β-catenin perturbations control differentiation programs in mouse embryonic stem cells

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Abstract

The Wnt/β-catenin pathway is involved in development, cancer and embryonic stem cell (ESC) maintenance; its dual role in stem cell self-renewal and differentiation is still controversial. Here, we applied an elegant *in vitro* system enabling conditional β-catenin control in β-catenin null mouse ESCs. We report that moderate induction of exogenous β-catenin enhances epiblast stem cell (EpiSC) derivation *in vitro*. Using a different genetic model and β-catenin chemical modulation, we derived a new protocol for ESCs to EpiSCs differentiation, based on NDiff227 and the GSK3α/β inhibitor Chiron, that is more efficient than standard ActivinA/Fgf2-based protocols. Finally, we report that moderate β-catenin levels favour early stem cell commitment towards mesoderm if the protein is overexpressed only in the 'ground state' of pluripotency conditions, or endoderm if the overexpression is maintained during the differentiation, unravelling the controversial role of this signalling pathway at the exit from pluripotency.

Introduction

Pluripotent Cells (PCs) are characterized by indefinite proliferative and differentiation potential and their identity is determined by the balance between signals promoting self-renewal and differentiation. The first step for stem cell differentiation is the exit from the pluripotent state, tightly controlled by underlying gene regulatory network dynamics which can drive specific lineage commitment. During murine development *in vivo*, embryonic stem cells (ESCs), that represent the naïve pluripotent state of the early epiblast[1-3], convert into the late epiblast and finally in terminally differentiated

somatic cells. ESCs can be derived from the pre-implantation epiblast and used to study cell pluripotency and differentiation, providing an excellent *in vitro* system for understanding signalling pathway interplay in cell fate decision making.

In serum-based cultures, mouse ESCs (hereafter called ESCs) are heterogeneous for the expression of pluripotency genes[1,2,4-10], while, when cultured in serum-free media supplemented with inhibitors of MEK1/2 (PD) and GSK3α/β (Chiron) and in presence or not of the Leukaemia Inhibitory Factor-LIF (2i or 2i+LIF)[3], a uniform self-renewal condition known as 'ground state' of pluripotency is established; it is characterized by homogenous gene expression[11-14], genome demethylation[15-17] and stable naïve pluripotency[18,19]. Mouse epiblast stem cells (hereafter called EpiSCs) also represent a relevant *in vitro* model because of their similarities with embryonic stem cells of human origin[20]. EpiSCs, derived from the post-implantation epiblast, are also capable of differentiating in all the germ-layers[20,21]; however, they differ from ESCs in morphology, clonogenicity, gene expression, epigenome status and, most importantly, ability to contribute to chimaeras[14,20-22]. EpiSCs require ActivinA and the fibroblast growth factor 2 (FGF2)[20,21] for *in vitro* expansion; of note, FGF signalling pathway activation, while promoting EpiSC self-renewal, induces ESC differentiation[23,24]. FGF2 treatment, in combination or not with ActivinA and LIF/STAT3 pathway inhibitors, has been used for ESCs differentiation into EpiSCs both in serum-based and serum-free culture conditions[25-28], although with low efficiency. Self-renewing EpiSCs have been recently established by simultaneous activation and inhibition of the Wnt/β-catenin pathway[29]; however, the effect of these perturbations on ESCs-EpiSCs direct transition has not been fully explored.

The Wnt/β-catenin is a highly conserved signalling pathway involved in ESCs self-renewal[30] and cell-cycle progression[31]. β-catenin levels are tightly controlled by the active transcription of negative regulators working at different levels of the signalling cascade[32]: Axin2[33-35] is part of the disruption complex whereas DKK1[36] binds the Wnt receptor complex attenuating cellular response upon activation of the pathway. These negative feedback loops contribute to the emergence of nonlinear dynamics in the Wnt/β-catenin pathway, proved to be important in different biological and developmental aspects (as reviewed in[37]), ESCs pluripotency[10] and somatic cell reprogramming[10,38-41]. The role of the canonical Wnt pathway in early *in vivo* developmental stages and the requirement of its activation for ESC self-renewal have

been a matter of intense research, often generating contradictory results[3,42-47]. Pluripotency incompetence has been reported in two independent studies using β-catenin$^{-/-}$ ECSs[42,44]; this phenotype was, however, contradicted in later studies with newly generated β-catenin$^{-/-}$ cell lines, which normally self-renew in both serum and 2i+LIF (hereafter called 2i/L), but present some differentiation defects when LIF-deprived[45-47]. Such knock-out models provide an excellent *in vitro* system to study β-catenin function on ESC decision making.

Here, we take advantage of the β-catenin$^{-/-}$ ESC line generated by Aulicino and colleagues[45], where the entire β-catenin coding sequence was removed to avoid possible compensatory mechanisms from aberrant truncated isoforms, to study the effect of β-catenin perturbations on the exit from pluripotency and differentiation. Different β-catenin doses have been indirectly achieved in the past by mutating the adenomatous polyposis coli gene (APC)[48]; teratomas from the mutants with the highest β-catenin transcriptional activity showed major differentiation defects in the neuroectoderm, dorsal mesoderm and endoderm lineages. Of note, results in[48] suggest that active β-catenin nuclear translocation (different across mutants) might also be involved in the observed differentiation impairment. Cellular models enabling direct modulation of β-catenin are necessary to systematically associate protein perturbations to pluripotency and differentiation phenotypes.

We tuned β-catenin levels in β-catenin$^{-/-}$ ESCs applying an improved inducible system[49] and measured both the global gene expression following 2i/L withdrawal, and the efficiency of ESC-EpiSC transition *in vitro*. Comparing the response to differentiation stimuli of ESCs expanded in serum/LIF or 2i/L, we demonstrated that moderate β-catenin overexpression in β-catenin$^{-/-}$ ESCs enhances the differentiation efficiency into EpiSCs. Moreover, short-term expansion in 2i/L predisposes ESCs to such transition, challenging the hypothesis of a synergistic effect of the ERK, Wnt/β-catenin and STAT pathways on EpiSCs derivation *in vitro* that would require further investigation. These results were recapitulated by exposing wild-type ESCs to low doses of the GSK3α/β inhibitor Chiron, further confirming our findings and providing an improved protocol for fast and efficient *in vitro* derivation of EpiSCs. Finally, the transcriptome of ESCs expressing different β-catenin levels confirmed what we and others reported about β-catenin dispensable requirement for pluripotency

establishment[45-47,49], while suggesting that specific β-catenin perturbations cause a bias towards the endoderm lineage.

Overall, our study highlights that synergistic effects of β-catenin doses and culture conditions control *in vitro* ESC fate decision making.

Results

Wnt/β-catenin pathway perturbations control *in vitro* generation of EpiSC

To study the role of the Wnt/β-catenin pathway in EpiSC derivation *in vitro*, we used the C1-EF1a-rtTA_TRE3G-DDmCherryβ-catenin^{S33Y} (hereafter called C1) ESC line we previously generated[49]. Briefly, β-catenin$^{-/-}$ ESCs[45] were modified to stably express a doxycycline-inducible fusion protein comprising the conditional destabilising domain (DD), the mCherry fluorescent protein and the constitutive active β-catenin^{S33Y}(Figure 1A)[50]. The inducer molecule doxycycline (Doxy) enables transcriptional initiation, while trimethoprim (TMP) allows protein stabilisation by inactivating the DD (Figure 1A)[49]. The use of a constitutively active and conditional β-catenin^{S33Y} form, uncoupled from upstream endogenous regulations and in a knock-out background, would help in dissecting β-catenin functions in the ESC-EpiSC transition, avoiding compensatory mechanisms and possible off-target effects of stable over-expression and chemical compounds.

We confirmed, in C1 cells, the correct induction (Figure 1A, inset and[49]), intracellular distribution and functionality of the exogenous protein upon input administration[49]. We previously confirmed the dispensable role of β-catenin in pluripotent culture conditions and showed, using Alkaline Phosphatase Staining, that moderate β-catenin overexpression (i.e., TMP10μM_Doxy10ng/mL) can protect cells from exiting pluripotency in the absence of both serum and LIF[49]. Following these results, we measured the efficiency of EpiSCs derivation when different doses of exogenous β-catenin are overexpressed in pluripotent conditions and/or during differentiation, adapting an existing differentiation protocol[29,51] (see Methods for details). To appreciate cellular response changes depending on the culture condition, C1 ESCs were expanded either in serum/LIF (hereafter called FBS/L) or in 2i/L. ESCs in the 'ground state' media (i.e. 2i/L)[3] have been extensively characterised for their transcriptional and epigenetic homogeneity[12,14] and resemblance of the pre-implantation epiblast[11,15-18]. However, prolonged culture in 2i/L results in epigenetic

changes impairing normal differentiation *in vitro* and development *in vivo*[52]. Therefore, we opted for a short-term culture in 2i/L (3 passages), sufficient to obtain cell homogeneity while avoiding possible aberrations.

ESCs from FBS/L or 2i/L (Figure 1B, C) were cultured for 48 hrs either in DMSO (Figure 1B) or in presence of maximum TMP (10μM) combined with low (10ng/mL) or saturating (100ng/mL) Doxy (Figure 1C). The concentrations of Doxy were extrapolated from flow cytometry measurements of the mCherry signal to provide two doses (moderate and high) of the exogenous protein (Figure 1A, inset). We next seeded 1.5×10^4 cells/cm^2 on fibronectin-coated plates in NDiff227[26] supplemented with ActivinA, FGF2[29] and different combinations of DMSO, TMP and Doxy (Figure 1B, C and Methods). Cells kept under these conditions for 4 days, with media refreshed after the first 2 culture days, were subsequently analysed for the expression of the Epiblast marker Fgf5 by qPCR (Figure 1B, C).

TMP/Doxy pre-treatment before differentiation (Figure 1C) did not alter the basal Fgf5 expression of FBS/L ESCs (T0 samples in Figure 1D), whereas that of TMP10μM_Doxy10ng/mL treated ESCs from 2i/L culture was higher compared to the TMP10μM_Doxy100ng/mL and the TMP10μM treated samples (T0 samples in Figure 1E).

Upon 4 days of differentiation, in all Doxy/TMP treated conditions, Fgf5 levels were higher when C1 ESCs were pre-cultured in 2i/L, suggesting that the latter predisposes cells to more efficiently differentiate into EpiSCs (Figure 1E). In both scenarios (i.e., from FBS/L or 2i/L), β-catenin overexpression in pluripotent conditions only (i.e., “Before” in Figure 1D, E), or both before and during differentiation (i.e., “Always” in Figure 1D, E), did not increase Fgf5 levels as compared to the control (i.e. TMP10μM-treated C1 ESCs, Figure 1D, E). Interestingly, FBS/L pre-cultured C1 ESCs induced with a low amount of Doxy (TMP10μM_Doxy10ng/mL “During Differentiation” sample, Figure 1D) converted into EpiSCs more efficiently as compared to all other treatments from FBS/L (Figure 1D).

Altogether, these results indicate that both the cell culture media and the doses of exogenous β-catenin strongly influence how cells respond to the ActivinA/FGF2 differentiation stimulus, with the 2i/L pre-culture enabling more homogeneous differentiation towards Epiblast, and moderate β-catenin overexpression in FBS/L-derived ESCs during the differentiation protocol only improving EpiSCs establishment *in vitro*.

