
Figures and figure supplements

Radical and lunatic fringes modulate notch ligands to support mammalian intestinal homeostasis

Preetish Kadur Lakshminarasimha Murthy *et al*

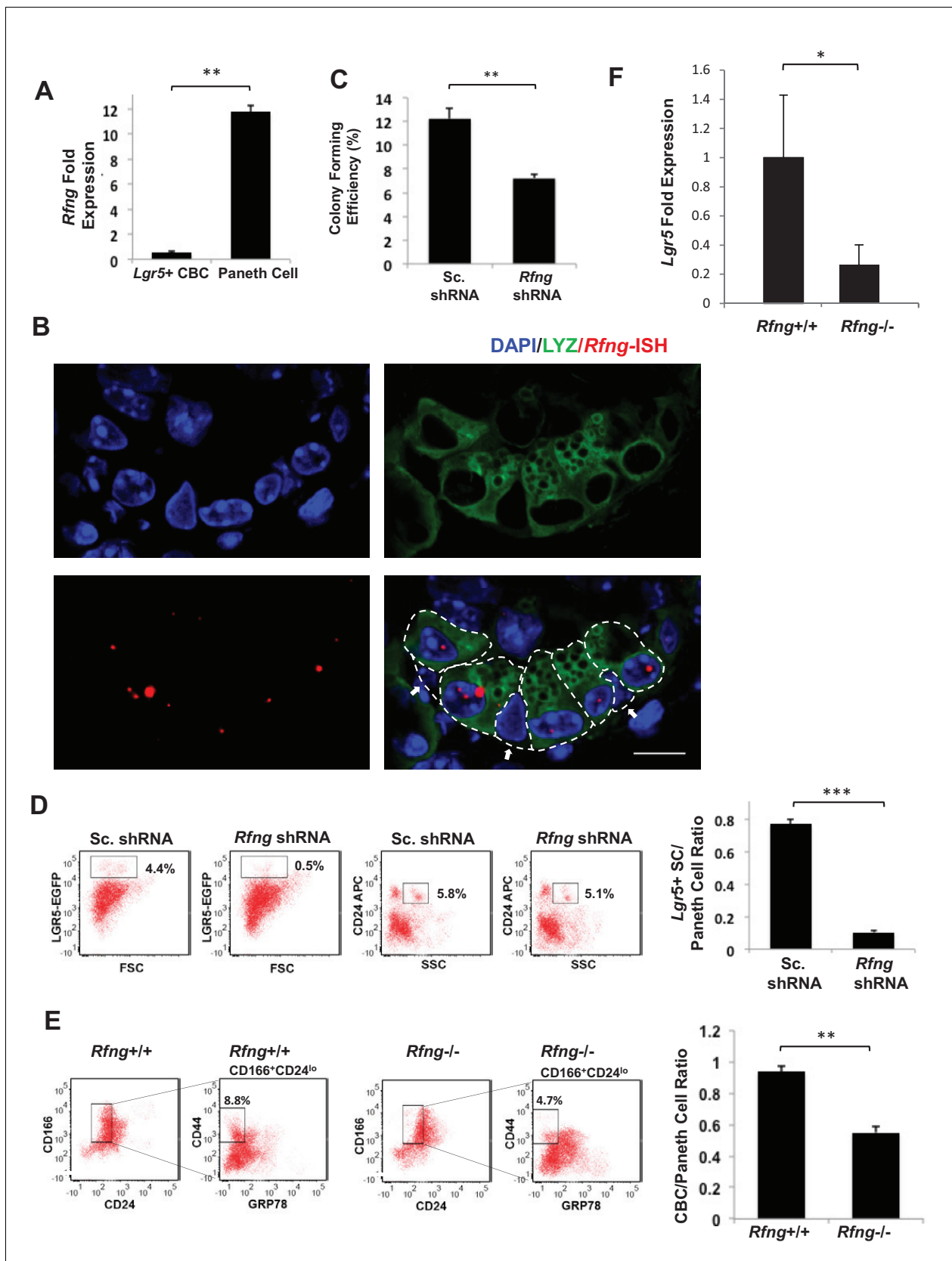


Figure 1. *Rfng* supports *Lgr5*⁺ stem cell self-renewal. (A) RT-qPCR quantification of *Rfng* in *Lgr5*⁺ CBC and Paneth cells isolated from *Lgr5*-GFP mouse intestines. The experiment was performed in triplicate and presented as mean \pm s.d. (standard deviation) (B) Representative image showing *Rfng*

Figure 1 continued on next page

Figure 1 continued

transcripts (red) and Lysozyme protein (green) expression at the bottom of the crypt of *Lgr5*-GFP mouse intestine. DAPI (Blue) labels the nuclei and scale bar represents 10 μ m. Arrows point to CBCs. (C–D) Single *Lgr5*-GFP CBCs were transduced with either Sc. shRNA or *Rfng* shRNA. The experiment was performed in triplicate. (C) Colony forming efficiency measured after 7 days. Quantitative analysis calculated from 1000 cells/replicate presented as mean \pm s.d. (D) Left: Representative flow cytometry plots indicating gated percentage of *Lgr5*⁺ (GFP^{high}) and Paneth cells (CD24^{high}/SSC^{high}). Right: Ratio of *Lgr5*-GFP⁺ CBCs/Paneth cells as determined by flow cytometry and presented as mean \pm s.d. (E) Left: Representative plots indicating gated population of CBCs (CD166⁺CD24^{lo}CD44⁺GRP78⁺) from the intestine of *Rfng*^{+/+} and *Rfng*^{-/-} mice. Percentage reflects fraction of total population. Right: Ratio of number of CBCs to Paneth cells of n = 3 mice and presented as mean \pm s.d. (F) RT-qPCR quantification of *Lgr5* in crypts extracted from *Rfng*^{+/+} and *Rfng*^{-/-} mice. n = 3 mice. Data is presented as mean \pm s.d. (*p<0.05; **p<0.01; ***p<0.001).

DOI: <https://doi.org/10.7554/eLife.35710.002>

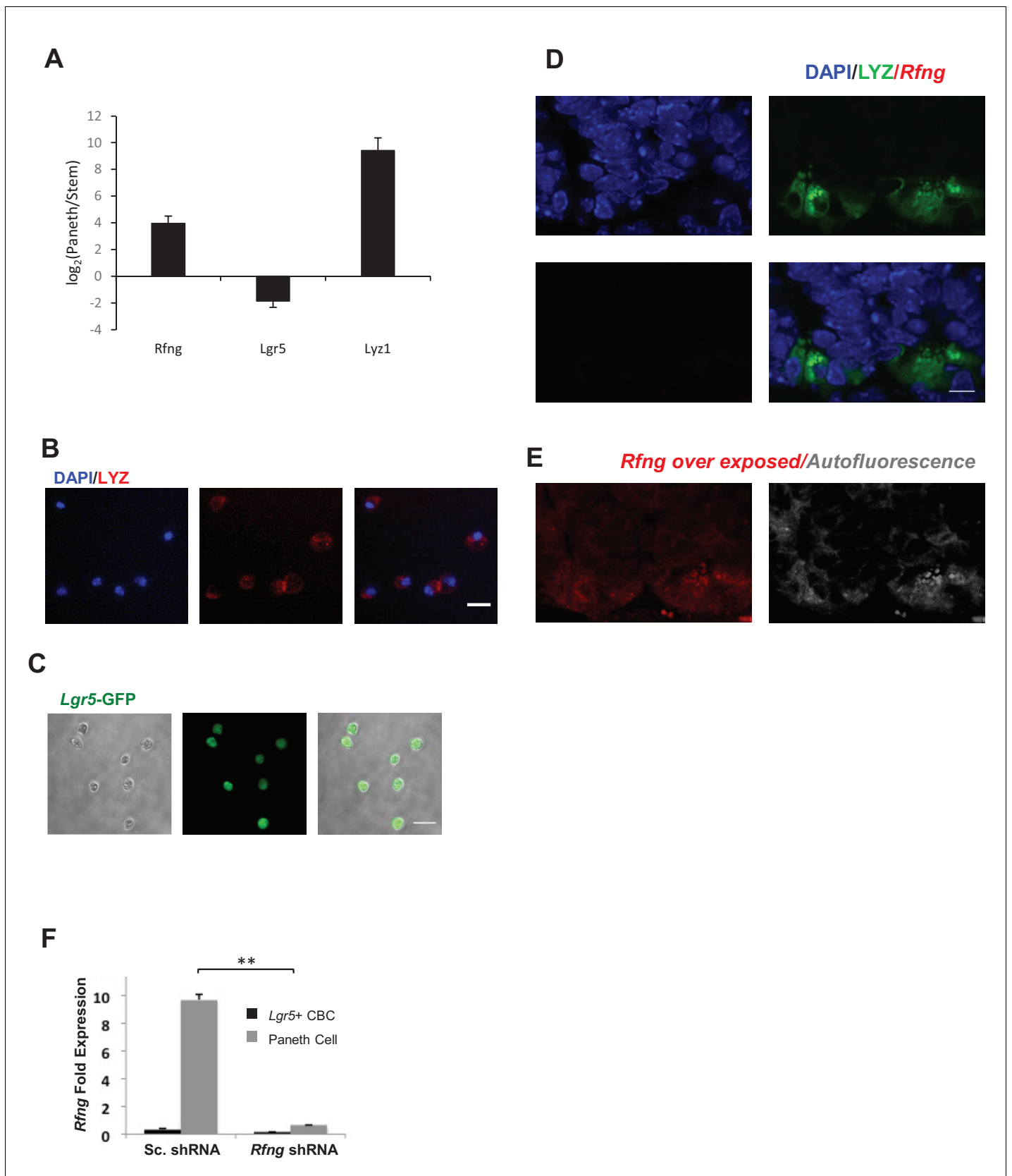


Figure 1—figure supplement 1. Paneth Cells express *Rfng*. (A) Ratio of gene expression in *Lgr5*+ CBC and Paneth cells from two independent microarrays published previously (Sato et al., 2011). Data presented as mean \pm s.d. (B) FACS Sorted Paneth cells were plated in Matrigel and stained Figure 1—figure supplement 1 continued on next page

Figure 1—figure supplement 1 continued

for Lysozyme (red). DAPI (Blue) shows the nuclei and scale bar represents 10 μm . (C) Sorted *Lgr5*-GFP cells were imaged. Endogenous GFP expression is shown in green. Scale bar represents 20 μm . (D–E) Representative image from RNAscope assay for *Rfng* on *Rfng*^{-/-} small intestinal section. DAPI (blue) shows nuclei and Lysozyme protein (green) marks the Paneth cells. Scale bar represents 10 μm . (D) No signal is seen in the red channel (*Rfng* channel) confirming the specificity of *Rfng* probes. (E) The region was then significantly overexposed to show the background signal. An additional image was taken by over exposing the region in the far-red channel (shown in grey; no probe/antibody present in this channel) to show tissue auto-fluorescence. (F) *Lgr5* + CBC and Paneth cell populations were isolated from organoids that were infected with scrambled or *Rfng* shRNA. The experiment was performed in triplicate. RT-qPCR quantification of *Rfng* presented as mean \pm s.d. in CBC and Paneth cells. (**p<0.01).

