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

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SUMMARY

The Wnt/β-catenin signaling pathway is a key regulator of embryonic stem cell (ESC) self-renewal and differentiation. Constitutive activation of this pathway has been shown to increase mouse ESC (mESC) self-renewal and pluripotency gene expression. In this study, we generated a novel β-catenin knockout model in mESCs to delete putatively functional N-terminally truncated isoforms observed in previous knockout models. We showed that aberrant N-terminally truncated isoforms are not functional in mESCs. In the generated knockout line, we observed that canonical Wnt signaling is not active, as β-catenin ablation does not alter mESC transcriptional profile in serum/LIF culture conditions. In addition, we observed that Wnt signaling activation represses mESC spontaneous differentiation in a β-catenin-dependent manner. Finally, β-catenin (ΔC) isoforms can rescue β-catenin knockout self-renewal defects in mESCs cultured in serum-free medium and, albeit transcriptionally silent, cooperate with TCF1 and LEF1 to inhibit mESC spontaneous differentiation in a GSK3-dependent manner.

INTRODUCTION

β-Catenin regulates different cellular processes spanning from development to cancer progression. In addition to its central role in adherens junctions, β-catenin is the key effector of the canonical Wnt signaling pathway. Exposure to canonical Wnt ligands, such as WNT3A or small-molecule inhibitors of GSK3 activity, triggers β-catenin stabilization and its nuclear translocation. In the nucleus, β-catenin acts as a scaffolding protein for transcriptional co-factors such as TCF/LEF family members, thereby activating the expression of Wnt target genes. Over the past years, accumulating evidence highlighted a key role for Wnt/β-catenin signaling in sustaining self-renewal, pluripotency, and cell-cycle progression of mouse embryonic stem cells (mESCs) (De Jaime-Soguero et al., 2017; Sato et al., 2004; Ten Berge et al., 2011) and in regulating somatic cell reprogramming (Aulicino et al., 2014; Lluis et al., 2008, 2011; Marucci et al., 2014). Despite its importance in mESC physiology, β-catenin knockout mESC lines developed so far show no defects in self-renewal or pluripotency marker expression when cultured in a medium containing serum plus the leukemia inhibitory factor (LIF), but display a strict LIF requirement for pluripotency maintenance and fail to efficiently differentiate *in vitro* (Lyashenko et al., 2011; Wray et al., 2011). Surprisingly, LIF dependency can be rescued by transcriptionally defective β-catenin isoforms (ΔC mutants), challenging the hypothesis that β-catenin transcriptional activity could be relevant for mESC pluripotency and self-renewal (Lyashenko et al., 2011; Wray et al., 2011). More recently, however, it has been demonstrated that the most widely used inducible β-catenin knockout alleles lead to the production of uncharacterized N-terminally truncated β-catenin isoforms (ΔN β-cat) during pre-implantation embryo development (Messerschmidt et al., 2016).

Here, we confirmed the production of ΔN β-cat isoforms in mESCs and generated a novel β-catenin full knockout model in mESCs using CRISPR/Cas9. We found that complete β-catenin deletion produces similar phenotypes observed in the previously described knockout models retaining ΔN β-cat fragments, suggesting that N-terminally truncated isoforms are biologically inactive. We furthermore analyzed the impact of β-catenin loss at transcriptomic level, in presence or absence of GSK3 chemical inhibition. Our results show that the Wnt/β-catenin pathway is not transcriptionally active in mESCs cultured in serum/LIF. However, upon GSK3 inhibition, we observed β-catenin-dependent inhibition of differentiation markers,

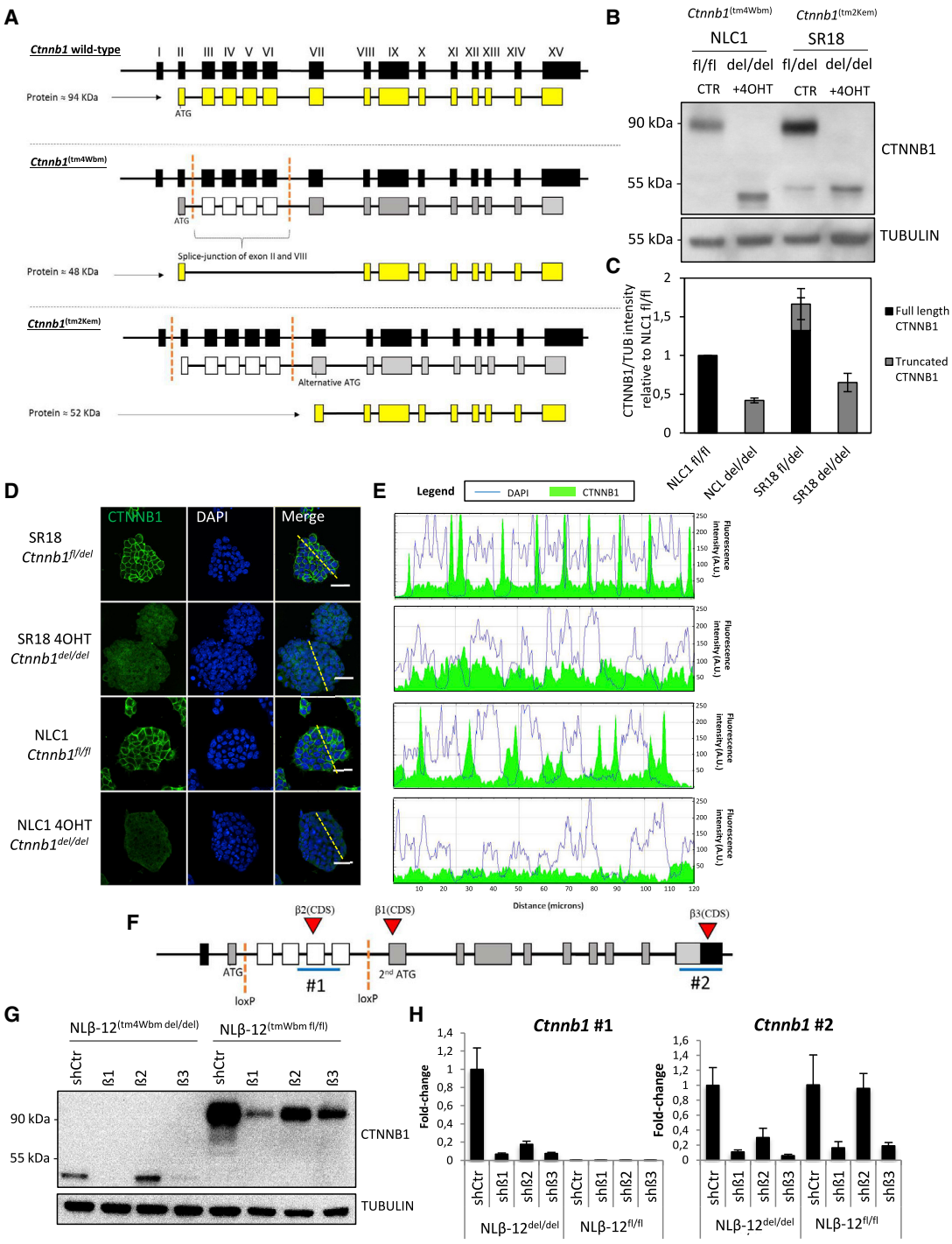

Figure 1. Inducible β-Catenin Knockout Alleles Produce N-Terminally Truncated Isoforms in mESCs

(A) Schematic representation of murine *β-catenin* (*Ctnnb1*) locus and the two *loxP* alleles used for β-catenin studies in mESCs. Black boxes represent exons, yellow boxes coding exons, dashed red lines indicate *loxP* sites, and white boxes represent exons excised upon CRE-mediated recombination of *loxP* sites.

(B and C) Western blot (B) and relative quantification (C) of NLC1 and SR18 cell lines upon 72-h 4′-hydroxytamoxifen treatment (+4OHT) and respective untreated controls (CTRs). SR18 untreated cell line is heterozygous for full-length β-catenin deletion. Western blot band intensities (C) are normalized on NLC1 full-length CTTNB1.

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while the expression of pluripotency genes remained unchanged. Finally, we showed that transcriptionally impaired C-terminally truncated β-catenin rescue isoforms (ΔC β-cat) can inhibit mESC differentiation in the absence of LIF when GSK3 is inhibited, as their full-length counterpart. However, this phenotype is impaired upon silencing of TCF1/LEF1, suggesting that ΔC β-cat isoforms are not entirely transcriptionally silent and their nuclear function could depend on TCF/LEF factors.

RESULTS

Inducible β-Catenin Knockout Alleles Generate N-Terminally Truncated Isoforms in mESCs

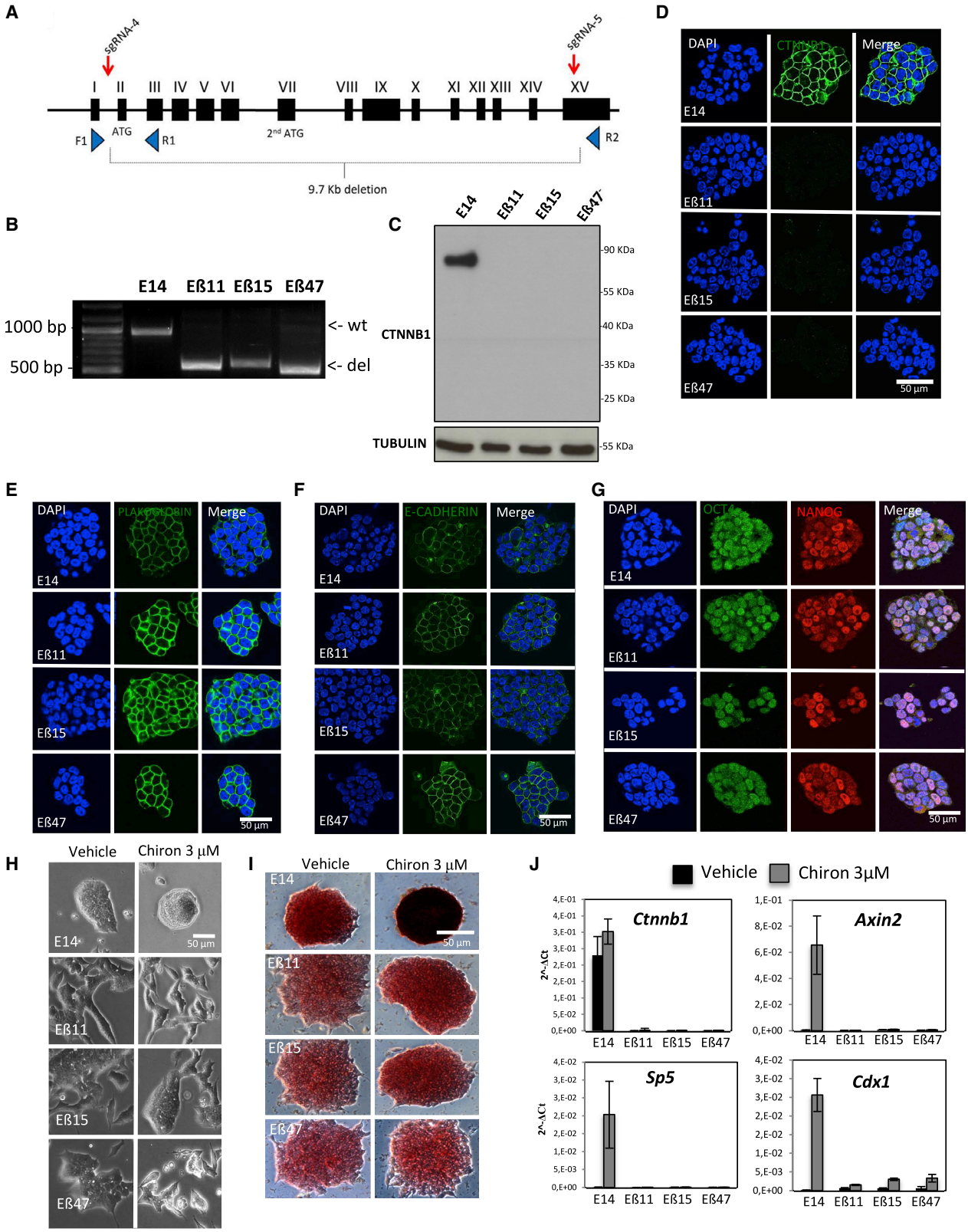
β-Catenin (*Ctnnb1*) mRNA includes 15 exons, with an open reading frame (ORF) spanning from exon 2 to exon 15. Previously reported β-catenin knockout models in mESCs were generated using CRE-mediated excision of a DNA fragment flanked by two *LoxP* sites encompassing exons 3–6 (*Ctnnb1*^{tm4Wbm}) or 2–6 (*Ctnnb1*^{tm2Kem}). Three groups have independently studied both alleles in mESCs and concluded that β-catenin is dispensable for mESC self-renewal (Lyashenko et al., 2011; Raggioli et al., 2014; Wray et al., 2011).

