

## SHORT REPORT

# Wnt/ $\beta$ -catenin and LIF–Stat3 signaling pathways converge on Sp5 to promote mouse embryonic stem cell self-renewal

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**ABSTRACT**

Activation of leukemia inhibitor factor (LIF)–Stat3 or Wnt/ $\beta$ -catenin signaling promotes mouse embryonic stem cell (mESC) self-renewal. A myriad of downstream targets have been identified in the individual signal pathways, but their common targets remain largely elusive. In this study, we found that the LIF–Stat3 and Wnt/ $\beta$ -catenin signaling pathways converge on Sp5 to promote mESC self-renewal. Forced Sp5 expression can reproduce partial effects of Wnt/ $\beta$ -catenin signaling but mimics most features of LIF–Stat3 signaling to maintain undifferentiated mESCs. Moreover, Sp5 is able to convert mouse epiblast stem cells into a naïve pluripotent state. Thus, Sp5 is an important component of the regulatory network governing mESC naïve pluripotency.

**KEY WORDS:** Embryonic stem cell, Self-renewal,  $\beta$ -catenin, Stat3, Sp5

**INTRODUCTION**

Mouse embryonic stem cells (mESCs) are pluripotent cell lines derived from pre-implantation embryos (Evans and Kaufman, 1981; Martin, 1981). They can be expanded in serum-containing medium with LIF, or serum-free N2B27 medium with 2i (hereafter N2B27/2i, see below) *in vitro* while retaining the capacity for multilineage differentiation (Smith, 2001; Williams et al., 1988; Ying et al., 2008). LIF binds to the LIF-receptor–gp130 heterodimer (gp130 is also known as IL6ST), leading to phosphorylation of Stat3 by JAK. Activated Stat3 then translocates into the nucleus and binds DNA (Niwa et al., 1998). Stat3 plays a key role in LIF-mediated mESC self-renewal. A chimeric Stat3 protein fused with the ligand-binding domain of the estrogen receptor (Stat3ER) maintains mESCs in an undifferentiated state in the presence of the synthetic ligand 4-hydroxytamoxifen (4-HT or 4-OHT) without LIF (Matsuda et al., 1999). By contrast, overexpressing a dominant-negative mutant of Stat3 in mESCs blocks LIF-induced activation of endogenous Stat3 and causes differentiation (Niwa et al., 1998). Furthermore, LIF fails to maintain Stat3-null mESC self-renewal (Ying et al., 2008). Many Stat3 targets have been identified recently, such as

*Klf4*, *Gbx2*, *Pim1*, *Pim3*, *Pramel7*, *Myc* and *Tfcp2l1* (Cartwright et al., 2005; Casanova et al., 2011; Hall et al., 2009; Martello et al., 2013; Tai and Ying, 2013; Ye et al., 2013). When overexpressed, these genes can bypass the LIF requirement for mESC maintenance. However, knockdown of any single gene does not abolish the self-renewal-promoting effect of LIF–Stat3 signaling, suggesting that LIF–Stat3 signaling triggers multiple downstream targets to promote mESC self-renewal.

2i contains two small molecules: CHIR99021 (CHIR) and PD0325901 (PD03), which inhibit glycogen synthase kinase 3 (GSK3) and mitogen-activated protein kinase (MAPK) kinases (MEK proteins), respectively (Huang et al., 2015; Ye et al., 2014; Ying et al., 2008). Inhibiting GSK3 results in the activation of the Wnt/ $\beta$ -catenin signaling pathway. Once in the nucleus,  $\beta$ -catenin liberates many Tcf3-repressed pluripotency genes (Wray et al., 2011), such as *Esrrb*, *Nr0b1*, *Tfcp2l1* and *Nanog* (Martello et al., 2012). It is noteworthy that 2i can sustain Stat3-null mESC self-renewal (Ying et al., 2008), whereas the identity of  $\beta$ -catenin-null mESCs can be maintained in medium containing LIF and PD03 (Lyashenko et al., 2011). Taken together, these results suggest CHIR and LIF might share common or cross-compensatory downstream targets in promoting mESC self-renewal. Here, we identified Sp5 as one of common targets of CHIR and LIF, and revealed its new function in promoting mESC self-renewal and reprogramming.

