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Supplemental Information

Competitive Interactions Eliminate Unfit Embryonic Stem Cells at the Onset of Differentiation

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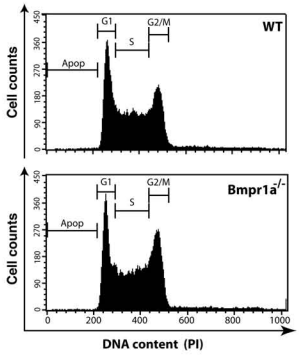
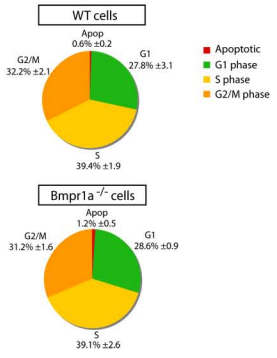
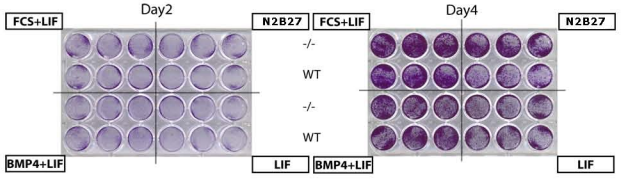
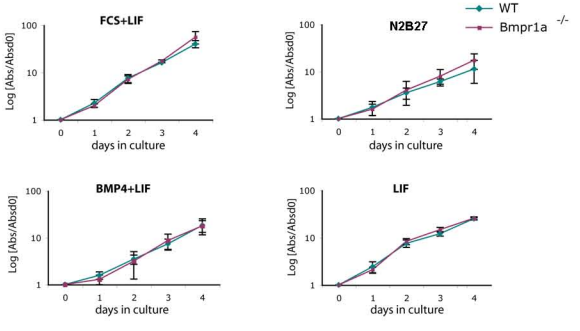
Inventory of Supplemental Information

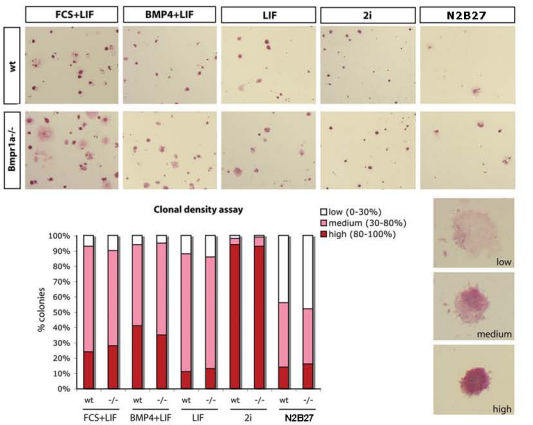
Figure S1. Sancho et al.

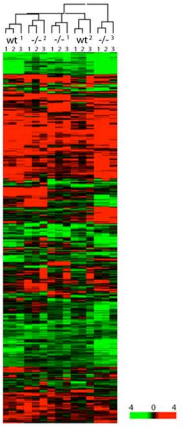












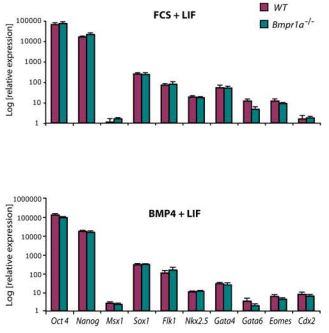


Figure S2. Sancho et al.

