Supplementary Information for

Driver mutations of the adenoma-carcinoma sequence govern the intestinal epithelial global translational capacity.

WL Smit, CN Spaan, RJ de Boer, P Ramesh, T Martins Garcia, BJ Meijer, JLM Vermeulen, M Lezzerini, AW MacInnes, J Koster, JP Medema, GR van den Brink, V Muncan, J Heijmans.

Corresponding Author:
Jarom Heijmans: j.heijmans@amsterdamumc.nl

This PDF file includes:

www.pnas.org/cgi/doi/10.1073/pnas.1912772117

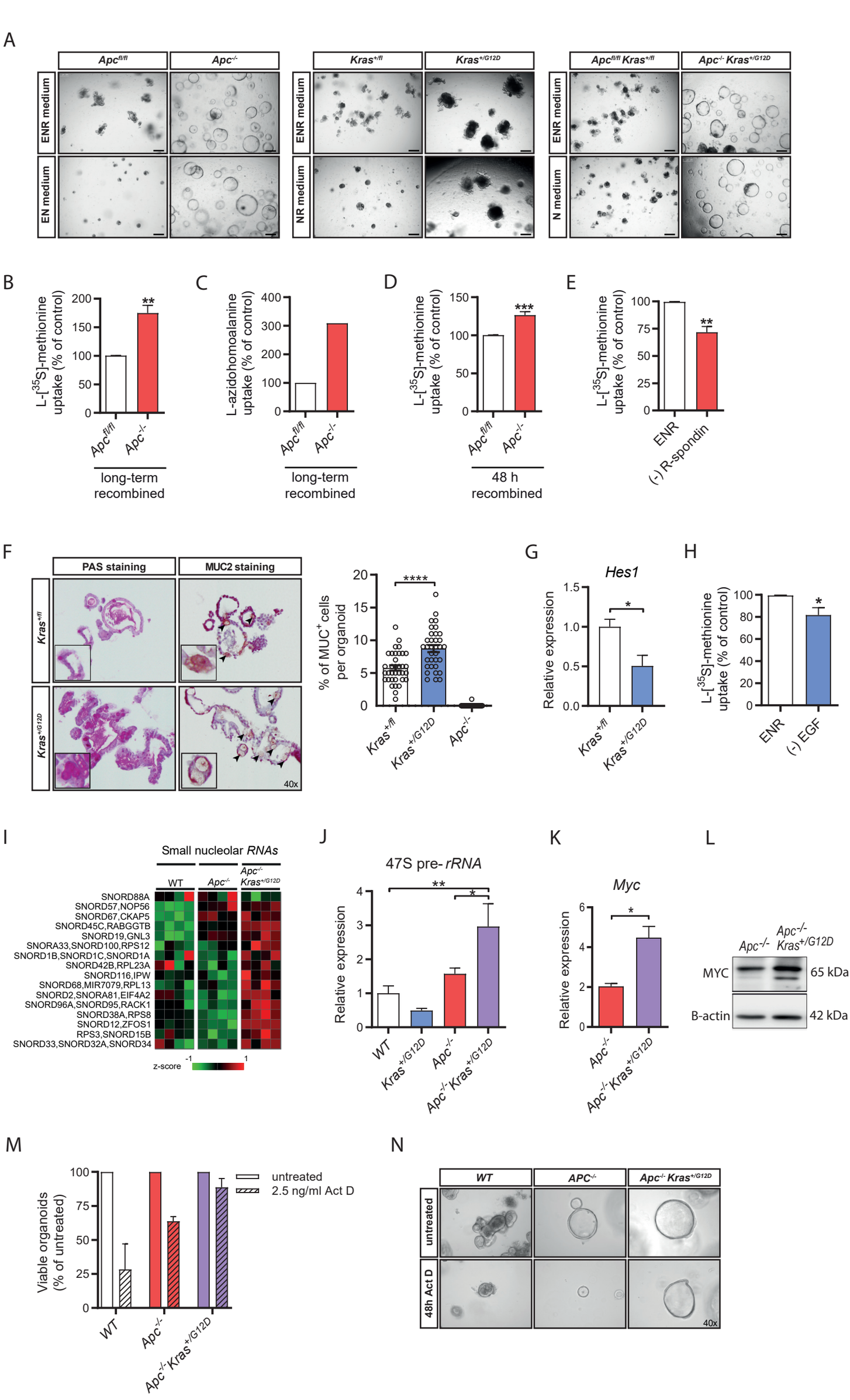

A
Apc^fl/fl
Apc^-/-
Kras^+/fl
Kras^+/G12D
Apc^fl/fl Kras^+/fl
Apc^-/- Kras^+/G12D
ENR medium
EN medium
NR medium
N medium
B
L-[35S]-methionine uptake (% of control)
long-term recombined
C
L-azidohomoalanine uptake (% of control)
long-term recombined
D
L-[35S]-methionine uptake (% of control)
48 h recombined
E
L-[35S]-methionine uptake (% of control)
ENR
(-) R-spondin
F
PAS staining
MUC2 staining
40x
% of MUC+ cells per organoid
G
Hes1
Relative expression
H
L-[35S]-methionine uptake (% of control)
ENR
(-) EGF
I
Small nucleolar RNAs
WT
Apc-/-
Apc-/- Kras+/G12D
SNORD88A
SNORD57,NOP56
SNORD67,CKAP5
SNORD45C,RABGGTB
SNORD19,GNL3
SNORA33,SNORD100,RPS12
SNORD1B,SNORD1C,SNORD1A
SNORD42B,RPL23A
SNORD116,IPW
SNORD68,MIR7079,RPL13
SNORD2,SNORA81,EIF4A2
SNORD96A,SNORD95,RACK1
SNORD38A,RPS8
SNORD12,ZFOS1
RPS3,SNORD15B
SNORD33,SNORD32A,SNORD34
z-score
J
47S pre-rRNA
Relative expression
K
Myc
Relative expression
L
MYC
65 kDa
B-actin
42 kDa
M
Viable organoids (% of untreated)
untreated
2.5 ng/ml Act D
N
WT
APC-/-
Apc-/- Kras+/G12D
untreated
48h Act D
40x

Fig. S1.

