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Supplemental Information

Canonical Wnt Pathway Controls mESC Self-Renewal Through Inhibition of Spontaneous Differentiation via β-Catenin/TCF/LEF Functions

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Supplemental information

Contents

Supplemental figures

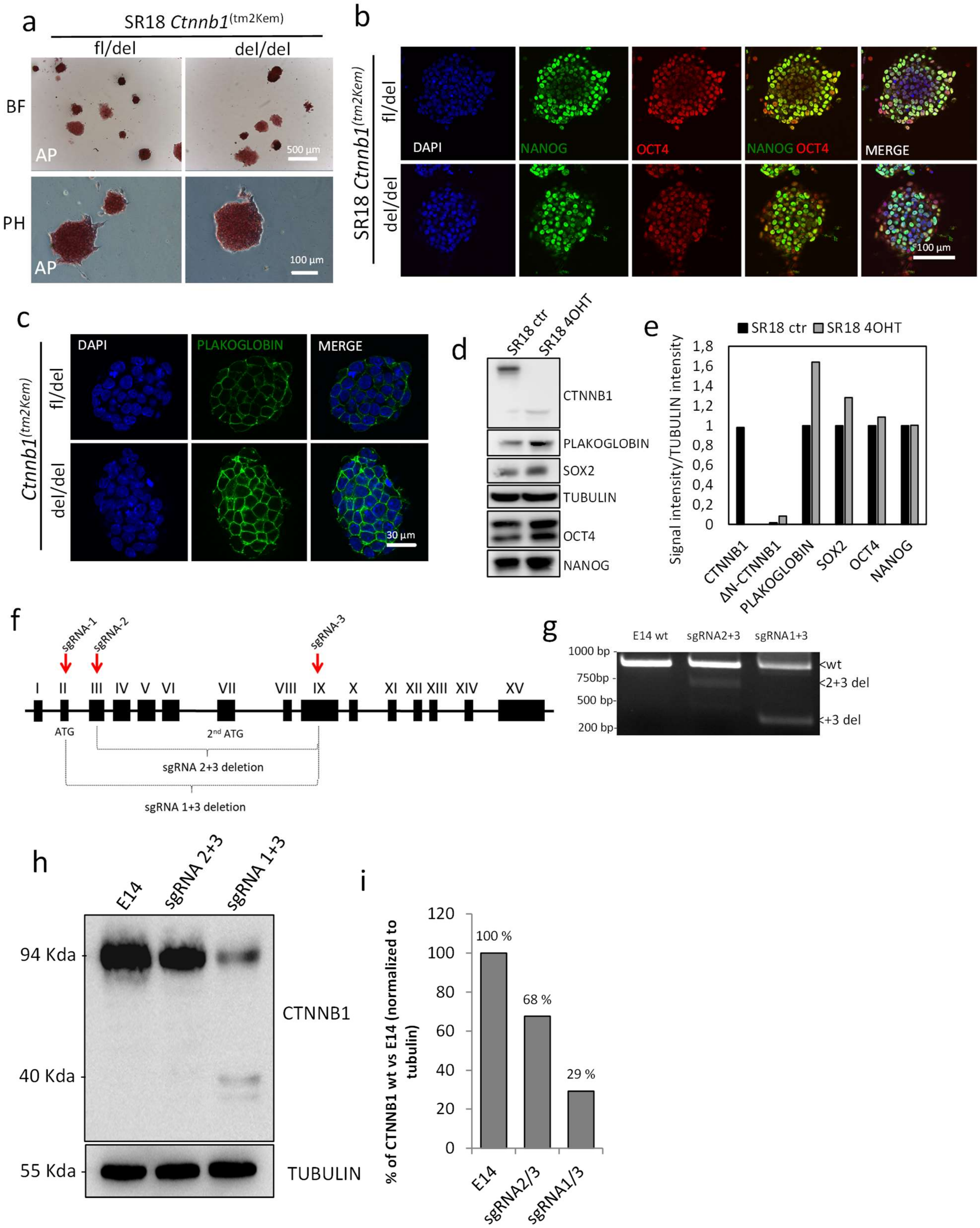


Figure S1

Figure S1 – Knock-out models producing N-terminally truncated isoforms display normal clonogenicity and pluripotency marker expression. Related to Figure 1.

a) SR18 *Ctnnb1*$^{fl/del}$ or *Ctnnb1*$^{del/del}$ display overall similar AP staining expression and morphology. BF=brightfield (scalebar=500 µm), PH=phase contrast (scalebar=100 µm). **b)** Immunofluorescence of Nanog and Oct4 on fixed SR18 *Ctnnb1*$^{fl/del}$ or *Ctnnb1*$^{del/del}$ cells. Scalebar=100 µM. DAPI was used to counterstain nuclei. **c)** Immunofluorescence of Plakoglobin on fixed SR18 *Ctnnb1*$^{fl/del}$ or *Ctnnb1*$^{del/del}$ cells. Scalebar=30 µM. DAPI was used to counterstain nuclei. **d, e)** Western blot (d) and quantification (e) of total protein extracts of SR18 *Ctnnb1fl/del* (SR18 ctr) or *Ctnnb1 del/del* (SR18 4OHT) cells. Protein extracts were probed for β-catenin (CTNNB1), PLAKOGLOBIN, SOX2, OCT4 and NANOG. TUBULIN was used as loading control. **f)** Schematic representation of sgRNAs target positions along the *β-catenin* locus. sgRNAs were used in pairwise combinations to excise different gene regions. sgRNAs are represented as red arrows, indicating the position and orientation of oligonucleotides used for PCR genotyping (3 oligos PCR). **g)** PCR-genotyping of E14 mESCs transiently transfected with Cas9 and pairwise combinations of sgRNAs as depicted in f). Untransfected cells were used as parental control. Expected amplicon size is 824 bp for wild-type, 595 bp for sgRNA2+sgRNA3, 278 bp for sgRNA1+sgRNA3. **h)** Western blot for β-catenin on total protein extract of E14 mESCs parental cell line, or upon transient transfection of Cas9 and pairwise combination of sgRNAs as in f). TUBULIN was used as a loading control. **i)** Quantification of full-length β-catenin deletion in g). β-catenin band intensity was normalized on Tubulin intensity for each sample and then rescaled as a percentage of the untrasfected parental cell line.

