Development 140, 1684-1692 (2013) doi:10.1242/dev.088690

E-cadherin is required for the proper activation of the Lifr/Gp130 signaling pathway in mouse embryonic stem cells

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SUMMARY

The leukemia inhibitory factor (Lif) signaling pathway is a crucial determinant for mouse embryonic stem (mES) cell self-renewal and pluripotency. One of the hallmarks of mES cells, their compact growth morphology, results from tight cell adhesion mediated through E-cadherin, β-catenin (Ctnnb1) and α-catenin with the actin cytoskeleton. β-catenin is also involved in canonical Wnt signaling, which has also been suggested to control mES cell stemness. Here, we analyze *Ctnnb1*$^{-/-}$ mES cells in which cell adhesion is preserved by an E-cadherin–α-catenin (Eα) fusion protein (*Ctnnb1*$^{-/-}$Eα mES cells), and show that mimicking only the adhesive function of β-catenin is necessary and sufficient to maintain the mES cell state, making β-catenin/Wnt signaling obsolete in this process. Furthermore, we propose a role for E-cadherin in promoting the Lif signaling cascade, showing an association of E-cadherin with the Lifr-Gp130 receptor complex, which is most likely facilitated by the extracellular domain of E-cadherin. Without Eα, and thus without maintained cell adhesion, *Ctnnb1*$^{-/-}$ mES cells downregulate components of the Lif signaling pathway, such as Lifr, Gp130 and activated Stat3, as well as pluripotency-associated markers. From these observations, we hypothesize that the changes in gene expression accompanying the loss of pluripotency are a direct consequence of dysfunctional cell adhesion. Supporting this view, we find that the requirement for intact adhesion can be circumvented by the forced expression of constitutively active Stat3. In summary, we put forward a model in which mES cells can be propagated in culture in the absence of *Ctnnb1*, as long as E-cadherin-mediated cell adhesion is preserved.

KEY WORDS: β-catenin, E-cadherin, Lif receptor, Embryonic stem cells, Pluripotency, Stat3, Mouse

INTRODUCTION

Embryonic stem cells are characterized by constant self-renewal and their pluripotent differentiation potential, i.e. the ability to populate ectoderm, mesoderm and endoderm as well as the germ line during embryonic development. Traditionally derived from the inner cell mass of the blastocyst, they express a specific set of pluripotency-associated transcription factors, e.g. Oct3/4 (Pou5f1), Nanog, Sox2 and Klf4. By overexpressing some of these factors in somatic cells, induced pluripotent stem cells can be generated, opening a new aspect to isogenic stem cell therapy (Takahashi and Yamanaka, 2006). Regardless of their mode of derivation, mouse embryonic stem (mES) cells depend on Lif (leukemia inhibitory factor) to maintain their characteristics. Lif signals through Lif receptor β (Lifr) and Gp130 (Il6st), activating multiple intracellular signaling pathways that sustain self-renewal and prevent differentiation (Hirai et al., 2011). One of these pathways leads to the activation of the signal transducer and activator of transcription 3 (Stat3) through phosphorylation by a Lifr-associated Janus tyrosine kinase. Phosphorylated Stat3 (pStat3) dimerizes and upon nuclear translocation induces the transcription of pluripotency-related genes (Ying et al., 2003). Blocking Stat3 activity with a dominant-negative form of Stat3 leads to the loss of self-renewal (Niwa et al., 1998; Raz et al., 1999), whereas the constitutive activation of Stat3 has been reported to be sufficient to maintain mES cells in an undifferentiated state even in the absence of Lif (Matsuda et al., 1999).

Besides Lif signaling, the canonical Wnt signaling pathway and E-cadherin-mediated cell adhesion have also been suggested to be involved in mES cell maintenance. In both processes, β-catenin plays a central role. Upon activation of canonical Wnt signaling, β-catenin is protected from degradation, accumulates in the nucleus and functions as a transcriptional co-activator together with Lef/Tcf (lymphoid enhancer factor/T-cell factor) transcription factors (Daniels and Weis, 2005). Furthermore, in adherens junctions, β-catenin interacts with the cytoplasmic domain of classical cadherins (E-cadherin, N-cadherin) and with α-catenin, linking the plasma membrane to the actin cytoskeleton (Kemler, 1993). Interestingly, β-catenin already binds to E-cadherin co-translationally at the endoplasmic reticulum (ER), shielding a PEST sequence (proline, glutamic acid, serine and threonine) in the C-terminal part of the transmembrane protein and thereby protecting it from degradation. E-cadherin is expressed on most epithelial cells, including embryonic stem cells, and interacts with E-cadherin molecules on neighboring cells, facilitating homophilic cell-cell contact (Huber et al., 1996). Interruption at any point of this chain of proteins results in cell adhesion defects. To investigate the role of β-catenin in mES cells, several overexpression and knockout studies have been performed. However, the results of these reports are complex and controversially discussed. For example, it was shown that canonical Wnt pathway activation, which stabilizes β-catenin, promotes the self-renewal ability of mES cells (Hao et al., 2006; Ogawa et al., 2006; Singla et al., 2006; Takao et al., 2007). However, it still remains to be resolved whether Wnt signaling alone is sufficient to support pluripotency, or if additional Lif pathway activation is required. It is generally believed that knockout of *Ctnnb1*, the gene encoding β-catenin, is adverse to mES cells identity, as the absence of β-catenin is associated with the loss of pluripotency marker expression and an adaption to an epiblast-like stem cell fate (Anton et al., 2007; Wagner et al., 2010). However, two recent works contradict this view by claiming that β-

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Accepted 7 February 2013

catenin is not required for the maintenance of mES cells (Lyashenko et al., 2011; Soncin et al., 2009). A proposed explanation for this observation was found in upregulated levels of plakoglobin (γ-catenin), which is structurally related to β-catenin and can substitute for it in E-cadherin-mediated cell adhesion (Lyashenko et al., 2011). These authors further hypothesize that sustained cell adhesion prevents the breakdown of the transcriptional network underlying pluripotency gene expression and self-renewal in a Lif-dependent manner. Interestingly, loss of cell adhesion in mES cells due to the knockout of E-cadherin also results in impaired expression of the pluripotency-associated transcription factors Nanog and Klf4, despite the presence of β-catenin (Hawkins et al., 2012). Therefore, intact cell adhesion seems to be a crucial determinant in preserving mES cell 'stemness'. More importantly, E-cadherin$^{-/-}$ mES cells have lost their ability to respond to Lif signaling, which is reflected in a defect in activation of Stat3 (Hawkins et al., 2012; Soncin et al., 2011).

The functional dualism of β-catenin imposes a scientific challenge to clearly distinguish the signaling contribution from the adhesive contribution of β-catenin to the maintenance of mES cells. Our work therefore aimed to refine the role of β-catenin in mES cells, focusing on its adhesive function. To this end, we used *Ctnnb1*$^{flox/-}$ mES cells to generate *Ctnnb1*$^{-/-}$ mES cells in which cell adhesion is maintained by the constitutive expression of an E-cadherin–α-catenin fusion protein (*Ctnnb1*$^{-/-}$Eα mES cells). Analyzing these cells, we found that preserving cell adhesion is sufficient to maintain the Lifr/Gp130/Stat3 pathway and the transcription factor network underlying mES cells characteristics. Moreover, we implicate E-cadherin as being crucially involved in this process by stabilizing the Lifr-Gp130 co-receptor complex. In summary, we provide evidence that only the adhesive function of β-catenin is absolutely required for the maintenance of pluripotency markers and the self-renewal capacity of mES cells.