(A) Representative images of *Apc*$^{-/-}$ (*left panel)*, *Kras*$^{+/G12D}$ (*middle panel*), and *Apc*$^{-/-}$ *Kras*$^{+/G12D}$ organoids (*right panel*) 3 days after passaging. Each panel also shows the method of selection for mutant cells using culture factor withdrawal; E = EGF, N = Noggin, R = R-spondin, scale bar = 250 μm (*B*) L-[^{35}S]-Methionine incorporation assay in *Apc*$^{-/-}$ organoids recombined for several weeks (>2 weeks) assessed at day 4 after passaging (n = 2) (*C*) Flow cytometric quantification of L-azidohomoalanine incorporation represented as relative fluorescence intensity compared to non-recombined controls assessed at day 4 after passaging. (*D*) L-[^{35}S]-Methionine incorporation assay in *Apc*$^{-/-}$ organoids 48 h after recombination, assessed at day 4 after passaging (n = 4). (*E*) L-[^{35}S]-Methionine incorporation assay in wildtype organoids cultured for 3 days in ENR followed by 24 hour withdrawal of R-spondin (n = 3). (*F*) Example of PAS and MUC2 staining in *Kras*$^{+/G12D}$ organoids and quantification of MUC2-positive cells, indicated by black arrows (40x objective). Data presented as number of MUC2-positive cells relative to the number of cells per organoid. (*G*) Quantitative RT-PCR analysis of Notch target gene *Hes1* in *Kras*$^{+/G12D}$ organoids (n = 3). (*H*) L-[^{35}S]-Methionine incorporation assay in wildtype organoids cultured for 3 days in ENR followed by 24 hour withdrawal of EGF (n = 3). (*I*) Heatmap of microarray *mRNA* expression data containing a set of genes encoding for small nucleolar *RNAs* in wildtype, *Apc*$^{-/-}$ and *Apc*$^{-/-}$*Kras*$^{+/G12D}$ organoids (n = 4). (*J*) Quantitative RT-PCR analysis of the 5' external transcribed spacer of 47S pre-*rRNA* as a by proxy measurement of *rRNA* synthesis (n = 3). (*K*) Quantitative RT-PCR analysis of *Myc* in *Apc*$^{-/-}$ and *Apc*$^{-/-}$*Kras*$^{+/G12D}$ organoids (n = 3). (*L*) Representative immunoblot of MYC levels in *Apc*$^{-/-}$ and *Apc*$^{-/-}$*Kras*$^{+/G12D}$ organoids (n = 2). (*M*) Quantification of the number of viable

