LETTER

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# C/EBPα poises B cells for rapid reprogramming into induced pluripotent stem cells

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**CCAAT/enhancer binding protein-α (C/EBPα) induces transdifferentiation of B cells into macrophages at high efficiencies and enhances reprogramming into induced pluripotent stem (iPS) cells when co-expressed with the transcription factors Oct4 (Pou5f1), Sox2, Klf4 and Myc (hereafter called OSKM)[1,2]. However, how C/EBPα accomplishes these effects is unclear. Here we find that in mouse primary B cells transient C/EBPα expression followed by OSKM activation induces a 100-fold increase in iPS cell reprogramming efficiency, involving 95% of the population. During this conversion, pluripotency and epithelial–mesenchymal transition genes become markedly upregulated, and 60% of the cells express Oct4 within 2 days. C/EBPα acts as a 'path-breaker' as it transiently makes the chromatin of pluripotency genes more accessible to DNase I. C/EBPα also induces the expression of the dioxygenase Tet2 and promotes its translocation to the nucleus where it binds to regulatory regions of pluripotency genes that become demethylated after OSKM induction. In line with these findings, overexpression of Tet2 enhances OSKM-induced B-cell reprogramming. Because the enzyme is also required for efficient C/EBPα-induced immune cell conversion[3], our data indicate that Tet2 provides a mechanistic link between iPS cell reprogramming and B-cell transdifferentiation. The rapid iPS reprogramming approach described here should help to fully elucidate the process and has potential clinical applications.**

Reprogramming of somatic cells into induced pluripotent stem (iPS) cells by the OSKM transcription factors (also called Yamanaka factors) can be divided into a stochastic and a deterministic phase[4,5]. During this process cells undergo a mesenchymal–epithelial transition (MET) and activate endogenous pluripotency genes, paralleled by changes in histone marks, nucleosome positioning and chromatin accessibility[6]. In addition, their promoters become de-methylated, preceded by oxidation of methylated CpGs through the dioxygenases Tet2 and Tet1, both of which have been implicated in the establishment of pluripotency[7,8]. Earlier work showed that co-expression of C/EBPα with OSKM increases the reprogramming efficiency of B cells ~15-fold, reaching ~3% of the population[1]. Here we describe that a pulse of C/EBPα followed by OSKM overexpression permits the rapid reprogramming of B cells into iPS cells by activating Tet2 and facilitating accessibility of pluripotency gene promoters to Oct4 binding. Highly efficient reprogramming of somatic cells has recently also been reported with a loss-of-function approach[9].

Committed B-cell precursors (hereafter referred to as B cells) can be induced to transdifferentiate into macrophages at 100% efficiency by forced C/EBPα expression, deregulating ~7,500 genes[10]. Reasoning that chromatin of cells in transition might be more 'open' than that of end stages, we tested the effect of transiently exposing B cells to C/EBPα, followed by OSKM expression. B cells were isolated from the bone marrow of reprogrammable mice (containing a tetracycline-controlled transactivator (rtTA) and a doxycycline-responsive OSKM cassette[11]), infected them with C/EBPα-ER-hCD4 retrovirus, sorted human $CD4^+$ cells 4 days later and incubated them for different times with β-estradiol (E2) followed by a wash-out (Fig. 1a). Subsequently, OSKM was induced by doxycycline treatment and $Nanog^+$ colonies scored 12 days post induction (d.p.i.). B cells continuously co-expressing C/EBPα with OSKM showed an 11-fold enhancement in reprogramming efficiency compared to cells induced with OSKM alone (B+OSKM cells), confirming earlier reports[1,12]. In contrast, cells treated for 18 h with E2 and then treated with doxycycline (Bα′+OSKM cells) exhibited a 103-fold colony increase, with cells pulsed for 6 h already showing a 74-fold increase (Fig. 1b–d). Clonal assays showed that 92–94% of viable colonies were $Nanog^+$ after 12 days (Extended Data Fig. 1a–c). OSKM induction of B cells pre-treated with E2 did not increase $Nanog^+$ colony numbers nor *Oct4* expression levels (Extended Data Fig. 2a). A mutant of C/EBPα (BRM-2 (ref. 13)) defective for DNA binding failed to enhance iPS cell generation (Extended Data Fig. 2b). Induced Bα′+OSKM cells remained >50% viable (Extended Data Fig. 2c). C/EBPα pulses after OSKM induction had no effect on reprogramming efficiency (Extended Data Fig. 2d), indicating that C/EBPα acts as a path-breaker for OSKM-induced reprogramming. Stable iPS cell lines derived from Bα′+OSKM cells (αiPS cells) displayed similar gene expression profiles as embryonic stem (ES) cells, differentiated into all three germ layers *in vitro* and *in vivo*, and efficiently contributed to coat colour chimaerism (Fig. 1e and Extended Data Fig. 3a–e).

Bα′+OSKM-derived iPS cell colonies could be identified as early as 4 d.p.i. and their numbers increased modestly after 8 days (Fig. 1f, g), whereas B+OSKM colonies continued to increase 8–10 d.p.i. (Fig. 1f, g). Retroviral hCD4 expression was found to be silenced within 2–4 days in Bα′+OSKM cells compared to ~8 days in B+OSKM cells (Fig. 1h). To test the effect of C/EBPα on OSKM-induced transgene independence, Bα′ and B cells were doxycycline-treated for different times (Fig. 1i). The first transgene-independent iPS cell colonies were observed after 4 days for Bα′+OSKM cells, compared to 9–10 days for B+OSKM controls (Fig. 1j). Together these results show that the C/EBPα pulse accelerates iPS cell reprogramming by 4–6 days.

Gene expression analyses of Bα′+OSKM cells 8 d.p.i. showed the upregulation of 764 out of 1,668 genes expressed more highly in ES cells than in B cells, including all well described pluripotency genes (Fig. 2a). Unsupervised hierarchical clustering analysis revealed that 8 d.p.i. Bα+OSKM cells clustered with ES/iPS cells (Extended Data Fig. 4a). A large part of known pluripotency genes were activated 2–6 d.p.i. (Fig. 2b, c and Extended Data Fig. 4b), reaching levels comparable to αiPS cells and ES cells within ~1 week (Fig. 2c and Extended Data Fig. 4c). In contrast, they remained essentially silent in B+OSKM cells (Fig. 2b, c). In addition, five genes described to be activated very late (21 d.p.i.) during fibroblast reprogramming[14] became upregulated within 2–4 days in Bα′+OSKM cells (Extended Data Fig. 4d). Using Oct4–GFP reporter mice[12] crossed with reprogrammable mice[11], Bα′+OSKM

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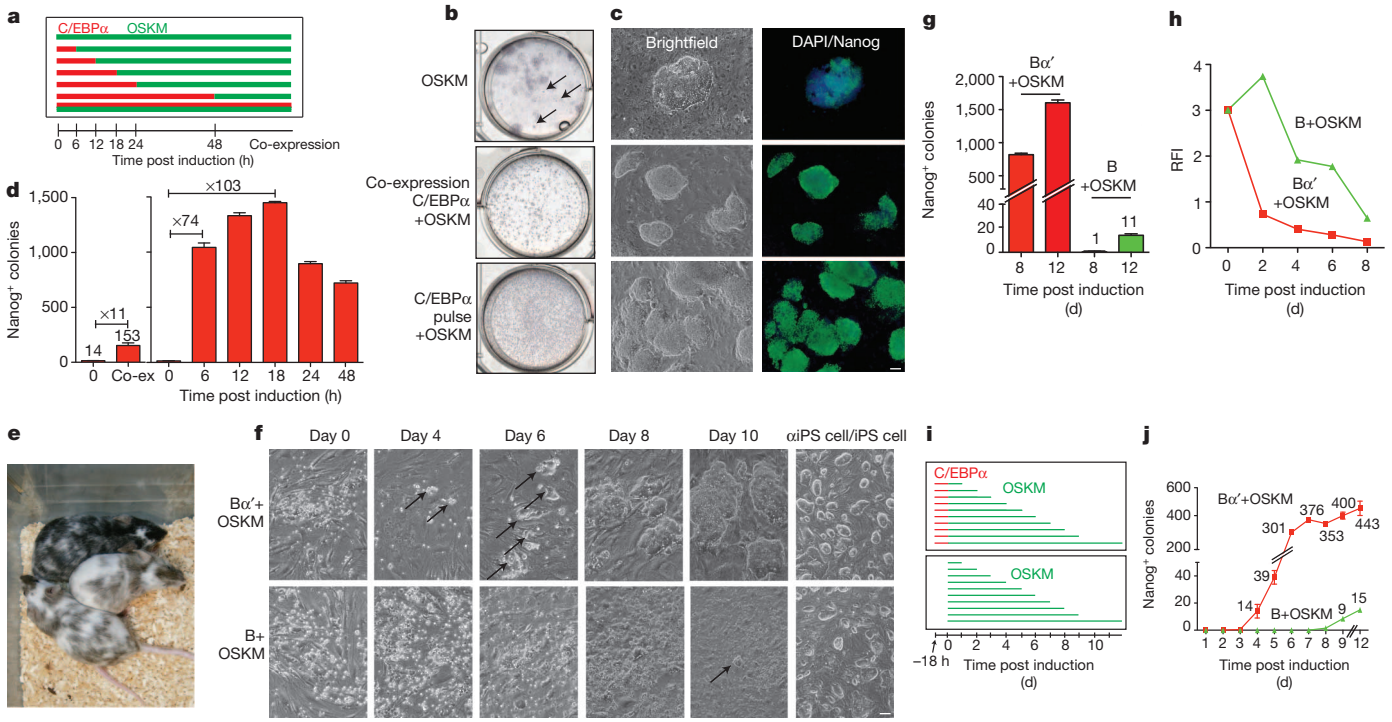


**Figure 1 | Effect of transient C/EBPα expression on OSKM-induced iPS cell reprogramming of B cells.** **a**, Experimental strategy. **b**, Six-well plates with alkaline-phosphatase-positive iPS cell colonies at 12 d.p.i. obtained from B-cell cultures treated with doxycycline to induce OSKM only (B+OSKM), cells co-expressing C/EBPα with OSKM, or cells treated for 18 h with E2 to activate C/EBPα followed by OSKM induction (Bα′+OSKM). **c**, Nanog$^+$ iPS cell colonies at 12 d.p.i. (scale bar, 100 μm). **d**, Quantification of 12 d.p.i. Nanog$^+$ colonies after OSKM induction in B cells pre-treated with C/EBPα for the indicated pulse lengths. Error bars indicate s.d. ($n = 3$). **e**, Chimaeric mice obtained after blastocyst injection of αiPS cell clone 12. **f**, Doxycycline-induced Bα′+OSKM cultures with arrows indicating earliest detectable ES-cell-like colonies (scale bar, 100 μm). **g**, Nanog$^+$ colonies of Bα′+OSKM and B+OSKM cultures at 8 and 12 d.p.i. Error bars indicate s.d. ($n = 3$). **h**, Retroviral silencing after induction of Bα′+OSKM and B+OSKM cells measured by hCD4 expression. RFI, relative fluorescence intensity. **i**, **j**, Experimental strategy to determine OSKM transgene independence (**i**) and formation of Nanog$^+$ iPS cell colonies (**j**) at 12 d.p.i. Error bars indicate s.d. ($n = 3$).

cells became 30% GFP$^+$ at 4 d.p.i. and 60% GFP$^+$ at 8 d.p.i., whereas B+OSKM cells remained negative (Extended Data Fig. 4e). Serum-free conditions further accelerated the process with 62% of the Bα′+OSKM cells becoming GFP$^+$ already at 2 d.p.i. and 95% at 4 d.p.i. (Fig. 2d, e), with a similar viability as in serum containing medium. Induced B+OSKM cells died without serum.