A recent study reported that, during pre-implantation embryo development, both these β-catenin knockout alleles are flawed by the production of N-terminally truncated (ΔN) proteins, possibly generated by alternative splicing (*Ctnnb1*^{tm4Wbm}) or by a secondary ATG within a Kozak consensus sequence downstream of the excised region (*Ctnnb1*^{tm2Kem}) (Figure 1A) (De Vries et al., 2004; Messerschmidt et al., 2016). Remarkably the production of these ΔN β-cat isoforms in mESCs has not been previously reported (Anton et al., 2007; Lyashenko et al., 2011; Wagner et al., 2010; Wray et al., 2011). We therefore tested the expression of ΔN β-cat isoforms by using an antibody raised against the C-terminal portion of β-catenin in protein extracts of NLC1 and SR18 mESCs, which harbor the *Ctnnb1*$^{tm4Wbm\ fl/fl}$ and *Ctnnb1*$^{tm2Kem\ fl/del}$ alleles, respectively, and stably express the CRE-ERT2. Full-length β-catenin was successfully excised upon 4-hydroxytamoxifen (4OHT) treatment in both cell lines, and ΔN β-cat isoforms with a molecular weight of approximately 48 and 52 kDa, respectively, were detected upon CRE recombination (Figures 1B and 1C). Immunofluorescence staining did not show any clear subcellular localization of ΔN isoforms, which instead appeared distributed among cytoplasm and nuclei showing little or no membrane localization (Figures 1D and 1E).

To assess if N-terminally truncated isoforms were a product of the *Ctnnb1* genomic locus and not a mere technical artifact, we designed three different short hairpins against three different regions of *β-catenin* mRNA (β1-3) (Figure 1F) to silence *β-catenin* in both knockout (del/del) and parental (fl/fl) mESCs (NLβ-12^{tm4Wbm} background). A scrambled short hairpin (shSCR) was used as control. All the short hairpins successfully induced knockdown of β-catenin at protein level (Figure 1G) in wild-type cells, while N-terminally truncated isoforms were depleted efficiently by β1 and β3 but not by β2, which targets the portion of mRNA excised upon CRE-mediated recombination. The same results were confirmed analyzing *β-catenin* mRNA levels by qRT-PCR using two different primer pairs targeting the CRE-excised region (Figure 1H, left panel), and the 3′ UTR (Figure 1H, right panel). The oligonucleotides targeting the excised region failed to detect *β-catenin* mRNA in knockout cells, while the ones designed on the 3′ UTR revealed that mRNA regions downstream of the excision had comparable expression levels in wild-type (NLβ-12 fl/fl shSCR) and knockout (NLβ-12 fl/fl del/del shSCR) cells and were efficiently silenced only by β1 and β3 but not, as expected, by β2 (Figure 1H). Importantly, we never observed ΔN isoforms in wild-type cells, including cells expressing short hairpins targeting *β-catenin*, indicating that they are only produced upon recombination of *Ctnnb1* locus.

Next, we characterized SR18 cells (*Ctnnb1*$^{tm2Kem\ fl/del}$) upon β-catenin CRE-mediated deletion (Raggioli et al., 2014). Upon 4OHT treatment and consequent β-catenin deletion, clone morphology and alkaline phosphatase (AP) expression (Figure S1A), as well as Nanog and Oct4

(D) β-Catenin immunofluorescence staining on fixed SR18 or NLC1 parental cell lines or upon 72-h +4OHT treatment. A primary antibody raised against the C-terminal portion of β-catenin was used. DAPI was used to counterstain nuclei. Scale bar represents 50 μm.
(E) Multichannel fluorescence intensity measurement of immunofluorescence images in (D). Image quantification has been performed across the dashed yellow line depicted in (D), merge panel.
(F) Schematic representation of short-hairpin targeted regions (red triangles, β1, β2, and β3) and qRT-PCR amplicons (blue lines, #1 and #2) along the *Ctnnb1*^{Tm4Wbm} allele.
(G) Western blot of β-catenin of mESCs harboring the Birchmeier β-catenin allele after (*Ctnnb1*$^{Tm4Wbmdel/del}$; left) or before (*Ctnnb1*$^{Tm4Wbm\ fl/fl}$; right) CRE-mediated recombination of the *loxP* sites. Cells were transduced with a control short hairpin (shCtr) or three different short hairpins against *β-catenin* mRNA (β1, β2, or β3).
(H) qRT-PCR on total mRNA extracts of *Ctnnb1*$^{Tm4Wbm\ fl/fl}$ or *Ctnnb1*$^{Tm4Wb\ del/del}$ cells transduced with the short-hairpin constructs used in Figure 1D. Two different amplicons were amplified to monitor deleted region (*Ctnnb1* #1) or 3′ UTR (*Ctnnb1* #2). *GAPDH* was used as housekeeping control. Error bars represents standard deviation of technical triplicates.



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expression patterns (Figure S1B), remained unaltered, confirming earlier results (Lyashenko et al., 2011; Wray et al., 2011). The lack of morphological changes upon β-catenin deletion was likely due to compensatory effects by the upregulation of PLAKOGLOBIN (Figure S1C), as reported by Lyashenko et al. (2011) and Wray et al. (2011). Furthermore, no changes were detected in the protein levels of pluripotency markers (NANOG, OCT4, SOX2) (Figures S1D and S1E). Although these results show that β-catenin loss does not affect morphology and pluripotency markers (confirming results in Lyashenko et al., 2011; Wray et al., 2011), we asked whether the ΔN isoforms, produced by *Ctnnb1*^{tm2Kem} or the *Ctnnb1*^{tm4Wbm} alleles, could be responsible for the maintenance of the observed mESC phenotype.

Generation of a New β-catenin Knockout Model in mESCs Using CRISPR/Cas9 Technology

We generated a full β-catenin knockout mESC line via CRISPR/Cas9 technology. As the presence of ΔN β-catenin isoforms could be due either to an alternative splicing or to the presence of a secondary ATG on exon VII (Figure 1A), we designed two different single-guide RNA (sgRNA) pairwise combinations (sgRNA1+sgRNA3 and sgRNA2+sgRNA3, resulting in 5,287-bp and 4,970-bp deletion, respectively, Figure S1F) to induce deletions spanning both alternative splicing sites and the entire exon VII. While the editing efficiency of sgRNA2+sgRNA3 was low, sgRNA1+sgRNA3 induced the deletion in a high percentage of cells (Figure S1G) with a significant loss of full-length β-catenin protein in the pool of transfected cells (Figures S1H and S1I). The deletion generated by sgRNA1+sgRNA3 resulted in the production of a new ΔN isoform with a lower molecular weight (40 kDa) with respect to the N-terminally truncated isoforms produced by *Ctnnb1*^{tm2Kem} (48 kDa) or the *Ctnnb1*^{tm4Wbm} (52 kDa) alleles (Figure S1H). The generation of this new ΔN isoform could only be explained by the presence of alternative downstream ATG sites, which, however, were not predictable *a priori*.

In order to eliminate any possible undesired gene-editing product, we sought to induce a 10-kb deletion encompassing the whole gene body. For this aim, we used an additional couple of sgRNAs (sgRNA4+sgRNA5), targeting the *Ctnnb1* locus upstream of the canonical ATG and within a portion of the last coding exon (XV), respectively (Figure 2A). Successful gene editing was confirmed at pool levels by PCR genotyping (Figure S2A). The pool of transfected cells did not show any expansion defect or cell detachment. Single-cell clones were picked, expanded, and finally three independent clones were isolated with homozygous deletions, Eβ11, Eβ15, and Eβ47. All three clones carried homozygous deletion of a 9.7-kb region as assessed by PCR genotyping and Sanger sequencing (Figures 2B and S2B and Supplemental DNA sequences) encompassing the whole *β-catenin* coding sequence (CDS), therefore preventing *Ctnnb1* locus rearrangement.

Western blot analysis with an antibody raised against the C-terminal portion of β-catenin revealed the absence of any ΔN isoform in all the analyzed clones (Figure 2C); this, together with the absence of any detectable signal by immunofluorescence (Figure 2D), confirmed the complete β-catenin depletion in the newly generated clones.

These data demonstrate that, regardless of the gene-editing technology used (CRE-mediated excision or CRISPR/Cas9), the gene-editing by-product cannot be easily predicted, and that complete locus ablation is a viable alternative to avoid undesired protein rearrangements.

Complete β-Catenin Loss Does Not Affect Self-Renewal and Pluripotency Marker Expression in mESCs Cultured in Serum/LIF

In order to characterize the impact of complete β-catenin ablation in mESCs, we analyzed the newly generated cell lines for proliferation, self-renewal, and pluripotency

Figure 2. CRISPR/Cas9-Mediated Excision of Whole Ctnnb1 Locus Results in a Complete β-Catenin Knockout Model in mESCs

(A) Schematic representation of sgRNA design for CRISPR/Cas9-mediated excision of whole β-catenin coding sequence. Red arrows indicate sgRNAs target sites. Blue triangles indicate position and orientation of oligonucleotides used for PCR genotyping.

(B) PCR genotyping of three homozygous β-catenin knockout clones (Eβ11, Eβ15, and Eβ47) and parental E14 mESCs. Expected amplicon size is 951 bp for wild-type (wt) alleles and 551 bp for knockout alleles (del).

(C) Western blot of total protein extracts from Eβ11, Eβ15, Eβ47, and wild-type E14 cells. Protein extracts were probed for β-catenin (using a C-terminally raised antibody), stripped and re-probed for TUBULIN as loading control.

(D–G) Immunofluorescence in fixed parental E14, Eβ11, Eβ15, and Eβ47 cells for β-catenin (D), PLAKOGLOBIN (E), E-CADHERIN (F), OCT4 and NANOG (G). DAPI was used to counterstain nuclei. Scale bar represents 50 μm.

(H) Phase contrast pictures of Eβ11, Eβ15, Eβ47, and parental E14 clones upon 72-h vehicle (0.3% DMSO, left) or Chiron 3 μM treatment (right) in serum/LIF. Cells were seeded at 4×10^5 cells/well density in 6-well plates. Scale bar represents 50 μm.

(I) Phase contrast pictures of AP staining on E14, Eβ11, Eβ15, and Eβ47 cells cultured in serum/LIF in presence of vehicle (0.3% DMSO, left) or Chiron 3 μM (right) for 5 days; 300 cells were seeded in each well of a 6-well plate. Scale bar represents 50 μm.

(J) Histogram of qRT-PCR data on total RNA extracts of E14, Eβ11, Eβ15, and Eβ47 cells exposed to vehicle (0.3% DMSO, black bars) or Chiron 3 μM; $2^{-\Delta Ct}$ are represented, and *GAPDH* was used as internal control. Error bars represents standard deviations of three technical replicates.

marker expression defects under serum/LIF culturing conditions. β-Catenin is normally found at the plasma membrane, where it physically interacts with E-CADHERIN and A-CATENIN connecting adherens junctions to the actin cortex. As in Lyashenko et al. (2011) and Wray et al. (2011), Eβ11, Eβ15, and Eβ47 clones did not show major morphological defects; PLAKOGLOBIN levels were upregulated in response to β-catenin loss (Figures 2E, S2C, and S2D), while E-CADHERIN localization and expression levels remained unchanged (Figures 2F and S2E). We did not detect any difference in the cell-cycle (Figures S2F and S2G) or proliferation rate (Figure S2H) in all three knockout clones with respect to the parental cell lines, which displayed overall comparable population doubling times (Figure S2I).