MATERIALS AND METHODS

Cell lines

For the generation of *Ctnnb1*$^{-/-}$Eα mES cells, the cDNA sequence of E-cadherin (E-cadh) and α-catenin lacking the β-catenin-binding domain (Imamura et al., 1999) was cloned into a LoxP-β-geo-LoxP-IRES-GFP plasmid and transfected into *Ctnnb1*$^{flox/-}$ mES cells using Lipofectamine 2000 (Invitrogen). Positive clones for *lacZ* expression were selected and stably transfected with a Cre-IRES-puro plasmid using Lipofectamine 2000 (Fig. 1A). Similarly, *Ctnnb1*$^{flox/-}$ or *E-cadh*$^{flox/-}$ mES cells were transfected with a Cre-IRES-puro plasmid to obtain *Ctnnb1*$^{-/-}$ or *E-cadh*$^{-/-}$ mES cells, respectively. Puromycin-resistant clones were genotyped to ensure the deletion of the endogenous, floxed *Ctnnb1* or *E-cadh* alleles.

In order to generate *E-cadh*$^{-/-}$*Ec* mES cells, *E-cadh*$^{flox/-}$ mES cells were simultaneously transfected with a Cre-IRES-puro plasmid and with a plasmid containing the extracellular and the transmembrane domain of E-cadherin (neo-IRES-Ec plasmid) (Ozawa, 2003). At least three resistant clones for G418 and puromycin selection were analyzed. Similarly, *E-cadh*$^{flox/-}$ mES cells were simultaneously transfected with a Cre-IRES-puro plasmid and a plasmid containing a constitutive active form of Stat3 (Stat3c-GFP) (Bromberg et al., 1999) or a GFP plasmid (control) in order to obtain *E-cadh*$^{-/-}$*Stat3c* mES cells. Positive cells for GFP labeling were then sorted and collected by fluorescence-activated cell sorting (FACS). In order to generate *E-cadh*$^{-/-}$+Eα and *Ctnnb1*$^{-/-}$+Eα mES cells, *E-cadh*$^{-/-}$ or *Ctnnb1*$^{-/-}$ mES cells were stably transfected with either a GFP plasmid (control) or with an E-cadherin-α-catenin-IRES-GFP plasmid and GFP-positive cells were sorted by FACS.

Ctnnb1$^{flox/-}$, *Ctnnb1*$^{-/-}$ and *Ctnnb1*$^{-/-}$Eα mES cells were genotyped using the following primers (5′-3′): RM41, AAGGTAGAGTGATG-AAAGTTGTT; RM42, CACCATGTCCTCTGTCTATCC; RM43, TACACTATTGAATCACAGGGACTT.

E-cadh$^{flox/-}$, *E-cadh*$^{-/-}$, *E-cadh*$^{-/-}$*Ec* and *E-cadh*$^{-/-}$*Stat3c* mES cells were genotyped with the following primers (5′-3′): pE10.2, CTTATACCGCTCGAGAGCCGGA; pI10as.3, TGACACATGCCTTTAC-TTTAGT; In5, TGTTCCAAGCCTGCTTTCTT.

In every set of experiments, at least three independent clones were analyzed to ensure reproducibility of results.

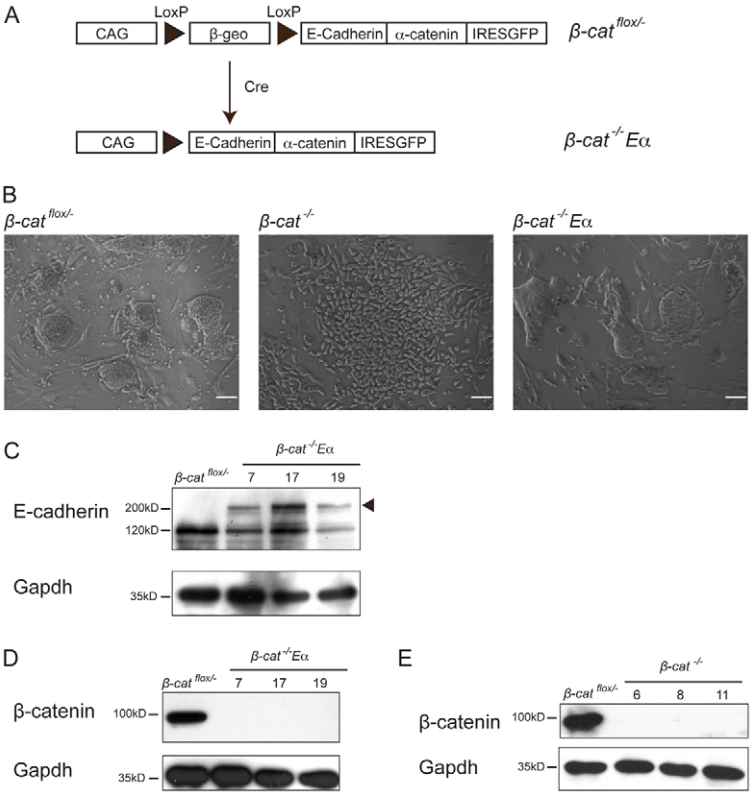

Fig. 1. Generation of *Ctnnb1*$^{-/-}$ and *Ctnnb1*$^{-/-}$Eα mES cells. (**A**) Schematic of the vector and recombination strategy used to generate *Ctnnb1*$^{-/-}$Eα mES cells. (**B**) Phase-contrast images of *Ctnnb1*$^{flox/-}$, *Ctnnb1*$^{-/-}$ and *Ctnnb1*$^{-/-}$Eα mES cells. Only *Ctnnb1*$^{-/-}$ mES cells grow isolated. (**C**) Immunoblot for E-cadherin in *Ctnnb1*$^{flox/-}$ mES cells and three clones of *Ctnnb1*$^{-/-}$Eα mES cells (7, 17 and 19). Gapdh was used as a loading control. The arrowhead indicates the protein band corresponding to the E-cadherin–α-catenin fusion protein (220 kDa). (**D**,**E**) Immunoblot for β-catenin in *Ctnnb1*$^{flox/-}$ mES cells, three clones of *Ctnnb1*$^{-/-}$Eα mES cells (D) or three clones of *Ctnnb1*$^{-/-}$ mES cells (6, 8 and 11) (E). Gapdh served as a loading control. Scale bars: 50 μm.

Cell culture conditions

All mES cell lines used in the present work were cultured on irradiated mouse embryonic fibroblasts (MEFs) or on gelatin-coated plates under standard conditions [DMEM supplemented with 15% fetal calf serum (FCS) and Lif]. For embryoid bodies differentiation experiments, cells were grown in bacteriological dishes for 5 days in the absence of Lif and then re-plated on gelatin-coated plates for 7-10 days. Cells were then assessed for the expression of differentiation markers. For long-term differentiation assays, mES cells were plated on gelatin dishes for 3 weeks in standard mES cell medium either in the absence or presence of Lif prior to RNA extraction or immunostaining experiments. To ensure homogeneity of the results, all experiments were performed between passages 20 and 30 unless otherwise specified.