We next scored the expression of MET genes[15,16]. Bα′+OSKM cells upregulated all epithelial genes tested and expressed E-cadherin 2–4 d.p.i., again with B+OSKM cells remaining negative (Extended Data Fig. 5a, b). In line with the possibility that the C/EBPα pulse is capable of initiating an epithelial–mesenchymal transition (EMT)[17], mesenchymal genes encoding TGF-β pathway members, transcription factors and collagens were first upregulated by the C/EBPα pulse and then switched off after OSKM induction (Extended Data Fig. 5a, e). To compare gene expression changes during OSKM-induced reprogramming of Bα′ cells with C/EBPα-induced transdifferentiation of B cells we selected genes selectively expressed in macrophages (263) and B cells (83) (Supplementary Table 1). Most of these genes were already up- or down-regulated in Bα′ cells, respectively, as expected from the induced conversion (Extended Data Fig. 6a). After OSKM induction, B-cell genes became further silenced whereas macrophage genes elicited a more heterogeneous response (Extended Data Fig. 6b, c). Bα′+OSKM cells at 2–4 d.p.i. co-expressed E-cadherin and the macrophage markers CSF-1R and Mac1, whereas B+OSKM cells remained negative and αiPS cells expressed exclusively E-cadherin (Extended Data Fig. 6d). Sorted Mac1$^+$E-cadherin$^+$ cells and Mac1$^-$E-cadherin$^+$ cells yielded similar proportions of iPS cell colonies, indicating that expression of Mac1 has no role in iPS cell reprogramming (Extended Data Fig. 6e). Analysis of the 18 h pulsed B cells by RNA-seq revealed 1,418 upregulated and 552 downregulated genes (Extended Data Fig. 7a). Gene set enrichment analysis (GSEA) showed a strong correlation between genes downregulated in Bα′ cells with sets of B-cell-specific genes, and upregulated genes with genes related to EMT, collagen expression and the TGF-β pathway (Extended Data Fig. 7b), confirming the array data. C/EBPα chromatin immunoprecipitation sequencing (ChIP-seq) analysis revealed 1,766 DNA regions bound by the protein in Bα′ cells of which ~20% were within 50 kb of genes whose expression changed (Extended Data Fig. 7c–g). These included two regulatory sites in *Tet2* (Fig. 3a, ref. 3) as well as sites close to lymphoid, MET and TGF-β pathway genes. *Tet2* became upregulated ~1.6-fold after the 18-h pulse and was further activated by OSKM, reaching levels comparable to ES cells within 2 days (Fig. 3b, c). In contrast, mouse embryonic fibroblasts (MEFs) pulsed with C/EBPα did not upregulate the gene (Extended Data Fig. 8a). Tet2 overexpression enhanced iPS cell reprogramming of B cells ~3-fold (Fig. 3d), supporting the reported role of Tet2 in iPS cell formation[7,8]. Because the Tet2-induced 5-hydroxymethylcytosine (5hmC) modification has been described to participate in the maintenance of pluripotency, we determined 5hmC levels by hydroxymethylated DNA immunoprecipitation followed by qPCR (hMeDIP-qPCR) in Bα′ cells at regulatory regions of nine pluripotency genes for which hydroxymethylation levels change during reprogramming[18]. 5hmC levels were significantly increased for all genes tested (Fig. 3e and Extended Data Fig. 8b) and Tet2 binding was detected at the same regions (Fig. 4f and Extended Data Fig. 8c). Unexpectedly, Tet2 was predominantly cytoplasmic in uninduced B cells and nuclear after the C/EBPα pulse (Fig. 3g). The nuclear localization was maintained after sustained expression of C/EBPα (72 h) (Extended Data Fig. 8d). No protein translocation was observed in B cells treated with E2 alone or infected with a control vector (Extended Data Fig. 8e) and MEFs exhibited predominantly nuclear Tet2 localization (Extended Data Fig. 8f). Because hydroxymethylation is required

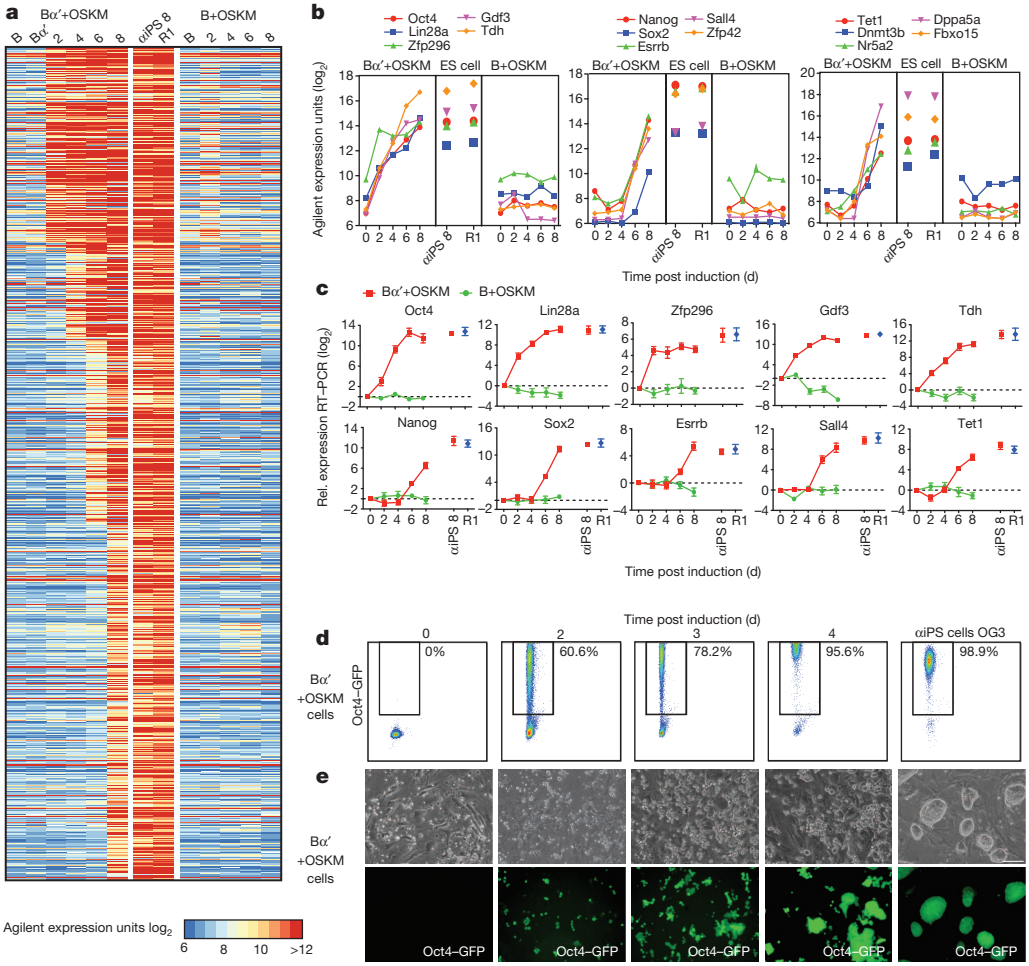


**Figure 2 | Upregulation of pluripotency genes.** **a**, Heat map of Agilent expression arrays showing upregulation of genes expressed >4-fold higher in ES cells than in B cells at the indicated time points after doxycycline treatment of Bα′+OSKM cells and B+OSKM cells, with αiPS cells (clone 8) and ES cells (R1) as controls. **b**, Array expression kinetics of three groups of pluripotency genes from Bα′+OSKM cells, αiPS cells and ES cells and B+OSKM cells. **c**, qRT–PCR validation of selected genes. Normalized against *Pgk* expression. Error bars indicate s.d. ($n = 3$). **d**, **e**, Oct4–GFP expression analysed by FACS and microscopy in Bα′+OSKM cells (scale bar, 100 μm).

for active DNA demethylation, we analysed the DNA methylation status of the *Oct4* and *Nanog* promoters using bisulphite sequencing (Fig. 3h). Whereas the *Oct4* promoter became partially demethylated in Bα′+OSKM as early as 2 d.p.i. (Fig. 3h), the *Nanog* promoter only became demethylated at 8 d.p.i., consistent with the observed delayed upregulation of the gene (Fig. 2b, c).

The 5hmC changes at pluripotency genes raised the possibility that the C/EBPα pulse eliminates epigenetic barriers, increasing chromatin accessibility to the OSKM transcription factors. To test this, we performed quantitative DNase I sensitivity assays on selected regulatory regions of pluripotency genes. This revealed increased accessibility to the enzyme of all sites tested in the 18-h pulsed B cells whereas control regions and pluripotency sites in control B+OSKM cells showed no difference (Fig. 4a). Interestingly, after prolonged induction of C/EBPα (72 h) the chromatin of pluripotency genes returned to the closed state as seen in untreated B cells (Extended Data Fig. 9a). In addition, no demethylation of the *Nanog* and *Oct4* promoters was observed (Extended Data Fig. 9b). We next tested Oct4 occupancy in OSKM-induced Bα′ cells at sites within enhancers and promoters of pluripotency genes bound by Oct4 in ES cells[19] (Fig. 4b). At 2 d.p.i. all of these sites showed significant Oct4 occupancy, whereas they remained unbound in B+OSKM cells (Fig. 4b). Together, these findings show that the C/EBPα pulse transiently induces chromatin remodelling at regulatory regions of pluripotency genes, making them rapidly accessible to Oct4 binding.

