## Supplemental Information

# Next generation surrogate Wnts support organoid growth and deconvolute Frizzled pleiotropy *in vivo*

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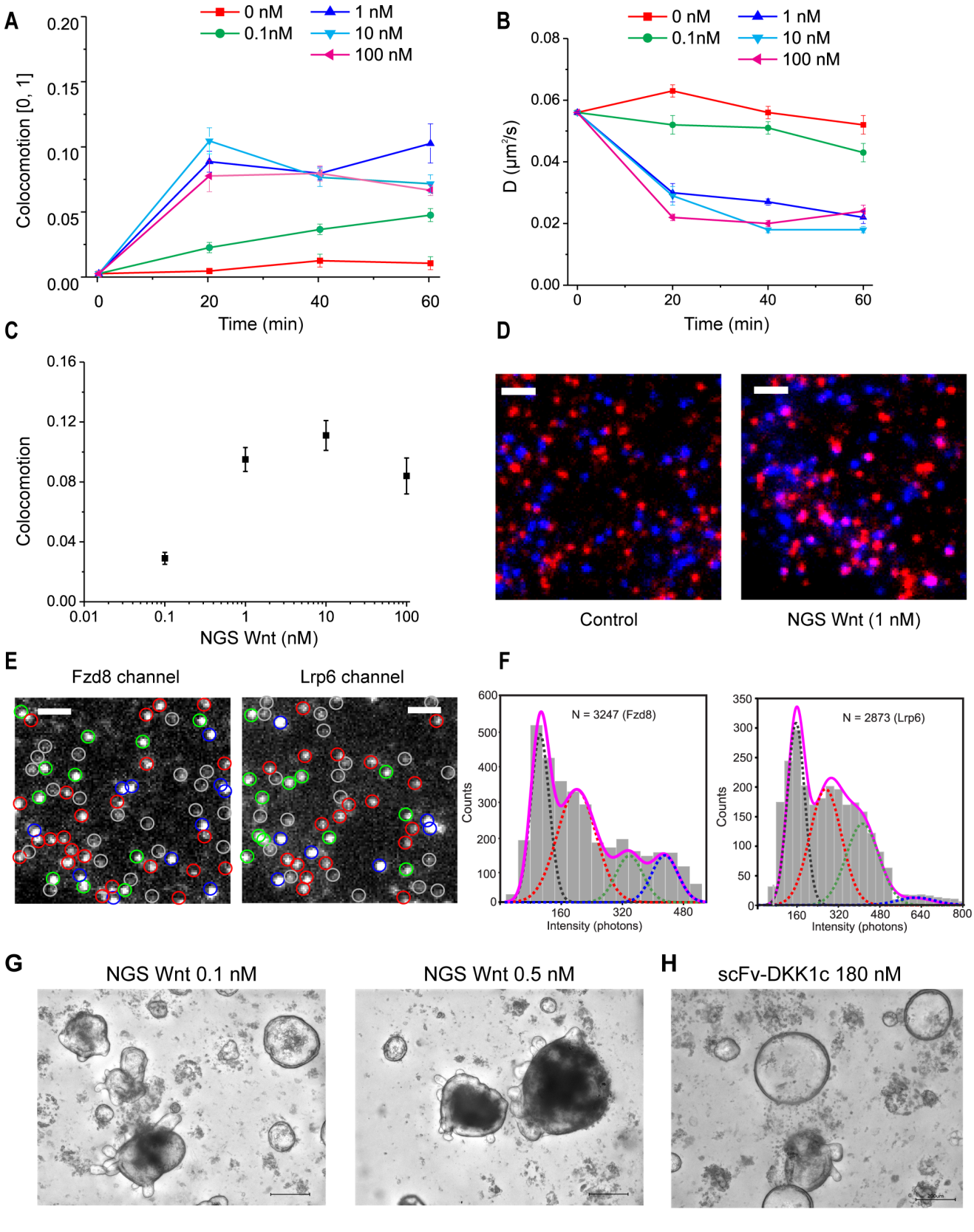
A
0 nM
0.1nM
1 nM
10 nM
100 nM
Colocomotion [0, 1]
Time (min)
B
D (μm²/s)
Time (min)
C
Colocomotion
NGS Wnt (nM)
D
Control
NGS Wnt (1 nM)
E
Fzd8 channel
Lrp6 channel
F
N = 3247 (Fzd8)
N = 2873 (Lrp6)
Counts
Intensity (photons)
G
NGS Wnt 0.1 nM
NGS Wnt 0.5 nM
H
scFv-DKK1c 180 nM

**Figure S1. Time-lapse measurement of co-locomotion and quantification of Lrp6 and Fzd8 using single molecule imaging analysis. (Related to Figure 1)**

(A) Time-lapse measurement of co-locomoting Lrp6 and Fzd8 using 0 nM, 0.1 nM, 1 nM, 10 nM and 100 nM of Fzd7/8 subtype NGS Wnt. Error bars in represent S.E. of measurements from n > 12 individual cells for each condition.