DOI: <https://doi.org/10.7554/eLife.35710.003>

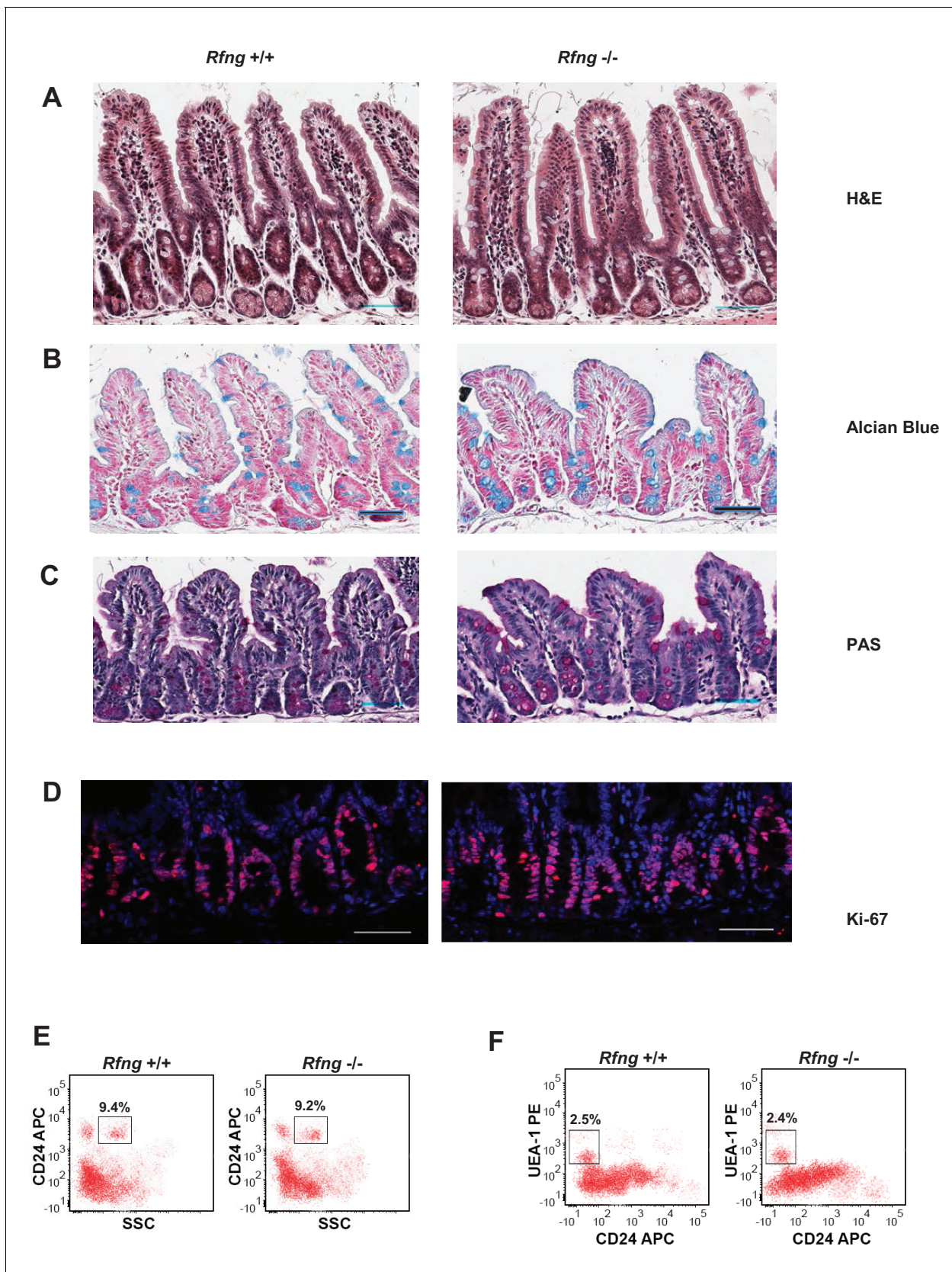


Figure 1—figure supplement 2. Histological and flow cytometric analysis of *Rfng* null intestines. (A–D) Representative images from the small intestine of *Rfng*^{+/+} and *Rfng*^{-/-} mouse strains stained using (A) Haematoxylin and Eosin (H and E) (B) Alcian Blue and Nuclear Fast Red (C) Periodic Acid-Schiff (PAS) (D) Ki-67. (E–F) Flow cytometry plots for CD24 APC vs SSC (E) and UEA-1 PE vs CD24 APC (F). The percentage of CD24⁺ cells is 9.4% in *Rfng*^{+/+} and 9.2% in *Rfng*^{-/-} (E). The percentage of UEA-1⁺ cells is 2.5% in *Rfng*^{+/+} and 2.4% in *Rfng*^{-/-} (F).
Figure 1—figure supplement 2 continued on next page

Figure 1—figure supplement 2 continued

(PAS) and Haematoxylin and (D) Ki-67 (red) and DAPI (blue). Scale bar represents 50 μm . (E–F) Representative flow cytometry plots indicating gated percentage of Paneth cells (E) or goblet cells (F) from the small intestine of *Rfng*^{+/+} and *Rfng*^{-/-} mice.

DOI: <https://doi.org/10.7554/eLife.35710.004>

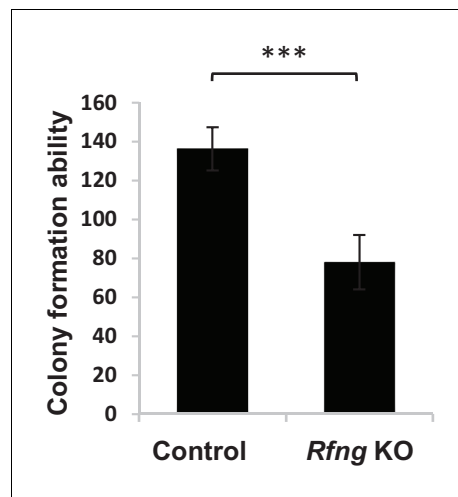


Figure 1—figure supplement 3. Colony formation ability of *Lgr5*⁺ CBCs when mixed with Paneth cells from control or *Rfng* null mice. *n* = 4 replicates with 8000 CBCs per replicate. Data is presented as mean ± s.d. (***p* < 0.001).

DOI: <https://doi.org/10.7554/eLife.35710.005>

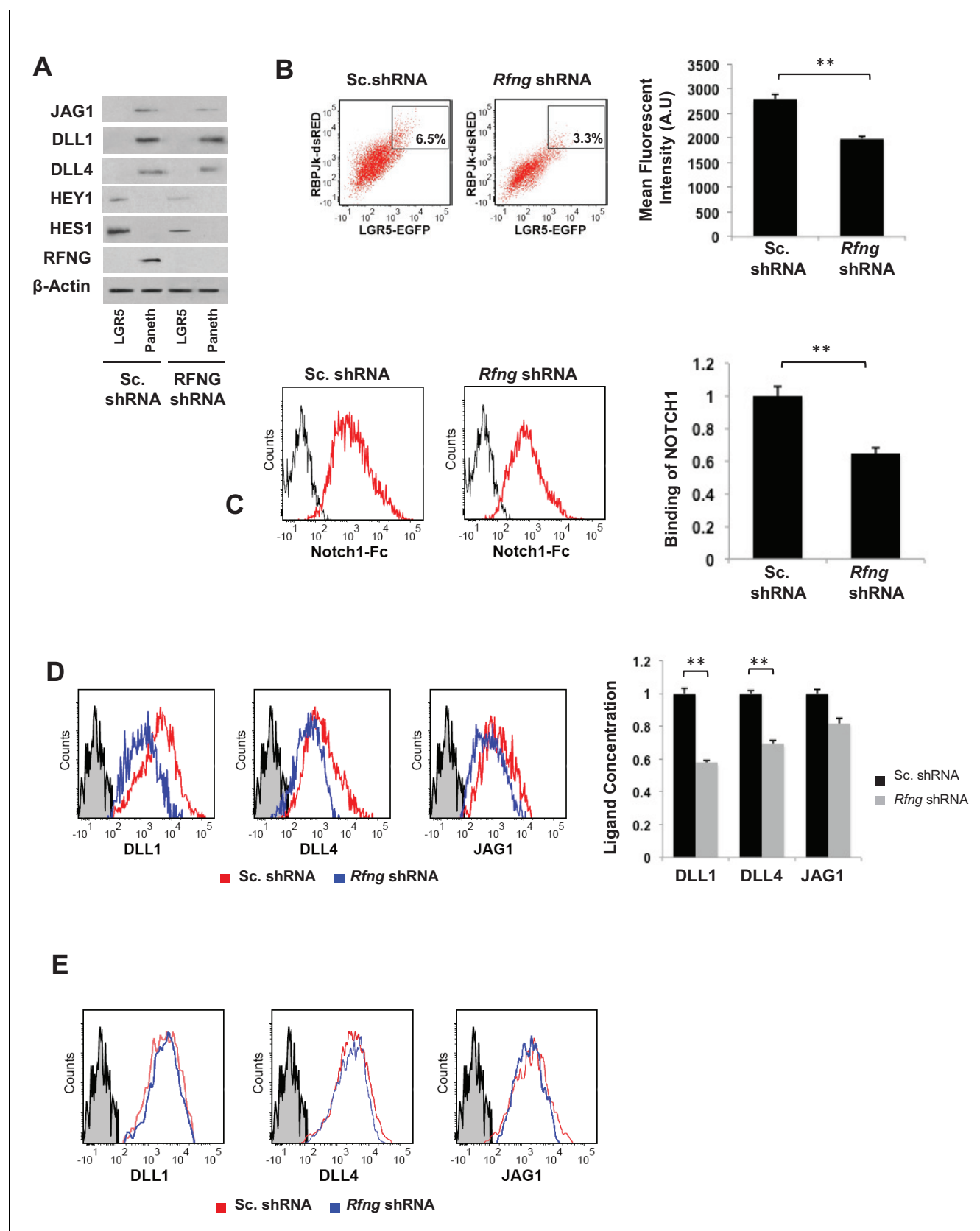


Figure 2. *Rfng* promotes Notch signaling in *Lgr5*⁺ CBC. (A) Western blot analysis of Notch signaling components in CBCs and Paneth cells sorted from *Rfng* KD and control organoids. (B) Left: Representative plots for RBPJk-dsRed and *Lgr5*-GFP expression indicating a gated double positive population. Right: Bar graphs showing Mean Fluorescent Intensity (A.U.) and Binding of NOTCH1 for *Sc. shRNA* and *Rfng shRNA*. (C) Flow cytometry histograms showing Notch1-Fc binding for *Sc. shRNA* and *Rfng shRNA*. (D) Flow cytometry histograms showing ligand concentration for DLL1, DLL4, and JAG1. Legend: *Sc. shRNA* (red), *Rfng shRNA* (blue). (E) Flow cytometry histograms showing ligand concentration for DLL1, DLL4, and JAG1. Legend: *Sc. shRNA* (red), *Rfng shRNA* (blue).