Similarly to previously reported knockout models (Lyashenko et al., 2011; Raggioli et al., 2014; Wray et al., 2011), complete β-catenin loss did not show any additional defects of NANOG and OCT4 pluripotency marker expression as their localization (Figure 2G) and expression levels (Figure S2C and S2D) remained similar to the parental E14 cell line in all the analyzed clones.

The addition of small-molecule inhibitors of GSK3 activity has been reported to promote mESC pluripotency and self-renewal, while inhibiting spontaneous differentiation, by increasing β-catenin levels and activating the expression of Wnt target genes (Sato et al., 2004).

We therefore monitored the morphological and transcriptional response of β-catenin knockout clones to CHIR99021 (Chiron), a selective small-molecule inhibitor of GSK3. Wild-type and knockout cells were cultured in serum/LIF supplemented with 3 μM Chiron or vehicle (DMSO 0.3 μL/mL). After 72 h of Chiron treatment, wild-type cells acquired a homogeneous round morphology with tight colony boundaries, while Eβ11, Eβ15, and Eβ47 mESCs did not show any major morphological changes with respect to vehicle (Figure 2H), confirming that morphological changes induced by GSK3 inhibition are mediated by β-catenin (Wray et al., 2011). Morphological defects were only evident when cells were reaching >50% confluency (Figure 2H, vehicle), while β-catenin knockout clones were indistinguishable from wild-type E14 if plated at clonal density (Figure 2I, vehicle).

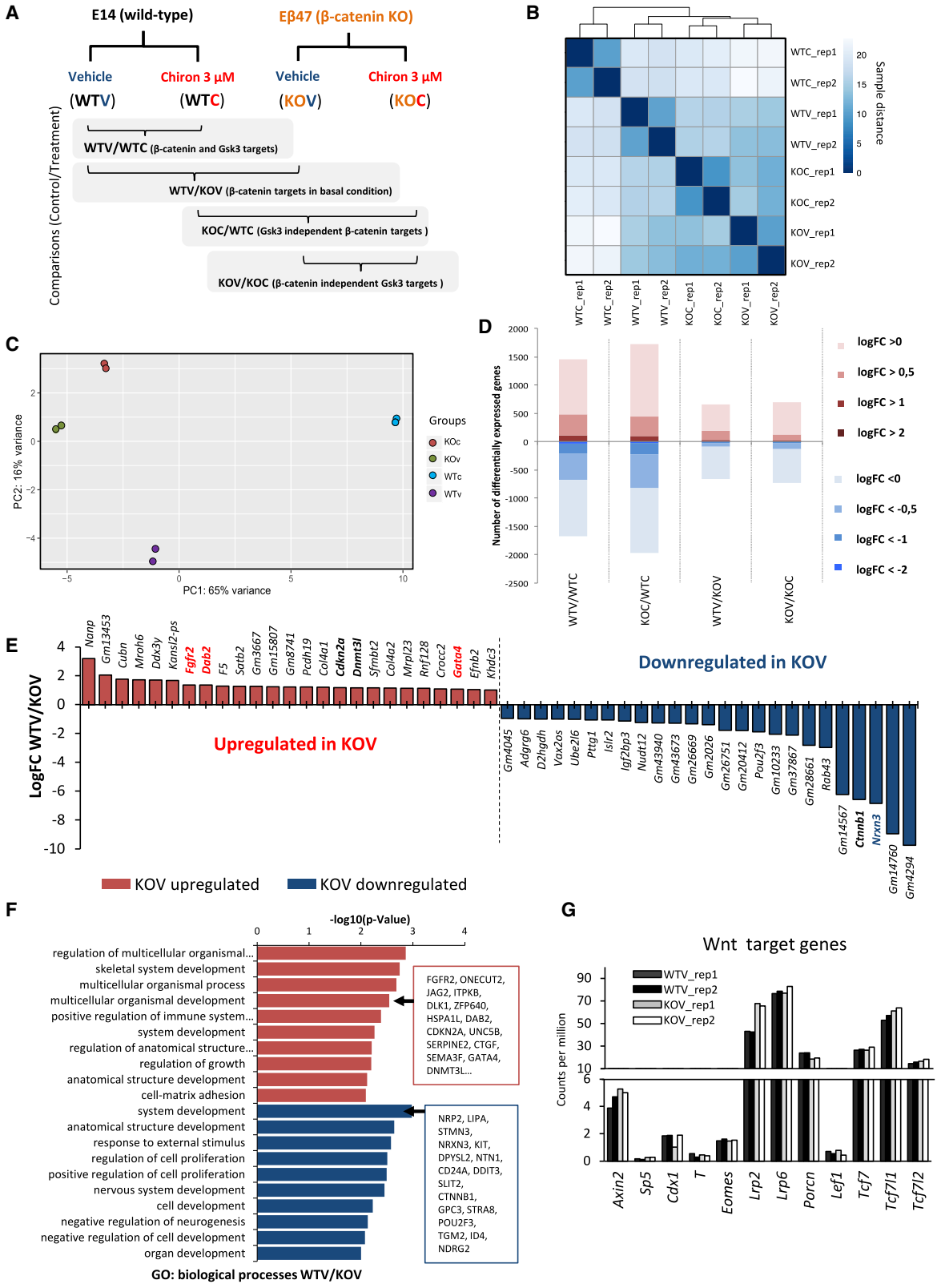
These data suggest that ΔN β-cat isoforms are biologically inactive as complete β-catenin depletion does not result in any additional phenotypic defect with respect to previously characterized knockout models.

Next, we assessed the colony formation capacity and AP expression of wild-type and β-catenin knockout cells in response to GSK3 inhibition. Cells were plated at clonal density and, while cultured in serum/LIF in presence of vehicle, wild-type E14 and β-catenin knockout clones were all positive for AP expression, although β-catenin knockout cells displayed a slightly lower AP staining intensity (Figures 2I, S2J, and S2K). In presence of Chiron, however, wild-type cells dramatically increased AP staining intensity. By contrast, AP staining intensity in β-catenin knockout cells was only moderately increased in response to Chiron treatment, probably as the result of slightly increased colony compaction (Figures 2I, S2J, and S2K). In addition, the AP transcript (*Alpl*) was upregulated upon Chiron treatment only in wild-type cells (Table S1, WTC/WTV differentially expressed genes [DEGs], row 89).

We then assessed the transcriptional response to GSK3 inhibition in wild-type and β-catenin knockout clones upon 72 h of Chiron treatment through qRT-PCR. As expected, *β-catenin* mRNA levels were undetectable in all knockout clones and remained unchanged upon Chiron treatment in wild-type cells. Instead, canonical Wnt targets such as *Axin2* and *Sp5* showed comparable levels between wild-type and knockout cells, while their expression was activated only in wild-type in response to Chiron treatment (Figure 2J). Of note, slightly higher levels of *Cdx1* mRNA were found in all knockout clones in basal conditions with respect to wild-type cells. In addition, although the absence of β-catenin severely impaired *Cdx1* upregulation upon Chiron treatment, it did not completely abrogate it, suggesting that downstream targets of GSK3 contribute to partially regulate its expression (Figure 2J).

β-Catenin Ablation Promotes a Weak Upregulation of Primitive Endoderm Genes

According to previously published works, the canonical Wnt pathway is active in mESCs (Ten Berge et al., 2011) and β-catenin nuclear translocation reinforces the pluripotency network by upregulating pluripotency genes such as *Nanog*, *Esrrb*, or *Tcfp2l1* (Martello et al., 2012; Pereira et al., 2006; Qiu et al., 2015) mainly by inhibiting TCF3 repressive activity on their promoters (Pereira et al., 2006). Based on this model, a reasonable consequence of β-catenin ablation would be the increase in TCF3 repressive activity on core-pluripotency network genes with an expected destabilization of self-renewal and pluripotency features. Complete β-catenin loss did not, however, affect Tcf3 levels or its subcellular localization pattern (Figure S2L), suggesting that, in absence of exogenous WNT3A or GSK3 inhibitors, β-catenin does not control TCF3 activity. On the other hand, a previous β-catenin knockout model did not reveal major transcriptomic defects (Lyashenko et al., 2011); due to the lack of characterization of truncated isoforms, a possible explanation for this apparently controversial phenotype could be that ΔN β-cat isoforms retain some of the functions of the full-length counterpart, as recently proposed in pre-implantation embryo development (Messerschmidt et al., 2016).



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We therefore sought to analyze the expression profile of E14 parental cell line and the Eβ47 knockout clone through high-throughput RNA sequencing (RNA-seq) in cells cultured in serum/LIF culture conditions upon treatment with either vehicle (0.3% DMSO) or 3 μM Chiron for 72 h (Figure 3A). Since the impact of complete β-catenin loss in mESCs has never been assessed before at transcriptomic level, we designed an assay to study, through different comparisons between samples, the β-catenin and GSK3-dependent transcriptional changes in mESCs (Figure 3A). As expected, *β-catenin* was not detectable at transcriptional level in Eβ47 clone (Figure S3A), confirming the difference with previous knockout models in which *β-catenin* mRNA was still detectable (Figure 1F).

The transcriptomic profile of E14 wild-type cells cultured in serum/LIF (WTV), was overall very similar to Eβ47, independently if cultured in presence of vehicle (KOV) or Chiron (KOC), while E14 wild-type cultured in presence of 3 μM Chiron (WTC) clustered away from all the other samples in the samples distance matrix (Figure 3B). Principal component analysis (PCA) showed that the highest amount of variance was due to GSK3 inhibition in presence of β-catenin (WTC samples), while only subtle differences could be spotted between KOC, KOV, and WTV samples (Figure 3C). These results confirmed that, similarly to what was observed in β-catenin knockout models producing N-terminally truncated isoforms (Lyashenko et al., 2011), β-catenin absence does not significantly alter the transcriptional profile of serum/LIF cultured mESCs.

We then analyzed differential expressed genes (DEGs) across different samples/treatments, considering a threshold for adjusted p value <0.05 and absolute log2 fold change >0.5 (logFC, defined as the log ratio of a transcript's expression values in two different conditions). Upon β-catenin depletion, 286 genes were differentially expressed with respect to E14 cells cultured in serum/LIF + vehicle (WTV/KOV comparison, of which 192 upregulated and 94 downregulated; Figures 3D and S3B and Table S1), and only 11 genes displayed a logFC higher than 2. Genes such as *Fgfr2*, *Dab2*, *Pdgfra*, and *Gata4* were slightly upregulated upon β-catenin depletion, although with very low fold changes (between 1.2- and 2-fold enrichment), suggesting a partial priming toward primitive endoderm (PrE), while *Neurexin 3* (*Nrxn3*) was downregulated (Figure 3E). We then performed Gene Ontology (GO) analysis on WTV/KOV DEGs (adjusted p value <0.05, logFC > 0.5); both upregulated and downregulated DEGs were enriched for developmental categories such as multicellular organism development and system development (Figure 3F and Table S2). Surprisingly, β-catenin depletion did not alter the expression of canonical Wnt target genes such as *Axin2*, *Sp5*, *Cdx1*, or *T/Brachyury* (Figure 3G), suggesting that canonical Wnt pathway is not transcriptionally active in mESCs cultured in serum/LIF, although *β-catenin* is highly expressed (Figure S3A). These results are in line with previous reports that failed to identify activity of the TOP/FOP reporter in serum/LIF cultured mESCs (Aulicino et al., 2014; De Jaime-Soguero et al., 2017; Faunes et al., 2013), while they stand at odds with the evidence that the Wnt/β-catenin pathway is active in the inner cell mass of the blastocyst, and the canonical Wnt signaling is required for mESC self-renewal in serum/LIF (Ten Berge et al., 2011).

Finally, when analyzing the expression levels of key lineage markers, we could not detect any change in pluripotency genes, while a slight overexpression of PrE lineage markers (*Gata4*, *Gata6*, *Foxa2*, *Dab2*, *Ihh*, *Cerberus*, and *Pdgfra*) was observed independently of Chiron treatment in absence of β-catenin (Figure S3C, bottom).