Our results show that C/EBPα poises B cells for highly efficient and almost immediate reprogramming into iPS cells by OSKM transcription factors, with MET and pluripotency network activation occurring concomitantly (summarized in Extended Data Fig. 9c). In addition, our data indicate that the C/EBPα pulse already initiates an EMT, reminiscent of the recently described EMT–MET transition during reprogramming of fibroblasts[17]. The single cell cloning and Oct4–GFP reporter experiments suggest that essentially 100% of the poised cells can be reprogrammed into iPS cells. Several lines of evidence suggest that the rapid demethylation of the *Oct4* promoter involves Tet2: the C/EBPα pulse directly binds to and upregulates the *Tet2* gene and shuttles the protein into the nucleus. Then Tet2 binds to regulatory sites of *Oct4* and other pluripotency genes, converting 5mC residues into 5hmC (summarized in Fig. 4c). This modification might directly cause the gene's de-repression or be linked to demethylation and/or induction of chromatin remodelling[8]. The effect of C/EBPα is highly specific as only the closely related factor C/EBPβ, but not the lineage-instructive factors GATA1 (ref. 20), nor Mash1 or MyoD expressed in fibroblasts, was active (Extended Data Fig. 10a–i). C/EBPα also sensitized T-cell precursors[21] but not fibroblasts (Extended Data Fig. 10j, k). However, when C/EBPα was co-expressed with PU.1, a condition that mediates transdifferentiation to macrophage-like cells[22], it induced a ~6-fold enhancement of iPS cell reprogramming, whereas PU.1 alone had no effect (Extended Data Fig. 10k). This indicates that a myeloid determinant sensitizes somatic cells for iPS cell reprogramming, in line with

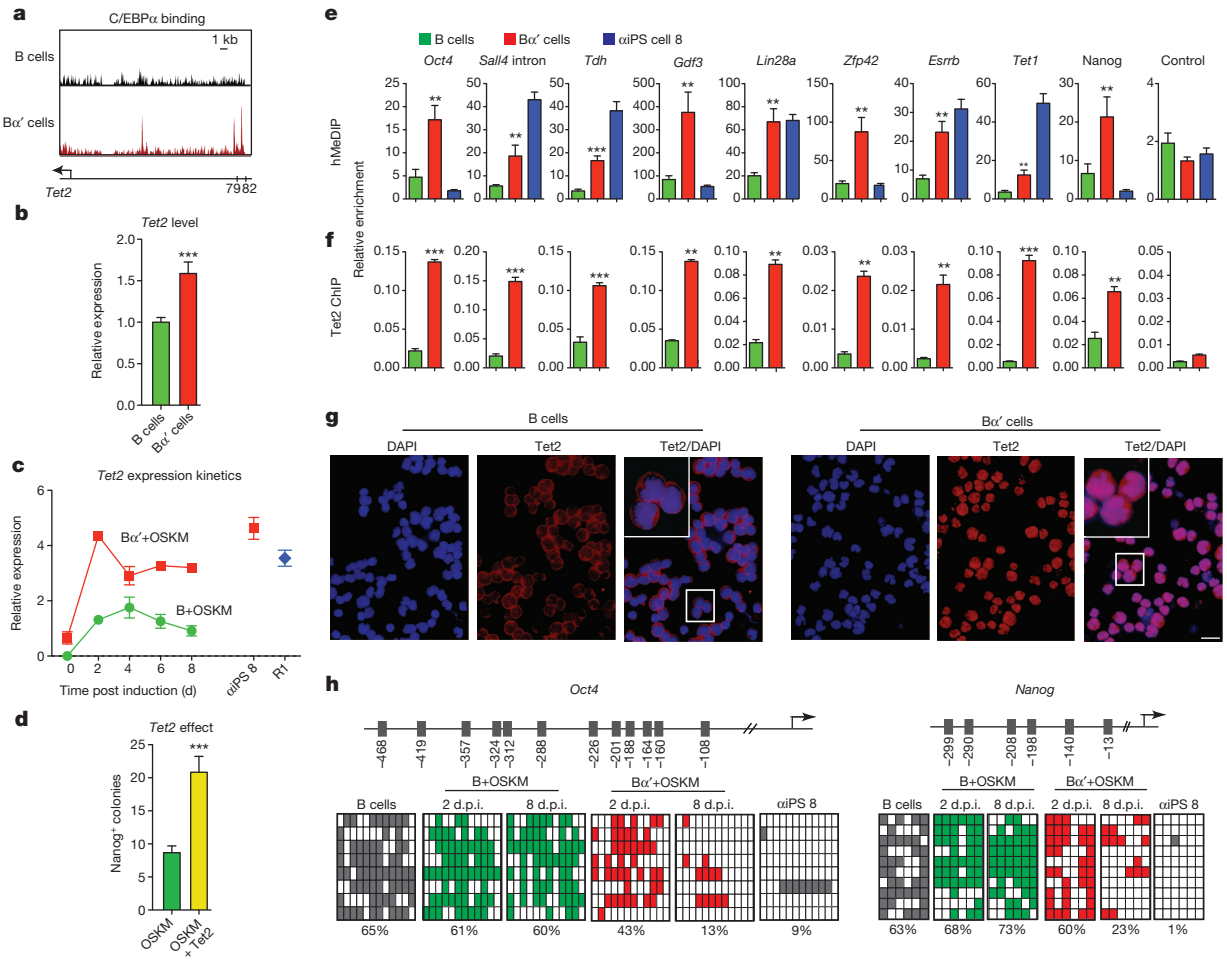


**Figure 3 | Tet2 activation, cytosine hydroxymethylation and DNA methylation.** **a**, C/EBPα binding at two enhancers upstream of the *Tet2* gene. **b**, **c**, qRT–PCR for Tet2 expression. Error bars indicate s.d. ($n = 6$). Student's *t*-test $^{***}P < 0.001$ relative to control. **d**, Tet2 overexpression effect on B-cell reprogramming. Error bars indicate s.d. ($n = 6$). Student's *t*-test $^{***}P < 0.001$ relative to control. **e**, hMeDIP followed by qPCR at regulatory regions of selected pluripotency genes. Error bars indicate s.d. ($n = 3$). Student's *t*-test $^{**}P < 0.01$, $^{***}P < 0.001$ relative to B cells. **f**, Tet2 binding sites determined by ChIP of the regions in **e**. Error bars indicate s.d. ($n = 3$). Student's *t*-test $^{**}P < 0.01$, $^{***}P < 0.001$ relative to B cells. **g**, Immunofluorescence of Tet2 protein in B cells and Bα′ cells (scale bar, 30 μm). **h**, CpGs analysed for methylation by bisulphite sequencing in the *Pou5f1* and *Nanog* promoters, with their position indicated on the top. Filled rectangles represent methylated CpGs; empty rectangles represent unmethylated residues.

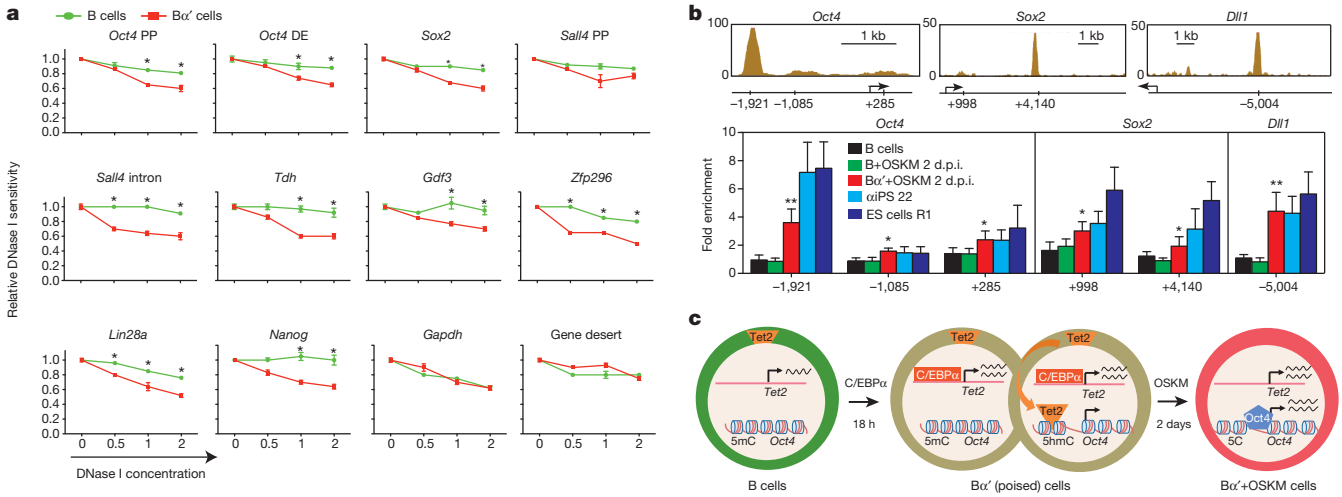


**Figure 4 | Chromatin accessibility and summary schemes.** **a**, DNase I sensitivity at regulatory regions in B cells and Bα′ cells determined by enzyme titration. Error bars indicate s.d. ($n = 3$). Student's *t*-test $^{*}P < 0.05$ relative to control. DE, distal enhancer; PP, proximal promoter. **b**, Oct4 binding sites examined at the *Pou5f1*, *Sox2* and *Dll1* loci, with Oct4 peaks corresponding to ES cells[19]. Oct4 binding by ChIP-qPCR analysis. Student's *t*-test $^{*}P < 0.05$, $^{**}P < 0.01$ relative to control. Error bars indicate s.d. ($n = 3$). **c**, Model of reprogramming mechanism involving Tet2.

the finding that macrophage/granulocyte progenitors, the formation of which is driven by C/EBPα[23], show the highest described reprogramming efficiency of somatic cells (25%)[12]. The C/EBPα effect might recapitulate an embryonic function of the gene as it is required, in combination with C/EBPβ, for the formation of functional trophoblasts[24], cells with intriguing similarities to macrophages[25].

## METHODS SUMMARY

Somatic cells used for iPS cell reprogramming were derived from the reprogrammable mouse[11]. ES cells and iPS cells were cultured on mitomycin-C-treated MEFs in ES cell medium containing 15% FBS and 1,000 U $ml^{-1}$ of LIF. For B- and T-cell reprogramming experiments the medium was supplemented with cell-type-specific cytokines[12]. OSKM expression was induced with 2 ng $ml^{-1}$ of doxycycline. αiPS cells and iPS cells correspond to individual iPS cell clones obtained after expansion into stable lines of OSKM induced Bα′ and B cells, respectively. The total number of iPS cell colonies was scored after alkaline phosphatase or Nanog staining. For qRT–PCR and microarray analyses, B cells at different time points during reprogramming, as well as ES cells and iPS cells, were trypsinized and FACS sorted to remove feeder cells and dead cells. Total RNA was extracted using the miRNeasy kit (Qiagen). Microarrays were done using Agilent 8X60K expression arrays. Details on injections into blastocysts to test for chimaerism are provided in Methods.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** T.G. and B.D.S. conceived the idea for this project, designed the experiments and wrote the paper. B.D.S. performed the cell culture, animal experiments and molecular biology and B.D.S. and J.L.S. the methylation analyses. J.L.S. performed the studies on Tet2 and J.L.S and C.v.O. the ChIP experiments. S.C., E.M.K. and D.T. were responsible for the bioinformatics and G.P.V. and M.B. for the DNase I experiments and analyses. J.L. provided the Tet2 overexpressing plasmids.

**Author Information** The array data are available from the Gene Expression Omnibus under the accession number GSE52397. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to T.G. (Thomas.Graf@crg.eu).

## METHODS

**Mice.** The reprogrammable mouse containing a tetracyclin-inducible OSKM cassette as well as rtTA has been described previously[11].

**Cell cultures.** ES cells and iPS cells were cultured on mitomycin-C-treated MEF feeder cells in KO-DMEM (Invitrogen) supplemented with L-glutamine, penicillin/streptomycin, non-essential amino acids, β-mercaptoethanol, 1,000 U ml$^{-1}$ LIF (ES cell medium) and 15% fetal bovine serum (FBS, Invitrogen).

MEF cultures were established by trypsin digestion of mouse embryos (embryonic day 13.5) and the resulting cells cultured in DMEM supplemented with 10% FBS, L-glutamine and penicillin/streptomycin. CD19$^{+}$ pro-B and pre-B cells were isolated from bone marrow using monoclonal antibodies to CD19 (1D3), obtained from BD Pharmingen, using MACS (Miltenyi Biotech). Double-negative (DN) T cells were prepared from thymii of 4-week-old mice by lineage depletion with Streptavidin microbeads. The following biotin-conjugated antibodies were used to label lineage (lin)-positive thymocytes before lineage depletion: CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), B220 (RA3-6B2), CD19 (1D3), Mac1/CD11b (M1/70), Gr1 (RB6-8C5), Ter119 (TER-119), I-A/I-E (2G9). The purity of the sorted cell fractions was confirmed by FACS using an LSR II machine (BD). After isolation, B cells were expanded in RPMI medium supplemented with 10% FBS and IL-7 for 1 week before inducing reprogramming. T cells were grown RPMI medium supplemented with 10% FBS supplemented with IL-7, Flt3 and SCF.