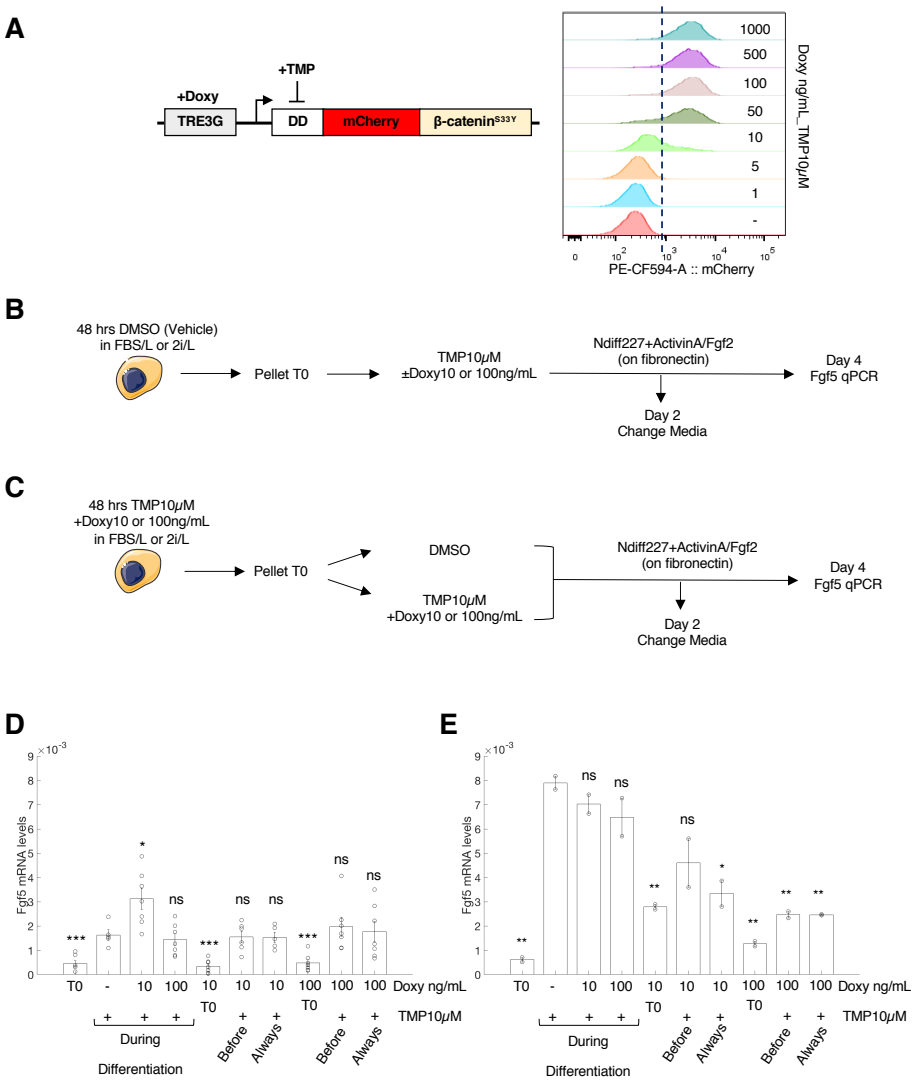


Figure 1. Dual-input control of β-catenin doses in EpiSC derivation *in vitro*.

A Dual-input regulation system consisting of the doxycycline responsive element and the conditionally destabilised mCherryβ-catenin^{S33Y} module. Doxycycline (Doxy) and trimethoprim (TMP) allow mCherryβ-catenin^{S33Y} transcription initiation and protein stabilisation, respectively. **(A, inset)** Flow cytometry profile of C1 ESCs treated for 24 hrs with TMP10μM and the indicated concentrations of Doxy. **B, C** Experimental scheme ESC to EpiSC differentiation. FBS/L and 2i/L C1 ESCs were pre-treated either with DMSO **(B)** or TMP10μM and Doxy10-100ng/mL **(C)**. Following 48 hrs of treatment, cells were seeded on fibronectin in NDiff227 and exposed to ActvinA/FGF2 and different combination of DMSO, Doxy and TMP for 4 days before being collected for RNA extraction. After 2 days, the media was changed, and the drugs were refreshed. **D, E** Fgf5 mRNA levels measured in FBS/L **(D)** and 2i/L **(E)** C1 ESCs, after 4 days of differentiation in NDiff227+ActivinA/FGF2 and different combination of DMSO, Doxy and TMP. Data are means±SEM (n=7, D (T0, Doxy10-100ng/mL "During Differentiation", Doxy10ng/mL T0, Doxy100ng/mL T0, Doxy100ng/mL "Before" ad Doxy100ng/mL "Always"); n=6, D (Doxy10ng/mL "Before"); n=5, D (TMP10μM "During

Differentiation"); n=2, E). p-values from two-tailed unpaired t test computed by comparing each sample with C1 TMP10μM "During Differentiation" are shown, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Dots represent individual data with similar values overlapping.

Chemical modulation of the canonical Wnt pathway enhances Epiblast derivation from ESCs

The above results (Figure 1) and the need to define a protocol for efficient derivation of EpiSCs *in vitro* motivated us to explore the differentiation potential of wild-type ESCs when deprived of pluripotency factors and exposed to different chemical perturbations of the Wnt/β-catenin pathway (Figure 2A). We took advantage of the *miR-290-mCherry/miR-302-eGFP*[53] ESC line (hereafter called dual reporter ESCs), which allows fluorescent tracking of the exit from pluripotency. Specifically, naïve dual reporter ESCs express the mCherry reporter only, and progressively start expressing also the GFP reporter when allowed to differentiate into EpiSCs[53].

We measured the transition from ESCs to EpiSCs using flow cytometry in at least 2 independent 4-day time-courses (Figure 2A). Before differentiation, dual reporter ESCs were cultured in FBS/L and treated for 48 hrs with DMSO (Figure 2B) or with Chiron (1-3μM, Figure 2C, D respectively) to pre-activate the canonical Wnt pathway, or were maintained in 2i/L for 3 passages (i.e., 1 week; Figure 2E). At Day 0, 1.5×10^4 cells/cm^2 were seeded on fibronectin-coated plates and exposed to different combination of drugs added to the NDiff227[26]: ActivinA+FGF2+DMSO (AFD); ActivinA+FGF2+Ch1μM (AFC1); ActivinA+FGF2+Ch3μM (AFC3); Ch1μM (C1); Ch1μM+XAV2μM (C1X2); Ch3μM (C3); Ch3μM+XAV2μM[29] (C3X2) (Figure 2A). The GFP signal was analyzed using flow cytometry every day for 4 days and the media changed after the first 2 culture days (Figure 2A).

Dual reporter ESCs pre-cultured in FBS/L in absence of β-catenin activation showed, already at Day 1, a GFP+ cell percentage increase in all but AFD treatment; AFD-treated cells efficiently transited towards the Epi state later in the experiment, but they were unable to rich other treatment GFP+ percentage levels (Figure 2B).

FBS/L Ch1μM pre-cultured dual reporter ESCs showed an overall increased percentage of GFP+ cells compared to DMSO-treated ESCs (compare Figure 2B, C). However, the fold-increase at Day 4 with respect to Day 0 of the best performing conditions (i.e., C1 and C1X2; Figure 2C) was lower in Ch1μM pre-treated cells (13- and 12-fold increase, respectively; Figure 2C) in comparison with the DMSO (23- and

22-fold increase, respectively; Figure 2B). This suggests, as in the experiments performed in a β-catenin null background (Figure 1), that the ESC to EpiSC conversion is favoured by β-catenin activation during the differentiation only, while its prolonged stimulation might reduce the differentiation efficiency. This was confirmed in Ch3μM pre-treated cells, where the best performing conditions (i.e., C3 and C3X2; Figure 2D) also showed an overall lower percentage of GFP+ cells as compared to DMSO-treated ESCs. Moreover, the simultaneous activation/inhibition of the pathway (i.e., C3X2 condition) had opposite effects given cells pre-culture conditions: the percentage of GFP+ cells increased over-time in DMSO pre-treated cells (20-fold increase at Day 4 with respect to Day 0; Figure 2B), while GFP expression was quite low at the end of the time-course in Ch3μM pre-treated ESCs (3.6-fold increase at Day 4 with respect to Day 0; Figure 2D). Interestingly, Chiron pre-treatment had no positive effect on the differentiation efficiency using the standard differentiation protocol (AFD), as the GFP+ percentage increase in AFD-treated cells at Day 4, as compared to Day 0, was almost the same in Ch1μM pre-cultured cells (7.4-fold increase; Figure 2C) and lower in Ch3μM (1.9-fold increase; Figure 2D) and 2i/L (1.1-fold increase; Figure 2E) pre-cultured cells as compared to the DMSO condition (7.4-fold increase; Figure 2B). In ESCs pre-cultured in 2i/L, there was not much difference across the differentiation protocols, as can be seen by the overall higher p-values computed through a one-way ANOVA test (compare Figure 2B-E). Finally, the combined treatment with ActivinA, FGF2 and Chiron (AFC1 and AFC3; Figure 2B-E) did not change EpiSCs derivation efficiency, as compared to the standard ActivinA/FGF2 treatment (Figure 2B-E).

The ESC-EpiSC transition was proven to be a highly heterogeneous process involving massive cell death[54]. Although we did not observe significant changes in cell viability across experiments, we noticed strong variability in the efficiency of *in vitro* EpiSCs derivation: in Supplementary Figure 2, we show the average of at least 2 experiments where the overall percentage of GFP+ cells was almost halved across conditions, as compared to data in Figure 2. Still, results in Supplementary Figure 2 confirm that the addition of Chiron during differentiation, in combination or not with XAV2μM (C1 and C1X2 or C3 and C3X2; Supplementary Figure 2A-D), enables a much more efficient conversion towards EpiSCs as compared to the other protocols. Also, we confirmed that, if pre-cultured in FBS/L, no pre-activation of the Wnt/β-catenin pathway and differentiation in media enriched with Ch1μM or Ch1μM±XAV2μM enables efficient

EpiSCs derivation (C1 and C1X2; Supplementary Figure 2A), in agreement with β-catenin genetic perturbation results in Figure 1.

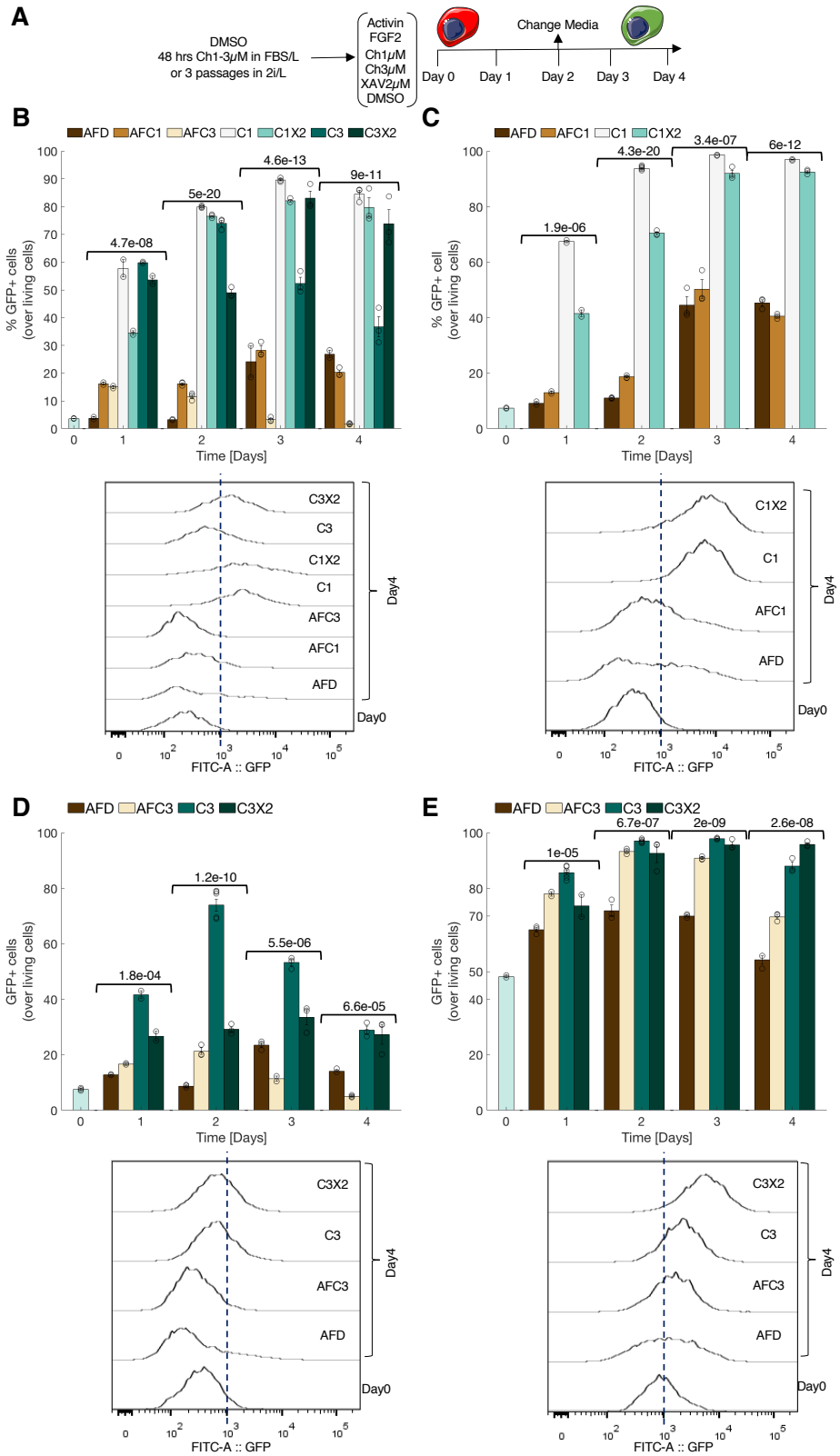


Figure 2. Chemical perturbation of the Wnt/β-catenin in EpiSC derivation *in vitro*. **A** Experimental scheme of Epiblast differentiation. Dual reporter ESCs cultured in FBS/L and pre-treated for 48 hrs with DMSO and Chiron (1-3 μM), or in 2i/L for 3 passages, were seeded