As colony formation and AP staining phenotypes are slightly ameliorated in β-catenin knockout mESCs in presence of Chiron with respect to vehicle (Figures 2I and S2J), we asked whether GSK3 inhibition per se could alter the transcriptome of mESCs independently of β-catenin. To address this question, we compared Eβ47 cells cultured in presence of vehicle (KOV) or 3 μM Chiron (KOC) for 72h (KOV/KOC comparison). Chiron treatment only resulted

Figure 3. β-Catenin Depletion Produces Minor Changes at Transcriptomic Level

(A) Schematic representation of experimental design for RNA-seq analysis. E14 parental cells (WT) or Eβ47 cells (knockout) were cultured in serum/LIF upon 72-h vehicle (0.3% DMSO, V) or Chiron 3 μM (C) treatment. Two biological replicates were analyzed for each sample. Pairwise sample comparisons are indicated as control/treatment.

(B) Sample distance matrix and hierarchical clustering of biological replicates (rep_1 and rep_2) for WTV, WTC, KOV, and KOC samples.

(C) PCA plot of indicated samples.

(D) Histogram of differentially expressed genes across pairwise comparisons as indicated in Figure 3A. Shades of red indicate overexpressed genes; shades of blue indicate downregulated genes. Shade intensity represents log fold-change cutoff from >0 (no fold-change cutoff, light), to absolute log fold change >2 (dark). Adjusted p value cutoff is 0.05.

(E) Top differentially expressed genes in WTV/KOV comparison ranked for log fold change. Adjusted p value <0.05.

(F) GO analysis of biological processes enriched in differentially expressed genes in WTV/KOV comparison (adjusted p value <0.05, absolute logFC >0.5). Upregulated features are shown in red, downregulated features are shown in blue.

(G) Histogram of RNA level (counts per million reads [CPM]) of canonical Wnt target genes and components across WTV (black and dark gray) or KOV (light gray and white) samples. Individual replicates are shown for each sample.

in minor changes as the transcriptome of KOV and KOC were overall very similar (Figures 3B and 3C). Only 254 genes were differentially expressed in cells lacking β-catenin and exposed to GSK3 chemical inhibition, again with overall low fold change (p value adjusted <0.05 and logFC >0.5, of which 124 upregulated and 130 downregulated) (Figures 3D and S3B and Table S1). Upregulated genes were enriched for biological processes such as stress response and metabolic features, while downregulated genes were enriched for metabolic and developmental processes (Figure S3D and Table S3). Nevertheless, the slight transcriptional changes induced by Chiron in absence of β-catenin revealed a certain degree of overlap with canonical Wnt target genes (Figure S3E). Canonical target genes such as *Myc* or *Axin2* were slightly inhibited by Chiron treatment in absence of β-catenin as well as *Tcf3* (*Tcf7l1*) mRNA levels (Figure S3F). These data confirm, as previously demonstrated (Doble et al., 2007), that GSK3 inhibition has little or no effect on mESC transcriptional landscape independently of β-catenin. Of note PLAKOGLOBIN can partially mimic β-catenin function (Mahendram et al., 2013), but, although PLAKOGLOBIN protein levels are upregulated in β-catenin knockout cells (Figures 2E, S2C, and S2D), *Plakoglobin* mRNA was not found among the differentially expressed genes upon β-catenin loss, suggesting the existence of a post-translational regulation mechanism for PLAKOGLOBIN that relies on β-catenin levels. In addition, our data demonstrate that Plakoglobin cannot replace nuclear β-catenin functions in response to Chiron in mESCs as no major transcriptional changes were observed in KOV/KOC comparison and, among the canonical Wnt/β-catenin targets, inhibition rather than activation was observed at best (Figures S3E and S3F).

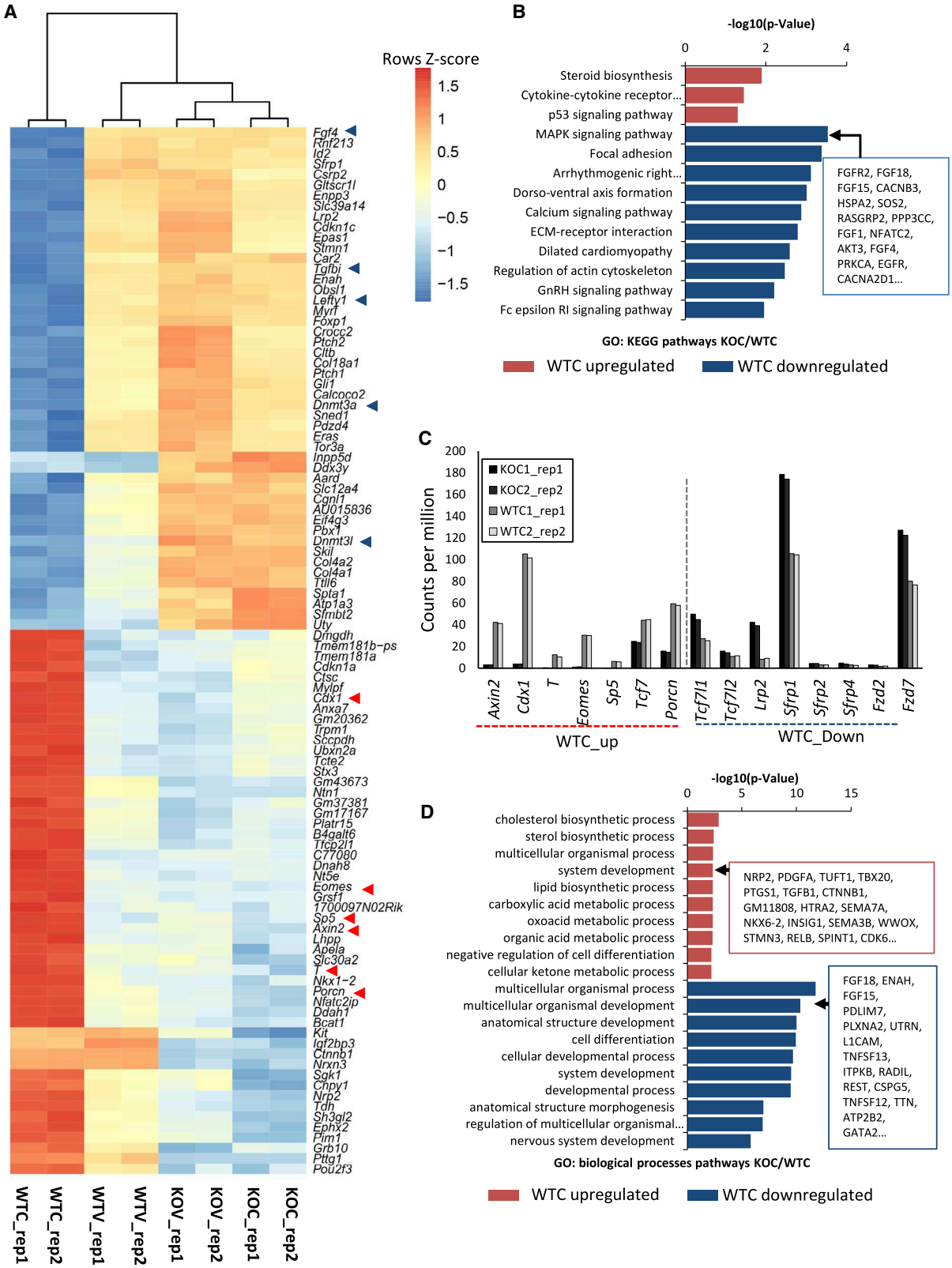
Canonical Wnt Signaling Inhibits Differentiation of mESCs Toward Ectoderm

Although we assessed that the transcriptional effects of Chiron in absence of β-catenin are negligible, the DEGs in the KOC/WTC comparison were dependent solely on the presence of β-catenin. A similar approach has been used previously (Zhang et al., 2013) by comparing mESCs treated with Chiron or XAV (a small-molecule inhibitor of the Wnt pathway) without considering possible off-target effects of both drugs. Our experimental approach, instead, allowed us to decouple β-catenin-dependent targets (DEGs in KOC/WTC) from GSK3-only-dependent targets (DEGs in KOV/KOC).

The highest number of DEGs between comparisons represented as control/treatment was found in the WTV/WTC (1,157 DEGs, 476 upregulated, 681 downregulated) and in the KOC/WTC (1,259 DEGs, 437 upregulated, 822 downregulated) comparisons (Figures 3D and S3B and Table S1). Wild-type mESCs treated with Chiron clustered far apart from all the other conditions, as assessed by sample distance matrix PCA (Figures 3B and 3C). Numerous reports associated Wnt activation with transcriptional activation of *Nanog*, *Klf4*, *Esrrb*, and *Tcfp2l1* in mESCs (Ai et al., 2016; Martello et al., 2012; Pereira et al., 2006; Qiu et al., 2015). However, with the exception of a slight *Tcfp2l1* upregulation and *Dppa3* downregulation, we were not able to identify any change at transcriptional levels of pluripotency marker genes upon Chiron treatment in wild-type cells (Figure S3C). Accordingly, we previously showed that *Nanog*, *Oct4*, and *Rex1* levels do not change in mESCs even upon prolonged (up to 8 passages) exposure to Chiron (De Jaime-Soguero et al., 2017). Nevertheless, lineage markers such as *Pax6*, *Fgf1*, *Nes*, *Otx2*, *Lefty1*, and *Pou3f1* were strongly inhibited by Chiron treatment in presence of β-catenin, indicating an overall reduction of differentiation commitment toward the ectoderm lineage (Figure S3C). Conversely, canonical Wnt target genes required for trophectoderm and mesoderm specification, including *Cdx1*, *T/Brachyury*, and *Eomes*, were strongly upregulated upon Chiron treatment.

We then clustered the top 100 DEGs in the KOC/WTC comparison across the various samples (Figure 4A). As expected, sample clustering did not change with respect to previous analysis (Figures 3B and 3C) and canonical Wnt targets such as *Axin2*, *Cdx1*, and *T/Brachyury* were only activated in wild-type cells treated with Chiron, while few or no differences were observed in the other samples (Figures 4A and 4C). Furthermore, this subset of genes was transcriptionally perturbed only in presence of both Chiron and β-catenin, once again confirming that the canonical Wnt pathway is not active in mESCs cultured in serum/LIF unless an external stimulus (i.e., chemical GSK3 inhibition) is applied (Figure 4A).

We next asked about the nature of transcriptional changes induced by GSK3 inhibition in presence of β-catenin. We focused once again on the KOC/WTC comparison and performed KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways enrichment and gene ontology (DEGs, KOC/WTC, adjusted p value 0.05, logFC > 0.5, Figures 4B and 4D and Table S5). Interestingly, Chiron treatment led to a perturbation of *Tcf/Lefs* levels, with the downregulation of *Tcf3* (*Tcf7l1*) and the upregulation of *Tcf1* (*Tcf7*) (Figure 4C). Furthermore, downregulated genes were strongly enriched for the mitogen-activated protein kinase (MAPK) signaling pathway (Figure 4B) and for focal adhesion. Both upregulated and downregulated genes were enriched for biological process categories associated with development. Nevertheless, the enrichment was stronger among DEGs downregulated upon Chiron treatment (Figure 4D and Table S5), confirming the general inhibition of spontaneous differentiation induced by the coupled action of GSK3 inhibition and β-catenin.



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Transcriptional β-Catenin Activity Is Required to Inhibit Differentiation in Absence of LIF

Previous reports have shown that transcriptional activity of β-catenin is dispensable for mESC self-renewal (Lyashenko et al., 2011; Wray et al., 2011). In order to validate or disprove these findings, we generated lentiviruses carrying different β-catenin isoforms. In addition to the full-length β-catenin (wild-type [wt] βcat), an N-terminally truncated (ΔN β-cat) and a C-terminally truncated isoform (ΔC β-cat) were generated. While ΔN β-cat isoforms mimic the isoforms generated from previous knockout models (Messerschmidt et al., 2016), ΔC β-cat carries a deletion of the transactivation domain, which results in an impaired transcriptional activity (Figure 5A).

Eβ47 cells were transduced with a lentivirus carrying the different β-catenin isoforms under the constitutive EF1α promoter, or an empty lentivirus as a control (EV). Upon isoform expression, we studied protein subcellular localization and expression levels using N-terminal and C-terminal antibodies against β-catenin. N-terminally truncated β-catenin isoforms were only detected with the C-terminal raised antibody, and their subcellular localization recapitulated the one observed in previous knockout models (Figures 5B and 1C). Furthermore, as in ΔN β-cat isoforms emerging from previous knockout models, the overall ΔN β-cat levels were reduced, as compared to total β-catenin levels in wild-type cells (Figure 5C).