**Reprogramming.** Reprogramming experiments were conducted in gelatinized plates seeded with a feeder layer of the OP9 stromal cell line, using ES cell medium supplemented with 2 μg ml$^{-1}$ of doxycycline and 15% FBS. For the reprogramming of B cells, IL-4 (10 ng ml$^{-1}$), IL-7 (10 ng ml$^{-1}$) and IL-15 (2 ng ml$^{-1}$) were added to the medium (ref. 13). B cells were seeded at a density of 500 cells cm$^{-2}$ in 6-well plates. For the conditioning of Bα′ cells, C/EBPα-infected sorted B cells were exposed for 18 h to 100 nM of β-estradiol (E2) followed by inducer wash-out and addition of ES cell medium supplemented with doxycycline and cytokines. Serum-free iPS reprogramming was performed in serum-free ES cell medium (KO-DMEM supplemented with 15% knockout serum replacement, L-glutamine, penicillin/streptomycin, non-essential amino acids, β-ME, 1,000 U ml$^{-1}$ LIF, N2 (100X), B27 (100X))[9] supplemented with 2 μg ml$^{-1}$ doxycycline to activate OSKM factors. 2i inhibitors were added to the medium 2 days after doxycycline induction.

For expansion of iPS cell lines, colonies with ES cell morphology were picked after doxycycline withdrawal at 12 d.p.i. OSKM MEFs were seeded on mitomycin-C-treated MEF feeders in ES cell medium containing FBS and induced with doxycycline.

**Vectors and virus production and infection.** C/EBPα-ER-GFP, C/EBPα-ER-CD4, C/EBPβ, PU.1, C/EBPα-BRM-2 retroviruses and shPax5 lentivirus have been described previously[1,2,13]. *Gata1* cDNA was cloned by PCR in the BglII/XhoI restriction sites of the MIG vector. The MyoD-ER plasmid was obtained from P. Muñoz, Mash1 from V. Broccoli. The retroviral vector for murine Tet2 overexpression was derived from MSCV-based pMIRWAY-puro-TET2[26] by replacing puro with EGFP. Virus production has been described previously[27]. Briefly, HEK293T cells were co-transfected with vector plasmid and packaging plasmids using calcium phosphate transfection. Viral supernatants were collected 48–72 h later and concentrated by ultracentrifugation at 20,000*g* for 2 h at 20 °C. Viral concentrates were re-suspended in 1 × PBS and stored at −80 °C. Infection of MEFs was carried out in medium containing 5 μg ml$^{-1}$ polybrene, and infection of B cells by centrifugation with concentrated virus for 2 h at 32 °C at 1000*g* in B-cell medium.

**Alkaline phosphatase staining.** For alkaline phosphatase staining, the cells were fixed in 2% paraformaldehyde and then incubated for 15 min in NTMT solution (NaCl 100 mM, Tris A 1 M, Tris B 1 M, $MgCl_2$ 50 mM, Tween 0.1%) supplemented with BCIP e NBT (Roche).

**Immunofluorescence assays.** The cells were fixed with 4% paraformaldehyde, blocked and incubated with primary antibodies overnight at 4 °C. On the next day, the cells were exposed to secondary antibodies (all Alexa Fluor from Invitrogen) at room temperature for 1 h. The primary antibodies used were Nanog (Calbiochem), Oct4 (SC-5279), SMA (obtained from P. Muñoz), Tuj1 (obtained from V. Broccoli), Foxa2 (obtained from S. Aznar Benitah) and Tet2 (SC-61). Nuclear staining was performed with DAPI (Invitrogen).

**Differentiation of iPS cells.** Embryoid bodies (EBs) were derived by plating iPS cells at a concentration of 1.3 × 10$^{6}$ cells ml$^{-1}$ in non-adherent dishes in ES medium without LIF. After 4 days in suspension, cell aggregates were plated on gelatine-coated dishes in DMEM medium supplemented with 10% FBS for 10 days.

**Teratoma assay.** One million iPS cells were injected subcutaneously into SCID BEIGE mice. Three weeks after the injection, the tumours were surgically removed and embedded in paraffin. Teratomas were sectioned into 10-μm-thick slices using a cryostat (Leica, CM1850 UV) and sections were stained with haematoxylin and eosin.

**Chimaeric mice.** For the chimaera formation assay, 10–15 iPS cells (C57BL/6J background, black coat colour) were injected into a 3.5 days post coitum (d.p.c.) blastocyst of CD1 mice (white coat colour) and transferred into pseudo-pregnant 2.5 d.p.c. CD-1 recipients for chimaera generation. Chimaerism was ascertained after birth by the appearance of black coat colour (from the C57BL/6J iPS cells) over the white background from the host pups.

**FACS.** Cell suspensions were stained with various antibodies from either BD Pharmingen or eBiosciences. Cells were analysed with an LSR II FACS (BD Biosciences) using Diva v6.1.2 (BD Biosciences) and FlowJo software v10.0.6 (TreeStar). Primary antibodies used were CD19, Mac1, B220, Cdh1, CD115 (all from BD Biosciences), hCD4 (eBioscience) and Nanog (Calbiochem).

**RNA isolation and quantification.** RNA isolation of MEFs and B cells was done with the miRNeasy mini kit (Qiagen). RNA was collected from sorted live cells at 0, 2, 4, 6 and 8 d.p.i. To remove the feeders, ES cells and iPS cell clones at passage 2 or higher were seeded on gelatinized plates and processed with the above kits. RNA was eluted from the columns using RNase-free water or TE buffer and quantified by Nanodrop. cDNA was produced with the High Capacity RNA-to-cDNA kit (Applied Biosystem).

**Gene expression arrays and qRT–PCR.** RNA samples (with an RNA integrity number (RIN) greater than 9) were subjected to transcriptional analyses using Agilent expression arrays. For hybridization, 500 ng of total RNAs were labelled using Agilent's QuickAmp labelling kit following manufacturer instructions and analysed using Agilent 8X60K expression arrays. For the expression data with multiple probes annotated to the same gene only the most dynamic probe as defined by all array hybridizations was considered. Genes with expression changes during reprogramming were defined as showing >4-fold differences between ES cells and uninduced B cells, and having a Student's *t*-test *P* value <0.05 between technical duplicates. B-cell- and macrophage-specific genes were defined by combining array data from a previous study[28]. Lineage-specific genes were defined as >4-fold upregulated in one lineage over all of the others. Macrophage-specific genes were selected on the basis of >4-fold upregulation in primary macrophages and 5-day C/EBPα-induced macrophages as compared to B cells.

Unsupervised clustering was performed on Pearson correlation coefficients calculated between the indicated array samples using all genes that changed expression at least 2-fold between B cells and ES cells ($n$ = 10,982). Data were analysed and visualized using R v2.15.1.

The array data are available from the Gene Expression Omnibus under the accession number GSE52397.

qRT–PCR reactions were set up in triplicate with the SYBR Green QPCR Master Mix (Applied Biosystem) and primers as listed in Supplementary Table 2. Reactions were run on an AB7900HT PCR machine with 40 cycles of 30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C.

**Chromatin immunoprecipitation.** ChIP experiments were performed as described previously[29]. Antibodies against Oct4 (SC-8626) and C/EBPα (SC-61) were purchased from Santa Cruz Biotechnologies. Tet2 ChIP was performed as in ref. 3. Data were obtained from three biological replicates, three independent immunoprecipitations and three technical triplicates.

For ChIP-seq analysis, reads were mapped onto mouse mm9 genome using bowtie2 (v2.1.0) (parameter:–very-sensitive). Reads filtering was done using SAMtools to keep reads that map only once, with a quality score of 10 or more, and to remove duplicates. Peaks were called using HOMER (v4.3) (parameter: style factor) and peaks were selected to control the false discovery rate at 0.001. Peaks annotation and genes association (Extended Data Fig. 7b–d) was performed using HOMER and BedTools (v 2.17).

Circular plot (Extended Data Fig. 7e) was done using Circos.

The sequencing data are available under the Gene Expression Omnibus number GSE52397.

**DNA methylation.** DNA was extracted using the Blood & Cell Culture DNA Mini kit (Qiagen). Bisulphite treatment of DNA was achieved using the EpiTect bisulphite kit (Qiagen) according to the manufacturer's instructions. The resulting modified DNA was amplified by polymerase chain reaction (PCR) using primer listed in Supplementary Table 2. The resulting amplified products were gel-purified (Qiagen), subcloned into the pGEM-T Vector Systems (Promega), and sequenced using the T7 and SP6 primers.

**DNase I assay.** Chromatin samples obtained as described before from two biological replicates were subjected to DNase I digestion. Briefly, 2 μg of chromatin were treated with 0.5, 1, and 2 units of DNase I (Roche) for 3 min at 37 °C in 1× DNase incubation buffer. Control samples were incubated in the absence of DNase I. Reactions were terminated by adding EDTA (40 mM final concentration) and the crosslinking was reversed by incubating the samples at 65 °C. After 6 h, proteinase K (40 μg ml$^{-1}$ final concentration) was added to each reaction and incubated overnight at 37 °C. After careful phenol-chloroform extractions, the DNA was quantified and used as template for Real Time-PCR reactions using specific primers.

**hMeDIP.** DNA extraction and IP (DIP) was carried out by a previously published protocol (http://www.epigenome-noe.net/, PROT33) with the following alterations.

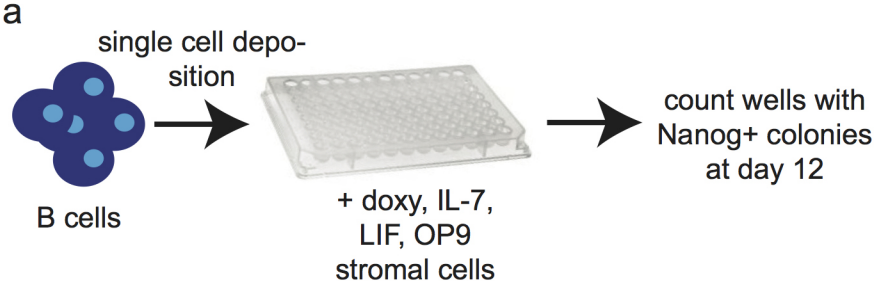
Genomic DNA was fragmented to a mean size of 350 bp using a Bioruptor (Diagenode) for 7×30 s cycles. DNA (re-suspended in 1× IP buffer) was incubated with 5 μg of anti-OHMeC antibody (Active Motif) at 4 °C for 2 h, and 40 μl of a 1:1 mixture of BSA-blocked Protein A (Millipore 16-125) and Protein G (Millipore 16-266) agarose beads were added, followed by an additional 2 h incubation at 4 °C. After washing 3 times in 1× IP buffer, immune complexes were released by incubation with 70 μg Proteinase K, and DNA was extracted once with phenol, extracted once with chloroform, and recovered by EtOH precipitation for qPCR analysis. The 5hmC enrichment in the samples was assessed by qPCR and the values were normalized against *Gapdh* promoter.