on fibronectin in NDiff227 supplemented with different combination of drugs (ActivinA+FGF2+DMSO (AFD); ActivinA+FGF2+Ch1μM (AFC1); ActivinA+FGF2+Ch3μM (AFC3); Ch1μM (C1); Ch1μM+XAV2μM (C1X2); Ch3μM (C3); Ch3μM+XAV2μM (C3X2)). The GFP signal from the EpiSC marker was measured by flow cytometry every 24 hrs for 4 consecutive days. After the first 2 days, the media was changed, and the drugs were refreshed. **B-E** Percentage of GFP+ cells calculated over the total amount of living cells in DMSO **(B)**, Ch1μM **(C)**, Ch3μM **(D)** and 2i/L **(E)** pre-cultured dual reporter ESCs. Histograms from Day 0 and Day 4 of each condition are shown as insets. Data are means±SEM (n=2, B (Day1, Day3 AFD, Day4 AFD), C (Day 1), D (Day1, Day3 AFC3), E (Day0, Day1 AFC3-C3X2); n=3, B (Day0, Day2, Day3 AFC1-AFC3-C1-C1X2-C3-C3X2, Day4 AFC1-AFC3-C1-C1X2-C3-C3X2), C (Day0, Day2 AFD-AFC1-C1X2, Day3, Day4), D (Day0, Day2 AFD-AFC3-C3X2, Day3 AFD-C3-C3X2, Day4), E (Day1 AFD, Day2 AFD-AFC3-C3X2, Day3, Day4); n=6, C (Day2 C1), D (Day2 C3), E (Day1 C3, Day2 C3). p-values from one-way ANOVA are shown across samples for each day. Dots represent individual data with similar values overlapping. Colour-blind safe combinations were selected using colorbrewer2 (https://colorbrewer2.org/#type=sequential&scheme=BuGn&n=3).

To confirm these results at single cell level, we monitored single-cell ESC-EpiSC transition in 71 hrs time-lapse microscopy experiments (Supplementary Movies 1-3; see Methods for details). FBS/L pre-cultured dual reporter ESCs were imaged every 60 minutes while constantly stimulated with AFD (Supplementary Movie 1) or Chiron 1-3μM (Supplementary Movies 2 and 3, respectively). In the AFD condition, a few GFP+ cells started to appear after 57 hrs (Supplementary Movie 1), whereas in the C1 and C3 culture conditions the transition started already after 10-14 hrs, with a peak around 40-43 hrs (Supplementary Movies 2 and 3). The GFP signal from cells differentiated in the presence of Chiron3μM was weaker as compared to the Chiron1μM treatment (compare Supplementary Movies 2 and 3).

These results confirm that strength and time of β-catenin stabilization are important to tip the balance between cell fates. In agreement with our previous observations in FBS/L pre-cultured knock-out cells (see TMP10μM_Doxy10ng/ml “During Differentiation” sample in Figure 1D), data collected using the dual reporter cell line confirm that high levels of β-catenin can be detrimental to efficiently derive EpiSCs *in vitro*. Indeed, a moderate activation or the simultaneous treatment with both an activator (i.e., Chiron) and an inhibitor (i.e., XAV) of the Wnt/β-catenin pathway

improve ESC direct differentiation into EpiSC, effectively replacing ActivinA and FGF2 requirement (Figure 2B, Supplementary Figure 2A).

Transcriptome and WGCNA analysis of ESC exit from pluripotency with varying β-catenin doses

Next, we studied C1 ESCs exit from the 'ground state' of pluripotency, i.e., upon 2i/L withdrawal, by RNA-sequencing; such monolayer differentiation protocol does not induce a specific cell fate and is ideal to observe any β-catenin dependent differentiation bias.

C1 ESCs cultured in 2i/L for 3 passages were treated for 48 hrs with saturating concentrations of Doxy and TMP (100ng/mL and 10μM, respectively) to induce the expression of the exogenous fusion protein (Figure 3A). Taking advantage of the mCherry-tag for exogenous β-catenin overexpression, Doxy/TMP treated C1 ESCs were sorted into two different subpopulations: C1 with Middle and High β-catenin levels (hereafter called C1M and C1H samples, respectively; Figure 3A and Supplementary Figure 3A). Cells were then seeded at 1.5×10^4 cells/cm^2 on gelatin-coated plates and cultured in NDiff227 media±inducers for 4 days before being transcriptionally profiled (see Methods for details; Figure 3A). Sequencing provided snapshots of the C1, C1M and C1H samples transcriptome in pluripotent condition, and of the differentiated counterparts (Day 4 samples) cultured in NDiff227 and DMSO (i.e., upon Doxy/TMP withdrawal; hereafter called C1DMV and C1DHV), or in NDiff227 and TMP/Doxy (hereafter called C1DMDT and C1DHDT) during differentiation. The C1 and C1D samples refer to cells treated only with TMP10μM in the pluripotent and differentiated states, respectively.

Principal Components Analysis (PCA, Figure 3B) showed three main clusters: one group included C1, C1M and C1H in the 'ground state' of pluripotency, another group included C1D ESCs and the final group contained all perturbed samples (C1DMV, C1DHV, C1DMDT and C1DHDT) after 4 days of differentiation.

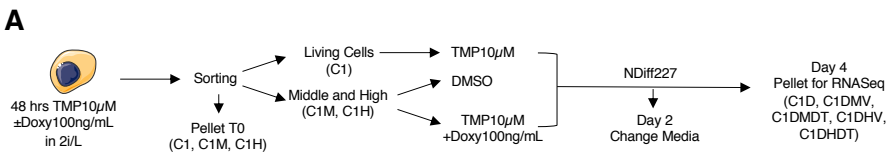
We next performed the Weighted Gene Correlation Network Analysis (WGCNA) to pinpoint the processes associated with β-catenin perturbations. The WGCNA[70] is a computational approach that allows the identification of gene modules based on their correlation and network-derived topological properties. We applied the WGCNA to our

sequencing data and identified 13 different modules (Supplementary Figure 3B; see Methods for details). We then defined the principal component of each module (i.e., eigenmodule, ME) and calculated the Pearson Correlation Coefficient (*r*) of each eigenmodule with time (Supplementary Figure 3C) or β-catenin doses (Figure 3C). Next, for each module, we performed a functional annotation analysis on the genes with a module membership |kME| ≥ 0.8 (Methods) to identify the biological processes and pathways enriched (Supplementary Table 1), focusing the attention on the modules having $r > 0.5$ and p-value < 0.05 with the examined traits (Figure 3C, Supplementary Figure 3C, Methods).

We found four modules significantly correlating (i.e., Green r = 0.92/p-value = 9e-14, Blue r = 0.82/p-value = 1e-08 , Black r = 0.67/p-value = 3e-05 and Brown r =0.57/p-value = 7e-04; Supplementary Figure 3C, Supplementary Table 1) and three anti-correlating (i.e., Turquoise r = -0.99/p-value = 2e-24, yellow r = -0.75/p-value = 1e-06 and Pink r = -0.56/p-value = 8e-04; Supplementary Figure 3C, Supplementary Table 1) with time, meaning that these modules were regulated during the differentiation. The biological processes (list of genes with a kME > 0.8; Supplementary Table 1) related to these modules with a FDR < 0.05 showed an enrichment of genes involved in translation, rRNA processing, ribosomal biogenesis and stem cell division (Supplementary Figure 3D, F Supplementary Table 1, Green and Brown), protein transport and processes associated with cellular respiration (Supplementary Figure 3E, Supplementary Table 1, Blue), positive regulation of growth, ncRNA processing and neuronal tube formation and development (Supplementary Figure 3F, Supplementary Table 1, Brown), regulation of tissue remodelling, embryonic and forebrain development, stem cell population maintenance and cell homeostasis (Supplementary Figure 3G, Supplementary Table 1, Turquoise), nuclear division and meiosis and organelle fission (Supplementary Figure 3H, Supplementary Table 1, Pink). For the pathway analysis, we found an enrichment in ribosome (Supplementary Table 1, Green and Blue), Hedgehog signalling pathway (Supplementary Table 1, Green), oxidative phosphorylation and diseases associated with oxidative stress (Supplementary Table 1, Blue and Turquoise), cancer (Supplementary Table 1, Brown and Turquoise) and regulation of the actin cytoskeleton (Supplementary Table 1, Yellow). Of note, there were no significantly enriched BPs and/or pathways for the genes from the Black, Yellow and Pink modules (Supplementary Table 1, Black, Yellow and Pink).

To gain more information about the effects of β-catenin at the exit from pluripotency, we correlated the eigenmodule of each module with β-catenin doses (Figure 3C, see Methods for details) and found three modules correlating with β-catenin doses (i.e., Tan r = 0.81/p-value = 2e-8, Purple r = 0.71/p-value = 7e-6 and Yellow/Green r = 0.69/p-value =1 e-5; Figure 3C, Supplementary Table 1). The biological processes (list of genes with a kME > 0.8; Supplementary Table 1) corresponding to these modules with a FDR < 0.05 showed enrichment in cell division (Figure 3D, Supplementary Table 1, Tan), metabolism and negative regulation of neuronal death (Figure 3E, Supplementary Table 1, Purple). Finally, the pathways analysis showed a significant enrichment only in Lysosome and N-Glycan biosynthesis from the Purple module, whereas pentose phosphate pathways and autophagy from the Green/Yellow (Supplementary Table 1, Purple and GreenYellow).

Overall, the WGCNA analysis confirmed the expected major transcriptional and metabolic changes associated with the exit from the pluripotent status, and confirmed previously reported β-catenin functions on cell survival and proliferation[31].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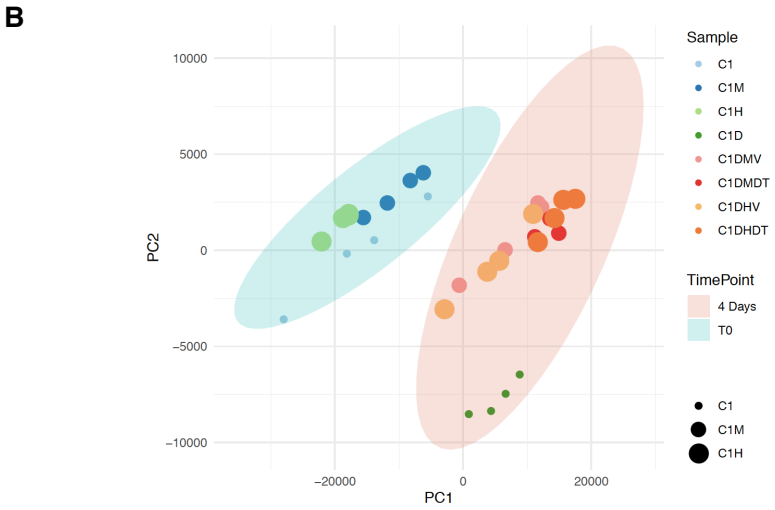
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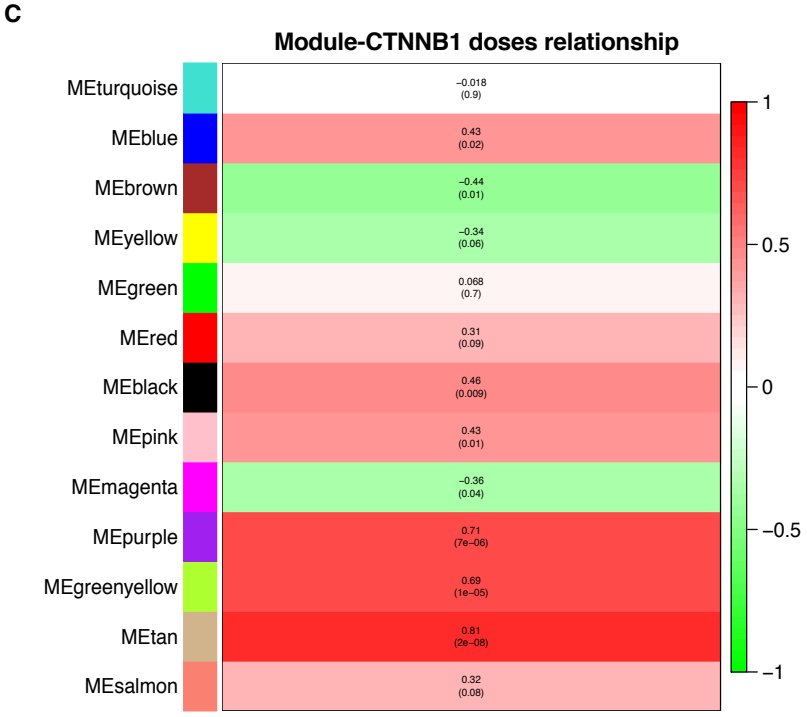
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(0.9)
0.43
(0.02)
−0.44
(0.01)
−0.34
(0.06)
0.068
(0.7)
0.31
(0.09)
0.46
(0.009)
0.43
(0.01)
−0.36
(0.04)
0.71
(7e−06)
0.69
(1e−05)
0.81
(2e−08)
0.32
(0.08)
1
0.5
0
−0.5
−1