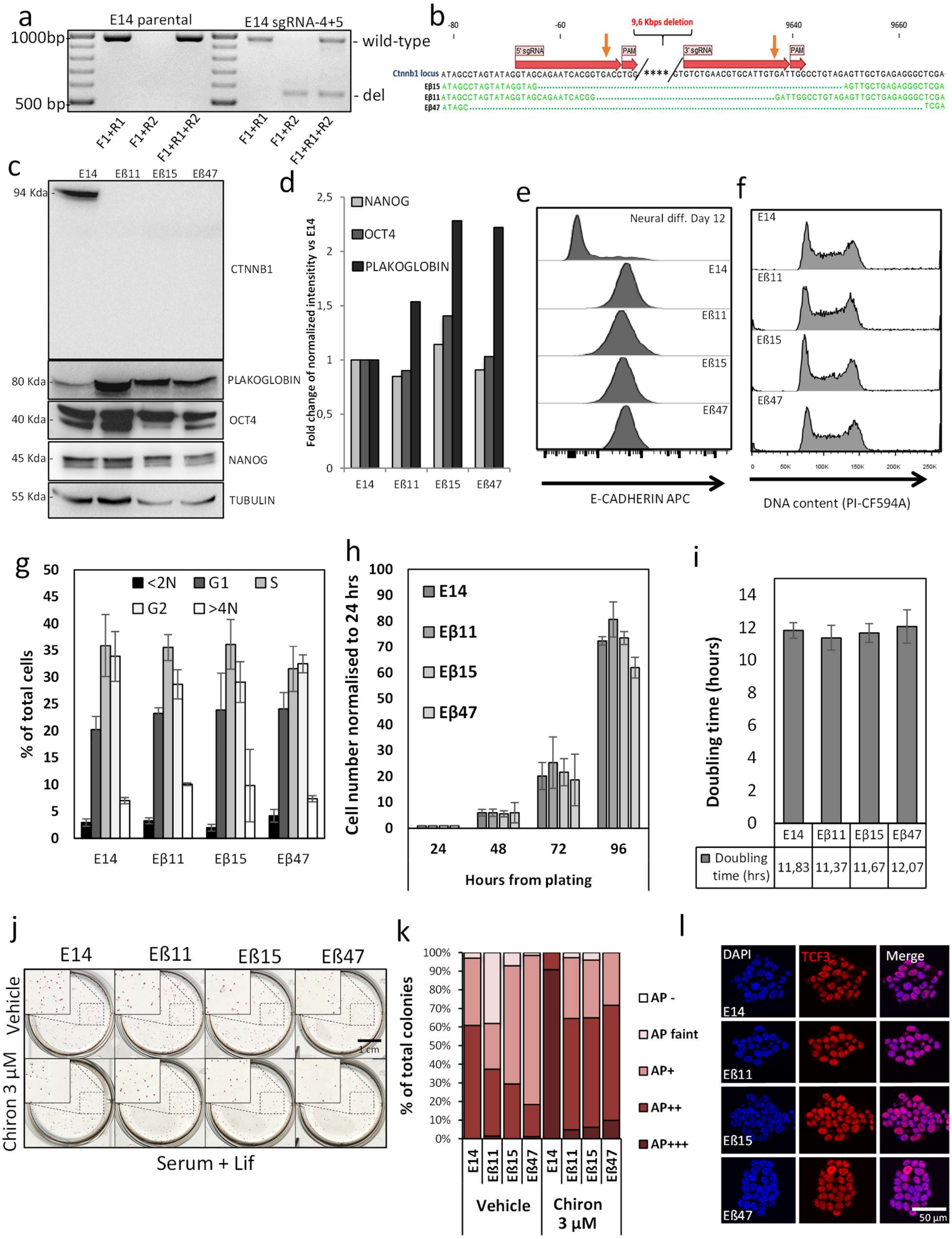
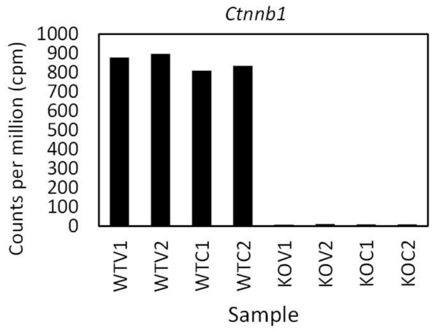

a
E14 parental
E14 sgRNA-4+5
1000bp
500 bp
- wild-type
- del
F1+R1
F1+R2
F1+R1+R2
b
9,6 Kbps deletion
5' sgRNA
PAM
3' sgRNA
Ctnnb1 locus
Eβ15
Eβ11
Eβ47
c
E14
Eß11
Eß15
Eß47
94 Kda
80 Kda
40 Kda
45 Kda
55 Kda
CTNNB1
PLAKOGLOBIN
OCT4
NANOG
TUBULIN
d
NANOG
OCT4
PLAKOGLOBIN
Fold change of normalized intensitity vs E14
e
Neural diff. Day 12
E-CADHERIN APC
f
DNA content (PI-CF594A)
g
<2N
G1
S
G2
>4N
% of total cells
h
Cell number normalised to 24 hrs
Hours from plating
i
Doubling time (hours)
Doubling time (hrs)
11,83
11,37
11,67
12,07
j
Vehicle
Chiron 3 µM
Serum + Lif
1 cm
k
% of total colonies
AP -
AP faint
AP+
AP++
AP+++
Vehicle
Chiron 3 µM
l
DAPI
TCF3
Merge
50 µm

Figure S2

Figure S2 – Characterization of β-catenin knock-out clones. Related to Figure 2.

a) PCR genotyping of E14 transiently transfected with Cas9, sgRNA4 and sgRNA5 (right) and parental cell line (left). Three different oligos combinations were used to detect wild-type allele (F1+F2), deleted alleles (F1+R2) or both (F1+R1+R2). **b)** Sanger sequencing of Eβ11, Eβ15 and Eβ47 *Ctnnb1* edited locus. Matching bases are represented as green letters, green dots are deleted bases. Orange arrows indicate expected Cas9 editing sites, sgRNAs sequences and PAM are shown as red arrows. **c)** Western blot of total protein extracts from E14, Eβ11, Eβ15 and Eβ47 mESCs. Protein extracts were probed for β-catenin, PLAKOGLOBIN, NANOG and OCT4 expression. TUBULIN was used as loading control. **d)** Band intensity quantification relative to western blot in figure (c). Band intensities were normalized on TUBULIN intensity for each sample and then rescaled as fold-change with respect to the parental cell line. **e)** Flow cytometry analysis of E-CADHERIN expression in Eβ11, Eβ15, Eβ47 and parental E14 cells. E14 cells undergoing neuro-ectodermal differentiation were used as negative control for E-Cadherin expression (top). **f)** Representative flow-cytometry DNA content histograms in fixed E14, Eβ11, Eβ15 and Eβ47 cells. PI was used to measure DNA content. **g)** Histogram of cell-cycle analysis on flow-cytometry data in (f). Error bars represent standard error of four independent biological replicates. No statistically significant differences were observed using Student's T-test **h**) Growth curve of E14, Eβ11, Eβ15and Eβ47 cells. Data are cell counts derived from flow-cytometry on living cells. Error bars represent standard deviation of three independent biological replicates. No statistically significant differences were observed using Student's T-test and doubling time analysis; **i)** Average population doubling times of E14, Eβ11, Eβ15 and Eβ47 cells, inferred from growth curve data in (h). Error bars represent standard deviation of three independent biological replicates. No statistically significant differences were observed using Student's T-test. **j)** AP staining of E14, Eβ11, Eβ15 and Eβ47 cells cultured in Serum/LIF in presence of Vehicle (0.3 % DMSO) or 3 μM Chiron for 5 days. Whole plate scanning and magnification inset (dashed boxes). Scalebar= 1 cm. **k)** AP staining intensity quantification relative to Figure 2i. **l)** Immunofluorescence of parental E14 cells, Eβ11, Eβ15 and Eβ47 for Tcf3 expression. DAPI was used to counterstain nuclei. Scalebar= 50 μm.

