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Differential requirement for the dual functions of β-catenin in embryonic stem cell self-renewal and germ layer formation

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Abstract

Canonical Wnt-signalling has been implicated in mouse and human embryonic stem cell (ESC) maintenance, however its requirement is controversial. β-catenin is the key component in this highly conserved Wnt pathway, acting as a transcriptional transactivator. Yet, β-catenin has additional roles at the plasma membrane regulating cell-cell adhesion, complicating the analyses of cells/tissues lacking β-catenin. We report here the generation of a *β-catenin* deficient mouse ESC (mESC) line and show that self-renewal is maintained in absence of β-catenin. Cell-adhesion is partially rescued by plakoglobin up-regulation, but fails to be maintained during differentiation. When differentiated as aggregates, wild-type mESCs form descendents of all three germ layers, while mesendodermal germ layer formation and neuronal differentiation are defective in *β-catenin* deficient mESCs. A Tcf/Lef-signalling defective β-catenin variant, which re-establishes cadherin-mediated cell-adhesion, rescues definitive endoderm and neuroepithelial formation, suggesting that β-catenin cell-adhesion function is more important than its signalling function for these processes.

Keywords

beta-catenin; plakoglobin; stemness; endoderm differentiation; cell- adhesion

ESCs are self-renewing, fully pluripotent cells. Descendents from all three germ layers can be produced *in vitro* by allowing ESCs to differentiate as aggregates, known as embryoid bodies (EBs) [1–3]. Various signalling pathways, including Leukemia inhibitory factor (LIF),

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Competing financial interest

The authors declare no competing financial interest.

Author Contributions

NL performed, analysed and interpreted experiments. MW and DM performed pull-down studies, TB and RM generated and provided BAR/fuBAR lentiviruses, and commented on the paper. CH supervised the study and wrote the paper together with NL.

bone morphogenetic protein 4, and Wnt/β-catenin signalling can maintain mouse ESCs self-renewal capacity [4–7]. Although cytokines required for human and mESCs differ substantially [8–11], activation of Wnt/β-catenin signalling is beneficial for both [12]. Wnt-ligand binding to its receptor complex, composed out of a serpentine receptor encoded by the *Frizzled* gene family and a co-receptor of the low-density lipoprotein related family encoded by the *Lrp5* and *Lrp6* genes, activates the pathway. Ligand-binding results in inhibition of the destruction complex composed out of Adenomatosis polyposis coli, Axin, Casein kinase 1α (CK1α) and the Glycogen synthase kinase 3β (GSK3β) [13, 14]. This changes the fate of β-catenin, which in absence of Wnt-ligands would be phosphorylated by CK1α and GSK3β, subsequently ubiquitinated and degraded via the proteosome pathway. In response to Wnt-signalling, β-catenin accumulates, translocates to the nucleus and transactivates target genes, such as *Axin2*, in a complex with TCF/LEF transcription factors [14].

Wnt/β-catenin requirement in ESC self-renewal has been addressed yielding somewhat contradictory results [15–18]. Inhibition of Wnt/β-catenin signalling in mESCs using a dominant-negative ΔNhLef1 did not affect self-renewal [16], while studies analyzing β-catenin deficient mESCs differed substantially. Two studies, using either a previously [19] or a newly established β-catenin deficient mESC line, reported defects in stemness [15, 18]. A third study, using β-catenin null mESCs of unclear origin, reported no such defects [17]. In two of the three studies the mESCs, according to their molecular characterization, were actually epiblast-like stem cells (EpiSCs) [15, 17]. Thus, there is clearly need to establish additional independent β-catenin deficient mESCs in order to shed light on the requirement of Wnt/β-catenin in ESC self-renewal. Yet, β-catenin is not only an essential component of the canonical Wnt pathway, it is also an integral component of adherens junctions linking classical cadherins to α-catenin and the actin cytoskeleton [20]. This second function of β-catenin can also be performed by the related family member plakoglobin, which is also an essential desmosomal component [19, 21–23]. Given the dual functions of β-catenin it is difficult to unambiguously distinguish between β-catenin requirement in Wnt-signalling versus cell-adhesion in cellular and developmental processes.

Here we established new *β-catenin* deficient mESCs with a deletion of exons 3–6 to address the role of β-catenin in mESC self-renewal and differentiation and show thatβ-catenin is not required for self-renewal. Under self-renewal conditions, plakoglobin partially substitutes for the β-catenin cell-adhesion function, but fails to do so during differentiation. In order to address to what extent β-catenin signalling versus cell-adhesion functions are required during *in vitro* differentiation of mESCs, we generated β-catenin-rescued mESCs expressing either wild-type or a TCF/LEF-signalling defective β-catenin variant. Our findings show that the cell-adhesion function of β-catenin is more important than its signalling function for definitive endoderm formation and promotion of neuronal differentiation.

Results

Characterization of β-catenin deficient mESCs

mESCs were derived from β-catenin fl/fl ($\beta\text{-}cat^{fl/fl}$) [24] blastocysts and individual feeder-free $\beta\text{-}cat^{fl/fl}$ mESCs were established using LIF and serum. Several *β-catenin* deficient mESC

subclones (referred to as *β-cat*$^{\Delta/\Delta}$) were established from one *β-cat*$^{fl/fl}$ mESC line (NLβ-12) using adenoviral mediated *Cre*-recombinase and two were used for further characterization (Fig. 1a). The parental *β-cat*$^{fl/fl}$ mESC line, NLβ-12, was fully pluripotent as it gave rise to chimeric mice and transmitted through the germline (see Supplementary Information, Fig. S1 online).

β-cat$^{\Delta/\Delta}$ and *β-cat*$^{fl/fl}$ mESCs showed an overall similar proliferation behaviour and colony appearance (Fig. 1b, c). *β-cat*$^{\Delta/\Delta}$ colonies stained negative for β-catenin by immunocytochemistry, while E-cadherin and plakoglobin staining and distribution were normal (Fig. 1d). Interestingly, staining intensity of the latter was increased, as were protein levels (Fig. 1d, e), but transcript levels were unchanged (Fig. 1f). Thus, similar to F9 teratocarcinoma stem cells, plakoglobin substitutes for β-catenin cell-adhesion function in mESCs [22]. This was confirmed by immunoprecipitation experiments, showing increased plakoglobin levels bound to E-cadherin in *β-cat*$^{\Delta/\Delta}$ versus *β-cat*$^{fl/fl}$ mESCs (Fig. 1g).

Plakoglobin has reportedly a weak *in vitro* signalling activity [25, 26]. Therefore, we analysed TCF/LEF-mediated gene expression using the sensitive lentiviral luciferase β-catenin activated reporter (BAR) system, with 12 multimerised TCF/LEF binding sites [27]. Without external stimulation minimal reporter activity was detected in control and *β-cat*$^{\Delta/\Delta}$ mESCs (Fig. 1h). Upon stimulation with the GSK3 inhibitor BIO, recombinant Wnt3a (rWnt3a), or Wnt3a conditioned medium (Wnt3aCM) BAR-reporter activity was increased in control, but not detectable above ground levels in *βcat*$^{\Delta/\Delta}$ mESCs (Fig. 1i, and data not shown). Likewise, *Axin2* levels were increased in control, but not in *β-cat*$^{\Delta/\Delta}$ mESCs (Fig. 1j). Stimulation had no effect on plakoglobin levels, nor did *plakoglobin* knock-down affect basal *Axin2* levels (data not shown). Thus, our data suggest that the 2-fold increase in plakoglobin cannot substitute for β-catenin/TCF/LEF-mediated signalling activity.