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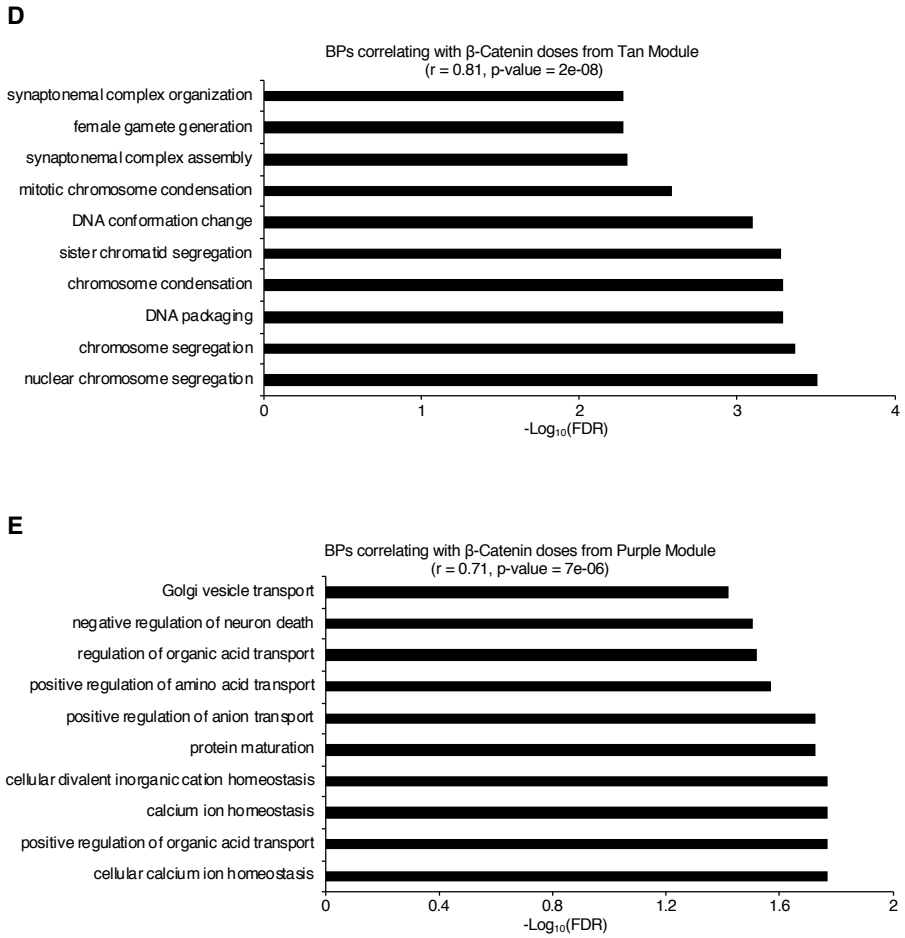


Figure 3. Transcriptome analysis of monolayer differentiation experiments upon β-catenin perturbations and WGCNA of the genes correlating with β-catenin doses. **A** Experimental scheme of the monolayer differentiation protocol. 2i/L C1 ESCs were pre-treated with TMP10μM and Doxy100ng/mL for 48 hrs; living cells were then sorted from the Dapi negative fraction of TMP-treated cells (C1), whereas β-catenin overexpressing cells from Doxy/TMP-treated samples were FACS-sorted from the mCherry fraction and divided into Middle (C1M) and High (C1H) subpopulations. 1.5×10^4 cells/cm^2 from each individual population were then seeded on gelatin in NDiff227 supplemented with DMSO or TMP±Doxy100ng/mL. After 4 days of differentiation in NDiff227, cells were collected and processed for the RNA sequencing. During the protocol, the media was changed, and the drugs refreshed after 2 culture days. **B** Principal Component Analysis (PCA) of all samples; the average of biological replica is shown. **C** Eigenmodules correlating with β-catenin doses; the Pearson Correlation Coefficient (*r)* and relative p-value are shown. **D, E** Bar-chart of the top-ten enriched biological processes (BP) with FDR < 0.05 from genes belonging to the Tan **(D)** and Purple **(E)** WGCNA modules.

Next, to look at comparisons between specific pairs of samples, we performed a Gene Ontology (GO) and Functional Annotation analysis on the differentially expressed

genes filtered for the false discovery rate (FDR < 0.05) and log fold change (logFC < -2 or > 2; see Methods for details). To identify gene ontologies specific for each experimental perturbation, we focused on the Biological Processes (BPs) enriched only in one comparison (red bars in Figure 4A-D, Supplementary Figure 4A, B). Starting from the pluripotent condition, when comparing C1M and C1H with C1 we found that the first 10 biological processes with a FDR < 0.05 were mainly enriched in cell cycles and metabolism (Supplementary Figure 4A, B, Supplementary Tables 2, 3). Interestingly, the genes exclusively upregulated in C1H compared to C1 were related to tissue differentiation (i.e., eye morphogenesis and urogenital system development) and DNA methylation involved in gamete generation (Supplementary Figure 4B, Supplementary Table 3). Only a few signalling pathways were enriched in C1M ESCs compared to the control cell line C1 (Supplementary Tables 2, 3). These results, together with the PCA in Figure 3B, confirm previous observations about the dispensable function β-catenin has in pluripotent culture conditions[45-47,49] and suggest a bias towards differentiation in C1H ESCs (Supplementary Figure 4B, Supplementary Table 3).

We then analysed the genes differentially expressed at Day 4 upon β-catenin perturbation as compared to C1D ESCs. Upregulated genes mostly contributed to the BPs significantly enriched (Figure 4A-D, Supplementary Tables 4-7), while downregulated genes only contributed to enrich a few processes, namely general metabolic processes (e.g., regulation of transporter and cation channel activity) and mesenchymal to epithelial transition (Figure 4A-D, Supplementary Tables 4-7). Genes exclusively upregulated in the C1DMV *vs* C1D comparison belonged to the mesoderm lineage (i.e., cardiovascular system development; Figure 4A and Supplementary Table 4), while, in the C1DMDT *vs* C1D comparison, upregulated genes were enriched for the endoderm lineage (i.e., urogenital system; Figure 4B and Supplementary Table 5). Nevertheless, mesoderm and endoderm lineages were represented in both comparisons. GO performed on the C1DHV and C1DHDT comparisons with C1D showed only a few differences in the enriched BPs, that indeed did not define a bias toward a specific lineage (Figure 4C, D, Supplementary Tables 6, 7).

These results suggest that the major changes in the differentiation program initiated upon 2i/L withdrawal are induced by moderate β-catenin doses and are influenced by the timing of protein overexpression. The pathway enrichment analysis showed the upregulation of protein metabolism in C1DMV and C1DHV (Supplementary Tables 4,

6), MAPK signalling pathway (Supplementary Table 4) in C1DMV, and ECM-receptor interaction and PI3K-AKT signalling pathway in C1DHV (Supplementary Table 6).

To gauge insights into specific differentiation programs, we selected sets of markers for naïve and general pluripotency, early post-implantation epiblast, ectoderm, mesoderm, endoderm, germ cell and trophectoderm[51], and clustered our samples according to the expression of these gene sets.

Naïve pluripotency genes were downregulated during differentiation in all samples, indicating the successful exit of cells from pluripotency (Figure 4E). Pluripotent C1M and C1H samples clustered together (Figure 4E) although close to C1 ESCs, in agreement with previous observations (Figure 3B). ESCs differentiated in presence of DMSO (i.e., C1DMV and C1DHV; Figure 4E) clustered together, similarly to samples differentiated in presence of Doxy and TMP (i.e., C1DMDT and C1DHDT, Figure 4E); still, a large number of genes (e.g., Klf5, Tcl1, Klf2 and Nr0b1) showed a different pattern among C1D, C1DMV, and C1DHV, discriminating ESCs with different β-catenin doses (Figure 4E). These results support the hypothesis of a β-catenin-dependent effect on transcriptional changes.

The clustering across samples was similar for general pluripotency markers (Figure 4F). In accordance with previous reports about the transition to primed pluripotency[55-58], in the majority of differentiated samples Sox2 was downregulated and Utf1, Zfp281 and Lin28 were upregulated (Figure 4F). Moreover, almost all the genes were lower in C1DMV, C1DMDT, C1DHV and C1DHDT as compared to C1D (exceptions were Lin28a, higher in C1DHDT and Sox2, higher in C1DHV; Figure 4F), and showed different behaviours in DMSO- (i.e., C1DMV and C1DHV; Figure 4F) *vs* Doxy/TMP-treated samples (i.e., C1DMDT, C1DHDT; Figure 4F), confirming that the duration of β-catenin overexpression has an influence on cell identity. Zfp281, Zic2 and Utf1 showed a similar pattern in β-catenin overexpressing cells as compared to C1D ESCs, all being downregulated (Figure 4F). Zfp281 is a Zinc finger transcription factor implicated in regulating stem cell pluripotency[59,60], and recently reported as a bidirectional regulator of the ESC-EpiSC transition in cooperation with Zic2, another zinc finger protein[61]. The undifferentiated embryonic cell transcription factor 1 (Utf1) is expressed in ESCs and is involved in many aspects of gene expression control (e.g., transcription factor[62] and epigenome organization[63,64]), playing an important regulatory role during the exit of ESCs from pluripotency[63,64]. The concomitant

reduction of Zfp281, Zic2 and Urf1 suggests a global change in the chromatin organization of β-catenin overexpressing ESCs en route to differentiation (Figure 4F). Early post-implantation epiblast genes were mostly upregulated in primed ESCs compared to the pluripotent condition with no evident differences across treatments in naïve ESCs (Figure 4G). The exception was Foxd3, downregulated in both naïve and primed β-catenin overexpressing cells as compared to the controls C1 and C1D ESCs (Figure 4G). Interestingly, Dnmt3a levels, although similar at T0, were very different at Day 4, with C1DMV/C1DHV and C1DMDT/C1DHDT showing 80% and 70% gene expression reduction as compared to the control C1D, respectively (Figure 4G); also, samples constantly exposed to Doxy/TMP (i.e., C1DMDT and C1DHDT) showed higher Dnmt3a expression than DMSO-treated ESCs (i.e., C1DMV and C1DHV; Figure 4G). Similar observations hold for Dnmt3b, where the reduction compared to C1D was of about 60% for C1DMV/C1DHV and 10% for C1DMDT/C1DHDT (Figure 4G). Dnmt3a, b and Foxd3 are DNA and chromatin remodelling factors, respectively; Dnmt enzymes methylate genomic regions, whereas Foxd3 reduces active and enhances inactive histone marks by recruiting the Lysine-specific demethylase 1 (Lsd1)[65]. The reduced expression of those genes in β-catenin overexpressing cells, including the pluripotent markers Utf1 discussed above, supports the hypothesis of differentially methylated DNA.