a



b

		noFC	logFC >0.5	logFC>1	logFC>2
WTV/WTC	Total	3134	1157	316	45
	UP	1454	476	111	14
	DOWN	1680	681	205	31
WTC/KOC	Total	3690	1259	318	47
	UP	1716	437	98	21
	DOWN	1974	822	220	26
WTV/KOV	Total	1318	286	51	11
	UP	658	192	30	2
	DOWN	660	94	21	9
KOV/KOC	Total	1423	254	44	6
	UP	697	124	19	3
	DOWN	726	130	25	3

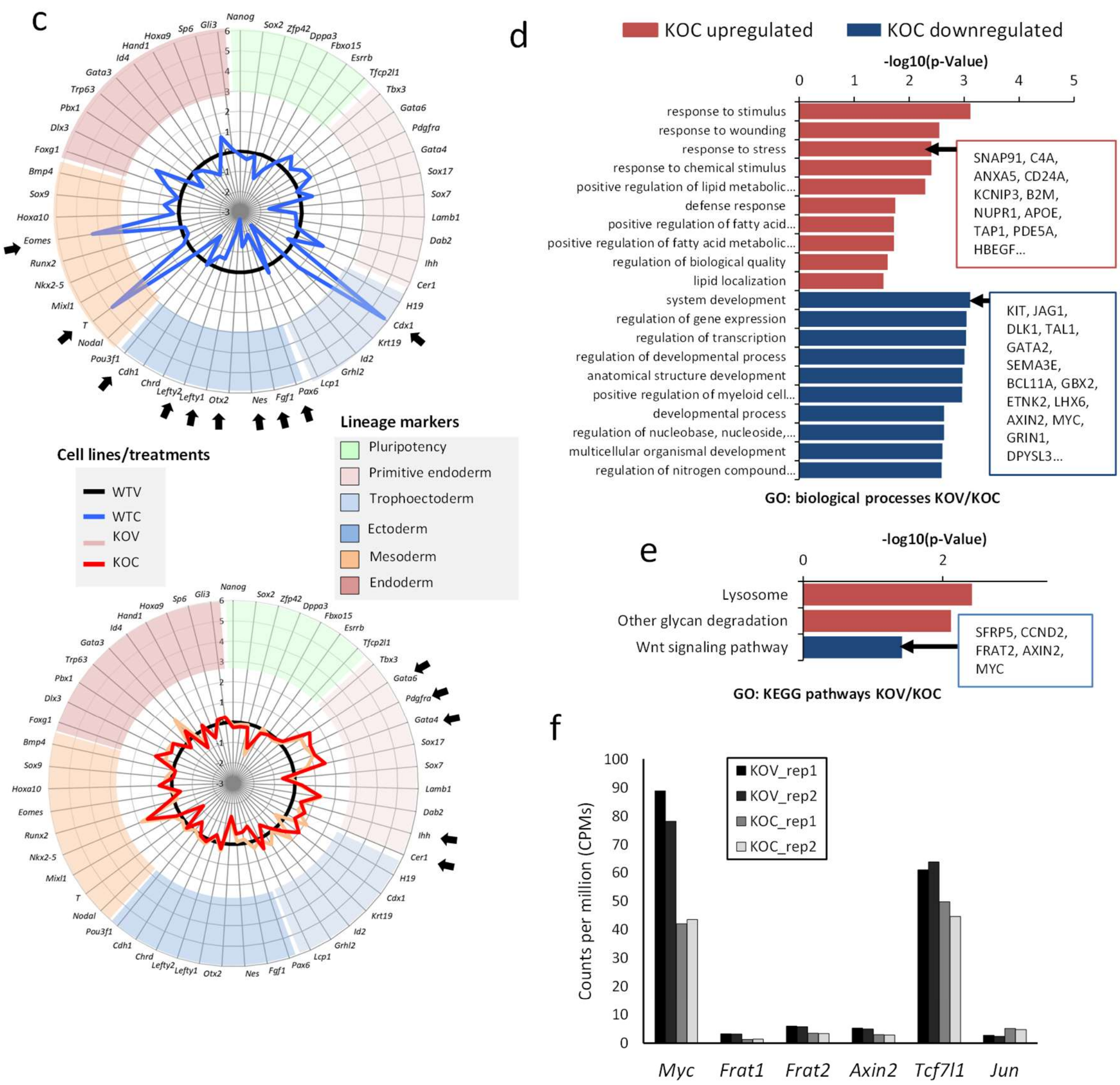


Figure S3

Figure S3 – RNA-seq analysis of β-catenin depletion and Gsk3 inhibition in mESCs. Related to Figure 3.

a) Histogram of *Ctnnb1* mRNA expression levels (raw counts) across WTV, WTC, KOV and KOC samples, individual replicates are shown. **b)** Number of differentially expressed genes in various comparison relative to Figure 3d. **c)** Radar plot showing the fold-change of pluripotency and lineage marker genes in WTC (top panel, blue line) or KOV, KOC samples (light and dark red lines respectively, bottom panel), versus WTV sample (black line, top and bottom panel). **d, e)** Gene ontology analysis of biological processes (d) and KEGG pathways (e) enriched in differentially expressed genes in the KOV/KOC comparison (adjusted -p-value <0.05, absolute logFC >0.5) **f)** Histogram of counts per million (CPMs) of canonical Wnt target genes with minor expression level changes in KOV/KOC comparison. Individual biological replicates are shown.