The expression and localization of ΔC β-cat and wt β-cat rescue isoforms were overall comparable, with intense membrane localization and similar expression levels (Figures 5B and 5C). Interestingly, expression of ΔC and wt β-cat, but not ΔN β-cat, restored normal Plakoglobin levels (Figures 5C, 5D, and S4A), indicating that impaired membrane functions, but not the transcriptional ones, are responsible for the increased Plakoglobin levels in β-catenin knockout cells. Similarly, ΔN β-cat isoforms could not rescue the loss of morphological changes induced by Chiron treatment, while both ΔC and wt rescued Eβ47 cells exhibited a round morphology (Figure 5E) indistinguishable from E14 wild-type cells upon Chiron addition (Figure 2H). Upon GSK3 inhibition, however, eGFP expression under the control of the synthetic Wnt reporter was only detectable in wild-type rescued Eβ47, confirming that both ΔN and ΔC β-cat isoforms are transcriptionally impaired for canonical Wnt signaling (Figure S4B).

We next assessed the colony formation capacity and AP expression of rescued β-catenin knockout cells in different culture conditions. In serum/LIF, only ΔC β-cat and wt β-cat rescued Eβ47 cells recapitulated the parental E14 cells phenotype, while ΔN β-cat overexpression did not alter the phenotype with respect to empty vector transduced cells (EV, Figure S4C, top panel). Eβ47 showed impair clonogenicity and self-renewal, in line with previously published knockout models. In serum-free media, Eβ47 could not be expanded in PD + LIF, Chiron + LIF, or PD + Chiron (2i), but could self-renew in 2i/LIF; again, ΔN β-cat rescued Eβ47 cells did not show any improvement of AP expression or self-renewal (Figure S4C, bottom panel). As previously reported, both ΔC β-cat and wt β-cat isoforms restored self-renewal and AP staining intensity defects (Figure S4C, bottom panel), suggesting that canonical Wnt pathway activity is dispensable for self-renewal in these culture conditions.

However, nuclear β-catenin activity appears to be required for inhibition of differentiation in serum and in absence of LIF (Ogawa et al., 2006; Sato et al., 2004). Therefore, we performed clonal assay and AP staining of Eβ47 rescued cells in serum without LIF. While all the cell lines quickly differentiated in absence of LIF, Chiron treatment maintained AP staining in ΔC β-cat and wt β-cat rescued cells, but not in ΔN β-cat rescued Eβ47 (Figures 5F and S4D). Furthermore, ΔC β-cat expression only partially rescued AP staining, indicating that canonical Wnt activity is required to counteract spontaneous differentiation upon LIF withdrawal.

These results suggest that the inhibition of spontaneous mESC differentiation in the absence of LIF could be due to combined nuclear and membrane-associated β-catenin functions. However, while the ΔC β-cat isoform fails to activate the synthetic Wnt reporter (Figure S4B), it has previously been observed that the ΔC β-cat isoform exhibit a certain degree of transcriptional activity, despite lacking

Figure 4. RNA-Seq Analysis of β-Catenin-Dependent Differentially Expressed Genes

(A) Heatmap clustering of the top 100 differentially expressed genes in KOC/WTC comparison across KOV, KOC, WTV, and WTC samples (p value adjusted <0.05, absolute logFC >0.5). Minimum and maximum are scaled across conditions on single genes; the heatmap represents logCPM rescaled on each gene for their *Z* scores (average of the values is the center of a normal distribution; color codes represent positive or negative deviations from the average).

(B) GO analysis of KEGG pathway categories enriched in differentially expressed genes in the KOC/WTC comparison (p value adjusted <0.05, absolute logFC >0.5).

(C) Histogram of CPMs of canonical Wnt target genes and components differentially expressed in KOC/WTC comparison. Individual biological replicates are shown.

(D) GO analysis of biological processe categories enriched in differentially expressed genes in KOC/WTC comparison (p value adjusted <0.05, absolute logFC >0.5).

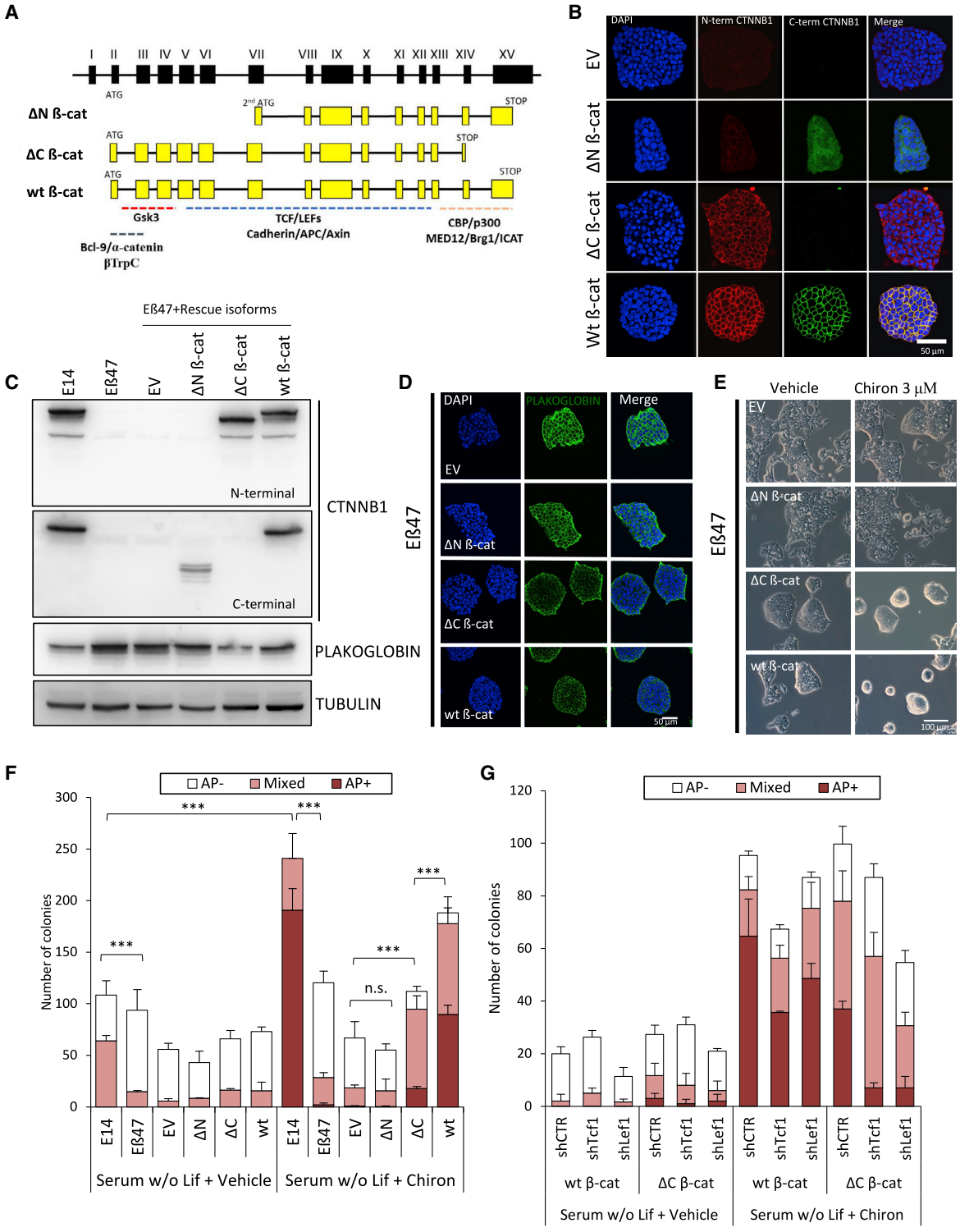

Figure 5. Canonical β-catenin Functions Are Required for Inhibition of Differentiation

(A) Schematic representation of β-catenin isoforms used for rescue experiments. N-terminally (ΔN β-cat) truncated β-catenin isoform mimics N-terminally truncated β-catenin isoforms obtained in previously published knockout models.

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the transactivation domain (Wray et al., 2011). A possible explanation could be that ΔC β-cat isoforms still interact with TCF3 and, like the full-length β-catenin, alleviate TCF3-mediated repression on a subset of target genes (Wray et al., 2011). On the other hand, it has been reported that ΔC β-cat isoforms can still form a transcriptionally active complex in association with LEF1 (Hsu et al., 1998) or TCF1. We therefore silenced *Lef1* or *Tcf1* in Eβ47 cells rescued with either full-length or ΔC β-cat (Figure S4E) and assessed AP staining intensity upon exposure to vehicle or Chiron in absence of LIF. *Tcf1* or *Lef1* depletion efficiently reduced the number of AP colonies formed in response to Chiron treatment in ΔC β-cat rescued cells and, to a lesser extent, also in wild-type β-catenin mESCs (Figures 5G and S4F).

These results suggest that ΔC β-cat could still interact with TCF1 and LEF1, and that nuclear β-catenin/TCF/LEF functions could be required to inhibit mESC spontaneous differentiation in the absence of LIF, while the membrane-associated β-catenin functions may have little or no role in this phenotype.

DISCUSSION

In this work, we showed that the inducible β-catenin knockout models currently available result in the production of N-terminally truncated isoforms in mESCs. While ΔN β-cat isoforms are not physiologically expressed, their appearance is a consequence of rather unpredictable gene rearrangement upon genetic manipulation. By using CRISPR-Cas9 technology, we isolated three mESC clones with no detectable β-catenin expression. Of note, only a paired sgRNA approach generating a deletion encompassing the whole β-catenin gene body was successful in generating knockout alleles (10-kb genomic DNA deletion), while standard strategies (ORF shifting and microdeletion) only produced ΔN-truncated isoforms at best. As CRISPR-Cas9 is boosting genetic engineering, the production of undesired isoforms with potential gain of functions, which could undermine all the conclusion of a study, should be carefully monitored and avoided. For this reason, we foresee that whole-gene deletion using CRISPR-Cas9 will be an advantageous approach for generating knockout models free of undesired side products.

We assessed that the appearance of ΔN β-cat isoforms in previously published β-catenin knockout mESC models is not detrimental for their phenotype, as no compensatory or overlapping effects were to be attributed to the truncated proteins. In addition, none of the impaired phenotypes observed upon complete β-catenin loss could be rescued by a ΔN β-cat isoform, thus recapitulating the phenotype observed in the previously generated knockout models (Lyashenko et al., 2011; Wray et al., 2011).

β-Catenin depletion does not alter self-renewal and pluripotency marker expression of mESCs under serum/LIF culturing conditions, with expected morphological changes probably rescued by Plakoglobin stabilization. Moreover, β-catenin depletion also does not alter the expression of known canonical Wnt targets in basal culturing conditions, suggesting that the Wnt/β-catenin pathway is not constitutively active in mESCs cultured in feeder-free media supplemented with serum/LIF. Accordingly, β-catenin loss does not globally alter the transcriptional profile of mESCs but induces a mild activation of PrE marker genes. The latter phenotype, coupled to the absence of self-renewal and proliferation defects in serum/LIF, could either not be significant or could potentially highlight the presence of a rare population of terminally differentiated cells committed toward PrE that cannot withstand β-catenin absence.

(B) Immunofluorescence of Eβ47 cells transduced with lentiviral vectors encoding empty vector (EV), wild-type (wt β-cat), ΔN β-cat, and C-terminally (ΔC β-cat) truncated β-catenin isoforms. Cells were stained with N-terminally (red) and C-terminally (green) β-catenin antibodies. Nuclei were counterstained with DAPI.

(C) Western blot of total protein extracts from E14 and Eβ47 untransduced cells and Eβ47 transduced with EV, ΔN β-cat, ΔC β-cat, and wt β-cat encoding lentiviruses. Membranes were probed with N-terminally or C-terminally raised β-catenin antibodies and anti-PLAKOGLOBIN. TUBULIN was used as loading control. Scale bar represents 50 μm.

(D) Immunofluorescence of Eβ47 cells transduced with EV, ΔN β-cat, ΔC β-cat, or wt β-cat encoding lentiviruses. Cells were stained for PLAKOGLOBIN. DAPI was used to counterstain nuclei (Scale bar represents 50 μm).