We then screened for a large panel of lineage-priming factors. Clustering based on the ectoderm lineage genes showed that pluripotent C1 and C1M are more similar than C1H (Figure 4H); following 2i/L withdrawal, the clustering resembled those of previous sets (Figure 4E-G), with C1DMV/C1DHV and C1DMDT/C1DHDT grouping together (Figure 4H). Genes from this lineage had different and sometimes opposite expression across samples, making difficult to identify a clear pattern associated with β-catenin perturbations. Nevertheless, we distinguished two groups of genes: one group (i.e., Dlx3, Pou3f2, Otx1 and Pou3f3) was mainly downregulated during differentiation, whereas the other group (i.e., Nes, Ascl1, Cdh2 and Pax6) had the opposite trend (Figure 4H).

When looking at mesoderm markers (Figure 4I), differentiated samples clustered similarly to the previous data set; however, C1DMV/C1DHV were more similar to C1D than in the previous comparisons (Figure 4E-H) and genes showed different expression patterns across conditions. Lhx1, Lefty1/2, Meox1, Hoxb1 and Bmp4 were mainly upregulated upon differentiation and no major differences were observed

across samples in pluripotent conditions. The exception in this group was Lhx1 that was higher in β-catenin overexpressing cells depending on the timing of overexpression (compare C1DMV/C1DHV with C1DMDT/C1DHDT in Figure 4I). In contrast, genes such as Nodal, Kdr, Mixl1, Gsc, Foxf1 and Zic1 got downregulated when exiting pluripotency and the levels across conditions were not significantly changing (Figure 4I). Of note, although the behaviour of individual genes is hard to interpret, C1D ESCs were very different from all differentiated samples with perturbations of β-catenin, stressing the relevance of the latter for mesoderm specification[46].

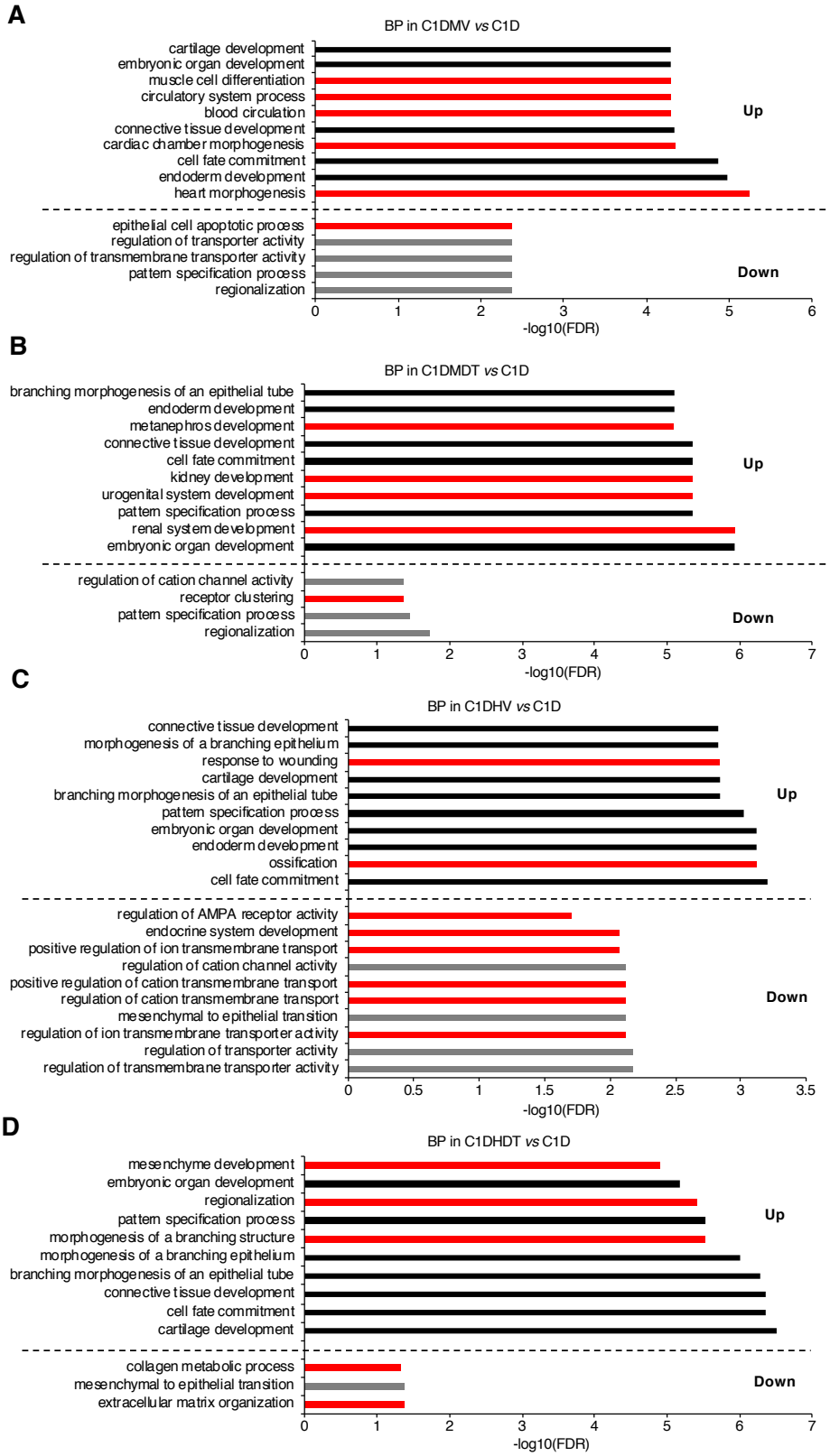
The endoderm lineage was the one most influenced by β-catenin perturbations: C1D cells were unable to induce the expression of endoderm-related genes (compare C1 and C1D in Figure 4J), whereas in all perturbed ESCs their expression increased over time. As previously observed (Figure 4E-I), samples clustered together based on the duration of β-catenin overexpression rather than on its dose (i.e., C1DMV/C1DHV and C1DMDT/C1DHDT, Figure 4J). Moreover, because of their impaired differentiation, C1D clustered together with pluripotent samples (Figure 4J). The C1DHDT showed the highest expression of the 50% of the endoderm-associated genes (namely, Cxcr4, Gata4 and Sox7) as compared to all other differentiated samples (i.e., C1D, C1DMV, C1DMDT and C1DHV). Slightly different was the behaviour of Cxcr4, that decreased over time; however, its expression is indicative of definitive endoderm differentiation[66] that most probably is not happening in the short protocol we applied. These observations, together with the incapability of C1D cells to embark differentiation toward this lineage, strongly support previous knowledge about the β-catenin requirement for endoderm organization[46,67].

In the analysis of the germ cell lineage markers, all genes showed a rather heterogeneous expression pattern across samples (Figure 4K). Pluripotent C1M and C1H clustered together and close to C1, and differentiated samples clustered based on the duration of β-catenin perturbation (i.e., C1DMV/C1DHV and C1DMDT/C1DHDT). Dazl, Prdm1 and Ddx4 showed a clearer pattern in pluripotent conditions, being upregulated in C1M and C1H compared to C1 ESCs (Figure 4K).

Finally, when looking at trophectoderm markers, clustering showed similarity of C1 and C1M, as for the ectoderm and endoderm lineages (Figure 4H, J, respectively). C1DMDT and C1DHDT were similar for the expression of trophectoderm genes, whereas C1DMV was part of a different branch clustering with C1D; C1DHV was more

closely related to C1DMDT and C1DHDT (Figure 4L). Moreover, 90% of trophectoderm genes got downregulated during differentiation (namely, Eomes, Elf3 and Cdx1) in all the conditions; the only exception was Cdx2 that was upregulated in C1D, C1MDV and C1DMDT in comparison with the corresponding T0 (Figure 4L). Eomes was recently reported to control the exit from pluripotency by acting on the chromatin status[68]; its behaviour in naïve C1M and C1H ESCs support the theory of a different chromatin conformation in pluripotent β-catenin overexpressing cells (Figure 4L).

Accounting for the fact that ectoderm is a default linage of the monolayer differentiation protocol we applied[69], sequencing results suggest that β-catenin overexpression in a knock-out background favours rescuing defects in differentiation towards endoderm more than mesoderm. Indeed, mesodermal genes were mostly downregulated when β-catenin was overexpressed, whereas endodermal genes were all upregulated as compared to the control (Figure 4I, J). Moreover, we observed that lineage differentiation was influenced by the duration of protein overexpression rather than the dose. According to that, there was a transition from mesoderm to endoderm following moderate but constant β-catenin overexpression (compare C1DMV and C1DMDT in Figure 4A, B). Nevertheless, endoderm was an enriched gene ontology in all considered comparisons (Figure 4A-D). Finally, the observed expression of pluripotency markers Zfp281, Zic2 and Utf1, the early post-implantation markers Dnmt3a-b and Foxd3 and the trophectoderm marker Eomes suggests a reorganization of the epigenome in naïve C1M and C1H ESCs and upon monolayer differentiation of C1DMV, C1DMDT, C1DHV and C1DHDT ESCs.



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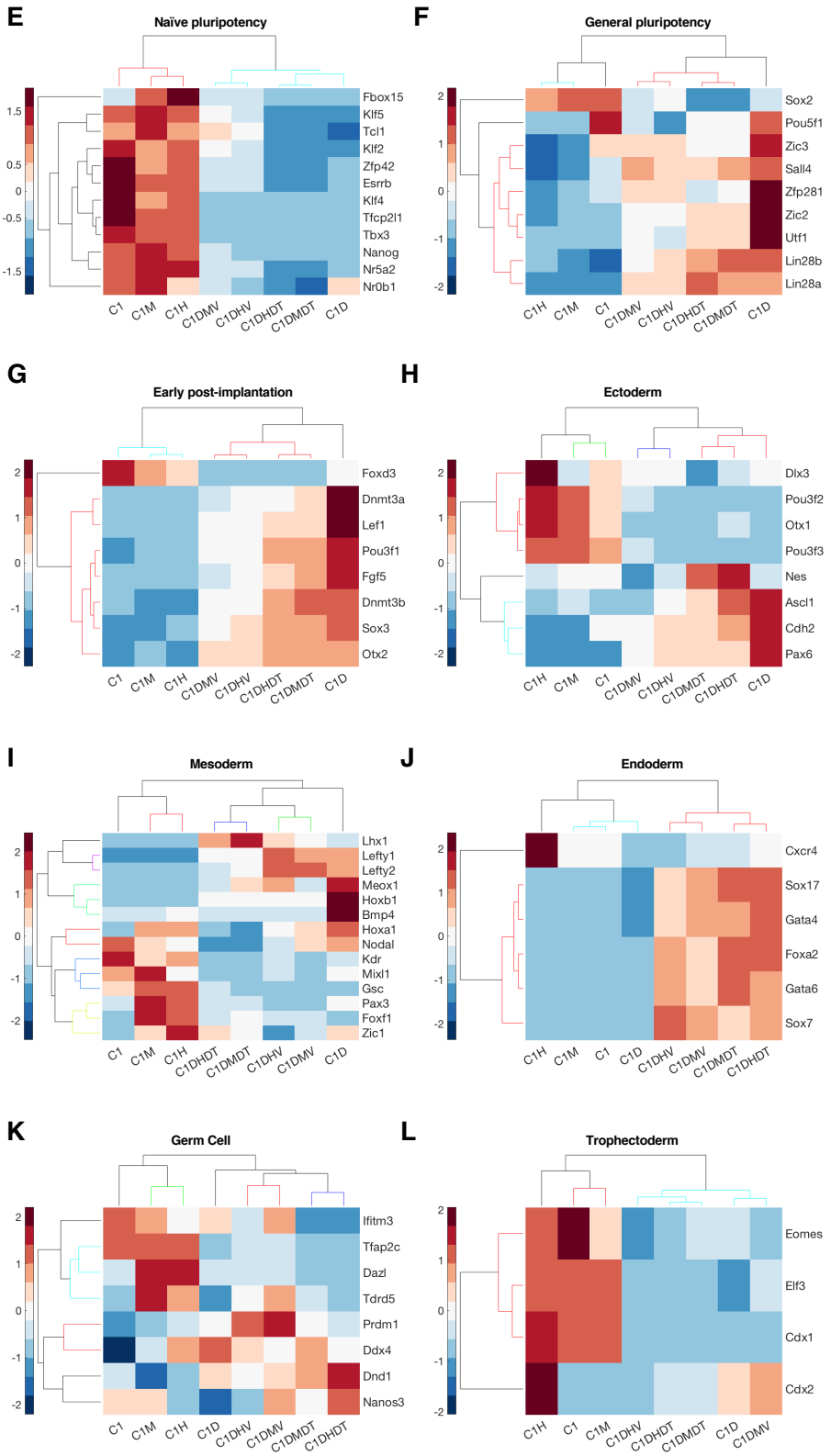

Figure 4. Gene ontology and clustergram of the differential expressed genes in control and perturbed ESCs.