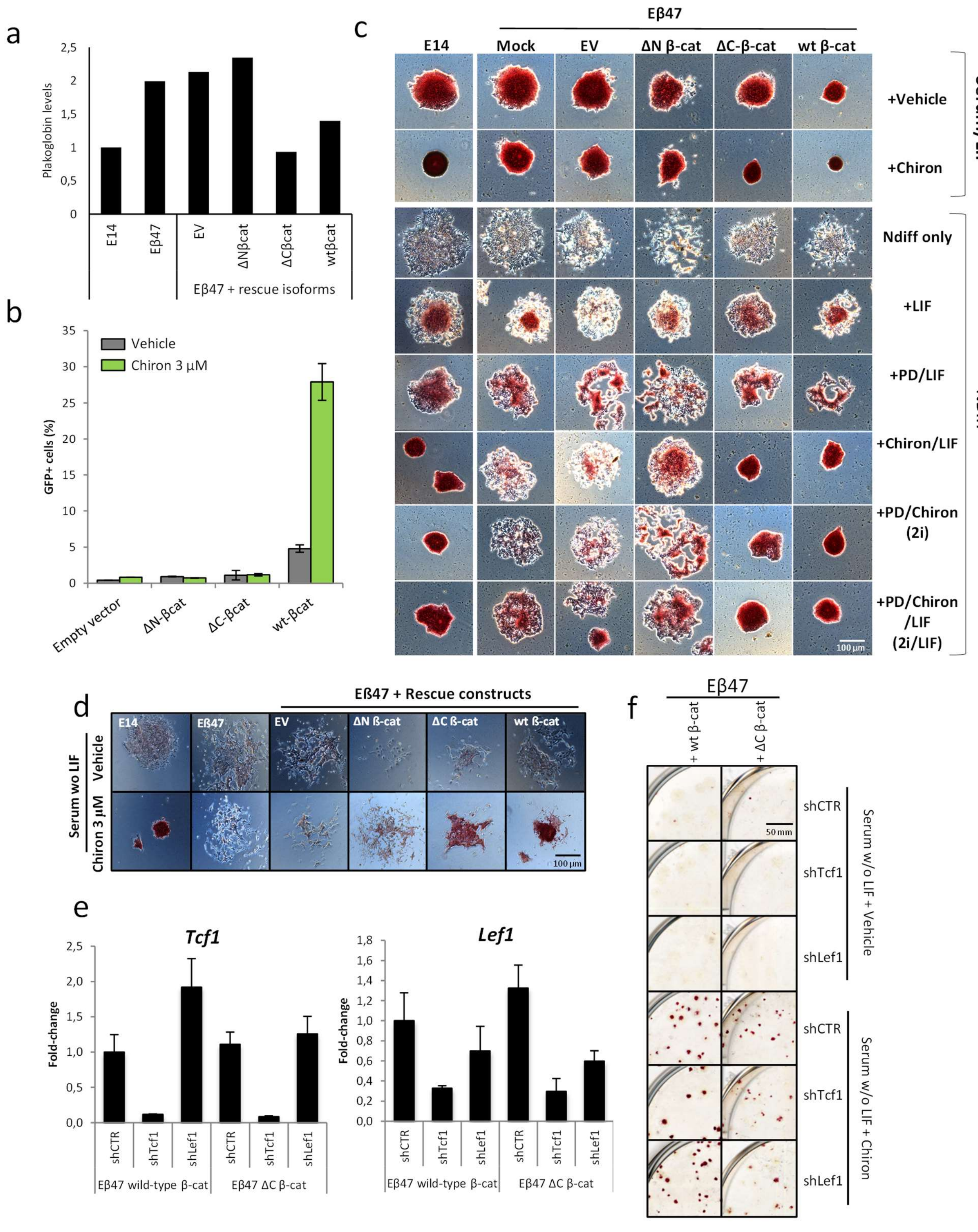

a
Plakoglobin levels
2,5
2
1,5
1
0,5
0
E14
Eβ47
EV
ΔNβcat
ΔCβcat
wtβcat
Eβ47 + rescue isoforms
b
GFP+ cells (%)
Vehicle
Chiron 3 μM
Empty vector
ΔN-βcat
ΔC-βcat
wt-βcat
c
Eβ47
E14
Mock
EV
ΔN β-cat
ΔC-β-cat
wt β-cat
+Vehicle
+Chiron
Serum/LIF
Ndiff only
+LIF
+PD/LIF
+Chiron/LIF
+PD/Chiron (2i)
+PD/Chiron /LIF (2i/LIF)
NDiff
100 μm
d
Eß47 + Rescue constructs
E14
Eß47
EV
ΔN ß-cat
ΔC ß-cat
wt ß-cat
Serum wo LIF
Vehicle
Chiron 3 μM
100 μm
e
Tcf1
Lef1
Fold-change
shCTR
shTcf1
shLef1
Eβ47 wild-type β-cat
Eβ47 ΔC β-cat
f
Eβ47
+ wt β-cat
+ ΔC β-cat
shCTR
shTcf1
shLef1
50 mm
Serum w/o LIF + Vehicle
Serum w/o LIF + Chiron

Figure S4

Figure S4 – Canonical β-catenin functions are required for inhibition of differentiation. Related to Figure 5.

a) Western blot quantification of PLAKOGLOBIN levels relative to Figure 5c. PLAKOGLOBIN/TUBULIN ratios are represented as fold change with respect to the E14 sample. b) Eβ47 cells were transduced with either EV, ΔN β-cat, ΔC β-cat or wt β-cat encoding lentiviruses. Cells were further transduced with the 7TGP Wnt reporter lentivirus and cultured for 48 hours in presence of 3 µM Chiron (green bars) or Vehicle (0.3 % DMSO, grey bars). The percentage of eGFP positive cells is represented for each sample. Error bars represents standard error of two technical replicates. c) AP staining of E14 and Eβ47 cells, and Eβ47 cells, transduced with rescue β-catenin isoforms encoding lentiviruses and cultured for 1 week in the indicated media. Scalebar= 100 µm. d) Exemplificative phase contrast pictures relative to Figure 5f. Scalebar = 100 µm. e) qRT-PCR of *Tcf1* and *Lef1* levels in Eβ47 cells rescued with either wt βcat or ΔC β-cat plasmids and transduced with lentivirus encoding short hairpin against *Tcf1* or *Lef1*. Error bars represents standard errors of technical triplicates. f) AP staining exemplificative pictures relative to Figure 5g. Scalebar= 50 mm.

Supplemental Tables

Table S1 - Differentially expressed genes across pairwise comparisons

Table S2 - WTV_KOV Gene ontology summary

Table S3 - KOV_KOC Gene ontology summary

Table S4 - WTV_WTC Gene ontology summary

Table S5 - KOC_WTC Gene ontology summary

Table S6 – List of oligonucleotides, antibodies and PCR genotyping assays

Supplemental experimental procedures

qRT-PCR

RNA was extracted and purified using with Maxwell LEV semi-automated RNA extraction kit (Promega) following manufacturer instructions. The cDNA was produced with iScript cDNA synthesis kit (BioRad). Real-time quantitative PCR reactions from 8,3 ng of cDNA were set up in triplicate using a LightCycler DNA SYBR Green I Master PCR machine (Roche). The oligonucleotides used in qRT-PCR experiments are provided in **Table S6**.

Western blot, immunofluorescence, flow-cytometry and alkaline phosphatase staining

For western blot experiments cells were harvested and washed twice with PBS. Cell lysis was performed on ice for 25 min, in RIPA buffer (150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris-HCl, pH 8.0) containing a protease inhibitory cocktail (Roche). Insoluble material was pelleted by centrifugation at 16,000×g for 3 min at 4°C. Protein concentrations were determined using the Bradford assay (Bio-Rad). Thirty micrograms extract was mixed with 4× sample buffer (40% glycerol, 240 mM Tris/HCl, pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% β-mercaptoethanol), denatured at 96ºC for 5 minutes, separated by SDS-PAGE, and transferred to nitrocellulose membranes (PROTRAN-Whatman, Schleicher&Schuell). The membranes were blocked with 5% non-fat dry milk in TBS-T for 60 min, incubated with primary antibodies overnight at 4°C, washed three times with TBS-T for 10 min, incubated with the peroxidase-conjugated secondary antibody (1:2000; Amersham Biosciences) in TBST with 5% non-fat dry milk for 60 min, and washed three times with TBST for 10 min. Immunoreactive proteins were detected using Supersignal West Dura HRP Detection kits (Pierce). A list of the primary antibodies is provided in **Table S6**.

For flow-cytometry analysis, cells were trypsinised, washed once in PBS, resuspended in PBS with 5% FBS + 4′,6-diamidino-2-phenylindole (DAPI) and analysed on BD Fortessa cytometer. For E-cadherin staining, incubation with the PE conjugated antibody was performed after the first wash. Neuronally differentiated mESCs were used as negative staining control.