Analysis of stem cell markers

No difference in *Oct4*, *Nanog*, *Rex1* and *Sox2* transcriptional (Fig. 2a), nor Oct4 and Nanog protein levels (Fig. 2b) were observed between *β-cat*$^{fl/fl}$ and *β-cat*$^{\Delta/\Delta}$ mESCs. FACS analysis showed no difference in SSEA-1 levels (data not shown). Furthermore, Stat3 phosphorylation kinetics was not altered (Fig. 2c). Late passages of *β-cat*$^{\Delta/\Delta}$ and *β-cat*$^{fl/fl}$ mESCs stained positive for the self-renewal marker alkaline phosphatase (AP) (Fig. 2d) and showed no differences in colony formation ability (data not shown). Concomitantly, transcriptomes analyses of *β-cat*$^{fl/fl}$ and *β-cat*$^{\Delta/\Delta}$mESCs under standard conditions revealed significant differences ($P<0.05$) in only five genes besides β-catenin; with *l7Rn6*, *H19*, and *Rhox5/Pem* being elevated, and *Trpv6* and *Daam1* being decreased (see Supplementary Information, Table S1).

Overall, our data suggest that β-catenin activity is not required for mESC self-renewal in presence of LIF and serum. To further establish that β-catenin is dispensable for mESC self-renewal, we tested *β-cat*$^{\Delta/\Delta}$ mESCs under serum-free conditions using the 2i+LIF system, inhibiting mitogen-activated kinase kinase (MEK) using PD0325901, and GSK3 using CHIR99021 [16]. *β-cat*$^{\Delta/\Delta}$ mESCs could readily be established in 2i+LIF from *β-cat*$^{fl/fl}$ mESCs using adenovirally provided *Cre*-recombinase, but displayed a different morphology (Fig. 2e). Yet, *Nanog*, *Oct4*, *Sox2* and *Rex1* expression levels were indistinguishable from

controls (Fig. 2f). *β-cat*$^{\Delta/\Delta}$ mESCs formed colonies also under serum-free conditions in presence of PD0325901+LIF or CHIR99021+LIF, but not under PD0325901+CHIR99021 conditions (see Supplementary Information, Fig. S2 online). This establishes that β-catenin deficient mESCs are absolutely LIF dependent and that the GSK3 inhibitor, CHIR99021, has weak, β-catenin-independent effects. However, *β-cat*$^{\Delta/\Delta}$ mESC propagation was only possible under serum-free conditions in presence of PD0325901+CHIR99021+LIF or PD0325901+LIF (data not shown).

Minor cell-adhesion defects in mESCs

β-cat$^{\Delta/\Delta}$ and control mESCs colonies looked similar in serum+LIF (Fig. 1c). However, we noticed subtle differences and analysed colonies at the ultrastructural level by transmission electron microscopy (TEM). This revealed cell-adhesion defects in *β-cat*$^{\Delta/\Delta}$ mESC colonies, with apparent regions lacking cell-cell contacts (Fig. 3a), suggesting that plakoglobin cannot fully substitute for β-catenin. To show that plakoglobin indeed substitutes in part for β-catenin cell-adhesion, we performed siRNA knock-down (Fig. 3b). Corresponding with transfection efficiency about 85% of *plakoglobin* siRNA treated *β-cat*$^{\Delta/\Delta}$ mESC colonies displayed a differentiated morphology, with cells being more dispersed and flattened. In *β-cat*$^{fl/fl}$ only 5% had a different morphological appearance, while control, *lamin A/C* siRNA treated cultures, showed no alterations (Fig. 3c; see Supplementary Information, Fig. S3b online). Concomitant to the morphological changes, the cell-adhesion molecules PECAM-1 and E-cadherin were lost from the membrane and their protein levels reduced upon *plakoglobin* siRNA treatment (Fig. 3b–d). Expression of self-renewal markers, *Nanog*, *Oct4* and *Sox2*, was, however not altered; only proliferation was slightly decreased (see Supplementary Information, Fig. S3c online). This shows that in absence of β-catenin plakoglobin is an essential component of cell-adhesion in mESCs, mediating adhesion via adherens junctions and desmosomes, and supports previous findings that cell-adhesion is not essential for self-renewal maintenance [16, 17]. Furthermore, our data establish no requirement for plakoglobin in self-renewal maintenance. Next, we performed rescue experiments expressing different β-catenin versions, wild-type (βcat^{WT}), C-terminal truncated (β-cat$^{\Delta C}$), and a point-mutated version (β-cat^{M6}) [28] from the *Rosa26* locus in *β-cat*$^{\Delta/\Delta}$ mESCs, referred to in the following as *β-cat*^{rescWT}, *βcat*$^{resc\Delta C}$, and *β-cat*^{rescM6}. All three variants showed membrane localization (see Supplementary Information, Fig. S4a online) and restored cell-adhesion, as well as membranous localization of E-cadherin and PECAM-1 upon *plakoglobin* siRNA treatment (Fig. 3c, d). All three interacted with E-cadherin and their presence resulted in a slight reduction of plakoglobin (see Supplementary Information, Fig. S4b online). A slight further reduction was observed using mESCs stably expressing β-catenin isoforms under the CAG promoter yielding slightly increased levels of β-catenin variants (see Supplementary Information, Fig. S4c, d online), supporting the notion that the increased plakoglobin levels are due to protein stabilization via its recruitment to adherens junctions substituting for the loss of β-catenin.

Cell-adhesion defects during EB differentiation

The *in vitro* differentiation potential of *β-cat*$^{\Delta/\Delta}$ mESCs was analysed using EB differentiation. This revealed cell-adhesion defects at day 5, reflected in an increase of single cells and associated with a size decrease of *β-cat*$^{\Delta/\Delta}$ compared to control EBs (Fig. 4a).

Controls formed cyst-like structures at day 9, while this did not occur with *β-cat*$^{\Delta/\Delta}$ cells (Fig. 4a, and data not shown). Concurrent with the appearance of cell-adhesion defects, plakoglobin levels decreased from day 5 onwards in *β-cat*$^{\Delta/\Delta}$, but also in control EBs (Fig. 4b). The reduced plakoglobin levels were probably insufficient to compensate for lack of β-catenin, while in control EBs β-catenin steadily increased. EB differentiation proceeded normally in the different Rosa26 rescued mESCs (Fig. 4a). To confirm previous observations suggesting that the βcat$^{\Delta C}$ and β-cat^{M6} variants are compromised in TCF/LEF transcriptional activity [28], we generated the corresponding stable BAR reporter lines and activated canonical Wnt-signalling using CHIR99021. Surprisingly low reporter activity was observed in *βcat*^{rescWT} and no activity in *β-cat*^{rescΔC} or *β-cat*^{rescM6} mESCs (Fig. 5a). Since Rosa26-transgene expression was relatively low and given that residual TOPFlash reporter activity had been observed previously [28], we also assayed BAR reporter activity in the stable CAG-rescue mESCs. Here, the CAG-WT^{resc} cells showed a more pronounced response and residual activity was observed in CAG-M6^{resc}, but not in CAG-ΔC^{resc} mESCs (Fig. 5a). Accordingly, expression of the endogenous Wnt/β-catenin/TCF regulated genes, *Axin2*, *Cdx1* and *Brachyury*, was not up-regulated in *β-cat*$^{\Delta/\Delta}$ and *βcat-CAG*^{rescΔC} mESCs (Fig. 5b). Together, our data show that the β-catenin ΔC variant is defective in TCF/LEF mediated signalling.

Subsequent EB differentiation studies were performed using the Rosa26 *βcat*^{rescWT} and *β-cat*^{rescΔC} mESCs, since *β-cat*^{M6} mESCs showed residual TCF/LEF- mediated activity and observed transgene-silencing in CAG-rescued mESCs during EB differentiation (data not shown),

Rescue of Endoderm and Neuronal Differentiation Defects

Genetic inactivation of *β-catenin* in mice results in an early developmental arrest around E7.0, with defects in anterior-posterior axis, mesoderm, definitive endoderm and ectoderm formation [19, 29]. Maternal β-catenin is provided until E4.5 and might therefore compensate for loss of zygotic β-catenin activity obscuring its absolute requirement in early mouse development [29, 30]. EB formation from mESCs allows differentiation of descendents of all three germ layers and resembles to a large degree normal postimplantation embryonic development [2, 3, 31, 32]. Thus, enabling us to examine the *in vitro* differentiation potential of *β-cat*$^{fl/fl}$, *β-cat*$^{\Delta/\Delta}$, *β-cat*^{rescWT}, and *βcat*^{rescΔC} mESCs. Expression profiles of the early ectodermal markers *Nestin*, *Fgf5* and *Pax6* were similar in *β-cat*$^{\Delta/\Delta}$ and *β-cat*$^{fl/fl}$ EBs during differentiation (Fig. 6a), suggesting that ectoderm induction occurs normally. Nevertheless, differentiation ofβ3-tubulin (TuJ) positive neurons was severely compromised in *β-cat*$^{\Delta/\Delta}$ EBs (Fig. 6b). In contrast, TuJ positive neurons were readily detected in EBs derived from *βcat*^{rescWT} and signalling-defective *β-cat*^{rescΔC} mESCs (Fig. 6b). Neuronal differentiation in monolayer cultures revealed that *β-cat*$^{\Delta/\Delta}$ mESCs have, in principle, the potential to differentiate into neurons, but compared to *β-cat*$^{fl/fl}$ mESCs this is strongly reduced (Fig. 6c). Again differentiation of TuJ positive neurons was restored in *β-cat*^{rescWT} and signalling-defective *β-cat*^{rescΔC} cells (Fig. 6c).