Figure 2 continued

fraction for *Rfng* KD and control CBCs transfected with RBPJκ-dsRed reporter. Right: Mean fluorescence intensity (MFI) of RBPJκ-dsRed expression. The experiment was performed in triplicate and presented as mean \pm s.d. (C) Ligand availability on *Rfng* KD and control Paneth cells. Representative traces (left) and MFI (right) showing ligand binding to NOTCH1 measured by flow cytometry. Unstained Paneth cells were used as a negative control. The experiment was performed in triplicate and presented as mean \pm s.d. (D) Cell surface DLL1, DLL4, and JAG1 concentration on *Rfng* KD and control unpermeabilised Paneth cells. Left: Representative traces measured by flow cytometry. Right: MFI measurements. The experiment was performed in triplicate and presented as mean \pm s.d. (E) Cell surface DLL1, DLL4, and JAG1 concentration on *Rfng* KD and control permeabilised Paneth cells. (**p<0.01).

DOI: <https://doi.org/10.7554/eLife.35710.006>

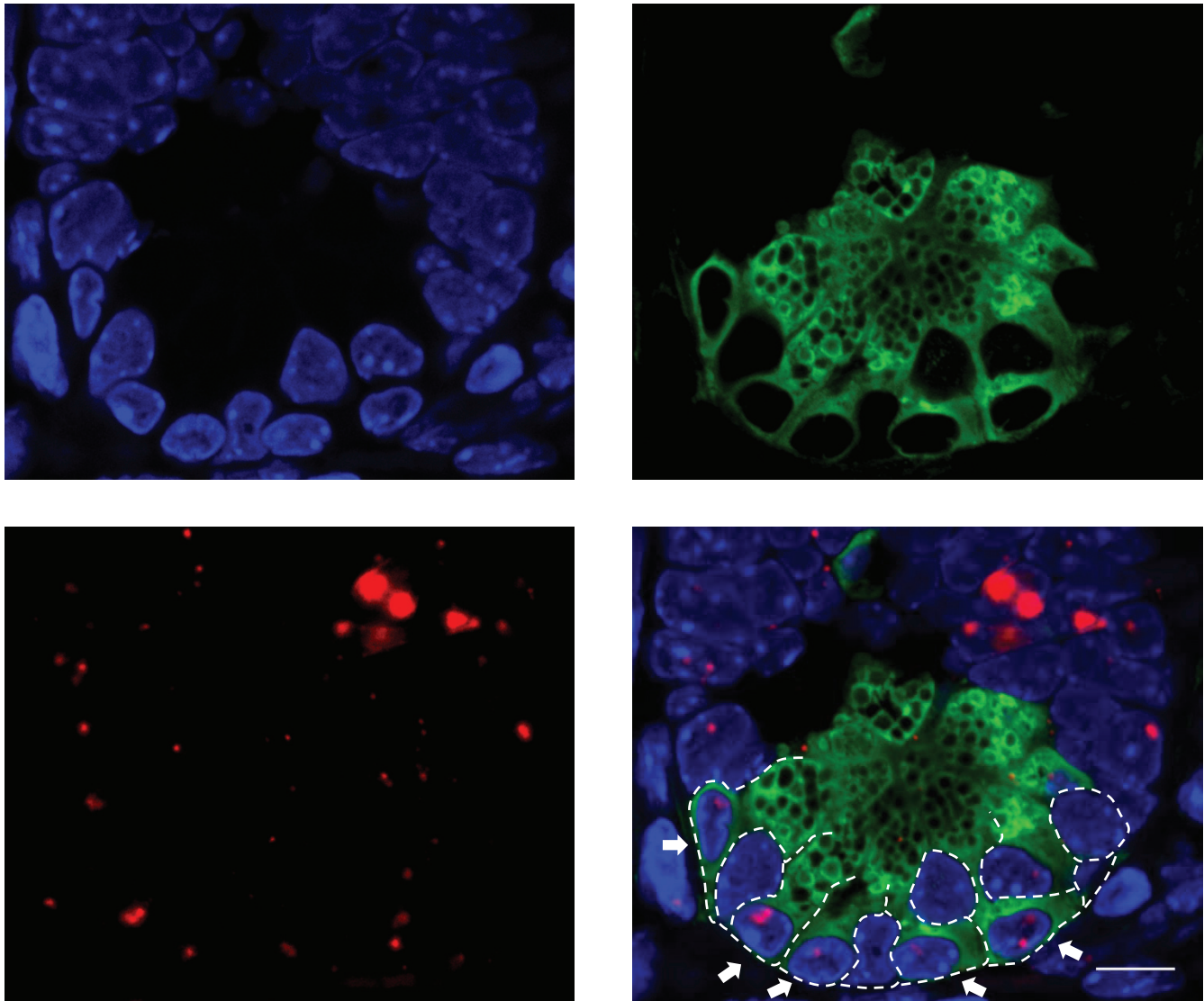
DAPI/LYZ/*Dll1*-ISH

Figure 2—figure supplement 1. *Dll1* expression in the crypts. Representative image showing *Dll1* transcripts (red) and Lysozyme protein (green) expression at the bottom of the crypt of *Lgr5*-GFP mouse intestine. DAPI (Blue) labels the nuclei and scale bar represents 10 μ m. Arrows point to Paneth cells.

DOI: <https://doi.org/10.7554/eLife.35710.007>

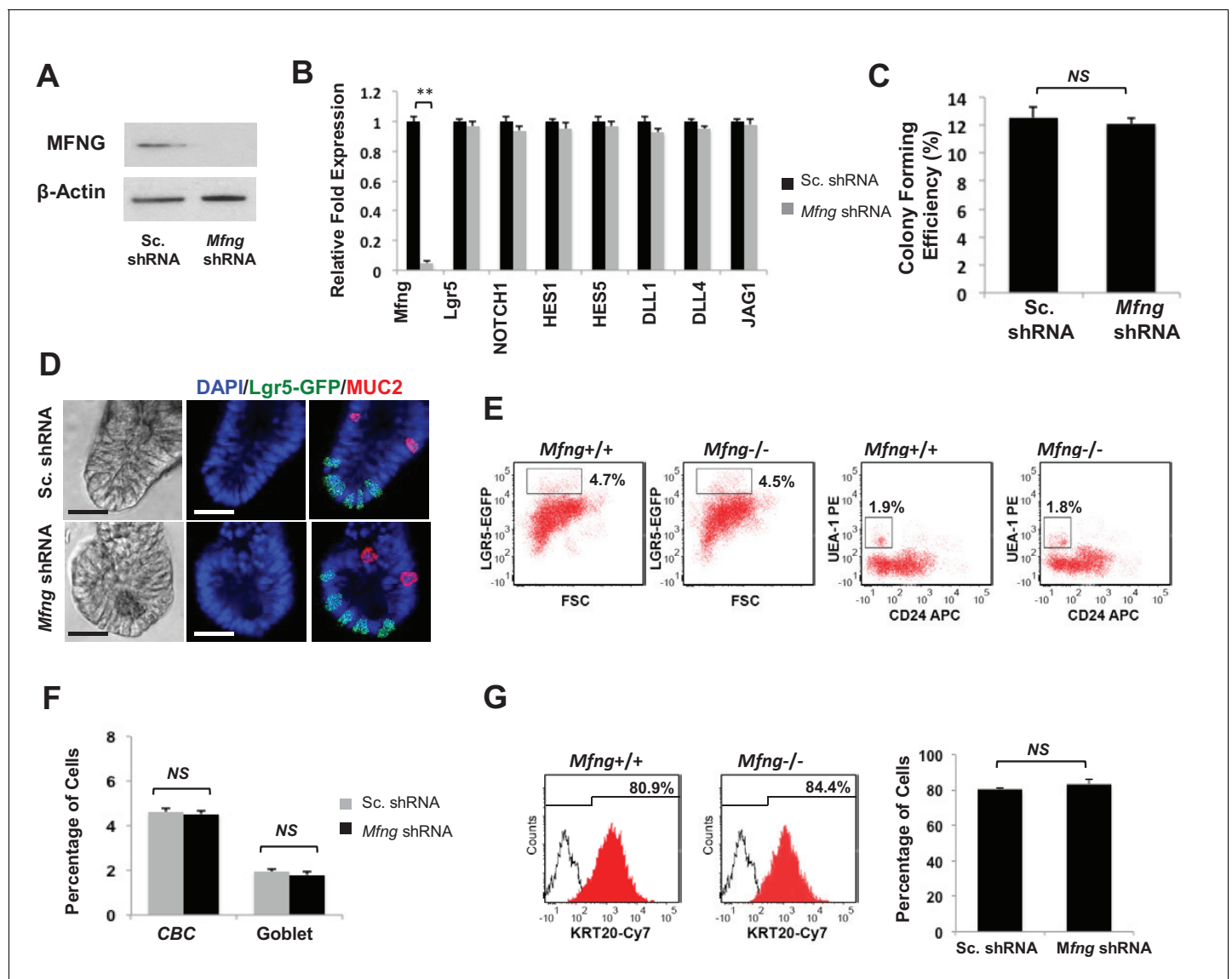


Figure 3. *Mfng* plays an insignificant role. Single *Lgr5*-GFP CBCs were transduced with either Sc. shRNA or *Mfng* shRNA. The experiment was performed in triplicate. (A) Western blot for *Mfng* expression. (B) RT-qPCR quantification of *Mfng* and Notch components in organoids. (C) Colony forming efficiency measured after 7 days. Quantitative analysis from 1000 cells/replicate. (D) Representative bright field and co-IF images indicating *Lgr5*-GFP (green) expression. MUC2 (red) marks Goblet cells. DAPI (blue) labels nuclei and scale bar represents 25 μ m. (E) Representative flow cytometry plots indicating gated percentage of *Lgr5*+ CBCs (GFP^{high}) and goblet cells (UEA-1⁺/CD24⁺). (F) Percentage of *Lgr5*+ CBCs and goblet cells as determined by flow cytometry and presented as mean \pm s.d. (G) Left: Representative flow cytometry histograms indicating KRT20+ (CK20+) cells. Right: Percentage of KRT20+ cells and presented as mean \pm s.d.