For immunofluorescence, mESCs were fixed with 4% paraformaldehyde for 20 min at room temperature, and then washed twice with PBS following incubation in blocking solution containing 10% goat serum or 3% Bovine Serum Albumin (Sigma) and 0.1% Triton X-100 (Sigma) for 1 h at room temperature. The cells were then left overnight at 4 °C in blocking solution containing the primary antibody. The next day, the cells were washed three times with PBS and then incubated with the secondary antibody for 1 h at room temperature in PBS. The primary antibodies used are provided in **Table S6**. Goat anti-mouse IgG, goat anti-rabbit IgG, (1:1000, Life Technologies) conjugated to Alexa Fluor-488 or Alexa Fluor-594 were used as secondary antibodies. Nuclear staining was performed with DAPI (Life Technologies). For multichannel fluorescence intensity plots in **Figure 1E**, the BAR plugin from FIJI on multi-channel composite images was used. Fluorescence intensities were measured across a section line of equal length as in (Lyashenko et al., 2011).

For AP staining, 600 cells per well were seeded on gelatin coated 6-well plates; fresh medium was added every two days. When distinguishable colonies were formed (usually 5 to 7 days after seeding) cells were washed twice with PBS fixed in 10% Neutral Formalin Buffer for 15 min at 4°C, and washed three times with distilled water. Fixed cells were then incubated for 45 min at room temperature in 2ml/well of the staining solution prepared as follows: 0,005g Naphthol AS MX-PO4 (Sigma, N5000), 0,03g Red Violet LB salt (Sigma, F1625), 200 µl N,N-Dimethylformamide (DMF, Fischer Scientific, D1191), 25 ml of Tris-HCl (MW=157.6, pH 8.3, 0.2M), and 25 ml of distilled water. Finally, the staining solution was removed, and cells were overlaid with 2 ml PBS and imaged using phase contrast and widefield microscopy. Whole plate scanning images were acquired through a high-resolution scanner. AP staining intensity was manually scored as previously described (Wray et al., 2011) using phase contrast microscopy.

Constructs

sgRNAs were cloned by annealed oligos cloning into px459-SpCas9-Puro (Addgene # 48138) as previously described (De Jaime-Soguero et al., 2017), oligonucleotides used for generating Ctnnb1 targeting vectors are listed in **Table S6**. pSpCas9(BB)-2A-Puro (PX459) was a gift from Dr Feng Zhang(Ran et al., 2013). Lentiviral vectors expressing wt or truncated β-catenin isoforms (pL-EF1a-Puro) were generated by Gibson assembly. pL-EF1a-Puro backbone was amplified by PCR from 7TGP vector (Addgene#24305) while hEF1a promoter was amplified from p1494 EF1a Ires Hygro vector (Aulicino et al., 2014). Wt β-cat CDS was amplified from mESCs cDNA using Superscript III first-strand cDNA synthesis kit. Mutant β-catenin CDSs were generated by PCR on the wild-type product. Sequences for pL-EF1a Puro based vectors expressing wt and mutant β-catenin isoforms are provided in **Supplemental DNA sequences**. 7TGP was a gift from Dr Roel Nusse(Fuerer and Nusse, 2010). Short-hairpins RNAs were cloned in pLKO Hygro as previously described (Aulicino et al., 2014). Oligonucleotides sequences for short-hairpin cloning are provided in **Table S6**. pLKO.1 hygro was a gift from Bob Weinberg (Addgene plasmid # 24150). The maps and the full-length sequences of all the constructs generated in this study are provided in **Supplemental DNA sequences**.
List of top oligonucleotides used for cloning sgRNAs into px459-spCas9-Puro digested with BbsI.

Supplemental DNA sequences

Sanger sequencing – related to Figure S2B
Forward sequencing oligo (5'-3'): CACCGTATGCCTACAATCTGTTTCTA
Reverse sequencing oligo (5'-3'): CTACACAATGTTACACGTCTCCAGAT

>Eβ11-Forward
NNNNNNNNCTTCCTGACCCGTGGCTGCTGTGTATTTTTAGTGTATGCCATGGTGAACTGGCTTTTGGTGTC
GGGGCACATAGCCTAGTATAGGTAGAGTTGCTGAGAGGGCTCGAGGGGTGGGCTGGTATCTCAGAAAGT
GCCTGACACACTAACCAAGCTGAGTTTCCTATGGGAACAGTCGAAGTACGCTTTTTGTTCTGGTCCTTTTT
GGTCGAGGAGTAACAATACAAATGGATTTGGGGAGTGACTCACGCAGTGAAGAATGCACACGAATGGAT
CACAAGATGGCGTTATCAAACCCTAGCCTTGCTTGTTCTTTGTTTTAATATCTGTAGTGGTGCTGACTTTGC
TTGCTTTTATTTTTTGCAGTAACTGTTAGTTTTTAAGTAGTGTTATGTTCTAGTGAACCTGCTACAGCAATT
TCTGATTTCTAAGAACCGAGTAATGGTGTAGAACACTAATTCATAATCACGCTAATTGTAATCTGGAGAC
GTGTACAATTGTGTAGANNNNA

>Eβ11-Reverse
TATAGCGTGATTTGATTCGTGTTCTACACCATTTACTCCGGTTTCTTAGAAATCAGAAATTTGCTGTAGCA
GGTTCACTAGAACATAACACTACTTAAAAACTAACAGTTACTGCAAAAAATAAAAGCAAGCAAAGTCAG
CACCACTACAGATATTAAAACAAAGAACAAGCAAGGCTAGGGTTTGATAACGCCATCTTGTGATCCATTC
GTGTGCATTCTTCACTGCGTGAGTCACTCCCCAAATCCATTTGTATTGTTACTCCTCGACCAAAAAGGACC
AGAACAAAAAGCGTACTTCGACTGTTCCCATAGGAAACTCAGCTTGGTTAGTGTGTCAGGCACTTTCTGA
GATACCAGCCCACCCCTCGAGCCCTCTCAGCAACTCTACCTATACTAGGCTATGTGCCCCGACACCAAAA
GCCAGTTCACCATGGCATACACTAAAAATACACAGCAGCCACGGTGTCAGGAAGCTCTTCTCAGTAGAAA
CAGATTGTAGGCATAACGGTGA

>Eβ15 Forward
CCCGNNANNNNNNNNGNCNNGTGGCTTGCNGTGTATTTTNAGTTGTATGCCATGGTGAACTGGCTTTTGG
TGTCGGGGCACATAGCCTAGTATAGGTAGCAGAATCACGGGATTGGCCTGTAGAGTTGCTGAGAGGGCTC
GAGGGGTGGGCTGGTATCTCAGAAAGTGCCTGACACACTAACCAAGCTGAGTTTCCTATGGGAACAGTCG
AAGTACGCTTTTTGTTCTGGTCCTTTTTGGTCGAGGAGTAACAATACAAATGGATTTGGGGAGTGACTCAC
GCAGTGAAGAATGCACACGAATGGATCACAAGATGGCGTTATCAAACCCTAGCCTTGCTTGTTCTTTGTTT
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TGTTCTAGTGAACCTGCTACAGCAATTTCTGATTTCTAAGAACCGAGTAATGGTGTAGAACACTAATTCAT
AATCACGCTAATTGTAATCTGGAGACGTGTACAATTTGTGTAGANNNNA