Mesendodermal marker, *Mixl1*, expression was absent in *β-cat*$^{\Delta/\Delta}$ EBs, but restored in *β-cat*^{rescWT} and signalling-defective *β-cat*^{rescΔC} EBs (Fig. 7a). Similarly, expression of the

endodermal markers, *Foxa2* and *Gata6*, was absent in *β-cat*$^{\Delta/\Delta}$ EBs (Fig. 7a), but restored in *β-cat*^{rescWT} and *β-cat*^{rescΔC} EBs (Fig. 7a, see also Supplementary Information, Fig. S5 online). Loss and rescue of endoderm formation in EBs derived from *β-cat*$^{\Delta/\Delta}$ and *β-cat*^{rescWT} and *β-cat*^{rescΔC} mESCs, respectively, was confirmed by immunofluorescent staining for the endodermal markers Gata4, Foxa2 and Cxcr4 (Fig. 7b, c). In *β-cat*$^{fl/fl}$ EBs endodermal cells stained positive for Gata4, E-cadherin, and Cxcr4 and located to the outer region associated with a fibronectin-positive basement membrane (Fig. 7b). In *β-cat*$^{\Delta/\Delta}$ EBs, Gata4 staining was severely reduced, staining for fibronectin and E-cadherin was very dispersed, and the Cxcr4 positive cells did not localize to the outer layer (Fig. 7b). In contrast, an outer layer with localized Gata4, E-cadherin and Cxcr4 staining was restored in *β-cat*^{rescWT} *and β-cat*^{rescΔC} EBs (Fig. 7b). The presence of Cxcr4 suggests that definitive endoderm is being formed [33]. Foxa2 positive cells were detected in dissociated EBs derived from *β-cat*$^{fl/fl}$, *β-cat*^{rescWT} *and β-cat*^{rescΔC} mESCs, but not in EBs derived from *β-cat*$^{\Delta/\Delta}$ mESCs (Fig. 7c). Thus, our data suggest that TCF/LEF-mediated signalling activity is not absolutely required for endoderm formation. In addition, our data suggest that the cell-adhesion function of β-catenin promotes neuron formation *in vitro*. In agreement with the *in vitro* data, we found that *in vivo β-cat*^{rescWT} *and β-cat*^{rescΔC} mESCs show a higher chimeric embryonic contribution compared to *β-cat*$^{\Delta/\Delta}$ mESCs and that they can in principle contribute to all three germ layers, but were preferentially found in endodermal and ectodermal layers (see Supplementary Information, Fig. S6 online).

Discussion

Canonical Wnt-signalling activation has been implicated in pluripotency and self-renewal maintenance in human and mouse ESCs [7, 34–36]. Nevertheless, its absolute requirement for mESCs self-renewal is still unclear. While nuclear localization of β-catenin has been reported in wild-type mESCs under standard conditions [15, 37], we and others failed to observe nuclear β-catenin or significant TCF/LEF mediated transcriptional activity in wild-type ESCs [7, 15, 34, 38]. Still, the possibility of β-catenin having positive effects on ESC self-renewal in a TCF/LEF-independent manner remains. This possibility was favoured in a recent study reporting that *β-cat*$^{-/-}$ mESCs, generated by Huelsken and colleagues, displayed defects in stemness marker expression [15, 19]. The overall changes in this *β-cat*$^{-/-}$ mESC line, however, exhibit an epiblast-like gene expression profile [15]. In contrast, the *β-cat*$^{\Delta/\Delta}$ mESCs described here, generated from a fully pluripotent *β-cat*$^{fl/fl}$ mESCs *in vitro* by *Cre*-mediated recombination, showed no alteration of self-renewal markers under serum and serum-free conditions and only few differences by transcriptomes analysis. Our data are in accordance to data in the accompanying study by Wray et al [39], which independently established *β-catenin* deficient mESCs. Collectively, our data show that mESC self-renewal does not absolutely require endogenous β-catenin activity and as we have shown does also not depend on plakoglobin in the absence of β-catenin. Furthermore, our two studies demonstrate that β-catenin deficient cultures are absolutely LIF-dependent. This is in contrast to a recent report showing that *β-cat*$^{-/-}$ mESCs maintain self-renewal in absence of LIF, but depend on activin [17]. Activin-dependence is one of the hallmarks of EpiSCs, which express Oct4, but are LIF independent [40]. Thus, the *β-cat*$^{-/-}$ mESCs used in the study by

Soncin and colleagues are probably EpiSCs [17], explaining the differences in LIF dependency.

In addition to its central role in Wnt-signalling, β-catenin is a component of adherens junctions connecting classical cadherins via α-catenin to the actin cytoskeleton [20]. Plakoglobin can also bind to cadherins and participate in adherens junctions [20, 41, 42] and has been shown to substitute there for β-catenin [19, 22, 23, 29]. Similar to F9 teratocarcinoma cells [22], membranous plakoglobin levels were increased in β-catenin deficient mESCs. This was not associated with altered transcription, ruling out a transcriptional feed-back and suggesting a post-transcriptional mechanism via prolonged protein stability due to augmented recruitment of plakoglobin to adherens junctions in absence of β-catenin. Posttranscriptional regulation of protein levels seems a common mechanism in cell-adhesion; e.g. α-catenin regulation by E-cadherin[43]. However, our study shows that plakoglobin cannot fully compensate for lack of β-catenin. In agreement with the observation that α-catenin levels influence the strength of E-cadherin bonds between cells [44–46], we propose that the α-catenin reduction in the E-cadherin-plakoglobin complexes weakens mESC cell-adhesion and furthermore our data suggest that plakoglobin and β-catenin might bind α-catenin differentially. In accordance with other *in vivo* finding[19, 47–50], but contrary to *in vitro* findings[25, 26, 51], we found no evidence that plakoglobin participates in TCF/LEF-mediated signalling activity.

Embryos lacking zygotic β-catenin activity show defects in A/P patterning, mesoderm and definitive endoderm formation [19] and increased ectodermal apoptosis [29]. Blastomere adhesion defects become apparent upon removal of maternal β-catenin [52], which are not substituted for by plakoglobin, as it is not present at early preimplantation stages, and associates with E-cadherin and α-catenin only from the early morula stage onwards [30]. Wnt/β-catenin pathway activation occurs in the embryo around E6.5 and in EBs around day 3–4 [32, 53]. Thus, maternal β-catenin may participate in cell-adhesion in embryos lacking zygotic β-catenin, but not in canonical Wnt-signalling. β-catenin deficient EBs displayed adhesion defects during differentiation, which were associated with plakoglobin down-regulation. Furthermore, mutant EBs showed, similar to what has been observed in *β-catenin* mutant embryos, defects in endoderm induction, while ectoderm induction occurred normally. The rescue of induction and formation of definitive endoderm in EBs derived from *β-cat*^{rescΔC} mESCs suggests that the cell-adhesion function of β-catenin is required at least for endoderm formation. Mesoderm formation was neither rescued in *β-cat*^{rescΔC} nor control *β-cat*^{rescWT} mESCs, as judged by the absence of *Brachyury* expression (data not shown). This is likely to be due to the moderate Rosa26 transgene levels, which are probably too low to allow participation in signalling. By addition of the CAG promoter [54] we augmented transgene expression from the Rosa26 locus and rescued *Brachyury* expression in EBs derived from Rosa26-CAG *βcat*^{rescWT}, but not from Rosa26-CAG *β-cat*^{rescΔC} mESCs (see Supplementary Information, Fig. S5 online). This is in agreement with the notion that *Brachyury* expression is under transcriptional β-catenin control [55, 56]. Thus, mesoderm induction may not require β-catenin cell-adhesion function to the same extend as its transcriptional activity.