organoids treated for 72 h with 2.5 ng/ml Actinomycin D and presented as relative growth reduction to their respective untreated control (n = 2). (*N*) Representative microscopic image of organoids treated with 2.5 ng/ml Actinomycin D.

Significance in all figures analyzed by Student t-test, *P < 0.05, **P < 0.01, ***P < 0.001.

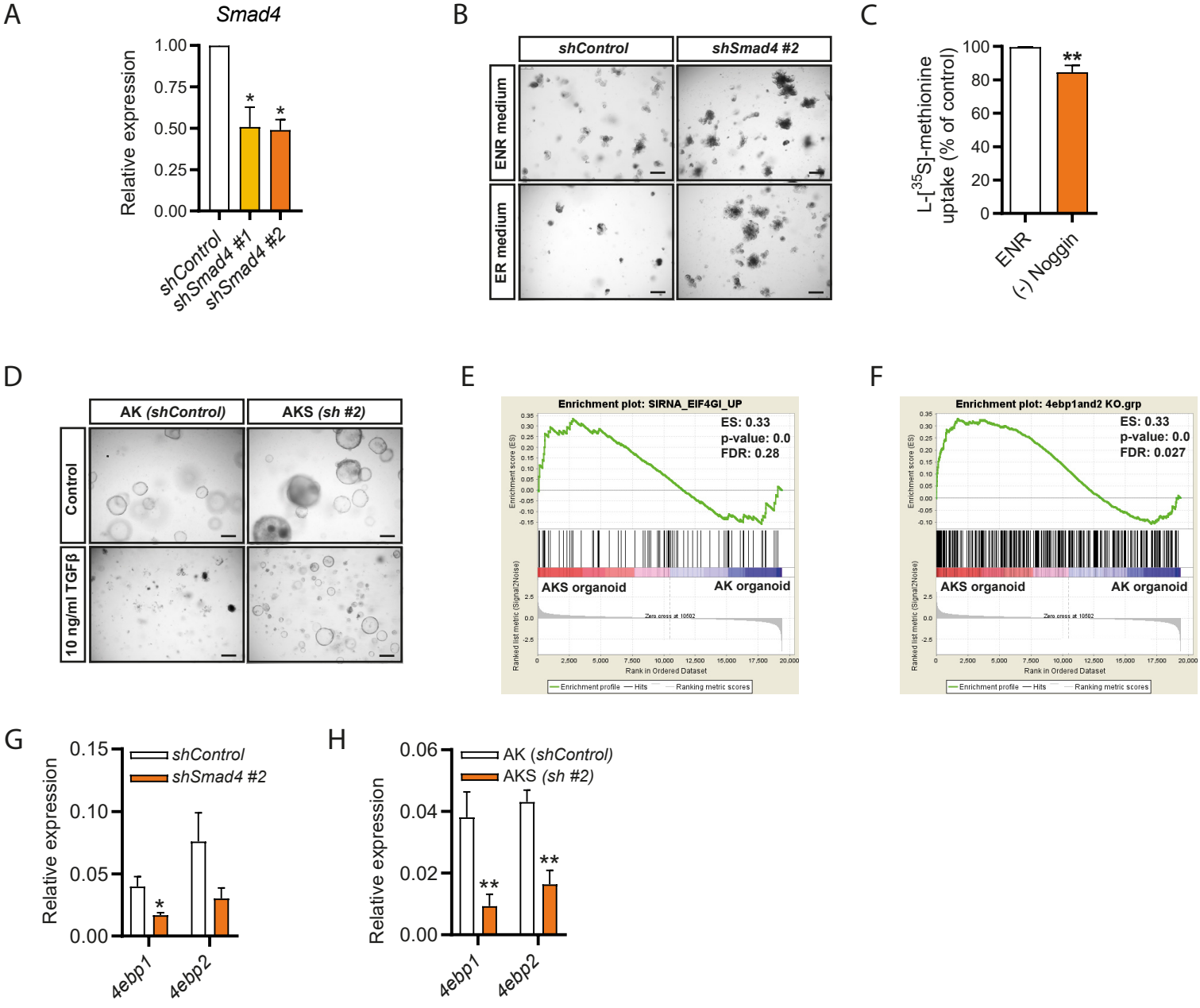
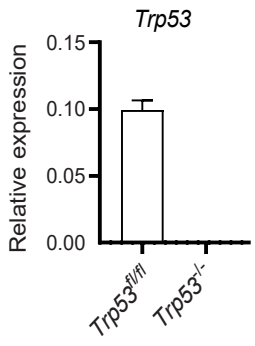