>Eβ15-Reverse
NNNNNNNNNNGTGNNNTGANNNGTNGTNCTACACCATTTACTCCGGTTNCTTAGAAATCAGAAATTNGCTG
TAGCAGGTTCACTAGAACATAACACTACTTAAAAACTAACAGTTACTGCAAAAAATAAAAGCAAGCAAA
GTCAGCACCACTACAGATATTAAAACAAAGAACAAGCAAGGCTAGGGTTTGATAACGCCATCTTGTGATC
CATTCGTGTGCATTCTTCACTGCGTGAGTCACTCCCCAAATCCATTTGTATTGTTACTCCTCGACCAAAAA
GGACCAGAACAAAAAGCGTACTTCGACTGTTCCCATAGGAAACTCAGCTTGGTTAGTGTGTCAGGCACTT
TCTGAGATACCAGCCCACCCCTCGAGCCCTCTCAGCAACTCTACAGGCCAATCCCGTGATTCTGCTACCTA
TACTAGGCTATGTGCCCCGACACCAAAAGCCAGTTCACCATGGCATACACTAAAAATACACAGCAGCCAC
GGTGTCAGGAAGCTCTTCTCAGTAGAAACAGATTGTAGCCNTTACGGGTGANNA

>Eβ47-Forward
NNNGNNNNNCTNCCTGACCNGTGGCTGCTGTGTATTTTTAGTGTATGCCATGGTGAACTGGCTTTTGGTGT
CGGGGCACATAGCTCGAGGGGTGGGCTGGTATCTCAGAAAGTGCCTGACACACTAACCAAGCTGAGTTTC
CTATGGGAACAGTCGAAGTACGCTTTTTGTTCTGGTCCTTTTTGGTCGAGGAGTAACAATACAAATGGATT
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TAGAACACTAATTCATAATCACGCTAATTGTAATCTGGAGACGTGTACATTNGTGTAGANNNA

>Eβ47-Reverse

NNTNNNNCGTGATTATGATTAGTGTTCTACACCATTACTCGGTTCTTAGAAATCAGAAATTGCTGTAGCAG
GTTCACTAGAACATAACACTACTTAAAAACTAACAGTTACTGCAAAAAATAAAAGCAAGCAAAGTCAGC
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GAACAAAAAGCGTACTTCGACTGTTCCCATAGGAAACTCAGCTTGGTTAGTGTGTCAGGCACTTTCTGAG
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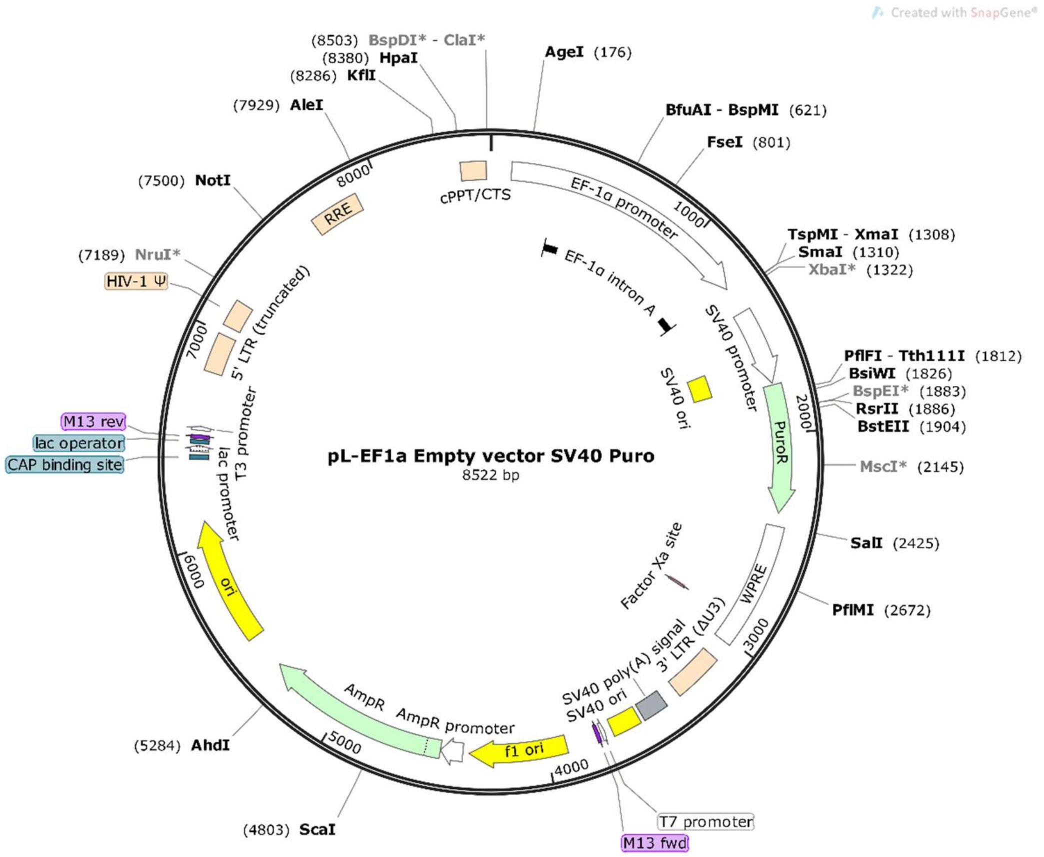
Plasmid maps and sequences



>pL-EF1a empty vector SV40 Puro
CTAGCCCCGATAAGCTTTGCAAAGATGGATAAAGTTTTAAACAGAGAGGAATCTTTGCAGCTAATGGACC
TTCTAGGTCTTGAAAGGAGTGGGAATTGGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCCACAG
TCCCCGAGAAGTTGGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGCGCGGGGTAAACT
GGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGTGCAGT
AGTCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGAACACAGGTAAGTGCCGTGTGTGGTTCCC
GCGGGCCTGGCCTCTTTACGGGTTATGGCCCTTGCGTGCCTTGAATTACTTCCACTGGCTGCAGTACGTGA
TTCTTGATCCCGAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTCGAGGCCTTGCGCTTAAGGAGCCCCTTC
GCCTCGTGCTTGAGTTGAGGCCTGGCCTGGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCACCTTCGC
GCCTGTCTCGCTGCTTTCGATAAGTCTCTAGCCATTTAAAATTTTTGATGACCTGCTGCGACGCTTTTTTTC
TGGCAAGATAGTCTTGTAAATGCGGGCCAAGATCTGCACACTGGTATTTCGGTTTTTGGGGCCGCGGGCG
GCGACGGGGCCCGTGCGTCCCAGCGCACATGTTCGGCGAGGCGGGGCCTGCGAGCGCGGCCACCGAGAA
TCGGACGGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTGGCCTCGCGCCGCCGTGTATCGCCCC
GCCCTGGGCGGCAAGGCTGGCCCGGTCGGCACCAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCT
GCTGCAGGGAGCTCAAAATGGAGGACGCGGCGCTCGGGAGAGCGGGCGGGTGAGTCACCCACACAAAG
GAAAAGGGCCTTTCCGTCCTCAGCCGTCGCTTCATGTGACTCCACGGAGTACCGGGCGCCGTCCAGGCAC
CTCGATTAGTTCTCGAGCTTTTGGAGTACGTCGTCTTTAGGTTGGGGGGAGGGGTTTTATGCGATGGAGTT
TCCCCACACTGAGTGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAATTTG

CCCTTTTTGAGTTTGGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCAAAGTTTTTTTCTTCCATTTC
AGGTGTCGTGAGGAATTCTGCAGTCGATCGACGGTACCGCGGGCCCGGGGCTCGAGATCTAGAGTCGAG
AAGCTTGATGATCTGCGCAGCACCATGGCCTGAAATAACCTCTGAAAGAGGAACTTGGTTAGGTACCTTC
TGAGGCGGAAAGAACCAGCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCA
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TGCAAAAAGCTTACCATGACCGAGTACAAGCCCACGGTGCGCCTCGCCACCCGCGACGACGTCCCCAGGG
CCGTACGCACCCTCGCCGCCGCGTTCGCCGACTACCCCGCCACGCGCCACACCGTCGATCCGGACCGCCA
CATCGAGCGGGTCACCGAGCTGCAAGAACTCTTCCTCACGCGCGTCGGGCTCGACATCGGCAAGGTGTGG
GTCGCGGACGACGGCGCCGCGGTGGCGGTCTGGACCACGCCGGAGAGCGTCGAAGCGGGGGCGGTGTTC
GCCGAGATCGGCCCGCGCATGGCCGAGTTGAGCGGTTCCCGGCTGGCCGCGCAGCAACAGATGGAAGGC
CTCCTGGCGCCGCACCGGCCCAAGGAGCCCGCGTGGTTCCTGGCCACCGTCGGCGTCTCGCCCGACCACC
AGGGCAAGGGTCTGGGCAGCGCCGTCGTGCTCCCCGGAGTGGAGGCGGCCGAGCGCGCCGGGGTGCCCG
CCTTCCTGGAGACCTCCGCGCCCCGCAACCTCCCCTTCTACGAGCGGCTCGGCTTCACCGTCACCGCCGAC
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ACCCGCAGCGCCCGACCGAAAGGAGCGCACGACCCCATGCCAGTCGACAATCAACCTCTGGATTACAAA
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CCTTTGTATCATGCTATTGCTTCCCGTATGGCTTTCATTTTCTCCTCCTTGTATAAATCCTGGTTGCTGTCTC
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ACTGGTTGGGGCATTGCCACCACCTGTCAGCTCCTTTCCGGGACTTTCGCTTTCCCCCTCCCTATTGCCACG
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ACGTCCTTCTGCTACGTCCCTTCGGCCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCTGCTGCCGGCTCT
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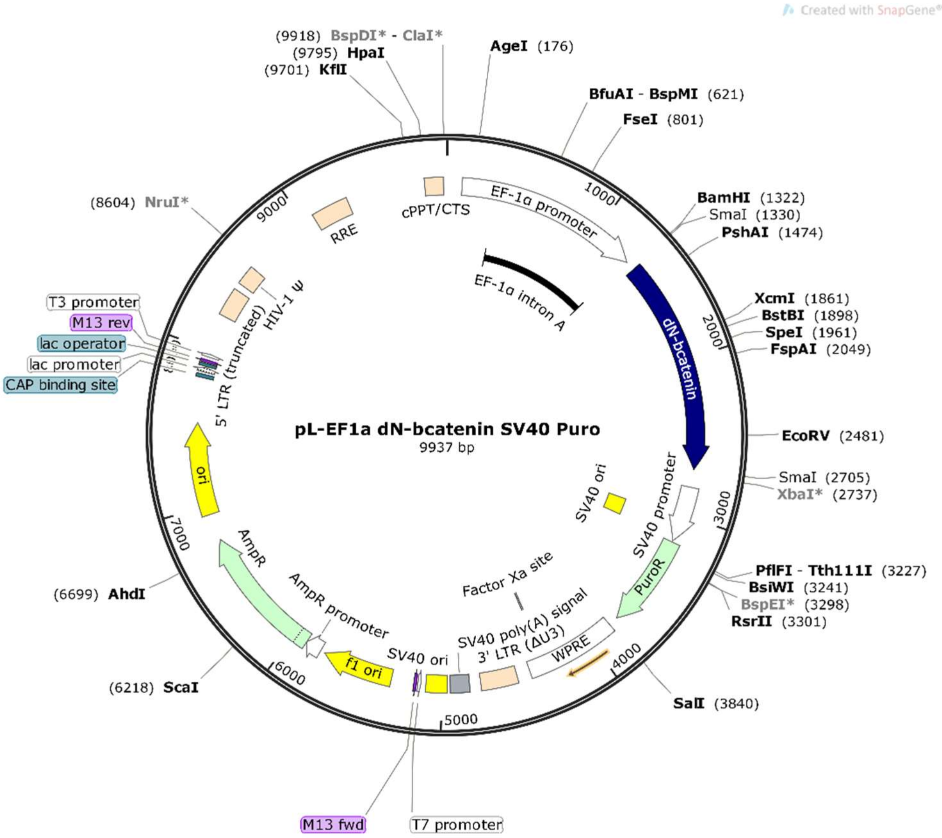

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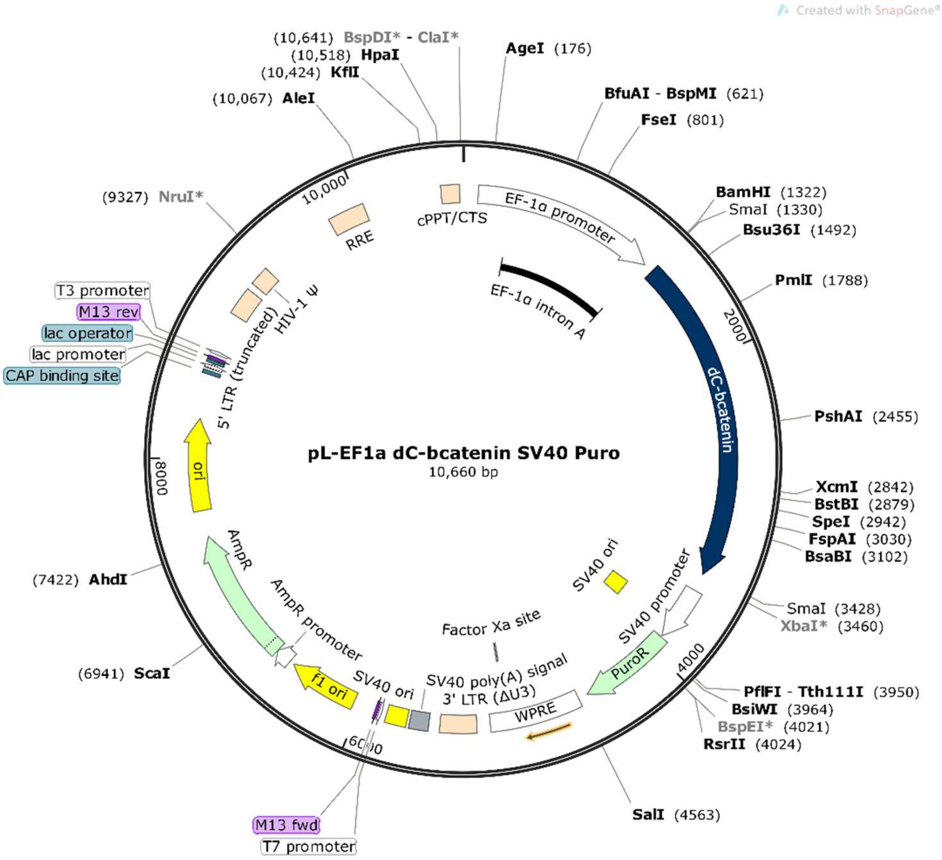