A-D Bar-chart of the top-ten enriched biological processes (BP) with FDR < 0.05 from differentially expressed genes in C1DMV **(A),** C1DMDT **(B),** C1DHV **(C)** and C1DHDT **(D)** compared to C1D ESCs. Black and grey bars represent upregulated and downregulated BPs,

respectively. In red bars, the BPs exclusively enriched in the indicated condition. **E-L** Clustergram over heatmaps of Naïve **(E)** and general pluripotency **(H)**, early post-implantation **(G)**, ectoderm **(H)**, mesoderm **(I)**, endoderm **(J)**, germ cell **(K)** and trophectoderm **(L)** lineages from pluripotent and differentiated ESCs expressing different β-catenin amount. Each column is the average of 4 biological replicates.

Discussion

The role of the Wnt/β-catenin pathway as a pluripotency gatekeeper has been matter of many studies and debates[30,42,48,71-74]; while modulation of the canonical Wnt pathway has been extensively proved to be important for EpiSC *in vivo* derivation[75,76], self-renewal[77] and *in vitro* lineage differentiation[78-80], its relevance for the exit from pluripotency and for ESC-EpiSC direct transition have not been explored thoroughly. In this work, we proved that genetic β-catenin manipulation or chemical perturbation of the canonical Wnt pathway controls ESC fate at the exit from pluripotency.

Using two different cellular models, we found that moderate β-catenin doses in β-catenin$^{-/-}$ ESCs or its stabilization in wild-type ESCs increases the differentiation efficiency of ESCs into EpiSCs, similarly to what was reported for EpiSCs self-renewal[29]: EpiSCs can be maintained in Chiron3μM/XAV2μM cultures, with self-renewal regulated by both Axin2 and β-catenin[29]; here we found that, in absence of pre-activation of the canonical Wnt pathway, the Chiron3μM/XAV2μM treatment has a better effect than Chiron3μM alone on ESC-EpiSC transition at the end of the differentiation experiments (Day 3 and 4 in Figure 2B and Supplementary 2A), while the opposite was observed in the early phases of the protocol (Day 1 and 2 in Figure 2B and Supplementary 2A). This could be explained by the fact that Axin2 expression is β-catenin-dependent, and β-catenin stabilization gets induced by Chiron treatment; therefore, the effect of XAV might be relevant only upon Axin2 stabilisation. Alternatively, it might also suggest a switch in the effect of Chiron3μM/XAV2μM stimulation in a specific moment during the differentiation process, probably when ESCs start to acquire a more stable EpiSC identity (as in[29]). Although both hypotheses are in agreement with what was reported by Kim and colleagues[29], the first one seems to be more plausible looking at the response in Ffg5 (Figure 1) and GFP expression (Figure 2B, Supplementary Figure 2A), i.e., when a moderate activation of the Wnt/β-catenin pathway was induced. Indeed, both β-catenin$^{-/-}$ exposed to a low concentration of the input Doxy (i.e., TMP10 μM _Doxy10ng/mL, “During Differentiation” in Figure

1D) and the dual reporter ESCs treated with Chiron1μM (i.e., C1 in Figure 2B) showed the highest differentiation efficiency, suggesting that Chiron1μM treatment alone is enough to keep β-catenin doses within a range that would instead require XAV treatment if using higher doses of Chiron (i.e., 3μM), and that Chiron1μM is mimicking the effect obtained by β-catenin genetic manipulation. Furthermore, XAV treatment in combination with Chiron1μM reduced the percentage of differentiated cells at Day 1 (Figure 2B and Supplementary 2A); this could be due to the stoichiometry between β-catenin, Ainx2 and the two inhibitors. We reason that, in Chiron1μM/XAV2μM, XAV is in excess with respect to the amount of transcribed Axin2, therefore all the translated protein gest stabilised, and the final effect is a strong inhibition of the signalling pathway. Nevertheless, this effect might get attenuated with time, probably when a balance between the drug and the target is achieved. This theory seems to be supported by the behaviour of cells treated with Chiron1μM or Chiron3μM; the first showed a GFP peak within the first 2 days followed by GFP stabilisation, while the second showed the opposite trend (i.e., GFP reduction over time), suggesting that prolonged β-catenin stabilization in FBS/L can impair ESCs differentiation towards EpiSCs. These results pose the question of what would have happened if cells were stimulated for longer with Chiron1μM. There are two possible scenarios; in the first, EpiSCs, derived from ESCs, would self-renew in Chiron1μM as well as in Chiron3μM/XAV2μM. In the second scenario, the transition from Chiron1μM to Chiron 3μM would be preferred for the maintenance of established EpiSCs. However, the latter scenario would be in contrast with the results showed in[29], where it was proved immediate differentiation or death of EpiSCs expanded in Chiron3μM±PD1μM. Of note, the basal medium used in[29] for EpiSC self-renewal is based on minimal essential medium (GMEM) supplemented with 10% FBS[29], therefore differences might be expected. Finally, we found that 2i/L pre-cultured ESCs better differentiate into EpiSCs, regardless of further modulation of the pathway during differentiation. It has been shown that, during the differentiation process, ESCs downregulate Oct4 expression and undergo a temporary cell cycle arrest[54]; when differentiation has been accomplished, Oct4 levels and cell cycle progression are restored to support the clonal expansion of the EpiSC population. However, rare Oct4-/Cdx2+ cells might appear during the initial phase of the differentiation process; this cellular fraction does not respond to the cell cycle arrest, and therefore has a proliferative advantage[54]. The simultaneous elimination of differentiating incompetent cells (i.e., Oct4-/Cdx2+) and

633 the re-established proliferation of differentiated cells (i.e., Oct4+/Cdx2-) ensure a
634 homogenous population of EpiSCs[54].
635 We and others reported a reduced proliferation rate of ESCs stimulated with Chiron
636 both in FBS/L[31] and 2i/L[13]. In agreement with these observations, we hypothesise that
637 Chiron treatment might reduce the proliferation of Oct4-/Cdx2+ cells, establishing a
638 balance between differentiating competent (i.e., Oct4-/Cdx2-) and incompetent (i.e.,
639 Oct4-/Cdx2+) cells. The 2i/L pre-culture might additionally homogenise the expression
640 of various genes, including Oct4[81] and reduce the appearance of Cdx2+ cells.
641 Overall, these results confirm the effect β-catenin has on preparing cells to
642 appropriately respond to the differentiation stimuli and suggest that both the duration
643 and the dose of β-catenin overexpression control cell differentiation *in vitro*. Moreover,
644 they enabled us to define an improved protocol for EpiSCs derivation *in vitro*, based
645 on NDiff227 and Chiron1μM.
646 RNA sequencing performed in ESCs during the exit from the naïve 'ground state' of
647 pluripotency[51] showed that, in β-catenin overexpressing cells (in particular in C1DMV),
648 Dnmt3a and b had an expression pattern similar to the one observed in Rex1-high
649 ESCs differentiated using a similar protocol[51], indicating that moderate β-catenin
650 overexpression in naïve ESCs influences DNA methylation associated with the exit
651 from pluripotency. Interestingly, we observed a significant upregulation of endodermal
652 genes in β-catenin overexpressing cells, indicating a requirement of β-catenin for this
653 specific fate. This phenotype was previously reported in the β-catenin null cell line
654 generated by Lyashenko and colleagues[46], where the defect in endoderm lineage
655 differentiation was rescued by overexpressing both wild-type or transcriptional
656 incompetent β-catenin[46]; in contrast, mesoderm and ectoderm induction seemed to
657 not require β-catenin[46]. With our approach that enables dose- and time-controlled β-
658 catenin overexpression, we were able to define the amount of protein and the optimal
659 window of overexpression to facilitate mesoderm (i.e., C1DMV; Figure 4A,
660 Supplementary Table 4) or endoderm (i.e., C1DMDT; Figure 4B, Supplementary Table
661 5) differentiation, and confirmed that the ectoderm lineage is not affected by β-catenin
662 loss and therefore is not influenced by its overexpression.
663 In the future, it will be of great interest to use our inducible system to interrogate the
664 effect of more complex β-catenin dynamics on stem-cell identity and to further
665 investigate the role of the β-catenin transcriptional activity in pluripotent and
666 differentiated cells of both murine and human origin.

Authors Contributions

E.P. designed and performed experiments; M.F. and G.G. performed the WGNCA analysis; M.F. performed the GO; R.D.C. performed the Differential Expression analysis; E.P., and L.M. analysed data; E.P., M.F., R.D.C. and L.M. wrote the paper; D.d.B. supervised the bioinformatics analysis; L.M. supervised the entire project.

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Methods

Cell line derivation

C1 cell lines were previously derived in[49] by a double lentiviral infection of β-catenin$^{-/-}$ ESCs[45] with the EF1a-rtTA (Neomycin) plasmid followed by the pLVX_TrE3G-DDmCherryβ-catenin^{S33Y}(Puromycin). Cells were selected with Neomycin after the first round and with Puromycin after the last infection. The dual reporter ESCs were a gift from Dr Blelloch[53].

ESCs were cultured on gelatin-coated dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS, Sigma), 1 x nonessential amino acids, 1 x GlutaMax, 1 x2-mercaptoethanol and 1000 U/mL LIF (Peprotech). To note, that for the 2i/L culture, cells were kept for 3 passages (around 1week) in serum-free NDiff227-based media supplemented with 1000 U/mL LIF, 3μM of the GSK-3α/β inhibitor Chiron-99021 (Selleck, S1263) and 1μM of the MEK inhibitor PD0325901 (Selleck, S1036).

Epiblast Differentiation

For EpiSC derivation *in vitro*, FBS/L and 2i/L 1.5×10^4 cells/cm^2 were seeded on 10µg/mL Fibronectin-coated 12-well plates in NDiff227 (Takara, Y40002) and, according to the experiment, stimulated with DMSO, TMP10µM (Sigma, T7883), Doxy 10-100ng/mL (Sigma, D9891), human ActivinA 10ng/mL (Peptrotech, 120-14E), human FGF2 10ng/mL (Peptrotech, 100-18B) Chiron1-3µM and the XAV939 2µM (Sigma, 575545) for 4 days with the media and drugs refreshed after the first 2 culture days (Figures 1B, C and 2A).

Monolayer differentiation

Sorted C1, C1M and C1H ESCs were plated at 1.5×10^4 cells/cm^2 on gelatin-coated 12-well plates in plain NDiff227 and stimulated with DMSO or TMP10µM±Doxy10-100ng/mL for 4 days with the media and drugs refreshed after the first 2 culture days (Figure 3A).

Drugs pre-treatment

Some experimental conditions required pre-treatment of cells. For β-catenin overexpression in Figure 1C, C1 ESCs were stimulated for 48 hrs with TMP10µM and Doxy10-100ng/mL before the EpiSC differentiation, whereas for pre-activation of the canonical Wnt pathway in Figure 2A, C-E and Supplementary Figure 2B-D, dual reported ESCs were exposed to Chiron1-3µM (Figure 2C, Supplementary Figure 2B and Figure 2D, Supplementary Figure 2C, respectively) for 48 hrs or cultured for 3 passages in 2i/L (Figure 2E, Supplementary Figure 2D), before the differentiation.

Flow cytometry analysis

ESCs from a 12-well plate were washed with sterile Phosphate-Buffered Saline (PBS, Gibco), incubated with 80 µL of trypsin for 2–3′ at room temperature and collected with 120 µL of PBS 2% FBS containing DAPI as cell viability marker. Cell suspension was analysed using the BD LSR Fortessa and 10,000 living cells were recorded for each sample. The % of GFP positive cells was calculated over living cells, gated as DAPI negative, using the FlowJo V10 software.

