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A membrane-associated β-catenin/Oct4 complex correlates with ground-state pluripotency in mouse embryonic stem cells

Fernando Faunes[1,*], Penelope Hayward[1,*], Silvia Muñoz Descalzo[1], Sujash S. Chatterjee[2], Tina Balayo[1], Jamie Trott[1], Andrew Christoforou[1,3], Anna Ferrer-Vaquer[4], Anna-Katerina Hadjantonakis[4], Ramanuj Dasgupta[2] and Alfonso Martinez Arias[1,‡]

SUMMARY

The maintenance of pluripotency in mouse embryonic stem cells (mESCs) relies on the activity of a transcriptional network that is fuelled by the activity of three transcription factors (Nanog, Oct4 and Sox2) and balanced by the repressive activity of Tcf3. Extracellular signals modulate the activity of the network and regulate the differentiation capacity of the cells. Wnt/β-catenin signaling has emerged as a significant potentiator of pluripotency: increases in the levels of β-catenin regulate the activity of Oct4 and Nanog, and enhance pluripotency. A recent report shows that β-catenin achieves some of these effects by modulating the activity of Tcf3, and that this effect does not require its transcriptional activation domain. Here, we show that during self-renewal there is negligible transcriptional activity of β-catenin and that this is due to its tight association with membranes, where we find it in a complex with Oct4 and E-cadherin. Differentiation triggers a burst of Wnt/β-catenin transcriptional activity that coincides with the disassembly of the complex. Our results establish that β-catenin, but not its transcriptional activity, is central to pluripotency acting through a β-catenin/Oct4 complex.

KEY WORDS: Oct4, Wnt signaling, Mouse embryonic stem cells, Pluripotency, β-Catenin

INTRODUCTION

Embryonic stem cells (ESCs) are clonal populations derived from single cells within the mammalian blastocyst that can be propagated in culture with the ability to give rise to derivatives of all germ layers of the developing embryo, i.e. they are pluripotent (Smith, 2001). An ESC culture represents a dynamic equilibrium between cells that self-renew and others that differentiate under the control of a small transcription factor network (TFN) with three core components: Nanog, Oct4 and Sox2 (for reviews, see Silva and Smith, 2008; Young, 2011), which are required to maintain pluripotency and, together with Klf4, are sufficient to reprogram differentiated cells into ESCs (Takahashi and Yamanaka, 2006; Okita et al., 2007). Nanog is central to the establishment of the pluripotent state and, in concert with Oct4, plays a key role in its maintenance (Chambers et al., 2007; Silva et al., 2008; Silva et al., 2009). Although the cell-autonomous activity of this TFN determines pluripotency, the balance between self-renewal and differentiation is influenced by extracellular signals (Ying et al., 2003). A combination of chemical inhibitors for MEK and GSK3 (2i conditions) allows robust propagation of pluripotency and aids reprogramming under minimal growth conditions (Silva et al., 2008; Ying et al., 2008).

Inhibition of MEK blocks FGF signaling and has been shown to promote differentiation and lineage choice of ESCs (Kunath et al., 2007; Stavridis et al., 2007); whereas, GSK3 targets multiple cellular networks, one of which, Wnt, is a major regulator of many cell fate decisions during development (Doble and Woodgett, 2003; Forde and Dale, 2007; Wu and Pan, 2010). Wnt proteins modulate the activity of a cytosolic pool of β-catenin (Angers and Moon, 2009). In the absence of Wnt, GSK3 mediates phosphorylation of specific residues at the N terminus of β-catenin and primes its proteasome-mediated degradation (Logan and Nusse, 2004; Clevers, 2006; MacDonald et al., 2009). Genetic or chemical inhibition of GSK3 results in an increase in the cytosolic levels of β-catenin, which can enter the nucleus and, through an interaction with members of the Tcf family of transcription factors, can promote gene expression. However, the levels of β-catenin might not be diagnostic of its transcriptional activity (Fagotto et al., 1997; Guger and Gumbiner, 2000; Lawrence et al., 2000; Staal et al., 2002; Tolwinski et al., 2003; Fodde and Brabletz, 2007; Hendriksen et al., 2008) and this has led to the suggestion that there is a transcriptionally competent form of β-catenin that represents a small fraction of the total pool (Staal et al., 2002; Maher et al., 2010; Muñoz Descalzo et al., 2011).

Wnt/β-catenin signaling has been shown to contribute to the maintenance of pluripotency in mESCs (Kielman et al., 2002; Ying et al., 2002; Sato et al., 2004; Ogawa et al., 2006; Singla et al., 2006; Takao et al., 2007; Ying et al., 2008; Wagner et al., 2010). Increases in β-catenin or addition of Wnt3A to the culture medium promotes pluripotency and aids the activity of Nanog and Oct4. Most significantly, the effects of GSK3 inhibitors in 2i conditions have

[1]Department of Genetics, University of Cambridge, Cambridge CB2 3EH, UK. [2]New York University School of Medicine, Department of Pharmacology and the NYU Cancer Institute, 522 First Avenue, SRB #1211, New York, NY 10016, USA. [3]Department of Biochemistry, University of Cambridge, Cambridge CB2 1GA, UK. [4]Developmental Biology Program, Sloan-Kettering Institute, New York, NY 10065, USA.

*These authors contributed equally to this work
‡Author for correspondence (ama11@hermes.cam.ac.uk)

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recently been shown to be mediated principally through β-catenin (Wray et al., 2011). A connection between Wnt signaling and pluripotency is fuelled further by the observation that Tcf3, a member of the family of nuclear effectors of Wnt/β-catenin signaling, is bound to the same regulatory regions as Oct4 and Nanog, and its loss of function results in high activity of the pluripotency network and its effectors, as well as in robust pluripotency (Cole et al., 2008; Tam et al., 2008; Yi et al., 2008; Guo et al., 2011). This has led to the suggestion that Tcf3 balances the effects of Oct4 and Nanog in the maintenance of the pluripotent state (Yi et al., 2008). In this model, the function of β-catenin appears to be to antagonize the repressive activity of Tcf3 on key regulators of pluripotency in a manner that does not require its transcriptional activity (Wray et al., 2011; Yi et al., 2011).

Although these results show that GSK3 inhibitors aid pluripotency through β-catenin, they do not provide information about the state of β-catenin in mESCs. Furthermore, Wnt/β-catenin signaling is also required for differentiation (Gadue et al., 2006; Lindsley et al., 2006; Hansson et al., 2009; Jackson et al., 2010) and a report has suggested that β-catenin transcriptional activity plays a role in preventing the exit from the pluripotent state (ten Berge et al., 2011). Altogether, these observations raise questions about the role of β-catenin in pluripotency and differentiation. Here, we address these issues and show that under self-renewal conditions the transcriptional activity of β-catenin is negligible due to the localization of the transcriptionally competent forms of β-catenin to the membrane, where some of them form a complex with E-cadherin and Oct4. During differentiation, we detect transcriptionally competent β-catenin in the cytosol and can measure its transcriptional activity, which is coincident with a decrease in the levels of Oct4 and E-cadherin. These results lead us to suggest that β-catenin promotes pluripotency by creating a balance between Tcf3 and the pluripotency factors, in particular Oct4.

MATERIALS AND METHODS

Cell culture and differentiation

For characterizaton of cell lines used and culture details see E14Tg2A, TNGA (Chambers et al., 2007), NG4 (Schaniel et al., 2010), TK215 (see supplementary material Fig. S3A for characterization), *Nanog*$^{-/-}$ (Chambers et al., 2007), ZHBTc4 (Niwa et al., 2002), AG23191 (Nishiyama et al., 2009), *β-catenin*$^{fl/-}$ and *β-catenin*$^{-/-}$ (Wray et al., 2011). In differentiation assays, 4×10^4 cells were plated in six-well plate in serum+LIF. After 6-12 hours, media were replaced with serum+LIF or serum+1 μM retinoic acid. Cells were fed daily and assessed after 4 days.

Colony forming assays

Six-hundred cells in serum+LIF were added to six-well plates. After 8-12 hours, media were replaced as indicated. Colonies were analyzed for alkaline phosphatase (Sigma) activity after 5 days.

Flow cytometry

GFP and RFP expression was assessed using a Fortessa Flow cytometer. Analysis of data from single live (DAPI-negative) cells was conducted using Flowjo software.

Transcriptional reporter assays

Cells were transfected with either TOP or FOP reporter + CMV-Renilla. After 24 hours, cells were exposed to media as indicated for a further 24 hours. Luciferase levels were assessed using Dual reagent (Promega).

qRT-PCR

qPCRs were performed using Quantifast SYBR Green PCR Master Mix (Qiagen) Primer sequences can be found in supplementary material Table S1.

siRNA knock down

siRNA knock down was carried out as described by Schaniel et al. (Schaniel et al., 2010).

Biochemistry

Cell lysates were fractionated using concanavalin A-sepharose (ConA, Sigma) at a ratio of 2:1 and incubated at 4°C by continuous rotating for 1 hour. Soluble proteins were denatured in 5× Laemelli's buffer. Membrane-associated proteins were isolated by washing the ConA pellet in PBS and denaturing in an equal volume of 2× Laemelli's buffer.

Immunoprecipitations

Immunoprecipitations were carried out on either whole-cell lysates or ConA-bound proteins. Anti-Oct4 and protein A/G magnetic beads were incubated with the input for 1 hour at room temperature; the magnetic beads were isolated against gravity (to prevent contamination with unbound ConA-sepharose) and washed in this manner three times in PBS.

Primary antibodies used

Anti-total-β-catenin (Sigma C2206 and Abcam Ab19450), anti-active-β-catenin ABC (Millipore 8E7), anti-PSer45-β-catenin (Cell Signaling 07/2010), anti-Nanog (eBioscience eBioMLC-51); anti-Oct4 (Santa Cruz sc5279), anti-Tcf3 (Santa Cruz sc8635), anti-SSEA1 (Santa Cruz 21702) or anti-β-Tubulin (DSHB E7) were used. All antibodies except anti-Oct4, were tested on mESC mutants for the relevant protein to verify specificity.

Immunofluorescence and image analysis

Immunofluorescence and image analysis were carried out as described previously (Muñoz Descalzo et al., 2012).

Western blots

Primary antibodies were used according to the manufacturer's instructions using the Odyssey Imaging System (LI-COR Biosciences).

RESULTS

The effects of Wnt/β-catenin signaling on pluripotency

Activation of Wnt signaling, either by chemical inhibition of GSK3α, β (e.g. with chiron) or by Wnt3A, contributes to pluripotency. This effect is reflected in the expression of *Nanog*::GFP, a reporter for pluripotency (Okita et al., 2007; Kurimoto et al., 2008; Silva et al., 2009) that can be used as a readout of the activity of the pluripotency TFN (Chambers et al., 2007; Kalmar et al., 2009). In minimal neural differentiation medium N2B27, cells exhibit a differentiation profile within 48 hours, but in the presence of chiron or Wnt3A, they maintain a pluripotent profile (Fig. 1A; supplementary material Fig. S1A). Wnt3A and chiron might not act on the transcription of Nanog and Oct4 directly, as the first changes that we observe when cells are placed in N2B27 are at the protein level (Fig. 1B); transcript levels change only after 1 or 2 days (Fig. 1C). Suppression of Wnt secretion in self-renewing conditions with IWP2, an inhibitor of the Wnt chaperone porcupine (Huang et al., 2009), has little effect on the Nanog::GFP profile under self-renewing conditions over a few days (supplementary material Fig. S1B), suggesting a minor role for endogenous Wnt secretion in these conditions.

As there is a report that the transcriptional activity of β-catenin is required for pluripotency (ten Berge et al., 2011), we analyzed the effects of knocking down components of Wnt signaling on the activity of a Nanog::GFP reporter (Fig. 1D) . This study confirmed and extended the observation that β-catenin is required for pluripotency under minimal culture conditions, i.e. loss of β-catenin function aids differentiation, whereas loss of function of APC or axin, which increase the total levels of β-catenin, promote pluripotency (Fig. 1D). However, in general, reductions in the levels of factors that mediate transcriptional activity of β-catenin (e.g. pygopous, Tcf1 and Lef1) had little or no effect on the expression

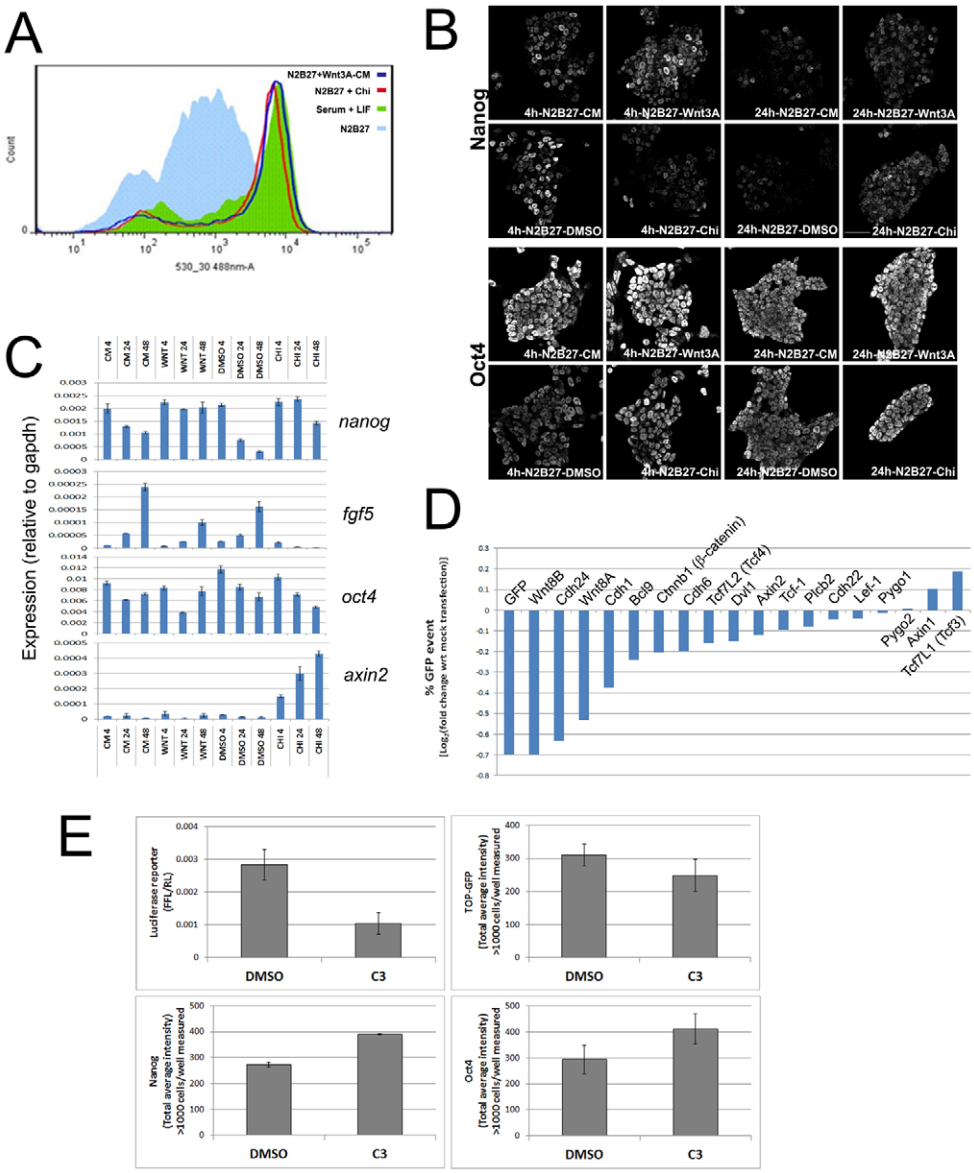

Fig. 1. Wnt signaling aids pluripotency. (**A**) Flow cytometry analysis of TNGA (Nanog:GFP) cells after 2 days under the indicated conditions. (**B**) Representative confocal images of Nanog and Oct4 protein expression in E14Tg2A cells grown in serum+LIF and exposed to the indicated culture conditions for 4 and 24 hours prior to fixation. Scale bar: 50 μm. (**C**) Real-time qRT-PCR analysis of selected genes in E14Tg2A cells grown in serum+LIF and exposed to 3 μM chiron or Wnt3A-conditioned medium (in N2B27) for 4, 24 or 48 hours, and their controls [DMSO and L-cell-conditioned medium (CM)]. The data shown are normalized to the expression of *Gapdh*. (**D**) Effect of knockdown of selected Wnt pathway components on Nanog-GFP expression. (**E**) Effects of ICRT3 (C3) on TOPFLASH reporter activity (top, left), GFP expression (top, right), and Nanog and Oct4 protein levels (bottom left and right, respectively) in TLG Wnt reporter mESCs grown in serum+LIF.