(B) Average diffusion constant of Fzd8 as function of time after treating with 0 nM, 0.1 nM, 1 nM, 10 nM and 100 nM of Fzd7/8 subtype NGS Wnt. Error bars in represent S.E. of measurements from n > 12 individual cells for each condition.

(C) Co-locomotion of Lrp6 and Fzd8 as function of Fzd7/8 subtype NGS Wnt concentrations after 20 min stimulation. Error bars represent S.E. of measurements from n > 12 individual cells for each condition.

(D) Representative raw images without treatment (control) and after treatment with 1 nM Fzd7/8 subtype NGS Wnt. Fzd8: blue. Lrp6: red. Scale bars represents 2 μm.

(E) Representative raw images of Fzd8 and Lrp6 after treatment with 1 nM Fzd7/8 subtype NGS Wnt. On the basis of their intensities, individual diffraction-limited spots in the raw images were classified as monomers (gray circle), dimers (red circle), trimers (green circle) and higher oligomers (blue circle), respectively. Scale bars represents 2 μm.

(F) Quantification of oligomer fractions by multicomponent Gaussian fitting of single molecule intensity histogram. More than 2800 individual complex intensities were examined in the pooled analyses for each receptors.

(G) Representative brightfield images of mouse colon organoids cultured with 0.1 nM and 0.5 nM Fzd7/8 subtype NGS Wnt. Scale bar represents 200 μm.

(H) Representative brightfield images of mouse colon organoids cultured with 180 nM Wnt surrogate scFv-DKK1c. Scale bar represents 200 μm.

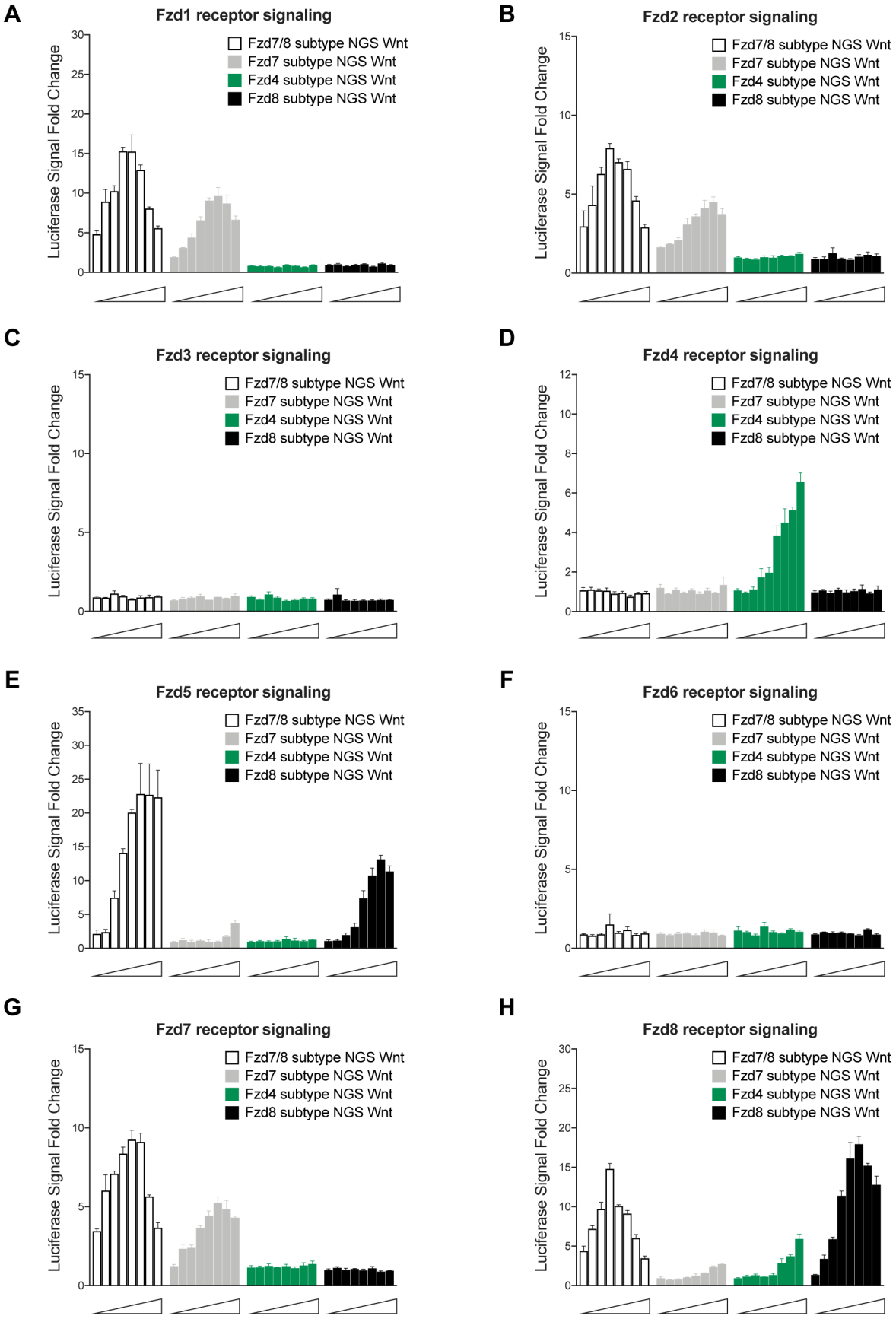
A
Fzd1 receptor signaling
B
Fzd2 receptor signaling
C
Fzd3 receptor signaling
D
Fzd4 receptor signaling
E
Fzd5 receptor signaling
F
Fzd6 receptor signaling
G
Fzd7 receptor signaling
H
Fzd8 receptor signaling
Fzd7/8 subtype NGS Wnt
Fzd7 subtype NGS Wnt
Fzd4 subtype NGS Wnt
Fzd8 subtype NGS Wnt
Luciferase Signal Fold Change

