate 70% ethanol solutions, two separate 100% ethanol solutions, and two separate xylene solutions, leaving them for 3 min each time.
61. To complete the IHC protocol, mount the slides with a cover slip and mounting media suitable for brightfield microscopy and leave them to dry in a fume hood for at least 2 h.

Histological analysis of phenotypic changes in ablated tissue

Timing: 1 week

This step describes methods for evaluating phenotypic changes in the intestinal epithelium following DT-mediated ablation of the Lgr5-expressing stem cell compartment. Hematoxylin and eosin (H&E) staining enables the visualization of large-scale disruptions in tissue architecture following ablation, while IHC performed against proliferation (Ki67) and lineage (Lysozyme, Chromogranin A and Muc2 labeling Paneth, enteroendocrine and goblet cells respectively) markers and RNAscope against Alpi (enterocyte marker) can offer insights into specific changes in the turnover pattern and cellular lineage composition within these ablated intestinal glands. These methods can also generally be adapted to other tissues of interest.

Continuing from step 34 of the previous section, slides can be used for H&E staining based on the following protocol:

62. Slot the slides into a slide rack and leave them baking in an oven set to 60°C for 10 min.
63. Deparaffinate the slides by dipping them sequentially in two separate xylene solutions, two separate 100% ethanol solutions, and two separate 70% ethanol solutions for 3 min each time.
64. Transfer the slides into a large container filled with running tap water and leave them submerged for 10 min.
65. Perform the staining by placing the slides in the following solutions:
    Richard Allan Hematoxylin for 5 min.
    Running water for 1 min.
    Acid alcohol for 30 s.
    Running water for 1 min.
    Scott's blue for 3 min.
    Running water for 1 min.
    Eosin for 10 s.
    Running water for 1 min.
66. Dehydrate the slides by dipping them successively in two separate 70% ethanol solutions, two separate 100% ethanol solutions, and two separate xylene solutions, leaving them for 3 min each time.
67. To complete the H&E protocol, mount the slides with a cover slip and mounting media suitable for brightfield microscopy and leave them to dry in a fume hood for at least 2 h.

Following step 34 of the previous section, slides can be used for IHC against proliferation and lineage markers, with the following changes to the earlier described protocol:

68. At step 39, use the pH6 Target Retrieval Solution for slides to be stained with Ki67 antibody.
69. At step 48, prepare the Ki67, Lysozyme, and Chga antibodies at a dilution of 1:200.
70. At step 53, use the mouse EnVision+ secondary antibody against the mouse anti-ChgA.

Following step 34 of the previous section, slides can also be used for RNAscope against Alpi, following manufacturer's instructions.

Part 2: *In vitro* DT ablations

Establishment of Lgr5-2A-DTR murine intestinal organoid cultures

Timing: 2 h

This section of the protocol describes steps taken to generate intestinal epithelial organoids from Lgr5-2A-DTR and wild-type mice.

71. 1 day prior to the start of the experiment, thaw Matrigel on ice in a 4°C fridge for at least 12–16 h.
72. Sacrifice a Lgr5-2A-DTR and a wild-type mouse by cervical dislocation or CO_2 asphyxiation.
73. Dissect out the duodenum from each mouse and collect the tissues in a 10 cm petri dish containing ice-cold PBS (without Mg^{2+} and Ca^{2+}).
74. Flush out the contents of the duodenum using ice-cold PBS (without Mg^{2+} and Ca^{2+}) with an oral gavage needle.
75. Cut the duodenum longitudinally with a pair of dissecting scissors and spread open the tissue on the lid of the petri dish with the lumen side up.
76. Gently scrape the tissue with a cover slip once longitudinally to remove any excess mucus or remaining contents.
77. Transfer the tissue into a 50 mL Falcon tube containing 50 mL ice-cold 2 mM EDTA in PBS (without Mg^{2+} and Ca^{2+}) and leave shaking on a rolling platform at 4°C for 45 min, to achieve

