Figures

a. Immunoblot showing Id1, pErk and p38 in ES cells cultured in N2B27 alone (N) or with LIF (L), LIF plus PD184352 (LP), SU5402 (LS), PS (LPS), BMP4 (LB) or BMP4 alone (B). Note that BMP does not inhibit activation of either Erk or p38 mitogen activated protein kinases.

b,c. (**b**) Oct4 and (**c**) Nanog immunostaining of ES cells cultured in N2B27 with LIF plus 5μM SU5402 or 1μM PD184352. Note flattened morphology of colonies.

d. Growth curve over five passages for Oct4GiP ES cells cultured in N2B27 supplemented with LIF plus BMP or with 3i.

e. qRT-PCR analysis of mRNA levels after 24 hours culture of ES cells in N2B27 only (N), serum plus LIF (GL), or N2B27 plus LIF and BMP4 (LB), PS, 3i or CHIR99021 (CHIR).

f. STAT3 activation is not detected in ES cells cultured in 3i. Immunoblot of phospho (tyr705)-STAT3 in ES cells cultured in N2B27 with LIF/BMP (LB) or with 3i for 24 hours (steady state), in Basal Medium (BM) for 24hrs or as BM stimulated with LIF for 20 mins (positive control).

g. STAT3 target gene SOCS3 is down regulated in wild type ES cells cultured in 3i.
We also found that episomal transfection with dominant negative STAT3Y705F or with the negative feedback regulator SOCS3 do not impede undifferentiated colony formation in 3i (data not shown), in contrast to their suppressive effect on LIF-mediated self-renewal[2, 3].

h. Immunoblot analyses of NDRG1 and of phosphorylated and total β-catenin steady state levels in ES cells cultured for 24 hours in the indicated conditions.

i. Ecadherin deficient ES cells maintain undifferentiated morphology and Oct4 expression in 3i.

j. TOPFlash activity in GSK3α/β deficient DKO or wild-type cells cultured in N2B27 alone (non) or in the presence of PS and/or CHIR99021. Dual luciferase assays were performed 48hrs after transfection. Error bars represent standard deviation of the mean for 3 biological replicates.

k. CHIR99021 does not induce expression of pro-survival genes. qRT-PCR analysis of mRNA levels after 12 hours culture in N2B27 alone followed by 8 hours exposure to N2B27 alone (N) or plus CHIR99021 (CHIR). Similar results were obtained with PS or 3i.

SUPPLEMENTARY TABLE

Contributions to chimaeras and germ line transmission of ES cells derived and expanded in 3i

Strain/Cell line	Embryos injected	Liveborn pups	Chimaeras	Test-mated	Transmitting
129/CPS1	64	16	12	8 (5♂, 3♀)	3♀[#]
129/CPS2	21	5	4	3 (1♂, 2♀)	2♀[#]
129/CPS3	20	15	11	4 (3♂, 1♀)	2♂
CBA/D6	16	8	2	2♀	2♀[#]
CBA/D7	16	11	7	1♀	1♂

[#] ES cells assumed to be XX.

Chimaerism and transmission of the ES cell genome are detected by coat colour.
Chimaeras resulting from blastocyst injection of 129 ES cells were identified by the appearance of sandy and/or agouti patches amongst the black hair derived from the C57BL/6 host embryos. Adult

chimaeras exhibiting a high level of contribution from the injected ES cells were test mated with albino MF1 outbred mice. Grey progeny confirmed transmission of the 129 genome. CBA ES cells were injected into C57BL/6 embryos at the 8 cell stage. Chimaeras were identified by the presence of agouti hair. Germline transmission was ascertained by the birth of agouti pups following mating of chimaeras to mice of the C57BL/6 strain.