**RESULTS AND DISCUSSION****Identification of downstream targets of CHIR and LIF in mESCs**

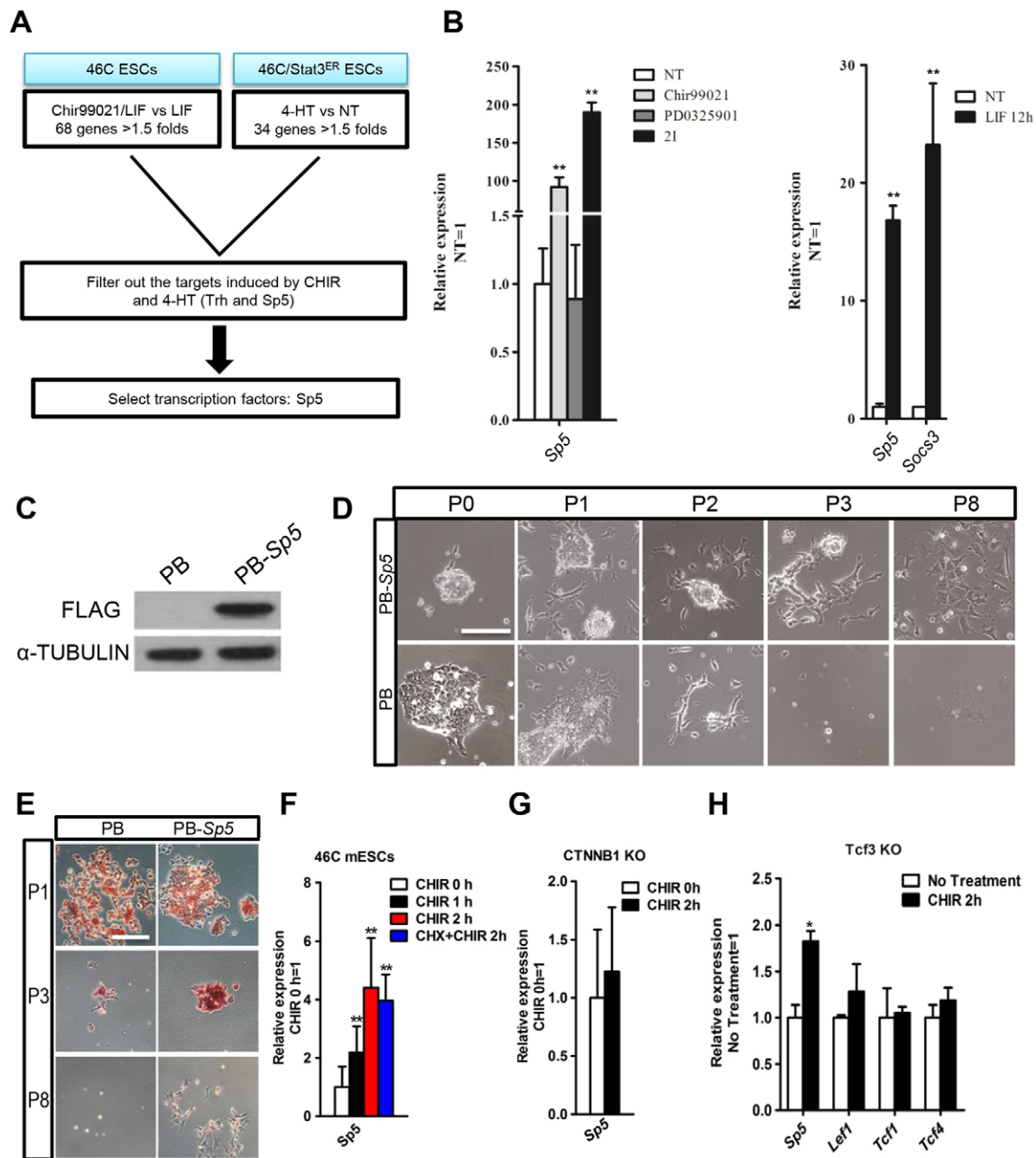
To screen the possible shared targets of the Wnt/ $\beta$ -catenin and LIF–Stat3 signaling pathways, we performed a DNA microarray analysis in mESCs treated with or without CHIR (GEO Number: GSE50393). We then looked for genes that were upregulated by 1.5 times or greater by CHIR treatment or by Stat3 stimulation (Bourillot et al., 2009). From this comparison, two common targets emerged: *Trh* and *Sp5* (Fig. 1A). *Trh*, or thyrotropin-releasing hormone, is a secretory protein. Sp5 (trans-acting transcription factor 5) belongs to the Sp1 transcription factor family (el-Baradi and Pieler, 1991; Wimmer et al., 1993) and harbors three zinc fingers that are similar to those found in members of the KLF gene family (Suske et al., 2005; Treichel et al., 2001). KLF family members, such as *Klf2*, *Klf4* and *Klf5*, play an important role in maintaining mESC self-renewal (Ema et al., 2008; Hall et al., 2009; Jiang et al., 2008; Qiu et al., 2015). We thus chose *Sp5* as our candidate gene. Quantitative real-time PCR (qRT-PCR) was used to confirm that both CHIR and LIF treatments could induce the expression of *Sp5* (Fig. 1B). *Socs3*, a direct target of LIF–Stat3 signaling, was used as a positive control and showed substantial induction upon LIF stimulation (Fig. 1B) (Niwa et al., 2009).

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**Fig. 1. Overexpressing *Sp5* partially mimics the effect of CHIR in medium with 2i.** (A) Flow chart showing the method used to identify candidate genes downstream of CHIR and Stat3. (B) qRT-PCR analysis of *Soc3* and *Sp5* expression induced by CHIR, PD03 and 2i (left), or LIF (right) for 12 h. Data represent mean $\pm$ s.d. of three biological replicates. \*\* $P$ <0.01 versus NT. NT, no treatment. (C) FLAG-tagged *Sp5* was introduced into 46C mESCs and the protein level of FLAG-tagged SP5 was determined by western blotting.  $\alpha$ -tubulin is used as a loading control. (D,E) Alkaline phosphatase staining images of PB-*Sp5* mESCs cultured in N2B27 medium with PD03 for the indicated number of passages (P). Scale bars: 100  $\mu$ m. (F–H) qRT-PCR analysis of the indicated gene expression after treatment with CHIR for the indicated time in 46C mESCs, *CTNNB1*-KO ( $\beta$ -catenin knockout) and *Tcf3*-KO mESCs. CHX, cycloheximide treatment. Data represent mean $\pm$ s.d. of three biological replicates. \*\* $P$ <0.01 versus 0 h CHIR.

### Overexpression of *Sp5* promotes mESC self-renewal in the absence of CHIR

We then investigated the function of *Sp5* in CHIR- and LIF-mediated self-renewal in mESCs. We generated an mESC line that overexpressed FLAG-tagged *Sp5* using a PiggyBac vector (PB-*Sp5*) in which *Sp5* expression was efficiently enhanced (Fig. 1C). Empty vector (PB) and PB-*Sp5* mESCs grew robustly in N2B27/2i, and in serum-containing medium with LIF. We then withdrew CHIR to test the ability of *Sp5* to replace the function of CHIR in N2B27/2i. Before passaging, PB-*Sp5* mESCs maintained an undifferentiated

state, whereas cells containing only PB began to die (Fig. 1D,E). Moreover, PB-*Sp5* mESCs could be split for three more passages, but then also collapsed and lost alkaline phosphatase activity (Fig. 1D,E). Therefore, overexpressing *Sp5* supports short-term mESC self-renewal when combined with PD03, indicating that *Sp5* partially reproduces the effect of CHIR in 2i.

*Sp5* is known to be a downstream target of the Wnt/ $\beta$ -catenin signaling pathway during vertebrate development and in cancer cells (Hoverter et al., 2012; Waaler et al., 2011; Weidinger et al., 2005). To determine whether *Sp5* is regulated through the

Wnt/ $\beta$ -catenin signaling pathway in mESCs, we examined the expression of *Sp5* in 46C ESCs after CHIR treatment for 2 h. *Sp5* exhibited significantly increased expression in response to CHIR, even in the presence of the protein synthesis inhibitor cycloheximide (Fig. 1F). Furthermore, CHIR failed to induce *Sp5* expression in  $\beta$ -catenin-null ESCs (Fig. 1G), indicating that *Sp5* is a direct target of  $\beta$ -catenin in mESCs. Notably, CHIR upregulated *Sp5* in the absence of *Tcf3* without a change in the expression of other members of the Tcf family, including *Lef1*, *Tcf1* and *Tcf4* (Fig. 1H), indicating that  $\beta$ -catenin can induce *Sp5* expression through interaction with Tcf factors but not Tcf3.