The fact that loss of β-catenin affected neuronal differentiation was unexpected, given that ectoderm induction occurred normally in β-catenin deficient EBs and accumulating *in vivo* and *in vitro* evidence suggests that active canonical Wnt- signalling inhibits neuronal differentiation [16, 53, 57–59]. Nevertheless, our finding coincide with other *in vitro* data, showing that Wnt/β-catenin signalling might actually promote neuronal differentiation [60, 61]. But, most excitingly our data from the Rosa26 *β-cat*^{rescΔC} EBs, support *in vivo* findings that β-catenin participating in cell-adhesion plays a permissive role in neuronal differentiation by supporting neuroepithelial formation [62, 63]. Given that α-catenin has been implicated in brain development [64], we can however, not completely rule out that neuronal differentiation defects are not in part due to the observed altered α-catenin distribution (Fig. 1g and see also Supplementary Information, Fig. S7 online). However, as nuclear levels were not altered (see Supplementary Information, Fig. S7a,b online), we exclude a potential role in the nucleus [65]. The previously proposed model implies, in agreement with our findings, that the number of functional adherens junctions controls brain development, and that this may involve a Hh-signalling feed-back loop [64]. Whether the latter is also the case in our system remains to be seen.

Cell adhesion is an important aspect of embryonic development. For bi-functional molecules such as β-catenin it is utterly important to decipher its individual functional contributions in development. Our data suggest, that β-catenin cell-adhesion and not its TCF/LEF-mediated signalling function is important for neuroepithelial and endoderm formation in EBs. Thus, despite plakoglobin up-regulation in *β-catenin* mutants at E7.0 [19], cell-adhesion defects may contribute to some phenotypic aspects, as others have recently observed[63]. In this context it is interesting that *Wnt3* mutants and double-mutants for *Lrp5/6* phenocopy *β-catenin* mutants, with the exception that anterior visceral endoderm (AVE) movement towards anterior still occurs [19, 66, 67]. Thus, the failure to proper position the AVE in *β-catenin* mutants might be associated with defects in cell-adhesion.

Methods

mESC Establishment, Culture Conditions, Blastocyst injections

Individual day 3.5 old blastocysts isolated from *β-cat*$^{fl/fl}$ females intercrossed with *β-cat*$^{fl/fl}$ males were seeded on irradiated MEFs in 4-well plates in ESC medium (high glucose DMEM, 15% FBS, 2mM L-glutamine, 2mM penicillin/streptomycin, 1mM sodium pyruvate, 1x MEM non-essential amino acids, 0.1mM β-mercaptoethanol, 1000 U/ml LIF). After 5–7 days the inner cell mass was dissociated using 0.05% trypsin-EDTA and plated on MEFs. From these cultures colonies with ESC-like morphology were selected, eventually adapted for feeder-free conditions and further characterized. *β-cat*$^{Δ/Δ}$ mESCs were established from the fully pluripotent NLβ-12 *β-cat*$^{fl/fl}$ mESC line through infection with an Adeno-Cre virus (University of Iowa) at MOI 150 for $5×10^4$ mESCs. 24 hrs later individual mESCs were seeded in 96-wells and PCR genotyped (primers: 5′-AGAATCACGGTGACCTGGGTTAAA-3′, 5′-CAGACAGACAGCACCTTCAGCACTC-3′, 5′-CAGCCAAGGAGAGCAGGTGAGG-3′). Two mESC clones of each genotype, *β-cat*$^{fl/fl}$ and *β-cat*$^{Δ/Δ}$, were further characterized. For serum-free cultures mESCs were cultured in N2B27 medium (StemCellSciences)

supplemented with 1000 U/ml EsgroLIF (Chemicon), 3μM CHIR99021 and 1μM PD0325901 (both StemGent). Blastocyst injections were performed with the mESC clone NLβ-12 (on feeder, passage 18). The resulting high-grade chimera was crossed to C57Bl/6 and the offspring was analysed by PCR for germ line transmission of the *β-catenin* floxed allele. Blastocyst injections were also performed for β-cat^{rescWT} and β-cat^{rescΔC} mESCs clones stably marked with H2B-Gfp (Addgene plasmid 11680) [68] injecting 10–15 mESCs per blastocyst. Embryos were recovered 6 days later, staged and visually examined for their grade of chimerism using a LSM 710 Spectral Confocal microscope (Zeiss).

Rosa26 and stable pCAGGS rescue mESCs and siRNA experiments

Targeting into the Rosa26 locus was performed using a modified Rosa26 targeting vector, pR26hygro (5′homology arm-SA-XbaI-SV40polyA-loxP-PGKhygro-loxP-3′ homology arm-PGKDTA) (kindly provided by A. Wutz) into which either wild-type full-length mouse β-catenin or N-terminal myc-tagged human versions of C-terminal truncated (ΔC) or M6 β-catenin (expression plasmids kindly provided by R. Grosschedl) had been inserted into the XbaI site by blunt-end cloning. For the Rosa26-CAG rescue lines myc-tagged wild-type and ΔC β-catenin were first cloned behind the CAG-promoter, fragments containing the CAG promoter and the β-catenin variants were then isolated and inserted into the XbaI site of pR26hygro by blunt-end cloning. Correctly targeted mESCs were identified by Southern blotting using Rosa26 5′ and 3′ external probes. For stable CAG-mESC lines, open reading frames were inserted by blunt-end cloning into the XhoI site of a modified pCAGGS vector containing a PGK neomycin selection cassette. Individual neomycin-resistant clones were established and tested by western for their β-catenin levels. For transient siRNA transfections mESCs were seeded onto 6-well plates at 2.5×10^4 cells/well the day before Lipofectamin2000 (Invitrogen) mediated transfection with 150 nM *plakoglobin* (5′-ACACCUACGACUCGGGCAU-3′, 5′-CCAAGCUGCUCAACGAUGA-3′, 5 ′-GGAACUACAGCUACGAGAA-3′, 5 ′-UGAGUGUAGAUGACGUCAA-3′) o r *LaminA/C* (5′-UGACCAUGGUUGAGGACAA-3′) OnTarget siRNA mix (Dharmacon).

Generation of Stable BAR Reporter Lines and Luciferase Assays

Stable BAR (beta-catenin activated reporter) and fuBAR (found-unresponsive beta-catenin activated reporter) mESCs were established by infecting mESCs with pBARLS and pFuBARLS lentiviruses followed by puromycin selection for 7 days. For TOPFLASH/FOPFLASH luciferase reporter assays BAR and fuBAR mESCs were seeded onto 12-well plates at a concentration of 5×10^4 cells/well. The Wnt pathway was activated the next day by treatment with 200 ng/ml rWnt3a (R&D Systems), 50% Wnt3aCM from stable Wnt3a producing L-cells, 2 μM BIO (Calbiochem) or 3 μM CHIR99021 respectively. Luciferase assays were performed in triplicates after 24 hrs using Dual-Glo luciferase assay (Promega) and the Synergy Fluorescence Plate Reader using at least two biological samples.

EB and neuron differentiation cultures

For EB differentiation, 1×10^6 feeder independent mESCs were plated onto 15 cm low attachment plates (Corning) and cultured in mESC medium without LIF (differentiation medium). For the hanging drop method 1×10^3 cells were plated per drop of differentiation

medium and transferred after 60 hrs to low attachment plates (Corning). EBs were collected at indicated time points fixed in 4% paraformaldehyde and processed for immunocytochemical stainings or embedded in OCT (TissueTek) for cryosectioning. For high-density monolayer differentiation, 4×10^4 cells/cm2 mESCs were plated on laminin-coated coverslips in N2B27 medium (StemCellSciences) and cultured for 14 days. Medium was renewed every second day. TuJ-positive cells were counted in random fields.