(E) Phase contrast pictures of Eβ47 cells transduced with EV, ΔN β-cat, ΔC β-cat, or wt β-cat encoding lentiviruses and cultured in serum/LIF in presence of 3 μM Chiron or vehicle (0.3% DMSO). Scale bar represents 100 μm.

(F) AP staining quantification of E14 and Eβ47 untransduced cells or Eβ47 transduced with EV, ΔN β-cat, ΔC β-cat, or wt β-cat encoding lentiviruses. Cells were plated in serum without LIF and supplemented with 3 μM Chiron (right) or vehicle (0.3% DMSO, left). Error bars represent standard error of three biological replicates. Student's t test was used to measure statistical significance as indicated, stars indicate p value (n.s. = not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

(G) AP staining of Eβ47 cells transduced with either wt βcat or ΔC β-cat encoding lentiviruses. Cells were further transduced with lentivirus encoding short hairpins against *Lef1* (shLef1), *Tcf1* (shTcf1), or shCTR. Cells were cultured in serum without LIF in presence of 3 μM Chiron or vehicle (0.3% DMSO) for 1 week and stained for AP expression. Error bars represent standard error of three biological replicates.

Furthermore, only chemical inhibition of GSK3 activates a transcriptional Wnt response in wild-type cells, while eliciting little or no response on β-catenin knockout mESCs, suggesting that Plakoglobin upregulation cannot recapitulate nuclear β-catenin functions. Nevertheless, activation of the canonical Wnt pathway shields mESCs from spontaneous epiblast/ectoderm differentiation by suppressing the expression of lineage-specific genes and not by enhancing the expression levels of pluripotency factors. This effect is particularly evident when cells are deprived of LIF, suggesting overlapping functions between LIF and Wnt signaling in mESC self-renewal. With respect to these functions, Chiron treatment can prolong, but not indefinitely sustain, mESC self-renewal in the absence of LIF. This phenotype is abrogated by β-catenin loss but can be rescued by wild-type β-catenin and, to a lesser extent, by ΔC β-cat isoforms, posing the question of whether nuclear β-catenin functions are truly required for differentiation inhibition. We demonstrated that TCF1 and LEF1 are involved in the observed phenotypes, as their depletion further impairs the colony formation capacity and AP staining of both wild-type and ΔC rescued β-catenin knockout cells. These results suggest that ΔC β-cat isoforms are not completely transcriptionally silent and can still interact not only with TCF3 (as previously suggested by Wray et al., 2011) but also with other TCF/LEFs; we cannot, however, exclude that other regulatory mechanisms might be involved. Finally, we proved that activation of the canonical Wnt pathway sustains mESC self-renewal through inhibition of spontaneous differentiation; nuclear β-catenin functions, in association with TCF/LEF family members, could play a role in this phenotype.

In the future, it will be particularly interesting to further investigate the transcriptional response of ΔC-rescued β-catenin knockout cells to Chiron, as it could pinpoint a subset of powerful transcriptional targets truly responsible for Wnt-mediated self-renewal enhancement. In addition, since most of the observed pluripotency phenotypes are inevitably dependent on the specific culture conditions we used (mostly, serum based and feeder free using gelatin coating), it might be of interest to extend the results presented in this work to study pluripotency phenotypes upon β-catenin deletion in serum-free media and MEK1/2 and GSK3β inhibitors (e.g., 2i/LIF), which can confer ground-state cell pluripotency to mESCs (Ying et al., 2008); notably, 2i/LIF cultured mESCs should better resemble *in vivo* features observed in the inner cell mass in the pre-implantation embryo, where Wnt signaling is active (De Jaime-Soguero et al., 2018; Ten Berge et al., 2011).

EXPERIMENTAL PROCEDURES

Detailed experimental procedures are provided in Supplemental Information.

Cell Culture

mESCs E14 (129/Ola strain) were cultured on gelatin-coated plates in ESC medium: DMEM supplemented with 15% FBS (Hyclone), 1× non-essential amino acids, 1× GlutaMax, 1× penicillin/streptomycin, 1× 2-mercaptoethanol, and 1,000 U/mL LIF ESGRO (Chemicon). mESCs cultured in serum + LIF medium were re-plated every 3 days at a split ratio from 1:30 following dissociation with trypsin 0.05% EDTA (Gibco). mESCs containing floxed alleles of β-catenin were a kind gift of Prof. Rolf Kemler (SR18 and NLC1 cell lines, stably expressing CRE-ERT2) and Prof. Christine Hartmann (NLβ-12^{tm4Wbm} fl/fl and del/del cells).

For serum-free cultures, mESCs were cultured without feeders or serum in pre-formulated N2B27 medium (NDiff N2B27 base medium, Stem Cell Sciences Ltd, cat. no. SCS-SF-NB-02) supplemented with small-molecule inhibitors PD0325901 (PD, 1 μM, Selleck) and CHIRON99021 (CH, 3 μM, Selleck) and 1000 U/mL LIF (ESGRO, Millipore). Cells were routinely propagated on 0.1% gelatin-coated plastic and re-plated every 3 days at a split ratio of 1 in 10 following dissociation with Accutase (Gibco) as previously reported (Wray et al., 2011).

Human embryonic kidney 293t (HEK293t) were purchased from ATCC (293T; ATCC CRL-3216) and cultured in DMEM supplemented with 10% FBS (Hyclone), 1× penicillin/streptomycin. HEK293t were re-plated every 3 days at a split ratio of 1 in 6 following dissociation with trypsin 0.05% EDTA (Gibco, Life technologies).

RNA-Seq and Data Analysis

Total RNA from mESCs was extracted wit RNAeasy kits (Qiagen) following manufacturer's instructions. RNA integrity check, Poly-A pulldown, and library preparation were performed by CRG genomic facility. Samples were sequenced in two biological replicates to a depth of 30 million reads (100 bp) per sample using Illumina HiSeq. Reads were mapped to a reference transcriptome (mouse transcriptome from Ensembl v80) using kallisto (v0.42.5) (Bray et al., 2016) to generate counts and transcripts per million (TPM) values. We tested for differentially expressed genes for all pairwise conditions using edgeR (Robinson et al., 2010) from raw counts, filtering for genes with adjusted p value <0.05 and logFC > 0.5. Table S1 reports the list of all DEGs across pairwise comparisons regardless of their logFC. GO and KEGG analyses were performed using DAVID (version 6.7) (Huang da et al., 2009; Huang et al., 2007) using differentially expressed genes with adjusted p value <0.05 and logFC > 0.5. For each pairwise comparison, the list of expressed genes in control and analyzed samples was obtained by filtering out all the genes with zero counts in at least one sample. Expressed genes for each pairwise comparison were set as background universe to estimate enriched categories among differentially expressed genes in DAVID (Timmons et al., 2015). A p value threshold of 0.05 was set to determine enrichment of gene ontology categories. Multiple test correction was not performed.

Data and Code Availability

RNA-seq data that support the findings of this study have been deposited in the NCBI/GEO database (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE143340.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2020.07.019.

AUTHOR CONTRIBUTION

F.A., L.M., and M.P.C. wrote the manuscript. F.A. and M.P.C. designed the experiments. M.P.C. supervised the project. F.A., F.S., E.P., and F.L.L. performed the experiments.

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SR18 and NLC1 mESCs cell lines were a kind gift from Professor Rolf Kemler. NLβ-12 (Ctnnb1$^{tm4Wbm\ fl/fl}$ and Ctnnb1$^{tm4Wbm\ del/del}$) were a kind gift from Professor Christine Hartmann.

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Supplemental Information

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

Francesco Aulicino, Elisa Pedone, Francesco Sottile, Frederic Lluis, Lucia Marucci, and Maria Pia Cosma

Supplemental information

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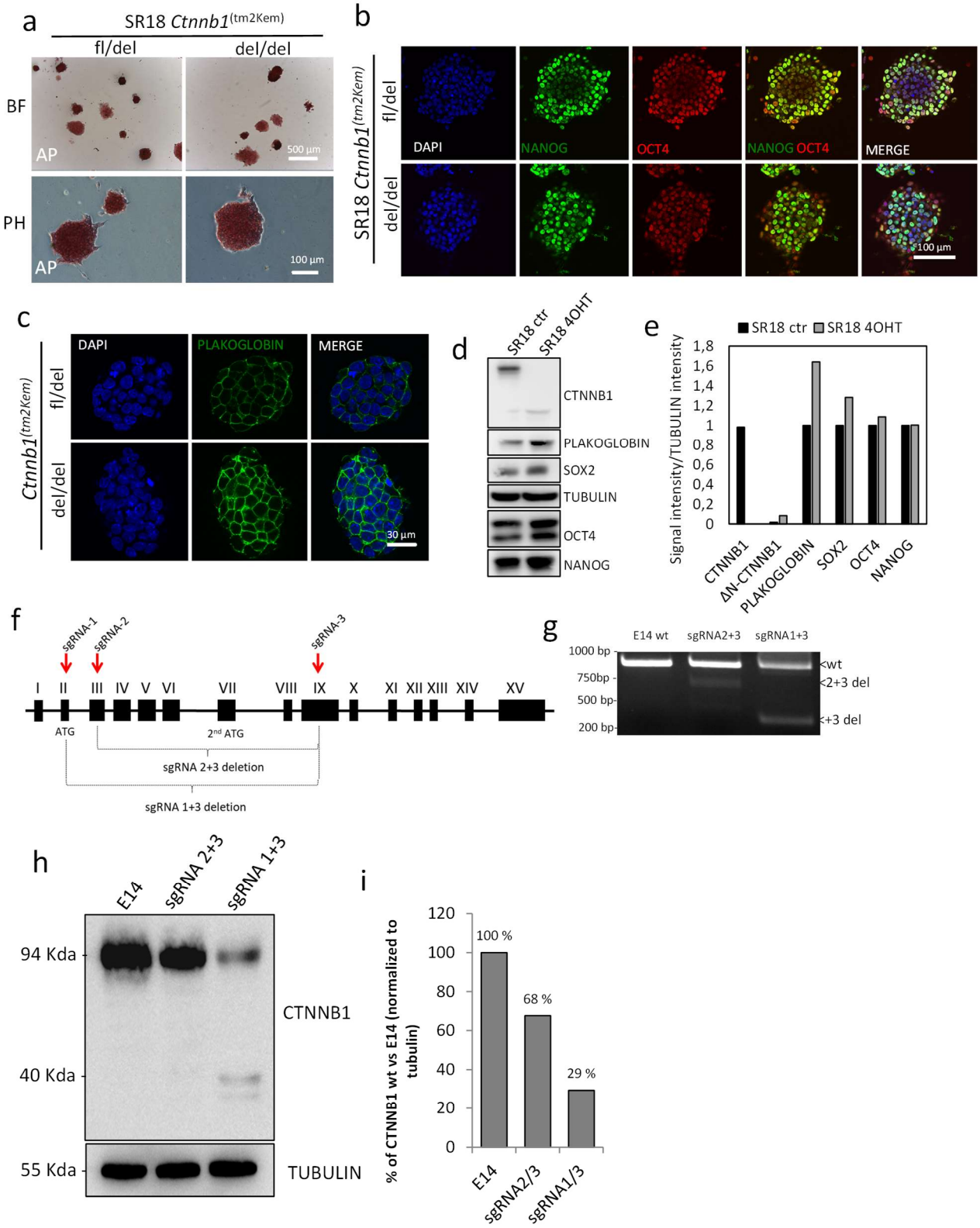


Figure S1

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.