of Nanog::GFP (Fig. 1D), and, as expected from previous studies, lowering the levels of Tcf3 results in a delayed exit from pluripotency (Pereira et al., 2006; Cole et al., 2008; Tam et al., 2008; Yi et al., 2008). These results suggest that β-catenin, but not its transcriptional activity, is required for the maintenance of pluripotency. Additional support for this conclusion is derived from the observation that iCRT3, an inhibitor of the interaction between β-catenin and Tcfs that interferes with the transcriptional activity of the complex (Gonsalves et al., 2011), has no effect on the expression of Oct4 and Nanog (Fig. 1E; supplementary material Fig. S1C).

The requirement of β-catenin for the maintenance of pluripotency might not be absolute, as some studies have shown that it is possible to maintain β-catenin mutant ESCs in culture (Lyashenko et al., 2011; Wray et al., 2011). However, closer scrutiny of their behavior reveals that pluripotency in these cells is not robust: β-catenin mutant cells express Nanog and Oct4 (Fig. 2A), but the levels, particularly their protein levels, are low (Fig. 2B; supplementary material Fig. S2A) and the cells exhibit a strong tendency to differentiate, even in 2i+LIF, which promotes ground state pluripotency (Ying et al., 2008) (Fig. 2C). This tendency is accentuated when the cells are placed in serum+LIF (Fig. 2C; supplementary material Fig. S2B). The fragility of pluripotency in these cells is exposed in the live recording of their patterns of growth in 2i+LIF. E14 cells rapidly form tight aggregates that are characteristic of growth in this medium, but β-catenin mutant cells are unable to form these aggregates and grow in a manner reminiscent of differentiating cells (supplementary material Fig. S2C, Movies 1-3). A similar phenotype is observed when E14 cells are treated with XAV939, an agonist of the function of axin that results in reduced levels of β-catenin (Fig. 2D).

These observations lead us to conclude that β-catenin, but not its transcriptional activity, is required for the maintenance of robust pluripotency.

mESCs exhibit low transcriptional activity of β-catenin under self-renewing conditions

The observation that the transcriptional activity and partners of β-catenin are dispensable for pluripotency (Fig. 1D,E) is consistent with observations that the transcriptional activation domain of β-catenin is not required for pluripotency (Lyashenko et al., 2011; Wray et al., 2011). This led us to analyze the state of Wnt/β-catenin signaling in ESCs.

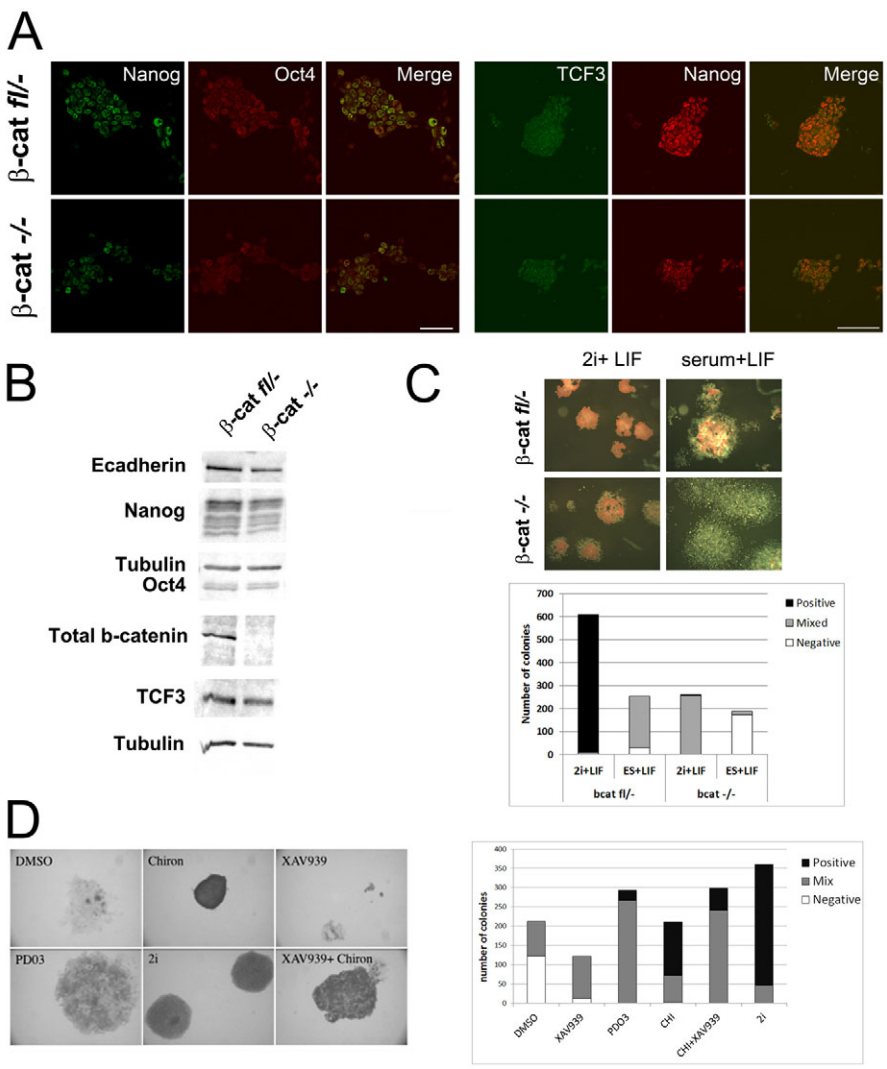

Fig. 2. β-Catenin is required for pluripotency. (**A**) Confocal images of β-catenin$^{fl/-}$ and β-catenin$^{-/-}$ cells grown in 2i+LIF stained for Nanog and Oct4 (left), and Tcf3 and Nanog (right). Scale bars: 50 μm (left); and 100 μm (right). (**B**) Western blot of β-catenin$^{fl/-}$ and β-catenin$^{-/-}$ cells grown in 2i+LIF. Quantification is in supplementary material Fig. S3A. (**C**) Colony-forming assays for β-catenin$^{fl/-}$ and β-catenin$^{-/-}$ cells under the indicated conditions. Colonies were assayed for alkaline phosphatase (a marker for pluripotency). Quantification of this assay is shown in the graph. (**D**) TNGA cells grown in serum+LIF and were FACS sorted to isolate cells with low levels of GFP (Low Nanog, LN); colony assays were undertaken in the indicated conditions (basal media was N2B27).

In a first set of experiments, we used Luciferase-based β-catenin/Tcf (TOP-FLASH) reporters, which have been shown to be activated by β-catenin and repressed by Tcf proteins (Merrill et al., 2001) (Fig. 3A). In ESCs, these reporters display the same low levels of activity without stimulation of Wnt signaling as they do in HEK293T cells. This was confirmed using a fluorescent reporter, TK215, which is inactive under self-renewal conditions (Fig. 3B). Stimulation of Wnt signaling, through Wnt3A or chiron, induces activity of both reporters in a concentration-dependent manner (Fig. 3A,B; supplementary material Fig. S3A). Under self-renewal conditions, it is not easy to activate the reporter; even with 6 μM chiron it is not possible to obtain levels of activation observed by simply transferring the cells into differentiation medium.

Recently, a sensitive β-catenin/Tcf reporter (TL) has been described that recapitulates Wnt signaling in the embryo (Ferrer-Vaquer et al., 2010). We established ES cell lines bearing Green (TLG) or mCherry (TLC) variants of this reporter and analyzed their behavior under self-renewing conditions. All lines exhibited heterogeneous low expression in culture; these basal levels of fluorescence can be increased by either Wnt or chiron (Fig. 3D; supplementary material Fig. S3B) and decreased by inhibitors of Wnt activity (supplementary material Fig S3B). We chose two of these variants, TLC2 and TLG2, for further study. Treatment of the TL reporter lines with iCRT3 under self-renewing conditions reduces, but does not abolish, their activity (Fig. 3E), raising the possibility that the TL reporters reflect loss of Tcf3, the main Tcf family member that is active in ESCs and has been shown to play a central role in the regulation of pluripotency by acting as a repressor (Pereira et al., 2006; Cole et al., 2008; Tam et al., 2008; Yi et al., 2008; Yi et al., 2011). Consistent with this, knock down of Tcf3 increases the expression of the reporter (not shown).

When mESCs are placed in neural differentiation conditions (with N2B27 or in serum with retinoic acid) (supplementary material Fig. S4A), they activate all β-catenin/Tcf reporters without external stimulation of Wnt activity, and become sensitized to Wnt3A and chiron (Fig. 3A,C). This activity is inhibited by iCRT3 (Fig. 3E), indicating that it is due to the transcriptional activity of β-catenin, which therefore represents an early event during the differentiation of mESCs and marks the exit from pluripotency. This activity is likely to be triggered by the increased expression of the ligands of the pathway during differentiation (Aiba et al., 2006; Nordin et al., 2008).

Transcriptionally competent β-catenin is limiting in self-renewing mESCs

To test the causes behind the low transcriptional activity of β-catenin in mESCs, we monitored the abundance and subcellular location of β-catenin and of some phospho-specific forms during self-renewal

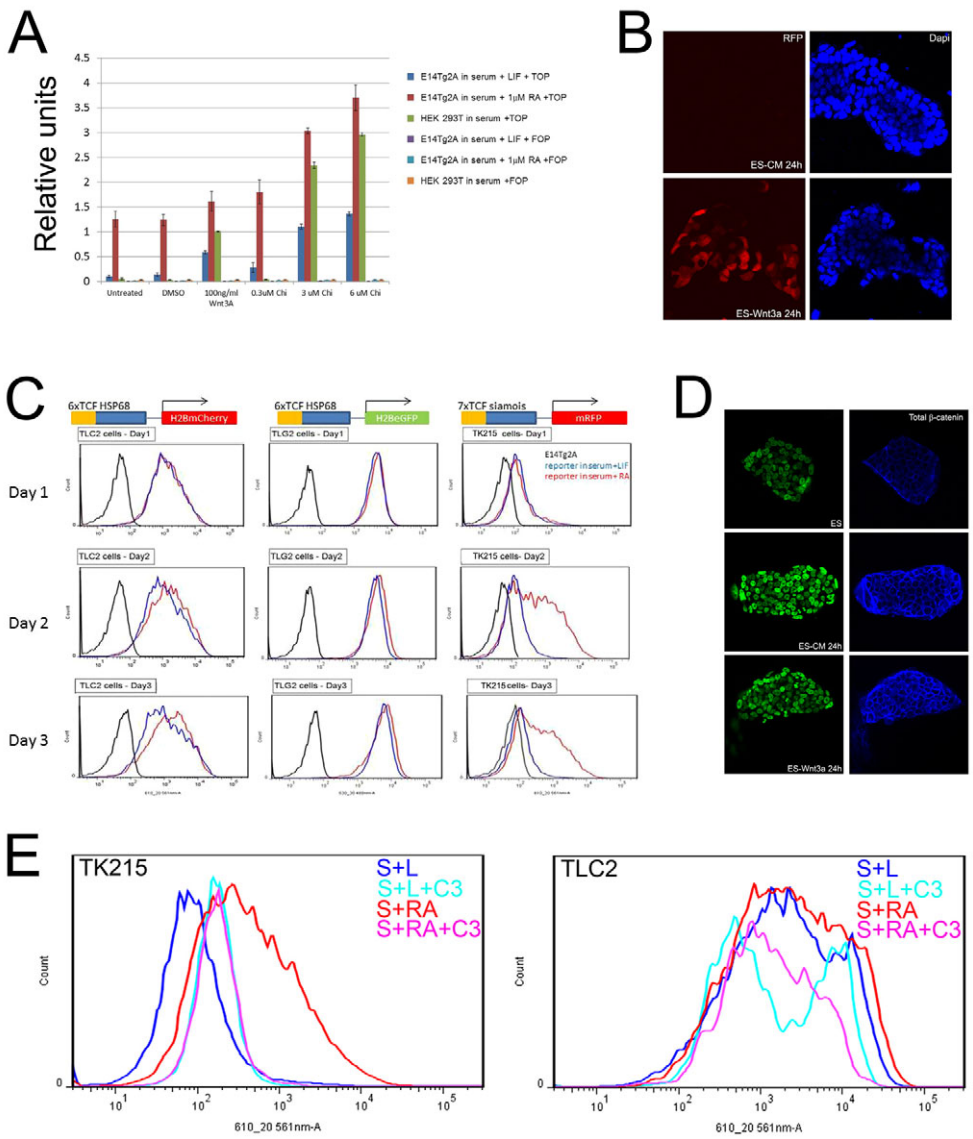

Fig. 3. Wnt reporter activity during self-renewal and differentiation of mESCs. (**A**) TopFlash assay assessing Wnt/β-catenin transcriptional activity in mESCs (E14Tg2A) under self-renewing (serum+LIF, blue bars) and differentiating conditions (SRA, red bars), or HEK293T cells (green bars). Results are representative of two experiments and the average of three replicates (**B**,**D**) Confocal images of Wnt reporter lines grown in serum+LIF and exposed to indicated conditions 24 hours prior to fixation. TK215 (RFP fluorescent, B) and TLG2 (eGFP fluorescence, D) were stained with DAPI to show nuclei (B) or for β-catenin to visualize cell outlines (blue channels, D). (**C**) Flow cytometry profiles of Wnt reporter lines TK215, TLC2 and TLG2 grown in serum+LIF (blue population) or SRA (red population) for the indicated number of days. (**E**) Characterization of the Wnt transcriptional inhibitor ICRT3 in TK215 and TLC2 cells. The response of the reporter to the indicated conditions was analyzed by flow cytometry after 72 hours. The increase in fluorescence of cells grown in serum+LIF+C3 is due to an increase in autofluorescence (see supplementary material Fig. S3C).

and differentiation. We used three different antibodies in these studies: one (total) recognizes most forms of β-catenin; a second one (PS45-β-catenin) recognizes, principally, β-catenin phosphorylated at Ser45, which primes for GSK3 phosphorylation (Aberle et al., 1997); and a third one (ABC) that detects the activated form of β-catenin, which is unphosphorylated in the N-terminal region (Staal et al., 2002) (see Materials and methods). The PS45 and ABC isoforms are estimated to represent about 1-5% of the total β-catenin (Lee et al., 2003; Maher et al., 2010).