**RNA-seq.** RNA-seq was performed as described previously[30]. For RNA-seq analysis, reads were aligned onto mouse mm9 genome using STAR and Refseq mm9 annotation for splicing. Reads were filtered to keep only uniquely mapped reads with a maximum of two mismatches. Gene read count was performed using HTseq-count (parameter: mode = union, stranded, features = exons, attribute = gene_id) with Refseq mm9 annotation, and data were normalized using Bioconductor DEseq library (parameter: method = blind, sharingMode = fit-only, fitType = local). Genes with a read count above 300 in all conditions (set as a minimal threshold for functional expression) and genes with a 1.5-fold difference in normalized reads count between B cells and Bα′ cells were considered as differentially expressed. The sequencing data are available under the Gene Expression Omnibus number GSE52397.

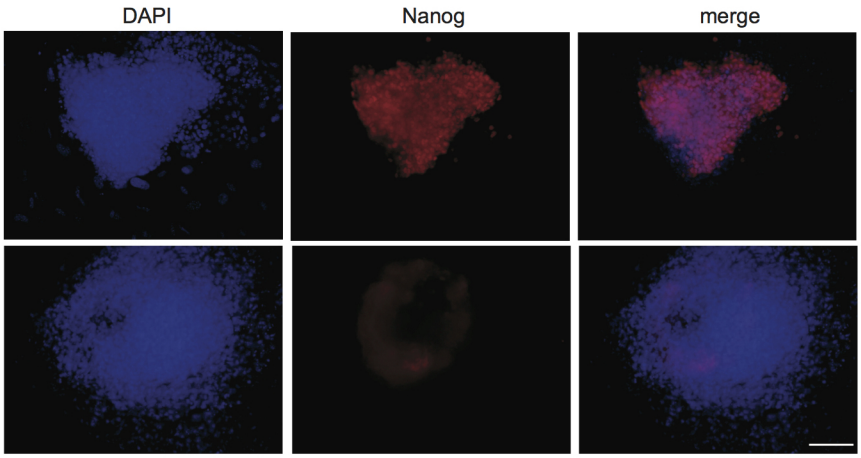
**Statistical analysis and replicates.** All data presented are representative of at least three independent experiments that yielded similar results. Statistical analyses were performed using Prism software (GraphPad).


26. Cheng, J. *et al.* An extensive network of TET2-targeting microRNAs regulates malignant hematopoiesis. *Cell Rep.* **5,** 471–481 (2013).
27. Di Stefano, B. *et al.* A microRNA-based system for selecting and maintaining the pluripotent state in human induced pluripotent stem cells. *Stem Cells* **29,** 1684–1695 (2011).
28. Di Tullio, A. & Graf, T. C/EBPα bypasses cell cycle-dependency during immune cell transdifferentiation. *Cell Cycle* **11,** 2739–2746 (2012).
29. van Oevelen, C. *et al.* A role for mammalian Sin3 in permanent gene silencing. *Mol. Cell* **32,** 359–370 (2008).
30. Tilgner, H. *et al.* Deep sequencing of subcellular RNA fractions shows splicing to be predominantly co-transcriptional in the human genome but inefficient for lncRNAs. *Genome Res.* **22,** 1616–1625 (2012).

a



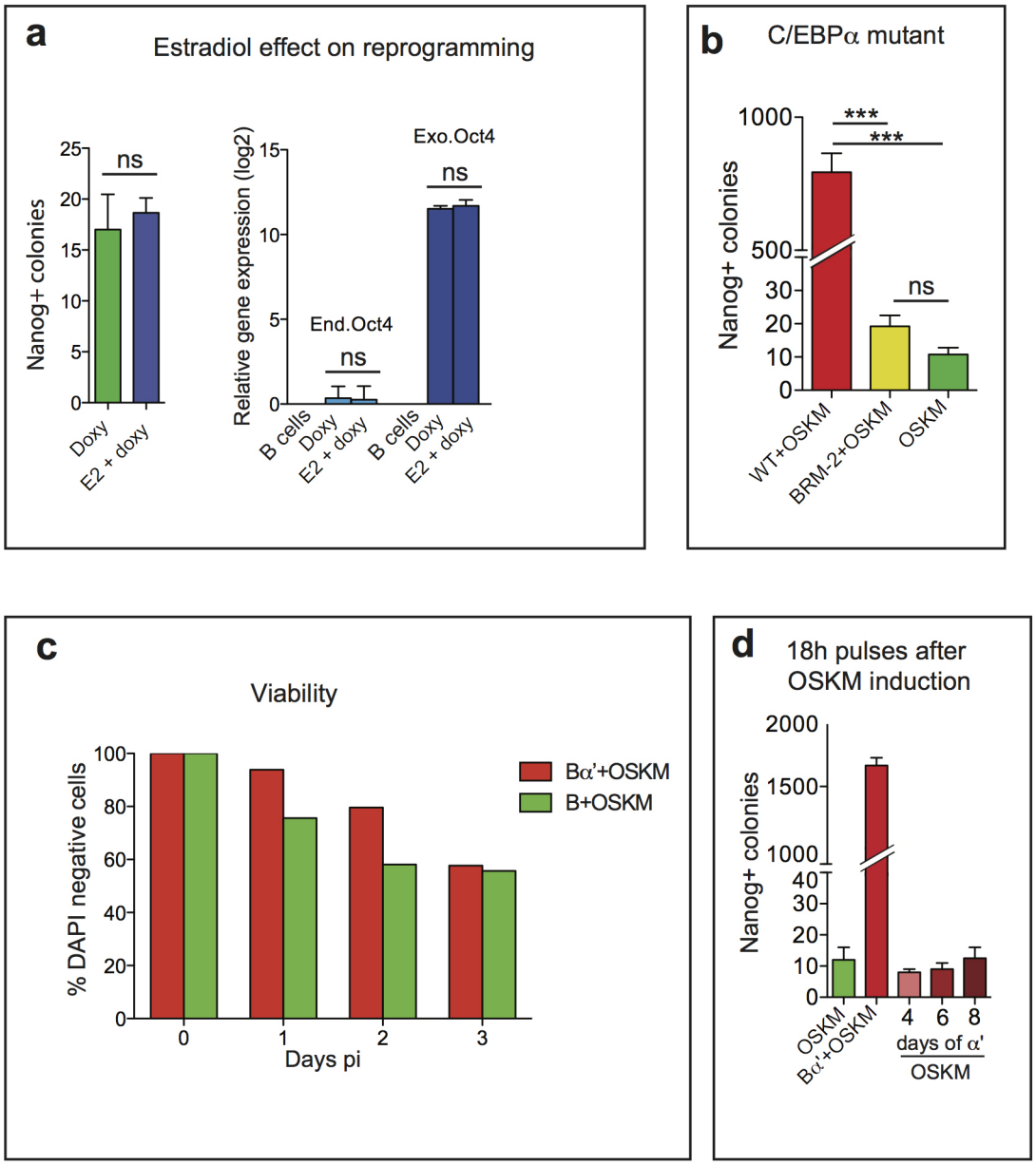

b



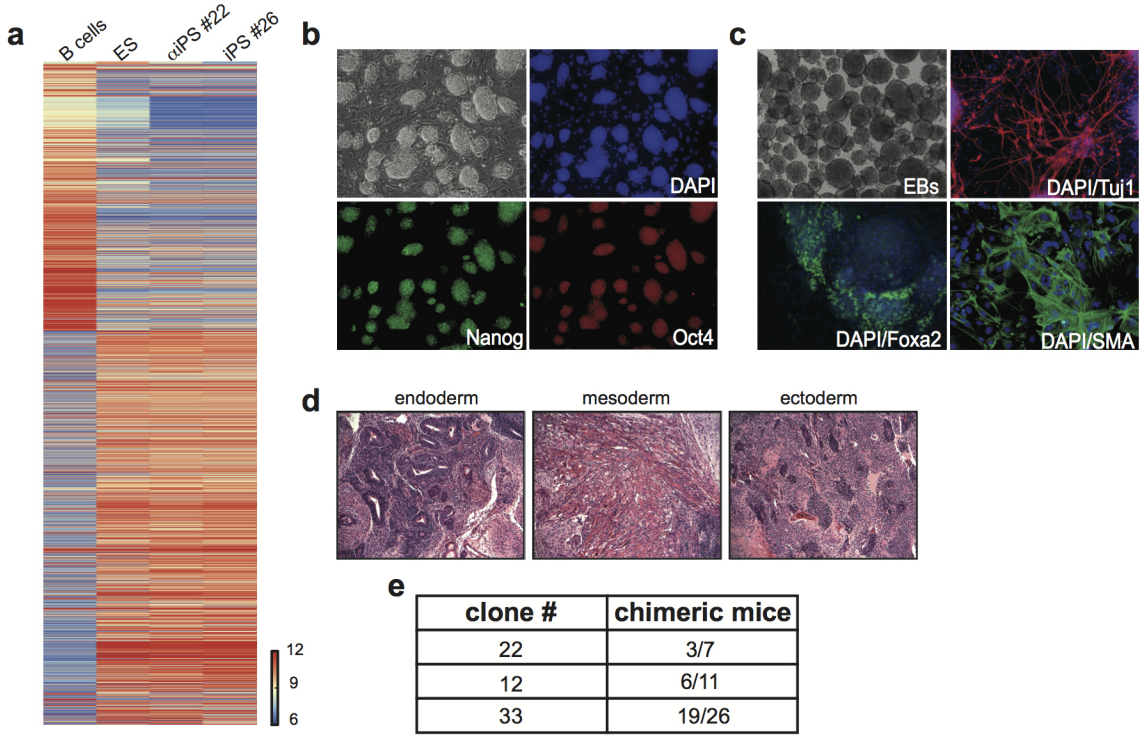

c

| | # of wells with cell growth | # of wells with Nanog+ colonies |
|---|---|---|
| Exp 1 | 114 | 105 |
| Exp 2 | 126 | 119 |

**Extended Data Figure 1 | Single cell reprogramming.** **a**, Bα′ cells were plated as single cells in 96-well plates on OP9 feeders in medium conditioned with IL-7, IL-15 and IL-4 cytokines. **b**, Representative Nanog-positive (upper panels) and Nanog-negative (lower panels) iPS cell clones in 96-well plates (scale bar, 100 μm). **c**, Efficiency of reprogramming obtained from two independent experiments.