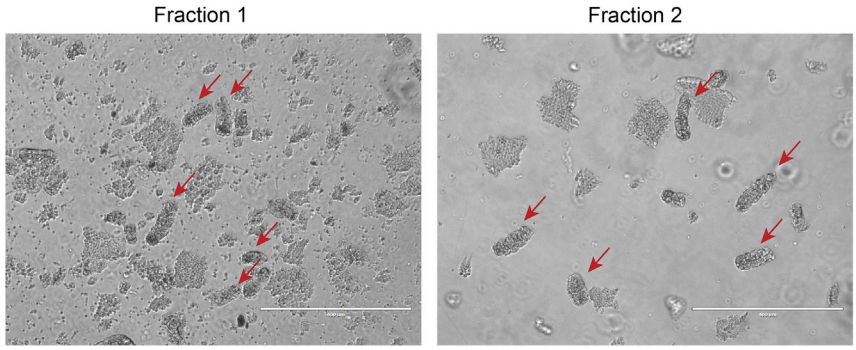

Figure 3. Examples of intestinal crypt suspension fractions as seen under a 10× objective of a standard brightfield microscope

Whole crypts present in each fraction are highlighted by the red arrows. Scale bars represent 400 μm.

efficient tissue dissociation into epithelial fragments that include crypts housing the intestinal stem cells.

78. Following incubation, transfer the epithelial fragments into a new 50 mL Falcon tube containing 10 mL PBS (without Mg^{2+} and Ca^{2+}) and shake the tube vigorously 20 times. This tube is retained as Fraction 1.
79. Transfer the remaining tissue to a second 50 mL Falcon tube containing 10 mL PBS (without Mg^{2+} and Ca^{2+}) and shake it vigorously 20 times. This tube is labeled as Fraction 2.
80. Pipette 10 μL of crypt suspension from each fraction into a glass slide and assess the quality of each fraction under the microscope. The fraction with a higher number of complete intestinal crypts with intact crypt bases is selected as the higher quality fraction with greater potential for organoid formation (Figure 3).
81. Filter the higher quality fraction through a 70 μm cell strainer into a new 50 mL Falcon tube.
82. Pipette 10 μL of the filtered crypt suspension into a hemocytometer and manually count the number of crypts under a light microscope.
83. Transfer an appropriate volume of crypt suspension into 1.5 mL protein LoBind tubes to obtain approximately 150 crypts per well of a 48-well plate. This can be adapted for other plate sizes:

Type of culture plate	Surface area per well (cm^2)	Number of crypts per well
6-well plate	9.6	1500
12-well plate	3.5	500
24-well plate	1.9	250
48-well plate	1.1	150
96-well plate	0.32	50

84. Spin the tubes in a microcentrifuge at 350 g for 5 min.
85. Carefully remove and discard the supernatant. Resuspend the remaining pellet in an appropriate amount of Matrigel sufficient to form a 50 μL drop per well.
86. Add 50 μL crypt-Matrigel suspension to the center of each well of a pre-warmed 48-well culture plate. Place the plate in a tissue culture incubator set to 37°C, 5% CO_2 for at least 5 min for the suspension to set.
87. Once the Matrigel has solidified, add 300 μL of Intesticult Organoid Growth Medium (OGM) to each well and leave the cultures to grow in a tissue culture incubator set to 37°C with 5% CO_2.

DT administration during initiation phase of intestinal organoid establishment

Timing: 30 min

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This step ablates Lgr5-expressing intestinal stem cells present in dissociated intestinal crypts at the point of plating, under culture conditions supporting organoid outgrowth. Wild-type intestinal crypts are plated under identical conditions in the presence of DT to account for non-specific effects of the DT toxin on organoid outgrowth. This *in vitro* ablation strategy allows for an assessment of the requirement of Lgr5-expressing cells during the organoid initiation phase.

88. Resuspend dissociated Lgr5-2A-DTR or wild-type intestinal crypts with Matrigel at a ratio of 150 crypts per 50 μL Matrigel and plate them into pre-warmed 48-well culture plates, as described in the earlier section.
89. Once the Matrigel suspension has set, add 300 μL of Intesticult OGM only or 300 μL of Intesticult OGM with a final concentration of 0.05 μg/mL DT to each organoid culture well [troubleshooting 5]. At least three wells per condition are required as technical replicates in each experiment.

Note: For culture plates of other sizes, the volume of media added can be adjusted as follows:

Type of culture plate	Volume of media per well (μL)
6-well plate	2000
12-well plate	1000
24-well plate	600
48-well plate	300
96-well plate	150

90. Leave the organoid cultures to grow in a standard tissue culture incubator set to 37°C and 5% CO_2.
91. After 5 days of culture, manually count the number of organoids that have grown out in each well under a light microscope and tabulate the data accordingly (Table 1).