Crude fractionation with concanavalin A (ConA) of the extracts reveals that, under self-renewal conditions, the cytosolic fraction (soluble) contains little β-catenin and barely detectable amounts of the PS45 and ABC isoforms (Fig. 4A). However, the membrane-associated ConA-bound fraction (pellet) contains all forms of β-catenin, including ABC, and this is supported by the localization of β-catenin in immunostaining experiments (Fig. 4B). An association of ABC and PS45 with membranes has been described before in other cell lines (Maher et al., 2009; Maher et al., 2010) and might reflect the existence of a specific hub for their regulation. In these experiments, we find some Nanog and Oct4, but not Tcf3, in membrane-containing fractions (Fig. 4A). This localization of Oct4 is corroborated with a different and more-stringent membrane fractionation protocol (supplementary material Fig. S5). Activation of Wnt signaling leads to an increase in total β-catenin and ABC in the soluble fraction within 4 hours of the stimulation, an effect that is more prominent with chiron (Fig. 4C). During RA-induced differentiation, we observe a similar increase in cytosolic total β-catenin and ABC (Fig. 4A,C; supplementary material Fig. S4C). We also notice that the increases of β-catenin in the soluble pool induced by Wnt3A or chiron in differentiating cells are larger than those induced during self-renewal (Fig. 4C), reflecting the increased sensitivity of the reporters in differentiating conditions (see Fig. 3A).

These results suggest that the reason for the low transcriptional activity of β-catenin in self-renewing mESCs is the limiting amounts of transcriptionally competent β-catenin in the cytosol. These levels change during differentiation, coincident with the onset of transcriptional activity. Immunostaining of differentiating cells under self-renewing conditions support this conclusion: differentiating cells with low levels of Nanog have high levels of ABC that can now be seen in the nucleus, coincident with an elevation in the activity of the TLG reporter (Fig. 4D).

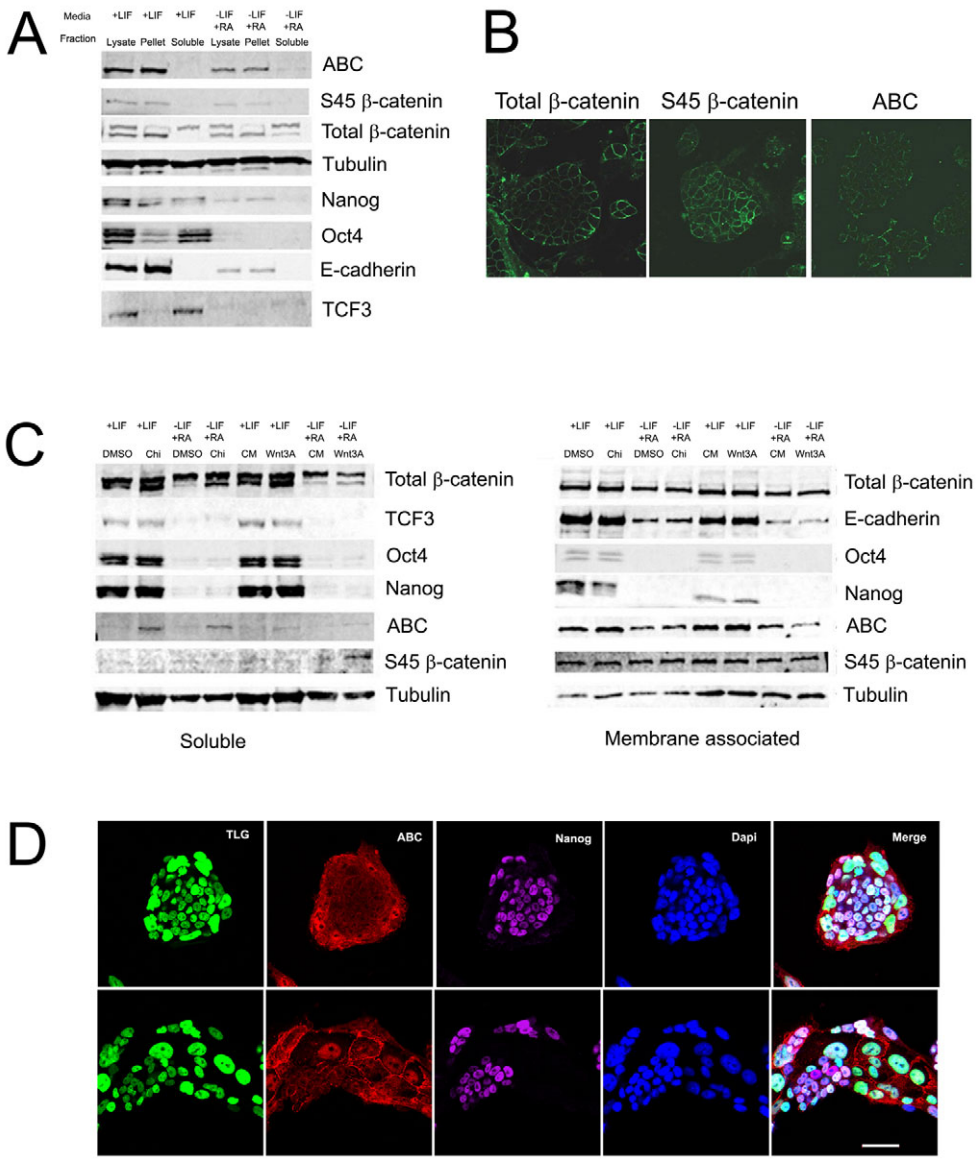

Fig. 4. The state of β-catenin during self-renewal and differentiation in mESCs. (**A**) E14Tg2A cells were grown in self-renewing (serum+LIF) or differentiating conditions (SRA) for 4 days. Cell lysates were fractionated with concanavalin A to separate membrane-associated proteins (pellet) from the soluble proteins (soluble). Expression of proteins was assessed by western blot and a quantitative fluorescent system. Analysis of total β-catenin in western blots from whole extracts of mESCs under self-renewal conditions shows two pools: one specific – β-catenin; and a second, slower running, one, which has an unspecified component (Fig. 4A; supplementary material Fig. S4B). Quantification can be found in supplementary material Fig. S4C. (**B**) E14Tg2A cells grown in serum+LIF were stained for the different isoforms of β-catenin with specific antibodies (see text for details). (**C**) Mouse ESCs grown and processed as described in A, with the additional treatment consisting of exposure to the indicated conditions 4 hours prior to lysis. Quantifications of western blots are in supplementary material Fig. S6. (**D**) Confocal images of TLG cells grown in serum+LIF. Twenty-four hours prior to fixation, cells were exposed to L cell-conditioned medium to allow differentiation. Cells were stained for activated β-catenin (ABC) and Nanog. Cells that lack Nanog expression (arrows) elevate expression of the reporter and of ABC, which now can be observed in the nucleus. Scale bar: 50 μm.

β-Catenin acts through a protein network during self-renewal

It has been suggested that the effect of β-catenin on pluripotency is mediated through an antagonism of Tcf3 (Wray et al., 2011). Therefore, genes whose expression is increased in *Tcf3* mutants should respond to β-catenin; this appears to be true for some, although the effect of Wnt activation is lower than that triggered by loss of Tcf3 (Yi et al., 2011). Many of the genes that are regulated by both Tcf3 and β-catenin require the activity of the pluripotency network (Yi et al., 2008; Yi et al., 2011), which raises the possibility that the effects of β-catenin on their expression are indirect.

To explore this, we compared the levels of Oct4 and Nanog protein 4 hours after stimulation of Wnt signaling by Wnt3A under self-renewing conditions. Measuring the protein levels using quantitative immunofluorescence (QIF), we observed changes in the mean levels and profiles of Oct4 and Nanog (Fig. 5A-E). This supports our contention that the immediate effects of β-catenin on pluripotency are not likely to be effected through transcriptional modulation of the pluripotency network (Fig. 1). Use of QIF reveals correlations between the expression of different proteins in single cells and avoids the population averaging associated with western blotting. This analysis reveals a correlation between Nanog and Oct4 at the level of single cells that is increased by Wnt/β-catenin signaling (Fig. 5E; supplementary material Fig. S7).

The activity of the pluripotency network is antagonized by Tcf3, which can interact with β-catenin (Lee et al., 2001). Although we did not observe any clear correlation between Tcf3 and total β-catenin (data not shown), the levels of Tcf3 are regulated by Wnt signaling: over 4 hours, Wnt signaling reduces the levels of Tcf3 (Fig. 5F; Fig. 4C), in agreement with the suggestion that β-catenin can titrate out Tcf3 (Wray et al., 2011). We also observed that the levels of Nanog, and particularly of Oct4, correlated with the expression of the TL reporters; to a lesser extent TL and total β-catenin also correlate (Fig. 5G). As this reporter is likely to reflect the degree of Tcf3-mediated repression, these observations provide further support for an inverse correlation between the expression of Nanog and Oct4, and the activity of Tcf3. They also suggest that correlations in the levels of the different elements of the pluripotency network are an important variable of pluripotency, and, furthermore, that one of the functions of β-catenin is to regulate these correlations.

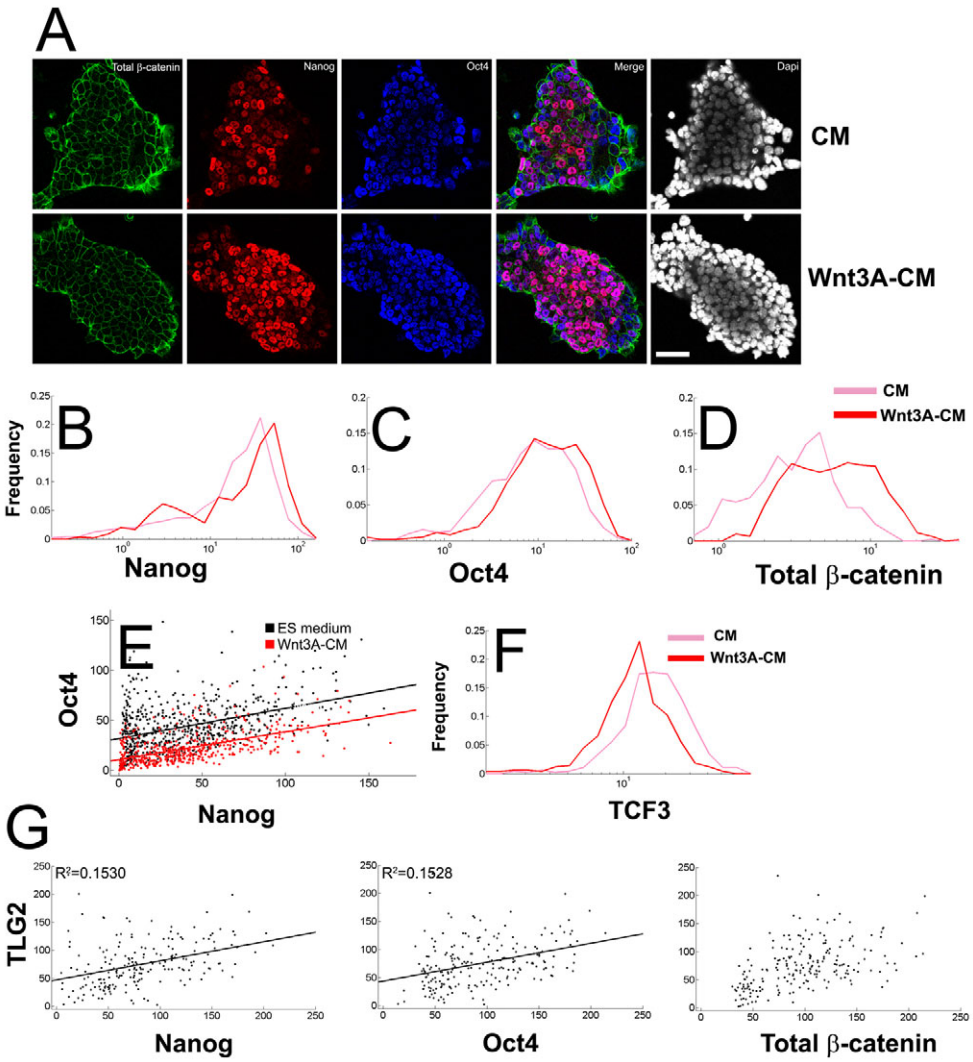

Fig. 5. Effects of Wnt/β-catenin signaling on Nanog and Oct4. (**A**) Representative confocal images of E14Tg2A cells treated for 4 hours with Wnt3A-CM or CM (in N2B27) and stained for total β-catenin (green), Nanog (red) and Oct4 (blue) used for quantitative immunofluorescence (QIF). DAPI was used to identify the nuclei (white). Scale bar: 50 μm. (**B-D**) Line plot distributions of Nanog (B), Oct4 (C) and total β-catenin (D) obtained by QIF analysis from single sections confocal images as described in A. Fluorescence levels (grayscale) were quantified for each individual cell from four colonies (see Material and methods), binned in 20 classes spaced logarithmically (*x*-axis); the frequency of each bin is shown on the *y*-axis. Wnt3A treatment of the cells for 4 hours increases the mean levels of Nanog (m_{CM}=38.68; m_{Wnt}=51.35), Oct4 (m_{CM}=81.4; m_{Wnt}=135.4) and total β-catenin (m_{CM}=8.41; m_{Wnt}=19.34). There is also a decrease in the variability of the levels upon Wnt3A treatment, as reflected by the coefficient of variation of Nanog (CV_{CM}=0.7; CV_{Wnt}=0.54), Oct4 (CV_{CM}=0.52; CV_{Wnt}=0.43) and total β-catenin (CV_{CM}=0.73; CV_{Wnt}=0.57). (**E**) Scatterplot of the expression of Nanog and Oct4 after 24-hour exposure to Wnt3A-CM relative to serum+LIF. The black line is the linear fitting of the data for the cells in serum+LIF medium (R^2: 0.1822); the red line is the linear fitting of the data for Wnt-treated cells (R^2: 0.2931), indicating that Wnt signaling increases the correlation between the expression of the proteins across the population (*P* value=0.017, Fisher r-to-z transformation). (**F**) Line-plot distribution of Tcf3 QIF analysis in E14Tg2A cells treated with Wnt-CM (red) or CM (pink) for 4 hours. Quantifications were as described in Fig. 4C-E. (**G**) Scatterplots showing the analysis of Nanog, Oct4 or total β-catenin (*x*-axis) versus GFP in TLG2 (*y*-axis) cells grown in serum+LIF. The black lines are the linear fitting of the data (TLG2 versus Nanog R: R^2=0.1530; TLG2 versus Oct4: R^2=0.1428).