Western blot and co-immunoprecipitation

Total protein was extracted from cultured mES cells plated on gelatin using lysis buffer and protein concentrations were determined using the BCA protein assay (Thermo Scientific). For western blotting assays, 20-50 μg of total extract was loaded per lane. Primary antibodies were probed against β-catenin (Cell Signaling; 1:1000), E-cadherin (BD Biosciences; 1:2000), Gp84 (1:2000) (Vestweber and Kemler, 1984), Lifr (Santa Cruz; 1:1000), Gp130 (Santa Cruz; 1:1000), plakoglobin (BD Biosciences; 1:4000), Stat3 (Cell Signaling; 1:1000), pStat3 (Abcam; 1:5000), Nanog (self-made affinity-purified rabbit antibody; 1:100), Akt (Cell Signaling; 1:1000), pAkt (Cell Signaling; 1:1000), Erk1/2 (Cell Signaling; 1:1000), pErk1/2 (Cell Signaling; 1:1000) and Gapdh (Calbiochem; 1:25,000). For immunoprecipitation analyses, 1 mg of pre-cleared lysate was incubated at 4°C overnight with 20 μl Dynabeads Protein G (Invitrogen). Incubations were carried out with 500 ng anti-E-cadherin antibody or with 2 μg anti-Lifr antibody.

Gene expression analysis

Expression assay was performed using the Affymetrix GeneChip Mouse Gene 1.0 ST array. The experiment included *Ctnnb1*$^{flox/-}$, *Ctnnb1*$^{-/-}$ and *Ctnnb1*$^{-/-}$Eα mES cells using three biological replicates for each mES cell line. Processing and statistical analysis of the microarray data was carried out in R programming environment using Bioconductor packages ‘oligo’ (Carvalho and Irizarry, 2010) and ‘limma’ (Smyth, 2004). Differentially expressed genes were obtained using linear models and the Benjamini and Hochberg method (Benjamini and Hochberg, 1995) was applied to adjust the *P*-values for multiple testing. The cutoff values for adjusted *P*-values and fold-changes were 0.05 and 2, respectively. Microarray data are available at GEO under accession number GSE44543.

Immunostaining

mES cells were fixed in 4% paraformaldehyde (PFA) w/v for 15 minutes prior to immunostaining as previously described (Messerschmidt and Kemler, 2010). The following primary antibodies were used: Nanog (self-made affinity-purified rabbit antibody; 1:100), Oct3/4 (Santa Cruz; 1:200), Sox2 (Chemicon; 1:500), Gp84 (1:200) (Vestweber and Kemler, 1984), nestin (Chemicon; 1:100), vimentin (Exbio; 1:200), Gata4 (Santa Cruz; 1:100), cytokeratin 8 (1:2) (Kemler et al., 1981), β-III-tubulin (Sigma; 1:500) and plakoglobin (BD Transduction Laboratories; 1:200). Secondary antibodies used were Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse or goat anti-rat (Molecular Probes; 1:500). For immunostaining experiments performed in living cells, mES cells were incubated with Gp84 antibody (1:200) for 1 hour at 4°C, washed and incubated with the corresponding secondary antibody for 45 minutes at 4°C. After washing, cells were fixed in 4% PFA w/v for 15 minutes and subsequently embedded. Nuclei were stained with 10 μM DAPI (Molecular Probes) added to the embedding medium.

Flow cytometry

Single cell suspensions obtained from mES cells were labeled with Gp84 (1:100) or SSEA-1 (1:100) antibodies in PBS supplemented with 0.5% BSA and 0.1% sodium azide, for 1 hour at 4°C. Cells were washed and subsequently incubated with the corresponding phycoerythrin-conjugated secondary antibody for 45 minutes at 4°C. Incubated cells with secondary antibody in the absence of primary antibody were used as control. Cells were analyzed using a Becton Dickinson FACS Calibur.

qRT-PCR

Total RNA was isolated using Trizol (Invitrogen) following the manufacturer’s instructions. RNA (1 μg) was reverse transcribed by oligo-dT primers (Roche), diluted 1:20 and amplified in qPCR using Absolute qPCR ROX Mix (Thermo Scientific) in combination with the mouse Universal Probe Library (Roche). Results were obtained from at least three experiments performed in triplicate using the ΔΔCT method. Error bars indicate s.e.m. Primers and probes used are listed in supplementary material Table S1.

Luciferase assay

mES cells were plated on gelatin-coated 24-well cell culture plates one day before transfection. Transfection was performed using jetPRIME transfection reagent (Polyplus) with 100 ng of *Renilla* luciferase reporter construct and 200 ng of Firefly reporter construct (TOP or FOP). Cells were collected 48 hours after transfection and lysed with 100 μl NP40 lysis buffer for 20 minutes at 4°C. Luciferase activity measurements were performed at a Centro-LB-960 luminometer, using 10 μl of the cell lysates. All measurements were carried out in triplicate.

RESULTS

The expression of Eα preserves cell adhesion in *Ctnnb1*$^{-/-}$ mES cells

Upon transient expression of Cre recombinase in *Ctnnb1*$^{flox/-}$ mES cells, we obtained several novel isogenic *Ctnnb1*$^{-/-}$ mES cells lines. After more than ten passages, all *Ctnnb1*$^{-/-}$ mES cell clones presented an adhesion defect compared with *Ctnnb1*$^{flox/-}$ cells (Fig. 1B). In order to have a means to preserve cell adhesion following the Cre-mediated deletion of β-catenin, we introduced a conditional E-cadherin–α-catenin (Eα) (Imamura et al., 1999) IRES-GFP fusion gene preceded by a floxed β-geo selection cassette into *Ctnnb1*$^{flox/-}$ mES cells (Fig. 1A). *Ctnnb1* knockout cells generated in this way were termed *Ctnnb1*$^{-/-}$Eα mES cells and retained a compact morphology similar to *Ctnnb1*$^{flox/-}$ mES cells (Fig. 1B; supplementary material Fig. S1A,B). The expression of Eα was assessed by western blot; an additional band of ~220 kDa corresponding to the fusion protein was detected only in *Ctnnb1*$^{-/-}$Eα cells (Fig. 1C; supplementary material Fig. S1C). In immunofluorescence, E-cadherin was uniformly localized at the cell surface of *Ctnnb1*$^{flox/-}$ and *Ctnnb1*$^{-/-}$Eα mES cells, whereas in *Ctnnb1*$^{-/-}$ mES cells E-cadherin detection was highly variable among cells and its expression pattern was rather spotty, scattered along the cell surface (supplementary material Fig. S2A-C). The varying expression pattern of E-cadherin in *Ctnnb1*$^{-/-}$ mES cells was also found in FACS plots (supplementary material Fig. S2D-F). The deletion of *Ctnnb1* was confirmed by genotyping (supplementary material Fig. S1D) and by western blot (Fig. 1D,E). We further investigated β-catenin/TCF transcriptional activity in the different mES cell lines using a TOP flash reporter assay. *Ctnnb1*$^{-/-}$Eα and *Ctnnb1*$^{-/-}$ mES cells lacked luciferase reporter activity compared with *Ctnnb1*$^{flox/-}$ mES cells (supplementary material Fig. S1E). Finally, as recently reported (Lyashenko et al., 2011), we tested whether plakoglobin was upregulated in our *Ctnnb1* knockout mES cell lines. We detected an interaction of plakoglobin and E-cadherin in *Ctnnb1*$^{-/-}$ mES cells by co-immunoprecipitation, but we did not observe an increase in plakoglobin protein levels compared with *Ctnnb1*$^{flox/-}$ mES cells (supplementary material Fig. S2G-I). Therefore, in our *Ctnnb1*$^{-/-}$ mES cells plakoglobin is not able to substitute for β-catenin. In summary, in the absence of β-catenin, Eα is able to maintain the compact growth morphology of mES cells. This finding encouraged

us to investigate in more detail, whether the maintenance of cell adhesion also promotes mES cell 'stemness'.