A
Smad4
Relative expression
1.00
0.75
0.50
0.25
0.00
shControl
shSmad4 #1
shSmad4 #2
B
shControl
shSmad4 #2
ENR medium
ER medium
C
L-[35S]-methionine uptake (% of control)
100
80
60
40
20
0
ENR
(-) Noggin
D
AK (shControl)
AKS (sh #2)
Control
10 ng/ml TGFβ
E
Enrichment plot: SIRNA_EIF4GI_UP
ES: 0.33
p-value: 0.0
FDR: 0.28
AKS organoid
AK organoid
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
F
Enrichment plot: 4ebp1and2 KO.grp
ES: 0.33
p-value: 0.0
FDR: 0.027
AKS organoid
AK organoid
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
G
Relative expression
0.15
0.10
0.05
0.00
shControl
shSmad4 #2
4ebp1
4ebp2
H
Relative expression
0.06
0.04
0.02
0.00
AK (shControl)
AKS (sh #2)
4ebp1
4ebp2

Fig. S2.

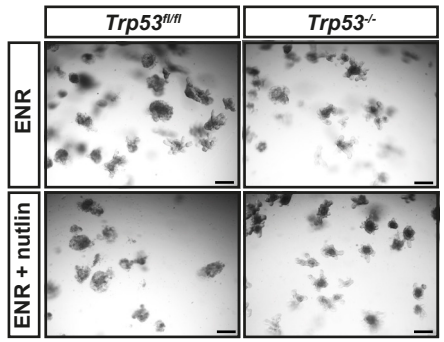
(*A*) Quantitative RT-PCR analysis of *Smad4* in wildtype organoids transduced with two *shRNAs* against *Smad4* and scrambled control *shRNAs* (n = 3). (*B*) Representative image of generated *shSmad4* organoids 3 days after passaging and the method of selection using long-term Noggin withdrawal; E = EGF, N = Noggin, R = R-spondin, scale bar = 250 µm. (*C*) L-[^{35}S]-Methionine incorporation assay in wildtype organoids cultured for 3 days in ENR followed by 24 h withdrawal of Noggin (n = 3). Student t-test, *P < 0.05. (*D*) Representative image of generated Apc$^{-/-}$*Kras*$^{+/G12D}$ *shSmad4 #2* (AKS) organoids and the method of selection showing the regenerative potential under incubation with 10 ng/ml human recombinant TGFß. Scale bar = 250 µm. (*E*) Gene set enrichment plot comparing AK and AKS organoids against a published gene set of a MCF10A cell line with high EIF4G1 expression. (*F*) Gene set enrichment plot comparing AK and AKS organoids against a published gene set of *4ebp1/4ebp2* double KO mouse fibroblasts. (*G*) Quantitative RT-PCR analysis of *4ebp1* and *4ebp2* expression in *shControl* and *shSmad4* organoids. (*H*) Quantitative RT-PCR analysis of *4ebp1* and *4ebp2* expression in AK and AKS organoids (n = 3).