AP staining, Immunohistochemistry and TEM

AP staining was performed using the alkaline phosphatase kit according to manufactures instructions (Sigma Aldrich). Immunohistochemistry was performed upon mESCs or EBs seeded on gelatin covered 12 mm glass coverslips (Novodirect) after 20 min fixation with 4% paraformaldehyde, followed by 10 min permeabilization in PBS with 0.5% Triton-X and a 1 hr blocking step with PBS/2% BSA. For immunohistochemistry on cryosection, EBs from hanging drop cultures were fixed for 30 min in 4% paraformaldehyde, embedded in OCT and sectioned at 8 μm. Incubation with primary antibodies against Nanog, CXCR4 (both 1:100; Abcam), β-catenin (anti-C-terminus 1:250; BD Transduction; anti-N-terminus 1:200; Alexis Biochemicals), E-cadherin, plakoglobin, PECAM-1 (all 1:200; BD-Transduction), Oct3/4 (1:100), Foxa2 (1:100), TuJ (1:200), GATA-4 (1:500) (all from Santa Cruz), Fibronectin (1:200; Sigma) was performed for 1 hr at RT or oN at 4°C in PBS/2% BSA. Incubation with corresponding secondary anti-mouse, anti-rabbit or anti-goat antibodies coupled with Alexa488 or Alexa674 (1:1000; Molecular Probes) were performed for 1 hr at RT in the dark in PBS/2% BSA. To stain nuclei, samples were incubated for 5 min in 10 μg/ml DAPI (Sigma) and mounted with ProLong® Gold antifade reagent (Molecular Probes). Pictures were taken using the LSM 510 Meta/Axiovert200M confocal microscope. For TEM, mESCs were grown for 2 days on gelatinized 12 mm UV-sterilized Aclar coverslips, fixed in 2% glutaraldehyde for 1 hr, rinsed 3 times with PBS, treated with 2% OsO_4, rinsed with PBS, dehydrated 10 min each in a graded series of EtOH (40, 60, 80, 95, 100%). For embedding coverslips were incubated in a 100% EtOH/epoxy resin mixture (1:1) for 30 min at RT, followed by two 30 min incubations of the coverslips on 100% epoxy resin after which the coverslips were placed onto resin filled lids of Beem capsules and polymerized for 48 hrs at 60 °C. Resin blocks were sectioned on a Leica Ultracut UCT microtome. Sections were collected on metal grids and post-stained with Reynolds lead citrate $(PbNO_3)_2$ and uranyl acetate. Images were taken on a FEI Morgagni 268 electron microscope.

PCR analyses, Microarrays, Western blots and Co-Immunoprecipitation

RNA was isolated using RNeasy Kit (Qiagen). 1–2 μg RNA was used to prepare cDNAs using the SuperscriptII first strand cDNA synthesis system (Invitrogen). Amounts equivalent to 10–20 ng of the initial total RNA input were used as templates for real-time and RT-PCR analyses using gene primer pairs spanning exon-intron boundaries. Real-time PCR was performed using SYBR greenI nucleic acid gel stain (Roche) and TAKARA rTaq at least in duplicates. Products were analysed by agarose gel electrophoresis and melting curves. Values were calculated using the comparative ΔC(t) method and normalized to HPRT values, for relative expression levels displayed in histograms the values of control fl/fl mESCs were always set to 1. For semiquantitative RT-PCR *mGAPDH* and *mHPRT* were

used as controls. All primers are listed in Supplementary Information, Table S2 online. In-house microarray platforms were used to compare total RNA from *β-cat*$^{fl/fl}$ and *β-cat*$^{\Delta/\Delta}$ mESCs. Cy3 and Cy5-labeled cRNA was prepared from 3–5 μg of total RNA by oligo-dT-primed reverse transcription. Activation/repression ratios for individual spots were calculated using GenePixPro5-chip software. Two biological replicas were analysed.

For western blots and immunoprecipitation (IP) analyses, protein was extracted from cultured mESCs or EBs. Protein concentrations were determined after Bradford (Biorad). For westerns, if not otherwise noted 10–20 μg extract was loaded per lane. For nuclear extracts cells were fractionated after [69] or the NE-PER Kit (Pierce) was used for nuclear and cytoplasmic protein extractions. For IPs Gammabind Plus Sepharose Beads (Amersham) and 1–2 mg of pre-cleared lysate were used. Incubations with 2 μg and 10 μg antibodies against E-cadherin and α-catenin, respectively, were performed at 4°C oN. Primary antibodies were directed against β-catenin (1:2000; anti-C-terminus, BD Transduction; anti-N-terminus, Alexis Biochemicals), plakoglobin (1:6000; (BD-Transduction), E-cadherin (BD-Transduction), α-catenin (Chemicon), tubulin (1:6000; Sigma), Stat3, phospho (705Tyr)-Stat3, myc-tag (Cell Signaling) and proteasome 20S-C2 (Abcam), HP1α (Cell Signaling), and used at dilution 1:1000, if not otherwise noted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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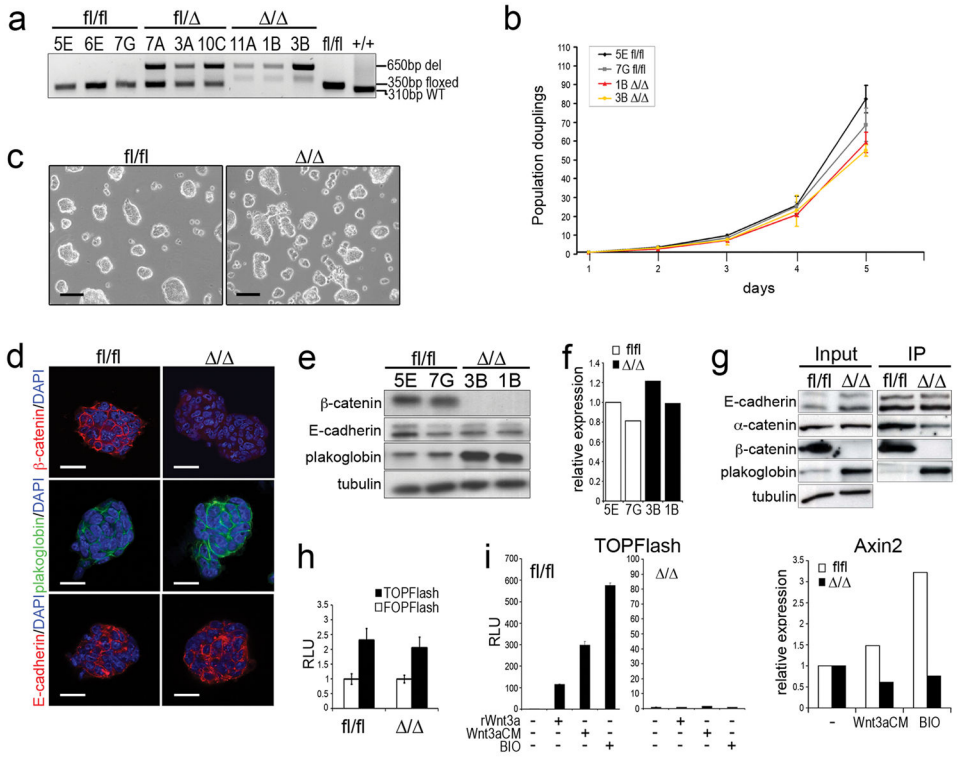

Figure 1.

Characterization of β-cat$^{fl/fl}$ and β-cat$^{Δ/Δ}$ mESCs.

(**a**) Genotyping PCR for β-cat$^{fl/fl}$, β-cat$^{fl/Δ}$ and β-cat$^{Δ/Δ}$ mESC clones.

(**b**) Population doublings of β-cat$^{fl/fl}$ and β-cat$^{Δ/Δ}$ mESC clones grown for 5 days. Cells were collected and counted each day (performed in triplicates).