Ctnnb1$^{-/-}$Eα mES cells have a marker profile associated with pluripotency, but are restricted in their differentiation potential

mES cells are characterized by the expression of the core pluripotency transcription factors Oct3/4, Sox2, Nanog and Klf4. When we assessed the abundance of transcript levels of these genes, we found no significant difference between our *Ctnnb1*$^{-/-}$Eα and *Ctnnb1*$^{flox/-}$ mES cells. In *Ctnnb1*$^{-/-}$ mES cells, by contrast, the expression levels of *Pou5f1* (*Oct3/4*) and *Sox2* were reduced and those of *Klf4* and *Nanog* barely detectable (Fig. 2A). By immunofluorescence, all mES cell lines showed nuclear localization of Oct3/4 and Sox2 (Fig. 2C). Nanog, however, was only found in the nuclei of *Ctnnb1*$^{flox/-}$ and *Ctnnb1*$^{-/-}$Eα mES cells, but was not detectable in *Ctnnb1*$^{-/-}$ mES cells by immunofluorescence or western blot (Fig. 2C,D). In addition, the ES cell markers SSEA-1 (also known as Fut4) and alkaline phosphatase, which are highly expressed in undifferentiated mES cells, showed similar expression patterns (Fig. 2B; supplementary material Fig. S3A). Cytokeratin 8 (CK8; also known as keratin 8), an early marker of mES cells exiting the pluripotent state, is occasionally found only in a small subset of differentiating mES cells under normal culture conditions. In line with the previous results, CK8 is upregulated in almost all *Ctnnb1*$^{-/-}$ mES cells, providing additional evidence for the loss of pluripotency in these cells (supplementary material Fig. S3B). To evaluate the full differentiation potential of the different mES cell types *in vitro*, we generated embryoid bodies (EBs) in the absence of feeders and Lif. After 2 days in suspension, *Ctnnb1*$^{flox/-}$ and *Ctnnb1*$^{-/-}$Eα cells had formed compact aggregates (supplementary material Fig. S3C), whereas *Ctnnb1*$^{-/-}$ EBs comprised loosely attached cells, most likely owing to the absence of cadherin-mediated cell adhesion (not shown). Moreover, upon plating of EBs, we did not observe changes in cell morphology and were unable to detect the expression of differentiation markers in these cells. On the contrary, EBs derived from *Ctnnb1*$^{-/-}$Eα or *Ctnnb1*$^{flox/-}$ mES cells gave rise to cells expressing the neuroectoderm markers nestin and β-III-tubulin or the mesenchyme-specific intermediate filament vimentin (supplementary material Fig. S3C,D). Differentiated

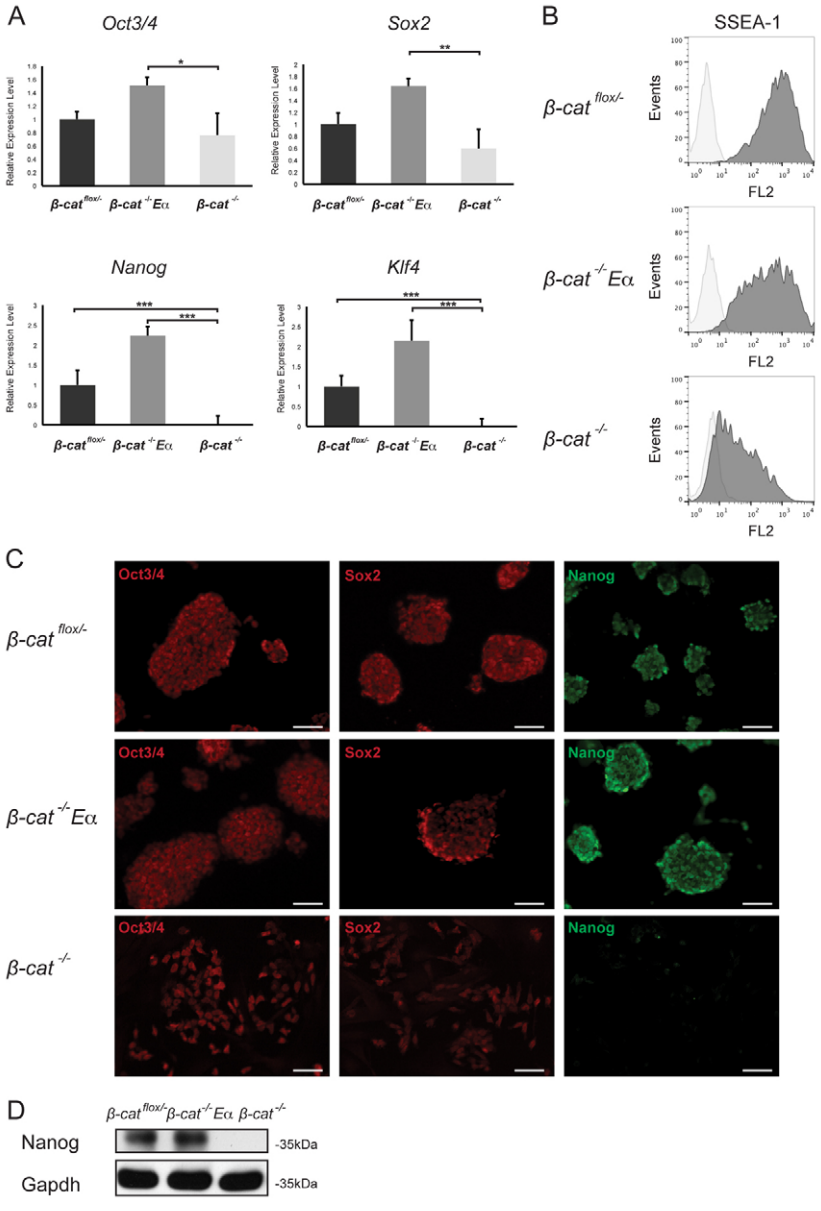

Fig. 2. *Ctnnb1*$^{-/-}$Eα mES cells retain the expression of pluripotency markers. (**A**) qRT-PCR analysis of the pluripotency markers *Oct3/4*, *Sox2*, *Nanog* and *Klf4* in *Ctnnb1*$^{flox/-}$, *Ctnnb1*$^{-/-}$Eα or *Ctnnb1*$^{-/-}$ mES cells. Only *Ctnnb1*$^{-/-}$ mES cells have significantly reduced levels of all four factors. (**B**) Flow cytometry plots for SSEA-1 show reduced expression levels on *Ctnnb1*$^{-/-}$ mES cells compared with *Ctnnb1*$^{flox/-}$ or *Ctnnb1*$^{-/-}$Eα mES cells. (**C**) Immunofluorescence staining of *Ctnnb1*$^{flox/-}$, *Ctnnb1*$^{-/-}$Eα and *Ctnnb1*$^{-/-}$ mES cells for Oct3/4, Sox2 and Nanog. Only *Ctnnb1*$^{-/-}$ mES cells do not express Nanog, whereas Oct3/4 and Sox2 are detected in the nuclei of all three cell lines. (**D**) Western blot analysis for Nanog revealed the absence of this transcription factor only in *Ctnnb1*$^{-/-}$ mES cells, whereas it is detected at equal amounts in *Ctnnb1*$^{flox/-}$ and *Ctnnb1*$^{-/-}$Eα mES cells. Gapdh was used as a loading control. Error bars represent s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bars: 25 μm.