DOI: <https://doi.org/10.7554/eLife.35710.008>

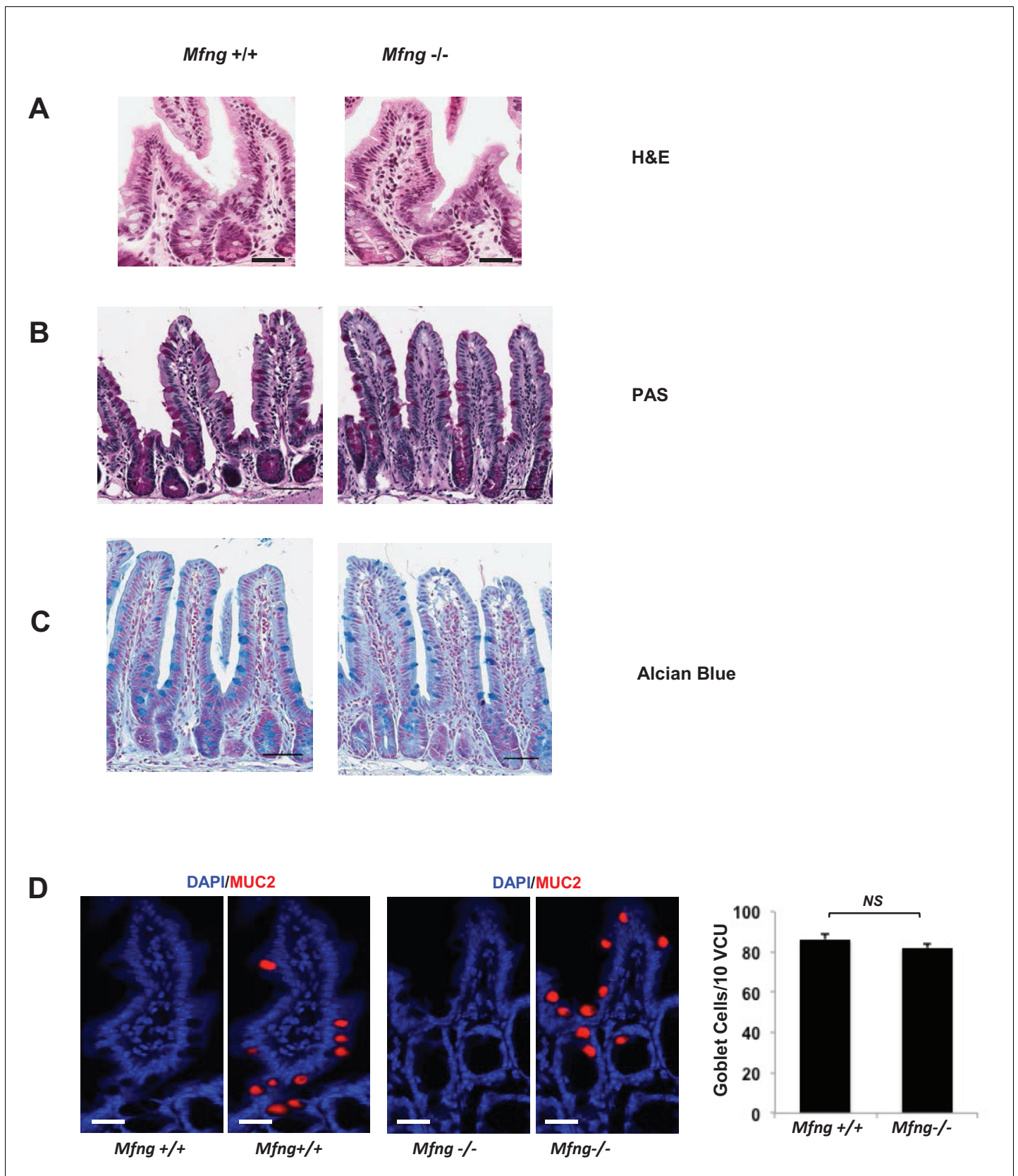


Figure 3—figure supplement 1. Histological analysis of *Mfng* null intestines. (A–C) Representative images from the small intestine of *Mfng* $^{+/+}$ and *Mfng* $^{-/-}$ mouse strains stained using (A) Haematoxylin and Eosin (H and E) (B) Periodic Acid Schiff (PAS) and Haematoxylin (C) Alcian Blue and Nuclear Figure 3—figure supplement 1 continued on next page

Figure 3—figure supplement 1 continued

Fast Red. Scale bar represents 50 μm . (D) Left: Representative IF images of villus-crypt units (VCU) from the small intestine of *Mfng*^{+/+} and *Mfng*^{-/-} mouse strains. MUC2 (red) marks goblet cells. DAPI (blue) labels nuclei. Right: Quantification of the number of goblet cells of $n = 4$ mice/condition. Data represents mean \pm s.d of $n = 500$ VCU per mouse. (NS (not significant)).

DOI: <https://doi.org/10.7554/eLife.35710.009>

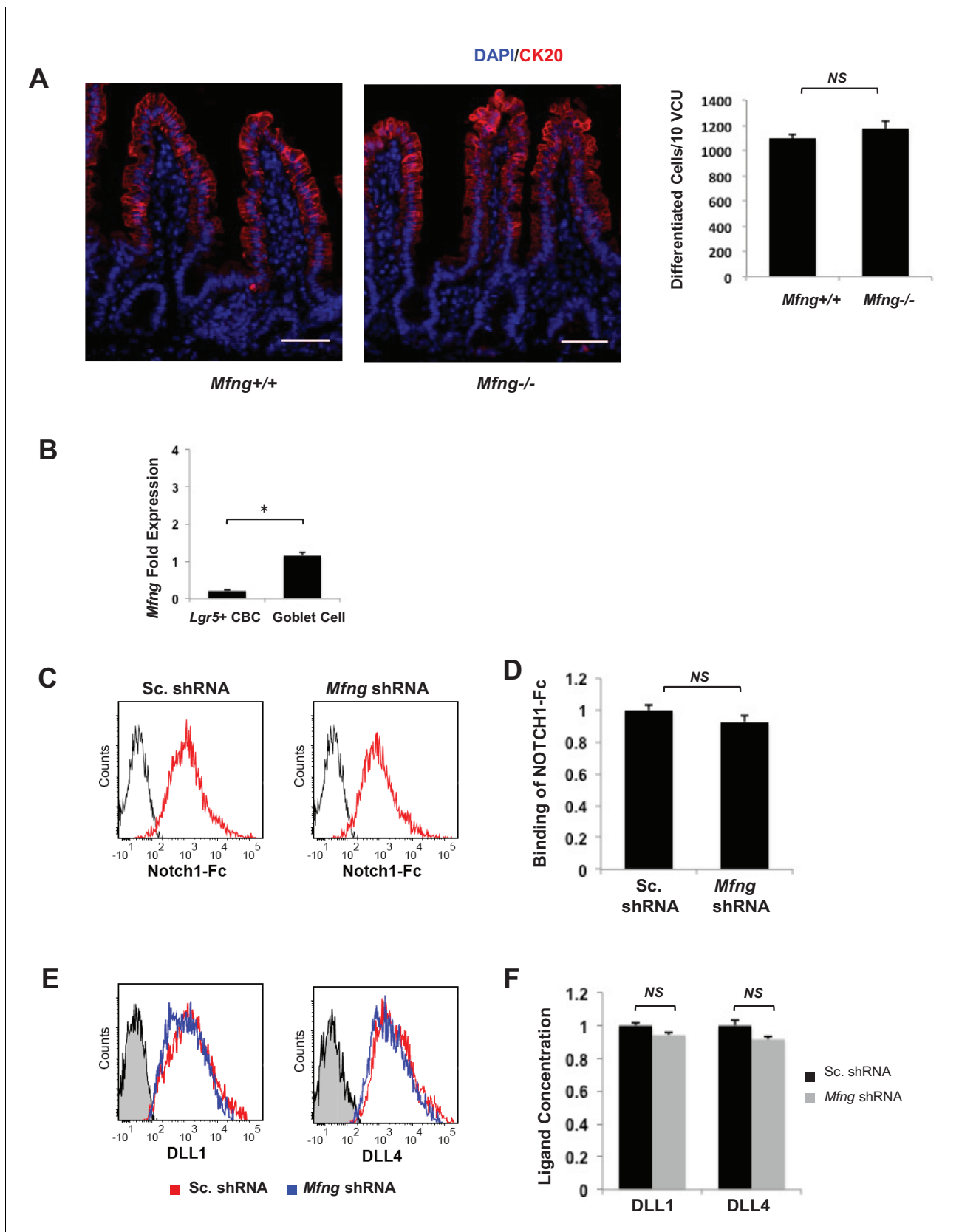


Figure 3—figure supplement 2. No significant phenotype detected upon loss of *Mfng*. (A) Left: Representative IF images of villus-crypt units (VCU) from the small intestine of *Mfng*^{+/+} and *Mfng*^{-/-} mouse strains. CK20 (red); DAPI (blue) labels nuclei; scale bar represents 50 μ m. Right: Quantification of Figure 3—figure supplement 2 continued on next page

Figure 3—figure supplement 2 continued

CK20+ differentiated cells in VCU of *Mfng*^{+/+} and *Mfng*^{-/-} mice based on IF. The data represents 500 VCU/mouse of n = 4 mice/condition and is presented as mean ± s.d. (NS) (not significant; Student t-test). (B) RT-qPCR quantification of *Mfng* in *Lgr5*+ CBC and goblet cell populations from small intestinal tissue of *Lgr5*-GFP mice. The experiment was performed in triplicate and presented mean ± s.d. (C) Single *Lgr5*-GFP CBCs were transduced with either Sc. shRNA or *Mfng* shRNA and propagated as organoids for seven days. Isolated goblet cells were incubated with 0.5 µg/ml NOTCH1-Fc. Shown are representative traces indicating ligand binding to Notch1 measured by flow cytometry in goblet cells. Unstained goblet cells were used as a negative control. (D) Ligand binding data to NOTCH1-Fc in *Mfng* shRNA-expressing goblet cells normalized to Sc. shRNA-transfected goblet cells using flow cytometry mean fluorescent intensity (MFI) measurements. The experiment was performed in triplicate and presented mean ± s.d. (NS). (E) Single *Lgr5*-GFP CBCs were transduced with either Sc. shRNA or *Mfng* shRNA and propagated as organoids for 7 days. Isolated goblet cells were incubated with antibodies directed against DLL1 and DLL4 ligands. Shown are representative traces showing surface ligand concentration measured by flow cytometry on goblet cells. Unstained goblet cells were used as a negative control. (F) Surface DLL1 and DLL4 ligand concentration on *Mfng* shRNA-expressing goblet cells normalized to Sc. shRNA-transfected goblet cells using flow cytometry mean fluorescent intensity (MFI) measurements. The experiment was performed in triplicate and presented mean ± s.d. (NS). (*p<0.05; **p<0.01).