### Overexpression of *Sp5* maintains mESC self-renewal in the absence of LIF

To determine the role of *Sp5* in LIF–Stat3 signaling, we performed experiments in serum-containing medium without LIF. Surprisingly, PB-*Sp5* mESCs could be continually passaged while retaining typical ESC morphology, positive alkaline phosphatase activity and high expression levels of the pluripotency markers OCT4, NANOG and KLF4, whereas empty vector (PB) control cells differentiated (Fig. 2A,B; Fig. S1A–C). The results were validated in OCG9 mESCs harboring an Oct4–GFP pluripotency reporter (Fig. S1D,E). We then tried culturing PB and PB-*Sp5* 46C ESCs in N2B27 medium with BMP4, which maintains mESC self-renewal when combined with LIF (Ying et al., 2003). Similarly, PB-*Sp5* mESCs maintained an undifferentiated state, whereas PB cells differentiated rapidly (Fig. 2C,D). We found that overexpression of *Sp5* led to an upregulation in the level of *Nanog*, whereas it repressed the expression of the differentiation markers *Gata4* and *Gata6*. Both of these markers are typically suppressed by *Nanog* in mESCs (Fig. 2E) (Chambers et al., 2003). These collective results suggest that elevated expression of *Sp5* recapitulates the self-renewal-promoting effect of the LIF–Stat3 signaling pathway, probably through induction of *Nanog* expression.

To examine whether mESCs maintained by *Sp5* overexpression retain pluripotency, we used a *loxP*-based excisable vector to overexpress *Sp5*. After expansion in the absence of LIF for five passages, the floxed-*Sp5* mESCs were transiently transfected with a *Cre* expression vector to excise the floxed *Sp5* transgene (Fig. 2F). After the excision of *Sp5* transgene, the *GFP* transgene was activated, and their maintenance became dependent on LIF (Fig. 2G). The revertant cells maintained the ability to differentiate into cells from the three germ layers after forming embryoid bodies (Fig. 2H,I), suggesting that *Sp5* overexpressing ESCs retain their pluripotency.

### Overexpressing *Sp5* sustains Stat3-null ESCs in an undifferentiated state

To determine whether Stat3 is dispensable for *Sp5*-promoted mESC self-renewal, we overexpressed *Sp5* in *Stat3*-null mESCs and cultured them in serum-containing medium without LIF. These transfectants remained undifferentiated, whereas *Stat3*-null mESCs transfected with an empty vector died or differentiated after two passages (Fig. S2). These results suggest that the self-renewal-promoting effect of *Sp5* is independent of Stat3.

### *Sp5* is a direct downstream target of the LIF–Stat3 signaling pathway

LIF mainly activates three intracellular signaling pathways: the JAK–Stat3, PI3K–AKT and MAPK pathways (Hirai et al., 2011). To examine whether *Sp5* is a direct target of LIF–Stat3 pathway, we tested the responsiveness of *Sp5* to the activation of Stat3. LIF stimulation for 1 h in 46C mESCs led to an accumulation of

phosphorylated STAT3 and acute transcript induction of *Socs3* and *Sp5* (Fig. 3A,B). However, their expression was suppressed by JAK inhibitor (Fig. 3A). This phenomenon was also observed in the presence of the protein synthesis inhibitor cycloheximide (Fig. 3A,B). A similar expression pattern of *Sp5* induction was observed in GY118F and Stat3ER transfectants (Fig. 3C–F). GY118F is a mutated chimeric receptor and only triggers JAK–Stat3 signaling in the presence of granulocyte colony stimulating factor (GCSF) (Burdon et al., 1999). Stat3ER stimulates Stat3 direct targets by translocating into the nucleus in the presence of 4-HT. This process occurs independently of endogenous STAT3, MAPK and AKT (Bourillot et al., 2009; Matsuda et al., 1999). Collectively, these data indicate that *Sp5* is a direct target of the LIF–Stat3 signaling pathway. However, knockdown of *Sp5* did not impair the self-renewal-promoting effect of LIF–Stat3 signaling (Fig. S3A,B). Interestingly, downregulation of *Sp5* significantly induced *Pim1* (a Stat3 direct target) expression (Fig. S3A) (Martello et al., 2013), suggesting that the self-renewal-promoting function of *Sp5* is redundant with other Stat3 targets in mESCs.

### LIF and CHIR upregulates *Sp5* expression independently

Given that *Sp5* is also a direct target of the Wnt/ $\beta$ -catenin signaling pathway (Dunty et al., 2014; Weidinger et al., 2005), we next wanted to examine whether LIF and CHIR depended on each other to exert their own upregulating effect. CHIR strongly induced *Sp5* expression in 46C mESCs in the presence of JAK inhibitor whereas LIF failed (Fig. 3G). By contrast, LIF stimulated *Sp5* expression in the absence of  $\beta$ -catenin, whereas CHIR-mediated induction of *Sp5* was dependent on  $\beta$ -catenin (Fig. 3H), indicating that LIF and CHIR induce *Sp5* transcription independently of one another.