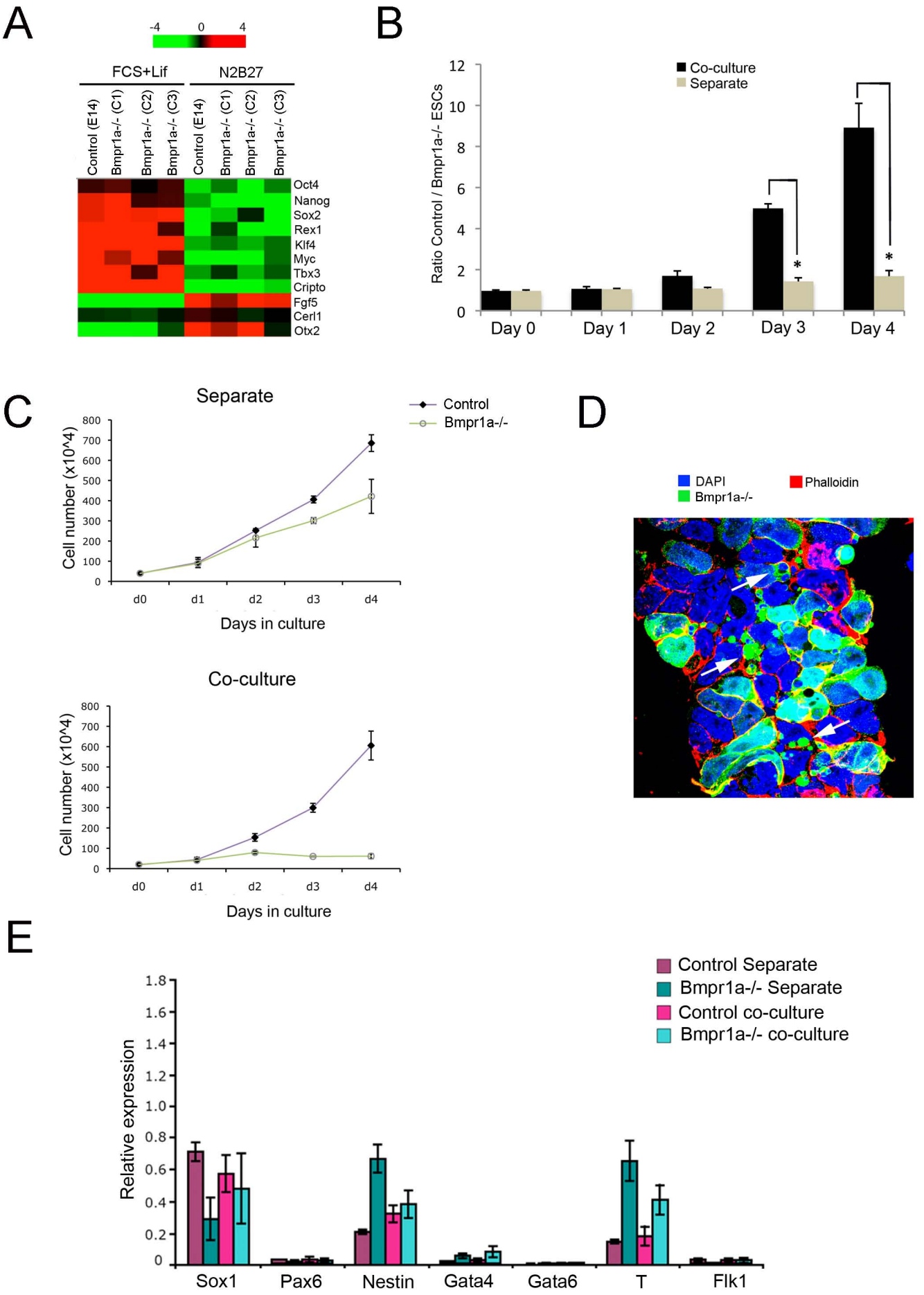


Figure S3. Sancho et al.

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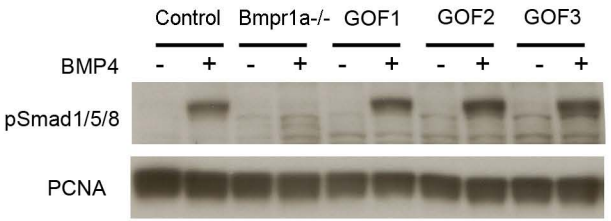

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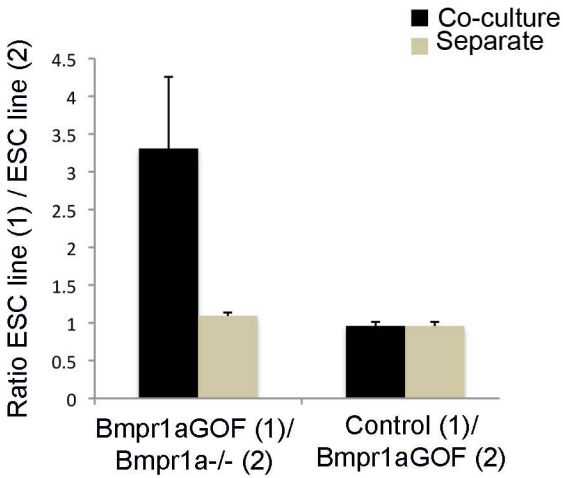