DOI: <https://doi.org/10.7554/eLife.35710.010>

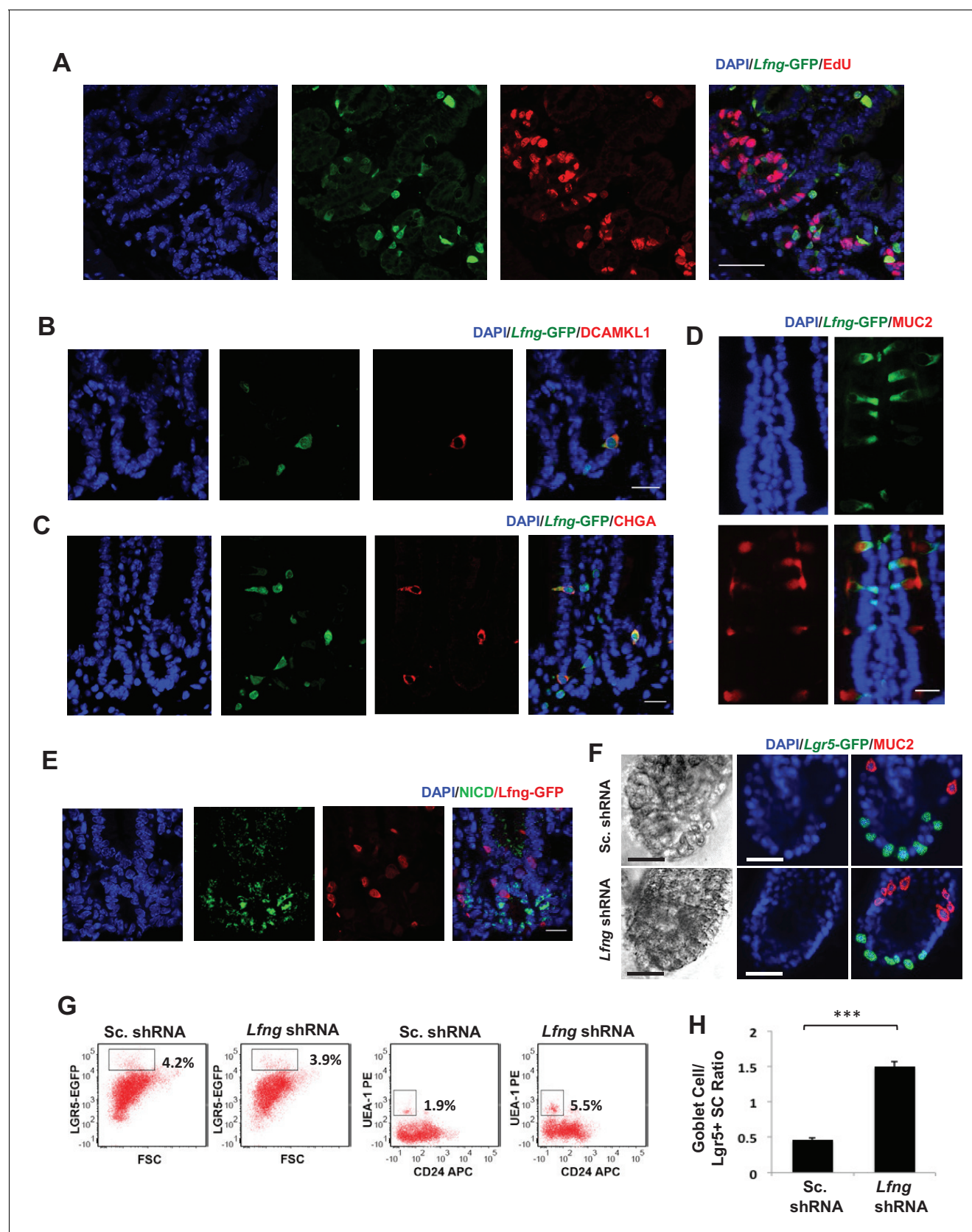


Figure 4. *Lfng* loss results in increased goblet cell differentiation in vitro. (A–E) Representative IF images of the small intestine of *Lfng*-GFP reporter mice. (A) GFP (green) shows the *Lfng* expression and EdU (red) marks the proliferating cells. DAPI (blue) labels nuclei. Scale bar represents 50 μ m. (B) Figure 4 continued on next page

Figure 4 continued

GFP (green) shows the *Lfng* expression and DCAMKL1 (red) marks the Tuft cells. Scale bar represents 20 μm . (C) GFP (green) shows the *Lfng* expression and CHGA (red) marks the enteroendocrine cells. Scale bar represents 20 μm . (D) GFP (green) shows the *Lfng* expression and MUC2 (red) marks the goblet cells. Scale bar represents 20 μm . (E) GFP (red) shows the *Lfng* expression and NICD (green) identifies the cells with NOTCH1 activity. Scale bar represents 20 μm . (F) Representative bright field and co-IF images of *Lfng* KD and control organoids indicating *Lgr5*-GFP (green) expression. MUC2 (red) marks goblet cells. DAPI (blue) labels nuclei and scale bar represents 25 μm . (G) Representative plots indicating gated percentage of *Lgr5*⁺ (GFP^{high}) and goblet cells (UEA-1⁺/CD24⁻) of *Lfng* KD and control organoids. (H) Ratio of goblet cells to *Lgr5*-GFP + CBCs as determined by flow cytometry. The experiment was performed in triplicate and presented mean \pm s.d. (**p<0.01, ***p<0.001).

DOI: <https://doi.org/10.7554/eLife.35710.011>

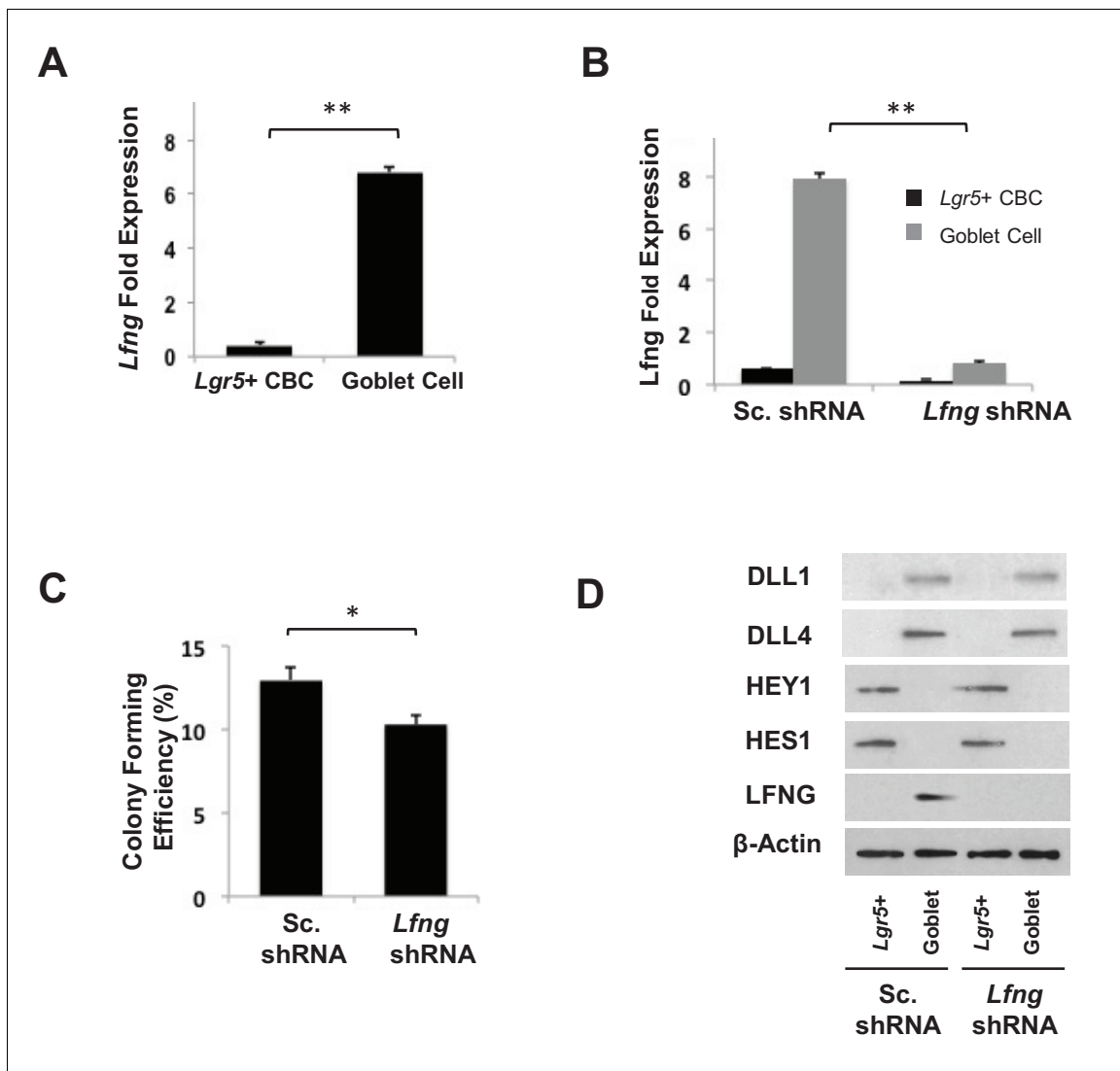


Figure 4—figure supplement 1. Characterisation of *Lfng* KD organoids. (A) RT-qPCR quantification of *Rfng* in *Lgr5*+ CBC and goblet cell populations from *Lgr5*-GFP murine intestinal tissue. The experiment was performed in triplicate and presented mean \pm s.d. (B) RT-qPCR quantification of *Lfng* in *Lgr5*+ CBC and goblet cell populations after organoids were infected with Scrambled shRNA (Sc. shRNA) or *Lfng* shRNA. The experiment was performed in triplicate and presented mean \pm s.d. (C) Single *Lgr5*-GFP CBCs were transduced with either Sc. shRNA or *Lfng* shRNA. Shown is colony forming efficiency measured after seven days. Quantitative analysis calculated from 1000 cells/replicate. The experiment was performed in triplicate and presented mean \pm s.d. (D) Single *Lgr5*-GFP CBCs were transduced with either Sc. shRNA or *Lfng* shRNA and propagated as organoids for seven days. Shown is Western blot analysis for LFNG and Notch signaling components. Actin was used as a loading control. (* $p < 0.01$; ** $p < 0.01$).