**Figure S2. Fzd subtype NGS Wnts activates canonical β-catenin signaling in a Frizzled-selective manner. (Related to Figure 1)**

Wnt luciferase reporter assays in HEK 293T Fzd KO cell line transfected with individual Fzd receptors. Fzd receptor knockout cells were treated with indicated NGS Wnts. Luminance signal fold change was normalized to control. All NGS Wnts were added at concentrations from 78 pM to 10 nM (expect 78 pM to 20 nM in Fzd4-transfected cells). Data represents mean ± S.E., N = 3 technical replicates. Experiments were performed twice.

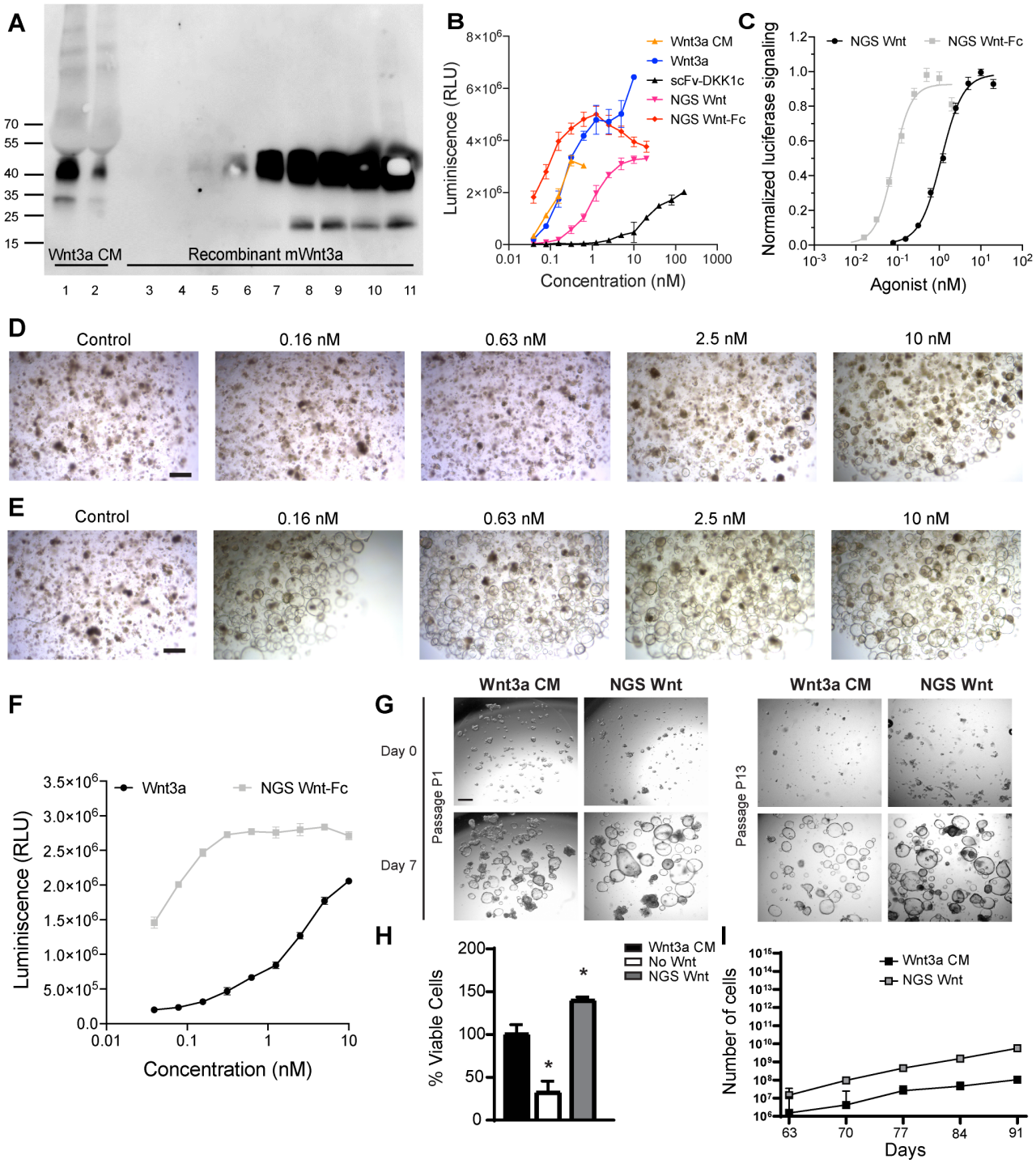
A
70
55
40
35
25
15
Wnt3a CM
Recombinant mWnt3a
1 2 3 4 5 6 7 8 9 10 11
B
Luminiscence (RLU)
Concentration (nM)
Wnt3a CM
Wnt3a
scFv-DKK1c
NGS Wnt
NGS Wnt-Fc
C
Normalized luciferase signaling
Agonist (nM)
NGS Wnt
NGS Wnt-Fc
D
Control
0.16 nM
0.63 nM
2.5 nM
10 nM
E
Control
0.16 nM
0.63 nM
2.5 nM
10 nM
F
Luminiscence (RLU)
Concentration (nM)
Wnt3a
NGS Wnt-Fc
G
Wnt3a CM
NGS Wnt
Wnt3a CM
NGS Wnt
Day 0
Day 7
Passage P1
Passage P13
H
% Viable Cells
Wnt3a CM
No Wnt
NGS Wnt
I
Number of cells
Days
Wnt3a CM
NGS Wnt