Ctnnb1$^{flox/-}$ cells were also positive for the primitive endodermal marker Gata4 (supplementary material Fig. S3C) and were able to generate cardiomyocyte precursors, a differentiation pattern never observed in *Ctnnb1*$^{-/-}$Eα EBs. Moreover, an alternative differentiation protocol, in which attached mES cells are deprived of Lif, delivered similar results (supplementary material Fig. S4A-C). From this, we conclude that *Ctnnb1*$^{-/-}$ mES cells have lost their ability to differentiate into derivatives of the three germlayers. Taken together, *Ctnnb1*$^{-/-}$Eα mES cells appear normal under mES cell culture conditions, but possess a restricted differentiation potential.

Lif signaling is lost in *Ctnnb1*$^{-/-}$, but preserved in *Ctnnb1*$^{-/-}$Eα mES cells

Although the impaired differentiation potential of *Ctnnb1*$^{-/-}$Eα mES cells could be ascribed to the lack of canonical Wnt signaling, the mechanism promoting the pluripotent state in these cells remained unclear. In a microarray expression study among our isogenic mES cell lines, we found that the loss of *Ctnnb1* led to a highly altered expression profile, regardless of cell adhesion status (supplementary material Fig. S5). However, the expression of pluripotency-associated genes was generally less affected in *Ctnnb1*$^{-/-}$Eα mES cells than in *Ctnnb1*$^{-/-}$ mES cells, verifying our analysis on pluripotency markers on a transcriptome-wide scale. Interestingly, *Lifr* was among the downregulated genes found in *Ctnnb1*$^{-/-}$ mES cells in comparison with *Ctnnb1*$^{-/-}$Eα or *Ctnnb1*$^{flox/-}$ mES cells (supplementary material Fig. S5B). Subsequent qRT-PCR analysis confirmed a reduction in the expression levels of *Lifr*, *Gp130* and *Stat3* in *Ctnnb1*$^{-/-}$ mES cells compared with *Ctnnb1*$^{flox/-}$ or *Ctnnb1*$^{-/-}$Eα mES cells (Fig. 3A). Furthermore, *Ctnnb1*$^{-/-}$ mES cells also had reduced protein levels of Lifr, Gp130 and Stat3, and, more importantly, failed to phospho-activate Stat3 (Fig. 3B). Other important signaling pathways in mES cells, such as activation of Akt or Erk1/2 (also known as Mapk3/1), were not affected (Fig. 3C). These findings led us to hypothesize that maintenance of cell adhesion suffices to sustain the regulatory network underlying the pluripotent state of mES cells downstream of Lif signaling.

E-cadherin promotes the activation of the Lif signaling pathway

Reduced levels of Lifr and Gp130 as well as the absence of pStat3 in *Ctnnb1*$^{-/-}$ mES cells clearly showed that Lif signaling and cell adhesion are connected. However, when we restored the adhesion defect in *Ctnnb1*$^{-/-}$ mES cells by introducing the Eα fusion protein (supplementary material Fig. S6A,B), we never observed an increase in the expression of *Lifr* or *Gp130* back to wild-type levels (supplementary material Fig. S6C), nor a recovery of pStat3 or Nanog (supplementary material Fig. S6D,E). This shows that permanent disruption of cell adhesion brings about irreversible changes in these cells, regardless of the restoration of E-cadherin-mediated cell adhesion. Thus, in order to shed more light on how cell adhesion contributes to the pluripotency network, we analyzed whether E-cadherin might be directly involved in activating the Lif signaling pathway. To test this hypothesis, we performed immunoprecipitation with an E-cadherin-specific antibody and analyzed the precipitated protein complexes for the presence of Lifr and Gp130. Indeed, we found an interaction of E-cadherin with the Lif co-receptor complex in total cell lysates of *Ctnnb1*$^{flox/-}$ and *Ctnnb1*$^{-/-}$Eα (Fig. 4A,B). However, this interaction was never observed for *Ctnnb1*$^{-/-}$ mES cells (Fig. 4C), which could be because of the low expression levels of Lifr and Gp130 in these cells (see Fig. 3B). In the reverse experimental setup using a Lifr-specific antibody for immunoprecipitation and an E-cadherin-specific antibody for western blot, we could also detect an interaction between both proteins in *Ctnnb1*$^{flox/-}$ and *Ctnnb1*$^{-/-}$Eα mES cells (supplementary material Fig. S7A,B). The ternary

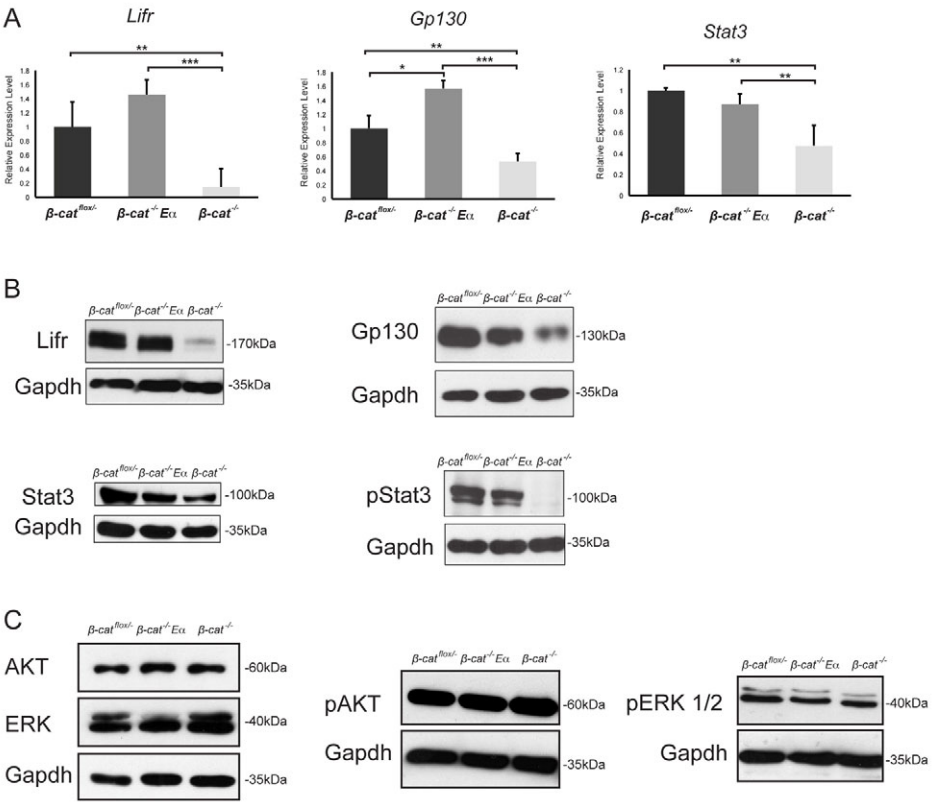

Fig. 3. *Ctnnb1*$^{-/-}$ mES cells fail to activate the Lifr/Gp130/Stat3 pathway. (**A**) qRT-PCR for *Lifr*, *Gp130* and *Stat3* shows significantly reduced expression levels in *Ctnnb1*$^{-/-}$ mES cells compared with *Ctnnb1*$^{flox/-}$ or *Ctnnb1*$^{-/-}$Eα mES cells. (**B**) Western blot analysis of whole cell extracts reveals decreased levels of Lifr, Gp130 and Stat3 in *Ctnnb1*$^{-/-}$ mES cells compared with *Ctnnb1*$^{flox/-}$ or *Ctnnb1*$^{-/-}$Eα mES cells. Phosphorylation of Stat3 was not detected in *Ctnnb1*$^{-/-}$ mES cells. (**C**) Furthermore, expression and phosphorylation status of Akt and Erk1/2 are unchanged among the three mES cell lines. Gapdh was used as a loading control. Error bars represent s.e.m. *P<0.05, **P<0.01, ***P<0.001.