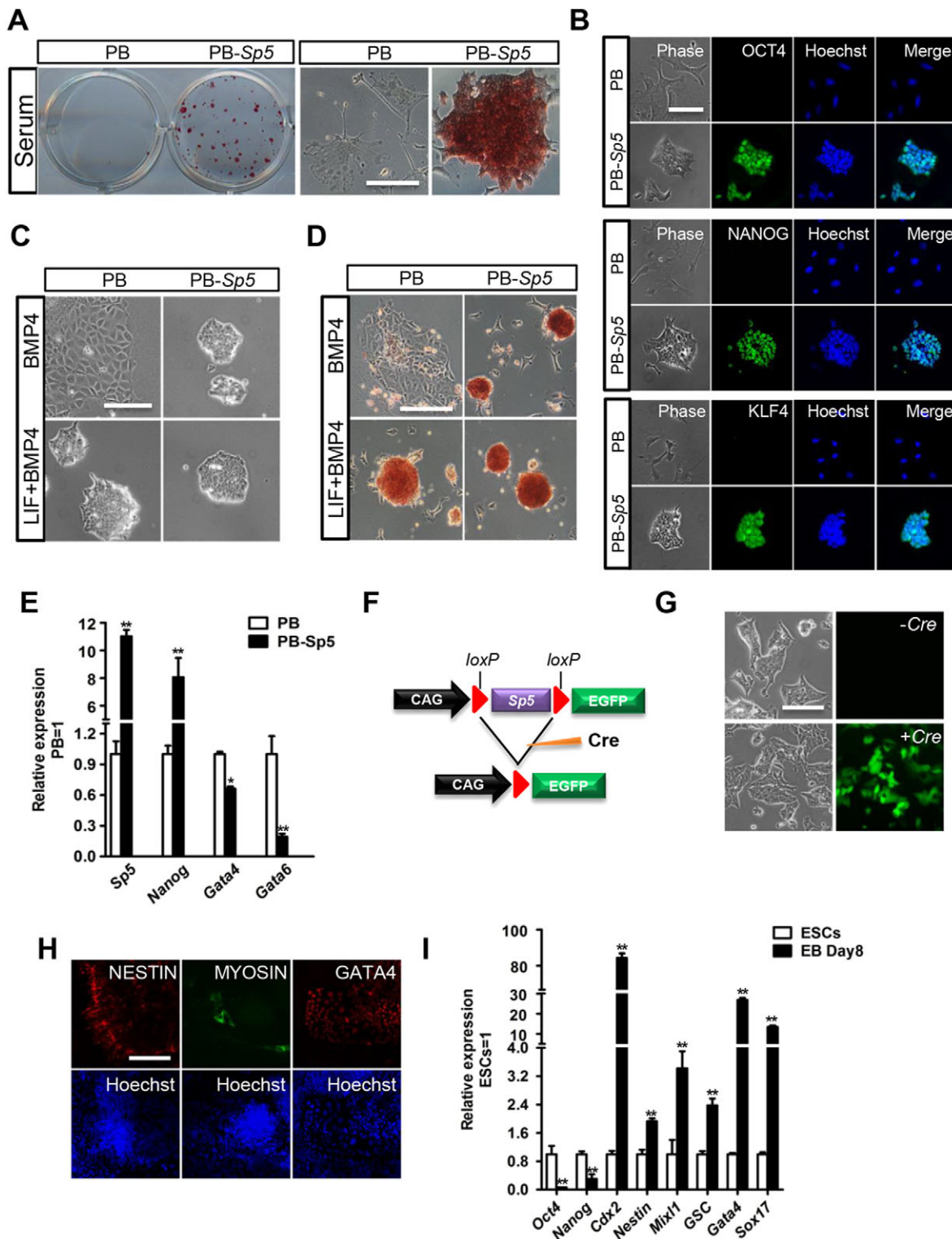
### *Sp5* is able to reprogram EpiSCs to a naïve state

Another distinguishing feature of LIF–Stat3 signaling is its ability to convert mouse epiblast stem cells (EpiSCs) into cells with a naïve state of pluripotency (van Oosten et al., 2012; Yang et al., 2010). Mouse EpiSCs are isolated from epiblasts of post-implantation mouse embryos and share many features with human ESCs (Brons et al., 2007; Tesar et al., 2007). *Stat3* and its multiple targets are expressed highly in mESCs, but are downregulated in EpiSCs. Importantly, their overexpression is sufficient to reprogram EpiSC into the mESC state (Guo et al., 2009; Tai and Ying, 2013; Yang et al., 2010). To test the role of *Sp5* in EpiSC reprogramming, we first differentiated 46C mESCs into EpiSCs (Fig. 4A), but observed no obvious change in *Sp5* expression (Fig. 4B). We then engineered 46C EpiSCs to overexpress *Sp5* (Fig. 4C) and cultured  $10^6$  transfectants in medium containing LIF, serum and 2i. After 12 days, five ESC-like colonies emerged, whereas empty vector transfectants died or differentiated (Fig. 4D). We then picked two colonies and these colonies continuously expanded in medium containing serum and LIF. They expressed high levels of the naïve pluripotency markers *Nanog*, *Stella*, *Nrobl1*, *Klf2* and *Rex1*, but low levels of the EpiSC-specific markers *Fgf5* and *Sox17* (Fig. 4E). We confirmed this experiment in E3 EpiSCs carrying an Oct4–GFP pluripotency reporter (Greber et al., 2010). The *Sp5*-reprogrammed stem cells were expanded in medium containing serum and LIF over multiple passages, showing undifferentiated morphology and stable expression of the Oct4–GFP reporter (Fig. S4A,B). These results imply that forced expression of *Sp5* is capable of converting EpiSCs into a naïve pluripotent state.