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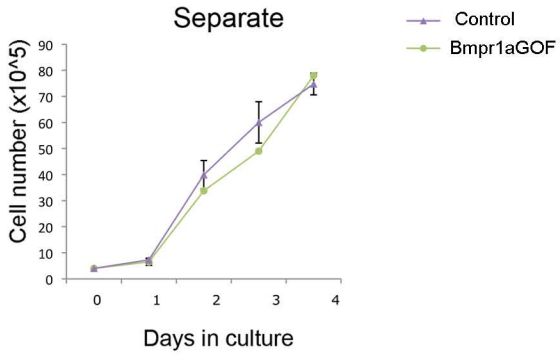

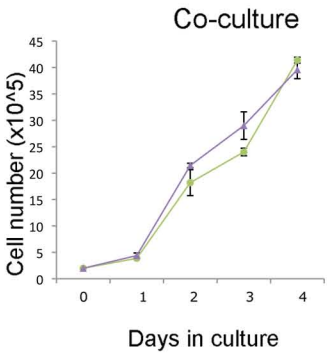

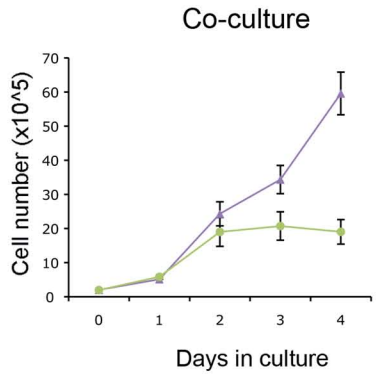

Figure S4. Sancho et al.

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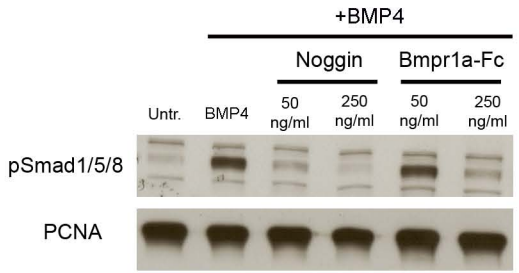

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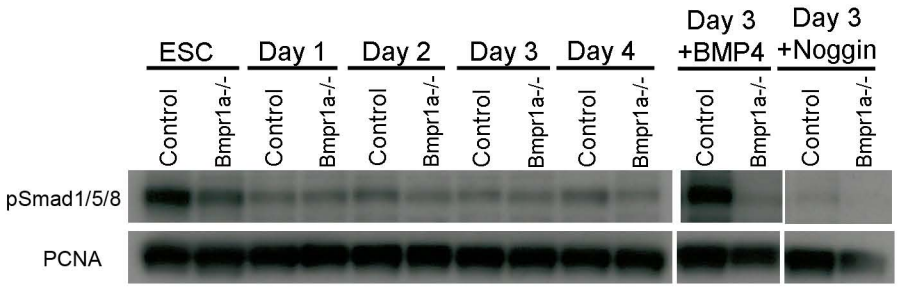

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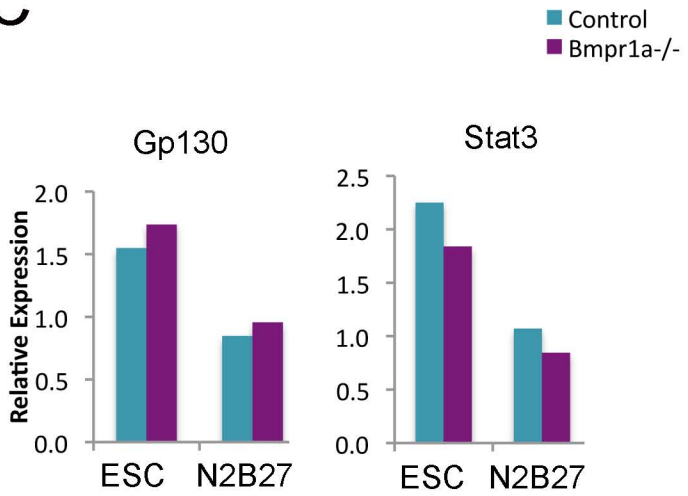