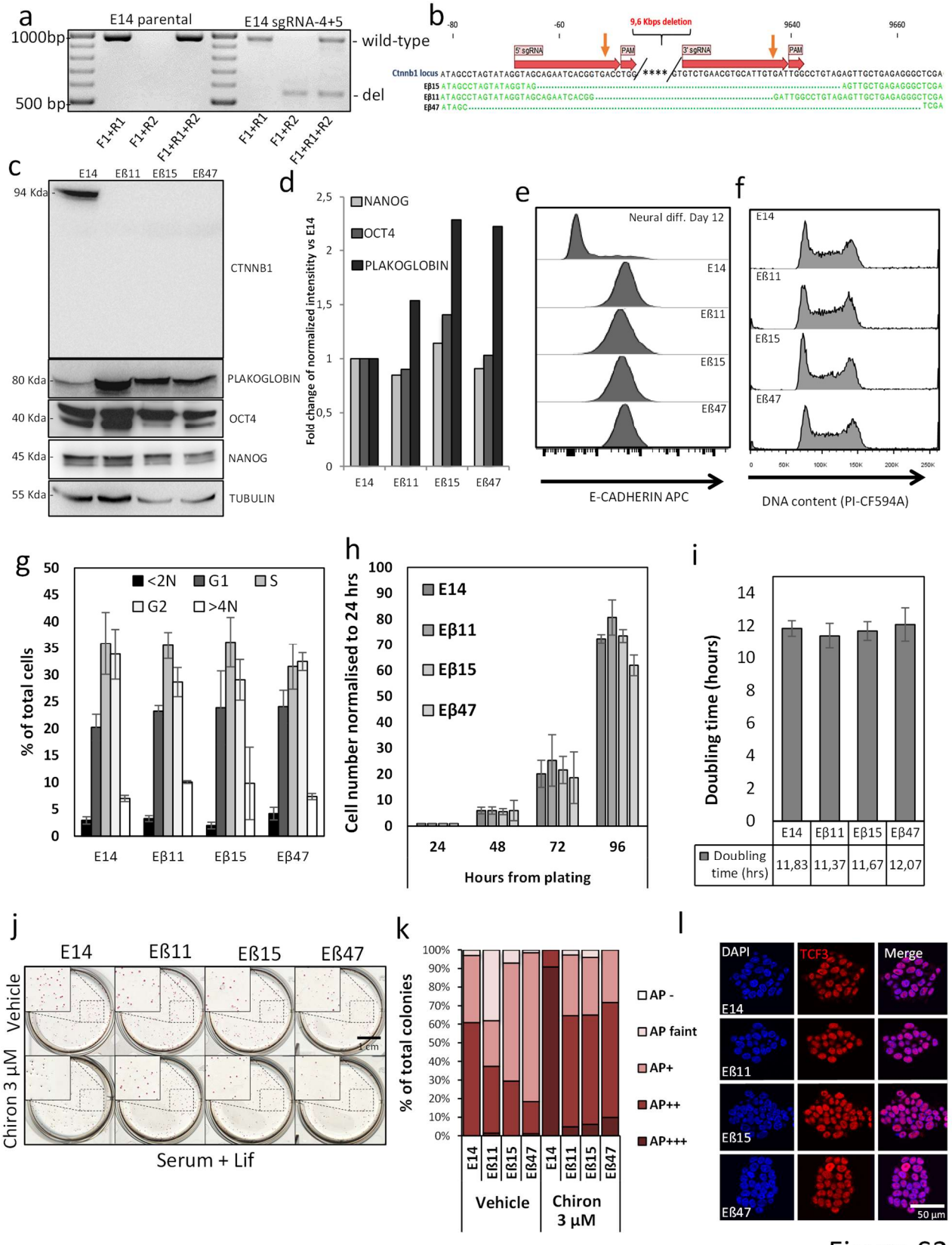
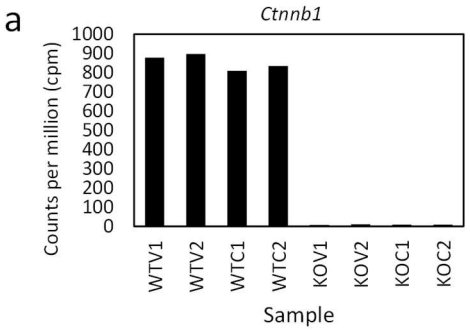

a
E14 parental
E14 sgRNA-4+5
1000bp
500 bp
- wild-type
- del
F1+R1
F1+R2
F1+R1+R2
b
9,6 Kbps deletion
5' sgRNA
3' sgRNA
PAM
Ctnnb1 locus
Eβ15
Eβ11
Eβ47
c
E14
Eß11
Eß15
Eß47
94 Kda
80 Kda
40 Kda
45 Kda
55 Kda
CTNNB1
PLAKOGLOBIN
OCT4
NANOG
TUBULIN
d
NANOG
OCT4
PLAKOGLOBIN
Fold change of normalized intensity 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

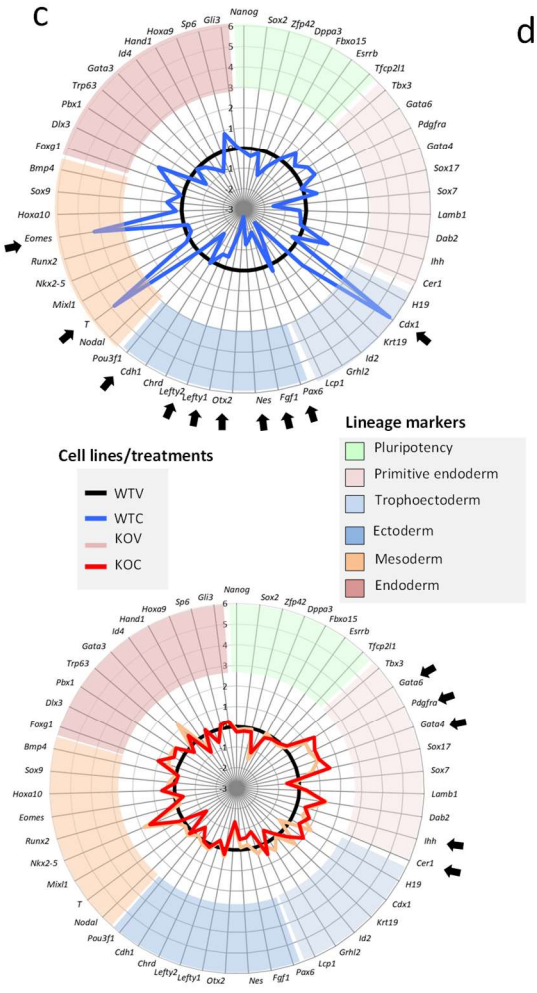
Figure S2 – Characterization of β-catenin knock-out clones. Related to Figure 2.

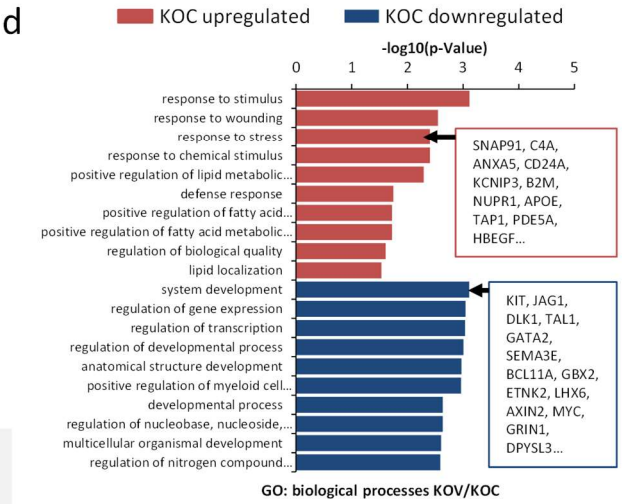
a) PCR genotyping of E14 transiently transfected with Cas9, sgRNA4 and sgRNA5 (right) and parental cell line (left). Three different oligos combinations were used to detect wild-type allele (F1+F2), deleted alleles (F1+R2) or both (F1+R1+R2). **b)** Sanger sequencing of Eβ11, Eβ15 and Eβ47 *Ctnnb1* edited 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.

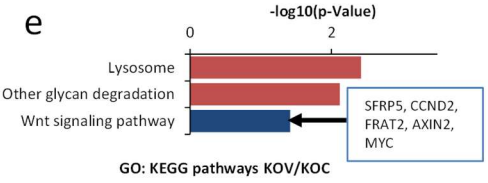


b

		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







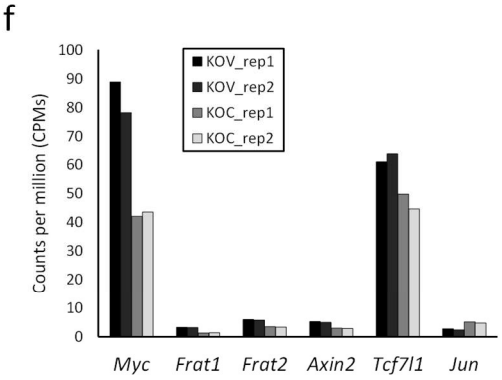
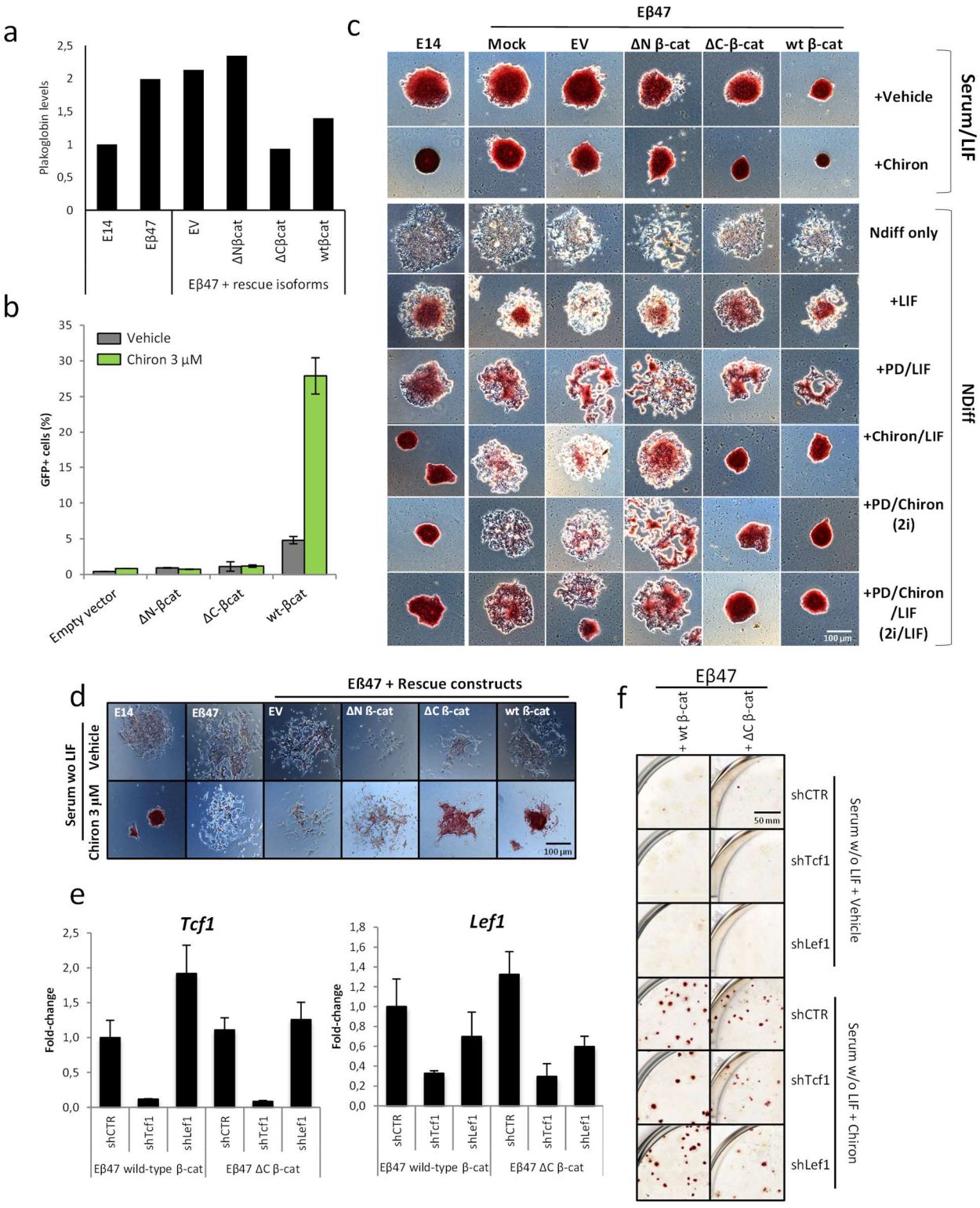