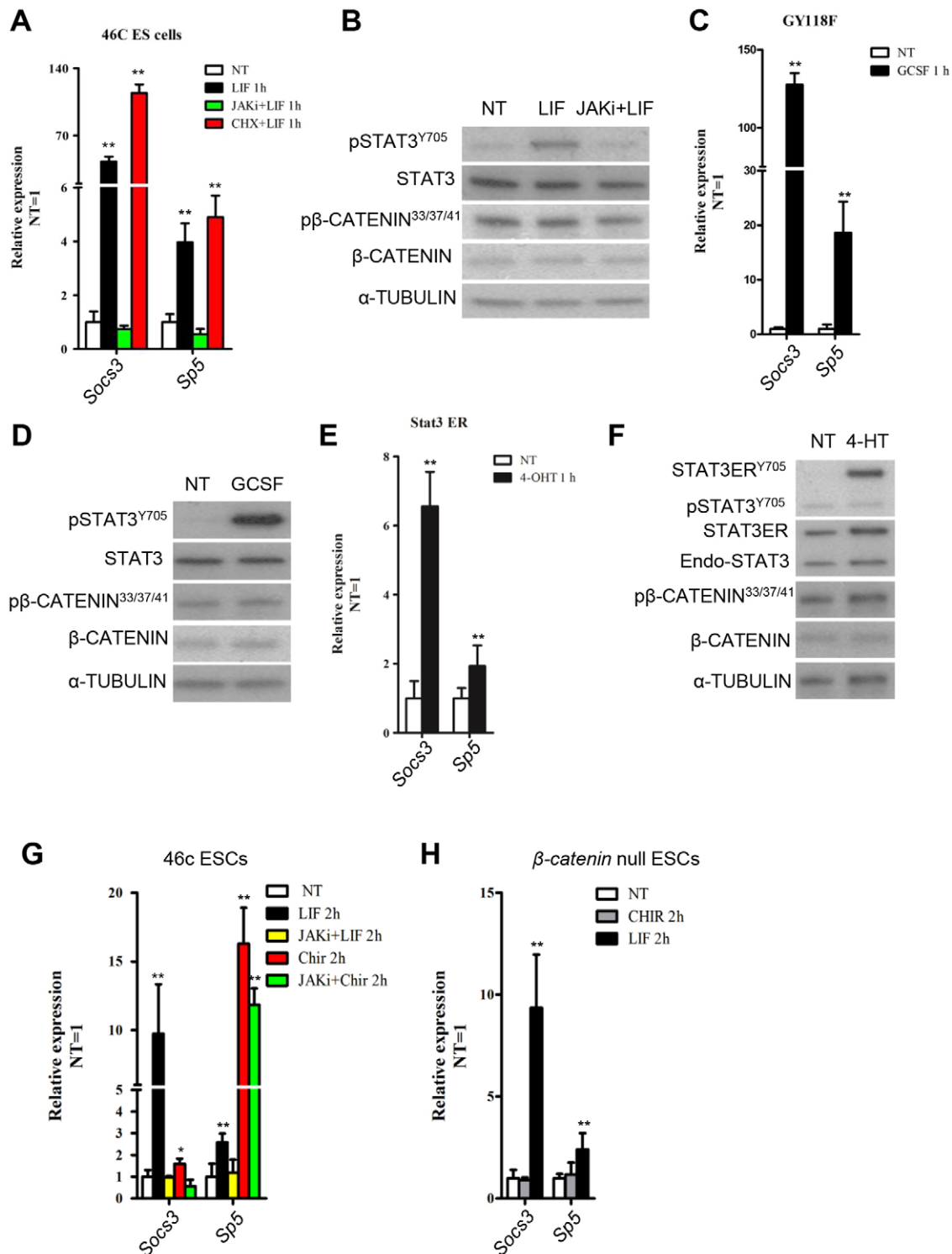
### Conclusions

CHIR and LIF have redundant function in mESC maintenance. We demonstrate that *Sp5* is a convergent target of the Wnt/ $\beta$ -catenin and





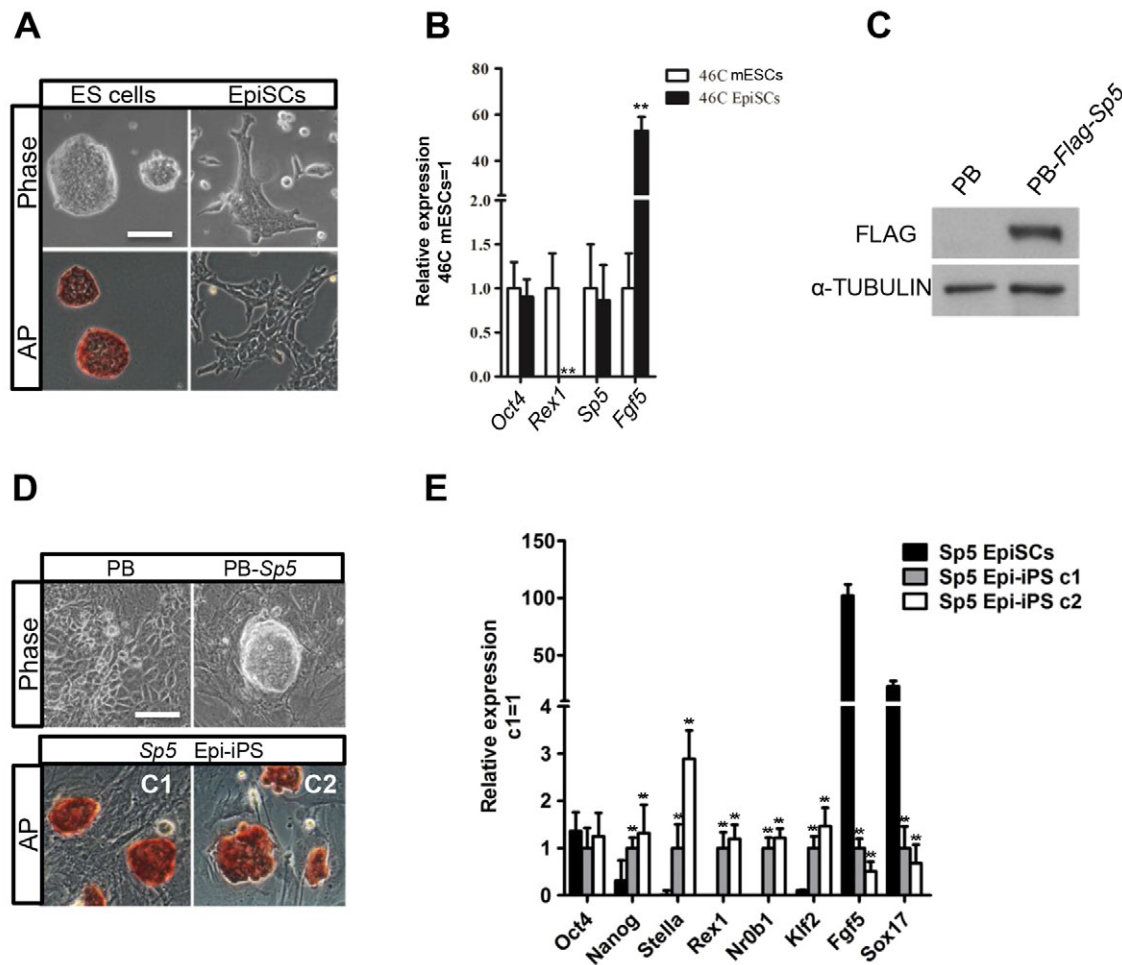
**Fig. 2. Forced *Sp5* expression promotes mESC self-renewal without LIF addition.** (A) Alkaline phosphatase staining of PB and PB-*Sp5* mESCs cultured in serum for five passages. Scale bar: 100  $\mu$ m. (B) Immunofluorescence staining of PB and PB-*Sp5* mESCs cultured in medium with serum for five passages. Scale bar: 100  $\mu$ m. Hoechst, Hoechst 33342. (C,D) Phase contrast (C) and alkaline phosphatase staining (D) images of PB and PB-*Sp5* mESCs cultured in N2B27 medium with BMP4 in the absence or presence of LIF for 8 days. Scale bars: 100  $\mu$ m. (E) qRT-PCR analysis of *Sp5*, *Nanog*, *Gata4* and *Gata6* expression in PB and PB-*Sp5* 46C ESCs cultured in medium with LIF and serum. Data represent mean $\pm$ s.d. of three biological replicates. \* $P$ <0.05, \*\* $P$ <0.01 versus PB. (F) Diagram showing the Cre-excisable construct used for *Sp5* overexpression. (G) Phase contrast and GFP images of loxP-*Sp5* mESCs after transfection with or without the *Cre* expression plasmid. ESCs were cultured in medium with LIF and serum. Scale bar: 100  $\mu$ m. (H) Immunofluorescence staining for the neural marker nestin, the cardiomyocyte marker myosin, and the primitive endoderm marker GATA4 in differentiated cells derived from loxP-*Sp5* mESCs in which the *Sp5* transgene has been removed by Cre. Hoechst 33342 was used for nuclear staining. Scale bar: 100  $\mu$ m. (I) qRT-PCR analysis of the indicated gene expression in ESCs and embryoid bodies (EB) derived from cells with an excised *Sp5* transgene. Pluripotency genes, *Oct4* and *Nanog*; trophoblast marker, *Cdx2*; ectoderm marker, nestin; mesoderm markers, *Mixl1* and *Gsc*; endoderm makers, *Gata4* and *Sox17*. \* $P$ <0.05, \*\* $P$ <0.01 versus ESCs.