A membrane-associated β-catenin/Oct4 complex is linked to ground-state pluripotency

The correlations that we observe using QIF might reflect molecular relationships between proteins in individual cells. Interactions between β-catenin and Tcf3 are well characterized (Lee et al., 2001) and there is evidence that Oct4, in addition to its interactions with Nanog, can bind β-catenin (Tam et al., 2008; Abu-Remaileh et al., 2010; Kelly et al., 2011). These complexes could provide a basis for interpreting the correlations that we observe (Fig. 5; supplementary material Fig. S7). To test this, we first sought to confirm the interactions between Oct4 and β-catenin, as in some cases the complex was not identified under self-renewal conditions (Tam et al., 2008; Abu-Remaileh et al., 2010) or its detection required the inhibition of GSK3 (Kelly et al., 2011). In agreement with earlier reports, a β-catenin/Oct4 complex was difficult to detect in whole lysates of ESCs under self-renewing conditions (Kelly et al., 2011) (supplementary material Fig. S8A). Therefore, we made two assumptions: first, that the β-catenin/Oct4 complex would be associated with ground-state pluripotency; and in serum+LIF the number of cells in the ground state is lower than in 2i (Wray et al.,

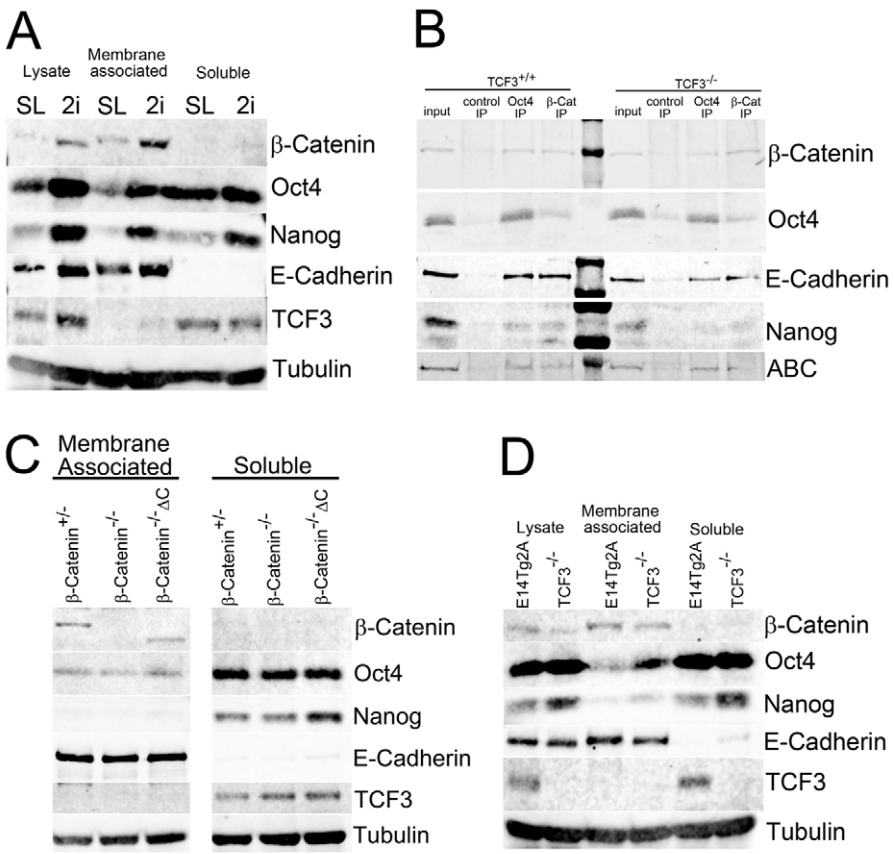

Fig. 6. A β-catenin/Oct4-containing complex is associated with membranes in pluripotent mESCs. (**A**) E14TgA cells grown in either serum+LIF or 2i for at least two passages prior to lysis and ConA fractionation. (**B**) Oct4- and β-catenin-containing complexes immunoprecipitated from membrane-associated protein extracts of *Tcf3*-null ($Tcf3^{-/-}$) and their wild-type parental ($Tcf3^{+/+}$) mESC cells were maintained in 2i media for at least two passages prior to lysis. (**C**) $\beta\text{-catenin}^{fl/-}$, $\beta\text{-catenin}^{-/-}$ and $\beta\text{-catenin}^{-/-}$ cells rescued with ΔC β-catenin ($^{-/-}$ ΔC) grown in 2i+LIF prior to lysis and ConA fractionation. (**D**) E14Tg2A and TCF3-null ($Tcf3^{-/-}$) cells grown in serum+LIF prior to lysis and ConA fractionation. Expression of proteins was assessed by western blot; quantification of blots is shown in supplementary material Figs S8, S9.

2010). The second assumption is that the complex is associated with membranes based on our observation that some Oct4 is associated with the pellet of ConA fractionations (Fig. 4A,C). Therefore, to increase the chance of detecting the complex, we selected membrane-associated proteins with ConA from cells grown in 2i (see Materials and methods). Under these conditions, we find an increase in the amount of Nanog and Oct4 in the ConA fraction of the lysate (Fig. 6A; supplementary material Fig. S8B) and an increase in the yield of the β-catenin/Oct4 complex obtained upon immunoprecipitation with either Oct4 or β-catenin (Fig. 6B; supplementary material Fig. S8C). The complex could be immunoprecipiated both in the presence and absence of Tcf3, and contains both E-cadherin and a small amount of Nanog (Fig. 6B); in wild-type cells, Tcf3 was not detected in these complexes and was abundant in the supernatants that correspond to the soluble fractions. Furthermore, a β-catenin/Oct4 complex could also be immunoprecipitated from the cytoplasmic fraction of cells grown in 2i (supplementary material Fig. S9A). These results establish a correlation between the presence of a β-catenin/Oct4 complex and ground state pluripotency.

A β-catenin/Oct4 complex enhances pluripotency

As a first step to test the function of the β-catenin/Oct4 complex, we analyzed the levels and location of its components in the absence of each of the main elements of the network.

We first assessed the localization of Oct4 in β-catenin mutant cells and mutant cells rescued with ΔC β-catenin, a transcriptionally impaired β-catenin protein (Hsu et al., 1998). In the absence of β-catenin, the levels of Oct4 associated with membranes are greatly reduced (Fig. 6C; supplementary material Fig. S9B). These cells exhibit reduced levels of E-cadherin, suggesting that although β-catenin is not an essential element of the complex, it is required for its efficient assembly. In support of this, ΔC β-catenin that has been shown to promote pluripotency (Kelly et al., 2011; Lyashenko et al., 2011; Wray et al., 2011) can maintain the levels of Oct4 at the membrane (Fig. 6C; and for quantifications see supplementary material Fig. S9B). The correlation between membrane levels of Oct4 and Nanog, and pluripotency is emphasized by the observation that *Tcf3* mutant cells, which have delayed differentiation, also have high levels of Oct4 associated with membranes (Fig. 6D; supplementary material Fig. S9C). Reduction of the levels of β-catenin in these cells results in lower pluripotency and an increased frequency of differentiation (Fig. 7A), indicating that β-catenin has roles in pluripotency that are additional to the downregulation of Tcf3.

Oct4 mutant ESCs cannot be maintained in culture as they rapidly differentiate (Niwa et al., 2000); therefore, to test the impact of Oct4 on the complex, we used ZHBTc4 cells, in which Oct4 is under doxycycline control (Niwa et al., 2002). Addition of doxycycline to a culture of these cells results in a rapid decrease in the levels of Oct4 allowing us to analyze the effects that this has on β-catenin. One day after the addition of doxycycline, Oct4 has disappeared and the levels of β-catenin in the membrane have been reduced (Fig. 7B). These cells also exhibit low levels of Nanog expression, suggesting that the cells are differentiating. These cells cannot respond to enhanced β-catenin levels induced with chiron (Fig. 7C), a feature that they share with β-catenin mutant cells (Wray et al., 2011) but not with Nanog mutant cells, which show some response to chiron, despite their tendency to differentiate (Fig. 7C). Furthermore, overexpression of Oct4 under self-renewal conditions induces differentiation, but this effect can be rescued by stabilizing β-catenin with chiron (Fig. 7D; supplementary material Fig. S10).

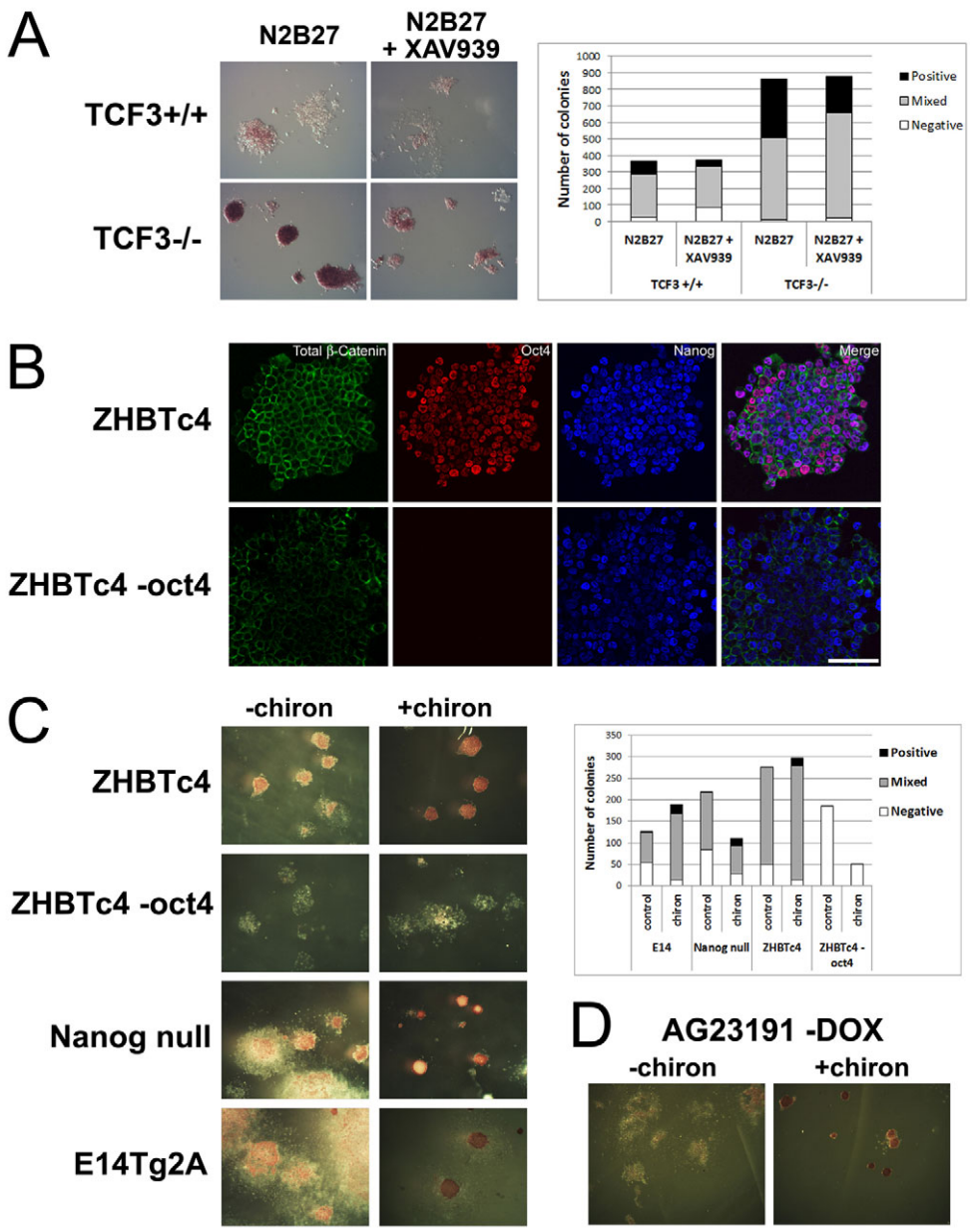

Fig. 7. Subcellular localization and function of β-catenin depends on Oct4. (**A**) Colony assays of *Tcf3*-null ($Tcf3^{-/-}$) and their wild-type parental ($Tcf3^{+/+}$) cells grown in N2B27 or N2B27+XAV939, as indicated. Quantification is shown on the right. (**B**,**C**) ZHBTc4 cells grown in serum+LIF with (ZHBTc4-Oct4) or without (ZHBTc4) doxycycline for 24 hours. (B) Cells were stained for total β-catenin (green), Oct4 (red) and Nanog (blue). Scale bar: 50 μm. (C) Colony assays of ZHBTc4 cells with (ZHBTc4-Oct4) or without (ZHBTc4) doxycycline. E14Tg2A and Nanog-null cells grown in serum+LIF in the presence or absence of chiron. Quantification is shown on right. (**D**) AG23191 cells overexpressing Oct4 from a TetOFF system were grown in serum+LIF in the presence of absence of chiron. Colonies were assayed for alkaline phosphatase activity and quantification of the assay is shown in supplementary material Fig S10.

These results suggest that β-catenin contributes to pluripotency not only by titrating the activity of Tcf3 but also by promoting the efficient membrane association of Oct4. The correlation between the levels of Oct4 and Nanog at the membrane and the degree of pluripotency of an ES cell population suggests, although does not prove, that this complex is likely to be functionally significant.

DISCUSSION

There is ample evidence that β-catenin, an effector of Wnt signaling, aids the maintenance of pluripotency (Kielman et al., 2002; Ying et al., 2002; Sato et al., 2004; Ogawa et al., 2006; Singla et al., 2006; Takao et al., 2007; Ying et al., 2008; Bone et al., 2009; Wagner et al., 2010). Most significantly, chiron, an inhibitor of GSK3 and an agonist of the pluripotency gene regulatory network (Bell et al., 2003; Sato et al., 2004; Umehara et al., 2007; Ying et al., 2008; Bone et al., 2009), has been shown to operate through β-catenin independently of its transcriptional activity (Wray et al., 2011). Here, we have shown that, under conditions that promote pluripotency, ESC contain high levels of β-catenin but its transcriptionally competent isoforms are tightly associated with membranes. This localization correlates with low β-catenin/Tcf transcriptional activity and a reduced requirement for the β-catenin transcriptional co-factors. Our experiments confirm that β-catenin not only contributes to pluripotency by antagonizing Tcf3 but also uncover an additional contribution through a complex involving Oct4 and, to a lesser extent, Nanog.