DOI: <https://doi.org/10.7554/eLife.35710.012>

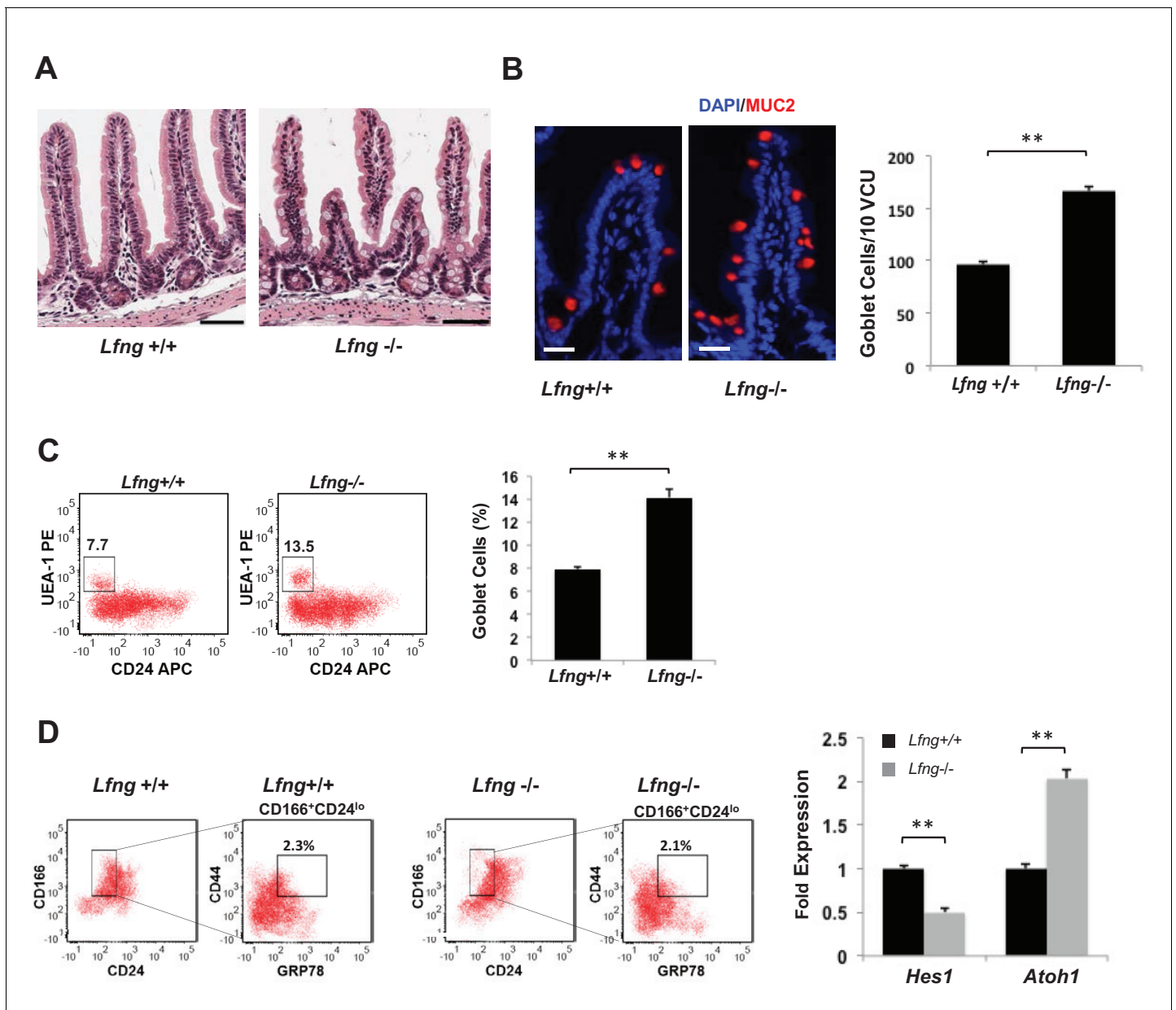


Figure 5. *Lfng* loss results in increased goblet cell differentiation in vivo. (A) Representative H and E sections from the small intestine of *Lfng*^{+/+} and *Lfng*^{-/-} mice. Scale bar represents 50 μ m. (B) Left: Representative IF images of intestine of *Lfng*^{+/+} and *Lfng*^{-/-} mice. MUC2 (red) marks goblet cells. DAPI (blue) labels nuclei. Right: Quantification of the number of goblet cells of $n = 4$ mice/condition and $n = 500$ VCU per mouse presented as mean \pm s.d. (C) Left: Representative plots indicating gated percentage of goblet cells (UEA-1⁺/CD24⁺) from intestinal tissue derived from *Lfng*^{+/+} or *Lfng*^{-/-} mice. Right: Percentage of goblet cells presented as mean \pm s.d. The data represent $n = 3$ mice/condition. (D) Left: Representative plots indicating gated population of intestinal progenitors from the intestine of *Lfng*^{+/+} and *Lfng*^{-/-} mice. Percentage reflects fraction of total population. Right: RT-qPCR measurements in progenitor cells from *Lfng*^{+/+} and *Lfng*^{-/-} mice. The experiment was performed in triplicate presented as mean \pm s.d. (** $p < 0.01$).

DOI: <https://doi.org/10.7554/eLife.35710.013>

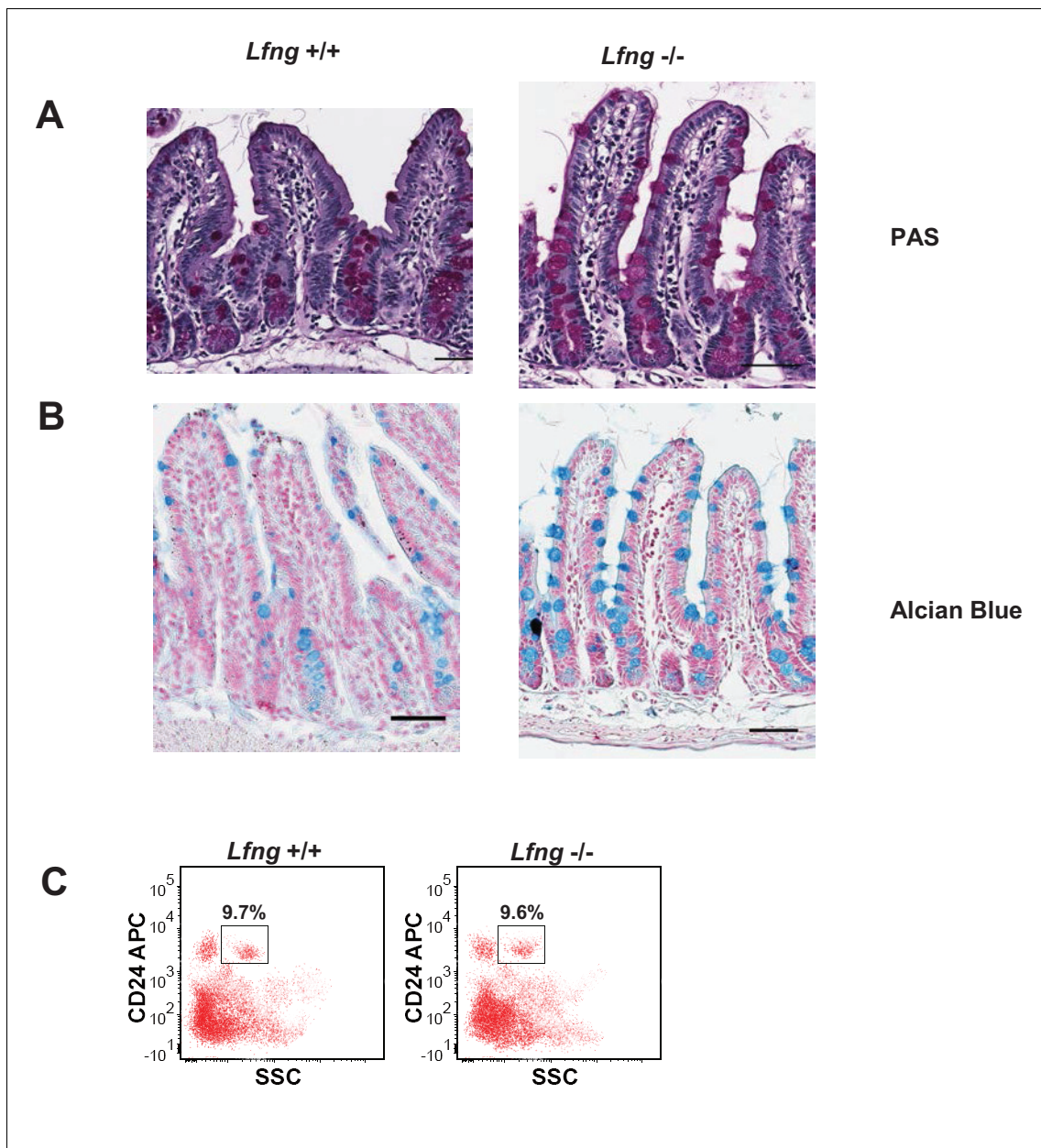


Figure 5—figure supplement 1. Histological and flow cytometric analysis of *Lfng* null intestines. (A-B) Representative images from the small intestine of *Lfng* $^{+/+}$ and *Lfng* $^{-/-}$ mouse strains stained using (A) PAS and Haematoxylin (B) Alcian Blue and Nuclear Fast Red.

Scale bar represents 50 μ m. (C) Representative flow cytometry plots indicating gated percentage of Paneth cells from the small intestine of *Lfng* $^{+/+}$ and *Lfng* $^{-/-}$ mice.