DT administration to propagating intestinal organoid cultures

Timing: 15 min

This step ablates Lgr5-expressing intestinal stem cells within propagating intestinal organoids in culture. Dissociated intestinal crypts are initially plated in the absence of DT under culture conditions supporting organoid outgrowth, and DT is subsequently administered to the established organoids. This strategy assesses the requirement of Lgr5-expressing cells for sustaining the propagation of established organoids.

Table 1. Tabulation of organoid counts following DT administration during organoid initiation phase

		Organoid counts			
Organoid line	Condition	Well1	Well2	Well3	Average
Lgr5-2A-DTR Line1	Untreated				
	DT treated				
Lgr5-2A-DTR Line2	Untreated				
	DT treated				
Lgr5-2A-DTR Line3	Untreated				
	DT treated				
WT Line1	Untreated				
	DT treated				
WT Line2	Untreated				
	DT treated				
WT Line3	Untreated				
	DT treated				

Table 2. Tabulation of organoid counts following DT administration to established organoid cultures during organoid propagation phase

			Organoid counts			
Organoid line	Condition	Timepoint	Well1	Well2	Well3	Average
Lgr5-2A-DTR Line1	Untreated	Day 0				
	Untreated	Day 1/2/3				
	DT-treated	Day 0				
	DT-treated	Day 1/2/3				
Lgr5-2A-DTR Line2	Untreated	Day 0				
	Untreated	Day 1/2/3				
	DT-treated	Day 0				
	DT-treated	Day 1/2/3				
Lgr5-2A-DTR Line3	Untreated	Day 0				
	Untreated	Day 1/2/3				
	DT-treated	Day 0				
	DT-treated	Day 1/2/3				
WT Line1	Untreated	Day 0				
	Untreated	Day 1/2/3				
	DT-treated	Day 0				
	DT-treated	Day 1/2/3				
WT Line2	Untreated	Day 0				
	Untreated	Day 1/2/3				
	DT-treated	Day 0				
	DT-treated	Day 1/2/3				
WT Line3	Untreated	Day 0				
	Untreated	Day 1/2/3				
	DT-treated	Day 0				
	DT-treated	Day 1/2/3				

92. Using an established Lgr5-2A-DTR and a wild-type intestinal organoid culture (typically 5 days after plating), manually count the number of organoids present in each well under a light microscope and the data tabulated accordingly (Table 2).
93. Remove the culture media in each well.
94. Add 300 μL of fresh Intesticult OGM only or 300 μL of fresh Intesticult OGM with 0.6 μg/mL DT to each organoid culture well [troubleshooting 5]. At least three wells per condition are required as technical replicates in each experiment.
95. Monitor the organoids daily. After 1/2/3 days post-DT administration, manually count the number of organoids in each well under a light microscope and tabulate the results accordingly (Table 2).

Note: Organoid counts over multiple timepoints can also be tabulated to quantify the dynamics of sustained DT ablation in the intestinal organoid cultures over time.

EXPECTED OUTCOMES

Efficient ablation of Lgr5-expressing cells *in vivo* should result in dramatic reductions in Lgr5 expression as determined by RT-qPCR on isolated tissue (approximately 90% decrease can be expected for the small intestine), and minimal (if any) detection of endogenous Lgr5 RNA transcripts within the intestinal stem cell compartment at the crypt base by RNAscope (Figure 4). Caspase 3 IHC within DT-ablated tissues should reveal evidence of cell death present at the intestinal crypt bases (Figure 4). With sustained ablation of Lgr5-expressing cells, phenotypic changes to intestinal architecture should also be apparent by hematoxylin and eosin (H&E) staining (Figure 4).