**Fig. 3. *Sp5* is a direct target of Stat3.** (A) qRT-PCR analysis of *Socs3* and *Sp5* expression levels in 46C mESCs deprived of LIF overnight and stimulated with LIF for 1 h in the presence or absence of JAK inhibitor I (JAKi) or cycloheximide (CHX, 50 mg/ml). Data represent mean±s.d. of three biological replicates. \*\**P*<0.01 versus 46C NT. NT, no treatment. (B,C) qRT-PCR analysis of *Socs3* and *Sp5* in GY118F and Stat3ER transfectants deprived of LIF overnight and stimulated with GCSF or 4-HT for 1 h. Data represent mean±s.d. of three biological replicates. \*\**P*<0.01 versus NT. (D–F) Western blot analysis of total and phosphorylated Stat3 and β-catenin in wild type, and GY118F- and Stat3ER-transfected mESCs. p, phosphorylated protein. (G,H) qRT-PCR analysis of *Socs3* and *Sp5* in β-catenin-null or 46C mESCs treated with LIF or CHIR for 2 h in the presence or absence of JAK inhibitor I. Data represent mean±s.d. of three biological replicates. \**P*<0.05, \*\**P*<0.01 versus NT.

LIF–Stat3 signaling pathways. In support of this, we present evidence that *Sp5* recapitulates features of Wnt/β-catenin and LIF–Stat3 signaling in promoting mESC self-renewal. Furthermore,

overexpression of *Sp5* is able to overcome the differentiation cues to convert EpiSCs into cells with naïve pluripotency. Our study therefore reveals a new function of *Sp5* in mESC self-renewal, which



**Fig. 4. Forced expression of *Sp5* promotes EpiSC reprogramming to naïve pluripotency.** (A) Phase contrast and alkaline phosphatase (AP) staining images of 46C mESCs and 46C mESC-derived EpiSCs. Scale bar: 100  $\mu$ m. (B) qRT-PCR analysis of expression patterns of *Sp5*, *Oct4*, *Rex1* and *Fgf5* in 46C mESCs and EpiSCs. Data represent mean  $\pm$  s.d. of three biological replicates. \*\* $P < 0.01$  versus 46C mESCs. (C) Western blot analysis for Sp5 in 46C EpiSCs transfected with vector expressing FLAG-tagged *Sp5*. (D) Phase contrast and alkaline phosphatase staining images of *Sp5* EpiSC transfectants and *Sp5* EpiSC transfectant-derived induced pluripotent stem cells (iPSCs). Scale bar: 100  $\mu$ m. (E) qRT-PCR analysis of *Oct4*, *Nanog*, *Stella*, *Nr0b1*, *Klf2*, *Rex1*, *Fgf5* and *Sox17* expression in *Sp5* EpiSCs and two *Sp5* Epi-iPS colonies. Data represent mean  $\pm$  s.d. of three biological replicates. \*\* $P < 0.01$  versus *Sp5* EpiSCs. C1 and C2 are the individual colonies of ESC-like cell. Epi-iPS, EpiSC-derived iPSCs.

is mediated by CHIR and LIF, and reprogramming of EpiSCs into naïve ESCs. In the future, understanding how *Sp5* cooperates with other downstream targets of  $\beta$ -catenin and Stat3 to maintain the pluripotent ESC state might facilitate the development of new culture conditions for the derivation of authentic ESCs from various species.

## MATERIALS AND METHODS

### Cell culture

46C mESCs, kindly provided by Austin Smith (Wellcome Trust-Medical Research Council Cambridge Stem Cell Institute, University of Cambridge, UK), were cultured on 0.1% gelatin-coated dishes. mESC medium contains Glasgow's minimum essential medium (GMEM; Sigma), 10% fetal calf serum (FCS; HyClone), 1% MEM nonessential amino acids (Invitrogen), 2 mM GlutaMax (Invitrogen), 0.1 mM  $\beta$ -mercaptoethanol (Invitrogen) and 100 units/ml LIF (prepared in-house). For serum-free culture, mESCs were maintained in N2B27 supplemented with 3  $\mu$ M CHIR99021 and 1  $\mu$ M PD0325901 (Sigma), or supplemented with 100 units/ml LIF and 10 ng/ml BMP4 (Peprotech).