Flow activated cell sorting (FACS)

ESCs were washed with sterile phosphate-buffered saline (PBS, Gibco), trypsinised for 2-3′ at room temperature and centrifuged at 1000 × g for 5′. Pelleted cells were resuspended in 500 µL of plain NDiff227 media supplemented with DAPI. The mCherry positive fraction was sorted from DAPI negative using the BD Influx high-speed 16-parameter fluorescence activated cell sorter.

qPCR

For quantitative PCR, the total RNA, extracted from cells using the RNeasy kit (Qiagen), was retrotranscribed (Thermo Fischer, RevertAid Reverse Transcriptase EP0441) and the cDNA used as template for each qPCR reaction in a 15 µL reaction volume. iTaq Universal SYBR Green Supermix (1725120, Bio-Rad) was used with the Qiagen Rotor-Gene System. To eliminate the contamination from genomic DNA, the RNeasy Plus Mini Kit (Qiagen, 74134) was used to purify the total RNA used for the RNA Sequencing. The primers used were: Actin-Fwd: ACGTTGACATCCGTAAAGACCT, Actin-Rev: GCAGTAATCTCCTTCTGCATCC; Fgf5-Fwd: AAAACCTGGTGCACCCTAGA, Fgf5-Rev: CATCACATTCCCGAATTAAGC).

Time-lapse experiments

1.5×10^4 cells were seeded on 10µg/mL fibronectin-coated µ-Slide 8 Well Glass Bottom (Ibidi, 80827) and imaged with a Leica DMI6000 inverted epifluorescence microscope equipped with the Photometrics Prime 95B sCMOS camera (1200x1200 11µm pixels, 8 ,12 bit or 16 bit, 70 fps full frame) and an environmental control chamber (Solent) for long- term temperature control and CO2 enrichment. The Adaptive Focus Control (AFC) ensures focus is maintained during multiple acquisition cycles. Images were acquired from two channels (phase contrast and green fluorescence) with a 20X objective every 60min for 71 hrs. ImageJ version: 2.0.0-rc-69/1.52p was used to improve the GFP signal, apply a Gaussian blur filter (Sigma radius 2) and combine channels.

QuantSeq 3’ RNA sequencing library preparation.

Preparation of libraries was performed with a total of 100ng of RNA from each sample using QuantSeq 3'mRNA-Seq Library prep kit (Lexogen, Vienna, Austria) according to manufacturer's instructions. Total RNA was quantified using the Qubit 2.0

fluorimetric Assay (Thermo Fisher Scientific). Libraries were prepared from 100ng of total RNA using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen GmbH). Quality of libraries was assessed by using screen tape High sensitivity DNA D1000 (Agilent Technologies). Libraries were sequenced on a NovaSeq 6000 sequencing system using an S1, 100 cycles flow cell (Illumina Inc.). Amplified fragmented cDNA of 300 bp in size were sequenced in single-end mode with a read length of 100 bp.

Illumina novaSeq base call (BCL) files are converted in fastq file through bcl2fastq [http://emea.support.illumina.com/content/dam/illuminasupport/documents/documentation/software_documentation/bcl2fastq/bcl2fastq2-v2-20-software-guide-15051736-03.pdf] (version v2.20.0.422).

QuantSeq 3' RNA sequencing data processing and analysis.

For analysis, sequence reads were trimmed using bbduk software (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/usage-guide/) (bbmap suite 37.31) to remove adapter sequences, poly-A tails and low-quality end bases (regions with average quality below 6). Alignment was performed with STAR 2.6.0a3[82] on mm10 reference assembly obtained from cellRanger website (https://support.10xgenomics.com/single-cell-gene-expression/software/release-notes/build#mm10_3.0.0; Ensembl assembly release 93). Expression levels of genes were determined with htseq-count[83] using Gencode/Ensembl gene model. We have filtered out all genes having < 1 cpm in less than n_min samples and Perc MM reads > 20% simultaneously. Differential expression analysis was performed using edgeR[84], a statistical package based on generalized linear models, suitable for multifactorial experiments. The threshold for statistical significance chosen was False Discovery Rate (FDR) < 0.05 (GSE148879). The lists of differentially expressed genes (DEGs), for each comparison, with a threshold of logFC > 2 for the induced and logFC < -2 for the inhibited transcripts (Supplementary Tables 2-7) were used for the Functional Annotation analysis.

The data were deposited in GEO with the accession number GSE148879.

Weighted Gene Correlation Network Analysis (WGCNA)

Quant-seq 3' mRNA data of 32 samples was used to construct a gene co-expression network by applying Weighted Gene Correlation Network Analysis (WGCNA)[70] from

802 the WGCNA package in the R statistical environment version 3.6. Briefly, we first
803 computed the Pearson correlation coefficient among all pairs of expressed genes and
804 then an appropriate value of the soft-thresholding power (β=6) giving a scale-free
805 topology fitting index (R^2) ≥ 0.85 was selected to build the weighted adjacency matrix.
806 The weighted adjacency matrix was further transformed into a topological overlap
807 matrix (TOM)[85] and the resulting dissimilarity matrix used for hierarchical clustering.
808 Gene modules were finally identified by cutting the hierarchical dendrogram with the
809 dynamic tree cut algorithm from dynamicTreeCut package in R[86] statistical
810 environment with standard parameters, except for cutHeight we set equal to 0.25 and
811 deepSplit we set equal to 1. The value of deepSplit parameter was selected after
812 performing a cluster stability analysis. Briefly, for each possible value of deepSplit
813 parameter (i.e., 0, 1, 2, 3 or 4), modules were identified for both the full dataset and
814 50 resampled datasets. Then, the clustering solution obtained for the full dataset was
815 compared with each resampled solution by mean of Adjusted Rand Index (ARI)[87]. The
816 solution giving the highest average ARI was used for the clustering analysis as
817 described above. Finally, to identify which clusters were correlated with β-catenin
818 expression doses or differentiation time we correlated the first principal component of
819 each gene module (i.e., the eigenmodule) with the traits of interest. The eigenmodule
820 can be considered as a "signature" of the module gene expression. Modules correlated
821 with the traits with a p-value < 0.01 were considered statistically significant and used
822 for further analyses.
823
824 **Functional Annotation Analysis**
825 Differentially expressed genes (either logFC > 2 or logFC < -2) and module "hubs"
826 having high module membership (also known as |KME| > 0.8) within the module were
827 analysed for the enrichment in GO Biological Processes[88] and KEGG Pathways[89] via
828 the clusterProfiler package in R statistical environment[90]. The threshold for statistical
829 significance was FDR < 0.05, the top-ten BPs were represented as -log10 (FDR;
830 Figure 3D-E, Supplementary Figure 3D-H, and Figure 4A-D, Supplementary Figure
831 4A, B).
832
833 **Statistical analysis**

Differences between samples were analysed by two-tailed unpaired t tstest and one-way ANOVA using Matlab (MathworksMatlab R2019a, update 9.6.0.1307630). A p-value lower than 0.05 was considered statistically significant.

Clustergram over heatmaps were generated using the clustergram function in Matlab (MathworksMatlab R2019a, update 9.6.0.1307630) that applies the Euclidean distance metric and average linkage. The data have been standardized across all samples for each gene and have 0 as mean and 1 as standard deviation.

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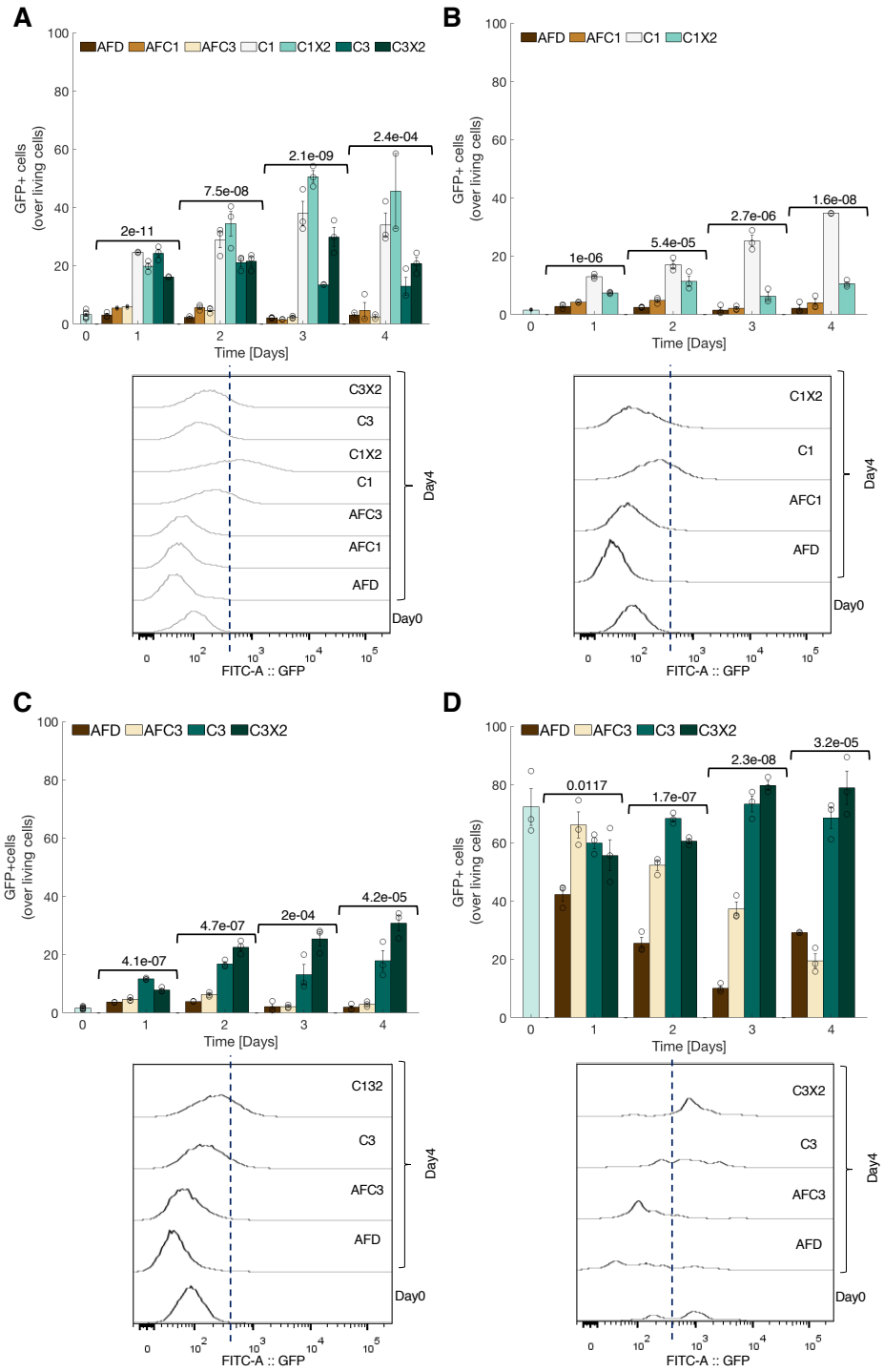
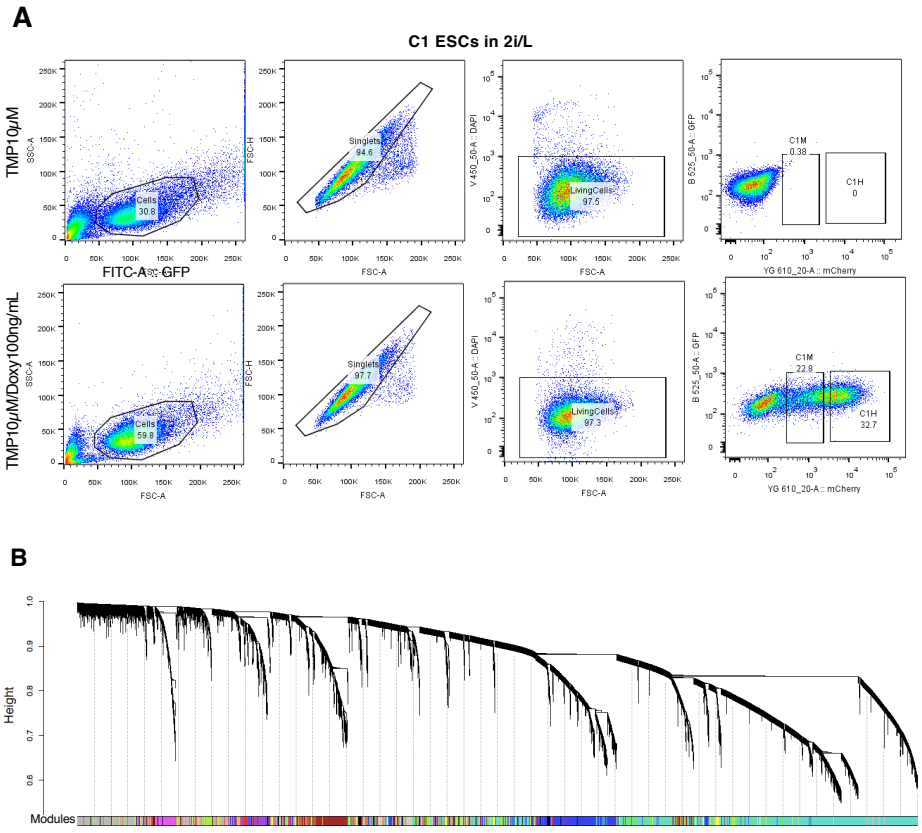
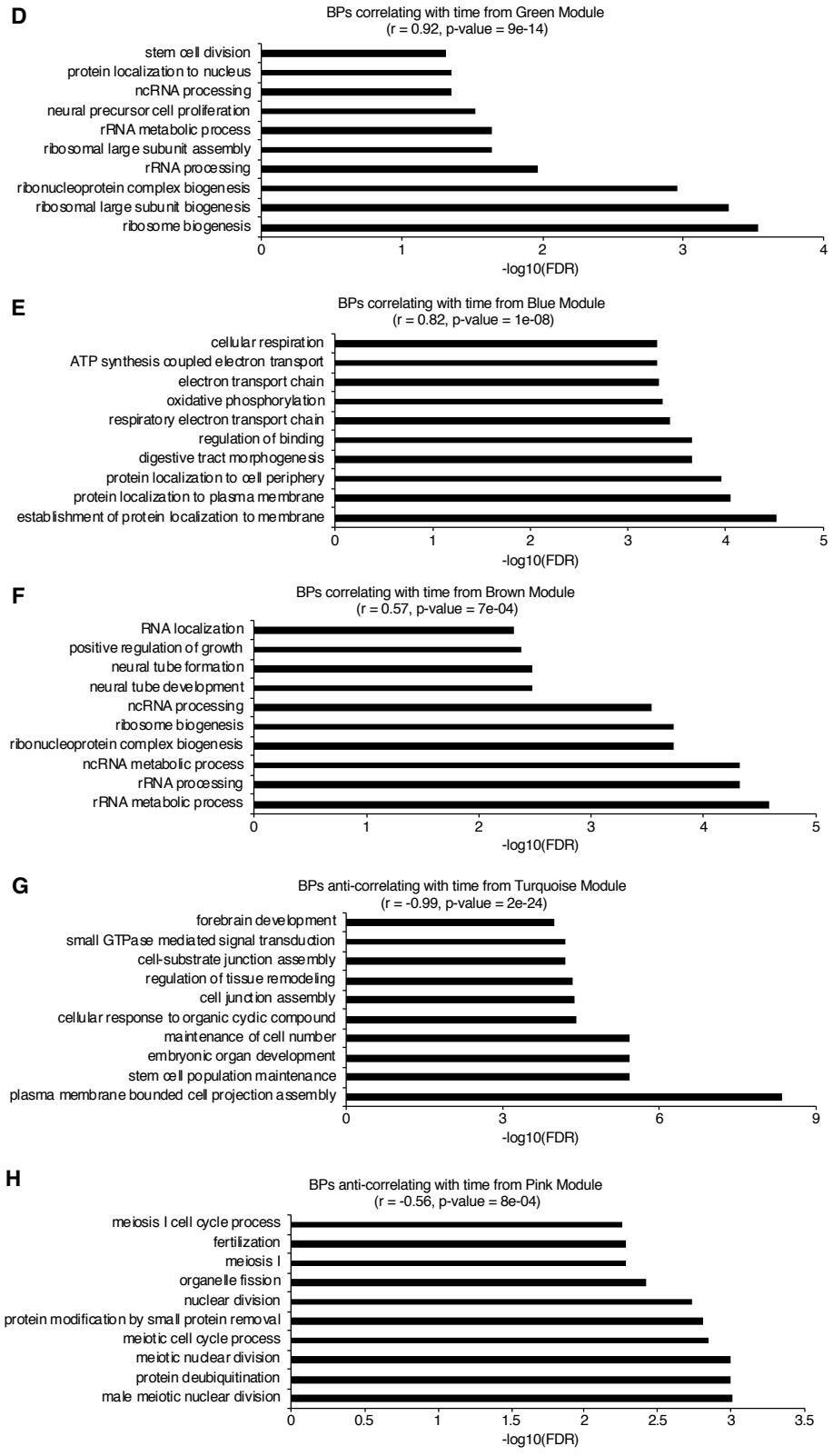