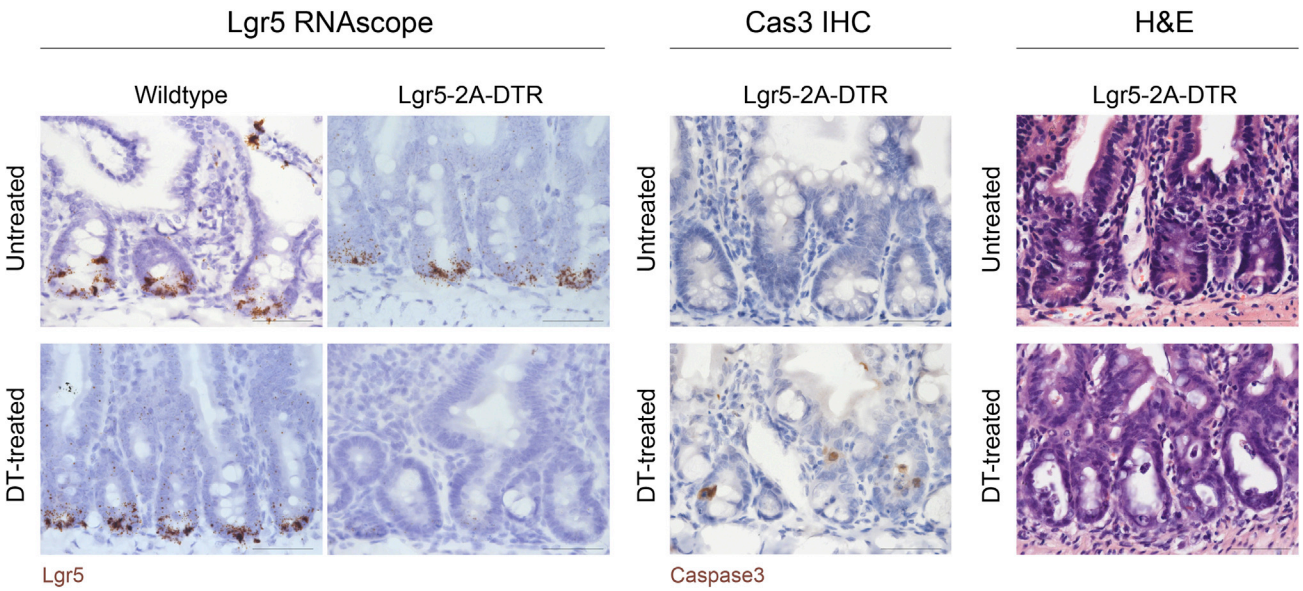

Figure 4. Expected outcomes from *in vivo* ablation of Lgr5-expressing cells within the native intestinal epithelium by DT administration

Wild-type control was used alongside Lgr5-2A-DTR tissue to account for non-specific toxicity effects of DT. Lgr5 transcript levels were significantly depleted in DT-treated Lgr5-2A-DTR tissue as determined by RNAscope (left), confirming the efficacy of the treatment. This is accompanied by an increase in numbers of Caspase3-positive apoptotic cells within intestinal crypt bases (middle) and altered intestinal epithelial architecture (right) in DT-treated mice. Scale bars represent 50 μm. Images used in this figure were adapted from Tan et al. (2021).

In vitro, ablation of the Lgr5-expressing intestinal stem cells is also expected to compromise both organoid initiation and growth (Figure 5). In contrast, the same DT treatment regimen performed on wild-type organoids should have negligible effects on organoid maintenance.

QUANTIFICATION AND STATISTICAL ANALYSIS

At least 3 biological replicates should be performed for each experiment. Technical replicates should also be performed for the *in vitro* ablation assays, with 3 organoid wells plated for each condition. The individual organoid counts obtained are then averaged. Tables 1 and 2 below illustrate how the data should be collected.

LIMITATIONS

Lgr5 has been identified as a marker of tissue-resident stem cells in multiple tissues, including intestine, stomach, hair follicle, uterus, ovary and kidney (Barker et al., 2007, 2010, 2012; Jaks et al., 2008; Ng et al., 2014; Seishima et al., 2019). The *in vivo* ablation method targeting Lgr5-expressing cells in Lgr5-2A-DTR mouse models thus exerts a wide-ranging effect on multiple tissues and stem cell compartments, leading potentially to phenotypes arising outside of the tissue of interest. To address this, intestinal epithelial organoids can be generated from Lgr5-2A-DTR mice to evaluate the specific requirement of Lgr5-expressing cells in this context. The three-dimensional, budding form of these intestinal organoids recapitulates to a significant extent the crypt-villus morphology of the intestinal epithelium, thus serving as a close approximation of the native tissue.

A second difficulty arises from the cellular plasticity of differentiated cell lineages within the intestine that are able to revert to a stem-like state (Buczacki et al., 2013; Jadhav et al., 2017). As such, ablation of Lgr5-expressing cells can trigger the conversion of other surviving cells into new Lgr5-positive cells, necessitating the use of successive rounds of DT administration in order to thoroughly eliminate Lgr5-expressing cells from the tissue and accordingly reveal associated phenotypes.