Significance in all figures analyzed by One-way ANOVA, *P < 0.05, **P < 0.01.

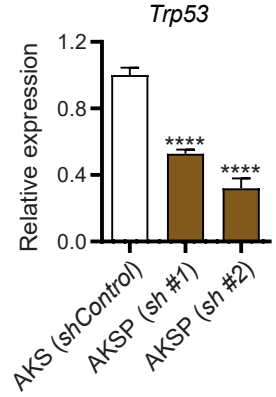
A



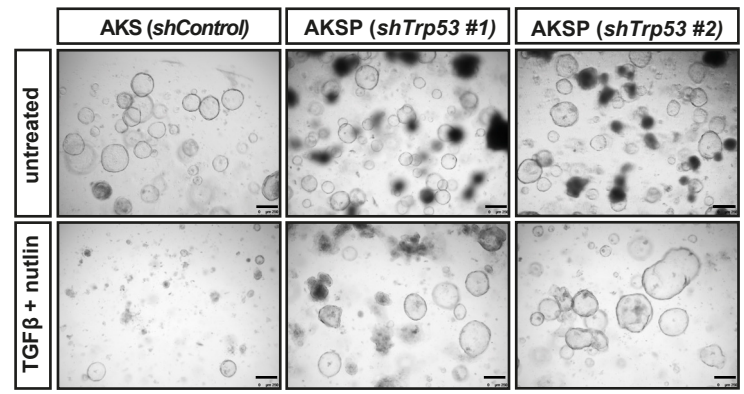
B



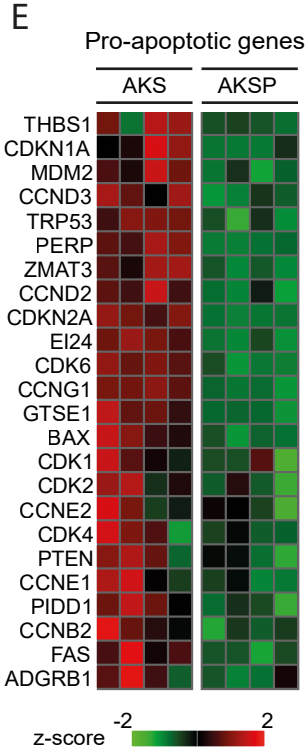
C



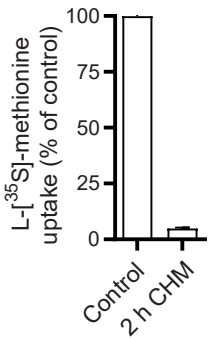
D