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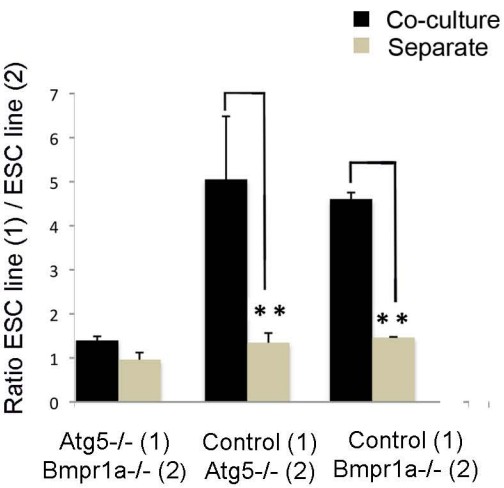


Figure S5. Sancho et al.

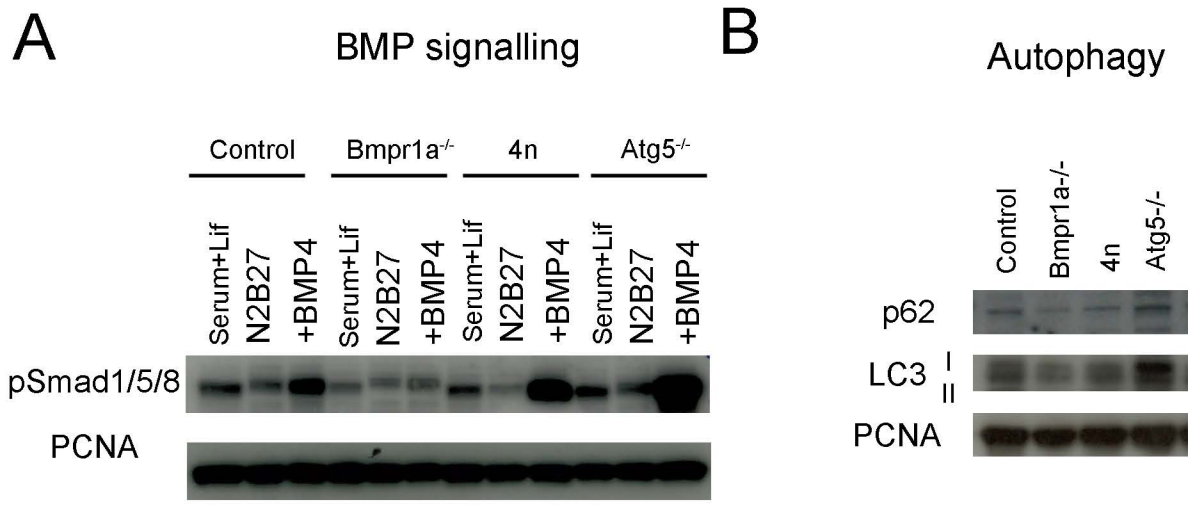
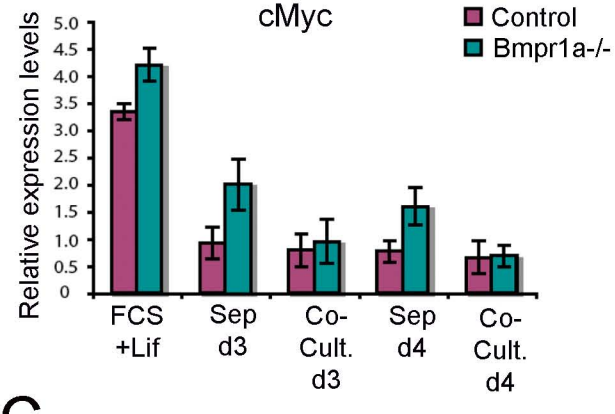


Figure S6. Sancho et al.

A



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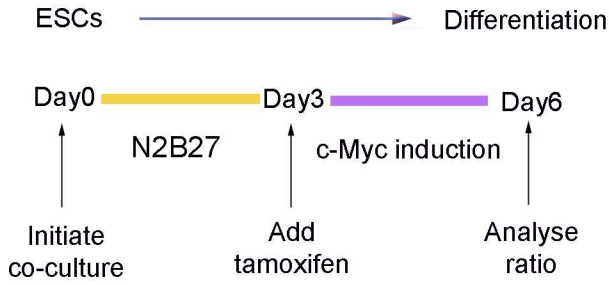