DOI: <https://doi.org/10.7554/eLife.35710.014>

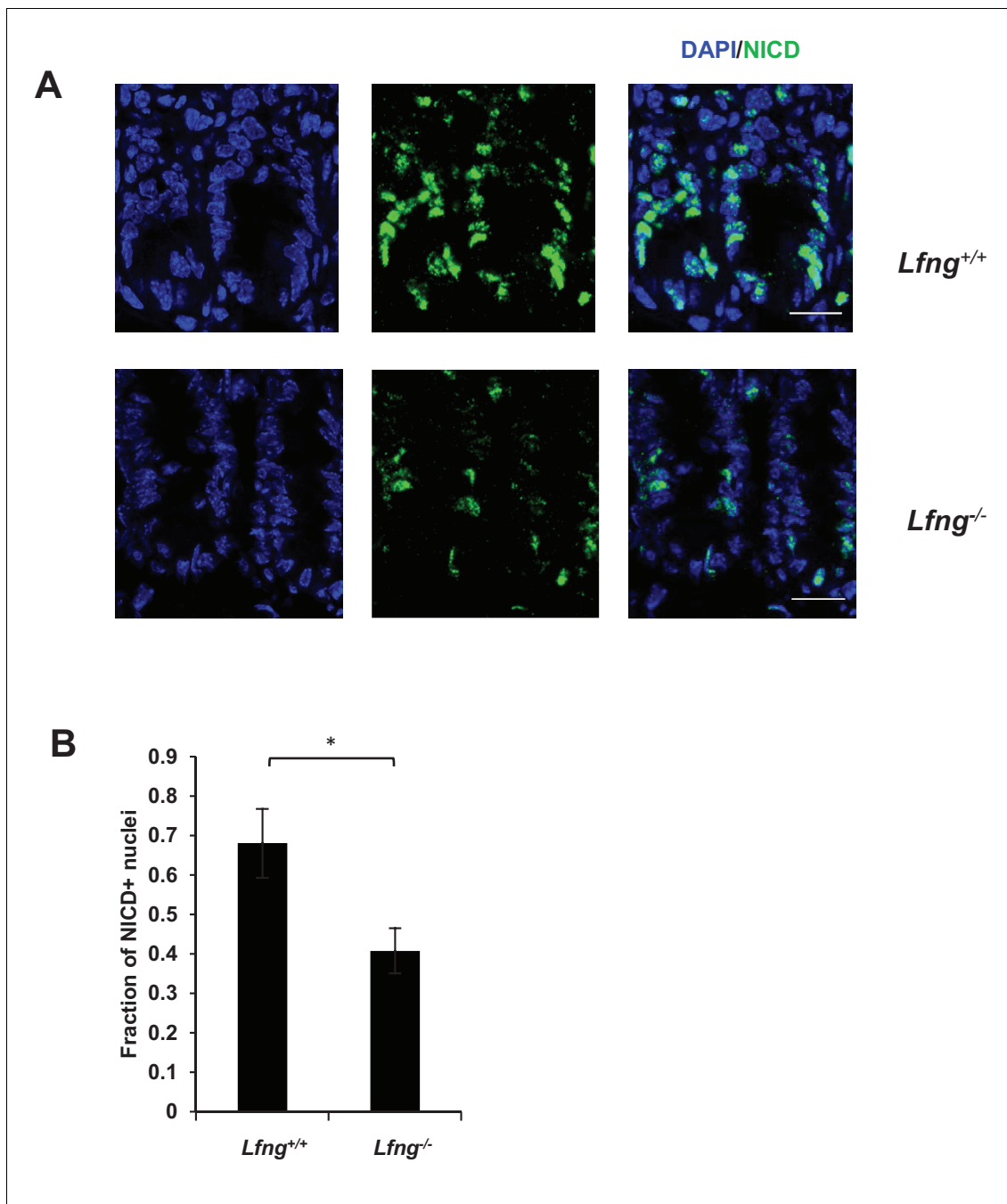


Figure 5—figure supplement 2. *Lfng* loss results in reduced Notch activity. (A) Representative images from the small intestine of *Lfng*^{+/+} and *Lfng*^{-/-} mouse strains stained for NICD. Scale bar represents 20 μ m. (B) Fraction of NICD + nuclei per crypt quantified from small intestines of *Lfng*^{+/+} and *Lfng*^{-/-} mice presented as mean \pm s.d. Data represents n = 3 mice/condition. (*, p<0.05).

DOI: <https://doi.org/10.7554/eLife.35710.015>

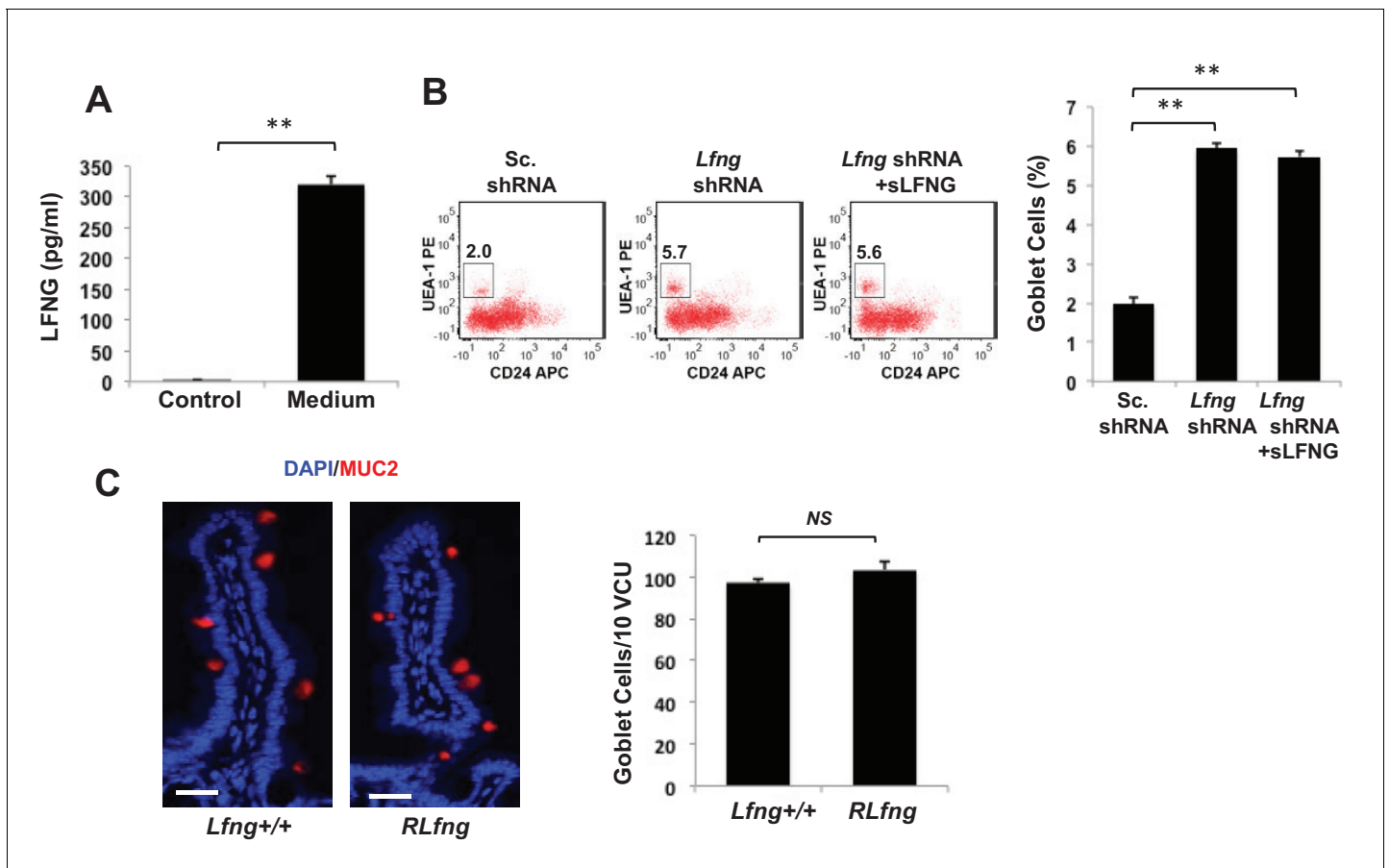


Figure 6. Secreted LFNG plays no apparent function. (A) ELISA of the secretion of LFNG in culture medium from *Lgr5*-GFP organoids. Culture medium (T = 0 days) was used as a control. The experiment was performed in triplicate and presented as mean \pm s.d. (B) Left: Representative plots indicating gated percentage of goblet cells (UEA-1⁺/CD24⁺) for organoids under Sc. shRNA control, *Lfng* KD and *Lfng* KD treated with sLFNG conditions. Right: Percentage of goblet cells in each condition. The experiment was performed in triplicate and presented as mean \pm s.d. (C) Left: Representative IF images of intestine of *Lfng*^{+/+} and *Lfng*^{RLfng/+} mice. MUC2 (red) marks goblet cells. DAPI (blue) labels nuclei. Right: Quantification of the number of goblet cells of n = 4 mice/condition and n = 500 VCU/mouse. Data presented as mean \pm s.d. (**p < 0.01).