Our results argue against the notion that Wnt/β-catenin transcriptional signaling is involved in pluripotency (Miyabayashi et al., 2007; Cole et al., 2008; Nusse et al., 2008; Wagner et al., 2010; ten Berge et al., 2011). It has been suggested that β-catenin-mediated transcription is necessary in ESCs to prevent their transition to Epi-stem cells, implicating this activity in the maintenance of pluripotency (ten Berge et al., 2011). Much of the evidence for this conclusion relies on experiments with cells grown on mouse embryonic fibroblasts (MEFs), which are an uncertain source of pluripotency enhancers, and the report of ten Berge et al.

lacks correlations between reporter activity and levels or specific isoforms of β-catenin in the manner that we have provided here. Notwithstanding, our observations agree that β-catenin is necessary for the maintenance of pluripotency and to prevent differentiation (see also Wray et al., 2011; Rudloff and Kemler, 2012); they also support a role for some Wnt proteins in the regulation of the levels of β-catenin in self-renewal. However, in contrast to the conclusions of ten Berge et al. (ten Berge et al., 2011), albeit in agreement with those of others (Lyashenko et al., 2011; Takao et al., 2007; Kelly et al., 2011; Wray et al., 2011; Rudloff and Kemler, 2012), our results suggest that this requirement is not mediated by nor requires the transcriptional activity of β-catenin.

Experimental increases in β-catenin concentration can trigger a transcriptional response under self-renewing conditions (Yi et al., 2011) (this work). In this situation, β-catenin can be recruited to the regulatory regions of target sites. However, this effect could be misleading in the context of pluripotency, as β-catenin recruitment to the regulatory regions of pluripotency genes was not observed in ESCs lacking Tcf3 and Tcf1, yet these cells were proficient at self-renewal (Yi et al., 2011), suggesting that recruitment, like transcriptional activity, is not necessary for pluripotency. A recent report has detected β-catenin at the promoter of *Tert* in association with Klf4 (Hoffmeyer et al., 2012). Although this event might be important for the long-term robustness of the cells, Tert is not an element of the core pluripotency network. Thus, we conclude that β-catenin-mediated transcription is not involved in pluripotency, a conclusion further supported by experiments using GSK3 mutants in which blocking the activation of transcriptional targets of β-catenin through ΔNTcf4 overexpression failed to affect enhanced pluripotency of GSK3 DKO cells (Kelly et al., 2011). However, the exit from pluripotency correlates with an onset of β-catenin transcriptional activity, which is coincident with a cytoplasmic rise in β-catenin and, specifically, a transcriptionally competent isoform. In agreement with this, addition of iCRT3 (an inhibitor of the interaction between β-catenin and Tcf) to cells grown in chiron or 2i, has no effect on pluripotency but eliminates differentiating cells (P.H., R.D. and A.M.A., unpublished).

Our study does not address the mechanisms that trigger the transition from the non-transcriptional to the transcriptional mode of β-catenin activity during differentiation. The exit from pluripotency is associated with an EMT-like process (Li et al., 2011) (S. Lowell, unpublished), and, in agreement with this, we observe correlated changes in the amount of ABC and E-cadherin at the plasma membrane during differentiation. As it has been shown that E-cadherin can regulate the transcriptional activity of β-catenin (Orsulic et al., 1999; Heuberger and Birchmeier, 2010) and we observe that reduction in cadherin expression affects the exit from pluripotency (Fig. 1D), it is possible that there is a causal relationship between the remodeling of cadherin-based complexes, differentiation and the activation of β-catenin-mediated transcription.

β-Catenin and the pluripotency network

The study of the effects of β-catenin on pluripotency have focused on its interactions with Tcf3 and its antagonism of the activity of Oct4, Nanog and Sox2 (Boyer et al., 2005; Cole et al., 2008; Tam et al., 2008; Yi et al., 2008; Guo et al., 2011; Yi et al., 2011). An epistatic analysis of the relationship between chiron, a GSK3 inhibitor, and Tcf3 has led to the suggestion that the main role of β-catenin in self-renewal is to compete the activity of Tcf3 and thus facilitate the function of Nanog, Oct4 and Sox2 (Wray et al., 2011). Our results provide additional support for this interaction and our sensitive Wnt reporter suggests that under self-renewal conditions the activity of Tcf3 fluctuates across the population, probably because of fluctuations in the concentration of β-catenin. However,

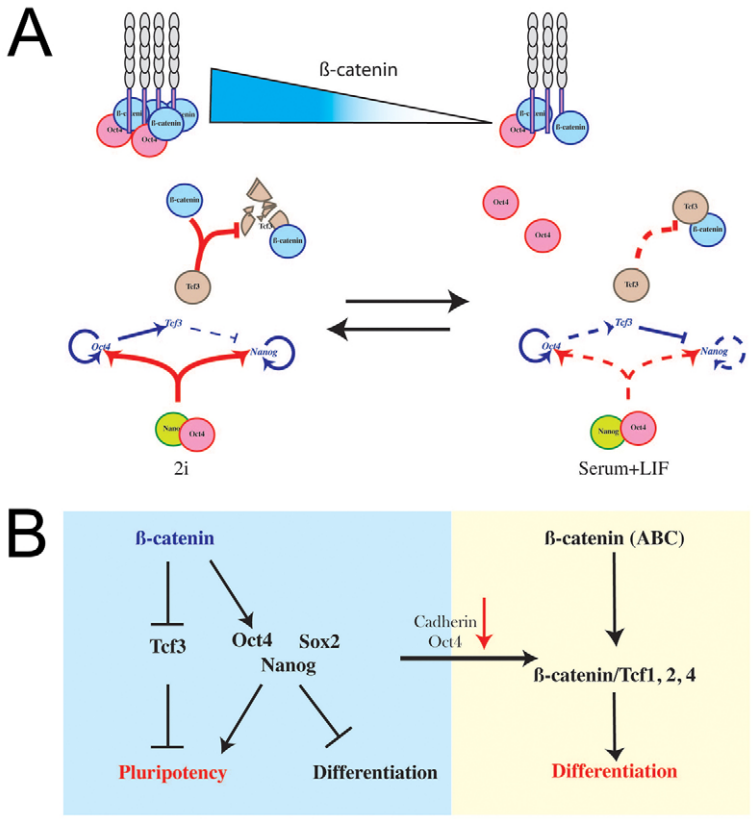

Fig. 8. A mechanism for β-catenin-mediated regulation of pluripotency. (**A**) The level of β-catenin is a major determinant of pluripotency through its role in the assembly of a complex with Oct4 and E-cadherin. In the ground state (enriched in 2i conditions), a membrane-associated complex between Oct4 (pink), β-catenin (blue) and cadherin limits the amount of Oct4 available for interactions with other proteins. Most of the Oct4 is available for interactions with Nanog (green), which is abundant in the ground state; the complex promotes pluripotency. In parallel, a different pool of β-catenin interacts with and inactivates Tcf3 (orange), thus allowing efficient activity of the Oct4:Nanog-driven network. If the levels of β-catenin drop, the amount of Oct4 in the membrane-associated complex is reduced and there is more Oct4 available for interactions with other factors, some of which promote lineage priming. The reduced amount of β-catenin limits its interaction with Tcf3, which is now available to repress pluripotency genes. In this state, as a consequence of looser regulation of Oct4 and Tcf3, cells have a higher probability of differentiating and exhibit a noisier pluripotency network. Red lines indicate protein-protein interactions; blue lines indicate transcriptional regulatory interactions. (**B**) The interactions and activity of β-catenin in these protein complexes does not involve or require its transcriptional activity (for details see text).

although there is evidence that β-catenin can lower the levels of Tcf3 (this work), it is not clear where and how this is achieved. Furthermore, we have shown the dampening of the function of β-catenin in the absence of Tcf3 still influences pluripotency and that this effect is likely to be associated with the β-catenin/Oct4 interaction.

Our results confirm reports of interactions between β-catenin and Oct4 (Tam et al., 2008; Abu-Remaileh et al., 2010; Kelly et al., 2011) but add that the complex is associated with membranes, that it does not include Tcf3 but contains E-cadherin and Nanog, and that its abundance correlates with ground state pluripotency. β-Catenin and Oct4 appear to regulate the amount and location of each other at the membrane and their relationship is reflected in the observation that the effect of chiron in the maintenance of pluripotency requires both β-catenin and Oct4. Although β-catenin is not essential for the localization of Oct4 to the membrane, it does increase its presence at this location, suggesting the involvement of other proteins, including cadherins, in the complex.

We surmise that the β-catenin/Oct4 complex lies at the core of a protein interaction network whose dynamics govern the activity of the pluripotency TFN. The network is defined through interactions between three complexes – Oct4/β-catenin, Oct4/Nanog and β-catenin/Tcf3 – which compete with each other for their individual shared components (Fig. 8). The output of these interactions might be a steady state ratio of the different complexes and free molecules, which determine the degree of pluripotency of the ES cell population: if Tcf3>Oct4, there is a higher probability of differentiation; if Oct4>Tcf3, there is a higher probability of self-renewal (Fig. 8). We surmise that the impact of β-catenin on the network is twofold. In agreement with previous reports (Wray et al., 2011; Yi et al., 2011), it limits the activity of Tcf3 and delays the exit from pluripotency but it also controls the amount of Oct4 that can be free by promoting its membrane localization to ensure that Oct4 interacts preferentially with Nanog rather than with lineage-priming factors. A prediction of this model is that self-renewal will be characterized by limiting amounts of Oct4 that, once they exceed a threshold value, will promote differentiation through their lineage-priming ability. This is in agreement with the sensitivity of pluripotency to the levels of Oct4 (Niwa et al., 2000).

Our model provides a mechanism for the observation that β-catenin can stimulate the transcriptional activity of Oct4 in pluripotency, even though it is difficult to detect β-catenin at Oct4 targets (Takao et al., 2007; Tam et al., 2008): it acts by ensuring limiting amounts of Oct4 for interaction with its preferred partners: Nanog and Sox2. A reciprocal interaction is possible, and Oct4 might be the reason for the inability of transcriptionally competent β-catenin to access the cytosol under self-renewal conditions: in the cadherin-based complex, Oct4 might block the release of the transcriptionally competent form of β-catenin. In this regard, there is evidence that Oct4 can antagonize the transcriptional activity of β-catenin (Anton et al., 2007; Marikawa et al., 2011), and, during differentiation, the activation of β-catenin reporters is concomitant with decreases in Oct4 and cadherin.

In summary, we suggest that a competitive dynamics of defined protein complexes between pluripotency factors is an important determinant of pluripotency and that the main function of β-catenin is to regulate these ratios and thus to control molecular heterogeneities that can and will be reflected in phenotypic heterogeneities at the level of the population (Martinez Arias and Hayward, 2006; Munoz Descalzo et al., 2011).

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Competing interests statement
The authors declare no competing financial interests.

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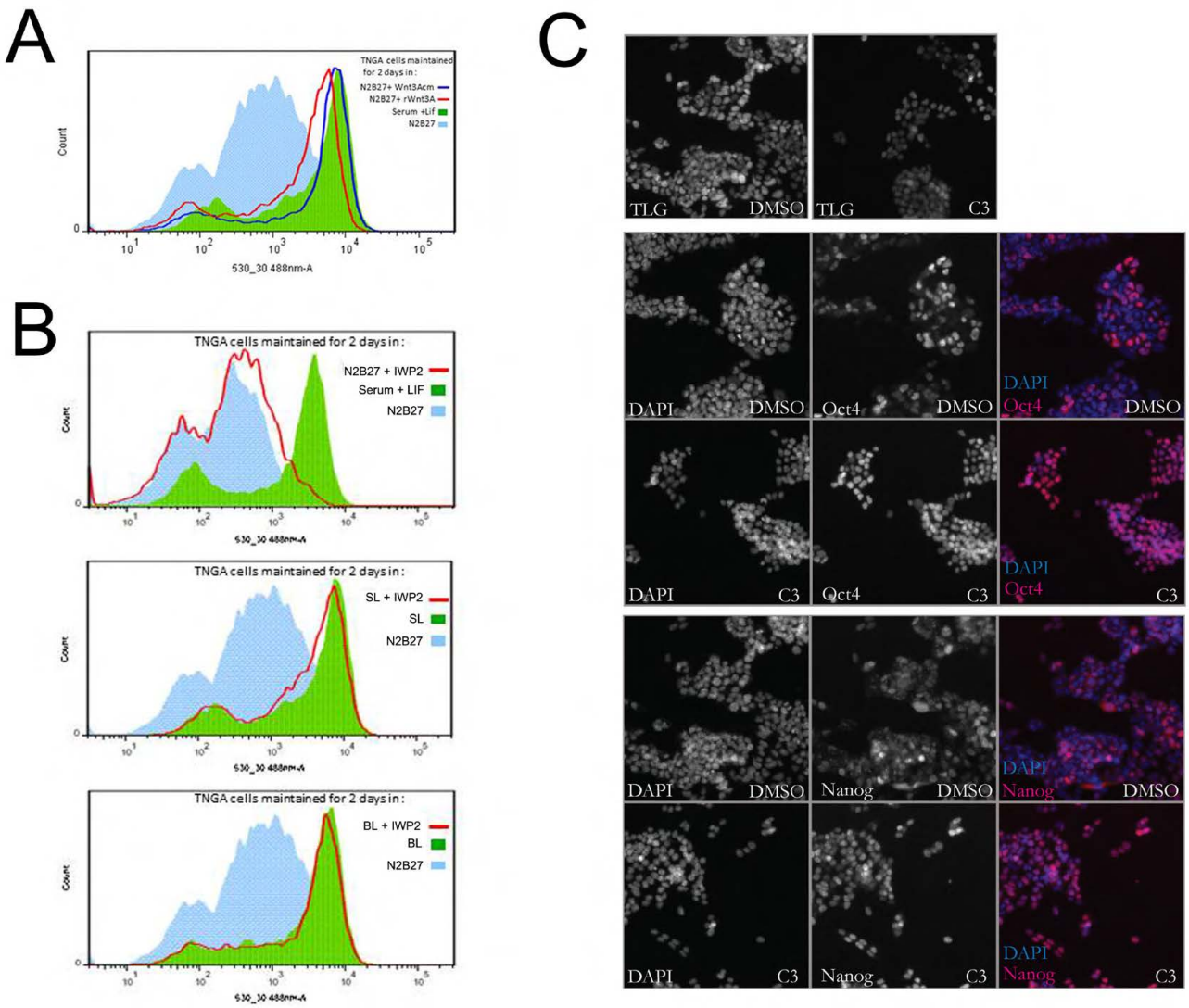


Fig. S1. Characterization of the effects of Wnt signalling inhibitors on mESC pluripotency and differentiation. (**A**) TNGA cells were grown in N2B27 supplemented with either Wnt3A-conditioned medium or recombinant Wnt3A for 2 days. (**B**) Profiles of TNGA (Nanog::GFP) cells comparing differentiation in N2B27 under the different conditions as indicated after 2 days in culture. The effect of IWP2 (an inhibitor of the Wnt chaperone porcupine) is dependent on the culture medium. Although there is little effect in LIF+BMP, there is a slight loss of pluripotency in serum+LIF. Over longer periods of time, IWP2 leads to a loss of pluripotency (not shown) in agreement with published results. We believe this is not due to the loss of pluripotency but to an increased flow of LN cells into differentiation that, slowly, drains the pool of pluripotent cells. (**C**) 10,000 cells/well (96-well plate) were plated on day 0. In parallel, 10,000 cells were transfected for the 14× TOP-Luciferase and SV40-Renilla control vectors. On day 1, media were replaced with 50 µg of C3 and control DMSO in serum+LIF media. Cells were processed on day 2 for immunostaining and luciferase assays.