Figure S3

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
Vehicle
Chiron 3 µM
GFP+ cells (%)
35
30
25
20
15
10
5
0
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
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1,0
0,5
0,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
shCTR
shTcf1
shLef1
Eβ47 wild-type β-cat
Eβ47 ΔC β-cat
f
Eβ47
+ wt β-cat
+ ΔC β-cat
50 mm
shCTR
shTcf1
shLef1
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
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TCTGATTTCTAAGAACCGAGTAATGGTGTAGAACACTAATTCATAATCACGCTAATTGTAATCTGGAGAC
GTGTACAATTGTGTAGANNNNA

>Eβ11-Reverse
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GATACCAGCCCACCCCTCGAGCCCTCTCAGCAACTCTACCTATACTAGGCTATGTGCCCCGACACCAAAA
GCCAGTTCACCATGGCATACACTAAAAATACACAGCAGCCACGGTGTCAGGAAGCTCTTCTCAGTAGAAA
CAGATTGTAGGCATAACGGTGA

>Eβ15 Forward
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>Eβ15-Reverse
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>Eβ47-Forward
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>Eβ47-Reverse

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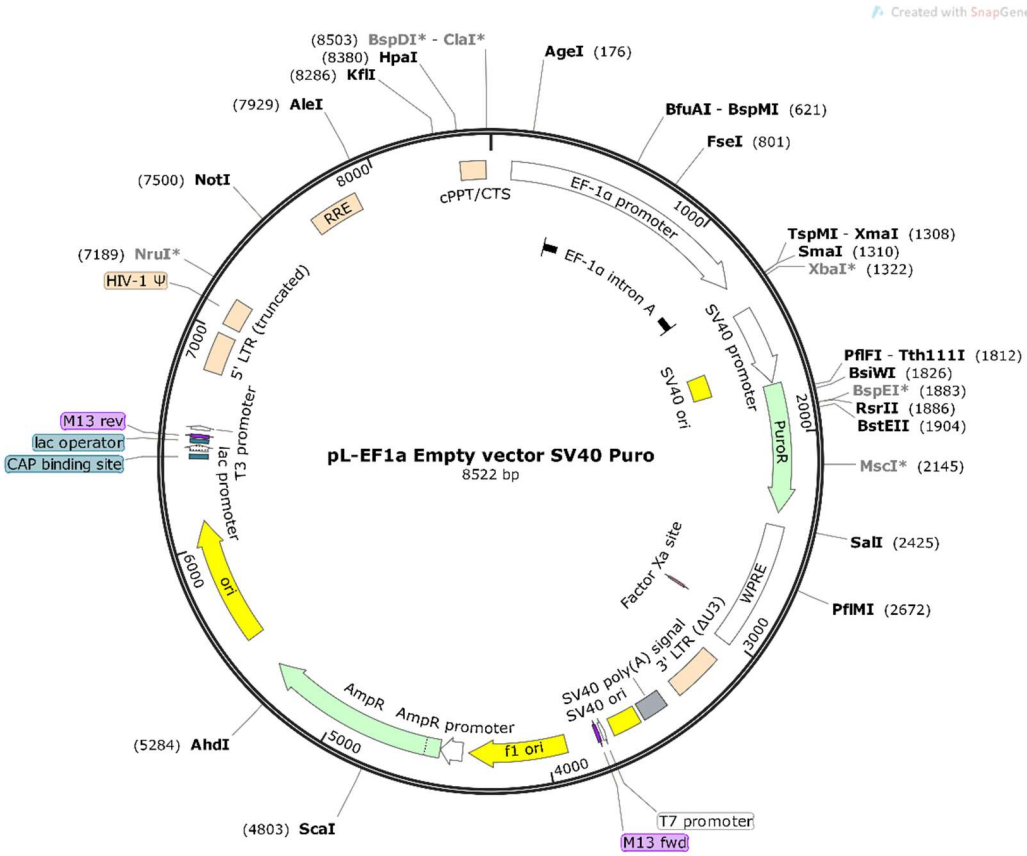
Plasmid maps and sequences



>pL-EF1a empty vector SV40 Puro

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TCGGACGGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTGGCCTCGCGCCGCCGTGTATCGCCCC
GCCCTGGGCGGCAAGGCTGGCCCGGTCGGCACCAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCT
GCTGCAGGGAGCTCAAAATGGAGGACGCGGCGCTCGGGAGAGCGGGCGGGTGAGTCACCCACACAAAG
GAAAAGGGCCTTTCCGTCCTCAGCCGTCGCTTCATGTGACTCCACGGAGTACCGGGCGCCGTCCAGGCAC
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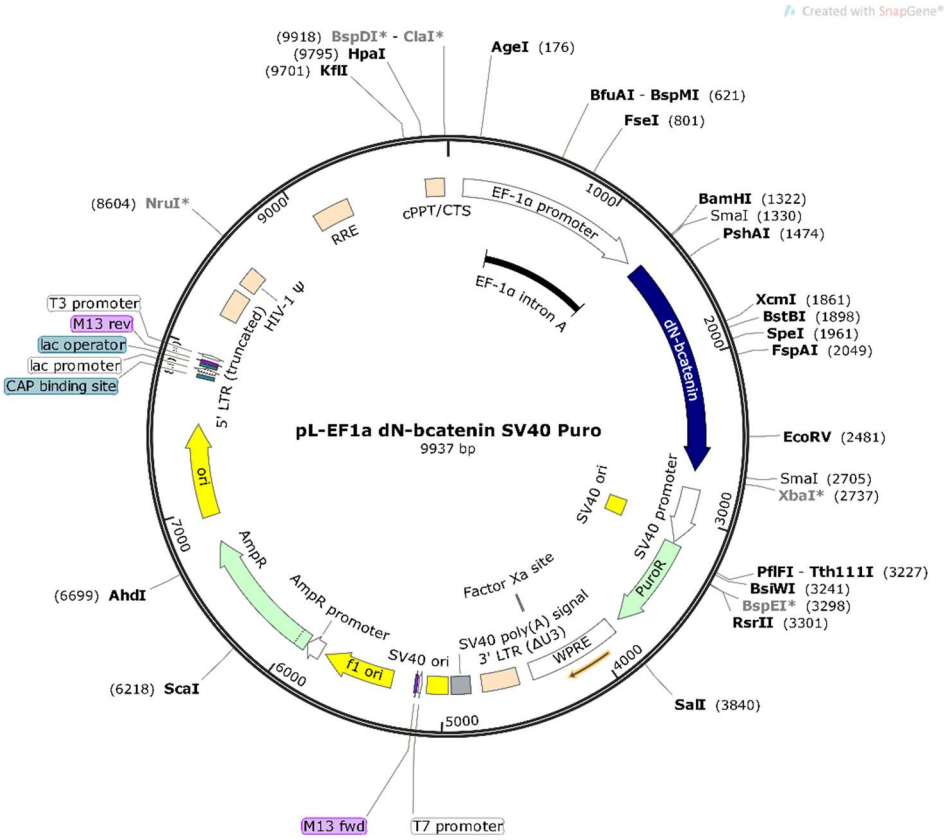

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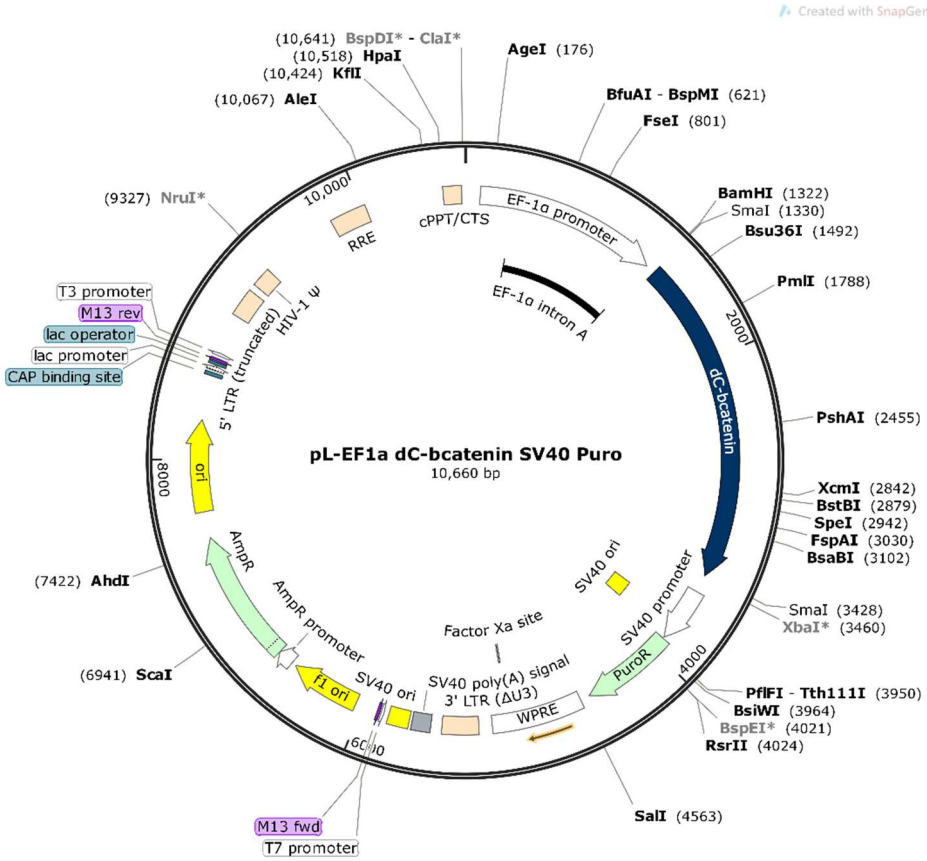

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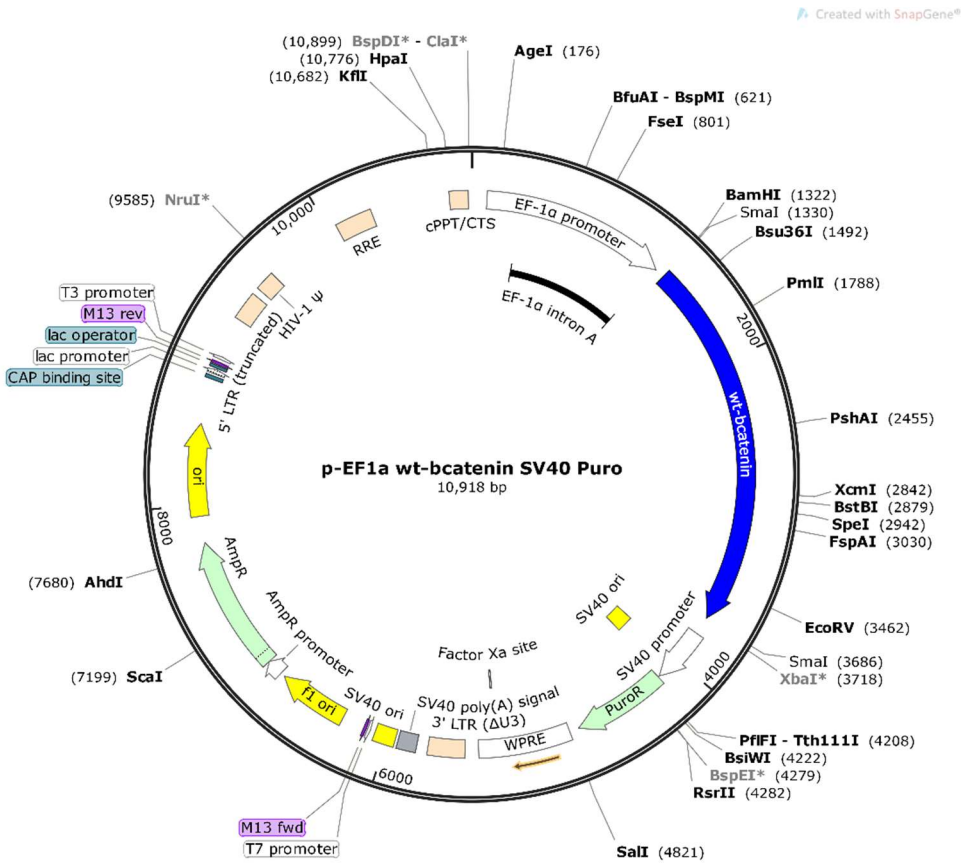

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Generation of Ctnnb1 KO cells

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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