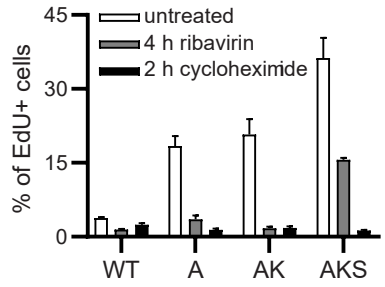
E



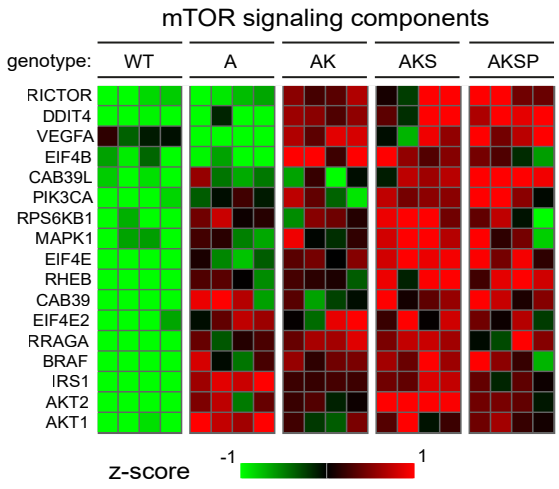
F



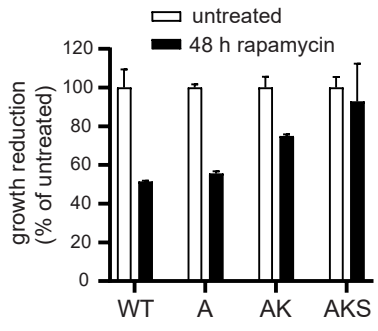
G



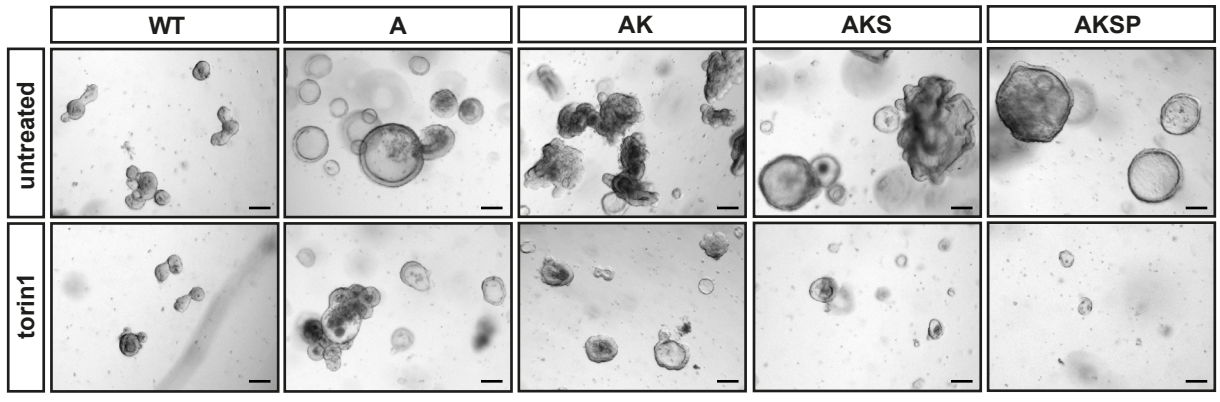
H



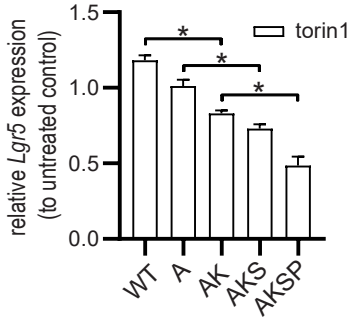
I



J



K



L

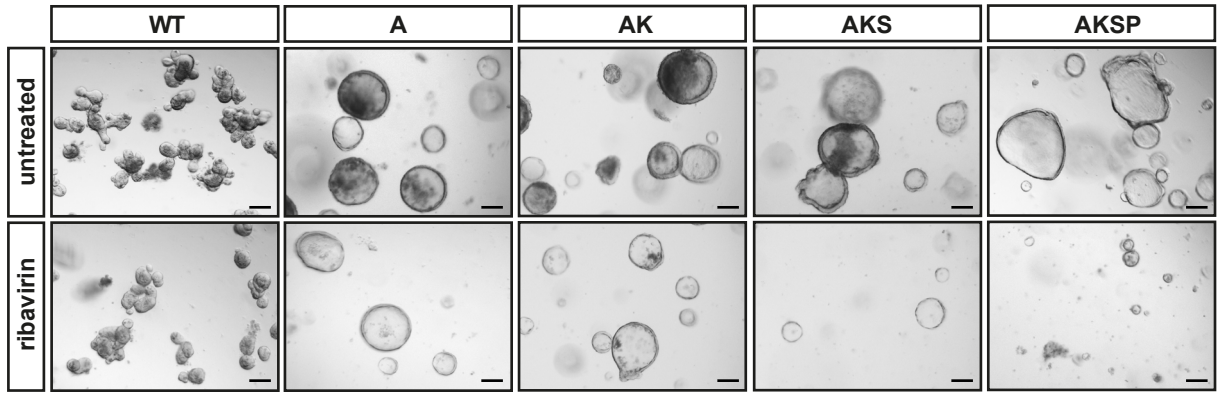


Fig. S3.