**Figure S3. Expansion of organoids using NGS Wnt, Wnt3a CM and recombinant Wnt3a. (Related to Figure 2)**

(A) Quantification of Wnt3a component in Wnt3a CM using Western blots. Lanes 1 to 11 correspond to 100% Wnt3a CM, 50% Wnt3aCM, 0.08 nM, 0.16 nM, 0.31 nM, 0.63 nM, 1.25 nM, 2.5 nM, 5 nM, 10 nM and 20 nM recombinant mWnt3a.

(B) Wnt reporter assays using scFv-DKK1c, Wnt3a CM, mWnt3a, NGS Wnt and Fc conjugated NGS Wnt in the presence of Rspo. Error bar represents S.E. from three technical repeats.

(C) NGS Wnt-Fc showed better $EC_{50}$ (80 pM) compared with NGS Wnt (1.2 nM) in the presence of Rspo. Error bar represents S.E. from three technical repeats.

(D-E) Representative brightfield images of human colon organoids cultured with recombinant mWnt3a (D) or NGS Wnt-Fc (E) using indicated concentrations. Scale bars represents 500 μm.

(F) Quantification of colon organoids cultured in mWnt3a or NGS Wnt-Fc using Cell Titer Glo assays. Error bar represents S.E. of three technical replicates.

(G) Representative brightfield images of normal human colon organoid cultures expanded for 13 passages in either Wnt3a CM (50% v/v) or NGS Wnt (0.5 nM). N=10 for normal human colon organoids. Scale bar is 100 μm.