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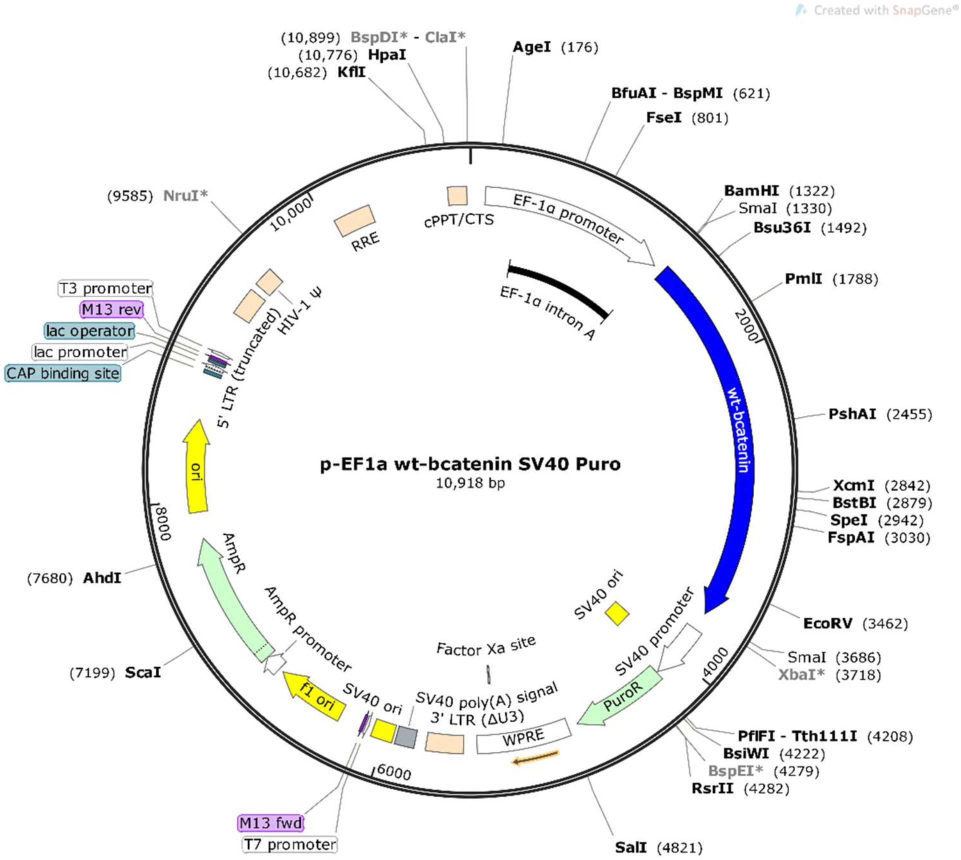

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AAAATTCAAAATTTTATCGATCACGAGACTAGCCTC

Generation of Ctnnb1 KO cells

CRISPR/Cas9 was used to induce small in-dels, microdeletions or complete deletion of the Ctnnb1 locus using single sgRNA or pairwise combinations. Briefly for each experiment $5x10^6$ mESCs (E14Tg2a from ATCC) per well were seeded onto gelatin-coated mw6 plates. 24 hours after seeding, 2 ml of fresh mESCs medium were provided at least 30 minutes before transfection. Transfection mix consisted of 5 ug of all-in-one vectors expressing Cas9 and previously subcloned sgRNA (px459-spCas9-Puro), 100 µl Optimem (Thermo-Fisher) and 20 µl Polyfectamine reagent (Qiagen). For co-transfection of two sgRNAs, 2.5 ug of each vector were used. Transfection mix was incubated 15 minutes at room temperature and then directly added to seeded mESCs. Fresh mESC medium was added to a final volume of 2,7 ml and 24 hours after, medium was replaced. 48 hours after transfection puromycin selection (5 ug/ml) was applied for additional 48 hours. Cells were then analysed at population level to assess the knock-out efficiency. For the establishment of β-catenin KO cell lines, transfected pools were replated at clonal density and single cell clones were manually picked and screened for homozygous Ctnnb1 deletion. For PCR assay of Ctnnb1, two or three oligonucleotides were used depending on the deletion strategy. Knock-out and screening strategies together with oligos for genotyping edited cells are summarised in **Table S6**.

Cell cycle and proliferation analysis

For cell cycle analysis, cells were seeded in equal number ($1x10^6$ cells) on gelatin coated 10 cm dishes. 3 days after plating cells were trypsinised and collected with complete medium by centrifugation at 300xg for 5 minutes. Cell pellet was washed with PBS twice and centrifuged again for 5 minutes at 300xg. The pellet was then resuspended in cold 70% Ethanol while vortexing and incubated overnight at 4C. The next day cells were stained with propidium iodide after RNAseI treatment using the Propidium Iodide Flow Cytometry Kit (Abcam ab139418) following manufacturer's instructions. DNA content was measured on a BD Fortessa cytometer. Finally, FCS files were processed in FlowJo using the built-in cell-cycle analysis plug-in.

For growth curve analysis $7x10^3$ mESCs per well were plated in triplicates in 96-well plates. For cell counts by FACS each day, for the following 96 hours, cells were detached, diluted in DAPI containing medium to stain dead cells and transferred into mw96 U-bottom plates (Falcon) and counted using FACS-canto. Exponential growth curves and population doubling time were calculated as previously described (De Jaime-Soguero et al., 2017).

Lentivirus production

For mESCs transduction, lentiviral particles were produced following the RNA interference Consortium (TRC) instructions for lentiviral particle production and infection in 6-well plates (http://www.broadinstitute.org/rnai/public/). Briefly, $5×10^5$ HEK293T cells/well were seeded in 6-well plates. The day after plating, the cells were co-transfected with 1 µg of lentiviral vector, 750 µg pCMV-dR8.9, and 250 µg pCMV-VSV-G, using Polyfect reagent (Qiagen). The day after transfection, the HEK293T culture medium was substituted with the ESC culture medium. Then $5×10^5$ ESCs/well were plated onto gelatin-coated 6-well plates the day before transduction. The lentiviral particles containing medium was harvested from HEK293T cells at 48, 72 and 96 hrs after transfection, filtered, and added to the ESC plates. The day after transduction, these ESCs were washed twice in PBS and hygromycin selection or puromycin selection were applied.

Supplemental references

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