(**c**) Morphological appearance of β-cat$^{fl/fl}$ and β-cat$^{Δ/Δ}$ mESCs. Scale bar: 100 μm.

(**d**) Confocal images of immunofluorescent stainings for β-catenin, plakoglobin and E-cadherin of β-cat$^{fl/fl}$ and β-cat$^{Δ/Δ}$ mESC colonies. Scale bar: 20 μm.

(**e**) Western blots for β-catenin, E-cadherin, plakoglobin and tubulin in mESCs.

(**f**) Relative plakoglobin expression levels in β-cat$^{fl/fl}$ and β-cat$^{Δ/Δ}$ mESCs (n=1).

(**g**) Western blot for E-cadherin, α-catenin, β-catenin and plakoglobin showing input and E-cadherin immunoprecipitats (IP) in β-cat$^{fl/fl}$ and β-cat$^{Δ/Δ}$ mESCs.

(**h**) Histogram showing representative example of BAR (TOPFlash) and fuBAR (FOPFlash) assays using stable β-cat$^{fl/fl}$ and β-cat$^{Δ/Δ}$ mESC lines.

(**i**) Histogram showing representative example of TOPFlash assay of stable BAR βcat$^{fl/fl}$ and β-cat$^{Δ/Δ}$ mESCs stimulated with rWnt3a, Wnt3aCM or BIO. Error bars ± s.d in (h) and (i) are calculated on the basis of three measurements.

(**j**) Histogram showing relative Axin2 expression level in β-cat$^{fl/fl}$ and β-cat$^{Δ/Δ}$ mESCs stimulated with Wnt3aCM and BIO. Data are mean of two biological replicates.

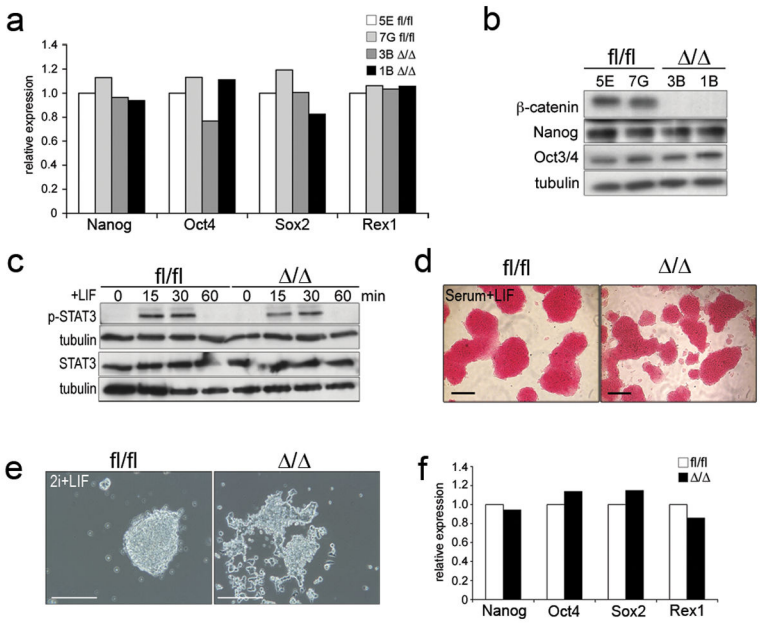

Figure 2.

Self-renewal markers are not affected in *β-cat*$^{fl/fl}$ and *β-cat*$^{\Delta/\Delta}$ mESCs under different culture conditions.

(**a**) Histogram showing relative expression levels of self-renewal markers *Nanog*, *Oct4*, *Sox2* and *Rex1* in *β-cat*$^{fl/fl}$ and *β-cat*$^{\Delta/\Delta}$ mESCs cultured in serum+LIF.

(**b**) Western blot of β-catenin, Nonog, Oct3/4 and tubulin in *β-cat*$^{fl/fl}$ and *β-cat*$^{\Delta/\Delta}$ mESCs cultured in serum+LIF.

(**c**) Western blots for Stat3 and p (Tyr705)-Stat3 levels in response to LIF stimulation.

(**d**) AP staining on passage 25 *β-cat*$^{fl/fl}$ and *β-cat*$^{\Delta/\Delta}$ mESCs cultured in serum+LIF.

(**e**) Morphology of *β-cat*$^{fl/fl}$ and *β-cat*$^{\Delta/\Delta}$ mESCs cultured in 2i+LIF without serum.

(**f**) Histogram showing relative expression levels of self-renewal markers *Nanog*, *Oct4*, *Sox2* and *Rex1* in *β-cat*$^{fl/fl}$ and *β-cat*$^{\Delta/\Delta}$ mESCs cultured in 2i+LIF.

Data in (a) and (f) are mean of two biological replicates. Scale bars in (d) and (e) are 100 μm.

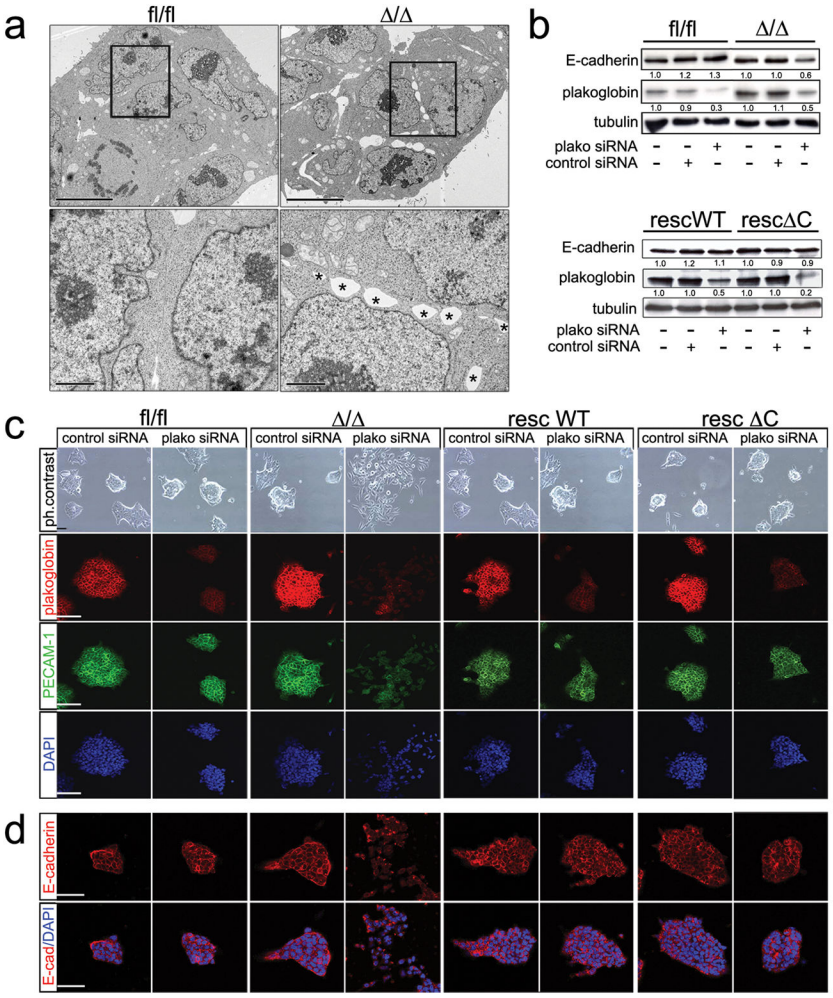

Figure 3.

Cell-cell adhesion defects in *β-cat*$^{fl/fl}$, *β-cat*$^{\Delta/\Delta}$ mESCs and upon knock- down of plakoglobin.

(**a**) TEM pictures of *β-cat*$^{fl/fl}$ and *β-cat*$^{\Delta/\Delta}$ mESC colonies at low magnification (scale bar 10 μm) and high magnification of the boxed areas (scale bar 2 μm), showing non-adherent spaces between adjacent *β-cat*$^{\Delta/\Delta}$ mESCs (asterisks) compared to the tight cell-cell adhesion between *β-cat*$^{fl/fl}$ mESCs.