(H) Quantification of viable cells (as percentage) of normal human colon organoids after first passage (7 days) cultured in Wnt3a CM (black), no Wnt source (white) or NGS Wnt (grey). * indicates $P<0.05$.

(I) Expansion rates and *in vitro* growth curves of normal colon organoids were analyzed at late passages from passage P9 to P13. Graphs illustrate the number of cells counted per well at each passage. Data represent mean ± S.D. of three independent assays. * indicates $P<0.05$.

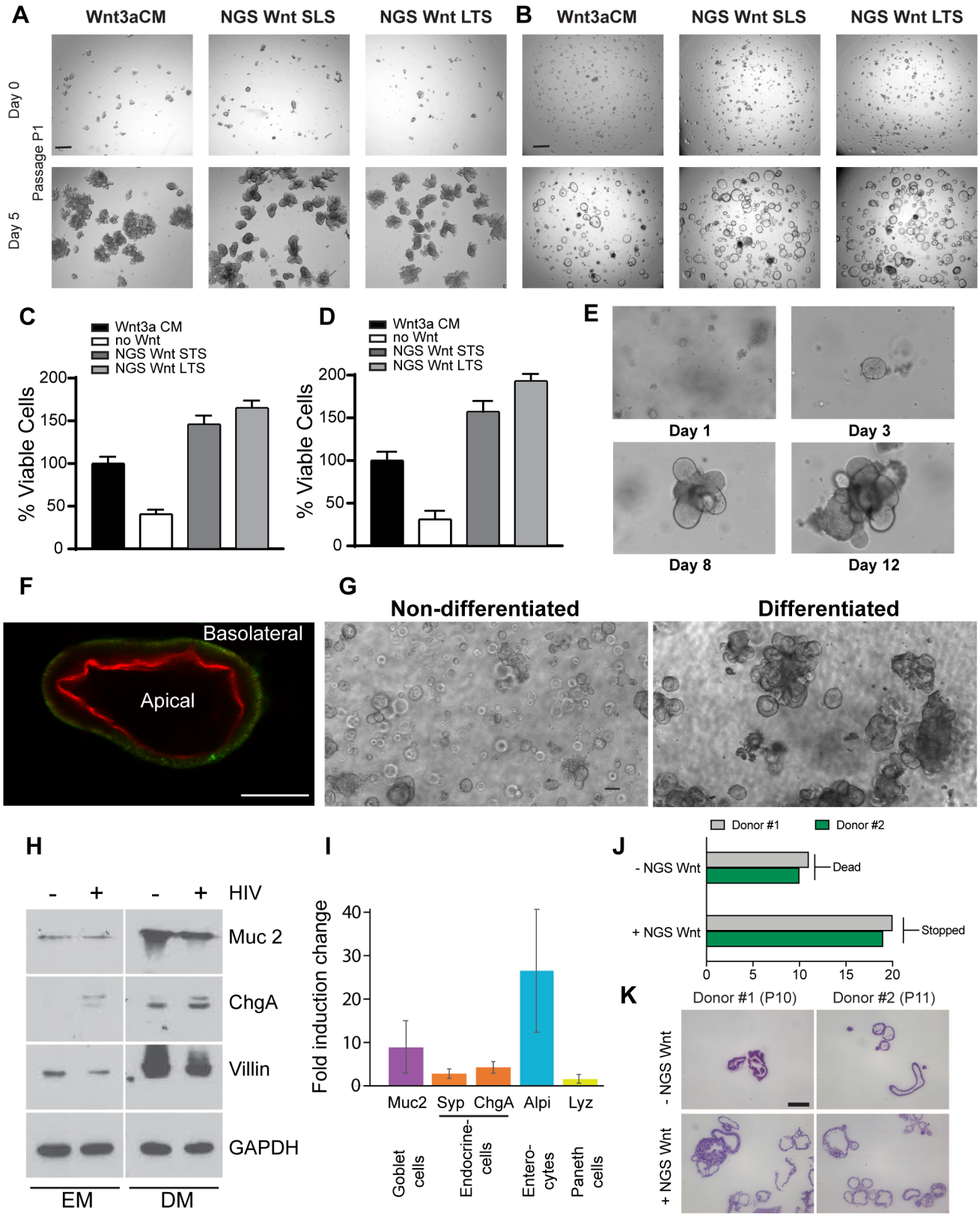
A
Wnt3aCM
NGS Wnt SLS
NGS Wnt LTS
B
Wnt3aCM
NGS Wnt SLS
NGS Wnt LTS
Day 0
Passage P1
Day 5
C
Wnt3a CM
no Wnt
NGS Wnt STS
NGS Wnt LTS
% Viable Cells
200
150
100
50
0
D
Wnt3a CM
no Wnt
NGS Wnt STS
NGS Wnt LTS
% Viable Cells
200
150
100
50
0
E
Day 1
Day 3
Day 8
Day 12
F
Basolateral
Apical
G
Non-differentiated
Differentiated
H
- + - + HIV
Muc 2
ChgA
Villin
GAPDH
EM
DM
I
Fold induction change
40
30
20
10
0
Muc2
Syp
ChgA
Alpi
Lyz
Goblet cells
Endocrine-cells
Entero-cytes
Paneth cells
J
Donor #1
Donor #2
- NGS Wnt
Dead
+ NGS Wnt
Stopped
0
5
10
15
20
K
Donor #1 (P10)
Donor #2 (P11)
- NGS Wnt
+ NGS Wnt