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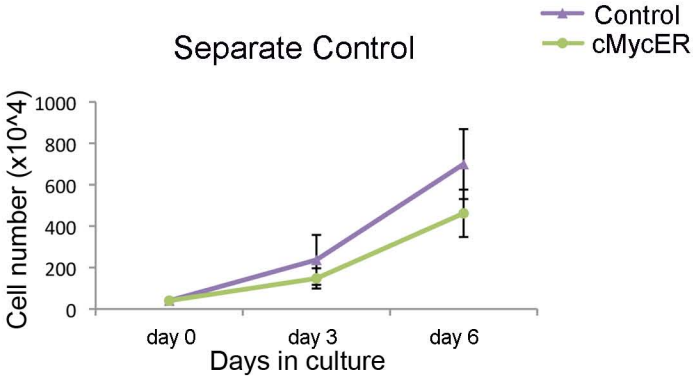

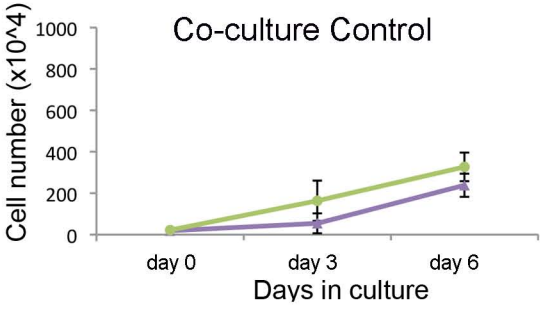

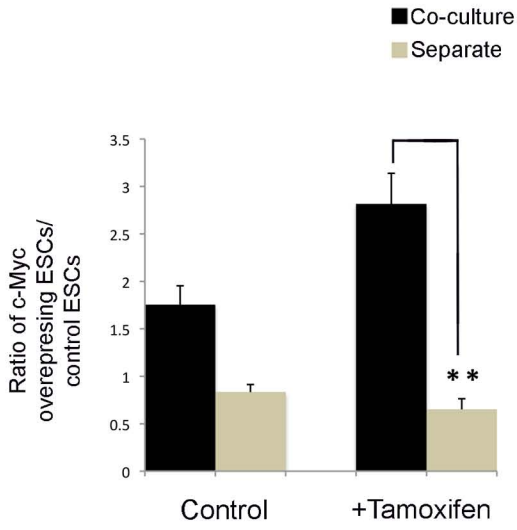

Supplemental Figure and Table legends.

Figure S1. Related to Figure 1. *Bmpr1a*$^{-/-}$ ESCs show similar self-renewal and pluripotency to control ESCs. (A) Cell cycle profiles, (B) crystal violet staining assays and (C) growth curves indicate that *Bmpr1a*$^{-/-}$ and control ESCs proliferate at similar rates in FCS+Lif, BMP4+Lif, N2B27 and when grown with Lif alone. (D) Clonal density assays and quantification of the level and proportion of alkaline phosphatase staining indicates that *Bmpr1a*$^{-/-}$ ESCs have similar pluripotent colony potential than control cells. (E) Microarray analysis and (F) analysis of pluripotency and lineage specific markers by qPCR indicates that *Bmpr1a*$^{-/-}$ ESCs show similar gene expression profiles to control cells. A minimum of 3 independent experiments were performed and the average ±SEM is plotted.

Figure S2. Related to Figure 2. ESCs with defective BMP signalling are eliminated by control cells. (A) Microarray analysis indicates that in N2B27 culture conditions, pluripotency gene expression decreases and epiblast markers increase. (B) Ratio and (C) growth curves of control-GFP/*Bmpr1a*$^{-/-}$ ESCs showing that *Bmpr1a*$^{-/-}$ ESCs are out-competed when cultured with control-GFP cells. (D) Confocal microscopy analysis reveals large amounts of GFP positive cellular debris (arrows) in *Bmpr1a*$^{-/-}$*-GFP* and control ESC cocultures. (E) qRT-PCR analysis indicating that the expression of differentiation genes does not change during cell competition. A minimum of 3 independent experiments were performed and the average ±SEM is plotted. * $p<0.05$ students paired t-test.