(A) Quantitative RT-PCR analysis of *Trp53 mRNA* expression MSCV-Cre *Trp53*$^{-/-}$ organoids 48 h after recombination (n = 2). (*B*) Representative image of *Trp53*$^{-/-}$ organoids 3 days after passaging, and the method of selection using 5 day incubation with 10 µM Nutlin-3. Scale bar = 250 µm. (*C*) Quantitative RT-PCR analysis of *Trp53* in AKS organoids transduced with two different *shRNAs* against *Trp53* (n = 3). (*D*) Representative image of generated AKSP organoids 3 days after passaging, and the method of selection for *Smad4* and *Trp53* knockdown showing the regenerative potential during incubation with a combination of 10 ng/ml recombinant TGFß and 10 µM Nutlin-3. Scale bar = 250 µm. (*E*) Heatmap of microarray *mRNA* expression data containing a set of pro-apoptotic genes in AKSP and AKS organoids (n = 4). (*F*) L-[^{35}S]-Methionine incorporation assay in wildtype organoids treated for 2 h treatment in the presence of 10 µg/ml cycloheximide (CHM). (*G*) EdU incorporation assay in organoids treated for 2 h with 10 µg/ml cycloheximide or 4 h with 1 mM ribavirin. Data presented as percentage of the number of EdU-positive cells to the total cell population (n = 2). (*H*) Heatmap of microarray *mRNA* expression data containing genes related to mTORC1 and mTORC1 signaling in organoids of all ACS genotypes (n = 4). (*I*) Representative experiment of growth in organoids with ACS mutations treated for 48 h with 100 nM rapamycin and presented as quantified growth reduction after treatment relative to untreated control (n = 2). (*J*) Representative images of organoids with ACS mutations treated for 48 h with 50 nM torin 1. (*K*) Quantitative RT-qPCR analysis of stem cell marker *Lgr5* in organoids with ACS mutations treated for 48 h with 50 nM Torin 1 (n = 2). (*L*) Representative images of organoids with ACS mutations treated for 48 h with 10 µM ribavirin.

Significance in all figures analyzed by One-way ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001.

Table 1. List of used RT-qPCR primers

Gene	Forward primer	Reverse primer
Lgr5	TGTGTCAAAGCATTTCCAGC	CAGCGTCTTCACCTCCTACC
Lyz1	ATGGAATGGCTGGCTACTATGG	ACCAGTATCGGCTATTGATCTGA
Muc2	GAAGCCAGATCCCGAAACCA	GAATCGGTAGACATCGCCGT
Chga1	GTCTCCAGACACTCAGGGCT	ATGACAAAAGGGGACACCAA
Alpi	CACAGCTTACCTGGCACTGA	GGTCTCTGACGACAGGGGTA
Cd44	TCTGCCATCTAGCACTAAGAGC	GTCTGGGTATTGAAAGGTGTAGC
Sox9	AGGAAGCTGGCAGACCAGTA	TCCACGAAGGGTCTCTTCTC
Smad4	GGTTGTCTCACCTGGAATTGA	GGCTGTCCTTCAAAGTCGTG
P53	AAAGGATGCCCATGCTACAG	CCCCACTTTCTTGACCATTG
Hes1	CCAGCCAGTGTCAACACGA	AATGCCGGGAGCTATCTTTCT
eIf4ebp1	GGGGACTACAGCACCACTC	CTCATCGCTGGTAGGGCTA
ETS (47S pre-*rRNA*)	TTTTGGGGAGGTGGAGAGTC	AGAGAACTCCGGAGCACCAC

Movie S1.

Cross sectional view of a wildtype small intestinal organoid transduced with scrambled control *shRNA*, three days after passaging. Visible is the central lumen containing cellular debris and normal buds consisting of stem cells and Paneth cells (cells with dark grey vesicles in the crypt bottom).

Movie S2.

Cross sectional view of a small intestinal organoid transduced with *shRNA* against *Smad4*, three days after passaging. Knockdown of *Smad4* results in extensive formation of buds compared to wildtype organoids (movie S1), indicating enrichment for both stem cell and Paneth cell populations.