**Figure. S4 NGS Wnt supports organoid expansion and long-term maintenance (Related to Figures 2 and 3)**

(A-D) Representative brightfield images of human cystic fibrosis (A) and healthy (B) colon organoid cultures expanded for 5 days with either Wnt3aCM (50% v/v), NGS Wnt 0.5 nM STS or NGS Wnt 0.5 nM LTS. Viable cells (as percentage) of human cystic fibrosis (C) and healthy (D) colon organoid cultures after 5 days in culture were quantified. STS: short term storage (NGS Wnt stored at -80°C less than 3 months) LTS: Long term storage (NGS Wnt stored at -80°C more than a year). Scale bar is 100 µm.

(E) Representative brightfield images of human colon organoid formation from isolated crypts.

(F) Immunofluorescence images of human colon organoids, Actin (red) and E-cadherin (green). Scale bar represents 100 µm.

(G) Withdrawal of growth factors from the media redirects the stem cells (left) toward a differentiated cell state (right). Scale bar is 100 µm.

(H) Western blot analysis of non-differentiated (EM) and differentiated organoids (DM). Crypts were isolated from HIV -/+ individuals and cultured in EM media containing R-spondin 1, NGS Wnt, Noggin and EGF. Withdrawals of these factors leads to an increase of differentiation markers e.g. Mucin 2 (Muc2) for goblet cells, Chromogranin A (ChgA) for endocrine cells and Villin for enterocytes. GAPDH is used as control.

(I) qRT-PCRs result of non-differentiated (EM) and differentiated organoids (DM) confirm an increase of differentiation markers in organoids cultured in DM, e.g. Mucin 2 (Muc2) for goblet cells, Chromogranin A (ChgA), Synaptophysin (Syp) for endocrine cells, intestinal alkaline phosphatase (Alpi) for enterocytes and Lysozyme 1 (Lyz) for Paneth cells. Expression levels are normalized to 18sRNA expression. Results represent mean and S.E. from N=3.

(J) Tubuloids failed to expand after 10-11 passages in the absence of NGS Wnt. In the presence of NGS Wnt, tubuloids were successfully expanded to passages 19-20 before experiments were stopped.

(K) Hematoxylin and eosin (H&E) staining of human tubuloids cultured without/with NGS Wnt. Scale bar represents 100 µm.

| Table S1. Primer sequences in qRT-PCR, related to STAR Methods. | |
|---|---|
| Mucin 2 Forward | TGGGTGTCCTCGTCTCCTACA |
| Mucin 2 Reverse | TGTTGCCAAACCGGTGGTA |
| ChgA Forward | TAAAGGGGATACCGAGGTGATG |
| ChgA Reverse | TCGGAGTGTCTCAAAACATTCC |
| Syp Forward | TGGTGTTCGGCTTCCTGAA |
| Syp Reverse | GCGGCCCAGCCTGTCT |
| Alpi Forward | GCAACCCTGCAACCCACCCAAGGAG |
| Alpi Reverse | CCAGCATCCAGATGTCCCGGGAG |
| Lyz Forward | TCAATAGCCGCTACTGGTGTA |
| Lyz Reverse | ATCACGGACAACCCTCTTTGC |
| 18s RNA Forward | GTAACCCGTTGAACCCCATT |
| 18s RNA Reverse | CCATCCAATCGGTAGTAGCG |