### Plasmid construction and cell transfection

The coding region of *Sp5* was inserted into a PiggyBac vector and transduced into cells combined with 2  $\mu$ g transposase using LTX

(Invitrogen). Selection was continued for 1 week by adding 2  $\mu$ g/ml puromycin. The short hairpin RNA (shRNA) plasmids were generated according to the Addgene PLKO.1 protocol. The target-specific sequence for *Sp5* is 5'-GGATTC AAAGGATTGCTTTC-3'.

### Alkaline phosphatase activity assay and qRT-PCR

The alkaline phosphatase activity assay and qRT-PCR analyses were performed according to our previous report (Ye et al., 2013). The primers used for the qRT-PCR analyses are listed in Table S1.

### Western blotting

Western blotting was performed according to a standard protocol. The primary antibodies used for probing were against  $\alpha$ -tubulin (32-2500, Invitrogen; 1:2000), phosphorylated Stat3<sup>Y705</sup> (9131S, Cell Signaling Technology; 1:1000), Stat3 (610190, BD Biosciences; 1:1000), phosphorylated  $\beta$ -Catenin<sup>Ser33/Ser37/Thr41</sup> (9561S, Cell Signaling Technology; 1:1000) and  $\beta$ -catenin (610153, BD Biosciences; 1:1000).

### Immunofluorescence staining

Immunostaining was performed with standard protocols. Primary antibodies used were against Oct4 (sc-5279, Santa Cruz Biotechnology; 1:200), Nanog (AF2729, R&D; 1:100), Klf4 (AF3158, R&D; 1:100), nestin (2Q178, Santa



Cruz Biotechnology, 1:100), GATA4 (G4, Santa Cruz Biotechnology, 1:100) and myosin (MF-20, Developmental Studies Hybridoma Bank, 1:50).

### mEpiSC derivation and reprogramming

For ESC-to-EpiSC differentiation, 5000 46C mESCs were plated into 0.1% gelatin-coated plates and cultured in serum medium supplemented with activin A (10 ng/ml, Peprotech), bFGF (10 ng/ml, Peprotech) and JW55 (20  $\mu$ m, Tocris). Cells were used after 10 passages. For reprogramming,  $10^6$  transfectants were seeded onto a 3.5-cm dish and cultured in mESC medium supplemented with LIF and 2i. The number of alkaline-phosphatase-positive clones was counted under a microscope. ESC-like clones were picked and subsequently expanded in medium containing LIF and serum.

### Accession numbers

Data and details of the method for the DNA microarray analysis are available in the Gene Expression Omnibus under accession number GSE50393.

### Statistical analysis

All data are reported as the mean $\pm$ s.d. A Student's *t*-test was used to determine the significance of differences in comparisons. Values of  $P < 0.05$  were considered as statistically significant.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

S.Y., D.Z. and F.C. designed the study and performed the experiments; D.W. and J.M. contributed reagents; F.L., S.H., K.H., Q.B. and D.L. analyzed data; S.Y. and Q.-L.Y. wrote the manuscript.

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### Supplementary information

Supplementary information available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.177675/-DC1>