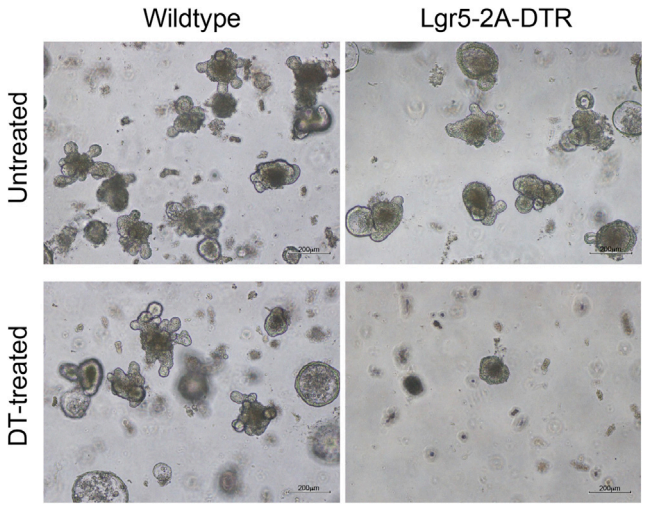

Figure 5. Expected outcomes from *in vitro* DT-mediated ablation of Lgr5-expressing cells within intestinal epithelial organoids

DT-treated Lgr5-2A-DTR cultures lacking viable Lgr5-expressing stem cells are not able to establish intestinal organoids, whereas DT-treated wild-type cultures retain the ability to robustly form organoids at similar levels as untreated cultures. Scale bars represent 200 μm.

Beyond the mouse models discussed in this protocol, the DT system may be less suited for cells that express the human heparin-binding epidermal growth factor (EGF)-like growth factor (hHB-EGF). When localized to the membrane, hHB-EGF can act as a receptor for DT, which could result in unintended ablation of these cell populations (Mitamura et al., 1995). In these systems, other targeted cell ablation methods such as laser ablations or inducible activation of caspases could be considered.

TROUBLESHOOTING

Problem 1

High frequency of lethality observed in mice receiving intraperitoneal injections of DT, 1–3 days following DT administration. In the Lgr5-2A-DTR mouse model, DT administration ablates not only the Lgr5-expressing crypt stem cells within the intestine but also other Lgr5-expressing cells in multiple tissues throughout the body. DT itself, when administered at high doses, could also induce non-specific toxicity effects and damage independent of its ability to ablate DTR-expressing cells (Chapman and Georas, 2013) (part 1: in vivo DT ablations, step 7).

Potential solution

Proper titration of DT concentrations and evaluation of ablation specificity by histological analysis are essential steps prior to starting any experiments. The lethality effects can further be overcome by varying the DT dosage and/or frequency of DT administration.

Problem 2

qPCR analysis indicates very low or undetectable levels of Lgr5 in both ablated and wild-type samples (part 1: in vivo DT ablations, step 18).

Potential solution

Lgr5 qPCR reactions should be run alongside a housekeeping gene such as Gapdh in order to normalize for variations in sample input across different experimental conditions. Gapdh is strongly expressed and should yield a low Ct value in the range of 15–20. If the Gapdh results deviate significantly from this value, there could be an issue with the amount of cDNA template used for the qPCR

reaction. Different dilutions of the template could be tested to improve the readout from this experiment.

Problem 3

Absence of RNAscope signal in tissue sections, even when using the positive control probe (part 1: in vivo DT ablations, step 35).

Potential solution

Proper processing and storage of FFPE tissues are critical steps in preserving high tissue and RNA quality for RNAscope analysis. Tissues should be processed in an RNase-free environment and RNAscope performed on freshly prepared tissue blocks where possible. Extended storage of tissues for more than 6 months may affect the quality of the RNAscope results.

Problem 4

IHC signal intensity is too strong, with non-specific background signal appearing (part 1: in vivo DT ablations, step 57).

Potential solution

One solution is to reduce the length of time for DAB staining. In general, when using a new primary antibody or performing IHC on a new type of tissue, different retrieval solutions and antibody concentrations should be tested to identify the optimal conditions. A known positive control should be used alongside the tissue of interest.

Problem 5

Impaired establishment or growth observed in wild-type organoid cultures receiving DT (part 2: in vitro DT ablations, steps 89 and 94).

Potential solution

Prior to starting the experiment, titrate DT dosage by evaluating a range of DT concentrations to determine an optimal concentration with minimal toxicity effects on wild-type organoids.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nick Barker (nicholas_barker@imcb.a-star.edu.sg).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets or code.

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AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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