(**b**) Western blot for E-cadherin, plakoglobin and tubulin on mESC lysates 48 hrs after treatment with control or plakoglobin RNAi. Quantified E-cadherin and plakoglobin protein levels normalized to tubulin are shown by the numbers below the corresponding lanes.

(**c**) Phase contrast and confocal images of immunofluorescent stainings (scale bars 50 μm) for plakoglobin, PECAM-1 and DAPI on control siRNA and plakoglobin siRNA treated mESCs 48 hrs after transfection.

(**d**) Confocal images (scale bar 50 μm) of immunofluorescent stainings for E-cadherin and E-cadherin/DAPI on control siRNA and plakoglobin siRNA treated mESCs 48 hrs after transfection.

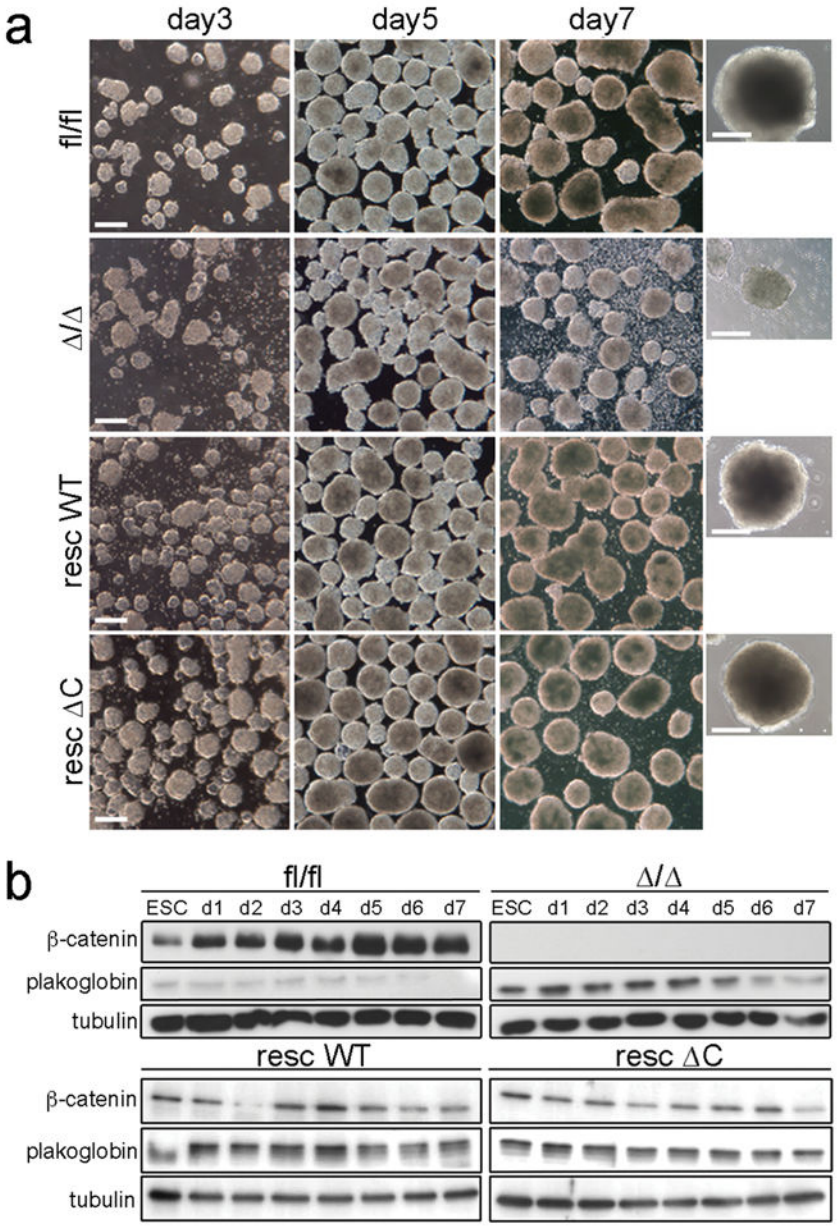

Figure 4.

Morphological appearance and plakoglobin levels of *β-cat*$^{fl/fl}$, *β-cat*$^{\Delta/\Delta}$, *β-cat*^{rescWT} and *β-cat*^{rescΔC} EBs during differentiation.

(**a**) Phase contrast pictures of EBs at days 3, 5 and 7 of differentiation. Scale bar: 200μm. Last row: magnified view of single EBs of the different genotypes on day 7.

(**b**) Western blot for β-catenin, plakoglobin and tubulin from mESC and EB lysates analysed at indicated time points during differentiation. d: day.

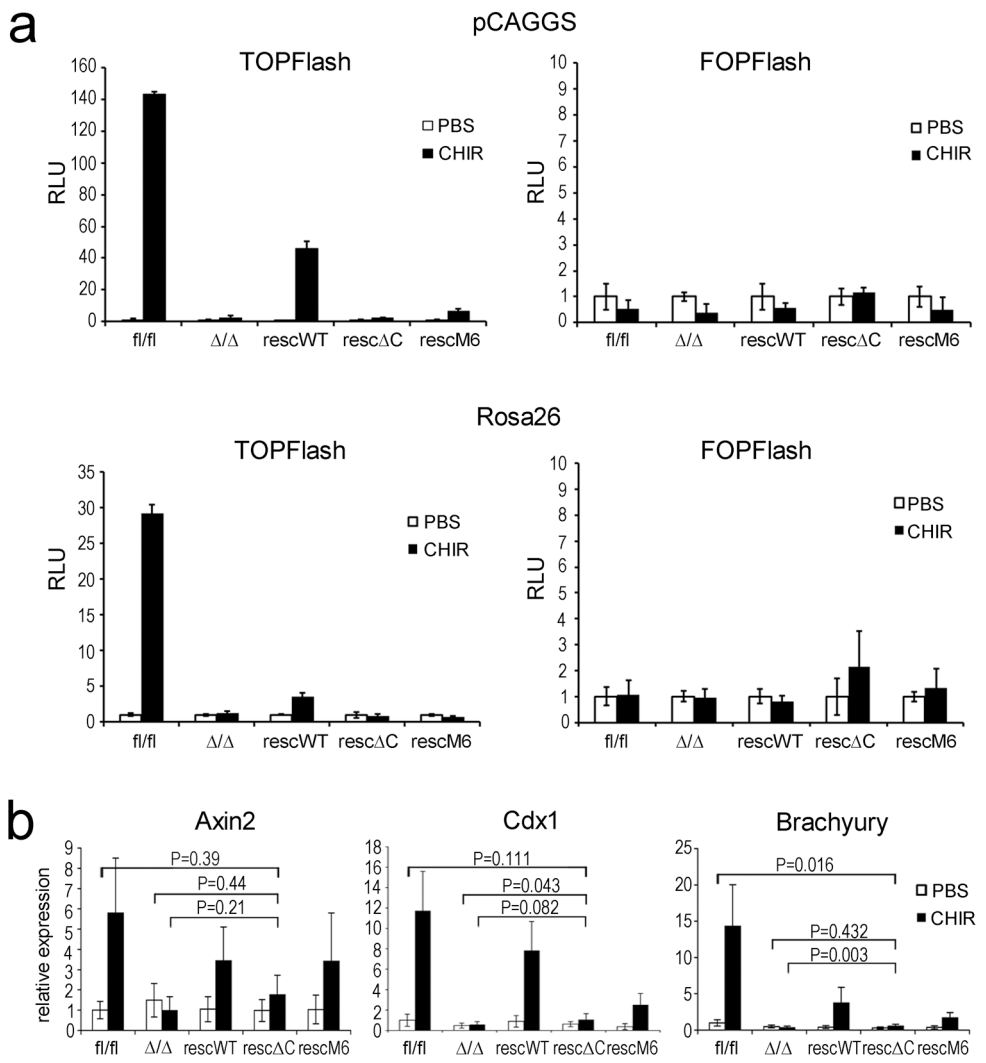

Figure 5.

Analyses of TCF/LEF mediated transcriptional activity.