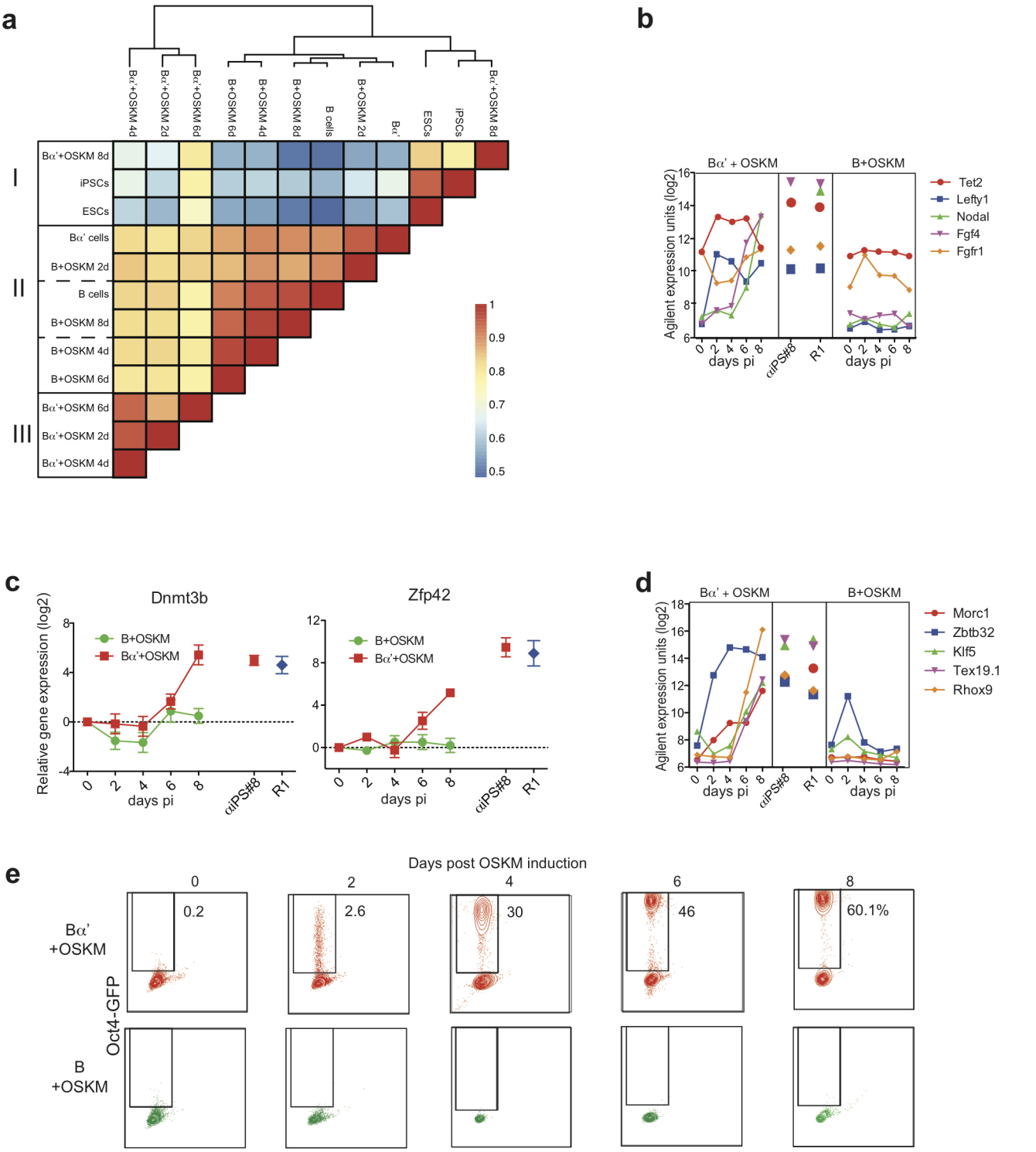

**Extended Data Figure 2 | C/EBPα mutant and cell viability during iPS cell reprogramming.** **a**, E2 has no effect on reprogramming efficiency and ectopic Oct4 expression. Error bars indicate s.d. ($n = 3$). **b**, Effect of a C/EBPα mutant for the DNA binding domain on iPS cell reprogramming efficiency. Student's *t*-test $^{***}P < 0.001$ relative to control. Error bars indicate s.d. ($n = 3$). **c**, Histogram showing percentages of live cells during the first 3 days of iPS cell reprogramming as analysed by FACS. Dead cells were scored as positive for DAPI staining. **d**, Effect of 18 h C/EBPα pulses after OSKM induction. Error bars indicate s.d. ($n = 3$).



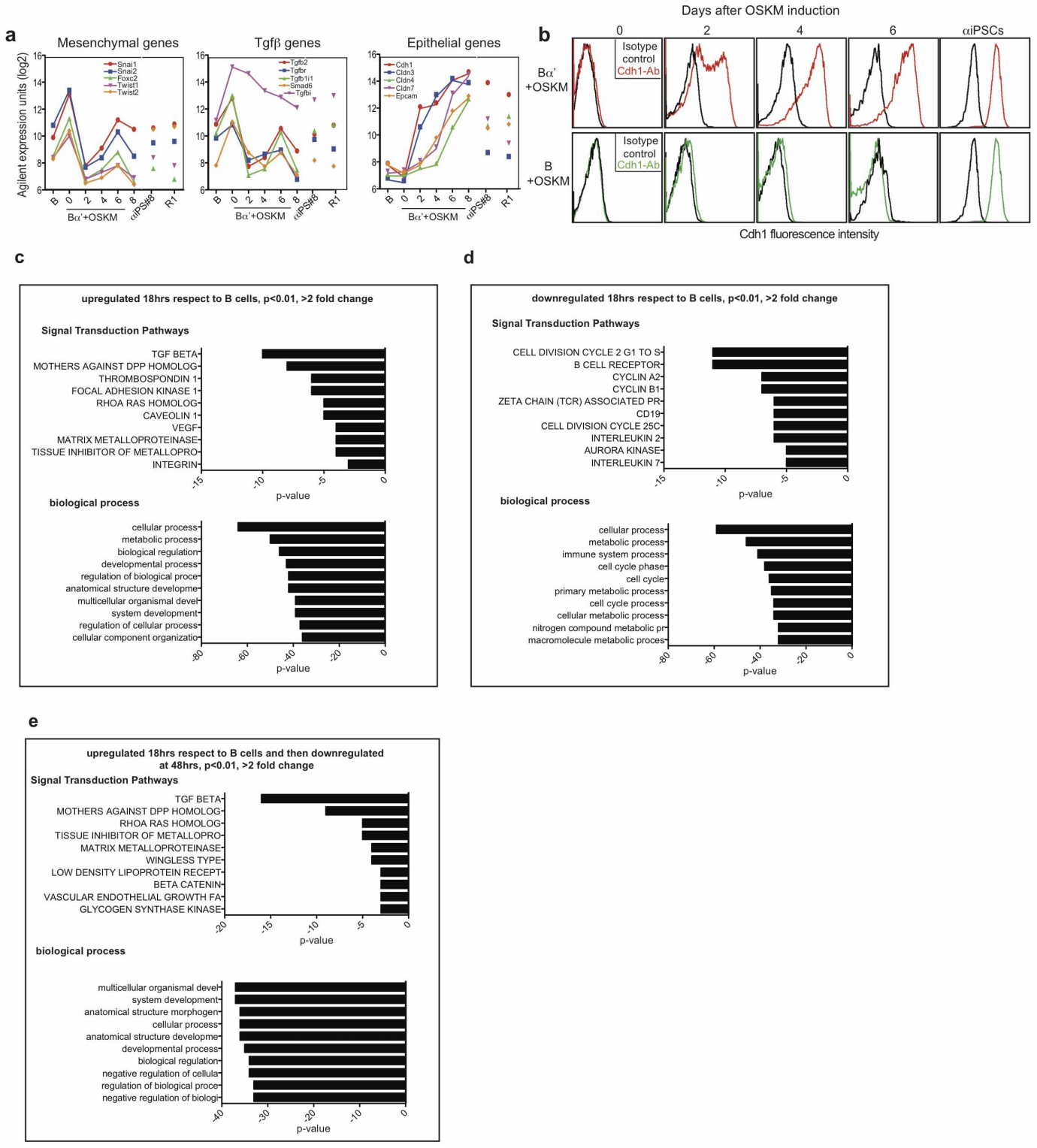

e

| clone # | chimeric mice |
|---|---|
| 22 | 3/7 |
| 12 | 6/11 |
| 33 | 19/26 |

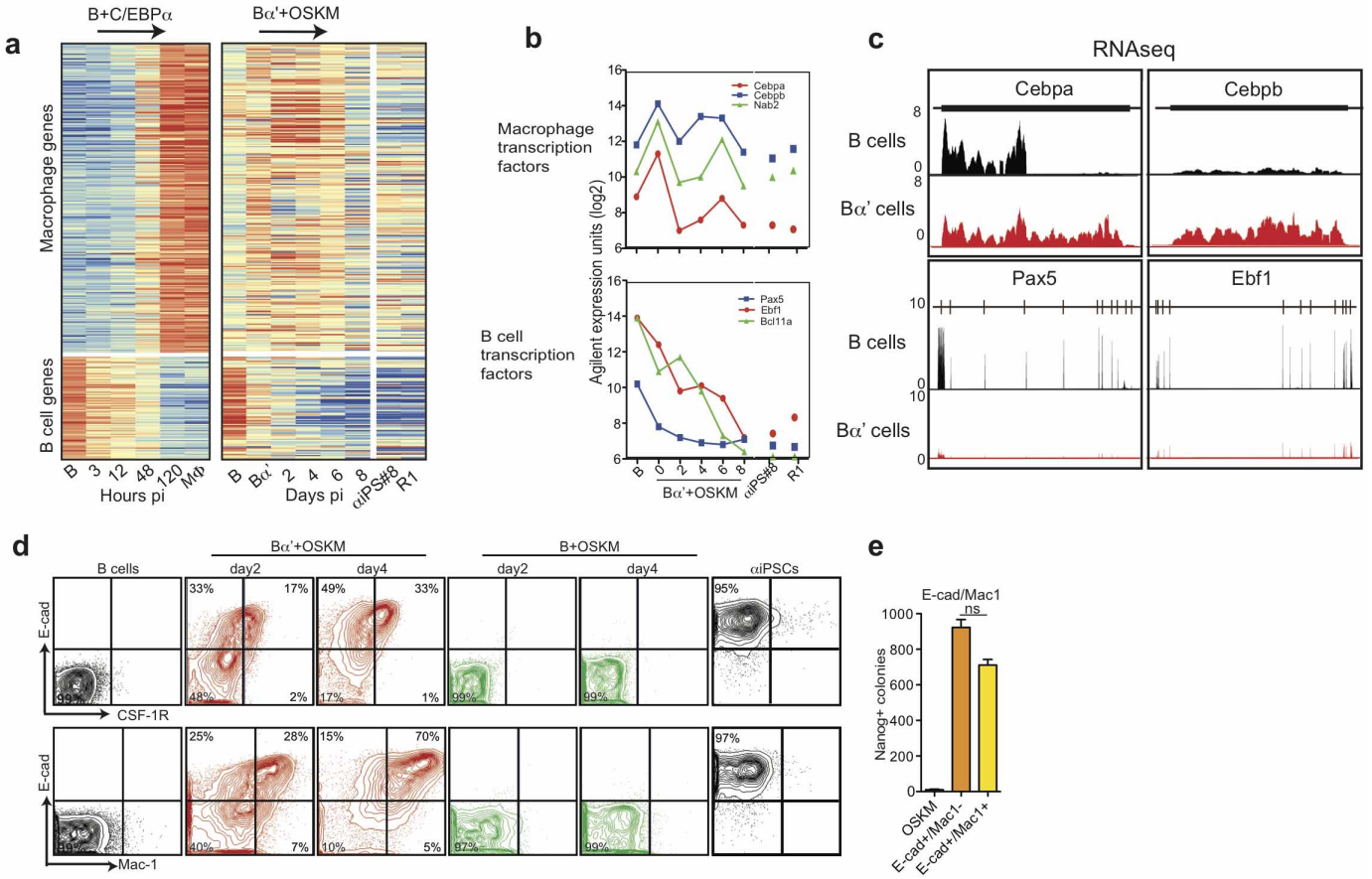
**Extended Data Figure 3 | Properties of αiPS cells.** **a**, Heat maps showing gene expression profiles of B cells, ES cells, αiPS cells (clone 22) and iPS cells (clone 26) derived from Bα′ and B cells, respectively. Genes were selected based on a >4-fold difference between B cells and ES cells. **b**, Micrographs (×10) of αiPS cell clone 22 growing on MEF feeders, showing colonies of ES-like morphology (bright field) and expression of Nanog (green) and Oct4 (red). **c**, Embryoid bodies generated from αiPS cell clone 22 (bright field) differentiated into Tuj1-positive cells (ectoderm), Foxa2-positive cells (endoderm) and SMA expressing cells (mesoderm). Original magnification, ×10. **d**, Section of a teratoma obtained from αiPS clone 22 stained with haematoxylin and eosin, showing differentiation into the major germ layers Original magnification, ×10. **e**, Proportion of chimaeric mice obtained after injection of CD1 blastocysts with three different αiPS cell clones.