Figure S3. Related to Figure 2. Defective BMP signalling determines the elimination of *Bmpr1a*$^{-/-}$ ESCs. (A) Western blot analysis indicating that *Bmpr1aGOF* ESCs activate Smad1/5/8 phosphorylation when treated for 1 hour with BMP4 after overnight starvation. (B) Plot of the ratio of the different ESC lines cocultured or grown separately for 4 days and (C) growth curves indicating that *Bmpr1aGOF* ESCs out-compete *Bmpr1a*$^{-/-}$ ESCs and are not out-competed by control ESCs. A minimum of 3 independent experiments were performed and the average ±SEM is plotted.

Figure S4. Related to Figure 3. Relative levels of BMP signalling do not mediate the elimination of *Bmpr1a*$^{-/-}$ ESCs. (A)Western blot analysis indicating that in wild-type ESCs Noggin and the dominant negative BMP receptor Bmpr1aFc inhibit Smad1/5/8

phosphorylation. (B) Time-course by western blot showing that BMP signalling is decreased in control and *Bmpr1a*$^{-/-}$ ESCs during coculture in N2B27, and that at the third day of coculture BMP4 increases pSmad1/5/8 levels in control cells and Noggin decreases it both ESC types. (C) Relative mRNA expression levels of *Gp130* and *Stat3* showing a decrease upon ESC culture for 3 days in N2B27. (D) Plots of the ratio of different ESC lines when cocultured or grown separately indicating that in *Atg5*$^{-/-}$ and *Bmpr1a*$^{-/-}$ ESC cocultures neither cell type is eliminated. A minimum of 3 independent experiments were performed and the average ±SEM is plotted. **p<0.005 students paired t-test.

Figure S5. Related to Figure 4. Neither defective BMP signalling or defective autophagy is a common mechanism to cell competition. (A) Western blot analysis of the levels of BMP signalling and (B) autophagy in control, *Bmpr1a*$^{-/-}$, *Atg5*$^{-/-}$ and tetraploid ESCs after 3 days culture in N2B27 indicating that neither defective BMP signalling or defective autophagy mediates the elimination of these cells upon coculture.

Figure S6. Related to Figure 6. cMyc overexpression induces the elimination of wild-type ESCs. (A) cMyc mRNA expression levels determined by qPCR show no difference between separate and coculture conditions. (B) Schematic representation of the strategy used to overexpress cMycER during ESC differentiation. Cells were grown as separate populations or cocultured for 3 days in N2B27 and then treated with tamoxifen for a further 3 days and analysed. (C) Growth curves and plot of the ratio of cMycER/control ESCs cultured under control conditions or when treated with tamoxifen indicates that even without tamoxifen cMycER ESCs appear to display a competitive advantage over control ESCs in coculture. A minimum of 3 independent experiments were performed and the average ±SEM is plotted. **p<0.005 students paired t-test.

Table S1. Ratios and growth rates of different embryonic stem cells lines used in separate and coculture experiments. A minimum of 3 independent experiments were performed and the average ±SEM is shown.

TABLE S1

	Co-culture	Separate
Figure 1D	Ratio Control / Bmpr1a$^{-/-}$ ESC	
N2B27 day 1	1.86±0.11	1.89±0.15
N2B27 day 2	2.54±0.14	1.88±0.06
N2B27 day 3	2.78±0.21	1.44±0.04
N2B27 day 4	4.60±0.15	1.46±0.01
Figure 2B	Ratio Control / Bmpr1a$^{-/-}$ ESC	
DMSO	4.02±0.34	1.66±0.26
ZVAD-FMK	1.85±0.18	2.01±0.24
Figure 2C	Ratio Control / Bmpr1a$^{-/-}$ ESC	
EpiSC day 1	1.01±0.08	1.04±0.1
EpiSC day 2	2.42±0.04	1.85±0.56
EpiSC day 3	7.54±1.32	2.12±0.53
EpiSC day 4	16.58±2.73	2.1±0.54
Figure 2D	Ratio Control / Bmpr1a$^{-/-}$ ESC	
N2B27	3.27±0.26	1.60±0.26
FCS+Lif	1.25±0.10	1.58±0.19
BMP4+Lif	1.42±0.14	1.51±0.16
N2B27	7.83±0.61	1.70±0.19
2i	1.71±0.44	1.56±0.21
CHIR99021	2.31±0.11	1.84±0.15
PD0325901	1.85±0.22	1.74±0.21
Figure 3B	Ratio Control / Bmpr1a$^{-/-}$ ESC	
N2B27	3.58±0.35	1.14±0.05
Noggin	4.80±0.50	1.21±0.09
Bmpr1aFc	4.30±0.42	1.21±0.04
N2B27	4.45±0.29	1.35±0.02
Bmp4	3.80±0.06	1.11±0.002
BMP7	4.47±0.15	1.30±0.07
Control	7.82±0.67	2.10±0.30
Fgf4	7.62±0.25	1.67±0.40
Fgf5	10.82±2.63	2.01±0.23
Control	4.98±0.35	1.42±0.07
Lif from d0	0.76±0.11	0.86±0.16
Control	7.38±1.01	1.65±0.15
Lif from D2	4.61±0.41	1.18±0.05