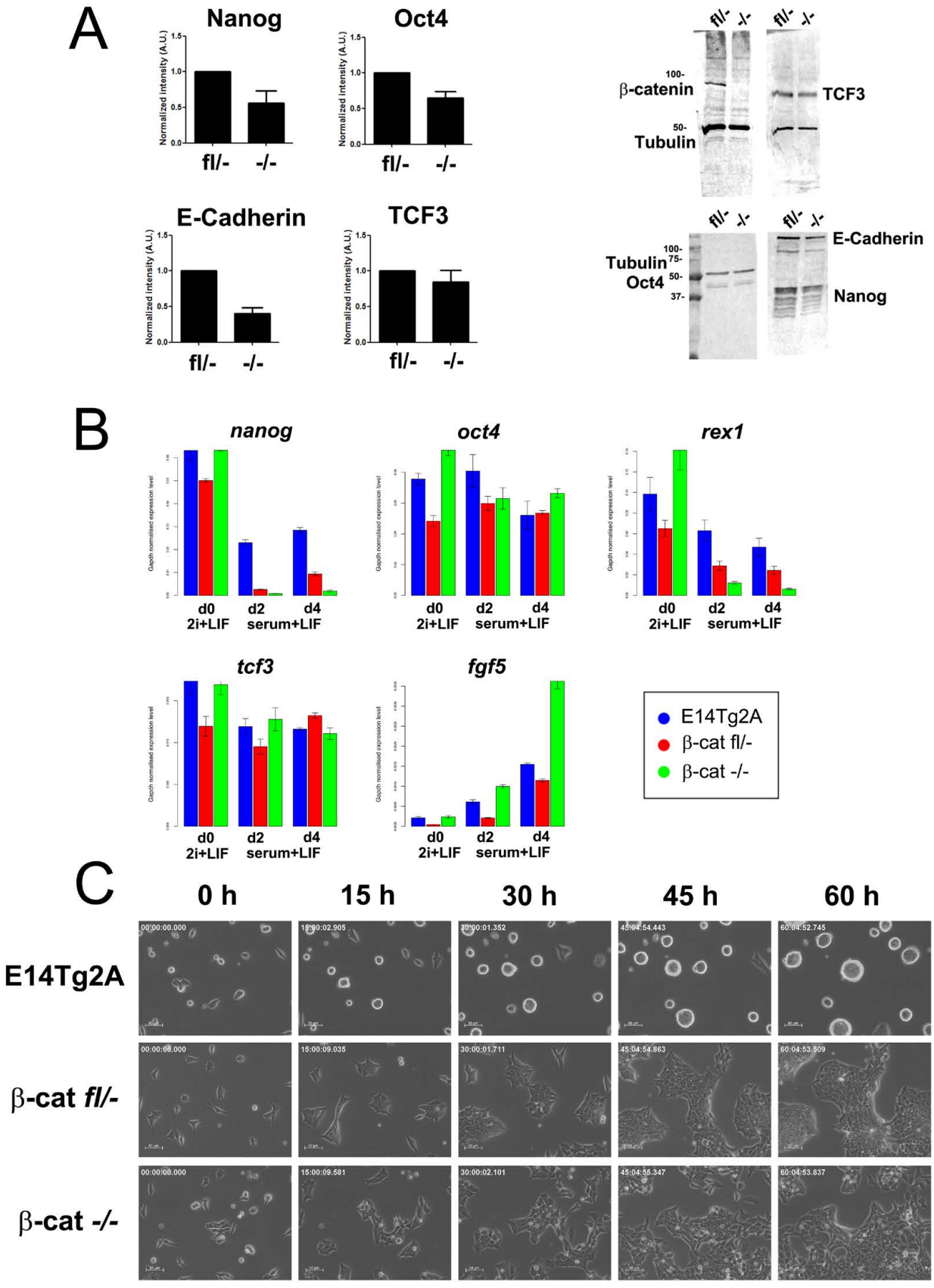


Fig. S2. Characterization of β-catenin$^{fl/-}$ and β-catenin$^{-/-}$ mESC lines. (**A**) Quantifications of western blots shown in Fig. 2B for β-catenin$^{fl/-}$ (fl/–) and β-catenin$^{-/-}$ (–/–) cells. Normalization was performed to tubulin and levels of β-catenin$^{fl/-}$ were arbitrarily assigned to one. Gels are shown on the right. (**B**) E14Tg2A, β-catenin$^{fl/-}$ and β-catenin$^{-/-}$ cells were grown in 2i+LIF (2i+LIF) and changed to serum+LIF for 2 and 4 days. Total RNA was isolated at day 0 (d0 in 2i + LIF) and then at day 2 (d2) and day 4 (d4) in serum+LIF. qPCR was performed for specific genes and levels were normalized to *Gapdh*. (**C**) E14Tg2A, β-catenin $^{fl/-}$ and β-catenin$^{-/-}$ cells were grown in 2i+LIF on fibronectin and filmed for 3 days (see Movies 1-3).

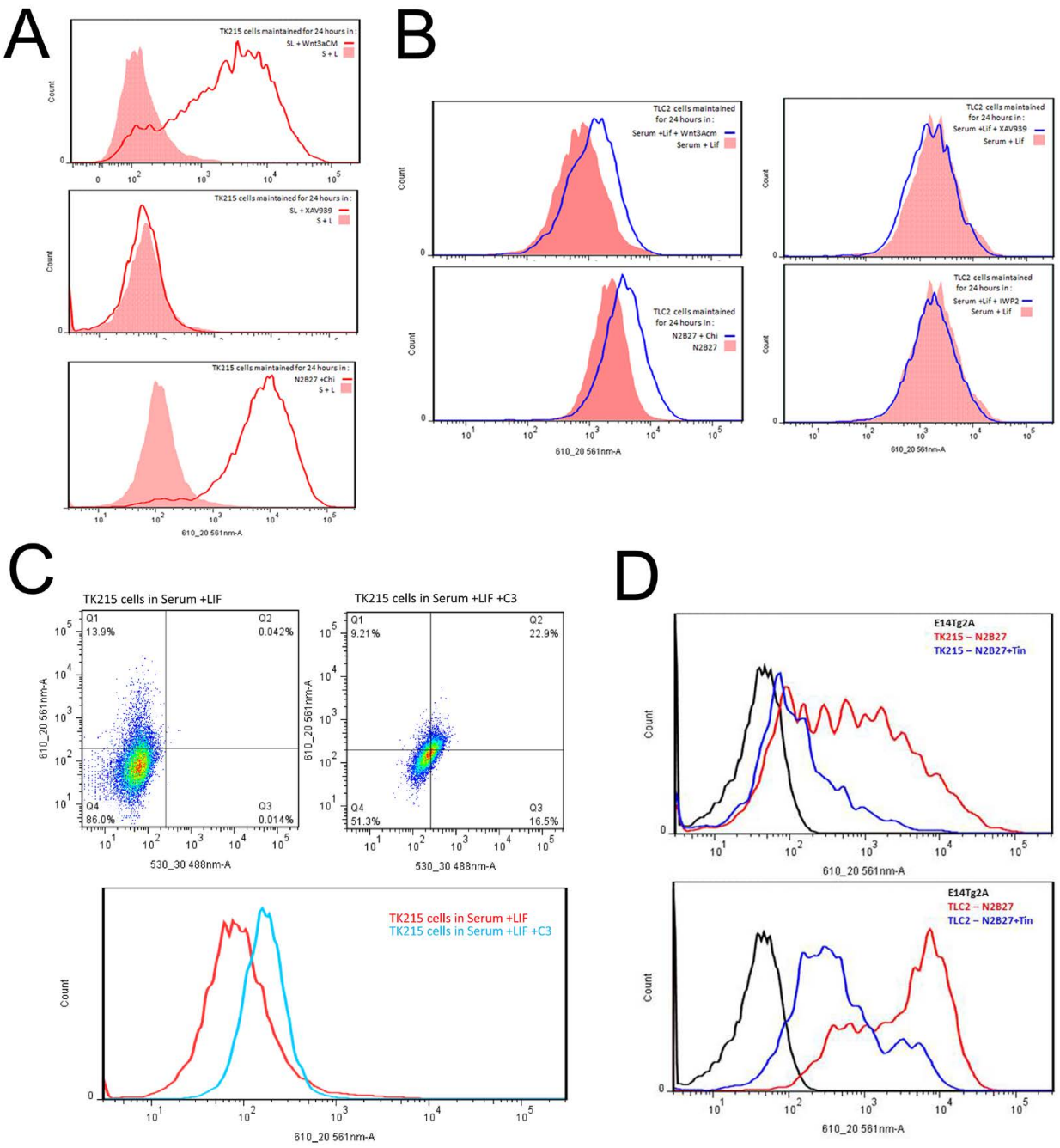


Fig. S3. Characterization of fluorescent Wnt reporter mESC lines. (**A**,**B**) Characterization of Wnt reporter lines in TK215 (A) and TLC2 (B) under conditions that promote or inhibit Wnt activity . (**C**) The addition of ICRT3 to the cells for 3 days causes an increase in the autofluorescence of the cells. Over shorter time periods, this increase is not apparent (see Fig. 1E).

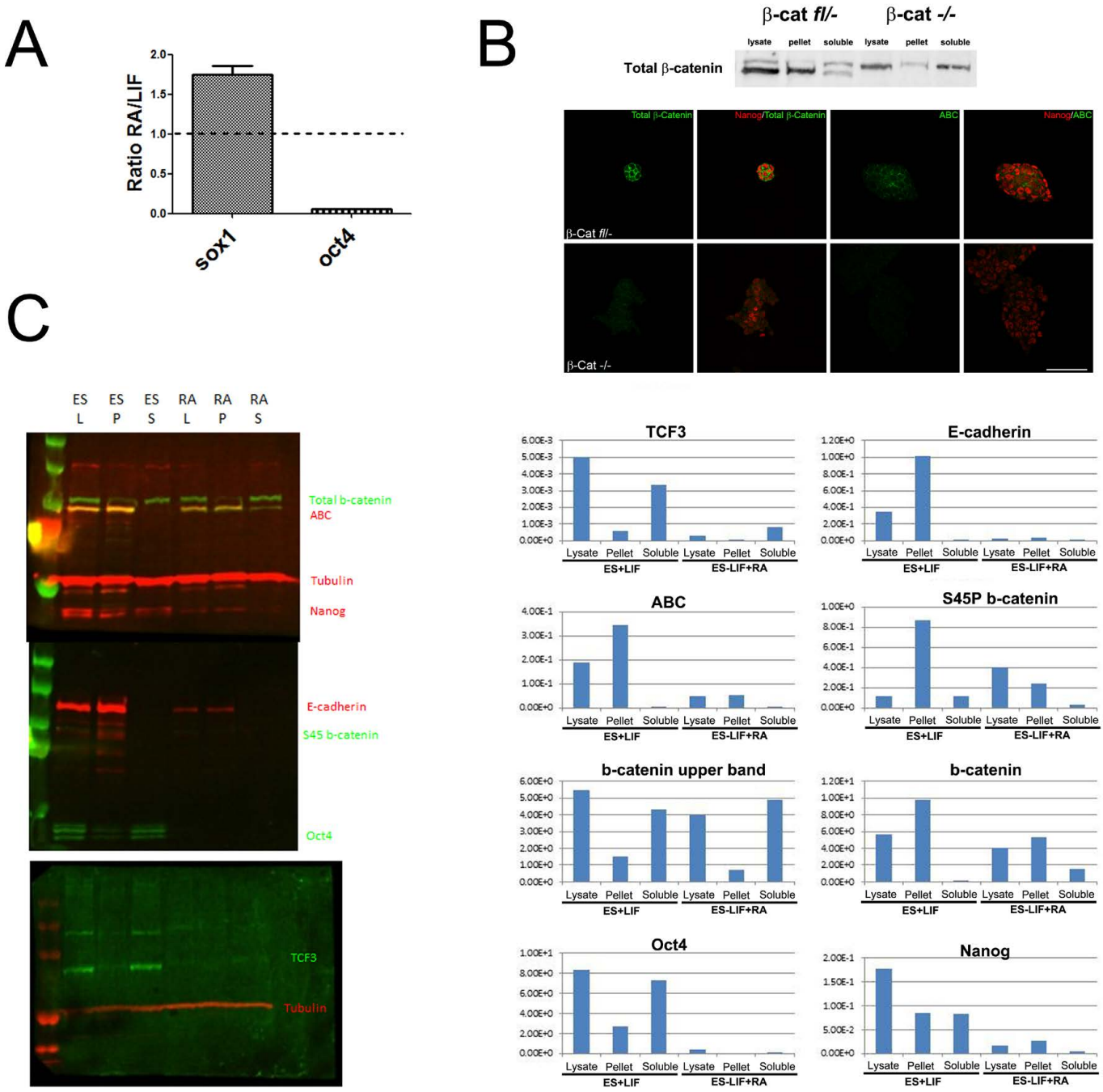


Fig. S4. Characterization of Serum+RA induced differentiation. (**A**) E14Tg2A cells were grown in self-renewing (serum+LIF) or differentiating conditions (serum+1 mM RA) for 3 days. Total RNA was isolated and qPCR was performed using specific primers for *Sox1* and *Oct4*. Normalization to *Gapdh* was performed and the ratio between serum+1 mM RA and serum+LIF was plotted. (**B**) Western blots of β-catenin$^{fl/-}$ and β-catenin$^{-/-}$ cells with a Total β-catenin antibody (Sigma cc2206). The upper band has a non-specific component (see main text). β-Catenin$^{fl/-}$ and β-catenin$^{-/-}$ cells were stained for total β-catenin (green channel) and Nanog (red channel), and for ABC (green channel) and Nanog (red channel) to show the specificity of these antibodies. This non-specific component of the upper band is also not recognized by total β-catenin (Abcam 19450, data not shown). For a further discussion of the specificity of β-catenin antibodies see Maher et al. (Maher et al., 2009a) (**C**) Gel images (left) and quantifications (right) of the experiment shown in Fig. 4A. Western blot membranes were scanned in the Oddysey System. The intensity of each band was normalized to tubulin. ES, serum+LIF; RA, serum+1 mM RA.