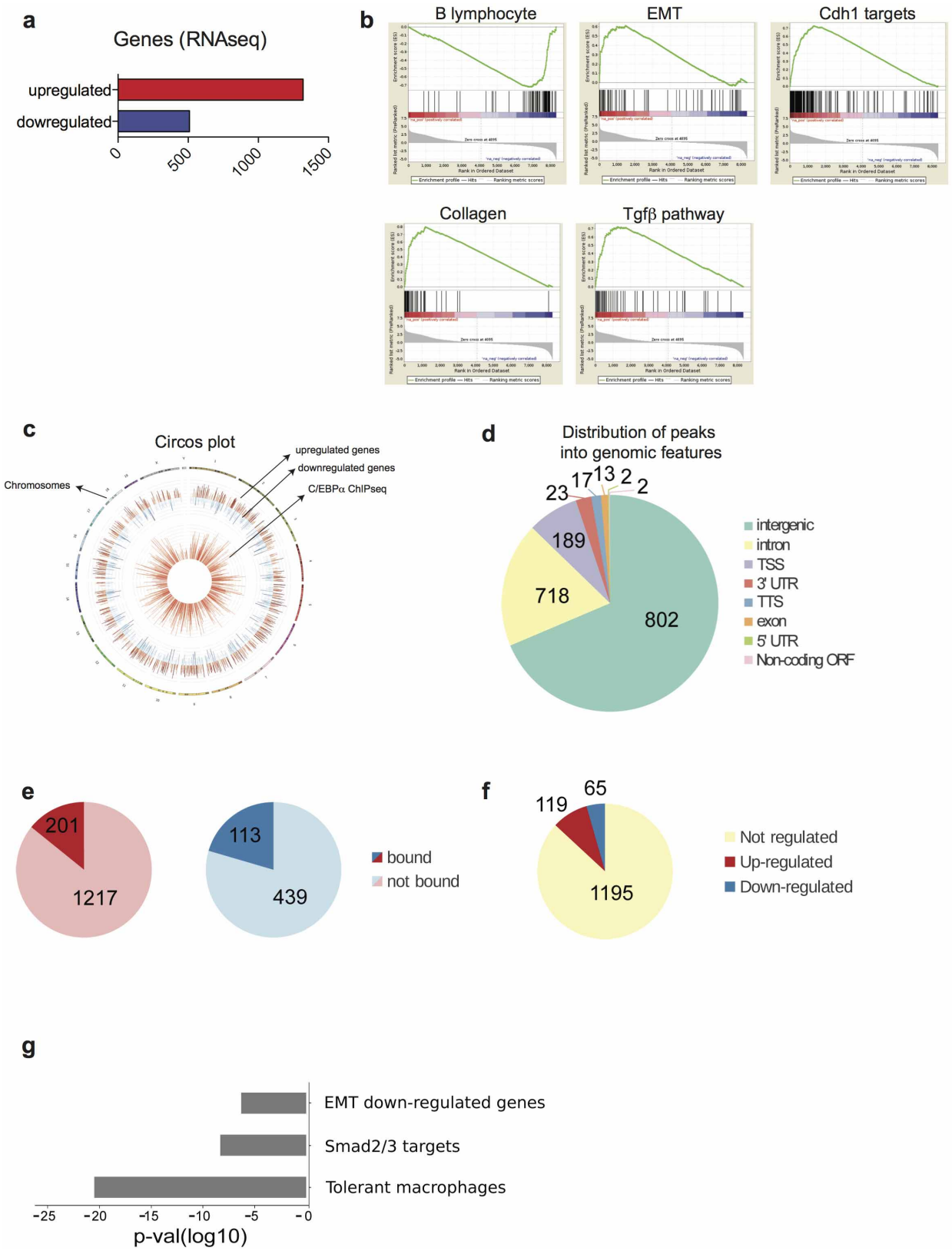

**Extended Data Figure 4 | Pluripotency gene regulation during iPS cell reprogramming.** **a**, Unsupervised hierarchical clustering analysis of gene expression array data. **b**, Agilent gene expression kinetics ($log_2$ expression units) of pluripotency genes from Bα′+OSKM cells (red shaded panels), αiPS cells and ES cells (white panels) and B+OSKM cells (green shaded panels). **c**, qRT–PCR confirming array results for *Dnmt3b* and *Zfp42* (*Rex1*) genes. Normalized against *Pgk* expression. Error bars indicate s.d. ($n = 3$). **d**, Agilent gene expression kinetics of late transition genes. **e**, Oct4–GFP expression during iPS cell reprogramming.



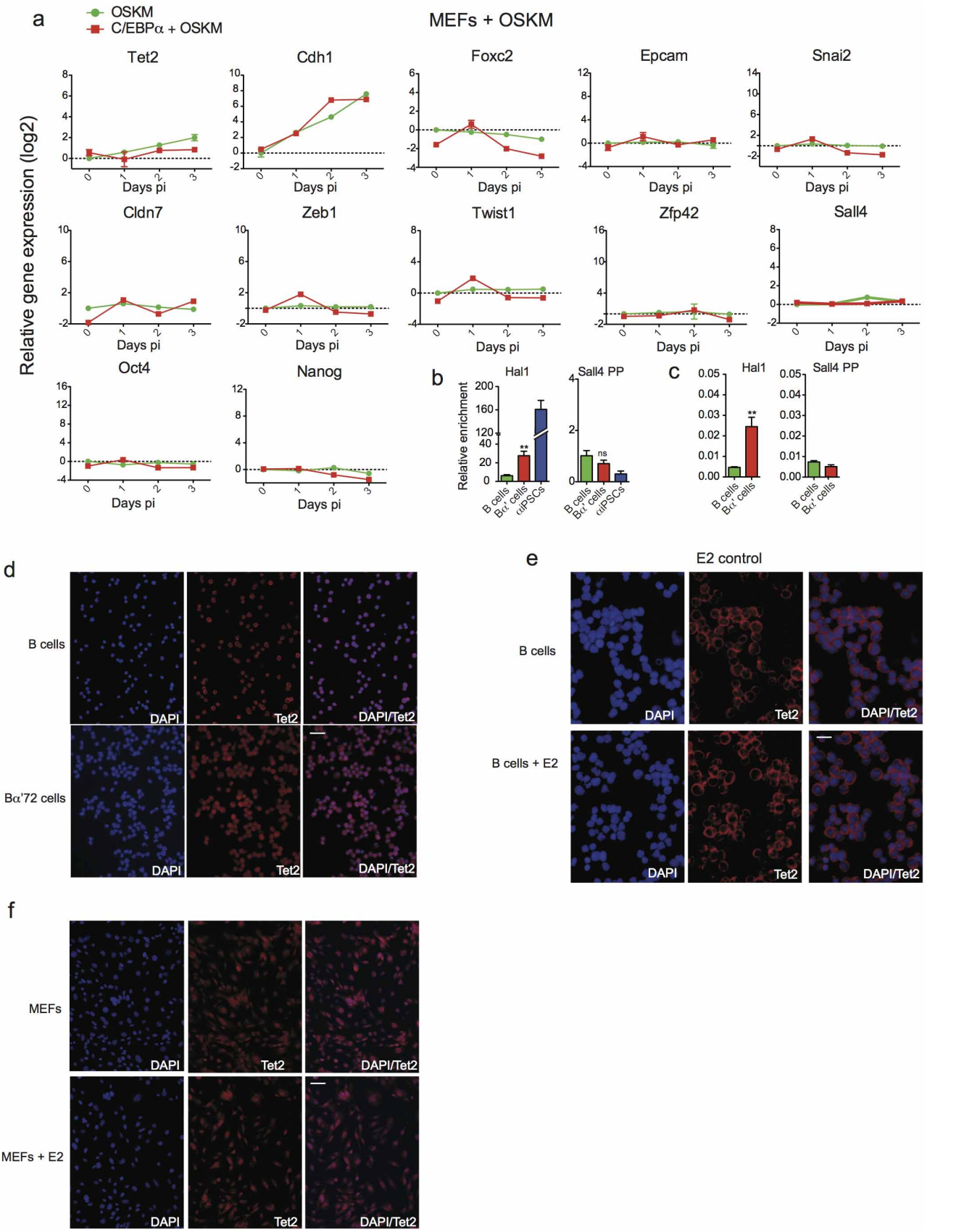

**Extended Data Figure 5 | Changes in the expression of mesenchymal–epithelial transition genes.** **a**, Agilent gene expression kinetics of mesenchymal–epithelial transition (MET) genes. **b**, E-cadherin expression by FACS at different times after induction. Isotype controls are shown in black; antibody-stained cells in red and green. **c**, GO analysis for genes upregulated >2-fold in Bα′ cells compared with B cells. **d**, GO analysis for genes downregulated >2-fold in Bα′ cells compared with B cells. **e**, GO analysis of genes upregulated >2-fold in Bα′ cells compared with B cells and subsequently downregulated >2-fold at 48 h.