DOI: <https://doi.org/10.7554/eLife.35710.016>

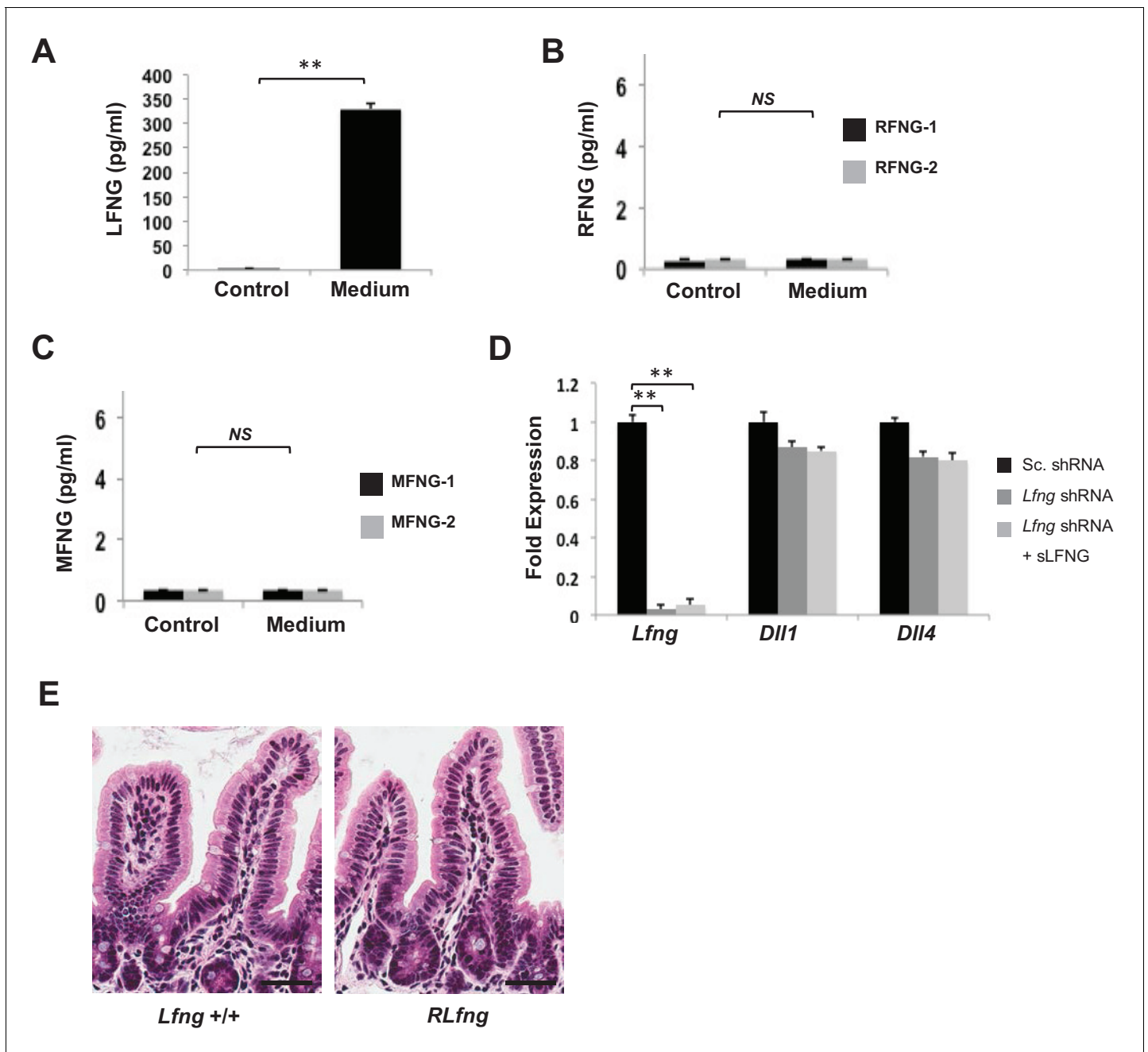


Figure 6—figure supplement 1. Secretion of Fringe proteins. (A) ELISA of the secretion of LFNG in culture medium from murine *Lgr5*-GFP+ CBCs propagated as organoids for 7 days using an additional primary LFNG antibody (LFNG-2). Culture medium (T = 0 days) was used as a control. The experiment was performed in triplicate and presented mean \pm s.d. (B) ELISA of the secretion of RFNG in culture medium from murine *Lgr5*-GFP+ CBCs propagated as organoids for 7 days using two separate primary RFNG antibodies (RFNG-1, RFNG-2). Culture medium (T = 0 days) was used as a control. The experiment was performed in triplicate and presented mean \pm s.d. (C) ELISA of the secretion of MFNG in culture medium from murine *Lgr5*-GFP+ CBCs propagated as organoids for 7 days using two separate primary MFNG antibodies (MFNG-1, MFNG-2). Culture medium (T = 0 days) was used as a control. The experiment was performed in triplicate and presented mean \pm s.d. (NS). (D) RT-PCR quantification of *Lfng* and Notch ligands (DLL1 and DLL4) in *Lgr5*-GFP+ organoids that were infected with Scrambled shRNA (Sc. shRNA) or *Lfng* shRNA. *Lfng* shRNA-expressing CBC organoids were subsequently incubated in conditioned medium containing soluble LFNG (sLFNG) for 24 hr. The experiment was performed in triplicate and presented mean \pm s.d. (E) Representative H and E sections from the small intestine of *Lfng*^{+/+} and *RLfng* mice. Scale bar represents 50 μ m. (**p<0.01).

DOI: <https://doi.org/10.7554/eLife.35710.017>

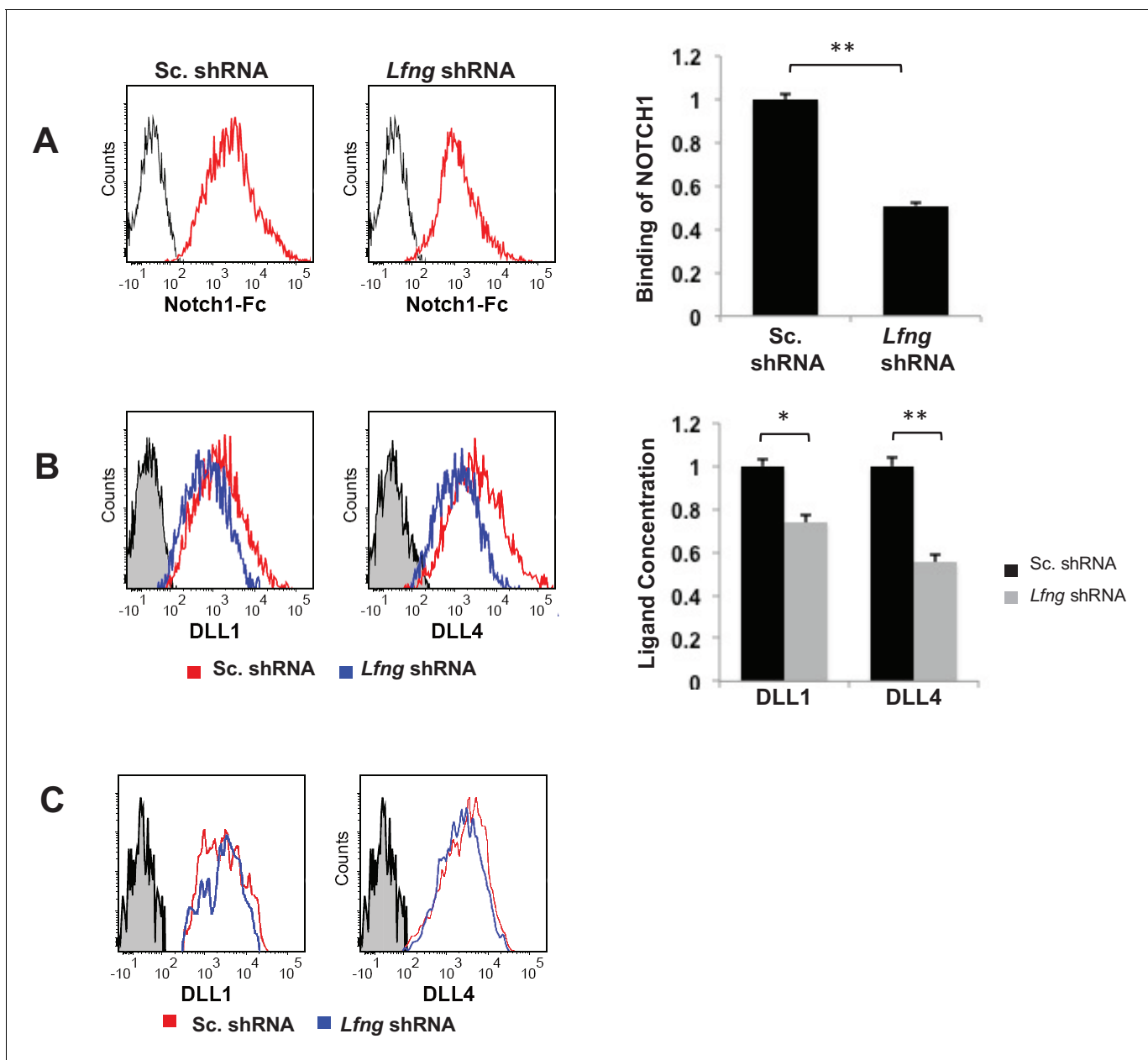


Figure 7. LFNG promotes cell surface expression of DLL. (A) Ligand availability on *Lfng* KD and Sc. Control goblet cells. Representative traces (left) and MFI (right) showing ligand binding to NOTCH1 measured by flow cytometry. Unstained goblet cells were used as a negative control. The experiment was performed in triplicate and presented as mean \pm s.d. (D) Cell surface DLL1 and DLL4 concentration on *Lfng* KD and Sc. Control unpermeabilised goblet cells. Left: Representative traces measured by flow cytometry. Right: MFI measurements. The experiment was performed in triplicate and presented as mean \pm s.d. (E) Cell surface DLL1 and DLL4 concentration on *Lfng* KD and Sc. Control permeabilised goblet cells. (** $p < 0.01$).

DOI: <https://doi.org/10.7554/eLife.35710.018>

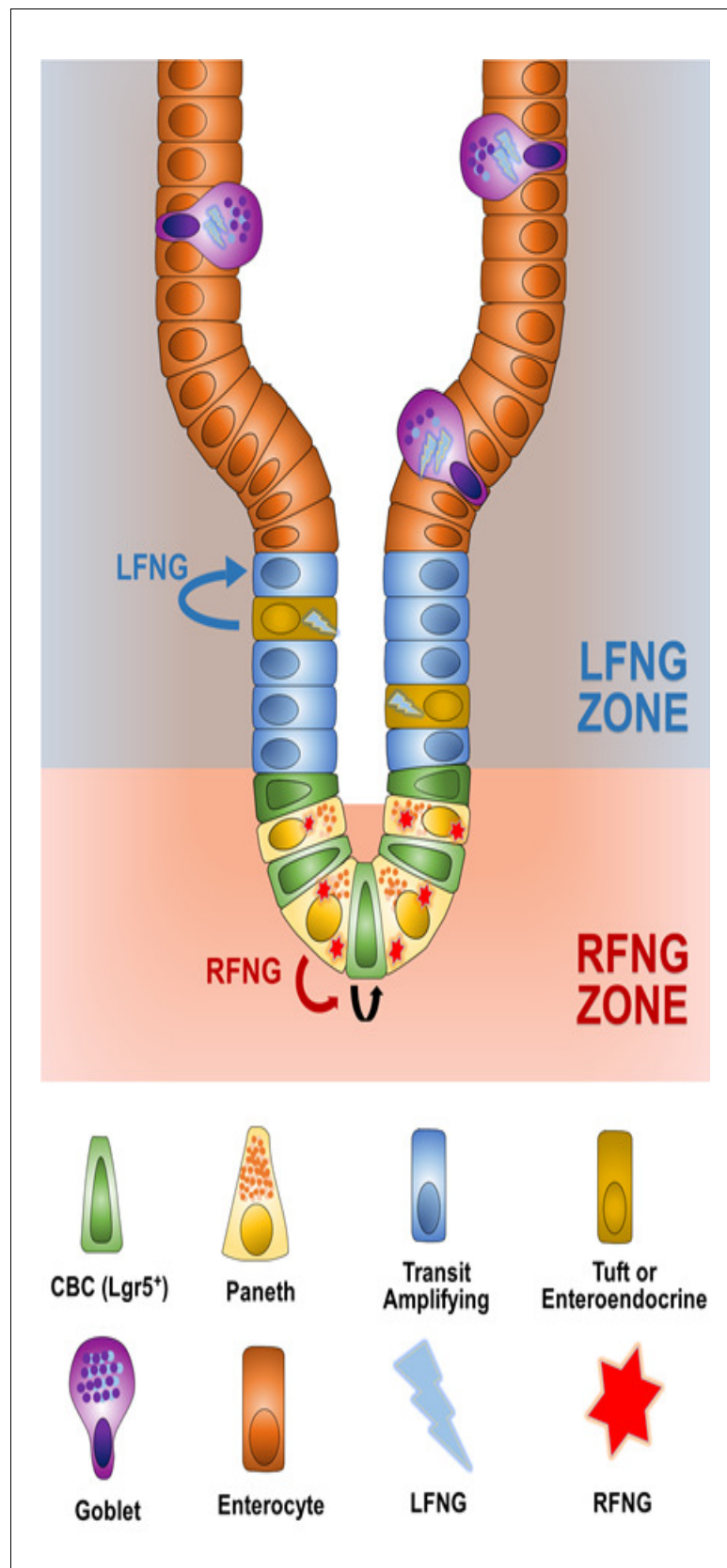


Figure 8. Summary. *Rfng* is enriched in the Paneth cells and promotes cell surface expression of DLL1 and DLL4. This promotes Notch activity in the neighbouring *Lgr5*⁺ CBCs assisting their self-renewal. *Mfng* does not appear

Figure 8 continued on next page

Figure 8 continued

to contribute significantly in maintaining the epithelium. *Lfng* on the other hand is expressed by enteroendocrine, Tuft, and goblet cells and suppresses the secretory lineage.

DOI: <https://doi.org/10.7554/eLife.35710.019>