Figure 4A	Ratio Control / Atg5$^{-/-}$ ESC	
Atg5 day 1	0.98±0.06	0.88±0.05
Atg5 day 2	1.15±0.06	0.87±0.04
Atg5 day 3	2.49±0.51	1.17±0.06
Atg5 day 4	5.05±1.43	1.35±0.22

Figure 4B	Ratio Control / Tetraploid ESC	
Tetraploid day 1	1.72±0.23	1.73±0.21
Tetraploid day 2	3.62±0.41	2.00±0.77
Tetraploid day 3	20.51±0.47	4.52±3.98
Tetraploid day 4	50.5±0.78	6.75±2.91

Figure 6E	Ratio cMyc overexpressing / control ESC	
cMycER	2.82±0.32	0.83±0.08
Figure S2B	Ratio Control / Bmpr1a$^{-/-}$ ESC	
N2B27 day 1	1.06±0.11	1.05±0.03
N2B27 day 2	1.69±0.24	1.08±0.06
N2B27 day 3	4.98±0.22	1.43±0.18
N2B27 day 4	8.92±1.18	1.68±0.27

Figure S3B	Ratio ESC(1) / ESC(2)	
Bmpr1aGOF(1)/Bmpr1a-(2)	3.31±0.95	1.09±0.04
Control(1)/BmprGOF(2)	0.96±.0.05	0.96±.0.05

Figure S4D	Ratio ESC(1) / ESC(2)	
Atg5(1)/Bmpr1a-(2)	1.40±0.09	0.96±0.16
Control(1)/Atg5-(2)	5.05±1.43	1.35±0.22
Control(1)/Bmpr1a-(2)	4.60±0.15	1.46±0.01

Figure S6C	Ratio cMyc overexpressing / control ESC	
N2B27	1.76±.20	0.83±0.08
Tamoxifen	2.82±0.32	0.65±0.11

Growth Rates

Growth Rate Control and Bmpr1a-/- (GFP) in N2B27

	Control		
	Separate	**Co-culture**	***t-test***
D0-1	1.25±0.28	1.65±0.01	0.31147
D1-2	1.53±0.1	1.45±0.03	0.54587
D2-3	0.63±0.2	0.77±0.16	0.52268
D3-4	0.39±0.05	0.63±0.02	**0.01605** In Figure 2A

	Bmpr1a-/- (GFP)		
	Separate	**Co-culture**	***t-test***
D0-1	0.64±0.03	1.05±0.28	0.01647
D1-2	1.37±0.007	0.99±0.1	0.02274
D2-3	0.1±0.11	0.65±0.2	0.06736

D3-4	0.54±0.07	-0.102±0.05	**0.03618**	In Figure 2A

Growth Rate Control (GFP) and Bmpr1a-/- in N2B27

	Control (GFP)		
	Separate	**Co-culture**	***t-test***
D0-1	1.178696826	1.44754493	0.75088
D1-2	1.47637097	3.65295345	0.03832
D2-3	0.687074209	1.95993636	0.09191
D3-4	0.754294117	2.01543695	**0.03239**

	Bmpr1a-/-		
	Separate	**Co-culture**	***t-test***
D0-1	1.115658599	0.94153041	0.23858
D1-2	1.283820275	0.98599732	0.20723
D2-3	0.512936869	-0.39725072	0.11643
D3-4	0.455455341	0.00539718	**0.01914**

Growth Rate Control and Bmpr1a-/- in EpiSC media

	Control		
	Separate	**Co-culture**	***t-test***
D0-1	1.76±0.17	1.78±0.11	0.88988
D1-2	2.18±0.3	2.49±0.06	0.38421
D2-3	1.53±0.18	2.19±0.16	0.09837
D3-4	0.85±0.11	0.63±0.05	0.27617