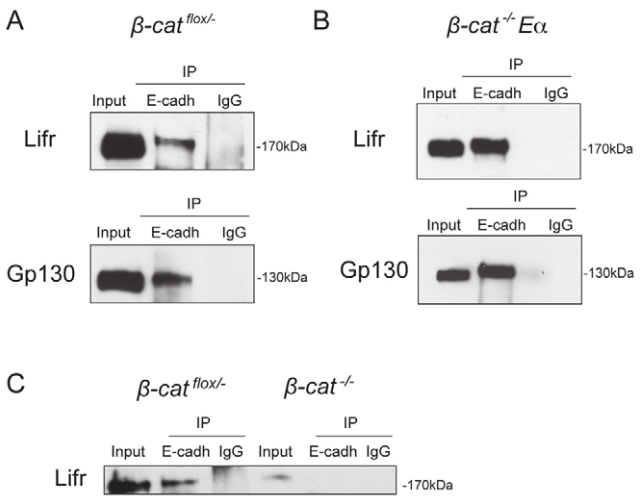

Fig. 4. E-cadherin interacts with Lifr and Gp130 in mES cells. (**A**,**B**) Co-immunoprecipitation of Lifr or Gp130 with E-cadherin in *Ctnnb1*$^{flox/-}$ (A) or *Ctnnb1*$^{-/-}$Eα (B) mES cells. Total cell extracts were immunoprecipitated with an anti-E-cadherin antibody and probed for Lifr or Gp130. An interaction between E-cadherin and Lifr or Gp130 was found in both mES cell lines. (**C**) Co-immunoprecipitation of Lifr with E-cadherin in *Ctnnb1*$^{-/-}$ mES cells. No interaction could be detected in these cells. *Ctnnb1*$^{flox/-}$ mES cells were used as a control in the same experiment.

complex was also found in wild-type mES cells (supplementary material Fig. S7C). From these results, we conclude that E-cadherin takes part in the formation of the Lif receptor complex. To confirm the importance of E-cadherin for Lif signaling, we generated E-cadherin$^{-/-}$ (*E-cadh*$^{-/-}$) mES cells from E-cadherin$^{flox/-}$ (*E-cadh*$^{flox/-}$) mES cells (supplementary material Fig. S8A-C). Similarly, we established E-cadherin null mES cells that express a transgene encoding only the extracellular and transmembrane domain of E-cadherin (Ec, 90 kDa), missing the intracellular part of E-cadherin, which is responsible for binding to β-catenin (*E-cadh*$^{-/-}$*Ec* mES cells; Fig. 5A,B). Comparable to *Ctnnb1*$^{-/-}$ mES cells, *E-cadh*$^{-/-}$*Ec* and *E-cadh*$^{-/-}$ mES cells exhibited a clear cell adhesion defect (Fig. 1B; Fig. 5A; supplementary material Fig. S8A). The lack of E-cadherin in *E-cadh*$^{-/-}$ mES cells was confirmed by western blot, flow cytometry and genotyping (supplementary material Fig. S8B-D). Further characterization of these E-cadherin null cells revealed also that expression of pluripotency markers, such as SSEA-1, Nanog or Klf4, was absent or reduced (supplementary material Fig. S8D-F). Similarly to *Ctnnb1*$^{-/-}$ mES cells, *E-cadh*$^{-/-}$ mES cells presented a differentiation defect in the absence of Lif compared with differentiated *E-cadh*$^{flox/-}$ mES cells (supplementary material Fig. S8G-I). Furthermore, in comparison to *E-cadh*$^{flox/-}$ mES cells, the activity of the Lif signaling pathway was severely compromised in *E-cadh*$^{-/-}$ mES cells, as both Lif receptors and pStat3 were downregulated (supplementary material Fig. S9A,B), whereas the levels of p-Akt or p-Erk1/2 were slightly upregulated or unchanged, respectively (supplementary material Fig. S9C). Introduction of Eα into *E-cadh*$^{-/-}$ mES cells was not able to revert these cells to a pluripotent state, despite the rescue of cell adhesion (supplementary material Fig. S9D-H). This again provides evidence that mES cells with continuous abrogation of cell adhesion irrevocably lose their pluripotent characteristics, even in the presence of β-catenin. However, in *E-cadh*$^{-/-}$*Ec* mES cells, despite their adhesion defect, the expression levels of components of the Lif receptor complex and the activation of Stat3 were unaltered compared with controls (Fig. 5C,D). Moreover, Nanog was present in the nuclei of these cells (Fig. 5E). Lastly, we could show an interaction between Ec and Lifr (Fig. 5F). Based on these results, we conclude that E-

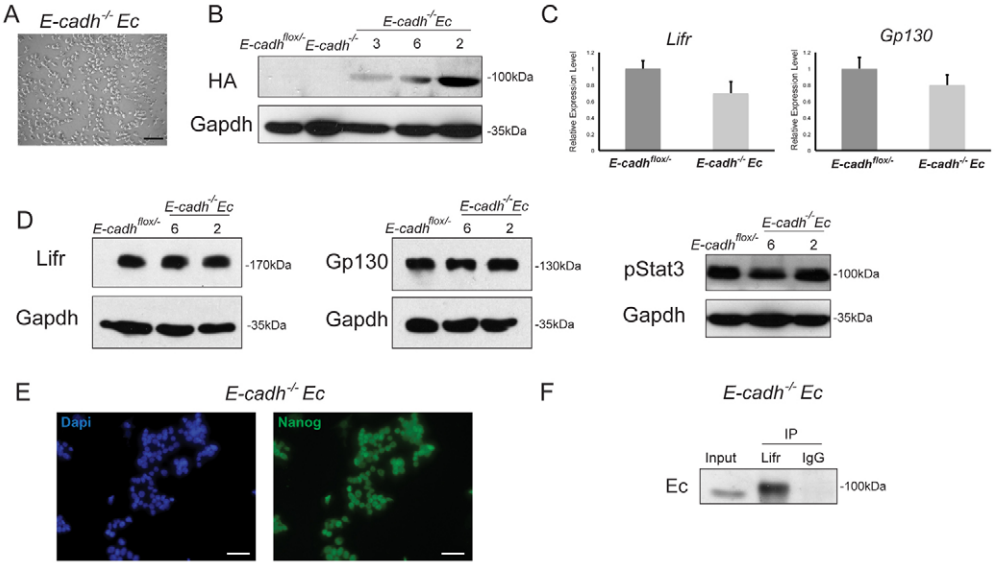

Fig. 5. The extracellular domain of E-cadherin maintains the expression levels of Lifr, Gp130 and pStat3 in mES cells. (**A**) Phase-contrast image of *E-cadh*$^{-/-}$*Ec* mES cells, exhibiting a clear cell adhesion defect. (**B**) Ec detected with an anti-HA antibody is only found in *E-cadh*$^{-/-}$*Ec* mES cells (clones 3, 6 and 2). (**C**) *E-cadh*$^{-/-}$*Ec* mES cells sustain the expression levels of *Lifr* and *Gp130* relative to *E-cadh*$^{flox/-}$ mES cells, as detected by qRT-PCR. (**D**) The protein levels of Lifr and Gp130, as well as the phosphorylation status of Stat3 are maintained in *E-cadh*$^{-/-}$*Ec* mES cell clones similar to *E-cadh*$^{flox/-}$ mES cells. Gapdh was used as a loading control. (**E**) Immunofluorescence for Nanog in *E-cadh*$^{-/-}$*Ec* mES cells clearly shows nuclear localization of this transcription factor. DAPI was used for nuclei counterstaining. (**F**) Co-immunoprecipitation of Lifr and Ec in *E-cadh*$^{-/-}$*Ec* mES cells using an anti-Lifr antibody for precipitation and an antibody recognizing the extracellular domain of E-cadherin for blotting. Error bars represent s.e.m. Scale bars: in A, 50 μm; in E, 25 μm.