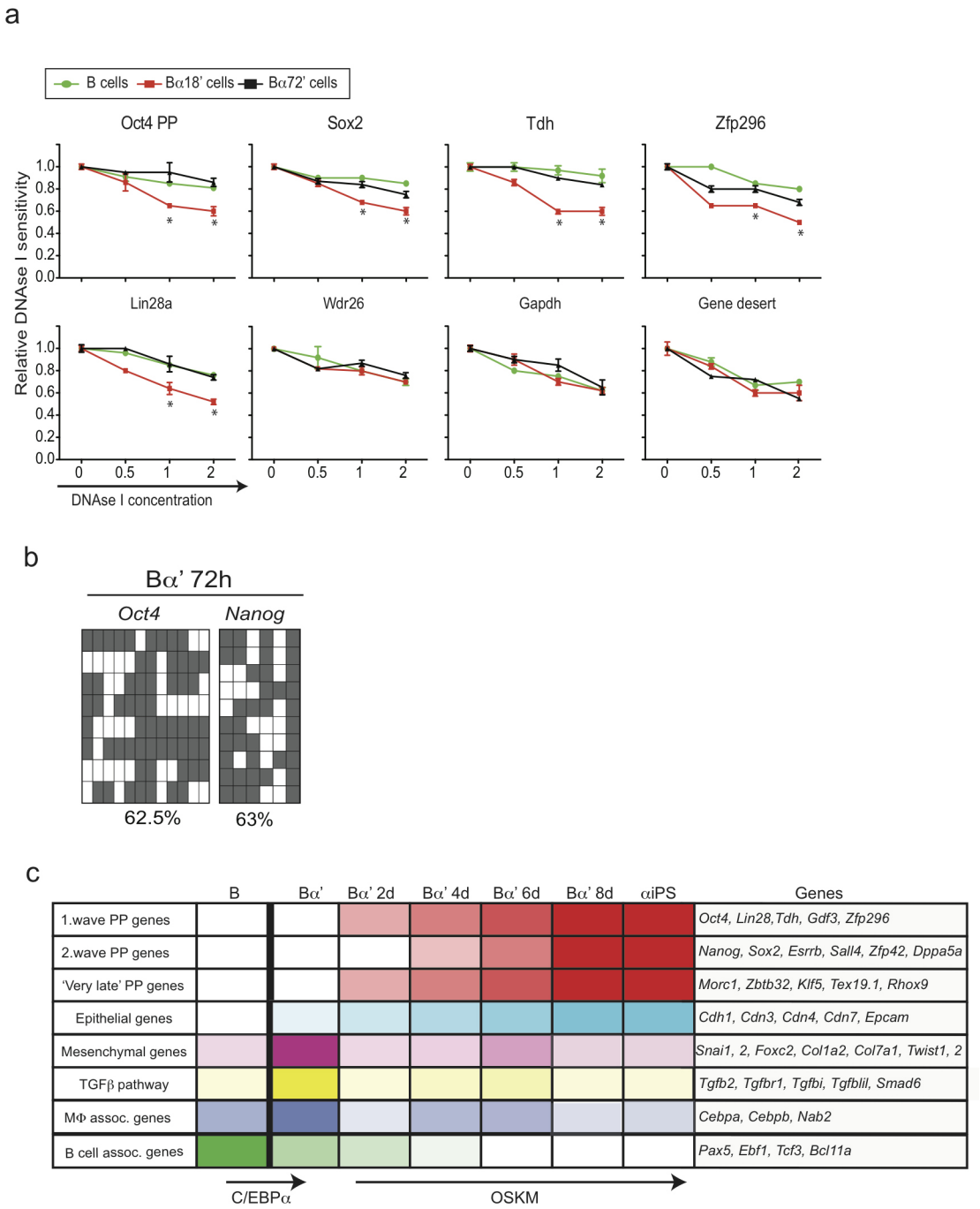

**Extended Data Figure 6 | Changes in the expression of B cell and macrophage genes.** **a**, Heat map of expression Affymetrix arrays with 263 macrophage and 83 B-cell-specific genes during C/EBPα-induced transdifferentiation of B cells and heat maps of the same genes using data from the Agilent expression array in Fig. 2. **b**, Agilent gene expression kinetics of selected macrophage and B-cell transcription factors. **c**, RNA-seq data for two representative macrophage and B-cell-specific genes each. **d**, FACS profiles of E-cadherin (Cdh1) expression, combined with either CSF-1R (upper panels) or Mac1 (lower panels) staining. **e**, Reprogramming potential of E-cadherin$^+$/Mac1$^-$ and E-cadherin$^+$/Mac1$^+$ cells. Error bars indicate s.d. ($n = 3$).



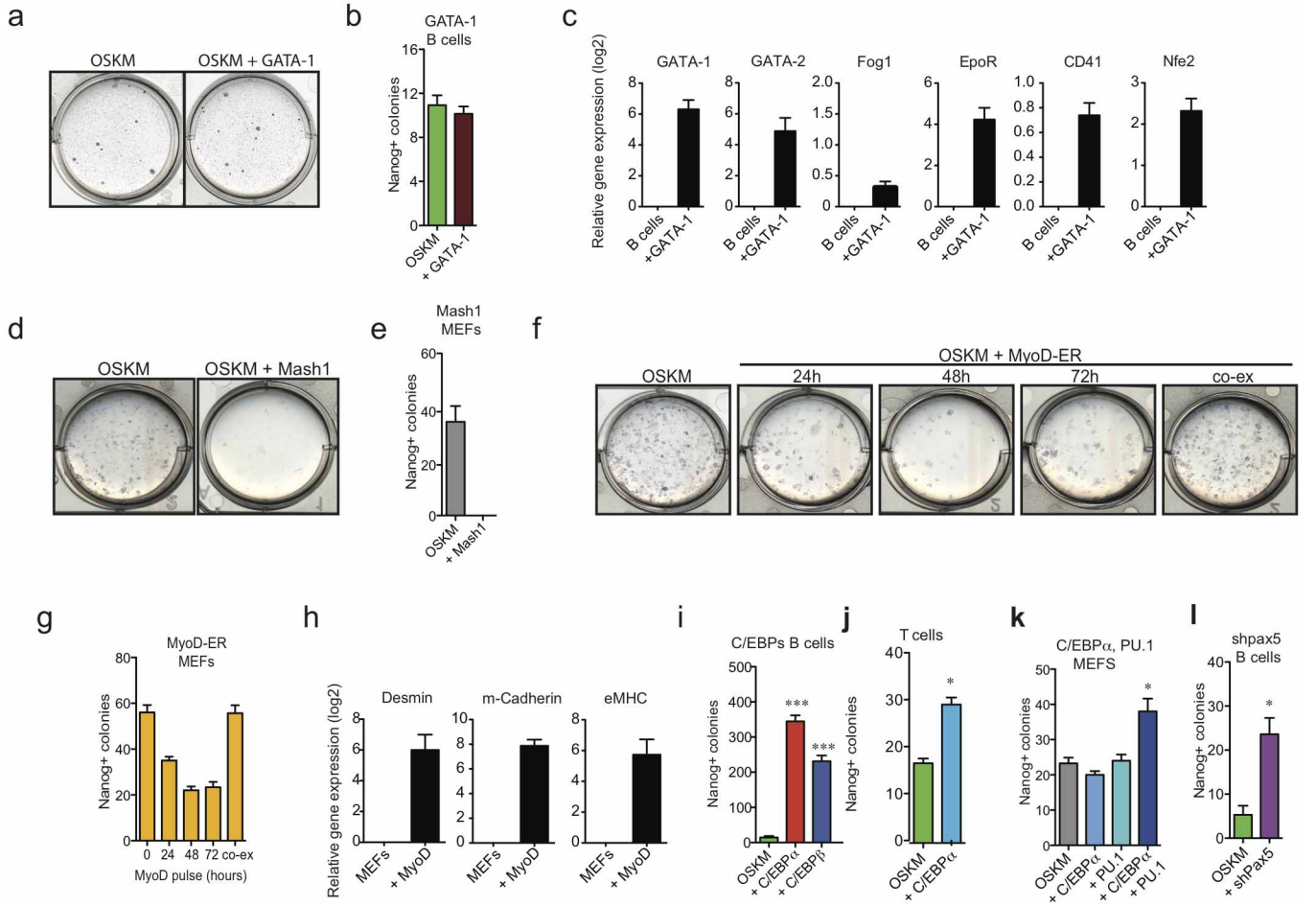

**Extended Data Figure 7 | ChIP-seq and RNA-seq analysis.** **a**, Number of upregulated and downregulated genes (>1.5-fold change) after a pulse of C/EBPα. **b**, Result of gene set enrichment analysis on RNA-seq data (ranked by fold change) for selected significantly enriched gene sets (from MSigDB cp.v4 database). **c**, Circular visualization of genomic distribution of differentially expressed genes (RNA-seq) and C/EBPα binding sites (ChIP-seq). **d**, Distribution of C/EBPα peaks into genomic features, on the basis of Refseq mm9 annotation. **e**, Fraction of upregulated (red chart) and downregulated genes (blue) after a pulse of C/EBPα showing a peak at 50 kb from TSS. **f**, Variation of expression after C/EBPα pulse of nearest genes to peaks. **g**, Selected gene sets significantly enriched in genes adjacent to C/EBPα peaks based on hypergeometric tests (from MSigDB cp.v4 database). Bonferoni procedure, $P$ value $<0.05$.




**Extended Data Figure 8 | Tet2 expression in B cells and MEFs.** **a**, RT–qPCR of Tet2, pluripotency and MET genes after induction of OSKM in MEFs pulsed or not with C/EBPα. Error bars indicate s.d. ($n = 3$). **b**, **c**, hMeDIP and Tet2 binding at regulatory regions of the Tet2 target gene *Hal1* (ref. 3) and *Sall4*. **d**, Tet2 localization in B cells pulsed with C/EBPα for 72 h. **e**, Oestradiol treatment has no effect on cellular localization of Tet2 protein in B cells (scale bar, 30 μm). **f**, Cellular localization of Tet2 protein in MEFs treated or not with E2 (scale bar, 100 μm).




**Extended Data Figure 9 | DNase I sensitivity and gene expression summary.** **a**, DNase I sensitivity at regulatory regions in B cells and Bα′ cells (data from Fig. 4a) and B cells pulsed for 72 h determined by enzyme titration. Error bars indicate s.d. ($n = 3$). Student's $t$-test $^*P < 0.05$ relative to control. **b**, CpGs analysed for methylation by bisulphite sequencing in the *Pou5f1* and *Nanog* promoters. Filled rectangles represent methylated CpGs, empty rectangles unmethylated residues. **c**, Summary of relevant gene expression changes during the transition from B cells to Bα′ cells and Bα′ cells to αiPS cells.




**Extended Data Figure 10 | Transcription factor and cell type specificity.** **a**, **b**, B+OSKM cells were infected with a retrovirus expressing GATA1 or left uninfected, induced with doxycycline and scored for 12 d.p.i. alkaline phosphatase$^+$ and Nanog$^+$ colonies. Error bars indicate s.d. ($n = 3$). **c**, qRT–PCR for megakaryocyte/erythroid-restricted genes in B cells infected with GATA1 retrovirus for 3 days. Error bars indicate s.d. ($n = 3$). **d**, **e**, Alkaline phosphatase$^+$ colonies at 15 d.p.i. in OSKM MEFs infected with a retrovirus expressing Mash1 or mock infected, and Nanog$^+$ colonies counted. Error bars indicate s.d. ($n = 3$). **f**, **g**, Effect of MyoD expression on iPS cell reprogramming of MEFs. Cells were infected with MyoD-ER and sequentially induced with E2 to activate MyoD and doxycycline to activate OSKM. Representative plates with alkaline phosphatase$^+$ iPS cell colonies 15 d.p.i. of MEFs pre-induced for 24, 48 or 72 h or continuously with E2. Error bars indicate s.d. ($n = 3$). **h**, Expression of muscle-restricted genes in OSKM MEFs 3 days after MyoD-ER induction. Error bars indicate s.d. ($n = 3$). **i**, Effects of C/EBPα and C/EBPβ in B+OSKM cells. Student's *t*-test $^{***}P < 0.001$ relative to control. Error bars indicate s.d. ($n = 3$). **j**, Effect of an 18-h C/EBPα pulse in pre-T+OSKM cells on the formation of 12 d.p.i. Nanog$^+$ colonies. Student's *t*-test $^{*}P < 0.05$ relative to control. Error bars indicate s.d. ($n = 3$). **k**, Effects of C/EBPα, PU.1 and their combination in MEFs. Student's *t*-test $^{*}P < 0.05$ relative to control. Error bars indicate s.d. ($n = 3$). **l**, Effect of Pax5 knock down in B-cell reprogramming. Student's *t*-test $^{*}P < 0.05$ relative to control. Error bars indicate s.d. ($n = 3$).