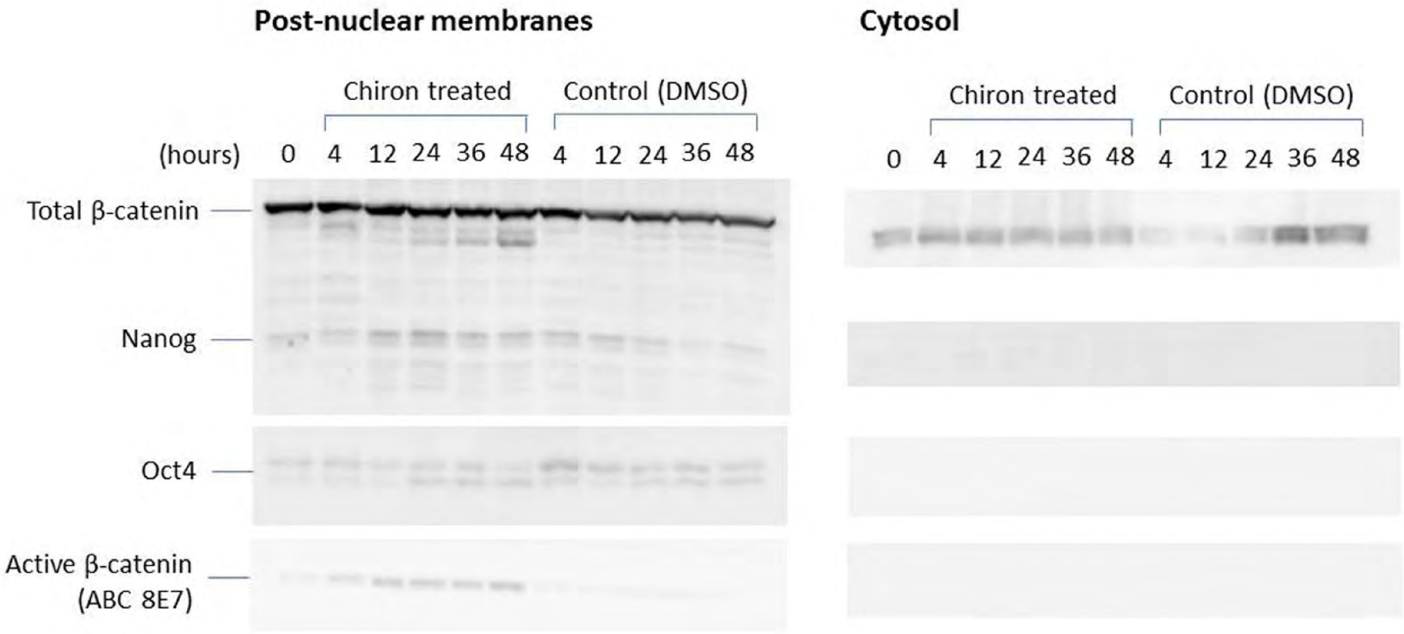


Fig. S5. Nanog and Oct4 are associated with membranes in mESCs. Post-nuclear membranes and cytosolic proteins fractionated by differential centrifugation as previously described (for details, see Berrios, 1997). Post-nuclear membranes samples were analyzed by mass spectrometry as previously described (for details, see Hall et al., 2009) to confirm they were free of nuclear contamination and strongly enriched in organellar proteins, including plasma membrane residents. Concentration of proteins was determined by BioRad DC assay and 2 µg protein was used for western blot analysis. Post-nuclear membranes were confirmed to be free of nuclear contamination and strongly enriched in organellar proteins, including plasma membrane residents by mass spectrometry analysis. Here, Oct4 and active β-catenin were found to localize to post-nuclear membranes but not the cytosol, whereas Nanog was readily detectable in post-nuclear membranes but almost undetectable in the cytosolic fraction.

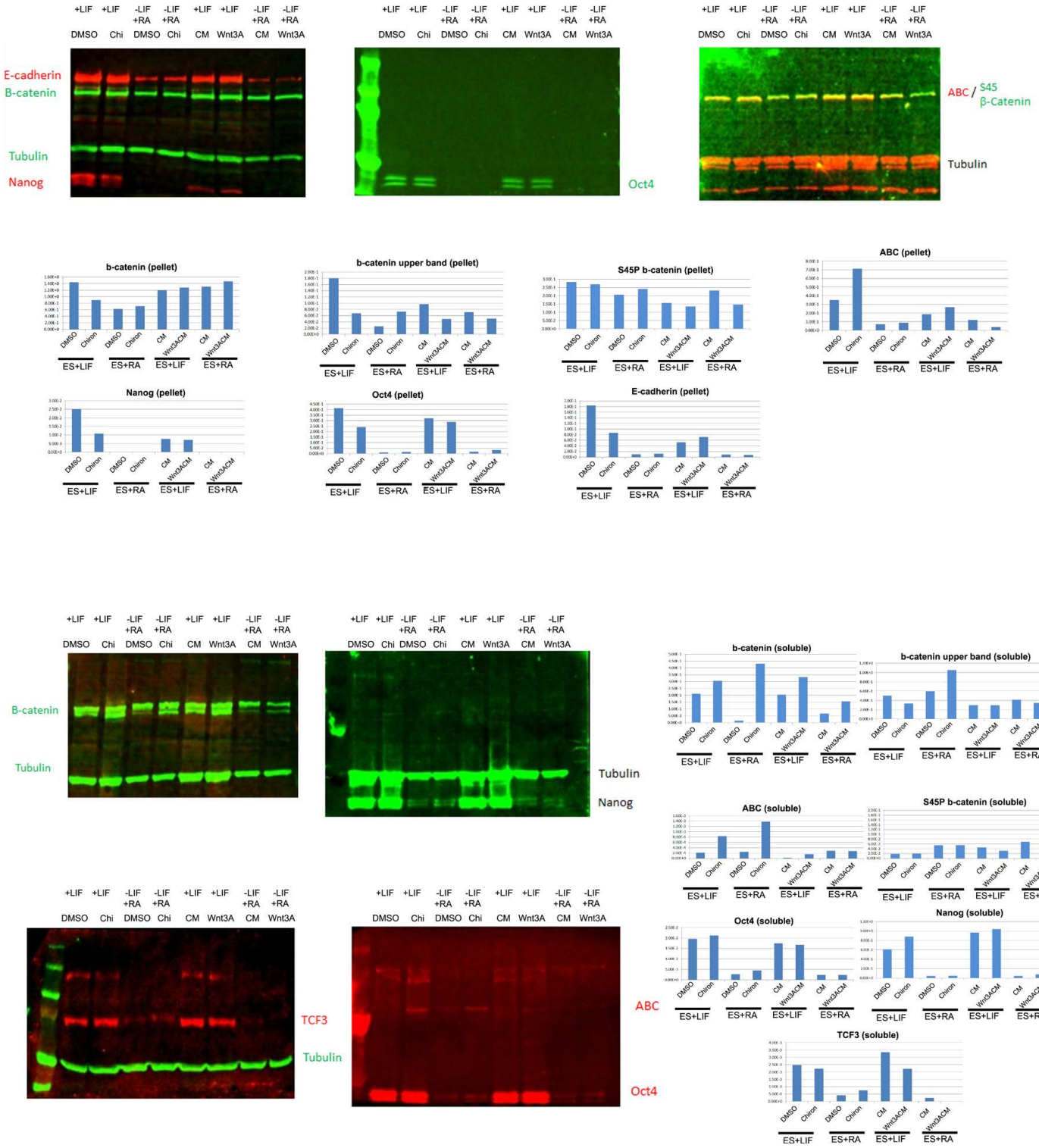


Fig. S6. Quantifications of western blots from Fig. 4C. Gel images and quantifications of western blots shown in Fig. 4C for membrane-associated proteins (pellet, upper panels) and soluble fractions (lower panels). Western blots membranes were scanned in the Oddysey System. Intensity of each band was normalized to tubulin. Primary antibodies used are: anti-Nanog (eBiosciences 14-5761, 1:100), anti-Oct4 (Santa Cruz 5279, 1:100); anti-SSEA1 (Santa Cruz 21702, 1:100); anti-active β-catenin (ABC) recognizing dephosphorylated residues in the interval 36-44 (Millipore 05-665, 1:300) (van Noort et al., 2007; Maher et al., 2009; Maher et al., 2010); anti-PSer45-β-catenin, recognizes PThr41 and PSer45 (Cell Signaling 9565, 1:200) (Maher et al., 2009; Maher et al., 2010); anti-total-β-catenin, recognizing epitopes in the C terminus of β-catenin residues 768-781 (Sigma C2206, 1:1000); anti-TCF3 (Santa Cruz sc-8635, 1:40 from the preabsorbed antibodies in *Tcf3*$^{-/-}$ cells). All antibodies, except anti-Oct4, were tested on mES cells mutant for the relevant protein to verify specificity. Secondary antibodies labeled with IRDye infrared dyes were used and membranes were scanned using the Odyssey Imaging System (LI-COR Biosciences). Quantification was performed using the LI-COR software normalizing with respect to tubulin intensity.

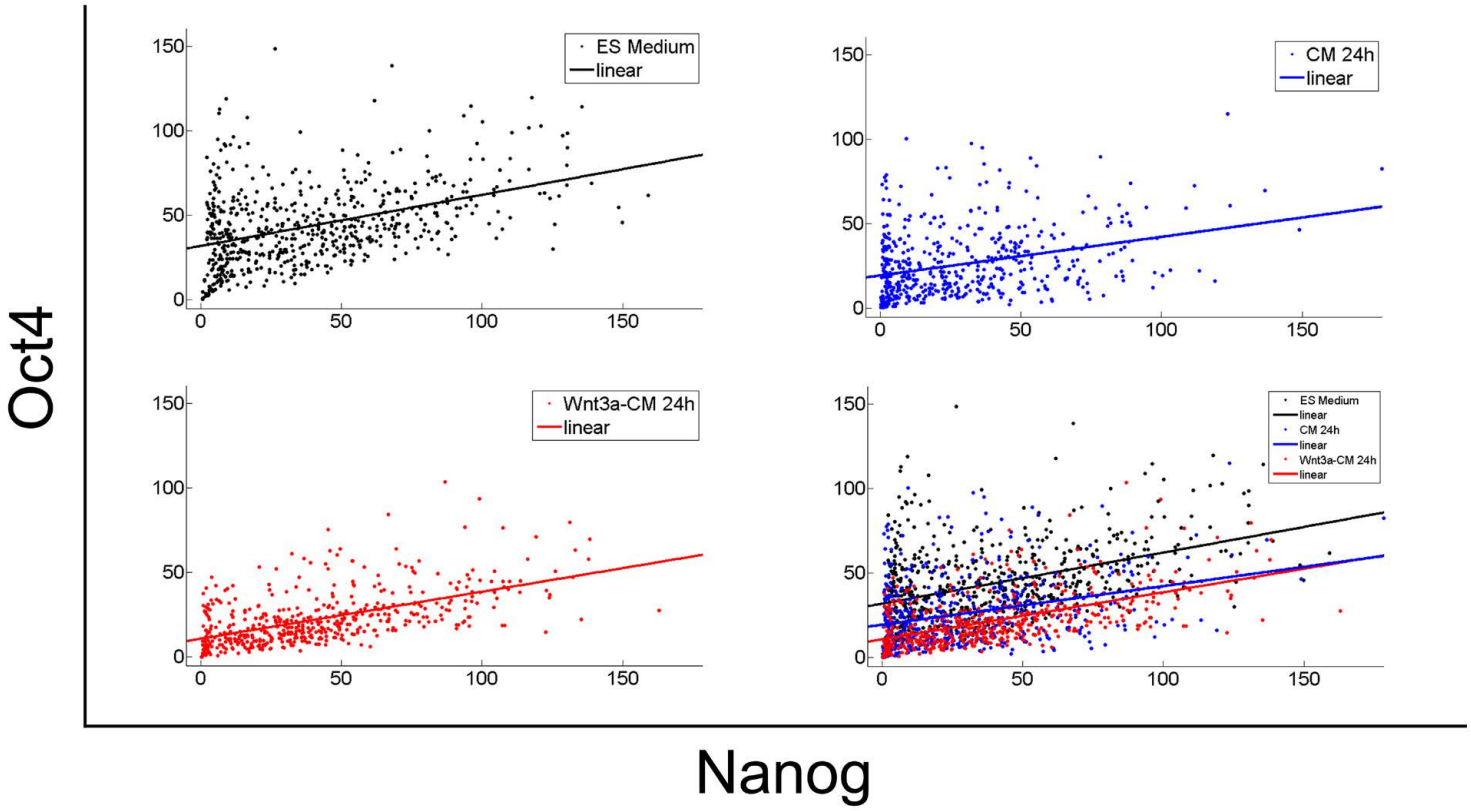


Fig. S7. The correlation between Nanog and Oct4 protein levels in single cells increases with Wnt signalling. Activation of Wnt signaling by treating E14Tg2A cells with Wnt3A-conditioned medium (Wnt, red dots) for 24 hours increases the correlation between Oct4 and Nanog (R^2: 0.2931). This effect is not mimicked by the control conditioned medium alone (CM, blue dots; R^2, 0.1033; *P* value=0.0001, Fisher r-to-z transformation). This result shows that the increased in correlation between Oct4 and Nanog is due to the activation of Wnt signaling. The black dots indicate cells grown in serum+LIF.