	Bmpr1a-/- (GFP)		
	Separate	**Co-culture**	***t-test***
D0-1	1.71±0.16	1.77±0.19	0.4013
D1-2	1.42±0.15	1.22±0.19	0.0554
D2-3	1.6±0.23	0.61±0.23	**0.0250**
D3-4	0.86±0.08	-0.51±0.04	**0.0083**

Growth Rate Control (GFP) and Atg5-/- ESCs

	Control (GFP)		
	Separate	**Co-culture**	***t-test***
D0-1	1.01±0.21	1.17±0.2	0.50491
D1-2	1.59±0.13	1.42±0.09	0.46955
D2-3	0.79±0.16	1.12±0.04	0.0632
D3-4	0.43±0.05	0.4±0.07	0.53284

	Atg5-/-		
	Separate	**Co-culture**	***t-test***
D0-1	1.16±0.25	1.04±0.27	0.75168
D1-2	1.48±0.12	1.19±0.15	0.16918
D2-3	0.34±0.29	0.07±0.23	0.4945

D3-4	0.21±0.23	-0.51±0.21	0.08041

Growth Rate Control and tetraploid (GFP) ESCs

	Control		
	Separate	**Co-culture**	***t-test***
D0-1	1.22±0.04	1.38±0.16	0.32434
D1-2	1.95±0.07	2.08±0.19	0.48013
D2-3	1.13±0.07	1.56±0.21	0.08865
D3-4	0.66±0.15	0.69±0.11	0.7033
	tetraploid (GFP)		
	Separate	**Co-culture**	
D0-1	0.0012±0.00	0.70±0.05	0.1397
D1-2	0.012±0.11	1.06±0.24	0.24326
D2-3	0.08±0.04	-0.97±0.06	**0.0024**
D3-4	0.016±0.13	-0.66±0.14	**0.02268**

Supplemental Experimental Procedures

ESC culture and manipulation

Clonal assays: Bmpr1a$^{-/-}$ and control ESCs were plated at 100 cells/cm^2 in serum-containing medium and 6 hours later this was replaced by ESGRO Basal medium (Milipore) alone or with LIF (1500U/ml), 15% FCS plus LIF, BMP4 (10 ng/ml) plus LIF, or 2i (PD0325901 + CHIR99021). After 6 days culture in these conditions, alkaline phosphatase activity was determined using the Leukocyte Alkaline Phosphatese kit (Sigma) and the level of staining assessed in 100 arbitrary ESC colonies.

AnnexinV staining: 2x10^5 cells were resuspended in annexin-binding buffer (0.1%BSA in 10mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH7.4), incubated with APC-conjugated annexin V (Molecular Probes) and 0.1mg/ml Propidium Iodide (Sigma), and then immediately analysed by flow cytometry. A minimum of 3 independent experiments were performed and the average ±SEM is shown.

Electroporation: pPyCAGIP-EGFP, Bmpr1a or cMycER expression vectors were electroporated into E14 ESCs and subjected to puromycin selection and colonies were screened for expression of the transgene and karyotyped.

Flow Cytometry: Analysis was performed using a FACSCalibur or a BD LSR2 cytometer and data analysis was performed with the FlowJo software (all from BD Biosciences).

Generation of chimeric embryos

Chimeras were generated by blastocyst injection as described (Nagy et al., 2003).

RNA Isolation and RT-PCR

Total RNA was extracted using RNeasy (Qiagen). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen), Random Nonamers (Sigma) and RNAse inhibitor (Roche). qPCR was performed with SYBR PCR Master Mix (Qiagen) in an Opticon II DNA engine (MJ Research Inc). Standard curves and melting curves were measured for each set of primers to confirm that only one amplicon was generated, b-actin, Hmbs and Hprt1 were used as house keeping genes to which data were normalised. The expression levels of mRNA were calculated using the comparative CT method. Primer sequences are indicated in Table S1.

Microarray analysis

RNA was obtained as previously described. Sample labelling, hybridization to the mouse Gene 1.0 ST Array system (Affymetrix), and data acquisition were performed by UCL Genomics at the Institute of Child Health. Normalisation and statistical analysis of the array data was performed using the GeneSpring software.

Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2003). Manipulating the Mouse Embryo, third edn (Cold Spring Harbor, New York, Cold Spring Harbor laboratory Press).