1189 **Supplementary Figure 2. Replica of *in vitro* ESC-EpiSC differentiation**
1190 **experiments upon chemical β-catenin perturbations**
1191 **A-D** Percentage of GFP+ cells calculated over the total amount of leaving cells in
1192 DMSO **(B)**, Ch1μM **(C)**, Ch3μM **(D)** and 2i/L **(E)** pre-cultured dual reporter ESCs.
1193 Histograms from Day 0 and Day 4 of each condition are shown as inset. Data are
1194 means±SEM (n=2, A (Day1 C1, Day3 AFC1, Day4 AFD-AFC3), B (Day 1 AFC1), D
1195 (Day4 AFD); n=3, A (Day1 AFD-AFC1-AFC3-C1X2-C3-C3X2, Day2, Day3 AFD-

AFC3-C1-C1X2-C3-C3X2, Day4 AFC1-C1-C1X2-C3-C3X2), B (Day1 AFD-C1-C1X2, Day2, Day3, Day4), C (Day1, Day2, Day3, Day4), D (Day0, Day1, Day2, Day3, Day4); n=6, A (Day0), B (Day0), C (Day0). p-values from one-way ANOVA are shown across samples for each day. Dots represent individual data with similar values overlapping. Colour-blind safe combinations were selected using colorbrewer2 (https://colorbrewer2.org/#type=sequential&scheme=BuGn&n=3).

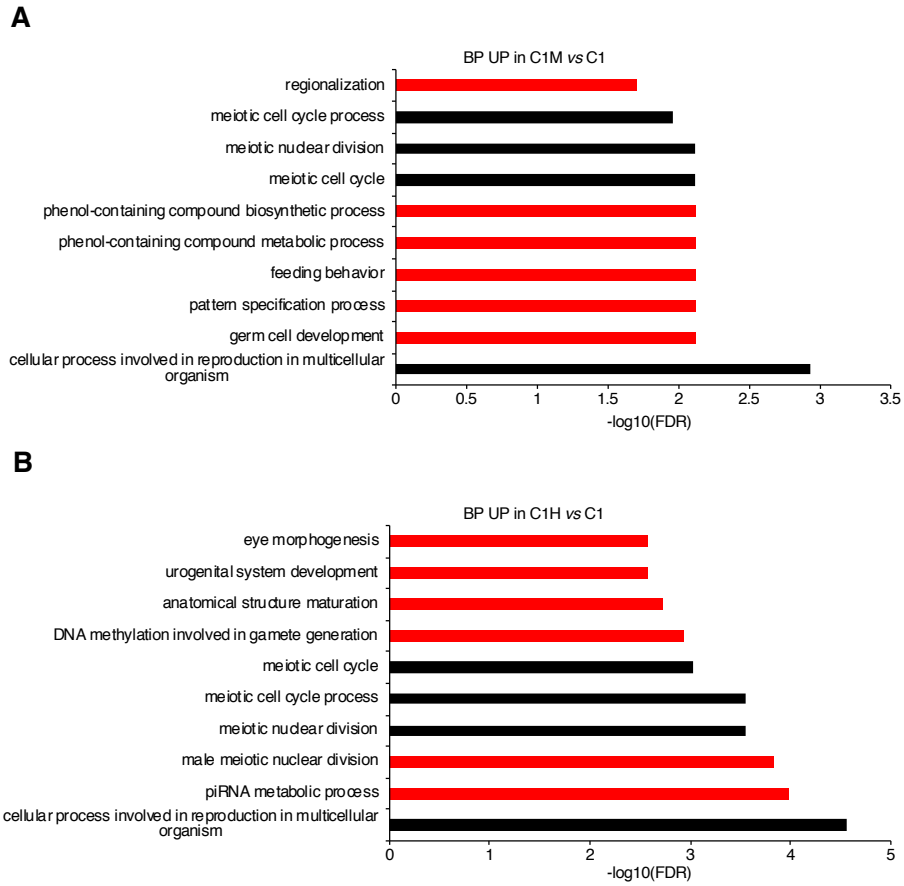

A
C1 ESCs in 2i/L
TMP10μM
TMP10μM/Doxy100ng/mL
SSC-A
FSC-H
FSC-A
V 450_50-A :: DAPI
B 525_50-A :: GFP
YG 610_20-A :: mCherry
Cells
30.8
59.8
Singlets
94.6
97.7
LivingCells
97.5
97.3
C1M
0.38
22.8
C1H
0
32.7
B
Height
Modules
C
Module-Time relationship
MEblack
MEblue
MEbrown
MEgreen
MEgreenyellow
MEmagenta
MEpink
MEpurple
MEred
MEsalmon
MEtan
MEturquoise
MEyellow
0.67
(3e−05)
0.82
(1e−08)
0.57
(7e−04)
0.92
(9e−14)
−0.26
(0.1)
0.35
(0.05)
−0.56
(8e−04)
0.27
(0.1)
0.3
(0.1)
−0.21
(0.3)
0.14
(0.4)
−0.99
(2e−24)
−0.75
(1e−06)
1
0.5
0
−0.5
−1



Supplementary Figure 3. FACS gating strategy and WGCNA of the genes correlating with time.

A Gating strategy used to sort C1M and C1H ESCs, following 48 hrs treatment with TMP10μM and Doxy100ng/mL. C1 ESCs treated with TMP10μM were used as negative control. **B** Clustering dendrogram of genes, with dissimilarity based on

topological overlap, together with the assigned module colours**;** grey genes are unassigned to any module**. C** Eigenmodules correlating with time; the Pearson correlation coefficient (*r*) and relative p-value are shown. **D-H** Bar-chart of the top-ten enriched biological processes (BP) with FDR < 0.05 from genes belonging to the Green (**D)**, Blue **(E**), Brown **(F)**, turquoise **(G)** and Pink **(H)** WGCNA modules.



Supplementary Figure 4. Gene ontology of the differential expressed genes in pluripotent ESCs.

A, B Bar-chart of the top-ten enriched biological processes (BP) with FDR < 0.05 from differentially expressed genes in in C1M **(A)** and C1H **(B)** compared to C1 ESCs. Black and grey bars represent upregulated and downregulated BPs, respectively. In red bars, the BPs exclusively enriched in the indicated condition.

Supplementary Table 1

List of identified module genes correlating with time (Supplementary Figure 3) or β-catenin doses (Figure 3) before and after the filtering for the |kME| ≥ 0.8; GO and pathway analysis.

Supplementary Table 2

List of differentially expressed genes before and after the filtering for the FDR (< 0.05) and log fold change (-2 > log > 2); GO and pathway analysis in pluripotent C1M vs C1 ESCs.

Supplementary Table 3

List of differentially expressed genes before and after the filtering for the FDR (< 0.05) and log fold change (-2 > log > 2); GO and pathway analysis in pluripotent C1H vs C1 ESCs.

Supplementary Table 4

List of differentially expressed genes before and after the filtering for the FDR (< 0.05) and log fold change (-2 > log > 2); GO and pathway analysis in differentiated C1DMV vs C1D ESCs.

Supplementary Table 5

List of differentially expressed genes before and after the filtering for the FDR (< 0.05) and log fold change (-2 > log > 2); GO and pathway analysis in differentiated C1DMDT vs C1D ESCs.

Supplementary Table 6

List of differentially expressed genes before and after the filtering for the FDR (< 0.05) and log fold change (-2 > log > 2); GO and pathway analysis in differentiated C1HV vs C1D ESCs.

Supplementary Table 7

List of differentially expressed genes before and after the filtering for the FDR (< 0.05) and log fold change (-2 > log > 2); GO and pathway analysis in differentiated C1DHDT vs C1D ESCs.

Supplementary Movie 1

71 hrs time-lapse of dual reporter ESCs without pre-activation of the canonical Wnt pathway, differentiated in NDiff227 supplemented with ActivinA+Fgf2+DMSO.

Supplementary Movie 2

71 hrs time-lapse of dual reporter ESCs without pre-activation of the canonical Wnt pathway, differentiated in NDiff227 supplemented with Chiron 1μM.

Supplementary Movie 3

71 hrs time-lapse of dual reporter ESCs without pre-activation of the canonical Wnt pathway, differentiated in NDiff227 supplemented with Chiron 3μM.