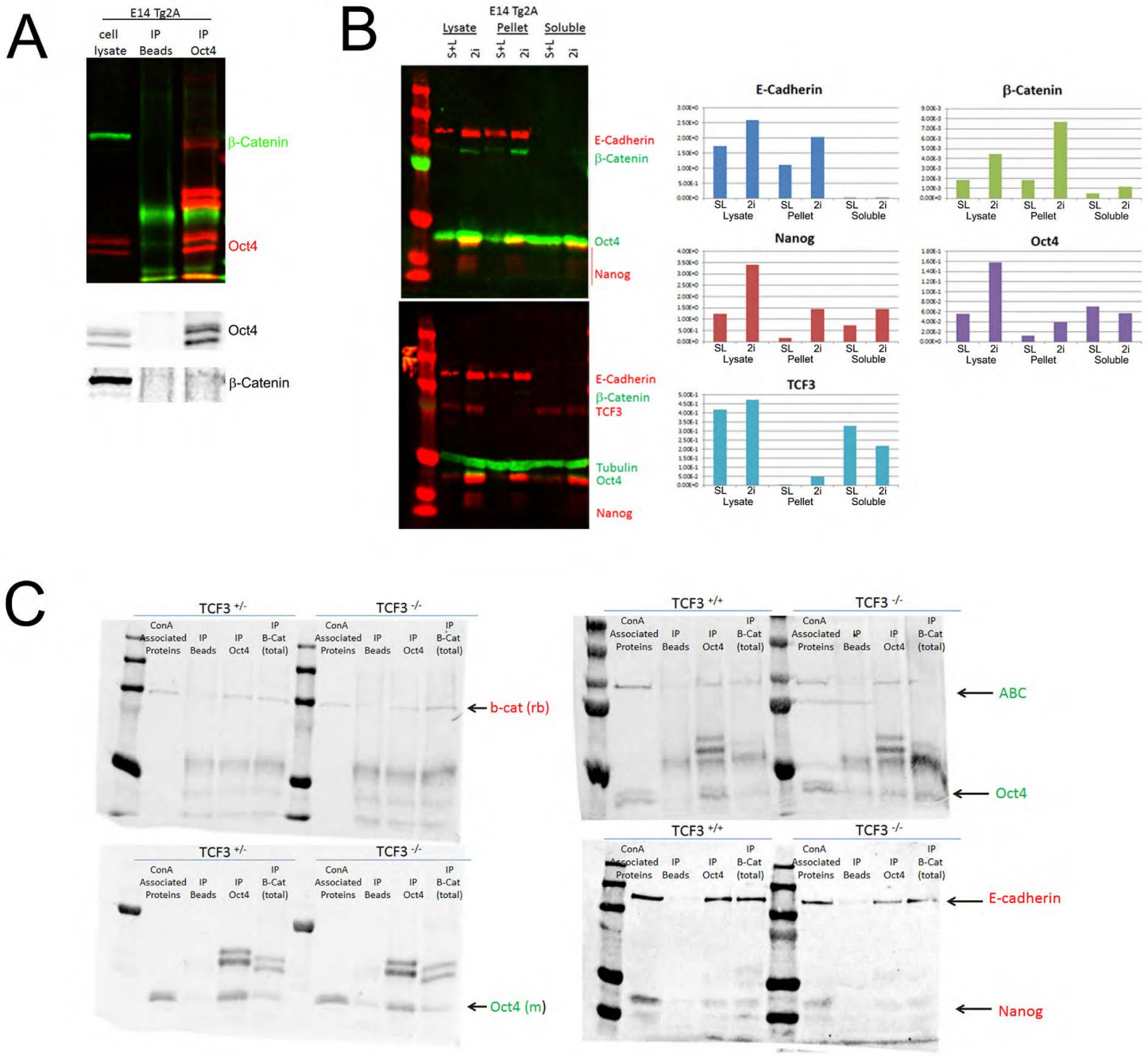


Fig. S8. Quantification of western blots from Fig. 6. (**A**) Immunoprecipitation of Oct4-containing protein complexes from total cell lysates of E14Tg2A cells grown in serum+LIF. There is a faint band of β-catenin in the Oct4 IP. (**B**) Uncropped blots from Fig. 6A. Protein expression was quantified and normalized to tubulin expression (see graphs alongside blots). These samples were obtained as follows. Immunoprecipitations were carried out on either whole-cell lysates or ConA-bound membrane-associated proteins. Anti-Oct4 (Santa Cruz sc5279) and protein A/G magnetic beads were incubated with the input for 1 hour at room temperature, the magnetic beads were then separated using a magnet against gravity (to prevent unbound ConA-sepharose beads contaminating the immunoprecipitate) and washed three times in PBS. Immunoprecipitated proteins were released in 2× Laemelli's buffer and identified by western blot (**C**) Uncropped blots from Fig. 6B.

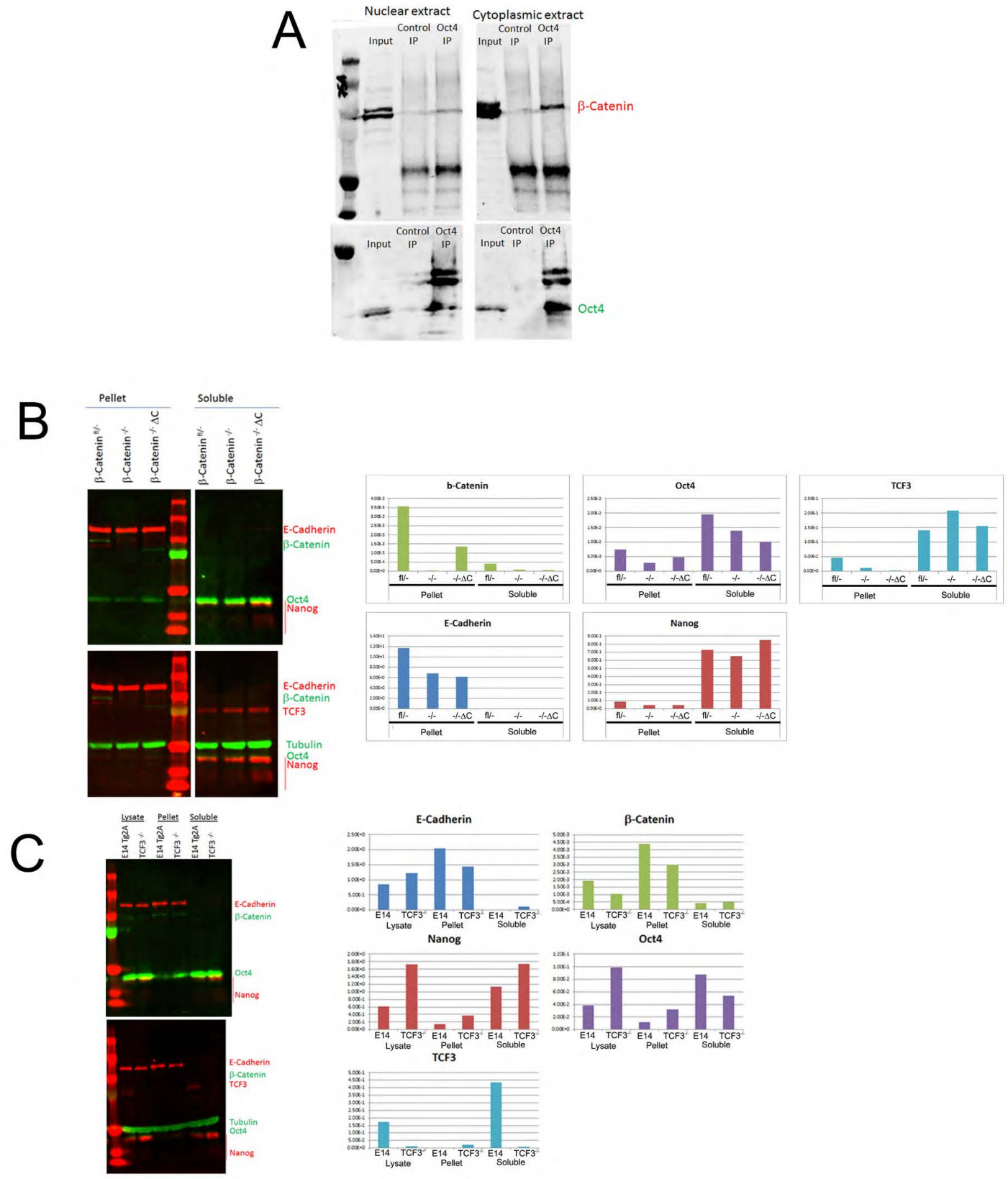


Fig. S9. A β-catenin and Oct4 complex is found in the cytoplasmic fraction of mESCs. (**A**) E14Tg2A cells maintained in 2i were fractionated using NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). Immunoprecipitation of Oct4 protein was then carried out using anti-Oct4 (Santa Cruz sc5279) and protein A/G magnetic beads. These were incubated with the input for 1 hour at room temperature, the magnetic beads were then separated using a magnet and washed three times in PBS. Immunoprecipitated proteins were released in 2× Laemelli's buffer and identified by western blot. A cytoplasmic Oct4/β-catenin complex is present in the Oct4 immunoprecipitate. E-cadherin and Nanog were not found to be associated with this complex under these conditions. Detection of E-cadherin in cytoplasmic, but not nuclear, fractions, and mSin3A in nuclear, but not cytoplasmic, extract confirmed effective nuclear/cytoplasmic fractionation (data not shown). (**B**,**C**) Uncropped blots from Fig. 6C,D. Protein expression was quantified and normalized to tubulin expression (see graphs alongside blots).

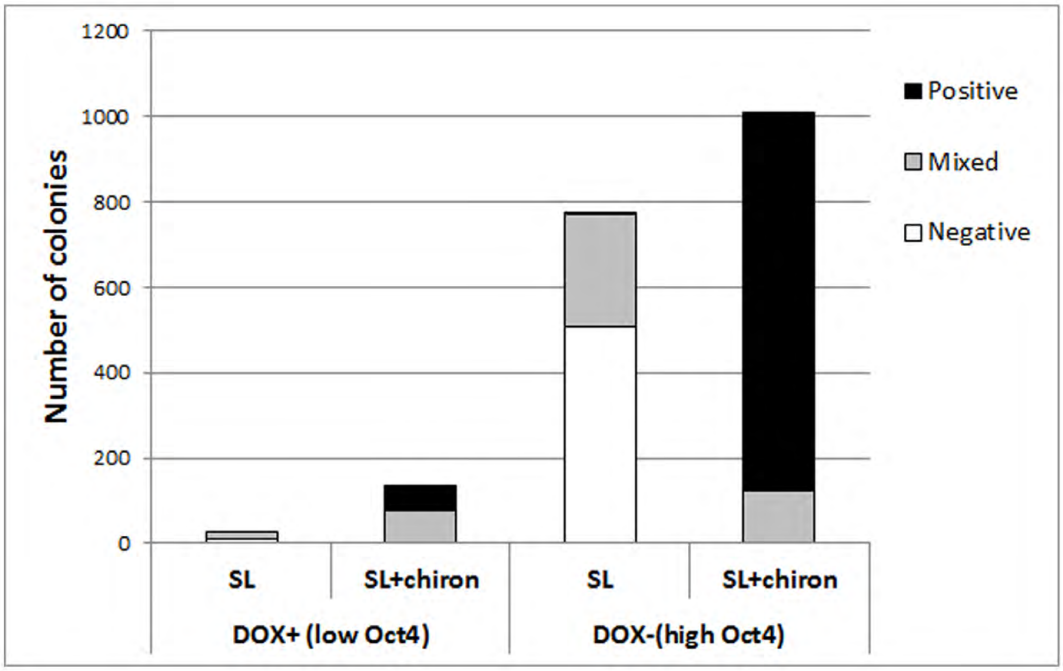
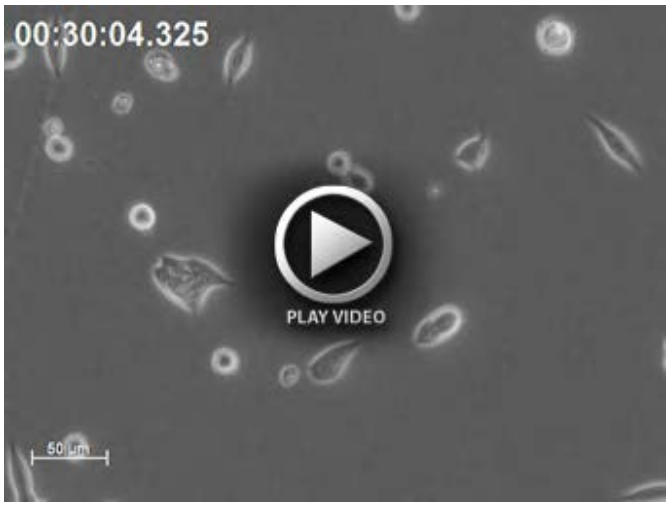
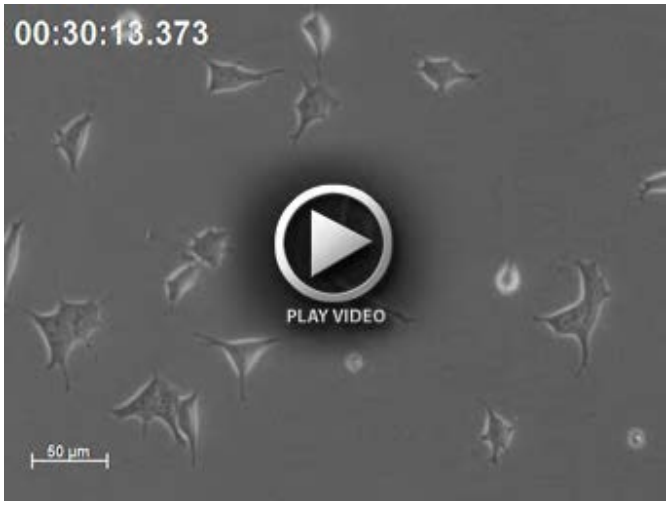


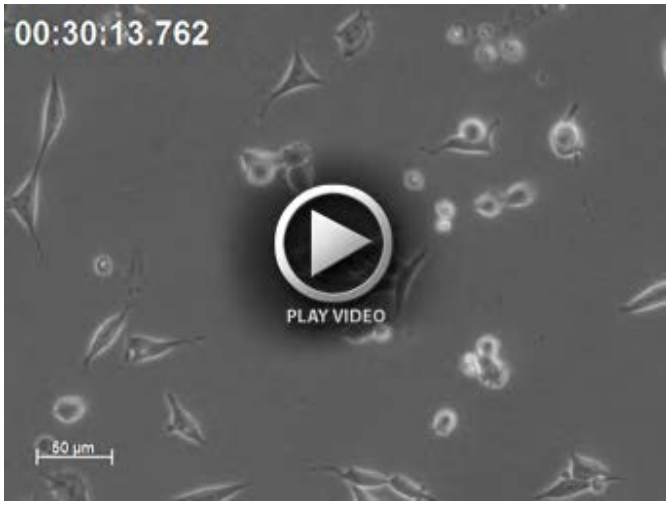
Fig. S10. Oct4 induced differentiation is inhibited by increasing β-catenin levels. AG23191cells overexpressing Oct4 from a TetOFF system were grown in serum+LIF in the presence of absence of chiron. Colonies were assayed for alkaline phosphatase and the quantification of the assay in Fig. 7D is shown.



Movie 1



Movie 2



Movie 3

Movies 1-3. Reduction or loss of b-catenin results in enhanced differentiation even in conditions that promote pluripotency. E14Tg2A (Movie 1), β-catenin$^{fl/-}$ (Movie 2) and β-catenin$^{-/-}$ (Movie 3) cells were grown in 2i+LIF on fibronectin and filmed during 3 days. Note in wild-type mESC (E14Tg2A) the cells grow in tight ball like colonies, with reduction or loss of β-catenin the cells grow in loose colonies and display a differentiated morphology.

Table S1. Primers used

Gene	Sequence
Nanog	TACCTCAGCCTCCAGCAGAT
	GCAATGGATGCTGGGATACT
Oct4	CCAATCAGCTTGGGCTAGAG
	CTGGGAAAGGTGTCCCTGTA
Sox2	CATGAGAGCAAGTACTGGCAAG
	CCAACGATATCAACCTGCATGG
Rex1	GCGGTGTGTACTGTGGTGTC
	GACAAGCATGTGCTTCCTCA
Fgf5	GCTCAATGATCAGAAGGAGGA
	TCAGCTGGTCTTGAATGAGG
Axin2	ACACATGCAGAAATGGGTCA
	ACGTACGGTGTAGCCTTTGG
β-catenin	GGTGGGCTGGTATCTCAGAA
	CTTGTGATCCATTCGTGTGC
Tcf3	ACGGTTCTGGATGAGACAGG
	AAGCAGGGAGCTGTTCAGTG
Gapdh	AACTTTGGCATTGTGGAAGG
	GGATGCAGGGATGATGTTCT