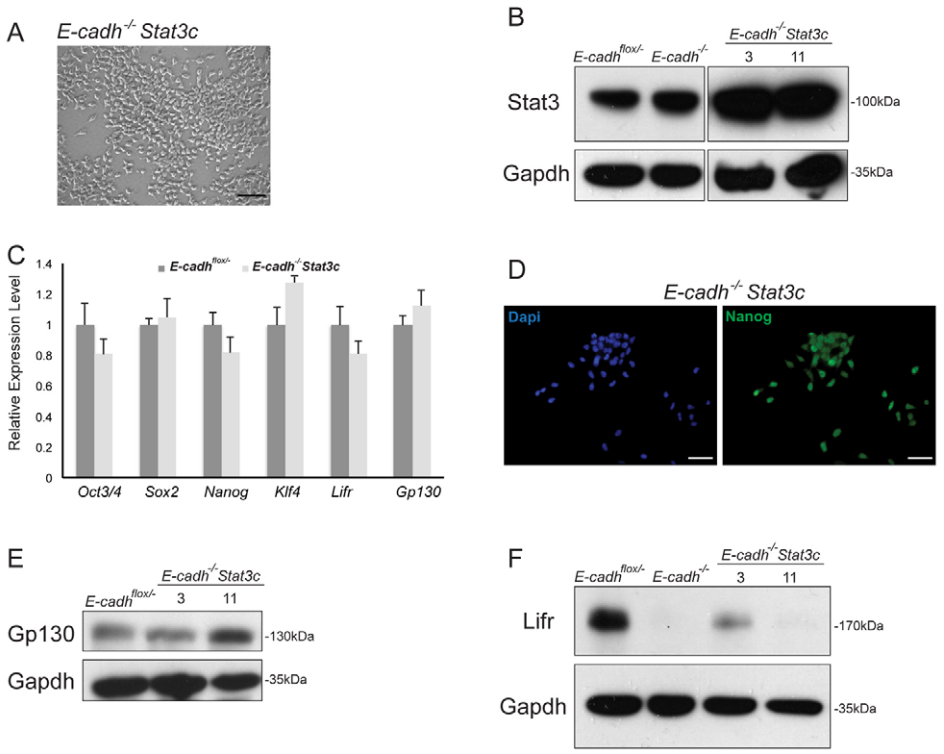

Fig. 6. Constitutive activation of Stat3 sustains pluripotent characteristics in the absence of E-cadherin. (**A**) Phase-contrast image of *E-cadh*$^{-/-}$*Stat3c* mES cells shows impaired adhesion of these cells. (**B**) Comparable levels of Stat3 are detected in *E-cadh*$^{flox/-}$ and *E-cadh*$^{-/-}$ mES cells by western blot. However, the expression of Stat3 is elevated in two independent *E-cadh*$^{-/-}$*Stat3c* mES cell clones (3 and 11). Gapdh was used as a loading control. (**C**) qRT-PCR analysis of *E-cadh*$^{-/-}$*Stat3c* mES cells shows that the expression of the pluripotency markers *Oct3/4*, *Sox2*, *Nanog*, *Klf4*, as well as *Lifr* and *Gp130* are retained at levels comparable to *E-cadh*$^{flox/-}$ mES cells. (**D**) Nanog is found in the nuclei of *E-cadh*$^{-/-}$*Stat3c* mES cells by immunofluorescence staining. DAPI was used to counterstain nuclei. (**E**,**F**) Constitutive activation of Stat3 in *E-cadh*$^{-/-}$*Stat3c* mES cells maintains Gp130 (E) but not Lifr (F) protein levels. Gapdh was used as a loading control. Error bars represent s.e.m. Scale bars: in A, 50 μm; in D, 25 μm.

cadherin is required to stabilize the Lif receptor complex at the cell membrane. Thereby, the extracellular part of E-cadherin seems to play a crucial role. Conversely, abrogation of E-cadherin leads to insufficient phosphorylation of Stat3 and cessation of pluripotency-associated gene expression.

Constitutive activation of Stat3 in the absence of E-cadherin is able to maintain mES cells in a pluripotent state

E-cadherin promotes the activation of the downstream Lif signaling effector Stat3. Based on this finding, we investigated whether E-cadherin-mediated cell adhesion could be substituted by constitutive activation of Stat3. To address this, we used *E-cadh*$^{flox/-}$ mES cells to generate E-cadherin null mES cells stably expressing a constitutively active form of Stat3 (Stat3c). *E-cadh*$^{-/-}$*Stat3c* mES cells exhibited a clear cell adhesion defect (Fig. 6A), and showed higher levels of Stat3 protein compared with *E-cadh*$^{flox/-}$ and *E-cadh*$^{-/-}$ mES cells (Fig. 6B). Importantly, the expression levels of pluripotency-associated genes, such as *Oct3/4*, *Sox2*, *Nanog*, *Klf4*, *Lifr* and *Gp130*, were not altered in comparison with *E-cadh*$^{flox/-}$ mES cells (Fig. 6C). Moreover, Nanog was present in the nuclei of *E-cadh*$^{-/-}$*Stat3c* mES cells (Fig. 6D). Interestingly, western blot analysis revealed a clear reduction of Lifr, but not Gp130, in these cells (Fig. 6E,F). From these findings, we propose that E-cadherin seems to specifically prevent Lifr internalization and degradation, and that permanent activation of Stat3 in mES is able to maintain these cells in a pluripotent state even in the absence of E-cadherin-mediated cell adhesion.

DISCUSSION

Cell adhesion and canonical Wnt signals have been independently ascribed to safeguard mES cells in a self-renewing and pluripotent state. A key player for both processes is β-catenin, which connects E-cadherin to the actin cytoskeleton via α-catenin, and which also mediates the transactivation of Wnt target genes. However, it still has been unresolved which of the two functions of β-catenin – adhesion or signaling – is absolutely required to maintain mES cells in culture. Now, our work provides novel insights into the molecular mechanisms underlying self-renewal and pluripotency of mES cells. We report that the adhesive role of β-catenin and, thus, E-cadherin plays the key role in retaining the expression of pluripotency-associated genes and that a nuclear function of β-catenin is absolutely not required in this process.

The functional significance of β-catenin for mES cells has been the subject of high controversy. Although we, and others, report that *Ctnnb1*$^{-/-}$ mES cells have lost their stemness characteristics (Anton et al., 2007; Hoffmeyer et al., 2012; Wagner et al., 2010), it was also recently reported that mES cells can be maintained without *Ctnnb1* (Lyashenko et al., 2011). Interestingly, these *Ctnnb1* null cells, in contrast to ours, maintained a compact morphology by upregulation of γ-catenin (plakoglobin). Plakoglobin, which is structurally very similar to β-catenin, binds to the same region of E-cadherin thereby restoring the integrity of the adhesion complex. Thus, similar to our *Ctnnb1*$^{-/-}$Eα mES cells, continuation of cell adhesion seems to be the road to mES cell stemness. However, it remains unclear whether the substitutive role of plakoglobin is functional in long-term culture. In another study, it was shown that a very low expression level of β-catenin is able to fulfill only the adhesive function of the protein, without evident canonical Wnt signaling activity (Rudloff and Kemler, 2012). In line with our results, in such mES cells the core pluripotency transcription factors are expressed at wild-type levels. However, β-catenin was still detectable in small amounts in the nuclear fraction of these mES cells, which could be sufficient for a potential submissive nuclear function of β-catenin in the maintenance of pluripotency other than canonical Wnt signaling. Conversely, the amount of β-catenin, and thereby the activity of the canonical Wnt pathway, become indispensable for the developmental potential of ES cells (Lyashenko et al., 2011; Rudloff and Kemler, 2012). The differentiation defect of *Ctnnb1*$^{-/-}$Eα mES cells can be explained by the absence of β-catenin in these cells.