(**a**) Histogram showing representative example of TOPFlash (BAR reporter) and FOPFlash (fuBAR reporter) activity in stable mESCs expressing rescue constructs from pGAGGS or Rosa26 locus under unstimulated (PBS) and stimulated (3μM CHIR99021) conditions. Error bars ± s.d are calculated on the basis of three measurements.

(**b**) Histogram showing relative expression of endogenous β-catenin/Tcf regulated genes *Axin2*, *Brachyury* (*T*), and *Cdx1* upon treatment with PBS or CHIR99021 (3μM). Data are mean ± s.d. of three biological replicates. P-values were calculated performing a two-tailed Student's t-test.

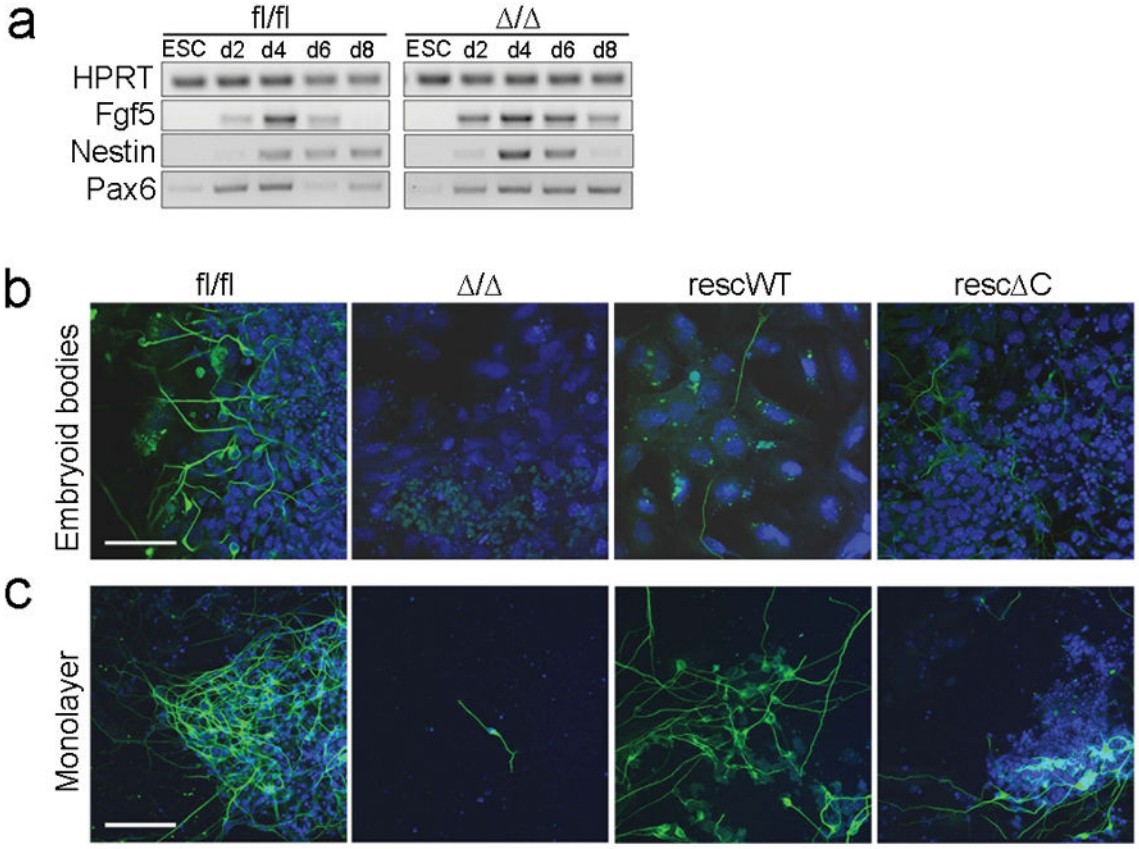

Figure 6.

Neuro-ectodermal differentiation potential of *β-cat*$^{fl/fl}$, *β-cat*$^{\Delta/\Delta}$, *β-cat*^{rescWT} and *β-cat*^{rescΔC} EBs.

(**a**) RT-PCR analyses of ectodermal markers *Fgf5*, *Nestin* and *Pax6* in mESCs and during EB differentiation time course of *β-cat*$^{fl/fl}$ and *β-cat*$^{\Delta/\Delta}$ mESCs.

(**b, c**) Immunofluorescent staining for TuJ positive neurons (b) in differentiated EBs at day 16; (c) in monolayer cultures of mESCs at day 14. Nuclei are stained for DAPI (blue). Scale bars 50 μm.

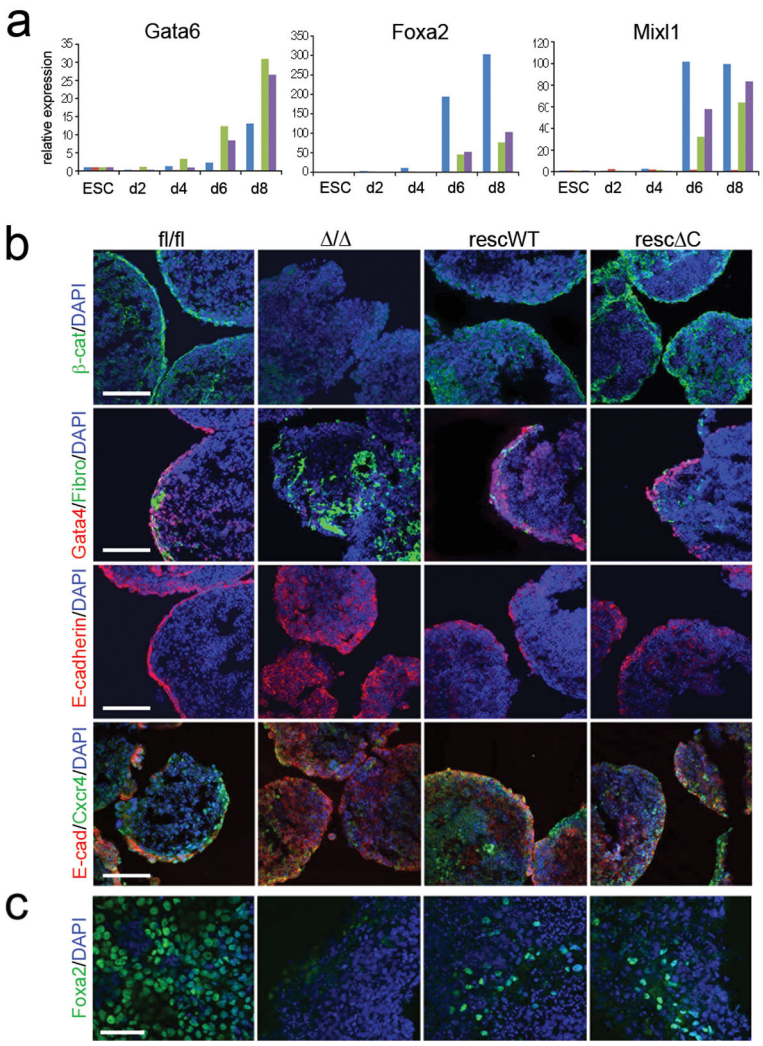

Figure 7.

Mesendodermal differentiation potential of *β-cat*$^{fl/fl}$, *β-cat*$^{\Delta/\Delta}$, *β-cat*^{rescWT} and *β-cat*^{rescΔC} EBs.

(**a**) Histograms showing relative expression of endodermal markers *Gata6*, *Foxa2* and *Mixl1* in mESCs and during EB differentiation. Data are mean of two biological replicates.

(**b**) Immunofluorescent staining for β-catenin/DAPI, Gata4/Fibronectin (Fibro)/DAPI, E-cadherin/DAPI, and E-cad/Cxcr4/DAPI in differentiated EBs at day 8. Arrowheads point at Gata4 positive cells in *β-cat*$^{\Delta/\Delta}$ EBs. Scale bar 100 μm.

(**c**) Immunofluorescent staining for Foxa2/DAPI on plated EBs at day 16. Scale bar 50 μm.