### References

- Bourillot, P.-Y., Aksoy, I., Schreiber, V., Wianny, F., Schulz, H., Hummel, O., Hubner, N. and Savatier, P. (2009). Novel STAT3 target genes exert distinct roles in the inhibition of mesoderm and endoderm differentiation in cooperation with Nanog. *Stem Cells* **27**, 1760–1771.
- Brons, I. G. M., Smithers, L. E., Trotter, M. W. B., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S. M., Howlett, S. K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R. A. et al. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448**, 191–195.
- Burdon, T., Chambers, I., Stracey, C., Niwa, H. and Smith, A. (1999). Signaling mechanisms regulating self-renewal and differentiation of pluripotent embryonic stem cells. *Cells Tissues Organs* **165**, 131–143.
- Cartwright, P., McLean, C., Sheppard, A., Rivett, D., Jones, K. and Dalton, S. (2005). LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* **132**, 885–896.
- Casanova, E. A., Shakhova, O., Patel, S. S., Asner, I. N., Pelczar, P., Weber, F. A., Graf, U., Sommer, L., Bürki, K. and Cinelli, P. (2011). Prmel7 mediates LIF/STAT3-dependent self-renewal in embryonic stem cells. *Stem Cells* **29**, 474–485.
- Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S. and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**, 643–655.
- Dunty, W. C., Jr, Kennedy, M. W. L., Chalamalasetty, R. B., Campbell, K. and Yamaguchi, T. P. (2014). Transcriptional profiling of Wnt3a mutants identifies Sp transcription factors as essential effectors of the Wnt/beta-catenin pathway in neuroesodermal stem cells. *PLoS ONE* **9**, e87018.
- el-Baradi, T. and Pieler, T. (1991). Zinc finger proteins: what we know and what we would like to know. *Mech. Dev.* **35**, 155–169.
- Ema, M., Mori, D., Niwa, H., Hasegawa, Y., Yamanaka, Y., Hitoshi, S., Mimura, J., Kawabe, Y.-I., Hosoya, T., Morita, M. et al. (2008). Krüppel-like factor 5 is essential for blastocyst development and the normal self-renewal of mouse ESCs. *Cell Stem Cell* **3**, 555–567.
- Evans, M. J. and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156.
- Greber, B., Wu, G., Bernemann, C., Joo, J. Y., Han, D. W., Ko, K., Tapia, N., Sabour, D., Sterneckert, J., Tesar, P. et al. (2010). Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells. *Cell Stem Cell* **6**, 215–226.
- Guo, G., Yang, J., Nichols, J., Hall, J. S., Eyres, I., Mansfield, W. and Smith, A. (2009). Klf4 reverts developmentally programmed restriction of ground state pluripotency. *Development* **136**, 1063–1069.
- Hall, J., Guo, G., Wray, J., Eyres, I., Nichols, J., Grotewold, L., Morfopoulou, S., Humphreys, P., Mansfield, W., Walker, R. et al. (2009). Oct4 and LIF/Stat3 additively induce Krüppel factors to sustain embryonic stem cell self-renewal. *Cell Stem Cell* **5**, 597–609.
- Hirai, H., Karian, P. and Kikyo, N. (2011). Regulation of embryonic stem cell self-renewal and pluripotency by leukaemia inhibitory factor. *Biochem. J.* **438**, 11–23.
- Hovertter, N. P., Ting, J.-H., Sundaresh, S., Baldi, P. and Waterman, M. L. (2012). A WNT/p21 circuit directed by the C-clamp, a sequence-specific DNA binding domain in TCFs. *Mol. Cell Biol.* **32**, 3648–3662.
- Huang, G., Ye, S., Zhou, X., Liu, D. and Ying, Q.-L. (2015). Molecular basis of embryonic stem cell self-renewal: from signaling pathways to pluripotency network. *Cell. Mol. Life Sci.* **72**, 1741–1757.
- Jiang, J., Chan, Y.-S., Loh, Y.-H., Cai, J., Tong, G.-Q., Lim, C.-A., Robson, P., Zhong, S. and Ng, H.-H. (2008). A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nat. Cell Biol.* **10**, 353–360.
- Lyashenko, N., Winter, M., Migliorini, D., Biechele, T., Moon, R. T. and Hartmann, C. (2011). Differential requirement for the dual functions of beta-catenin in embryonic stem cell self-renewal and germ layer formation. *Nat. Cell Biol.* **13**, 753–761.
- Martello, G., Sugimoto, T., Diamanti, E., Joshi, A., Hannah, R., Ohtsuka, S., Göttgens, B., Niwa, H. and Smith, A. (2012). Esrrb is a pivotal target of the Gsk3/Tcf3 axis regulating embryonic stem cell self-renewal. *Cell Stem Cell* **11**, 491–504.
- Martello, G., Bertone, P. and Smith, A. (2013). Identification of the missing pluripotency mediator downstream of leukaemia inhibitory factor. *EMBO J.* **32**, 2561–2574.
- Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78**, 7634–7638.
- Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T. and Yokota, T. (1999). STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J.* **18**, 4261–4269.
- Niwa, H., Burdon, T., Chambers, I. and Smith, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev.* **12**, 2048–2060.
- Niwa, H., Ogawa, K., Shimosato, D. and Adachi, K. (2009). A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature* **460**, 118–122.
- Qiu, D., Ye, S., Ruiz, B., Zhou, X., Liu, D., Zhang, Q. and Ying, Q.-L. (2015). Klf2 and Tfcp2l1, two Wnt/beta-catenin targets, act synergistically to induce and maintain naive pluripotency. *Stem Cell Rep.* **5**, 314–322.
- Smith, A. G. (2001). Embryo-derived stem cells: of mice and men. *Annu. Rev. Cell Dev. Biol.* **17**, 435–462.
- Suske, G., Bruford, E. and Philipson, S. (2005). Mammalian SP/KLF transcription factors: bring in the family. *Genomics* **85**, 551–556.
- Tai, C.-I. and Ying, Q.-L. (2013). Gbx2, a LIF/Stat3 target, promotes reprogramming to and retention of the pluripotent ground state. *J. Cell Sci.* **126**, 1093–1098.
- Tesar, P. J., Chenoweth, J. G., Brook, F. A., Davies, T. J., Evans, E. P., Mack, D. L., Gardner, R. L. and McKay, R. D. G. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* **448**, 196–199.
- Treichel, D., Becker, M.-B. and Gruss, P. (2001). The novel transcription factor gene Sp5 exhibits a dynamic and highly restricted expression pattern during mouse embryogenesis. *Mech. Dev.* **101**, 175–179.
- van Oosten, A. L., Costa, Y., Smith, A. and Silva, J. C. R. (2012). JAK/STAT3 signalling is sufficient and dominant over antagonistic cues for the establishment of naive pluripotency. *Nat. Commun.* **3**, 817.
- Waler, J., Machon, O., von Kries, J. P., Wilson, S. R., Lundenes, E., Wedlich, D., Gradl, D., Paulsen, J. E., Machonova, O., Dembinski, J. L. et al. (2011). Novel synthetic antagonists of canonical Wnt signaling inhibit colorectal cancer cell growth. *Cancer Res.* **71**, 197–205.

- Weidinger, G., Thorpe, C. J., Wuennenberg-Stapleton, K., Ngai, J. and Moon, R. T.** (2005). The Sp1-related transcription factors sp5 and sp5-like act downstream of Wnt/beta-catenin signaling in mesoderm and neuroectoderm patterning. *Curr. Biol.* **15**, 489-500.
- Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A. and Gough, N. M.** (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* **336**, 684-687.
- Wimmer, E. A., Jäckle, H., Pfeifle, C. and Cohen, S. M.** (1993). A Drosophila homologue of human Sp1 is a head-specific segmentation gene. *Nature* **366**, 690-694.
- Wray, J., Kalkan, T., Gomez-Lopez, S., Eckardt, D., Cook, A., Kemler, R. and Smith, A.** (2011). Inhibition of glycogen synthase kinase-3 alleviates Tcf3 repression of the pluripotency network and increases embryonic stem cell resistance to differentiation. *Nat. Cell Biol.* **13**, 838-845.
- Yang, J., van Oosten, A. L., Theunissen, T. W., Guo, G., Silva, J. C. R. and Smith, A.** (2010). Stat3 activation is limiting for reprogramming to ground state pluripotency. *Cell Stem Cell* **7**, 319-328.
- Ye, S., Li, P., Tong, C. and Ying, Q.-L.** (2013). Embryonic stem cell self-renewal pathways converge on the transcription factor Tfc2l1. *EMBO J.* **32**, 2548-2560.
- Ye, S., Liu, D. and Ying, Q.-L.** (2014). Signaling pathways in induced naive pluripotency. *Curr. Opin. Genet. Dev.* **28**, 10-15.
- Ying, Q.-L., Nichols, J., Chambers, I. and Smith, A.** (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* **115**, 281-292.
- Ying, Q.-L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P. and Smith, A.** (2008). The ground state of embryonic stem cell self-renewal. *Nature* **453**, 519-523.



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