SUPPLEMENTARY MATERIALS AND METHODS

Antibody Details

	Antibody	Source	Cat. No.	Dilution (1/)
Primary	β-catenin	BD Transduction Laboratories	610153	1000
	p-β-catenin(ser33/37/thr41)	Cell signalling	9561	1000
	p44/42 MAPK (ERK)	Cell signalling	9102	1000
	p-p44/42 MAPK(thr202/tyr204)	Cell signalling	9101	1000
	AKT (PKB)	R and D systems	MAB2055	1000
	p-AKT(ser473)	R and D systems	AF887	1000
	p38	Cell Signalling	9212	1000
	p-p38(thr180/tyr182)	Cell signalling	9211	500
	Nanog	Abcam	ab21603	200
	Oct3/4 (c10)	Santa Cruz Biotechnology	sc-5279	200
	Eed	Gift from Arie Otte		10
	Gata4 (c-20)	Santa Cruz Biotechnology	sc-1237	100
	Myc	Santa Cruz Biotechnology	sc-42	200
	NRDG1	Cohen laboratory[4]		1μg/ml
	p-NRDG1	Cohen laboratory[4]		1μg/ml
	Alpha-Tubulin	Abcam	Ab7291	5000
	Tuj1	Covance	MMS-435P	400
	Stat3	BD Transduction Laboratories	610189	1000
	pStat3 (Tyr705)	Cell Signaling Technology	9131	1000
Secondary	ECL Mouse IgG, HRP-Linked Whole Ab	Amersham	NA931	2000-5000
	ECL Rabbit IgG, HRP-Linked Whole Ab	Amersham	NA934	2000-5000
	Alexafluor (for IF)			500-1000

RT-PCR Primers

Gene	5'-primer:	3'-primer:
Gapdh	CCCACTAACATCAAATGGGG	CCTTCCACAATGCCAAAGTT
Nanog	ATGAAGTGCAAGCGGTGGCAGAAA	CCTGGTGGAGTCACAGAGTAGTTC
Oct3/4	GGCGTTCTCTTTGGAAAGGTGTTC	CTCGAACCACATCCTTCTCT
Rex1	GACACGTGGCAAAAGAAGATAGTC	AGTGAGGCGATCCTGCTTTC
Sox1	CCTCGGATCTCTGGTCAAGT	TACAGAGCCGGCAGTCATAC
Brachyury	GTGACTGCCTACCAGAATGA	ATTGTCCGCATAGGTTGGAG

Real-Time PCR Primers

Real-time quantitative PCR was performed using the Universal Probe Library (UPL) System (Roche). Details of primers and corresponding probes are given below.

Gene	Forward Primer	Reverse Primer	Probe #
Actb	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA	64
Axin2	GCAGGAGCCTCACCCTTC	TGCCAGTTTCTTTGGCTCTT	50
Bcl2	AGTACCTGAACCGGCATCTG	GGGGCCATATAGTTCCACAAA	75
Bcl-xl	TGACCACCTAGAGCCTTGGA	TGTTCCCGTAGAGATCCACAA	2
Cdx1	ACGCCCTACGAATGGATG	CTTGGTTCGGGTCTTACCG	70
Egr1	CCCTATGAGCACCTGACCAC	TCGTTTGGCTGGGATAACTC	22
Fgf5	AAAACCTGGTGCACCCTAGA	CATCACATTCCCGAATTAAGC	29
Gata4	GGAAGACACCCCAATCTCG	CATGGCCCCACAATTGAC	13
Gata6	GGTCTCTACAGCAAGATGAATGG	GGTCTCTACAGCAAGATGAATGG	40
mcl	TAACCCAGCCATGGAAGTTT	CAGCTTTCATTTCACCCTTTG	33
Myc	CCTAGTGCTGCATGAGGAGAC	TCTTCCTCATCTTCTTGCTCTTC	77
Nanog	TTCTTGCTTACAAGGGTCTGC	AGAGGAAGGGCGAGGAGA	110
nMyc	CCTCCGGAGAGGATACCTTG	TCTCTACGGTGACCACATCG	69
Pou5f1	GTTGGAGAAGGTGGAACCAA	CTCCTTCTGCAGGGCTTTC	95
Rex1	TCTTCTCTCAATAGAGTGAGTGTGC	GCTTTCTTCTGTGTGCAGGA	71
Socs3	ATTTCGCTTCGGGACTAGC	AACTTGCTGTGGGTGACCAT	83
T	CAGCCCACCTACTGGCTCTA	GAGCCTGGGGTGATGGTA	100
Tbx3	TTGCAAAGGGTTTTCGAGAC	TGCAGTGTGAGCTGCTTTCT	51
Tcl1	CGTGTACTTGGATGAGTTTCGT	GCAAGATCACCTGGAATTTTTC	71
Zfx	ACCGTCCGGTGCGTATAA	TTCTCATCAGCCAGAACACCT	104

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