Thus, *Ctnnb1*$^{-/-}$Eα mES cells cannot respond to canonical Wnt stimulation, which is well known to induce gastrulation and the specification of the mesendodermal cell fate (ten Berge et al., 2008; Gadue et al., 2006; Lindsley et al., 2006). However, low expression levels of β-catenin or blockade of Wnt signaling still promote the formation of neural ectoderm (Cajánek et al., 2009; Rudloff and Kemler, 2012). Lastly, another prerequisite for the formation of mesodermal cells is epithelial-mesenchymal transition (EMT), requiring the downregulation of E-cadherin (Takeichi, 1988). However, in our *Ctnnb1*$^{-/-}$Eα mES cells E-cadherin is constitutively expressed, which might also prohibit the differentiation of these cells along the mesodermal lineage.

Loss of cell adhesion in ES cells, by contrast, leads to a profound downregulation of pluripotency-associated markers. The recognition that E-cadherin-mediated cell adhesion seems to play the principal role to preserve the stemness state of mES cells was further investigated in our *Ctnnb1*$^{-/-}$Eα mES cells. We describe for the first time that E-cadherin directly contributes to maintain mES cells in culture through its interaction with the Lifr-Gp130 co-receptor complex. This association is crucial for the integrity of the Lif signaling pathway, proper phosphorylation of Stat3, and, thus, the transcription of pluripotency-associated genes. Several groups have proposed that cadherins take part in multiple cellular signaling processes in addition to their function in mediating calcium-dependent cell adhesion. For example, interactions with receptor tyrosine kinases (RTKs), such as Egfr or Fgfr2, were repeatedly observed. Such interactions seem to enable the cells to modulate their response to growth factor signaling (Fedor-Chaiken et al., 2003; Pece and Gutkind, 2000; Qian et al., 2004; Suyama et al., 2002). Supporting this notion, our group recently showed an association of E-cadherin with insulin-like growth factor I receptor (Igf1r) in the trophectodermal cell layer, which is essential for proper blastocyst formation (Bedzhov et al., 2012). The major finding of our work, that E-cadherin interacts with the Lif receptor in mES cells, adds another potential regulatory role to this adhesion molecule. Because Lifr is expressed in the inner cell mass (ICM) of pre-implantation mouse embryos (Nichols et al., 1996), it is very likely, even *in vivo*, that an interaction between E-cadherin and Lifr might contribute to maintenance of the pluripotent characteristics of the ICM. Moreover, enhanced cell adhesion might be a possible approach to promote the naive state of human ES cells, although they do not depend on Lif.

The binding interface of Lifr seems to lie within the extracellular or transmembrane domain of E-cadherin as the Lif signaling pathway is still intact in *E-cadh*$^{-/-}$*Ec* mES cells. From this, we conclude that the truncated Ec molecule is sufficient to stabilize the Lif receptor at the cell membrane. Likewise, the PEST degradation sequence located in the intracellular domain of E-cadherin cannot be shielded by β-catenin in *Ctnnb1*$^{-/-}$ mES cells, which in turn might lead to increased trafficking of E-cadherin away from the membrane and destabilization of the Lif receptor. Moreover, in the absence of E-cadherin neither β-catenin nor plakoglobin is able to rescue the mES cell state. This provides further evidence that E-cadherin is crucial for transduction of the Lif signal by stabilizing the Lifr-Gp130 co-receptor complex. Interestingly, in mammary epithelial cells, E-cadherin-mediated cell adhesion was shown to promote Stat3 phosphorylation (Arulanandam et al., 2009). However, when these authors competitively disrupted the cell-cell contacts with the help of a recombinant E-cadherin fragment that included the two distal extracellular domains of E-cadherin, pStat3 levels decreased.

In mES cells, the Stat3 pathway is activated exclusively upon Lif signaling, although the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) cascades can also potentially be triggered by Lif (Hirai et al., 2011). Stat3 activation is necessary and sufficient for self-renewal and pluripotency of mES cells cultured in the presence of serum and feeders (Niwa et al., 1998; Raz et al., 1999). In this regard it is noteworthy that constitutive activation of Stat3 is able to bypass the requirement for Lif (Matsuda et al., 1999). We have observed similar results in our *E-cadh*$^{-/-}$*Stat3c* mES cells, in which the permanent signaling activity of Stat3 is able to substitute for the loss of E-cadherin-mediated cell adhesion. These results are in agreement with a recently published work claiming that the reintroduction of full-length E-cadherin molecule into *E-cadh*$^{-/-}$ mES cells is able to rescue Stat3 activation (Hawkins et al., 2012). However, restoration of E-cadherin-mediated cell adhesion in our *Ctnnb1*$^{-/-}$ or *E-cadh*$^{-/-}$ mES cells did not lead to a recovery of *Lifr*, *Gp130* or *Nanog* expression. This can be explained by the altered transcription profile of *Ctnnb1*$^{-/-}$ or *E-cadh*$^{-/-}$ mES cells (Soncin et al., 2011). Furthermore, our microarray analysis supports the finding of large changes in transcription profiles associated with the deletion of β-catenin (~800 genes were differentially expressed), but also shows that preserving E-cadherin-mediated cell adhesion results in less severe alterations (~300 genes were differentially expressed). Interestingly, we found many pluripotency-associated transcription factors and components of the Lif signaling cascade to be still expressed at wild-type levels in *Ctnnb1*$^{-/-}$Eα mES cells.

An increasing number of reports demonstrate a role for E-cadherin in cellular signaling processes. Here, we propose a model in which E-cadherin stabilizes and promotes the Lif receptor complex at the cell membrane, thereby preserving the pluripotent characteristics of mES cells. Loss of adhesion, as observed in *Ctnnb1*$^{-/-}$ or in *E-cadh*$^{-/-}$ mES cells, first leads to fewer receptors at the membrane followed by a reduction in pStat3 levels and, subsequently, in the transcriptional downregulation of pluripotency-associated genes. Finally, in the long term, adhesion-deficient mES cells fail to respond to Lif signaling. Given the significance of stem cells, it will be of great interest to investigate whether E-cadherin or cell adhesion in general might contribute to preserve the stemness characteristics of other stem cell systems.

Acknowledgements
We thank Drs Masayuki Ozawa (Kagoshima University, Japan), Verdon Taylor and Dirk Junghans (University of Basel) for providing reagents and helpful discussions. We thank Caroline Johner and Benoit Kanzler from the animal and transgenic facilities.

Funding
This work was funded by the Max-Planck Society.

Competing interests statement
The authors declare no competing financial interests.

Author contributions
I.d.V. contributed to experimental design, performed experiments, contributed to data analysis and wrote the manuscript. S.R. contributed to experimental design, performed experiments and wrote the manuscript. A.C. and Y.L. contributed to data analysis. E.L. and R.V. performed experiments. R.K. contributed to experimental design, contributed to data analysis and wrote the manuscript. All authors read and approved the final manuscript for publication.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.088690/-/DC1

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